TPL2 enforces *RAS*-induced inflammatory signaling and is activated by point mutations

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Supplementary Information

The authors have declared that no conflict of interest exists

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METHODS (continued)

Reverse-phase protein array (RPPA)

All lysates were prepared according to sample preparation guidelines provided by MD Anderson Cancer Center (MDACC). HPNE-*KRAS*^{G12D} were infected with retroviral particles containing AU1-tagged IRAK4 WT or vector control in presence of 8ug mL^{-1} polybrene, selected with blasticidin (10ug mL^{-1}) and RPPA was performed. For drug treatment, IRAK4 overexpressing cells were treated with AS2444697 2μM or vehicle (DMSO) for 24 hours. For evaluation of TPL2 WT and E188K, 293T cells were transfected in duplicates with Vector (pbabe-puro), TPL2 WT or TPL2 E188K along with equal amount of polyethylenimine. Cells were harvested 48hrs post-transfection and lysates were prepared and sent for RPPA. For both RPPA experiments, lysates were quantified by Bradford assay (Thermo Scientific), equalized for concentration, and denatured and reduced by adding 4X SDS sample buffer (without bromophenol blue) and boiling for 5 minutes.

Immunoblots and Immunoprecipitations

Standard immunoblotting procedure was followed. Cells were washed twice with ice-cold 1X PBS and lysed with ice-cold triton-X lysis buffer (25mM Tris, pH 7.4, 150mM NaCl, 5mM EDTA, 1% Triton-X) containing 1X protease (10μg mL^{-1} leupeptin, 700ng mL^{-1} pepstatin, 170ng mL^{-1} aprotinin, 1mM PMSF) and phosphatase (10mM NaF, 1mM Na_3VO_4, 1mM $Na_4P_2O_7$, 5mM Na β-glycerophosphate) inhibitors. Lysates were quantified by Bradford assay (Thermo Scientific), normalized for concentration, and reduced and denatured with 6X SDS sample buffer followed by boiling for 5 minutes. 20-40μg of protein per sample was resolved by SDS-PAGE, blotted on PVDF membrane and probed with primary antibodies (Cell Signaling Technologies: p-ERK1/2 (#4370), p-MEK1/2 (#9154), ERK1/2 (#4695), MEK1/2 (#8727), p-p105 (#4806), NF-

kB1 p105/p50 (#3035), p-IRAK4 (#11927), IRAK4 (#4363), ubiquitin (#43124), anti-HA (#3724), p-p90RSK (#11989), RSK1/2/3 (#9355), p-TPL2 (#4491), PARP (#9532), p-B-RAF S445 (#2696), BRAF (#9433S). Santa Cruz Biotechnology: GAPDH (#sc-32233), p-p65 S536 (#sc101752). Thermo Fisher Scientific: p-TPL2 (#PA5-36635). EMD Millipore: IRAK4 (#07-418). R&D systems: TPL2/MAP3K8 (#MAB4586). Novus Biologicals: anti-AU1 (#NB600-453). Abcam: p-p65 S276 (#ab194726)). Membranes were incubated with appropriate HRP-conjugated secondary antibodies (anti-mouse or anti-rabbit, (1:5000 dilution), Jackson Laboratory) and imaged using chemiluminescent substrate. Additional steps for immunoprecipitation include incubation of soluble fraction lysate with anti-HA magnetic beads (Thermo Scientific cat# 88837) overnight at 4°C, washing, and elution using 1X SDS sample buffer and boiling as mentioned in manufacturer's protocol.

Immunohistochemistry (IHC) and immunofluorescence (IF)

IHC and IF staining were performed using the following antibodies: p-IRAK4 (T345S) (ABNOVA, A8A8, 1:200), p-ERK (CST, 4370, 1:200) and TPL2 (Sigma-Aldrich, HPA017962, 1:100). For tissue micro-array, entire slides were scanned at 20X magnification using automated Zeiss Axio Scan Z1 Slide Scanner and analysed using HALO software (Indica Labs) TMA module with area quantification v1.0 algorithm to quantify staining area and intensity (weak, moderate, strong). Histology-score (H-score) was computed as (3 x strong intensity area %) + (2 x moderate intensity area %) + (1 x weak intensity area %). Depleted tissue cores were invalidated and excluded from data set and analysis.

Pharmaceutical compounds

Gemcitabine was purchased from the Siteman Cancer Center Pharmacy. Details of other agents: oxaliplatin (Sigma cat#O9512), 5-fluorouracil (Sigma cat#F6627), AS2444697 (Tocris,

cat#5430) TPL2 kinase inhibitor (Tocris #5240), IMD-0354 (Tocris, cat#2611), PF06650833 (Tocris cat#6373), SN-38 (Tocris, cat#2684), BVD-523(Biomed Valley), GDC-0941 (Selleckchem, cat#S1065), Trametinib (Selleckchem, cat#S2673), Selumetinib (Selleckchem, cat#S1008), PLX-4720 (Selleckchem, cat#S1152), PLX-4032 (Selleckchem, cat#S1267), Dabrafenib (Selleckchem, cat#S2807), GDC-0994 (Selleckchem, cat#S7554), Paclitaxel (Selleckchem, cat#S1150), anti-mouse IL-1β neutralizing antibody (Invivogen, clone 7E3), anti-human IL-1β neutralizing antibody (Invivogen, clone 4H5).

Plasmids

pCLXSN-HA-TPL2/COT was a gift from Shao-Cong Sun (Addgene plasmid # 27558). HA-TPL2 was subcloned into pBabe expression vector which was utilized for majority of experiments. TPL2 R442H, L444V, R459W and truncated mutants were generated using mutated 3'end reverse PCR primers. TPL2 R397H was were generated by site-directed mutagenesis using Platinum SuperFi Green PCR Master Mix (Invitrogen, cat#12359-010 or 14001011)**.** TPL2 E188K was purchased as a synthetic gene insert and cloned into pBabe vector. All constructs except HA-TPL2 E188K contained KOZAK sequence for robust expression. AU1-tagged IRAK4 WT and IRAK4 KD were expressed in pCMV-puro expression vector. All target sequences for sgRNA and shRNA are listed in **Supplementary Table 4**.

Lentiviral and retroviral production and transduction

To generate lentivirus, shRNA encoding plasmid (pLKO.1 or Tet-pLKO.1) or sgRNA encoding plasmid (LentiCRISPRv2 or TLCV2) was mixed with packaging plasmids psPAX2 and pMD2.G in 4:2:1 (6μg:3μg:1.5μg) ratio in serum free DMEM. Polyethelyneimine (PEI) transfection reagent (42μL, 4:1 PEI to total DNA ratio) was added, mixture was incubated at room temperature for 20 minutes and added dropwise onto 293TV cells in 100mm dish. Media

was replaced with 6mL fresh 10% FBS DMEM 12-16 hours post-transfection. Virus was collected at 48 hours and 72 hours post-transfection and cleared by 0.45μM filter. Target cells were transduced with virus in presence of 8μg mL^{-1} polybrene (Sigma) for 16 hours before being washed, cultured for 24-48 hours and then selected with 2μg mL^{-1} puromycin (Sigma) for 48 hours. After initial selection bout, cells were cultured without puromycin for 48-72 hours after which puromycin was re-added and maintained in culture until cells were used for experiments. Similar procedure was followed for retrovirus production and transduction, except virus was generated by co-transfecting expression vector and packaging plasmid pCL10A1 in 1:1 ratio.

Anchorage independent soft agar growth assays.

Cells were seeded at 5000-15000 cells in 0.3% noble agar-DMEM suspension per well, in triplicate, in 24 well plate. For drug treatment, compound was added to cell suspension at 1X concentration. Seeded cells were fed with fresh 0.6% agar-DMEM mix once every 7-12 days. In case of drug treatment, compound was added at 2X concentration in 0.6% agar-DMEM mix and applied over existing agar layer (resulting in final drug concentration of 1X) to prevent desiccation and replenish inactive drug compounds. After 3-6 weeks, colonies were counted under microscope and imaged. Colony count was normalized to control and graphed in GraphPad Prism v7/8.

Organoid 3D assays

48 well plates were coated with 0.6% agar in 10% serum DMEM. Cells were counted, suspended in 1:1 mixture of media and regular Matrigel (Corning), and seeded at 4000-5000 cells (depending on cell line) per well in triplicate or quadruplicate. For IGROV1, TPL2 knockdown cells were transiently transfected with TPL2 WT or TPL2 R442H using XtremeGene9 transfection reagent (Roche) ~24 hours prior to seeding. 10-15 days later, organoids were

counted and imaged. Organoid count was normalized to control and graphed in GraphPad Prism v7/8.

Clonogenic 2D assays

Cells were seeded at 500-1000 cells per well (6 well plate format) or 200-1000 cells per well (12 well format) in 10% serum media. For drug treatment, compound was added at time of seeding at indicated concentration. Media was replenished as needed over course of incubation. After 3-5 weeks, colonies were fixed with 4% formaldehyde, stained with 0.5% crystal violet and scanned using document scanner. Images were quantified using particle analyzer on ImageJ software and values were normalized to vehicle.

Drug response and viability assays

Cells were seeded at 1500-2500 cells per well in triplicates, in 96 well format. Next day, appropriate inhibitor was added in serial dilution from $1.00x10^{2}\mu M$ or indicated single doses. Vehicle (DMSO) was added to match volume of inhibitor used for highest concentration. After 96-120 hours, 5X AlamarBlue® reagent (resazurin sodium salt, Sigma) was added to wells and incubated for 2-4 hours. Fluorescence at 585nm (excitation 555nm) was measured by SpectraMax i3 Microplate Reader. Raw values were normalized to vehicle, analyzed and graphed using GraphPad Prism v7/8 software.

Drug synergy assays

HPAC cells were plated in 12-by-12 matrix at ~1000 cells per well. Next day, serial dilutions of SN-38 ($2\mu M$ to $1.95x10^{-3}\mu M$) and TPL2i ($20\mu M$ to $19.5x10^{-3}\mu M$) was added in matrix format. Cells were incubated at 37°C for 96 hours and viability was measured by AlamarBlue® as described above. Three independent experiments were performed, data was compiled, and

synergy scores by Loewe additivity model were computed using SynergyFinder software, exported and graphed in GraphPad Prism v8.

Cell proliferation assays

Cells were seeded in triplicate or quadruplicate at indicated density per well. When applicable, appropriate drug compound was added to cells the next day. Viable cells were counted using trypan blue dye exclusion and BioRad TC20 Automated Cell Counter at each time point.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed using BioLegend® Human IL-1β ELISA MAX™ Deluxe Set kit (Cat# 437004). Cells were cultured in 6cm^2 dish (2 biological replicates in separate dishes) with 2mL media, starting at ~55% confluency. 0.75mL fresh media was added to cells 24 hours later. Next, entire 48-hour-old 2.75mL media supernatant was collected and 50μL was used for ELISA, performed according to manufacturer's protocol. Final values that were undetected in HEK T/tH cells were reported as the minimum detectable amount of hIL-1β protein (0.5pg/mL).

Reporter assays

Cells stably expressing plasmid encoding serum-response-element (SRE) or NFκB promoter driven *Firefly* luciferase and constitutive *Renilla* luciferase were seeded at equal density per well and treatment was initiated the next day for 16-24 duration. Alternatively, 293T reporter cells were transfected in duplicate or triplicate with 1μg of appropriate plasmid DNA. Medium was replaced 16-18 hours later and at 48 hours post-transfection reporter activity was measured using Dual-Glo® Luciferase Assay System. Data was acquired using SpectraMax i3

Microplate Reader and analysis was performed by calculating ratio of firefly:renilla. Values were normalized to control and graphed using GraphPad Prism v7/8 software.

RNA sequencing

Samples were prepared according to library kit manufacturer's protocol, indexed, pooled, and sequenced on an Illumina HiSeq. Basecalls and demultiplexing were performed with Illumina's bcl2fastq software and a custom python demultiplexing program with a maximum of one mismatch in the indexing read. RNA-seq reads were then aligned to the Ensembl release 76 top-level assembly with STAR version 2.0.4b1. Gene counts were derived from the number of uniquely aligned unambiguous reads by Subread:featureCount version 1.4.52. Isoform expression of known Ensembl transcripts were estimated with Sailfish version 0.6.133. Sequencing performance was assessed for the total number of aligned reads, total number of uniquely aligned reads, and features detected. The ribosomal fraction, known junction saturation, and read distribution over known gene models were quantified with RSeQC version 2.34. All gene counts were then imported into the R/Bioconductor package EdgeR5 and TMM normalization size factors were calculated to adjust for samples for differences in library size. Ribosomal genes and genes not expressed in the smallest group size minus one samples greater than one count-per-million were excluded from further analysis. The TMM size factors and the matrix of counts were then imported into the R/Bioconductor package Limma6. Weighted likelihoods based on the observed mean-variance relationship of every gene and sample were then calculated for all samples with the voomWithQualityWeights7. The performance of all genes was assessed with plots of the residual standard deviation of every gene to their average log-count with a robustly fitted trend line of the residuals. Differential expression analysis was then performed to analyze for differences between conditions and the results were filtered for only those genes with Benjamini-Hochberg false-discovery rate adjusted p-values less than or

equal to 0.05.For each contrast extracted with Limma, global perturbations in known Gene Ontology (GO) terms and KEGG pathways were detected using the R/Bioconductor package GAGE8 to test for changes in expression of the reported log 2 fold-changes reported by Limma in each term versus the background log 2 fold-changes of all genes found outside the respective term. The R/Bioconductor package heatmap39 and Pathview10 was used to display heatmaps or annotated KEGG graphs across groups of samples for each GO term or KEGG pathway (respectively) with a Benjamini-Hochberg false-discovery rate adjusted p-value less than or equal to 0.05.

TCGA data analysis

TCGA data was accessed via CBioportal.org and graphs were generated and exported for publication.

Gene set enrichment analysis

Genes in RNAseq differential expression data were ranked by Log_2 fold change, and pre-ranked gene set enrichment analysis was performed using ranked lists. Gene sets associated with TPL2, PDAC and IL-1 were downloaded from the Broad Institute Molecular Signature database (MSigDB) and are listed in **Supplementary Table 3**. For leading edge analysis, regular (not pre-ranked) gene set enrichment was performed with phenotype permutation in order to preserve gene-to-gene correlation. Ranking metric was set to “difference-of-classes” since expression data was in Log_2 units. Otherwise, GSEAv.4 was used for analysis in default format. Data generated was exported and graphed in GraphPad Prism v8 software.

Quantitative (real-time) PCR

Total RNA was isolated using RNAzol RT (Sigma), cDNA was generated using High Capacity cDNA reverse transcription kit (Thermo Fisher Scientific, 4368814) and qPCR was performed using SYBR-Green reagent (Applied Biosystems, cat#4309155). Primer sequences are listed in **Supplementary Table 5**. All experiments were performed in biological duplicates or triplicates (n=2,3).

Flow cytometry

HPAC cells were stained using Annexin V-FITC and propidium iodide (PI) (BD bioscience #556547) and followed by FACSCalibur (BD bioscience) analysis. The results were further analyzed and quantified by FlowJo software (BD bioscience). Briefly, HPAC cells were plated in the 12-well plates and then treated with TPL2 inhibitor, SN38 or combination for 48 hours. Cells were then trypsinized and washed with PBS twice with centrifugation at 500g for 5 minutes between each trypsinization and wash. Cells were stained using Annexin V-FITC and PI for 20 minutes on ice and then analyzed by FACSCalibur. After acquiring the data, compensation using non-stain cells, Annexin V-FITC and PI single-stain cells, and gating quantification were performed in FlowJo. The gating area was defined as Q4 (Annexin V^- and PI^-) containing the main cell population within vehicle cells and the quantitative apoptosis ratio was calculated by adding early apoptosis (Annexin V^+ and PI^-) and late apoptosis (Annexin V^+ and PI^+) cells.

Proximity Ligation Assay (PLA)

PLA was performed using Duolink® *in situ* Red Starter Kit Mouse/Rabbit (DUO92101, Sigma) per manufacturer's protocol. Briefly, cells were seeded on cover slips in 6 well plate at ~50% confluency per well. Next day cells were treated with SN-38 (10μM) and 16 hours later PLA was performed using p-IRAK4 (ABNOVA) and TLR9 (CST) primary antibodies. Stained cells were imaged using Nikon C2+ fluorescent microscope paired with NIS-Element software.

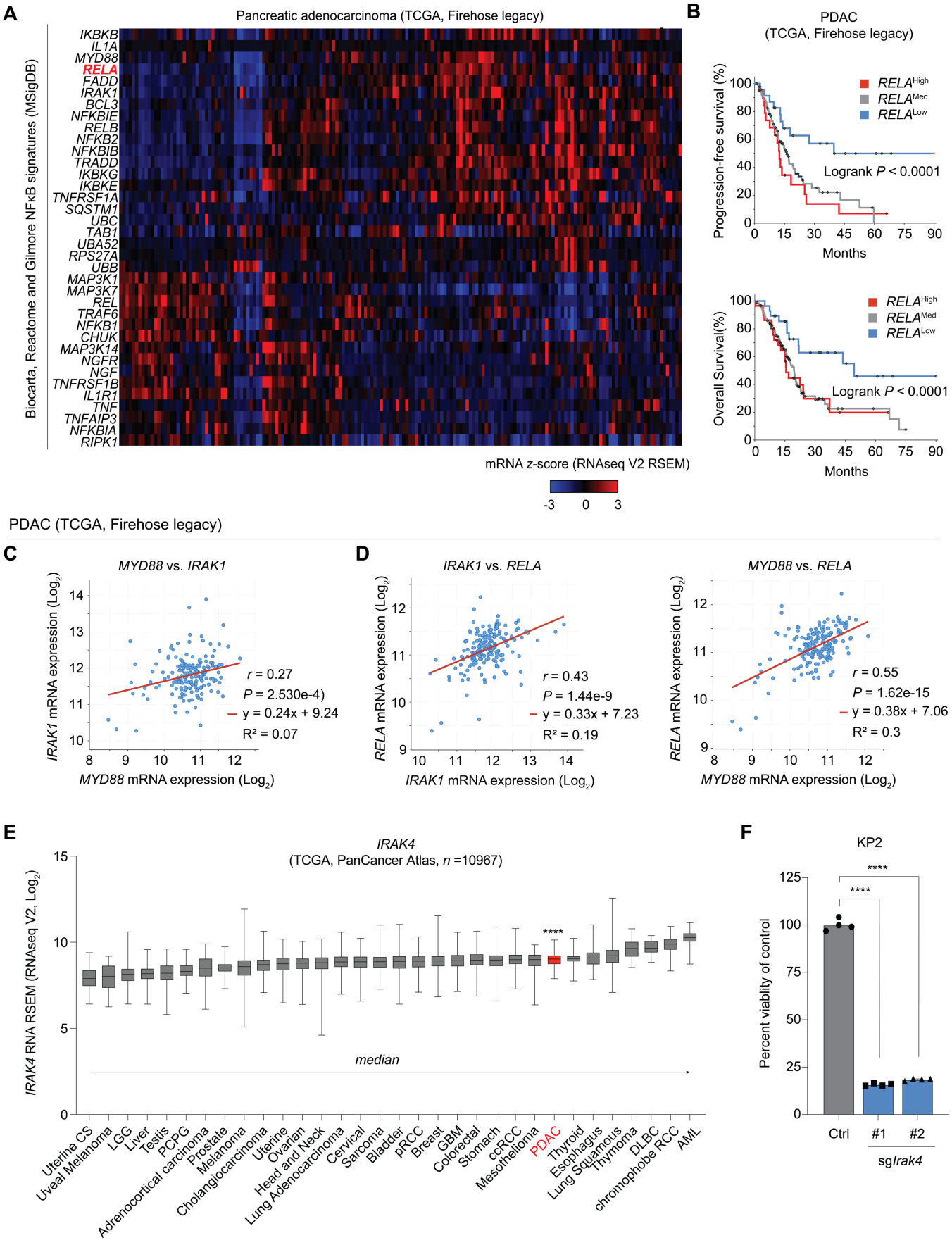
Number of puncta per field was quantitated for six 400X fields per condition using Find Maxima tool in ImageJ software. Data was exported and graphed in GraphPad Prism v8.

Supplementary Figure Legends and Figures

Supplementary Figure 1 (Supports Figure 1)

(A) Heatmap of mRNA RNAseq *z*-scores for NFκB related genes from the Broad Institute Molecular Signatures Database evaluated in PDAC patient samples in the TCGA Firehose Legacy project. Gene-set lists are provided in **Supplementary Table 3**. **(B)** Progression-free and overall survival of patients with high (z-score > 1), medium (z-score -1 to 1) and low (z-score < -1) mRNA expression of *RELA.* **(C)** Scatter plot showing correlation (Pearson *r*) between *MYD88* and *IRAK1* RNA expression. **(D)** Scatter plot showing correlation (Pearson *r*) between *RELA* and *IRAK1* (left) or *RELA* and *MYD88* RNA expression. For **(B**-**D)** expression was analyzed in samples in the PDAC TCGA Firehose Legacy study. **(E)** Graph depicting *IRAK4* RNA expression in multiple cancers in the TCGA PanCancer Atlas project. Data are arranged by increasing median expression, from left to right. Disease of interest, PDAC, is in red color. *P* values are from one-way ANOVA with Dunnett's multiple comparison test and are listed for each comparison in **Supplementary Table 1**. **(F)** Relative viability of KP2 cells after *Irak4* knockout. Data shows average of four replicates, *P* values from one-way ANOVA with Dunnett's multiple comparison test and error bars indicate mean ± SEM. ****$P<0.0001$, ***$P<0.0002$, **$P<0.0021$, *$P<0.0332$.

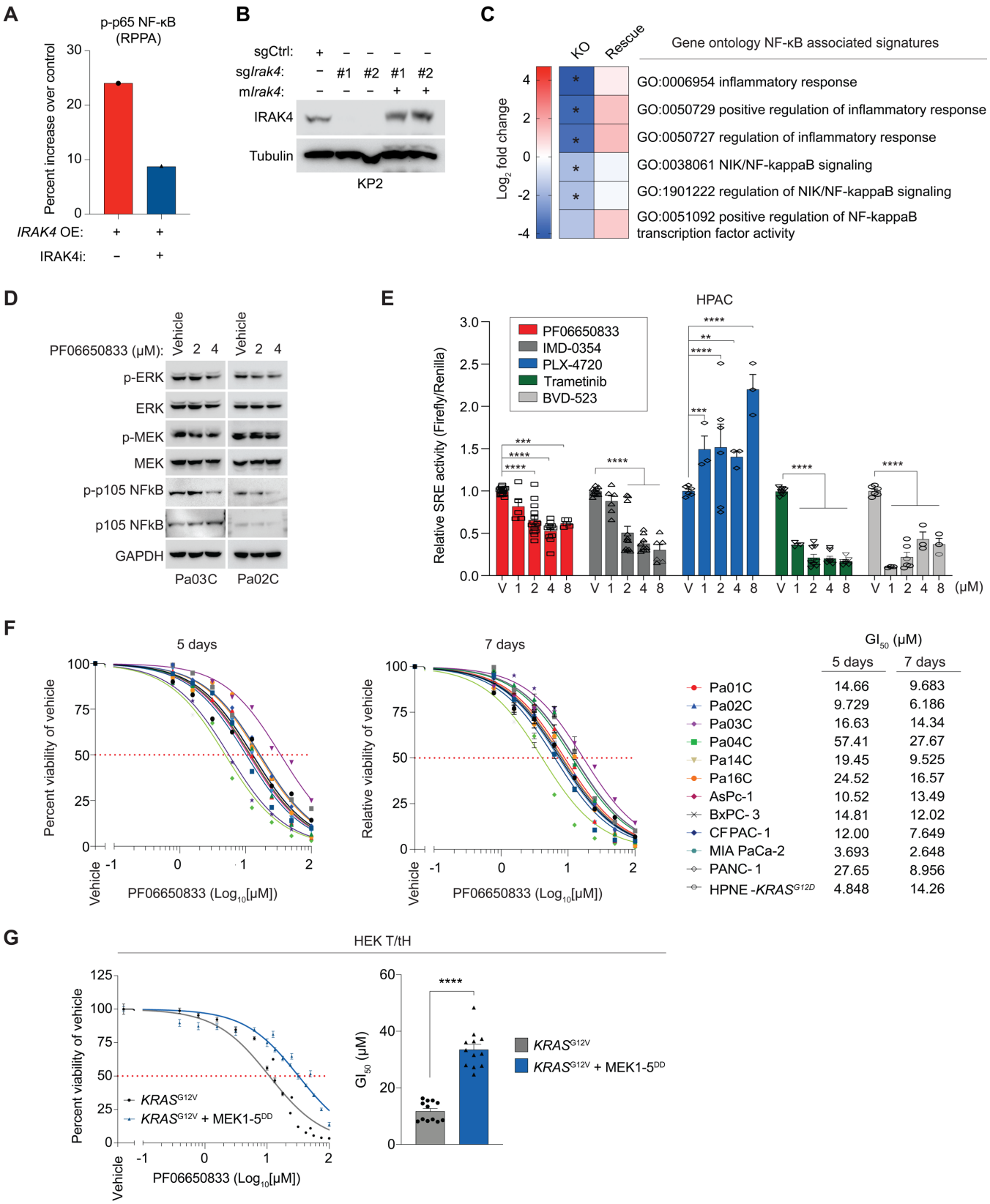
Supplementary Figure 1 (Supports Figure 1)



Supplementary Figure 2 (Supports Figure 2)

(A) Graph depicting percent change (compared to vector control) in p-p65 levels from RPPA on HPNE-*KRAS*^{G12V} cells, as in **Figure 2A**. **(B)** Immunoblots of WT, *Irak4*-KO, and m*Irak4* rescued (KO + m*Irak4*) KP2 cells. **(C)** Heat map showing fold change in NF-κB associated GO gene signatures in *Irak4*-KO and rescue KP2 cells. Signatures significantly ($P < 0.05$) depleted (blue) or enriched (red) are marked with an asterisk (*). **(D)** Immunoblots of three human PDAC cell lines treated with IRAK4i as in **Figure 2J. (E)** Relative SRE activity of HPAC cells treated with IRAK4i (PF06650833), IKKi (IMD-0354), BRAFi (PLX-4720), MEKi (trametinib) and ERKi (BVD-523). Data represents three or more independent experiments, each with technical replicates. *P* values from two-way ANOVA with Dunnett's multiple comparison test. **(F)** Viability of 12 human PDAC cell lines after treatment with IRAK4i for 5 days (left) and 7 days (middle). GI_{50} values for each cell line are listed on right. **(G)** Viability of HEK-*KRAS*^{G12V} cells with or without ectopic MEK1-5^{DD} expression treated with IRAK4i (PF06650833) for 96 hours. Data represents 12 replicates from 4 independent experiments. Graph on right shows GI_{50} with *P* value from two-sided paired t-test. All error bars indicate mean ± SEM; ****P<0.0001, ***P<0.0002, **P<0.0021, *P<0.0332

Supplementary Figure 2 (Supports Figure 2)

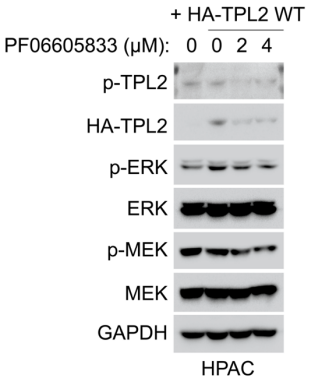


Supplementary Figure 3 (Supports Figure 3)

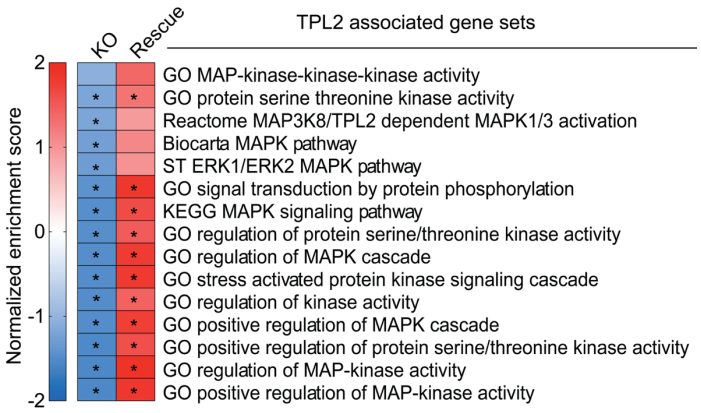
(A) Immunoblot of HPAC cells ectopically expressing HA epitope-tagged TPL2 WT and treated with IRAK4i (PF06650833) for 24 hours in serum-free media. **(B)** Heat-map of normalized enrichment scores (NES) for TPL2 associated gene signatures from gene set enrichment analysis of RNAseq differential expression in KP2 IRAK4 knockout and rescue cells. Significantly ($P < 0.05$) depleted (blue) or enriched (red) signatures are marked with an asterisk (*). TPL2 associated gene set lists are provided in **Supplementary Table 3**.

Supplementary Figure 3 (Supports Figure 3)

A



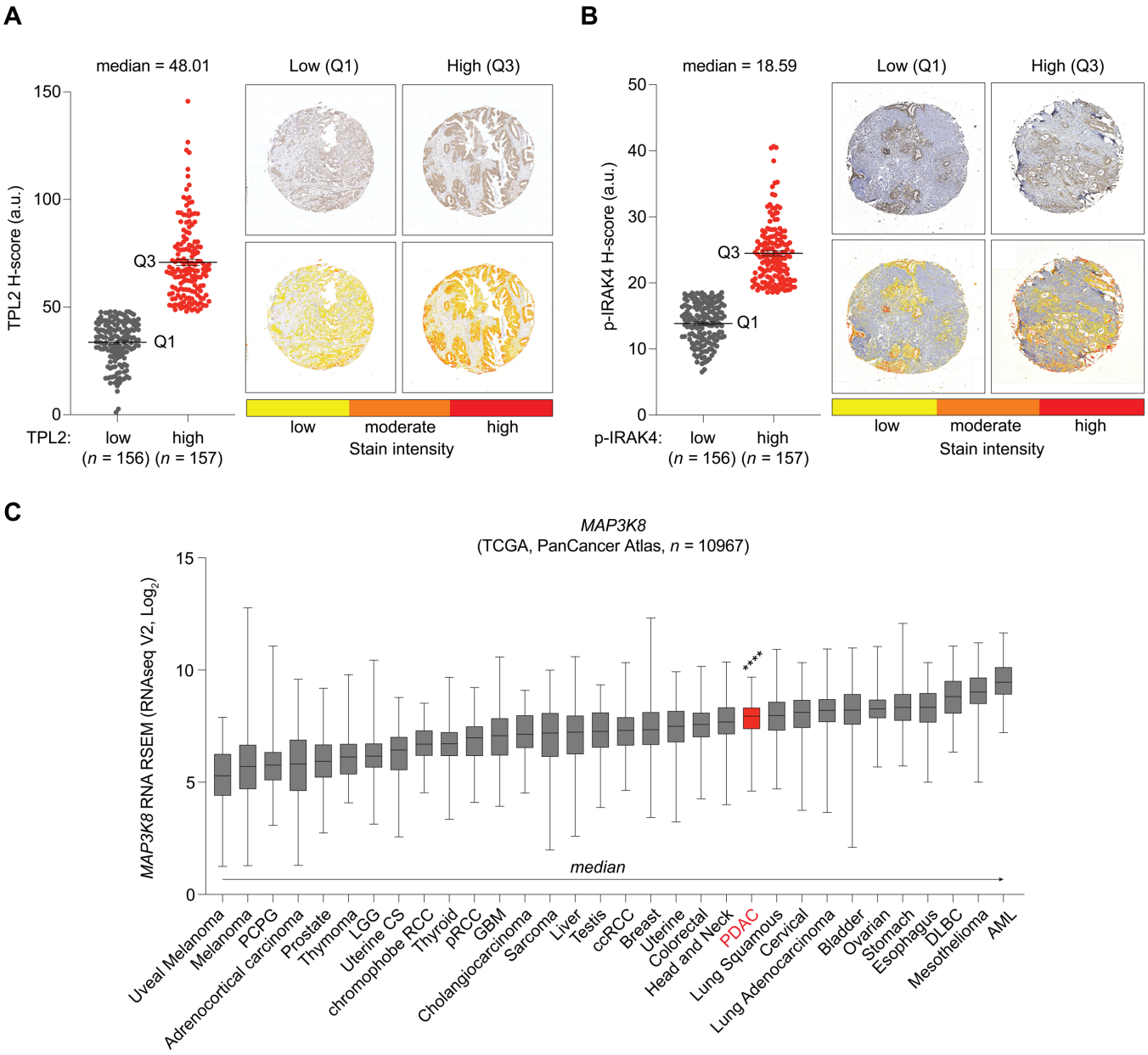
B



Supplementary Figure 4 (Supports Figure 4)

(A) Scatter plots and representative images (original and with HALO analyzed markup) depicting "high" and "low" TPL2 H-scores for tissue-micro-array (TMA) analysis. Images shown represent Q1 and Q3 quartile H-score by median. **(B)** Scatter plots and representative images (original and with HALO analyzed markup) depicting "high" and "low" p-IRAK4 H-scores for tissue-micro-array (TMA) analysis. Images shown represent Q1 and Q3 quartile H-score by median. **(C)** Graph depicting *MAP3K8* (encoding TPL2) RNA expression in multiple cancers in the TCGA PanCancer Atlas project. Data are arranged by increasing median expression, from left to right. Disease of interest, PDAC, is in red color. *P* values are from one-way ANOVA with Dunnett's multiple comparison test and are listed for each comparison in **Supplementary Table 2**. All error bars indicate mean ± SEM; ****$P<0.0001$, ***$P<0.0002$, **$P<0.0021$, *$P<0.0332$.

Supplementary Figure 4 (Supports Figure 4)



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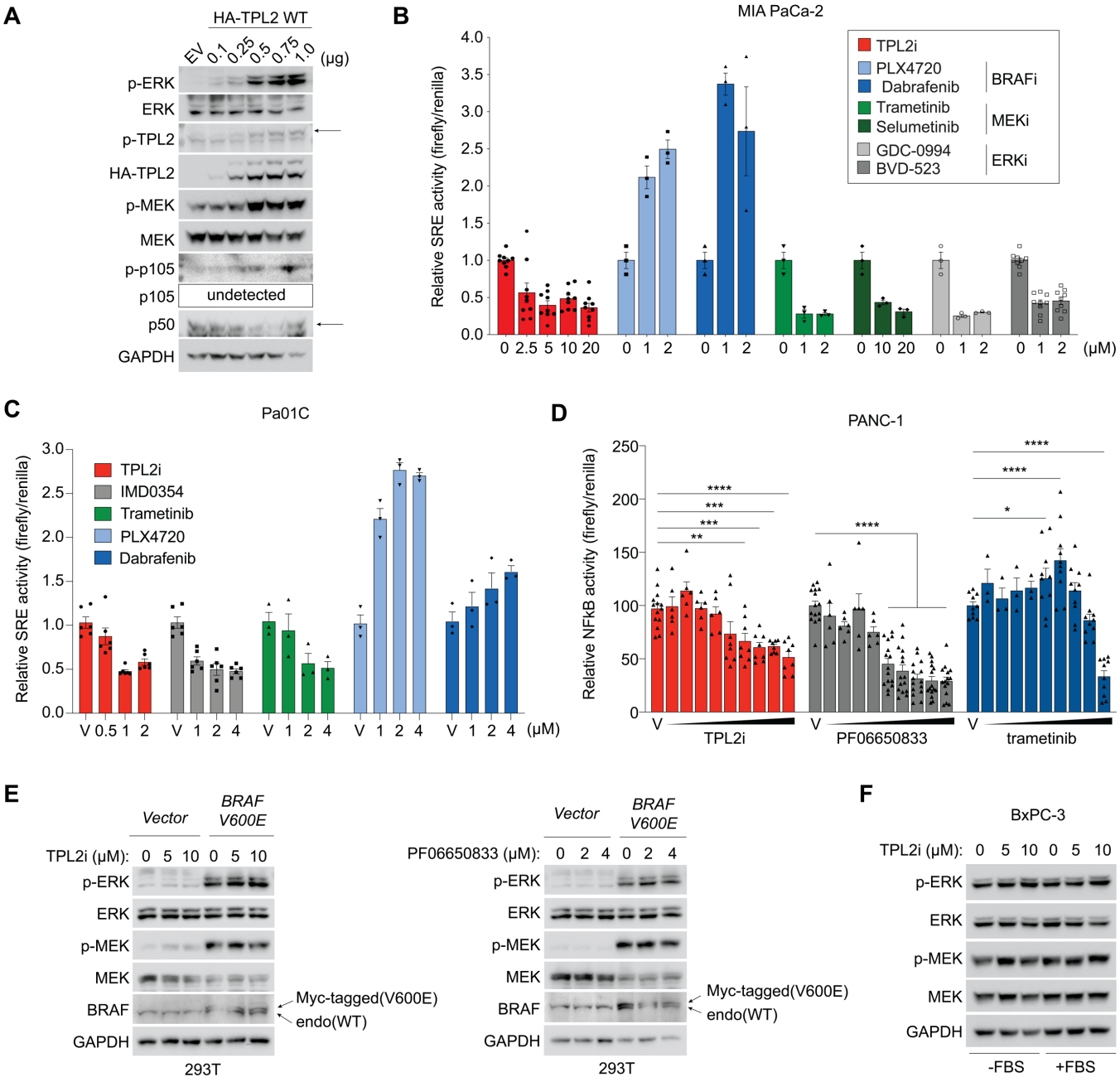
Supplementary Figure 5 (Supports Figure 5)

(A) Immunoblot of 293T cells transfected with 1μg of empty vector or indicated amounts of plasmid encoding HA epitope-tagged TPL2 WT for 48 hours. **(B)** Serum-response element (SRE) reporter activity of MIA-PaCa2 cells treated with TPL2i, BRAFi, MEKi or ERKi for 24 hours. Data for TPL2i and ERKi is from three independent experiments each done in technical triplicate. Rest are from one independent experiment. **(C)** SRE reporter activity of Pa01C cells treated with TPL2i, IKKi (IMD0354), MEKi, and BRAFi. Data for TPL2i and ERKi is from two independent experiments each done in technical triplicate. Rest are from one independent experiment. **(D)** NF-κB reporter activity of PANC-1 cells treated with serial dilutions from 20μM of TPL2i, IRAK4i (PF06650833) and MEKi (trametinib) for 18 hours . Data are from three or more independent experiments, each done in technical triplicate. *P* values from two-way ANOVA and Dunnett's multiple comparison test. **(E)** Immunoblots of 293T cells transiently transfected with empty vector or MYC epitope-tagged BRAF V600E, and after 16 hours, treated with TPL2i (on left) or IRAK4i (PF06650833; on right) for 24 hours. Experiment with TPL2i was performed twice, IRAK4i was done once. **(F)** Immunoblots of BxPC-3 cells treated with TPL2i for 16 hours, with or without FBS in media. One representative data of four independent experiments is shown. All error bars indicate mean ± SEM; ****P<0.0001, ***P<0.0002, **P<0.0021, *P<0.0332.

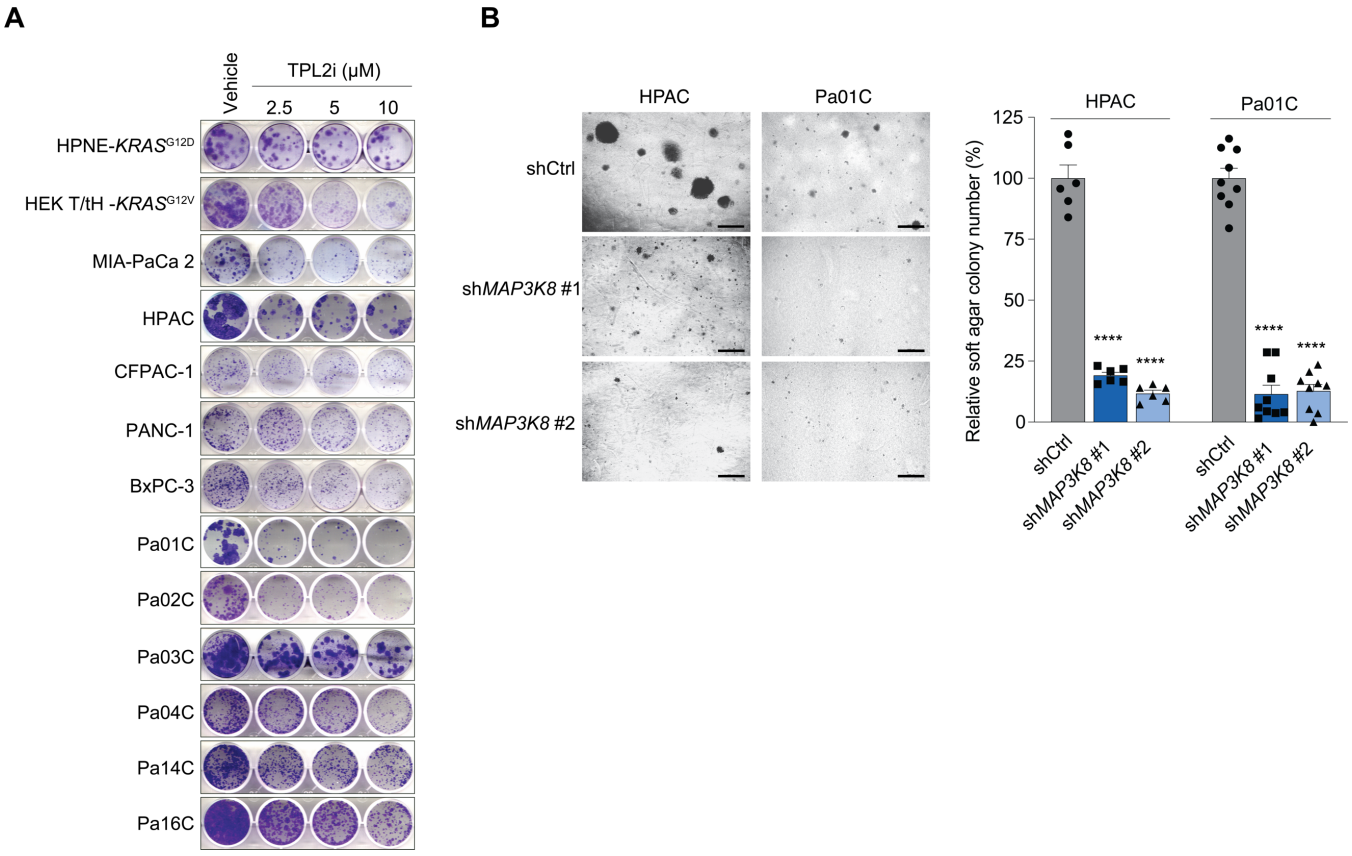
Supplementary Figure 5 (Supports Figure 5)



Supplementary Figure 6 (Supports Figure 5)

(A) Images of 2D clonogenic colony formation assays of multiple *KRAS* mutant cell lines treated with TPL2i. Two independent experiments were performed per cell line. **(B)** Quantification and representative images depicting soft agar colony formation of HPAC and Pa01C cells stably expressing non-targeting shRNA (shCtrl) or shRNA targeting *MAP3K8* (encoding TPL2). Data is from three independent experiments (two for HPAC) each with technical triplicates. Scale bar is 500μm. *P* values from two-way ANOVA with Dunnett's multiple comparison test. All error bars are mean ± SEM; ****$P<0.0001$, ***$P<0.0002$, **$P<0.0021$, *$P<0.0332$.

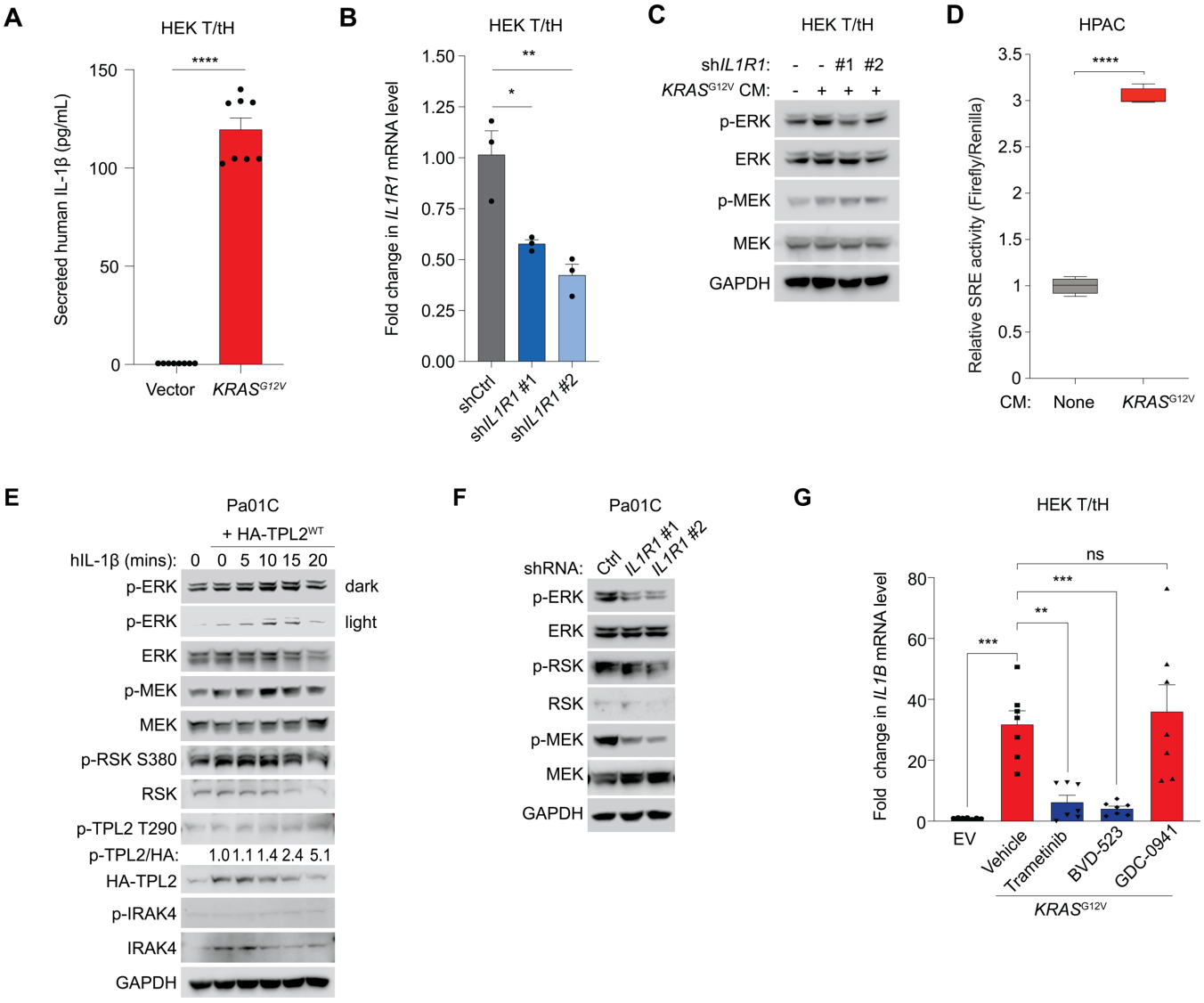
Supplementary Figure 6 (Supports Figure 5)



Supplementary Figure 7 (Supports Figure 6)

(A) ELISA for secreted human IL-1β in HEK T/tH cells expressing empty vector or *KRAS*^{G12V}. Data represents two biological replicates, each with four technical replicates. *P* value from two-sided unpaired t-test. **(B)** Quantification of *IL1R1* mRNA level in HEK T/tH *KRAS*^{G12V} cells after knockdown of *IL1R1* (encoding IL-1R1) with shRNA. **(C)** Immunoblots of WT and IL-1R1 (*IL1R1*) knock-down HEK T/tH cells stimulated with conditioned media (CM) from HEK T/tH *KRAS*^{G12V} cells. **(D)** SRE reporter assay of HPAC cells serum starved for 24 hours and stimulated with serum-free HEK T/tH *KRAS*^{G12V} CM for 16 hours. Data represent four replicates. *P* values from two-sided unpaired t-test**. (E)** Immunoblot of Pa01C cells overexpressing empty vector or HA epitope-tagged TPL2 WT serum starved for 24 hours and then stimulated with 100ng mL^{-1} recombinant human IL-1β. **(F)** Immunoblots of WT and IL-1R1 (*IL1R1)* knockdown Pa01C cells. **(G)** *IL1B* expression by qRT-PCR of HEK vector and HEK-*KRAS*^{G12V} cells treated with MEKi (trametinib), ERKi (BVD523) and PI3Ki (GDC0941) for 16 hours. Data shows seven replicates from three independent experiments. *P* values from two-way ANOVA with Dunnett's multiple comparison test. All error bars indicate mean ± SEM; ****P<0.0001, ***P<0.0002, **P<0.0021, *P<0.0332.

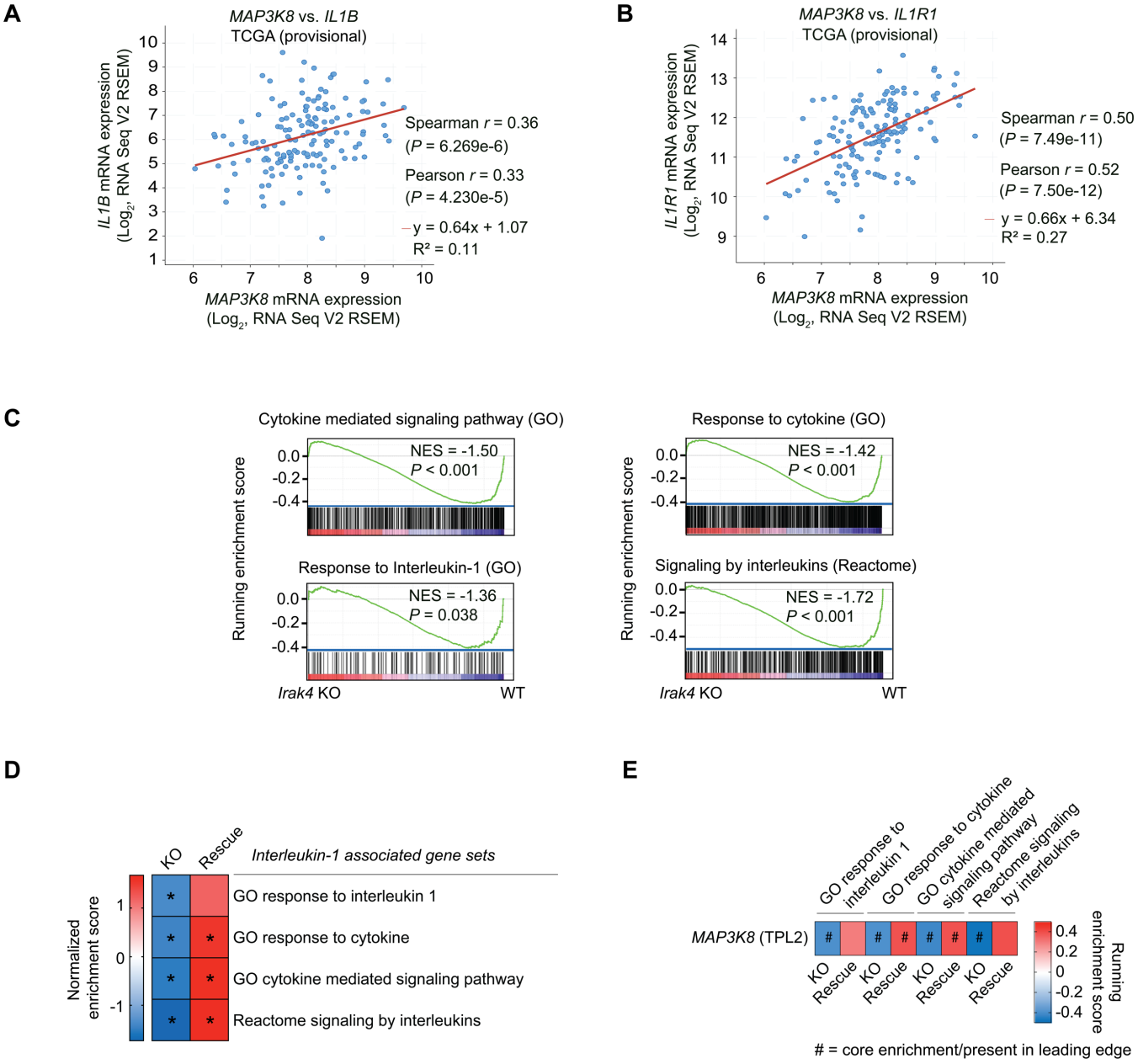
Supplementary Figure 7 (Supports Figure 6)



Supplementary Figure 8 (Supports Figure 6)

(**A**) Scatter plot, linear regression line, and Spearman and Pearson coefficients depicting positive correlation between *MAP3K8* (encoding TPL2) and *IL1B* mRNA expression (in Log_2 units) obtained from TCGA database. **(B)** Scatter plot, linear regression line, and Spearman and Pearson coefficients depicting positive correlation between *MAP3K8* and *IL1R1* mRNA expression (in Log_2 units) obtained from TCGA database. **(C)** Individual gene set enrichment plots of IL-1 associated gene sets in KP2 *Irak4* KO cells. Genes are from the MSigDB and are listed in **Supplementary Table 3**. **(D)** Heatmap showing normalized enrichment scores (NES) in KP2 *Irak4* KO and *Irak4* rescue cells for gene sets in **(C)**. Signatures that were significantly ($P < 0.05$) depleted (blue) or enriched (red) are marked with an asterisk (*). **(E)** Heatmap showing running enrichment score for *MAP3K8* in each gene set in **(C)** and **(D)** after *Irak4* KO and rescue. #: *MAP3K8* is present in the leading edge, indicating that changes in *MAP3K8* expression contributed significantly to the enrichment result shown in **(C)** and **(D).**

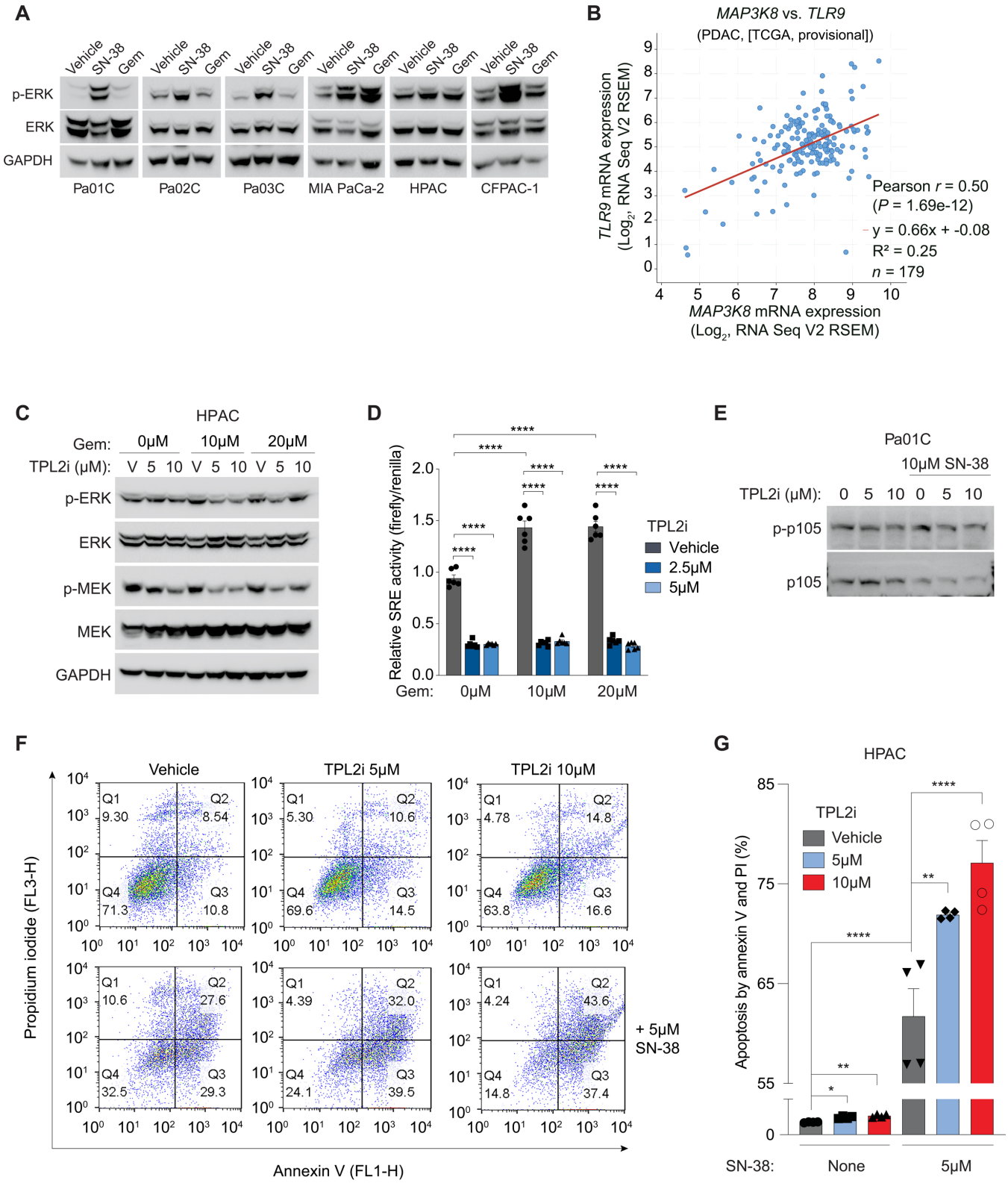
Supplementary Figure 8 (Supports Figure 6)



Supplementary Figure 9 (Supports Figure 7)

(A) Immunoblots depicting induction of p-ERK in six PDAC cell lines after 16-hour treatment with chemotherapy agents, SN-38 (10μM) and gemcitabine (20μM) in 10% serum DMEM. **(B)**, Scatter plot depicting correlation between mRNA expression of *MAP3K8* (encoding TPL2) and *TLR9* based on TCGA data. **(C)** Immunoblot of HPAC cells treated with TPL2i, gemcitabine or the combination for 16 hours in 10% serum DMEM. **(D)** Quantification of serum-response element (SRE) reporter activity in HPAC cells treated with TPL2i, TPL2i, gemcitabine or the combination for 16 hours in 10% serum DMEM. Data shows six replicates from two independent experiments. *P* value from two-way ANOVA with Dunnett's multiple comparison test. **(E)** Immunoblot depicting p-p105 levels in Pa01C cells treated with TPL2i, SN-38 or the combination from same corresponding samples showing in **Figure 7E. (F** and **G)** Flow cytometry scatter plots and bar graph respectively of HPAC cells treated with TPL2i, SN-38 or the combination for 48 hours**.** Data shows four replicates from two independent experiments. *P* values from one-way ANOVA with Tukey's multiple comparison test. All error bars indicate mean ± SEM; ****$P<0.0001$, ***$P<0.0002$, **$P<0.0021$, *$P<0.0332$.

Supplementary Figure 9 (Supports Figure 7)

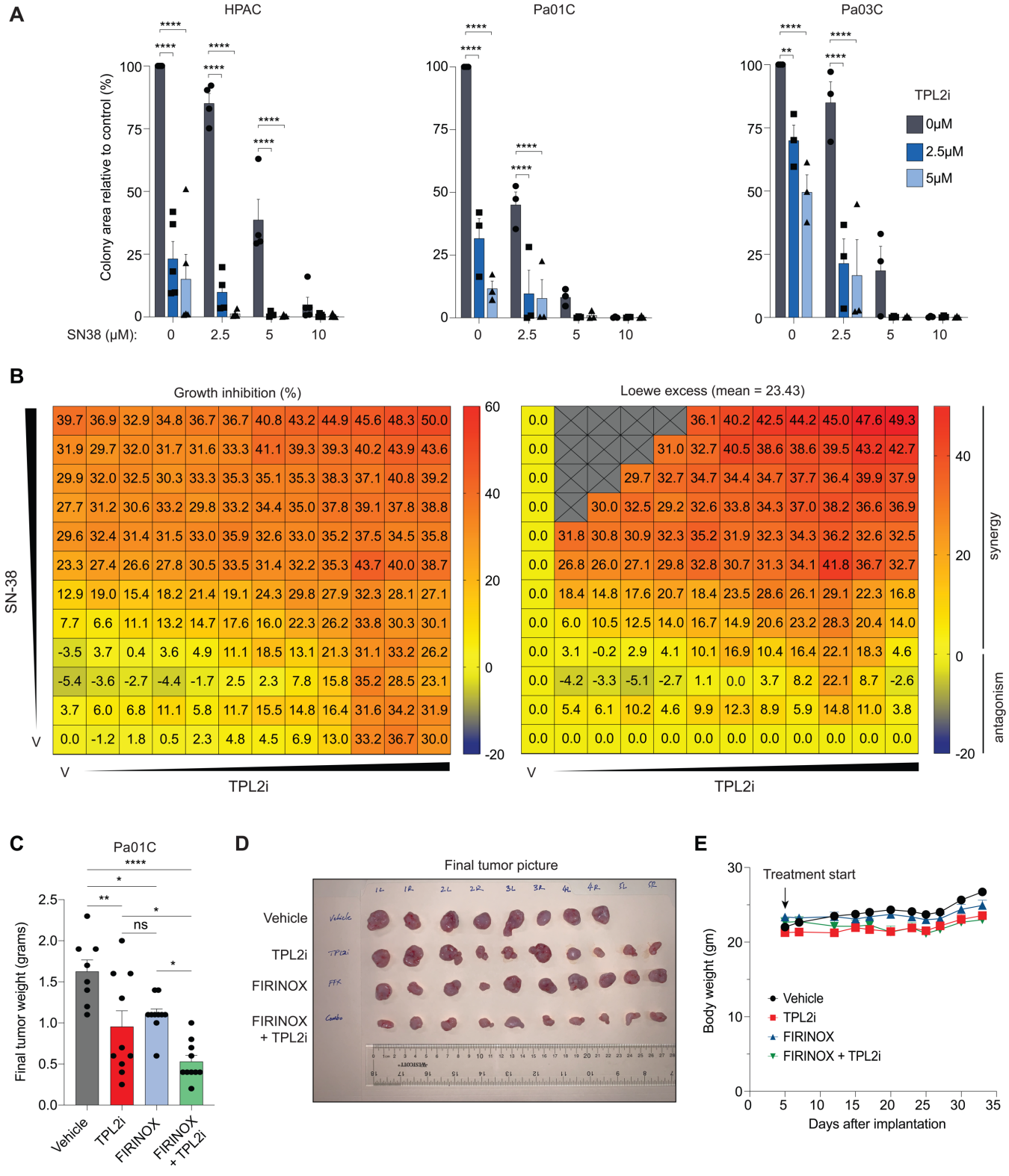


665

Supplementary Figure 10 (Supports Figure 7)

(A) Quantification of 2D clonogenic colony formation of three PDAC cell line treated with TPL2i, SN-38 or the combination for data shown in Figure 7F. Data represents *n* = 3 (Pa01C and Pa03C) and *n* = 5 (HPAC) independent experiments. *P* values from two-way ANOVA with Dunnett's multiple comparison test. **(B)** Growth inhibition and Loewe additivity excess matrices of TPL2i inhibitor added to SN-38 for HPAC cells. Darker colors indicate greater cytotoxicity (growth inhibition) and higher synergy scores (Loewe excess). Data shown is compilation of three independent experiments. Concentrations used are stated in method section. **(C)** Final weight of subcutaneous Pa01C tumors for experiment in **Figure 7G**. One outlier was removed by Grubb's, alpha = 0.01, and replaced with average value. *P* value from one-way ANOVA with Holm-Sidak's multiple comparison test. **(D)** Pictures of all Pa01C subcutaneous tumors isolated from mice treated with vehicle, TPL2i, FIRINOX or combination. Data is from same experiment in **(C)** and **Figure 7G. (E)** Average body weight over time of nude mice for experiments in **(C)**, **(D)** and **Figure 7G.** All error bars indicate mean ± SEM; ****$P<0.0001$, ***$P<0.0002$, **$P<0.0021$, *$P<0.0332$

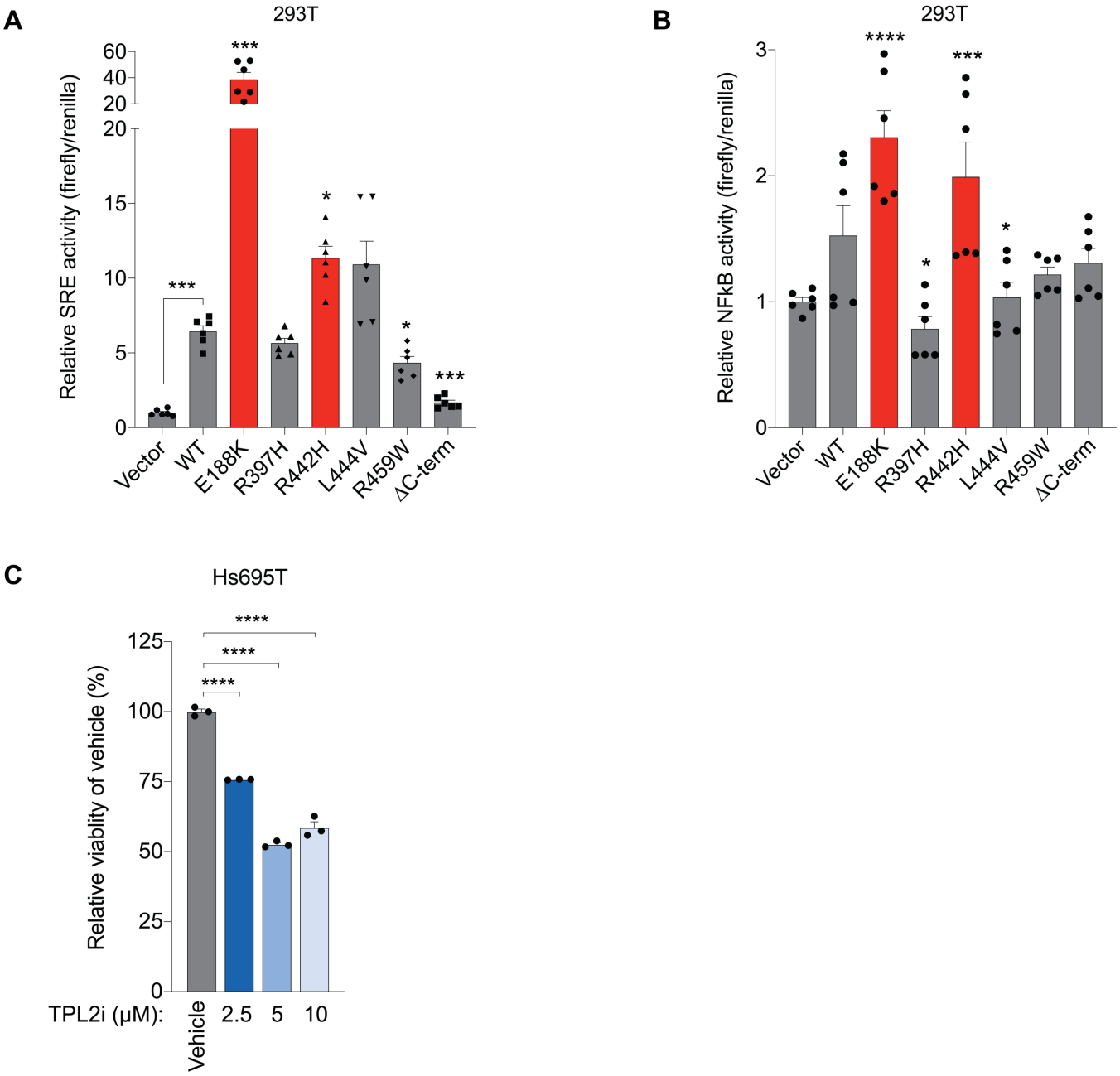
Supplementary Figure 10 (Supports Figure 7)



Supplementary Figure 11 (Supports Figures 8, **9** and **10)**

(A) Relative serum-response element (SRE) activity in 293T cells transfected with empty vector or vector encoding HA epitope-tagged TPL2 (wild-type or mutants) for 48 hours. Data is from two independent experiments, each of technical triplicate. **(B)** Relative NFκB reporter activity in 293T cells transfected with empty vector or vector encoding HA epitope-tagged TPL2 (wild-type or mutants) for 48 hours. Data is from two independent experiments, each of technical triplicate. **(C)** Relative viability of Hs695T cells treated with TPL2i for 120 hours. Experiment was done once in technical triplicate *P* values from one-way ANOVA with Dunnett's multiple comparison test. For **(A)** and **(B)** repeated measures one-way ANOVA with Dunnett's multiple comparison test (others vs. WT) was performed. All error bars denote mean ± SEM; ****P<0.0001, ***P<0.0002, **P<0.0021, *P<0.0332

Supplementary Figure 11 (Supports Figures 8, 9 and 10)

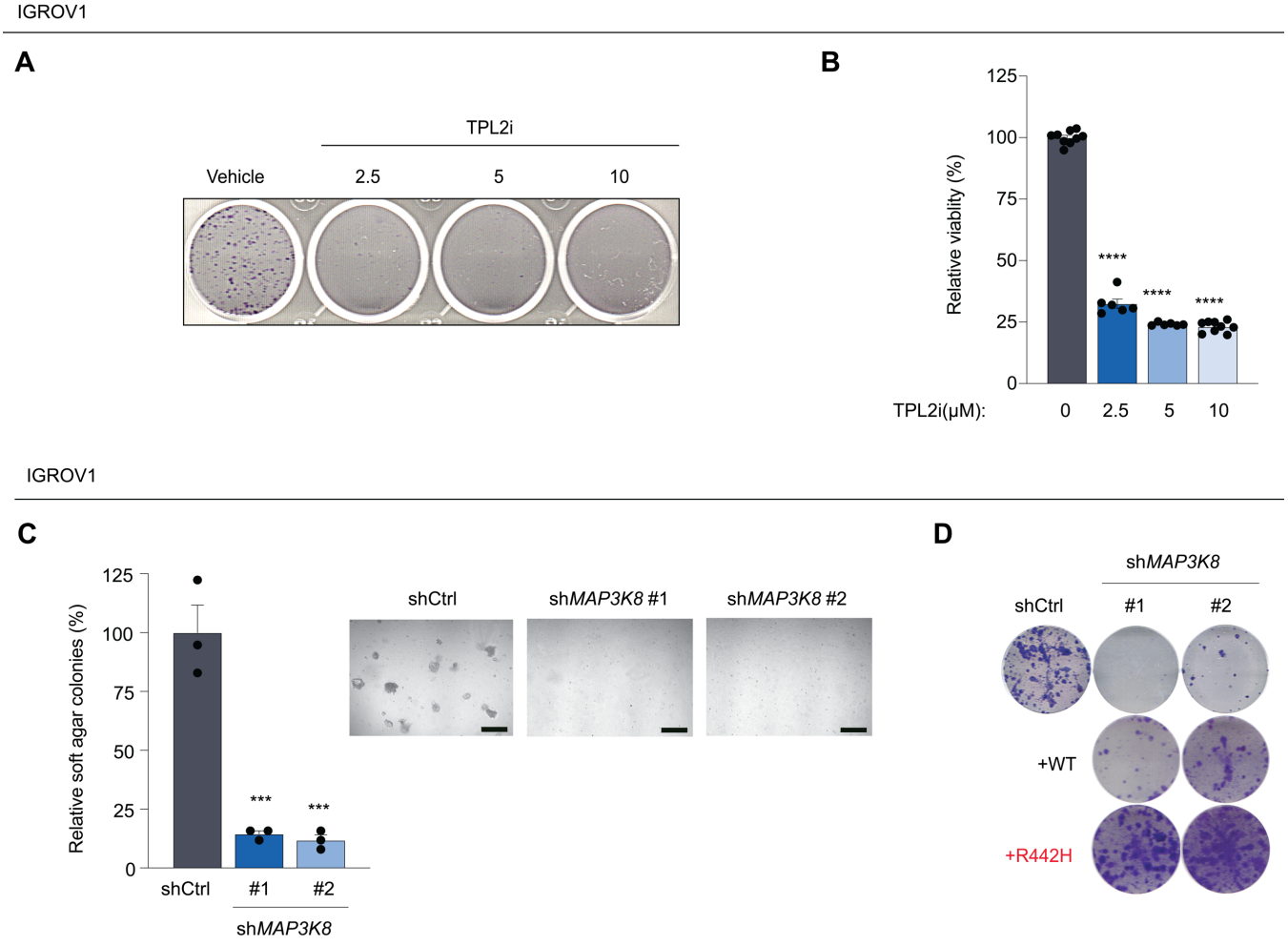


750

Supplementary Figure 12 (Supports Figure 10)

(A) 2D clonogenic colony formation of IGROV1 cells treated with TPL2i. **(B)** Relative viability of IGROV1 cells treated with TPL2i for 96 hours. Data is average of nine replicates from two independent experiments. **(C)** Quantification of soft agar colonies formed by IGROV1 cells after shRNA mediated TPL2 depletion. Data is average of three replicates from a single experiment. Scale bar is 500μm. **(D)** Image showing 2D clonogenic growth of IGROV1 cells after TPL2 knockdown as well as restoration of TPL2 expression with TPL2 WT or TPL2 R442H. For **(B)** and **(C)**, *P* values from one-way ANOVA with Dunnett's multiple comparison test. All error bars indicate mean ± SEM; ****P<0.0001, ***P<0.0002, **P<0.0021, *P<0.0332

Supplementary Figure 12 (Supports Figure 10)

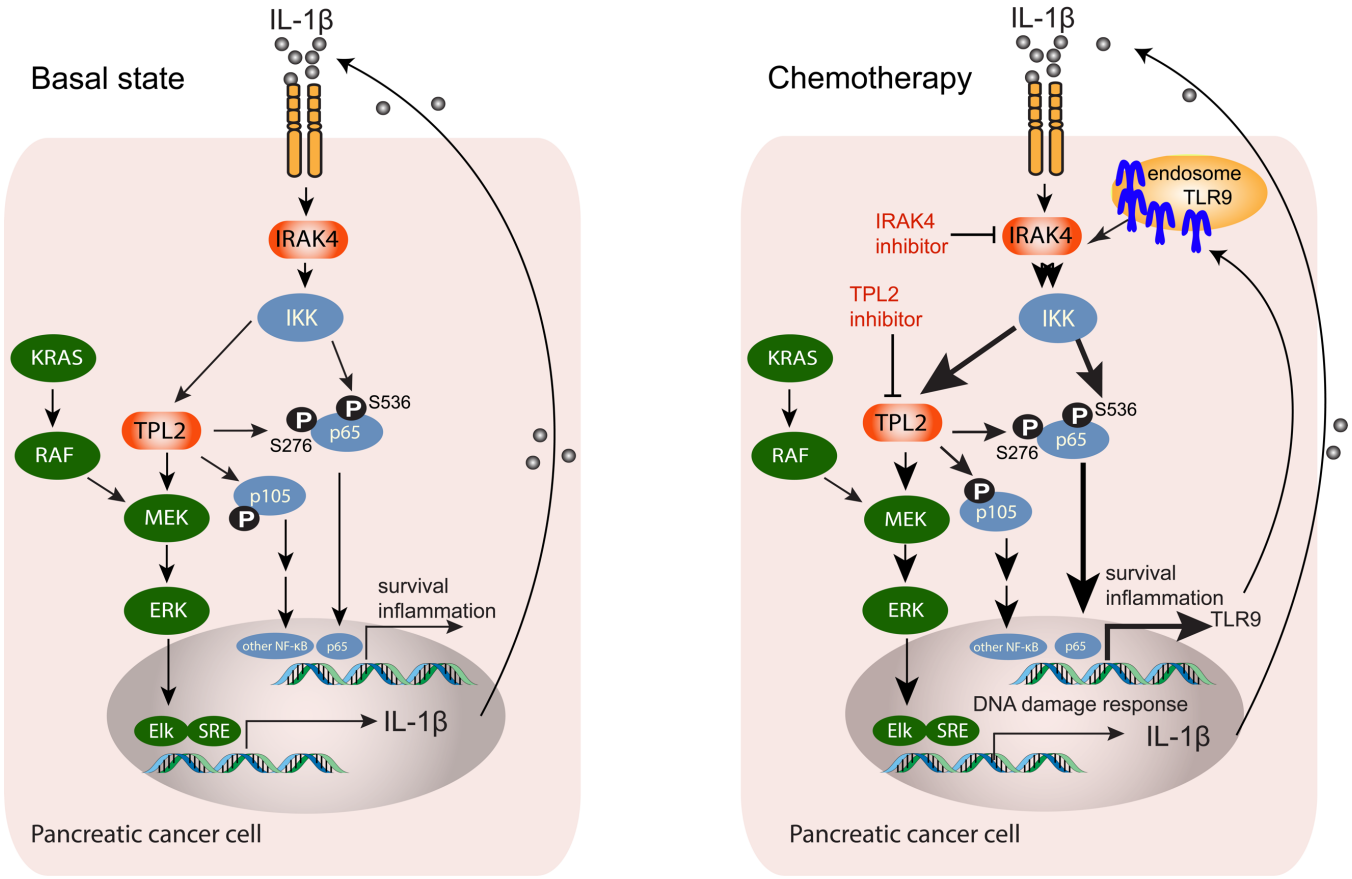


Supplementary Figure 13. Proposed signaling mechanism graphic

(A) Schematic cartoon depicting our proposed mechanism that leads to activation of p-IRAK4 and TPL2 signaling in basal state and in context of chemotherapy induced genotoxic stress.

Supplementary Figure 13

TPL2 drives *RAS*-induced inflammatory signaling and promotes survival under genotoxic stress



Supplementary Table 1. *IRAK4* pan-cancer mRNA expression *P*-values

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
PDAC vs. Adrenocortical carcinoma	0.4806	0.2721 to 0.6890	Yes	****	<0.0001
PDAC vs. Bladder	0.1298	-0.008270 to 0.2679	No	ns	0.0815
PDAC vs. Breast	0.08398	-0.04037 to 0.2083	No	ns	0.4463
PDAC vs. Cervical	0.1362	-0.009685 to 0.2821	No	ns	0.0858
PDAC vs. Cholangiocarcinoma	0.3248	0.04444 to 0.6053	Yes	*	0.0115
PDAC vs. Colorectal	0.03366	-0.09773 to 0.1650	No	ns	0.9988
PDAC vs. DLBC	-0.6839	-0.9335 to -0.4343	Yes	****	<0.0001
PDAC vs. Esophagus	-0.09136	-0.2535 to 0.07076	No	ns	0.7085
PDAC vs. GBM	0.05462	-0.1127 to 0.2219	No	ns	0.9936
PDAC vs. Head and Neck	0.2036	0.06997 to 0.3372	Yes	***	0.0002
PDAC vs. chromophobe RCC	-0.8905	-1.113 to -0.6681	Yes	****	<0.0001
PDAC vs. ccRCC	-0.04327	-0.1771 to 0.09053	No	ns	0.9938
PDAC vs. pRCC	0.1246	-0.02233 to 0.2716	No	ns	0.1607
PDAC vs. AML	-1.277	-1.441 to -1.113	Yes	****	<0.0001
PDAC vs. LGG	0.8688	0.7351 to 1.002	Yes	****	<0.0001
PDAC vs. Liver	0.8025	0.6621 to 0.9429	Yes	****	<0.0001
PDAC vs. Lung Adenocarcinoma	0.1298	-0.004041 to 0.2636	No	ns	0.0643
PDAC vs. Lung Squamous	-0.2042	-0.3390 to -0.06951	Yes	***	0.0002
PDAC vs. Mesothelioma	0.0478	-0.1530 to 0.2486	No	ns	0.9989
PDAC vs. Ovarian	0.2411	0.09569 to 0.3864	Yes	****	<0.0001
PDAC vs. PCPG	0.6489	0.4861 to 0.8117	Yes	****	<0.0001
PDAC vs. Prostate	0.4648	0.3304 to 0.5992	Yes	****	<0.0001
PDAC vs. Sarcoma	0.1332	-0.01707 to 0.2835	No	ns	0.1224
PDAC vs. Melanoma	0.5222	0.3858 to 0.6586	Yes	****	<0.0001
PDAC vs. Stomach	0.04626	-0.09158 to 0.1841	No	ns	0.9933
PDAC vs. Testis	0.7555	0.5850 to 0.9261	Yes	****	<0.0001
PDAC vs. Thyroid	-0.06449	-0.1987 to 0.06973	No	ns	0.8805
PDAC vs. Thymoma	-0.6417	-0.8235 to -0.4599	Yes	****	<0.0001
PDAC vs. Uterine	0.2865	0.1532 to 0.4197	Yes	****	<0.0001
PDAC vs. Uterine CS	1.067	0.8338 to 1.301	Yes	****	<0.0001
PDAC vs. Uveal Melanoma	1.032	0.8251 to 1.238	Yes	****	<0.0001

Supplementary Table 2. *MAP3K8* (TPL2) pan-cancer mRNA expression *P*-values

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
PDAC vs. Adrenocortical carcinoma	2.094	1.670 to 2.518	Yes	****	<0.0001
PDAC vs. Bladder	-0.3828	-0.6637 to -0.1019	Yes	**	0.0012
PDAC vs. Breast	0.4233	0.1704 to 0.6762	Yes	****	<0.0001
PDAC vs. Cervical	-0.2063	-0.5031 to 0.09046	No	ns	0.4038
PDAC vs. Cholangiocarcinoma	0.6492	0.07882 to 1.219	Yes	*	0.014
PDAC vs. Colorectal	0.269	0.001805 to 0.5363	Yes	*	0.0472
PDAC vs. DLBC	-1.072	-1.580 to -0.5645	Yes	****	<0.0001
PDAC vs. Esophagus	-0.4516	-0.7814 to -0.1219	Yes	**	0.0011
PDAC vs. GBM	0.731	0.3907 to 1.071	Yes	****	<0.0001
PDAC vs. Head and Neck	0.1313	-0.1405 to 0.4031	No	ns	0.8761
PDAC vs. chromophobe RCC	1.122	0.6693 to 1.574	Yes	****	<0.0001
PDAC vs. ccRCC	0.5142	0.2421 to 0.7864	Yes	****	<0.0001
PDAC vs. pRCC	0.9496	0.6506 to 1.249	Yes	****	<0.0001
PDAC vs. AML	-1.72	-2.054 to -1.387	Yes	****	<0.0001
PDAC vs. LGG	1.559	1.287 to 1.831	Yes	****	<0.0001
PDAC vs. Liver	0.7123	0.4266 to 0.9980	Yes	****	<0.0001
PDAC vs. Lung Adenocarcinoma	-0.3938	-0.6660 to -0.1217	Yes	***	0.0004
PDAC vs. Lung Squamous	-0.1167	-0.3907 to 0.1573	No	ns	0.953
PDAC vs. Mesothelioma	-1.136	-1.544 to -0.7276	Yes	****	<0.0001
PDAC vs. Ovarian	-0.4374	-0.7330 to -0.1417	Yes	***	0.0003
PDAC vs. PCPG	2.047	1.716 to 2.379	Yes	****	<0.0001
PDAC vs. Prostate	1.866	1.592 to 2.139	Yes	****	<0.0001
PDAC vs. Sarcoma	0.8	0.4943 to 1.106	Yes	****	<0.0001
PDAC vs. Melanoma	2.134	1.857 to 2.412	Yes	****	<0.0001
PDAC vs. Stomach	-0.5443	-0.8247 to -0.2640	Yes	****	<0.0001
PDAC vs. Testis	0.6364	0.2896 to 0.9833	Yes	****	<0.0001
PDAC vs. Thyroid	1.131	0.8576 to 1.404	Yes	****	<0.0001
PDAC vs. Thymoma	1.678	1.307 to 2.048	Yes	****	<0.0001
PDAC vs. Uterine	0.4139	0.1429 to 0.6849	Yes	***	0.0002
PDAC vs. Uterine CS	1.635	1.160 to 2.110	Yes	****	<0.0001
PDAC vs. Uveal Melanoma	2.496	2.076 to 2.917	Yes	****	<0.0001