Cell Reports, Volume *22*

Supplemental Information

Tet2 Rescues Age-Related Regenerative Decline and Enhances Cognitive Function in the Adult Mouse Brain

Geraldine Gontier, Manasi Iyer, Jeremy M. Shea, Gregor Bieri, Elizabeth G. Wheatley, Miguel Ramalho-Santos, and Saul A. Villeda

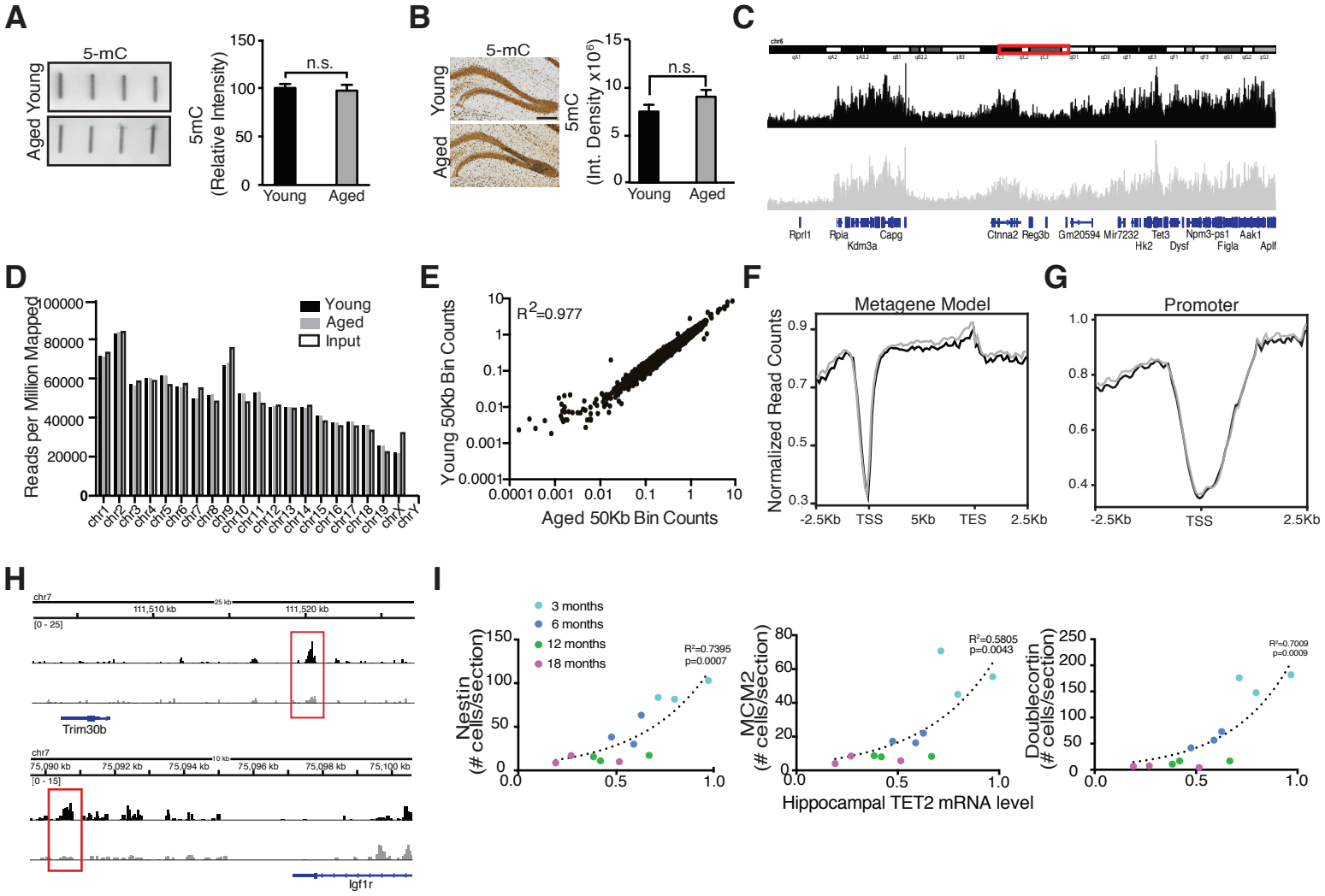

Figure S1. Characterization of 5mC levels, hMeDIP-sequencing, and adult neurogenesis in the young and aged hippocampus. Related to Figure 1.

A, Representative slot-blot and quantification of isolated hippocampal DNA probed with anti-5mc antibodies from young (3 months) and aged (18 months) mice. (n=4 per group; t-test; n.s. not significant)

B, Representative field and quantification of 5mc expression in the dentate gyrus (DG) of the young and aged hippocampus. (n=4 per group; scale bar, 100μm; t-test; n.s. not significant)

C, IGV Browser track of a 20mB region of chromosome 6. At the top is an ideogram of chromosome 6 with highlighted region in red box. Normalized read counts from young (3 months, black) and aged (18 months, grey) hippocampi from this region show increased reads over genes (signified at the bottom).

D, Chromosome coverage of hMeDIP-Seq reads from young, aged, and input samples normalized to reads per million mapped.

E, Scatterplot of young versus aged hippocampi using 50kb bins. Reads are normalized as FPKM.

F, Metagene read coverage in FPKM for RefSeq genes. The gene body was scaled to 5kb for all genes. 2.5kb upstream of the TSS and 2.5kb downstream of the TES are shown.

G, Read coverage in FPKM for RefSeq promoters. 2.5kb upstream and downstream of the TSS is shown.

H, Two examples of lost aging DhMRs that are proximal to Trim30b and Igf1r. Young (3 months, black) and aged (18 months, grey) samples are shown.

I, Temporal relationship between levels of adult neurogenesis and Tet2 expression in contralateral hippocampi with age (3, 6, 12, and 18 months). Quantification of Nestin-positive, MCM2-positive, and Doublecortin (DCX)-positive cells by immunohistochemistry was correlated with Tet2 mRNA levels assessed by quantitative reverse-transcription PCR. (n=3 per group)

Data are represented as mean±SEM.

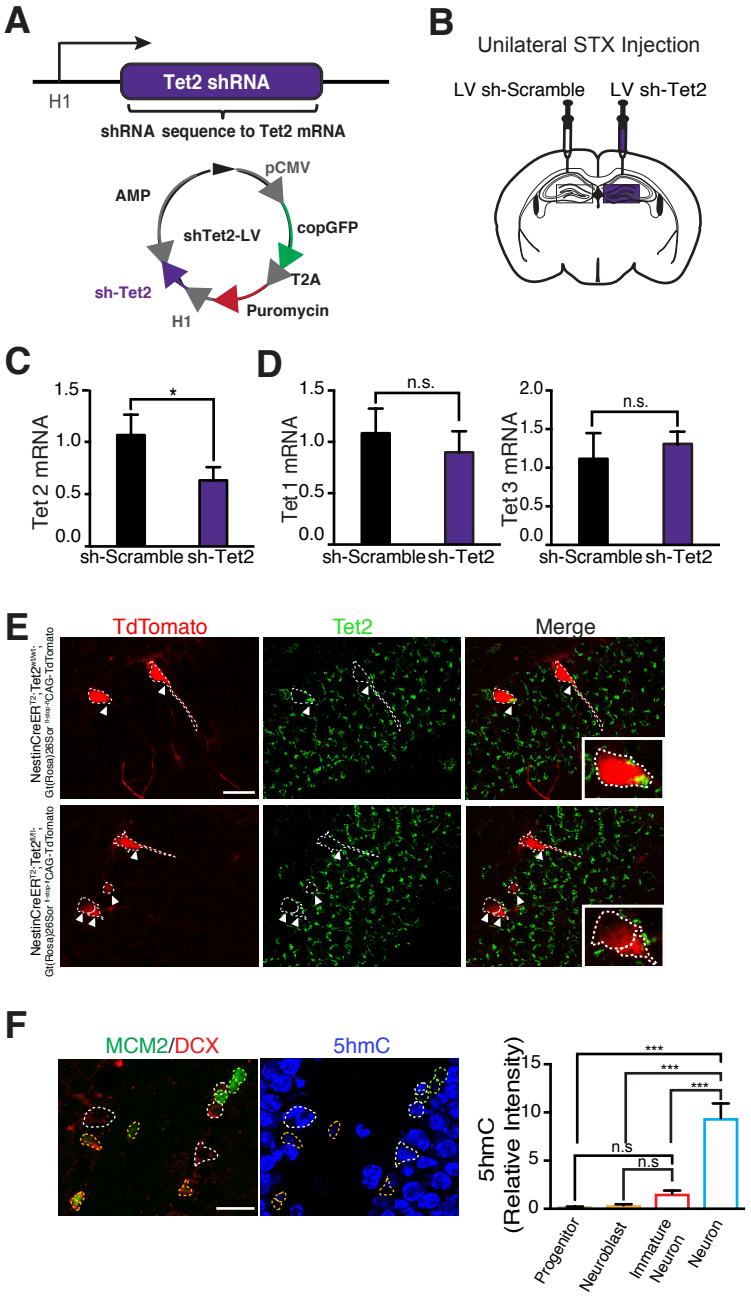

Figure S2. Abrogation of Tet2 by lentiviral-mediated shRNA approach and 5hmC expression during neuronal differentiation. Related to Figure 2.

A,B, Young adult (3 months) mice were given unilateral stereotaxic (STX) injections of lentivirus (LV) encoding either shRNA targeting Tet2 or scramble control sequences in tandem with a green florescent protein (GFP) reporter into contralateral dentate gyrus (DG). Schematic of lentiviral vector generated to express small hairpin RNAs (shRNA) targeting Tet2. Abbreviations: AMP, ampicillin, pCMV, cytomegalovirus promoter (A). Schematic illustrating unilateral STX injection paradigm into the DG (B).

C,D, Quantitative reverse-transcription PCR of Tet1, Tet2, and Tet3 mRNA from hippocampal lysates following STX injection. (n=5 per group; t-test; *P< 0.05, n.s. not significant)

E, A cell type specific temporally controlled Tet2wt/wt/NestinCre-ERT2/Gt(Rosa)26Sorflox-stop-floxCAG-TdTomato or Tet2flox/flox/Nestin-Cre-ERT2/Gt(Rosa)26Sorflox-stop-floxCAG-TdTomato genetic model was generated, in which TdTomato is expressed in adult neural stem/progenitor cells (NPCs) and Tet2flox/flox excised upon tamoxifen administration. Representative field of TdTomato-positive cells and Tet2 expression by immunostaining and confocal microscopy in the young adult (3 months) hippocampus (scale bar, 20μm).

F, Representative field and quantification of 5hmC expression in neural progenitors (MCM2+/DCX-), neuroblasts (MCM2+DCX+), immature neurons (MCM2-DCX+) and mature neurons by immunostaining and confocal microscopy in the young adult (3 months) hippocampus. (n=4; scale bar, 20μm; ANOVA with Dunnett's post hoc test; ***P<0.001, n.s. not significant)

Data are represented as mean±SEM.

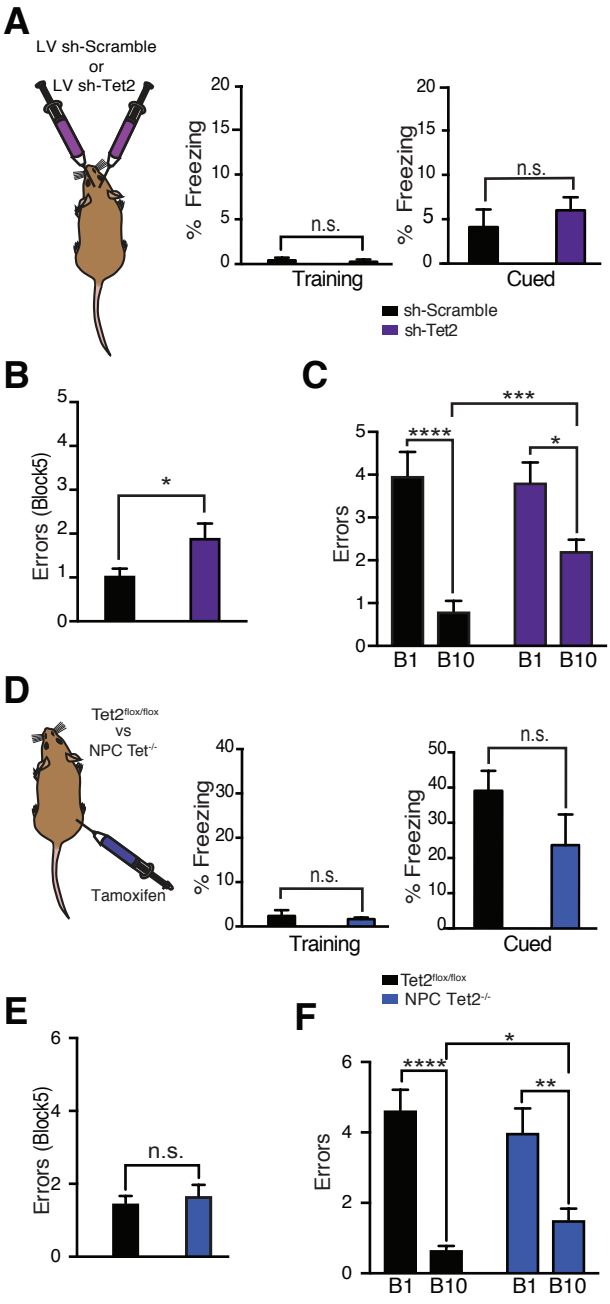

Figure S3: Abrogation of Tet2 in the adult dentate gyrus, but not loss of Tet2 in adult NPCs, selectively impairs short-term hippocampal-dependent spatial memory. Related to Figure 3.

A, Young adult (3 months) wild type mice were given bilateral stereotaxic injections of lentivirus (LV) encoding either shRNA targeting Tet2 (sh-Tet2) or scramble control sequences (sh-Scramble) into the dentate gyrus (DG). Cued fear memory was assessed in sh-Tet2 and sh-Scramble control injected mice using contextual fear conditioning. Quantification of percent freezing 24 hours after training. (n=14 per group; t-test; n.s not significant; t-test)

B,C, Hippocampal-dependent spatial learning and memory was assessed in sh-Tet2 and sh-Scramble control injected mice using RAWM. Quantification of the number of entry errors during RAWM short-term (B) and long-term (C) learning and memory testing. (n=14 per group; t-test; *P<0.05, ***P< 0.001, ****P< 0.0001)

D, Young adult Tet2flox/flox/NestinCre-ERT2 NPC-specific knockout (Tet2-/-) or littermate control (Tet2flox/flox) mice were administered tamoxifen. Cued fear memory was assessed in Tet2-/- and Tet2flox/flox control mice using contextual fear conditioning. Quantification of percent freezing 24 hours after training. (n=9-10 per group; t-test; n.s not significant)

E,F, Hippocampal-dependent spatial learning and memory was assessed in in Tet2-/- and Tet2flox/flox control mice using RAWM. Quantification of the number of entry errors during RAWM short-term (E) and long-term (F) learning and memory testing. (n=9-10 per group; t-test; *P<0.05, **P<0.01, ****P<0.0001, n.s not significant)

Data are represented as mean±SEM

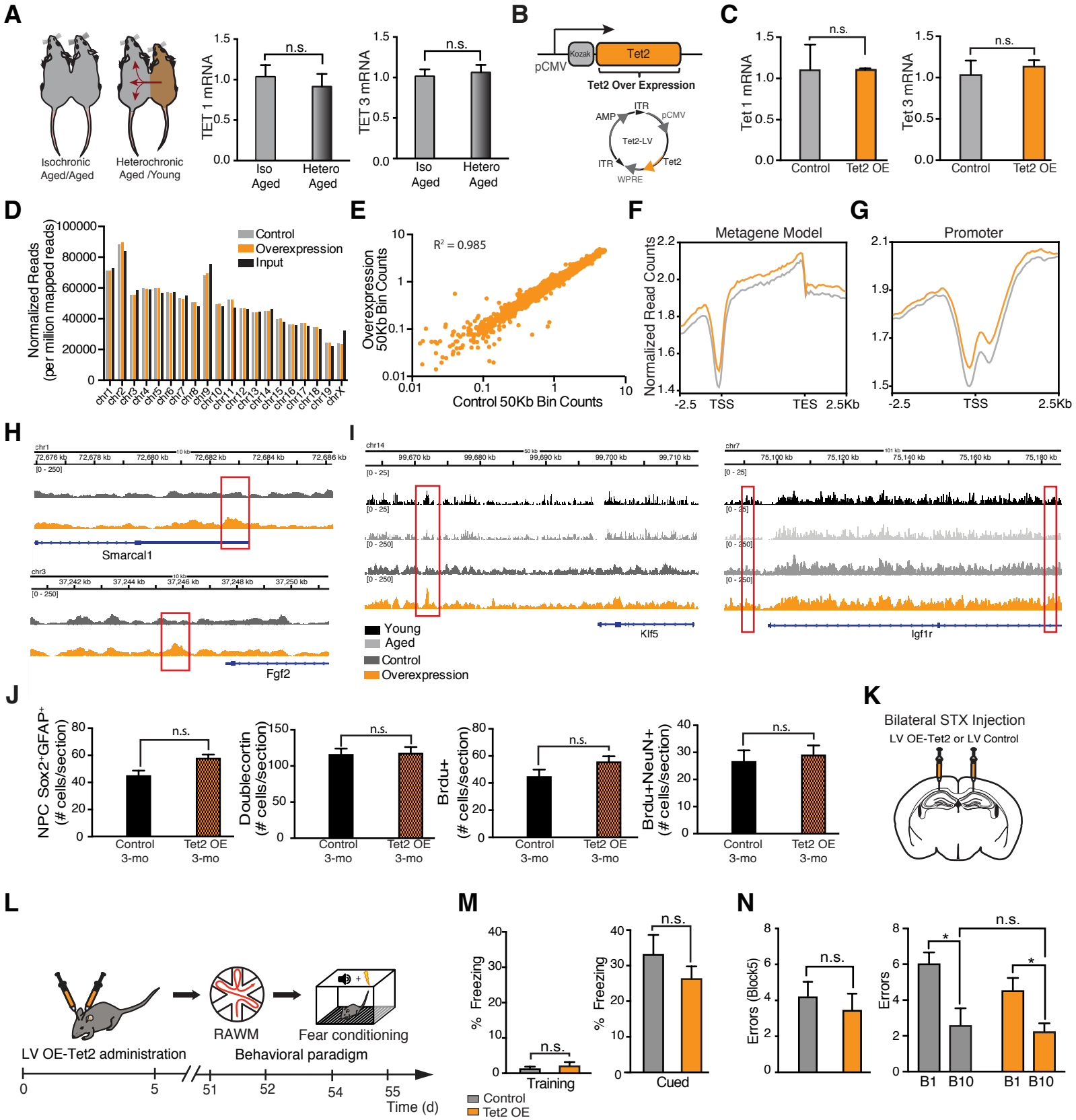

Figure S4. Characterization of hMeDIP-sequencing, adult neurogenesis, and cognitive function in the mature hippocampus following Tet2 overexpression. Related to Figure 4.

A, Schematic and quantitative reverse-transcription PCR of Tet2 mRNA from hippocampal lysates of aged (18 months) isochronic and heterochronic parabionts five weeks after parabiosis. (n= 5 per group; t-test; n.s not significant)

B,C, Mature adult (6 months) mice were given unilateral stereotaxic (STX) injections of lentivirus (LV) encoding Tet2 or control LV into contralateral dentate gyrus (DG). Schematic of lentiviral vector generated to overexpress Tet2. Abbreviations: AMP, ampicillin, pCMV, cytomegalovirus promoter (**B**). Quantitative reverse-transcription PCR of Tet1 and Tet3 mRNA from hippocampal lysates following STX injection (**C**). (n=5 per group; t-test; n.s. not significant)

D, Chromosome coverage of hMeDIP-Seq reads from control (dark grey), overexpression (orange), and input samples (black) normalized to reads per million mapped.
E, Scatterplot of control versus overexpression hippocampi using 50kb bins. Reads are normalized as FPKM.
F, Metagene read coverage in FPKM for RefSeq genes of control (dark grey) and Tet2 overexpression (orange). The gene body was scaled to 5kb for all genes. 2.5kb upstream of the TSS and 2.5kb downstream of the TES are shown.
G, Read coverage in FPKM for RefSeq promoters. 2.5kb upstream and downstream of the TSS is shown.
H, Two examples of DhMRs that were gained after Tet2 overexpression.
I, Two examples of genes that were associated with DhMRs that were lost with aging and gained after Tet2 overexpression are shown. In the example near Klf5, the DhMR is shared, while the example with Igf1r has two associated DhMRs. Examples of young (3 month, black), aged (18 month, grey), control (dark grey), and Tet2 overexpression (orange) samples are shown.
J, Young adult (3 months) mice were given unilateral stereotaxic (STX) injections of lentivirus (LV) encoding Tet2 or control LV into contralateral dentate gyrus (DG). Neurogenesis was analyzed by immunostaining and confocal microscopy after LV administration. All mice were administered BrdU by intraperitoneal injections for six days and euthanized 30 days later. Quantification of GFAP/Sox2-positive, Dcx-positive, BrdU-positive, and NeuN/BrdU-positive cells. (n=4 per group; t-test; n.s. not significant)
K,L, Schematic of experimental paradigm (**K**) and cognitive testing timeline (**L**). Mature adult (6 months) wild type mice were given bilateral stereotaxic (STX) injections of lentivirus (LV) encoding Tet2 or control LV into the dentate gyrus (DG). Hippocampal-dependent learning and memory was assessed by radial arm water maze (RAWM) and contextual fear conditioning paradigms.
M, Cued fear memory was assessed in Tet2 and control injected mice using contextual fear conditioning. Quantification of percent freezing 24 hours after training. (n=12-15 per group; t-test; ***P< 0.001)
N, Hippocampal-dependent spatial learning and memory was assessed in Tet2 and control injected mice using RAWM. Quantification of the number of entry errors during RAWM short-term and long-term learning and memory testing. (n=6-9 per group; t-test; *P<0.05, n.s not significant)
Data are represented as mean±SEM.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animal models. The following mouse lines were used: C57BL/6 (The Jackson Laboratory), C57BL/6 aged mice (The Jackson Laboratory), *Tet2*$^{flox/flox}$ (The Jackson Laboratory; stock number: 017573) mice, Gt(Rosa)26Sor$^{flox\text{-}stop\text{-}flox}$CAG-TdTomato (The Jackson Laboratory; stock number: 007905), and *NestinCreER*^{T2} mice (The Jackson Laboratory; stock number: 016261). All mice used were on a C57BL/6 genetic background. Mice carrying a *Tet2*$^{flox/lox}$ gene were crossed with mice carrying an inducible *NestinCre-ERT*^{2} gene to obtain *Tet2*$^{flox/flox}$/*NestinCreER*$^{T2+/0}$ mice. A subset of *Tet2*$^{flox/flox}$/*NestinCreER*$^{T2+/0}$ mice were crossed with Gt(Rosa)26Sor$^{flox\text{-}stop\text{-}flox}$CAG-TdTomato mice to obtain *Tet2*$^{flox/flox}$/*NestinCreER*$^{T2+/0}$/Gt(Rosa)26Sor$^{flox\text{-}stop\text{-}flox}$CAG-TdTomato. All studies were done in male mice. The numbers of mice used to result in statistically significant differences was calculated using standard power calculations with $\alpha = 0.05$ and a power of 0.8. We used an online tool (http://www.stat.uiowa.edu/~rlenth/Power/index.html) to calculate power and sample size on the basis of experience with the respective tests, variability of the assays and inter-individual differences within groups. Mice were housed under specific pathogen-free conditions under a 12-h light-dark cycle and all animal handling and use was in accordance with institutional guidelines approved by the University of California San Francisco Institutional Animal Care and Use Committee (IACUC) and the VA Palo Alto Committee on Animal Research.

PCR genotyping. Tet2 floxed and NestinCreER^{T2} alleles were genotyped from skin biopsies using PCR with Tet2 primers and NestinCreER^{T2} primers. Primers specific for the myogenin gene were included in the reaction as a control.

Primer Set	Forward Primer	Reverse Primer
Tet2 Floxed	AAGAATTGCTACAGGCCTGC	TTCTTTAGCCCTTGCTGAGC
Cre	GAACCTGATGGACATGTTCAGG	AGTGCGTTCGAACGCTAGAGCCTGT
Myogenin	TTACGTCCATCGTGGACAGC	TGGGCTGGGTGTTAGCCTTA

Tamoxifen administration. All experimental genetic mouse models (*Tet2*$^{flox/flox}$ control and *Tet2*$^{flox/flox}$/*NestinCreER*$^{T2+/0}$ mutant mice) received tamoxifen. At 2 months mice were administered tamoxifen to induce *Tet2* excision specifically in adult neural stem/progenitor cells termed NPC *Tet2*$^{-/-}$. Tamoxifen (T5648, Sigma-Aldrich) was dissolved in sunflower seed oil/ethanol (10:1) at 30 mg ml^{-1}, and was administered intraperitoneally at 180 mg kg^{-1} body weight once per day for 5 days. Animals were euthanized 2 months after the last injection.

RNA extraction, cDNA synthesis and qPCR. Total RNA was isolated using column from the RNeasy kit (QIAGEN, cat#74104). To quantify Tet2 mRNA expression levels, equal amounts of cDNA were synthesized using the High-Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific, Cat# 4374966) and mixed with the Fast Taqman master mix (ThermoFisher Scientific), or the SYBR Fast mix (Kapa Biosystems), and Tet2 primers. GAPDH mRNA was amplified as an internal control. Quantitative RT-PCR was carried out in a CFX384 Real Time System (Bio-Rad).

Primer Set	reference	Forward Primer	Reverse Primer
Gapdh		AACAGCAACTCCCACTCTTC	CCTGTTGCTGTAGCCGTATT
Tet2		AGTAGGACTGAGAAGGGAAAGT	CGGTTGTGCTGTCATTTGTTT
Igf1r		GCCAGAAGTGGAGCAGAATAA	ACTTGTTGGCATTGAGGTAGG
Ptpn5		CGAGGAGATGAACGAGAAGTG	TCCGTGTGGATGACTTTCTG
Zfp521		AGTCCGATGAGAAGAAGACCTA	CTCATGGTTCAGCCCTTCAT
Dscam		GACACAGACCGAGCAAGAAG	TCCATCTTGGCGTGTTCATAG
Lrrc4c		GTCAAGACCGTGCATTCTTTC	TCTGGAGTTCAAACAGTTGTATTTC
Sorl1		CCTGACCAGGACTTGTTCTAC	AGCTTGCTTAGGCTGTACTC
Park2		CGATGCTCAACTTGGCTACT	TGGTACCTAGTGTACTGCTCTT
Cdk6		CTTCTGAAATGCCTGACGTTTAAT	GAGTTCAGGTTGTCCTTGTATCT
Sipa1l1		TCTGGACCTAGGACTTTCTACC	CACTCTGATGTCGCTTTCGT
Tet1	Mm01169087_m1		
Tet2	Mm00524395_m1		
Tet3	Mm00805756_m1		
Gapdh	Mm99999915_g1		

Slot blot analysis. Genomic DNA samples were prepared in TE buffer and then denatured in 0.0.5 M NaOH/12,5 mM EDTA at 95 °C for 10 min and followed by adding an equal volume of cold 2 M ammonium acetate (pH 7.2).

Denatured DNA samples were spotted on a charged nitrocellulose membrane in an assembled in the slot blot apparatus according to the manufacturer's instructions. The membrane was washed with TE buffer and ultraviolet-crosslinked for 3 min. Then the membrane was blocked with 1X PBS-0.1% Tween-20 (TBST) + 5% milk for 30 minutes at room temperature and incubated with rabbit anti-5hmC (1:500, Active Motif, Cat#39769) or mouse anti-5mC (1:500, Epigentek, Cat# A-1014-100) for HRP-conjugated secondary antibodies and enhanced chemiluminescence detection. Bands were analyzed using ChemiDoc (Bio-Rad) and quantified with ImageJ software analysis.

Viral plasmids. We generated lentiviruses that express shRNA sequences targeting Tet2 mRNA or full length Tet2. We used a lentiviral shRNA expression system (pGreenPuro shRNA, System Biosciences) according to the manufacturer's instructions. Tet2 shRNA sequences (Tet2, 5′- GTCTGAATCCATCTGTACATA-3′) were subcloned into the pGreenPuro vector. Scramble shRNA sequences (scramble, 5'- GGACGAACCTGCTGAGATAT-3') were subcloned as a control using the same protocol. Plasmid quality was tested with real time PCR analysis and sequencing. We generated lentiviral plasmids expressing full length Tet2 under a cytomegalovirus (CMV) promoter. Tet2 sequence was isolated using the Phusion Hot Start Flex DNA Polymerase (NEB,) using the following PCR primers: 5′- GAGGAGCAGAAGGAAGCAAGA-3′ and 5′- TGCCCTTGCATAGGATGCTC-3′, and subsequently subcloned into the pTB CMV plasmid using the restriction enzymes NheI and AscI.

Lentiviruses production. 293T cells were lipotransfected with 4:3:1 ug of lentiviral vector:psPax2:pCMV-VSVG. After 48 hours lentivirus-containing media was centrifuged and filtered to remove cellular debris. Media underwent utracentrifugation to concentrate virus. Lentiviral titers were between 2.5×10^7 and 5×10^8 viral particles per ml.

Stereotaxic injections. Animals were placed in a stereotaxic frame and anesthetized with 2% isoflurane (2 liters/min oxygen flow rate) delivered through an anesthesia nose cone. Ophthalmic eye ointment (Puralube Vet Ointment, Dechra) was applied to the cornea to prevent desiccation during surgery. The area around the incision was trimmed. Solutions were injected bilaterally into the DG of the dorsal hippocampi using the following coordinates: (from bregma) anterior = −2 mm, lateral = 1.5 mm, (from skull surface) height = −2.1 mm. A 2-µl volume was injected stereotaxically over 10 min (injection speed: 0.20 µl/min) using a 5-µl 26s-gauge Hamilton syringe. To limit reflux along the injection track, the needle was maintained *in situ* for 10 min, slowly pulled out halfway and kept in position for an additional 5 min. The skin was closed using silk suture. Each mouse was injected subcutaneously with analgesics. Mice were singly housed and monitored during recovery.

Parabiosis. Parabiosis surgery followed previously described procedures (Smith et al., 2015). Mirror-image incisions at the left and right flanks were made through the skin, and shorter incisions were made through the abdominal wall. The peritoneal openings of the adjacent parabionts were sutured together. Elbow and knee joints from each parabiont were sutured together and the skin of each mouse was stapled (9-mm Autoclip, Clay Adams) to the skin of the adjacent parabiont. Each mouse was injected subcutaneously with Baytril antibiotic and Buprenex as directed for pain and monitored during recovery. For overall health and maintenance behavior, several recovery characteristics were analyzed at various times after surgery, including paired weights and grooming behavior.

BrdU administration. For long-term BrdU labeling 50 mg/kg of BrdU (Sigma-Aldrich, Cat# B5002) was injected into mice once a day for 6 days and animals were euthanized 30 days after first administration.

Immunohistochemical Analysis. Tissue processing and immunohistochemistry was performed on free-floating sections according to standard published techniques (Smith et al., 2015). Briefly, mice were anesthetized with 87.5 mg/kg ketamine and 12.5 mg/mg xylazine and transcardially perfused with phosphate buffer saline. Brains were removed and fixed in phosphate-buffered 4% paraformaldehyde, pH 7.4, at 4 °C for 48 h before they were sunk through 30% sucrose for cryoprotection. Brains were then sectioned coronally at 40 µm with a cryomicrotome (Leica Camera, Inc.) and stored in cryoprotective medium. Primary antibodies were: goat anti-DCX (1:7500; Santa Cruz Biotechnology; sc-8066), rat anti-BrdU (1:1000, Abcam, AB6326), mouse anti-Nestin (1:200; Millipore; MAB353), mouse anti-MCM2 (1:250, BD Biosciences; 610700), mouse anti-NeuN (1:1000, Millipore, MAB377), rabbit anti-GFAP (1:1000; DAKO; Z0334), goat anti-Sox2 (1:200, Santa Cruz Biotechnology, sc17320), rabbit anti-GFP (1:10,000, Thermoscientific, PA5-22688), rabbit anti-5hmC (1:500, Active Motif, 39769), mouse anti-5mC (1:500, Epigentek, A-1014-100), and rabbit anti-Tet2 (1:500, Proteintech, 21207-1-AP). After overnight incubation, primary antibody staining was revealed using biotinylated secondary antibodies (Vector) and the ABC kit (Vector) with DAB (Sigma-Aldrich) or fluorescence-conjugated secondary antibodies (Life Technologies). For BrdU labeling, brain sections were pre-treated with 2 N HCl at 37 °C for 30 min and washed three times with Tris-buffered saline with

Tween (TBST) before incubation with primary antibody. For Nestin, MCM2, 5mC, 5hmC labeling, brain sections were pre-treated three times with 0.1M Citrate at 95 °C for 5 min, tree times and washed three times with TBST before incubation with primary antibody. To determine the number of mature differentiated neurons generated in the DG following first BrdU injection, we co-stained BrdU and the neuronal marker NeuN. All cells were counted in the DG of every sixth coronal hemibrain section through the hippocampus and analyzed by confocal microscopy using a Zeiss LSM800 microscope.

Contextual fear conditioning. In this task, mice learned to associate the environmental context (fear conditioning chamber) with an aversive stimulus (mild foot shock; unconditioned stimulus, US) enabling testing for hippocampal-dependent contextual fear conditioning. To also assess amygdala-dependent cued fear conditioning, the mild foot shock was paired with a light and tone cue (conditioned stimulus, CS). Conditioned fear was displayed as freezing behavior. Specific training parameters are as follows: tone duration is 30 seconds; level is 70 dB, 2 kHz; shock duration is 2 seconds; intensity is 0.6 mA. This intensity is not painful and can easily be tolerated but will generate an unpleasant feeling. More specifically, on day 1 each mouse was placed in a fear-conditioning chamber and allowed to explore for 2 min before delivery of a 30-second tone (70 dB) ending with a 2-second foot shock (0.6 mA). Two minutes later, a second CS-US pair was delivered. On day 2, each mouse was first placed in the fear-conditioning chamber containing the same exact context, but with no CS or foot shock. Freezing was analyzed for 1–2 minutes. One hour later, the mice were placed in a new context containing a different odor, cleaning solution, floor texture, chamber walls and shape. Animals were allowed to explore for 2 minutes before being re-exposed to the CS. Freezing was analyzed for 1–3 minutes using a FreezeScan video tracking system and software (Cleversys, Inc).

Radial arm water maze. Spatial learning and memory was assessed using the radial arm water maze (RAWM) paradigm according to the protocol described by (Alamed et al., 2006). In this task the goal arm location containing a platform remains constant throughout the training and testing phase, while the start arm is changed during each trial. Entry into an incorrect arm is scored as an error, and errors are averaged over training blocks (three consecutive trials). On day 1 during the training phase, mice are trained for 15 trails, with trials alternating between a visible and hidden platform for blocks 1-4 and then switching to only a hidden platform in block 5. On day 2 during the testing phase, mice are tested for 15 trials with a hidden platform for block 6-10. Investigators were blinded to genotype and treatment when scoring.

hMeDIP-Seq Library Preparation. For each library, 1.5ug of DNA was sonicated to 300-500 base pairs. DNA was precipitated, and resuspended in TE buffer. After end cleanup (Lucigen, ER81050) and A-tailing (Klenow Exo- NEB, M0212L), barcoded adapters were ligated onto the samples, followed by phenol/chloroform extraction and ethanol precipitation. Samples were then divided into input and immunoprecipitation samples. DNA was denatured by heating at 95°C for 10 minutes, followed by plunging the samples on ice. Ice-cold 10x hMeDIP buffer (100mM $NaPO_4$ pH 7.0, 1.4 mM NaCl, and 0.5% Triton X-100) was added to the immunoprecipitation sample to a final concentration of 1x. 2uL of 5hmC DNA antibody (Active Motif, 39769) was added to the samples. The samples were rotated overnight at 4°C. Protein A magnetic beads (NEB) were added to the reaction, and rotated at 4°C for 2 hours. The samples were collected on a magnetic rack, and the samples were washed three times (with 10 minute incubations) with 1x hMeDIP buffer. DNA was eluted from the beads by shaking the samples in lysis buffer (50mM Tris pH 8.0, 10mM EDTA, and 0.5% SDS) with proteinase K (100ug/ml) at 55°C for 3+ hours. The DNA was purified by phenol/chloroform extraction followed by ethanol precipitation. After purification, the libraries were PCR amplified (KAPA HiFi, kk2602) for 14-16 cycles using paired-end primers. Libraries were pooled and purified with 1.8X Agencourt AMpure XP beads (Beckman Coulter, A63881). Pooled libraries were quantified with KAPA Library Quantification Kit (kk4824).

Primer Set	Forward Primer	Reverse Primer
BC1-ACT	P-AGTAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACTT
BC2-TGA	P-TCAAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGAT
BC3-CTG	P-CAGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGT
BC4-GAC	P-GTCAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGACT
BC5-AGC	P-GCTAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCT
BC6-TCG	P-CGAAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGT
BC7-CAT	P-ATGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCATT
BC8-GTA	P-TACAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTAT
BC9-GAA	P-TTCAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAT
BC10-GTC	P-GACAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTCT
BC11-AAC	P-GTTAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACT
BC12-ATT	P-AATAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATTT
BC13-TAG	P-CTAAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGT
BC14-TTA	P-TAAAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTAT
BC15-CCA	P-TGGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCAT
BC16-CGG	P-CCGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGGT
BC17-GCC	P-GGCAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCT
BC18-GGT	P-ACCAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTT
PE	CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCC	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCT

hMeDIP Library Sequencing and Mapping. Pooled hMeDIP libraries were sequenced on an Illumina HiSeq 2500. FastQ files were demultiplexed using the Barcode Splitter on Galaxy (all mapping programs were run using Galaxy (Afgan et al., 2016). Demultiplexed samples were mapped to the mouse genome (mm9) using Bowtie2 (Langmead and Salzberg, 2012) with default settings. Duplicate reads were removed from the Bam files.

Differentially 5-hydroxymethylated Region (DhMR) Detection. Regional differences in hMeDIP enrichment between samples was determined using MACS2 (Feng et al., 2012). Aging-induced DhMRs used 3 month old mice and 18 month old mice. Overexpression (OE)-induced DhMRs used control lentivirus-injected brains and OE injected brains. A q-value of 0.05 was used as a cutoff for determining the significance of a peak. DhMRs located within chr*N*_random and chrUn_random (regions that cannot be confidently anchored to specific locations within the mouse genome build), and chrM were excluded from further analysis.

Genetic Element Enrichment. DhMRs identified by MACS2 for each sample, as well as those lost during aging or gained by Tet2 overexpression were mapped to a list of genetic elements (intragenic, intergenic, 5K promoter, exons, and introns). The enrichment of DhMRs in identified elements over the expected number was calculated. The expected DhMRs per element was calculated by finding the fraction of the genome covered by that element, multiplying that by the total number of DhMRs to derive the expected number of DhMRs for that element, then dividing the number of DhMRs mapped to each element by the expected number in that element to find the enrichment value. As a control, randomized DhMRs for each sample underwent these calculations, showing no enrichment over the expected values.

Gene Ontology Analysis. DhMRs were paired with overlapping RefSeq genes, and if the DhMRs was intergenic, it was paired with the nearest gene. Gene ontology analysis was performed using PANTHER (Mi et al., 2017) (www.geneontology.org) with RefSeq genes as the reference list (background). P-values were corrected using the Bonferroni method. Gene ontology terms that had greater than or equal to two-fold enrichment and a P-value of 0.05 or less after multiple hypothesis correction were considered significant; the top ten terms were shown in the figures.

hMeDIP-Seq Metagene Models. Metagene models were constructed using Deeptools (Ramirez et al., 2016). A 5bp binned BigWig file that was FPKM normalized (excluding ChrM, ChrY, Chr*N*_Random and ChrUn) was generated for each sample using bamCoverage. For generating a coverage scatterplot, the hMeDIP BigWig files were reads were quantified in 50kb bins using multiBigwigSummary. Metagene models were generated by scoring the BigWig files over RefSeq genes (that were scaled to the same size) or promoters using computeMatrix. Scores were averaged for every gene to arrive at the metagene model.

Data and statistical analyses. All experiments were randomized and blinded by an independent researcher before stereotaxic injection or assessment of genetic mouse models. Researchers remained blinded throughout histological,

biochemical and behavioral assessments. Groups were un-blinded at the end of each experiment upon statistical analysis. Data are expressed as mean ± s.e.m. The distribution of data in each set of experiments was tested for normality using D'Agostino-Pearson omnibus test or Shapiro-Wilk test. Statistical analysis was performed with Prism 7.0 software (GraphPad Software). Means between two groups were compared with two-tailed, unpaired Student's *t*-test. Comparisons of means from multiple groups with each other or against one control group were analyzed with one-way ANOVA followed by appropriate *post hoc* tests (indicated in figure legends).

SUPPLEMENTAL REFERENCES

Afgan, E., Baker, D., van den Beek, M., Blankenberg, D., Bouvier, D., Cech, M., Chilton, J., Clements, D., Coraor, N., Eberhard, C., et al. (2016). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. Nucleic Acids Res 44, W3-W10.

Alamed, J., Wilcock, D.M., Diamond, D.M., Gordon, M.N., and Morgan, D. (2006). Two-day radial-arm water maze learning and memory task; robust resolution of amyloid-related memory deficits in transgenic mice. Nat Protoc 1, 1671-1679.

Feng, J.X., Liu, T., Qin, B., Zhang, Y., and Liu, X.S. (2012). Identifying ChIP-seq enrichment using MACS. Nat Protoc 7, 1728-1740.

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat Methods 9, 357-359.

Mi, H., Huang, X., Muruganujan, A., Tang, H., Mills, C., Kang, D., and Thomas, P.D. (2017). PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. Nucleic Acids Res 45, D183-D189.

Ramirez, F., Ryan, D.P., Gruning, B., Bhardwaj, V., Kilpert, F., Richter, A.S., Heyne, S., Dundar, F., and Manke, T. (2016). deepTools2: a next generation web server for deep-sequencing data analysis. Nucleic Acids Res 44, W160-165.

Smith, L.K., He, Y., Park, J.S., Bieri, G., Snethlage, C.E., Lin, K, Gontier, G., Wabl, R., Plambeck, K.E., et al. (2015). β2-Microglobulin is a systemic pro-aging factor that impairs cognitive function and neurogenesis. Nat Medicine 21, 932-937.