Supplemental Methods

Cell viability and cell death assay

Cells were seeded into 96-well plates and incubated with the indicated treatments. Subsequently, 100 µl fresh medium was added to cells containing 10 µl Cell Counting Kit-8 (CCK-8) solution (Dojindo Laboratories, Tokyo, Japan) and incubated for 1 h (37℃, 5% CO_2). Absorbance at 450 nm was measured using a microplate reader (Tecan, Morrisville, NC, USA). Cell death was analyzed using propidium iodide (#P4170, Sigma-Aldrich, St. Louis, MO, USA) staining.

Clonogenic cell survival assay

Long-term cell survival was monitored in a colony formation assay. In brief, 1,000-2,000 cells were reseeded into 24-well plates after treatment with indicated drugs or inhibitors for 24 hours. The cells were allowed to grow for the next 10 to 12 days to allow colony formation and the colonies were visualized using crystal violet staining.

Time-lapse microscopy assay

Living-cell imaging of PANC1 cells was performed on six-well plates using an EVOS FL Auto Cell Imaging System (Invitrogen) with incubation chambers at 5% CO_2 and 37˚C. Images were acquired every 30 min for 24 hours, and movies were made via the system's built-in software.

Western blot analysis

Proteins in the cell lysate were resolved on 4%-12% Criterion XT Bis-Tris gels (Bio-Rad, Hercules, CA, USA) and transferred to a nitrocellulose membrane (pore size 0.22 µM). After blocking with 5% milk, membranes were incubated for two hours at 25℃ or overnight at 4℃ with various primary antibodies (1:500-1:1000). After incubation with peroxidase-conjugated secondary antibodies (1:3000) for one hour at routine temperature, the signals were visualized using enhanced or super chemiluminescence (Pierce, Rockford, IL, USA) and by exposure to X-ray films. Relative band intensity was quantified using the Gel-pro Analyzer® software (Media Cybernetics, Bethesda, MD, USA).

Immunoprecipitation analysis

Cells were lysed at 4°C in ice-cold radioimmunoprecipitation assay buffer (Millipore, USA) and cell lysates were cleared by brief centrifugation (12,000 g, 10 min). Concentrations of proteins in the supernatant were determined using BCA assay.

Prior to immunoprecipitation, samples containing equal amounts of proteins were pre-cleared with protein A or protein G agarose/sepharose (Millipore, USA) (4°C, 3 h), and subsequently incubated with various irrelevant IgG or specific antibodies (5 μg/mL) in the presence of protein A or G agarose/sepharose beads for 2 h or overnight at 4°C with gentle shaking. Following incubation, agarose/sepharose beads were washed extensively with phosphate buffered saline (PBS) and proteins were eluted by boiling in 2 × sodium dodecyl sulfate sample buffer before sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Cell cycle analysis

Cells were collected, trypsinized, and fixed in 70% ethanol overnight. Cells were centrifuged at 1,000 rpm for 5 min, washed once with pre-cooled PBS, and incubated with propidium iodide staining solution for at least 30 min at room temperature before analysis. Analysis of the percentage of total cells for each phase of the cell cycle (G0/G1, S, and G2/M) was performed using a Muse® Cell Analyzer (EMD Millipore) in accordance with the manufacturer's guidelines.

Caspase-3 activity assay

The activity of caspase-3 in cell lysates was assayed by the Caspase-3 Activity Assay Kit (#5723, Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's protocol. It contained a fluorogenic substrate (N-Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin or Ac-DEVDAMC) for caspase-3. During the assay, activated caspase-3 cleaved this substrate between DEVD and AMC, generating highly fluorescent AMC that can be detected using a fluorescence reader with excitation at 380 nm and emission between 420-460 nm.

Hematoxylin and eosin (H&E) tissue staining

Tissues were embedded in optimum cutting temperature cryomedium (Sakura Finetek, Zoeterwoude, the Netherlands) and cut into 4 μm sections. The deparaffinized sections were then stained using the standard H&E method. The stained slides were observed using an EVOS FL Auto Cell Imaging System (Invitrogen).

ELISA analysis

Alanine aminotransferase ("ALT", Bioo Scientific Corporation, #3460-01), blood urea nitrogen ("BUN", Bioo Scientific Corporation, #5602-01), high mobility group box 1 ("HMGB1", Shino-Test Corporation, #326054329), and adenosine triphosphate ("ATP", Abcam, #ab83355) concentrations in serum or cell culture medium were

measured using ELISA according to the manufacturer's protocol.

Flow cytometry

The FITC- or allophycocyanin (APC)-conjugated antibodies specific to CD8a (#100705) and IFN-γ (#505809) were purchased from BioLegend. Single-cell suspensions were prepared from isolated tumor samples and stained with anti-CD8 in the presence of FcR-Block (BioLegend). After the wash, cells were fixed, permeabilized, and stained with IFN-γ-specific or control isotype antibodies for 30 min on ice. Multiple-color FACS analysis was performed using an Accuri C6 flow cytometer (BD). Dead cells were excluded by the Fixable Aqua Dead Cell Stain Kit (Invitrogen).

Bioinformatics analysis

Kaplan-Meier analysis of the prognosis of pancreatic cancer patients was performed on the R2 website (http://r2.amc.nl). This online tool can be used to analyze a large collection of public genomic data. The dataset was from the AMC cohort study of 130 pancreatic cancer patients.

RNAi and gene transfection

The human RIPK1-shRNA-1 (5'-CCGGCGGAACAGATTCTGGTGTCTTCTCGAGAAGACACCAGAATCTGTTCCGTTTTTTG-3'), RIPK1-shRNA-2 (5'-CCGGGCAGTCTTCAGCCCATTAAATCTCGAGATTTAATGGGCTGAAGACTGCTTTTTTG-3'), RIPK3-shRNA-1 (5'-CCGGCTGAGAGACAAGGCATGAACTCTCGAGAGTTCATGCCTTGTCTCTCAGTTTTT-3'), RIPK3-shRNA-2 (5'-CCGGGCACTCTCGTAATGATGTCATCTCGAGATGACATCATTACGAGAGTGCTTTTT-3'), MLKL-shRNA-1 (5'-CCGGCCTCTGACAGTAACTTTGATACTCGAGTATCAAAGTTACTGTCAGAGGTTTTTTG-3'), MLKL-shRNA-2 (5'-CCGGCCTTCGGCATTGGGTTATCTACTCGAGTAGATAACCCAATGCCGAAGGTTTTT-3'), AURKA-shRNA-1 (5'-CCGGCACATACCAAGAGACCTACAACTCGAGTTGTAGGTCTCTTGGTATGTGTTTTT-3'), AURKA-shRNA-2 (5'-CCGGCCTGTCTTACTGTCATTCGAACTCGAGTTCGAATGACAGTAAGACAGGTTTTT-3'), and control empty shRNA (#SHC001) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The stable knockdown cells were selected by adding puromycin. The expression plasmids for pBABE-puro-AURKA-WT,

pBABE-puro-AURKA-D274A, pcDNA3-HA-GSK3β-WT, and pcDNA3-HA-GSK3β-S9A were purchased from Addgene. A synthetic Flag-tag sequence was added at the N-terminus of AURKA. Transfection with shRNA or cDNA was performed with Lipofectamine 3000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions.

Quantitative real time polymerase chain reaction assay (Q-PCR)

Total RNA was extracted using RNeasy Plus Mini Kit (#74134, QIAGEN, Germantown, MD, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of RNA using the iScript cDNA Synthesis kit (#1708890, Bio-Rad, Hercules, CA, USA). Described briefly, 20 μl reactions were prepared by combining 4 μl iScript Select reaction mix, 2 μl gene-specific enhancer solution, 1 μl reverse transcriptase, 1 μl gene-specific assay pool (20×, 2 μM), and 12 μl RNA diluted in RNase-free water. cDNA from various cell samples was then amplified using Q-PCR with specific primers (human *RIPK1*: 5'-TATCCCAGTGCCTGAGACCAAC-3' and 5'-GTAGGCTCCAATCTGAATGCCAG-3'; human *RIPK3*: 5'-GCTACGATGTGGCGGTCAAGAT-3' and 5'-TTGGTCCCAGTTCACCTTCTCG-3'; human *MLKL*: 5'-TCACACTTGGCAAGCGCATGGT-3' and 5'-GTAGCCTTGAGTTACCAGGAAGT-3'; human *AURKA*: 5'-GCAACCAGTGTACCTCATCCTG-3' and 5'-AAGTCTTCCAAAGCCCACTGCC-3'; human *GADPH* (5'-GTCTCCTCTGACTTCAACAGCG-3' and 5'-ACCACCCTGTTGCTGTAGCCAA-3') using a CFX96 Touch™ Real-Time PCR Detection System (#1855195, Bio-Rad) with SsoFast™ EvaGreen® Supermix (#1725201, Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data are expressed as means ± SD or presented as median value (black line), interquartile range (box), and minimum and maximum of all data (black line). Unpaired Student's t tests were used to compare the means of two groups. One-way Analysis of Variance (ANOVA) was used for comparison among the different groups. When ANOVA was significant, *post hoc* testing of differences between groups was performed using the Least Significant Difference (LSD) test. The Kaplan-Meier method was used to compare differences in mortality rates between groups. A p-value < 0.05 was considered statistically significant.

Supplemental Figures

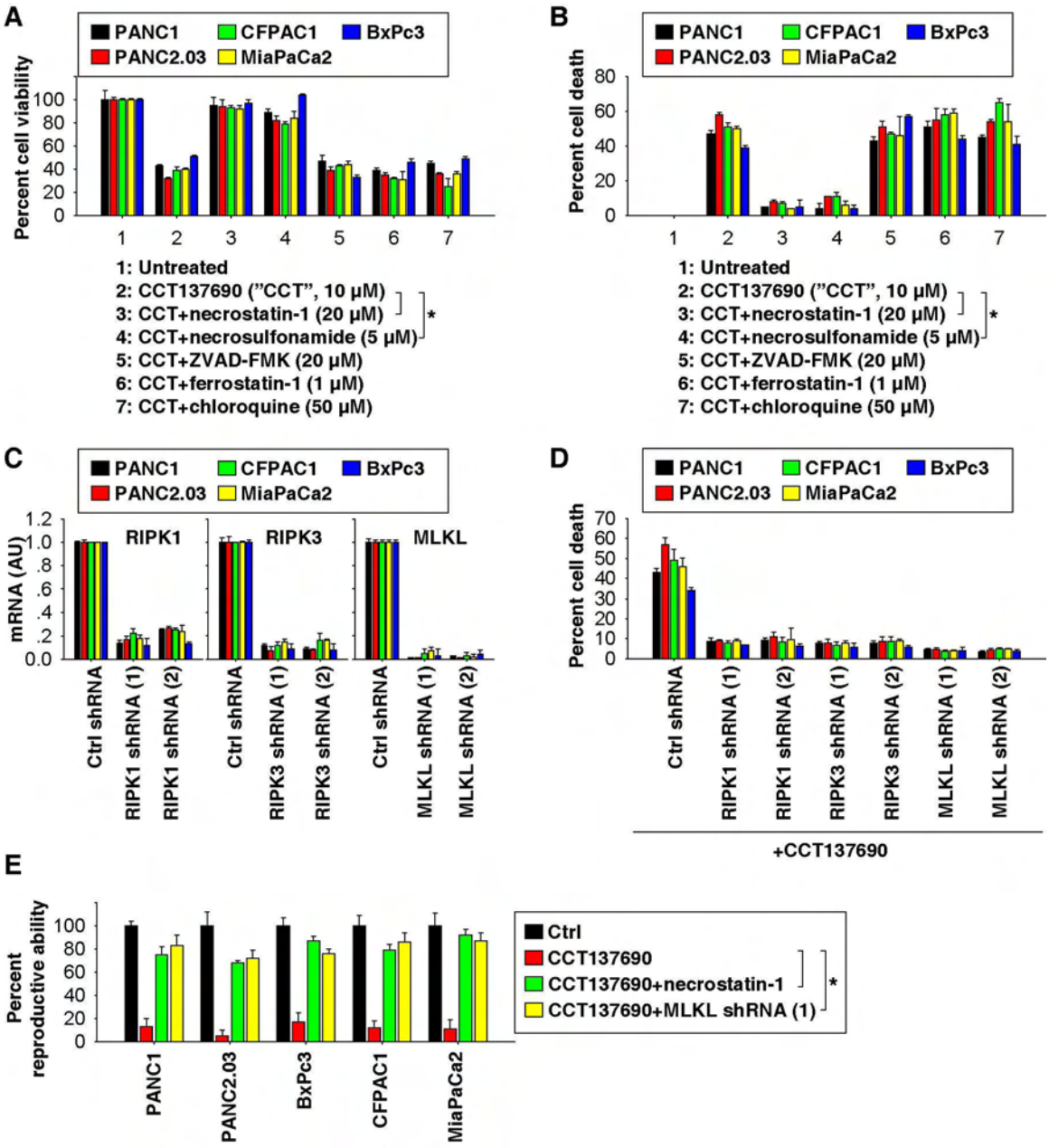

Figure S1. Induction of necroptosis contributes to the anticancer activity of CCT137690. (A, B) Indicated PDAC cells were treated with CCT137690 in the absence or presence of indicated cell death inhibitors for 24 hours. Cell viability and cell death was assayed (n=3, *p < 0.05). (C, D) Knockdown of RIPK1, RIPK3, and MLKL by two different shRNAs inhibited CCT137690 (10 μM, 24 hours)-induced cell death. (E) The effect of pharmacological or genetic inhibition of necroptosis by necrostatin-1 or knockdown of MLKL on long-term clonogenic survival in indicated PDAC cells following CCT137690 treatment (n=3, *p < 0.05).

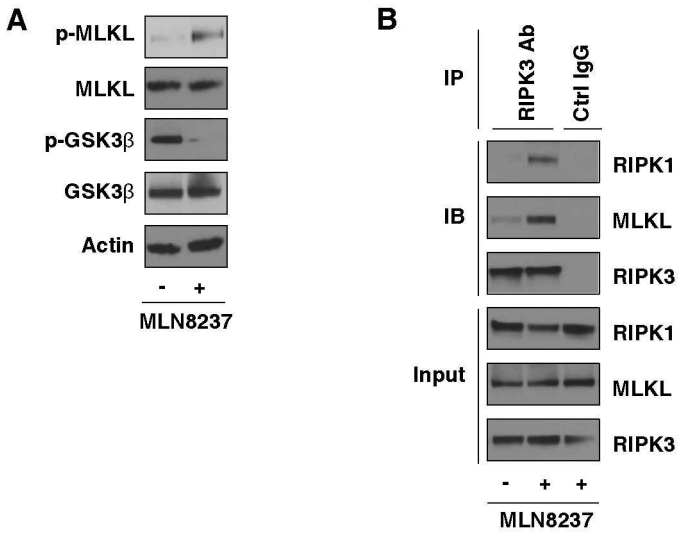

Figure S2. MLN8237 induces necroptosis by promoting necrosome formation. (A) Western blot analysis of indicated proteins in PANC1 cells following treatment with MLN8237 (10 μM) for 24 hours. (B) Immunoprecipitation (IP) analysis of the levels of RIPK3 binding to RIPK1 and MLKL in PANC1 cells following treatment with MLN8237 (10 μM) for 24 hours.

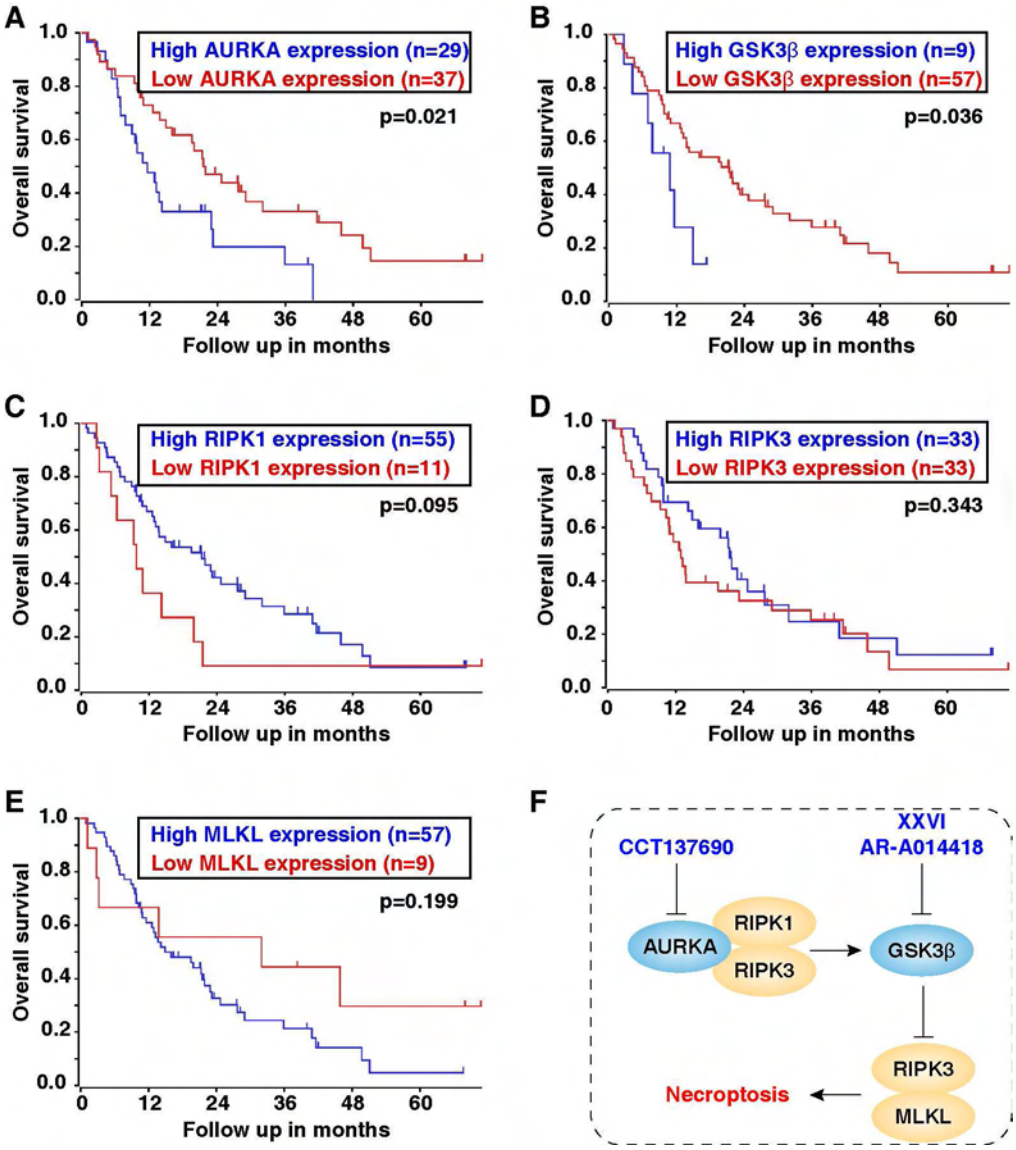

Figure S3. AURKA and GSK3β are associated with poor prognosis in human pancreatic cancer patients. (A-E) Prognostic value of AURKA (A), GSK3β (B), RIPK1 (C), RIPK3 (D), and MLKL (E) in pancreatic cancer patients. (F) Schematic depicting the role of AURKA and GSK3β in the negative regulation of necroptosis.

Supplemental VIDEOS

Video S1. PANC1 cells were treated with CCT137690 (10 μM) for 0-24 hours in six-well plates. Images were acquired every 30 min for 24 hours with the EVOS FL Auto Cell Imaging System and videos were captured via built-in system software.