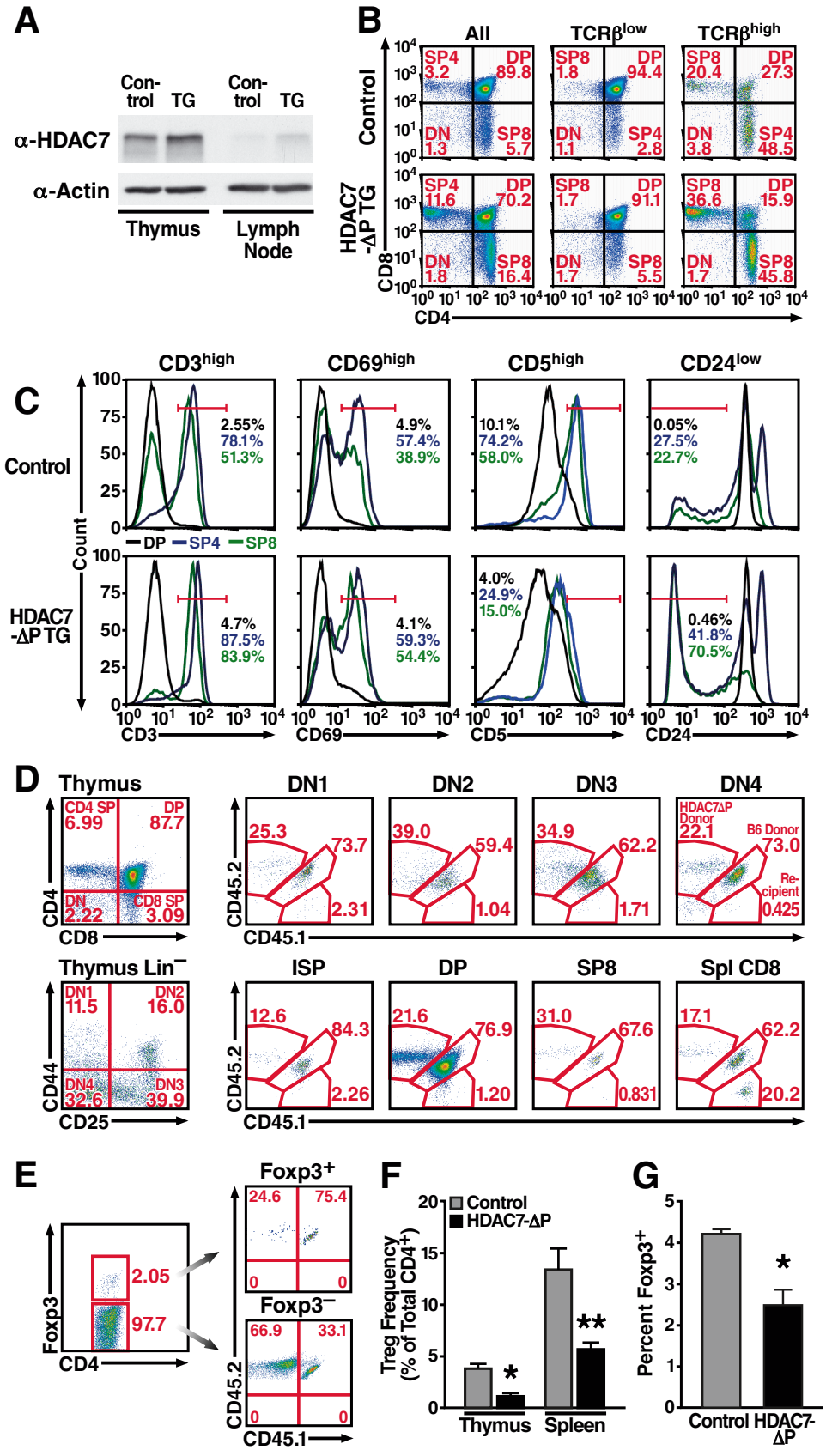
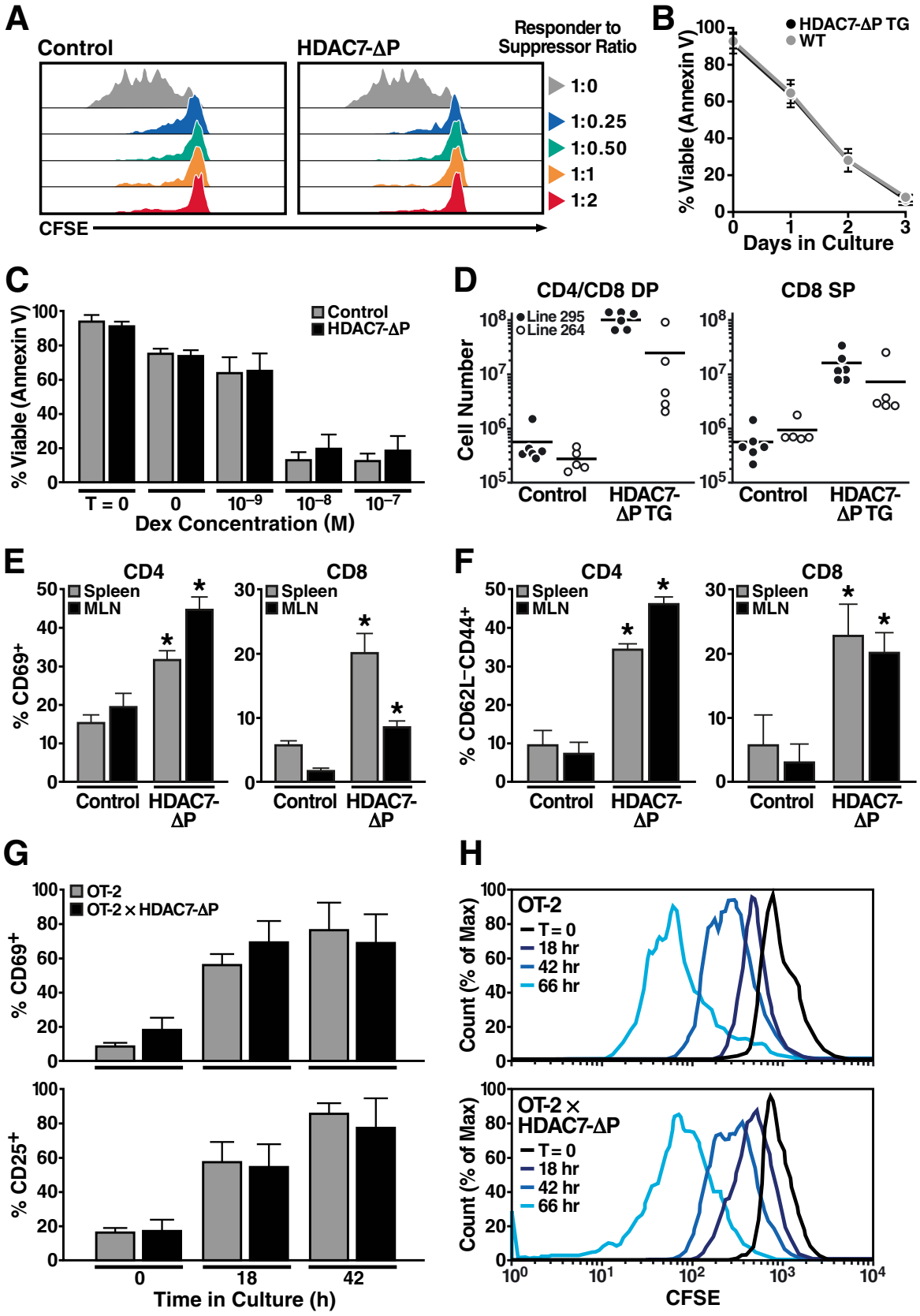
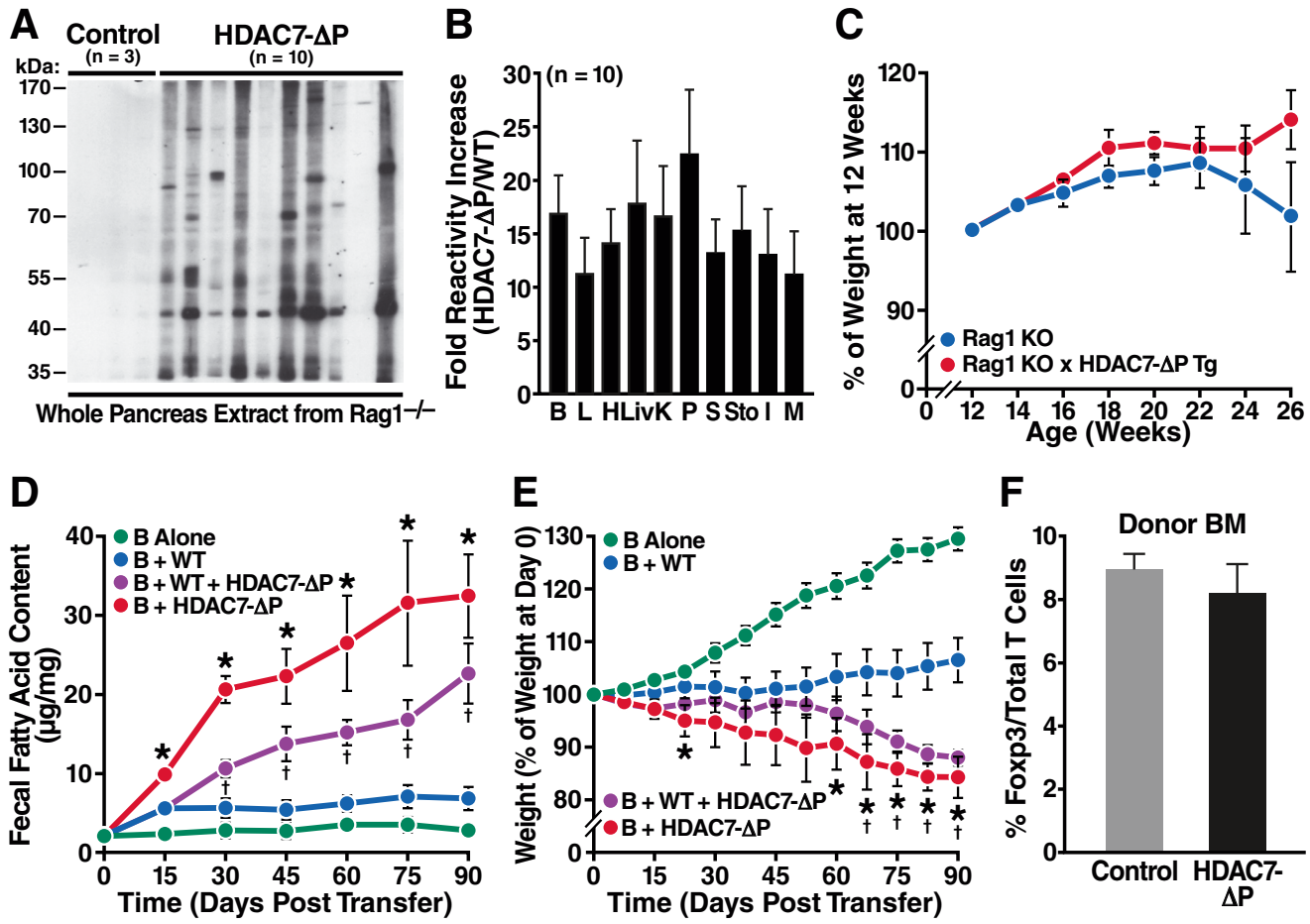
Kasler *et al*, Supplementary Figure 1



Kasler *et al*, Supplementary Figure 2



Kasler *et al*, Supplementary Figure 3



SUPPLEMENTARY FIGURE LEGENDS

Figure S1: Effect of HDAC7-ΔP Expression on Thymocyte Subsets. **A:** Expression of HDAC7 in thymocytes (left) and lymph node cells (right) of WT littermate control and HDAC7-ΔP transgenic mice. **B**: Representative flow scatter plots showing CD4 and CD8 expression for all cells (left), TCRβ-low cells (center), and TCRβ-high cells (right), in littermate control (top) and HDAC7-ΔP transgenic (bottom) mice. **C**: Representative flow histograms showing gating for differential expression of CD3ε, TCRβ, CD5, CD69, and CD24, as quantified in Fig. 1C. **D**: Representative flow data for analysis of T cell development in 1:1 HDAC7-ΔP: B6 bone marrow chimeras. CD45.2 single-positive cells are HDAC7-ΔP donor derived, cells expressing both CD45.1 and CD45.2 are derived from wild type donors, and cells expressing only CD45.1 are derived from the irradiated recipients (see top right panel). **E**: Representative flow data showing percentage of wild type ($CD45.1^+$/ $CD45.2^+$) and HDAC7-ΔP transgenic ($CD45.1^-$/$CD45.2^+$) cells present among $Foxp3^-$ (bottom) and $Foxp3^+$ (top) populations in CD4SP thymocytes of HDAC7-ΔP:wild-type bone marrow chimeras. **F:** Frequency of $Foxp3^+$ Tregs in wild type littermate control and HDAC7-ΔP transgenic-derived populations in thymus and spleen of 1:1 mixed radiation chimeras. Data for thymus are from 6 animals; data for spleen are from 12 animals. *: $P = 6.6X10^{-4}$, paired 2-tailed T-test, **: P = 0.013, paired 2-tailed T-test. **G**: Frequency of $Foxp3^+$ Tregs among CD4 SP thymocytes from wild type littermate (gray) and HDAC7-ΔP transgenic (black) mice. Data are from three WT/ HDAC7-ΔP littermate pairs.* P = 0.031, paired 2-tailed T-test.

Figure S2: Effect of HDAC7-ΔP on Treg Function, Thymic Selection, Thymocyte Survival and Peripheral Activation A: Representative flow histograms showing CFSE staining in anti-CD3/CD28-treated T splenic cells, co-cultured with Tregs from wild type (left) or HDAC7-ΔP transgenic (right) donors, at the indicated responder: suppressor ratios. Data are representative of three independent repetitions of the experiment. **B:** Viability (as measured by Annexin V staining) of WT control (gray circles) and HDAC7-ΔP transgenic (black circles) thymocytes over 72 hours of *ex-vivo* culture. **C:** Viability (as measured by Annexin V staining) of WT control (dark gray bars) and HDAC7-ΔP transgenic (light gray bars) 18 hours after *ex-vivo* treatment with the indicated concentrations of dexamethasone. **D:** Quantification of CD4/CD8 DP (left) and CD8 SP (right) thymocytes in littermate male H-Y TCR and H-Y TCR X HDAC7-ΔP transgenic mice. Results for 5 or 6 littermate pairs from 2 independent lines of HDAC7-ΔP transgenic mice are shown. $P < 0.01$ for all WT/ HDAC7-ΔP comparisons, Mann-Whitney U test. **E:** Frequency of activated ($CD69^{hi}$) CD4 (left) and CD8 (right) T cells for WT and HDAC7-ΔP transgenic cell populations in spleen and mesentery lymph node of 1:5 WT: HDAC7-ΔP mixed radiation chimeras. Results shown are for 5 animals. *: $P = 4.84X10^{-5} – 0.02$, 2-tailed paired T-test. **F:** Frequency of effector memory ($CD44^{+}$, $CD62L^{-}$) T cells for WT and HDAC7-ΔP transgenic populations in spleen and MLN of 1:5 WT: HDAC7-ΔP radiation chimeras. Results shown are for 5 animals. *: $P = 0.0002 – 0.017$, 2-tailed paired T-test. **G:** Upregulation of CD69 (top) and CD25 (bottom) in OT-2 (dark gray bars) and OT-2 X HDAC7-ΔP (light gray bars) splenic CD4 T cells, stimulated *ex-vivo* for the indicated times with APC, Ova peptide, and IL-2. Bars represent mean +/- std. dev. of 4 independent experiments. **H:** Representative flow

histograms showing Dilution of CFSE by cells stimulated *ex-vivo* as in Fig. S3G, at indicated time points. Data are representative of 4 independent experiments.

Figure S3: Effect of HDAC7-ΔP on Immune Self Tolerance. **A**: Western blot reactivity against $Rag1^{-/-}$ pancreas extracts of 3 wild type and 10 HDAC7-ΔP transgenic-derived sera. **B:** Quantification of autoreactivity by optical densitometry of 10 parallel western blots, as in Fig. 6B, for paired wild type control and HDAC7-ΔP transgenic sera. Error bars represent s.e.m.; $P < 0.05$ for all tissues shown, paired 2-tailed T-test. **C:** Mean weights (in % of weight at 12 wks age) of cohorts of 6 Rag1 KO littermate control (blue symbols) and 6 Rag1 KO/HDAC7-ΔP transgenic mice (red symbols) from 12 to 26 weeks of age. **D:** Change in fecal fat content over time in 8 Rag1 KO mice transferred with wild type B cells and T cells from WT and HDAC7-ΔP transgenic mice as indicated in legend. **E:** Mean weights (in % of weight at 12 wks age) of 4 cohorts, each of 8 Rag1 KO mice transferred with wild type B cells and T cells from WT and HDAC7-ΔP transgenic mice as indicated in legend. *: $P < 0.05$, 2-tailed T-test, HDAC7-ΔP T cells only vs. WT T cells only; †: $P < 0.05$, 2-tailed T-test, HDAC7-ΔP T cells + WT T cells vs. WT T cells only. **F**: Percentage of $Foxp3^{+}$ cells present among $CD3^{+}$ T cells in peripheral lymphoid tissues (Spleen plus lymph nodes) of radiation chimeras reconstituted with only wild type bone marrow cells (left) and in chimeras reconstituted with wild type and HDAC7-ΔP transgenic bone marrow at a 1:5 ratio (right). Data are shown for 5 animals in each group, +/- s.e.m.

DETAILED METHODS:

*Mouse Strains and animal handling***:** Animals were housed under standard conditions in a specific pathogen-free barrier facility at UCSF. Unless otherwise stated, all animals were analyzed at between three and six weeks of age, and controls were sex-matched animals from the same litter. All experimental strains were made on a C57BL/6 (B6) genetic background. B6, BoyJ, OT-2, and $Rag1^{-/-}$ mice were obtained from Jackson Laboratories. H-Y mice were obtained from Taconic. Mice expressing the HDAC7-ΔP transgene were prepared at the Gladstone Institutes Transgenics Core Facility by pronuclear injection of linearized p1013 LCR constructs into B6 recipients according to standard protocols. P1013 LCR is described elsewhere {Kasler, 2011 #1507}. The full-length coding sequence for HDAC7-ΔP (Human HDAC7, with the mutations S155A, S358A, and S486A) was inserted into the *Bam* H1 site of p1013LCR.

Antibodies: Antibodies used for Western blotting were as follows: HDAC7: H-273 rabbit polyclonal (Santa Cruz Biotech), β-Actin: clone C-4 (MP Biomedicals). Phospho-Erk: D13.14.4E Rabbit monoclonal (Cell Signaling). Phospho-JNK: 81E11 Rabbit monoclonal (Cell Signaling). Phospho-p38: rabbit polyclonal cat # 9211 (Cell Signaling). Erk: 3A7 mouse monoclonal (Cell Signaling). JNK: rabbit polyclonal cat # 9252 (Cell Signaling). P38: rabbit polyclonal cat # 9212 (Cell Signaling). Antibodies used for flow cytometry and cell sorting were as follows: CD4: clone GK1.5, fluorescein isothiocyanate (FITC), phycoerythrin (PE), or Alexa-Flour 647 (AF647)-conjugated (UCSF hybridoma core facility). CD8α: clone YTS169.4, conjugated to AF647 (UCSF

HCF) or clone 53-6.7, conjugated to PE (BD). CD3ε: clone 145-2C11, conjugated to FITC (UCSF HCF), or allophycocyanin (APC) (BD). CD44: clone IM7.8.1, FITC-conjugated (Invitrogen). CD25: clone 3C7, PE-conjugated (BD). CD2: clone RM2-5, FITC-conjugated (BD). CD5: clone 53-7.3, APC-conjugated (eBioscience). CD62L: clone MEL-14, PE-conjugated (BD). CD147: clone RL73, PE-conjugated (eBioscience). CF11b (Mac-1): clone M1/70, APC-conjugated (eBioscience). Ly-6G (Gr-1): RB6-8C5, APC-conjugated (eBioscience). Ter-119: clone TER-119, APC-conjugated (eBioscience). NK1.1: clone PK136, APC-conjugated (eBioscience). Control rat IgG_1: Clone R3-34, PE-conjugated (BD). CD45.1: clone A20.1-7, Alexa 647-conjugated (UCSF hybridoma core facility). CD45.2: clone 104, PerCP-conjugated (Biolegend).

Analysis of lymphocyte subsets by flow cytometry: Lymphoid cells were prepared from mouse thymus, spleen, and lymph nodes by standard techniques, stained with fluorochrome-conjugated antibodies for 30 minutes at 4ºC (see Antibodies and Western Blotting below), and fixed with PBS containing 2% paraformaldehyde. For isolation of pancreas-infiltrating were isolated as follows: After removal of pancreatic lymph nodes, whole pancreas was minced finely and sequentially digested at 37˚C in 1ml DMEM media containing collagenase IV (Sigm) at 1 mg/ml for 1min, at 0.5mg/ml for 10min, and at 0.2mg/ml for 5min, with collection of suspended calls at each step. Cells from the combined supernatants were washed 3 times with ice-cold PBS and stained for flow cytometry. Events were gated by forward and side scatter to exclude dead cells. For analysis of early thymic and bone marrow subsets (Fig. 1d, 1e, s1a), T cell precursors were identified by gating on cells with no expression of CD3, CD4, CD8, NK1.1, Mac-1,

Gr-1, B220, or Ter119 (i.e lin^-). Within the lin^- populaton, DN1-DN4 stages were identified by expression of CD25 and CD44, and the CLP population was identified by expression of IL7Rα, c-Kit, and Sca-1. Analytical flow cytometry was performed using a FACS Calibur flow cytometer (BD). Subsequent data processing and preparation for presentation were done using the FlowJo 7.0 data analysis package (Treestar Inc.). Cell sorting was performed using the BD Facs-Aria platform.

DNA microarray analysis: Differential gene expression data for loss of HDAC7, positive selection, and negative selection, used for comparison purposes in this study, were published elsewhere {Kasler, 2011 #1507}, and are archived in the GEO database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE26488. Raw microarray data associated with this work have also been deposited in GEO, under accession number GSE26488. DP thymocytes were isolated from 3 littermate pairs of OT-2 and OT-2, HDAC7-ΔP transgenic animals injected with agonist peptide (chicken ovalbumin, residues 323-339, 200 microliters i.p. of a 50 μM solution in PBS) 3 hours previously. Total RNA was prepared from these thymocytes using RNeasy kit reagents (Qiagen) and used to make microarray probes. Probes were prepared using Affymetrix GeneChip Whole Transcript cDNA synthesis, amplification, and cleanup modules, and hybridized to Affymetrix mouse Gene 1.0 ST arrays. Arrays were scanned using an Affymetrix GCS3000 scanner and the GCOS 1.4 data acquisition software, and data were normalized using the RMA algorithm in Affymetrix Expression Console. Normalized data were then assessed for significant differential expression using the Stanford University SAM analysis package {Tusher, 2001 #475}. Significance was assessed using the 2-class

unpaired t-test method. Genes were scored as differentially expressed based on a SAM q-value < 20% and a mean fold differential expression value of at least 1.5.

Northern and Western blotting: Thymocyte lysates for Western blotting were prepared using RIPA buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholate) with protease inhibitor cocktail (Sigma) and phosphatase inhibitors (1mM NaF, 100μM $NaVO_4$). For *ex-vivo* activation, thymocytes were isolated by magnetic bead sorting for CD8 expression, followed by 2 hours resting in RPMI/10% FBS. Cells were then plated at $2X10^6$ cells/ml, with 2μg/ml α-CD28, in 6-well dishes coated overnight at 4°C with α-CD3 (10μg/ml in PBS, overnight). SDS PAGE-resolved proteins were transferred to nitrocellulose membranes (Amersham), and visualized using ECL detection reagents (Amersham). For demonstration of autoantibodies, pancreatic and other tissue extracts were prepared from B6/$Rag1^{-/-}$ mice and homogenized in 1% SDS containing 0.2M Tris-HCl (pH 6.8) with protease inhibitor cocktail (Sigma). Protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce). Aliquots of pancreas lysate (50 μg of total protein per lane) were resolved by SDS-PAGE (7.5% gel) and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked in TBST (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20) supplemented with 4.0% BSA (Sigma). Autoantibodies were detected by incubating membrane with serum samples (1:250 dilution) followed by HRP-conjugated goat anti-mouse IgG (1/5000; Jackson ImmunoResearch Laboratories). Signal was detected using ECL (Amersham). For quantitative Western analysis shown in FigS3B and Fig4B, films were optically scanned. For Fig4D-E, membranes were scanned with a LiCor Odyssey

scanner. Band intensity was quantified using ImageJ program (Wayne Rasband, National Insititutes of Health). For Northern blotting, total RNA was prepared from thymocytes of HDAC7-deficient and littermate control animals using Trizol (Invitrogen). Fifteen micrograms of RNA/lane were resolved by formaldehyde agarose electrophoresis, and RNA transferred to Hybond XL (Amersham) charged nylon membranes. Membranes were hybridized with specific end-labeled 40-mer DNA oligonucleotide probes overnight at 42°C in Ultrahyb Oligo hybridization buffer (Ambion), washed, exposed to BAS IIIs storage phosphor screens (Fuji), and imaged using a Molecular Imager FX scanner (Bio-Rad) at 50 micron resolution. Raw 16-bit TIFF image data for each image were uniformly and linearly re-scaled and mapped to 8-bit grayscale values in order to allow for visual interpretation using Adobe Photoshop CS3 (Adobe). A minimal, uniform background tone was also added to each image in order to define the image boundary. Probe sequences for Northern blotting were as follows: GAPDH: 5'-gtcattgaga gcaatgccag ccccggcatc gaaggtggaa-3'. Nor-1: 5'-agcttcaggt agaagatgcg ctggaggccc tgggtacaga-3'. GADD45β: 5'-tctcagtctc ctcttgcctg aggtgccctc cttccgacct-3'.

Adoptive transfer of lymphocytes into Rag1$^{-/-}$ recipients: CD3^{+} T cells were isolated from the spleens of HDAC7-ΔP mice or littermate controls. CD19^{+} B cells were isolated from spleens of littermate controls only. Isolation was performed using magnetic beads and the AutoMacs platform (Miltenyi Biotech). Rag1$^{-/-}$ recipients (8 for each group) were injected via tail vein with 5X10^{6} B cells alone, 5X10^{6} B cells together with 5X10^{6} HDAC7-ΔP T cells, 5X10^{6} B cells together with 5X10^{6} littermate control T cells, or 5X10^{6} B cells together with 2.5X10^{6} HDAC7-ΔP and 2.5X10^{6} littermate control T cells.

The body weight of recipients was measured weekly until 90 days post-transfer. The loss of 20% of starting body weight was treated as a study endpoint, after which mice were sacrificed and prepared for histological analysis. Fecal samples were collected weekly and fecal fatty acid quantified as follows: Briefly, 100ug of feces were extracted 3 times with chloroform-methanol, followed by precipitation of the supernatant with $CaCl_2$. Acid dichromate reagent (5ug of $K_2Cr_2O_7$ in 1 ml of concentrated H_2SO_4) was added to the supernatants, followed by incubation at 100 ˚C for 10min. OD values were measured at 450nm.

Production of mixed radiation chimeras: Eight to ten week-old BoyJ (CD45.1) mice were given 1200 Rads of total body gamma irradiation from a ^{137}Cs source (J.S. Shepherd & Associates), in separate doses of 700 and 500 Rads, given 3 hours apart. Two hours after the second dose, mice were reconstituted with $5X10^6$ bone marrow cells from WT (CD45.1/.2 heterozygote) or HDAC7-ΔP transgenic (CD45.2) donors, injected retro-orbitally in 200 µl of PBS. Mice were given neomycin sulfate (USP, Invitrogen) at 2 mg/ml in drinking water for 2 weeks following irradiation.

Histology/ Immunofluorescence imaging: For histologic analysis, whole animals were given an overdose of tribromoethanol (400mg, i.p.) and after all withdrawal reflexes were absent transcardially perfused with cold PBS, followed by PBS with 3% paraformaldehyde (PFA). Perfused animals were stored 18 hours at 4°C in PBS/3% PFA and then transferred to 70% ethanol for long-term storage. Tissue specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin according to

standard protocols. Bright-field images for presentation were acquired using a Zeiss Axio Observer.z1 microscope, using Axiovision v4.8 software. In order to emphasize salient features for presentation, images were cropped and also uniformly adjusted for brightness and color balance using Adobe Photoshop CS4. For indirect Immunofluorescence staining, Frozen tissue sections were fixed in cold acetone (-20^{o}C) for 5 minutes, air dried, washed with PBS+ 0.1% Tween 20, blocked with PBS, 1% BSA, 0.1% Tween 20, then incubated overnight at 4°C with diluted (1:50 in PBS) sera as indicated (Fig. 5c). Slides were incubated with Alexa 488-conjugated anti-mouse secondary antibodies diluted in PBS+ 1% BSA, for 1 hr at RT, washed, then incubated with TOPRO3 dye at 2 mg.mL in PBS for 30 min. at room temperature. After washing, slides were mounted with Mowiol mounting medium and analyzed with a Leica TCS SP5 laser-scanning confocal microscope.

Assay of ex-vivo proliferation and Treg function: $CD4^{+}CD25^{hi}$ suppressor cells from HDAC7-ΔP and littermate control mice were sorted by FACS (BD FACS Aria II). $CD4^{+}CD25^{-}CD44^{-}CD69^{-}$ responder cells were isolated from Boy/J mice using magnetic beads (AutoMACS, Miltenyi Biotech). CFSE-labeled responder cells ($5X10^{4}$) were seeded onto round-bottom 96-well plate with a 1:1 mixture of αCD3 and αCD28 microbeads (Invitrogen) as well as the indicated number (Fig. S1d) of $CD4^{+}CD25^{hi}$ suppressor cells. Cells were harvested after 3 days, stained for CD4 and CD45.1 to distinguish responder and suppressor cells, and analyzed by flow cytometry (BD FACSCalibur). To assay *ex-vivo* proliferation of OT-2/ HDAC7-ΔP cells, splenocytes were prepared from OT-2 and OT-2/ HDAC7-ΔP mice, CFSE-labeled and cultured at $5X10^{6}$/ml in RPMI/5% FBS supplemented with 20U/ml IL-2 and 0.5μM $Ova_{323-339}$.

Surface marker expression and CFSE dilution were measured by flow cytometry at indicated intervals (Fig. S2G-H).