Supporting Information

Fletcher *et al.* 10.1073/pnas.0808691105

SI Materials and Methods

Cell Lines and Cell Viability assays. For short-term assays, cells were infected with retroviruses coexpressing BH3-only proteins and GFP, or treated with 0–10 μM etoposide (Amersham Pharmacia) or 0–1 μM ABT-737 (Abbott Laboratories) (1). After 24 h the cells were harvested, washed with buffer, and stained with propidium iodide (PI). Viability was assessed by flow cytometry (GFP^{+ve}, PI^{-ve} for the retrovirus-infected cells, and PI^{-ve} for drug-treated cells).

For long-term colony assays using MEF, cells were infected with GFP-expressing retroviral constructs, then treated with qVD.OPH (Enzyme Systems) to prevent cell death. After culture for 24 h, 200 GFP^{+ve} cells were sorted into 6-well plates. Colonies were stained and counted 6 d later. Colony assays with suspension FDC-P1 cells were similar, except that 100 cells were resuspended in a 1:1 mixture of 0.6% agar and Dulbecco's Modified Eagle's Medium (DME) with 40% FCS supplemented with IL-3, and plated onto 3-cm dishes.

Details of the cell lines used are available from the authors; specifically, the MEF were generated from E13–14.5 embryos and immortalized at passage 2–4 with SV40 large T antigen. All of the mice used were of C57BL/6 origin or have been backcrossed (>10 generations) to this background.

Assays on T and B Cell Blasts. Single cell suspensions prepared from the spleens of 8-week-old C57BL/6 mice were cultured in IL-2 and concavalin A to generate T cell blasts or in IL-2, IL-4, IL-5, and LPS to generate B cell blasts. The viability of the retrovirus-infected GFP^{+ve} T ($CD4^+$ $CD8^+$) or B ($B220^+$) cell blasts was determined flow cytometrically by PI exclusion 48 h after infection. Antibodies used included mouse monoclonal anti-CD4 (H1289), -CD8 (YTS169), and -B220 (RA3–6B2). The samples were analyzed using a FACSCalibur (BD Biosciences).

Yeast Colony Assays. *Saccharomyces cerevisiae* W303α cells were cotransformed with the indicated plasmids and grown under selection. For the survival assays, the cells were spotted as 5-fold serial dilutions onto glucose (repressing, "OFF") or galactose (inducing, "ON") plates as previously described (2). Plates were incubated for 48 h at 30°C and then photographed.

Immunoprecipitation and Immunoblotting. Tagged proteins were transiently coexpressed in HEK293T cells, along with the broad-spectrum baculoviral caspase inhibitor p35 to inhibit apoptosis, as previously described (3, 4). Cell lysates were prepared in lysis buffer (20 mM Tris·HCl [pH 7.4], 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, and 1% Triton X-100) supplemented with protease inhibitors (Roche or Sigma). Immunoprecipitation was performed using mouse monoclonal anti-HA (HA.11; Covance), anti-FLAG (M2; Sigma) antibodies or a control antibody (anti-Glu-Glu; MMS-115R; Covance). Proteins were resolved using SDS-PAGE (NuPage gels; Invitrogen), transferred onto nitrocellulose membranes, and detected by immunoblotting using rat monoclonal anti-HA (clone 3F10; Roche) or anti-FLAG (clone 9H1; ref. 5) antibodies. Secondary antibodies included HRP-conjugated goat anti-mouse or anti-mouse IgG (Southern Biotech). Proteins were detected using enhanced chemiluminescence (GE Healthcare).

Other primary antibodies used include mouse monoclonal anti-HSP70 (N6; gift of R. Anderson and W. Welch); -VDAC1 31HL (89; Calbiochem); -GAPDH (6C5; Chemicon); -Bcl-xL (2H12; BD Biosciences); -mouse Bcl-2 (3F11; BD Biosciences), and rat monoclonal anti-Apaf-1 (clone 2E12; ref. 6).

Subcellular Fractionation. Cells were permeabilized in digitonin fractionation buffer (20 mM Hepes [pH 7.2], 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, and 0.05% digitonin) for 10 min on ice and then pelleted at 13,000 rpm for 5 min to generate the soluble fraction. The pellet was treated with 0.1 M sodium carbonate (pH 11.5) for 20 min on ice and then neutralized with 0.1 M HCl, supplemented with nuclease buffer (140 mM Tris·HCl, 10 mM $MgSO_4$, and 1 mM $CaCl_2$) and treated with DNAseI for 10 min at 37°C. The alkali-resistant membrane integrated fraction was isolated by further centrifugation.

Recombinant Proteins and Peptides. Recombinant human Bcl-xL (45–84 "loop-deleted," ΔC24 C-terminal truncation, and a C-terminal hexahistidine tag) (7), mouse Bcl-xL C24 (mouse Bcl-xL amino acids 1–209 expressed as GST fusion protein and purified as described) (8), human Bcl-2 ΔC22 (5), Bcl-w ΔC29 (5), and "humanized" Mcl-1 ΔN171 ΔC23 (9) have been previously described. The proteins were purified by affinity chromatography followed by size-exclusion chromatography. Fractions corresponding to the correct molecular weight were pooled and protein concentration determined by absorbance at 280 nm. The 26-mer Bim BH3 peptide (10) and the 34-mer Bax BH3 peptides (DPVPQDASTKKLSECLKRIG**D**ELDSNMELQRMIA; D68 boldface) were synthesized by Mimotopes (Victoria, Australia) and purified by reversed-phase HPLC to >90% purity.

1. Oltersdorf T, *et al.* (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* 435:677–681.
2. Jabbour AM, *et al.* (2006) Human Bcl-2 cannot directly inhibit the *Caenorhabditis elegans* Apaf-1 homologue CED-4, but can interact with EGL-1. *J Cell Sci* 119:2572–2582.
3. Huang DCS, O'Reilly LA, Strasser A, Cory S (1997) The anti-apoptosis function of Bcl-2 can be genetically separated from its inhibitory effect on cell cycle entry. *EMBO J* 16:4628–4638.
4. Huang DCS, Cory S, Strasser A (1997) Bcl-2, Bcl-xL and adenovirus protein E1B19kD are functionally equivalent in their ability to inhibit cell death. *Oncogene* 14:405–414.
5. Wilson-Annan J, *et al.* (2003) Proapoptotic BH3-only proteins trigger membrane integration of prosurvival Bcl-w and neutralize its activity. *J Cell Biol* 162:877–888.
6. Hausmann G, *et al.* (2000) Pro-apoptotic apoptosis protease-activating factor 1 (Apaf-1) has a cytoplasmic localization distinct from Bcl-2 or Bcl-xL. *J Cell Biol* 149:623–634.
7. Muchmore SW, *et al.* (1996) X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. *Nature* 381:335–341.
8. Day CL, Dupont C, Lackmann M, Vaux DL, Hinds MG (1999) Solution structure and mutagenesis of the caspase recruitment domain (CARD) from Apaf-1. *Cell Death Diff* 6:1125–1132.
9. Czabotar PE, *et al.* (2007) Structural insights into the degradation of Mcl-1 induced by BH3 domains. *Proc Natl Acad Sci USA* 104:6217–6222.
10. Chen L, *et al.* (2005) Differential targeting of pro-survival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* 17:393–403.
11. Liu X, Dai S, Zhu Y, Marrack P, Kappler JW (2003) The structure of a Bcl-xL/Bim fragment complex: Implications for Bim function. *Immunity* 19:341–352.
12. Suzuki M, Youle RJ, Tjandra N (2000) Structure of Bax: Coregulation of dimer formation and intracellular localization. *Cell* 103:645–654.
13. Dewson G, Snowden RT, Almond JB, Dyer MJ, Cohen GM (2003) Conformational change and mitochondrial translocation of Bax accompany proteasome inhibitor-induced apoptosis of chronic lymphocytic leukemic cells. *Oncogene* 22:2643–2654.

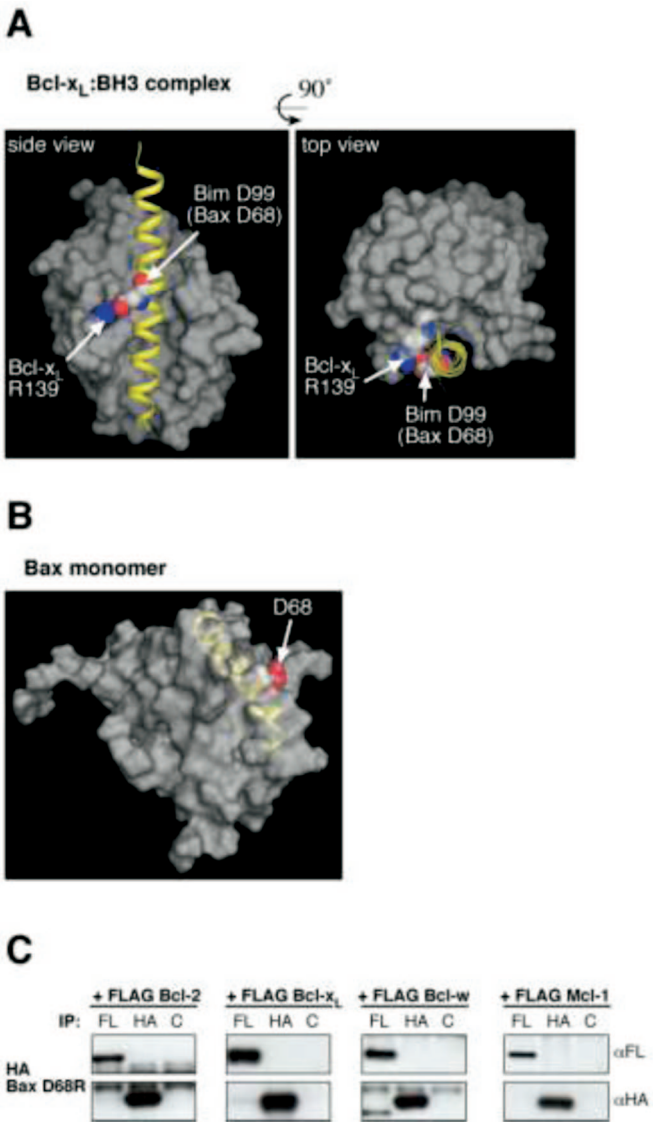

Fig. S1. The D68R mutation compromises binding to prosurvival proteins. (*A*) Interaction of the conserved aspartate (D99, red) of the Bim BH3 (yellow ribbon) with the arginine (R139, blue) of Bcl-xL (surface representation of 1PQ1; ref. 11). The equivalent Bax BH3 residue, D68, is predicted to interact similarly with Bcl-xL R139. (*B*) The invariant Asp residue (D68) is solvent exposed in the Bax monomer. The BH3 domain (αhelix 2) is shown as a yellow ribbon in a surface representation of Bax (1F16) (12). (*C*) Lysates prepared from cells coexpressing HA-tagged human Bax D68R and FLAG-tagged mouse prosurvival Bcl-2 proteins were immunoprecipitated with mouse monoclonal antibodies recognizing the HA, FLAG (FL), or an irrelevant control (C) tag. The immunoprecipitates were subjected to SDS-PAGE, transferred onto membranes, and the blots probed with rat anti-HA or -FLAG antibodies. Similar to the results observed with the human prosurvival proteins (Fig. 1*B*), binding of Bax D68R to the mouse prosurvival proteins was below the limit of detection.

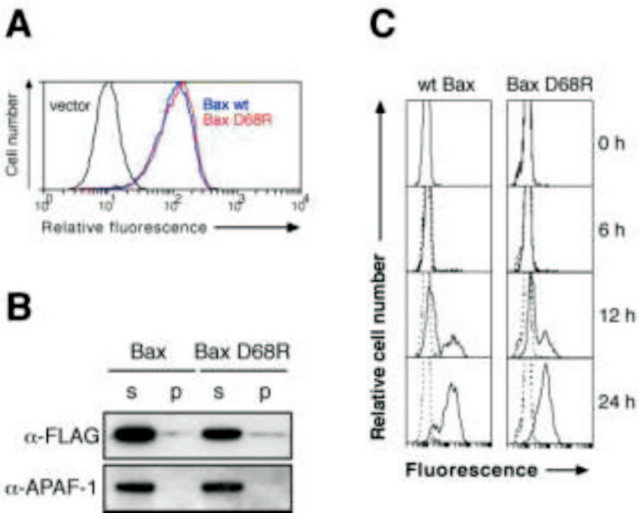

Fig. S2. The expression level, localization, and apoptotic function of the D68R mutant and WT Bax are comparable. (*A*) FLAG-tagged WT Bax or Bax D68R were stably expressed in *bax*$^{-/-}$*bak*$^{-/-}$ MEF. Protein expression was evaluated using flow cytometry after staining fixed cells with an anti-FLAG antibody, followed by an anti-mouse FITC secondary antibody. (*B*) Like WT Bax, the Bax D68R mutant is predominantly cytosolic in healthy cells. FLAG-tagged WT Bax or Bax D68R was stably expressed in *bax*$^{-/-}$*bak*$^{-/-}$ MEF. The cells were permeabilized with digitonin and separated into soluble (s) and pellet (p) fractions. (*C*) Bax D68R, like WT Bax, changes conformation after etoposide treatment. The reconstituted *bax*$^{-/-}$*bak*$^{-/-}$ MEF were fixed and stained with the anti-Bax clone 3 antibody to detect the conformationally altered (activated) form of Bax (13) 0–24 h after treatment with 10 μM etoposide.

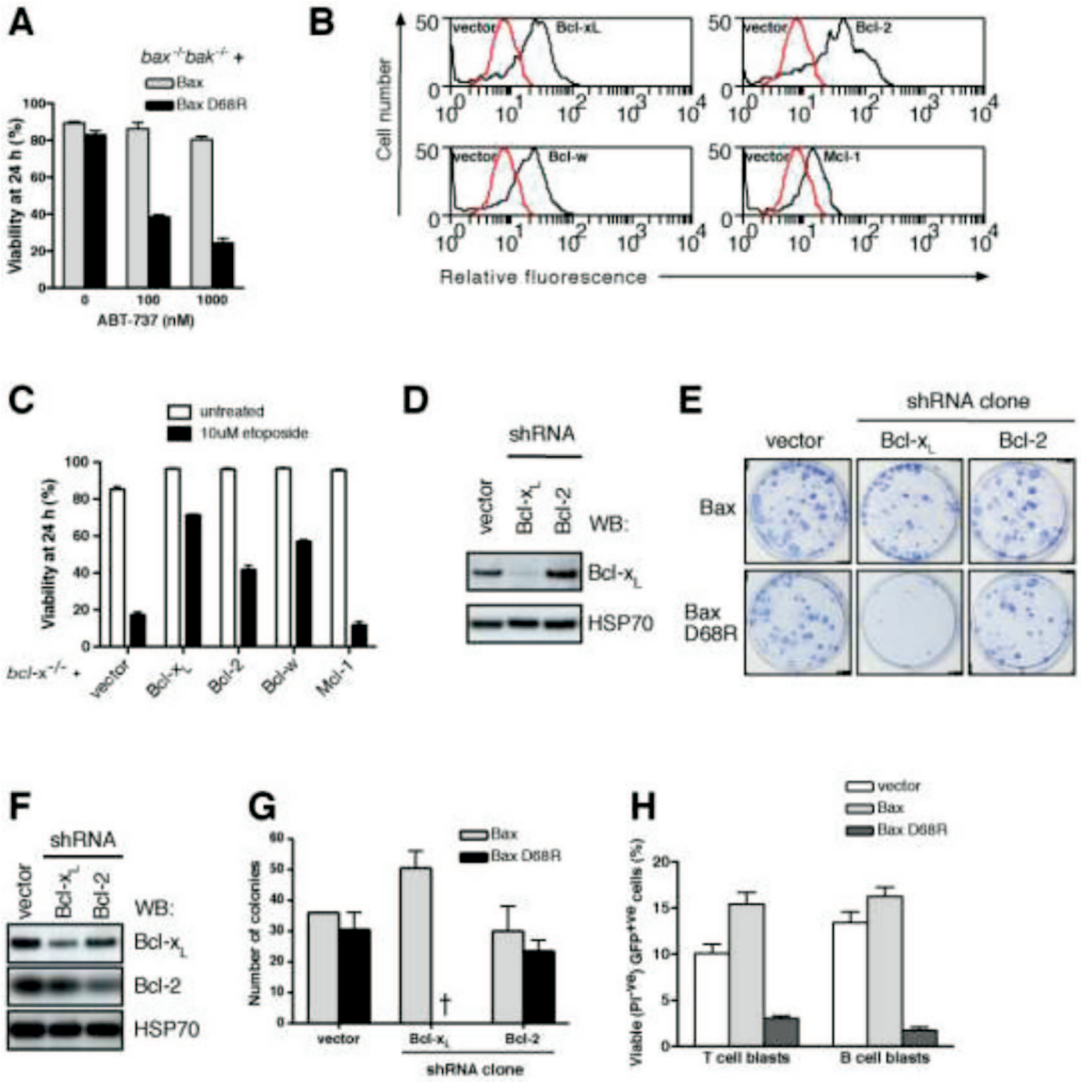

Fig. S3. Bax D68R is constitutively active in the absence of Bcl-xL. (*A*) The Bad-like BH3-mimetic compound ABT-737 can activate Bax D68R but not WT Bax. The viability of *bax*$^{-/-}$*bak*$^{-/-}$ MEF stably expressing WT Bax or Bax D68R was determined 24 h after treatment with 0–1 μM ABT-737. (*B*) FLAG-tagged prosurvival proteins were stably expressed in *bcl*-x$^{-/-}$ MEF. Protein expression was evaluated using flow cytometry after staining fixed cells with the anti-FLAG antibody, followed by an anti-mouse FITC secondary antibody. Mcl-1 is consistently expressed at low levels. (*C*) Overexpression of Bcl-xL, Bcl-2, and Bcl-w delay cell killing induced by etoposide in MEF lacking endogenous Bcl-xL. The viability of *bcl*-x$^{-/-}$ MEF stably expressing the indicated prosurvival proteins was determined 24 h after treatment with 10 μM etoposide. (*D*) Efficient downregulation of Bcl-xL by shRNA. Equivalent lysates from *bax*$^{-/-}$*bak*$^{-/-}$ MEF stably infected with shRNA constructs targeting *bcl*-x or *bcl*-2, or with the empty parental vector, were immunoblotted for Bcl-xL or HSP70 (loading control). (*E*) Downregulation of Bcl-xL triggers killing by Bax D68R. Colony formation 6 d after *bax*$^{-/-}$*bak*$^{-/-}$ MEF stably expressing Bax (*Top*) or D68R Bax (*Bottom*) were infected with retroviral shRNA constructs targeting Bcl-xL or Bcl-2, or with the empty parental vector. (*F*) Downregulation of Bcl-xL in mouse FDC-P1 myelomonocytic cells using shRNA. After infection with retroviral shRNA constructs targeting *bcl*-x or *bcl*-2 or the empty parental vector, the lysates were immunoblotted for Bcl-xL, Bcl-2, or HSP70 (loading control). (*G*) FDC-P1 cells with reduced Bcl-xL cannot tolerate Bax D68R. Colony formation in soft agar of FDC-P1 cells stably expressing Bax or the D68R mutant 6 d after infection with shRNA retroviral vectors that target *bcl*-x or *bcl*-2, or with a vector control. †No colonies. (*H*) Lymphocytes tolerate overexpression of Bax but not Bax D68R. Primary T or B cell blasts were infected with the parental EGFP-expressing retrovirus (vector), or ones coexpressing Bax or Bax D68R; the proportion of infected (GFP^{+ve} cells) that remained viable (PI^{-ve}) was determined 48 h later. Data in *A*, *C*, *G*, and *H* represent means ± 1 SEM of 3 or more independent experiments.

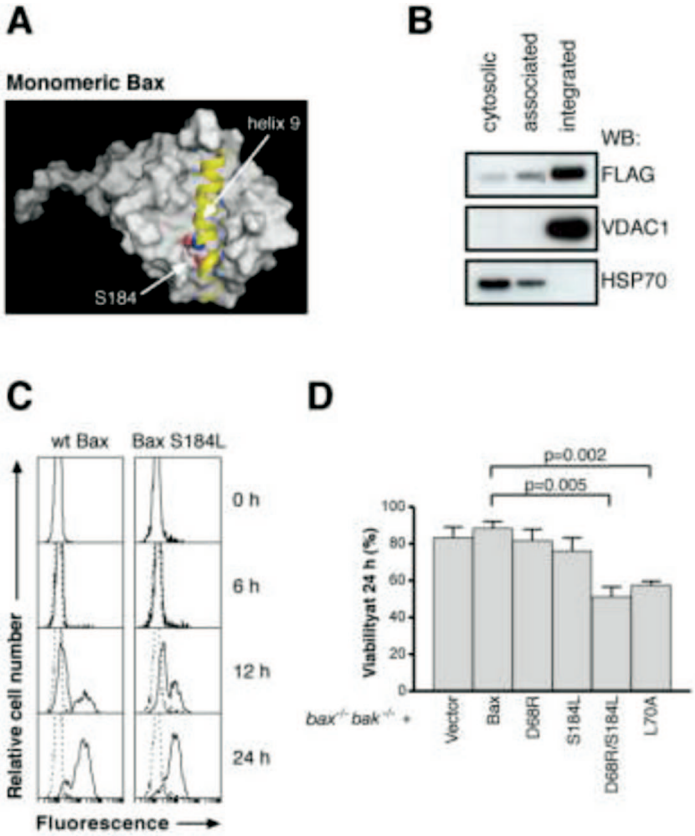

Fig. S4. Enforced membrane localization increases the potency of the Bax D68R mutant. (*A*) Bax S184 (space filling) constrains the C-terminal α-helix (yellow ribbon) of Bax (12), thus keeping monomeric Bax in the cytosol of healthy cells. (*B*) Bax S184L is an integral membrane protein. Subcellular fractionation and analyses were as in Fig. 2*A*. VDAC1, integral mitochondrial membrane protein; HSP70, cytosolic marker. (*C*) Like WT Bax, Bax S184L changes conformation in response to etoposide. The altered Bax conformation in reconstituted $bax^{-/-}bak^{-/-}$ MEF was detected by FACS analyses after staining with the conformation-specific mouse anti-Bax clone 3 antibody (13). (*D*) Expression of Bax D68R/S184L is not tolerated in $bax^{-/-}bak^{-/-}$ MEF. The MEF were infected with retroviral constructs expressing Bax or the indicated Bax mutants and viability measured by PI exclusion 24 h later. Data represent means $\pm$ 1 SEM of at least 3 independent experiments and were compared using two-tailed unpaired Student's *t* tests.