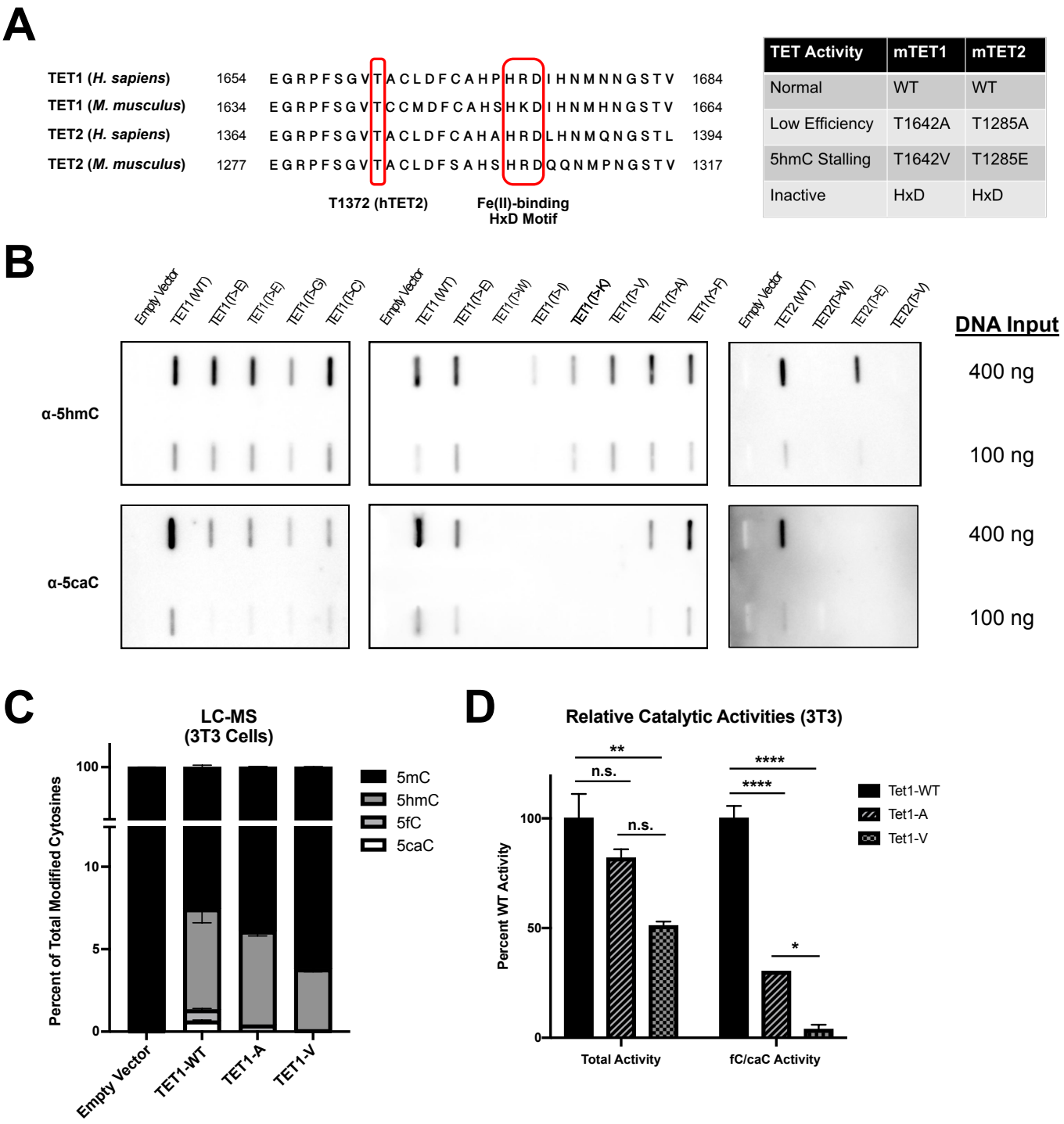


Figure S1 related to Figure 1. (A) Alignment of human and mouse TET1 and TET2 protein sequences (human TET2 T1372 site and Fe(II)-binding HxD motif indicated by red boxes) with summary of mouse TET1 and TET2 mutants used in this study. **(B)** Representative slot blots for 5hmC and 5caC in genomic DNA isolated from HEK cells transfected with a broader array of TET1 or TET2 catalytic variants. TET1 variants carry mutations at either T1642 (E, G, C, W, I, K, V, A) or Y2049F, while TET2 variants carry mutations at T1285 (W, E, V). **(C)** Genomic levels of modified cytosines in transfected NIH3T3 cells, quantified by LC-MS/MS and expressed as the percentage of total modified cytosines present in each sample (mean ± SEM; n=2-4). **(D)** Relative catalytic activities of TET1 variants. Modified cytosines were normalized to their mean levels in cells transfected with WT TET1 to determine the relative catalytic activities of each variant. Relative catalytic activities are presented as either total TET activity (5hmC + 5fC + 5caC) or fC/caC activity (mean relative catalytic activities ± SEM; n=2-3; one-way ANOVA with Tukey multiple comparisons; n.s. = not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

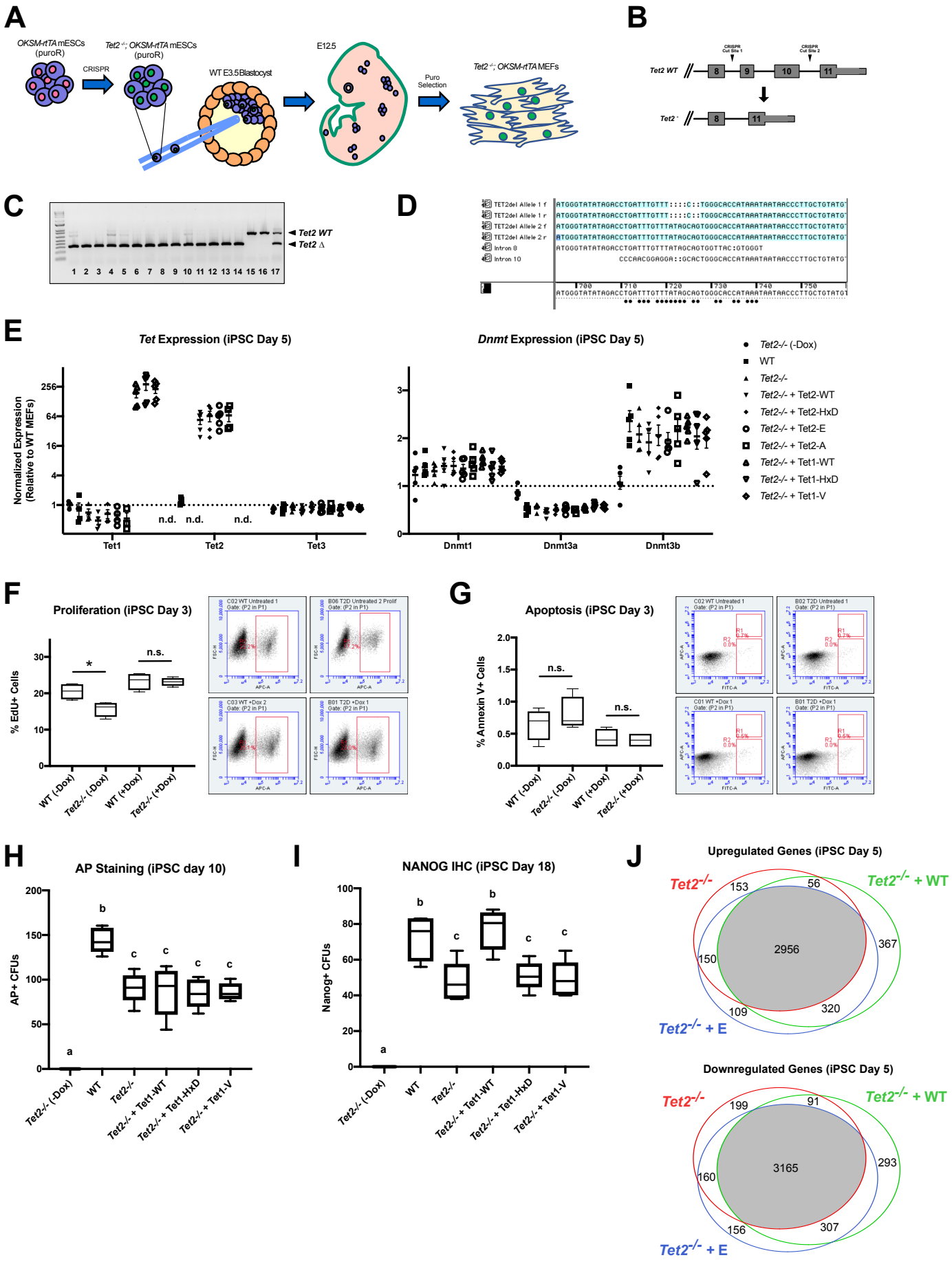
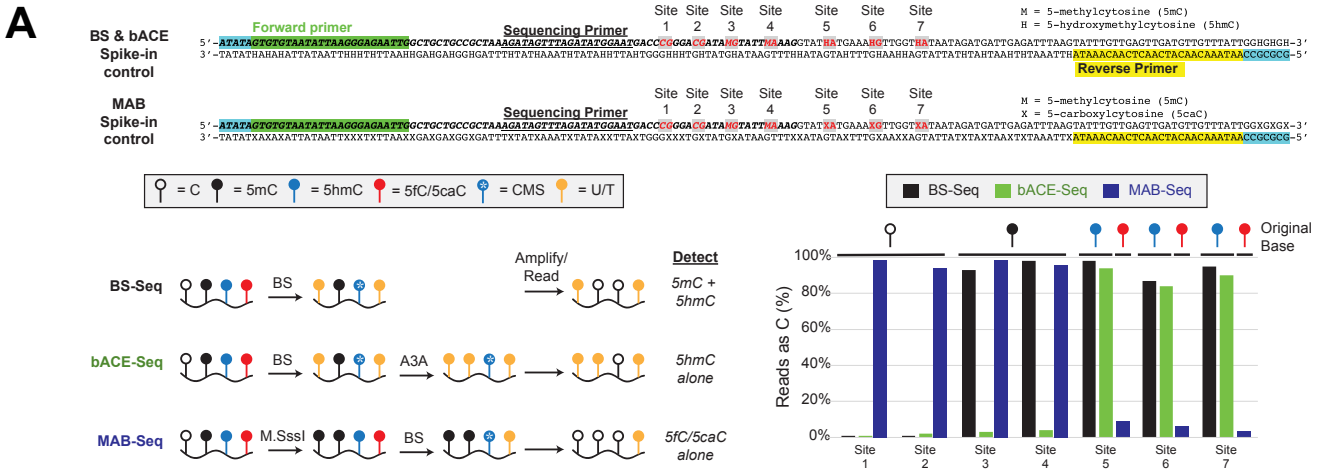
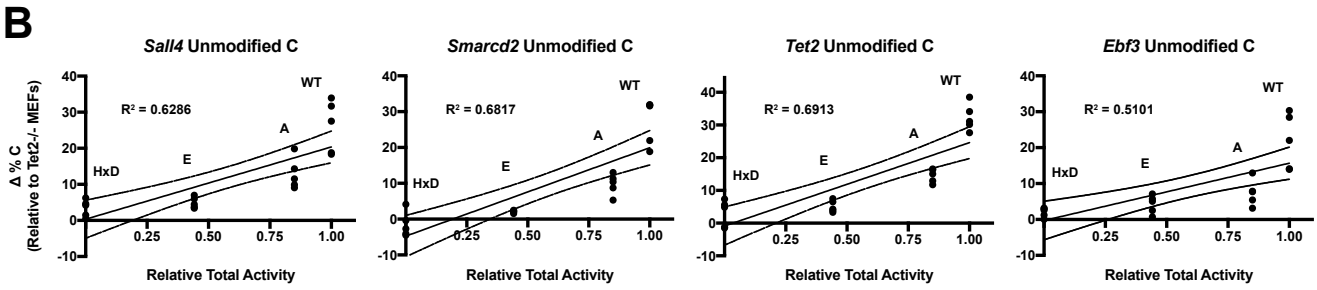
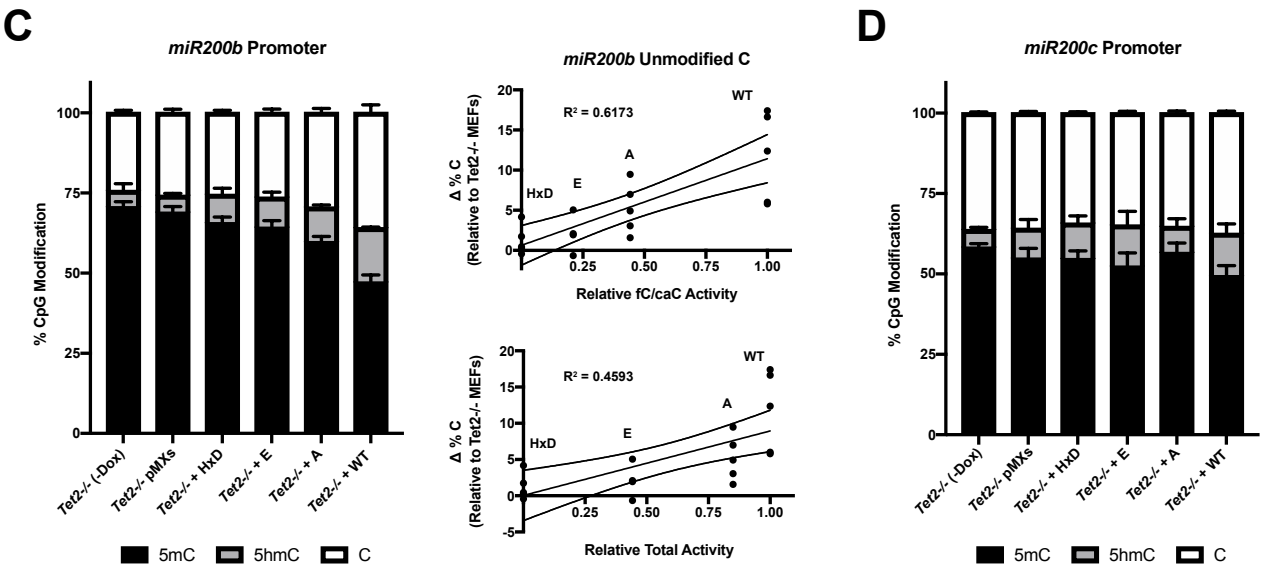

A
OKSM-rtTA mESCs (puroR)
CRISPR
Tet2 -/-; OKSM-rtTA mESCs (puroR)
WT E3.5 Blastocyst
E12.5
Puro Selection
Tet2 -/-; OKSM-rtTA MEFs
B
Tet2 WT
CRISPR Cut Site 1
CRISPR Cut Site 2
8
9
10
11
Tet2 -
8
11
C
Tet2 WT
Tet2 Δ
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
D
TET2del Allele 1 f
TET2del Allele 1 r
TET2del Allele 2 f
TET2del Allele 2 r
Intron 8
Intron 10
E
Tet Expression (iPSC Day 5)
Dnmt Expression (iPSC Day 5)
Normalized Expression (Relative to WT MEFs)
n.d.
n.d.
n.d.
Tet1
Tet2
Tet3
Dnmt1
Dnmt3a
Dnmt3b
Tet2-/- (-Dox)
WT
Tet2-/-
Tet2-/- + Tet2-WT
Tet2-/- + Tet2-HxD
Tet2-/- + Tet2-E
Tet2-/- + Tet2-A
Tet2-/- + Tet1-WT
Tet2-/- + Tet1-HxD
Tet2-/- + Tet1-V
F
Proliferation (iPSC Day 3)
% EdU+ Cells
*
n.s.
WT (-Dox)
Tet2-/- (-Dox)
WT (+Dox)
Tet2-/- (+Dox)
G
Apoptosis (iPSC Day 3)
% Annexin V+ Cells
n.s.
n.s.
H
AP Staining (iPSC day 10)
AP+ CFUs
I
NANOG IHC (iPSC Day 18)
Nanog+ CFUs
J
Upregulated Genes (iPSC Day 5)
Tet2-/-
Tet2-/- + WT
Tet2-/- + E
153
56
2956
367
150
109
320
Downregulated Genes (iPSC Day 5)
199
91
3165
293
160
156
307

Figure S2 related to Figure 2. (A) Outline of *Tet2*$^{-/-}$; *OKSM-rtTA* MEF derivation procedure. Wild-type, Dox-inducible *OKSM-rtTA* mouse ESCs (mESCs) were subjected to CRISPR-Cas9 mutagenesis to delete both endogenous alleles of *Tet2*. The resultant *Tet2*$^{-/-}$; *OKSM-rtTA* mESCs were subcloned and injected into mouse embryonic day 3.5 (E3.5) blastocysts to generate chimeric embryos. Embryos were transferred to pseudopregnant dams and allowed to develop to E12.5, at which point they were harvested and processed for MEF isolation. Pure *Tet2*$^{-/-}$; *OKSM-rtTA* MEF cultures were obtained through puromycin selection. **(B)** CRISPR-Cas9 mutagenesis strategy for *Tet2* knockout. Double-stranded breaks at the indicated cut sites and subsequent non-homologous end-joining (NHEJ) results in the excision of exons 9 and 10 of the *Tet2* catalytic domain. **(C)** Genotyping PCR assay for *Tet2* in *Tet2*$^{-/-}$; *OKSM-rtTA* MEFs following 48 hours of puromycin selection. Lanes 1-14 represent MEF clones derived from individual chimeric embryos, while lanes 15-17 represent WT (clones 15, 16) and heterozygous (clones 17) *Tet2*$^{+/-}$ mESC samples. Clones 2, 13, and 14 were used for all subsequent experiments. **(D)** Forward (f) and reverse (r) sequencing of *Tet2* in *Tet2*$^{-/-}$; *OKSM-rtTA* MEF clone 14. Note the fusion of *Tet2* introns 8 and 10 resulting from CRISPR/Cas9 NHEJ, as well as the presence of two distinct mutagenized alleles. **(E)** qRT-PCR for *Tet* (left) and *Dnmt* (right) expression relative to untreated WT MEFs after 5 days of Dox treatment. Cells were retrovirally transduced with either empty vector or the indicated *Tet1* or *Tet2* variant. Expression levels were normalized to *Nono*. Data points represent independent experiments, with mean expression levels +/- SEM indicated (n=5; n.d. = not detected). **(F)** EdU labelling of WT and *Tet2*$^{-/-}$ MEFs either untreated (-Dox) or following 3 days of Dox treatment. Box plots indicate median percentage of EdU^{+} cells for each condition (one-way ANOVA with Sidak's multiple comparisons; *p< 0.05; n.s. = not significant; n=4). Representative sorting plots for each condition are included on the right (EdU^{+} cells indicated by the red box). **(G)** Annexin V labelling of apoptotic WT and *Tet2*$^{-/-}$ MEFs either untreated (-Dox) or following 3 days of Dox treatment. Box plots indicate median percentage of Annexin V^{+} cells for each condition (one-way ANOVA with Sidak's multiple comparisons; n=4). Representative sorting plots for each condition are included on the right (Annexin V^{+} cells indicated by the lower-right red box). **(H)** Alkaline phosphatase (AP) staining of pluripotent colonies after 10 days of Dox treatment for TET1-transduced cells. Box plots indicate median AP^{+} colony forming units (CFUs) for each treatment group, with letters designating statistically distinct groups (one-way ANOVA with Tukey multiple comparisons; n=5). **(I)** Immunohistochemistry for Nanog-positive stable pluripotent colonies after one week of Dox withdrawal (median Nanog^{+} CFU counts; one-way ANOVA with Tukey multiple comparisons; n=5). **(J)** Venn overlap of differentially expressed genes following 5 days of Dox treatment relative to untreated *Tet2*$^{-/-}$ MEFs (RNA-seq; n=3-4; FDR < 0.05; fold-change > 1.5).


A
BS & bACE Spike-in control
MAB Spike-in control
Forward primer
Sequencing Primer
Reverse Primer
Site 1 Site 2 Site 3 Site 4 Site 5 Site 6 Site 7
M = 5-methylcytosine (5mC)
H = 5-hydroxymethylcytosine (5hmC)
X = 5-carboxylcytosine (5caC)
= C = 5mC = 5hmC = 5fC/5caC = CMS = U/T
BS-Seq
bACE-Seq
MAB-Seq
BS
A3A
M.SssI
Amplify/ Read
Detect
5mC + 5hmC
5hmC alone
5fC/5caC alone
Reads as C (%)
Original Base


B
Sall4 Unmodified C
Smarcd2 Unmodified C
Tet2 Unmodified C
Ebf3 Unmodified C
R² = 0.6286
R² = 0.6817
R² = 0.6913
R² = 0.5101
WT
A
E
HxD
Δ % C (Relative to Tet2-/- MEFs)
Relative Total Activity


C
miR200b Promoter
miR200b Unmodified C
% CpG Modification
Tet2-/- (-Dox)
Tet2-/- pMXs
Tet2-/- + HxD
Tet2-/- + E
Tet2-/- + A
Tet2-/- + WT
5mC
5hmC
C
R² = 0.6173
R² = 0.4593
Relative fC/caC Activity
Relative Total Activity

D
miR200c Promoter
% CpG Modification
5mC
5hmC
C

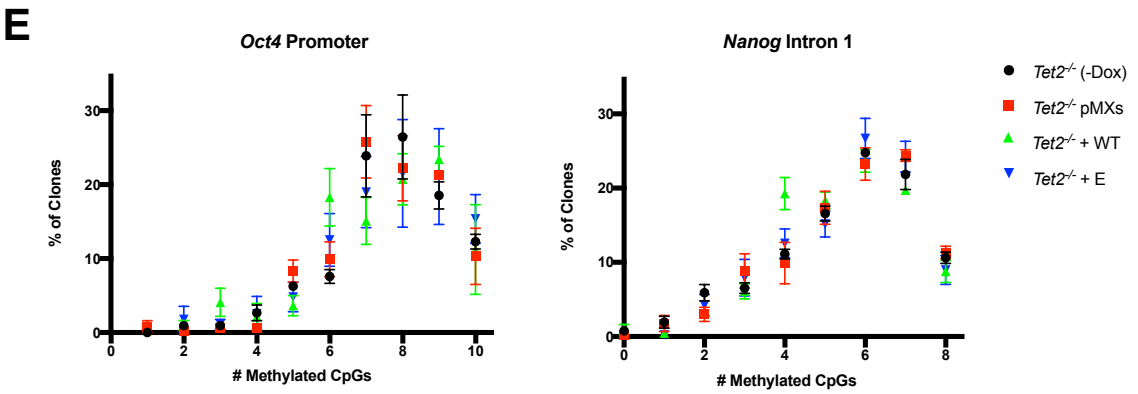

E
Oct4 Promoter
Nanog Intron 1
% of Clones
# Methylated CpGs
Tet2-/- (-Dox)
Tet2-/- pMXs
Tet2-/- + WT
Tet2-/- + E

Figure S3 related to Figure 3. (A) bACE- and MAB-Seq conversions of PCR-generated spike-in controls. The spike-ins were generated by PCR amplification of a template using a spike-in generation forward primer (bold sequence), which contains 5mC, a reverse primer, and the 5hmCTP or 5caCTP in lieu of dCTP. After bisulfite (BS), bisulfite + APOBEC3A (bACE), or M.SssI + bisulfite (MAB) treatment, the products were amplified using forward and reverse primers. Pyrosequencing with the indicated sequencing primer was then used to analyzed the number of C versus T reads at each site. In the bACE-Seq reaction, unmodified cytosines are fully deaminated following bisulfite reaction (site 1 and site 2). 5mC bases are resistant to deamination (site 3 and site 4), and 5hmC bases are converted into 5-methylenesulfonate (CMS, sites 5-7 of the bACE spike-in). Following APOBEC3A treatment, 5mCs are now subjected to deamination while the original 5hmC bases, converted to CMS, are protected from deamination. In the MAB-Seq reaction, unmodified cytosines are first converted to 5mC by M.SssI, thereby protecting them from deamination by bisulfite. Following bisulfite treatment, only 5fC or caC (sites 5-7 of the MAB spike-in) are deaminated. **(B)** Alternative model for TET2 activity at the *Sall4*, *Smarcd2*, *Tet2*, and *Ebf3* reprogramming enhancers. Data points represent independent experiments plotted against total TET2 activity (5hmC + 5fC + 5caC) values determined from HEK overexpression experiments (Fig. 1C). Simple linear regressions were performed for each enhancer, with the line of best fit (solid) and 95% confidence intervals (dotted) indicated (n=4-5). **(C-D)** Relative levels of unmodified cytosine, 5hmC, and 5mC at the **(C)** miR200b and **(D)** miR200c cluster promoters in untreated *Tet2*$^{-/-}$ MEFs or following 5 days of Dox treatment. Cells were retrovirally transduced with empty vector or the indicated *Tet2* variant. For the right panel of **(C)**, data points represent independent experiments plotted against either the relative fC/caC or total activity values determined from HEK overexpression experiments (Fig. 1C). Simple linear regressions were performed, with the line of best fit (solid) and 95% confidence intervals (dotted) indicated. (n=4-5). **(E)** Relative proportions of bisulfite clonotypes at the *Oct4* promoter and *Nanog* intron 1 in untreated *Tet2*$^{-/-}$ MEFs or following 5 days of Dox treatment, as determined by bisulfite sequencing. Data points represent the mean percentage of clones with the indicated clonotype +/- SEM (n=3-4). Trending increases were observed in the proportion of lowly methylated clones (< 5 methylated CpGs) in *Tet2-WT* cells.

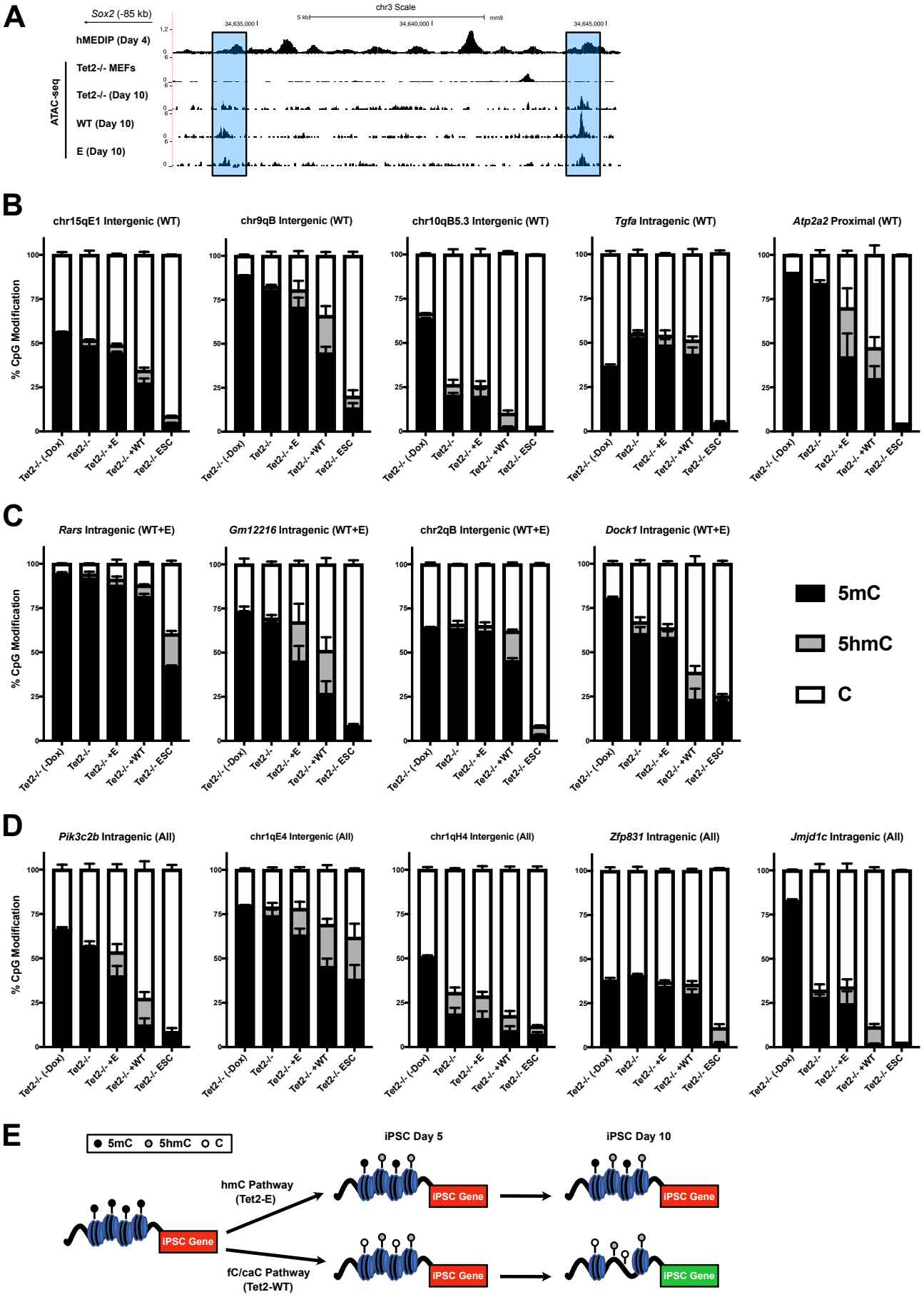

A
Sox2 (-85 kb)
chr3 Scale
5 kb
mm9
34,635,000
34,640,000
34,645,000
hMEDIP (Day 4)
ATAC-seq
Tet2-/- MEFs
Tet2-/- (Day 10)
WT (Day 10)
E (Day 10)
B
chr15qE1 Intergenic (WT)
chr9qB Intergenic (WT)
chr10qB5.3 Intergenic (WT)
Tgfa Intragenic (WT)
Atp2a2 Proximal (WT)
% CpG Modification
Tet2-/- (-Dox)
Tet2-/-
Tet2-/- +E
Tet2-/- +WT
Tet2-/- ESC
C
Rars Intragenic (WT+E)
Gm12216 Intragenic (WT+E)
chr2qB Intergenic (WT+E)
Dock1 Intragenic (WT+E)
5mC
5hmC
C
D
Pik3c2b Intragenic (All)
chr1qE4 Intergenic (All)
chr1qH4 Intergenic (All)
Zfp831 Intragenic (All)
Jmjd1c Intragenic (All)
E
5mC 5hmC C
iPSC Day 5
iPSC Day 10
hmC Pathway
(Tet2-E)
fC/caC Pathway
(Tet2-WT)
iPSC Gene

Figure S4 related to Figure 4. (A) Differential ATAC peaks overlapping 5hmC signal at two distal *Sox2* enhancers. UCSC genome browser snapshots include ATAC signal (merge of 4 biological replicates) for each condition at reprogramming day 10, as well as hMeDIP signal at iPSC day 4 (Sardina et al., 2018). Direction and distance to *Sox2* are indicated by the arrow in the top-left corner. **(B-D)** Relative levels of unmodified cytosine, 5hmC, and 5mC at regions showing **(B)** WT-specific (WT), **(C)** WT- or E-specific (WT+E), or **(D)** shared (All) iPSC ATAC peaks overlapping 5hmC signal. The relative levels of each cytosine derivative were measured in untreated *Tet2*$^{-/-}$ MEFs (-Dox), untreated *Tet2*$^{-/-}$ mESCs, or *Tet2*$^{-/-}$ MEFs following 5 days of Dox treatment. Cells were retrovirally transduced with empty vector or the indicated *Tet2* variant. Bar graphs represent the mean percentage of each cytosine derivative +/- SEM (n=4). **(E)** Model for fC/caC pathway's role in iPSC reprogramming. In contrast to the hmC pathway, the fC/caC pathway drives significant DNA demethylation during the early stages of iPSC reprogramming. This demethylation facilitates chromatin opening at these loci at later iPSC stages, thereby generating active chromatin environments in regions critical for the reprogramming process.

Expression vectors for HEK/3T3 transfections
Tet1-CD WT pLEXm
Tet1-CD T1642E pLEXm
Tet1-CD T1642V pLEXm
Tet1-CD T1642A pLEXm
Tet1-CD T1642G pLEXm
Tet1-CD T1642C pLEXm
Tet1-CD T1642W pLEXm
Tet1-CD T1642I pLEXm
Tet1-CD T1642K pLEXm
Tet1-CD Y2049F pLEXm
Tet2-CD WT pLEXm
Tet2-CD T1285E pLEXm
Tet2-CD T1285A pLEXm
Tet2-CD T1285W pLEXm
Tet2-CD T1285V pLEXm

Retroviral vectors
Tet1-CD WT pMXs
Tet1-CD WT pMXs
Tet1-CD HxD pMXs
Tet2-CD WT pMXs
Tet2-CD T1285E pMXs
Tet2-CD T1285A pMXs
Tet2-CD HxD pMXs

CRISPR/Cas9 Vectors
Tet2 Int8a pX330
Tet2 Int10a pX330

Table S1 related to STAR Methods.