Supplementary information for:

Differential Activity of GSK-3 Isoforms Regulates NF-κB and TRAIL- or TNFα-Induced Apoptosis in Pancreatic Cancer Cells

Supplementary information contains:

Detailed description of

Cell Surface DR4 and DR5 expression by flow cytometer

Proximity ligation assay

Table S1 (shRNA target sequences)

Table S2 (qRT-PCR primer sequences)

Table S3 (ChIP PCR primers sequences)

Figure legends (S1−S5)

Analysis of Cell Surface DR4 and DR5 Expression

Aliquots containing 2 × 10^5 pancreatic cancer cells were fixed with 4% (w/v) paraformaldehyde for 30 min. After 2 washes in PBS containing 2% (v/v) FBS (PBS/FBS), cells were incubated with saturating amounts (1.0 μg) of DR4, or DR5 monoclonal antibodies on ice for 45 min, washed twice with PBS/FBS, incubated with PE- or APC-conjugated anti-mouse IgG for an additional 30 min on ice, washed, and subjected to flow microfluorimetry. To quantitate cell surface antibody binding, beads conjugated with known amounts of PE (Quantibrite^{TM} PE beads, BD Biosciences) were run in parallel, and fluorescence histograms were analyzed as suggested by the supplier.

Proximity ligation assay (PLA)

PLA was used to determine the interactions of p50/p65 in MEF cells. Briefly, WT and GSK-3β null MEFs were cultured on 10-well 7 mm HTC Super Cured glass slides (Erie Scientific Company, Portsmouth, NH, USA) and stimulated with 2

μg/ml of TNFα for the indicated periods of time. Cells were fixed and permeablized as described[53] and blocked for 1 h by incubation at 37°C with the blocking solution in a pre-heated humidity chamber, followed by incubation with the following primary antibodies: mouse mAb against p50 (SC8414, 1:50; Santa Cruz) and rabbit mAb against p65 (1:300; Cell Signaling Technologies) for 2 hr. The cells were washed with buffer A (10 mM Tris, 150 mM NaCl, and 0.05% Tween-20) at room temperature and incubated for 2 h in a pre-heated humidity chamber at 37°C with PLA probes detecting rabbit or mouse antibodies (Duolink II PLA probe anti-Rabbit plus and Duolink II PLA probe anti-mouse minus diluted in the antibody diluent to a concentration of 1:5). After washing with wash buffer A at room temperature, cells were incubated in a pre-heated humidity chamber at 37°C for 30 min with the ligation solution (Duolink II Ligation stock 1:5 and Duolink II Ligase 1:40). Detection of the amplified probe was accomplished with the Duolink II Detection Reagents Red Kit by PCR amplification for 90 min in 37°C humidity chamber. After extensive washing with wash buffer B (200 mM Tris and 100 mM NaCl) and finally with 100x diluted wash buffer B, the cells were mounted using the mounting medium with DAPI. The samples were observed by confocal microscopy. p50/p65 heteromers was visualized as punctate red fluorescent spots detected by confocal microscopy. As negative controls for the technique, the same procedure was done but omitting the primary antibodies.

Table S1. pLKO-shRNA expression plasmids

Target Gene Names	Target sequences *(Data shown in figures is based on **target-A** unless specified)*
GSK-3α (A)	5′-GACTAGAGGGCAGAGGTAAAT-3′
GSK-3α (B)	5′-CCGGGAACAAATCCGAGAGAT-3′
GSK-3α (C)	5′-CAAGCACCCTTCCACTTCCAT-3′
GSK-3β (A)	5′-CATGAAAGTTAGCAGAGACAA-3′
GSK-3β (B)	5′-CACTGGTCACGTTTGGAAAGA-3′
GSK-3β (C)	5′-CCACTCAAGAACTGTCAAGTA-3′
XIAP/BIRC4(A)	5′-AGCTGTAGATAGATGGCAATA-3′
XIAP/BIRC4	5′-GCACTCCAACTTCTAATCAAA-3′
cIAP2/BIRC3 (A)	5′-GCACTACAAACACAATATTCA-3′
cIAP2/BIRC3 (B)	5′-CAGTTCGTACATTTCTTTCAT-3′
BCL-xL/BCL2L1 (A)	5′-GTGGAACTCTATGGGAACAAT-3′
BCL-xL/BCL2L1 (B)	5′-CGACGAGTTTGAACTGCGGTA-3′

Table S2. qRT-PCR primers

Gene names	Primer sequences
RPLP0	Forward 5′-AGATCCGCATGTCCCTTC**-3′** Reverse 5′-CCTTGCGCATCATGGTGTT**-3′**
IκBα/NFKBIA	Forward 5′-AAAGCCAGGTCTCCCTTCAC**-3′** Reverse 5′-CAGCAGCTCACCGAGGAC**-3′**
BCL-XL/BCL2L1	Forward 5′-CTGCTGCATTGTTCCCATAG**-3′** Reverse 5′-TTCAGTGACCTGACATCCCA**-3′**
cIAP2/BIRC3	Forward 5′-GTCAAATGTTGAAAAAGTGCCA**-3′** Reverse 5′-GGGAAGAGGAGAGAGAAAGAGC**-3′**
RPLP0	Forward 5′-GAAACTGCTGCCTCACATCCG**-3′** Reverse 5′-CTGGCACAGTGACCTCACACG**-3′**
mIκbα	Forward 5′-GTCTCCCTTCACCTGACCAA**-3′** Reverse 5′-CAGCAGCTCACGGAGGAC**-3′**
mBcl-xL	Forward 5′-GCTGCATTGTTCCCGTAGAG**-3′** Reverse 5′-GTTGGATGGCCACCTATCTG**-3′**
mcIAP2/Birc3	Forward 5′-TCTGGGGATGTAGTTTTGTGC**-3′** Reverse 5′-CCGGAGATCAGAGGTCATTG**-3′**

Table S3

ChIP-primers	
Bcl-xL (p1)	Forward 5′-AGGAAGGCATTTCGGAGAAG-3′ Reverse 5′-TTTGTGGGTCTTACGAAGGTC-3′
Bcl-xL (p2)	Forward 5′-AGAGCAAACCAGCGGCATTTGT-3′ Reverse 5′-TTCAAGGTTTCAGTGAGGGACGCA-3′
Bcl-xL (p3)	Forward 5′-TAGCTCAACGAGAGAGGTTGGT-3′ Reverse 5′-AGAAGAAGCATCGCCTTGTGGT-3′
cIAP2 (p1)	Forward 5′-CGCGAAGATATGCCACGGTTAA-3′ Reverse 5′-GGGAACTCCAGCGGTAATAACCAC-3′
cIAP2 (p2)	Forward 5′-TGTAAAGTGTATGGCGGATGG-3′ Reverse 5′-TTCATTGCTCGTGGGTCAG-3′
cIAP2 (p3)	Forward 5′-TGGACAGCCAAGTCCACCTAAACA-3′ Reverse 5′-TTCTGTGGAAACAGCATCCAGTGC-3′
IκBα/NFKBIA (p1)	Forward 5′-CCAGCTCAGGGTTTAGGCTTCTTT-3′ Reverse 5′-GGAATTTCCAAGCCAGTGAGACCA-3′
IκBα/NFKBIA (p2)	Forward 5′-TGCAAAGAGCCTGGTATAGGCAGA-3′ Reverse 5′-ACGCAAGAGTGGAAATGATGGCTG-3′
IκBα/NFKBIA (p3)	Forward 5′-AATTCAGTCCATGGCTTGCAG-3′ Reverse 5′-GTTGGACCCATTCACGCCAC-3′

Figure legends

Figure S1. GSK-3i inhibits pancreatic cancer cell proliferation. (a-e) Cells were treated with diluent or indicated concentrations of GSK-3i (LY2064827) for the indicated periods of time. MTS assay was performed to determine cell proliferation. (f) IC_{50} values of GSK-3i for inhibition of cell proliferation in tested pancreatic cancer cell lines.

Figure S2. **GSK-3i sensitizes TRAIL-induced apoptosis.** (a) Pancreatic cancer cells were treated with indicated concentrations of TRAIL for 24 h to determine cell proliferation by MTS assay. (b-d) Cells were treated with TRAIL alone or in combination with GSK-3i for 24 h, then stained with PI for analysis of subdiploid population by flow cytometery. Results of three independent experiments are shown as mean ± SD.

Figure S3. GSK-3i and TRAIL receptor DR4 and DR5 expression. (a) DR4 and DR5 mRNA expression determined by qRT-PCR in HupT3 and Panc04.03 cells. (b) Flow cytometric analysis to determine the membrane DR4 and DR5 expression. Pancreatic cancer cells were treated with 0.5 μM of GSK-3i for 24 h before RNA extraction for qRT-PCR (a) or stained with DR4 and DR5 antibodies for flow cytometer analysis (b).

Figure S4. Suppression of GSK-3α or GSK-3β sensitizes TRAIL-induced apoptosis. (a) Efficient lentiviral shRNA-mediated knockdown of GSK-3α or GSK-3β in Panc04.03 cells with distinct shRNA target sequences was confirmed by western blot analysis. (b, c) HupT3 cells suppressed of either GSK-3α or GSK-3β isoform by lentiviral shRNA expression sensitize to TRAIL-induced apoptosis as demonstrated by increased PARP and Caspase3 cleavage (b) and annexin V/PI staining (c). Percentage of apoptotic cells was calculated based on PI/Annexin V staining and flow cytometer analysis(c).

Figure S5. Effect of GSK-3β on TNFα-induced BCL-XL, cIAP2 and IKBα expression. WT and GSK-3β null MEFs were treated with mouse TNFα (2 ng/ml) for the indicated periods of time. Results of one representative experiment are shown as mean of triplicates ±SD and normalized to RPLP0 expression.