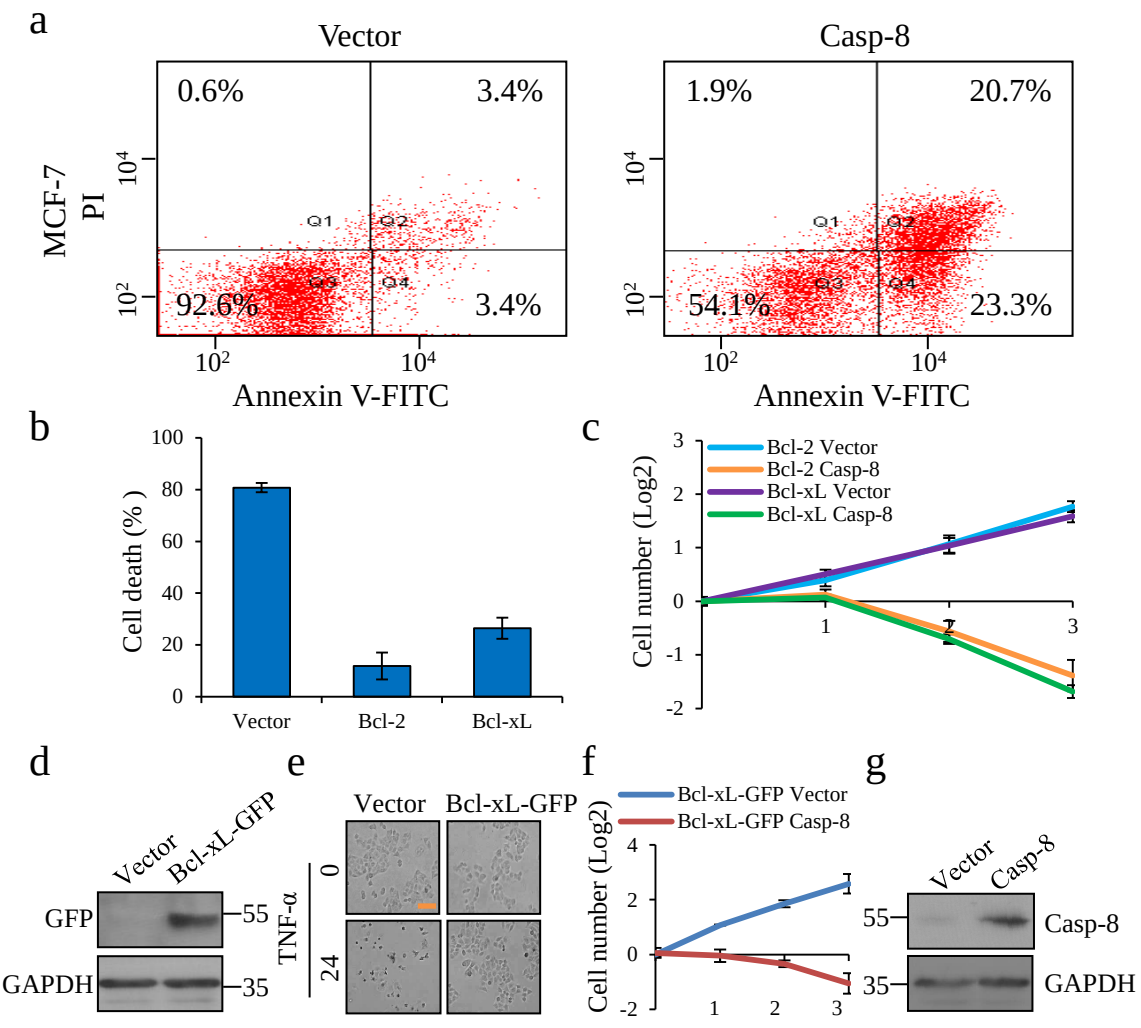
YMTHE, Volume *28*

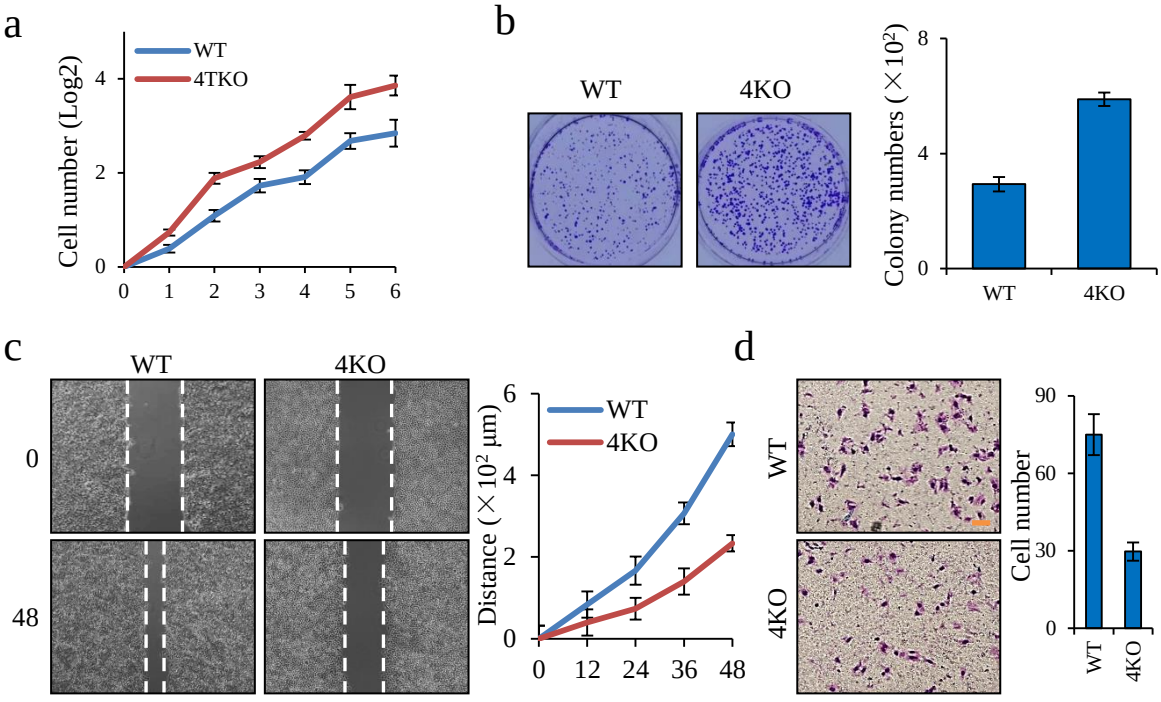
Supplemental Information

Caspase-8 Induces Lysosome-Associated Cell Death in Cancer Cells

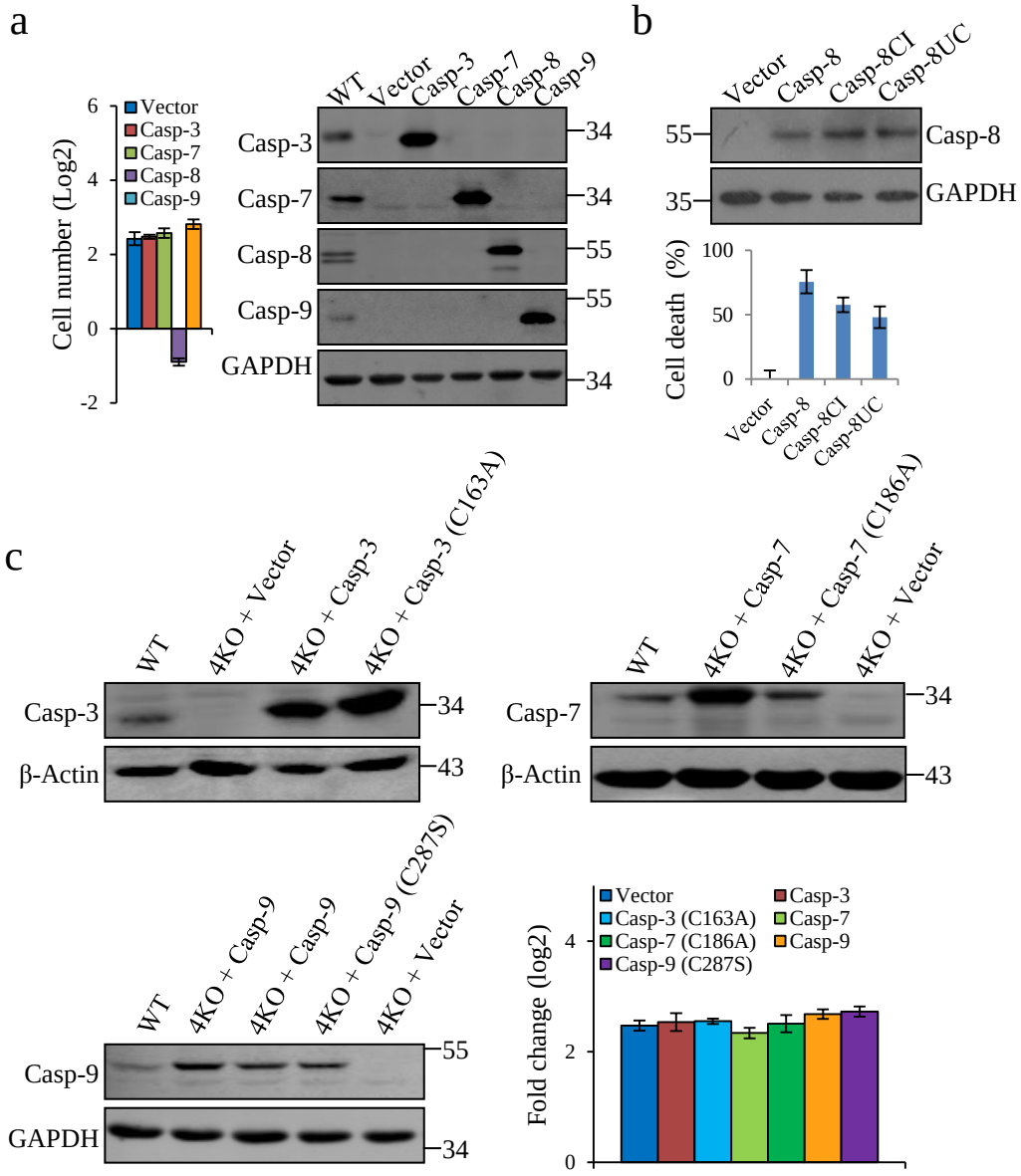
Benfu Zhong, Miao Liu, Changsen Bai, Yuxia Ruan, Yuanyuan Wang, Li Qiu, Yang Hong, Xin Wang, Lifang Li, and Binghui Li



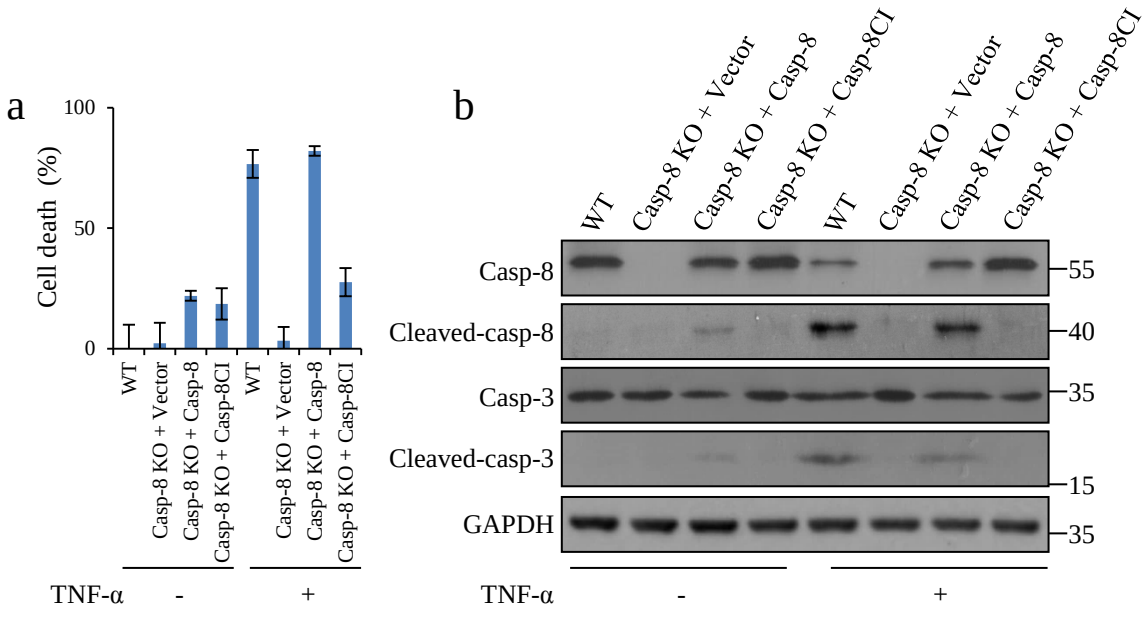
Supplementary Fig. 1 Caspase-8 expression induces apoptosis in MCF-7 cells and exogenous caspase-8 induces cell death independent of mitochondria in HeLa cells. (a) Caspase-8 overexpression induced apoptosis in MCF-7 cells, as measured by Annexin V/PI flow cytometric analyses. MCF-7 cells were transfected with vector or caspase-8, and apoptotic cells (Annexin V^+ PI^- and Annexin V^+ PI^+) were detected by the binding of Annexin V to externalized phospatidylserine in conjunction with PI, which is a dye excluded from viable cells. (b) HeLa cells stably expressing vector, Bcl-2, or Bcl-xL were treated with TNF-α (50 ng ml^{-1}) plus cycloheximide (CHX) (10 μg ml^{-1}) for 12 h, and cell death was evaluated based on the negative correspondence to ATP level. (c) Proliferation of HeLa cells stably expressing Bcl-2 or Bcl-xL was examined after transfection with vector or caspase-8 for 3 days. Each bar represents the mean ± S.D. for triplicate experiments. (d) The efficiency of lentivirus-mediated Bcl-xL-GFP expression in HeLa cells was verified by western blot. (e) Images of HeLa cells stably expressing vector or Bcl-xL-GFP after treatment with TNF-α (50 ng ml-1) plus CHX (10 μg ml-1) for 24 h. Scale bar, 100 μm. (f) Proliferation of HeLa cells stably expressing Bcl-xL-GFP after transfection with vector or caspase-8 for 3 days. Each bar represents the mean ± S.D. for triplicate experiments. (g) The efficiency of caspase-8 overexpression in HeLa cells stably expressing Bcl-xL-GFP was verified by western blot.



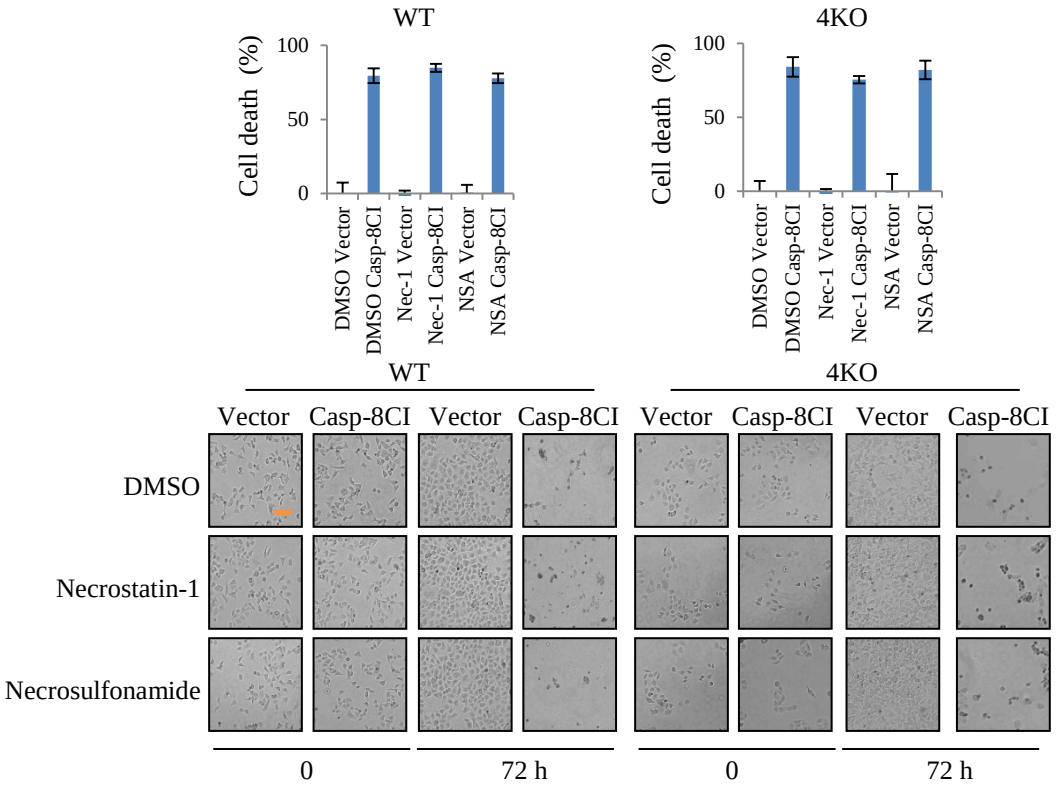
Supplementary Fig. 2 Biological characteristics of 4KO HeLa cell line. (a) Proliferation of wild-type and 4KO HeLa cells in 6 days. (b) Wild-type and 4KO HeLa cells were maintained for 14 days before stained with crystal violet and counted for colony numbers in colony formation assay. (c) Wound healing test of wild-type and 4KO HeLa cells from 0-48 h. Representative images and migrated distance were showed. (d) Cell invasion assays were performed in wild-type and 4KO HeLa cells, and the invaded cells were stained and counted. Each bar represents the mean ± S.D. for triplicate experiments. Scale bar, 100 μm.



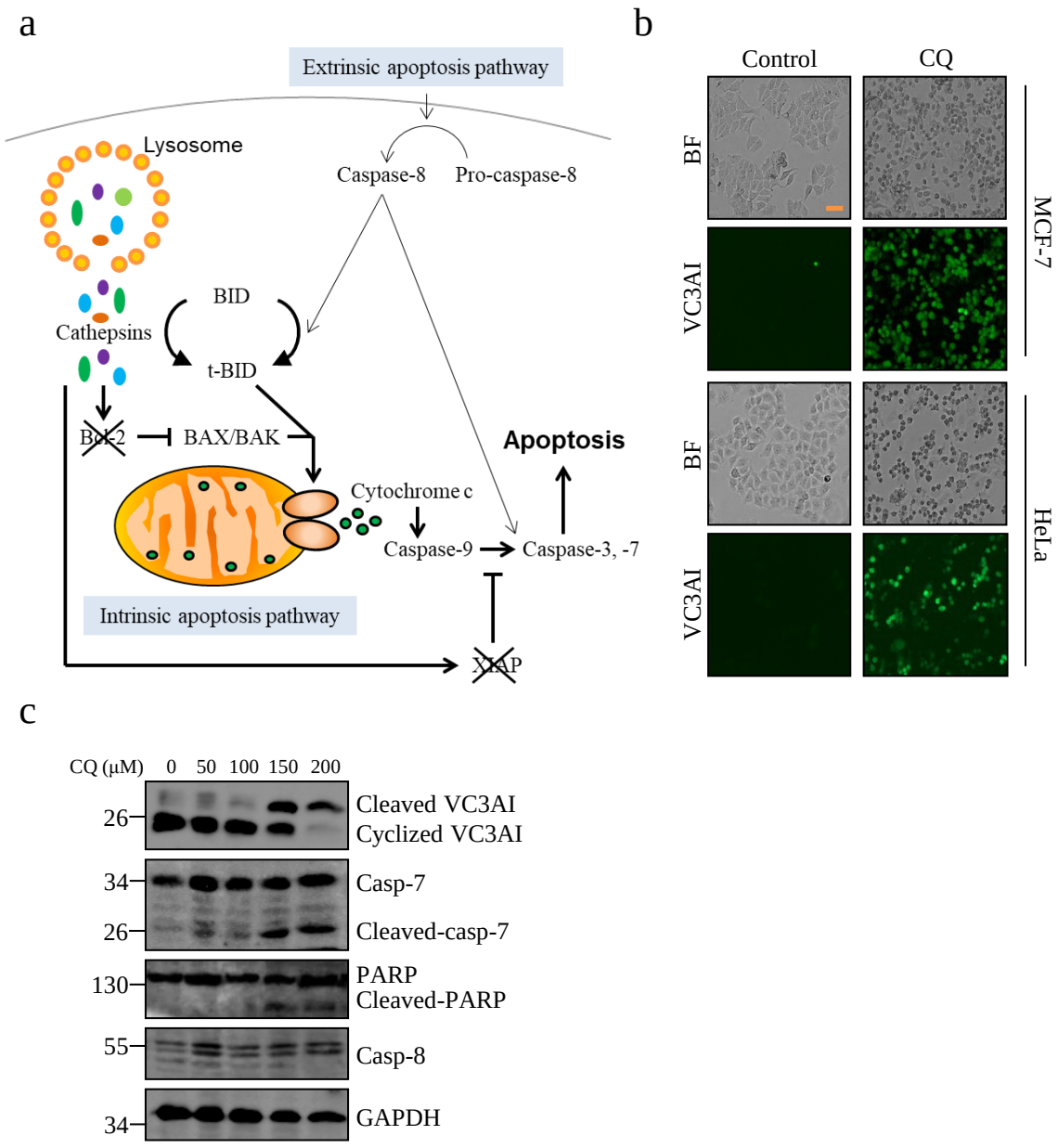
Supplementary Fig. 3 Achievement of 4KO cells stably re-expressed caspase-3, -7, -9, or their catalytic inactive point mutants. (a) Proliferation of 4KO HeLa cells transiently re-introduced with caspase-3, -7, -8, or -9 was measured in 72 hours (left). The re-introduction of the indicated proteins was verified by western blot in 48 hours (right). (b) 4KO HeLa cells were transfected with vector, caspase-8, casp-8CI, or casp-8UC for 3 days. The re-introduction of caspase-8 or its mutants was verified by western blot in 48 hours , and cell death was evaluated in 72 hours based on the negative correspondence to ATP level. (c) 4KO HeLa cells were infected with lentiviruses carrying caspase-3, -7, -9, or their catalytic inactive point mutants, and selected with G418. The re-expression of the proteins in the preserved cells was verified by western blot, and fold change in cell number in 3 days was shown.



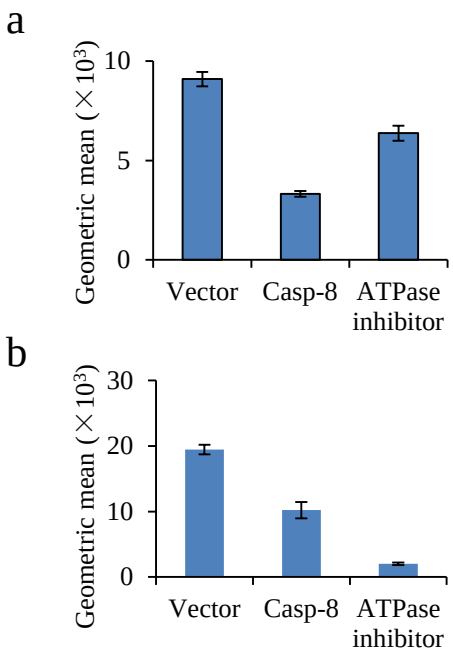
Supplementary Fig. 4 Enzymatic activity of capsase-8 is necessary to the canonical extrinsic apoptosis. (a) Casp-8 KO HeLa cells were transfected with wild-type caspase-8 or mutant casp-8CI, and treated post-transfection 8h with or not with TNF-α (50 ng ml^{-1}) plus CHX (10 μg ml^{-1}) for 16h. The level of ATP was examined, and the percentage of dead cells was calculated by subtracting the percentage of living cells from 100%. Each bar represents the mean ± S.D. for triplicate e experiments. (b) Western blot analysis of cleaved caspase-8 and caspase-3 in the wild-type HeLa cells or the casp-8 KO HeLa cells re-expressed wildtype caspase-8 or casp-8CI after treatment with TNF-α (50 ng ml^{-1}) plus CHX (10 μg ml^{-1}) for 16h.



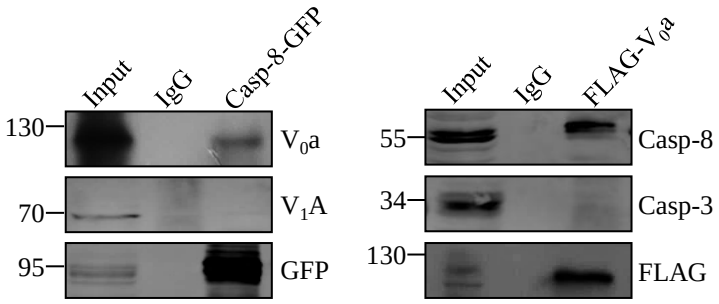
Supplementary Fig. 5 Cell death induced by exogenous casp-8CI is not necroptosis or pyroptosis. Wild-type or 4KO HeLa cells were treated with the RIPK1 inhibitor necrostatin-1 (Nec-1) (10 μM) or the MLKL inhibitor necrosulfonamide (NSA) (5 μM) for 24 hours, and then transiently transfected with vector or casp-8CI. Cell death was evaluated based on the negative correspondence to ATP level. Scale bar, 100 μm. Each bar represents the mean ± S.D. for triplicate experiments.



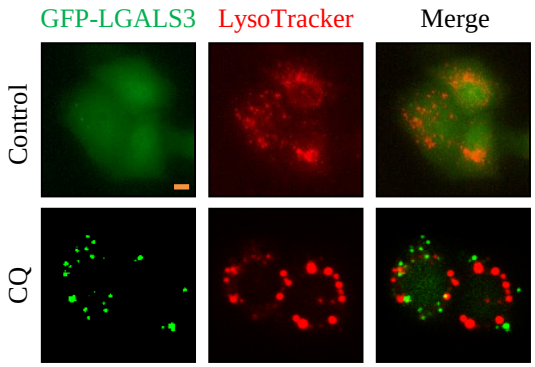
Supplementary Fig.6 lysosome rupture is able to elicit apoptosis. (a) Model of lysosome rupture in eliciting apoptosis. (b) Fluorescence images of MCF-7/VC3AI or HeLa/VC3AI cells after treatment with 150 μM of chloroquine (CQ) for 24 h. Green fluorescence indicates apoptosis. Scale bar, 100 μm. (c) Western blot analysis of cleavage of VC3AI and classical apoptotic proteins (caspase-7 and PARP) in MCF-7/VC3AI cells after treatment with CQ..



Supplementary Fig. 7 The geometric mean intensities in Fig. 4d and Fig. 4f. (a) 4KO HeLa cells re-introduced with vector or caspase-8 were stained with LysoSernser Green (1 μM) for 15 min, and fluorescence was measured by flow cytometer. The geometric mean is indicated. (b) 4KO HeLa cells re-introduced with vector or caspase-8 were stained with 1.5 μg/ml of acridine orange (AO) for 30 min, and red fluorescence was analyzed by flow cytometer. The geometric mean is indicated. Each bar represents the mean ± S.D. for triplicate experiments.



Supplementary Fig. 8 Co-immunoprecipitation experiments in HeLa cells with caspase-8-GFP or FLAG-V_0a expression. Whole-cell lysates from cells were immunoprecipitated with antibodies against IgG, GFP, or FLAG followed by immunoblotting with the antibodies against the indicated proteins.



Supplementary Fig. 9 CQ induces lysosome rupture in 4KO HeLa cells. Fluorescence images of 4KO HeLa cells expressing GFP-LGALS3, after treatment with 100 μM of CQ for 2 h. Changing of GFP-LGALS3 from diffuse to punctate after CQ treatment represents lysosome rupture. The expanded LysoTracker Red spots indicate lysosomal swelling. Scale bar, 5 μm.