Supplemental Information

Maintaining proton homeostasis is an essential role of glucose metabolism in cell survival

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Supplemental figures and legends

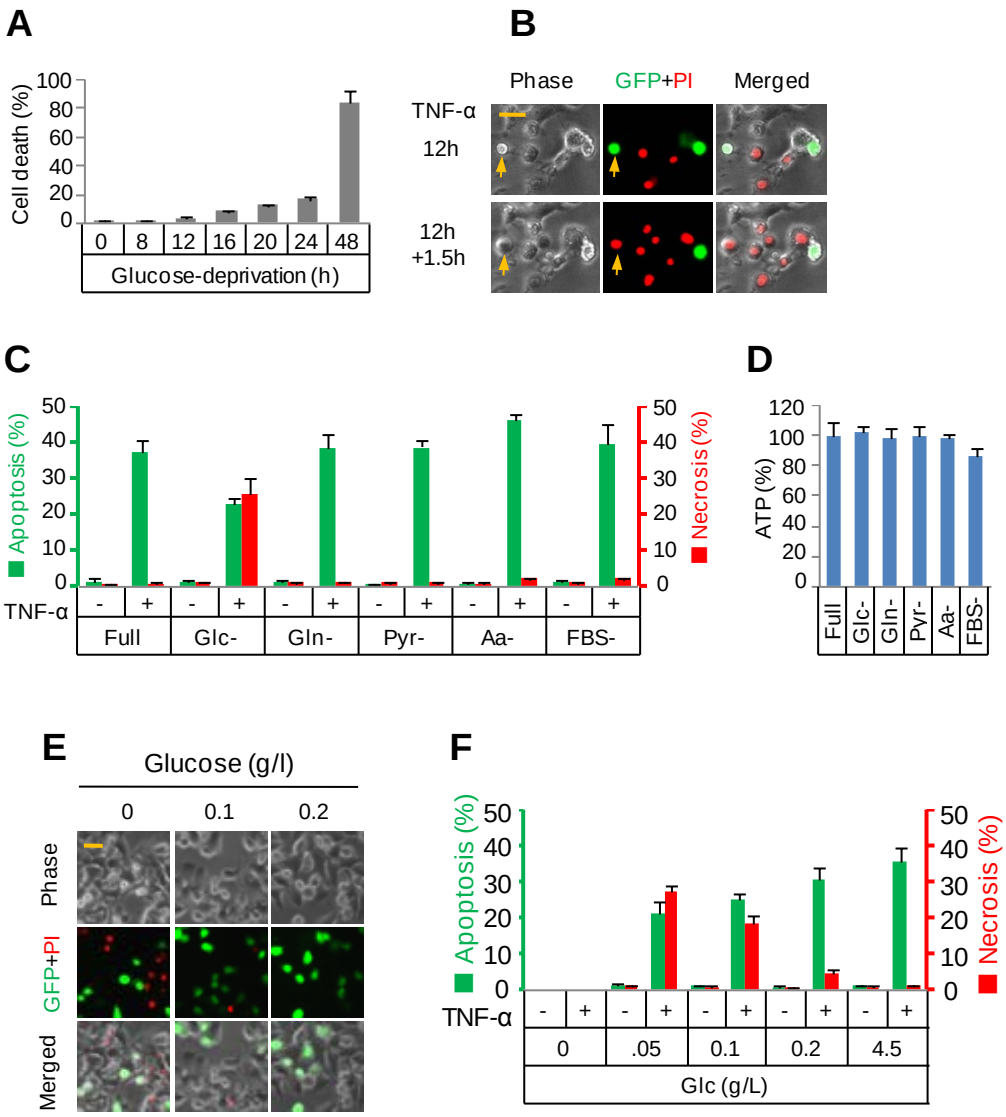

Figure S1, Related to Figure 1. TNF-α-induced necrosis depends on glucose deprivation. (A) The fraction of dead MCF-7/GC3AI cells in the absence of glucose, including apoptotic and necrotic cells. Glucose deprivation mainly induces necrosis. Error bars indicate ± SD (n=3). (B) Fluorescent images of MCF-7/GC3AI cells after treatment with 50 ng/ml of TNF-α for the indicated time in the absence of glucose. Arrow indicates the switch of apoptosis to necrosis and morphological change. (C) The fraction of apoptotic and necrotic MCF-7/GC3AI cells after treatment with 50 ng/ml of TNF-α for 6h in the absence of glucose, glutamine, pyruvate, amino acids or serum. Error bars indicate ± SD (n=3). (D) ATP levels of MCF-7/GC3AI cells after culture in the medium without glucose, glutamine, pyruvate, amino acids or serum for 3h. Error bars indicate ± SD (n=4). (E and F) The apoptotic and necrotic death in MCF-7/GC3AI cells after treatment with 50 ng/ml of TNF-α for 6h in the presence of different concentrations of glucose. Error bars indicate ± SD (n=3). Scale bar in all panels, 20 μm.

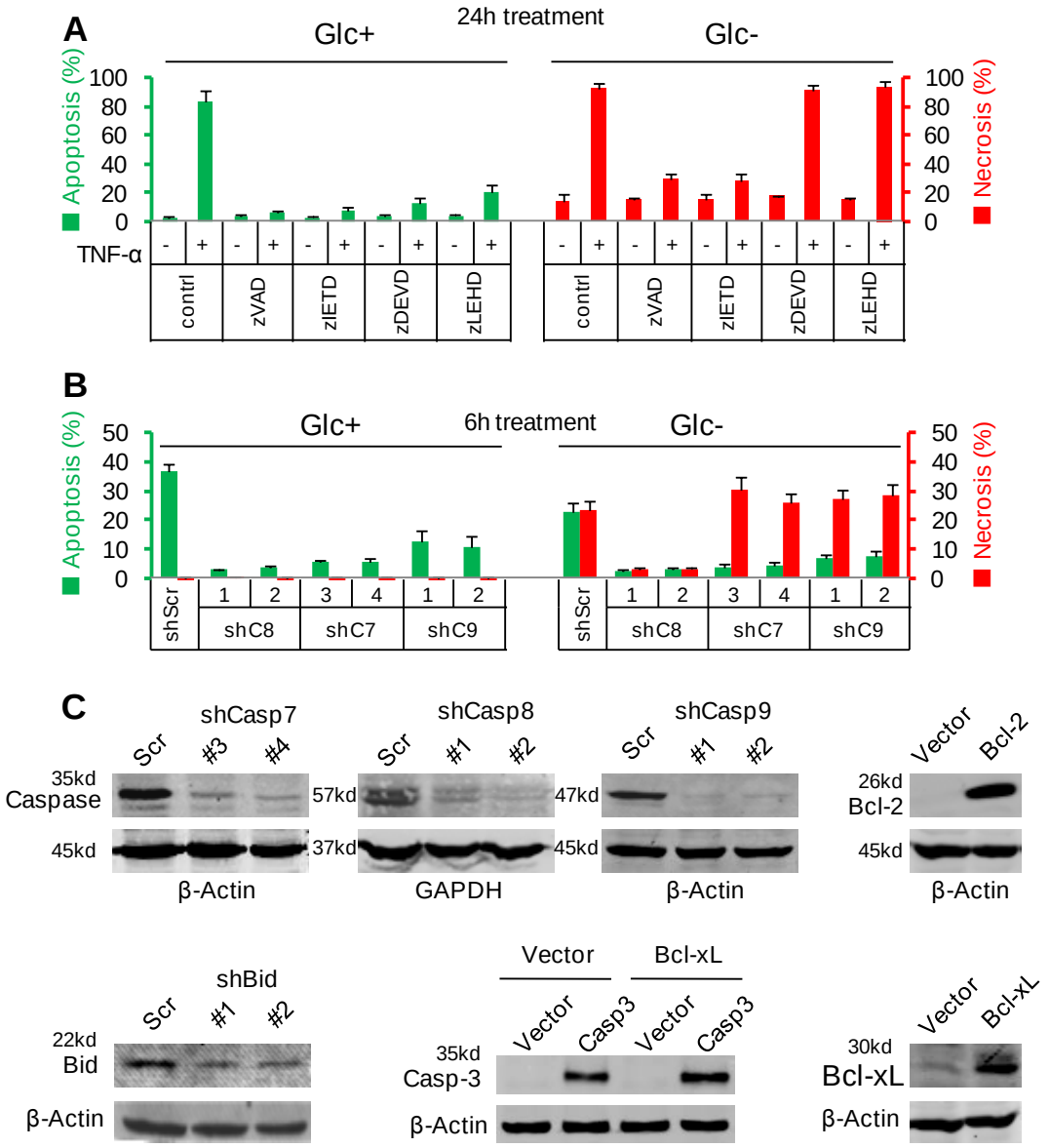

Figure S2, Related to Figure 2. Caspase-8 plays a critical role in TNF-α-induced necrosis in the absence of glucose. (A) The fraction of apoptotic or necrotic MCF-7/GC3AI cells after treatment without or with 50 ng/ml of TNF-α for 24h in the presence or absence of glucose. Cells were pre-treated with 50 μM of z-VAD, z-IETD, z-DEVD, or z-LEHD. Error bars indicate ± SD (n=3). (B) The fraction of apoptotic and necrotic MCF-7/GC3AI cells with knockdown of caspase-7, caspase-8 or caspase-9 after treatment without or with 50 ng/ml of TNF-α for 6h in the presence or absence of glucose. Error bars indicate ± SD (n=3). (C) Western blots of knockdown of caspase-7/8/9 and Bid, and over-expression of Bcl-xL, Bcl-2 and caspase-3 in MCF-7/GC3AI cells.

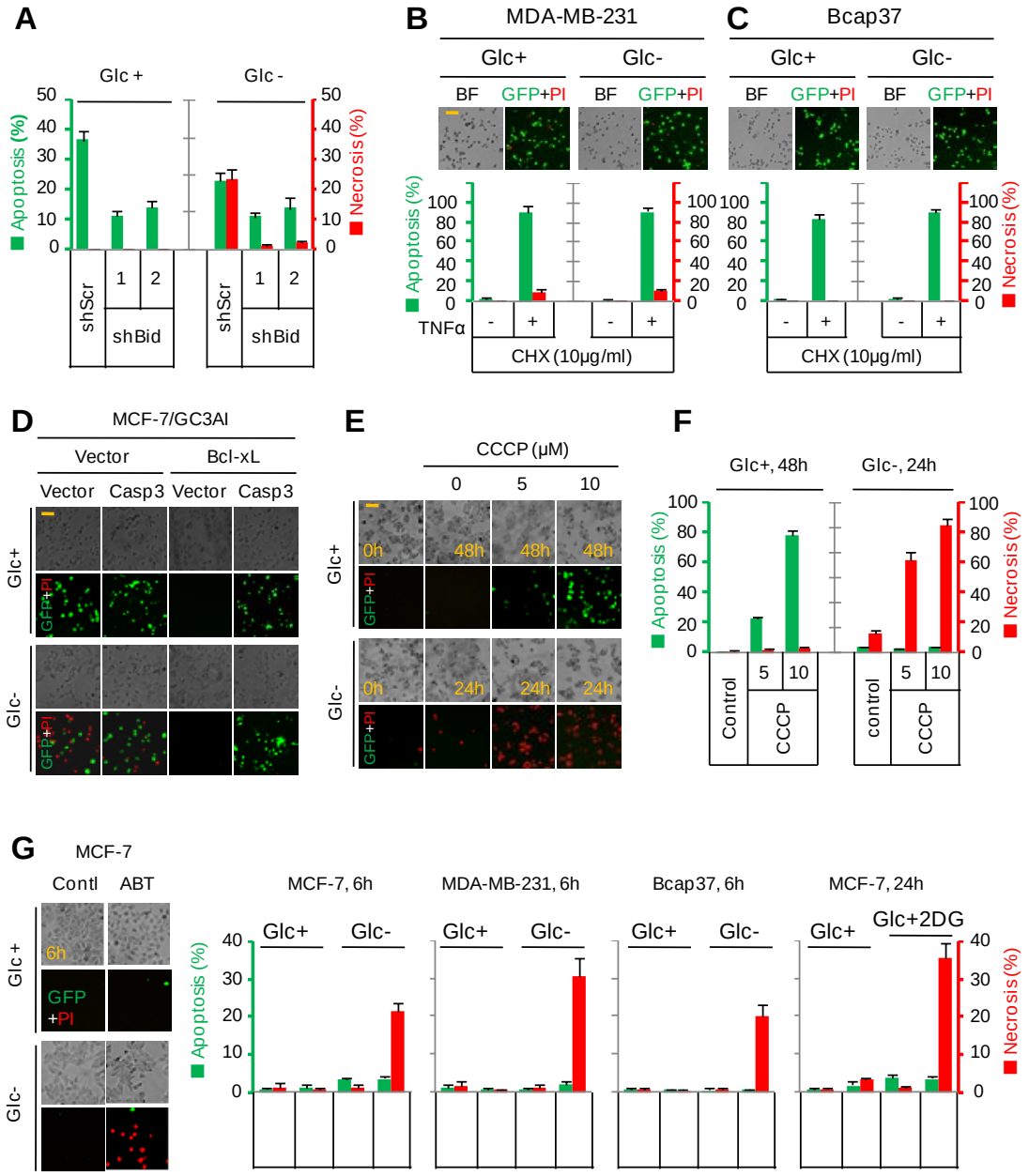

Figure S3, Related to Figure 2. Mitochondrial damage is required for TNF-α-induced necrosis in the absence of glucose. (A) The fraction of apoptotic and necrotic MCF-7/GC3AI cells without or with Bid knockdown. Cells were treated with 50 ng/ml of TNF-α for 6h in the presence or absence of glucose. (B) The fraction of apoptotic and necrotic MDA-MB-231 cells after treatment with 50 ng/ml of TNF-α plus 10 μg/ml of cycloheximide (CHX) for 6h in the presence or absence of glucose. (C) The fraction of apoptotic and necrotic Bcap37 cells after treatment with 50 ng/ml of TNF-α plus 10 μg/ml of CHX for 7h in the presence or absence of glucose. (D) Fluorescent images of MCF-7/GC3AI cells stably expressing Bcl-xL and/or caspase-3. Cells were treated with 50 ng/ml of TNF-α for 6h in the presence or absence of glucose. (E) Fluorescent images of MCF-7/GC3AI cells after treatment with CCCP for 48h in the presence of glucose or 24h in the absence of glucose. (F) The fraction of apoptotic and necrotic MCF-7/GC3AI cells treated as described in (E). (G) The fraction of apoptotic and

necrotic cells after treatment with 5 μM of ABT-737 for 6h in the presence or absence of glucose or with 20 mM of 2DG (2-Deoxy-D-glucose) for 24h. Error bars in all panels indicate ± SD (n=3). Scale bar in all panels, 40 μm.

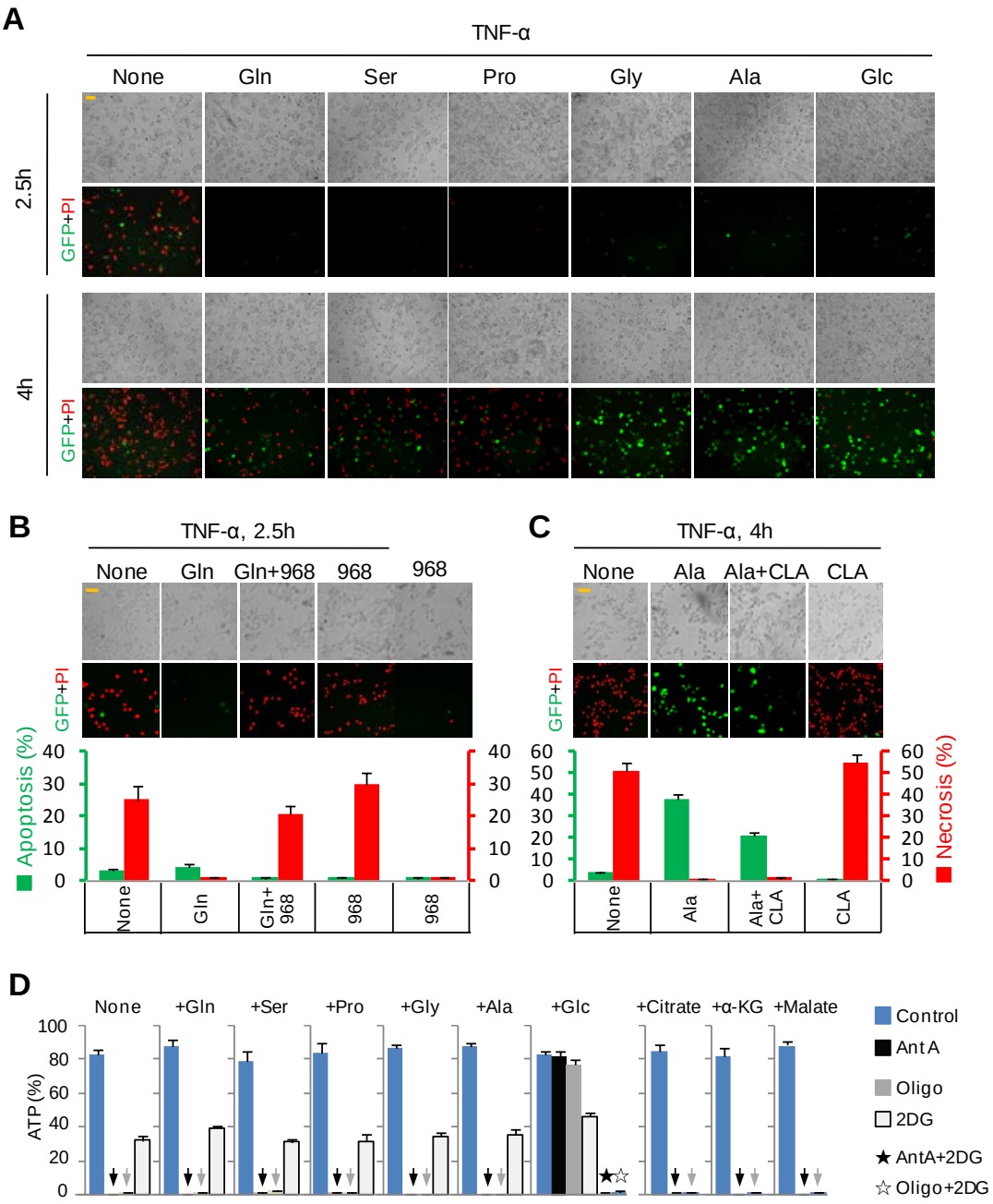

Figure S4, Related to Figure 3. Metabolism of some amino acids switches TNF-α-induced necrosis to apoptosis. (A) Fluorescent images of MCF-7/GC3AI cells in the nutrients-defined medium after treatment with 50 ng/ml of TNF-α for 2.5h or 4h. The nutrient-deficient medium was supplied with nothing (none), 2 mM of glutamine (Gln), 2 mM of serine (Ser), 2 mM of proline (Pro), 2 mM of glycine (Gly), 2 mM of alanine (Ala), or 2 mM of glucose (Glc). (B) Effect of compound 968 on cell death in MCF-7/GC3AI cells in the nutrient-deficient medium with the addition of 2 mM of glutamine. Cells were pre-treated with 100 μM of compound 968, an inhibitor of glutaminase, before they were treated with 50 ng/ml of TNF-α for 2.5h. Error bars indicate ± SD (n=3). (C) Effect of β-chloro-L-alanine on cell death in MCF-7/GC3AI cells in the nutrient-deficient medium with the addition of 2 mM of alanine. Cells were

pre-treated with 10 mM of β-chloro-L-alanine (CLA), an inhibitor of alanine aminotransferase, before they were treated with 50 ng/ml of TNF-α for 4h. Error bars indicate ± SD (n=3). (D) ATP levels of MCF-7/GC3AI cells in the nutrient-defined medium after they were treated with antimycin A (1 μM), oligomycin A (1 μM), 20 mM of 2DG or in combinations for 1h. The nutrient-deficient medium was supplied with nothing (none), 2 mM of glutamine (Gln), 2 mM of serine (Ser), 2 mM of proline (Pro), 2 mM of glycine (Gly), 2 mM of alanine (Ala), 2 mM of glucose (Glc), 50 mM of citrate, 50 mM of α-ketoglutarate (α-KG) or 50 mM of malate. Error bars indicate ± SD (n=4). Scale bar in all panels, 40 μm.

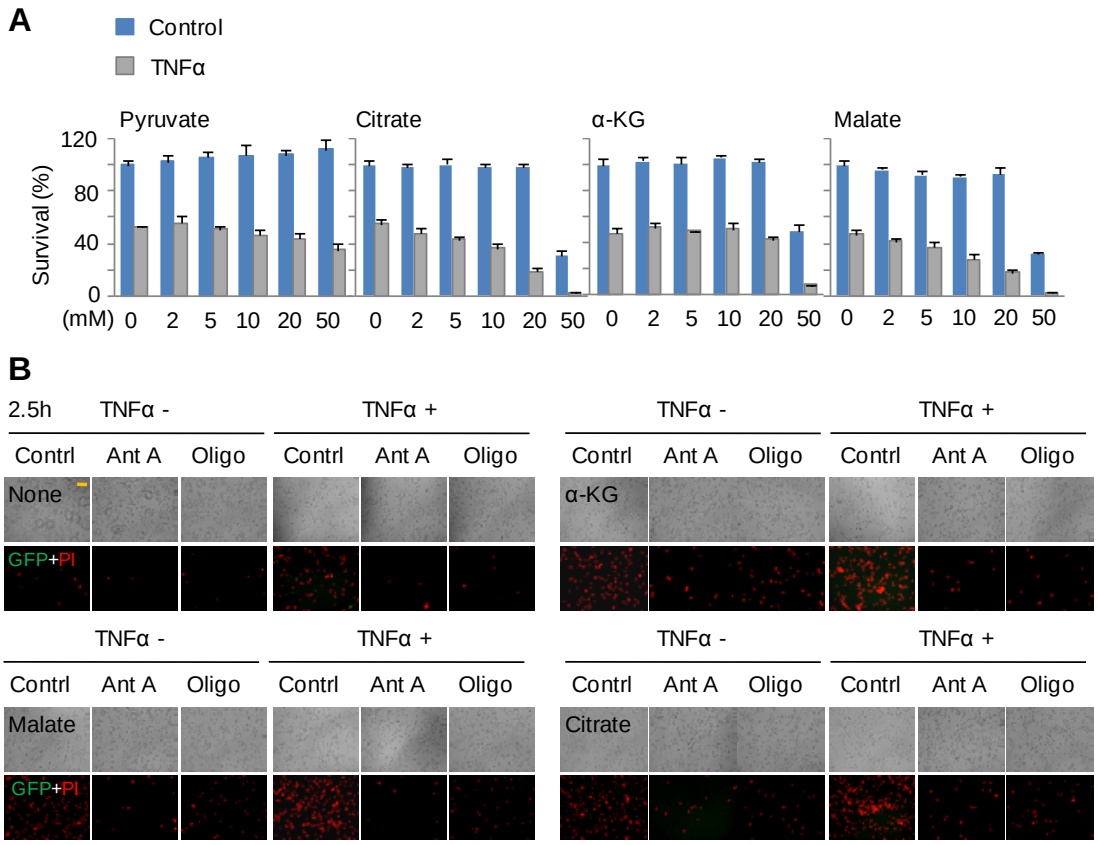

Figure S5, Related Figure 3. Metabolism of metabolites in tricarboxylic acid cycle exacerbates TNF-α-induced necrosis. (A) Survival of MCF-7/GC3AI cells in the nutrients-defined medium after treatment without or with 50 ng/ml of TNF-α for 4h. The nutrient-deficient medium was supplied with metabolites in tricarboxylic acid cycle as described. Error bars indicate ± SD (n=4). (B) Fluorescent images of MCF-7/GC3AI cells in the nutrients-defined medium after treatment without or with 50 ng/ml of TNF-α for 2.5h. The nutrient-deficient medium was supplied without (none) or with 50 mM of metabolites in tricarboxylic acid cycle as described, and cells were pre-treated with antimycin A (1 μM) or oligomycin A (1 μM) for 1h. Scale bar, 40 μm.

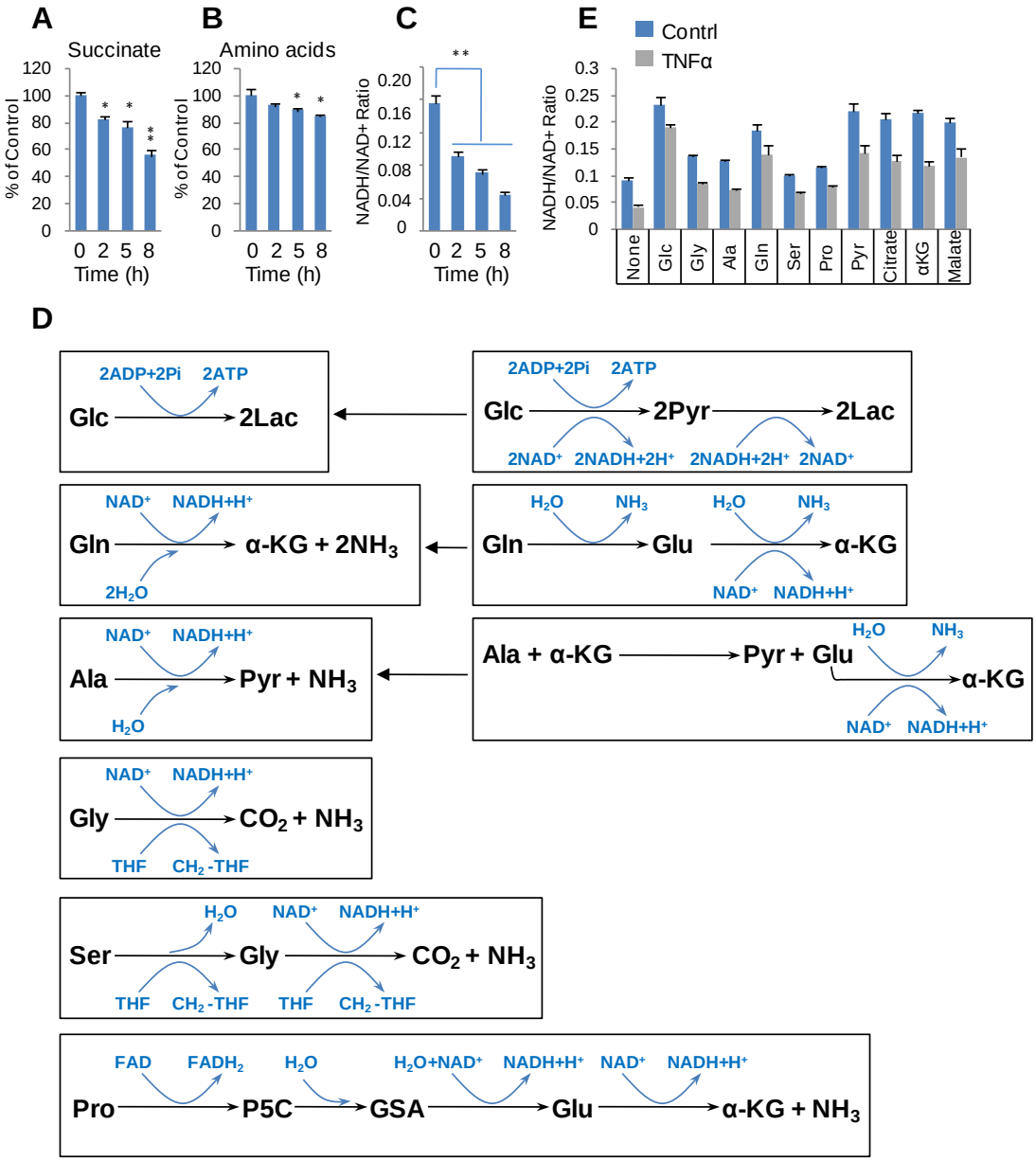

Figure S6, Related Figure 3. Consumption of intracellular metabolites and metabolism of NADH. (A-C) The intracellular succinate level, total amino acid level and NADH/NAD$^+$ ratio of MCF-7/GC3AI cells cultured in the nutrient-deficient medium for different times as indicated. Succinate was one of the most active components of the tricarboxylic acid cycle. Measurement of succinate level represents the level of metabolites in the tricarboxylic acid cycle. Error bars indicate ± SD (n=3). * P<0.05. * * P<0.01 (t-test). (D) Catabolic pathways of glucose and amino acids. (E) The NADH/NAD$^+$ ratio values of MCF-7/GC3AI cells cultured in the nutrient-defined medium after treatment without or with 50 ng/ml of TNF-α for 2h. The nutrient-deficient medium was supplied without (none) or with glucose (2 mM), glycine (2 mM), alanine (2 mM), glutamine (2 mM), serine (2 mM), proline (2 mM), pyruvate (2 mM), citrate (50 mM), α-ketoglutarate (50 mM), or malate (50 mM). Error bars indicate ± SD (n=3).

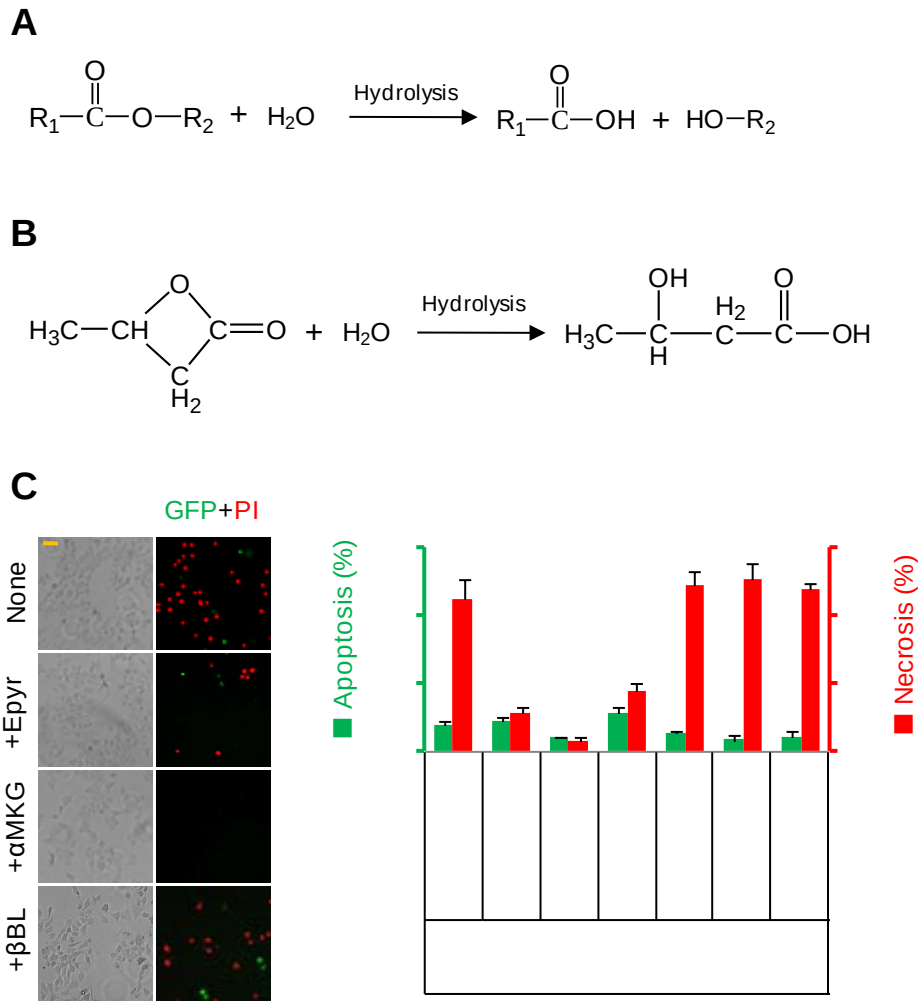

Figure S7, Related Figure 4. Catabolism of esters protects TNF-α-induced necrosis. (A) Hydrolysis of ester to acid and alcohol produces protons. (B) Hydrolysis of β-butyrolactone to β-hydroxybutyric acid produces protons. (C) The fraction of apoptotic and necrotic MCF-7/GC3AI cells after treatment with 50 ng/ml of TNF-α for 2.5h, and cells were treated in the nutrient-deficient medium supplied without (none) or with 5mM of ethyl pyruvate (EPyr), 5 mM of dimethyl α-ketoglutarate (αMKG), 5 mM of β-butyrolactone (βBL), 5mM of pyruvate plus 5mM of ethanol, 5 mM of α-ketoglutarate plus 10 mM of methanol and 5 mM of β-hydroxybutyric acid (βHB). The pH values of all the media were adjusted to pH 7.4. Error bars indicate ± SD (n=3). Scale bar, 40 μm.

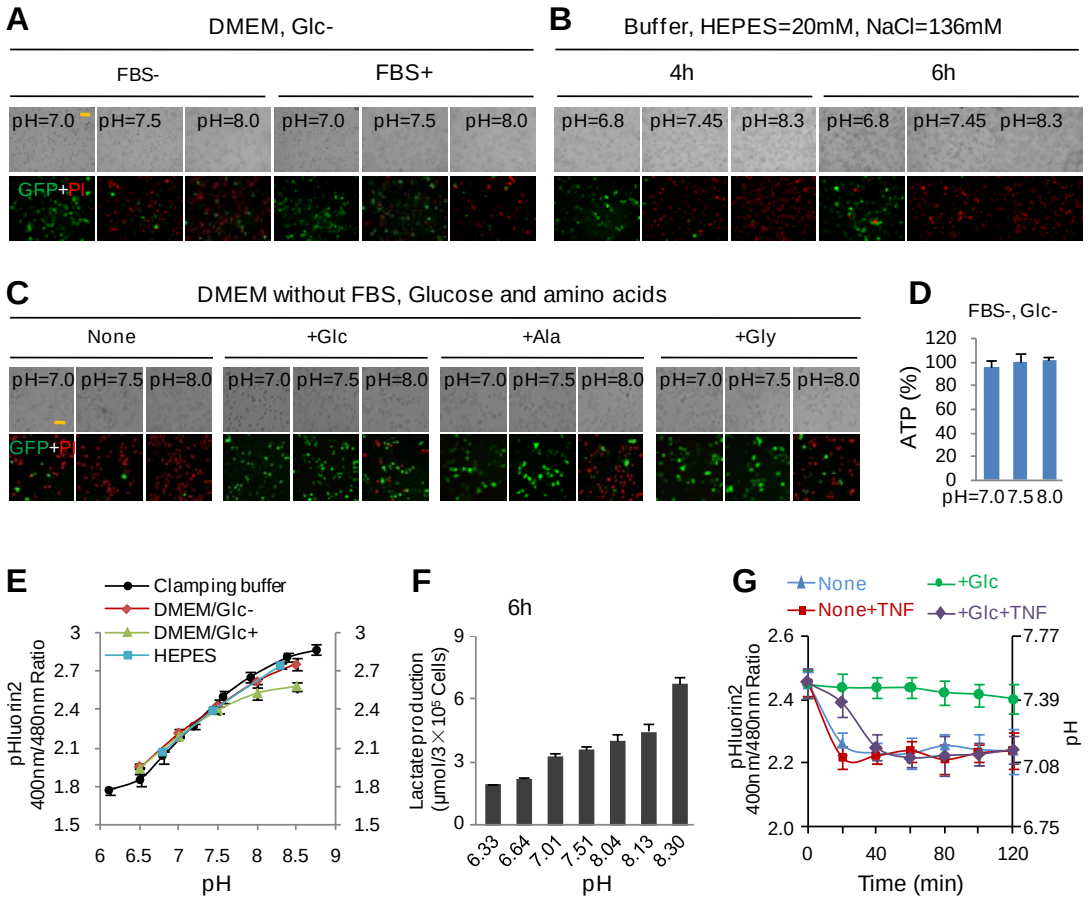

Figure S8, Related Figure 4. Effect of the extracellular pH on TNF-α-induced cell death and effect of TNF-α on the intracellular pH. (A) Fluorescent images of MCF-7/GC3AI cells after treatment with 50 ng/ml of TNF-α for 6h in the glucose-free medium with different pH values. (B) Fluorescent images of MCF-7/GC3AI cells after treatment with 50 ng/ml of TNF-α for 4h or 6h in the buffer solutions with different pH values. (C) Fluorescent images of MCF-7/GC3AI cells after treatment with 50 ng/ml of TNF-α for 4h in the nutrient-defined medium with different pH values. The nutrient-deficient medium was supplied with vehicle (none), glucose (2 mM), alanine (2 mM) or glycine (2 mM). The pH values of the media were adjusted as indicated. Scale bar in all panels, 40 μm. (D) ATP levels of MCF-7/GC3AI cells after they were incubated with the glucose-free and serum-free medium with different pH values for 6h. Error bars indicate ± SD (n=4). (E) The relationship between the 400nm/480nm ratio values of pHluorin2 fluorescence intensity and pH values of media or buffer solutions. MCF-7/pHluorin2 cells were incubated with the media or buffers for 4h. The 400nm/480nm ratio values of intracellular pHluorin2 fluorescence intensity were calibrated in the pH clamping buffer. Error bars indicate ± SD (n=3, 180 cells in total). (F) Lactate production of MCF-7 cells after they were incubated with the complete medium with different pH values for 6h. Error bars indicate ± SD (n=4). (G) The intracellular pH of single MCF-7 cells after treatment without or with 50 ng/ml of TNF-α in the nutrient-deficient medium supplied without or with 2mM of glucose. The mean values of data from 20 cells are shown here. Error bars indicate ± SD.

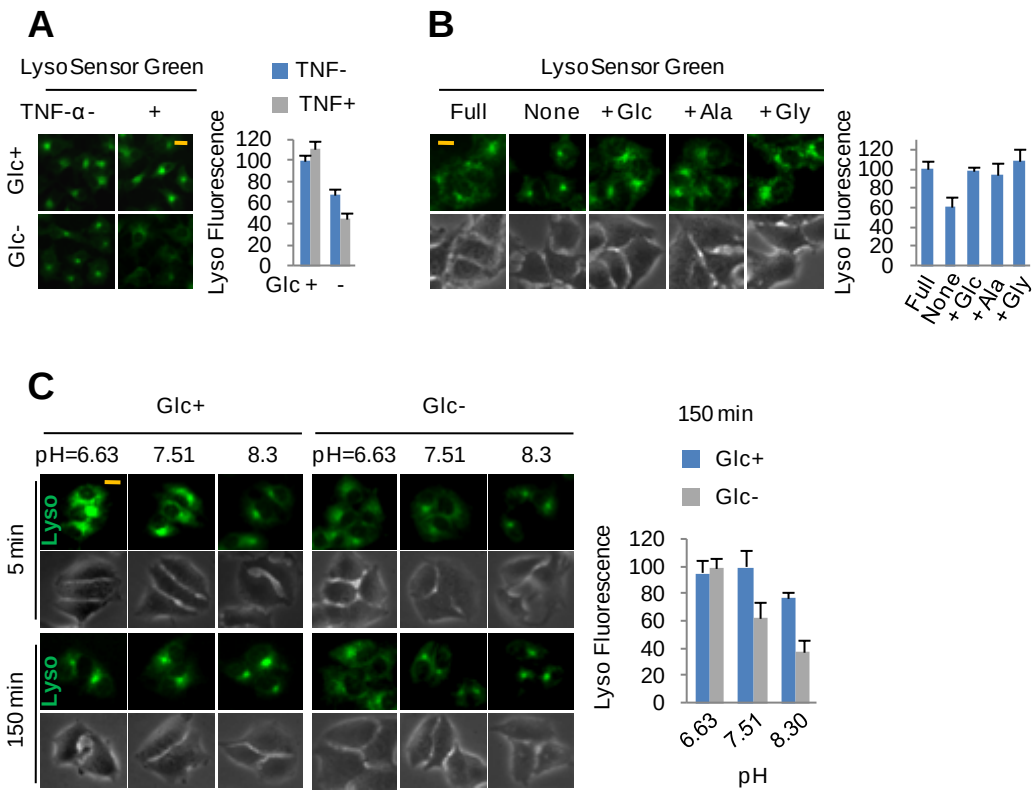

Figure S9, Related Figure 5. lysosomal alkalinization is associated with TNF-α-induced necrosis. (A) Fluorescent images of MCF-7/wt cells pre-stained with LysoSensor Green after treatments without or with 50 ng/ml of TNF-α for 5h in the presence or absence of glucose. The histogram shows the quantified fluorescence intensity normalized by the number of cells. Error bars indicate ± SD (n=3, 180 cells in total). (B) Fluorescent images of MCF-7/wt cells pre-stained with LysoSensor Green in the nutrient-deficient medium supplied without or with 2 mM of glucose, 2 mM of alanine or 2 mM of glycine. Images were captured after cells are cultured in the media for 2h. The complete medium (full) was used as the control. The histogram shows the quantified fluorescence intensity normalized by the number of cells. Error bars indicate ± SD (n=3, 180 cells in total). (C) Fluorescent images of MCF-7/wt cells pre-stained with LysoSensor Green in the presence or absence of glucose. The pH of the medium was adjusted to the indicated value. Images were captured after cells are cultured in the media for 50 min or 150 min. The histogram shows the quantified fluorescence intensity normalized by the number of cells. Error bars indicate ± SD (n=3, 180 cells in total). Scale bar in all panels, 10 μm.

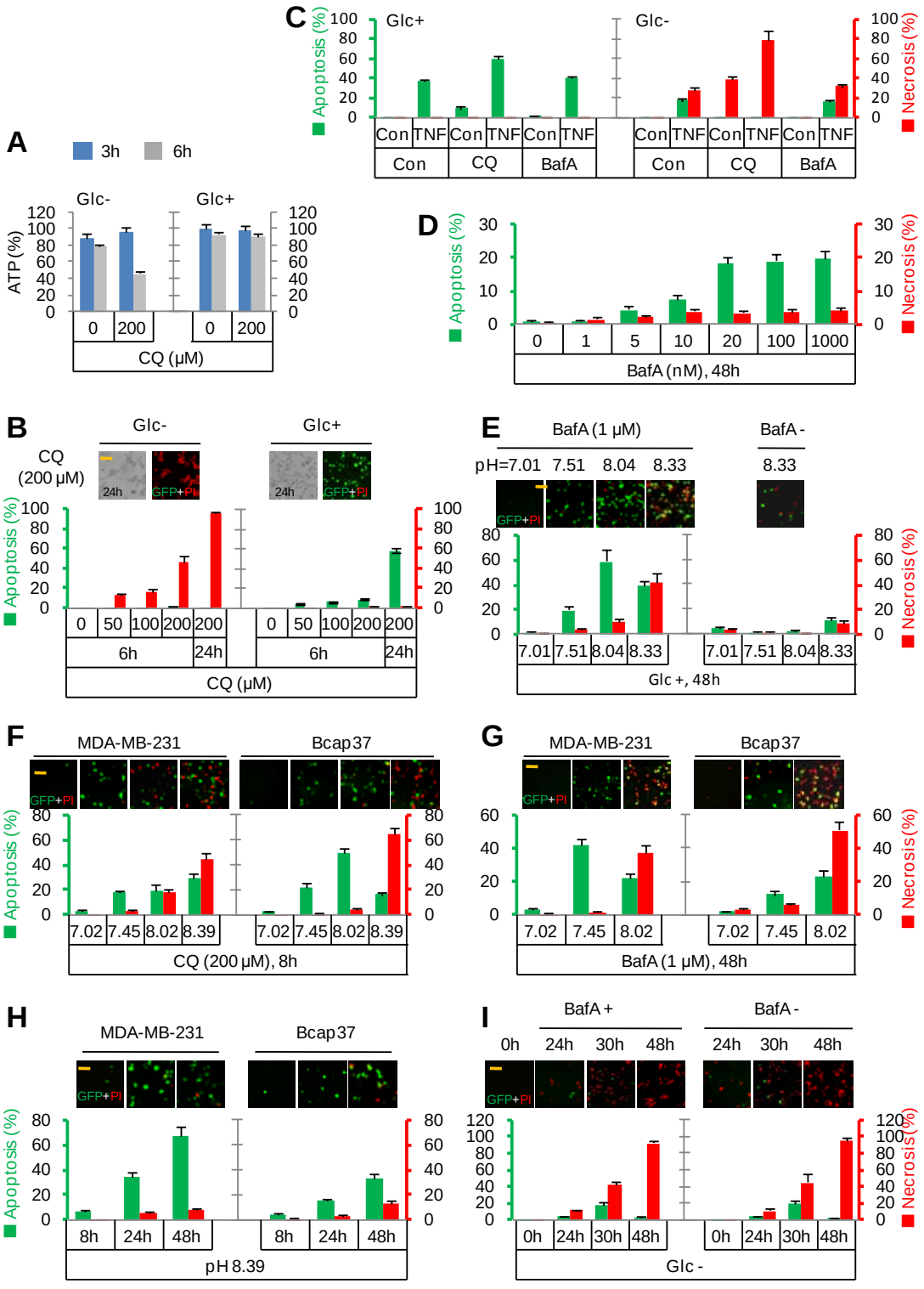

Figure S10, Related Figure 5. Effects of chloroquine and bafilomycin A on cell death. (A) ATP levels of MCF-7/GC3AI cells after treatment with or without chloroquine for the indicated time in the presence or absence of glucose. In the absence of glucose, the residual ATP content (about 50%) was consistent with the fraction of survived cells (B) at 6h. Error bars indicate ± SD (n=4). (B) The apoptotic and necrotic death in MCF-7/GC3AI cells after treatment with different concentrations of chloroquine for the

indicated time in the presence or absence of glucose. Error bars indicate ± SD (n=3). (C) The apoptotic and necrotic death in MCF-7/GC3AI cells after treatment with 50 ng/ml of TNF-α alone or in combination with balifomycin (1 μM) A or chloroquine (200 μM) for 6h in the complete or glucose-free medium. Error bars indicate ± SD (n=3). (D) The apoptotic and necrotic death in MCF-7/GC3AI cells after treatment with different concentrations of balifomycin A for 48h in the complete medium. Error bars indicate ± SD (n=3). (E) The apoptotic and necrotic death in MCF-7/GC3AI cells after treatment with 1 μM of balifomycin A for 48h in the complete medium with different pH values. Error bars indicate ± SD (n=3). (F) The apoptotic and necrotic death in MDA-MB-231/GC3AI and Bcap37/GC3AI cells after treatment with 200 μM of chloroquine for 8h in the complete medium with different pH values. Error bars indicate ± SD (n=3). (G) The apoptotic and necrotic death in MDA-MB-231/GC3AI and Bcap37/GC3AI cells after treatment with 1 μM of bafilomycin A for 8h in the complete medium with different pH values. Error bars indicate ± SD (n=3). (H) The apoptotic and necrotic death in MDA-MB-231/GC3AI and Bcap37/GC3AI cells cultured in the complete medium with pH 8.39 for different times as indicated. Error bars indicate ± SD (n=3). (I) The apoptotic and necrotic death in MCF-7/GC3AI cells after treatment without or with 1 μM of bafilomycin A for the indicated time in the absence of glucose. Error bars indicate ± SD (n=3). Scale bar in all panels, 40 μm.

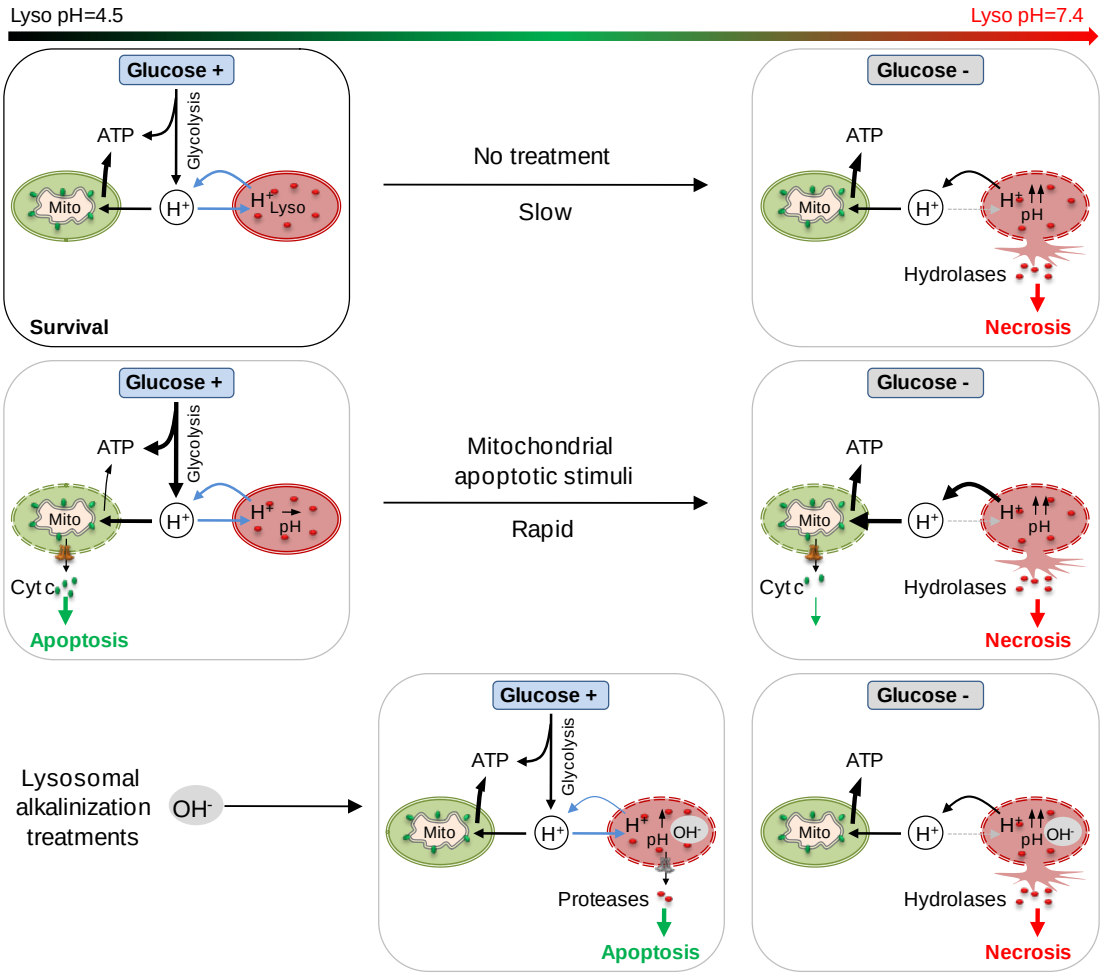

Figure S11, Related Figure 6. A model for cell death triggered by metabolism-driven proton homeostasis. In the absence of glucose, mitochondria consume protons to generate ATP and finally induce severe lysosomal alkalinization and necrosis. This process is promoted by mitochondrial apoptotic stimuli, such as TNF-α and CCCP. Alkaline treatments or lysosomotropics directly deacidify lysosomes, and this effect can be attenuated by glycolysis. See Discussion for details.

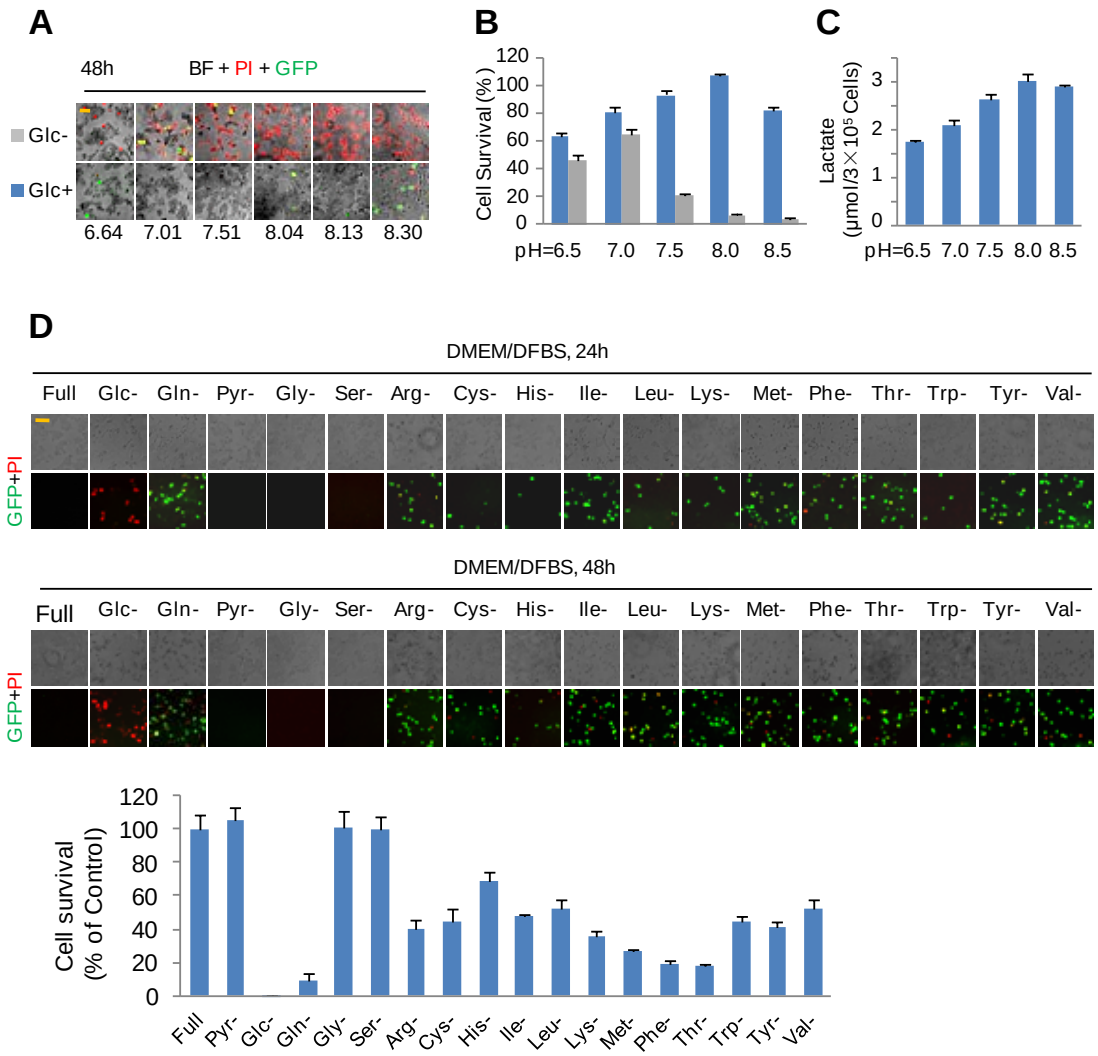

Figure S12, Related Figure 6. Glucose deprivation induces necrosis in MCF-7/GC3AI. (A) Images of MCF-7/GC3AI cells cultured in the complete medium or glucose-free medium with different pH values for 48h. (B) Survival of MCF-7/GC3AI cells cultured in the complete medium or glucose-free medium with different pH values in the CO_2-free incubator for 48h. (C) Lactate production of MCF-7 cells was measured after they were incubated with the complete medium with different pH values in the CO_2-free incubator for 6h. (D) The apoptotic and necrotic death in MCF-7/GC3AI cells in the complete medium with deprivation of glucose, pyruvate or one of amino acids as indicated. The medium was supplemented with dialyzed FBS to avoid the interference of metabolites in FBS. The histogram shows the survival of MCF-7/GC3AI cells after 72h of treatment. Error bars in all panels indicate ± SD (n=4). Scale bar in all panels, 40 μm.

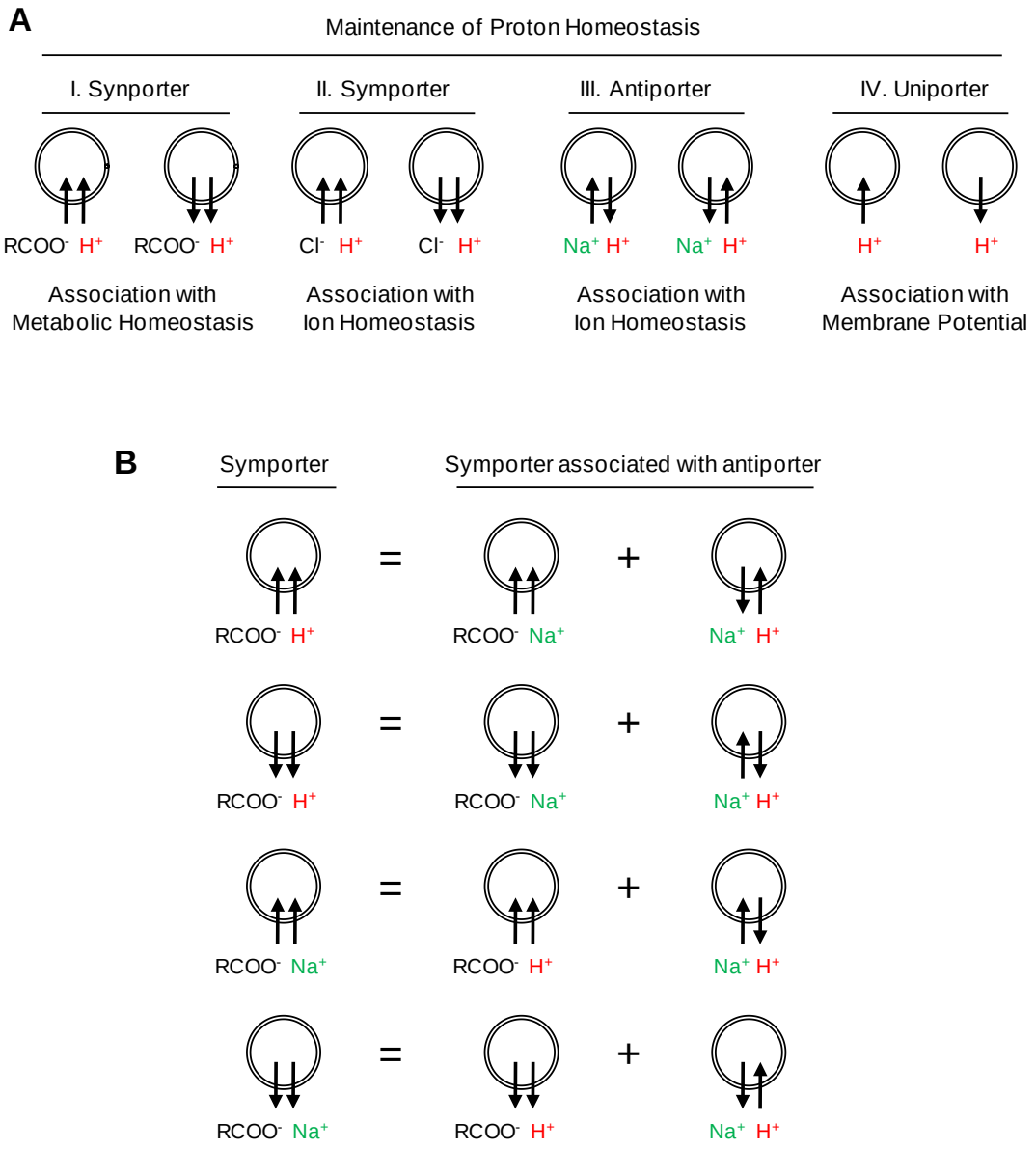

Figure 13, Related to Figure 6. Maintenance of cellular proton homeostasis by transporters. (A) Proton transporters. (B) Carboxylate transport by a single symporter or symporter associated with antiporter. $RCOO^-$, carboxylates, mainly including amino acids and lactates; Cl^- represents anion; Na^+ represents cation.

Supplemental Experimental Procedures.

General reagents and methods. β-Chloro-L-alanine, oligomycin A and bafilomycin A1 are obtained from Santa Cruz. LysoTracker Blue and LysoSensor Green were purchased from Invitrogen. Compound 968 was from Millipore. z-VAD-FMK, z-IETD-FMK, z-DEVD-FMK, z-LEHD-FMK, and ABT-737 were from BioVision. Puromycin and blasticidin are purchased from Invivogen while G418 is from Gibco, and these compounds are used for establishment and maintenance of stable cell lines. Other reagents including TNF-α, amino acids, glucose, carboxylates, esters, antimycin A, propidium iodide, cycloheximide, chloroquine, carbonyl cyanide m-chlorophenyl hydrazone, 2-Deoxy-D-glucose, nigericin, and all general chemicals are obtained from Sigma unless otherwise described.

Cell culture. MCF-7, MDA-MB-231, Bcap37, HeLa and A549 cells are obtained from ATCC. Stable cell lines were generated by lentivirus infection. Cells were maintained in high glucose DMEM supplemented with 10% fetal bovine serum (BioInd, Israel) and 50 IU penicillin/streptomycin (Invitrogen, USA) in a humidified atmosphere with 5% CO_2 at 37℃.

As for cells cultured in the CO_2-buffed incubator, the different pH of the medium was adjusted using sodium bicarbonate whose concentration ranges from 2.0 to 120 mM. As for experiments carried out in the CO_2-free incubator, the pH of solution or DMEM without sodium bicarbonate was modified by HCl and NaOH.

High glucose DMEM, glucose-free DMEM and dialyzed serum were obtained from

Gibco. The nutrient-deficient medium was made based on DMEM with a little modification. The compositions are as following: 20 mM HEPES, 8 g/l NaCl, 15 mg/l phenol red, 200 mg/l $CaCl_2$, 0.1 mg/l $Fe(NO_3)_3 \cdot 9H_2O$, 97.7 mg/l $MgSO_4$, 400 mg/l KCl, and Vitamin solution (100X) (Sigma, M6895) was used as 25X. Glucose and amino acids were dissolved in the nutrient-deficient medium as stocks.

Imaging of fixed cells. For fixed cell imaging, after the desired treatments, cells grown in 6-well plates were fixed with 4% paraformaldehyde in PBS for 30 minutes, and then carefully washed twice with PBS buffer. The fixed cells were incubated with 2 μg/ml of Hoechst33258 for 5 minutes for nuclear DNA staining. After cells were washed twice with PBS, the fluorescence and phase-contrast imaging was captured with a Nikon Eclipse TE2000-U fluorescence microscope. The filter sets for imaging were: GFP: Ex480/30, DM505, BA535/40; PI (RFP): Ex535/50, DM575, BA590; Hoechst (UV): Ex355/50, DM400, BA420.

Lysosomal acidification assessment. Cells were grown in six–well plates, and after the desired treatments, cells were stained with 0.5 μM of LysoTracker Blue dissolved in the treatment medium for 15 min and then washed twice with the treatment medium. As for the pre-staining experiments, cells were stained with 0.5 μM of LysoSensor Green in the complete medium for 15 min, and then washed twice with the complete medium before treatments. The cells were rapidly imaged with a Nikon Eclipse TE2000-U fluorescence microscope with filter sets as described above. The

fluorescence intensity was analyzed by ImageJ software (1.47).

Cell death assay. The culture medium contained 2 μg/ml of PI that did not affect cell growth. Cells expressing GC3AI were grown in twelve–well plates, and after the desired treatments, cells were then rapidly imaged with an EVOS® FL digital inverted fluorescence microscope with 10× objective lens. The filter sets for imaging were: GFP: Ex470/22, Em525/50; PI (RFP): Ex531/40, Em593/40. GFP-positive cells were counted as apoptosis, and cells only stained with PI were taken as necrotic cells. Five random areas in each well were imaged, and each area contained more than two hundreds of cells. Cell counting was performed with ImageJ software (1.47) by analyzing these pictures.

Cell survival assay. For the experiments where ATP generation was affected by inhibitors, cell survival was determined based on cell counting, as described in “Cell Death Assay”.

When the ATP generation by glycolytic or mitochondrial pathway was not blocked, survived cells were positively corresponding to the ATP level. Therefore, in such a condition, cell survival analysis was performed using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, Cat#7570), as described in “Intracellular ATP assay”. To avoid the potential minor influence of nutrient deprivation on ATP generation, cells were recovered with the complete medium for half an hour before undergoing ATP assay.

Intracellular ATP assay. The intracellular ATP was measured using the CellTiter-Glo Reagent (Promega, Cat#7571) following the user instruction with minor modification. In brief, 15×10^4 cells per well were seeded in twelve-well plates for 24h, and then were incubated with the treatment medium for the desired period. CellTiter-Glo reagent was mixed with one volume of PBS and prepared as assay mix. 400 μL of assay mix was added to each well of twelve-well plates after the medium was removed. Subsequently, plates were placed on a shaker for 10 min and were then incubated at room temperature for an additional 10 min. Luminescent reading was carried on a SpectraMax M2 reader (Molecular Devices). The measured ATP content was normalized by the number of survival cells.

Intracellular pH assay. A pH-sensitive fluorescent protein, pHluorin2 (modified pHluorin), was used to monitor the intracellular pH, and it displays a bimodal excitation spectrum with peaks at 395 and 475 nm and an emission maximum at 509 nm[17, 18]. Upon acidification, pHluorin2 excitation at 395 nm decreases with a corresponding increase in the excitation at 475 nm. pHluorin2 was stably expressed in cells using lentivirus. After desired treatments, cells were imaged with a Nikon Elicipse TE2000-U fluorescence microscope (Nikon, Japan). Dual-excitation ratio imaging used excitation filters 400DF10 and 480DF15. A 505DRLP dichroic mirror and an emission filter, 535DF20, were used for both excitations. Raw data were exported to ImageJ software (1.47) as 8-bit TIF for analysis. Background correction

was performed by subtracting the intensity of a nearby cell-free region from the signal of the imaged cell and a threshold was set to avoid ratio-created artifacts. Fluorescence excitation ratios were obtained from manually selected portions of intact whole cells in the ratio images by dividing the 400 nm picture by the 480 nm image pixel by pixel. The 400/480 ratio value for each treatment was the mean of ratios obtained from of 60 cells.

For pH clamp experiments, cells expressing pHluorin2 were incubated with a clamping buffer (120 mM KCl, 5 mM NaCl, 20 mM HEPES, 1 mM CaCl2, 1 mM MgCl2 and 25 μM nigericin) of desired pH for 30 min. In the clamping buffer, the 400/480 ratio value of intracellular pHluorin2 were quantitatively corresponding to the pH of buffer.

Lactate production assay. Measurement of lactate concentration in cell culture medium was performed using the Lactate Assay kit from BioVision (Cat# K627-100) according the user instruction. 40×10^4 cells per well were seeded in six-well plates, and then were incubated with the treatment medium. Cell media were collected after the desired period as indicated. Aliquots of 1 μl medium were used to measure lactate concentration. The lactate production was normalized by the number of cells.

Intracellular amino acids, succinate and NADH/NAD$^+$ assay. 40×10^4 cells per well were seeded in six-well plates for 24h, and then were incubated with the treatment medium for the desired period. Cells were washed twice with cold PBS and then underwent measurement using the Succinate Colorimetric Assay Kit (BioVision,

Cat#K649-100), the L-Amino Acid Quantitation Colorimetric Kit (BioVision, Cat#K639-100) and the NAD+/NADH Quantification Colorimetric Kit (BioVision, Cat#K337-100) according to the user instructions. Briefly, cells were collected in 400 μL cold assay/extraction buffer and then homogenized by freeze/thaw two cycles (20 min on dry-ice, then 10 min at room temperate). After being centrifuged at 12000 rpm for 5 min, the supernatant was filtered through 10 kDa molecular weight cut off filters and then used to measure metabolites with kits. The measured amount of amino acid and succinate was normalized by total protein content.

Oxygen consumption assay. An XF24 Analyzer (Seahorse Biosciences, USA) was used to measure oxgen consumption in MCF-7 cells. 4×10^4 MCF-7 cells per well were seeded into Seahorse Bioscience XF24 cell culture plates and allowed to adhere and grow for 24 h in a 37 °C humidified incubator with 5% CO_2. Before the start of oxygen consumption assay, the media was changed to unbuffered nutrient-deficient media (see Cell culture section) and incubated at 37 °C without CO_2. After the desired treatments for 1 hour, oxygen consumption was measured. The oxygen consumption data of cells with the control treatment were expressed as 100%.

Western blot. After desired treatments as specified as indicated, cells were washed twice with PBS and lysed in buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM b-glycer-ophosphate, 1 mM sodium vanadate, 1 mg/ml leupeptin, 1 mM phenylmethyl-sulfonylfluoride).

Equal amounts of protein (30μg) were loaded onto 15% SDS-PAGE gels. Western detection was carried out using a Li-Cor Odyssey image reader (Li-Cor, USA). The goat anti-mouse immunoglobulin G (IgG) and goat anti-rabbit IgG secondary antibodies were obtained from Li-Cor. The final concentration of the secondary antibodies used was 0.1 μg/ml. The sources of antibodies used are as following: Bax (Abcam, Cat#ab53154), Bid (CST, Cat#2002P), Bcl-2 (Abcam, Cat#ab692), Bcl-xL (Abcam, Cat#ab77571), caspase-3 (CST, Cat#9665), caspase-7 (CST, Cat#9494), caspase-9 (CST, Cat#9508), caspase-8 (CST, Cat#4790) anti-β-Actin (CST, Cat#8457). All these primary antibodies were used with a dilution of 1:1000.

Gene construction. The humanized pHluorin2, LGALS3 and GC3AI were commercially synthesized (Genewiz, China) according to the previous report [9, 17, 34]. All cDNAs were cloned into lentiviral expression vectors, pCDH-puro-CMV or pCDH-Neo-CMV using the eFusion Recombinant Cloning Kit (Biophay, China). The pLKO.1 lentiviral RNAi expression system was used to construct lentiviral shRNA for genes. The sequences of shRNA used in this study included the following:

shScramble: CCTAAGGTTAAGTCGCCCTCG

shCaspase-7#3: TTTGACGTGATTGTCTATAAT

shCaspase-7#4: GCTTCGCCTGCATCCTCTTAA

shCaspase-8#1: CCTGGCCGATGGTACTATTTA

shCaspase-8#2: GCCTTGATGTTATTCCAGAGA

shCaspase-9#1: CTTTGTGTCCTACTCTACTTT

shCaspase-9#2: CAGCTTCCAGATTGACGACAA

shBid#1: GTGAGGAGCTTAGCCAGAAAT

shBid#2: CAGGGATGAGTGCATCACAAA

shBax: GACGAACTGGACAGTAACATG

Lentivirus production. Viral packaging was done according to a previously described protocol [35]. Briefly, knockdown plasmids pLKO.1-shRNA or expression plasmids pCDH-CMV-cDNA, pCMV-dR8.91, and pCMV-VSV-G were co-transfected into 293T cells using the calcium phosphate coprecipitation at 20:10:10 μg (for a 10-cm dish). The transfection medium containing calcium phosphate and plasmid mixture was replaced with fresh complete medium after incubation for 5 h. Media containing virus was collected 48 h after transfection and then concentrated using Virus Concentrator Kit (Biophay, China). The virus was resuspended in the appropriate amount of complete growth medium and stored at -80℃. Cancer cells were infected with the viruses at the titer of 100% infection in the presence of polybrene (10 μg/ml) for 48 h, and then cells were selected with puromycin or neomycin.

Statistics. Data are given as means ± SD. Statistical analyses were performed using unpaired, two-tailed Student's t test for comparison between two groups. Asterisks in the figure indicated statistical significances (* , $p < 0.05$; * * , $p < 0.01$).