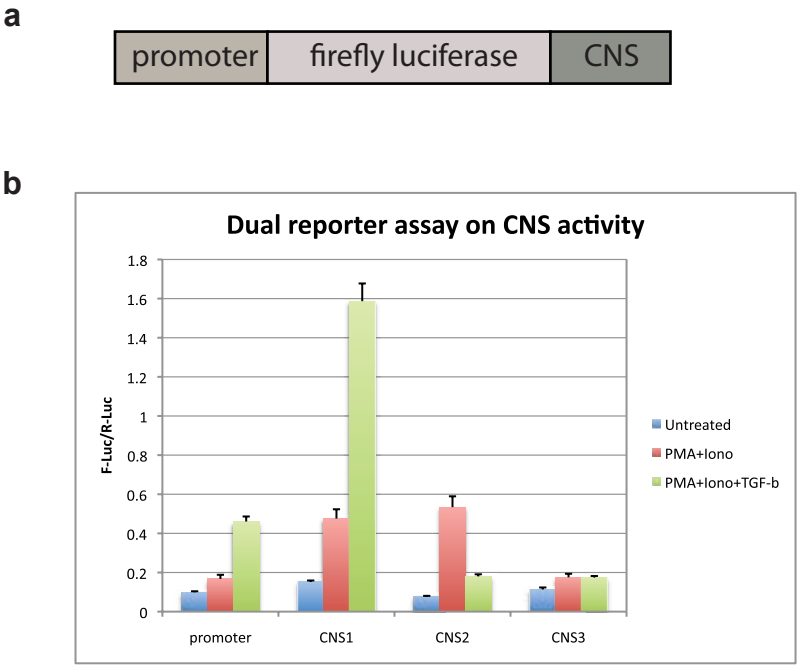
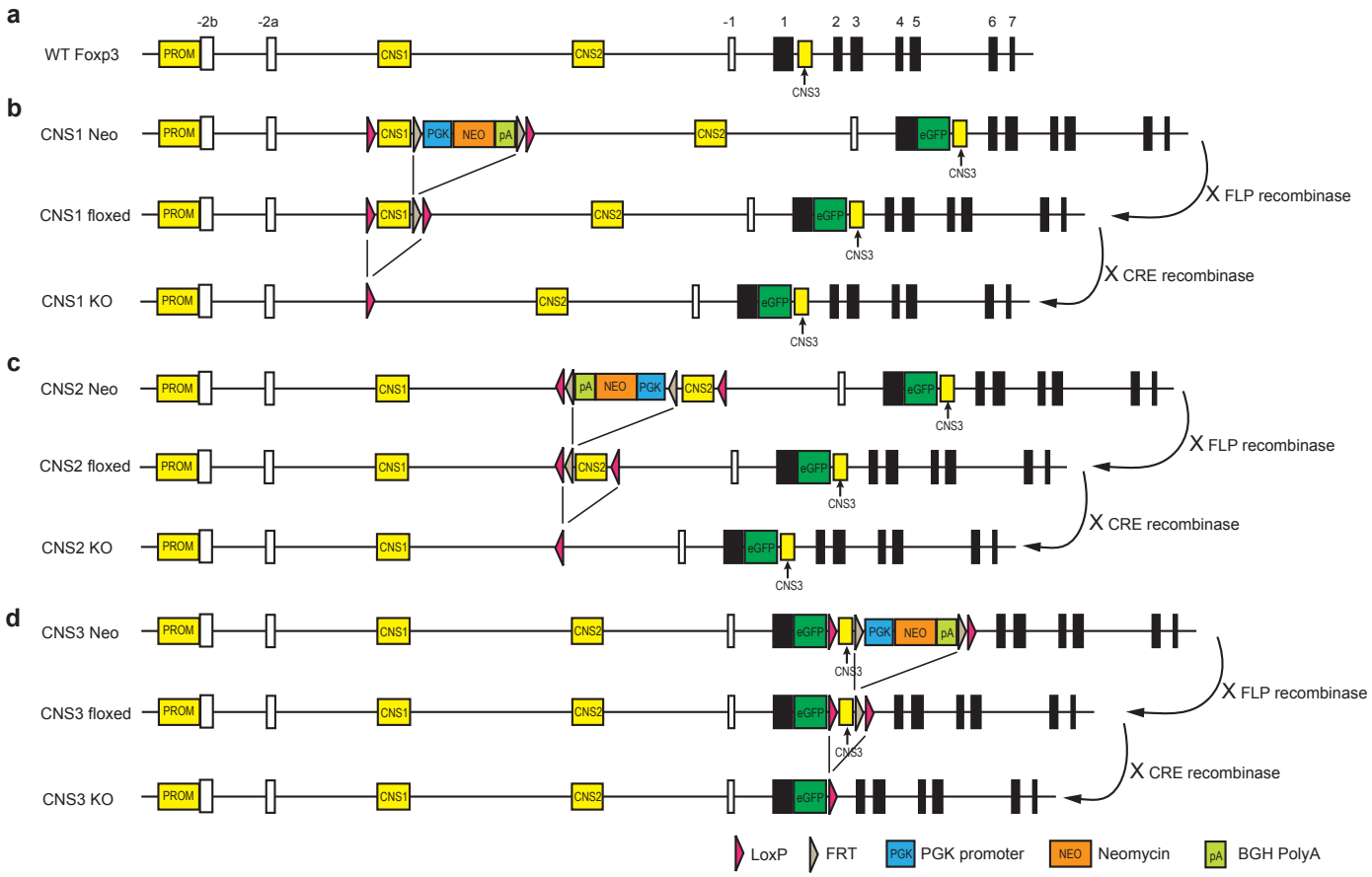
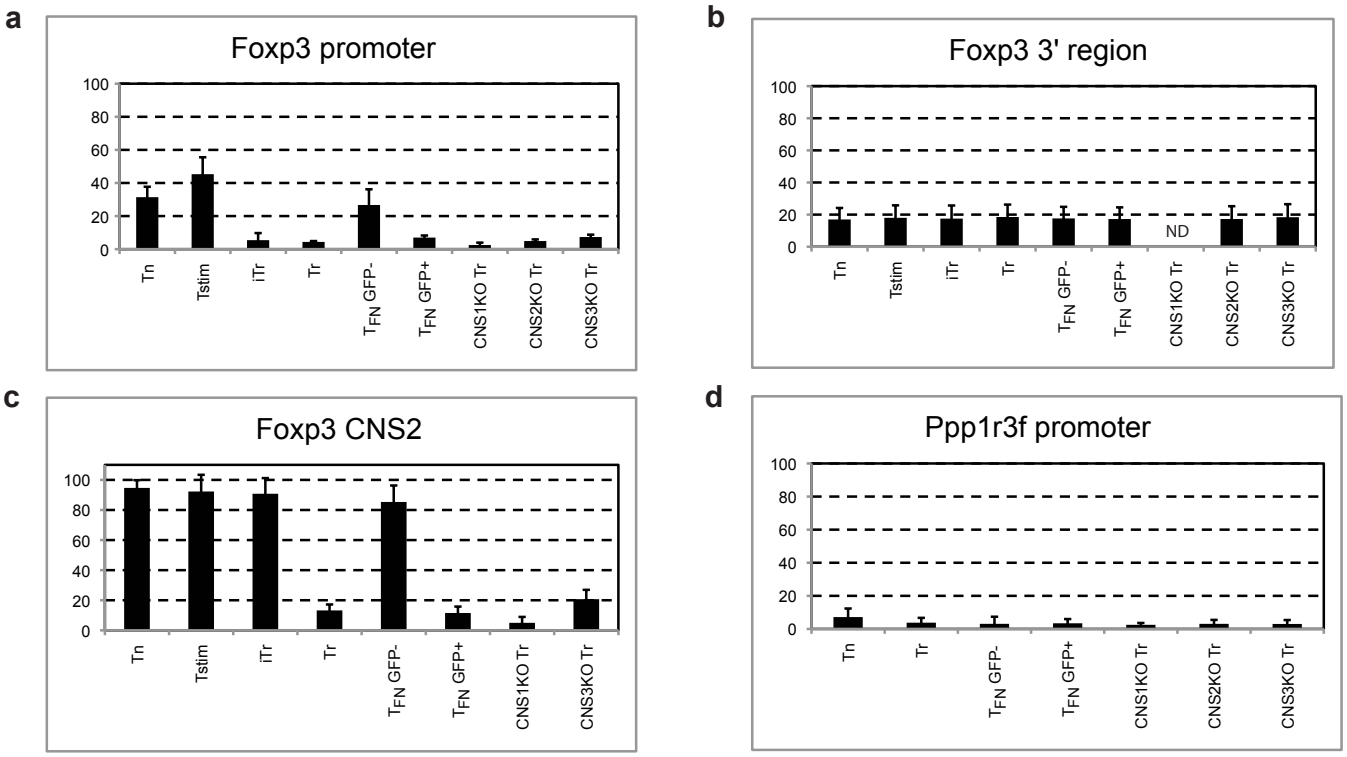
SUPPLEMENTARY INFORMATION



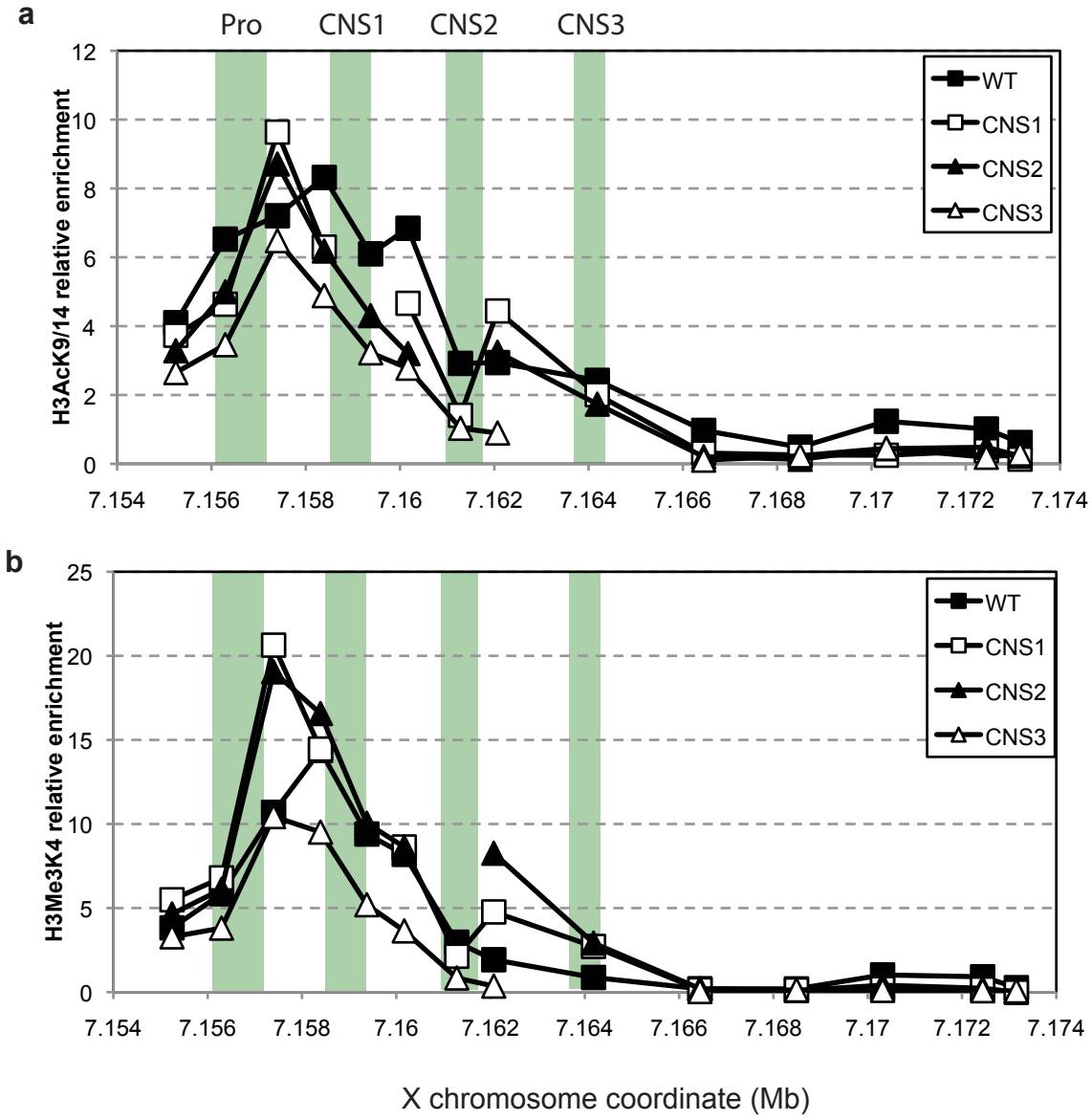
Supplementary Figure 1. **Dual luciferase assays on enhancer activity of Foxp3 CNS1, 2, and 3. a**. Foxp3 promoter sequence was inserted upstream of firefly luciferase gene in pGL3 vector. CNS1, 2, and 3 were inserted individually at the 3'-end of the luciferase gene. **b**. The luciferase reporter vectors were electroporated into EL-4 T cells along with a renilla luciferase vector (pRLTK) as internal control. Electroporated EL-4 cells were either untreated or incubated with PMA (50 ng/ml)+ Ionomycin (200 ng/ml) or PMA+Ionomycin+TGF-β (2 ng/ml) for 24 hrs before cells were harvested and luciferase activity was measured.



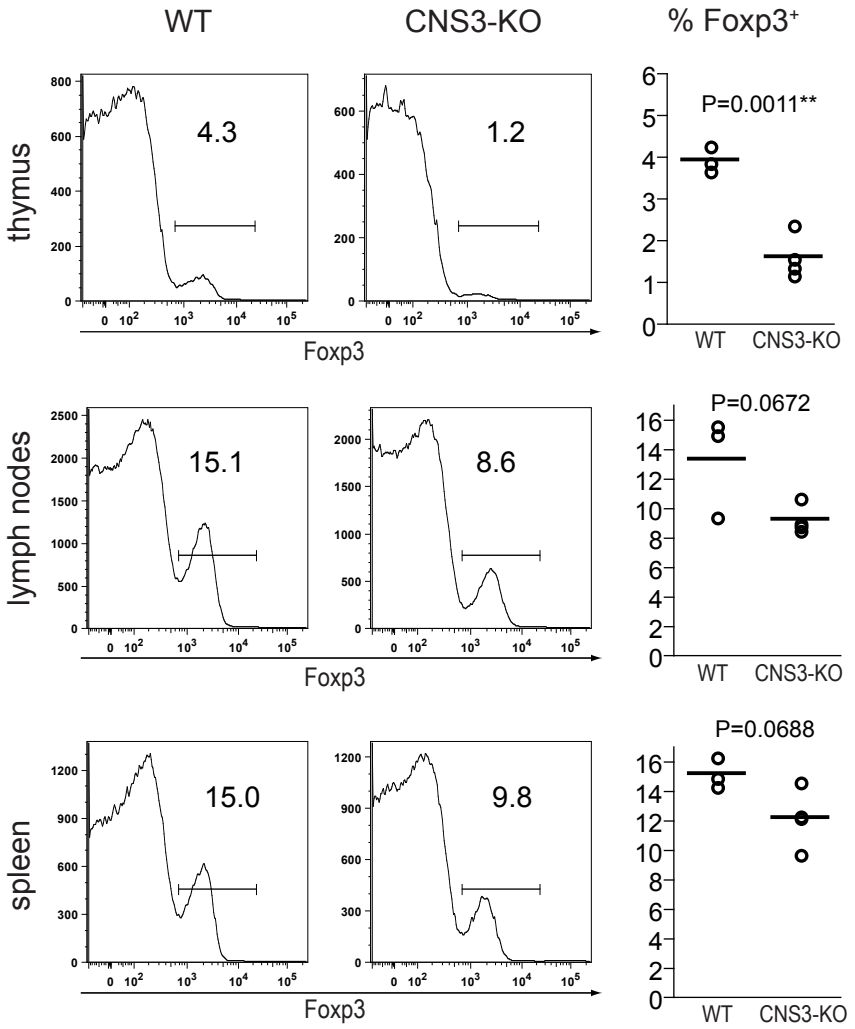
Supplementary Figure 2. Construct design for targeted deletion of three *Foxp3* CNS regions. Two LoxP sequences were placed flanking each CNS. A FRT flanked neomycin resistance cassette was inserted near each CNS to facilitate ES cell selection. Addtionally, an eGFP cassette was inserted in-frame into the first coding exon of the *Foxp3* gene. Mice with germ-line transmission of targeted mutations were crossed with FLPeR mice (with FLP recombinase expressed under control of ROSA26 locus) to remove the neomycin cassette and generate CNS floxed mice, which were then crossed with Mox2-Cre mice (with CRE recombinase expressed under control of *Mox2* locus) to induce germline deletion and generate CNS knockout (CNS-KO) mice.



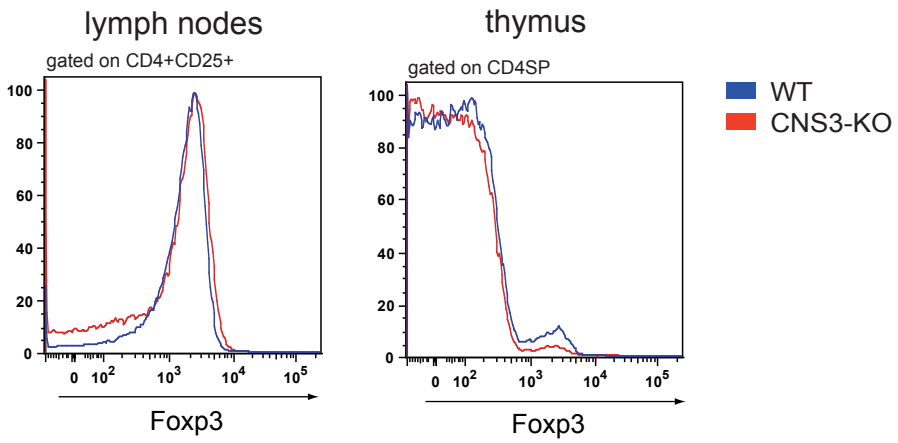
Supplementary Figure 3. CpG dinucleotide methylation at the Foxp3 locus. CpG methylation was determined by bisulfite treatment and pyrosequencing of genomic DNA from Foxp3-GFP$^-$ naïve CD4$^+$ T cells (Tn), CD3 and CD28 antibody stimulated Tn cells (Tstim), Foxp3-GFP$^+$ *in vitro* induced Treg cells (iTr), *ex vivo* isolated Foxp3-GFP$^+$ Treg (Tr), Tn (GFP$^-$) cells from *Foxp3^{gfpko}* mice (T_{FN} GFP$^-$), Foxp3-null GFP$^+$ T cells (T_{FN} GFP$^+$), Foxp3-GFP$^+$ Treg cells from CNS1, CNS2, and CNS3-KO mice (CNS1/CNS2/CNS3-KO Tr) at **a**, Foxp3 promoter, **b**, an intronic non-conserved sequence in 3' region of Foxp3, **c**, CNS2, the Foxp3 intronic CpG island, and **d**, a CpG island ~7kb upstream of Foxp3 in the promoter of the ubiquitously expressed *Ppp1r3f* gene.



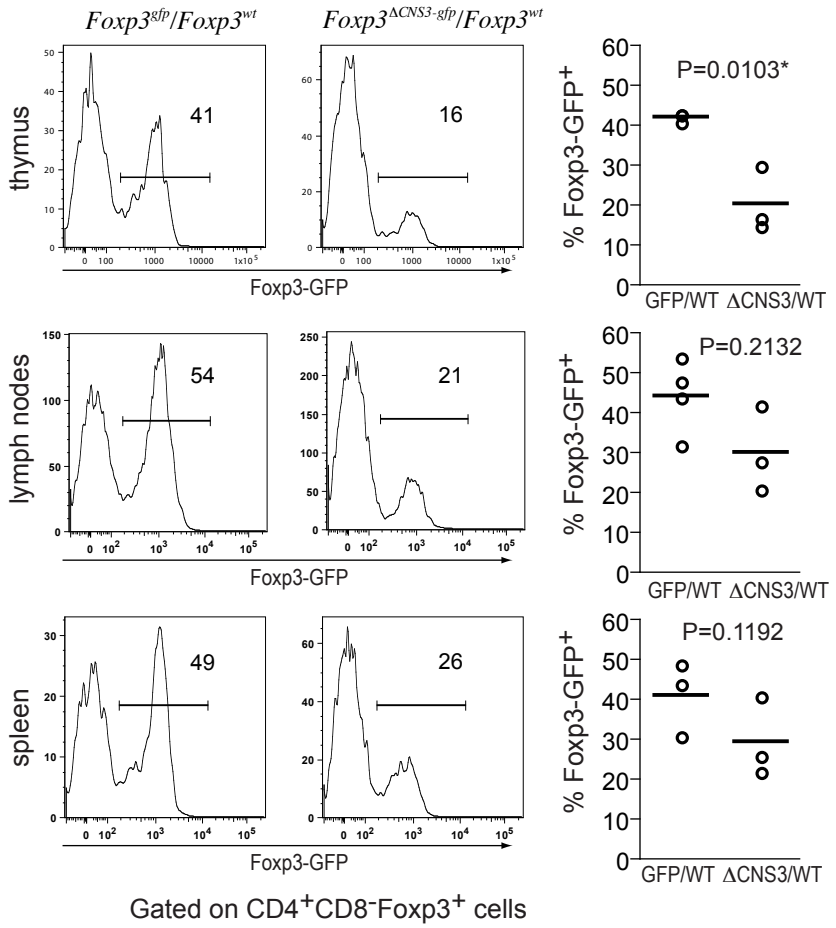
Supplementary Figure 4. Lack of gross alterations in *Foxp3* locus chromatin configuration in CNS-KO Treg cells. a, H3K9/14Ac and **b**, H3K4me3 histone modifications at the *Foxp3* locus in WT, CNS1-KO, CNS2-KO, and CNS3-KO Treg cells. ChIP was followed by qPCR with *Foxp3* locus tiling primers. Locations of Foxp3 promoter and CNS1, 2, 3 are shown in green shades.



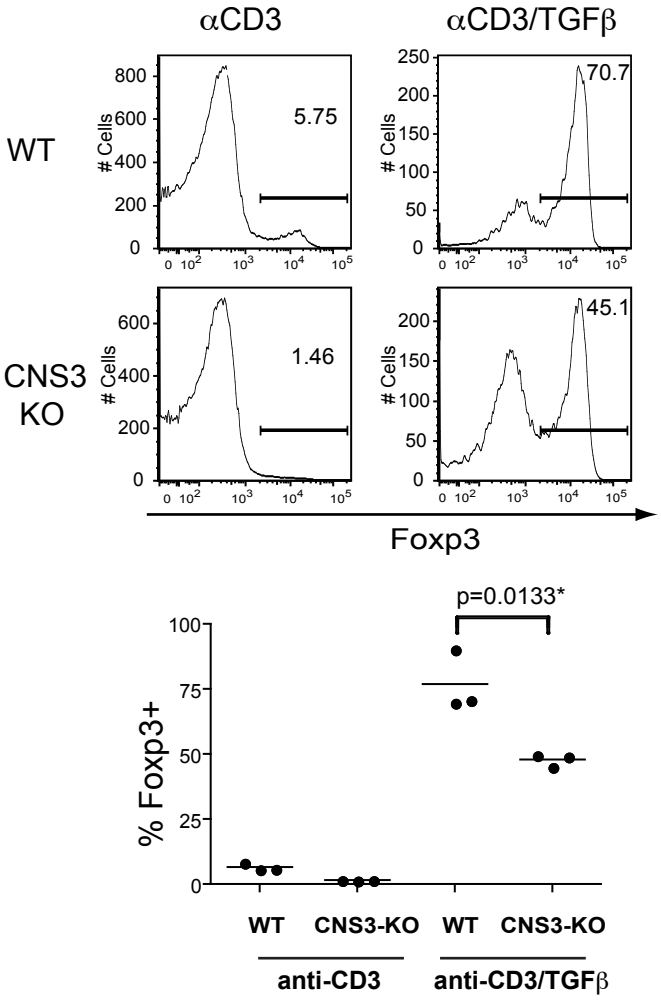
Supplementary Figure 5. Impaired differentiation of Foxp3$^+$ Treg cells in the thymus of adult CNS3-KO mice. Frequency of Foxp3$^+$ Treg cells among CD4SP thymocytes and CD4$^+$ cells in lymph nodes and spleen in six-week old CNS3-KO mice or littermate controls.



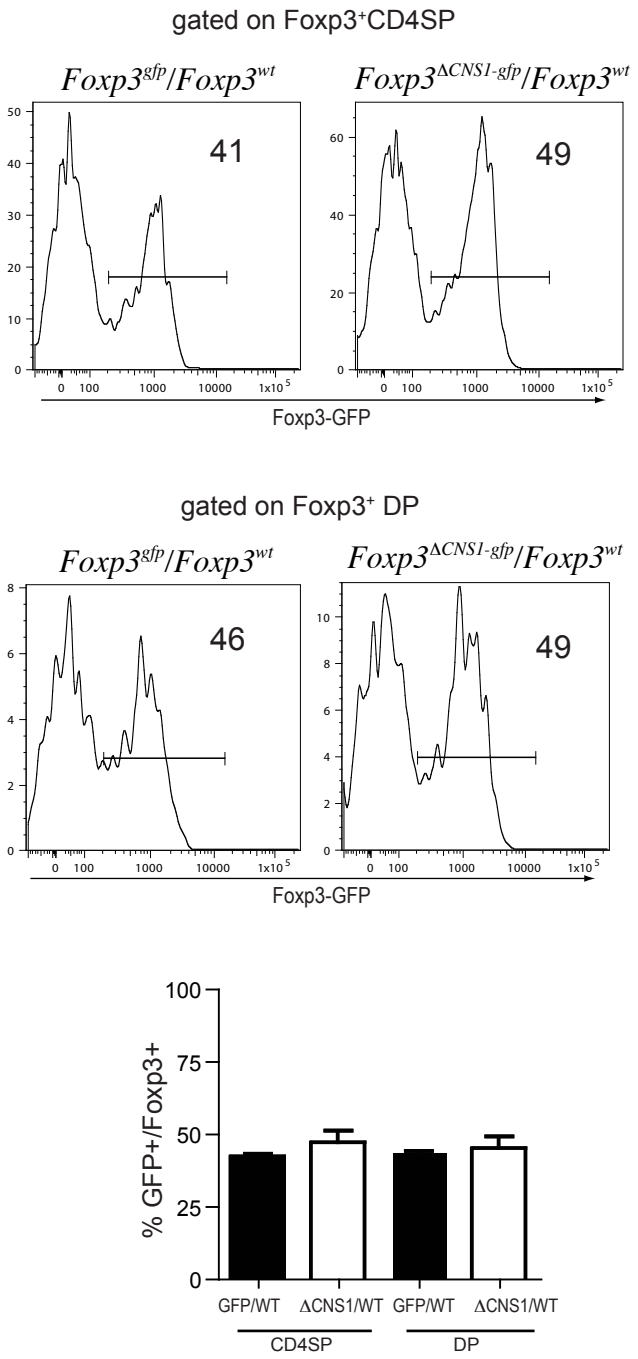
Supplementary Figure 6. CNS3 does not alter the expression level of Foxp3 on a per cell basis. FACS analysis of Foxp3 protein expression in $CD4^{+}CD25^{+}$ peripheral Treg cells (left) and CD4SP thymocytes (right) from CNS3-KO mice and littermate controls.



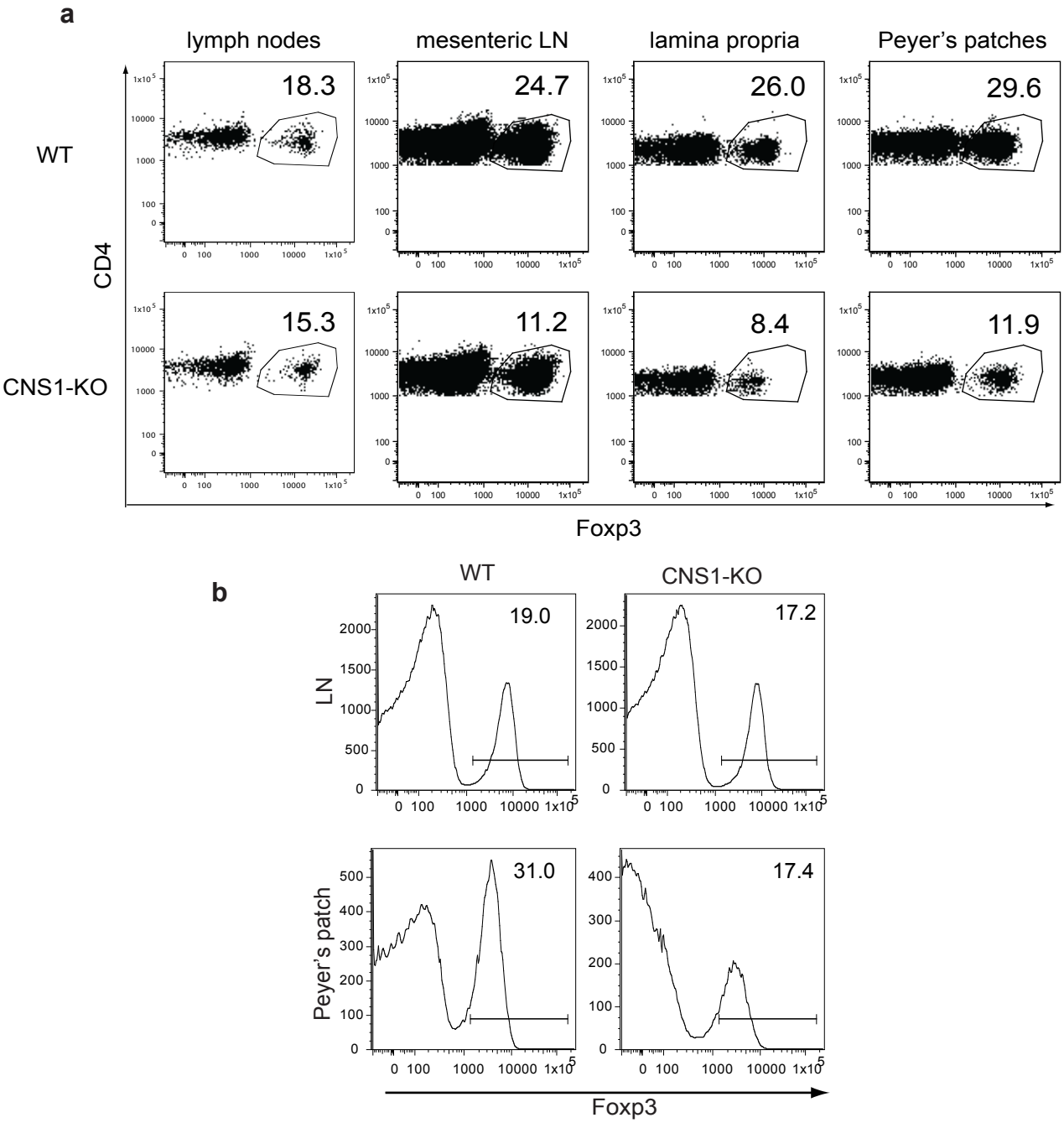
Supplementary Figure 7. CNS3-deficient Treg precursor cells are defective in the induction of Foxp3 compared with wild-type precursors in heterozygous female mice. Frequency of Treg cells expressing the Foxp3-GFP allele in *Foxp3*^{gfp}/*Foxp3*^{wt} (control) or *Foxp3*$^{\Delta CNS3\text{-}gfp}$/*Foxp3*^{wt} female mice. Foxp3^{+}CD4^{+}CD8^{-} thymocytes or Foxp3^{+}CD4^{+} peripheral T cells were gated on for determination of the frequencies of GFP^{+} cells among total Foxp3^{+} Treg cells.



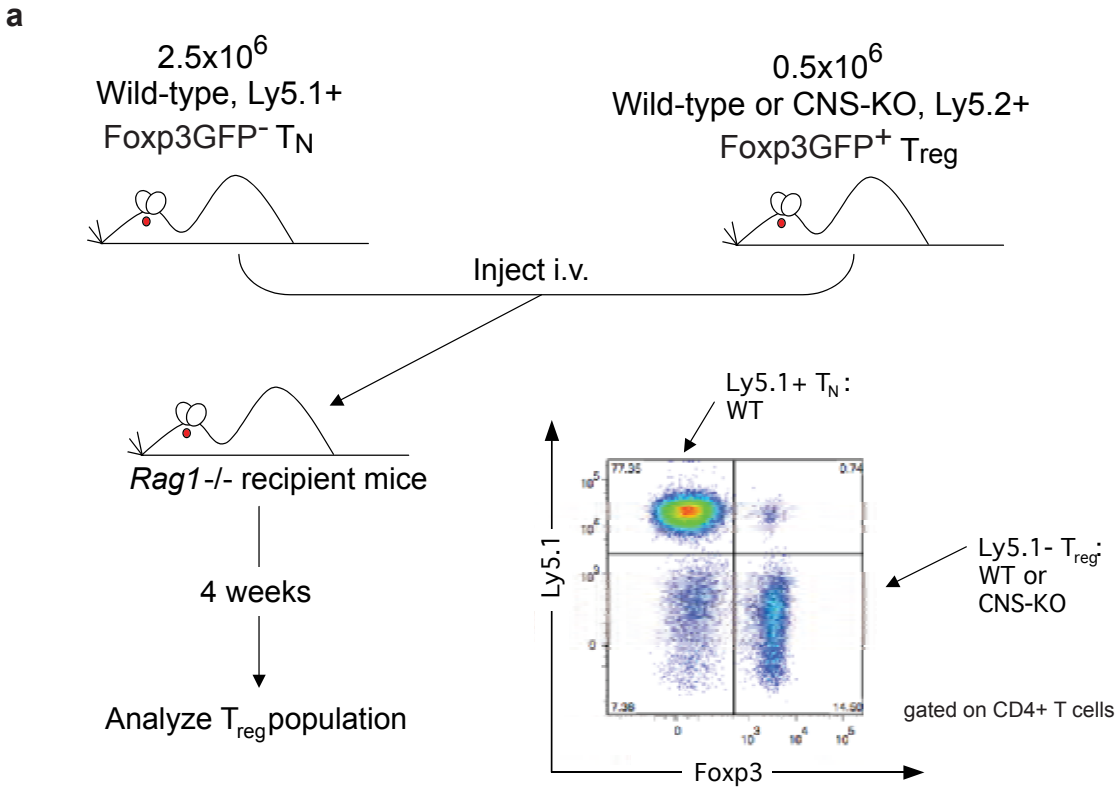
Supplementary Figure 8. iTreg differentiation is defective in CNS3-KO mice
Frequency of *in vitro* induced Foxp3^{+} cells after naïve CD4^{+}Foxp3^{-} T cells from CNS3-KO mice or littermate controls were stimulated with Ly5.1^{+} antigen presenting cells (APC) and anti-CD3 (1 μg/ml) for 3 days with or without TGF-β (2 ng/ml).

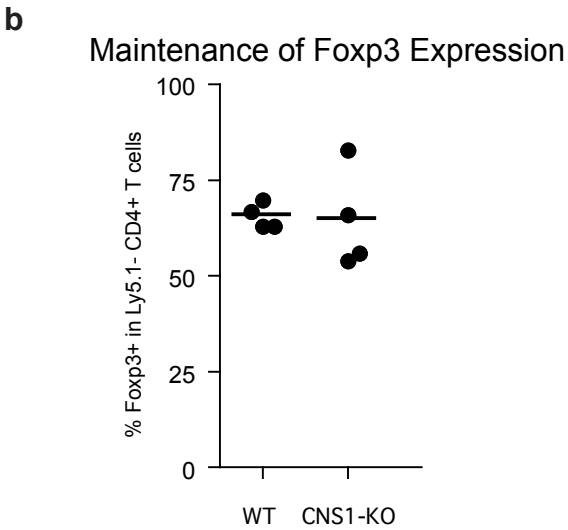


Supplementary Figure 9. CNS1 is dispensable for the differentiation of Foxp3$^+$ Treg cells in the thymus. Frequency of Treg cells expressing the Foxp3-GFP allele in heterozygous *Foxp3^{gfp}/Foxp3^{wt}* (control) or *Foxp3$^{\Delta CNS1\text{-}gfp}$/Foxp3^{wt}* mice. Foxp3$^+$ CD4SP thymocytes or Foxp3$^+$CD4$^+$CD8$^+$ (DP) thymocytes were gated for determination of the frequencies of GFP$^+$ cells among total Foxp3$^+$ Treg cells.

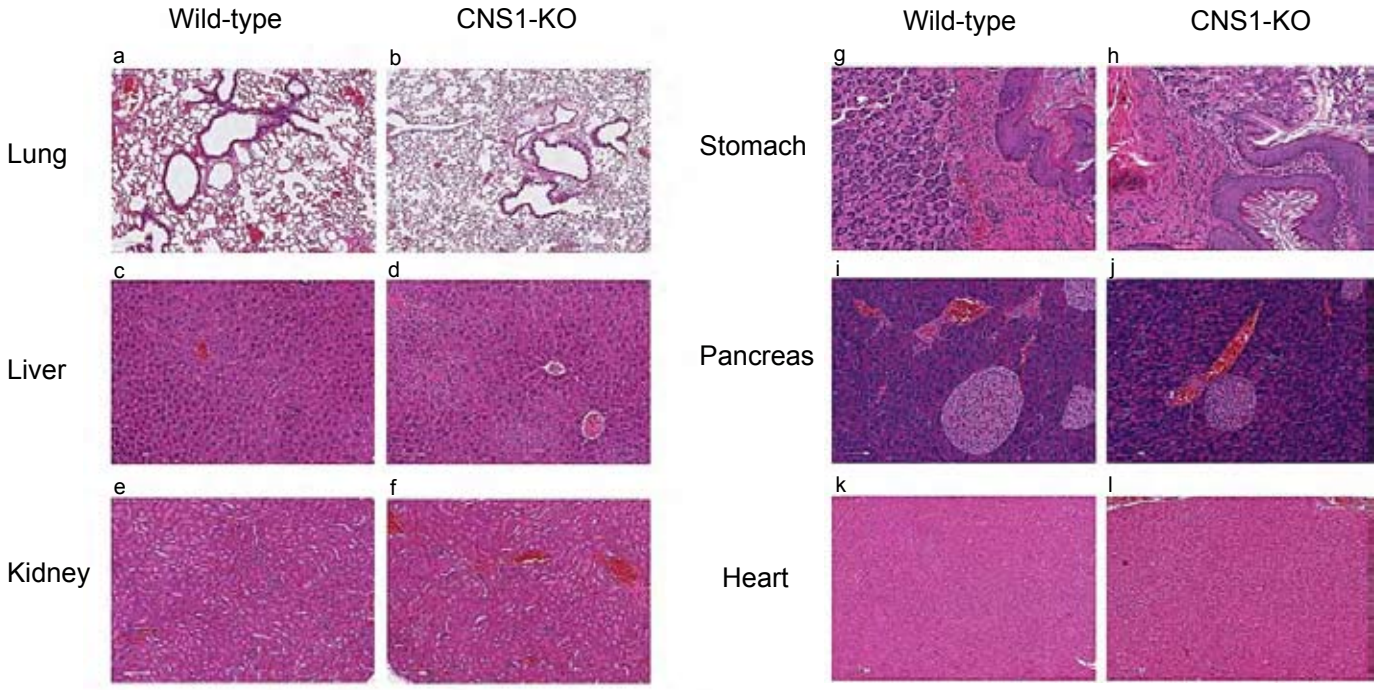


Supplementary Figure 10. Treg frequencies are reduced in the mesenteric lymph nodes and gut associate lymphoid tissues (GALT) of *Foxp3*$^{\Delta CNS1\text{-}gfp}$ (CNS1-KO) mice. **a**, Frequencies of Foxp3^{+} Treg cells among CD4^{+} T cells in "non-mesenteric" lymph nodes, mesenteric lymph nodes, lamina propria, and Peyer's patches from 4-month-old *Foxp3*$^{\Delta CNS1\text{-}gfp}$ and littermate control mice. **b**, Frequency of Foxp3^{+} Treg cells among CD4^{+} T cells in peripheral lymph nodes (non-mesenteric), and Peyer's patches of 8-11 month old *Foxp3*$^{\Delta CNS1\text{-}gfp}$ or littermate control mice; FACS plots are representative data from Fig 3d.

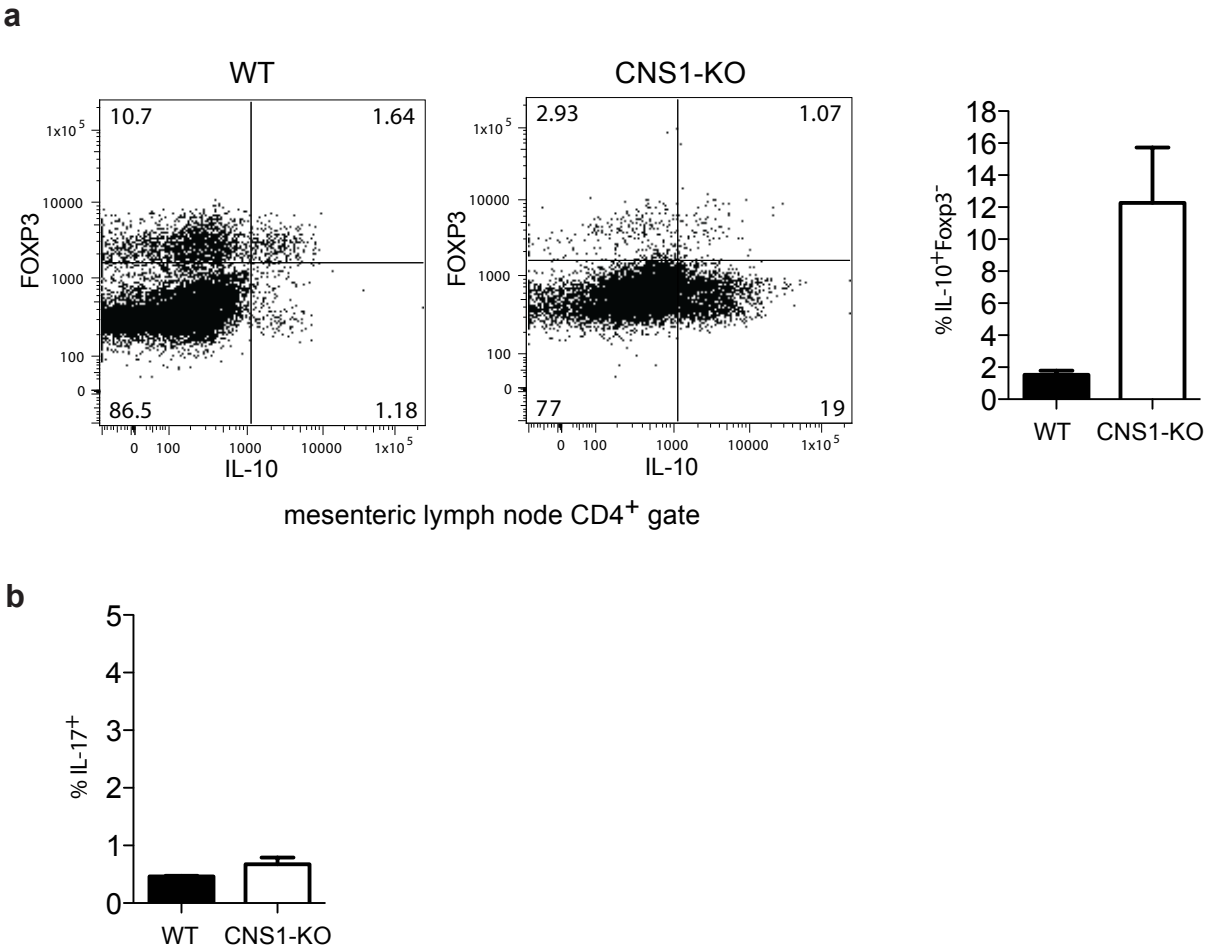




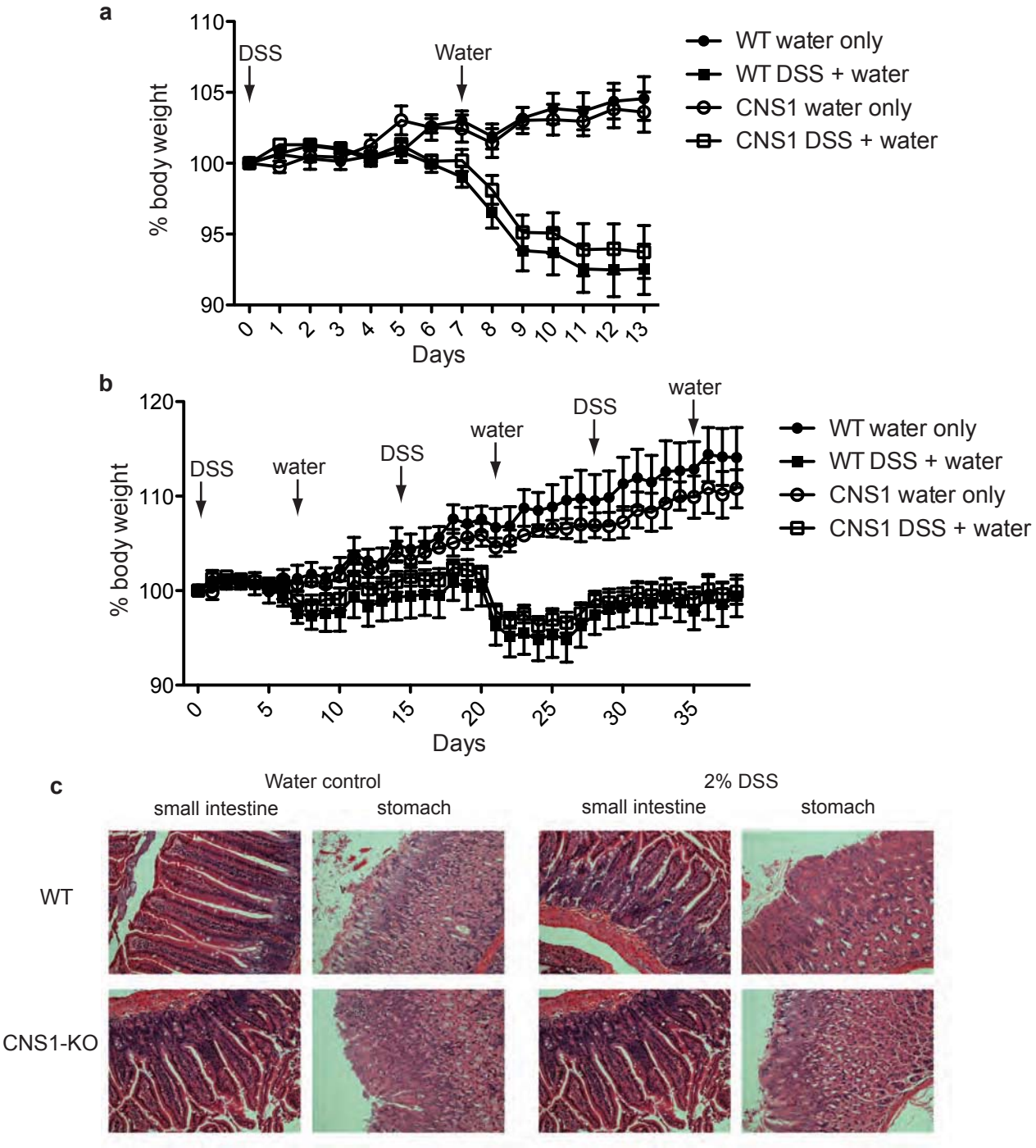
Supplementary Figure 11. CNS1-KO Treg cells are unimpaired in their ability to maintain Foxp3 expression. a, Schematics of the experimental approach. **b**, Maintenance of Foxp3 expression among wild-type, CNS1-KO Ly5.1$^-$ Treg cells isolated 4 weeks after transfer into T-cell-deficient recipients along with Ly5.1$^+$ wild-type T_N cells.



Supplementary Figure 12. Absence of significant inflammation in CNS1-KO mice. Representative examples of hematoxylin and eosin-stained tissue sections from 8-month-old wild type and CNS1-KO mice. There were no detectable differences in inflammation in the tissues examined between the two genotypes. **a** and **b**, Lung, original magnification 10X. **c** and **d**, Liver, original magnification 20X. **e** and **f**, Kidney, original magnification 10X. **g** and **h**, Limiting ridge of the stomach, original magnification 20X. **i** and **j**, Pancreas, original magnification 20X. **k** and **l**, Left ventricle of the heart, original magnification 10X.

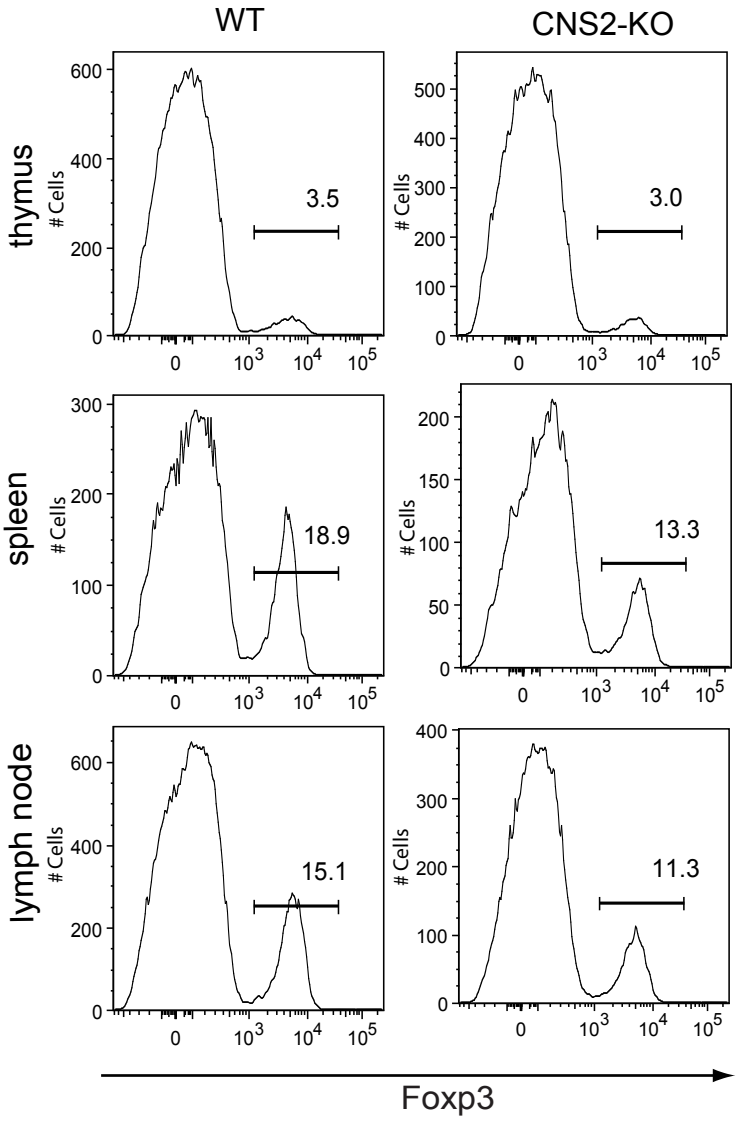


Supplementary Figure 13. CNS1-KO mice are enriched for IL-10 producing Foxp3$^-$ CD4$^+$ T cells in mesenteric lymph nodes (mLN). Cells were isolated from mLN and stimulated for 72 hours with plate-coated CD3 and CD28 antibodies, and cultured with 100 U/mL IL-2 for additional 48 hours prior to re-stimulation with PMA (50 ng/mL) and Ionomycin (250 ng/mL) for 5 hours. The frequencies of IL-10 (**a**) and IL-17 (**b**) expressing cells in CD4$^+$Foxp3$^-$ population were analyzed by flow cytometry.

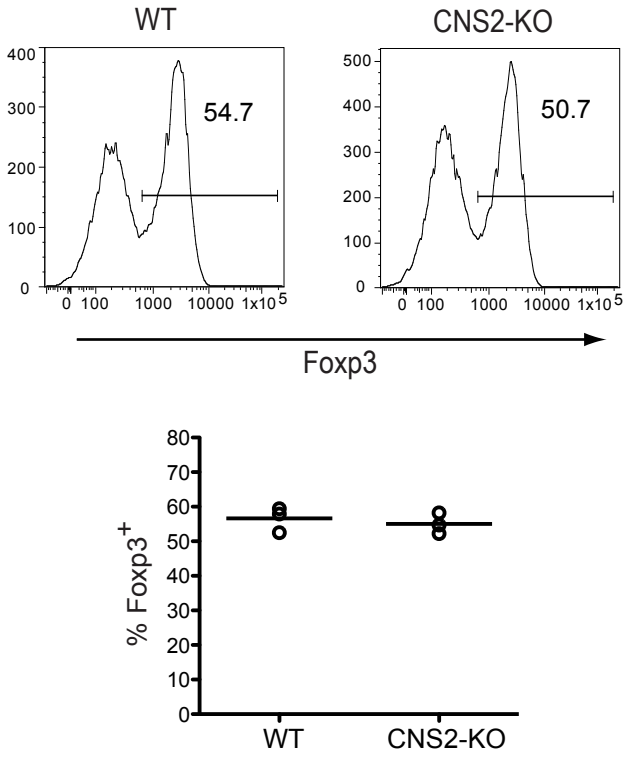


Supplementary Figure 14. Comparable weight loss and tissue inflammation in experimental model of DSS induced colitis in wild-type and CNS1-KO mice. Ability of regulatory T cells to suppress DSS induced colitis was previously demonstrated[1]. **a**, Acute phase weight loss in mice given DSS water. Mice were given 2% DSS water for 7 days and then given fresh water for another 6 days. Weight loss was monitored daily. **b**, **c**, chronic phase of DSS water induced colitis. Mice were alternatively given 2% DSS for 7 days and water without DSS for another 7 days. Weight loss was monitored daily. After 3 cycles of treatment, mice were sacrificed for histology analysis of gut lesions.

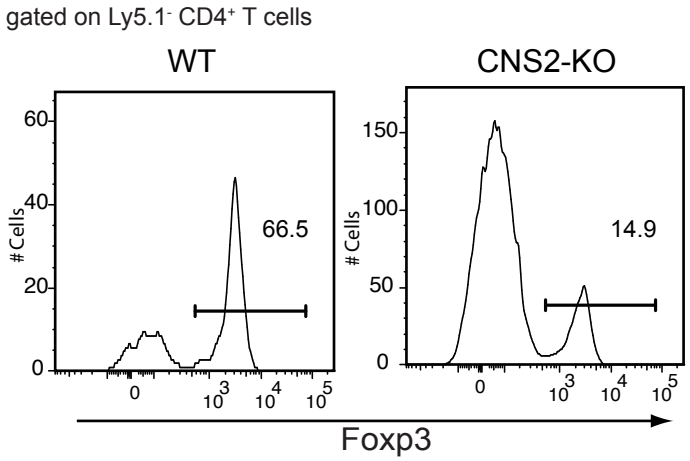
[1] Transcriptional repressor Blimp-1 regulates T cell homeostasis and function. Martins GA, et al., *Nature Immunology* 2006 vol. 7 (5) pp. 457-65.



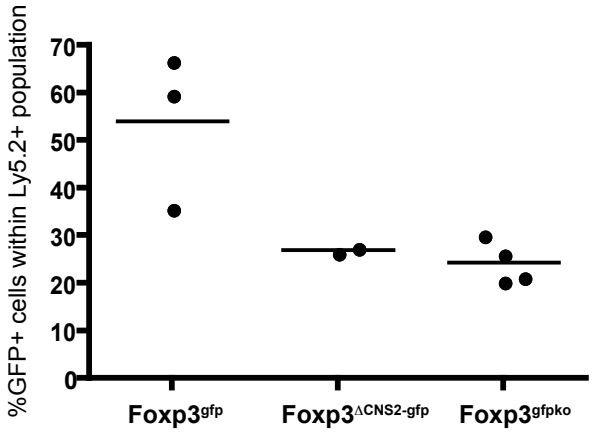
Supplementary Figure 15. CNS2 regulated maintenance of Foxp3 expression. Frequency of Foxp3^{+} Treg cells among CD4SP or CD4^{+} cells from thymus, lymph nodes, and spleen in six-month-old CNS2-KO mice or littermate controls; representative FACS plots from Fig 4a.



Supplementary Figure 16. Normal *in vitro* induction of Foxp3$^+$ iTreg cells from CNS2-KO naïve CD4$^+$ T cells. Frequency of Foxp3$^+$ iTreg cells generated upon stimulation of Foxp3GFP$^-$ CD4$^+$ T_N cells from CNS2-KO mice or littermate controls in the presence of CD3 antibody (1 μg/ml), TGF-β (2 ng/ml) and irradiated Ly5.1$^+$ APC. Cells were cultured for 3 days.



Supplementary Figure 17. Foxp3 and CNS2 control maintenance of Foxp3 expression. Maintenance of Foxp3 expression in Treg cells from Ly5.2^{+} CNS2-KO or wild-type mice 4 weeks after co-transferred with wild-type Ly5.1^{+}CD4^{+}Foxp3^{-} cells into T cell deficient recipient mice. Representative FACS plot from Fig 4b.



Supplementary Figure 18. Similar defect in maintenance of Foxp3 expression in Treg cells lacking CNS2 and T_{FN} cells lacking Foxp3 protein. Ly5.2^{+}Foxp3GFP^{+} Treg cells from *Foxp3*^{gfp}, *Foxp3*$^{\Delta CNS2\text{-}gfp}$ mice, or GFP^{+} T_{FN} cells from *Foxp3*^{gfpko} mice were co-transferred with Ly5.1^{+}CD4^{+} T cells into T-cell-deficient recipient mice. 12 days after transfer, CD4^{+} T cells from spleen were analyzed for Ly5.2^{+}GFP^{+} population.

Supplementary Figure 19. Runx1/AML1 motif in CNS2. Runx1/AML1 sequence motif at location +33 to +43 from the start of CNS2 sequence (Supplementary Table 1), top, and the Runx1/AML1 matrix, below, adapted from the JASPAR database (jaspar.genereg.net).

Supplementary Table 1. Sequences of double-stranded biotinylated DNA probes (sense strand)

Probe Name	Probe Sequence
Promoter	GCTTCAGATCCCTTCTTCTGTTCAACCCAGCGATCCTCCAACGTCTCACAAACAC AATGCTGTCTCTACCTGCCTCGGGATGCCTTTGTGATTTGACTTATTTTCCCTCAG TTTTTTTTTTCTGACTCTACACACTTTTGTTTAAGAAATTGTGGTTTCTCATGAGC CCTGTTATCTCATTGATACCTTTTACCTCTGTGGTGAGGGGAAGAAATCATATTTT CAGATGACTTGTAAAGGGCAAAGAAAAAACCCAAAATTTCAAAATTTCCGTTTA AGTCTCATAAGAAAAGAATAAACAAAGTAAGAGAGCAAAGAAAAAAAAACTAC AAGAACCCCCCCCCCACCCTGCAATTATCAGCACACACACTCATCAAAAAAAAA TTGGATTATTAGAAGAGCGAGGTCTGCGGCTTCCAC
CNS1	TAGATTACTCTTTTCTTGTGGGGCTTCTGTGTATGGTTTTGTGTTTTAAGTCTTTT GCACTTGAAAATGAGATAACTGTTCACCCCATGTTGGCTTCCAGTCTCCTTTATG GCTTCATTTTTTCCATTTACTGCAGAGGTCAAAAGTGTGGGTATGGGAGCCAGAC TGTCTGGAACAACCTAGCCTCAACTCAAGTCATCTGTGTGAATTTTACCCAGGCT CTTAACCTCTCTGTACCTCCATTTCCTCGTATGTACTGTGATGATTATAACAGTACC TACCTCAGAGGATCTTTCTGAGGATTATTTTTATTAATGATGGTAGGTGCTCAGCA CAAGGCC
CNS2	TGGGTTTTGCATGGTAGCCAGATGGACGTCACCTACCACATCCGCTAGCACCCAC ATCACCCTACCTGGGCCTATCCGGCTACAGGATAGACTAGCCACTTCTCGGAACG AAACCTGTGGGGTAGATTATCTGCCCCCTTCTCTTCCTCCTTGTTGCCGATGAAG CCCAATGCATCCGGCCGCCATGACGTCAATGGCAGAAAAATCTGGCCAAGTTCA GGTTGTGACAACAGGGCCCAGATGTAGACCCCGATAGGAAAACATATTCTATGT CCCAGAAACAACCTCCATACAGCTTCTAAGAAACAGTCAAACAGGAACGCCCC AACAGACAGTGCAGGAAGCTGGCTGGCCAGCCCAGCCCTCCAGGTCCCTAGTA CCACTAGACAGACCATATCCAATTCAGGTCCTCTTTCTGAGAATGTA
CNS3	GTGAGGCCCGGGGCCCAGAATGGGGTAAGCAGGGTGGGGTACTTGGGCCTATA GGTGTCGACCTTTACTGTGGCATGTGGCGGGGGGGGGGGGGGGCTGGGGCA CAGGAAGTGGTTTATGGGTCCCAGGCAAGTCTGACTTATGCAGATATTGCAGGG CCAAGAAAATCCCCACTCTCCAGGCTTCAGAGATTCAAGGCTTTCCCCACCCCT CCCAATCCTCATCCCGATAG
CNS3$^{\Delta c\text{-}Rel}$	GTGAGGCCCGGGGCCCAGAATGGGGTAAGCAGGGTGGGGTACTTGGGCCTATA GGTGTCGACCTTTACTGTGGCATGTGGCGGGGGGGGGGGGGGGCTGGGGCA CAGGAAGTGGTTTATGGGTCCCAGGCAAGTCTGACTTATGCAGATATTGCAGGG CCAAGAAAAGGTAGACTCTCCAGGCTTCAGAGATTCAAGGCTTTCCCCACCCCT CCCAATCCTCATCCCGATAG
CNS3 minimal c-Rel motif	CAAGAAAATCCCCAC
IL-2 CD28RE	TAAAGAAATTCCAGA
IL-2 ΔCD28RE	GTGGGGCTGGTACGA

Supplementary Table 2. Realtime PCR Primers for ChIP assays.

Primer Name	Primer Sequence
Foxp3_1kB_forward	TTCCTCCCGCTCTCTGACTCT
Foxp3_1kB_reverse	AAGCGCCAGTTGTGTACAAATATC
Foxp3_2kB_forward	ACTTAGTTTATGAGCATGCATGTTCTTC
Foxp3_2kB_reverse	TGAGATCCCACACCATCTTCTG
Foxp3_3kB_forward	TGTCCTGCACTGTTCCTCATG
Foxp3_3kB_reverse	AGAGTAGAAAACCGTGGCAGAGA
Foxp3_4kB_forward	GACCCAGGAGGCCATTAACA
Foxp3_4kB_reverse	AGATTTGGCCCCATGCTATG
Foxp3_5kB_forward	GTTGCCGATGAAGCCCAAT
Foxp3_5kB_reverse	ATCTGGGCCCTGTTGTCACA
Foxp3_6kB_forward	AGCCCCAGACATGATAGCAAA
Foxp3_6kB_reverse	TTGGGCATGTAGCTTCTGAGAA
Foxp3_7kB_forward	GTCATTGGAATAAAAAGATGAGAAGAGA
Foxp3_7kB_reverse	CCAGTACCCCCTGCACTCTGT
Foxp3_8kB_forward	AATGAATGAGACACAGAACTATTAAGATGA
Foxp3_8kB_reverse	CAGACGGTGCCACCATGAC
Foxp3_9kB_forward	GAGCTCTTGCCTCTCTGCTCTCT
Foxp3_9kB_reverse	GCAAACACGCACCATTGC
Foxp3_10kB_forward	CCCTGCCCTGTACAGACCAT
Foxp3_10kB_reverse	GTTTTGGAGGTCAGAAGGTTTGTAA
Foxp3_11kB_forward	GCTCACAGCTATCTCTAACTCCAGTTC
Foxp3_11kB_reverse	TGTCTGGGCATCGCATGT
Foxp3_12kB_forward	GGCTACAATGAAATGACAAGCTTAAG
Foxp3_12kB_reverse	TGGCTACGATGCAGCAAGAG
Foxp3_13kB_forward	TACGGCGGGTACTCAGTAAACAG
Foxp3_13kB_reverse	AATCCAAGGTCTCAATTCCTACCA
Foxp3_14kB_forward	GGCGATGATGTGCCTGCTA
Foxp3_14kB_reverse	GGCACCCCTCTCAGCTGTAA
Foxp3_15kB_forward	CACTGGGCTTCTGGGTATGTC
Foxp3_15kB_reverse	AGCTTGCGGCTCCTAATGC
Foxp3_16kB_forward	GCCATCAATCAGAGCCACAGT
Foxp3_16kB_reverse	ACTCAGCGCATCCTGGAGAT
Foxp3_17kB_forward	CTCGGCCACACTGAGTTCACT
Foxp3_17kB_reverse	GATCGTGGGCTGTGTGTATGA
Foxp3_upstream_1kB_forward	CTGAGGTTTGGAGCAGAAGGA
Foxp3_upstream_1kB_reverse	TCTGAAGCCTGCCATGTGAA
Foxp3_upstream_2kB_forward	GAGCCGGTCTGTGCCAAAT
Foxp3_upstream_2kB_reverse	GACTCCTCTGGAACTTGATGTTTGT
Foxp3_CNS1_forward	GTTTTGTGTTTTAAGTCTTTTGCACTTG
Foxp3_CNS1_reverse	CAGTAAATGGAAAAAATGAAGCCATA
Foxp3_CNS2_forward	GTTGCCGATGAAGCCCAAT
Foxp3_CNS2_reverse	ATCTGGGCCCTGTTGTCACA
Foxp3_CNS2.1_forward	TTGGGCTCTGGGACATCAAT
Foxp3_CNS2.1_reverse	GCCAACGGATTTTAGAATCTCAA
Foxp3_CNS2.2_forward	CCCTCTGGCATCCAAGAAAG
Foxp3_CNS2.2_reverse	GGGTGCTAGCGGATGTGGTA
Foxp3_CNS2.3_forward	GTTGCCGATGAAGCCCAAT
Foxp3_CNS2.3_reverse	ATCTGGGCCCTGTTGTCACA
Foxp3_CNS2.4_forward	CCCAACAGACAGTGCAGGAA
Foxp3_CNS2.4_reverse	AAAGAGGACCTGAATTGGATATGG

Primer Name	Primer Sequence
Foxp3_CNS2.5_forward	GATCCGCATTTGCTTGAGCTA
Foxp3_CNS2.5_reverse	AGCCTCCAGAACGCTAACATG
Ikzf2_forward	CCGTAAATAGAGGCTGCAGAAAG
Ikzf2_reverse	TGCTGCAGTGTTTTCCGAGTT
Pde3b_forward	TTTGGGCCGCATAGAGAAAA
Pde3b_reverse	CAGTGAATCATCAGCAGCACAA
Nt5e_forward	GCACAGCGTGCATCGCTAT
Nt5e_reverse	CAGGGCTTTCGGTTAATATCGT
Gmpr_forward	CAGCTGGAACAGCCTTGGAA
Gmpr_reverse	AAATGTCAAGGCCCCTGTGA