

Figure S1

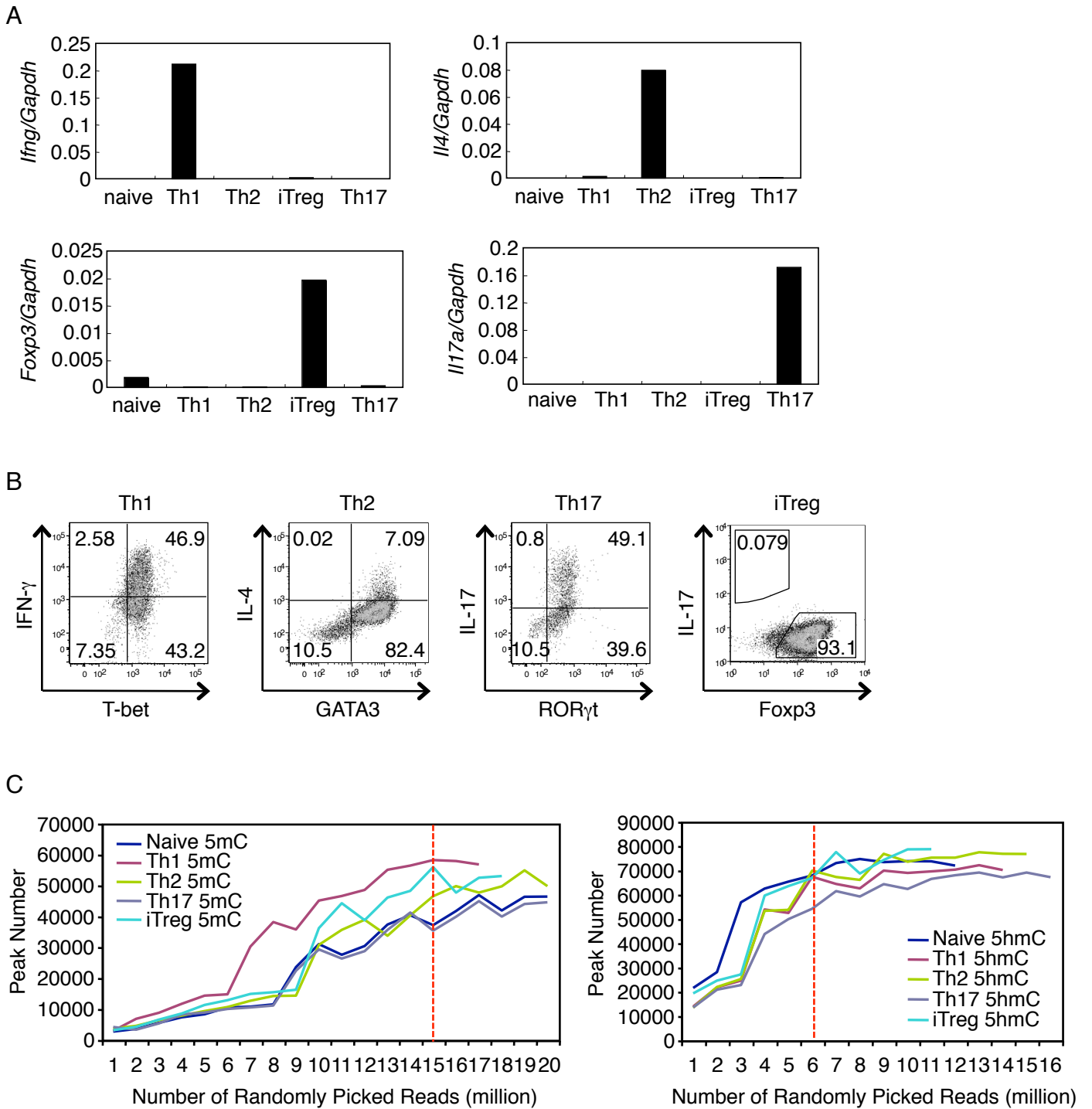
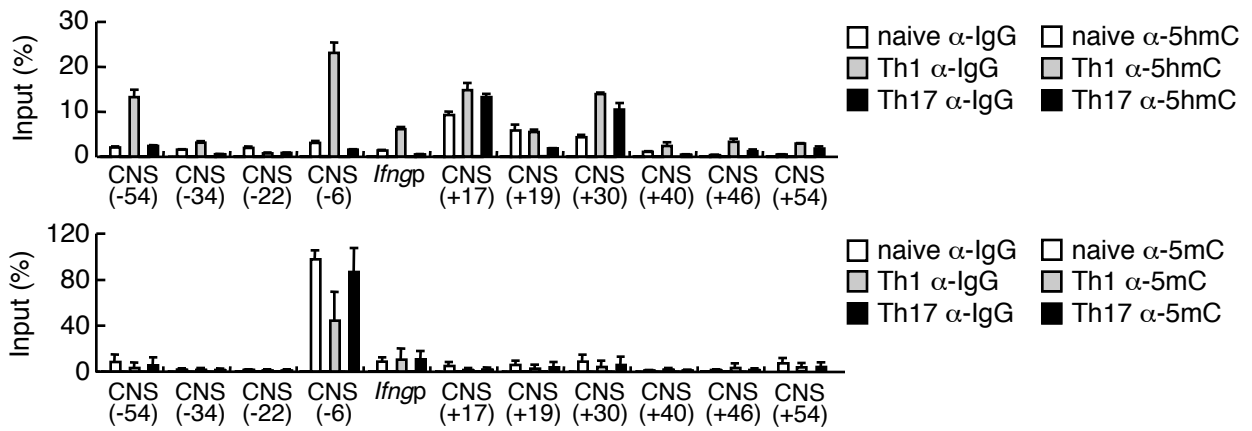
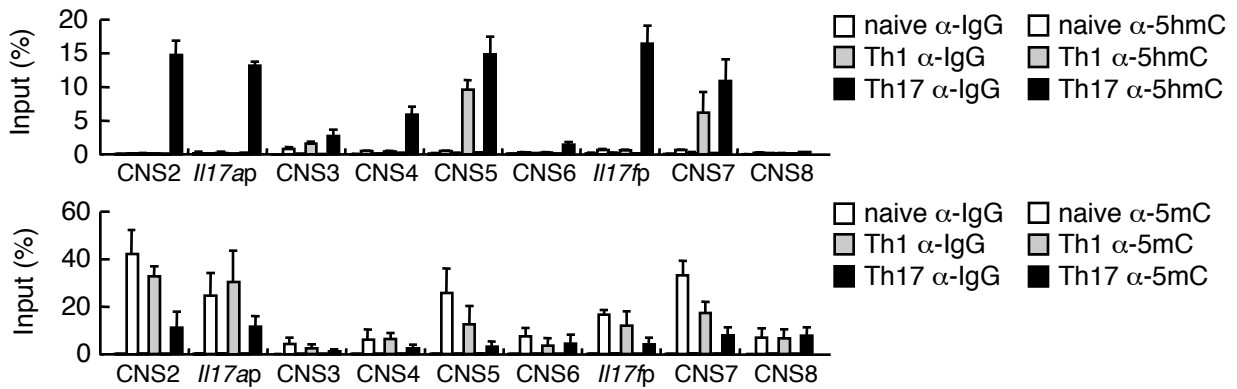


Figure S2

A



B



C

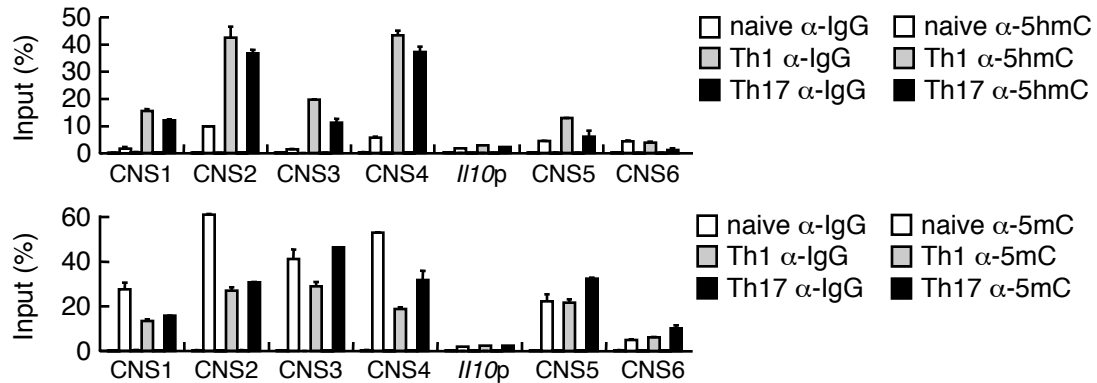
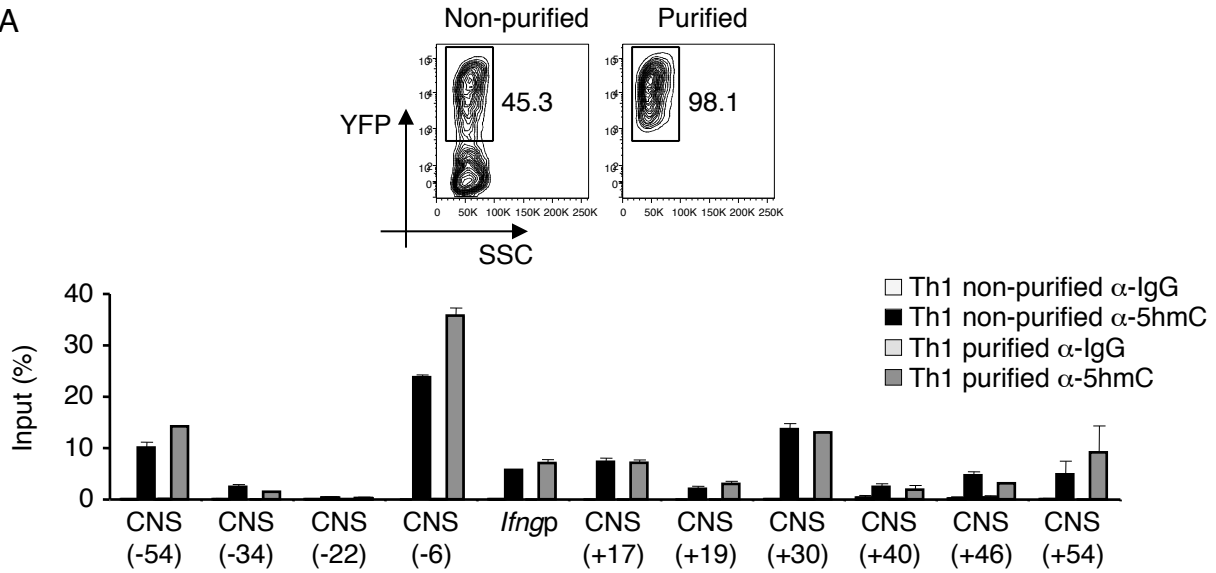
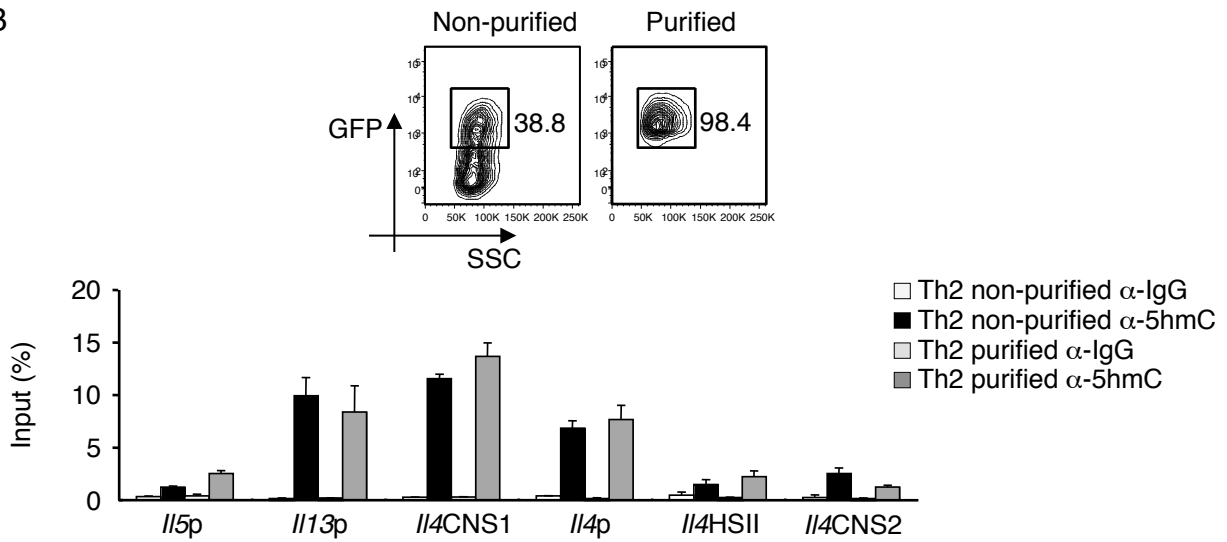


Figure S3

A



B



C

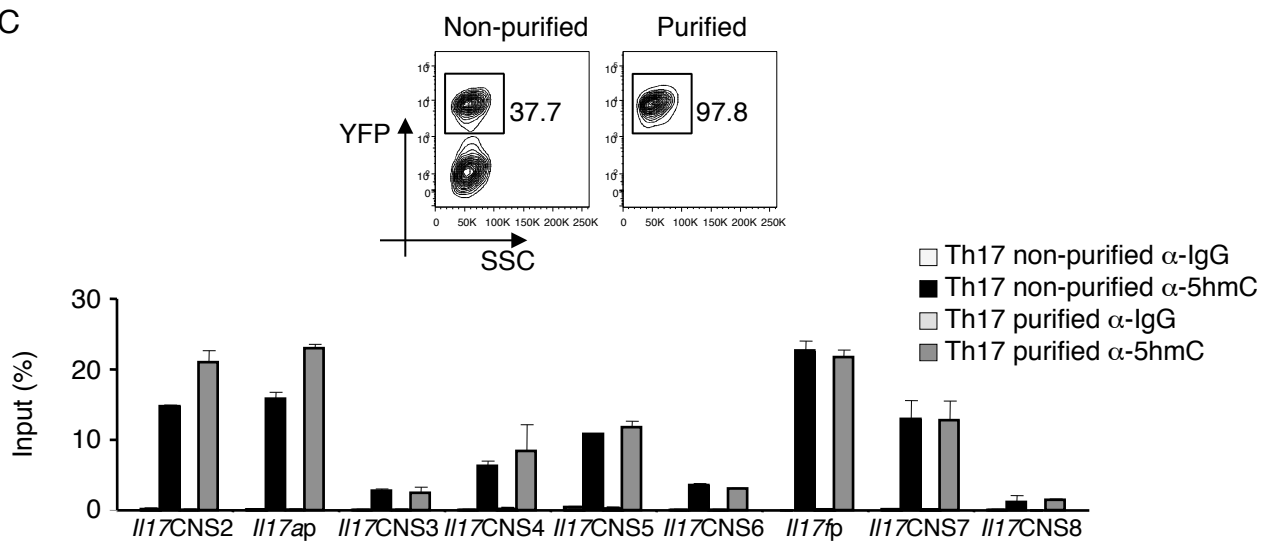
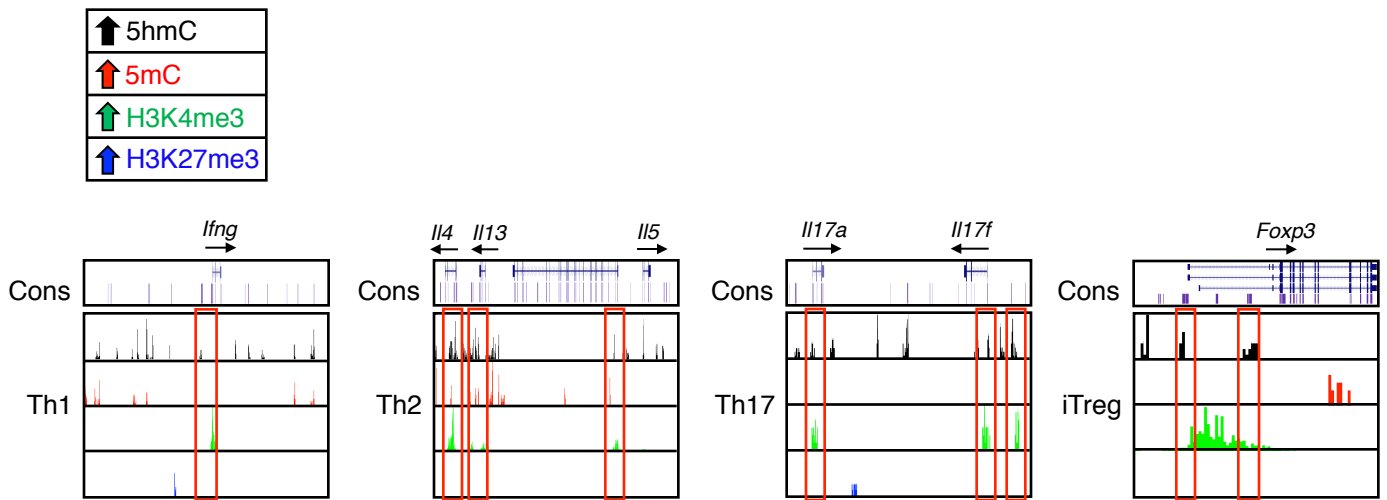
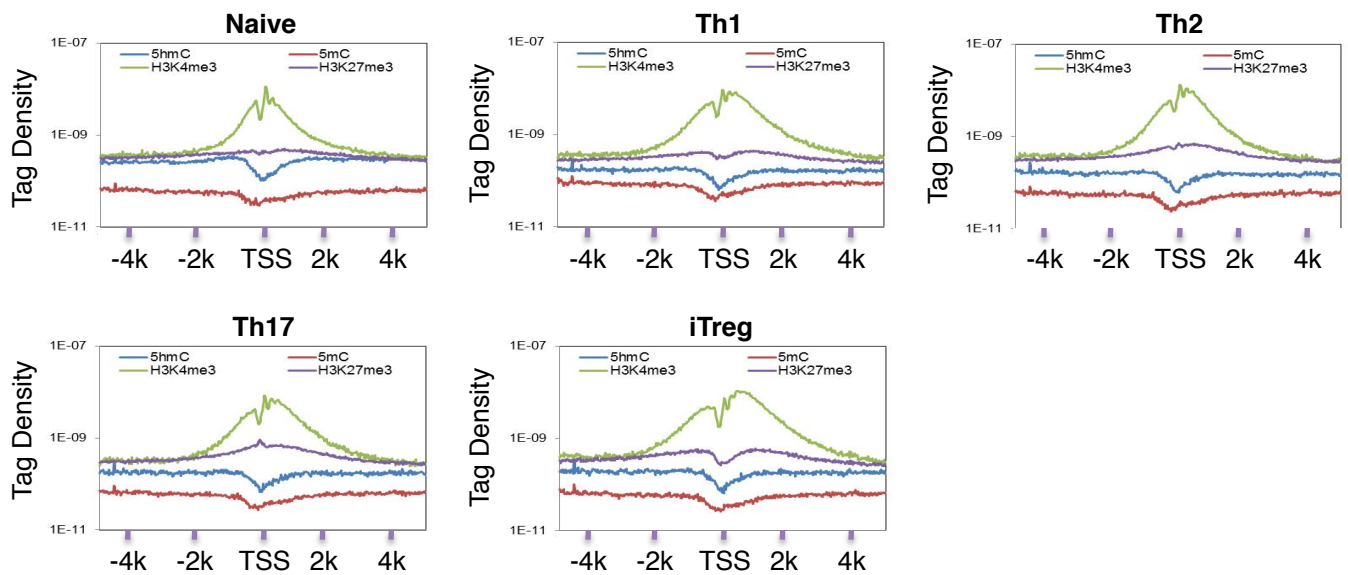


Figure S4

A



B



C

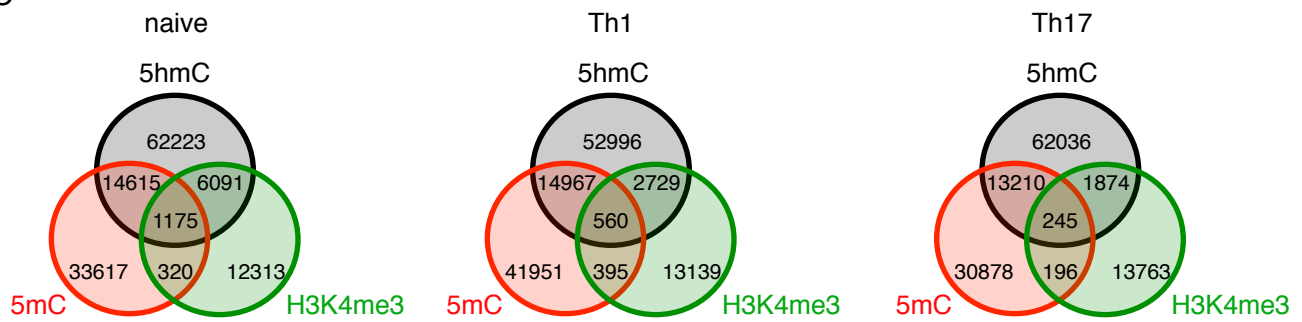


Figure S5

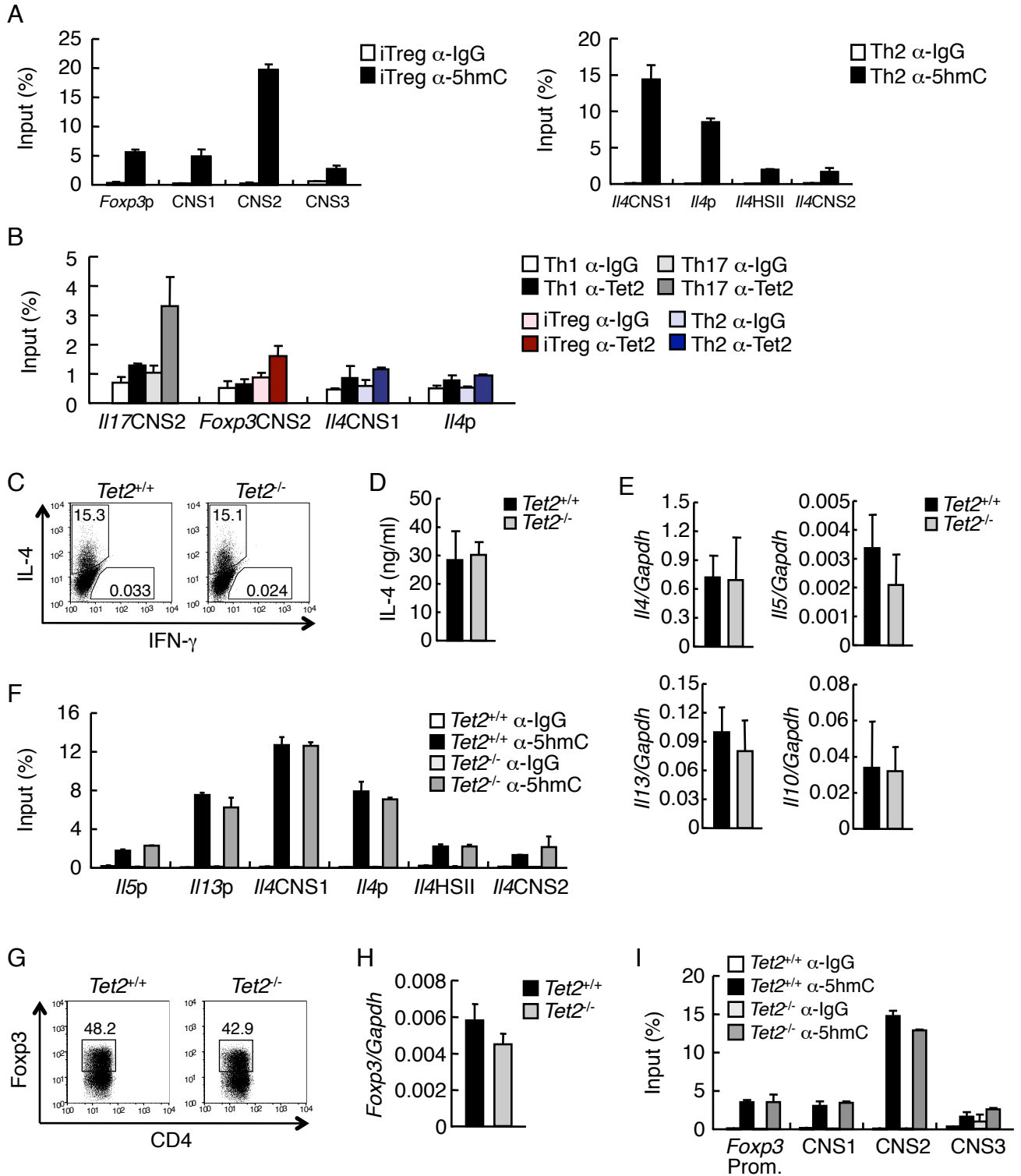


Figure S6

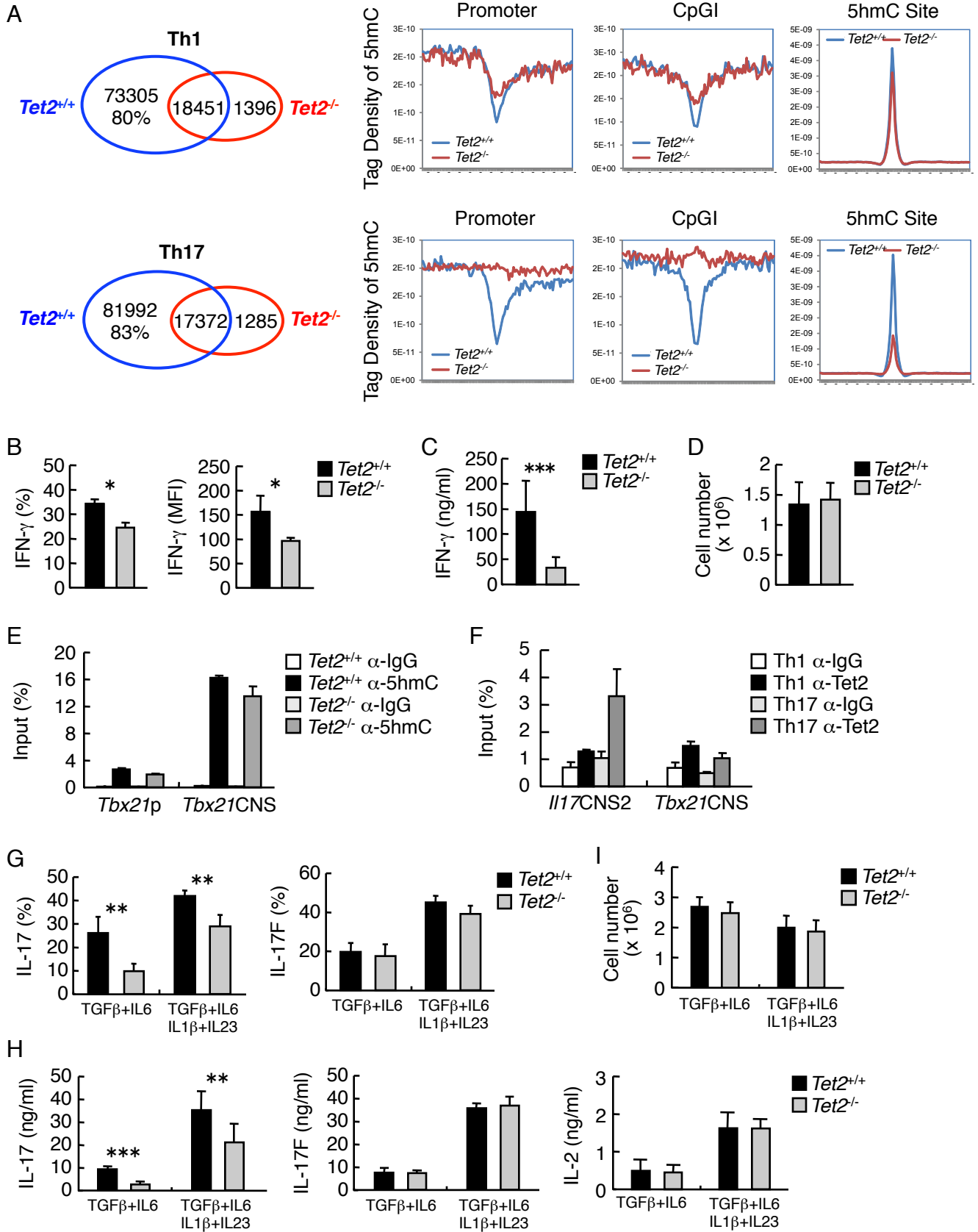
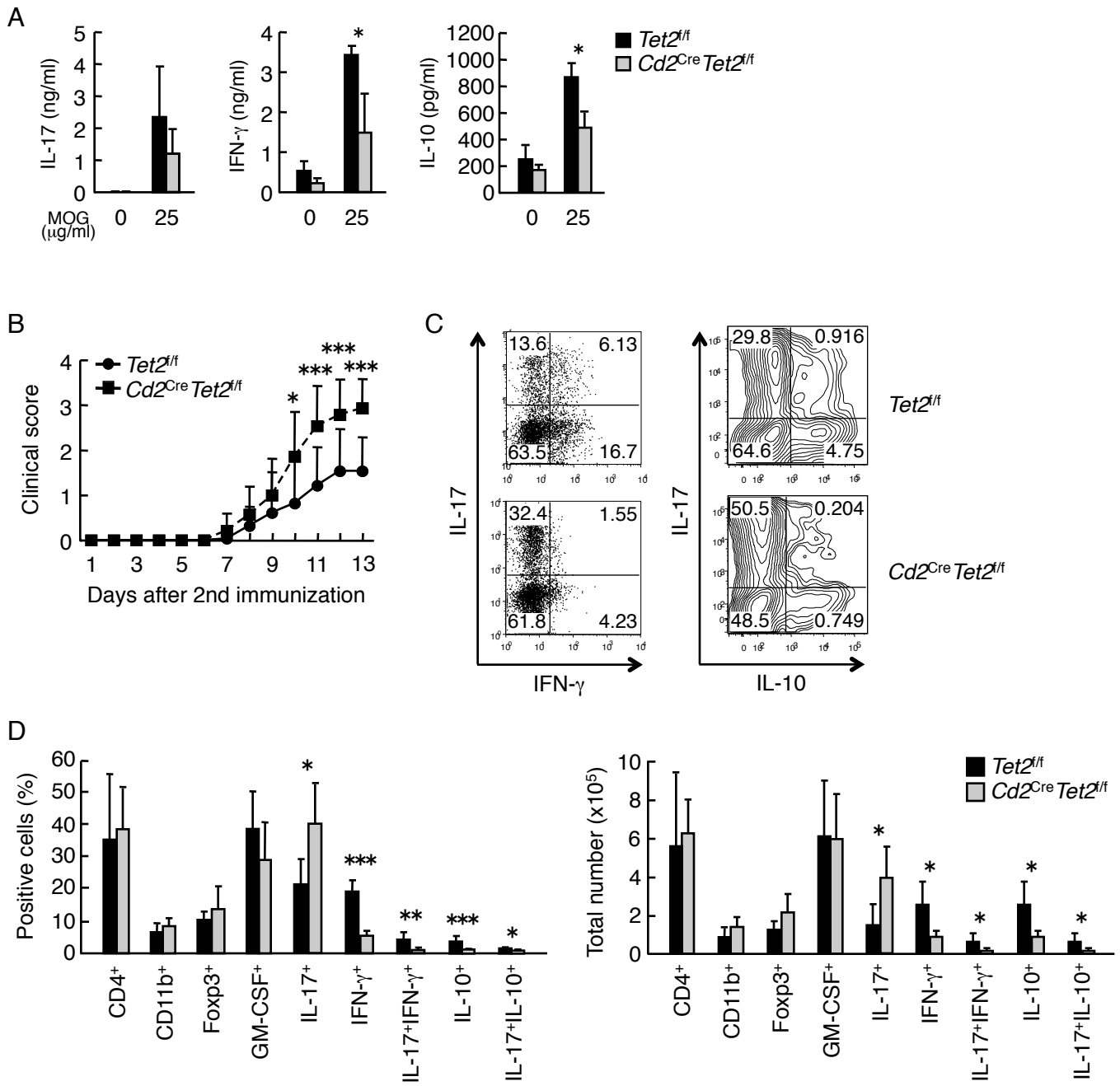


Figure S7



Supplemental Figure Legends

Figure S1, Related to Figure 1: Quality verification of DIP-sequencing samples and reads.

(A) Naive T cells were cultured under iTreg, Th1, Th2 and Th17 conditions. Expression of signature genes in differentiated Th cells was measured by real-time RT-PCR. (B) Proportions of transcription factor positive cells as well as and cytokine-producing cells in cell cultures were determined by intracellular staining. (C) Randomly sampled fractions of reads from each sample were subjected to peak identification using SICER. A red line was drawn at the number of reads with which the number of peaks identified would be saturated for 5hmC and 5mC libraries.

Figure S2, Related to Figure 2: Confirmation of 5mC and 5hmC distribution

in the *Ifng*, *Il17a*, *Il17f* and *Il10* loci by qPCR. The DIP-PCR assay on the *Ifng*

(A), *Il17a*, *Il17f* (B) and *Il10* (C) loci was performed in naïve, Th1 and Th17 cells.

Cell lysates were immunoprecipitated with α -5mC, α -5hmC or control IgG. The

results were normalized to the input control. Data are a representative of at least

three individual experiments and shown as mean \pm SD.

Figure S3, Related to Figure 2: Comparison of 5hmC distribution between

purified and non-purified cells. Naive T cells were from *Ifng*^{Yfp} (A), *Il4*^{Yfp} (B) and

Il17f^{Cre}*Rosa26*^{Yfp} (C) mice cultured under Th1, Th2 and Th17 conditions for 4-6

days, respectively. Reporter positive cells were purified by a FACSAria. 5hmC

modification in the purified and non-purified cells was analyzed by hMeDIP-PCR.

Data are a representative of two individual experiments and shown as mean \pm SD.

Figure S4, Related to Figure 3: Comparison of genome-wide DNA modifications with histone modifications. (A) Distribution of 5hmC, 5mC, H3K4me3 and H3K27me3 islands along the *Ifng*, *Il4*, *Il13*, *Il5*, *Il17a*, *Il17f* and *Foxp3* genomic regions in naïve and appropriate Th subsets is shown. The regions of overlapping peaks are highlighted by red squares. (B) Compiled tag density profiles for 5hmC, 5mC, H3K4me3 and H3K27me3 in each T cell subset are shown. The diagrams represent all genes that showed positive signals for the respective marks, and show tag density profiles across promoter ± 5 kb flanking regions with 25 bp resolution. (C) The concordance between 5hmC/5mC modifications and H3K4me3 modifications in naïve, Th1 and Th17 cells are shown.

Figure S5, Related to Figure 5 and 6: The role of Tet2-mediated active DNA demethylation in Th2 and iTreg development. (A) A MeDIP-PCR analysis on the *Foxp3* and *Il4* loci was performed in iTreg and Th2 cells, respectively. Cell lysates were immunoprecipitated with α -5hmC or control IgG. (B) A ChIP-PCR analysis on the *Foxp3*, *Il4* and *Il17* loci were performed in Th1, Th2, iTreg and Th17 cells. Cell lysates were immunoprecipitated with α -Tet2 or control IgG. The results were normalized to the input control. Data are a representative of two

individual experiments and shown as mean \pm SD. (C, D) Naïve T cells from *Tet2^{ff}* and *Cd2^{Cre}Tet2^{ff}* mice were cultured under Th2 condition for 7 days. IL-4 expression was analyzed by flow cytometry (C) and by ELISA (D). (E) The expression of *Il4*, *Il5*, *Il13* and *Il10* mRNA were analyzed by real-time RT-PCR. (F) A hMeDIP assay of the *Il4* gene was performed in Th2 cells from *Tet2^{ff}* and *Cd2^{Cre}Tet2^{ff}* mice. Cell lysates were immunoprecipitated with α -5hmC or control IgG. The results were normalized to the input control. (G, H) Naïve T cells from *Tet2^{ff}* and *Cd2^{Cre}Tet2^{ff}* mice were cultured under iTreg condition for 4 days. Foxp3 expression was analyzed by flow cytometry (G) and by real-time RT-PCR (H). (I) A hMeDIP assay of the *Foxp3* gene was performed in iTreg cells from *Tet2^{ff}* and *Cd2^{Cre}Tet2^{ff}* mice. Cell lysates were immunoprecipitated with α -5hmC or control IgG. The results were normalized to the input control. Data are a representative of at least two individual experiments and shown as mean \pm SD.

Figure S6, Related to Figure 5 and 6: The role of Tet2-mediated active DNA demethylation in Th1 and Th17 development. (A) The venn diagram showing the numbers of 5hmC peaks that appear in only *Tet2^{+/+}* or *Tet2^{-/-}* cells or in both cells. The normalized tag density profiles for 5hmC around \pm 5 kb regions flanking TSS (Promoter), CpG island centers (CpGI), and 5hmC peak center (5hmC Site) with 200 bp resolution are shown. Th1 cells (upper), Th17 cells (lower). (B) The statistics of intracellular staining in Figure 5A. (C) Naïve T cells from *Tet2^{ff}* and *Cd2^{Cre}Tet2^{ff}* mice were cultured under Th1 condition for 5 days and restimulated

with α -CD3 for overnight. The amount of IFN γ in supernatants was measured by ELISA. All groups are analyzed in triplicates. (D) The number of viable cells in Figure S6B. (E) A MeDIP assay of the *Tbx21* gene was performed in Th1 cells from *Tet2^{ff}* and *Cd2^{Cre}Tet2^{ff}* mice. Cell lysates were immunoprecipitated with α -5hmC or control IgG. (F) A ChIP-PCR analysis on the *Tbx21* and *Il17* loci were performed in Th1 and Th17 cells. Cell lysates were immunoprecipitated with α -Tet2 or control IgG. The results were normalized to the input control. Data are a representative of two individual experiments and shown as mean \pm SD. (G) The statistics of intracellular staining in Figure 6A. (H) Naïve T cells from *Tet2^{ff}* and *Cd2^{Cre}Tet2^{ff}* mice were cultured under Th17 condition for 4 days and restimulated with α -CD3 for overnight. The amount of IL17, IL17F and IL-2 in supernatants was measured by ELISA. All groups are analyzed in triplicates. (I) The number of viable cells in Figure S6G. (B-D, G-I) All data represent the average of at least three independent experiments and are shown as mean \pm SD.

Figure S7, Related to Figure 7: Tet2 regulates *in vivo* immune responses.

(A) *Tet2^{ff}* and *Cd2^{Cre}Tet2^{ff}* mice (n = 4) were immunized with MOG₃₅₋₅₅ peptide on day 0 and then analyzed on day 7 for IL17, IFN γ and IL10 production. (B) Age-matched *Tet2^{ff}* and *Cd2^{Cre}Tet2^{ff}* mice were immunized twice with MOG₃₅₋₅₅. Mean clinical scores are shown versus days after second MOG immunization. Shown here is the combinational result of three EAE experiments (the total number of mice used: WT = 16, KO = 16). (C) The IL17, IFN γ and IL10 levels

were determined in the infiltrates of CNS of MOG₃₅₋₅₅-immunized mice. (D) Statistics of the cell frequencies and absolute numbers. All the data are shown as mean \pm SD.

Table S1, Related to Figure 1: Distribution of 5mC and 5hmC. The mouse genome was divided into four kinds of regions: proximal promoter (1 kb upstream and downstream of transcription start site), exon, intron, and intergenic regions. The number of islands for each sample was listed as the total identified islands (Total Peak), followed by the islands among genomic regions with the percentage listed in the parenthesis. Normal distribution of promoter, exon, intron, and intergenic regions in mouse genome was calculated based on RefSeq Genes downloaded from UCSC Genome Browser on Sep 17, 2014.

Table S2, Related to Figure 1: Colocalization of both 5hmC and 5mC. The numbers of peaks uniquely associated with 5hmC, 5mC, or both modifications are shown.

Table S3, Related to Figure 2: Distribution of lineage specific 5hmC. All 5hmC peaks that only appeared in one of naïve, Th1, Th2, Th17, or iTreg lineages were identified as the lineage-specific 5hmC modification.

Table S4, Related to Figure 5 and 6: The list of genes regulated by Tet2 in Th1 and Th17 cells. The genes with at least 1.5 fold differences in their expression between wild-type and Tet2-deficient Th1 and Th17 cells are shown, respectively. 5hmC peaks that were present on their promoter plus gene body regions (-3K to TTS) are shown.

Supplemental Experimental Procedures

Flow Cytometry

For intracellular cytokine staining, cells were stimulated for 4-6 hr in complete medium with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (500 ng/ml; both from Sigma-Aldrich) in the presence of brefeldin A (eBioscience). Intracellular staining was performed with the Fixation and Permeabilization kit (BD Biosciences and eBioscience) according to the manufacturer's instructions. Data were acquired through a BD FACSAria or BD FACSCalibur and were analyzed with FlowJo software (Tree Star, Ashland, OR).

ELISA and Quantitative Real-Time RT-PCR

Supernatants were collected after the indicated periods of cell culture and were analyzed with an ELISA kit (BD Biosciences and R&D Systems) according to the manufacturer's instructions.

The cells were lysed in Trizol reagent (Invitrogen), which allowed RNA extraction. cDNAs were synthesized with Superscript reverse transcriptase and oligo(dT)

primers (Invitrogen) and analyzed with an iCycler Optical System and an iQ SYBR green real-time PCR kit (Bio-Rad). The data were normalized to a *Gapdh* control. The following genes were amplified with primers as described: *Gapdh* (Ichiyama et al., 2008); *Il10*, *Il4*, *Il5* and *Il13* (Angkasekwinai et al., 2010); *Il17a*, *Il17f*, *Rorc*, *Ifng*, *Tbx21* and *Foxp3* (Yang et al., 2008).

Chromatin immunoprecipitation (ChIP) Assay

The chromatin immunoprecipitation (ChIP) assay was performed as previously described (Ichiyama et al., 2011) using a ChIP assay kit (Millipore, Cat# 17-295). Briefly, cells were fixed with 1% formaldehyde at 37°C for 10 min and then suspended in an SDS lysis buffer. After sonication, samples were incubated with antibodies against p300 (SantaCruz), acetylated histone H3 (H3Ac) (Millipore), trimethylated histone H3K4 (H3K4me3) (Millipore), trimethylated histone H3K27 (H3K27me3) (Millipore), Tet2 (SantaCruz), RORc (SantaCruz), T-bet (SantaCruz) or control IgG overnight at 4°C. Antibody-DNA complexes were captured by protein A agarose/Salmon sperm DNA. The immunoprecipitated DNA was purified and quantified by real-time PCR. The results were normalized relative to the input control.

DNA Immunoprecipitation (DIP) Assay

The DNA immunoprecipitation (DIP) assay was performed as previously described (Vucic et al., 2009). In brief, the genomic DNA was purified and

sonicated. DNA fragments (4 μ g) were denatured and incubated with antibodies against 5mC (Eurogentec), 5hmC (Active Motif) or control IgG at 4°C overnight. Antibody-DNA complexes were captured by protein A agarose/Salmon sperm DNA. The immunoprecipitated DNA was purified and quantified by real-time PCR. The results were normalized relative to the input control.

MOG Immunization and Induction of EAE

Both MOG immunization and EAE induction were performed by immunizing mice with 300 μ g MOG₃₅₋₅₅ peptide (amino acids 35-55; MEVGWYRSPFS ROVHLYRNGK) emulsified in CFA and analyzed as previously described (Wang et al., 2012). For neutralization of IL10, α -IL10R (1B1.3A; BioXCell) or α -Rat IgG1 (HRPN; BioXCell) as control was intraperitoneally injected at every 4 days after MOG/CFA immunization. The disease scores were assigned on a scale of 0–5 as follows: 0, none; 1, limp tail or waddling gait with tail tonic; 2, wobbly gait; 3, hindlimb paralysis; 4, hindlimb and forelimb paralysis; 5, death.

CD4⁺ T Cell Transfer

Total CD4⁺ T cell populations were isolated from the spleens and lymph nodes of *Tet2^{ff}* and *Cd2^{Cre}Tet2^{ff}* mice. CD4⁺ T cells were further purified using CD4 microbeads and autoMACS (Miltenyi). Cells were counted and washed 3X in PBS and then injected intravenously into tail veins of *Rag1^{-/-}* mice at 8×10^6 cells per mouse. EAE was induced the day following CD4⁺ transfer.

Microarray

Two independent sets of microarray assays were performed. Total cellular RNA was extracted from Th1 and Th17 cells with Trizol reagent (Invitrogen) in *Tet2^{ff}* and *Cd2^{Cre}Tet2^{ff}* mice. DNA microarray labeling and analysis were performed in the microarray core at the Institute for Systems Biology. Approximately 10 µg RNA was labeled and hybridized to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix) according to the manufacturer's protocols. Expression values were defined with GeneChip Operating Software (GCOS; Affymetrix).

Primers

The primers used for real-time RT-PCR, ChIP-PCR, DIP-PCR and genotyping are listed below.

Primer name (forward)	Sequence (5' → 3')	Primer name (reverse)	Sequence (5' → 3')
<i>Cd2Cre</i>	AGATGCCAGGACAT CAGGAACCTG	<i>Cd2Cre</i>	ATCAGCCACACCAGA CACAGAGATC
<i>Tet2flox</i>	AAGAATTGCTACAG GCCTGC	<i>Tet2flox</i>	TTCTTTAGCCCTTGCT GAGC
<i>Tet1</i>	GAAGGAACAGGAAG CTGCAC	<i>Tet1</i>	CTGGCCAAACCTAGT CTCCA
<i>Tet2</i>	GATCCAGGAGGAGC AGTGAG	<i>Tet2</i>	TGGGAGAAGGTGGT GCTATC
<i>Tet3</i>	CCGGATTGAGAAGG TCATCTAC	<i>Tet3</i>	AAGATAACAATCAGG GCGTTCT
<i>H19</i>	GCATGGTCCTCAAAT TCTGCA	<i>H19</i>	GCATCTGAACGCCCC AATTA
<i>Ifng</i> CNS(-54)	CACACTCGAAGCTTT CCACA	<i>Ifng</i> CNS(-54)	CTAAGCCGGACCAGA ATGAG
<i>Ifng</i> CNS(-34)	CCCAGGCACTTTCCT ACTGA	<i>Ifng</i> CNS(-34)	TGGTTGACACTCTGG GCTTT

<i>Ifng</i> CNS(-22)	CACAGGAAGGAGAT GGGAAG	<i>Ifng</i> CNS(-22)	ACCTGCCTCCTGTCT TTTGA
<i>Ifng</i> CNS(-6)	ATCTGTCTGAACGG GTGGAG	<i>Ifng</i> CNS(-6)	AGCAGGTCGGTCCTC TGTA
<i>Ifng</i> p	GGGGGAGACGTAAA AGCAAT	<i>Ifng</i> p	GGGCTCTCTGACGAT GAGAC
<i>Ifng</i> CNS(+17)	AGCAAGAGCAAAGA GGATGC	<i>Ifng</i> CNS(+17)	CACCAAGTCTCAGGC ATTGA
<i>Ifng</i> CNS(+19)	AGCCAAACCACAGA CTGGAT	<i>Ifng</i> CNS(+19)	AGTTTGGGAAGTTGG GTGTG
<i>Ifng</i> CNS(+30)	AAGCCTTGTACTTCC GACGA	<i>Ifng</i> CNS(+30)	CCAGTTTTACCACCC ACACC
<i>Ifng</i> CNS(+40)	CAGCATTGTCTGAC GACCAC	<i>Ifng</i> CNS(+40)	ATGTGAGCAAGCCAT CTTCC
<i>Ifng</i> CNS(+46)	CATGTGGAACCCAC AACAAG	<i>Ifng</i> CNS(+46)	GTGCTCCTAGCGACT CTTGC
<i>Ifng</i> CNS(+54)	TGGGCTGCTCTTCC AGTATT	<i>Ifng</i> CNS(+54)	GACAGTTTCAGTGCC ACCAA
<i>Tbx21</i> p	CCCAATCTGGGAAA GAGTCA	<i>Tbx21</i> p	AGGCGTGAGAATGCT CAGAT
<i>Tbx21</i> CNS	CCACTTATCACCCCA GATGC	<i>Tbx21</i> CNS	TACACACTGGGTCGC TGAGA
<i>I17</i> CNS2	TGTGGTTGTCTAAGC CATGC	<i>I17</i> CNS2	CAGCAACTGACTGGG TTTCA
<i>I17</i> ap	CAGCTCCCAAGAAG TCATGC	<i>I17</i> ap	TGAGGTCAGCACAGA ACCAC
<i>I17</i> CNS3	TTAGTGAGGTCGGG GAAGTG	<i>I17</i> CNS3	TTTGATGGCAGCACA TTCAT
<i>I17</i> CNS4	TCGCCTCTTGACAAA CAGTG	<i>I17</i> CNS4	TTCGTCCCTGTGATT TCCTC
<i>I17</i> CNS5	AGGCCACAATGTA GGTCAG	<i>I17</i> CNS5	CAGGCTGGGAAGTCT CTCTG
<i>I17</i> CNS6	TGCCTAGGGCTTGA GTGTCT	<i>I17</i> CNS6	CAGGTCCGCTTTGGT ATGTT
<i>I17</i> fp	TCCCTGTTTCCACTG ACCTC	<i>I17</i> fp	TTAGGGTCCCCCTTT GATTC
<i>I17</i> CNS7	CTGAGTTGGGGGCT GTGTAT	<i>I17</i> CNS7	CATATCGAGGGTGTC GGACT
<i>I17</i> CNS8	CAGCAGACACACAT GCAAGA	<i>I17</i> CNS8	CCTCAGGGGAGGGA ATTAAG
<i>I10</i> CNS1	AACCCTGGCTAGAA GGGAAG	<i>I10</i> CNS1	GGAGGGAGGAACAG AAAAGG
<i>I10</i> CNS2	GGTGTTGGTGTGTG CAAGTC	<i>I10</i> CNS2	GTCGGAACCTTGATT GAGGA

<i>//10CNS3</i>	CACACCCTCCCCTCT AACAA	<i>//10CNS3</i>	CTACTGTGGCTGGGC TTCTC
<i>//10CNS4</i>	CTTGAGGAAAAGCC AGCATC	<i>//10CNS4</i>	GGTGCTTTGAGGTTG CAGTT
<i>//10p</i>	AGGGAGGAGGAGCC TGAATA	<i>//10p</i>	TGTGGCTTTGGTAGT GCAAG
<i>//10CNS5</i>	CAGTCGGGGCATCT ATGTCT	<i>//10CNS5</i>	CCAGAACCGTTGCAC AAGTA
<i>//10CNS6</i>	CCAAAGGATGCAAC CACTCT	<i>//10CNS6</i>	CCCCTTATCCTCAA GTCACG
<i>//5p</i>	GGGTGGGGGTCAAG ATGTTA	<i>//5p</i>	AAGGCGAGTCCTGAA ACTCA
<i>//13p</i>	GTGGCCTTAGCCTG TTGAAG	<i>//13p</i>	CTGTCCCAGACCCTT CTCAA
<i>//4CNS1</i>	GGGGAGGAAGAGAG AGCAAC	<i>//4CNS1</i>	CATTCATTTCTCGGC TCCAC
<i>//4p</i>	TCCTGCAGAGAAGG AAGAGG	<i>//4p</i>	CCAGAGTCAGCTTTC CCAAG
<i>//4HSII</i>	GAAAAGCAGGCAGT CTGGAG	<i>//4HSII</i>	TTTGTAGTGGGAGGG GACAG
<i>//4CNS2</i>	CCAAACACCGTTATG GCTCT	<i>//4CNS2</i>	TCGGCTGTAAAGTCC CAGTT
<i>Foxp3p</i>	CCTCCAACGTCTCAC AAACA	<i>Foxp3p</i>	CCCCTCACCACAGAG GTAAA
<i>Foxp3CNS1</i>	CTTTTCTTGTGGGGC TTCTG	<i>Foxp3CNS1</i>	GACAGTCTGGCTCCC ATACC
<i>Foxp3CNS2</i>	ATCTGGCCAAGTTCA GGTTG	<i>Foxp3CNS2</i>	GGCGTTCCTGTTTGA CTGTT
<i>Foxp3CNS3</i>	TAAGCAGGGTGGGG TACTTG	<i>Foxp3CNS3</i>	CTCTGAAGCCTGGAG AGTGG

Supplemental References

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