Supporting Information

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SI Materials and Methods

Reagents and Antibodies. Antibodies to Ubc12, phospho-Erk1/2, phospho-p38, p38, and phospho-PLCγ1 were purchased from Cell Signaling Technology. Antibodies to ZAP-70, IκB-α, Erk2, Cullin-1, Shc, Grb2, and HA were purchased from Santa Cruz Biotechnology. Anti-FLAG antibody was purchased from Sigma-Aldrich. All fluorochrome-tagged antibodies were purchased from eBioscience. MLN4924 was purchased from Active Biochem. CellTrace Violet and EdU (5-ethynyl-2′-deoxyuridine) were purchased from Invitrogen.

DNA Constructs. Plasmids to express HA-NEDD8, RGS-Ubc12, and p66Shc were obtained from Addgene. To generate the FLAG-tagged versions of p66Shc, p52Shc, and p46Shc, the p66Shc cDNA was PCR-amplified and subcloned into pCS2-FLAG vector. Point mutation was generated using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. WT p52Shc and K3R p52Shc cDNAs were cloned into pMIG retroviral vectors and used to transduce primary $CD4^+$ T cells. For construction of shRNA expression vectors, oligonucleotides were cloned in the LMP vector according to the manufacturer's protocol (Open Biosystems). Oligonucleotide sequences were as follows: Ubc12 shRNA, 5′-TGCTGTTGACAGTGAGCGCCCTGATGAAGGCTTCTACAAATAGTGAAGCCACAGATGTATTTGTAGAAGCCTTCATCAGGATGCCTACTGCCTCGGA-3′; Nedd8 shRNA, 5′-TGCTGTTGACAGTGAGCGAACAGACAAGGTGGAGCGAATCTAGTGAAGCCACAGATGTAGATTCGCTCCACCTTGTCTGTGTGCCTACTGCCTCGGA-3′; Nae1 shRNA, 5′- TGCTGTTGACAGTGAGCGCGCTCATGTTTGCTTAATAAATTAGTGAAGCCACAGATGTAATTTATTAAGCAAACATGAGCATGCCTACTGCCTCGGA -3′.

T-Cell Proliferation and IL-2 Production. FACS-sorted $CD4^+$ T cells were activated with various concentrations of anti-CD3 (clone 145–2C11; BioLegend) and anti-CD28 (clone 37.1; Bio X Cell). T-cell proliferation was assessed by EdU labeling according to the manufacturer's instructions (Invitrogen), followed by flow cytometry or, alternatively, the addition of 1 μCi/mL of ^{3}H-thymidine, with cell-incorporated radiation monitored by a β-plate counter. Cell division was analyzed by prelabeling $CD4^+$ T cells with 5 μM CellTrace Violet (Invitrogen). IL-2 production was analyzed by ELISA or intracellular staining.

Th-Cell Differentiation in Vitro. Purified naïve $CD4^+$ T cells from control or shUbc12-expressing chimeric mice were stimulated with plate-bound anti-CD3 and soluble anti-CD28 in the presence of irradiated splenocytes. Cells were polarized into Th1 cells by culturing with human rIL-2 (100 U/mL), mouse IL-12 (10 ng/mL; Peprotech), and anti-mouse IL-4 antibody (10 μg/mL; BD Biosciences), or into Th2 cells by culturing with human rIL-2 (100 U/mL), mouse IL-4 (20 ng/mL; Peprotech), anti-mouse IL-12 antibody (10 μg/mL; BD Biosciences), and anti-mouse IFN-r antibody (10 μg/mL; BD Biosciences).

Generation of Stable Cell Lines by Lentiviral Infection. Full-length human NEDD8 cDNA (aa 1–76) was subcloned into pLENTI6/V5-DEST lentiviral expression vector (Invitrogen) in frame with an N-terminal 3× FLAG epitope. The resulting plasmids were packaged into virus particles using the ViraPower Lentiviral Expression System (Invitrogen). Jurkat E6.1 cells were infected with lentivirus together with 5 μg/mL polybrene by centrifuging cells at 420 × *g* for 60 min. After infection, cells were selected in the presence of blasticidin for 2 wk.

Immunoprecipitation and Immunoblotting. Cell pellets were lysed in 2× Laemmli sample buffer. Cell lysates were resolved by SDS/PAGE and immunoblotted with the appropriate antibodies. For detection of NEDD8 conjugate proteins, cells were lysed with 1% SDS in Tris-buffered saline [50 mmol/L Tris (pH 7.5) and 150 mmol/L NaCl] supplemented with 10 mmol/L *N*-ethylmaleimide, incubated at 95 °C for 10 min, and then diluted to 0.1% SDS with Tris-buffered saline containing 1% Nonidet P-40. NEDD8-modified proteins were immunoprecipitated from diluted lysates using an anti–FLAG-M2 agarose bead (Sigma-Aldrich) for 4 h at 4 °C. The immunoprecipitates were eluted with triple-FLAG peptide. The eluates were subjected to Western blot analysis using the indicated antibodies.

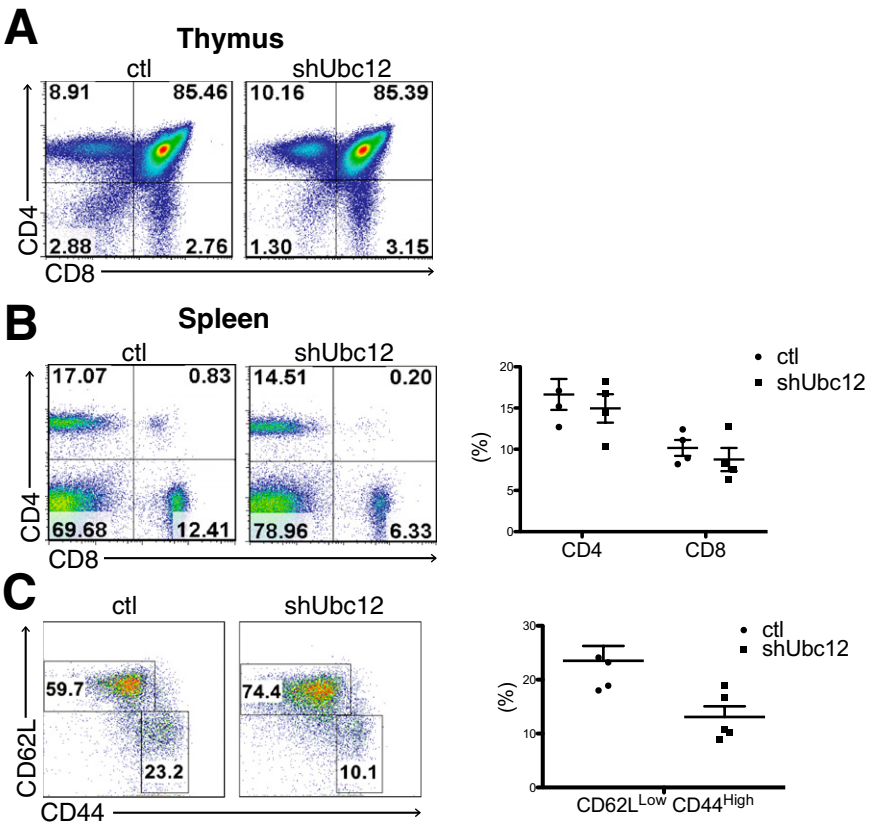


Fig. S1. Normal T-cell development in Ubc12 knockdown mice, as demonstrated by phenotypic analysis of Ubc12 shRNA-expressing chimeric mice. (*A*) Flow cytometry analysis of thymocytes in control and Ubc12 shRNA-expressing chimeric mice stained with anti-CD4 and anti-CD8 ($n = 4$). (*B*) Flow cytometry analysis of splenocytes from control and Ubc12 shRNA-expressing chimeric mice for CD4 and CD8 expression ($n = 4$). (*C*) Representative FACS plot of $CD4^+$-gated cells showing staining for CD62L and CD44. Lower percentages of $CD4^+$ memory T cells ($CD62L^{low}$ and $CD44^{high}$) were observed in the spleens of Ubc12 knockdown chimeric mice compared with WT mice.

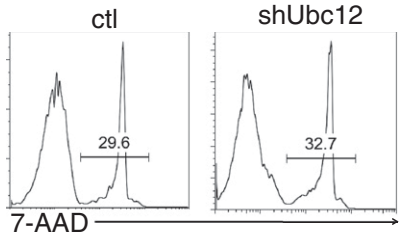


Fig. S2. Normal T-cell apoptosis by Ubc12 knockdown. Detection of 7-amino-actinomycin D (7-AAD)-positive apoptotic cells after 24 h of stimulation of $CD4^+$ T cells with anti-CD3/CD28 mAbs. Numbers above the bracketed lines indicate the percentage of 7-AAD–positive cells.

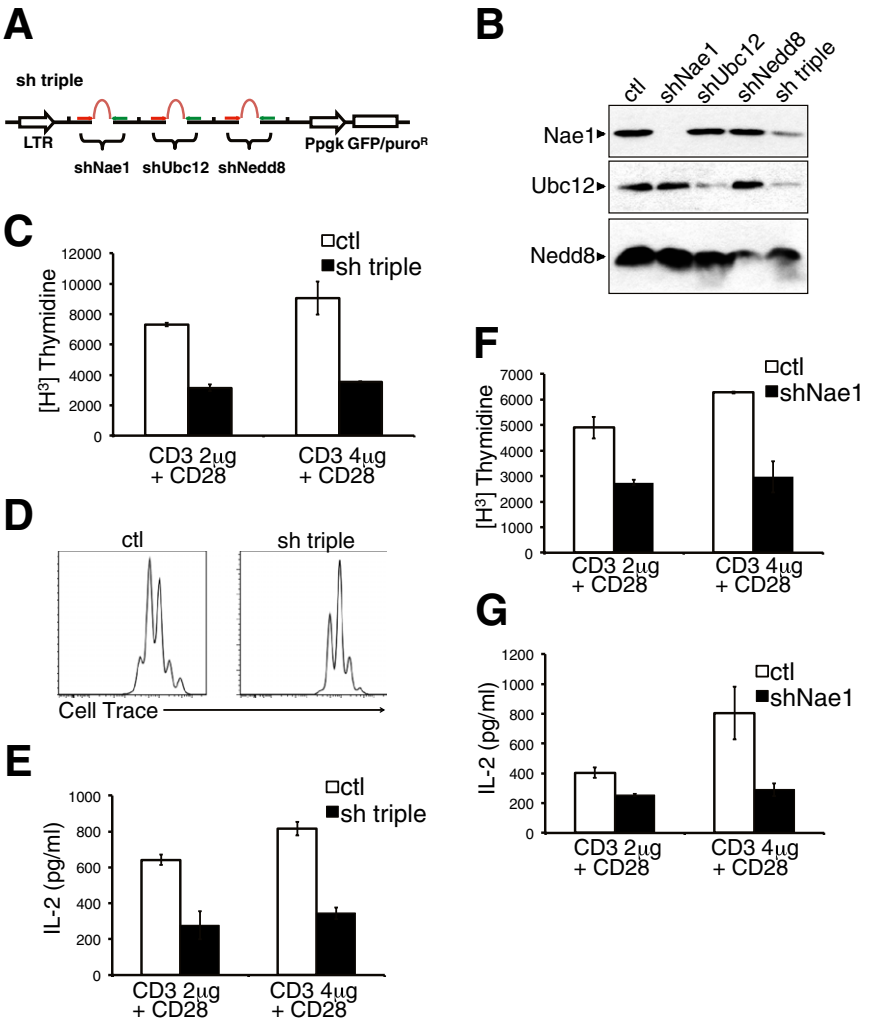


Fig. S3. Knockdown of the NEDD8 components affects T-cell function. Inhibition of the NEDD8 pathway by shRNA-mediated simultaneous knockdown of Nae1, Ubc12, and NEDD8 is shown. (*A*) Retroviral vector bearing multiple shRNAs targeting multiple genes (Nae1, Ubc12, and NEDD8). The pLMP vector encodes for a miR30-based target-specific shRNA under LTR promoter control. (*B*) Western blots of cell lysates from GFP^+CD4^+ T cells expressing Nae1, Ubc12, or NEDD8 shRNA individually or combined (shTriple). (*C*) Decreased proliferation of $CD4^+$ T cells sorted from shTriple-expressing chimeric mice. $CD4^+$ T cells from control and shTriple-expressing chimeric mice were stimulated in vitro with anti-CD3/CD28 mAbs for 3 d. ^{3}H-thymidine was added to the culture during the last 8 h, followed by measurement of incorporation. (*D*) Proliferation of control (*Left*) and shTriple-expressing (*Right*) $CD4^+$ T cells assessed by CellTrace Violet division assay after stimulation with anti-CD3/CD28 mAbs. (*E*) Production of IL-2 from control and shTriple-expressing $CD4^+$ T cells assessed by ELISA after stimulation with anti-CD3/CD28 mAbs for 2 d. (*F*) Proliferation of $CD4^+$ T cells from Nae1 shRNA-expressing chimeric mice, measured as described above. (*G*) IL-2 production of $CD4^+$ T cells from Nae1 shRNA-expressing chimeric mice, measured as described above.

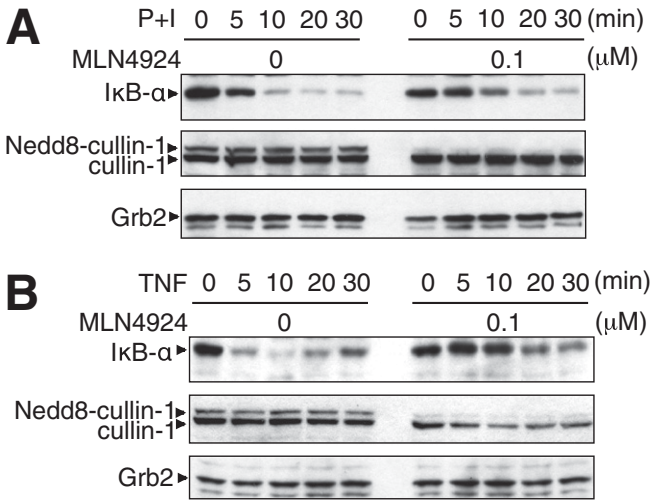
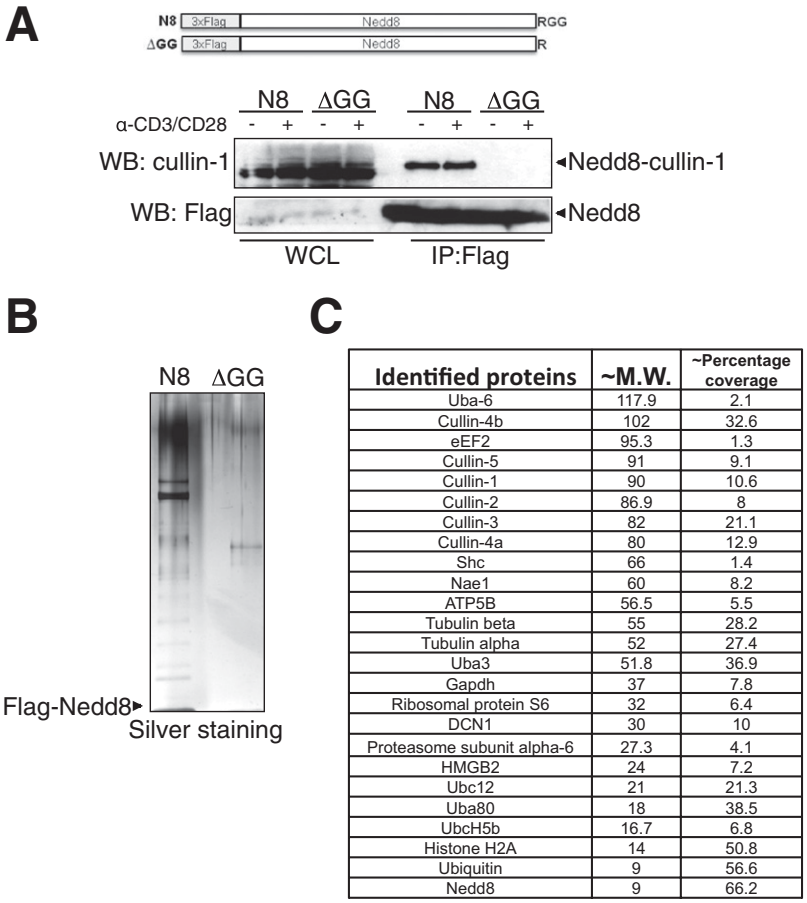


Fig. S4. Effect of MLN4924 on NF-κB activation. (*A*) $CD4^+$ T cells were preactivated with anti-CD3 in the presence of 0.1 μM MLN4924 for 16 h and then stimulated with phorbol 12-myristate 13-acetate (100 ng/mL) and ionomycin (1 μM) for the indicated time periods. Cell lysates were separated by SDS/PAGE and immunoblotted with the indicated antibodies. Grb2 was used as a loading control. (*B*) Jurkat cells pretreated with 0.1 μM MLN4924 for 16 h were stimulated with TNF-α (10 ng/mL).



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Identified proteins	~M.W.	~Percentage coverage
Uba-6	117.9	2.1
Cullin-4b	102	32.6
eEF2	95.3	1.3
Cullin-5	91	9.1
Cullin-1	90	10.6
Cullin-2	86.9	8
Cullin-3	82	21.1
Cullin-4a	80	12.9
Shc	66	1.4
Nae1	60	8.2
ATP5B	56.5	5.5
Tubulin beta	55	28.2
Tubulin alpha	52	27.4
Uba3	51.8	36.9
Gapdh	37	7.8
Ribosomal protein S6	32	6.4
DCN1	30	10
Proteasome subunit alpha-6	27.3	4.1
HMGB2	24	7.2
Ubc12	21	21.3
Uba80	18	38.5
UbcH5b	16.7	6.8
Histone H2A	14	50.8
Ubiquitin	9	56.6
Nedd8	9	66.2

Fig. S5. Identification of NEDD8-conjugated proteins by MS. (*A*) The 3× Flag-NEDD8 ΔGG denotes the NEDD8 mutant in which the last two glycine residues are deleted. To purify NEDD8-modified proteins under denaturing conditions, a JE6.1 stable cell line expressing 3× FLAG-NEDD8 or 3× FLAG-NEDD8 mutant (ΔGG) was lysed in 1% SDS lysis buffer and denatured at 100 °C, after which the SDS was diluted 10-fold with 1% Triton X-100 buffer and then immunoprecipitated with anti-FLAG–conjugated beads, followed by elution with triple FLAG peptide. The eluates were subjected to Western blot and MS analyses. (*B*) Silver staining of affinity-purified FLAG-NEDD8–modified proteins. (*C*) Proteins identified in the FLAG-NEDD8 sample, but not in the FLAG-NEDD8 mutant (ΔGG) sample.