nature
cell biology

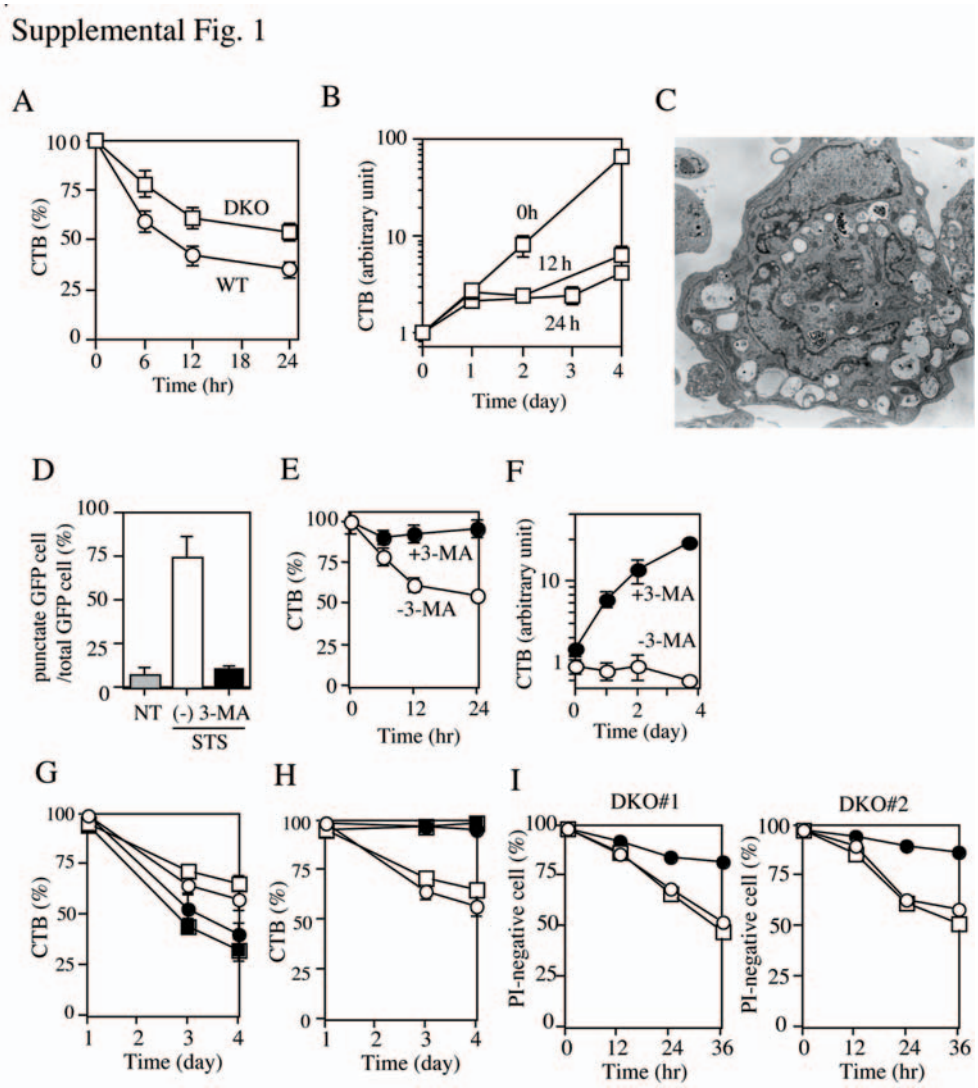

Figure S1 Induction of non-apoptotic death of SV40-transformed and primary DKO MEFs, and DKO thymocytes. (A-F) STS-induced non-apoptotic death of DKO MEF. (A, B) Reduced viability of DKO MEFs after exposure to STS. WT and DKO MEFs were treated with 1 μM STS. Then cell viability was measured (A) by the CTB assay and (B) by the same procedure as in (Fig. 1D). Data are shown as mean ± SD (n=4). (C) Electron micrograph of DKO MEFs treated with STS (x 17,000). Cells were incubated with STS (1 μM) for 12 hours, and then examined by electron microscopy. (D-F) Inhibition of STS-induced death of DKO MEFs by blocking autophagy. (D) GFP-LC3-transfected DKO MEFs were treated with 1 μM STS in the absence or presence of 10 mM 3-MA. The percentage of cells with punctate GFP-LC3 fluorescence was calculated relative to all GFP-LC3-positive cells. (E, F) DKO MEFs were treated with 1 μM STS in the absence or presence of 10 mM 3-MA for the indicated times (E) and 12 hr (F). Cell viability was measured by the CTB assay (E), and cell proliferation assay (F). Data are shown as mean ± SD (n=4). (G-I) Induction of non-apoptotic death of primary DKO MEFs and DKO thymocytes. (G) Reduced viability of primary DKO MEFs after exposure to etoposide. WT (closed squares), Bak-/- (closed circles) and DKO#1, 2 (open circles and squares, respectively) primary MEFs were treated with 50 μM etoposide. Then cell viability was measured by the CTB assay, and expressed as a percentage of the value at 1 day after etoposide treatment. This was because all the primary MEFs proliferated normally for 1 day after etoposide treatment (approximately 4-fold increase in cell numbers). (H) Inhibition of etoposide-induced cell death by 3-MA. Primary DKO MEFs (#1, #2) were treated with 50 μM etoposide in the absence (open symbols) or presence of 10 mM 3-MA (closed symbols) for the indicated day. Cell viability was measured by the CTB assay. (I) Reduced viability of DKO thymocytes after exposure to STS. DKO thymocytes (isolated from 2 different mice #1 and #2) were treated with 0.5 μM STS in the absence (open squares) or presence of 100 μM zVAD-fmk (open circles), or 10 mM 3-MA (closed circles). Then cell viability was measured by the PI staining and expressed as the percentage of PI-negative cells per total cells.

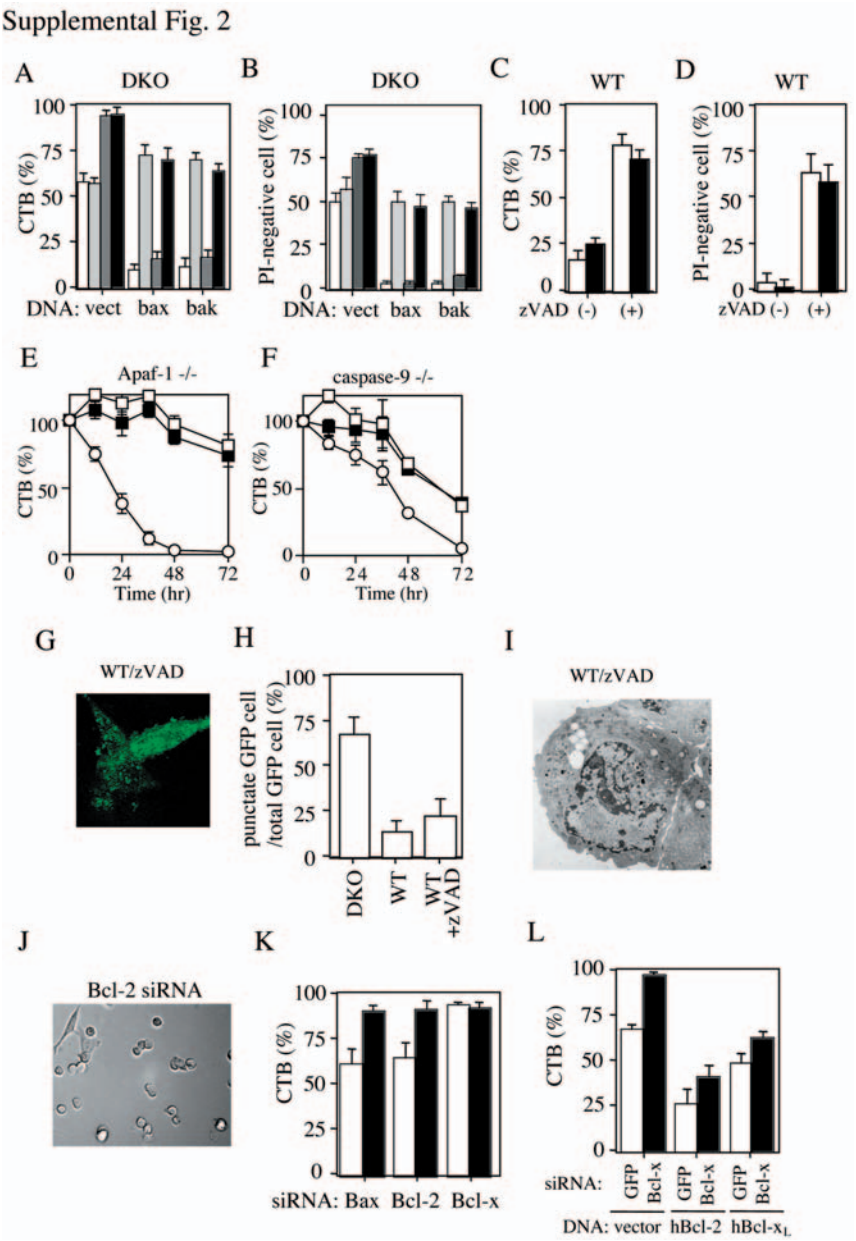

Figure S2 Absence of etoposide-induced 3-MA-inhibitable non-apoptotic death in MEFs by caspase inhibition, and no inhibition of non-apoptotic death in etoposide-treated DKO MEFs by siRNA for Bcl-2. (A-I) Absence of etoposide-induced 3-MA-inhibitable non-apoptotic death in MEFs by caspase inhibition. (A, B) DKO MEFs were transiently transfected with 1 μg of the indicated DNAs. After 24 hours, cells were treated with 20 μM etoposide in the absence (white columns) or presence of 100 μM zVAD (gray columns), 10 mM 3-MA (darkgray columns), or 100 μM zVAD plus 10 mM 3-MA (black columns), and then cell viability was measured by the CTB assay after 24 hours (A) and PI staining after 48 hours (B). Data are shown as mean ± SD for n=4. (C, D) Failure of 3-MA to inhibit death of WT MEFs treated with etoposide in the presence of a caspase inhibitor. WT MEFs with (closed columns) or without (open columns) 10 mM 3-MA were treated with etoposide in the absence or presence of 100 μM zVAD-fmk, and then cell viability was measured by the CTB assay after 24 hours (C) and PI staining after 48 hours (D). Data are shown as mean ± SD (n=4). (E, F) No effect of 3-MA on cell viability in Apaf-1- and caspase-9-deficient MEFs treated with etoposide. Apaf-1-deficient and caspase-9-deficient (squares) and their control MEFs (circles) were treated with 20 μM etoposide in the absence (open symbols) or presence (closed symbols) of 10 mM 3-MA, and then cell viability was assessed by the CTB assay. Data are shown as mean ± SD (n=4). (G-I) Mild induction of autophagy in MEFs after etoposide treatment. (G, H) WT MEFs that were transfected with GFP-LC3 were incubated with 20 μM etoposide in the absence or presence of 100 μM zVAD for 24 hours, and then were examined by confocal fluorescent microscopy. A representative photograph is shown (G), in which cells exhibit mild punctate distribution of GFP-LC3. The percentage of cells with punctate GFP-LC3 fluorescence was calculated relative to all GF-LC3-positive cells (H). Data are shown as mean ± SD. (I) WT MEFs were incubated with 20 μM etoposide in the presence of 100 μM zVAD for 18 hours, and then were analysed by electron microscopy (x 8,500). (J-L) No inhibition of non-apoptotic death in etoposide-treated DKO MEFs by siRNA for Bcl-2. (J) DKO MEFs that were transfected with the siRNA for Bcl-2 were treated with 20 μM etoposide for 24 hours, and then were examined by phase-contrast microscopy. (K) DKO MEFs that were transfected with the indicated siRNAs were treated with 20 μM etoposide in the absence (open symbols) or presence (closed symbols) of 10 mM 3-MA, and cell viability was assessed by the CTB assay at 24 hours. Data are presented as mean ± SD (n=4). (L) DKO cells that were transfected with the indicated siRNA together with plasmid (vector, human Bcl-2 or human Bcl-x_L) were treated with 20 μM etoposide for 24 hr and then cell viability was assessed by the CTB assay. Data are presented as mean ±SD (n=3).

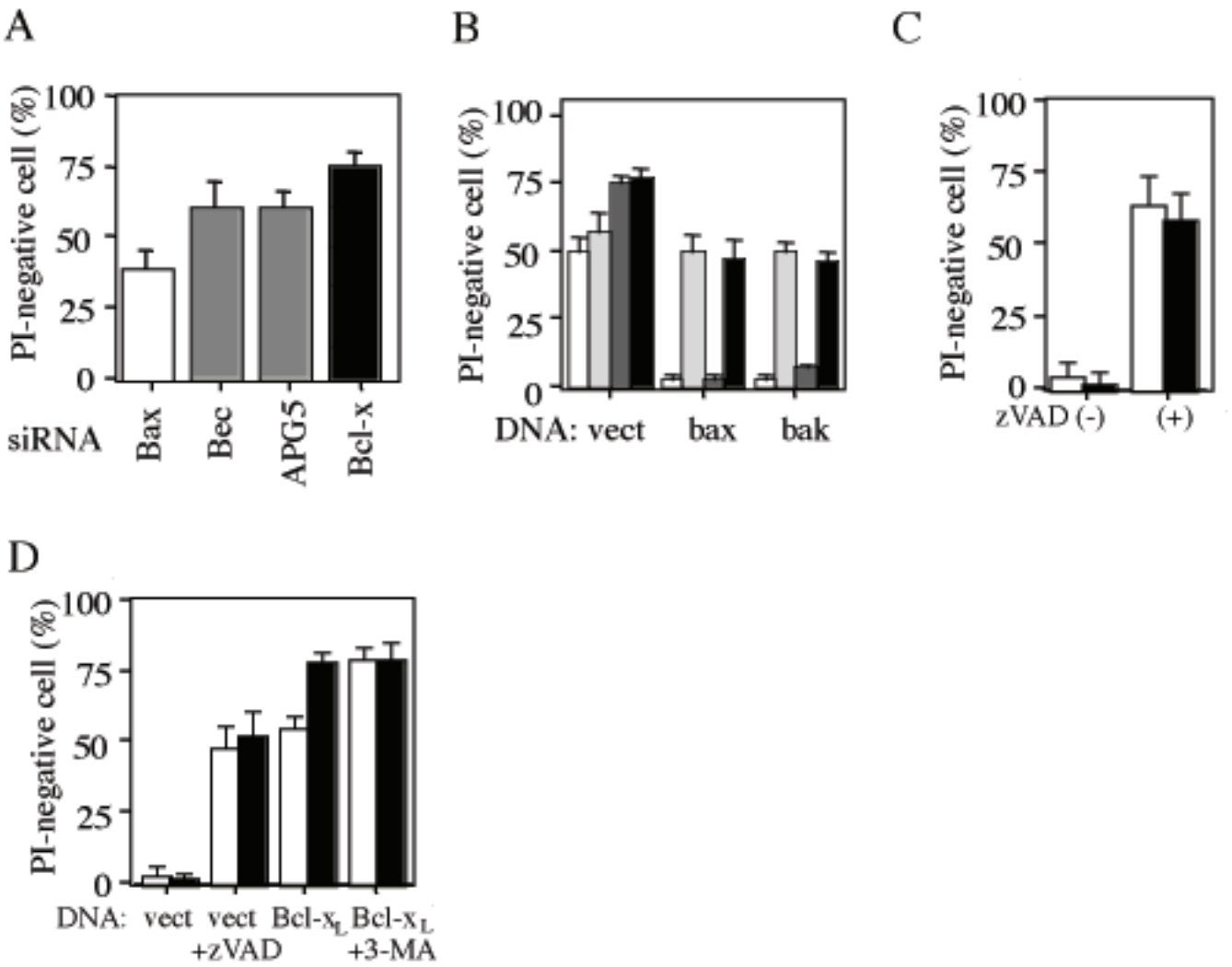

Figure S3 Assessment of cell viability by the PI staining **(A)** Inhibition of etoposide-induced death of DKO MEFs by silencing of Beclin 1, APG5, and Bcl-x. DKO MEFs which were treated with the indicated siRNAs were incubated with 20 μM etoposide for 48 hours. Cell viability was measured by PI staining. Data are shown as mean ± SD (n=3). **(B)** Similar experiments as shown in Fig. 3A were performed, and cell viability was assessed by PI staining after 48 hours. White columns: no inhibitor, gray columns: 100 μM zVAD, darkgray columns: 10 mM 3-MA, or black columns:100 μM zVAD plus 10 mM 3-MA. Data are shown as mean ± SD for n=3. **(C)** Similar experiments as shown in Fig. 3B were performed, and cell viability was assessed by PI staining after 48 hours. White columns: 3-MA (-), black columns: 10 mM 3-MA. **(D)** Similar experiments as shown in Fig. 3J were performed, and cell viability was assessed by PI staining after 48 hours. White columns: APG+/+ MEFs, black columns: APG-/- MEFs.

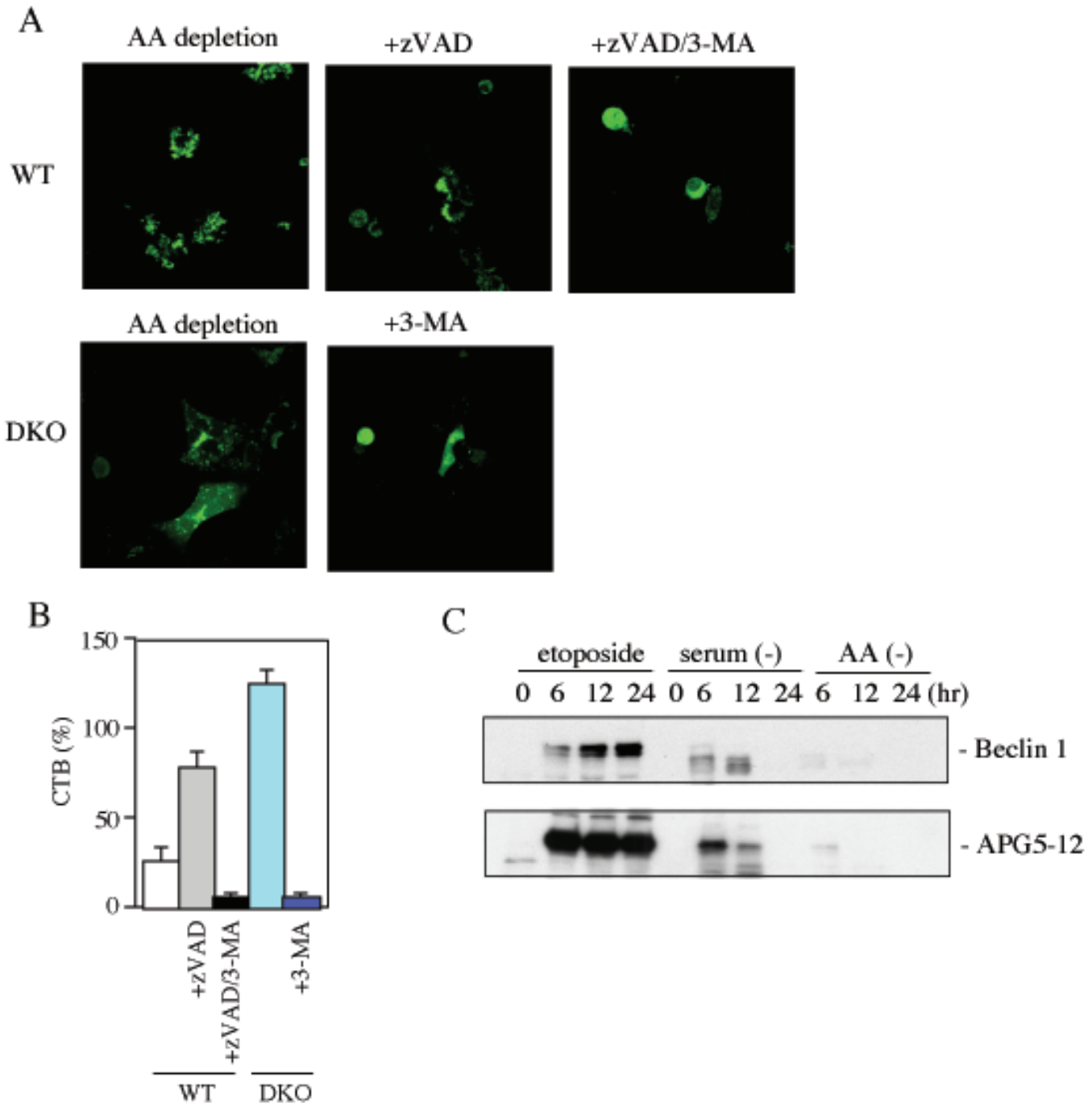

Figure S4 Apoptotic death of MEFs by amino acid starvation. **(A)** 3-MA-inhibitable punctate GFP-LC3 fluorescence in WT and DKO MEFs after deprivation of amino acids. WT and DKO MEFs that were transfected with GFP-LC3 were cultured in the starvation medium (Hanks balanced solution supplemented with 10% glucose, 1 mM sodium pyruvate, 10 mM Hepes/Na^+, (pH 7.4), 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin) with or without 10 mM 3-MA and/or 100 μM zVAD-fmk for 24 hours, and then were examined by confocal fluorescent microscopy. Representative photographs are shown. Both WT and DKO MEFs showed punctate GFP-LC3 fluorescence, which were inhibited by 3-MA, **(B)** Death of WT and DKO MEFs by amino acid starvation. WT and DKO MEFs were cultured in the starvation medium with or without 10 mM 3-MA and/or 100 μM zVAD-fmk. After 24 hours, cells were harvested and cell viability was assessed by CTB assay. Similar results were also obtained with PI staining. **(C)** No accumulation of Beclin 1 and APG5-APG12 complex in DKO MEFs after starvation. DKO MEFs were incubated with normal medium with 20 μM etoposide, serum-depleted medium (which also activates autophagy), and starvation medium for 24 hours. Expression of Beclin 1 and APG5-APG12 complex was analysed by Western blot analysis.

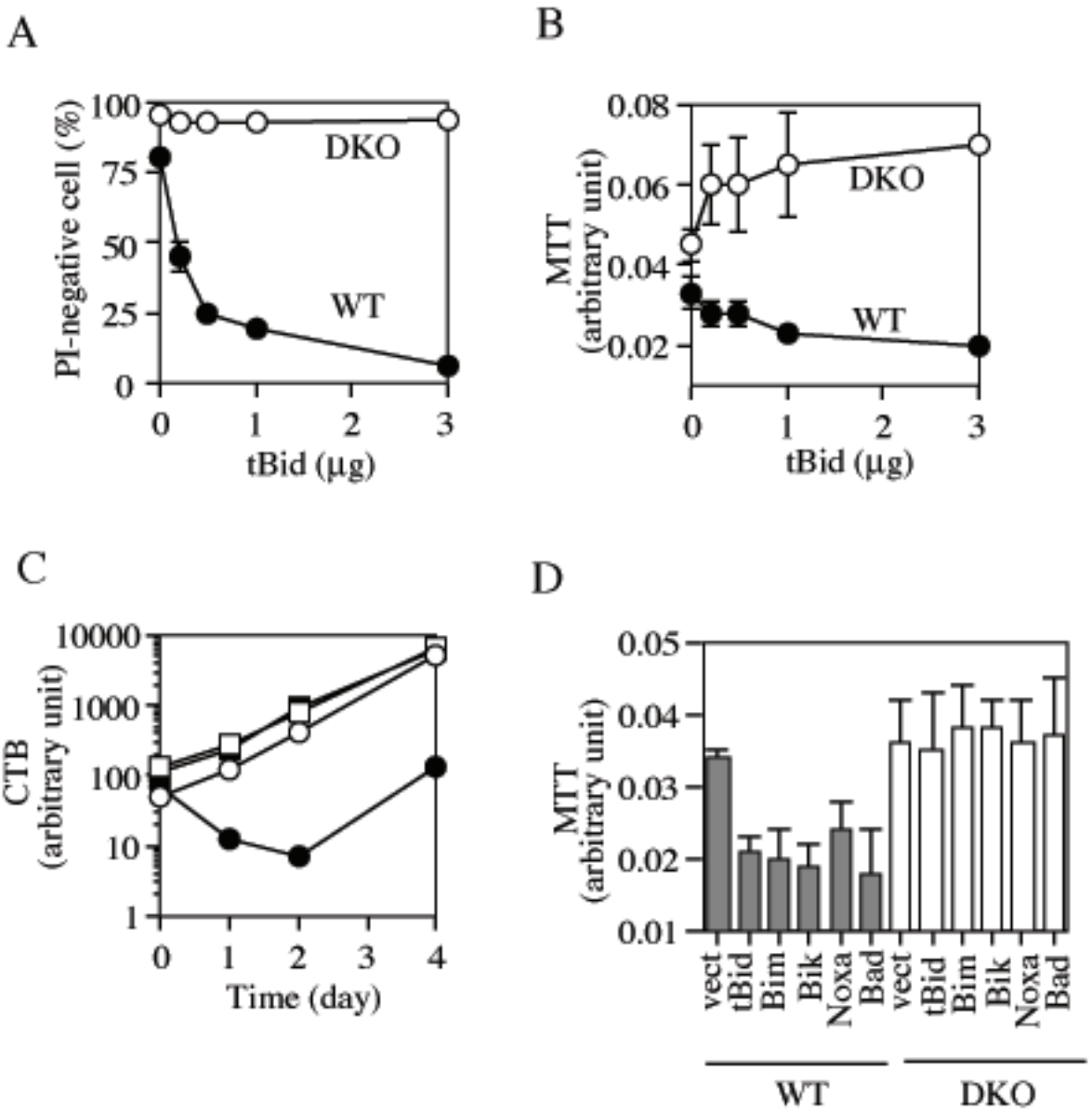

Figure S5 DKO MEFs died by etoposide, but not by BH3-only proteins. (**A**, **B**) WT (closed circles) and DKO MEFs (open circles) were transfected with the indicated amounts of tBid expression DNA, and cell viability was assessed by PI staining (A) and the MTT assay (B) at 24 hours. The CTB assay gave the virtually identical results as those with the MTT assay. (**C**) WT (closed symbols) and DKO (open symbols) MEFs were transfected with 3 μg of tBid expression DNA (circles) or control vector (squares), and viable cell numbers were measured by the CTB assay at the indicated times. Data are shown as mean ± SD (n=4). (**D**) No death of DKO MEFs by various BH3-only proteins. WT and DKO MEFs were transfected with 1 μg of the expression DNA for the indicated proteins, and cell viability was assessed by the MTT assay at 24 hours. Data are shown as mean ± SD (n=4).

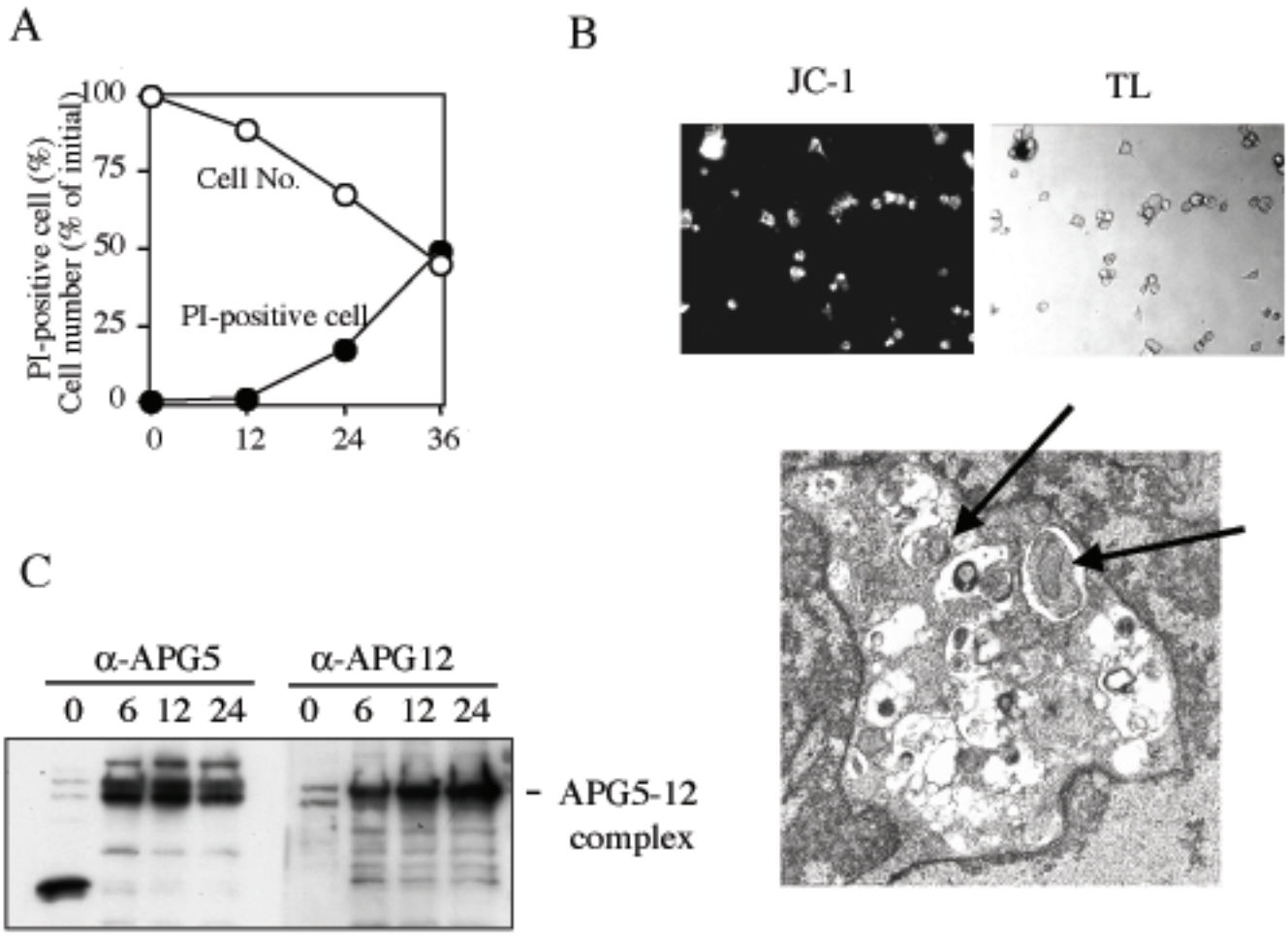

Figure A. Loss of etoposide-treated DKO MEFs in flow cytometric analysis. DKO MEFs were treated with 20 μM etoposide, and PI-positive cells were counted under fluorescence microscope. Cells were also harvested and the number of cell was counted by flow cytometer. **Figure B. Maintenance of mitochondrial membrane potential in etoposide-treated DKO MEFs.** (upper panel) DKO MEFs were treated with 20 μM etoposide for 36 hours, stained with JC-1 (10 μM), and the red fluorescence was observed under a fluorescence microscope. (Lower panel) DKO MEFs were treated with 20 μM etoposide for 18 hours, and analysed by electron microscopy. As shown in an EM photograph, some mitochondria were found in autophagosomes (arrows). **Figure C. Production of APG5/12 complex in etoposide-treated DKO MEFs** DKO MEFs were treated with 20 μM etoposide for the indicated hours. Expression of APG5/12 complex was analyzed by Western blot analysis using anti-APG5 and APG12 antibodies. Both antibodies reacted with the same band.

Movie 1 Phase-contrast microscopic analysis of etoposide-treated DKO MEFs. DKO MEFs were treated with 20 μM etoposide and observed for 40 hours under a phase-contrast microscope. Cells rounded around 12-18 hr, ruffled around 18-30 hr, and ballooned around 24-40 hr.
Movies 2, 3 Phase-contrast and PI-staining analysis of etoposide-treated DKO MEFs. DKO MEFs were treated with 20 μM etoposide in the presence of 2 μM PI and observed from 8 hours to 36 hours under a phase-contrast (2) and fluorescence (3) microscope.

Movies 4, 5 Phase-contrast microscopic and PI-staining analysis of etoposide-treated DKO MEFs with 3-MA. DKO MEFs were treated with 20 μM etoposide plus 10 μM 3-MA in the presence of 2 μM PI and observed from 12 hours to 48 hours under a phase-contrast (6) and fluorescence (7) microscope. The number of PI-positive cells was greatly reduced compared with that in Movie 3