Supplementary Information

Roles of ErbB3 binding protein 1 (EBP1) in embryonic development and gene-silencing control

Hyo Rim Ko[1,2], Inwoo Hwang[1,2], Eun-Ju Jin[1,2], Taegwan Yun[1,2], Dongryeol Ryu[1], Jong-Sun Kang[1,2,3], Kye Won Park[4], Joo-Ho Shin[1,2,3], Sung-Woo Cho[5], Kyung-Hoon Lee[3,6], Keqiang Ye[7] and Jee-Yin Ahn[1,2,3]*

***Correspondence should be addressed to** Jee-Yin Ahn, Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, 2066, Seobu-ro, Jangan-gu, Suwon 16419, Korea Phone: 82-31-299-6134; Fax: 82-31-299-6139; E-mail: jeeahn@skku.edu

This PDF file includes:

Supplementary text

Figures S1 to S6

Legends for Dataset S1 to S4

www.pnas.org/cgi/doi/10.1073/pnas.1916306116

Materials and Methods

Animal

The mouse Ebp1 gene is located on chromosome 10 (NM_011119). The *Ebp1* gene is composed of 10 exons and extends over 8.4kb. The mouse *Ebp1* knock mouse was generated in collaboration with genOway (Lyon, France). Ebp1 conditional knock-out mice were generated by introduction of two *loxp* sites flanking Ebp1 exons 6 to 10 and enable the monitoring of Ebp1 expression cells thanks to the insertion of an IRES-reporter (eGFP) cassette downstream of the stop codon. Insertion of the construct selected for the neomycin marker in the targeting vector and the neo cassette was flanked by frt sites. The construct was transfected into ES cells according to genOway's standard electroporation procedures. The selected ES cells by Polymerase chain reaction (PCR) and Southern Blot based screenings were injected into blastocyst.

To neuronal- specific deletion of *Ebp1*, homozygous mutant *Ebp1* allele ($Ebp1^{flox/flox}$) were crossed with the Nestin-cre mice. Offspring were weaned after 4 weeks and marked by ear clipping.

The genomic DNA was prepared from the tail (Sigma Aldrich, Saint Louis, MO, USA) and was genotyped using PCR analysis. The primer pair was designed for the detection of the cre-mediated excision event.

All animal experimentation was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Sungkyunkwan University School of Medicine (SUSM, SKKUIACUC 17-6-4-1). All experimental procedures were carried out in accordance with the regulations of the IACUC guideline of Sungkyunkwan University.

Antibodies

Anti-Bax (cat. 2772), and anti-Bcl2 (cat. 2870s) antibodies were acquired from Cell Signaling (Danvers, MA, USA). Anti-β-actin (cat. sc-47778), anti-survivin (cat. sc-17779), anti-p21 (cat. sc-6246), and anti-GFP (cat. sc-9996) antibodies were acquired from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-Ebp1 (cat. ab33613, ab186846), anti-BrdU (cat. ab6326), anti-DNMT1 (cat.

ab13537), anti-annexin V (cat. ab14196), and anti-ki67 (cat. ab8191) antibodies were obtained from Abcam (Cambridge, MA, USA). Anti-PCNA was acquired from Calbiochem. Anti-H3k9me3 (cat. 07-442) was obtained from Millipore (Burlington, Massachusetts, USA).

Public Dataset analyses

The covariation among genes (Fig. 3*B* and 6*D*) were evaluated with Pearson correlation coefficient and Spearman's Rank-Order Correlation with corresponding p values (two-tailed). All raw data of gene expression profile are publicly available on NCBI Gene Expression Omnibus (NCBI GEO, www.ncbi.nlm.nih.gov/geo) and GeneNetwork (www.genenetwork.org). The gene expression profiles were obtained from the human prefrontal cortex transcriptomes (NCBI GEO No. GSE36192), the monkey (*Macaca Fascicularis*) prefrontal cortexes transcriptomes (GeneNetwork Accession No. GN251), the brain transcriptomes of BXD mouse genetic reference population (GeneNetwork Accession No. GN123), and the brain transcriptomes of B6D2-F2 intercross mouse population (GeneNetwork Accession No. GN123). (PMID: 22433082, 20485568; 16783646; 22939713; 16783644; 27933521) Correlogram and interaction network were generated using RStudio (R Consortium Inc, Boston, MA) as described in the previous study (PMID: 27986797).

Western blotting

MEF and 293t cells were washed with 15 mL of PBS and immediately added to lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1.5 mM Na_3VO_4, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM beta-glycerolphosphate, 1 mM PMSF, and protease cocktail). The cell lysates were mixed with 5× SDS sample buffer, boiled, and analyzed by immunoblotting. Protein levels were quantified by densitometry and normalized to actin (ImageJ software).

Reverse transcriptase polymerase chain reaction and qPCR

Total RNA was extracted using the TAKARA miniBEST Universal RNA Extraction kit (TAKARA, Japan) according to the manufacturer's instructions. The reverse transcription reaction was performed using the PrimeScript 1st strand cDNA Synthesis kit (TAKARA, Japan). Data normalization of transcript concentrations was performed using GAPDH.

Cresyl violet staining

Cresyl violet acetate crystal powder was dissolved in distilled water. The solution was filtered using Whitman paper. Paraffin section slides were immersed in xylene and rehydrated by passing the tissue through a series of decreasing concentrations of ethanol (100% to 70% ethanol). The slides were immersed in cresyl violet staining solution for 5 min and washed in distilled water. The slices were dehydrated by passing the tissue through a series of increasing ethanol concentrations (70% to 100% ethanol). The final two immersions were in xylene solution. The slides were examined with a microscope (Aperio ScanScope slide scanner), and their image was captured using ImageScope software.

FACS analysis

MEF cells were fixed with 70% ethanol and store at -20°C then cells washed twice with cold PBS. Cells were incubated in PBS containing 10 μg/ml RNase A and 20 μg/ml PI and transfer to FACS tubes and incubate at room temperature in the dark for 30 minutes. Cells were analyzed by a FACS Canto II.

Immunofluorescence

Mouse embryos were fixed in 4% paraformaldehyde for 15 min, permeabilized in PBS containing 0.25% Triton X-100 for 1 h, and blocked in 1% BSA for 1 h. The slices were immunostained using primary antibodies and the appropriate Alexa Fluor 594 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse secondary antibodies. Nuclei were counterstained with DAPI stain.

Immunostained images were acquired using a laser scanning confocal microscope (LSM 710, Carl Zeiss, Germany). Fluorescent images were quantified on a pixel-by-pixel basis using a microscope and Zeiss ZEN software.

Global methylation assay

Genomic DNA was extracted from E13.5 *Ebp1* wild-type (*Ebp1*$^{(+/+)}$), heterozygous (*Ebp1*$^{(+/-)}$), and homozygous mutant (*Ebp1*$^{(-/-)}$) embryonic brain using Accuprep® Genomic DNA Extraction kit (Cat. No. K-3032, Bioneer, Korea). Global DNA methylation levels were measured by Imprint®Methylated DNA Quantification kit (Cat. No. MDQ1, Sigma-Aldrich, St Louis, MO, USA), according to manufacturer's instruction.

ChIP assay

A ChIP assay was performed using a ChIP assay kit (cat. 17-259, Millipore, Temecula, CA 92590, USA) according to the manufacturer's instructions. In brief, a histone was crosslinked to DNA by adding formaldehyde to the culture medium for 10 min at room temperature. Glycine was added for 5 min to quench any unreacted formaldehyde. The cells were scraped into e-tubes and centrifuged at 700 × *g* at 4°C for 1 min. The supernatant was removed, and the cell pellet was resuspended in SDS lysis buffer containing 1× protease inhibitor cocktail. For immunoprecipitation, the dilution buffer, chromatin, protein G agarose, and antibody were mixed and stored overnight at 4°C with rotation. The protein G agarose-antibody/chromatin complex was washed by resuspending the beads in 1-mL cold buffers (low salt immune complex wash buffer, high salt immune complex wash buffer, LiCl immune complex wash buffer, and TE buffer in regular sequence). Elution buffer was added to the antibody/agarose complex, mixed by flicking the tube gently, and incubated at room temperature for 15 min. To reverse crosslink of the protein/DNA complexes to free DNA, 5 M NaCl was added, and the mixture was incubated at 65°C for 6 h. DNA was purified using spin columns.

Embryo brain slice culture

Embryo brain slice cultures were prepared from E14.5 mouse brains. The 280-µm thick brain slices were obtained by vibratome sectioning (Leica VT1200, Leica Biosystems) in chilled MEMp [50% (vol/vol) minimum essential medium (MEM), 25 mM HEPES, and 2 mM glutamine without antibiotics, adjusted to pH 7.2–7.3 with 1 M NaOH]. The slices were transferred onto semi-porous membrane inserts (Millipore, 0.4-µm pore diameter, Schwalbach, Germany). Intact slices were cultured at 37°C and 5% CO_2 in maintaining medium [neurobasal medium, 2% B27, 2 mM glutamine, 1% penicillin/streptomycin solution, 0.5 % Glucose, and 1mg/ml gentamycin]. The medium was changed every other day. The slices were infected after DIV 2 and cultured for an additional 12 days. The slices were fixed with 4% PFA at DIV 14.

Micro-CT

E13.5 Ebp1 WT and knockout embryo samples were immersed in Lugo's solution. We used the Siemens Inveon Micro-CT scanner, which is designed as an *in-vivo* system (Siemens Medical Solutions, Knoxville, USA). All samples were scanned with 1.5-mm aluminium filter, using the following settings: 180° total rotation and 600 rotation steps, 70 kV and 400 µA source setting, and 3,000 ms exposure time per step. Pixels were binned by 2, resulting in an effective pixel size or resolution of approximately 19.98 µm. For each scan, the dataset was reconstructed with a downsample factor of 1 using the Inveon Acquisition Workplace (IAW) software package (IAW, Siemens Medical Solutions, Knoxville, TN, USA), implementing the modified Feldkamp filtered back projection algorithm (Shepp-Logan filter). The reconstructed images were imported using the Inveon Research Workplace (IRW) into the accompanying two-dimensional (2D) and three-dimensional (3D) biomedical image analysis software package (IRW, CT Bone Visualization and Analysis, Siemens Medical Solutions, Knoxville, TN, USA) for visualization and analysis.

Methylation-specific PCR

Genomic DNA was extracted from *Ebp1*$^{(+/+)}$ and *Ebp1*$^{(-/-)}$ MEF cells using the Accuprep® Genomic DNA Extraction kit (Bioneer, Daejeon, Korea). After digestion with either MSPl or Hpall restriction enzymes using the EpiJET DNA Methylation Analysis kit (Thermo Scientific, Massachusetts, USA) the endogenous survivin promoter was amplified. The Hpall and MSPl restriction enzymes recognized sites containing CCGG sequence. The Hpall restriction enzyme was unable to cut methylated cytosine, while Mspl cut all CCGG sites. The survivin promoter on endogenous genomic DNA was amplified by using a survivin promoter-specific 5' primer (ACCGCAGCAGAAGGTACAACTC) and a 3' primer (AAGGGCCAGTTCTTGAAG). PCR parameters were as follows: 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s.

Genotype

The genomic DNA was prepared from the tail (Sigma Aldrich, Saint Louis, MO, USA) and was genotyped using PCR analysis. The primer pair was designed for the detection of the cre-mediated excision event.

Genotype primer sets

Primer name	Primer sequnce	Primer product size(bp)		
		Wild-type	recombined	Cre excised
Ebp1-F	ATTGATGGAGAGAAGACGATTATCCAGAACC	3205	6298	404
Ebp1-R	ACTTGTAAGCCCAGATAGCCCTTCAGTTG			
Cre-F	GCGGTCTGGCAGTAAAAACTATC	.	.	100
Cre-R	GTGAAACAGCATTGCTGTCACTT			

RNA microarray

The RNA microarray service was provided by Macrogen Inc.(Seoul, Republic of Korea). RNA purity and integrity were evaluated using a ND-1000 Spectrophotometer (NanoDrop, Wilmington, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). RNA

labeling and hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis Protocol (Agilent Technology, V 6.5, 2010). Briefly, 100 ng of total RNA from each sample was linearly amplified and labeled with Cy3-dCTP. The labeled cRNAs were purified by RNAeasy Mini kit (Qiagen). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured using NanoDrop ND-1000 (NanoDrop, Wilmington, USA). Each labeled cRNA (600 ng) were fragmented by adding 5 μL of 10× blocking agent and 1 μL of 25 × fragmentation buffer and heated at 60°C for 30 min. Finally, 25 μL 2× GE hybridization buffer was added to dilute the labeled cRNA. Hybridization solution (40 μL) was dispensed into the gasket slide and assembled for Agilent SurePrint G3 Mouse GE 8X60K, Microarrays (Agilent®). The slides were incubated for 17 h at 65°C in an Agilent hybridization oven and washed at room temperature by using the Agilent One-Color Microarray-Based Gene Expression Analysis Protocol (Agilent Technology, V 6.5, 2010). The hybridized array was immediately scanned using the Agilent Microarray Scanner D (Agilent Technologies, Inc.)

MBD sequencing

The MBD-seq service was provided by Macrogen Inc.(Seoul, Republic of Korea). Methylated DNA was obtained using the MethylMiner Methylated DNA Enrichment kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, fragmentation of 1 μg of genomic DNA was performed using adaptive focused acoustic technology (AFA; Covaris) and captured by MBD proteins. The methylated DNA was eluted in high-salt elution buffer. DNA in each eluted fraction was precipitated using glycogen, sodium acetate, and ethanol, and resuspended in DNase-free water. The eluted DNA was used to generate libraries following the standard protocols of TruSeq Nano DNA Library Prep kit (Illumina). The eluted DNA was repaired, an A was ligated to the 3' end, and TruSeq adapters were ligated to the fragments. Once ligation was assessed, the adapter-ligated product was PCR amplified. The final purified product was quantified using qPCR according

to the qPCR Quantification Protocol and qualified using Agilent Technologies 4200 TapeStation (Agilent technologies). We sequenced using the HiSeq™ 2500 platform (Illumina). Paired-end sequencing reads (101 bp) generated from MBD sequencing were verified using FastQC (version 0.10.0). Before starting the analysis, Trimmomatic (version 0.32) was used to remove any adapter sequences and bases with base qualities lower than 3 from the end reads. Using the sliding window trim method, bases that did not qualify for window size = 4 and mean quality = 15 were removed. Thereafter, reads with a minimum length of 36 bp were removed to produce clean data. The cleaned reads were aligned to the human genome (UCSC mm10) using Bowtie (version 1.1.2 parameter set -n 2 -m 1 -X 600), allowing up to 2 nucleotide mismatches to the reference genome per seed and returning only uniquely mapped reads. Mapped data (SAM file format) were performed sorting and indexing using SAMtools (version 0.1.19). PCR duplicates were removed using Picard Mark Duplicates (version 1.118). The analysis of the MBD data was performed using the MEDIPS package (version 1.16.0). For each sample, the aligned reads were extended in the sequencing direction to a length of 300 nt. The sequencing read coverage of the extended reads was calculated at genome-wide 250 bp window size. Subsequently, the resulting coverage profiles (read count, RPKM, and RMS) at each genomic bin were calculated. Each DMR was annotated using the table browser function of the UCSC genome browser. Annotation included gene structures, transcripts, promoter regions (defined as −2 kb upstream of the transcription start site), exons, introns, and CpG islands.

ChIP sequencing

The ChIP-seq service was provided by Macrogen Inc.(Seoul, Republic of Korea). ChIPed DNA was quantified using Quant-IT PicoGreen (Invitrogen) and qualified using the Agilent High Sensitivity DNA kit on an Agilent 2100 Bioanalyzer (Agilent Technologies). The sequencing libraries were prepared according to TruSeq ChIP Sample Preparation kit manufacturer's instructions (Illumina, Inc., San Diego, CA, USA). Briefly, 10 ng of ChIPed DNA was end-repaired to create 5'-phosphorylated,

blunt-ended dsDNA molecules. Following end-repair, DNA was size-selected using a bead-based method. These DNA fragments went through the addition of a single A base and ligation of the TruSeq indexing adapters. The products were purified and enriched with PCR to create the final DNA library. The libraries were quantified using qPCR according to the qPCR Quantification Protocol (KAPA Library Quantification kits for Illumina Sequencing platforms) and qualified using the TapeStation D1000 ScreenTape (Agilent Technologies, Waldbronn, Germany). Indexed libraries were sequenced using the HiSeq2500 platform (Illumina, San Diego, USA by the Macrogen Incorporated). Library preparation was carried out according to the TruSeq ChIPSeq method (Illumina). Paired-end sequencing reads (101 bp) generated using a HiSeq4000 instrument were verified for sequence quality using FastQC (version 0.10.0). Before starting the analysis, Trimmomatic (version 0.32) was used to remove adapter sequences and bases with base qualities lower than 3 from the end reads. Using a sliding window trim method, bases that did not qualify for window size = 4 and mean quality = 15 were removed. Thereafter, reads with a minimum length of 36 bp were removed to produce clean data.

Primer list

Reverse transcriptase polymerase chain reaction and qPCR			
GENE	**Primer sequnce**	**GENE**	**Primer sequnce**
Bcl2	F: AAG CTG TCA CAG AGG GGC TA	Kdm3a	F:CAGGTTGGAGCTGGAGACT
Bcl2	R: GAC GGT AGC GAC GAG AGA AG	Kdm3a	R: TCT GCA GTT CAG GAG TGG TG
Bax	F: TGC AGA GGA TGA TTG CTG AC	Prdm5	F: AAG GGC TTT GCT CAC AGA AA
Bax	R: GAT CAG CTC GGG CAC TTT AG	Prdm5	R: GCC TTA TTG CAC AGC TCA CA
Vegf A	F: CAG GCT GCT GTA ACG ATG AA	Dnmt1	F: ACG GAA ACC CAA GGA AGA GT
Vegf A	R: GCA TTC ACA TCT GCT GTG CT	Dnmt1	R: TTC CGG TCT TGC TTC TCT GT
Gata 4	F: GCA GCA GCA GTG AAG AGA TG	Suv39H1	F: CAG GTA GCT GTT GGC TGT GA
Gata 4	R: GCG ATG TCT GAG TGA CAG GA	Suv39H1	R: AGT GCG GAA GAT GCA GAG AT
Cd31	F: TGC AGG AGT CCT TCT CCA CT	Dot1	F: TGG CAA GCC TGT CTC CTA CT
Cd31	R: ACG GTT TGA TTC CAC TTT GC	Dot1	R: TGG CAG CAC TCA TTT TCT TG
Sox18	F: AAC AAA ATC CGG ATC TGC AC	Ezh	F: ATC TGA GAA GGG ACC GGT TT
Sox18	R: GGT AGG CTC CAG TTG CTC TG	Ezh	R: TCA GGG TCT TTA ACG GGA TG
Sox17	F: CTC GGG GAT GTA AAG GTG AA	Survivin	F: ATC GCC ACC TTC AAG AAC TG
Sox17	R: GCT TCT CTG CCA AGG TCA AC	Survivin	R: CAG GGG AGT GCT TTC TAT GC
Endoglin	F: CTT CCA AGG ACA GCC AAG AG	Sox18	F: AAA GAA TTC ATG CAG AGA TCG CCG CCC
Endoglin	R: GTG GTT GCC ATT CAA GTG TG	Sox18	R: AAA GGA TCC CTC CGC ACC CAG AGT GGG
Ebp1	F: AGA GCA TTT GAA GAT GAG	Ebp1	R: TCAGTCCCCAGCTTCATT
ChIP assay			
Primer	**Primer sequence**	**Primer**	**Primer sequence**
Dnmt1 (-500F)	CTG GCT TTT GCA TTC TGA	Dnmt1 (+200 R)	CAA ACG CTG CCC CGC GCA
Survivin (-966F)	ACGTGACAAAACCCCTCTTG	Survivin (-365R)	CAG GTT CTT CCT GCC TCA AG
Survivin (-364F)	CAG AGC ACA TGG GAC TTG CAG	Survivin (-115R)	TGC CTT CTG GGA GTG GAC
Survivin (-132F)	GTC CAC TCC CAG AAG GCA	Survivin (+79R)	AAG GGC CAG TTC TTG AAG

Supplementary Figures

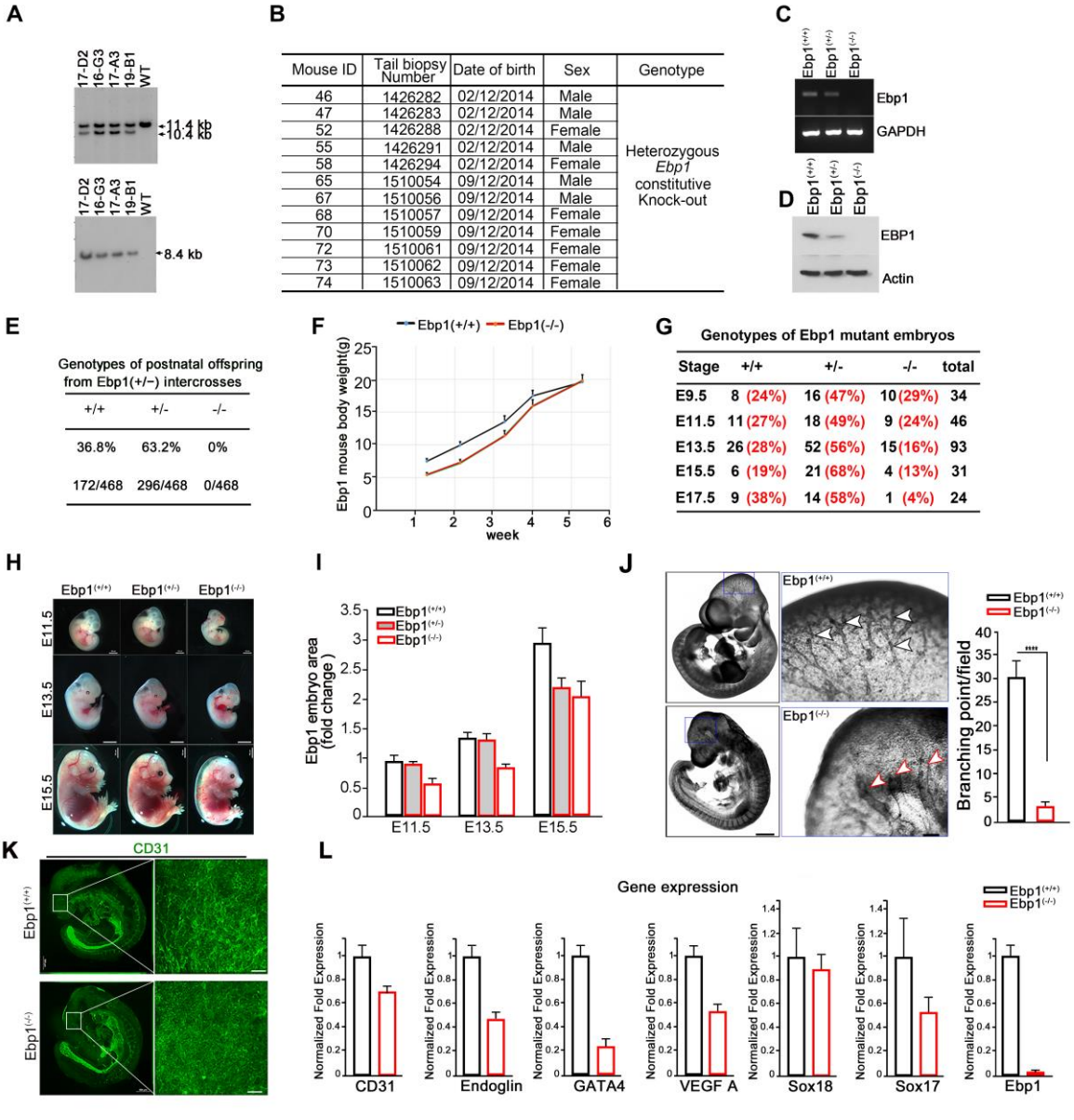
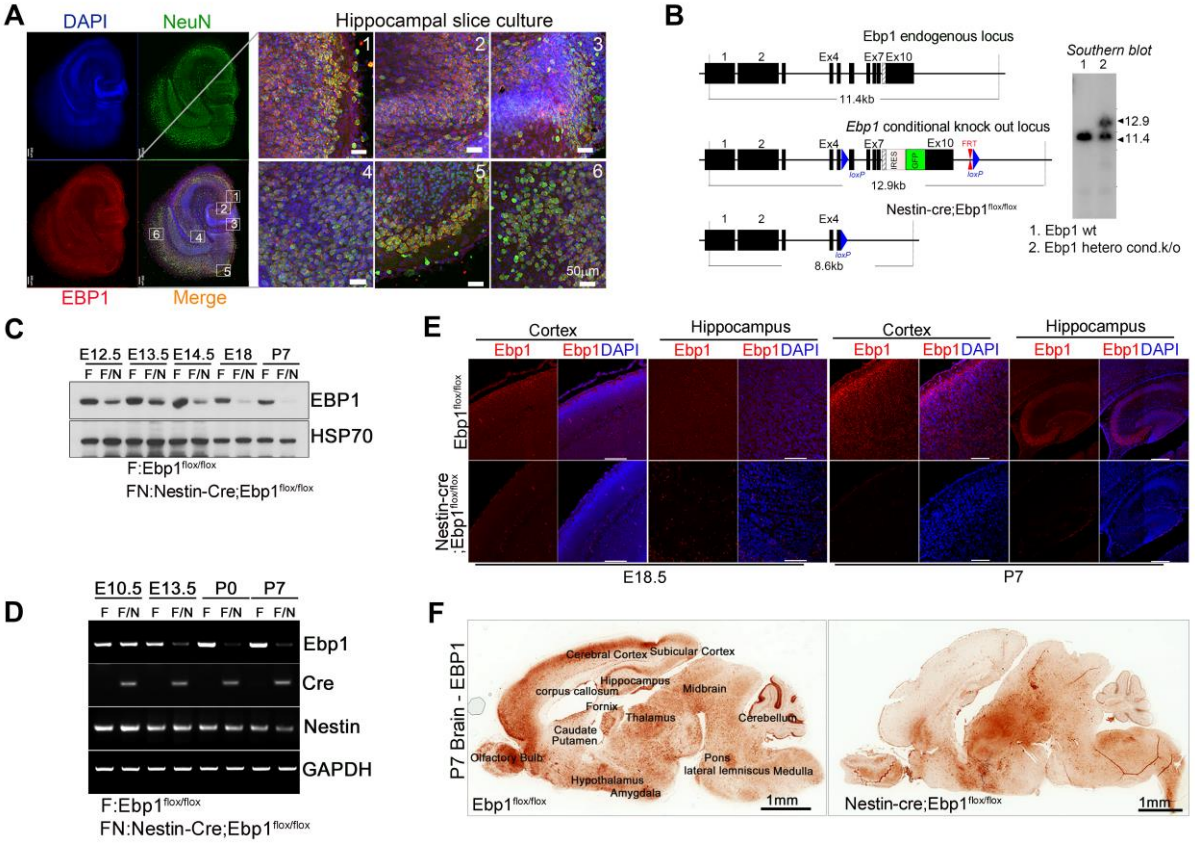


Fig. S1 Genetic ablation of *Ebp1* in mice.

(A) Generation of *Ebp1* knockout mice. Selected ES clones by PCR and Southern blot analysis for blastocyst injection. (B) Generation of heterozygous *Ebp1* constitutive knockout mice. (C and D) Ebp1 levels were checked in E13.5 mouse brains with the indicated genotypes using RT-PCR (C) and immunoblotting (D). The total RNA concentration was determined to GAPDH mRNA, and an equal amount of total proteins were detected using anti-Actin antibody. (E) Ebp1$^{(-/-)}$ mice exhibit embryonic

lethality (>468 pups analyzed). The ratio between wild-type and heterozygous mice was approximately1:1.7(36.8% and 63.2%, respectively). (F) Body weights in Ebp1 wild-type and hetero mice. Data shown are body weight over 6 weeks. (G) Calculation of the percentage of embryo genotypes at each time point. (H) Gross morphology of *Ebp1* wild-type ($Ebp1^{(+/+)}$), hetero ($Ebp1^{(+/-)}$), and knockout ($Ebp1^{(-/-)}$) mouse littermate from E11.5 to E15.5. Scale bar 500 μm. (I) Body area of embryonic *Ebp1* wild-type ($Ebp1^{(+/+)}$), hetero ($Ebp1^{+/-}$), and knockout ($Ebp1^{(-/-)}$) mouse from E11.5 to E13.5. (J) Whole-mount CD31 staining of E11.5 of $Ebp1^{(+/+)}$ and $Ebp1^{(-/-)}$ brain reveals comparable microvascular networks (left). Black arrows indicate the branching points and red arrows indicate blood vessels. Scale bar 100 μm. Quantification of branching point measurements from three independent experiments is shown on the right. Data are shown as mean ± SEM; ****$p < 0.0001$ versus the $Ebp1^{(+/+)}$. (K) Whole-mount CD31 staining of E11.5 of $Ebp1^{(+/+)}$ and $Ebp1^{(-/-)}$. The enlargement of the brain reveals microvascular networks. Scale bar 500 μm, Scale bar 50 μm (Magnification). (L) Quantitative RT-PCR analysis of angiogenesis-related genes from E13.5 $Ebp1^{(+/+)}$ or $Ebp1^{(-/-)}$ Yolk sacs. The mRNA levels of these genes were normalized to that of GAPDH. The relative fold changes were quantified and shown in the bar graphs.



239

240 **Fig. S2 Generation of [Nestin-Cre; *Ebp1*$^{flox/flox}$] mice.**

241 (A) The entorhinal cortex-hippocampus (EH) organotypic slices from P7 mice were cultured for 10
242 days and used for immunostaining. Immunofluorescence images were obtained after double-
243 immunostaining using anti-EBP1 (red) and NeuN (green). DAPI was used for nuclear staining. Scale
244 bar 50 μm. (B) Schematic representation of the targeting strategy for the generation of Ebp1
245 conditional knockout mice (left). The genomic DNA of the heterozygous conditional knockout tested
246 was compared to wild-type DNA by Southern blot analysis (right). (C) Mouse brains with indicated
247 genotypes were checked EBP1 levels by immunoblotting. (D) mRNA levels of Ebp1 were detected
248 using RT-PCR from E10.5 to P7. The total RNA concentration was determined to GAPDH mRNA. (E
249 and F) The paraffin-embedded section of the genotypes indicated was immunostained with anti-EBP1
250 antibody at E18.5, and postnatal 7 days (F).

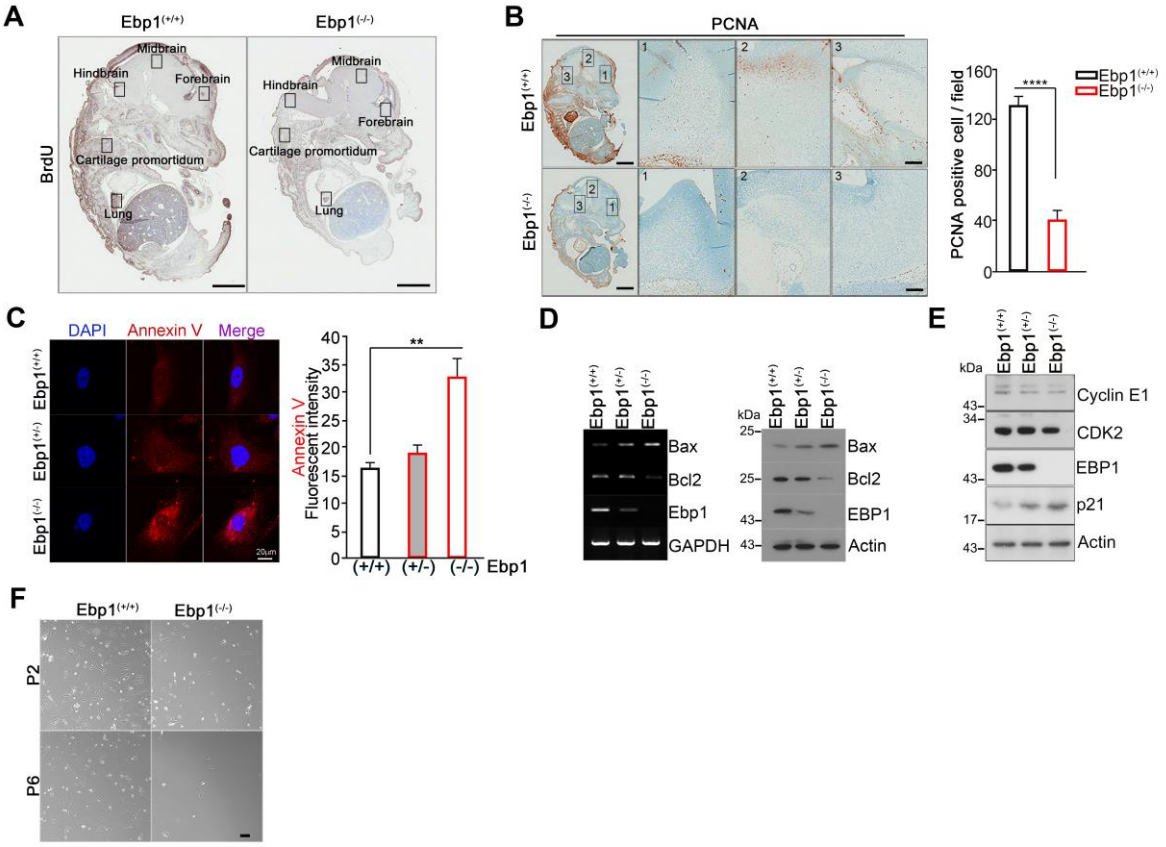


Fig. S3 Effect of *Ebp1* loss on apoptosis and cell proliferation.

(A) Paraffin section with indicated genotypes was stained with anti-BrdU antibody (brown). Scale bar 1 mm. (B) Paraffin section was stained with anti-PCNA antibody. Scale bar 1 mm, scale bar 100 μm (Enlargement). Quantification of PCNA positive cells is shown as a bar graph. Images shown here are representative of at least three independent experiments, and each value represents the mean ± SEM of triplicate measurements. *****$p < 0.00005$ versus wild-type. (C) MEF cells were stained with annexin V (red) and DAPI (blue). The bar graph shows annexin V fluorescence intensity. Data represent the mean ± SEM of three independent experiments. **p < 0.005 versus Ebp1 wild-type. (D) RNA and proteins were isolated from MEF cells (passage 2) of the genotypes indicated. The mRNA levels and protein levels of Bax and Bcl2 were determined by RT-PCR and immunoblot. The total

RNA concentration was determined to GAPDH mRNA, and an equal amount of total proteins were detected using anti-actin antibody. (E) Cell cycle-related protein expression was checked in MEF cells (passage 2) by immunoblotting. Actin served as the loading control. (F) The representative image shows *Ebp1*$^{(+/+)}$ and *Ebp1*$^{(-/-)}$ MEF cells at passage 2 and passage 6. Scale bar 100 µm.

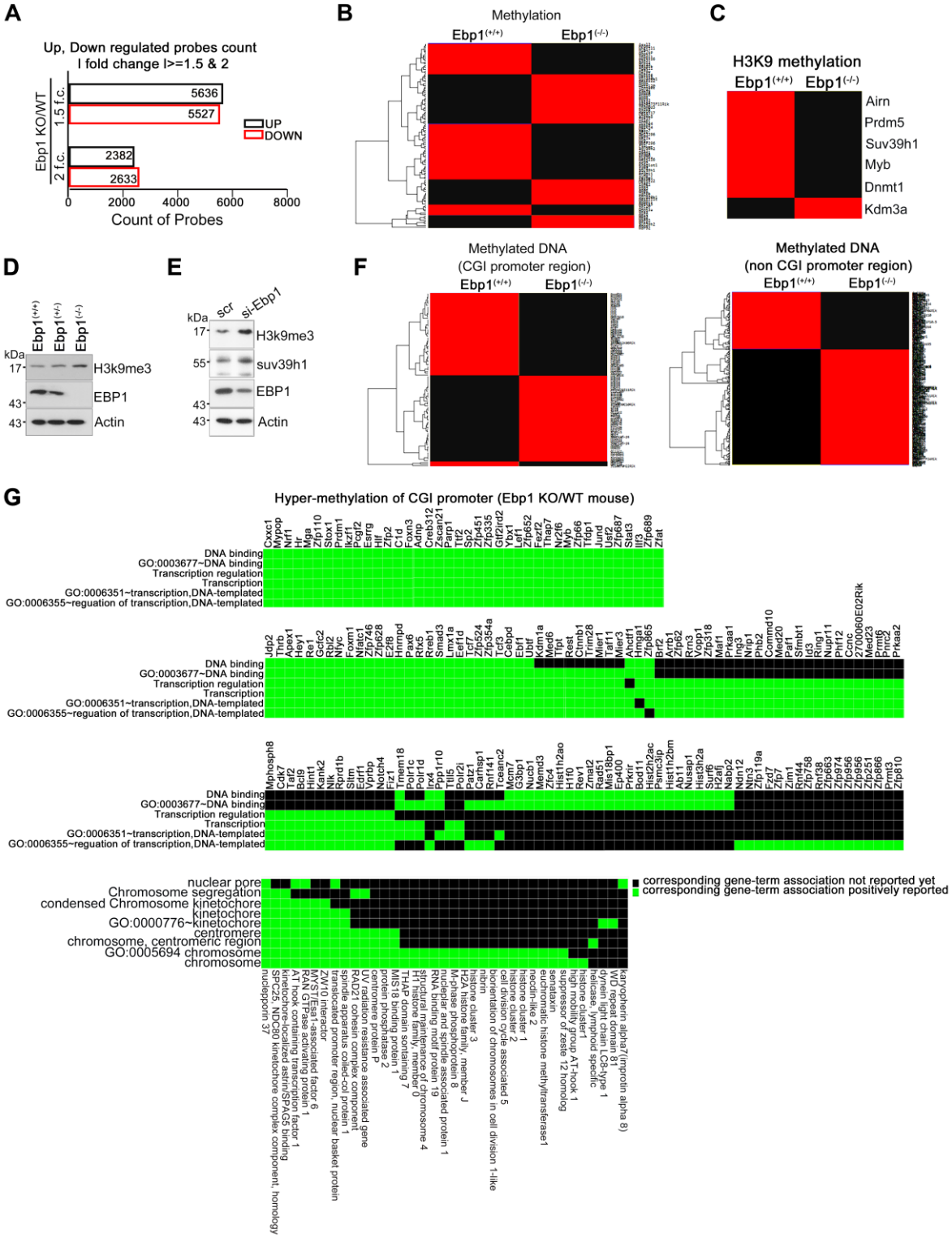
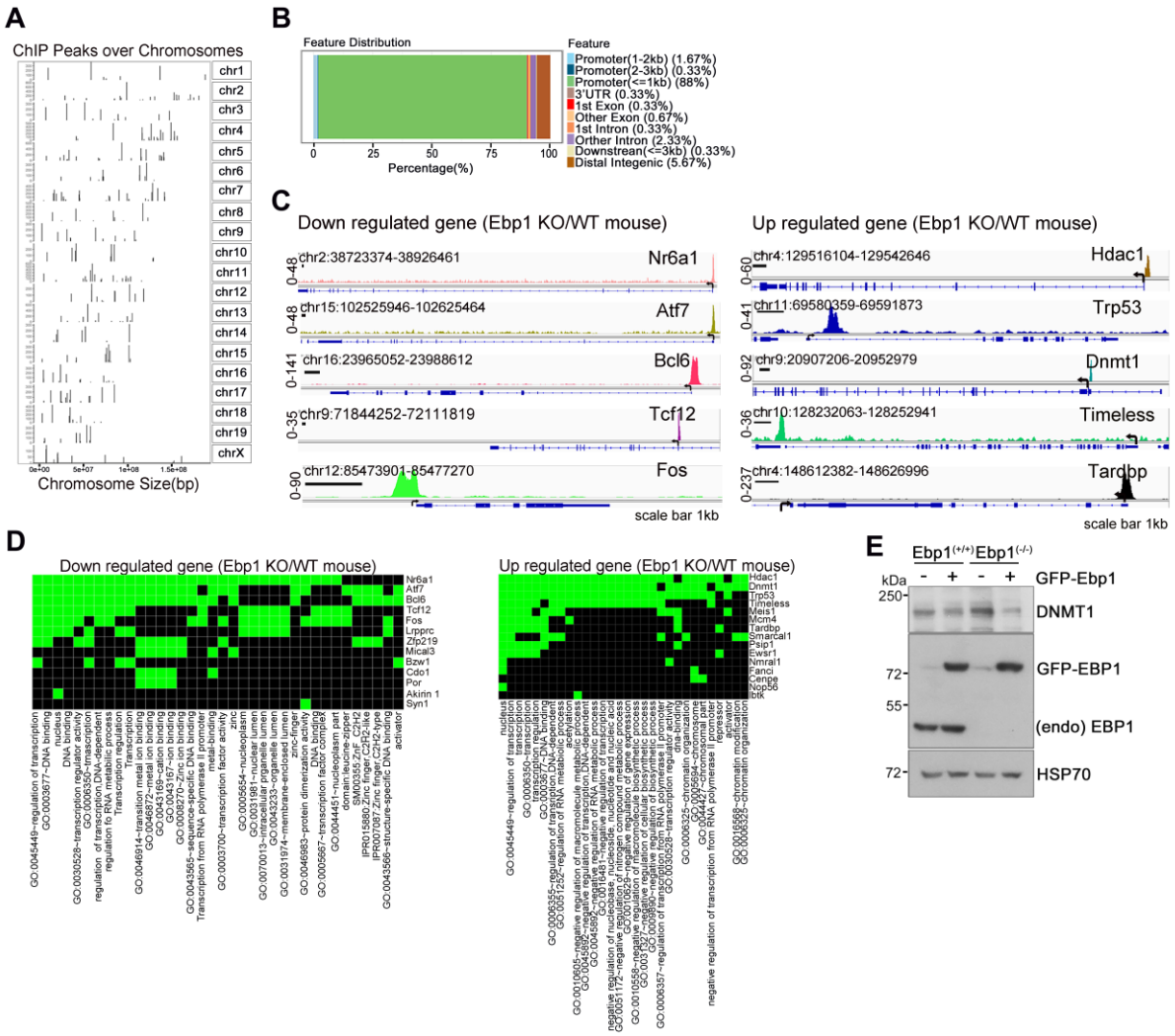


Fig. S4 Differential DNA methylation region in *Ebp1*$^{(+/+)}$ and *Ebp1*$^{(-/-)}$ mice.

(A) Microarray analysis was conducted by using mouse brain and displayed genes whose expression was ≥ 1.5 or 2.0. fold up- or down-regulated between *Ebp1*$^{(+/+)}$ and *Ebp1*$^{(-/-)}$ groups. (B) Heatmap is a representation of microarray data related to methylation between *Ebp1*$^{(+/+)}$ and *Ebp1*$^{(-/-)}$ groups. The values of the data are represented in color. Black indicates high expression, and red indicates low expression. (C) The heatmap shows the level of H3K9 trimethylation related genes between *Ebp1*$^{(+/+)}$ and *Ebp1*$^{(-/-)}$ groups. Black indicates high expression, and red indicates low expression. (D) MEF cell (passage 2) lysates were subjected to western blot. Methylation at histone H3 lysine 9 tri-methylation (H3K9me3) was detected anti-H3K9me3 antibody. Amount of total proteins were detected using anti-Actin antibody. (E) 293T cells were transfected with scramble RNA or si-Ebp1 RNA. Twenty-four hours after transfection, cell lysates were performed western blot. The indicated antibody detected protein levels. (F) MBD sequencing was performed using *Ebp1*$^{(+/+)}$ and *Ebp1*$^{(-/-)}$ embryonic brain (E15.5). The heatmap shows methylated DNA distribution in CGI promoter (left) and non-CGI promoter region (right) in *Ebp1*$^{(-/-)}$ / *Ebp1*$^{(+/+)}$ mouse. Block represents high methylation level, and red indicates low methylation level. (G) Functional GO-term annotation of hyper-methylated CGI promoter region in *Ebp1*$^{(-/-)}$. Gene lists derived from MBD-seq analysis of *Ebp1*$^{(-/-)}$ / *Ebp1*$^{(+/+)}$ mouse were applied to DAVID to identify functional ontological groups. The heatmaps are derived from the report generated by DAVID and are an annotated-term-focused view that lists annotated genes and their GO-term. Black color represents gene-term associations not reported yet in DAVID and green color represents gene-term associations positively reported in DAVID.



288

289 **Fig. S5 Analysis of EBP1-ChIP-seq data.**

290 (A and B) ChIP assay was performed with anti-EBP1 antibody using mouse embryo fibroblast
291 (passage 2). Coverage plot shows EBP1 chromosomal-binding site over the entire chromosome (A)
292 and genomic distribution of EBP1-binding sites visualized by ChIP seeker (B). See *SI Appendix*
293 Dataset 2. (C) MEF cells were used for ChIP-seq analysis with the anti-EBP1 antibody. Integrative
294 Genomics Viewer (IGV) track view of ChIP seq density profile for EBP1. (D) Functional GO-term
295 annotation of ChIP-seq data analysis. Gene lists derived from ChIP-seq analysis of *Ebp1* were applied
296 to DAVID to identify functional ontological groups. Black color represents gene-term associations not
297 reported yet in DAVID and green color represents gene-term association positively reported in

DAVID. (E) MEF with the indicated genotypes was transfected with control or GFP-Ebp1. DNMT1 expression levels were determined by immunoblotting. HSP70 served as the loading control.

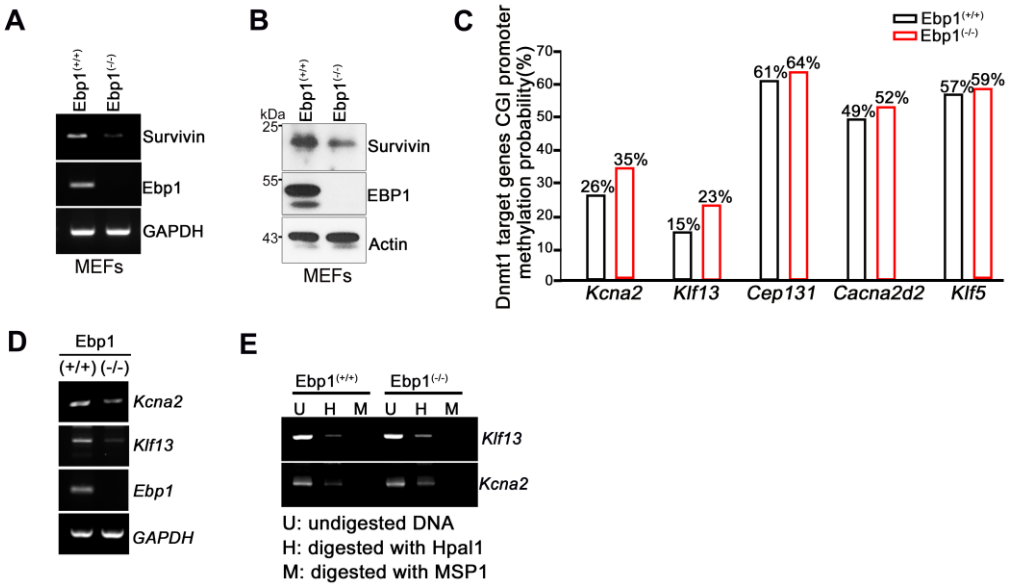


Fig. S6 EBP1 impairs DNMT1 mediated promoter methylation.

(A and B) RNA and proteins were isolated from MEF cells (passage 2) with the indicated genotypes. The mRNA levels (A) and protein levels (B) of Survivin and Ebp1 were determined by RT-PCR and immunoblot. The total RNA concentration was determined to GAPDH mRNA, and an equal amount of total proteins were detected using anti-actin antibody. (C) MBD sequencing was performed in E15.5 *Ebp1*$^{(+/+)}$ and *Ebp1*$^{(-/-)}$ embryonic brain. The bar graph shows the methylation probability of the Dnmt1 target genes CGI promoter region. See *SI Appendix* Dataset S3. (D) RNA was isolated from the brain with the indicated genotypes. The mRNA levels of Kcna2 and Klf13 were determined by RT-PCR. The total RNA concentration was determined to GAPDH mRNA. (E) Genomic DNA (gDNA) was extracted from *Ebp1*$^{(+/+)}$ and *Ebp1*$^{(-/-)}$ embryonic brain. The extracted gDNA was subjected to methylation-specific PCR (MSP). U: undigested DNA, H: digested with Hpal1, M: digested with MSP1.

Dataset S1. Correlation matrices showing Pearson's r between *Ebp1* and the other genes.

Correlation matrices are shown Pearson's r between *Ebp1* and the other genes (*i.e. Dnmt1, Suv39h1, Ezh2, Prdm5, and Dot1*) in the brain of the monkey (*Macaca Fascicularis*, n = 147), BXD mouse genetic reference population (n = 50) and B6D2-F2 intercross mouse population (n = 56), and in the hypothalamus of Mouse diversity panel (n = 17), with the depth of shading of correlogram according to the magnitude of the correlation and positive and negative correlations represented in blue and red, respectively.

Dataset S2. EBP1 ChIP-seq data.

ChIP assay was performed with anti-EBP1 antibody using mouse embryo fibroblast (passage 2).

Dataset S3. EBP1 MBD-seq data.

MBD sequencing was performed using $Ebp1^{(+/+)}$ and $Ebp1^{(-/-)}$ embryonic brain (E15.5).

Dataset S4. Scatterplots showing the negative correlation between *Ebp1* and *Dnmt1* expression.

Scatterplots are shown the negative correlation between *Ebp1* and *Dnmt1* expression in the brain of human (n = 147; Pearson's r = -0.46, $p < 0.0001$; Spearman's rho = -0.38, $p < 0.0001$), monkey (*Macaca Fascicularis*, n = 64; Pearson's r = -0.43, $p < 0.0001$; Spearman's rho = -0.47, $p < 0.0001$), BXD mouse genetic reference population (n = 50; Pearson's r = -0.30, $p < 0.033$; Spearman's rho = -0.31, $p < 0.026$), and B6D2-F2 intercross mouse population (n = 56; Pearson's r = -0.33, $p < 0.012$; Spearman's rho = -0.28, $p < 0.034$).