Supporting information

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SI Materials and Methods

Cell Culture. The cells were resuspended in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin for culture at 37 °C in 5% CO_2.

Animals. $p53^{-/-}$ animals were purchased from Jackson Laboratory and bred in-house. $IKK\beta^{+/-}$ $TNFR1^{-/-}$ animals (gift from Inder Verma) were bred to generate $IKK\beta^{-/-}$ $TNFR1^{-/-}$ and $IKK\beta^{+/+}$ $TNFR1^{-/-}$ pups. $p73^{+/-}$ animals were bred to generate $p73^{-/-}$ and $p73^{+/+}$ pups (gift from Frank McKeon, University of Strasbourg, France). $Bim^{-/-}$ mice (gift from Andreas Strasser, Walter and Eliza Hall, Melbourne, Australia) and $Mdm2^{-/-}$ $p53^{-/-}$ mice (gift from Charles Sherr, St. Jude Children's Research Hospital, Memphis, TN) were bred in our animal facility and housed under specific pathogen-free conditions at the La Jolla Institute for Allergy and Immunology (La Jolla, CA) and at St. Jude Children's Research Hospital (Memphis, TN).

Protein Extraction, Coimmunoprecipitation, and Western Blot Analysis. Cell extracts were prepared using boiling lysis solution (1% SDS, 1 mM Na-orthovanadate, 10 mM Tris, pH 7.4) or RIPA (buffer enables protein extraction from cytoplasm, membranes, and nucleus) and analyzed by SDS/PAGE and immunoblotting with specific antibodies: anti-MDM2 (SMP14), anti–MDM2-HRP (SMP14), and Hsc-70 (Santa Cruz Biotechnology), anti-p73 full (1) or anti-p73 full-HRP coupled using AbD Serotec Lynx Rapid HRP Antibody Conjugation Kit, anti-p73 (Calbiochem), anti-Actin (ICN), anti-lactate dehydrogenase (Sigma).

For coimmunoprecipitation studies, primary T cells were untreated or treated with anti-CD3 plus anti-CD28 in the presence or absence of MDM2 inhibitor (chalcone). Cells were harvested, homogenized in lysis buffer (25 mM Tris-HCl, pH 7.5; 150 mM NaCl; 10% glycerol; 1% Nonidet P-40; protease inhibitor mixture; Roche). Samples were precleared with protein A/G PLUS-agarose beads (Santa Cruz Biotechnology). Anti-MDM2 antibody or anti-MDM2 AC beads (Santa Cruz Biotechnology) or anti-IgG1 antibody-coated beads were added to cell extract overnight on a wheel at 4 °C. Samples were washed, resuspended in 50 μL lysis buffer, and analyzed by Western blotting for MDM2 and p73.

EMSA. Extracts and probes were prepared as previously described (2). Probe sequences are: human κB1 dm2 promoter (forward sequence, 5′-ATT TGA ATT TCC TGC TTT-3′; reverse sequence, 5′-AAA GCA GGA AAT TCA AAT-3′); human κB2 dm2 promoter (forward sequence, 5′-TAA GGA AGT TTC CTT T-3′; reverse sequence, 5′-AAA GGA AAC TTC CTT A-3′); and human Bim P2 promoter (forward sequence, 5′-GCT GCC AGA CCT TCC CCA GAC TTG CTG CCC TC-3′; reverse sequence, 5′-GAG GGC AGC AAG TCT GGG GAA GGT CTG GCAGC-3′). Neutralizing antibodies were p50NLS (sc-114 X), p65/Rel A (sc-109 X), c-Rel (N) (sc-70 X), and RelB (C-19) (sc-226 X; Santa Cruz Biotechnology).

Assessment of Apoptosis. To assess apoptosis, cells were stained with Hoechst stain and examined by fluorescence microscopy (at least 300 cells/condition were counted). Alternatively, apoptosis was assessed by subdiploid analysis (propidium iodide) or annexin V-APC (Caltag) and analyzed by FACS (Becton Dickinson).

Transient Transfection and Reporter Assays. A 4-kb promoter region of human *dm2* gene has been cloned into pCR-XL-TOPO vector (Invitrogen) using 5′-CTTTTTAAGTGATGACATGCACTT-3′ and 5′-CCTCCGCAAAGCCACGTGC-3′ as forward and reverse primers, respectively. Using pCR-XL-TOPO-human dm2 construct as template, a κB1–κB2 construct of 2,080 bp (forward, 5′-TGT GAC CCA TCA GGA CCC AGA G-3′; reverse, 5′-TCT CTG GCC AGT AAG TGA TTA GC-3′), a κB1 construct of 431 bp (forward, 5′-TGT GAC CCA TCA GGA CCC AGA G-3′; reverse, 5′-GAG AGC TGG GAG GAG ACG GCA GAC-3′) and a κB2 construct of 1,673 bp (forward, 5′-GTC TGC CGT CTC CTC CCA GCT CTC-3′; reverse, 5′-TCT CTG GCC AGT AAG TGA TTA GC-3′) and no κB site construct (forward, 5′-GTC TGC CGT CTC CTC CCA GCT CTC-3′; reverse, 5′-TTA AAC ATA GTG GTC ACT C -3′), respectively, were generated. Amplified fragments were cloned directly into pGLOW vector (Invitrogen). Murine *bim* p73 site promoter constructs were generated by PCR using the 5′-ACG ACT GAC GGC C-3′ and the 5′-GGT ACC ATT GTT TGC C-3′ primers, 5′-ACG ACT GAC GGC CGC TGC CAA ACC TTC TCC AGA CTT GCT GCC CTC AGC ATT TTC GGC AAA CAA TGG TAC CA-3′ as WT Bim promoter template, and 5′-ACG ACT GAC GGC CGC TGC CAA ACA TTT TCC AGA ATT TCT GCC CTC AGC ATT TTC GGC AAA CAA TGG TAC CA-3′ as mutant Bim promoter template. Amplified fragments were cloned directly into pGLOW vector (Invitrogen). Primary T cells (1×10^6) were transfected using Superfect with 500 ng of total vector and incubated after transfection for 6 to 13 h in 800 μL of complete RPMI medium in a 12-well plate. After the incubation time and homogenization, 200 μL of the cell suspension was analyzed (FACS, apoptosis assessment, and/or protein extraction) and the remaining 600 μL was transferred into a 96-well plate coated with anti-CD3 and anti-CD28 antibodies (100 μL of cell suspension plus 100 μL of fresh complete RPMI medium per well) and incubated for further studies. Using this technique, we were able to obtain up to 47% of transient transfection efficiency. (Superfect toxicity may vary among lots and may need adjustments.)

ChIP. Jurkat T cells were unstimulated or stimulated with anti-CD3 (OKT3) for 2 h. After cross-linking with formaldehyde, chromatin was extracted and sonicated. One percent of the supernatant was saved as input and the remaining supernatant was immunoprecipitated with antibodies against RelA/p65, RelB, c-Rel, p50NLS, or HA (irrelevant antibody; Santa Cruz Biotechnology). After washing, cross-linking was reversed, and the precipitated chromatin was extracted by ethanol precipitation. An aliquot of 1/3 immunoprecipitated DNA was subjected to qPCR using specific *hdm2κB1* or *hIκBα* promoter primers: *hdm2κB1* forward, 5′-TGACCACA-CCGTTGCTGG-3′; reverse, 5′-GATGATAATTTTCCCCCGG-G-3′; *hIκBα* forward, 5′-GGCTCATCGCAGGGAGTTT-3′; reverse, 5′-GAACTGGCTTCGTCCT CTGCTA-3′ (3).

Plasmid Constructs and Transfections. mdm2 forward primer was 5′-AAGCTTATGTGCAATACCAACATGTCTGTGTCTACC-3′ and mdm-2 reverse primer was 5′-CTCGAGCTAGTTGAAG-TAAGTTAGCACAATC-3′ at respective ATG start and stop codons. The amplified fragment was cloned directly into pcDNA3.1V5-TOPO vector (Invitrogen) or subcloned into the episomal pCEP4 vector using HindIII and XhoI digestion. LXSN- and IκBM-Jurkat cells were electroporated with empty pCEP4 vector or pCEP4 encoding *mdm2* and selected for 1 mo in the presence of 0.3 mg/mL Hygromycin B (Roche).

1. Bouchier-Hayes L, Muñoz-Pinedo C, Connell S, Green DR (2008) Measuring apoptosis at the single cell level. *Methods* 44:222–228.
2. Busuttil V, et al. (2002) Blocking NF-kappaB activation in Jurkat leukemic T cells converts the survival agent and tumor promoter PMA into an apoptotic effector. *Oncogene* 21:3213–3224.
3. Edelstein LC, Lagos L, Simmons M, Tirumalai H, Gélinas C (2003) NF-kappa B-dependent assembly of an enhanceosome-like complex on the promoter region of apoptosis inhibitor Bfl-1/A1. *Mol Cell Biol* 23:2749–2761.

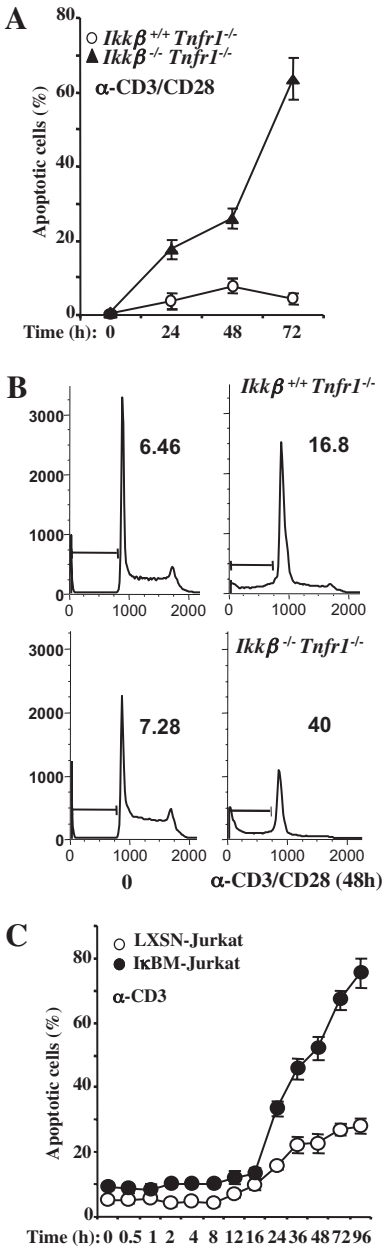

Fig. S1. (*A*) Primary lymphocytes from *Ikkβ*$^{+/+}$*Tnfr1*$^{-/-}$ and *Ikkβ*$^{-/-}$*Tnfr1*$^{-/-}$ mice were activated with plate-bound anti-mCD3 plus anti-mCD28 for the indicated times and apoptotic T cells were visually assessed determined by nuclear chromatin staining with Hoechst 33342. One representative of three independent experiments is shown. (*B*) Primary lymphocytes from *Ikkβ*$^{+/+}$*Tnfr1*$^{-/-}$ and *Ikkβ*$^{-/-}$*Tnfr1*$^{-/-}$ mice were activated with plate-bound anti-mCD3 plus anti-mCD28 for indicated times. Sub-G1 percentages were determined (number shown on each graph). (*C*) LXSN- and IκBM-Jurkat T cells were stimulated with soluble anti-hCD3 (OKT3) for the indicated time and apoptotic cells were counted as in *A*.

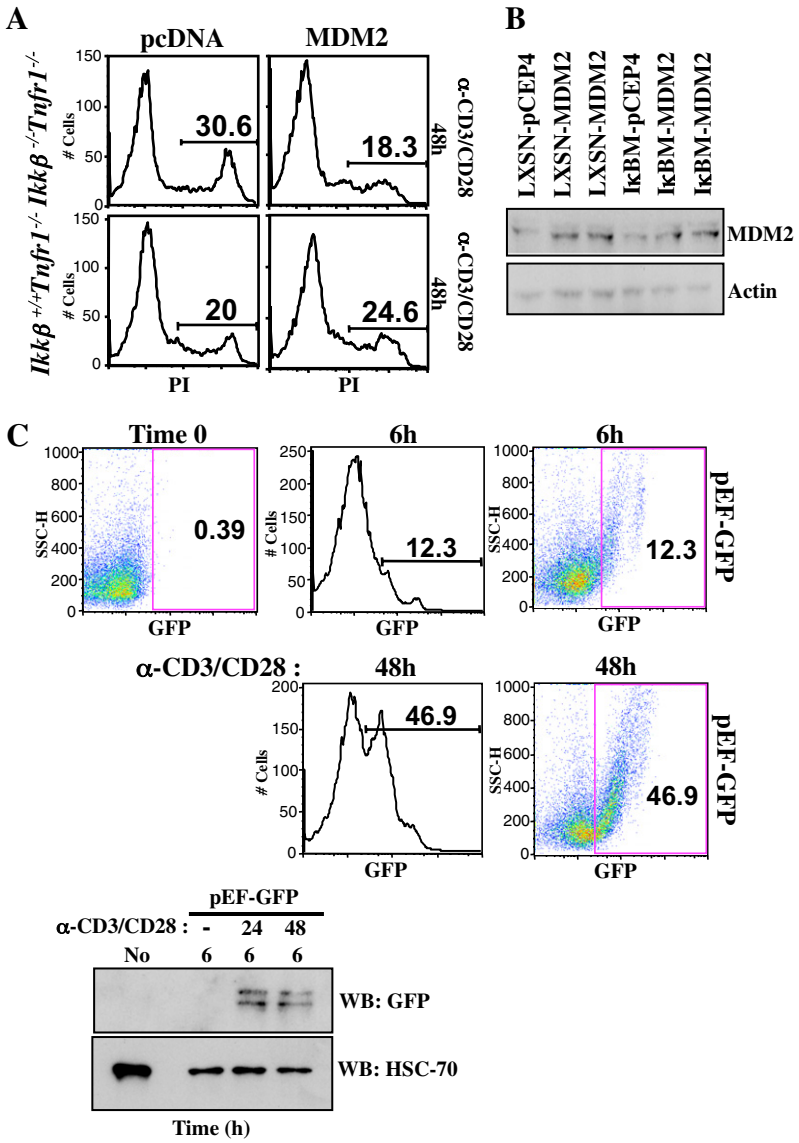

Fig. S2. Mdm2-enforced expression rescues primary *Ikkβ*$^{-/-}$*Tnfr1*$^{-/-}$ and IκBM-Jurkat T cells from TCR induced-death. (*A*) Primary *Ikkβ*$^{+/+}$*Tnfr1*$^{-/-}$ and *Ikkβ*$^{-/-}$*Tnfr1*$^{-/-}$ lymphocytes were transiently transfected with pcDNA or pcDNA encoding MDM2 during 13 h and then activated for 48 h with anti-mCD3 plus anti-mCD28. Propidium iodide uptake was measured on these primary transfected T cells to quantify the cell death. One representative experiment of several is shown. (*B*) LXSN- and IκBM-Jurkat T cells were stably transfected with pCEP4 or pCEP4 encoding MDM2. Western blots of extracts from indicated cell lines were probed with antibodies against MDM2 and actin. (*C*) Transient transfection of primary T cells using GFP expressing vector to show the efficiency of transfection without or with plate-bound anti-mCD3 plus anti-mCD28 for indicated times. Analysis was done by flow cytometry (five top panels) and by Western blot (*Bottom*) using anti-GFP antibody (HSC-70 is used as a loading control).

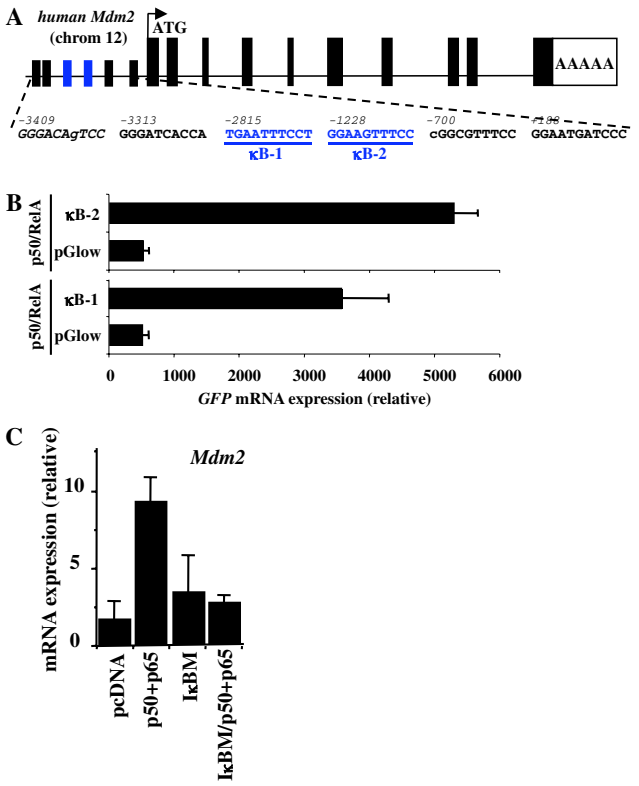

Fig. S3. (*A*) Identification and schematic representation of two NF-κB functional binding sites (κB1 and κB2, respectively) in *human Mdm2* (chromosome 12) *promoter 1*. (*B*) LXSN-Jurkat T cells were cotransfected with different constructs expressing human p50 and p65/RelA with the indicated GFP reporter plasmids with schematic κB1- or κB2-binding sites in the *Hdm2* P1 promoter region. *GFP* mRNA was analyzed by real-time RT-PCR and normalized to *LacZ* mRNA expression as a transfection efficiency control. (*C*) Primary thymocytes prepared from C57BL/6 mice were transfected with different constructs expressing empty vector, human p50 and p65, IκBM alone or in presence of p50/p65. Twenty hours later, total mRNA was isolated from transfected thymocytes and analyzed for *Mdm2* mRNA by real-time RT-PCR. Absolute mRNA values were determined, normalized to *18S* RNA, and reported as arbitrary units.

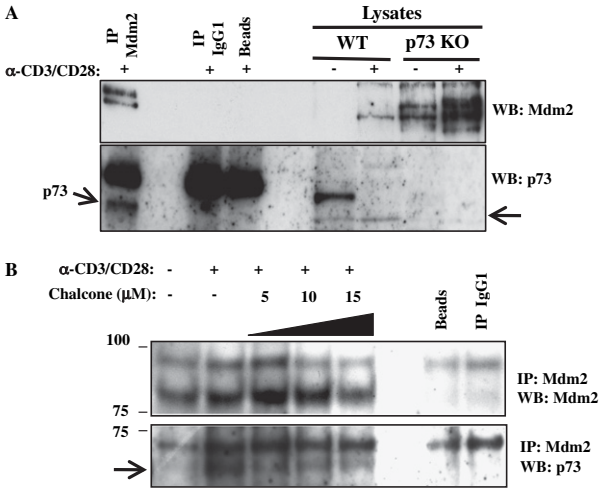

Fig. S4. (*A*) Primary T cells from C57BL/6 mice, p73 WT, and p73 KO mice were untreated or stimulated for 24 h with plate-bound anti-mCD3 plus anti-mCD28. Immunoprecipitations (IP) with an anti-MDM2 Ab or isotype control, or whole-cell lysates were followed by immunoblot detection of MDM2 and p73 with HRP-coupled antibodies. Controls include the Ab isotype alone (IP IgG1) and beads incubated in the absence of Ab (beads). (*Upper*) IP, anti-Mdm2; WB, anti-Mdm2, (*Lower*) IP, anti-Mdm2; WB, anti-p73. (*B*) Primary T cells from C57BL/6 mice were untreated or stimulated for 24 h with plate-bound anti-mCD3 plus anti-mCD28 in the presence of 0, 5, 10 or 15 mM of MDM2 inhibitor (chalcone). IP with an anti-MDM2 Ab or isotype control were followed by immunoblot detection of MDM2 and p73. Controls are the same as in *A*. (*Upper*) IP, anti-Mdm2; WB, anti-Mdm2. (*Lower*) IP, anti-Mdm2; WB, anti-p73.

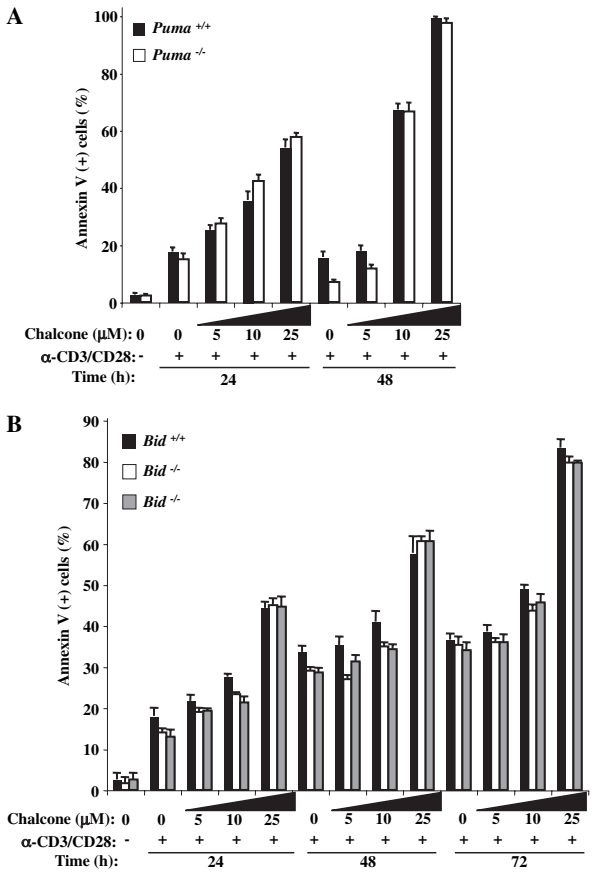

Fig. S5. Primary T cells from *Puma*$^{+/+}$ and *Puma*$^{-/-}$ mice (*A*) or from *Bid*$^{+/+}$ and *Bid*$^{-/-}$ mice (*B*) were activated with plate-bound anti-mCD3 plus anti-mCD28 for indicated times in presence or not of indicated concentration of MDM2 inhibitor (chalcone) and were analyzed by flow cytometry for APC-conjugated annexin V staining. Percentages of annexin V-positive cells are indicated.

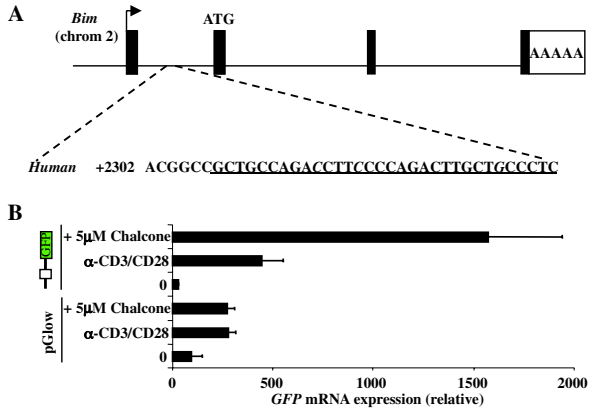

Fig. S6. (*A*) Identification and schematic representation of p73 binding site in *human* (chromosome 2) *promoter 2*. (*B*) Murine primary T cells isolated from C57BL/6 mice were cotransfected with the indicated GFP reporter plasmids without or with schematic p73 site from *Bim P2 promoter* region. *GFP* mRNA was analyzed by real-time RT-PCR and normalized to *LacZ* mRNA expression as a transfection efficiency control. Cells were activated with anti-mCD3 plus anti-mCD28 with or without chalcone for 12 h as indicated.

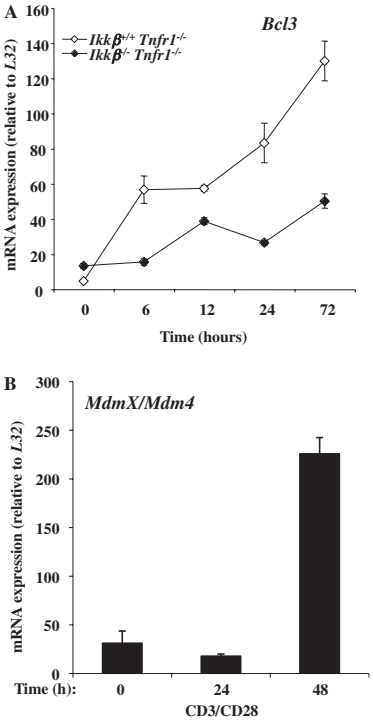

Fig. S7. Expression of Bcl3 and MdmX/Mdm4 in activated T cells. (*A*) Primary *Ikkβ*$^{+/+}$*Tnfr1*$^{-/-}$ and *Ikkβ*$^{-/-}$*Tnfr1*$^{-/-}$ lymphocytes were stimulated with anti-mCD3 plus anti-mCD28 for the indicated times and total mRNA was isolated and analyzed for *Bcl3* mRNA by real-time RT-PCR. Absolute mRNA values were determined, normalized to *L32* RNA, and reported as arbitrary units. (*B*) Primary murine T cells were stimulated with anti-mCD3 plus anti-mCD28 for the indicated times and total mRNA was isolated and analyzed for *MdmX/Mdm4* mRNA by real-time RT-PCR. Absolute mRNA values were determined, normalized to *L32* RNA, and reported as arbitrary units.