Supporting Information

Mortensen et al. 10.1073/pnas.0913170107

Materials and Methods

Genotyping of Excision. To check for excision between loxP sites at the Atg7 locus, PCR was performed on whole-blood DNA extracted using the Blood and Tissue DNeasy kit (Qiagen, Hilden, Germany). The $Atg7^{Flox}$, $Atg7^{WT}$, and $Atg7^{Null}$ alleles were detected as previously described (1).

Immunoblotting. For Atg7 immunoblotting, whole-cell lysates were prepared from single-cell suspensions by homogenizing cells in Nonidet P-40 lysis buffer with protease inhibitors (Roche Complete, Roche, Basel), and proteins were then resolved by SDS/PAGE (Invitrogen, Carlsbad, CA; NuPAGE 12% Bis-Tris gels, Mops buffer), immunoblotted with rabbit polyclonal anti-Atg7 (a kind gift of Dr. Ueno, Japan) followed by anti-rabbit-HRP (Sigma, St Louis, Missouri), and detected with the ECL detection system (Amersham Biosciences).

LC3 Immunofluorescence. BM cells were fixed in 4% PFA, permeabilized in 0.1% Triton X-100, and stained with a rabbit anti-LC3b antibody at 4°C overnight (Sigma, St Louis, Missouri), followed by secondary antibody labeling with an anti-rabbit AlexaFluor-488 for 30 min at room temperature (Molecular Probes).

Colony-Forming Cell Assays. BM cells (1×10^5) were cultured in complete methylcellulose medium containing growth factors and cytokines (MethoCult GF-3434; Stem Cell Technologies, Vancouver), and the blind counting of colonies was undertaken after 12 days by light microscopy.

Nuclear Staining Using LDS751. After surface marker staining, cells were incubated in 2 μg/mL LDS751 solution (Invitrogen) for 30 min at RT, and then diluted with 150 μL FACS buffer and directly analyzed by FACS.

Flow Cytometry. For blood developmental stages, whole-blood or single-cell suspensions obtained from spleen and femoral BM were stained with anti-mouse Ter119 and anti-mouse CD71 (either PE- or FITC-conjugated). Absolute cell numbers were determined using TruCount tubes (BD Pharmingen, San Diego, CA) on whole blood and the samples were analyzed on a BD FACSCalibur machine. For intracellular cell staining, cells were surface-stained followed by fixation and permeabilization (eBioscience kit), and stained with FITC-conjugated anti-active caspase 3 monoclonal antibody (BD Pharmingen). Annexin V-PE (BD Pharmingen) staining was performed according to the manufacturer's instructions following cell surface marker staining. Mitochondrial stains were performed after surface marker staining by incubating at 37°C for 30 min with either 100 nM MitoTracker Green, 100 nM NaO, 100 nM TMRM, or 5 μM MitoSOX Red (all from Molecular Probes, Invitrogen, Eugene, Oregon) and directly analyzed without fixing. For leukocyte analysis, red blood cells were lysed using a 1× RBC lysis buffer (eBioscience, San Diego, CA) in single-cell suspensions from spleens. Splenocytes were then stained with the following antibodies: CD4-PE, CD4-FITC, CD8-APC, CD11c-PECy7, CD80-PE, CD86-PE, and class II-APC. All antibodies, unless stated otherwise, were purchased from eBioscience.

Red Blood Cell Lifespan. Blood was withdrawn from host mice, after 24 h and then weekly, and stained with anti-Ter119 and -CD71, and the percent of $CFSE^+$ cells was determined by flow cytometry. To ensure comparability between the weekly stainings, negative fluorescence values were set on an unstained control.

Reticulocyte Staining and Sorting. Cells were stained for surface markers and then incubated for 30 min at RT with thiazole orange (AnaSpec, Fremont, CA) at 100 ng/mL in 2 mM EDTA and 0.02% azide.

RBC Membrane Preparation. A total of 100×10^8 RBCs from each mouse were pelleted, and cells were resuspended in −500 μL ice-cold distilled water for lysis followed by centrifugation at 13,000 rpm for 15 min at 4°C. This procedure was repeated twice.

1. Mizushima N, et al. (2003) Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. *J Cell Sci* 116:1679–1688.

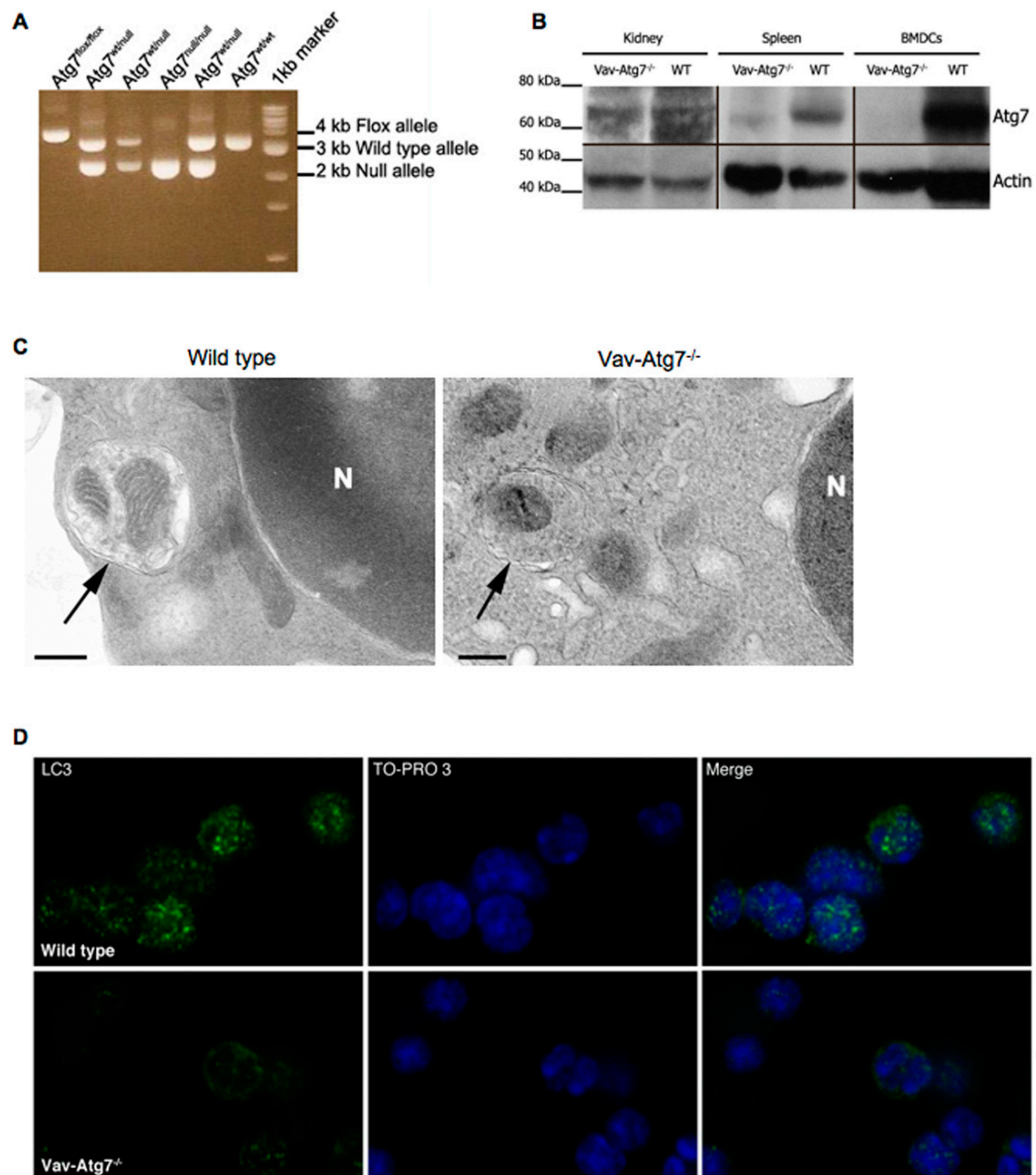

Fig. S1. Vav-Atg7$^{-/-}$ mice: conditional *Atg7* knockouts in the hematopoietic system. (*A*) Excision of the loxP-flanked region in blood genomic DNA from *Atg7*$^{Flox/Flox}$; Vav-iCre^{+} mice. Flox: nonexcised loxP-flanked *Atg7* allele; wt: wild-type *Atg7* allele; null: mutant *Atg7* allele resulting from excision of the loxP-flanked region. (*B*) Atg7 immunoblotted on kidney, spleen, and bone marrow-derived dendritic cell lysates. (*C*) EM micrographs of bone marrow cells from wild-type or Vav-Atg7$^{-/-}$ mice. Arrows indicate autophagosomal structures: a closed vesicle in the wild type, but incomplete and open in Atg7$^{-/-}$ cells (N, nucleus). (Scale bars, 100 nm.) (*D*) LC3-stained bone marrow cells directly ex vivo to look for constitutive autophagy levels.

PNAS PNAS PNAS

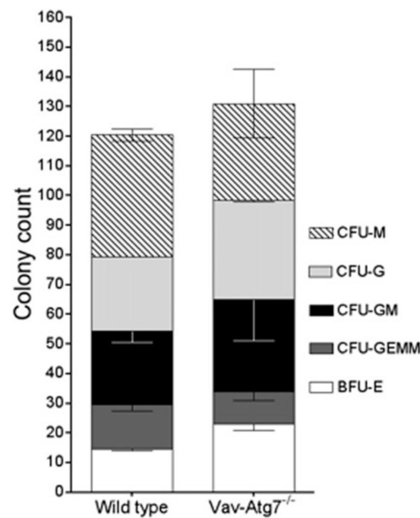

Fig. S2. Colony-forming cell assay in complete medium containing erythropoietin. Bone marrow cells (1×10^5) from 9-week-old mice were plated and colonies were counted after 12 days in culture. CFU, colony-forming unit; M, macrophage; G, granulocyte; GM, granulocyte-macrophage; GEMM, granulocyte-erythroid-megakaryocyte-macrophage; BFU-E, blast-forming unit erythroid.

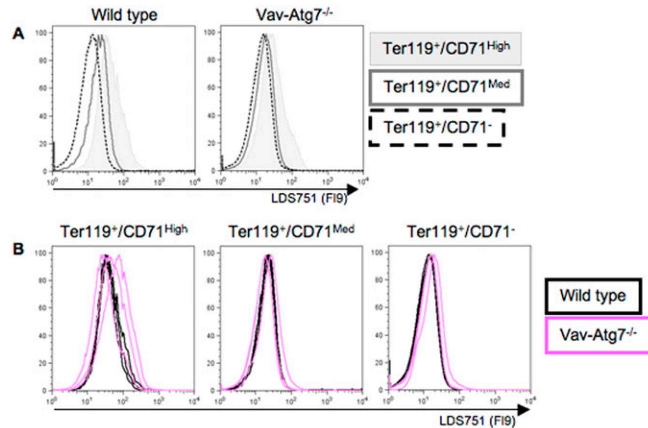

Fig. S3. *Atg7*$^{-/-}$ and wild-type peripheral blood erythroid stages have comparable levels of nucleic acid. (*A*) LDS751 levels on peripheral blood erythroid stages (gated as in Fig. 2*C*) overlaid from 9-week-old wild-type and Vav-Atg7$^{-/-}$ mice. (*B*) Overlays of three wild-type and three Vav-Atg7$^{-/-}$ LDS751 levels gated on the indicated peripheral blood erythroid stages.

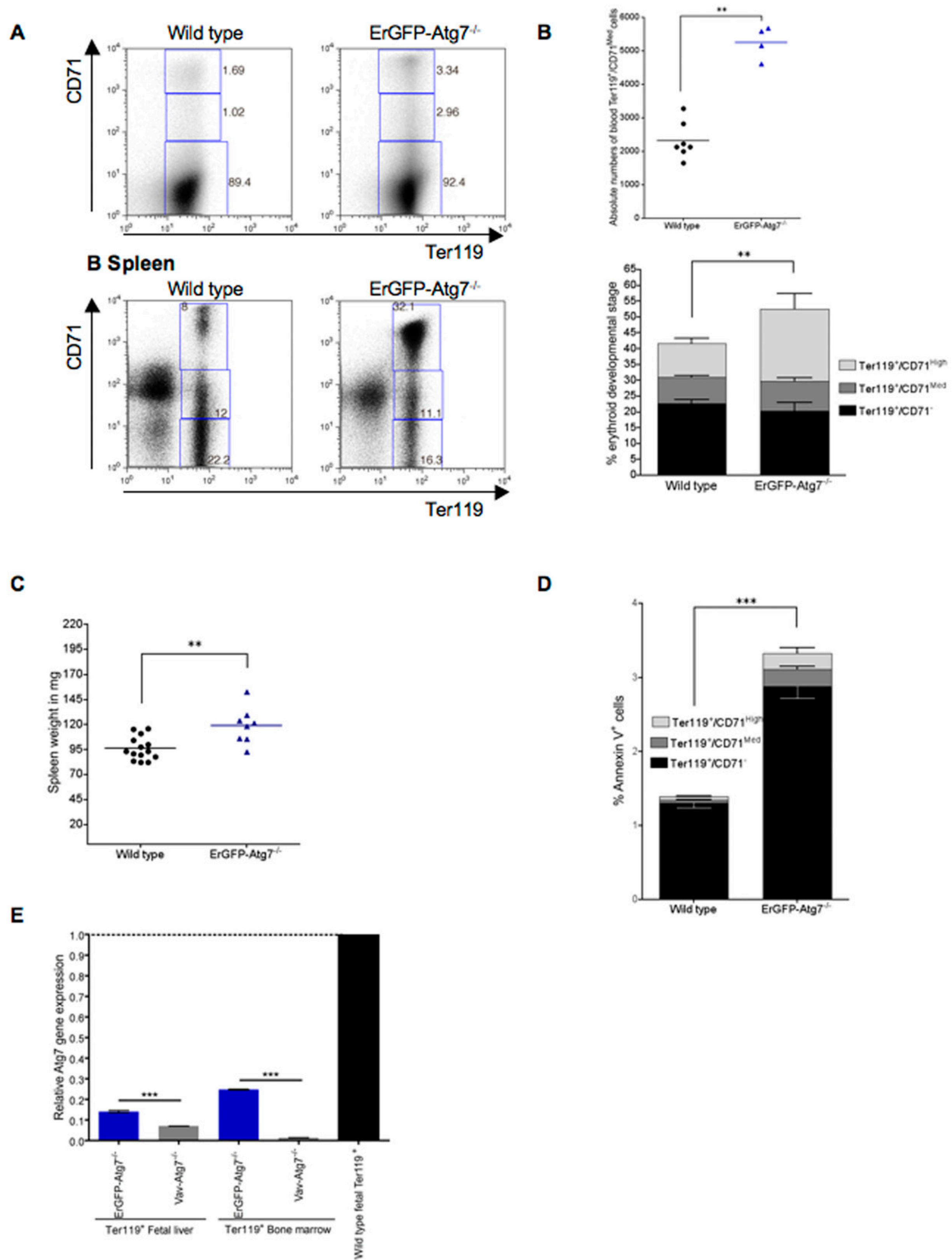

Fig. S4. ErGFP-Atg7$^{-/-}$ mice develop anemia and their RBCs show increased levels of death. (*A*) Peripheral blood erythroid cells from 7-week-old wild-type and ErGFP-Atg7$^{-/-}$ mice (left panels) and absolute count of blood Ter119^{+}/CD71^{Med} cells/μL (***P* = 0.0061). (*B*) Distribution of erythroid developmental stages in the spleen of 7-week-old mice (**interaction: *P* = 0.0021, two-way ANOVA, *n* = 5). (*C*) Spleen weight, 7-week-old wild-type and ErGFP-Atg7$^{-/-}$ mice (***P* = 0.0046). (*D*) Percent ex vivo Annexin V^{+} erythroid cells from peripheral blood, gated as in Fig. 3*C* (***interaction: *P* < 0.0001, two-way ANOVA, *n* = 3). (*E*) Relative Atg7 gene expression on fetal liver and adult bone marrow sorted Ter119^{+} cells from ErGFP-Atg7$^{-/-}$ and Vav-Atg7$^{-/-}$ mice (error bars: SD, *n* = 3, ****P* < 0.001, unpaired *t* test). Values were normalized to GAPDH and relative gene expression was determined by comparison with wild-type fetal Ter119^{+} cells.

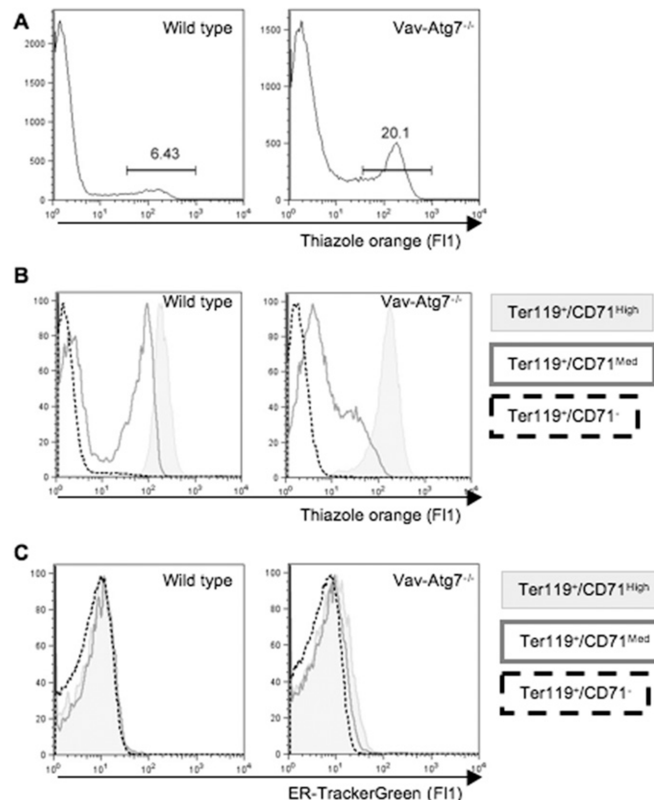

Fig. S5. RNA content at the Ter119+/CD71^{Med} is lower in *Atg7*$^{-/-}$ than in wild-type peripheral blood RBCs. (*A*) TO staining of peripheral blood RBCs of 6-week-old mice. (*B*) TO levels on RBCs (gated as in Fig. 2*C*) overlaid from 6-week-old wild-type or Vav-Atg7$^{-/-}$ mice. (*C*) ER-Tracker Green staining of peripheral RBCs overlaid from 9-week-old wild-type or Vav-Atg7$^{-/-}$ mice.

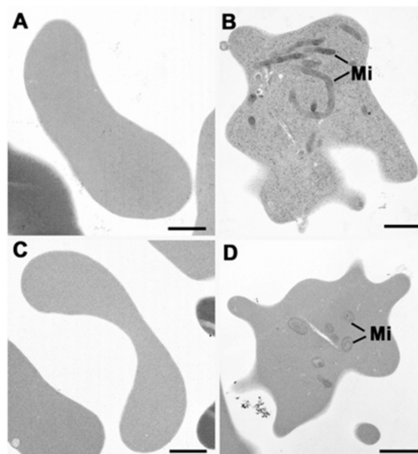

Fig. S6. Both peripheral blood *Atg7*$^{-/-}$ reticulocytes and mature RBCs show an accumulation of mitochondrial remnants. (*A*) Representative electron micrograph of a wild-type thiazole orange+ cell (TO+; reticulocyte); 15% of cells contained mitochondria. (*B*) Representative electron micrograph of a TO+ cell from Vav-Atg7$^{-/-}$ peripheral blood (Mi, mitochondria); 77% contained mitochondria. (*C*) Representative electron micrograph of a wild-type TO− cell (erythrocyte); 4% contained mitochondria. (*D*) Representative electron micrograph of an *Atg7*$^{-/-}$ TO− cell; 15% of cells contained mitochondria. (Scale bars, 1 mm.) One hundred cells were counted. Note that EM preparations do not always conserve the biconcave shape of mature erythrocytes, nor can damaged mitochondria be identified reliably.

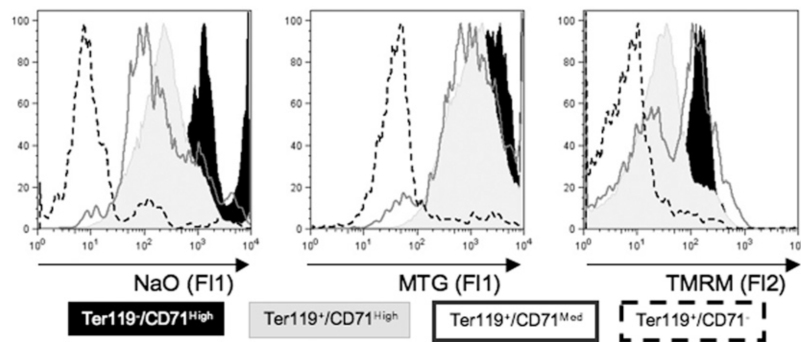

Fig. S7. Progressive mitochondrial loss during normal erythroid development. Ter119/CD71-stained wild-type bone marrow cells further stained with either NaO, MTG, or TMRM for 30 min at 37°C. Histograms were plotted on gates set according to Fig. *2B*.

Fig. S8. Vav-Atg7$^{-/-}$ mice are severely lymphopenic and *Atg7*$^{-/-}$ T lymphocytes display high levels of cell death. (*A*) Absolute numbers of CD19^{+} and TCRβ^{+} cells in the peripheral blood of 9-week-old mice. (*B*) Percent CD4^{+} and CD8^{+} cells in 9-week-old spleens. (*C*) *Left*: histograms of active caspase 3 levels gated on either CD4^{+} or CD8^{+} splenocytes ex vivo or after 5 h in culture. *Right*: active caspase 3 levels, gated as shown on the histograms on the left, in 9-week-old wild-type and Vav-Atg7$^{-/-}$ splenocytes. *$P < 0.05$, two-tailed Mann–Whitney test, $n = 4$ in all experiments. These results were repeated at least three times.

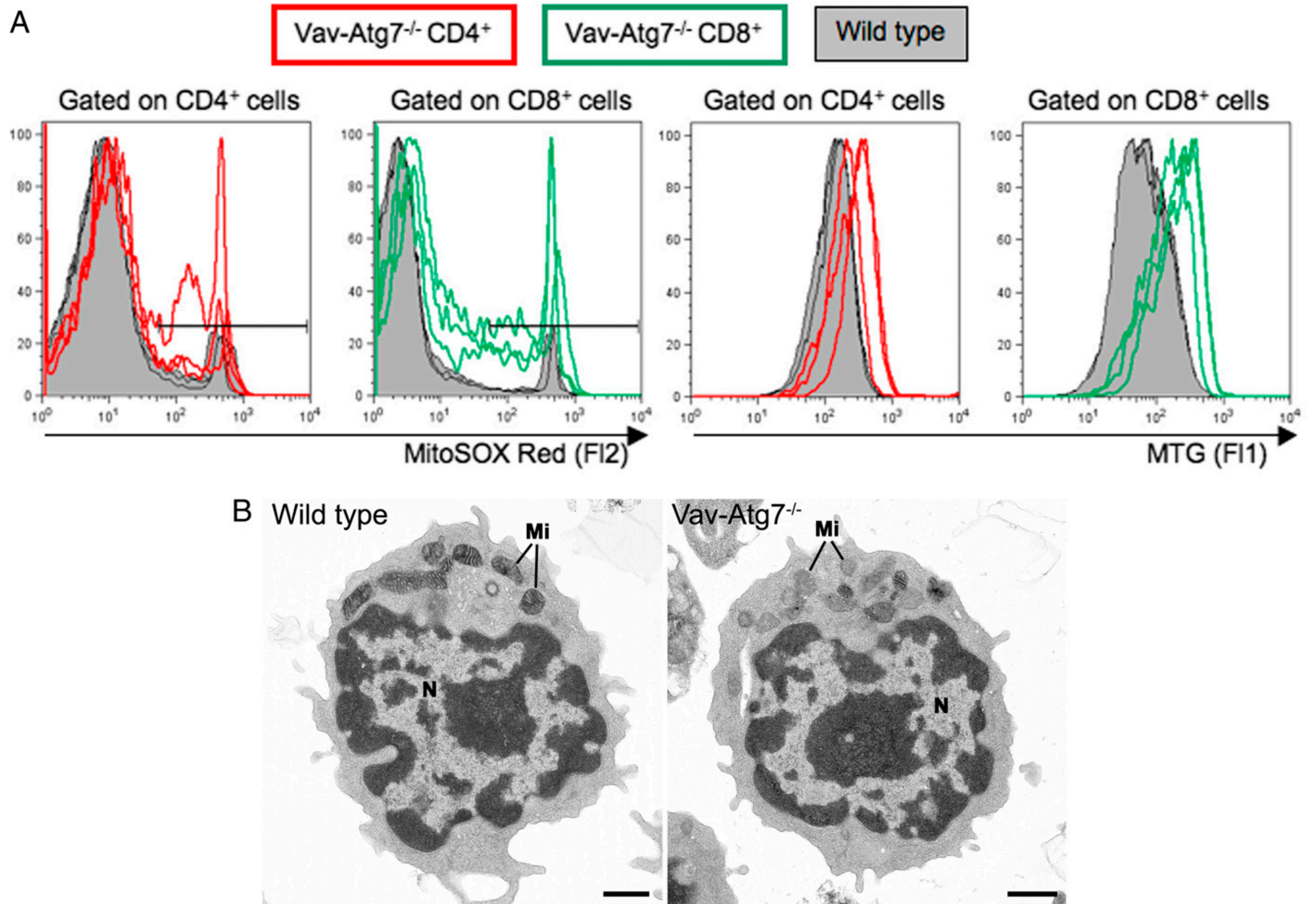

Fig. S9. Vav-Atg7$^{-/-}$ T lymphocytes accumulate mitochondria and have higher levels of mitochondrial superoxide. (*A*) Overlays of three wild-type and three Vav-Atg7$^{-/-}$ MitoSOX Red and MTG levels gated on either CD4$^+$ or CD8$^+$ splenocytes ex vivo from 9-week-old mice. (*B*) Mitochondrial count on EM from MACS-sorted CD4$^+$ splenocytes from 9-week-old mice (left) and representative EM of CD4$^+$ splenocytes Right. (Scale bars, 1 μm.) N, nucleus, Mi, mitochondria.

Table S1. Unique proteins identified in *Atg7*$^{-/-}$ RBC membrane preparations by mass spectrometry

Protein Name	Accession Number (Swiss-Prot)	PLGS Score*	Sequence coverage (%)**	Number of MS/MS queries***	Number of Atg7$^{-/-}$samples it was found in (n = 3)	Mascot Score (MudPIT scoring)
60 kDa heat shock protein mitochondrial precursor	P63038	181.2	28.2	11	3	127
Transferrin receptor protein 1 (1)	Q62351	145.6	28	19	3	50
Serotransferrin precursor	Q921I1	127.8	32	20	3	49
ATP synthase subunit alpha mitochondrial precursor	Q03265	37.84	23.9	11	3	29
Malate dehydrogenase mitochondrial precursor	P08249	34.3	31.7	10	2	ND
Acylglycerol kinase mitochondrial precursor	Q9ESW4	27.1	14.5	5	2	ND
Clathrin heavy chain 1	Q68FD5	25.05	11.3	13	1	ND
Coproporphyrinogen III oxidase mitochondrial precursor	P36552	23.5	17.2	6	2	ND
Autophagy related protein 12	Q9CQY1	19.64	15.6	4	1	ND
Lysosomal alpha glucosidase precursor	P70699	15.4	0.8	1	1	ND
Lysosomal associated transmembrane protein 4A	Q60961	15.19	11.6	1	2	ND

*Highest obtained score when protein identified in more than one sample and among the three replicates for each sample.
**Highest sequence coverage achieved when protein identified in more than one sample and within three replicates of each sample.
***Highest number of MS/MS queries obtained among all samples and triplicates of each sample in which the given protein was identified.
(1) Upregulation confirmed by flow cytometry (see Fig. 2 *A, B* and *C*).
ND: not determined.