Supplemental Data

Supplemental Figures

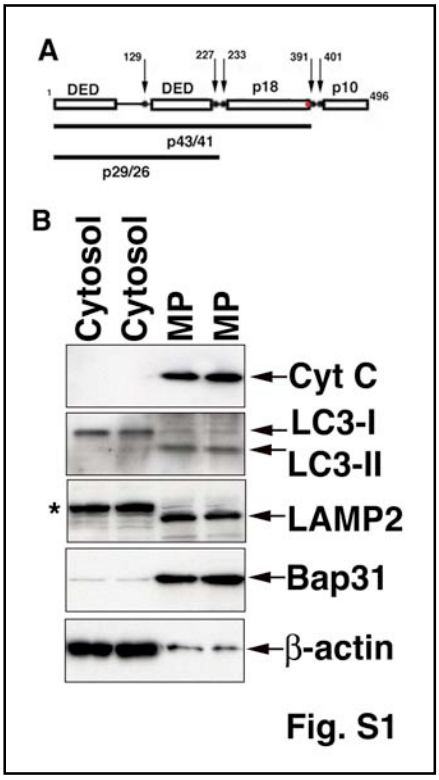


Fig. S1. (A) Schematic presentation of caspase-8 processing. The red dot at Asp391 represents the recognition epitope of the active caspase-8 specific Ab, suggesting that this Ab recognizes either the p18 subunit or its p43/41 precursor. (B) Presence of LC3-II and LAMP2-expressing organelles in the membrane pellet fraction obtained through digitonin permeabilization of the plasma membrane. $Bax^{-/-}$ Hct116 cells were fractionated into cytosol and membrane pellet fractions as described in the Supplemental Methods. The fractions were assessed by immunoblotting for the expression of the indicated proteins. The asterisk indicates an unidentified protein band.

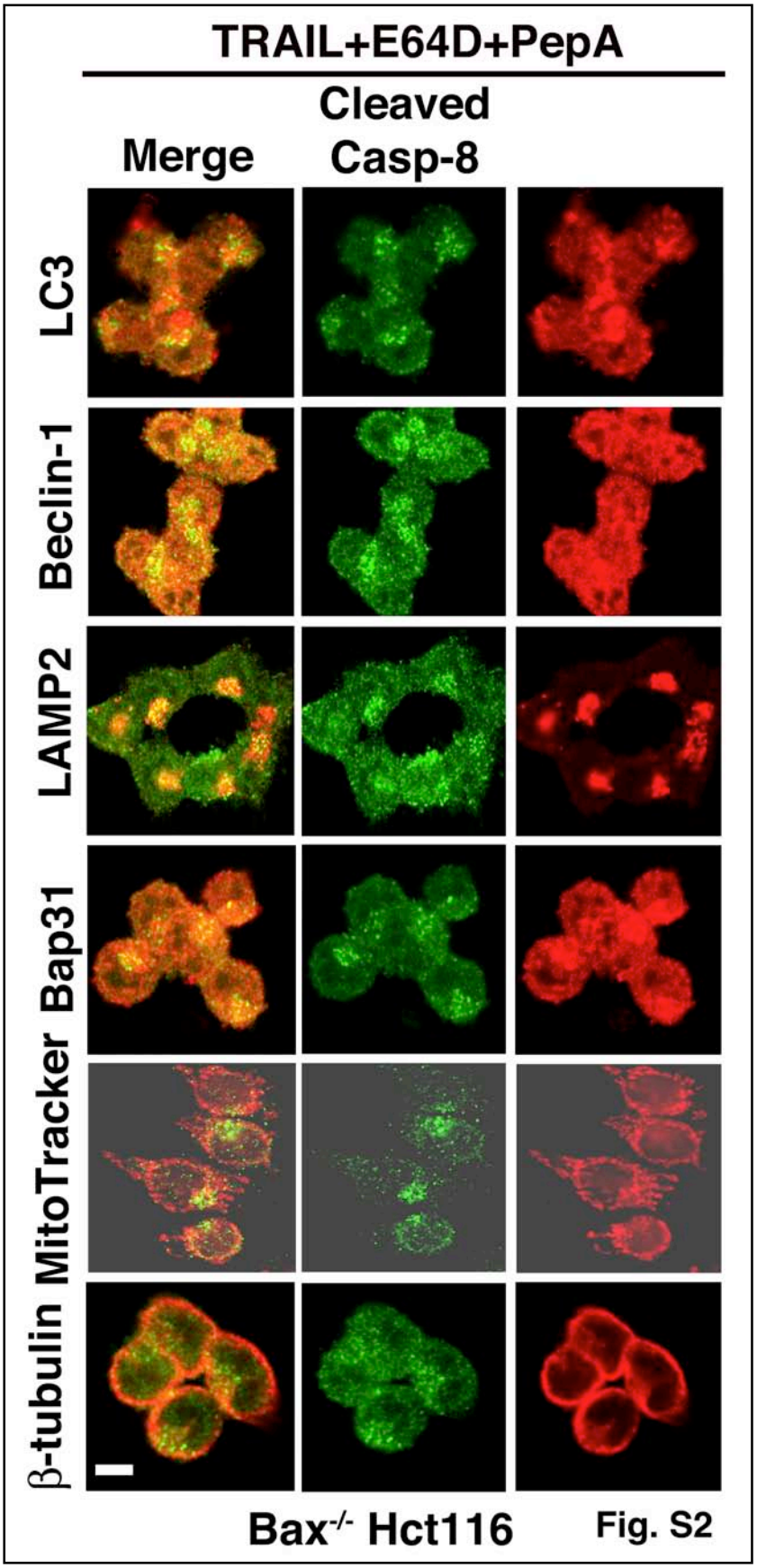


Fig. S2. Enlargement of images shown in Fig. 3A of $Bax^{-/-}$ Hct116 cells treated with TRAIL in the presence of E64/D/PepA. Scale bar = 40 μm.

Fig. S3. Differential subcellular localization of cleaved caspase-8 or cleaved caspase-3 subunits as compared to their respective prodomains or to each other. (A) Association of cleaved caspase-8, but not full-length caspase-8 with LAMP2 in TRAIL treated $Bax^{-/-}$ Hct116 cells. (B) Partial association of cleaved caspase-3, but no association of full-length caspase-3 with LAMP2 in TRAIL treated $Bax^{-/-}$ Hct116 cells. Whereas the majority of cleaved caspase-8 co-localizes with LAMP2, only a fraction of cleaved caspase-3 demonstrates such co-localization. Scale bar = 10 μm.

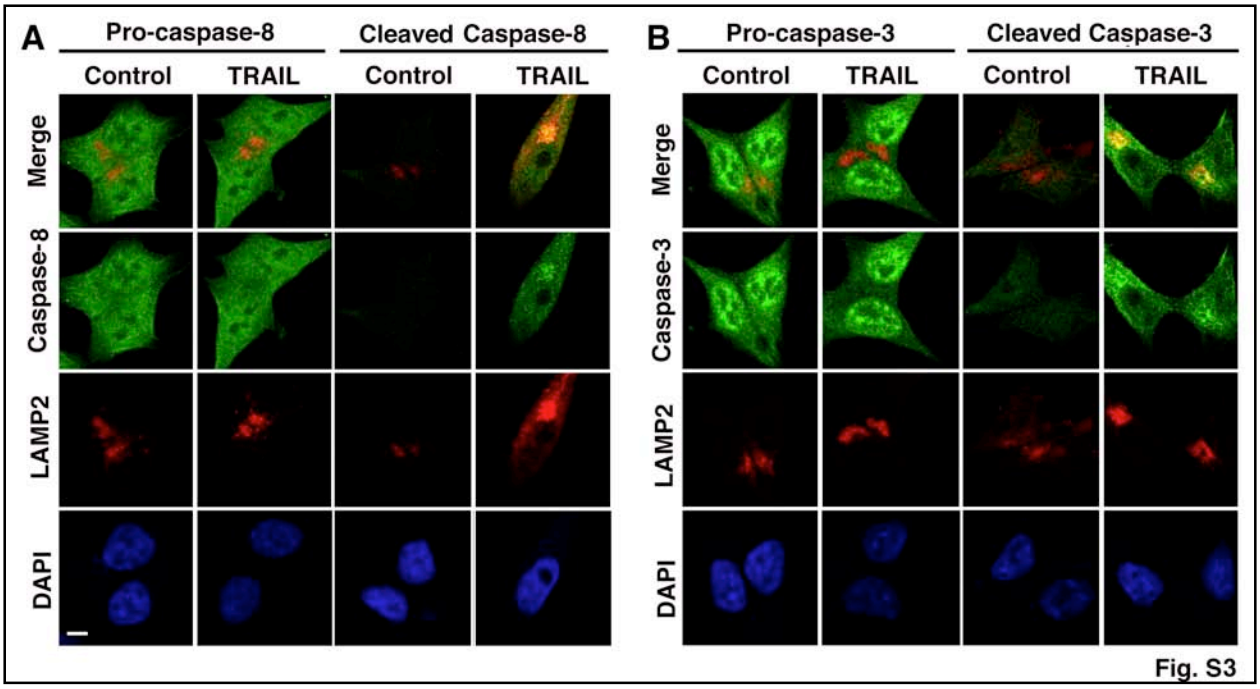


Fig. S4. Altered subcellular localization of cleaved caspase-8 under apoptotic conditions. (A) Increased cytosolic expression of cleaved caspase-8 in TRAIL-treated Jurkat cells (25 ng/ml, 2h) is associated with fragmented nuclei, lost expression of Beclin-1 and β-tubulin, and partial colocalization of cleaved caspase-8 with enhanced LAMP2. Please note the loss in expression of Beclin-1 and β-tubulun in TRAIL-treated Jurkat cells that express cleaved caspase-8 (green). (B) Treatment of $Bax^{-/-}$ Hct116 cells with a combination of TRAIL and Beclin-1 RNAi enhances the expression and the cytosolic localization of cleaved caspase-8. Please note the reduced expression level of Beclin-1 following its KD, and the fragmented nuclei (white arrows) in cells treated with TRAIL and Beclin-1 siRNA. Scale bar = 40 μm.

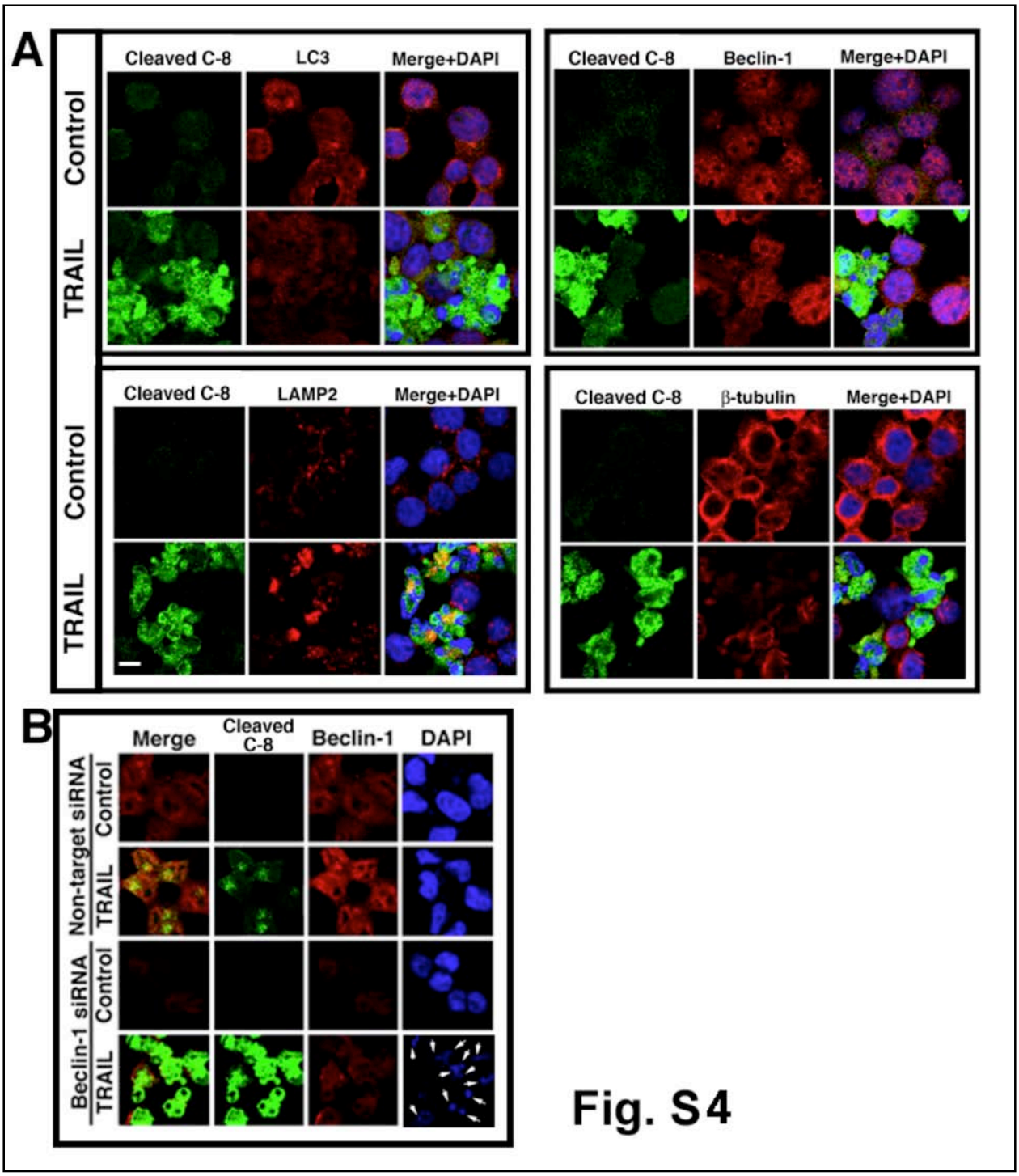
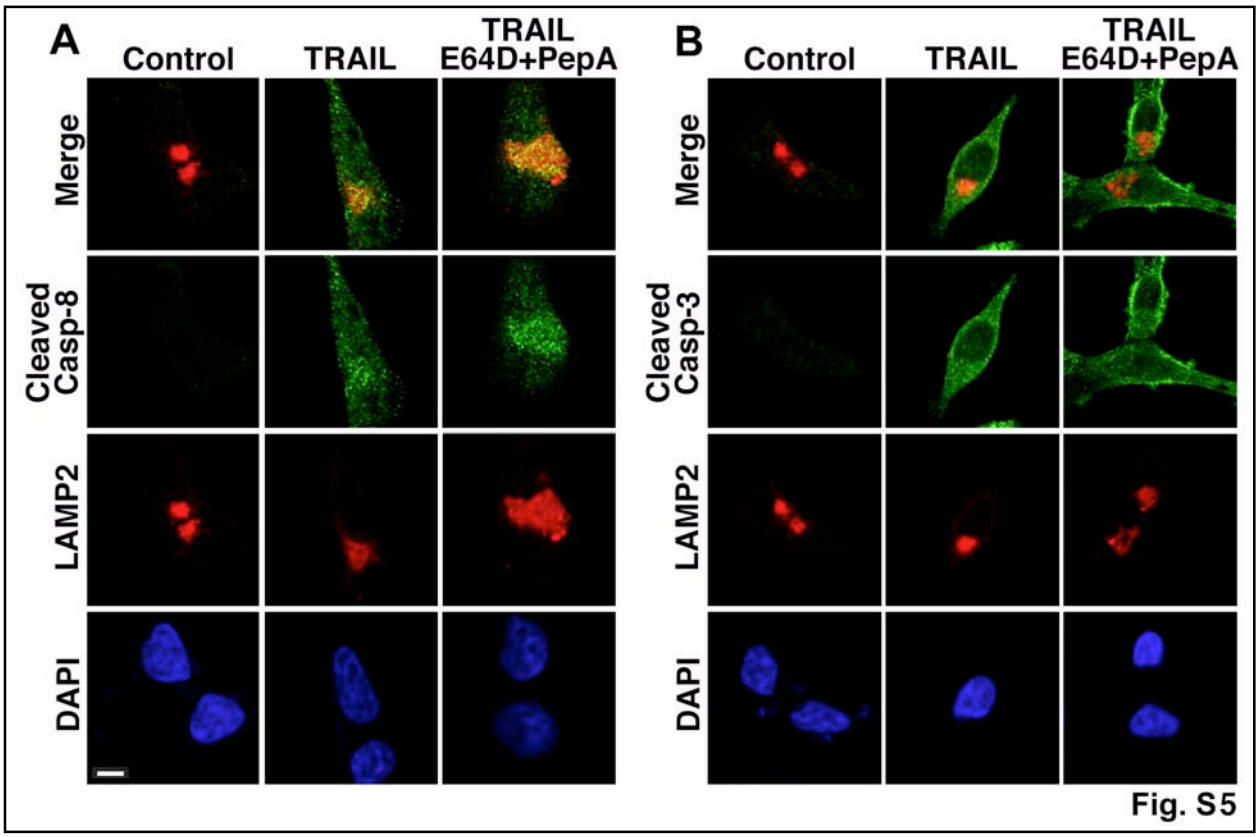


Fig. S5. Differential cellular localization of cleaved caspase-8 (A) vs. cleaved caspase-3 (B) in Bax$^{-/-}$ Hct116 cells treated with TRAIL for 24 hr in the presence or absence of E64D/pepstatin A. Scale bar = 10 μm.



Supplemental Materials and Methods

Cell Lines, Cell Lysates and Cell Extract. MCF7 (ATCC), WT Hct116, and $Bax^{-/-}$ Hct116 cells (generous gifts from Dr. Bert Vogelstein, Johns Hopkins University) were grown in DMEM medium containing 15% FCS, 20 mM L-glutamine, and 100 units/ml each of penicillin and streptomycin. Jurkat T leukemic cells (ATCC) were grown in RPMI 1640 medium containing 10% FCS, 20 mM HEPES, 2 mM L-glutamine, and 100 units/ml each of penicillin and streptomycin. Cell lysates were prepared with 1% NP-40, 20 mM Tris-HCl pH 7.4, 137 mM NaCl, 10% glycerol, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin. To prepare cell extracts, cultured cells were washed twice with PBS and then resuspended in ice-cold buffer (20 mM HEPES, pH 7.0, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 250 mM sucrose, and protease inhibitors). After incubation on ice for 20 min, cells ($2.5x10^6$/0.5 ml) were disrupted by Dounce homogenization. Nuclei were removed by centrifugation at 650 g for 10 min at 4°C. Cellular extracts were obtained as the supernatants resulting from centrifugation at 14,000 g at 4°C for 30 min.

Cytosol and Membranous Pellet Fractionation. Following TRAIL treatment, cytosolic and membranous pellet (MP) fractions were separated using a digitonin-based subcellular fractionation technique as described previously (1). Briefly, 10^7 cells were harvested by centrifugation at 800 g, washed in PBS pH 7.2, and re-pelleted. Cells were digitonin-permeabilized for 5 min on ice at a density of $3x10^7$/ml in cytosolic extraction buffer (250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 pH 7.2, 100 M PMSF, 10 μg/ml leupeptin, 2 g/ml aprotinin, containing 200 μg/ml digitonin). Plasma membrane permeabilization of cells was confirmed by staining with a 0.2% trypan blue solution. Cells were then centrifuged at 1000 g for 5 min at 4°C. The supernatant (cytosolic fraction) was saved and the membraneous pellet (MP) was solubilized in the same volume of lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2 % Triton X-100, 0.3% NP-40, 100 M PMSF, 10 μg/ml leupeptin, 2 μg/ml aprotinin), followed by the precipitation of materials to be discarded at 10,000 g for 10 min at 4°C. The cytosolic fraction was enriched in β-actin and LC3-I, whereas the MP fraction was enriched in mitochondria (cytochrome c), ER (Bap31), lysosomes (LAMP2) and autophagosomes (LC3-II) (Fig. S1B).

Image Cytometry. The Cellomics ArrayScan HCS Reader (Cellomics/ ThermoFisher, Pittsburgh, PA) was utilized to collect information on the distribution of fluorescently labeled components in the treated cells. The ArrayScan HCS system scans multiple fields in individual wells, acquiring and analyzing each of the cell images according to defined algorithms. The scanner is equipped with emission and excitation filters (XF93, Omega Optical, Brattleboro, VT, USA) for selectively imaging fluorescent signals. Data were captured, extracted, and analyzed with ArrayScan II Data Acquisition and Data Viewer version 3.0 (Cellomics), Quattro Pro version 10.0.0 (Corel, Ottawa, Ontario, Canada), and Office Excel

(Microsoft, Redmond, WA).

Supplemental Reference

1. Adrain C, Creagh EM, Martin SJ. Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. EMBO J 2001;20:6627-36.