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Supplemental Information

Requirement of Mitochondrial Transcription Factor A in Tissue-Resident Regulatory T Cell Maintenance and Function

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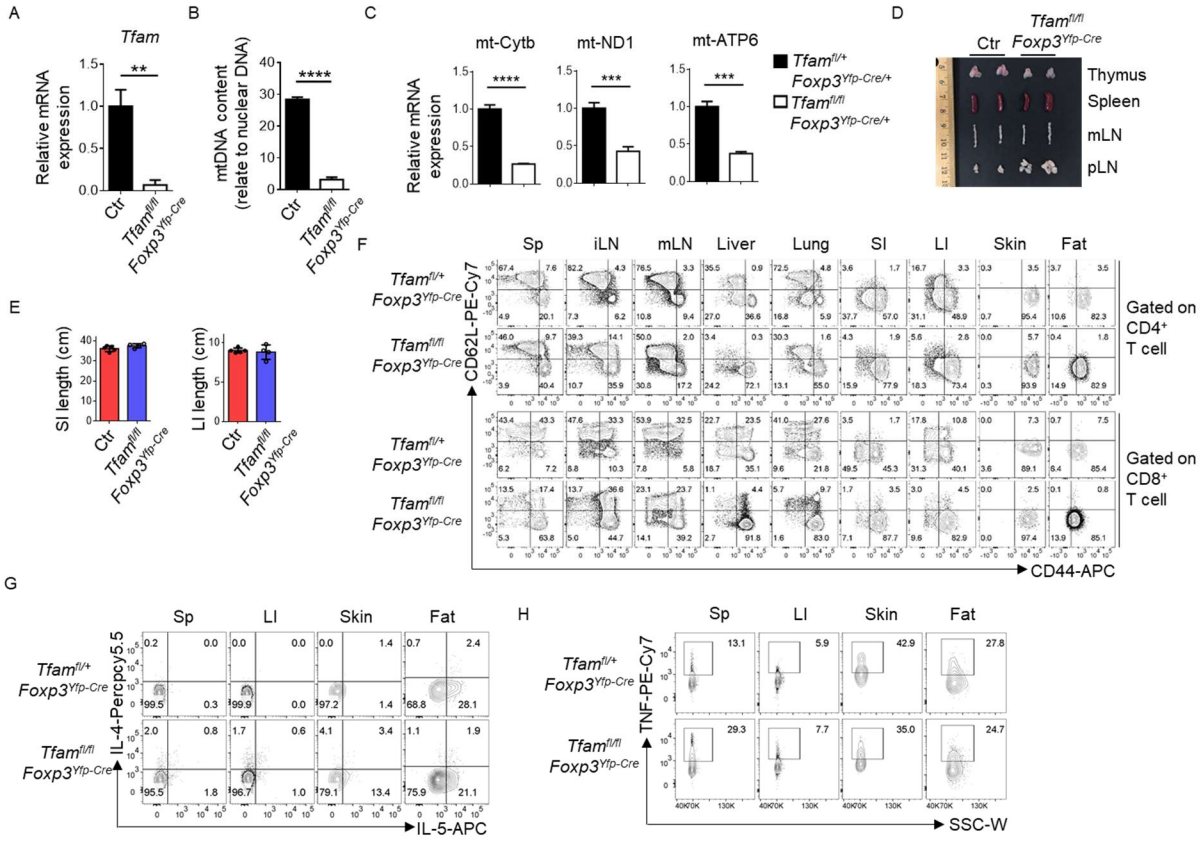

Figure S1. Treg-specific deletion of *Tfam* results in severe systemic inflammation in mice. Related to Figure 1.
Genotype of control (Ctr) group includes $Tfam^{+/+}Foxp3^{Yfp\text{-}Cre}$ and $Tfam^{fl/+}Foxp3^{Yfp\text{-}Cre}$ unless otherwise noted.
(A) Q-RT-PCR of *Tfam* (compared to *Actb* expression) in splenic Tregs from 6-week-old ctr (n=3) and $Tfam^{fl/fl}Foxp3^{Yfp\text{-}Cre}$ (n=3) mice (mean ± SD).
(B) Q-PCR of mitochondrial DNA content (mt-ND1 compared to nuclear gene *Hbb*) in splenic Tregs from 6-week-old ctr (n=3) and $Tfam^{fl/fl}Foxp3^{Yfp\text{-}Cre}$ (n=3) mice (mean ± SD).
(C) Q-RT-PCR of mitochondrial gene mt-Cytb, mt-ND1, mt-ATP6 expression (compared to *Actb* expression) in splenic YFP$^+$Tregs from 6-week-old female mice of indicated genotypes ($Tfam^{fl/+}Foxp3^{Yfp\text{-}Cre/+}$, n=3; $Tfam^{fl/fl}Foxp3^{Yfp\text{-}Cre/+}$, n=3) (mean ± SD).
(D) Representative picture of thymus, spleen, mesenteric lymph nodes (mLN) and peripheral lymph nodes (pLN) from 6-week-old control and $Tfam^{fl/fl}Foxp3^{Yfp\text{-}Cre}$ mice.
(E) Small intestine (SI) and large intestine (LI) length of 6-week-old ctr (n=5) and $Tfam^{fl/fl}Foxp3^{Yfp\text{-}Cre}$ (n=4) mice (mean ± SD).
(F) CD44 and CD62L staining in CD4$^+$ (up) and CD8$^+$ (down) T cells in 6-week-old mice with indicated genotypes by flow cytometry. Data were representative of three independent experiments.
(G) IL-4 and IL-5 expression in CD4$^+$ T cells in 6-week-old $Tfam^{fl/+}Foxp3^{Yfp\text{-}Cre}$ and $Tfam^{fl/fl}Foxp3^{Yfp\text{-}Cre}$ mice. Data were representative of three independent experiments.
(H) TNF expression in CD4$^+$ T cells in 6-week-old $Tfam^{fl/+}Foxp3^{Yfp\text{-}Cre}$ and $Tfam^{fl/fl}Foxp3^{Yfp\text{-}Cre}$ mice. Data were representative of three independent experiments.

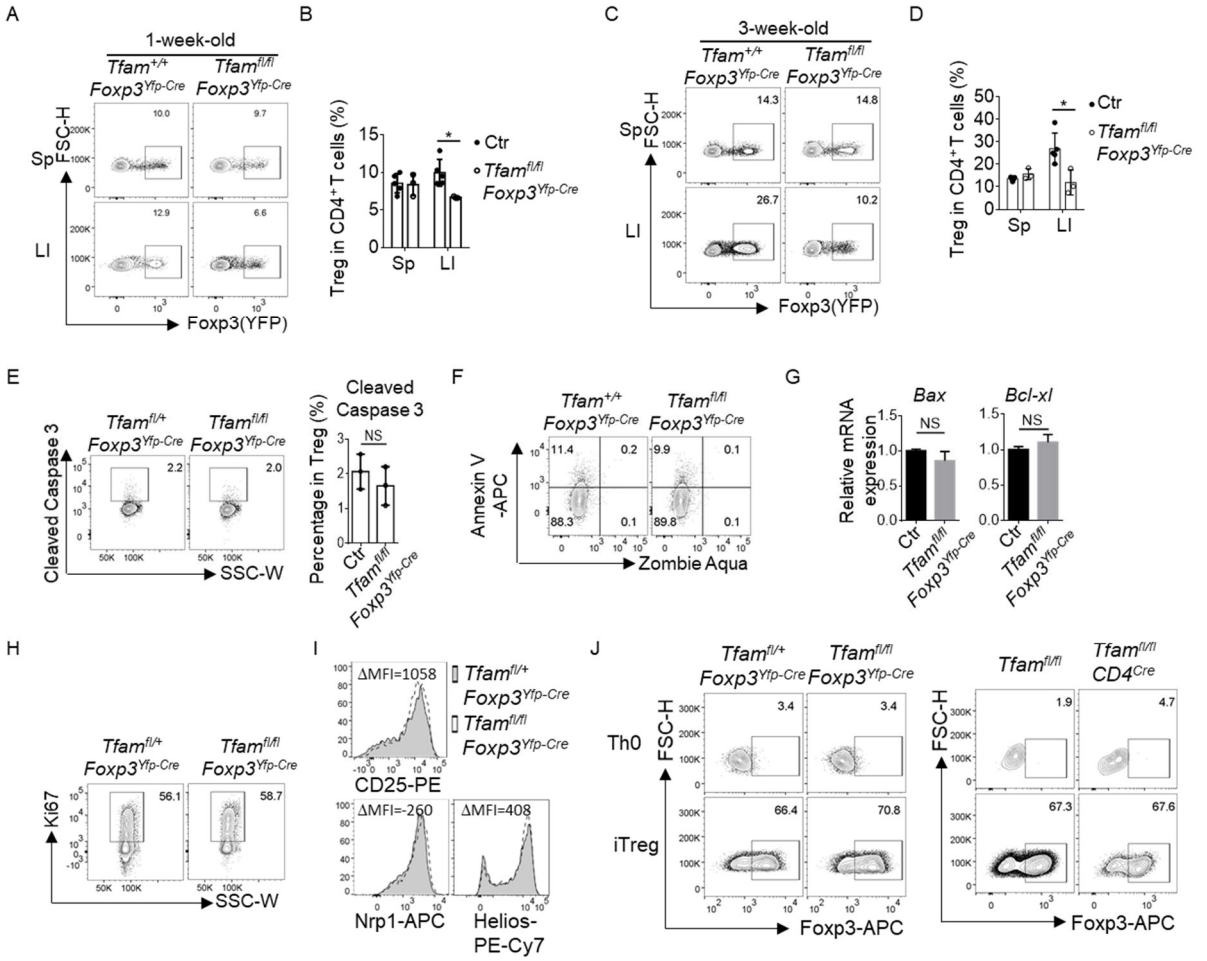

Figure S2. Tfam-deficiency in Tregs affects Treg maintenance. Related to Figure 2. Genotype of control (Ctr) group includes *Tfam*$^{+/+}$*Foxp3*$^{Yfp\text{-}Cre}$ and *Tfam*$^{fl/+}$*Foxp3*$^{Yfp\text{-}Cre}$ unless otherwise noted.

(A) Foxp3 (YFP) expression in CD4$^+$ T cells in Sp and LI of 1-week-old mice of indicated genotypes by flow cytometry. Data were representative of two independent experiments.

(B) Statistical analysis of percentage of Tregs in CD4$^+$T cells showed in A (Ctr, n=5; *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre}$, n=3, mean ± SD).

(C) Foxp3 (YFP) expression in CD4$^+$ T cells in spleen (Sp) and large intestine (LI) of 3-week-old mice of indicated genotypes by flow cytometry. Data were representative of two independent experiments.

(D) Statistical analysis of percentage of Tregs in CD4$^+$ T cells showed in C (Ctr, n=5; *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre}$, n=3, mean ± SD).

(E) Left, representative FACS of cleaved Caspase3 staining in large intestinal Tregs of 3- to 4-week-old mice with indicated genotypes. Data were representative of two independent experiments. Right, statistical analysis of cleaved caspase 3 staining in Tregs (n=3, mean ± SD).

(F) Annexin V/Zombie Aqua staining in YFP$^+$ Tregs from large intestine of 3- to 4-week-old mice with indicated genotypes. Data were representative of three independent experiments.

(G) Q-RT-PCR of *Bax* and *Bcl-xl* expression (compared to *Actb* expression) in splenic Tregs from 6-week-old mice with indicated genotypes (n=3, mean ± SD). Data were representative of two independent experiments.

(H) Representative figure of Ki67 staining in large intestinal Tregs from 3- to 4-week-old mice with indicated genotypes. Data were representative of three independent experiments.

(I) Flow staining of CD25, Nrp1 and Helios in splenic Tregs in 6-week-old mice with indicated genotypes. Data were representative of two independent experiments.

(J) Left, *in vitro* Treg (iTreg) differentiation from splenic naïve CD4$^+$ T cells isolated from *Tfam*$^{fl/+}$*Foxp3*$^{Yfp\text{-}Cre}$ and *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre}$ mice by TGF-β (5 ng/ml). Right, iTreg differentiation from splenic naïve CD4$^+$ T cells isolated from *Tfam*$^{fl/fl}$ and *Tfam*$^{fl/fl}$*CD4*^{Cre} mice. Data were representative of two independent experiments.

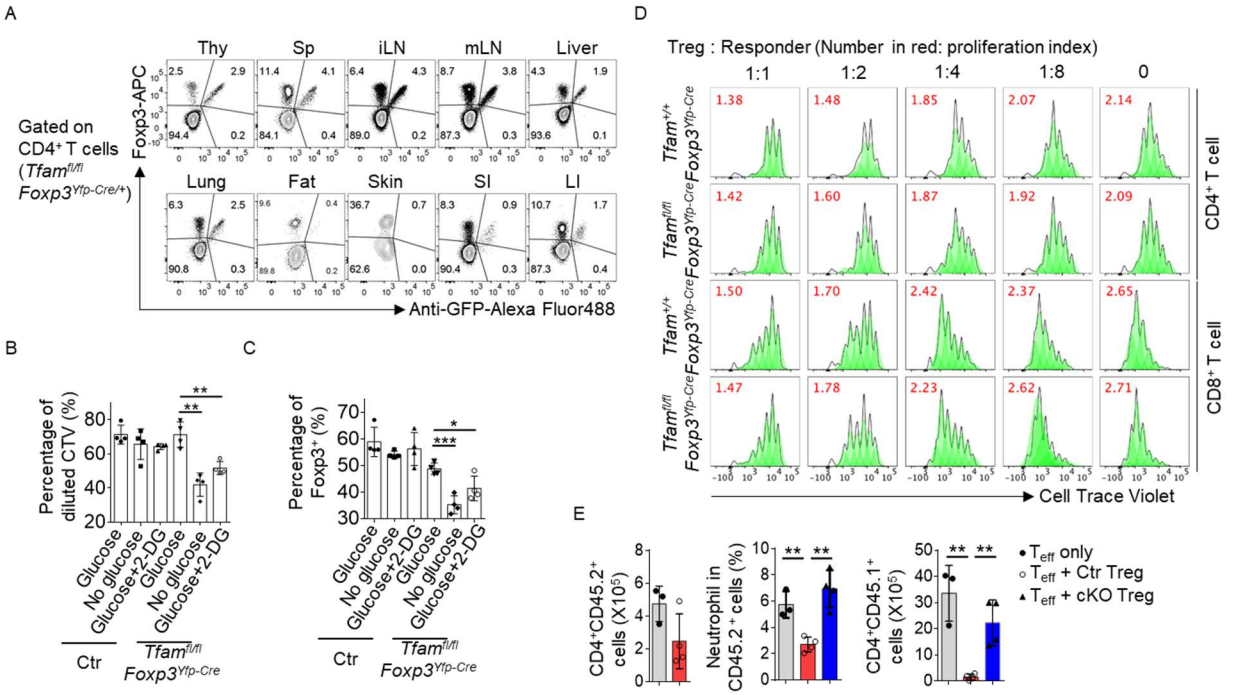

Figure S3. Tfam-deficiency in Tregs affects Treg maintenance and function. Related to Figure 2 and Figure 3. Genotype of control (Ctr) group includes *Tfam*$^{+/+}$*Foxp3*$^{Yfp\text{-}Cre}$ and *Tfam*$^{fl/+}$*Foxp3*$^{Yfp\text{-}Cre}$ while cKO indicates *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre}$.

(A) Foxp3 and YFP (anti-GFP antibody) staining in CD4$^+$ T cells from indicated organs of *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre/+}$ mice. Data were representative of three independent experiments.

(B) Statistic analysis of cell tracer violet (CTV) dilution as shown in Figure 2D.

(C) Statistic analysis of Foxp3(YFP) expression as shown in Figure 2E.

(D) *In vitro* Treg suppression assay for *Tfam*$^{+/+}$*Foxp3*$^{Yfp\text{-}Cre}$ and *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre}$ Tregs. Responder T cells were labeled with CTV and cocultured with *Tfam*$^{+/+}$*Foxp3*$^{Yfp\text{-}Cre}$ or *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre}$ splenic Tregs at different ratios. CTV dilution signals were analyzed at Day3 by flow cytometry. Data were representative of two independent experiments.

(E) Quantification of CD4$^+$CD45.2$^+$ cells (recovered donor Tregs) (left), neutrophils (middle) and infiltrating lymphocytes (right) in the large intestine of T cell-induced colitis model (T_{eff} only, n=3; T_{eff} + Ctr Treg, n=4; T_{eff} + cKO Treg, n=4) (mean ± SD).

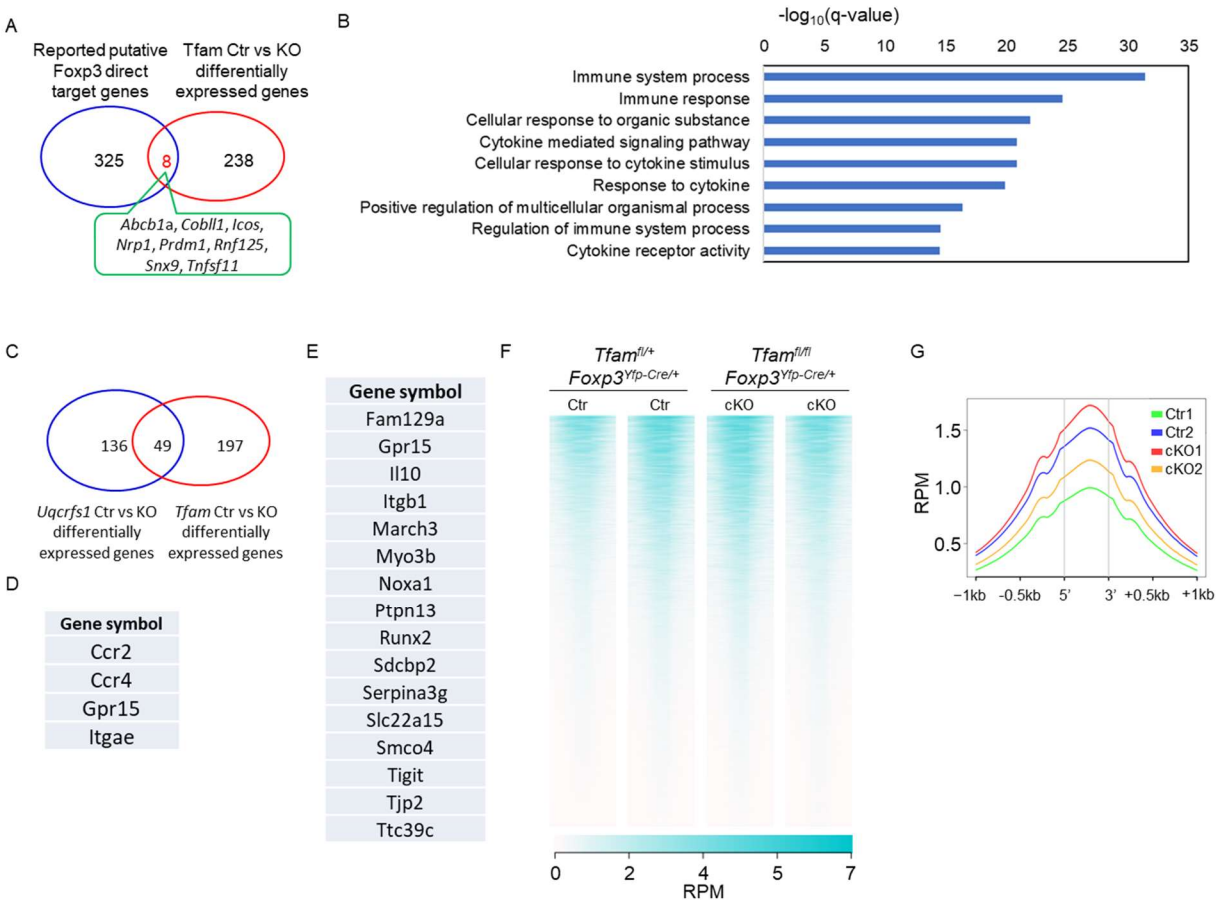

Figure S4. Bioinformatic analysis of RNA-seq and ATAC-seq data. Related to Figure 4.

(A) Comparison of reported Foxp3 direct target genes (a total of 333 genes) and differentially expressed genes (DEGs) identified between control and Tfam-deficient Tregs by the RNA-seq analysis (a total of 246 genes).

(B) GO analysis of RNA-seq identified 246 differentially expressed genes between *Tfam*$^{fl/+}$*Foxp3*$^{Yfp\text{-}Cre/+}$ (Ctr) and *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre/+}$ (cKO) Tregs.

(C) Comparison of DEGs between control and RISP-deficient Tregs (a total of 185 genes, fold change≥1.5, q≤0.05) and DEGs between control and Tfam-deficient Tregs (a total of 246 genes, fold change≥1.5, q≤0.05). DEG analysis between control and RISP-deficient Tregs and between control and Tfam-deficient Tregs were performed with same analysis method and criteria as described in the methods.

(D) Chemokine receptor and adhesion molecules among the shared 49 genes shared by DEGs between control and RISP-deficient Tregs and DEGs between control and Tfam-deficient Tregs.

(E) Gene list of 16 differentially expressed genes in RNA-seq that had significant changes in chromatin remodeling revealed by ATAC-seq.

(F) ATAC-seq signal (Reads Per Million mapped reads; RPM) across all peak locations comparing YFP^{+} Tregs from *Tfam*$^{fl/+}$*Foxp3*$^{Yfp\text{-}Cre/+}$ (Ctr) and *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre/+}$ (cKO) mice.

(G) Average ATAC-seq peak signal (RPM) centered on all peak locations (signal was calculated from 5' to 3' end of the peaks ± 1kb) of Tregs.

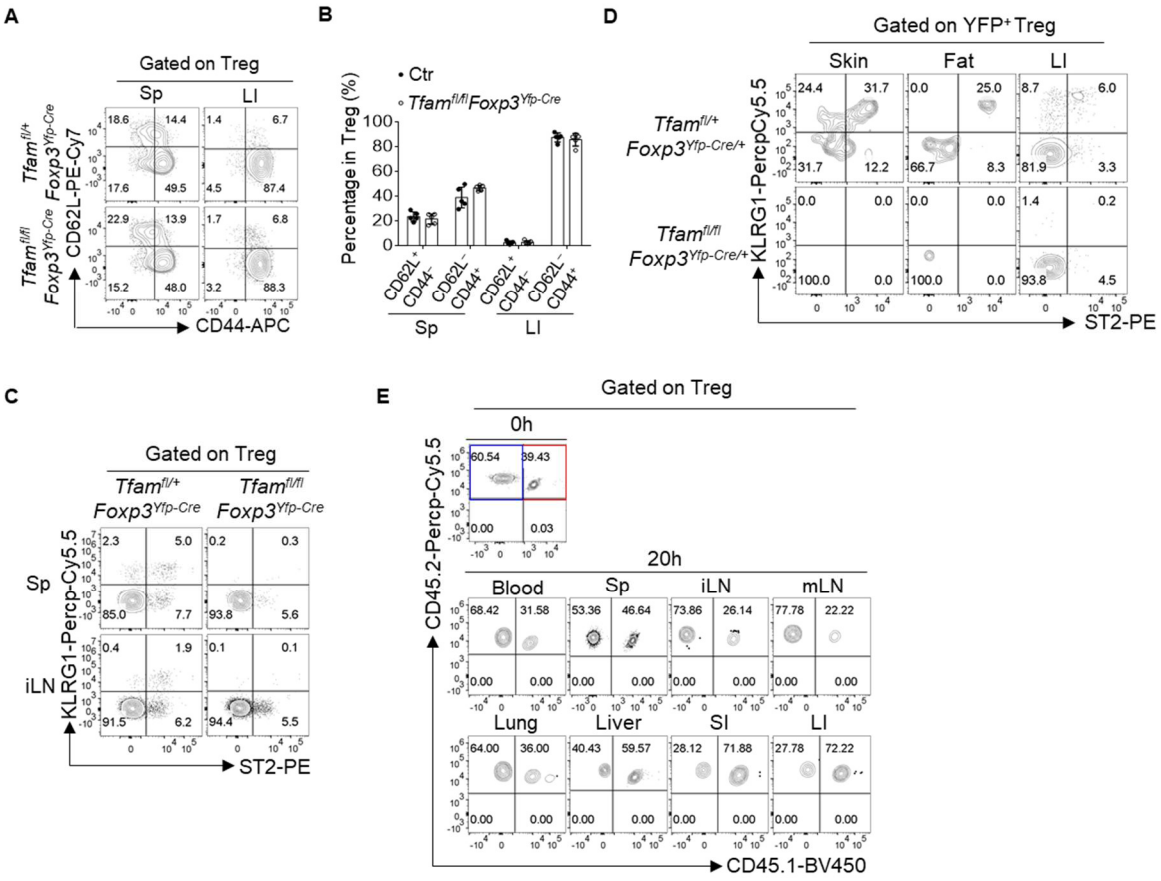

Figure S5. Tfam is essential for ST2$^+$KLRG1$^+$ Treg generation and controls Treg homing. Related to Figure 5. Genotype of control (Ctr) group includes *Tfam*$^{+/+}$*Foxp3*$^{Yfp\text{-}Cre}$ and *Tfam*$^{fl/+}$*Foxp3*$^{Yfp\text{-}Cre}$ while cKO indicates *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre}$.

(A) CD44 and CD62L staining in Tregs from spleen and large intestine of 6-week-old *Tfam*$^{fl/+}$*Foxp3*$^{Yfp\text{-}Cre}$ and *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre}$ mice. Data were representative of two independent experiments.

(B) Statistic analysis of percentage of CD62L$^+$CD44$^-$ and CD62L$^-$CD44$^+$ Tregs within Tregs (mean ± SD, n=5 for each group).

(C) ST2 and KLRG1 staining in Tregs from spleen and iLN of 6-week-old *Tfam*$^{fl/+}$*Foxp3*$^{Yfp\text{-}Cre}$ and *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre}$ mice. Data were representative of two independent experiments.

(D) ST2 and KLRG1 staining in YFP$^+$ Tregs from 6-week-old *Tfam*$^{fl/+}$*Foxp3*$^{Yfp\text{-}Cre/+}$ and *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre/+}$ female mice. Data were representative of two independent experiments.

(E) Representative FACS plot of *in vivo* Treg homing analysis of control and Tfam-deficient Tregs. Data were representative of four independent experiments. Calculation formula for migration index: ($Ratio_{\text{after transfer}}$ ($Treg_{cKO}$/ $Treg_{ctr}$))/($Ratio_{\text{before transfer}}$($Treg_{cKO}$/ $Treg_{ctr}$)).

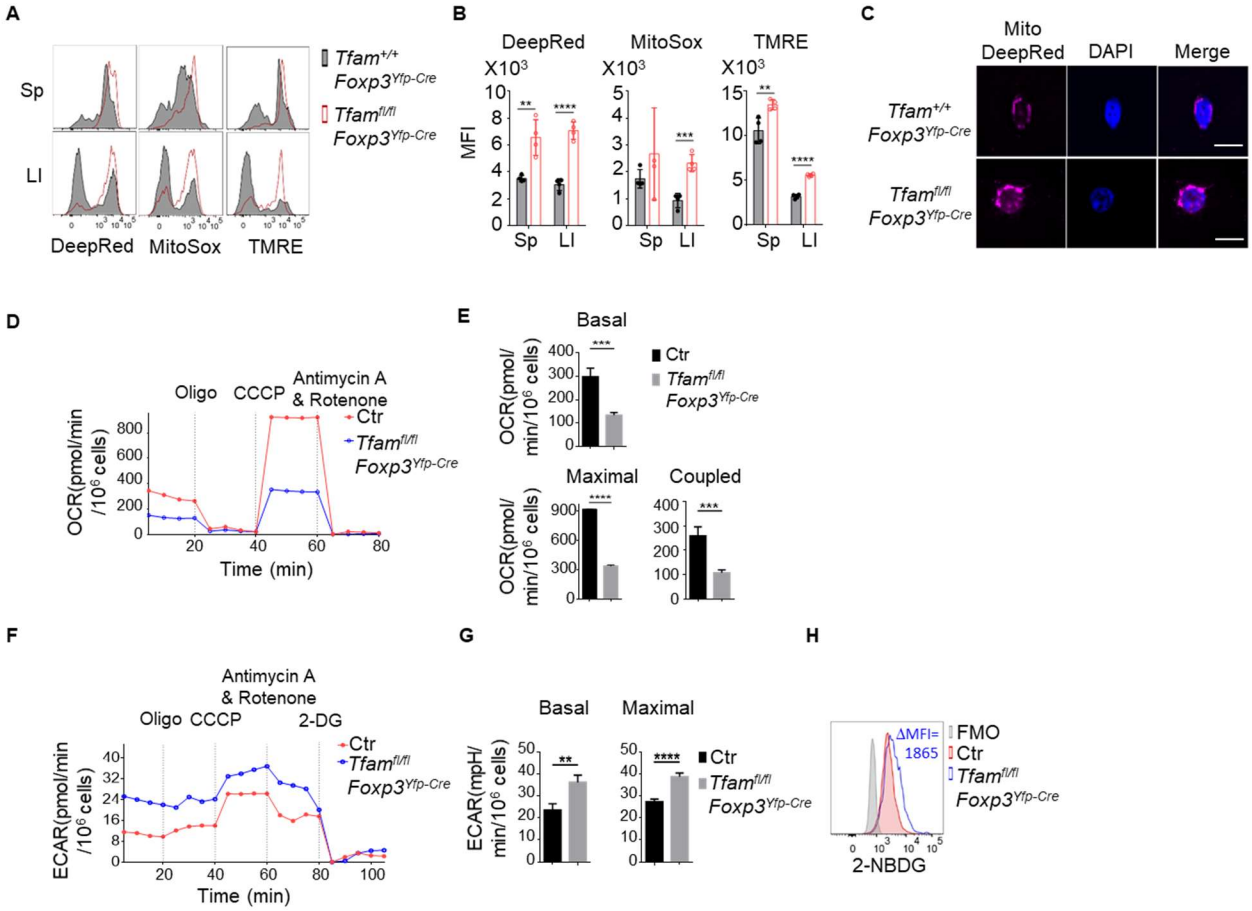

Figure S6. Tfam-deficiency in Tregs switches oxidative phosphorylation towards glycolysis. Related to Figure 6. Genotype of control (Ctr) group includes *Tfam*$^{+/+}$*Foxp3*$^{Yfp\text{-}Cre}$ and *Tfam*$^{fl/+}$*Foxp3*$^{Yfp\text{-}Cre}$ unless otherwise noted.

(A) Mitochondria staining by Mitotracker-Deepred, MitoSox and TMRE in Tregs from the spleen (Sp) and large intestine (LI) of mice with the indicated genotypes. Data were representative of three independent experiments.

(B) Statistic analysis of Mitotracker-DeepRed, TMRE and MitoSox staining as shown in A (mean ± SD).

(C) Confocal microscopy analysis of Mitotracker-DeepRed staining in *Tfam*$^{+/+}$*Foxp3*$^{Yfp\text{-}Cre}$ and *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre}$ splenic Tregs. Data were representative of three independent experiments.

(D) OCR measurement of Tregs pooled from spleens and pLNs of 6-week-old ctr (n=3) and *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre}$ mice (n=3). Representative data of two independent experiments. Each sample contained Tregs sorted from pooled spleen and pLNs of 3 to 5 mice.

(E) Quantification of basal, maximal and coupled OCR of Tregs. Representative data of two independent experiments and are shown as mean ± SD (n=4 time points).

(F) ECAR measurement of Tregs from ctr and *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre}$ mice. Representative data of two independent experiments. Each sample contained Tregs sorted from pooled spleen and pLNs of 3 to 5 mice.

(G) Quantification of basal and maximal ECAR of Tregs. Representative data of two independent experiments and are shown as mean ± SD (n=4 time points).

(H) 2-NBDG incorporation of splenic Tregs from 6-week-old ctr and *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre}$ mice. ΔMFI was calculated by subtracting MFI value of ctr Tregs from MFI value of *Tfam*$^{fl/fl}$*Foxp3*$^{Yfp\text{-}Cre}$ Tregs. Representative data of two independent experiments.

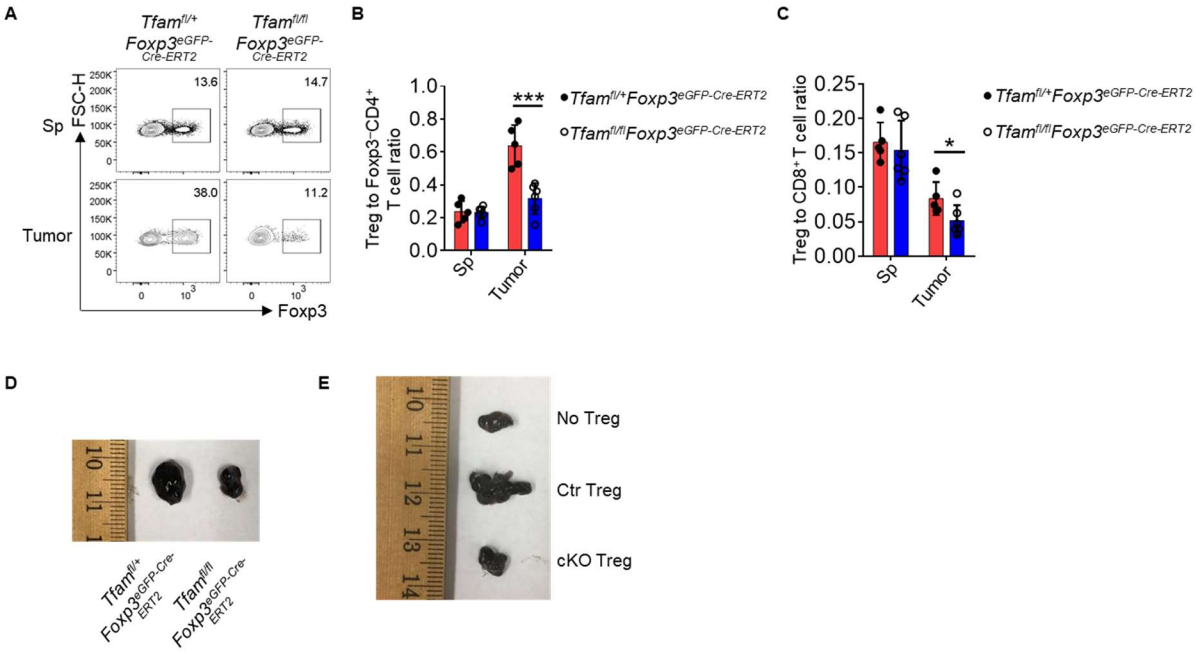

Figure S7. Tfam-deficiency in Tregs affects Treg maintenance in tumor. Related to Figure 7.

(A) Representative FACS plot of Foxp3 staining in CD4$^+$ T cells in the spleen and tumor from *Tfam$^{fl/+}$Foxp3$^{eGFP\text{-}Cre\text{-}ERT2}$* or *Tfam$^{fl/fl}$Foxp3$^{eGFP\text{-}Cre\text{-}ERT2}$* mice. Data were representative of two independent experiments.

(B) Tregs to Foxp3$^-$CD4$^+$ T cells ratio in the spleen and tumor as shown in A (*Tfam$^{fl/+}$Foxp3$^{eGFP\text{-}Cre\text{-}ERT2}$*, n=5; *Tfam$^{fl/fl}$Foxp3$^{eGFP\text{-}Cre\text{-}ERT2}$*, n=6; mean ± SD).

(C) Tregs to CD8$^+$ T cells ratio in the spleen and tumor as shown in A (*Tfam$^{fl/+}$Foxp3$^{eGFP\text{-}Cre\text{-}ERT2}$*, n=5; *Tfam$^{fl/fl}$Foxp3$^{eGFP\text{-}Cre\text{-}ERT2}$*, n=6; mean ± SD).

(D) Representative picture of tumors in *Tfam$^{fl/+}$Foxp3$^{eGFP\text{-}Cre\text{-}ERT2}$* or *Tfam$^{fl/fl}$Foxp3$^{eGFP\text{-}Cre\text{-}ERT2}$* mice as described in Figure 7D. Data were representative of two independent experiments.

(E) Representative picture of tumors in *Rag1$^{-/-}$* mice transferred with CD45.1$^+$ CD25-depleted total splenic lymphocytes alone or together with ctr (*Tfam$^{+/+}$Foxp3$^{Yfp\text{-}Cre}$* and *Tfam$^{fl/+}$Foxp3$^{Yfp\text{-}Cre}$*) or cKO (*Tfam$^{fl/fl}$Foxp3$^{Yfp\text{-}Cre}$*) Tregs. Data were representative of two independent experiments.

Table S1. Forty-nine overlapped genes shared by differentially expressed genes (DEGs) between control and Tfam-deficient Tregs and DEGs between control and RISP-deficient Tregs. Related to Figure 4.

Gene Name
Adam12, Ahr, Akr1e1, Ankrd6, Aplp1, Arrdc4, Atp2b4, Bag3, Baiap3, Bmp7, Ccr2, Ccr4, Crmp1, Csf1, Epcam, Fgl2, Gbp2b, Glrx, Gm4841, Gpr15, Gpr68, Hic1, Il18r1, Itgae, Itpripl2, Kif23, Maf, March3, Marveld2, Matk, Mmp9, mt-Cytb, mt-Nd4, mt-Nd6, Myo3b, Naip2, Naip5, Noxa1, Nrp1, Plscr1, Rnase4, Rorc, Rrm2, Sccpdh, Serpina3g, Slc22a15, Smim10l2a, Tnfsf14, Vsig10

Table S2. Primer sequences used. Related to Figure 3, Figure S1 and Figure S2.

Primer name	Sequence (5' - 3')
Tfam-F	CCAAAAAGACCTCGTTCAGC
Tfam-R	ATGTCTCCGGATCGTTTCAC
Atp6-F	CCATAAATCTAAGTATAGCCATTCCAC
Atp6-R	AGCTTTTTAGTTTGTGTCGGAAG
Cytb-F	CATTTATTATCGCGGCCCTA
Cytb-R	TGGGTTGTTTGATCCTGTTTC
mt-Nd1-F	CAAACACTTATTACAACCCAAGAACA
mt-Nd1-R	TCATATTATGGCTATGGGTCAGG
Il10-F	AGTGGAGCAGGTGAAGAGTGATT
Il10-R	TTCGGAGAGAGGTACAAACGAG
Bcl-xl-F	ATGACCACCTAGAGCCTTGGA
Bcl-xl-R	GAAGAGTGAGCCCAGCAGAAC
Bax-F	AACTGGTGCTCAAGGCCCT
Bax-R	GAGGACTCCAGCCACAAAGA
Actb-F	TGTGACGTTGACATCCGTAA
Actb-R	GCTAGGAGCCAGAGCAGTAA
Areg-F	GGTCTTAGGCTCAGGCCATTA
Areg-R	CGCTTATGGTGGAAACCTCTC
genome-mt-ND1-F	TGCCAGCCTGACCCATAGCC
genome-mt-ND1-R	ATGGGCCGGCTGCGTATTCT
genome-β-globin-F	GAGTTGAGACTGTGCTTGGC
genome-β-globin-R	TCTGCACCCAAATCATTGTT