Supplementary methods

Construction of KI mice.

A 9kb fragment containing the long arm of homology, short arm of homology, and target region of interest of SLP76 was subcloned into a pSP72-derived backbone vector using a Genebridges Red/ET recombineering kit (Dresden, Germany). Cloning was accomplished by amplifying the vector using chimeric oligos containing 20bps of homology to the vector and 50bps of homology to the ends of the 9kb fragment. A selection cassette containing neomycin flanked by LoxP and FRT sites was then introduced 3′ to exon 7 using recombineering techniques. During this step, a unique BsiW1 site was inserted at the 5′ end of the selection cassette. The A→T point mutations in exon 7 were generated by overlapping PCR mutagenesis. Thus, unique BsiW1 sites were introduced at the ends of the PCR products, which allowed for insertion into the targeting vector by conventional ligation techniques into the unique BsrG1 site 5′ to exon 7 and the inserted BsiW1 site 5′ to the selection cassette.

Linearized DNA was electroporated into 129 SvEv iTL ES cells that were selected with G418. ES cell DNA was initially screened by PCR and verified by Southern blot. For Southern blots, ES cell DNA was digested with EcoRV and probed with the 3′ probe depicted in Figure 1. Targeted ES cells were injected into C57BL/6×129 blastocysts. The neomycin gene was excised from targeted Y145F ES prior to injection into blastocysts by electroporating cells with 30μg of CMV-Cre plasmid (Haase et al., 2001). Y145F ES cells with neomycin deleted were screened by PCR as described in Figure S1. The neomycin gene was

excised from targeted Y112/128F mice by breeding them to E2a-cre mice (Jax Mice), which express Cre recombinases in the early mouse embryo resulting in Cre-mediate recombination in germ cells. Genomic DNA from established KI lines was isolated and sequenced to verify the presence of the KI mutations. WT and KI SLP76 protein levels were equivalent in thymocytes as measured by Western blot analysis.

Intracellular SLP76 staining.

Thymocytes were stained with antibodies to CD4 and CD8, washed, then fixed and permeabilized using the eBioscience Fix/Perm solution. Cells were stained with anti-SLP76-Fitc (eBioscience) or a FITC Ig isotype control.

Immunoprecipitation of SLP76 with Vav1 and Itk.

Cells used for SLP76 and Itk co-immunoprecipitations were lysed in a 1% NP40, 100mM NaCl, 50mM Tris pH 8.0, 10% glycerol and inhibitors as described in Materials and Methods. SLP76 was co-immunoprecipated using a His-myc-tagged human Fab against amino acids 1-120 of murine SLP76 generated by Antibodies by Design. This antibody was conjugated to magnetic Talon Dyna beads according to manufacture's instructions (Invitrogen). Thymocyte lysates were incubated with conjugated beads for 2 h, washed four times and resuspended in 2x NuPage sample buffer. Samples were analyzed by Western blot using antibodies to Vav1 (Cell Signaling), 4G10 (Upstate), and SLP76 (eBioscience). Itk was immunoprecipitated from thymocyte lysates as described

in Materials and Methods using a rabbit polyclonal anti-Itk antibody (Upstate). Western blots were probed for Itk using a mouse anti-Itk antibody (Chemicon) and anti-SLP76 antibody (eBioscience).

Figure S1. Schematic of Y145F and Y112/128F KI targeting construct. A. Point mutations were introduced into exon 7 of SLP76 by genetic recombination. The neomycin gene was excised either in targeted ES cells or by mating KI mice with E2a-cre mice. B. Mice were screened by PCR using the strategy shown. C. Histograms show intracellular SLP76 staining on total thymocytes from WT (shaded) or KI (black line) mice. The isotype control is shown in the gray line (n=3).

Figure S2. SLP76 KI mice demonstrate defective negative selection. The thymic cellularity and percent of DP and SP populations present in WT and KI HY TCR transgenic mice were determined (n=2-6 mice per genotype).

Figure S3. ERK phosphorylation is diminished in purified DP thymocytes. FACS purified DP thymocytes were stimulated with cross-linked CD3 and CD4 for the times indicated. Lysates were Western blotted for phospho-ERK and total PLCγ1 as a loading control (n=3).

Figure S4. Preserved binding of Vav1 and Itk to SLP76 tyrosine mutants. A. SLP76 was immunoprecipitated from thymocyte lysates. Vav1 co-

immunoprecipitated with SLP76 in lystaes from unstimulated cells and cells stimulated for 1 min and 5 min. SLP76 phosphoylation was monitored by phospho-tyrosine immunoblotting and total SLP76 protein was used as a loading control (n=3). B. SLP76 co-immunoprecipitated with Itk from thymocyte lysates under resting and TCR stimulated conditions (n=2).

Figure S5. Normal JNK phosphorylation in SLP76 KI thymocytes. Thymocytes were stimulated with anti-CD3, and lysates were immunoblotted with an anti-phospho-JNK antibody or anti-PLCγ1 antibody for a loading control (n=3).