Supporting information

Azithromycin enhances the cytotoxicity of DNA-damaging drugs via lysosomal membrane permeabilization in lung cancer cells

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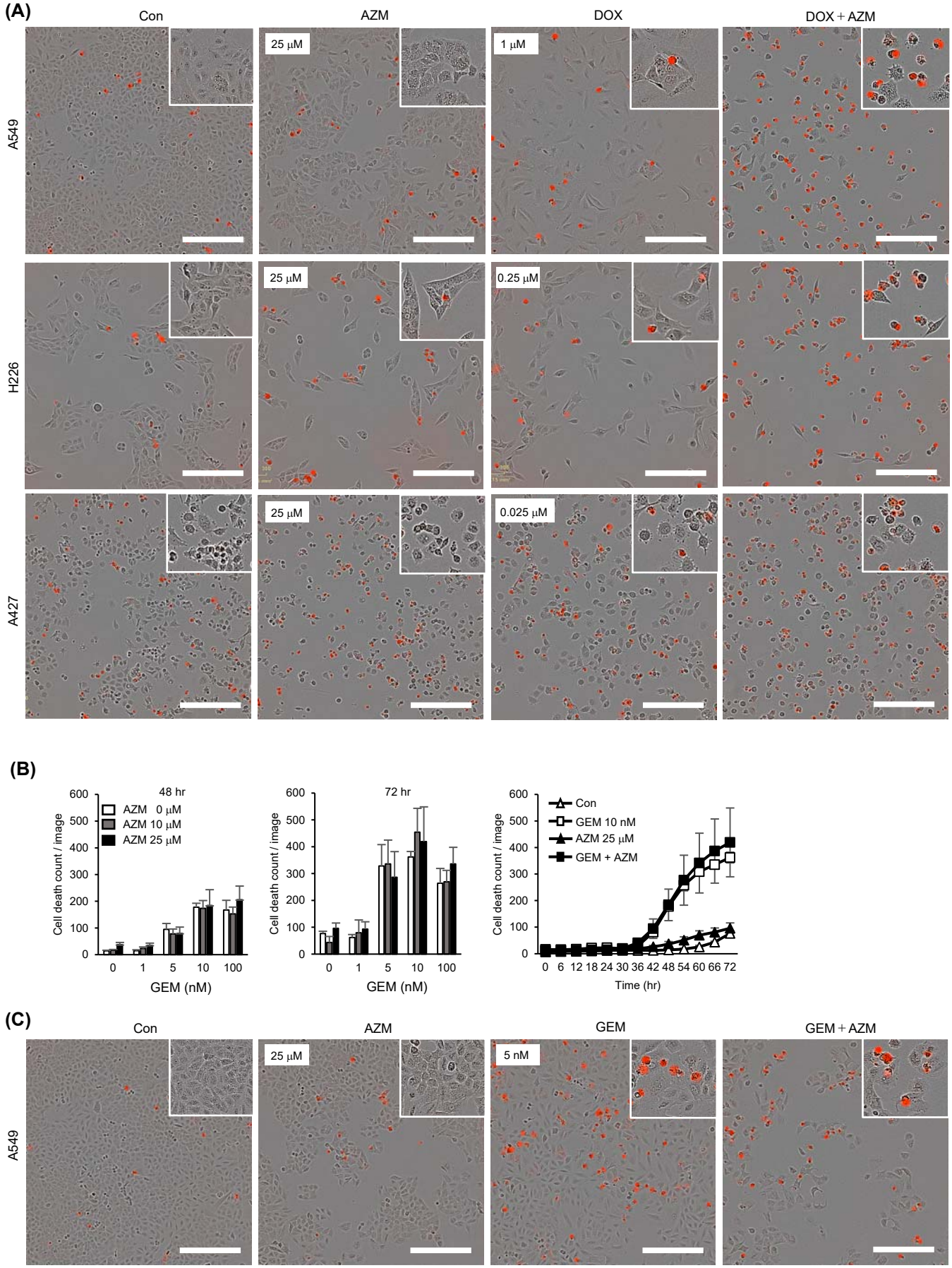

Figure S1. DOX- or GEM- ± AZM treated A549, H226 and A427 cells. (A) A549, H226 and A427 cells treated with DOX ± AZM for 72 h. Scale bar = 300 μm. Dead cells became positive with PI and show red fluorescence. (B) A549 cells were treated with GEM ± AZM for 72 h. Number of dead cells was assessed by PI staining. Dose dependent and time dependent cell death number are shown. n = 4, bar = mean ± S.D. (C) A549 cells treated with GEM + AZM for 72 h. Cells were stained with PI and dead cells show red fluorescence. Scale bar = 300 μm.

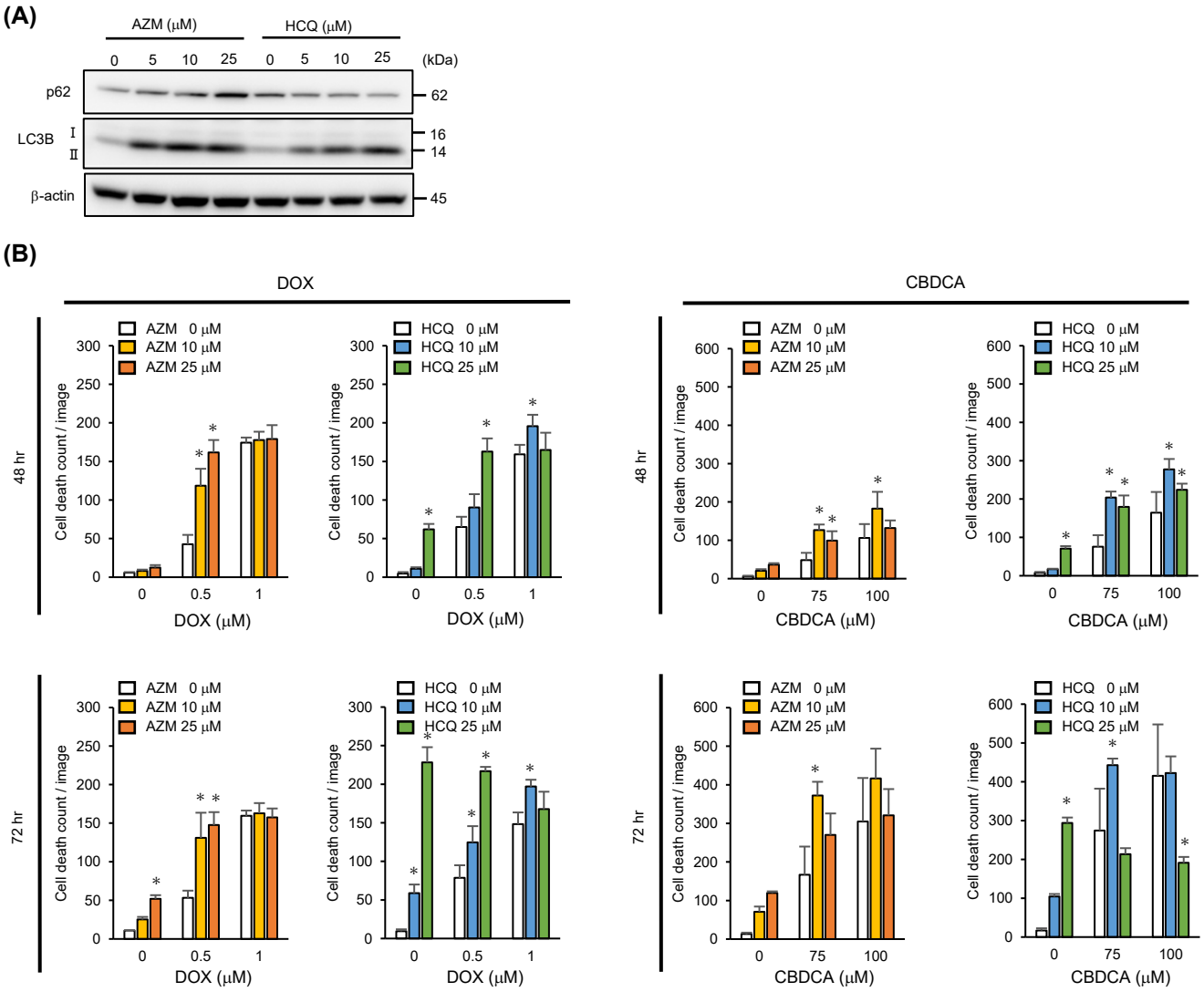

Figure S2. HCQ inhibited autophagy similar to that of AZM and enhanced the cytotoxicity of DOX and CBDCA. (A) A549 cells were treated with AZM or HCQ at the indicated concentration for 24 h. The expression levels of p62 and LC3B were assessed by western blotting. (B) A549 cells were treated with DOX ± AZM or HCQ, or CBDCA ± AZM or HCQ for 72 h. The number of dead cells was assessed by PI staining. n = 4, bar = mean ± S.D., *p<0.05.

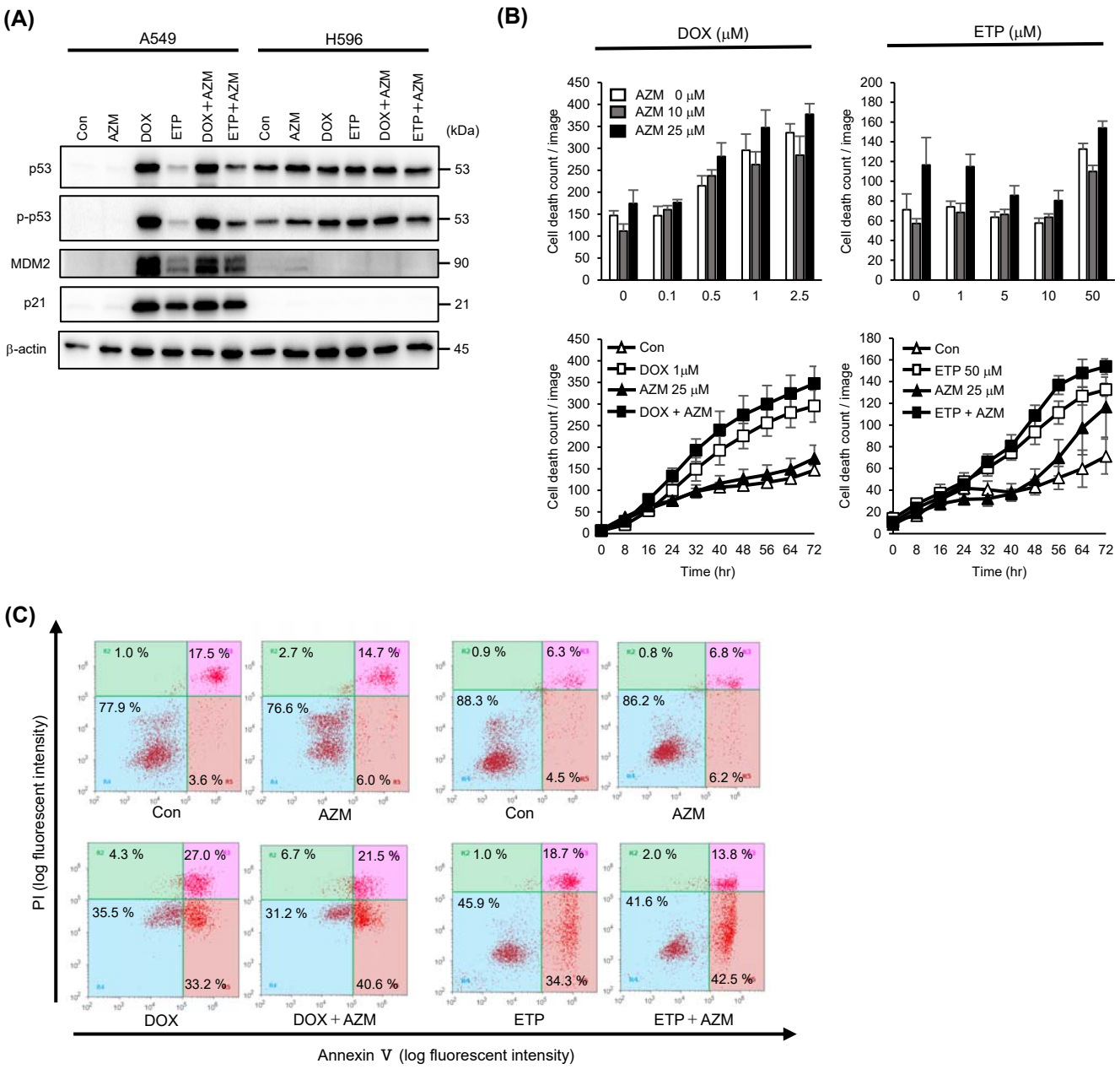

Figure S3. AZM did not enhance DOX-induced cell death in H596 cells carrying p53 mutation. (A) Western blotting of p53, phospho-p53, MDM2, and p21 expression in A549 and H596 cells after DOX (1 μM) or ETP (5 μM) ± AZM (25 μM) treatment for 24 h. (B) H596 cells were treated with DOX (1 μM) or ETP (5 μM) in combination with AZM (25 μM) for 72 h. Number of dead cells was assessed by IncuCyte with PI staining. Representative result is shown. n=4, bar = mean ± S.D., *p<0.05. v.s. 0 μM AZM treatment. (C) Flowcytometric analysis of Annexin V/PI double-stained H596 cells treated with DOX (1 μM) or ETP (5 μM) ± AZM (25 μM) for 72 h.

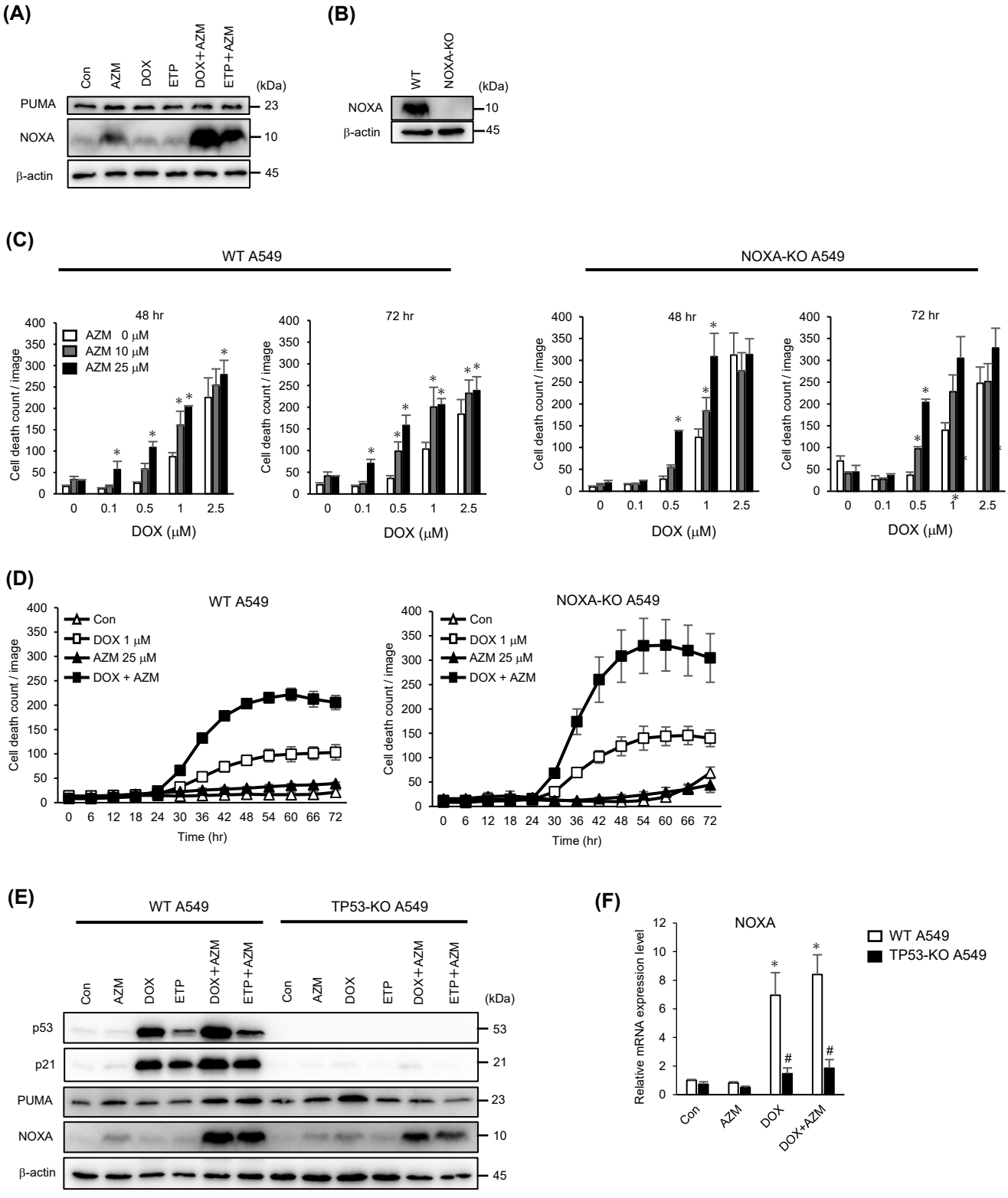

Figure S4. NOXA was strongly induced by combination treatment of DOX or ETP with AZM but independent from the induced cell death. (A) Protein expression of PUMA and NOXA was assessed by Western blotting. A549 cells were treated with DOX (1 μM) or ETP (5 μM), with or without AZM (25 μM), for 24 h. β-actin was used as loading control. (B) NOXA-KO was confirmed by Western blotting. WT or NOXA-KO A549 cells were treated with 1 μM DOX with 25 μM AZM for 24 h. (C–D) WT or NOXA-KO A549 cells were treated with DOX+ AZM for 72 h. Number of dead cells was assessed by IncuCyte with PI staining. Representative dose dependent (C) and time dependent (D) cell death numbers are shown. n=4, bar = mean ± S.D., *p<0.05. v.s. 0 μM AZM treatment. (E) Western blotting of NOXA and PUMA expression in WT or p53-KO A549 cells after DOX or ETP ± AZM treatment for 24 h. (F) Relative NOXA mRNA expression level in WT or TP53-KO A549 cells was measured by real-time PCR. Cells were treated with DOX (1 μM) ± AZM (25 μM) for 24 h. n=4, bar = mean ± S.D., *p<0.05 v.s. control. #p<0.05 v.s. WT cells.

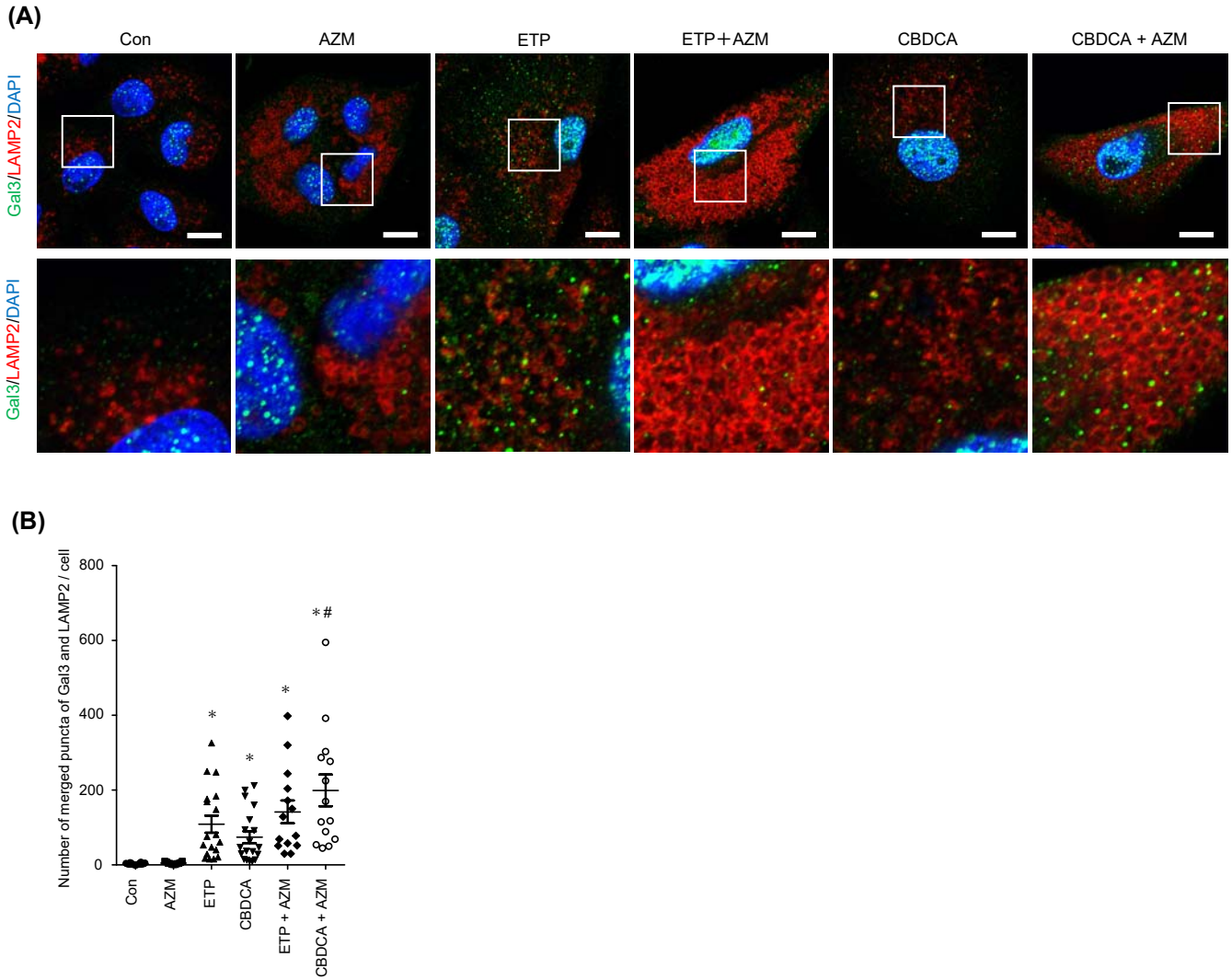

Figure S5. Coadministration of AZM with ETP or CBDCA enhanced LMP. (A) Colocalization of Gal3 and LAMP2 was assessed by immunofluorescent staining. A549 cells were treated with ETP (5 μM) or CBDCA (75 μM) ± AZM (25 μM) for 48 h, and then stained for Gal3 (Green), LAMP2 (Red), and nuclei (blue) with DAPI. Scale bar = 10 μm. The boxed area was enlarged in the bottom panels. (B) Colocalized signals of LAMP2 and Gal3 were calculated and summarized. n for Con, AZM, ETP, CDBCA, ETP + AZM, and CDBCA + AZM are 24, 11, 18, 19, 14, and 14, respectively, bar = mean ± S.E., *p<0.05 v.s. control, #p<0.05 v.s. CBDCA.

Table S1. Oligo DNA sequence used for knockout vector construction and real-time PCR.

Designation	Sequence	Reference
DNA sequence for pCas9 vector		
sgNega	5'-CACCGGTAGCGAACGTGTCCGGCGT-3'	[1]
sgTP53	5'-CACCGTCCATTGCTTGGGACGGCAA-3'	[2]
sgNOXA	5'-CACCGCGGCACCGGCGGAGATGCCTGGG-3'	CRISPR direct[3]
sgATG5	5'-CACCGAAGAGTAAGTTATTTGACGT-3'	[1]
DNA sequence for real-time PCR		
hLAMP1 Fwd	5'-CAGATGTGTTAGTGGCACCCA-3'	Harvard Primer Bank
hLAMP1 Rev	5'-TTGGAAAGGTACGCCTGGATG-3'	Harvard Primer Bank
hLAMP2 Fwd	5'-GCACAGTGAGCACAAATGAGT-3'	Harvard Primer Bank
hLAMP2 Rev	5'-CAGTGGTGTGTATGGTGGGT-3'	Harvard Primer Bank
hATG2A Fwd	5'-ACACGGAGATCCTGACCTTTC-3'	Harvard Primer Bank
hATG2A Rev	5'-GGTCGGCTCTTAGGCACAC-3'	Harvard Primer Bank
hATG2B Fwd	5'-AGCCAGTAAGAGGGTCAACAT-3'	Harvard Primer Bank
hATG2B Rev	5'-GTTCTGTGACGTTTCAGGTGG-3'	Harvard Primer Bank
hCTSD Fwd	5'-CACCACAAGTACAACAGCGAC-3'	Harvard Primer Bank
hCTSD Rev	5'-CCCGAGCCATAGTGGATGT-3'	Harvard Primer Bank
hMAP1LC3B Fwd	5'-AAGGCGCTTACAGCTCAATG-3'	Harvard Primer Bank
hMAP1LC3B Rev	5'-CTGGGAGGCATAGACCATGT-3'	Harvard Primer Bank
hHPRT Fwd	5'-GAAAAGGACCCCACGAAGTGT-3'	Harvard Primer Bank
hHPRT Rev	5'-AGTCAAGGGCATATCCTACAACA-3'	Harvard Primer Bank

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Reagents. DOX and L-Leucyl-L-Leucine methyl ester (LLOMe) were purchased from Cayman Chemical (Ann Arbor, MI, USA). ETP Lastet Injection was purchased from Nippon Kayaku (Tokyo, Japan). Carboplatin (CBDCA; carboplatin injection) was purchased from NICHI-IKO (Toyama, Japan). Gemcitabine hydrochloride (GEM) and azithromycin (AZM) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Z-VAD-FMK was purchased from Peptide Institute (Osaka, Japan). Puromycin dihydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell lines and culture conditions. Human lung adenocarcinoma-derived A549, H226, and A427 cells, and lung adenosquamous carcinoma-derived H596 cells were obtained from the American Type Culture Collection (ATCC). A549, H226, and H596 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. A427 cells were cultured in minimum essential medium (MEM) supplemented with 10% FBS and 1% penicillin/streptomycin. All cell lines were cultured in a humidified incubator containing 5% CO_2 and 95% air, at 37 °C. A549/mCherry-EGFP-LC3 cells were generated as previously described.[4] All cell line experiments were conducted within 10 passages after thawing. Mycoplasma contamination was tested routinely using the e-Myco^{TM} MYscoplasma PCR Detection kit ver.2.0 (iNtRON Biotechnology, Inc.).

Establishment of knockout cell lines. To establish the knockout cells, the CRISPR-Cas9 system was used. A549 cells were transfected with pSpCas9 (BB)-2A-Puro (PX459) V2.0 plasmid vector (a gift from Dr. Feng Zhang; plasmid cat. no. 48139; Addgene) [5]

containing a guide sequence for NOXA and TP53. Thereafter, cells were selected with puromycin for 2 d, and grown without puromycin to select single colonies. ATG5-KO A549 cells were established as previously described. [6]

Assessment of cell death. Cell death was assessed by staining with propidium iodide (PI) (Wako Pure Chemicals Corporation) and counting the number of red fluorescent signals using the IncuCyte ZOOM (Essen Bioscience, Ann Arbor, MI, USA) automated live cell imaging system. The cells were treated with DOX, ETP, CBDCA, or GEM with or without AZM (10 or 25 μM) in the presence of PI (2.5 μg/mL) for 72 h in 96-well plates in quadruplicate. [7]

Flow cytometry. To assess apoptosis, cells were stained with Annexin V and PI using the Annexin V-FITC Apoptosis Detection kit (Nacalai Tesque, Kyoto, Japan), according to the manufacturer's instructions, and subjected to flow cytometry using the Attune® Acoustic Focusing Cytometer (Life Technologies, Carlsbad, CA, USA).

Morphological assessment. Cells were spread on glass slides using a Cytospin 4 Centrifuge (Thermo Fisher Scientific) to prepare glass slides, stained with May-Grünwald-Giemsa, and examined under a digital microscope (BZ-X800; Keyence Co., Osaka, Japan).

Western blotting. Cells were lysed with RIPA lysis buffer (Nacalai Tesque, Kyoto, Japan) supplemented with a protease and phosphatase inhibitor cocktail (Nacalai Tesque). Equal amounts of proteins were loaded onto the gels, separated by sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto Immobilon-P membranes (Millipore Corp., Bedford, MA, USA). These membranes were probed with primary antibodies, such as anti-p53 antibody (Ab) (sc-126, 1:1,000), anti-p62 Ab (sc-28359, 1:1,000), anti-β-actin Ab (sc-47778, 1:1,000), anti-LAMP1 Ab (sc-20011, 1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-PUMA Ab (GTX109675, 1:1,000) (Genetex, Irvine, CA, USA), anti-NOXA Ab (ab13654, 1:1,000) (Abcam, Cambridge, MA, USA), anti-microtubule-associated protein 1 light chain 3 (LC3) B Ab (NB600-1384, 1:4000; Novus Biologicals, Inc., Littleton, CO, USA), anti-PARP Ab (#9542S, 1:1000), anti-Caspase-3 Ab (#9665S, 1:1000), anti-Caspase-7 Ab (#12827S, 1:1000), anti-Caspase-8 Ab (#9746S, 1:1000), anti-Phospho-p53 Ab (#9286T, 1:1000), anti-TFEB Ab (#4240S, 1:1000), anti-ATG5 Ab (#12994S, 1:1000), anti-MDM2 Ab (#86934S, 1:1000) and anti-p21 Ab (#2947S, 1:1000) (Cell Signaling Technology, Danvers, MA, USA)

Immunofluorescence staining and confocal microscopy. Cells were seeded on 13-mm glass coverslips in a 12-well culture plate, and then treated with AZM, DOX, and DOX in combination with AZM for 24 or 48 h. Coverslips were washed twice with phosphate-buffered saline (PBS), and then fixed for 10 min with ice-cold methanol at –20 °C. After washing twice with PBS and once with Tris-buffered saline with Tween 20 (TBST), coverslips were exposed to TBST containing 10 % normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature to block nonspecific binding. The cells were incubated with primary antibodies such as anti-Galectin 3 (Gal3) Ab (#87985S, 1:200), anti-TFEB Ab (#4240S, 1:100) (Cell Signaling Technology), and anti-LAMP2 Ab (sc-18822, 1:100) (Santa Cruz Biotechnology) at 4 °C overnight. The

coverslips were washed three times with TBST at room temperature, and subsequently incubated with Alexa Fluor® 488 goat anti-rabbit IgG (H+L) or Alexa Fluor® 555 goat anti-mouse IgG (H+L) secondary antibody (1:250; Thermo Fisher Scientific) for 1 h at room temperature. The coverslips were washed with TBST, and mounted in ProLong® Diamond Antifade Mountant (Thermo Fisher Scientific). Nuclei were stained with DAPI (Sigma #D9542, 1:1000), and the cells were imaged using a confocal laser scanning fluorescence microscope (LSM 700, Carl Zeiss, Germany). Since DOX possesses autofluorescence, experiments without antibodies were performed as negative control, to confirm that the detected fluorescence signal was not derived from DOX.

Quantification of autophagic puncta. To measure the autophagic structure in A549 cells expressing mCherry-EGFP-LC3, cells were observed using a confocal laser scanning fluorescence microscope (LSM 700) without fixation. The obtained images were analyzed using Imaris (Carl Zeiss), as previously reported.[8] Number of $mCherry^+$ ($mCherry^+/EGFP^+$ and $mCherry^+/EGFP^-$) puncta was measured and standardized with the number of cells in each image.

Measurement of lysosomal membrane permeability (LMP). The LMP was assessed by fluorescence immunocytochemistry by double staining with anti-Gal3 Ab and anti-LAPM2 Ab, as previously described.[9] As a positive control for LMP, cells were treated with 1 mM LLOMe for 4 hr. Colocalization of Gal3 and LAMP2 was analyzed using Image J.[10] Alternatively, we assessed the cytosolic and total enzymatic activities of cathepsin and β-N-acetyl glucosaminidase (NAG), according to previous reports, with several modifications. [11, 12] Cells were seeded in a 12-well plate (5×10^4 cells/well) and

then treated with DOX ± AZM for 24 or 48 h. After washing with PBS, cells were solubilized with 200 μL of 15 μg/mL (for cytosol) or 200 μg/mL (for total) digitonin in DE buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, pH 7.5, with 0.5 mM Pefabloc) on a rocking table for 15 min on ice. The optimal digitonin concentrations were determined according to a previous report.[11] The lysates were transferred to new tubes, and used for the following cathepsin or NAG activity assay, and for measuring protein concentration with a BCA kit (Thermo). Cathepsin activity was measured in cathepsin reaction buffer (50 mM sodium acetate, 4 mM EDTA, pH 6.0) with 0.5 mM Pefabloc, 8 mM DTT, and 50 μM z-FR-AMC (Peptide Institute). Ten microliters of lysate, 40 μL of DE buffer, and 50 μL of the reaction buffer were mixed, and the fluorescent intensity (Ex 400/ Em 489 nm) was measured every minute for 1 h at 30 °C. For the NAG activity assay, 5 μL of cell lysate was mixed with 100 μL of NAG reaction buffer (0.2 M sodium citrate buffer, 300 μg/mL 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (Sigma-Aldrich), pH4.5) and incubated at 37 °C for 30 min. Then, 100 μL of Tris buffer (0.5 M Tris-HCl, pH 10.4) was added, and fluorescence (Ex 360/ Em 440 nm) was measured. Each enzymatic activity was standardized according to protein concentration, and the cytosol/total cathepsin or NAG activity was calculated. All experiments were performed in six replicates for each condition, and statistical analysis was performed by excluding the highest and lowest values at each point.

Real-time PCR. To determine the gene expression level, total RNA was extracted from A549 cells using a NucleoSpin RNA kit (Takara Bio, Inc., Otsu, Japan), and cDNA was synthesized using PrimeScript RT Master Mix (Takara Bio, Inc.). Gene expression was

determined by qPCR using TB Green Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio, Inc.). Changes in target mRNA expression were calculated using the $\Delta(\Delta C_T)$ method. HPRT was used as an internal control. Primer sequences were obtained from Harvard Primer Bank, [13-15] and are listed in **Table S1**.

Statistical analysis. All quantitative data are expressed as mean ± standard deviation (SD). Statistical analyses of cell death assay and qPCR were performed with two-way ANOVA, followed by Bonferroni's multiple comparison test. For all other assays, one-way ANOVA, followed by Bonferroni's multiple comparison test was used. Statistical significance was set at $p < 0.05$. All analyses were performed using the GraphPad Prism 5 software (GraphPad Software, Inc.).

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