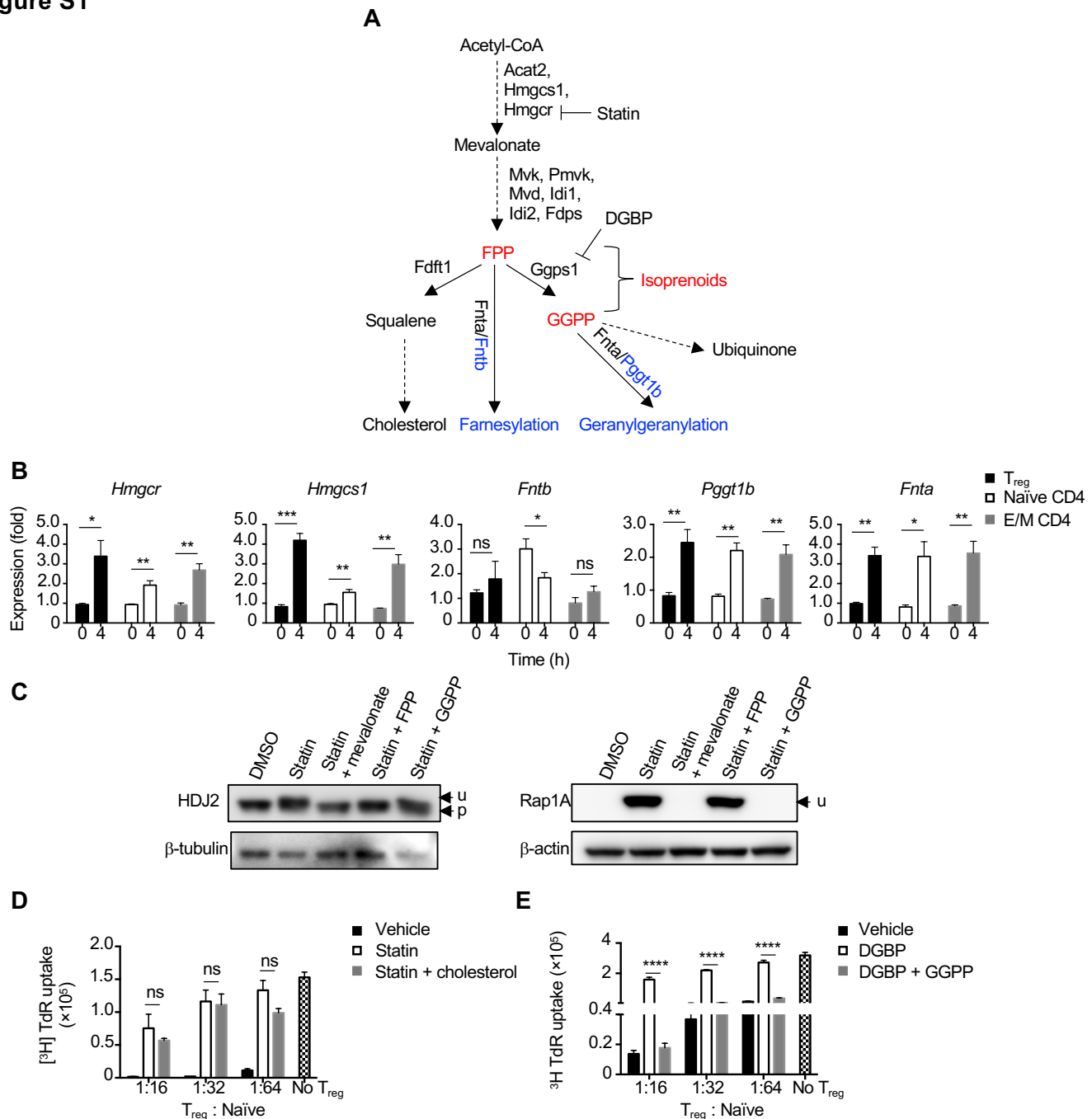# Figure S1

Figure S1. Upregulation of mevalonate metabolism supports protein prenylation for T_{reg}-cell suppressive activity. Related to Figure 1

(A) Schematic of the mevalonate pathway for cholesterol, isoprenoid, and ubiquinone synthesis. (B) Foxp3-YFP+ T_{reg} cells, Foxp3-YFP−CD4+CD44loCD62Lhi naïve T cells (naïve CD4), and Foxp3-YFP−CD4+CD44hiCD62Llo effector/memory T cells (E/M CD4) were isolated from *Foxp3*Cre-YFP reporter mice and stimulated with anti-CD3/28 for 0 or 4 h for analysis of indicated gene expression. (C) T_{reg} cells were treated with simvastatin (statin) with or without the indicated metabolites in the presence of IL-2 for overnight. Immunoblot analysis of unprenylated (u) or prenylated (p) HDJ2 (unprenylated HDJ2 is the upper band) and unprenylated (u) Rap1A (unprenylated Rap1A accumulates in the soluble cell lysate whereas prenylated Rap1A accumulates in the insoluble membrane fraction and is below detection limit). β-tubulin or β-actin was used as a loading control. (D and E) T_{reg} cells activated *in vitro* by anti-CD3/28 plus IL-2 for 3 days in the presence of DMSO or simvastatin supplemented with or without 5 μg/ml cholesterol (D) or in the presence of DMSO or 25 μM DGBP supplemented without or with 5 μM GGPP, followed by co-culture with conventional CD4+ T cells. T_{reg}-cell suppressive activity was then assessed after 3 days.

Data are representative of three (C) or two (D and E), or compiled from two (B) independent experiments, with 4 replicates (2 technical replicates from each independent experiment) (B) or 3 technical replicates per group (D and E). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ ns, not significant; two-tailed unpaired Student's *t*-test (B) or one-way ANOVA (D). Data are mean ± s.e.m.

Figure S2

A WT / *Foxp3*^Cre*Fntb*^fl/fl

Fntb, *Pggt1b* — mRNA (fold)

B WT / *Foxp3*^Cre*Pggt1b*^fl/fl

Pggt1b, *Fntb* — mRNA (fold)

C WT / *Foxp3*^Cre*Fntb*^fl/fl

HDJ2 — u / p; β-actin

D WT / *Foxp3*^Cre*Pggt1b*^fl/fl

Rap1A — u; β-Actin

E WT / *Foxp3*^Cre*Fntb*^fl/fl — [³H] TdR uptake (×10⁴); T_reg : Naïve

F WT / *Foxp3*^Cre*Pggt1b*^fl/fl — [³H] TdR uptake (×10⁴); T_reg : Naïve

G Skin (20×) / Skin (20×) — CD4 / CD8

H Skin (20×) / Skin (20×) — Arginase1 / Iba1

I Colon (40×) / Small intestine (20×) — Mcpt1 / Mcpt1

J WT (d7) / *Foxp3*^Cre*Fntb*^fl/fl (d7)

K WT (d7) / *Foxp3*^Cre*Fntb*^fl/fl (d7)

L WT (d7) / *Foxp3*^Cre*Pggt1b*^fl/fl (d7)
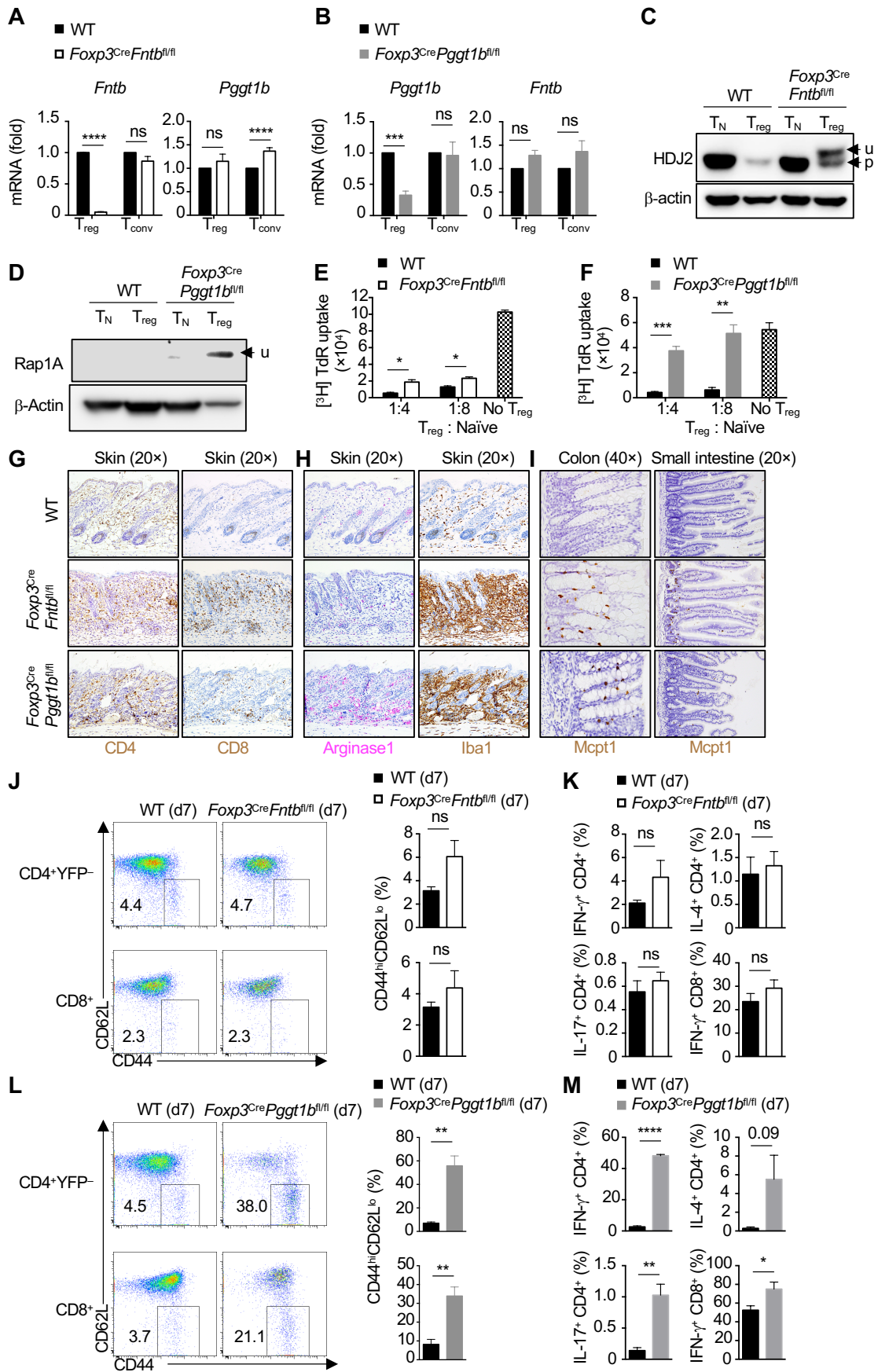
M WT (d7) / *Foxp3*^Cre*Pggt1b*^fl/fl (d7)

Figure S2. T$_{reg}$ cells require Fntb and Pggt1b for homeostasis. Related to Figure 2.

(A and B) Quantitative real-time PCR analysis of *Fntb* or *Pggt1b* expression in T$_{reg}$ cells or conventional CD4$^+$ T cells (T$_{conv}$) from WT and *Foxp3^{Cre}Fntb$^{fl/fl}$* mice (A) or WT and *Foxp3^{Cre}Pggt1b$^{fl/fl}$* mice (B). (C and D) Immunoblot analysis of unprenylated (u) or prenylated (p) HDJ2 (C) and unprenylated (u) Rap1A (D) in naïve CD4$^+$ T (T$_N$) and T$_{reg}$ cells isolated the indicated mice. (E and F) *In vitro* T$_{reg}$-cell suppressive assay by co-culture of T$_{reg}$ cells isolated from WT and *Foxp3^{Cre}Fntb$^{fl/fl}$* (E) or WT and *Foxp3^{Cre}Pggt1b$^{fl/fl}$* (F) mice with naïve CD4$^+$ T cells. (G–I) Immunohistochemistry analysis of CD4$^+$ and CD8$^+$ T cells (G), arginase 1- or Iba1-expressing macrophages (H), or Mcpt1-expressing mucosal mast cells (I) in different tissues from indicated mice at 3 weeks. (J and K) Flow cytometry analysis and quantification of CD44^{hi}CD62L^{lo} CD4$^+$Foxp3-YFP$^-$ (upper) or CD8$^+$ T cells (lower) (J), or frequency of splenic IFN-γ^+, IL-4$^+$, or IL-17$^+$ CD4$^+$ and IFN-γ^+ CD8$^+$ T cells (K) in neonatal WT and *Foxp3^{Cre}Fntb$^{fl/fl}$* mice at day 7 (d7). (L and M) Flow cytometry analysis and quantification of splenic CD44^{hi}CD62L^{lo} CD4$^+$Foxp3-YFP$^-$ or CD8$^+$ T cells (L), or frequency of IFN-γ^+, IL-4$^+$, or IL-17$^+$ CD4$^+$ and IFN-γ^+ CD8$^+$ T cells (M) in neonatal WT and *Foxp3^{Cre}Pggt1b$^{fl/fl}$* mice at d7.

Data are representative of two (C, E) or one (D, F), or compiled from two (A) or three (B, J–M) independent experiments, with 3 (A, B), or 4 (J–M) mice, or 3 technical replicates (E, F) per group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, ns, not significant; two-tailed unpaired Student's *t*-test (A, B, E, F, J–M). Data are mean ± s.e.m. Numbers indicate percentage of cells in gates.
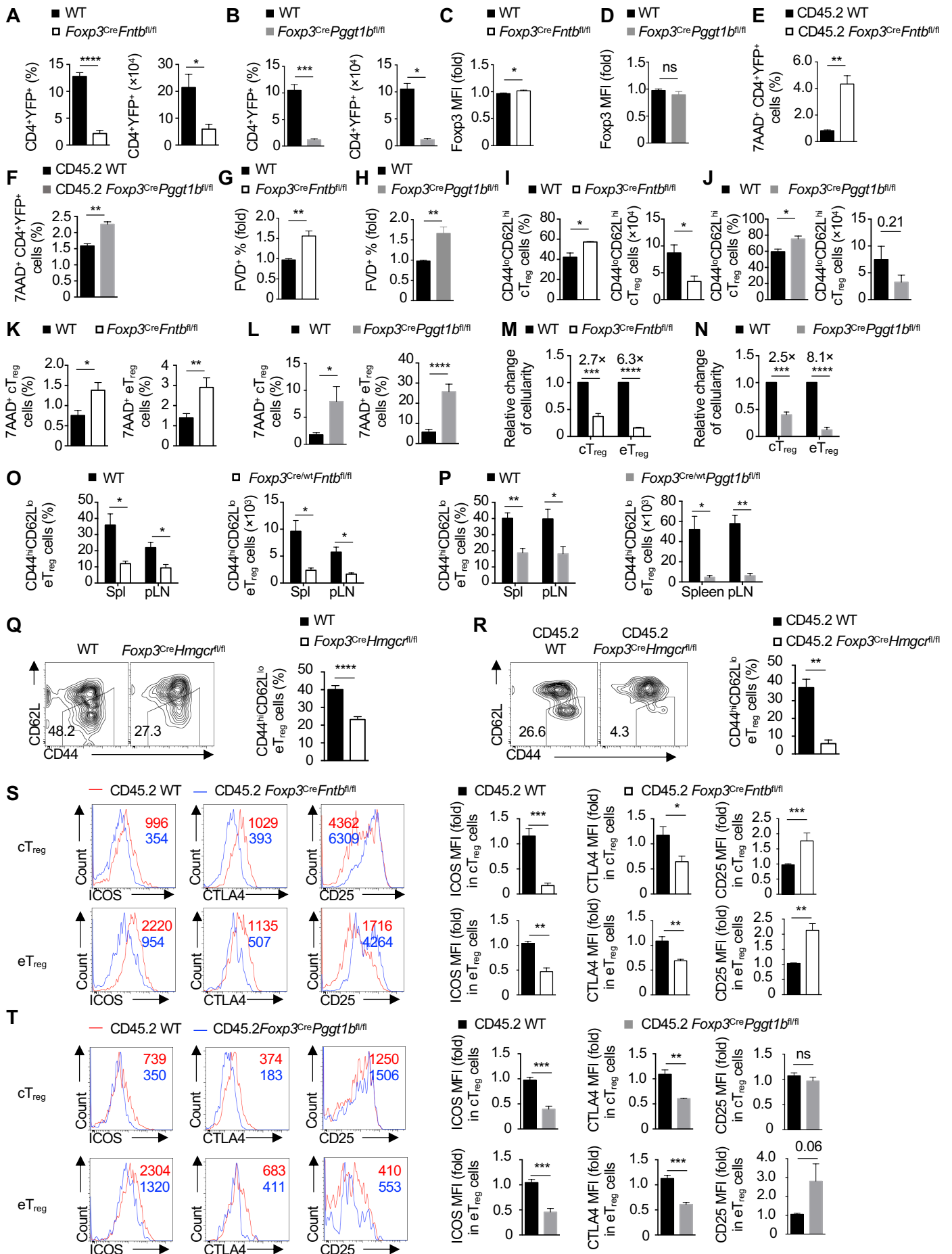
Figure S3

Figure S3. Deficiency of Fntb and Pggt1b contributes to severe loss of eT$_{reg}$ cells. Related to Figure 3.
(A and B) Quantification of the frequency and number of splenic Foxp3-YFP$^+$ T$_{reg}$ cells in WT and *Foxp3*^{Cre}*Fntb*$^{fl/fl}$
mice (A) or WT and *Foxp3*^{Cre}*Pggt1b*$^{fl/fl}$ mice (B). (C and D) Relative mean fluorescence intensity (MFI) of Foxp3 in
splenic T$_{reg}$ cells from the indicated mice. (E and F) Quantification of frequency of splenic 7AAD$^+$CD45.2$^+$Foxp3-
YFP$^+$ T$_{reg}$ cells from WT and *Foxp3*^{Cre}*Fntb*$^{fl/fl}$ (E) or WT and *Foxp3*^{Cre}*Pggt1b*$^{fl/fl}$ (F) mixed bone marrow chimeras.
(G and H) Quantification of percentage of fixable viability dye (FVD)$^+$ cells in cT$_{reg}$ cells activated by anti-CD3/28
plus IL-2 for 3 days. cT$_{reg}$ cells were isolated from the indicated mice. (I and J) Quantification of frequency and
number of splenic CD44^{lo}CD62L^{hi} cT$_{reg}$ cells in the indicated mice. (K and L) Quantification of frequency of
splenic 7AAD$^+$ cT$_{reg}$ (left) and eT$_{reg}$ (right) cells from the indicated mice. (M and N) Quantification of the relative
numbers (fold change) of splenic cT$_{reg}$ and eT$_{reg}$ cells from the indicated mice. WT mice are normalized to 1 for
each subset. (O and P) Quantification of frequency and number of eT$_{reg}$ cells in WT and *Foxp3*$^{Cre/wt}$*Fntb*$^{fl/fl}$ (O) or
WT and *Foxp3*$^{Cre/wt}$*Pggt1b*$^{fl/fl}$ (P) 'mosaic' mice. (Q and R) Flow cytometry analysis and quantification of splenic
CD44^{hi}CD62L^{lo} eT$_{reg}$ cells from neonatal WT and *Foxp3*^{Cre}*Hmgcr*$^{fl/fl}$ mice (Q) or WT and *Foxp3*^{Cre}*Hmgcr*$^{fl/fl}$ mixed
bone marrow chimeras (R). (S and T) Flow cytometry analysis and quantification of ICOS, CTLA4, and CD25
expression on splenic CD45.2$^+$ cT$_{reg}$ and eT$_{reg}$ cells from indicated mixed bone marrow chimeras.
Data are representative of seven (C), three (F) or four (P), or compiled from three (A, D, G–I, M, O, Q, R, T for
CD25), two (B, E, J, N, S for ICOS and CTLA4, T for ICOS and CTLA4), or four (K, L, S for CD25) independent
experiments, with 3–4 (A, C, I, P), 3 (B, D, F, J), 3 (M, N), 4–6 (E), 4 (E, G–H, O, Q, S for ICOS and CTLA4), 5 (T
for CD25), 6 (R, S for ICOS and CTLA4), 5–6 (S for CD25) or 9 (K, L) mice per group. *p < 0.05, **p < 0.01, ***p <
0.001, ****p < 0.0001, ns, not significant; two-tailed unpaired Student's *t*-test (A–T). Data are mean ± s.e.m.
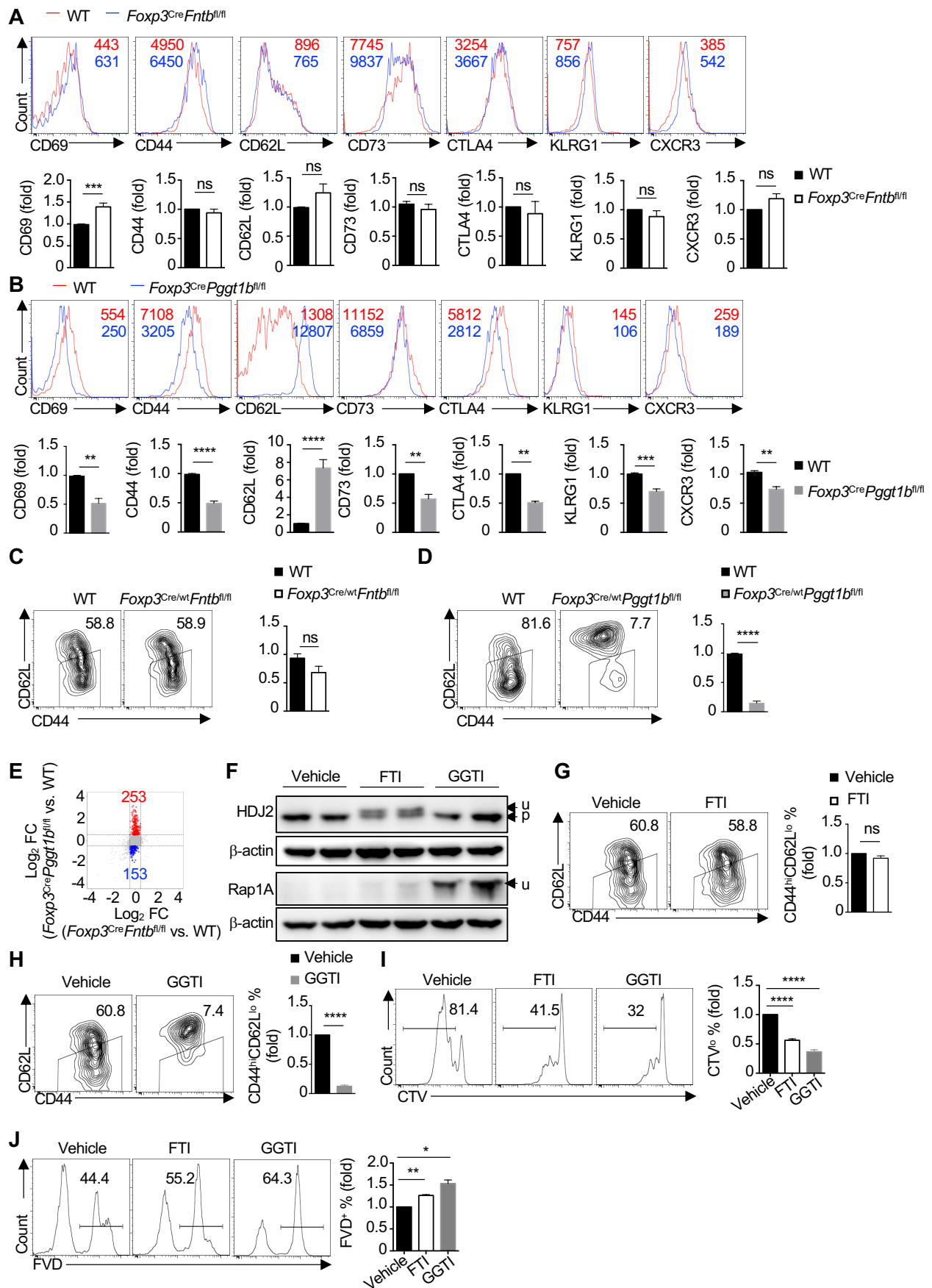Controls are normalized to 1 for each comparison (black bars).

Figure S4

Figure S4. Farnesylation is dispensable for eT$_{reg}$-cell induction and activation. Related to Figure 4.
(A and B) Flow cytometry analysis and quantification of expression of CD69, CD44, CD62L, CD73, CTLA4, KLRG1, or CXCR3 on cT$_{reg}$ cells isolated from WT or *Foxp3*^{Cre}*Fntb*$^{fl/fl}$ mice (A) or WT or *Foxp3*^{Cre}*Pggt1b*$^{fl/fl}$ mice (B) and stimulated with anti-CD3/28 plus IL-2 for 3 days. (C and D) Flow cytometry analysis and quantification of eT$_{reg}$-like cell generation in cT$_{reg}$ cells isolated from the indicated 'mosaic' mice and stimulated as in (A and B). (E) Fold-change/fold-change analysis of Pggt1b-deficient vs. WT compared with Fntb-deficient vs. WT T$_{reg}$ cells, using CD45.2$^+$Foxp3-YFP$^+$ T$_{reg}$ cells from WT and *Foxp3*^{Cre}*Fntb*$^{fl/fl}$ or WT and *Foxp3*^{Cre}*Pggt1b*$^{fl/fl}$ mixed bone marrow chimeras. Fold-change, FC. (F) Immunoblot analysis of unprenylated (u) or prenylated (p) HDJ2 and unprenylated (u) Rap1A in T$_{reg}$ cells treated with DMSO, FTI or GGTI in the presence of IL-2 for overnight. (G and H) Flow cytometry analysis and quantification of the frequency of CD44^{hi}CD62L^{lo} eT$_{reg}$-like cells. WT cT$_{reg}$ cells were stimulated as in (A and B) in the presence of vehicle, FTI (G), or GGTI (H). (I) Flow cytometry analysis and quantification of proliferated (Celltrace Violet (CTV)^{lo}) T$_{reg}$ cells stimulated as in (A and B) in the presence of FTI or GGTI. (J) Flow cytometry analysis and quantification of apoptotic/dead [fixable viability dye (FVD)$^+$] cells in T$_{reg}$ cells stimulated as in (A and B) in the presence of FTI or GGTI.
Data are representative of two (F), or compiled from at least two (A–D, I, J) or six (G, H) independent experiments, with 3–8 (A), 3–7 (B), 8 (G, H), 4 (C), or 3 (D, I, J) biological replicates per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns, not significant; two-tailed unpaired Student's *t*-test (A–D, G, H) or one-way ANOVA (I, J). Data are mean ± s.e.m. Numbers indicate percentage of cells in gates. Numbers in histograms indicate mean fluorescence intensity of proteins or frequency of cells in gates. Controls were normalized to 1 for each comparison (black bars).
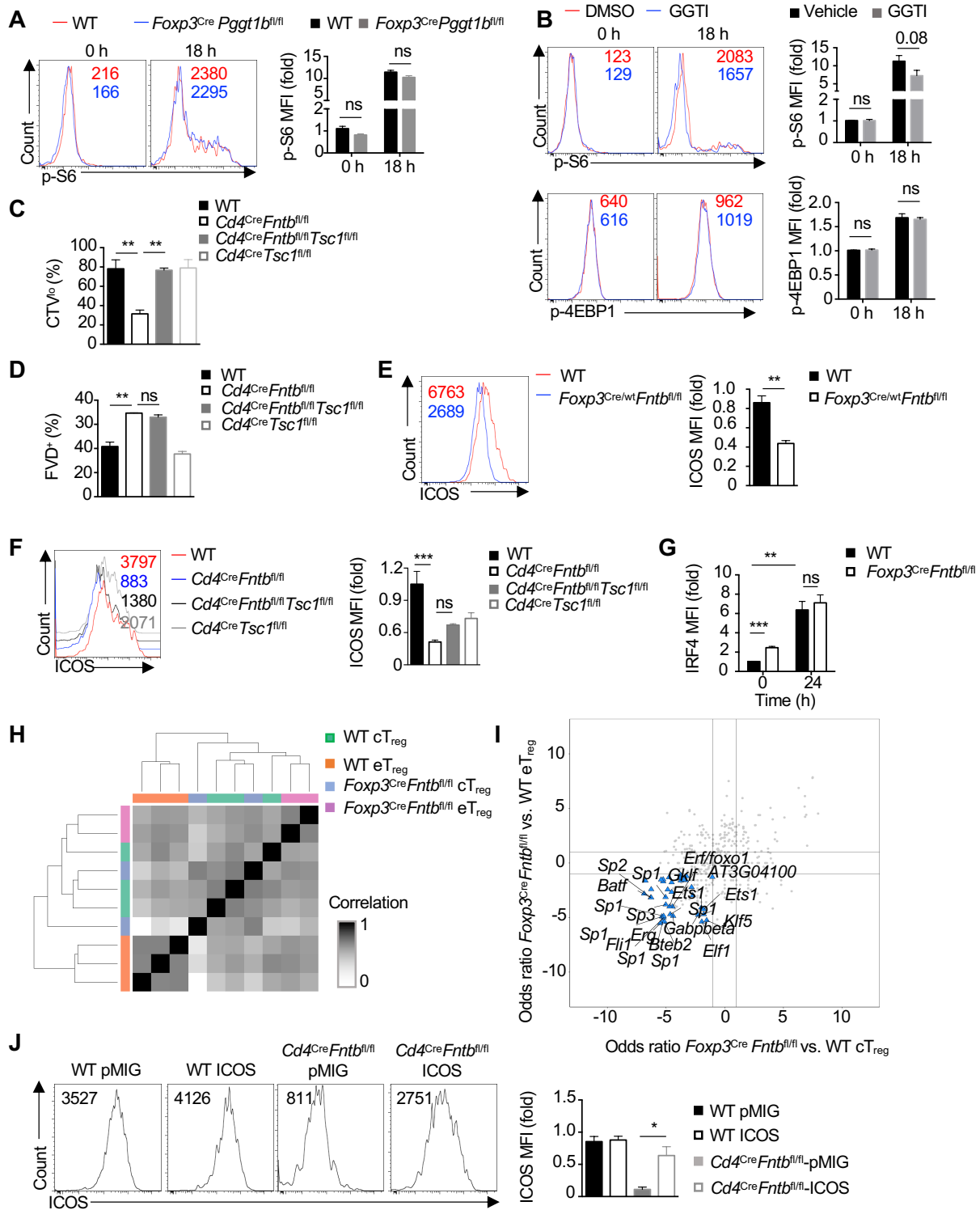
Figure S5

Figure S5. Pggt1b is not required for the activation of mTORC1 and hyper-activation of mTORC1 signaling restores T$_{reg}$-cell proliferation upon Fntb deletion. Related to Figure 5.

(A) Flow cytometry analysis and quantification of relative mean fluorescence intensity (MFI) of phosphorylated S6 (p-S6) in cT$_{reg}$ cells isolated from WT and *Foxp3*^{Cre}*Pggt1b*$^{fl/fl}$ mice and stimulated with anti-CD3/28 plus IL-2 for 18 h. (B) Flow cytometry analysis and quantification of relative p-S6 and p-4EBP1 MFI in WT cT$_{reg}$ cells stimulated as in (A) in the presence of vehicle or GGTI. (C and D) cT$_{reg}$ cells isolated from WT, *Cd4*^{Cre}*Fntb*$^{fl/fl}$, *Cd4*^{Cre}*Fntb*$^{fl/fl}$*Tsc1*$^{fl/fl}$ or *Cd4*^{Cre}*Tsc1*$^{fl/fl}$ mice and stimulated as in (A) for 3 days. Quantification of the percentage of proliferated cells [Celltrace Violet (CTV^{lo}); C] or the frequency of apoptotic [fixable live dead (FVD$^+$)] cells (D). (E) Flow cytometry analysis and quantification of ICOS expression on cT$_{reg}$ cells isolated WT or *Foxp3*$^{Cre/wt}$*Fntb*$^{fl/fl}$ 'mosaic' mice following stimulation as (A) for 3 days. (F) Flow cytometry analysis and quantification of ICOS expression on splenic T$_{reg}$ cells from WT, *Cd4*^{Cre}*Fntb*$^{fl/fl}$, *Cd4*^{Cre}*Fntb*$^{fl/fl}$*Tsc1*$^{fl/fl}$ or *Cd4*^{Cre}*Tsc1*$^{fl/fl}$ mice. (G) cT$_{reg}$ cells isolated from WT or *Foxp3*^{Cre}*Fntb*$^{fl/fl}$ mice were stimulated by anti-CD3/28 plus IL-2 for 0 or 24 h. The expression of IRF4 was measured by flow cytometry. (H) Similarity matrix plot of nucleosome-free reads of cT$_{reg}$ and eT$_{reg}$ cells from WT or *Foxp3*^{Cre}*Fntb*$^{fl/fl}$ mixed bone marrow chimeras. (I) Odds ratio/odds ratio plot indicating the predicted downregulated motifs in *Foxp3*^{Cre}*Fntb*$^{fl/fl}$ vs. WT eT$_{reg}$ cells compared with *Foxp3*^{Cre}*Fntb*$^{fl/fl}$ vs. WT cT$_{reg}$ cells from mixed bone marrow chimeras. (J) Flow cytometry analysis and quantification of ICOS expression on splenic T$_{reg}$ cells in retrogenic mice [WT or *Cd4*^{Cre}*Fntb*$^{fl/fl}$ bone marrow cells were transduced with pMIG-GFP empty vector (pMIG) or pMIG-GFP-ICOS (ICOS) retroviral vector; see Methods].

Data are representative of three (G) or compiled from one (A), three (B for p-S6, F, J) or two (B for p-4EBP1, C–E) independent experiments, with 2 (A), 6 (B for p-4EBP1, J for Cre-pMIG and Cre-ICOS), 9 (B for p-S6), 3 (C–F, J for *Cd4*^{Cre}*Fntb*$^{fl/fl}$ pMIG) or 4 (G, J for *Cd4*^{Cre}*Fntb*$^{fl/fl}$ ICOS) biological replicates per group. *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant; two-tailed unpaired Student's *t*-test (A, B, E, J) or one-way ANOVA (C, D, F, G). Data are mean ± s.e.m. Controls were normalized to 1 for each comparison (black bars).

Figure S6

A

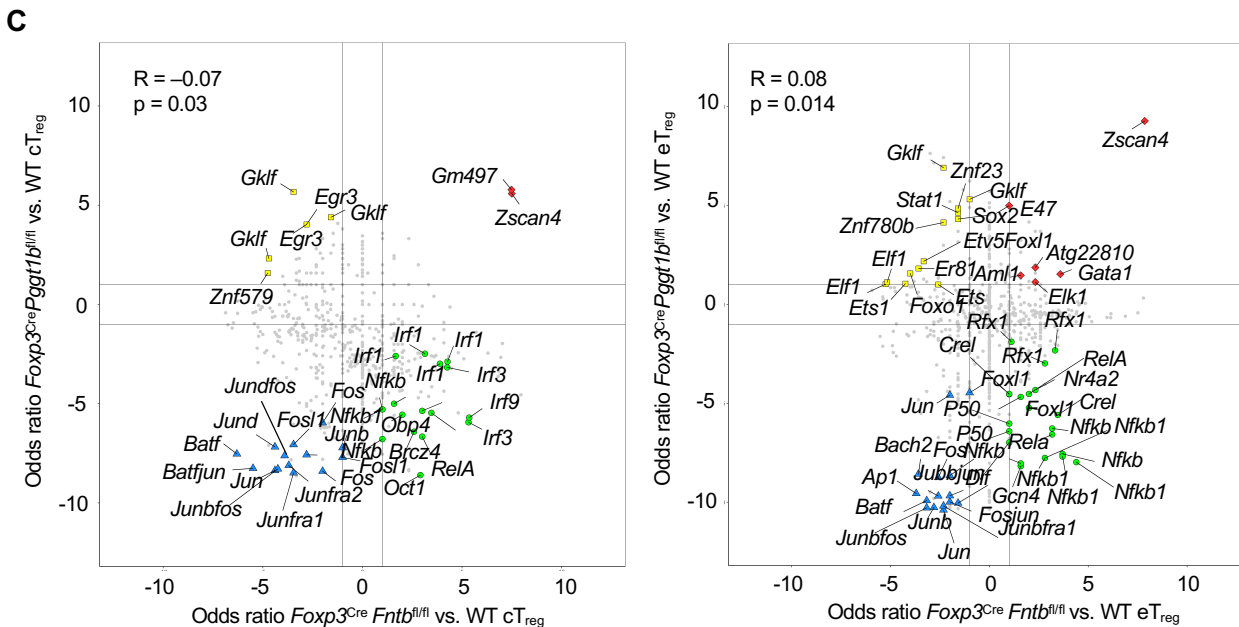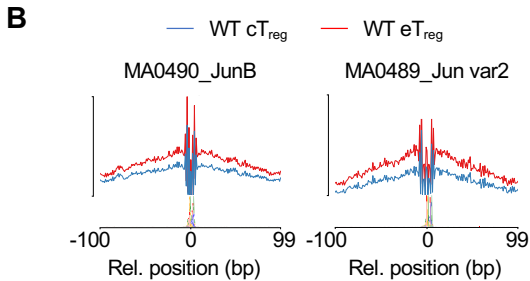| Rank in list	Transcription factor name	*Foxp3*^Cre*Pggt1b*^fl/fl_vs_WT_cT_reg_Fisher_*p*_val	*Foxp3*^Cre*Pggt1b*^fl/fl_vs_WT_eT_reg_Fisher_*p*_val
	Comparison of *Foxp3*^Cre*Pggt1b*^fl/fl vs WT cT_reg- and eT_reg-cell populations. The top transcription factors are filtered using FDR < 0.0001 and log₂OddsRatio < 1		
1	FRA1	1.52E-92	< 0.001
2	FOSL1	2.42E-88	< 0.001
3	JUN	1.19E-97	< 0.001
4	DBX1	1.46E-104	< 0.001
5	GCN4	8.41E-107	< 0.001
6	AP1	1.51E-95	< 0.001
7	JUND	6.07E-89	< 0.001
8	ATHB23	1.74E-117	< 0.001
9	JUNB	1.93E-75	< 0.001
10	JUNBFOSB	6.11E-83	< 0.001
11	JUNBFOS	3.03E-92	< 0.001
12	JUNDFRA2	4.85E-82	< 0.001
13	JUNBFRA1	9.19E-106	< 0.001
14	JUNBFRA2	3.05E-86	< 0.001
15	FOS	3.84E-87	< 0.001
16	FRA2	3.76E-66	2.04E-316
17	JUNFRA2	1.93E-81	6.53E-315
18	JUNDM2	7.50E-66	2.19E-307
19	FOSJUN	3.85E-78	1.15E-301
20	JDP2	3.06E-77	7.87E-291
64	NFKB	8.40E-32	1.73E-77
65	RELA	3.97E-29	3.41E-74
67	NFKB1	2.26E-35	1.05E-69
69	NFKB1	7.43E-11	5.11E-64
70	NFKB1	7.43E-11	5.11E-64
71	NFKAPPAB	2.61E-22	3.18E-62
72	NFKB1	3.09E-28	9.96E-61
74	NFKB	1.02E-21	2.39E-55
94	RELA	8.00E-16	2.44E-27
104	NFKB	7.90E-09	5.21E-22
122	CREL	1.32E-22	1.77E-13
146	NFATC1	0.62499662	4.07E-09
174	CREL	1.77E-13	2.98E-06
192	RELA	1.45E-10	2.10E-05

B

C

Figure S6. ATAC-Seq analysis reveals enrichment of Jun transcriptional programming in eT$_{reg}$ cells. Related to Figure 6.

(A) Ranking of downregulation of AP-1 family, NF-κB and NFATc1 transcription factors [ranked based on FDR < 0.0001 and odds ratio (*Foxp3*^{Cre}*Pggt1b*$^{fl/fl}$ vs. WT eT$_{reg}$ cells) < 1] as predicted from motif enrichment analysis of ATAC-Seq data. (B) ATAC-Seq analysis was performed for WT cT$_{reg}$ and eT$_{reg}$ cells from mixed bone marrow chimeras. Footprinting analysis revealed binding profiles of transcription factors from Jun family are enriched in eT$_{reg}$ cells compared to cT$_{reg}$ cells. (C) Odds ratio/odds ratio plot indicated the predicted motifs in *Foxp3*^{Cre}*Pggt1b*$^{fl/fl}$ vs. WT cT$_{reg}$ cells compared with *Foxp3*^{Cre}*Fntb*$^{fl/fl}$ vs. WT cT$_{reg}$ cells (left), or *Foxp3*^{Cre}*Pggt1b*$^{fl/fl}$ vs. WT eT$_{reg}$ cells compared with *Foxp3*^{Cre}*Fntb*$^{fl/fl}$ vs. WT eT$_{reg}$ cells (right).
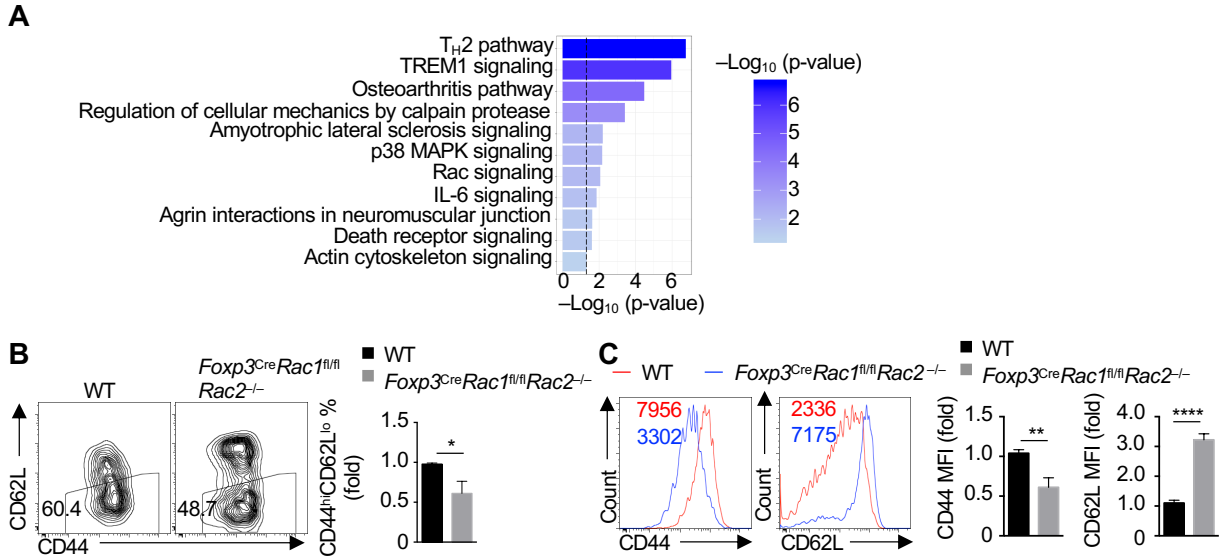
A

B

C

Figure S7. Rac promotes eT$_{reg}$-like cell generation. Related to Figure 7.
(A) Ingenuity pathway analysis of predicted downregulated pathways in transcriptomes of *Foxp3*^{Cre}*Pggt1b*$^{fl/fl}$ T$_{reg}$ cells compared to WT T$_{reg}$ cells. (B and C) cT$_{reg}$ cells were isolated from WT and *Foxp3*^{Cre}*Rac1*$^{fl/fl}$*Rac2*$^{-/-}$ mice and activated by anti-CD3/28 plus IL-2 for 3 days. Flow cytometry analysis and quantification of the percentage of eT$_{reg}$-like cells (B) and expression of CD44 and CD62L (C).
Data are compiled from three (B, C) independent experiments, with 3–5 (B, C) mice per group. Numbers indicate percentage of cells in gates. Numbers in histograms indicate mean fluorescence intensity of proteins.