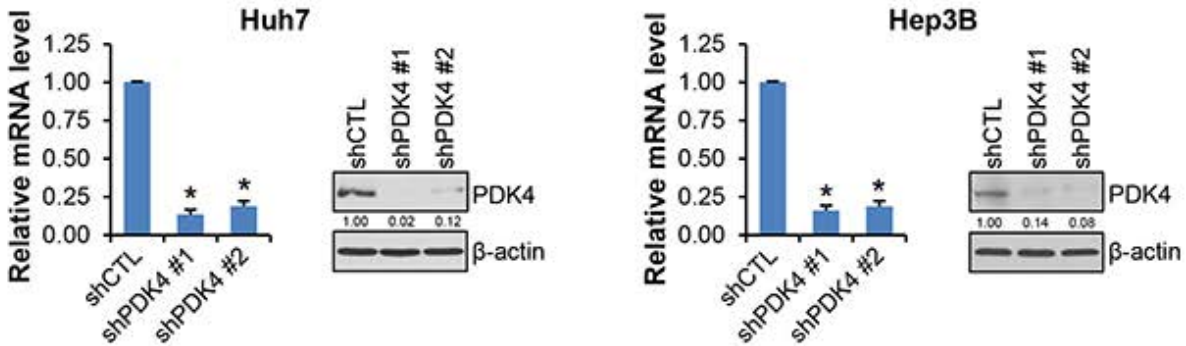
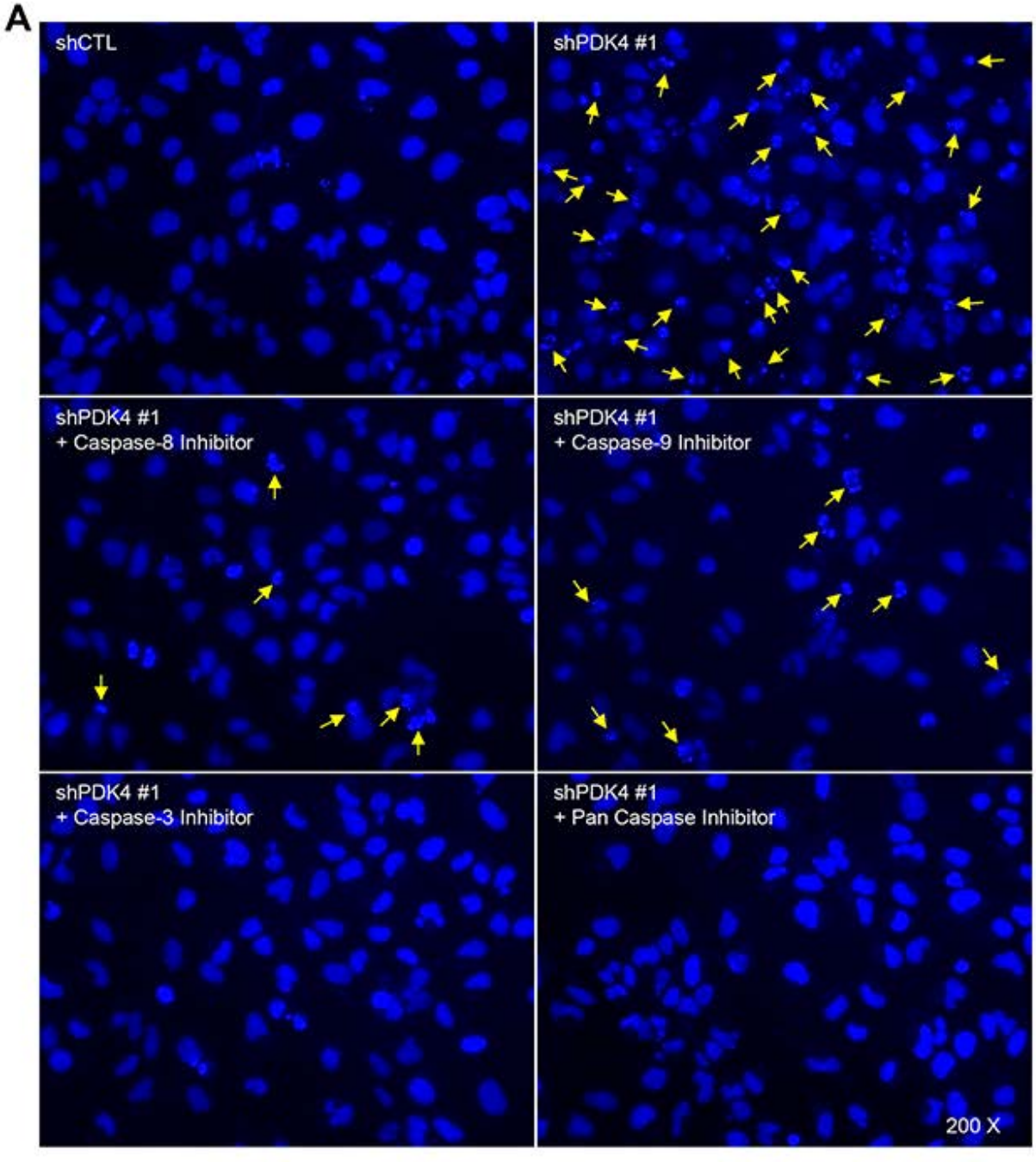
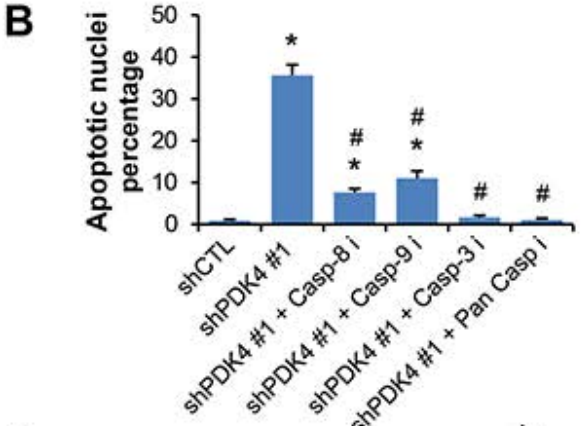
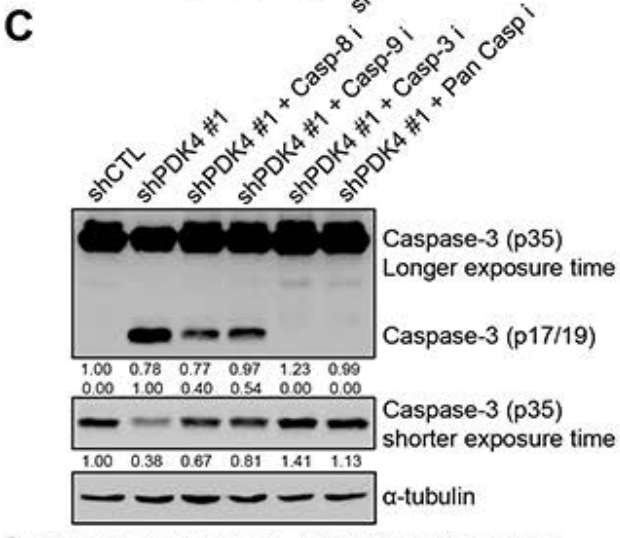


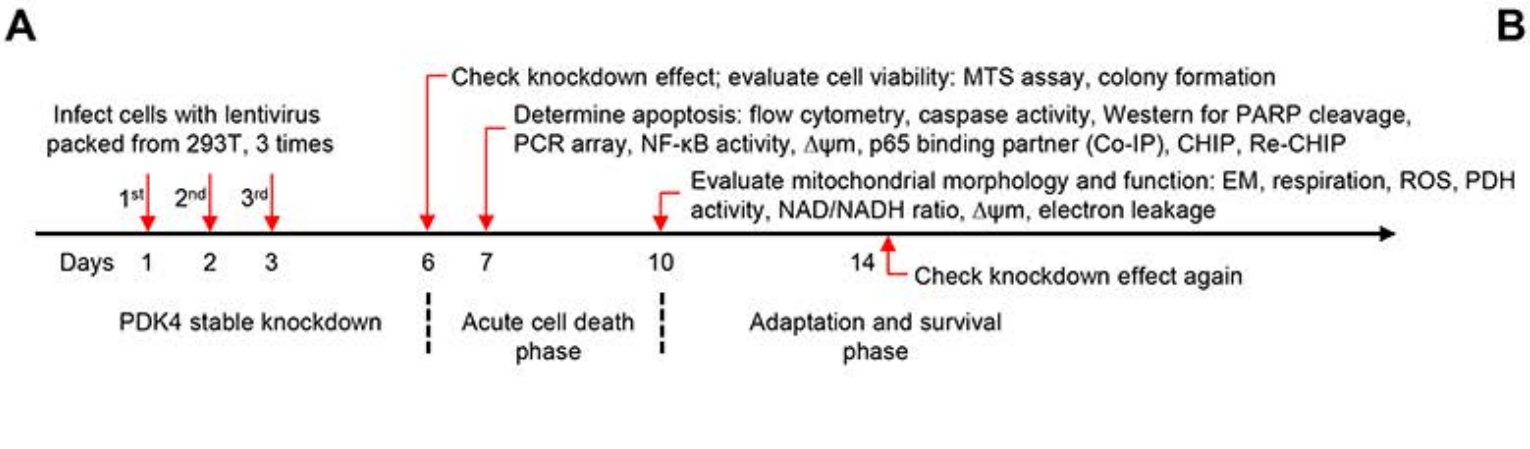
Supporting Figure 1. Knockdown of PDK4 in HCC cells. qPCR and WB were performed to detect PDK4 mRNA and protein respectively in control (shCTL) and PDK4 knockdown (shPDK4 #1 or #2) Huh7 (left) and Hep3B (right) cells 72 hours after lentiviruses infection. Data are shown as mean ± SEM. **P* < .01 vs shCTL.

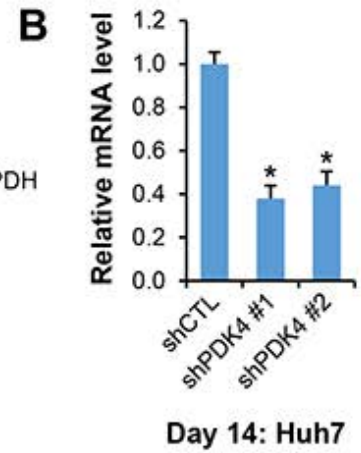






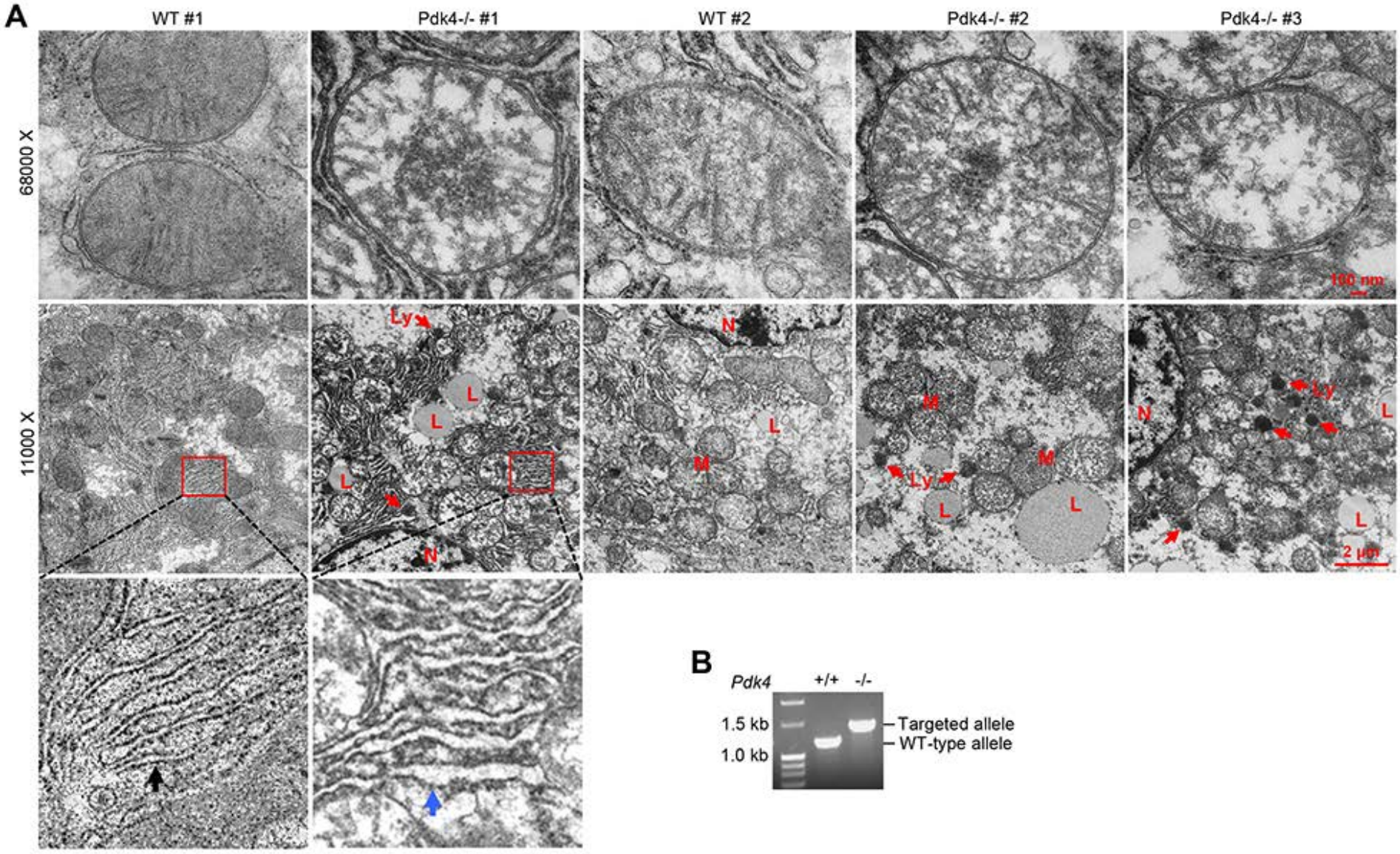
Supporting Figure 2. Inhibition of caspase prevented PDK4 knockdown-induced apoptosis. (A) PDK4 was knockdown in the presence of caspase-3, -8, -9, or pan caspase inhibitors (50 μM) in Huh7 cells. Cells were stained with 1 μg/mL Hoechst 33342 and photographed in a fluorescence microscope. Arrows indicate apoptotic nuclei. (B) Quantification of apoptotic nuclei from five fields of the same group of cells shown in (A). **P* < .01 vs shCTL; #*P* < .01 vs shPDK4 #1. (C) WB analysis of caspase-3.



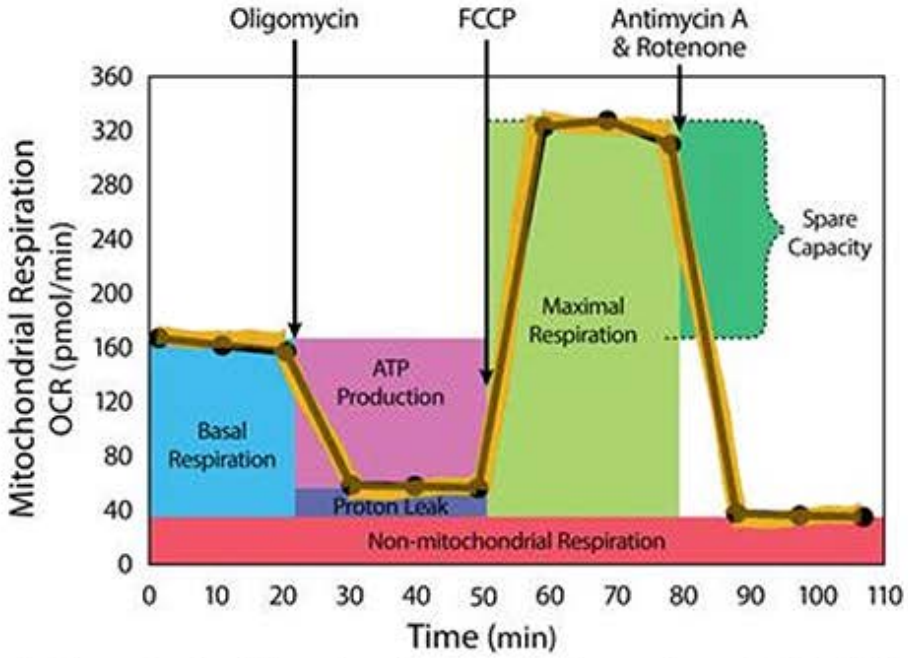


Supporting Figure 3. (A) A schematic representation showing the time points of experimental design. (B) qPCR of PDK4 mRNA in Huh7 cells at day 14 following the first application of shCTL and shPDK4 lentiviruses. Data are shown as mean ± SEM. **P* < .01 vs shCTL.

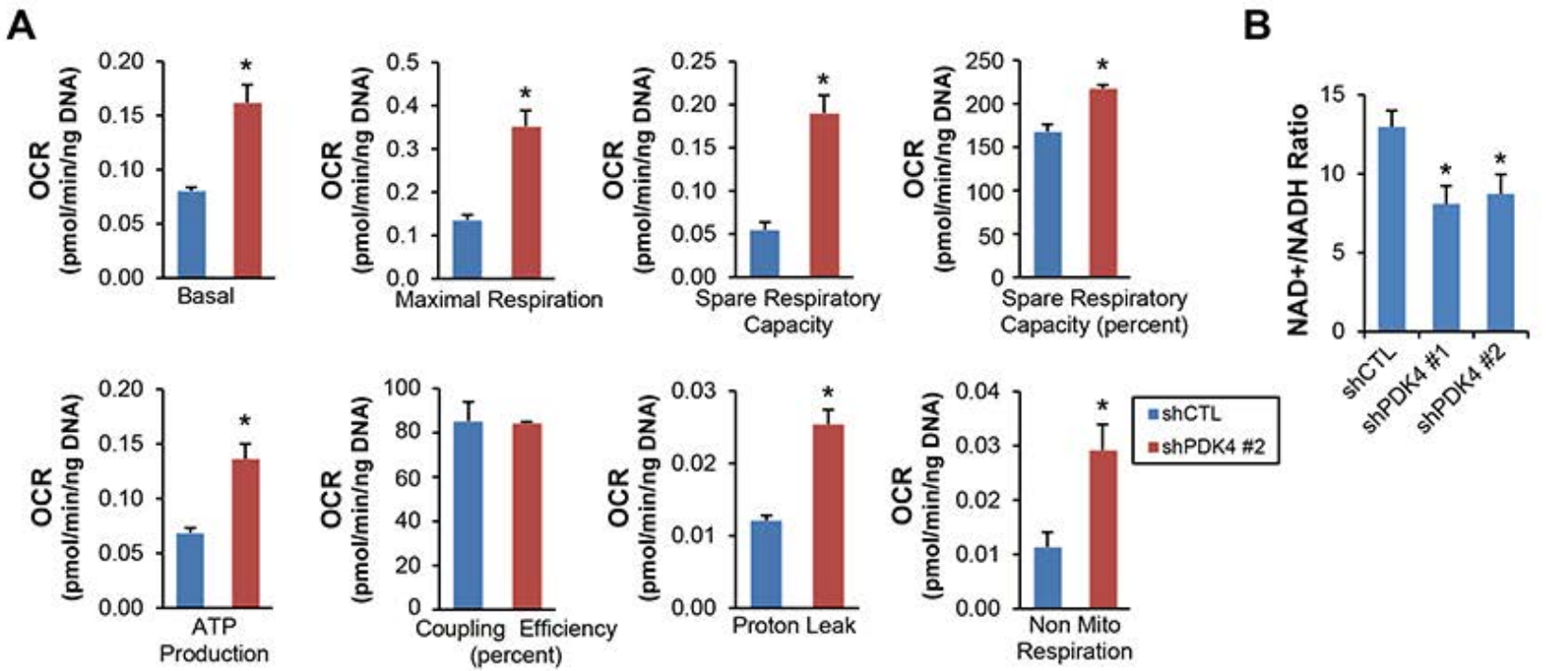
A note: To better evaluate mitochondrial function and respiration, we used cells after the acute cell death phase to evaluate mitochondrial respiration-related function such as reparation, ROS, NAD/NADH ratio, PDH activity, as well as EM.



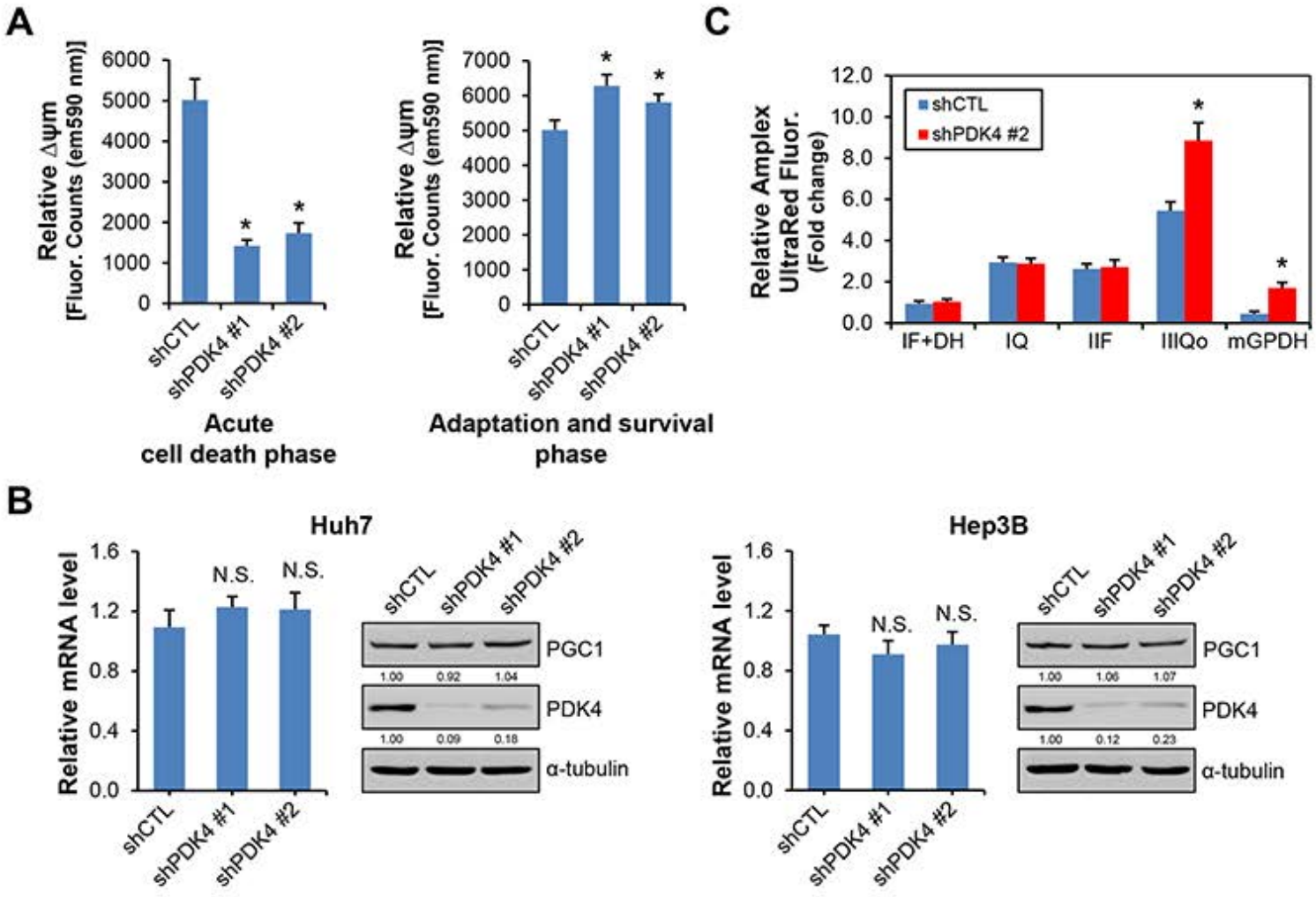
Supporting Figure 4. (A) Transmission electron microscopy (TEM) of mitochondria morphology in hepatocytes of 12 months old WT and *Pdk4*$^{-/-}$ mouse livers. A minimum of 10 cells per condition were examined. Red arrow: lysosome; black or blue arrow: normal or enlarged endoplasmic reticulum (ER), respectively. M: mitochondrion; Ly: lysosome; L: lipid droplet; N: nucleus. (B) Genotyping of *Pdk4*$^{-/-}$ mice.



Supporting Figure 5. The illustration and calculation of each parameter in a seahorse XF Cell Mito Stress Test using the 24-well Seahorse XFe24 Extracellular Analyzer. Non-mitochondrial respiration (non-mitochondria-derived OCR): A subset of cellular or mitochondrial enzymes (e.g., plasma membrane NADPH oxidase, cytochrome p450 *etc.*) can also consume oxygen. This rate is the minimal measurement after rotenone and antimycin A addition. Therefore, the rates of respiration solely attributable to mitochondria can be calculated by subtracting this rate. Basal respiration (Measurement prior to oligomycin addition – non-mitochondria-derived OCR) is used to meet the cell's ATP demand and drive mitochondrial proton leak. This measurement gives information about the resting energetics of the cell. Energetically active cell will have a high rate of base respiration. Maximal respiration occurs when the uncoupler FCCP stimulates the respiratory chain to operate at its maximum speed and mimics a physiological "energetic crisis" and is maximal measurement after FCCP addition through to before rotenone and antimycin A injection minus non-mitochondria-derived OCR. Spare respiratory capacity refers to the difference between the maximal FCCP-stimulated rate of respiration minus basal respiration and reflects the cells ability to deal with an energetic crisis. Spare respiratory capacity percentage, as a percentage that provides information about how closely during basal respiration the election transport chain is working to its maximal capacity, is the ratio of maximal and basal respiration. ATP production: this respiration is being used to drive ATP production under basal condition, calculated as basal respiration minus proton leak. Proton leak is minimal measurement after oligomycin injection minus non-mitochondrial-derived OCR; Proton leak can regulate physiological processes including non-shivering thermogenesis and attenuation of reactive oxygen species (ROS) production. Coupling efficiency: the coupling efficiency provides information about what fraction of basal respiration is used to drive ATP synthesis versus proton leak. For many cell types, it can be a rough estimate of bioenergetics health (most cell types have a coupling efficiency of around 80%); calculated as 100 × [ATP-linked respiration/basal].



Supporting Figure 6. (A) Seahorse assay in shCTL and shPDK4 Huh7 cells. Individual parameters from the Mito Stress assay were presented. Oxygen consumption rate (OCR) was normalized with the amount of DNA (ng) in each well. (B) Cellular NAD+/NADH ratio in shCTL and shPDK4 Huh7 cells. Data are shown as mean ± SEM of three independent experiments with triplicate assays. *$P < .01$ vs shCTL.



Supporting Figure 7. (A) Mitochondrial membrane potential (ΔΨm) was determined in shCTL and shPDK4 Huh7 cells at different time points after PDK4 knockdown. Also see schematic in Supporting Figure 3A. (B) qPCR and WB of PGC1α mRNA and protein in shCTL and shPDK4 Huh7 (left) and Hep3B (right) cells at day 10 following the first application of shCTL and shPDK4 lentiviruses. (C) Mitochondrial electron leakage sites were determined in shCTL and shPDK4 Huh7 cells (day 10) in Amplex UltraRed assay. The fluorescence values were normalized to I_{F+DH} site. Data are shown as mean ± SEM. *P < .01 vs shCTL. N.S. denotes no significance (P > .05 vs shCTL).

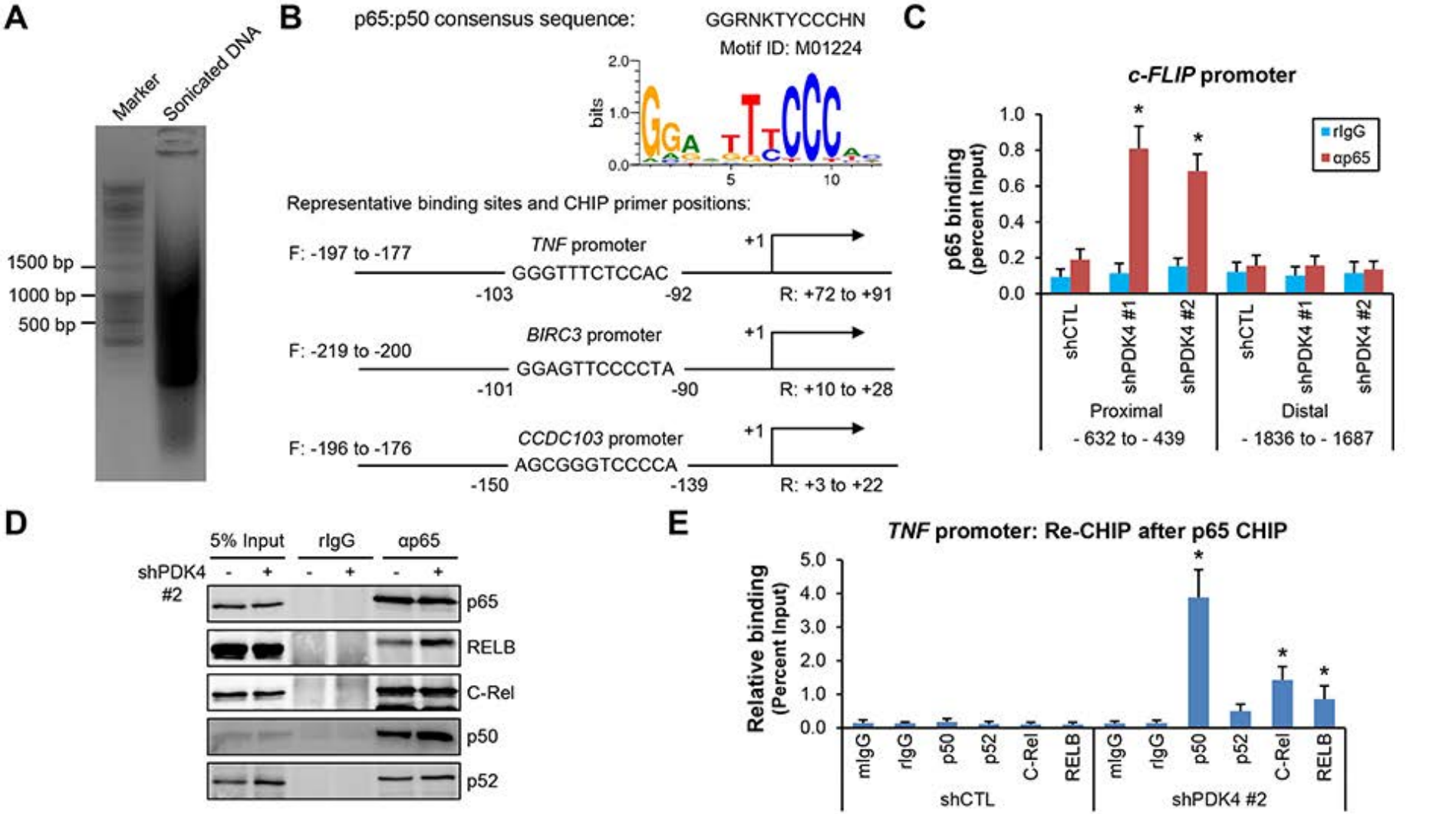
Table 1. Huh7 cells

Layout	1	2	3	4	5	6	7	8	9	10	11	12
A	ABL1	AKT1	APAF1	APP	ATG12	ATG16L1	ATG3	ATG5	ATG7	ATP6V1G2	BAX	BCL2
	-1.12	1.06	1.08	2.73	-4.15	1.14	-1.47	1.03	-1.39	1.5	-1.56	2.04
B	BCL2A1	BCL2L1	BCL2L11	BECN1	BIRC2	BIRC3	BMF	C1orf159	CASP1	CASP2	CASP3	CASP6
	-24.81	-1.7	1.29	1.05	-1.05	14.14	3.75	-1.91	-1.76	-1.64	-1.33	-1.61
C	CASP7	CASP9	CCDC103	CD40	CD40LG	CFLAR	COMMD4	CTS8	CTSS	CYLD	DEFB1	DENND4A
	1.03	-1.54	4.63	-1.04	-1.04	-1.27	-1.36	1.12	1.76	-1.18	-1.04	1.12
D	DFFA	DPYSL4	EIF5B	ESR1	FAS	FASLG	FOXI1	GAA	GADD45	GALNT5	GRB2	HSPBAP1
	-1.26	-2.63	-1.22	-16.46	-1.07	8.33	-1.04	1	2.09	2	-1.58	1.22
E	HTT	IFNG	IGF1	IGF1R	INS	IRGM	JPH3	KCNIP1	MAG	MAP1LC3A	MAPK8	MCL1
	1.26	-1.04	1.5	1	1.4	-1.04	-6.93	-1.04	-1.04	-2.39	-1.05	1.75
F	NFKB1	NOL3	OR10J3	PARP1	PARP2	PIK3C3	PVR	RAB25	RPS6KB1	S100A7A	SNCA	SPATA2
	-1.16	1.58	-1.04	-1	-1.4	-1.03	-1.8	1.01	-1.49	-14.66	1.33	-1.6
G	SQSTM1	SYCP2	TMEM57	TNF	TNFRSF10A	TNFRSF11B	TNFRSF1A	TP53	TRAF2	TXNL48	ULK1	XIAP
	1.45	-12.43	-1.78	125.83	1.41	-1.36	-1.56	-1.26	-1.09	-1.67	-1.02	-1.21

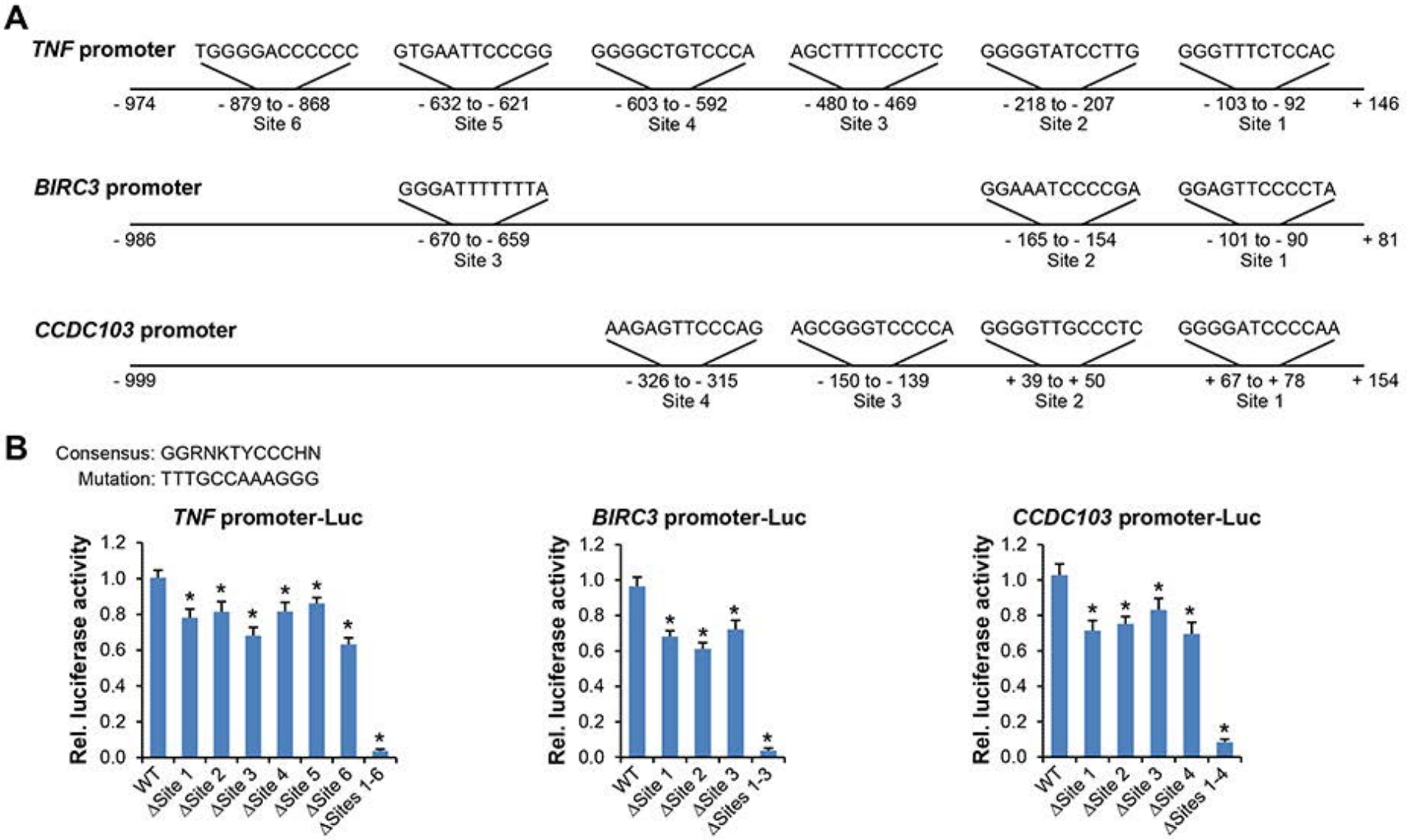
Table 2. Hep3B cells

Layout	1	2	3	4	5	6	7	8	9	10	11	12
A	ABL1	AKT1	APAF1	APP	ATG12	ATG16L1	ATG3	ATG5	ATG7	ATP6V1G2	BAX	BCL2
	1.23	1.46	1.32	1.44	-1.9	1.97	1.77	1.65	1.16	7.51	-1.2	-2.08
B	BCL2A1	BCL2L1	BCL2L11	BECN1	BIRC2	BIRC3	BMF	C1orf159	CASP1	CASP2	CASP3	CASP6
	31.64	1.19	1.19	1.84	1.42	3.83	-1.97	-1.29	-8.5	-1.21	1.19	1.03
C	CASP7	CASP9	CCDC103	CD40	CD40LG	CFLAR	COMMD4	CTS8	CTSS	CYLD	DEFB1	DENND4A
	1.76	-1.02	4.68	-19.01	1.6	1.08	1.64	1.11	2.87	1.46	-10.91	1.09
D	DFFA	DPYSL4	EIF5B	ESR1	FAS	FASLG	FOXI1	GAA	GADD45	GALNT5	GRB2	HSPBAP1
	1.51	1.77	1.46	1.6	1.6	1.6	1.6	1.29	1.12	31.68	1.44	1.56
E	HTT	IFNG	IGF1	IGF1R	INS	IRGM	JPH3	KCNIP1	MAG	MAP1LC3A	MAPK8	MCL1
	2.22	1.6	18.97	1.21	1.6	2.36	5.74	1.6	-4.9	-1.01	1.8	3.01
F	NFKB1	NOL3	OR10J3	PARP1	PARP2	PIK3C3	PVR	RAB25	RPS6KB1	S100A7A	SNCA	SPATA2
	1.2	2.37	1.6	-1.03	1.17	1.11	2.04	-1.95	1.45	1.6	-1.98	-1.17
G	SQSTM1	SYCP2	TMEM57	TNF	TNFRSF10A	TNFRSF11B	TNFRSF1A	TP53	TRAF2	TXNL48	ULK1	XIAP
	4.17	1.23	-1.04	64.12	-1.22	2.05	-1.11	-1.07	1.43	1.57	-1.11	1.19

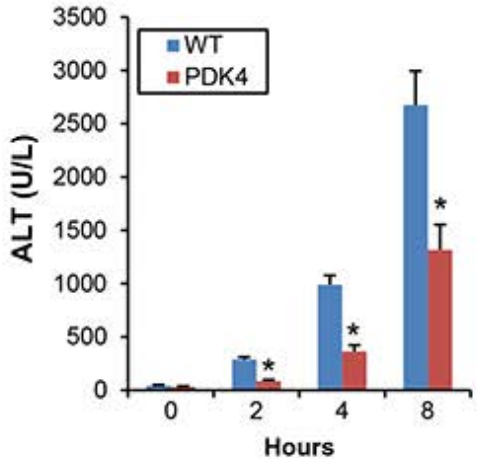
Supporting Figure 8. RT^2 Profiler PCR array in Huh7 and Hep3B cells transfected with shCTL and shPDK4. The number under each gene represents fold change in shPDK4 vs shCTL cells. There are 8 upregulated genes (APP, BCL2, BIRC3, BMF, CCDC103, FASLG, GADD45A and TNF) and 9 downregulated genes (ATG12, BCL2A1, DPYSL4, ESR, JPH3, MAP1LC3, S100A7A, SYCP2 and RPLP0) in Huh7 cells. In Hep3B cells, 16 genes (ATP6V1G2, BCL2A1, BIRC3, CTSS, GALNT5, HTT, IGF1, IRGM, JPH3, MCL1, NOL3, PVR, SQSTM1, TNF and TNFRSF11B) were upregulated and 5 genes (BCL2, CASP1, CD40, DEFB1 and MAG) were downregulated. Cut-off: 2.0. The commonly upregulated genes are TNF, BIRC3 and CCDC103, highlighted with red color.



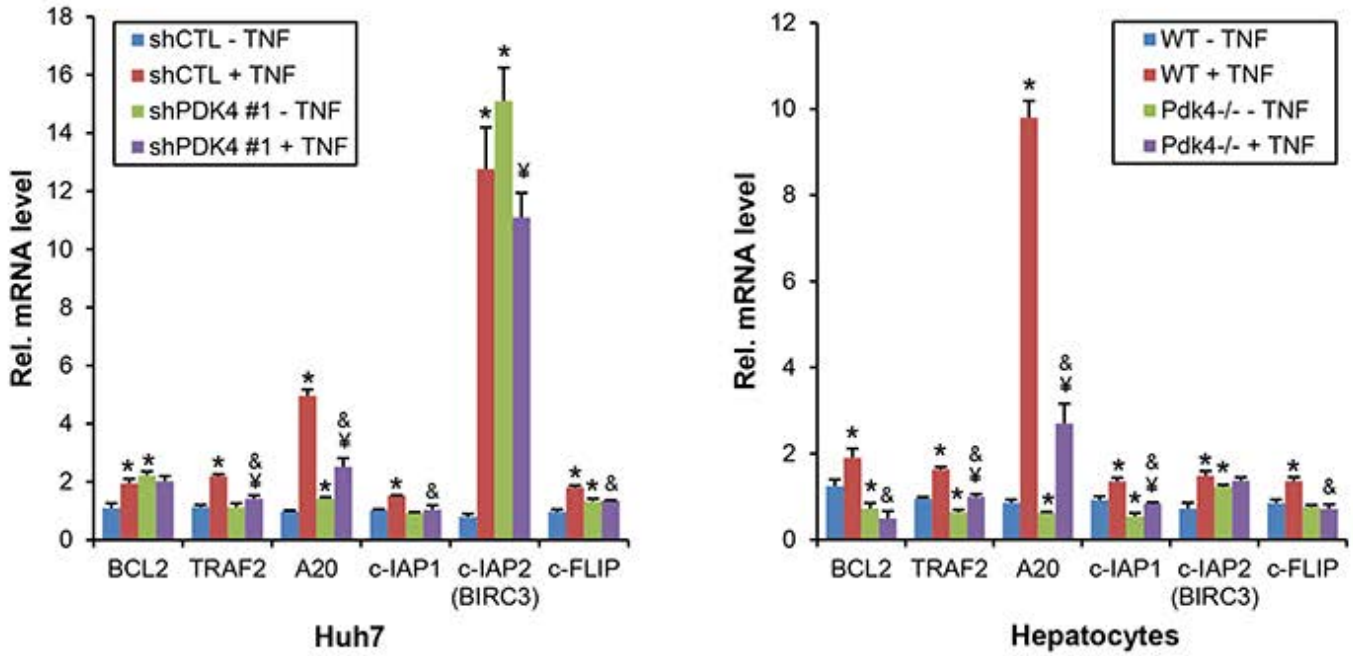
Supporting Figure 9. (A) Sheared chromatin size for ChIP assay. The chromatin was sheared by sonication, reverse cross-linked and resolved in a 1% agarose gel. (B) Top, the consensus motif used to predict NF-κB/p65-binding sites in the *TNF, BIRC3 and CCDC103* promoters was retrieved from MotifMap (http://motifmap.ics.uci.edu/). R: any purine; Y: any pyrimidine; N: any; H: not G; K: not C. Bottom, a representative binding site of p65 on each gene promoter is shown and the primer positions used in CHIP-qPCR assay is schematically labeled. (C) CHIP-qPCR assay was performed to examine p65 occupancy in the *c-FLIP* promoter in shCTL and shPDK4 Huh7 cells. The result was determined by qPCR and quantitated as the ratio of precipitated DNA to input DNA. (D) Co-immunoprecipitation using p65 antibody to define p65 binding partners in PDK4 knockdown Huh7 cells. To avoid heavy chain in immunoblot, a light chain specific secondary antibody was used. (E) sequential CHIP (Re-CHIP) was performed to detect NF-κB subunits co-occupancy on *TNF* promoter with p65 in Huh7 cells. *$P < .01$ vs shCTL.



Supporting Figure 10. Analysis of NF-κB binding sites in the *TNF, BIRC3 and CCDC103* promoters. (A) Predicted NF-κB/p65 binding sites within the *TNF, BIRC3 and CCDC103* promoters. The prediction is based the sense strands and consensus motif shown in Supporting Figure 9*B*. The results are from combined analysis of four data bases: Tfsitescan (http://www.ifti.org), JASPAR (http://jaspar.genereg.net), TFBIND (http://tfbind.hgc.jp) and PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). (B) Mutational analysis of NF-κB/p65 binding sites in the *TNF, BIRC3 and CCDC103* promoters. The predicted binding sites were mutated to the indicated sequence. Luciferase reporter assays were performed to determine the activity of single site mutation and full sites mutation. **P* < .05 vs WT.



Supporting Figure 11. Plasma ALT levels of WT and PDK4 overexpression mice, collected at the indicated time points after Jo2 (10 μg) injection, were determined. *$P < .05$ vs WT.



Supporting Figure 12. NF-κB pro-survival target genes were less responsive to TNF under PDK4-deficient conditions. shCTL and shPDK4 Huh7 cells, or WT and *Pdk4*$^{-/-}$ primary hepatocytes were treated with 10 ng/ml TNF for 24 hours. The mRNAs of the indicated genes were determined by qPCR. Huh7: $^{*}P < .05$ vs shCTL - TNF; $^{¥}P < .05$ vs shPDK4 #1 - TNF; $^{\&}P < .05$ vs shCTL + TNF. Hepatocytes: $^{*}P < .05$ vs WT - TNF; $^{¥}P < .05$ vs *Pdk4*$^{-/-}$ - TNF; $^{\&}P < .05$ vs WT + TNF.

Loss of PDK4 switches the hepatic NF-κB/TNF pathway from pro-survival to pro-apoptosis

Jianguo Wu, Yulan Zhao, Young-Ki Park, Ji-Young Lee, Ling Gao, Jiajun Zhao, Li Wang

Supporting Information

Supplementary Materials and Methods

Cell culture, transfection and reagents

The human hepatocellular carcinoma cell lines Huh7 and Hep3B, human embryonic kidney cell line HEK293T were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 U/ml penicillin at 37°C in a humidified incubator with 5% CO2. Transfection was performed with X-tremeGENE™ HP DNA Transfection Reagent (#06366236001; Roche, Basel, Switzerland) according to the manufacturer's instruction. Caspase-3 inhibitor Z-DEVD-FMK (#FMK004), caspase-9 inhibitor Z-LEHD-FMK (#FMK008), caspase-8 inhibitor Z-IETD-FMK (#FMK007) and pan caspase inhibitor Z-VAD-FMK (#FMK001) were from R&D systems (Minneapolis, MN). Hoechst 33342 (#H3570) and Amplex™ UltraRed Reagent (#A36006) were from Thermo Fisher Scientific (Waltham, MA).

Plasmids and small hairpin RNAs (shRNAs)

Flag-PDK4 was constructed by subcloning the coding sequence of PDK4 from pDONR223-PDK4 (Addgene plasmid 23577) to p3xFlag-CMV-10 vector through NotI and BamHI restriction enzymes. GFP-PDK4 was constructed by subcloning the coding sequence of PDK4 to pLVX-AcGFP-N1 vector through XhoI and BamHI restriction enzymes. pCMV3-Flag-p65/RelA was from Sino Biological Inc. (Beijing, China). MISSION® pLKO.1 lentiviral shRNAs for human PDK4 (#1: TRCN0000006264, Clone ID: NM_002612.2-2954s1c; #2: TRCN0000194917, Clone ID: NM_002612.2-1297s1c1), TNFR1/TNFRSF1A (TRCN0000378351, Clone ID: NM_001065.2-329s21c1), TNFR2/TNFRSF1B (TRCN0000373788, Clone ID: NM_001066.2-602s21c1), BIRC3 (TRCN0000003776, Clone ID: NM_001165.x-4821s1c1), CCDC103 (TRCN0000168319, Clone ID: NM_213607.1-153s1c1) and p65/RELA (TRCN0000014686, Clone ID: NM_021975.1-1067s1c1), for mouse TNFR1/Tnfrsf1a (TRCN0000066106, Clone ID: NM_011609.2-1538s1c1) and p65/Rela (TRCN0000235832, Clone ID:NM_009045.4-2313s21c1) were all from Sigma-Aldrich (St. Louis, MO). The promoter reporters used were pGL3-enhancer-TNF-Luc, pGL3-enhancer-BIRC3-Luc and pGL3-enhancer-CCDC103-Luc, which were constructed by cloning each promoter (the sequence lengths and the primers were indicated in the Supporting Figure 10A and Supporting Table 1 respectively) from genomic DNA to pGL3-enhancer vector (#E1771; Promega, Madison, WI). Renilla Luciferase control reporter vector is pRL-TK (#E2241; Promega).

Lentivirus preparation and infection

The second-generation lentiviral system (pLKO.1, psPAX2 and pMD2.G) was used to deliver shRNA. Briefly, a lentiviral vector containing the shRNA along with packing vector and envelope vector were cotransfected into 293T cells using X-tremeGENE™ HP DNA Transfection Reagent according to the manufacturer's protocol. The supernatants containing virus particles were collected, filtered and concentrated. Resuspended virus was applied to target cells with 6 μg/ml polybrene (#TR-1003-G; EMD Millipore, Billerica, MA).

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated with TRIzol® RNA Isolation Reagents (#15596018; Thermo Fisher Scientific) and reversely transcribed into cDNA using High-Capacity cDNA Reverse

Transcription Kit with RNase Inhibitor (#4374967; Thermo Fisher Scientific) according to the manufacturer's instruction. Real-time PCR was performed using CFX384 Touch™ Real-Time PCR Detection System and SsoAdvanced™ Universal SYBR® Green Supermix (#1725275; Bio-Rad, Hercules, CA). The data were normalized to the amount of the β-actin transcript. The primer sequences are provided in Supporting Table 1.

Western blot and antibodies

Cells were rinsed twice with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% Na-deoxycholate and 1 mM EDTA) supplemented with Pierce™ Protease and Phosphatase Inhibitor Mini Tablet (#88669; Thermo Fisher Scientific). Concentration was determined by the Pierce™ BCA Protein Assay Kit (#23227; Thermo Fisher Scientific). Protein samples were separated by SDS-PAGE and transferred onto PVDF membrane (#162-0177; Bio-Rad). The membrane was blocked with 5% defatted milk in PBS/T (0.2% Tween-20 in PBS) and incubated overnight with the indicated primary antibodies. After extensive washing with PBS/T, the membrane was incubated with HRP-conjugated secondary antibody. The chemiluminescence detection procedure was performed using Pierce ECL western blotting substrate (#32209) or SuperSignal West Femto Maximum Sensitivity Substrate (#34095). The primary antibodies against PARP (46D11) (#9532; 1:500), GAPDH (D4C6R) (#97166; 1:1000), TNF-R1 (C25C1) (human specific) (#3736; 1:500), TNF-R2 (#3727; 1:500), BIRC3/c-IAP2 (58C7) (#3130; 1:500), PCNA (PC10) (#2586; 1:1000), Caspase-3 (#9662; 1:500), Cytochrome c (#4272; 1:500), Smac/Diablo (D5S3R) (#15108; 1:500), SAPK/JNK Antibody (#9252; 1:500), phospho-SAPK/JNK (Thr183/Tyr185) (81E11) (#4668; 1:500), c-Jun (60A8) (#9165; 1:500), phospho-c-Jun (Ser63) (#9261; 1:500), TRAF2 (C192) (#4724; 1:500), c-IAP1 (#4952; 1:500), A20/TNFAIP3 (D13H3) (#5630; 1:500), c-FLIP (D5J1E) (#56343; 1:500), RelB (C1E4) (#4922T; 1: 1000) and c-Rel (#4727T; 1: 1000) were from Cell Signaling Technology (Danvers, MA). The primary antibodies against β-actin (sc-47778, C4; 1:500), p65/RELA (sc-372; 1:500), Lamin A/C (sc-6215; 1:500), α-Tubulin (sc-5286; 1:1000), TNF-R1 (H-5) (sc-8436; 1:500), p50 (E-10) (sc-8414: 1: 500) and p52 (sc-7386, 1:500) were from Santa Cruz Biotechnology (Santa Cruz, CA). Other primary antibodies were against CCDC103 (#SAB1104041-200UL; Sigma-Aldrich; 1:500), Flag (F3165, M2; Sigma-Aldrich; 1:3000), PDK4 (AP7041B; ABGENT, San Diego, CA; 1:200), PGC1 (#NBP1-04676SS; Novus Biologicals, Littleton, CO; 1:500) and cleaved Bid (ab10640; Abcam, Cambridge, MA; 1: 500). Goat Anti-Mouse IgG (H + L)-HRP Conjugate (#1706516) and Goat Anti-Rabbit IgG (H + L)-HRP Conjugate (#1706515) were from Bio-Rad Laboratories. Rabbit anti-Goat IgG HRP-conjugated Antibody (#HAF109) was from R&D Systems. Peroxidase AffiniPure Goat Anti-Mouse IgG, Light Chain Specific for Western blotting after IP (#115-035-174) was from Jackson Immunoresearch Labs Inc (West Grove, PA).

MTS assay

Cells were plated in 96-well tissue culture plates at a density of 1 x 10^3 cells per well. The cell viability was assessed by using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (#3580; Promega) according to the manufacturer's instruction. Experiments were performed in triplicate, and four independent experiments were performed.

Colony formation assay

Briefly, equal number of control and PDK4-shRNA cells were seeded in 6-well plates at low density (2 x 10^3 cells per well) and cultured for 14 days. Then, medium was removed; adherent colonies were slightly rinsed with PBS, fixed in fixation buffer containing 45% methanol and 10% acetic acid, and stained with 0.25% Coomassie Brilliant Blue R250 in fixation buffer. The images of each well were acquired by scanning the plates under a laser scanner.

Caspase colorimetric assay
Caspase-3 Colorimetric Assay Kit was from BioVision, Inc. (#K106-200; Milpitas, CA). Caspase-8 (#KHZ0061) and Caspase-9 (#KHZ0101) Colorimetric Protease Assay Kits were from Thermo Fisher Scientific. The assays were performed according to the manufacturers' instructions. Briefly, cells were lysed in chilled cell lysis buffer provided in each colorimetric assay kit. Cytosolic extracts (200 µg proteins in 50 µl cell lysis buffer) and 5 µl chromophore substrate were added to 50 µl reaction buffer. The mixtures were placed in wells of a 96-well plate and assayed for caspase activity in terms of the absorbance of 405 nm after incubation.

Flow cytometry
The entire cell population was labeled with FITC-Annexin V Apoptosis Detection Kit with PI (#640914; Biolegend, San Diego, CA) according to the manufacturer's instruction and analyzed on a BD LSRFORTESSA X-20 flow cytometer. All flow cytometry data were analyzed with FlowJo software.

Transmission electron microscopy (TEM)
TEM was performed at the Bioscience Electron Microscopy Laboratory of the University of Connecticut with standard protocols. In brief, cells were detached with trypsin, washed in PBS and resuspended in 2.0% glutaraldehyde, 1.0% paraformaldehyde, and 3 mM $MgCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.4 for 30 minutes. Cells were gently pelleted and resuspended in 500 µl of 0.1 M sodium cacodylate buffer and enrobed by adding 500 µl of 2% Ultra-low Gelling Temperature agarose (#A5030; Sigma-Aldrich) and centrifuged for 6 minutes at 100 g. After gelling for 30 minutes in ice, pellet was cut and rinsed twice in 0.1 M sodium cacodylate buffer, pH 7.4 for 15 minutes each. Secondary fixation was conducted in 1% osmium tetroxide, 0.8% potassium ferricyanide, and 3 mM $MgCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.4 for 1 hour avoiding light. After rinse in cold Milli-Q distilled water, pellets were dehydrated through a series of graded ethanol. Enblock fixation was conducted with 0.5% uranyl acetate in 70% ethanol for one hour. Dehydration continued through a series of graded ethanol. After clearing twice with acetone for 5 minutes each, cells were embedded in epoxy resin through gradient infiltration and then molded with PELCO® Double End Mold (#10590; Ted Pella, Inc., Redding, CA). Ultrathin (~100 nm) sections were cut with an ultra 45° Diatome™ diamond knife on a Leica Ultracut UCT microtome and collected on 200 mesh copper grids. Sections were counterstained with 4% uranyl acetate in 50% ethanol, rinsed with distilled water, stained with 2.5% Sato's lead citrate, and rinsed again with distilled water. For mouse liver, fresh liver fragments were collected in fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) and chopped into 1 mm cubes. The tissue blocks were rinsed twice in 0.1 M sodium cacodylate buffer, pH 7.4 for 15 minutes each. Secondary fixation was performed in 1% osmium tetroxide, 0.1 M sodium cacodylate buffer, pH 7.4 for 1 hour avoiding light. After sequential rinse with sodium cacodylate buffer and maleate buffer, the tissue blocks were fixed in 1% aqueous uranyl acetate, 0.05% maleate buffer, pH 5.2 for 1 hour. After rinse with maleate buffer, samples were subjected to dehydration, clearing, infiltration, embedding, polymerization and counterstaining steps. Images were obtained using a bright field FEI Tecnai Biotwin G2 transmission electron microscope operated at an accelerating voltage of 80 kV and equipped with an AMT 2k (4 megapixel) XR40 CCD camera.

Seahorse Mito Stress experiment
Kinetic metabolic profiling was done in real time on the fully integrated 24-well Seahorse XFe24 Extracellular Analyzer (Agilent Technologies, Santa Clara, CA) by using the seahorse XF Cell Mito Stress Test Kit (#103015-100) and the SeahorseXFe24 FluxPak (#102340-100) to determine the oxygen consumption rate (OCR) as a surrogate for mitochondrial respiration and the extracellular acidification rate (ECAR) as an indicator for glycolytic capacity of the cells.

Control or PDK4-shRNA cells (40 X 10^3/well) were seeded overnight in Seahorse XF24 V7 PS Cell Culture Microplates (#100777-004) in growth medium. Per the manufacturer's user guide, cells were washed with Seahorse XF Base Medium (#102353-100) and cultured for 1 h at 37°C without CO_2 in mito stress assay media (base medium supplemented with 25 mM glucose, 1 mM pyruvate and 2 mM glutamine). Sensor cartridge was hydrated overnight and calibrated. Afterward, metabolic profiles were determined by injecting oligomycin (1 µmol/L), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 1 µmol/L), rotenone/antimycin A (0.5 µmol/L). All of the above supplies were from Agilent Technologies. After the completion of the assay, medium was removed and cells in the plate were subjected to DNA preparation with a NucleoSpin® Tissue kit (#740952.50; Macherey-Nagel, Düren, Germany). Data were normalized with DNA content and analyzed in Wave Desktop 2.3.

Mitochondrial membrane potential assay

JC1-Mitochondrial Membrane Potential Assay Kit (ab113850; Abcam) was used to determine mitochondrial membrane potential according to the manufacturer's instruction. The emission of fluorescence at 590 nm was determined in a Bio-Tek Clarity™ Microplate Reader.

Identification of electron leakage sites

The measurement of electron leakage sites was determined by Amplex UltraRed assay which has been reported previously.(1, 2) In brief, 10 µl mitochondria (2.2 mg protein/ml) freshly isolated from shCTL and shPDK4 Huh7 cells were laid in a 96-well clear bottom black plate in KHEB medium (120 mM KCl, 5 mM HEPES, 1 mM EGTA (pH 7.2) and 0.3% (w/v) bovine serum albumin). Then, 100 µl assay medium was added to incubate for 15 min at room temperature before fluorescence of the resorufin product of Amplex UltraRed oxidation was read on a Bio-Tek Clarity™ Microplate Reader (λex = 540 nm, λem = 590 nm). Individual assay medium for assay of each site was KHEB medium containing Amplex UltraRed (50 µM), superoxide dismutase (5 U/ml) and horseradish peroxidase (1 U/ml) plus one of the following specific sets of components to drive H_2O_2 production predominantly from a single mitochondrial sites: site I_Q, 5 mM succinate alone; site I_F+DH, 5 mM glutamate plus 5 mM malate with 4 µM rotenone; site II_F, 1 mM succinate with 2.5 µM antimycin A, 4 µM rotenone and 2 µM myxothiazol; site III_{Qo}, 5 mM succinate with 2.5 µM antimycin A and 4 µM rotenone; mGPDH, 25 mM glycerolphosphate with 4 µM rotenone, 2.5 µM antimycin A,1 mM malonate ,and 2 µM myxothiazol. Superoxide dismutase (#S9697), peroxidase from horseradish (#P8250) and all above compounds were from Sigma-Aldrich.

Detection of ROS

Production of reactive oxygen species (ROS) in live cells was measured using DCFDA Cellular Reactive Oxygen Species Detection Assay Kit (ab113851; Abcam). Briefly, control or PDK4 knockdown Huh7 cells (2.5 x 10^4/well) were seeded in a dark, clear bottom 96-well microplate to allow to adhere overnight. DCFDA (25 µM) working solution was loaded and incubated with cells for 1 h at 37°C. Cell fluorescence was immediately analyzed in the presence of 100 µl PBS/well using a fluorescent plate reader with excitation wavelength at 485 nm and emission wavelength at 535 nm. A luminol analogue L-012 (#5085, R&D Systems) was used to detect ROS production in mice liver. Right after mice were sacrificed, liver tissues were immediately homogenized and protein concentration was quantified. 2 mg of tissue lysate was loaded in a 96-well plate in 250 µl PBS, 50 mM L-012 was injected and immediately assayed for luminescence activity in a Bio-Tek Clarity™ Luminescence Microplate Reader.

PDH activity assay

Pyruvate dehydrogenase (PDH) Enzyme Activity Microplate Assay Kit (ab109902; Abcam) was used to evaluate PDH activity. Briefly, Huh7 cells (5 x 10^6) were grown in regular high glucose

DMEM to 70% confluency in 100 mm dish and continued to culture for 48 hours in fresh medium containing either high (4.5 g/L) or low (1.0 g/L) level of glucose. Then, about $1x10^7$ cells were washed with ice-cold PBS and resuspended with 500 µl sample buffer (PBS containing protease and phosphatase inhibitors). Proteins were extracted by adding detergent solution supplied in the kit to samples and centrifuging after incubation. Protein concentration in each sample was determined with the Pierce™ BCA Protein Assay Kit. 1 mg extracted proteins in 200 µl sample buffer were loaded onto each well of the testing plate. Triplicate assays were performed on each sample, and blank reactions were included as negative controls. The subsequent steps were stringently performed according to the manufacturer's instructions. The plate was measured with a plate reader in kinetic mode at 450 nm for 30 min at interval of 30 seconds in room temperature. PDH activity is expressed as Rate (mOD/min) = (Absorbance 2 - Absorbance 1)/Time (minutes) per the manufacturer suggested.

Intracellular NAD+/NADH ratio quantification

The ratio of NAD+ and NADH was determined with NAD+/NADH Quantification Colorimetric Kit (#K337-100; BioVision) according to the manufacturer's instruction. In brief, cells were lysed in NAD+/NADH extraction buffer and filtered through a 10 kDa cut-off Amicon Ultra-0.5 ml Centrifugal Filter (#UFC501096, Emd Millipore). The half of lysates were incubated at 60 °C for 30 min and used to determine NADH levels, and the other half was used to determine total NADt (NAD and NADH) levels. The reactions were prepared in 96-well plates and read at 450 nm using a spectrophotometer.

RT² Profiler PCR Array assay

Total RNA was isolated with TRIzol® RNA Isolation Reagent (#15596018; Thermo Fisher Scientific) and Direct-zol™ RNA MiniPrep Plus (#R2072; Zymo Research, Orange, CA), and then reversely transcribed into cDNA using RT² First Strand Kit (#330401; Qiagen, Germantown, MD). Real time PCR were carried out with proprietary kits Human Cell Death PathwayFinder PCR Array (#PAHS-212ZE-4) and RT² SYBR® Green qPCR Mastermix (#330501) from Qiagen in a Bio-Rad CFX384 C1000 Touch™ Real-Time PCR Detection System. The results were uploaded onto Data Analysis Center on Qiagen website for analysis.

Enzyme-linked immunosorbent assay (ELISA)

Cellular and secretion forms of TNF were determined with a Human TNF alpha ELISA Ready-SET-Go kit (#88-7346-22; eBioscience, San Diego, CA). 2 mg of whole cell lysate per well in 100 µl volume was loaded for the detection of cellular form. 20 ml of culture medium was collected and concentrated using a 3 kDa cut-off Amicon Ultra-15 Centrifugal Filter (#UFC900308, Emd Millipore) to detect the secreted form of TNF. The detection procedures were performed according to the manufacturer's instruction.

Preparation of nuclear and cytoplasmic extracts

Tissue or cultured cells were collected, washed, and lysed in hypotonic buffer (10 mM HEPES-KOH [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM Pefabloc® SC, 0.5% NP-40). After incubation on ice for 5 min, cell lysates were centrifuged for 10 s at 16,000 g at 4 °C. The supernatants (cytoplasmic extracts) were collected and the pellets were washed once with hypotonic buffer, and lysed with high-salt buffer (50 mM Tris [pH 7.4], 450 mM NaCl, 1% NP-40, 1 mM PMSF, 0.2 mM Na_3VO_4, 5 mM β-glycerophosphate, 20% glycerol, 2 mM DTT). Following incubation for 10 min on ice, cell lysates were centrifuged for 15 min at 16,000 g at 4 °C, and the supernatants (nuclear extracts) were collected.

Measurement of NF-κB (p65) activity with nuclear extracts

The transcriptional activity of NF-κB (p65) was measured with a commercial kit (#10007889; Cayman Chemical, Ann Arbor, MI) following the manufacturer's standard protocol with 20 μg prepared nuclear protein. The activity is determined based on a sensitive colorimetric readout at 450 nm.

Immunofluorescence and confocal laser scanning microscopy

The primary antibody used was p65/ RELA (sc-372, Santa Cruz) as 1:200 dilution. Mitochondria were labeled with 50 nM Mito Red (#53271; Sigma-Aldrich) for 30 min in 37°C before fixation. The secondary antibodies were Goat anti-Rabbit IgG (H+L), Alexa Fluor® 488 conjugate (#A31556) and Goat anti-Rabbit IgG (H+L), Alexa Fluor® 405 conjugate (#A11034), all of which were from Thermo Fisher Scientific and used as 1:200 dilution. Coverslips were mounted onto slides with ProLong® Gold Antifade Mountant (#P36934 or #P36935 (with DAPI) from Thermo Fisher Scientific. The immunofluorescence procedure has been described previously.(3) Images were taken under a Leica TCS SP8 confocal microscope with oil immersion lens in sequential scanning mode.

Chromatin immunoprecipitation-qPCR (CHIP-qPCR) and Re-CHIP-qPCR

Enriched chromatin DNA fragments were purified with QIAquick PCR Purification Kit (#28106; Qiagen), and subjected to qPCR using primers listed in the Supporting Table 1. The detailed experimental procedure has been described previously.(4, 5) The data was analyzed with Percent Input Method.

Fractioning of cytosolic, mitochondrial and nuclear extracts

The Mitochondria Isolation Kit for Cultured Cells (#89874; Thermo Fisher Scientific) was used to separate the nucleus, cytosol and mitochondria. The fractions were either lysed/diluted in NETN for co-immunoprecipitation assay or subjected to GSH measurement.

Co-immunoprecipitation (Co-IP) assay

Cells were lysed in NETN lysis buffer (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) containing protease and phosphatase inhibitors. Cellular extracts were incubated with appropriate primary antibody at 4°C overnight, followed by addition of Protein A/G PLUS-Agarose (sc-2003; Santa Cruz) beads for 2 hours at 4°C. Beads were then washed and the immune complexes were subjected to SDS-PAGE followed by immunoblotting with indicated antibodies.

GST Pull-down assay

pGEX-4T1 and pGEX-4T1-PDK4 were used to generate GST and GST-PDK4 proteins in BL21 (DE3) Escherichia coli cells by isopropy-β-D-thiogalactoside (IPTG) induction (37°C 3 hr, 0.1 mM IPTG). The protein purification and GST pull-down experiment were performed as previously described.(6) Briefly, equal amounts (5 μg) of GST and GST-PDK4 proteins were immobilized on 50 μl of Gutathione-Sepharose 4B beads (#17075601; GE Healthcare Bio-Sciences, Pittsburgh, PA). The coated beads were then incubated with 25 μl of flag-p65 generated from in vitro transcription/translation using TnT® T7 Quick Coupled Transcription/Translation System (#L1171; Promega) in GST pull-down binding buffer (10 mM HEPES [pH 7.9], 3 mM $MgCl_2$, 100 mM KCl, 5 mM EDTA, 5% glycerol, 0.5% CA-630) for 2 h at 4°C. After wash with GST pull-down binding buffer, the proteins bound to the beads were eluted with SDS loading buffer and analyzed by immunoblotting with indicated antibodies.

Promoter mutagenesis

The mutagenesis was performed using Q5® Site-Directed Mutagenesis Kit (#E0554S; New England BioLabs, Ipswich, MA) following manufacturer's instruction. The primers for substituting

the predicted NF-κB sites in *TNF*, *BIRC3* and *CCDC103* promoters with a non-consensus sequence (TTTGCCAAAGGG) were designed using NEBaseChanger. The primer sequences were included in Supporting Table 1.

Luciferase reporter assay

293T cells were seeded in 24-well plates at 80% confluence and transiently transfected with indicated plasmids using X-tremeGENE™ HP DNA Transfection Reagent. In all transfections, the total quantity of DNA used was kept constant and the Renilla luciferase expression vector was included as an internal control. 24 hr after transfection, cells were harvested and luciferase activities were determined with a Dual-Luciferase® Reporter Assay System (#E1910; Promega) in a Bio-Tek Clarity™ Luminescence Microplate Reader. Firefly luciferase values in each well were normalized to the Renilla luciferase activity. Experiments were performed in triplicate.

Mice information

Wild-type (WT) and *Pkd4*$^{-/-}$ mice were handled in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut. All mice were of C57BL/6J (inbred strain) genetic background. In brief, *Pdk4*$^{-/-}$ mice were backcrossed with the C57BL/6J WT to the 10th generation with >99.99% pure C57BL background. The heterozygous *Pdk4*$^{-/+}$ mice (11th generation) were intercrossed with each other to generate offspring including homozygous *Pdk4*$^{-/-}$ and *Pdk4*$^{+/+}$ (WT) mice for most experiments. Additional mice were from littermate mating of the same generation of *Pdk4*$^{-/-}$ and *Pdk4*$^{-/-}$ mice, and *Pdk4*$^{+/+}$ and *Pdk4*$^{+/+}$ mice. We purchased 3-4 week-old C57BL/6J (Stock No.000664) WT from Jackson Laboratory when encountering shortage of WT mice and acclimated the mice until 8-week-old under the same conditions in the same room with other mice at the UConn animal facility. To minimize potential microbiome effects, WT or *Pkd4*$^{-/-}$ mice were cohoused in clean and ventilated cages bedded with Teklad aspen sani chips (#7090A; Envigo Bioproducts, Inc, Madison, WI) in the Animal Care Services Facility at the University of Connecticut. Breeding mice were fed with irradiated sterile Teklad S-2335 mouse breeder diet (#7904; Envigo Bioproducts, Inc.). Experimental adult mice were fed with irradiated Teklad Global 18% Protein (#2918; Envigo Bioproducts, Inc.). Mouse diet composition is available on http://www.envigo.com). Mice were maintained in a 24-hour light/dark cycle with free access to water.

Mice primary hepatocytes isolation, culture and treatment

Primary hepatocytes isolation and culture has been described previously.(7) In brief, mice were anesthetized with Isofluorane. Liver was sequentially perfused with pre-warmed solution I (HBSS containing 0.1 mM EGTA, 5.5 mM Glucose, 1% Pen/Strep) for 10 min, and solution II (HBSS containing 1.5 mM $CaCl_2$, 5.5 mM Glucose, 1% Pen/Strep and 0.04% collagenase I) for 15 min through PV (portal vein) cannulation with IVC (inferior vena cava) drainage method. Strained hepatocytes were washed and cultured with William E medium containing 2 mM glutamine, 1% Pen/Strep and 5% FBS in type I collagen-coated tissue culture plates. Hepatocytes were treated with H2O2 or TNF of different concentrations as indicated. 10 μg/ml cycloheximide was used to sensitize the apoptosis-inducing effect of TNF.

In vivo transfection, anti-CD95 injection and liver histology analysis

Two-month-old male mice were intravenously (tail) injected with plasmids (50 μg per mouse) using TurboFect in vivo Transfection reagent (#R0541; Thermo Fisher Scientific) according to manufacturer's general protocol. 3 days after plasmids injection, mice were injected i.p. with purified hamster Anti-Mouse CD95 (Jo2) (#554255; BD Pharmingen, San Diego, CA) diluted in 200 μl sterile PBS. Mice were sacrificed at different time points after injection. At the time of sacrifice, livers were rapidly spliced into several pieces; some were snap frozen for subsequent

RNA or protein extraction, some were homogenized for cellular extracts preparation, and others were formalin-fixed for H&E or TUNEL staining.

ALT measurement
Serum ALT was detected by Infinity™ ALT (GPT) Liquid Stable Reagent (#TR71121; Thermo Fisher Scientific).

H&E staining
Mice liver samples were fixed in 10% buffered formalin (#23-245684; Fisher Scientific, Pittsburgh, PA) and sent to the Department of Pathology at University of Connecticut for embedding, sectioning and hematoxylin-eosin staining.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)
The staining was performed in an In Situ Cell Death Detection Kit, Fluorescein (#11684795910; Sigma-Aldrich) following the manufacturer's instruction. The nucleus was counterstained with DAPI. The fluorescence was examined and images were taken under a Leica TCS SP8 confocal microscope with 40 or 63 × oil immersion lens in sequential scanning mode.

Measurement of reduced glutathione (GSH)
Levels of reduced GSH in subcellular fractions or mouse liver tissues were measured using GSH-Glo™ Glutathione Assay kit (#V6911; Promega) according to the manufacturer's protocol.

Statistical Analysis
Data are shown as the mean ± standard error of the mean (SEM) and are representative of at least three independent experiments from triplicate assays. Statistical analysis was carried out using Student's t test between two groups and one way ANOVA among multiple groups. $P < .01$ was considered statistically significant.

Supplementary References

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Author names in bold designate shared co-first authorship.

Supporting Table 1. Primers used in the study

Primers used in RT-qPCR	
name	sequence (5>3)
hGAPDH	F: 5'-GAAATCCCATCACCATCTTCCA-3';
	R: 5'-CAGCATCGCCCCACTTG-3';
hPDK4	F: 5'-TCCCGGAATGCTCCTTTG-3';
	R: 5'-CTTTGCATACAGACGAGAAATTGG-3';
hBIRC3 (hcIAP2)	F: 5'-AGACAGAGTGGCTTGCTTTGC-3';
	R: 5'-GCATTATCCTTCGGTTCCCAAT-3';
hCCDC103	F: 5'-TGCAAGGGCTTGTTTCAGAA-3';
	R: 5'-GCCCCTCCTTCACGGATCT-3';
hTNF	F: 5'-TGCTCCTCACCCACACCAT-3';
	R: 5'-GGAGGTTGACCTTGGTCTGGTA-3';
hACTb	F: 5'-GCGCGGCTACAGCTTCA-3';
	R: 5'-CTTAATGTCACGCACGATTTCC-3';
hBCL2	F: 5'-CATGTGTGTGGAGAGCGTCAA-3';
	R: 5'-TCATCCACAGGGCGATGTT-3';
hTRAF2	F: 5'-TGGCTGGCCGCATACC-3';
	R: 5'-TGTAGCCGTACCTGCTGGTGTA-3';
hA20	F: 5'-AACGGTGACGGCAATTGC-3';
	R: 5'-TGAACGCCCCACATGTACTG-3';
hcIAP1	F: 5'-TGCCTGTGGTGGGAAGCT-3';
	R: 5'-CCGGTGTTCTGACATAGCATCA-3';
hc-FLIP	F: 5'-TGGGCCGAGGCAAGATAA-3';
	R: 5'-TTTCTCCAACTCAACCACAAGGT-3';
mTnf	F: 5'-CAGCCGATGGGTTGTACCTT-3';
	R: 5'-GGCAGCCTTGTCCCTTGA-3';
mBirc3 (mcIAP2)	F: 5'-TGGGTCAGTCTGCTTCGAGAT-3';
	R: 5'-AATACGGGCTGCGTGTGTCT-3';
mCcdc103	F: 5'-AGCCATGCAGAGCGAGAGA-3';
	R: 5'-TGCTCATGGCTTGCAACTTC-3';
mActb	F: 5'-CGATGCCCTGAGGCTCTTT-3';
	R: 5'-TGGATGCCACAGGATTCCA-3';
mBcl2	F: 5'-ATCTTCTCCTTCCAGCCTGAGA-3';
	R: 5'-ACGTCCTGGCAGCCATGT-3';
mTRAF2	F: 5'-AGATCCCTCGGGAGACGTTT-3';
	R: 5'-AGAGAACCCGGCATTTGCT-3';
mA20	F: 5'-TGAAAACCAATGGTGATGGAAA-3';
	R: 5'-GAACACCCCACATGTACTGACAA-3';
mcIAP1	F: 5'-TGGTTAAAGCAGCCTTGGAAA-3';
	R: 5'-TGCCGCTGAACCGTCTGT-3';
mc-FLIP	F: 5'-GGACCACCTGCGCAGAAA-3';
	R: 5'-TCTCCATCAGCAGGACCCTATAA-3';
Primers used in CHIP-qPCR assay	
name	sequence (5>3)
CHIP TNF	F: 5'-TGTCCCCAACTTTCCAAATCC-3';
	R: 5'-GTGGCGTCTGAGGGTTGTTT-3';
CHIP BIRC3	F: 5'-TTCAGTAAATGCCGCGAAGA-3';
	R: 5'-CGCGAGTCTCACGCTGTCT-3';

Supporting Table 1. Continued

CHIP CCDC103	F: 5'-AGGACCAGTTCCGAGGTATGC-3';
	R: 5'-CAGCTGTGGTTGCCTAGCAA-3';
CHIP c-FLIP-proximal	F: 5'-GAGACCAGCCTTGCCAACAT-3';
	R: 5'-CTGTCGCCCAGGCTAGAAG-3';
CHIP c-FLIP-distal	F: 5'- CCAGGCTGGTCTTGAACTCC-3';
	R: 5'- GCTAGGTGTGCCACTACAGG-3';
Primers used in cloning	
name	sequence (5>3)
promTNF	F: 5'-AGTCGGTACCTGTAGCGGCTCTGAGGAATG-3';
	R: 5'- AGTCAAGCTTGCCGTGGGTCAGTATGTGAG-3';
promBIRC3	F: 5'-AGTCGGTACCTCAGCTCAGTCCAATTGCGT-3';
	R: 5'- AGTCAAGCTTAGCCCAGTCTTTTCAAGCGA-3';
promCCDC103	F: 5'-AGTCGGTACCACTCTCCAACTTCCCACTTTCA-3';
	R: 5'- AGTCAAGCTTACTAACCCTTCCGTCCCCTT-3';
Flag-PDK4	F: 5'-AGCTGCGGCCGCGAAGGCGGCCCGCTTCGTG-3';
	R: 5'-AGCTGGATCCTCACATGGCCACTTCTTTTGC-3';
GFP-PDK4	F: 5'-AGCTCTCGAG**CCACC**ATGAAGGCGGCCCGCTTCGTG-3'
	R: 5'-AGCTGGATCCTCACATGGCCACTTCTTTTGC-3';
Primers used in mutagenesis	
name	sequence (5>3)
TNF ∆site1	F: 5'-aaagggCAAGGAAGTTTTCCGCTG-3';
	R: 5'-ggcaaaATGAGCTCATCTGGAGGAAG-3';
TNF ∆site2	F: 5'-aaagggATGCTTGTGTGTCCCCAAC-3';
	R: 5'-ggcaaaTCACACTCCCCATCCTCC-3';
TNF∆site3	F: 5'-aaagggCAACCCCGTTTTCTCTCC-3';
	R: 5'-ggcaaaGTGTTGAGTCCTGAGGCC-3';
TNF ∆site4	F: 5'-aaagggGGCTTGTCCCTGCTACCC-3';
	R: 5'-ggcaaaGGGGAGTGAAATCACCCC-3';
TNF ∆site5	F: 5'-aaagggGGGTGATTTCACTCCCCGG-3';
	R: 5'-ggcaaaAGACCCCACTGGGGCAGG-3';
TNF ∆site6	F: 5'-aaagggCTTAACGAAGACAGGGCC-3';
	R: 5'-ggcaaaTACTCGACTTCCATAGCC-3';
BIRC3 ∆site1	F: 5'-aaagggAGTCCTAAAAGGAAAGCAC-3';
	R: 5'-ggcaaaAGCGGTAATAACCACACAC-3';
BIRC3 ∆site2	F: 5'-aaagggGTGGGTTTGCCAGGCCAC-3';
	R: 5'-ggcaaaATGACCCAAAAGCATGACTCTTAAC-3';
BIRC3 ∆site3	F: 5'-aaagggAGTAGCCAACTCCACCCT-3';
	R: 5'-ggcaaaTCCTTTATAGCCTTTGATCTCAC-3';
CCDC103 ∆site1	F: 5'-aaagggAGAAAGCAAGGGGACCAA-3';
	5'-ggcaaaTGATAAACCAGAACAGGAGG-3';
CCDC103 ∆site2	F: 5'-aaagggCTGTTCTGGTTTATCAGGGGATCCCCAAAGAAAG-3';
	R: 5'-ggcaaaGCGCAGACCACGCCCGCA-3';
CCDC103 ∆site3	F: 5'-aaagggAGACGAGTAGCCCAAGACG-3';
	R: 5'-ggcaaaACTCCGCCTTGCCAGTCC-3';
CCDC103 ∆site4	F: 5'-aaagggGCCGCTGCCGGAGATCGT-3';
	R: 5'-ggcaaaAGCTGAGCAGGCGAGAGG-3';

h, human; m, mouse; prom, promoter; red color indicates restriction enzyme site; bold indicates Kozak sequence.