Supporting Information

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SI Materials and Methods

Generation of Conditional *Pik3c3* KO Mice. Mouse genomic DNA of the *Pik3c3* gene was isolated from C57BL/6 BAC clone RP24-303P17 (from Children's Hospital Oakland Research Institute). The targeting vector was constructed by PCR cloning of three genomic fragments into pEasy-FLIRT: a 5′ homology arm (2.4 kb) into the AscI site, a floxed region containing exon 4 (1.5 kb) into SalI/NheI-Xho I sites, and a 3′ homology arm (3.2 kb) into Bam HI/NheI-Not I sites. The final targeting construct was verified by a restriction digest and DNA sequencing. After linearization with Not I, the targeting vector was electroporated into JM8 ES cells (C57BL/6 origin). Correctly targeted clones were identified by PCR screening, injected into blastocysts, and implanted into foster mothers. Chimeric mice were bred to C57BL/6 mice, and the F1 generation was screened for germ line transmission with primers around *LoxP* site (primers E1 and F1; see Fig. S1). The *neo* gene was removed by breeding to FLP recombinase transgenic mice (JAX). The mice were then bred to either *Cd4*-Cre (1) or *Cd8a*-Cre (2) transgenic mice.

Genomic PCR and Quantitative RT-PCR. Genomic DNA was isolated from tail biopsy specimens and purified cell populations. WT, floxed, and deleted *Pik3c3* alleles were distinguished with the following primers: GTATCCGCACTTTGGGCTTC (primer E1), TCTCCAGGTCACTCTTTTCTCC (primer F1), and CCATTATCCTTAGGCAGCCA (primer C1R). Total RNA was extracted from purified T cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions and used for cDNA synthesis with the SuperScript First-Strand Synthesis System (Invitrogen). Quantitative RT-PCR was performed on an ABI 7500 Fast Real-Time PCR system with primer probe sets (Applied Biosystems). Expression values were calculated using the comparative threshold cycle method and normalized to *Hprt*.

Mixed BM Chimeras. BM cells from Vps34 HET or KO mice ($CD45.2^+$) were mixed at a ratio of 1:1 with WT C57BL/6 cells ($CD45.1^+$). A 2×10^6-BM cell mixture was injected i.v. into sublethally irradiated (6 Gy) *Rag1* KO recipient mice ($CD45.1^+$). Mice received prophylactic antibiotics (Sulfatrim) in drinking water for 3 wk posttransplantation and were analyzed at 8 wk postreconstitution. Donor-derived lymphocytes were detected by flow cytometry using the congenic markers CD45.1 and CD45.2.

Flow Cytometry and Cell Sorting. Single-cell suspensions were prepared from thymus, spleen, and peripheral lymph nodes (LNs) of mice at 6–16 wk of age. Lysis of RBCs was performed using ACK Lysis Buffer (Lonza). Samples were then stained with the following fluorochrome-labeled mAbs (all from BioLegend): CD4 (RM4-5), CD8α (53-6.7), TCRβ (H57-597), CD24 (M1/69), CD44 (IM7), CD62L (MEL-14), CD45.1 (A20), and CD45.2 (104). A biotinylated mAb against IL-7Rα (A7R34; eBioscience) was used in conjunction with PE-conjugated streptavidin (BD Biosciences) to detect IL-7Rα cell surface expression. Samples were analyzed on a LSRII flow cytometer (BD Biosciences). Thymocyte subpopulations were sorted based on CD4 and CD8α expression on a FACSAria cell sorter (BD Biosciences). Peripheral T cells were purified from spleen and LN by negative immunomagnetic selection using a mixture of biotinylated mAbs against B220, CD11b, CD11c, NK1.1, and CD25 (all from BD Biosciences), followed by streptavidin-coupled beads (Miltenyi Biotec). The positive fraction ($B220^+$ $CD11b^+$ $CD11c^+$ $NK1.1^+$ $CD25^+$) was collected ("non-T cells"). The negative fraction was further purified into CD4 and CD8 T cells by cell sorting.

Cell Culture and Western Blot Analysis. Purified CD4 and CD8 T cells were either used fresh or activated with 10 μg/mL of plate-bound α-CD3 mAb and 5 μg/mL of soluble CD28 mAb for 24 h in vitro. Cells were cultured in RPMI medium 1640 supplemented with 10% (vol/vol) FCS, 2 mM L-glutamine, 1% (vol/vol) penicillin-streptomycin, and 55 μM β-mercaptoethanol at 37 °C/5% CO_2. For the last 4 h of culture, either 400 nM BafA1 (Sigma-Aldrich) or 1 μM rapamycin was added. DMSO was used as a solvent control. Cell lysates were prepared in cell lysis buffer (Cell Signaling), and proteins were separated by SDS/PAGE using 12% or 4–12% Bis-Tris gels (Invitrogen). The following antibodies were used for immunoblotting: Vps34 (N-terminus; Sigma-Alrich), Vps34 (C-terminus; Cell Signaling), Vps15 (Novus Biologicals), Beclin-1 (Cell Signaling), p62 (Sigma-Aldrich), LC3B (MBL), phospho-$S6^{Ser235/236}$ and S6 (Cell Signaling), and actin (Santa Cruz Biotechnology).

Mitochondria and ROS. A total of 2×10^6 LN cells were loaded with 10 nm of MitoTracker Green (Invitrogen) in RPMI 1640 for 15 min at 37 °C/5% CO_2. After washing, cells were surface-stained on ice, washed, and then incubated with Annexin V-APC (BD Biosciences) for 15 min at room temperature before analysis. For ROS detection, 2×10^6 LN cells were loaded with 1 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA; Invitrogen) in PBS for 10 min at 37 °C/5% CO_2. After washing, cells were surface-stained on ice and analyzed immediately.

1. Lee PP, et al. (2001) A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* 15:763–774.

2. Maekawa Y, et al. (2008) Notch2 integrates signaling by the transcription factors RBP-J and CREB1 to promote T cell cytotoxicity. *Nat Immunol* 9:1140–1147.

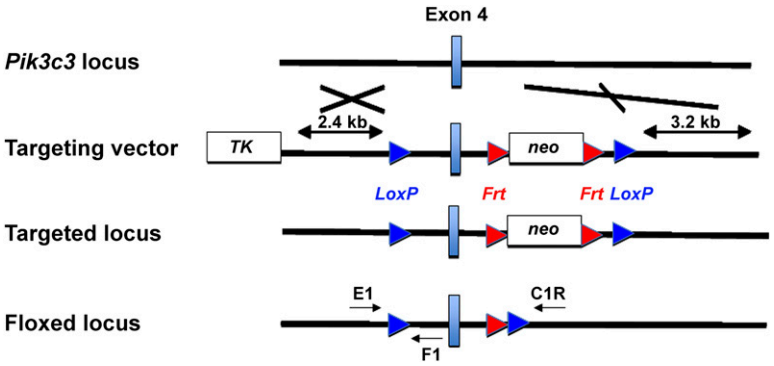

Fig. S1. Targeting of Pik3c3 locus to generate conditional Vps34 KO mice. The sizes of the homology arms are shown in kilobases. *LoxP* and *Frt* sites are depicted as blue and red arrows, respectively. TK, thymidine kinase gene; neo, neomycin resistance gene cassette. The locations of primers E1, F1, and C1R used to distinguish floxed and deleted *Pik3c3* alleles (Fig. 1*A*) are indicated by arrows.

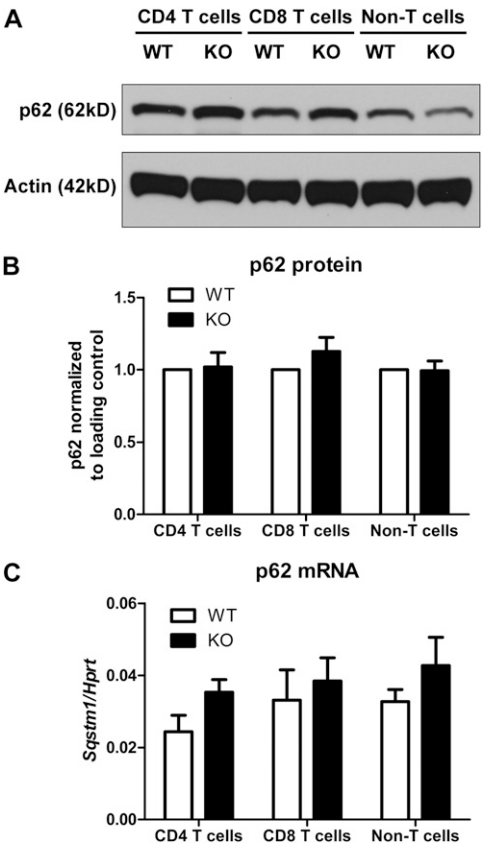

Fig. S2. Expression of p62 in Vps34-deficient T cells. (*A* and *B*) Western blot analysis (*A*) and densitometry analysis (*B*) of p62 protein expression in CD4 and CD8 T cells as well as non-T cells from *Cd4*-Cre*Pik3c3*$^{+/+}$ or *Pik3c3*$^{flox/flox}$ (WT) and *Cd4*-Cre*Pik3c3*$^{flox/flox}$ (KO) mice. Actin was used as a loading control. Amounts of p62 protein were normalized to actin and are relative to the amounts in WT cells. (*C*) Quantitative RT-PCR analysis of p62 mRNA expression in CD4 and CD8 T cells as well as non-T cells from Vps34 WT and KO mice ($n = 3$). mRNA expression was normalized to *Hprt*. Data are from three (*C*) or six (*A* and *B*) independent experiments.

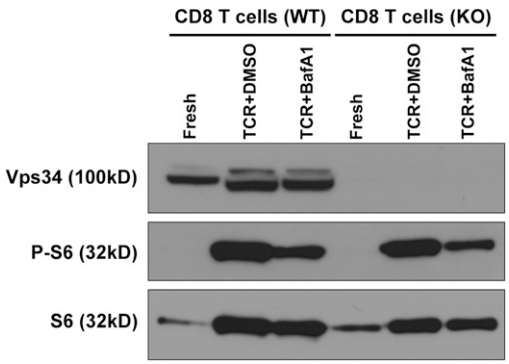

Fig. S3. Normal TCR-induced mTORC1 activation in Vps34-deficient T cells. Western blot analysis of ribosomal S6 protein phosphorylation in resting and activated CD8 T cells from Vps34 WT or KO mice. T cells were used either freshly isolated or activated with α-CD3 and α-CD28 mAbs for 24 h in vitro.

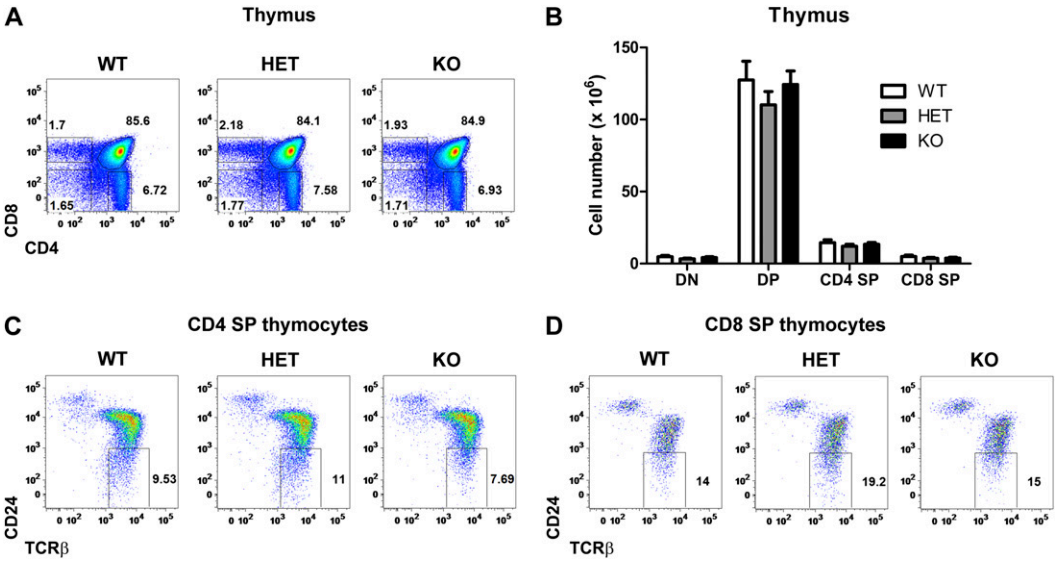

Fig. S4. Normal T-cell development in *Cd4*-Cre*Pik3c3*$^{flox/flox}$ mice. (*A*) Representative flow cytometry analysis of thymocytes from *Cd4*-Cre*Pik3c3*$^{+/+}$ or *Pik3c3*$^{flox/flox}$ (WT), *Cd4*-Cre*Pik3c3*$^{flox/+}$ (HET), and *Cd4*-Cre*Pik3c3*$^{flox/flox}$ (KO) mice. Numbers next to outlined areas indicate percentages of cells. (*B*) Numbers of DN, DP, CD4 SP, and CD8 SP thymocytes from Vps34 WT, HET, and KO mice (n = 8–13). (*C* and *D*) Representative FACS analysis of CD4 (*C*) and CD8 (*D*) SP thymocytes from Vps34 WT, HET, and KO mice. Numbers next to outlined areas indicate percentages of mature (TCRβ^{hi}CD24^{lo}) SP thymocytes. Results are cumulative from three independent experiments.

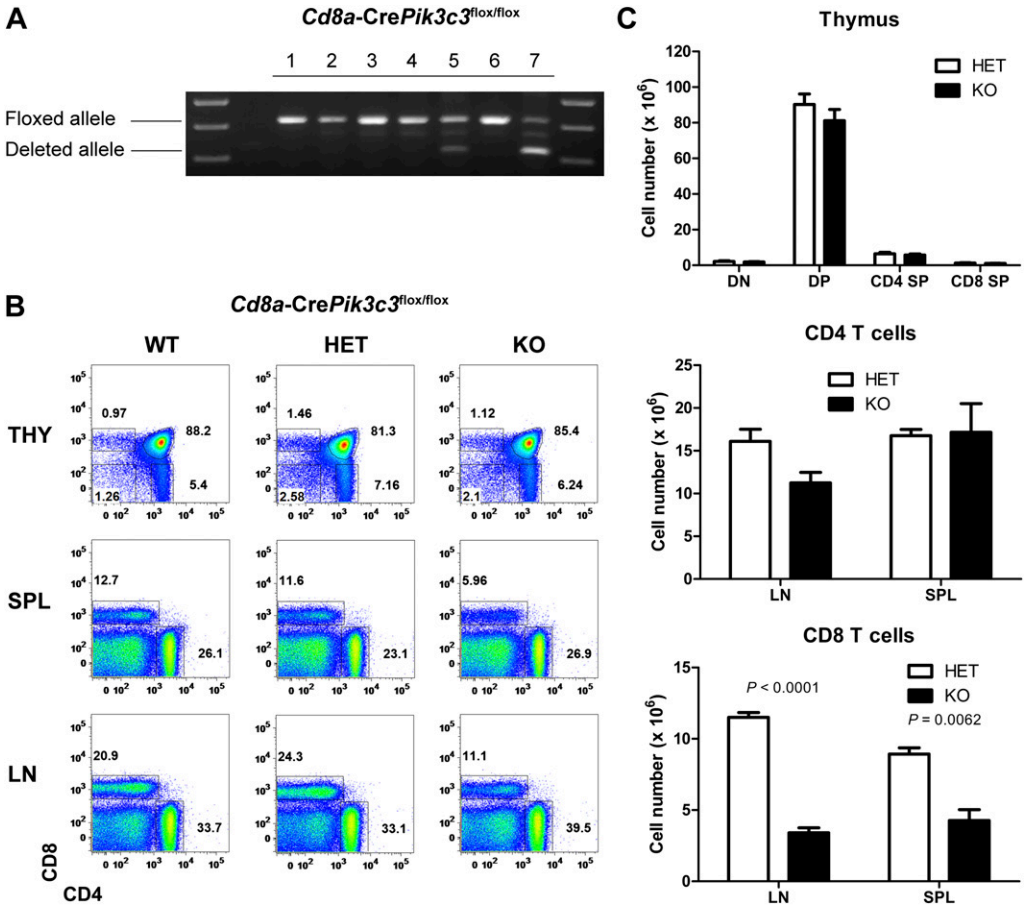

Fig. S5. Vps34 regulates peripheral CD8 T-cell homeostasis in a thymus-independent manner. (*A*) PCR analysis of genomic DNA from various cell populations isolated from *Cd8a*-Cre*Pik3c3*$^{flox/flox}$ mice. 1, tail; 2, DN; 3, DP; 4, CD4 SP; 5, CD8 SP thymocytes; 6, peripheral CD4 T cells; 7, peripheral CD8 T cells. (*B*) Representative FACS analysis of thymus (THY), spleen (SPL), and LN T cells from *Cd8a*-Cre*Pik3c3*$^{+/+}$ (WT), *Cd8a*-Cre*Pik3c3*$^{flox/+}$ (HET), and *Cd8a*-Cre*Pik3c3*$^{flox/flox}$ (KO) mice. Numbers next to outlined areas indicate percentages of cells. (*C*) Numbers of various T-cell populations from *Cd8a*-Cre*Pik3c3*$^{flox/+}$ (HET) and *Cd8a*-Cre*Pik3c3*$^{flox/flox}$ (KO) mice (n = 3). Data are from two independent experiments.

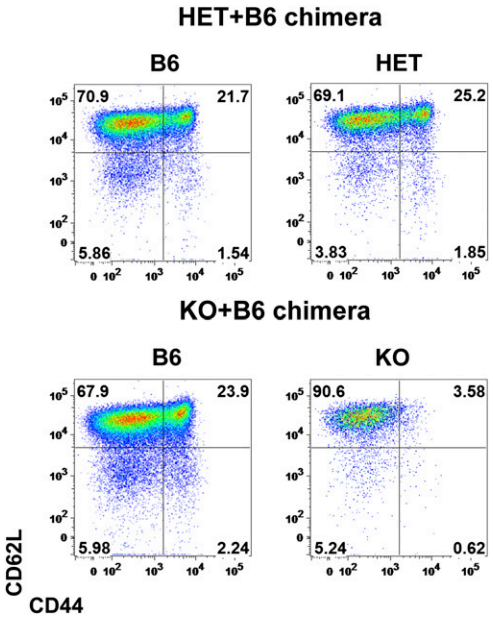

Fig. S6. Vps34-deficient CD8 T cells do not up-regulate CD44 in a nonlymphopenic environment. Shown is FACS analysis of CD44/CD62L expression by CD8 T cells from Vps34 HET/B6 and Vps34 KO/B6 mixed BM chimeras at 9 wk postreconstitution. Plots are gated on $CD8^{+}CD4^{-}$ cells from BM-derived HET or KO ($CD45.2^{+}$) and B6 ($CD45.1^{+}$) cells. Data are representative of three independent experiments (n = 16–17).

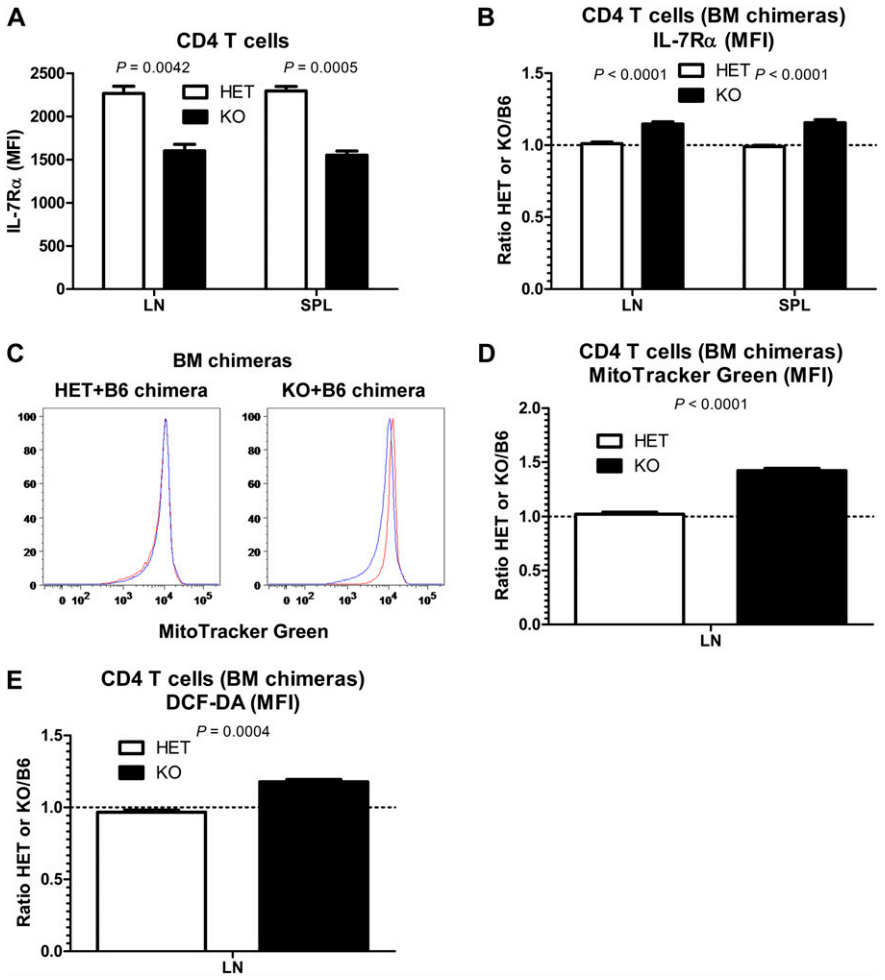

Fig. S7. Vps34 ensures mitochondrial homeostasis in CD4 T cells. (*A*) Cell surface expression of IL-7Rα by naïve ($CD44^{lo}$) CD4 T cells from Vps34 HET and KO mice was determined using FACS ($n = 3$). MFI, mean fluorescence intensity. (*B*) Cell surface expression of IL-7Rα by LN and spleen (SPL) naïve ($CD44^{lo}$) CD4 T cells from Vps34 HET/B6 and Vps34 KO/B6 mixed BM chimeras ($n = 15–17$). IL-7Rα expression (MFI) by Vps34 HET and KO CD4 T was normalized to expression by WT B6 cells in the same individual chimera (i.e., ratio Vps34 Het or KO/WT B6). A ratio of 1.0 represents equal IL-7Rα expression by Vps34 HET or KO cells compared with WT B6 cells. (*C*) Representative FACS analysis of MitoTracker Green staining in LN CD4 T cells from Vps34 HET/B6 and Vps34 KO/B6 mixed BM chimeras. Blue and red histograms represent B6 and HET or KO cells, respectively. (*D*) Quantification of MitoTracker Green staining in LN CD4 T cells from Vps34 HET/B6 and Vps34 KO/B6 mixed BM chimeras ($n = 16–17$). Staining was normalized to WT B6 cells as described in *B*. (*E*) Quantification of DCF-DA staining in LN CD4 T cells from Vps34 HET/B6 and Vps34 KO/B6 mixed BM chimeras ($n = 8–10$). Staining was normalized to WT B6 cells as described in *B*. Data are from one experiment (*A*) or combined from two (*E*) or three (*B–D*) independent experiments.