# Supporting Information

## Park et al. 10.1073/pnas.1221925110

### SI Materials and Methods

**Immunoprecipitation and Immunoblotting.** Cells were lysed with Nonidet P-40 lysis buffer [1% Nonidet P-40, 20 mM Tris·HCl, (pH 7.5), 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 2 mM $Na_3VO_4$, and 10 μg/mL each of aprotinin and leupeptin) or with 1× SDS sample buffer [50 mM Tris·HCl (pH 6.8), 100 mM DTT, 2% SDS, and 10% glycerol]. Cell lysates were adjusted to 0.1% Nonidet P-40 and incubated with anti-FLAG M2 agarose beads (Sigma-Aldrich) for 4 h at 4 °C. The immune complexes were recovered by low-speed centrifugation, and the beads were washed extensively with the binding buffer with 0.1% Nonidet P-40 and then eluted with buffer containing 20 mM Tris·HCl (pH 8.0) and 2% SDS. Immunoprecipitated proteins were separated by SDS/PAGE and transferred to nitrocellulose membrane (Bio-Rad). Membranes were visualized by Western blot analysis with the enhanced chemiluminescence detection system (ECL; GE Healthcare). When necessary, membranes were stripped by incubation in stripping buffer (Thermo Fisher Scientific) for 15 min with constant agitation, washed, and then reprobed with various other antibodies.

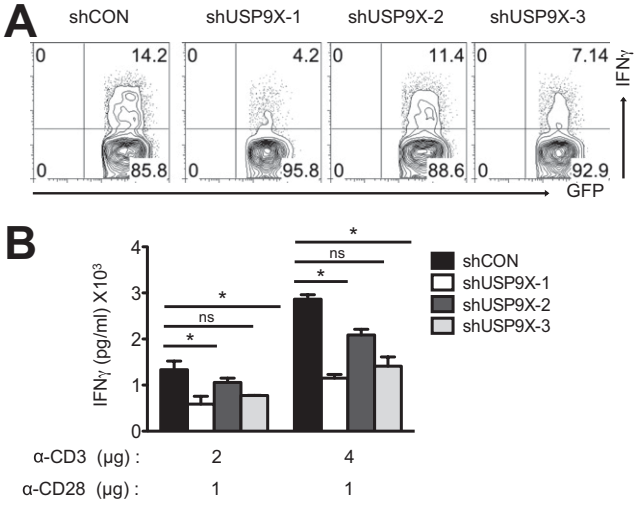


**Fig. S1.** IFN-γ production of ubiquitin-specific protease 9X (USP9X) shRNA-expressing chimeric mice. IFN-γ production by control and USP9X shRNA-expressing $CD4^+$ T cells was stimulated with anti-CD3 + anti-CD28 for 48 h and analyzed by intracellular cytokine staining (*A*) or ELISA (*B*). The data are compiled from three independent experiments. Error bars indicate mean ± SD. *$P < 0.05$, two-tailed unpaired *t* test.

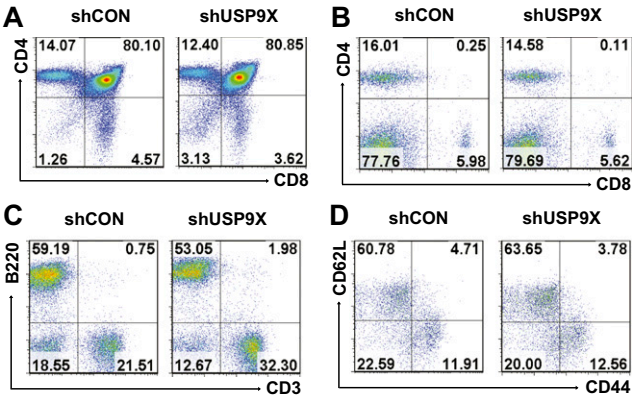


**Fig. S2.** Phenotypic analysis of USP9X shRNA-expressing chimeric mice. (*A*) Flow cytometry analysis of thymocytes in control or USP9X shRNA-expressing chimeric mice stained with anti-CD4 and anti-CD8 ($n = 5$ per group). (*B* and *C*) Flow cytometry analysis of splenocytes in control or USP9X shRNA-expressing chimeric mice stained with anti-CD4 and anti-CD8 (*B*) or with anti-CD3 and anti-B220 (*C*) ($n = 5$ per group). (*D*) Expression of CD62L and CD44 on CD4-gated splenic T cells from control or USP9X shRNA-expressing chimeric mice ($n = 5$ per group).

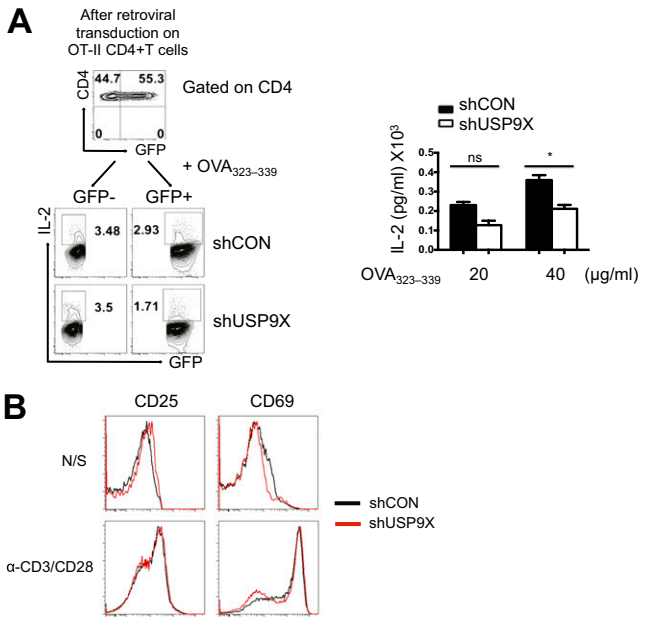


**Fig. S3.** T-cell activation in USP9X-deficient T cells. (*A*) IL-2 production by ovalbumin (OVA)-specific $CD4^+$ T cells in vitro. Splenocytes from OVA-specific, MHC class II restricted TCR (OT-II) transgenic mice were stimulated with concanavalin A (1.5 μg/mL) for 24 h and then transduced with control or shUSP9X-expressing retroviruses. At 2 d after transduction, splenocytes were stimulated with $OVA_{323–339}$ peptide for 8 h and analyzed by intracellular staining and flow cytometry (*Left*), or isolated OT-II $CD4^+$ T cells were stimulated with $OVA_{323–339}$ peptide for 24 h and analyzed by ELISA (*Right*). The data are representative of and compiled from three independent experiments. Error bars indicate mean ± SD. $*P < 0.05$, two-tailed unpaired *t* test. (*B*) Expression of activation markers in control or USP9X shRNA-expressing $CD4^+$ T cells. Purified $CD4^+$ T cells were stimulated with anti-CD3 + anti-CD28 for 16 h and then analyzed for CD25 and CD69 expression by flow cytometry. The data are representative of three independent experiments.

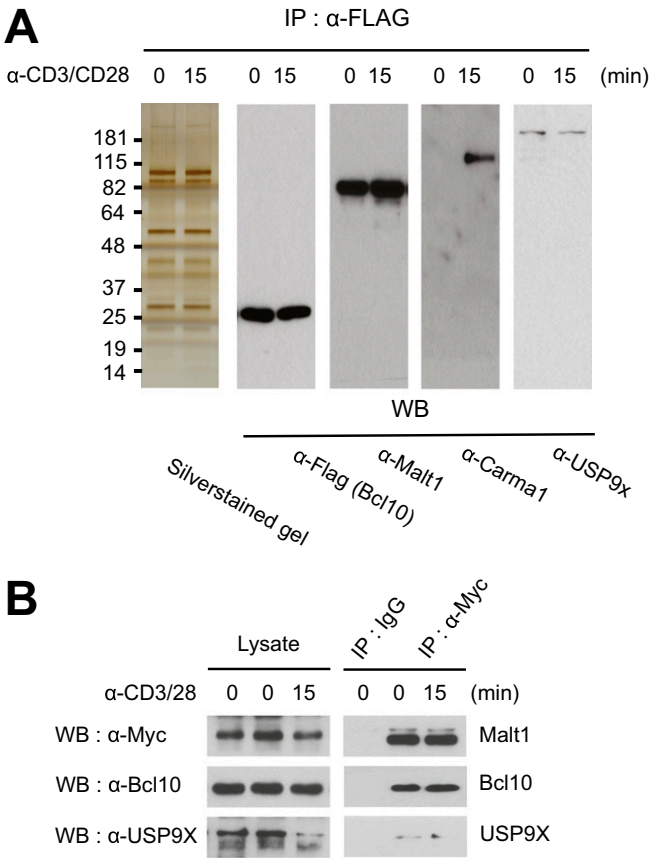


**Fig. S4.** Immunoaffinity purification of Bcl10 and Malt1. (*A*) Immunoaffinity purification of the FLAG-Bcl10 complex was performed with anti-FLAG antibody in the presence or absence of anti-CD3 + anti-CD28 stimulation. The purified FLAG-Bcl10 samples were subjected to SDS/PAGE (gradient of 4–20% polyacrylamide), visualized by silver staining, and immunoblotted with the indicated antibodies. Protein size markers (in kDa) are indicated on the left. The data are representative of two independent experiments. (*B*) Interaction between USP9X and Malt1. Jurkat cell line carrying SV40 large T antigen cells were transfected with Myc-Malt1 and USP9X and stimulated with anti-CD3 + anti-CD28 for the indicated times. Myc-Malt1 was immunoprecipitated with anti-Myc antibody. The immunoprecipitates were separated by SDS/PAGE and immunoblotted with indicated antibodies. The data are representative of two independent experiments.

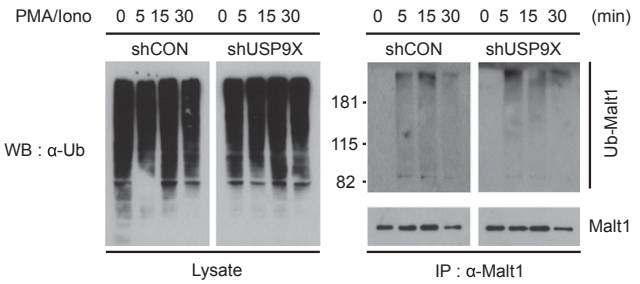


**Fig. S5.** USP9X does not exert deubiquitinase activity on Malt1. TCR-induced ubiquitination of Malt1 in control and USP9X-shRNA expressing cells is shown. JE6.1 cells were left untreated or were stimulated with anti-CD3 + anti-CD28 for the indicated times. Cell lysates were generated by the addition of SDS lysis buffer (2% SDS) and immunoprecipitated with anti-Malt1 antibody. The whole-cell lysates (*Left*) and immunoprecipitates (*Right*) were subjected to SDS/PAGE and analyzed by immunoblotting with anti-ubiquitin antibody. The data are representative of two independent experiments.

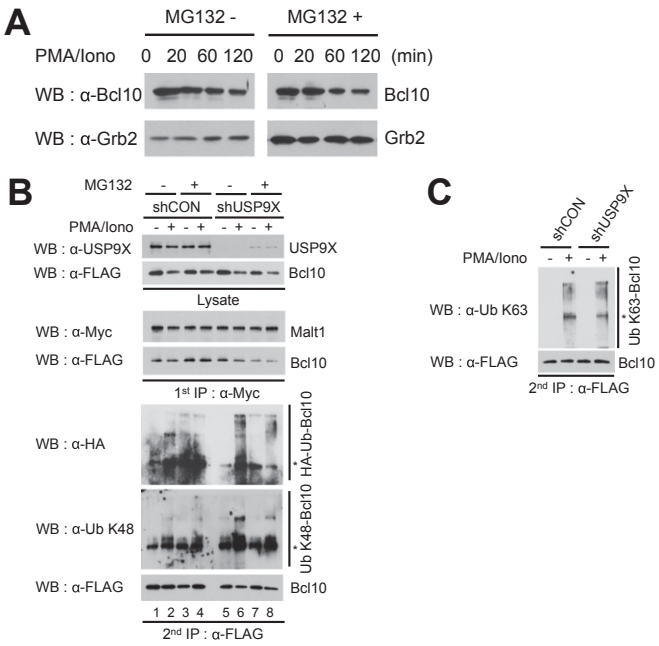


**Fig. S6.** Nonproteasomal degradation of Bcl10 on TCR stimulation. (*A*) Jurkat (clone JE6.1) T cells were incubated with or without MG132 (25 μM) for 3 h. Then the cells were washed and stimulated with PMA + ionomycin (Iono) for the indicated times. Cell lysates were subjected to immunoblotting with the indicated antibodies, with anti-Grb2 blot used as a loading control. The data are representative of two independent experiments. (*B* and *C*) JTAg cell lines stably expressing control or shUSP9X were transfected with CD28, Flag-Bcl10, Myc-Malt1, and HA ubiquitin, and then incubated with or without MG132 for 3 h. Cells were washed and stimulated with PMA + Iono. Cell lysates were immunoprecipitated with anti-Myc [first immunoprecipitation (IP)], and the immunoprecipitates were eluted by heating in the presence of SDS. The eluates were reimmunoprecipitated with anti-FLAG (second IP), and the immunoprecipitates were subjected to SDS/PAGE and analyzed by immunoblotting with anti-HA or anti-K48 polyubiquitin antibodies (*B*) or anti-K63 polyubiquitin antibody (*C*). *IgG. The data are representative of two independent experiments.