

**Supplementary Material and Methods:*****Global Proteome and Phosphoproteome Profiling using TMT-based Mass Spectrometry.***

Cell pellets were resuspended in lysis buffer (1% Sodium deoxycholate, 100mM Tris-HCl pH 8, 150mM NaCl, 1mM EDTA, 40mM chloroacetamide, 10mM dithiothreitol, phosphatase inhibitor cocktail 2 and 3 (Sigma)), heated for 10 minutes at 95°C, cooled down and treated with Benzonase® (Merck, 50 units) for 30 min at 37°C. Endopeptidase LysC (Wako) and sequence-grade trypsin (Promega) were added to 200 µg protein extract (enzyme-to-protein ratio of 1:50), followed by an incubation over night at 37°C. A reference mix sample with equal peptide amount from all samples was prepared and processed in parallel. Samples were acidified with formic acid (1% final concentration), desalted with Sep-Pak C18 cc Cartridges (Waters), resuspended in 50 mM HEPES and labeled with 16-plex tandem mass tag reagents (TMTpro, Fisher Scientific) following the vendors instructions. TMT channel assignment was randomized between two plexes, where reference was placed to the same channel (channel 16) in both plexes. Samples and references were combined, fractionated by high-pH reversed phase off-line chromatography (1290 Infinity, Agilent) and pooled into 30 fractions. 10% of each fraction was taken out for global proteome measurements. The remaining 90% were further pooled onto 15 fractions and applied to IMAC based phosphopeptide enrichment using Fe(III)-IMAC cartridges and the AssayMAP Bravo Platform (Agilent Technologies).

For LC-MS/MS measurements, peptides were reconstituted in 3% acetonitrile with 0.1% formic acid and separated on a reversed-phase column (20 cm fritless silica microcolumns (inner diameter of 75 µm, packed with ReproSil-Pur C18-AQ 1.9 µm resin (Dr. Maisch GmbH)), using a 98-min gradient of increasing Buffer B (90% ACN, 0.1% FA) concentration (from 2% to 60%) with a 250 nl/min flow rate on a High-Performance Liquid Chromatography (HPLC) system (Thermo Fisher Scientific) and analyzed on an Exploris 480 instrument (Thermo Fisher Scientific). The mass spectrometer was operated in data-dependent acquisition mode using 60K resolution, 375–1500 m/z scan range, maximum injection time of 50 ms and 300 % AGC target value. MS2 scans were obtained at 45K resolution with an 0.4 m/z isolation window, 100 % AGC target value and a maximum injection time of 86 ms. Cycle time was set to 1 s. Dynamic exclusion was set to 20 s and only precursor with a charge state between 2-6 were selected for fragmentation. For analysis of phosphopeptide enriched samples the parameters were the same, with the exception of MS2 maximum injection time that was set to 120 ms.

RAW data were analyzed with MaxQuant software package (v 1.6.10.43) using the Uniprot human databases (UP000005640\_2022\_03) and default protein contaminants. Reporter ion MS2 for TMT16 was selected (internal and N-terminal) and TMT batch specific corrections factors were specified. Minimum reporter precursor intensity fraction was set to 0.5. The search included variable modifications of methionine oxidation, N-terminal acetylation, deamidation (NQ), phosphorylation (STY) and fixed modification of carbamidomethyl cysteine. The FDR (false discovery rate) was set to 1% for peptide and protein identifications.

The resulting text files were used for data analyses. Reverse hits, potential contaminants and proteins only identified by site were excluded. Protein groups and phosphosite tables were further filtered for 100 % valid value. Corrected reporter ion intensities were log2 transformed and normalized by subtraction of the internal reference channel contained in each TMT plex. The resulting TMT ratios were normalized via median z-score within each sample. Differences in

protein and phosphosite abundance between experimental groups were calculated using Student's T-Test. Signals passing the significance cut-off of FDR 5%.

**Note on Large Language Model (LLM) use.** This manuscript was generated by human researchers (author list), but parts of the manuscript have been corrected and edited by a Large Language Model (LLM) such as ChatGPT. The LLM was used to assist with the formatting, organization, and clarity of the text, but did not contribute to the conceptualization, design, or interpretation of the experiments.

**Ethics, Consent to Participate and to Publish declarations.** All animal experiments were performed by EPO (Experimental Pharmacology and Oncology, Berlin-Buch) and were approved by the local responsible authorities and performed in accordance with the German Animal Protection law. The primary human PDAC models were established/analyzed in accordance with the declaration of Helsinki. The study was approved by the local ethics committee of the University Medical Center Göttingen (UMG) (vote 11/5/17). Written informed consent from the patients for research use was obtained prior to the investigation. Both sequencing data and expression profiles / count tables are not provided in the manuscript to prevent identification if any individuals. Therefore, consent to publish is not applicable.

**Single cell RNA sequencing Meta-analysis.** We collected raw scRNAseq data from a publicly available PDAC dataset (GEO: GSE205049) <sup>1</sup> and analyzed immune cell populations and phenotype using a common analysis pipeline. The gene expression matrix and metadata were imported into R (version 4.4.2) using Seurat (version 5.1.0). Cells with fewer than 100 detected genes, and more than 25% mitochondrial gene content were filtered out. Data were normalized using log-normalization, scaled, and subjected to PCA for dimensionality reduction. To address batch effects, Harmony (version 1.2.3) was applied. Cell clustering was performed using the Louvain algorithm (resolution=0.5), and UMAP was used for visualization. Double-negative (DN) T cells were identified by low expression of CD4, CD8 and NK markers, but positive CD3E expression. Code available upon request.

**Patient-derived organoids.** Excised tumor tissue was minced into small fragments using a scalpel and transferred to a 15 mL conical tube containing Human Digestion Medium. This medium consisted of Advanced DMEM/F-12 (#12634028, Gibco), 10 mM HEPES (#15630080, Gibco), 1× GlutaMAX (#35050061, Gibco), 0.1% BSA (#A9576, Sigma-Aldrich), 10% R-spondin1-conditioned medium (R-spondin1-expressing HEK293T cells), 50% Wnt-3a-conditioned medium (HEK293-WNT3A cells), 1× B27 (#17504001, Thermo Fisher), 10 mM Nicotinamide (#N0636, Sigma-Aldrich), 1.25 mM N-acetylcysteine (#A9165, Sigma-Aldrich), 100 µg/mL Primocin (#ant-pm-2, Invivogen), 100 ng/mL mNoggin (#250-38, Peprotech), 50 ng/mL hEGF (#PHG0313, Invitrogen), 100 ng/mL hFGF10 (#100-26, Peprotech), 10 nM hGastrin I (#3006, Tocris), and 500 nM A83-01 (#2939, Tocris). Medium was supplemented with 5 mg/mL Collagenase Type XI (#C9407, Sigma-Aldrich), 10 µg/mL DNase I (#D5025, Sigma-Aldrich), and 10.5 µM Y-27632 (#Y0503, Sigma-Aldrich). Digestion was performed at 37°C for 15 minutes on a rotator set to 300 rpm. Digested tissue fragments were allowed to settle, and supernatant was collected as first fraction. Fresh digestion medium was added to undigested tissue fragments for a second round of digestion, and resulting supernatant was combined with the first fraction. Combined fractions were centrifuged at 500 g for 5 minutes at 4°C, and the pellet was retained for further processing. If necessary, red blood cells were lysed by incubating the sample with ACK lysis buffer (#A10492-01, Gibco) for 3 minutes at room temperature. Following centrifugation at 500 g for 5 minutes at 4°C, the pellet was resuspended in Matrigel Growth Factor Reduced Basement Membrane Matrix (#356231, Corning), and 50 µL Matrigel domes were plated into pre-warmed 24-well plates. Plates

were incubated at 37°C for 30 minutes to allow Matrigel solidification, after which 500 µL of Human Complete Feeding Medium supplemented with 10.5 µM Y-27632 was added to each well. The plates were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere.

For propagation, patient-derived organoids (PDOs) were cultured in a medium containing Advanced DMEM/F-12 (#12634028, Gibco), 10 mM HEPES (#15630080, Gibco), 1× GlutaMAX (#35050061, Gibco), 0.1% BSA (#A9576, Sigma-Aldrich), 10% R-spondin1-conditioned medium, 1× B27 (#17504001, Thermo Fisher), 10 mM Nicotinamide (#N0636, Sigma-Aldrich), 1.25 mM N-acetylcysteine (#A9165, Sigma-Aldrich), 100 µg/mL Primocin (#ant-pm-2, Invivogen), 100 ng/mL mNoggin (#250-38, Peprotech), 100 ng/mL hFGF10 (#100-26, Peprotech), 10 nM hGastrin I (#3006, Tocris), and 500 nM A83-01 (#2939, Tocris).

For organoid passaging, the Matrigel was mechanically disrupted by pipetting, and the suspension was collected in a 15 mL conical tube kept on ice. Sample was centrifuged at 500 g for 5 minutes at 4°C, and the pellet was resuspended in Express Enzyme (#12605028, Gibco) supplemented with 10 µg/mL DNase I (#D5025, Sigma-Aldrich) and 10.5 µM Y-27632 (#Y0503, Sigma-Aldrich). After incubation at 37°C for 15 minutes, the reaction was stopped by adding 5 mL of ice-cold wash medium (Advanced DMEM/F-12 supplemented with 10 mM HEPES, 1× GlutaMAX, and 0.1% BSA). The pellet was then resuspended in Cultrex Reduced Growth Factor Basement Membrane Extract (#3536-005-02, R&D Systems) and plated as 50 µL domes in pre-warmed 24-well plates. Plates were incubated for 1 hour at 37°C for Cultrex solidification, after which 500 µL of Human Complete Feeding Medium supplemented with 10.5 µM Y-27632 was added. Organoids were cultured at 37°C in a humidified incubator with 5% CO<sub>2</sub>.

**Cell culture and treatment, Chemicals, Viral Infection.** MiaPaCa-2 (CVCL\_0428) and Panc-1 (CVCL\_0480) were cultured in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific #41965062) supplemented with 10% Fetal Bovine Serum (Thermo Fisher Scientific #10270106). BxPc3 (CVCL\_0186), PSN1 (CVCL\_1644), AsPc1 (CVCL\_0152) and DanG (CVCL\_0243) were cultured in Roswell Park Memorial Institute 1640 Medium (Thermo Fisher Scientific #21875091) supplemented with 10% Fetal Bovine Serum (Thermo Fisher Scientific #10270106). 9091PPT (kindly provided by D. Saur) and 53631PPT murine PDACs cell lines were cultured in DMEM (Thermo Fisher Scientific #41965062). Cells were cultured in a humidified incubator at 37°C with 5% CO<sub>2</sub>. Cells were regularly tested for Mycoplasma contamination by PCR and confirmed to be negative. Cell identity was analyzed by short tandem repeat (STR) profiling.

**Generation of copanlisib-resistant cells.** MiaPaCa-2 cells were continuously cultured for six months. The adaptation process involved supplementing the culture medium with increasing concentrations of copanlisib. Treatment commenced at a sub-lethal concentration of 10nM and was gradually escalated every two weeks reaching a final concentration of 1000nM. Cells were routinely passaged every 3-4 days. The successful development of copanlisib-resistant cells was evidenced by the increased cell viability and reduced apoptosis under copanlisib treatment. For comparison parental cells were kept in culture in parallel for the same period of time.

**Chemicals.** Subasumstat was either purchased from MedChemExpress or provided by Millennium Pharmaceuticals, Inc., a wholly owned subsidiary of Takeda Pharmaceutical Company Limited. Subasumstat doses and treatment durations are indicated in the figure legends. Pictilisib (RG7321) was purchased from Selleckchem. Copanlisib (HY-15346), zVAD-fmk (HY-16658B) and necrostatin-1 (HY-15760) were purchased from MedChemExpress, Parsaclisib (HY-109068), Eganelisib (HY-100716), GSK2636771 (HY-15245), Idelalisib (HY-13026). Alpelisib was purchased from TargetMol (T1921).

**Viral infection.** For the generation of lentiviral particles, HEK293T cells were co-transfected with the indicated lentiviral plasmids and viral packaging plasmids (Lipofectamine 2000, Invitrogen

#11668027). Virus supernatants were collected 48h after transfection and used to transduce the indicated cell line in the presence of 1ug/ml polybrene (Sigma-Aldrich, #TR-1003-G).

**Cyclic Immunofluorescence.** To prepare slides for cyclic immunofluorescence (cyclIF) staining, tissue sections were deparaffinized by baking at 65°C for 30 min a laboratory oven. Subsequently, slides were immersed in xylene and then rehydrated in a graded series of ethanol to distilled water (100%, 95%, 70%). To retrieve epitopes masked during tissue fixation, slides were subjected to heat and pH-mediated antigen retrieval: Slides were immersed in pH 6 citrate buffer (Antigen Retrieval Citra Plus, BiogeneX HK080-9K), heated in a pressure cooker and then transferred to warm Tris/EDTA buffer (Target Retrieval Solution, Dako S2368). Endogenous peroxidases were blocked using 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, Sigma-Aldrich H1009) for 10 minutes with illumination and initial fluorescence bleaching was performed to reduce background signal before the first cycle. From now, washing of slides in 1x PBS with agitation was performed 3 times with 2-10 minutes each between further steps.

Subsequent iterative cyclIF rounds consisted of staining, imaging and quenching of fluorophores. Fluorophore-conjugated anti-mouse antibodies used in cyclIF are: CD3ε (Cell Signaling Technology; CST, E4T1B, 56611), CD4 (CST, D7D2Z, 93960), CD8α (CST, D4W27, 98941), PD1 (CST, D7D5W, 61237), Ki67 (CST, D3B5, 12075), CC3 (CST, D3E9, 97774S), CD68 (CST, E3O7V, 17846). Antibodies were diluted in blocking buffer (10% Normal Goat Serum, GIBCO #16210), 1% Bovine Serum Albumin, Sigma-Aldrich A9647; in 1x PBS) and incubated on the slides for 2 hours at room temperature in a humidified chamber protected from light. Cover glass was mounted using mounting medium (SlowFade™ Gold Antifade Mountant with DAPI, Invitrogen S36938) and images acquired with an automated slide scanner (Zeiss AxioScan Z1, Carl Zeiss Germany). Cover glass was removed by gentle agitation of slides in 1x PBS and fluorophores bleached twice with freshly prepared quenching solution (3% H<sub>2</sub>O<sub>2</sub>, 20 mM Sodium Hydroxide in 1x PBS) for 25 minutes each whilst being illuminated.

DNA content measured by DAPI staining and expression of each marker was quantified from images by signal coverage using Fiji (NIH Image to ImageJ). Staining of each single marker was manually threshold-ed to a binary black/white scale excluding background signal.

**Genome-wide CRISPR/Cas9 knockout library screen.** The human Brunello library (Addgene, #73178) was used to identify genes for synthetic lethal interactions with subasumstat in PDAC cells. First, stable Cas9-expressing cell lines were established by lentiviral transduction (Addgene, #181981). The expression of Cas9 was confirmed by western blotting and the function was validated by electroporation with specific guide RNAs and target knockout analysis. The final dose of subasumstat used was the GI30 dose (30% Growth Inhibition). Cas9 expressing cells were transduced with the lentiviral libraries and selected with Puromycin (Gibco™, #A1113803, final concentration 1ug/ml) for 5 days. Cells were harvested for day0 condition, and the rest was then split into control (vehicle, DMSO) and treatment groups (120nM subasumstat). Cells were passaged every 3-4 days for 2 weeks and drug treatment was refreshed each time. On day 14, cells were harvested, and genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, #69504) according to the manufacturer's instructions.

**Flow cytometry. Apoptosis assay.** For detection of apoptotic cell death, cells were stained with AnnexinV/PI by flow cytometry. Briefly, 1x10<sup>6</sup> cells were centrifuged at 300g for 5min, resuspended in 300uL 1X Annexin V binding buffer (BD Pharmingen, #8008652), added 4uL APC Annexin V (BioLegend #640941) and 10ug/mL PI (Sigma, #P4864) and incubated for 15min at 4°C in the dark. Cells were analyzed by flow cytometry using a CytoFLEX S Flow Cytometer (Beckman Coulter). Annexin V staining was quantified as early apoptotic (Annexin V+, PI-) and apoptotic-dead cells (Annexin V+, PI+). Analysis was performed using FlowJo™ v10.6.0 software.



**Cell cycle assay.**  $5 \times 10^5$  were collected and washed with PBS and fixed with 70% ethanol and stored at 4°C overnight. Cells were resuspended in PBS containing 0,25% Triton X-100 and incubated on ice for 15 minutes. Pellets were washed with PBS containing 0,5% BSA and resuspended finally with PBS containing 10 µg/ml of RNase A and 20 µg/ml of PI, incubated at room temperature for 30 minutes in the dark then analyzed by flow cytometry. Analysis was performed using FlowJo™ v10.6.0 software.

**Western Blot.** Cells were seeded at a density of  $2 \times 10^6$  in 10 cm dishes. The following day, dishes were washed with PBS and medium replaced with medium plus corresponding treatment. At indicated timepoints, cells were harvested in RIPA buffer and fractioned on SDS-PAGE gels. Protein lysates were transferred to PVD-FL membranes (Immobilon-Merck) and incubated with specific primary antibodies at 4°C overnight. For ECL measurements, membranes were incubated for 1h at room temperature with HRP-conjugated secondary antibodies (Cell Signaling Technologies #7076S and #7074P) and HRP-substrate (Merck Millipore Immobilon Western #24191B4) was used. Membranes were developed with the OdysseyM imager (LiCor Biosciences). A list of antibodies used can be found in **STable3**. All the quantification of the blots was analyzed by EmpiriaStudio3.1.

**In vivo drug efficacy analysis in mice and Immunohistochemistry.** All animal experiments were performed by EPO (Experimental Pharmacology and Oncology, Berlin-Buch) and were approved by the local responsible authorities and performed in accordance with the German Animal Protection law. Subcutaneous MiaPaCa-2 xenograft experiments were performed in *NMRI nu/nu* mice. Tumor growth was monitored daily by measurement with a caliper. Once the tumor volume reached 0.2 cm<sup>3</sup>, mice were randomized into the different groups and treated with vehicle, subasumstat, copanlisib, or the combination for 18 days. For the orthotopic pancreatic tumor model, cancer cells were transplanted into the pancreas of syngeneic immunocompetent *C57Bl/6J* mice. When tumor volume reached 0.1 cm<sup>3</sup>, mice were randomized into the different treatment cohorts. Animals were sacrificed when individual mouse reached the human endpoint or at completion of treatment.

**Library preparation, Next-Generation Sequencing and MaGECK analysis.** The PCR was set up according to manufacturer's protocol. One PCR reaction (100 µL) contained up to 10 µg of gDNA, 50 µL NEBNext High-Fidelity 2X MasterMix (NEB, #M0541S) and 1 µL of each primer (P7 with unique sequencing-barcodes and P5 with staggers). AMPure XP purification beads (Beckman Coulter #A63881) were used according to manufacturer's instructions. All samples were sent to Novogene (Cambridge, UK) where they were sequenced on a NovaSeq Illumina machine. Sequencing analysis was conducted using the MaGECK pipeline <sup>2</sup>.

**RNA isolation.** MiaPaCa-2 cells were seeded at day 0 onto 6-well dishes at a density of  $5 \times 10^5$  cells/well. On day 1, cells were treated for 6 or 24 hours. Cells were lysed and RNA isolated with the RNeasy Isolation kit (Qiagen, #74104).

**GSEA and ssGSEA.** Gene Set Enrichment Analysis (GSEA) was performed using the GenePattern platform from the Broad Institute under standard settings. Single-sample GSEA (ssGSEA) was conducted using GenePattern with the sample normalization method set to log.rank <sup>3</sup>. Additionally, GSEA analysis was carried out using the GeneTrail web tool <sup>4</sup>, and the resulting data were visualized as dot plots generated in R.

**Colony formation assay.** Cells were seeded onto 6-well or 24-well plates, grown for 24h, and then treated with indicated compounds for 96 hours. Wells were washed with PBS, fixed and stained with Crystal violet. Plates were either scanned and cell growth was assessed by readout

with the OdysseyM imager (LiCor Biosciences) or first solubilized and optical density was measured at 590nm. Density of vehicle-treated cells was arbitrarily set to 100% and dose-response was calculated as relative viability.

### **Single-cell library preparation**

Three 50 µm FFPE sections per sample were dissociated following the 10x Genomics protocol for FFPE tissue sections (CG000632\_RevD), using the gentleMACS Octo Dissociator workflow. Library preparation was performed using the Fixed RNA Profiling protocol (CG000527\_RevF, 10x Genomics). For each sample, 550,000 cells were used for probe and barcode hybridization, which was carried out for 22 hours at 42°C. After hybridization, samples were pooled, and 210,000 cells were loaded into a single GEM well of a Chip Q. Sample index PCR was performed with 10 cycles.

### **Sequencing and alignment**

The library was sequenced on a NovaSeq X Plus 25B flow cell (Illumina) with 150 bp paired-end reads. Reads were demultiplexed with bcl2fastq v4.2.7 (Illumina). FASTQ files were processed with Cell Ranger v9.0.1 (10x Genomics) using the multi pipeline with the mouse reference (refdata-gex-GRCm39-2024-A) and the Chromium Mouse Transcriptome Probe Set v1.1.1.

### **Preprocessing of GEM Flex scRNA-seq data**

Ambient RNA was removed using scAR<sup>5</sup> on the raw feature barcode matrix with default parameters. Further analysis was performed using scanpy v1.10.3<sup>6</sup>. Calculation of quality metrics and filtering of low-quality cells based on median absolute deviations (MAD) was performed in adherence to single-cell best practices<sup>7</sup>. Samples were integrated using scVI<sup>8</sup> on the intersection of highly variable genes per sample, with the following parameters: n\_hidden=256, n\_latent=12, n\_layers=3, and gene\_likelihood='zinb'. Dimensionality reduction was performed using Uniform Manifold Approximation and Projection (UMAP), with the number of neighbors set to the number of cells divided by 1,000 (rounded to the nearest integer). Finally, cells were grouped based on leiden clustering and marker genes were derived with the scanpy rank\_genes() function using the Wilcoxon test. The marker genes were then used to annotate the clusters.

### **Downstream analysis GEM Flex scRNA-seq data**

Gene set scores were calculated using the scanpy score\_genes() function. ((either here or list in supplementary: For the M1 score, the following genes were used: Cd86, H2-Ab1, Tlr4, Fcgr1, Itgax, Il1b, Tnf, Cxcl9, Nos2, Stat1. For the M2 score, Mrc1, Arg1, Chil3, Stab1, Tgfb1, Il10, Mmp12, Clec10a, Stat3, Klf4.)) Pseudobulk profiles were generated to perform differential gene expression analysis between treatment groups. For each sample, cells were randomly shuffled and evenly split into two groups, forming two pseudobulk replicates. The raw counts of each group were summed to generate the pseudobulk profiles. Lowly expressed genes were removed by filtering for genes with at least 50 counts in 70% of pseudoreplicates. Subsequent analyses were performed following the standard DESeq2 workflow<sup>9</sup>. The decoupler package<sup>10</sup> was utilized to compute pathway enrichment scores across cells. Gene Set Enrichment Analysis (GSEA) was conducted using gseapy employing the signal-to-noise ratio as the ranking metric <sup>11</sup>.

### **STable 3a Western Blot Antibodies:**

Target	Company	Article number
SUMO1	Abcam	ab133352
SUMO2-3	Abcam	ab81371
panAKT	Cell Signaling Technology	#9272
phosphoAKT (Ser479)	Cell Signaling Technology	#9271

phosphoAKT(Thr308)	Cell Signaling Technology	#9275
UBLE1A/SAE1	Cell Signaling Technology	#13585
UBA2	Cell Signaling Technology	#8688
UBE2I/Ubc9	Cell Signaling Technology	#4786
P110 $\alpha$	Cell Signaling Technology	#4249
P110 $\delta$	Cell Signaling Technology	#34050
$\beta$ -actin	Sigma-Aldrich	#A1978
vinculin	Cell Signaling Technology	#4650

**Table 3b Flow Cytometry/IF Antibodies:**

Epitope	Reactivity	Fluorochrome	Company	Article number
AnnexinV	Human	APC	Biolegend	#640941
DAPI	all	-	Biolegend	#422801
Cleaved caspase-3	Human-mouse	Alexa 488	Cell Signaling Technology	#9603
Anti-CD3	Human	PE	Biolegend	#344805
Anti-CD3	Human	APC	Biolegend	#344811
Anti-CD14	Human	APC	Biolegend	#325607
Anti-CD56	Human	PE	Biolegend	#362507
Anti-CD16	Human	PE	Biolegend	#302007

**Table 4 CRISPR/Cas9 sgRNA sequences:**

sgRNA target	sgRNA sequence
PIK3CA ex2_fw	taatacgactcactataGGTTCACCTGATGATGGTTCGgttttagagctagaaatagc
PIK3CA ex2_rv	taatacgactcactataGGCTTTAGAATGCCTCCGTGgttttagagctagaaatagc
PIK3CD ex3_fw	taatacgactcactataGGTTGGCATTGCGGGACACAgtttagagctagaaatagc
PIK3CD ex3_rv	taatacgactcactataGGTGAGCTTTTGTACCCGCgttttagagctagaaatagc

## Supplementary References

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## Supplemental Figure Legends

### SFig.1: Both SUMOylation and PI3K pathways are PDAC vulnerabilities.

**A.** Top: OncoPrint diagram of mutational frequencies and types of alterations of PDAC driver gene and *PI3K* genes across independent PAAD datasets: Biankin et al. (Nature, 2012), Bailey et al. (Nature, 2016), TCGA Firehose Legacy, TCGA GDC, TCGA Pan-Cancer Atlas, Witkiewicz et al. (Nat Commun, 2015), and Cao et al. (Cell, 2021). Bottom, left: Expression of mRNA in pancreatic epithelial cells with and without expression of mutant Kras<sup>G12D</sup> (Data derived from GSE154543). Bottom, middle: Expression of mRNA in normal pancreas and PDAC samples from human patients (Data derived from Badea et al., 2008). Bottom, right: Protein expression in human normal tissue (N) and PDAC (T) samples (CPTAC data derived from Cao et al., 2021).

**B.** MSigDb (Hallmark) ssGSEA analysis of ICGC PDAC patient data (Bailey et al. (Nature, 2016) indicates an overrepresentation of PI3K/AKT/MTOR and MYC gene sets in the squamous subtype (blue) compared to other subtypes (red).

**C.** Heat map depicting the results of a pharmacological screen conducted in *KRAS* knockout cells, confirming an increased PI3K dependency of *KRAS* deficient cells. Data derived from Muzumdar *et al.* (Nat Commun, 2015)<sup>24</sup>.

**D.** Landscape plots depicting the synergistic area of concentrations of RMC-6236 (*KRAS* inhibitor) and GDC-0941 (pictilisib) combination treatment (Bliss synergy score). Viability was measured by ATP quantification using by CellTiterGlo® 72h after treatment with indicated concentrations in depicted cell lines.

**E.** Quantification of immunoblots from Figs. 1E, F. Top: Relative SUMO1, SUMO2/3, SAE1 and UBA2 expression was determined by normalization to loading controls. MiaPaCa-2 cells were treated with 1000nM pictilisib in for the indicated time points (0, 6, 12, 24, 48, 72h). Bottom: Relative SUMO1, SUMO2/3, SAE1, UBA2 and UBE2I protein expression levels in MiaPaCa-2 control cells (parental) or in copanlisib resistant cells, generated by increasing concentrations of copanlisib over a period of 6 months, resulting in the resistant cell lines R-500nM and R-1000nM. Protein bands were normalized to the corresponding loading controls.

**F.** Immunoblot analysis of SUMO1 and SUMO2/3 upon indicated treatment (left). Quantified relative expression of SUMO1 and SUMO2-3 (right). P-values were determined by ANOVA \**P* < .05, \*\*\**P* < .001.

**G.** Relative protein expression levels determined by mass-spec of indicated Sentrin/SUMO-specific proteases (SENPs) after 6h (top) or 24h (bottom) of copanlisib treatment (1000nM) in MiaPaCa-2 cells.

P-values were determined by ANOVA

**H.** Immunoblot analysis of indicated PI3K downstream targets upon copanlisib (1000nM for 6 and 24hours) and vehicle (DMSO) treatment in MiaPaCa-2 cells.

**I.** GSEA (MSigDb – Reactome) of transcriptome data from PDOs, derived from chemotherapy treated PDAC patients compared to PDOs, derived from non-treated (naïve) PDAC patients, display significantly enriched SUMOylation gene sets Zhou et al. (Nature Cancer, 2023)<sup>23</sup>.

**J.** Heatmap showing the expression pattern (mRNA) of indicated SUMO core machinery components in SOC treated PDAC with different (indicated) mutations in the *KRAS* gene. Mean expression of the entire SUMO core machinery was compared in treatment groups versus treatment naïve PDOs. P-value was determined by Student's t-test \* $P < .05$ .

### **SFig.2: The PI3K pathway cooperates with SUMOylation in PDAC.**

Landscape plots depicting the synergistic area of concentrations of copanlisib and subasumstat combination treatment in PDAC cells. Cells were treated with single and combination treatments using a 4 × 6 matrix for 96h. Cell confluency was assessed with clonogenic assay quantification by readout with the Odyssey M imaging system (LiCor Biosciences). Data depicted are mean values from n=3 independent experiments (ZIP synergy score is depicted as mean with corresponding P values).

### **SFig.3: Insights into the mode of action of the copanlisib and subasumstat combination.**

**A.** Landscape plots depicting the synergistic area of concentrations of PI3K isoform specific inhibitors eganelisib, idelalisib, alpelisb, GSK2636771 or parsaciclib (indicated on the plots) and subasumstat combination treatment with indicated concentrations in MiaPaCa-2 cells. Cells were treated with single and combination treatments using a 4 × 6 matrix for 96h. Cell confluency was assessed with clonogenic assay quantification by readout with the Odyssey M imaging system (LiCor Biosciences). Data depicted are mean values from n=3 independent experiments (ZIP synergy score).

**B.** Immunoblot analysis of indicated CRISPR/Cas9 mediated p110 isoform MiaPaCa-2 knockout cells (two different clones, each), probed with SUMO1, SUMO2/3 and beta-actin as loading control.

**C.** Immunoblot analysis of SUMO2/3, cleaved PARP, pAKT (Ser473), AKT, MCL1 and BCL-xL in MiaPaCa-2 cells supplemented with subasumstat (200nM), pictilisib (1000nM) or the combination of both. Alpha-Tubulin served as loading control.

**SFig.4: Effects of combination treatment on cell death and cell cycle.**

**A.** Representative images of cleaved caspase 3 (green) and DAPI (blue) upon combination treatment in MiaPaCa-2 cells for the indicated timepoints (subasumstat 200nM, copanlisib 1000nM or combination). Scale bars, 100µm (top). Quantification of imaging results with MiaPaCa-2 cells equipped with a cleaved-caspase reporter system upon indicated treatment (subasumstat 200nM, copanlisib 1000nM or combination) (bottom).

**B.** Flow cytometry analysis of cell cycle upon single or combination treatment for 72h in PSN1 and MiaPaCa-2 cells (subasumstat 200nM, pictilisib 1000nM or combination). The percentage of cells in each phase of the cell cycle is shown. Data are mean  $\pm$  SD; n=3. P-value of one-way ANOVA with Tukey's post hoc test \*\*\*\*< .0001, \*\*\*< .001, \*\*< .01.

**SFig.5: Disease progression in xenograft model upon combined treatment.**

Outline of experimental setup for the investigation of the *in vivo* effects of combined subasumstat and copanlisib treatment. The human MiaPaCa-2 cells were used to generate murine xenograft model. Mice were treated with vehicle, subasumstat, copanlisib and the combination (left). Tumor volume was measured over time (n=4 mice in each group). Data are mean  $\pm$  SD; n=4. P-value determined by two-way ANOVA with Tukey's post hoc test (right).

**SFig.6: Single cell RNA Sequencing from orthotopically transplanted mice treated with SUMOi, PI3Ki or combination of both.**

**A.** Uniform manifold approximation and projection (UMAP) all cells detected in the 4 treatment groups: vehicle (n=4), subasumstat (n=4), copanlisib (n=4), combination (n=4) are annotated by cell type.

**B.** Mean expression of mRNA of cell type specific markers to dissect tumor and stroma cell populations derived from all 4 treatment groups.

**C.** Mean expression of mRNA of T cell specific markers to dissect tumor and stroma cell populations derived from all 4 treatment groups.

**D.** Mean expression of mRNA of macrophage specific markers to dissect tumor and stroma cell populations derived from all 4 treatment groups.

**E.** Gene set enrichment analysis of the tumor cell population using the Hallmark set of the Molecular Signature Database in indicated treatment groups, each compared to vehicle control. Only significant pathways (Nominal P-value < .05) are displayed with corresponding normalized enrichment scores.

**SFig.7: Combination of subasumstat and copanlisib *in vivo* does not induce signs of toxicity or intolerability.**

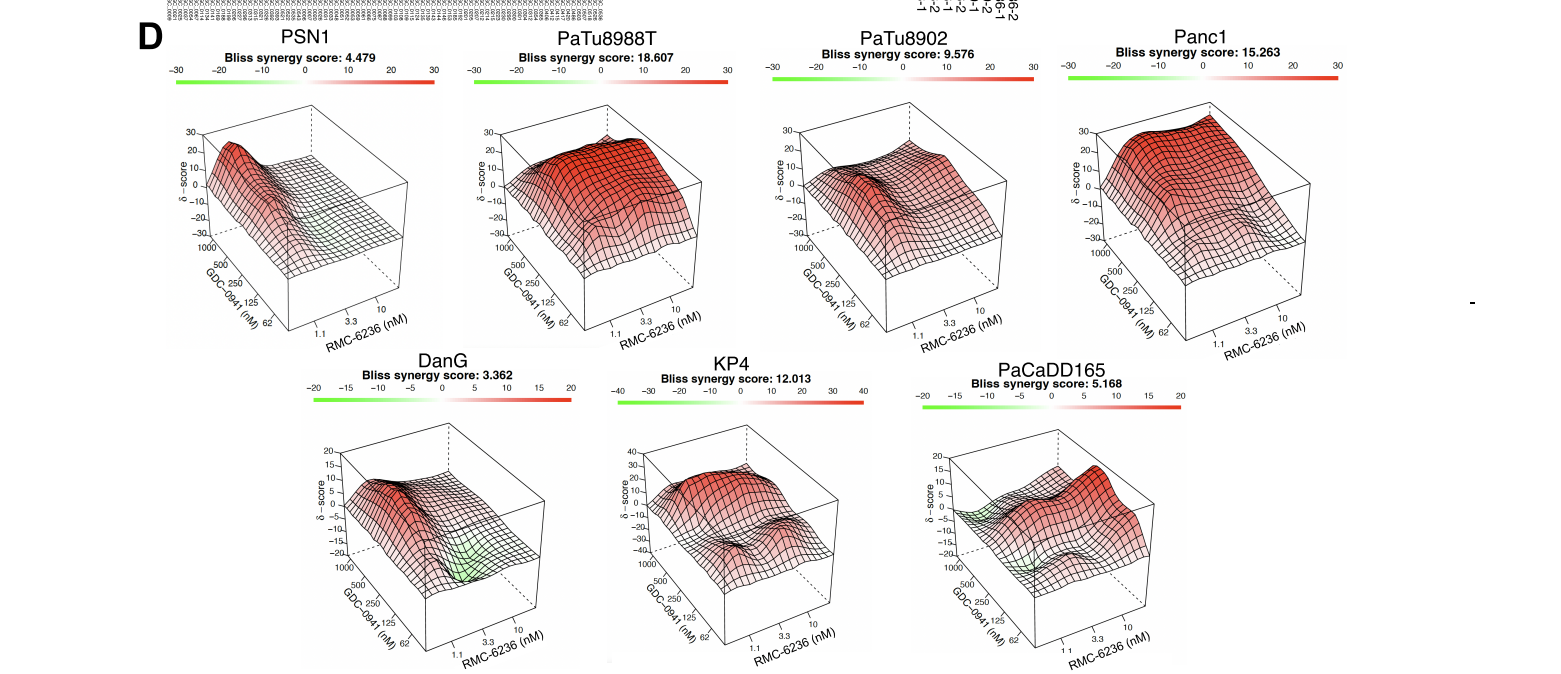
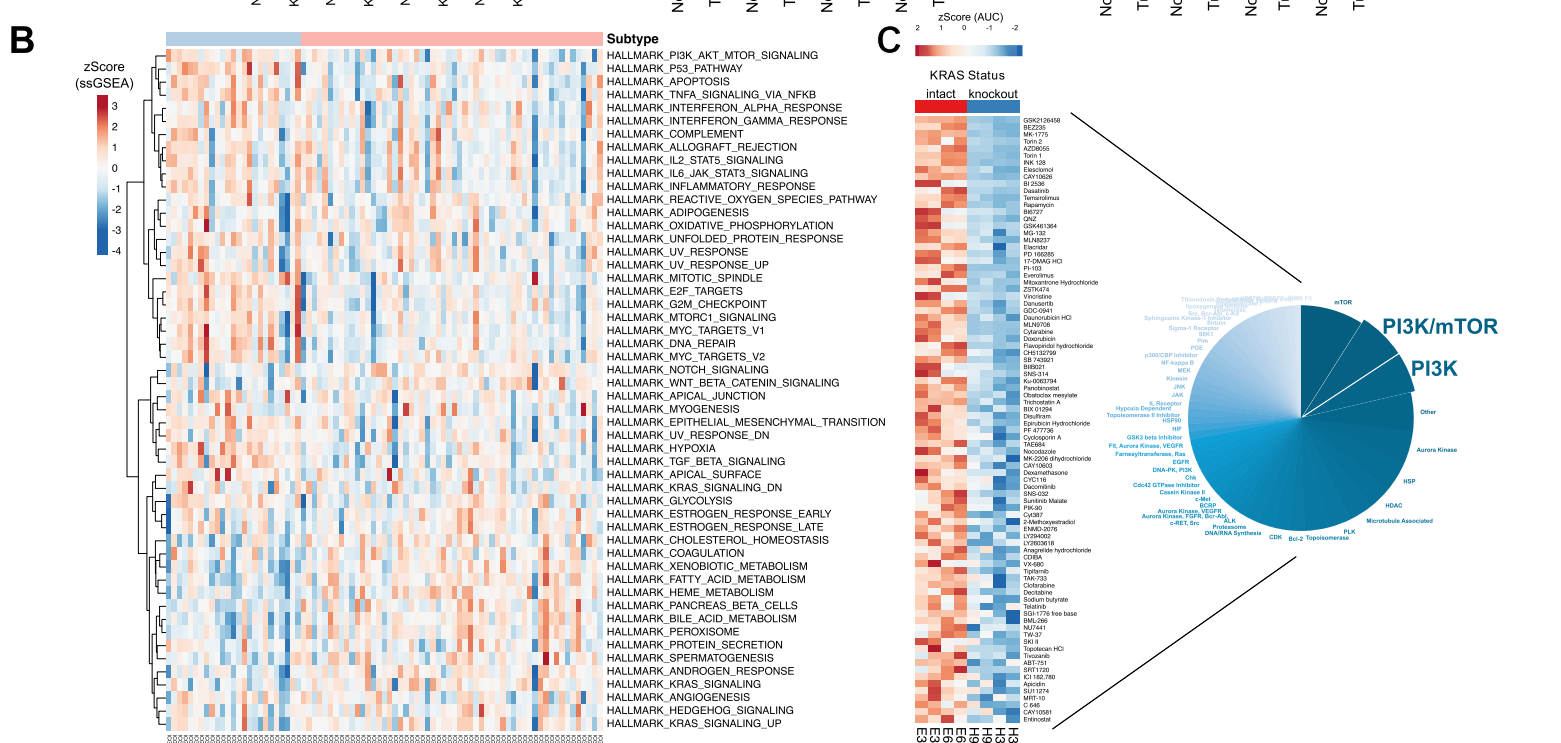
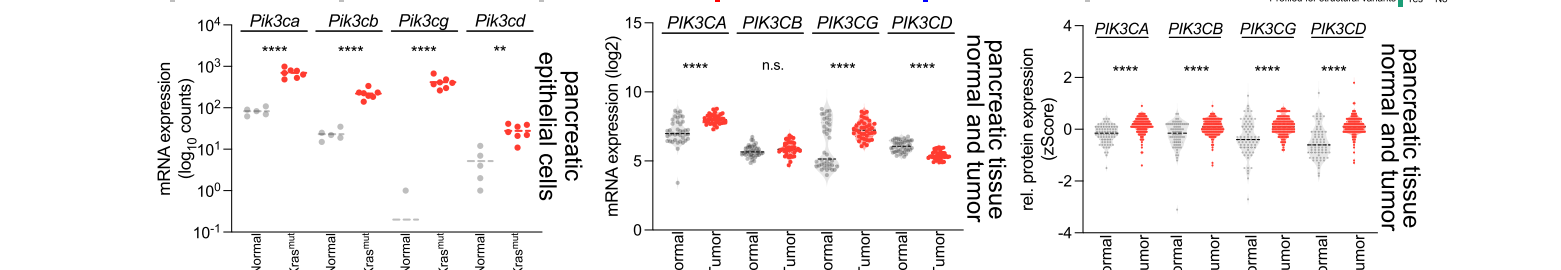
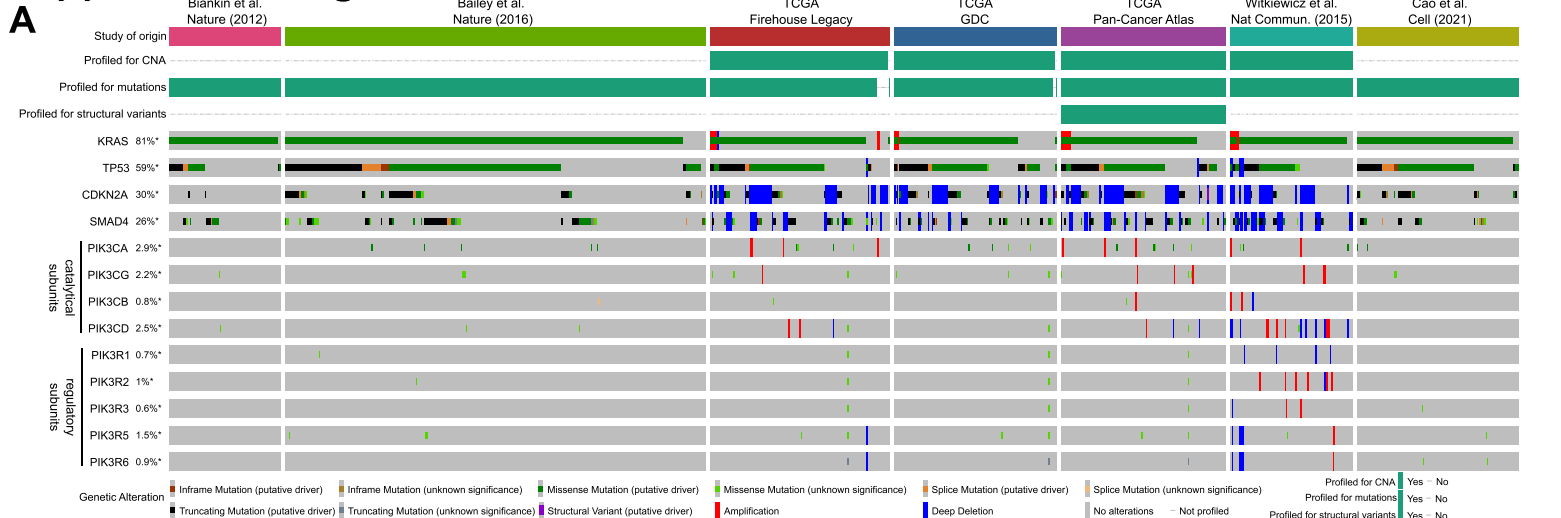
**A.** Body weight of *C57BL6/J* mice treated with the indicated regimens. Comparisons have been done at the endpoint. Significance determined with one-way ANOVA with Tukey's post hoc test \* < .05.

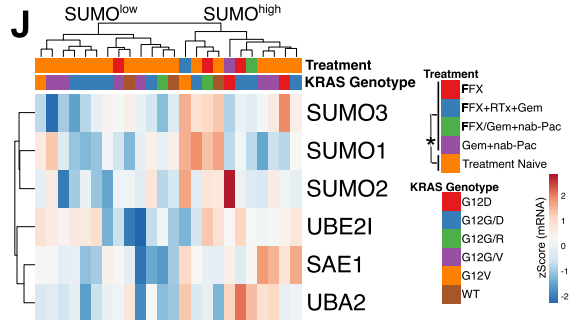
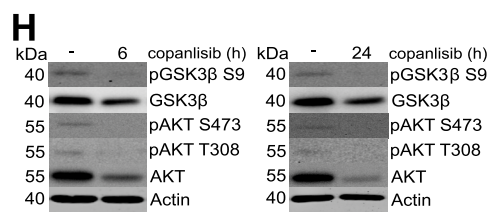
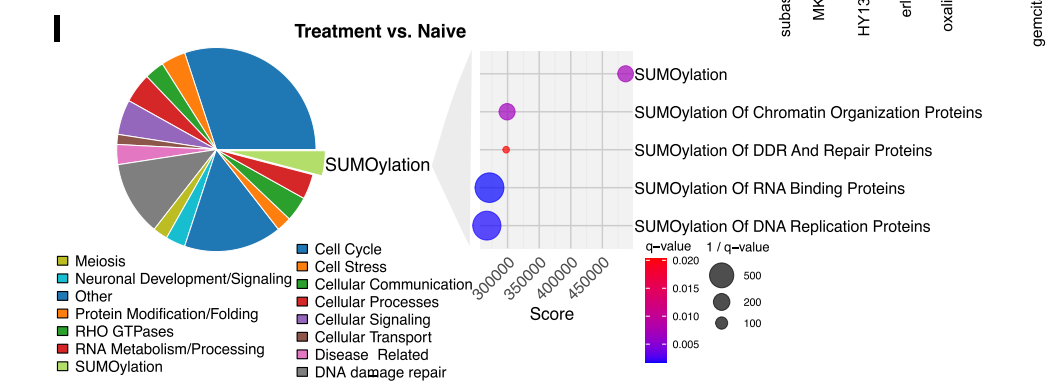
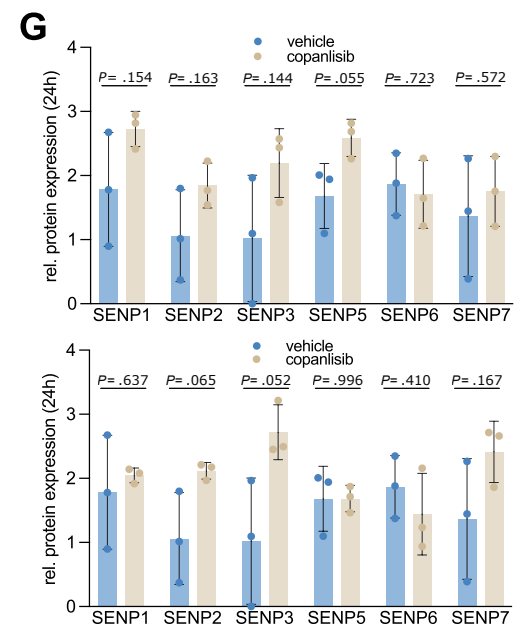
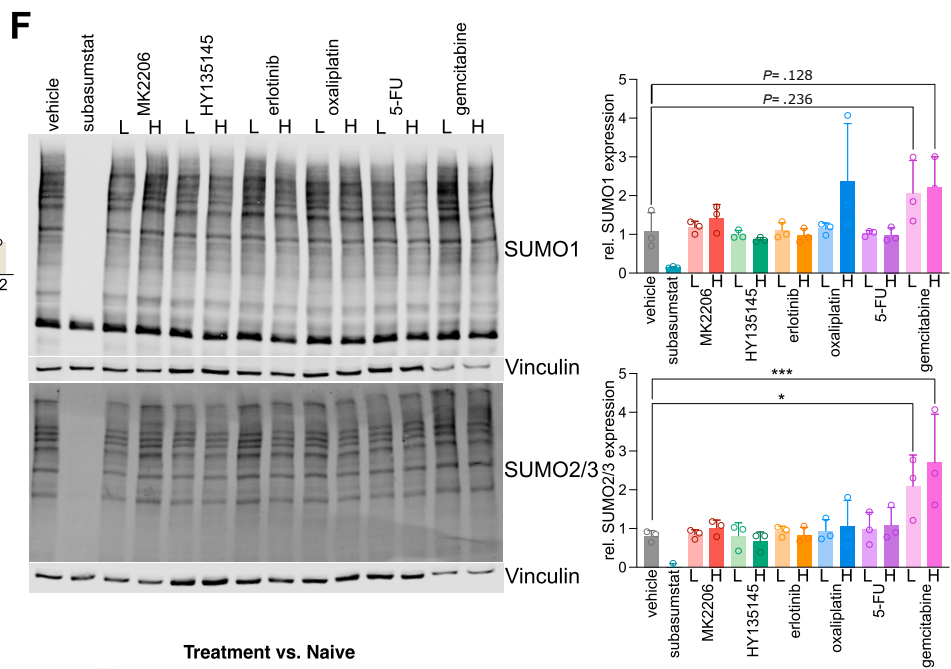
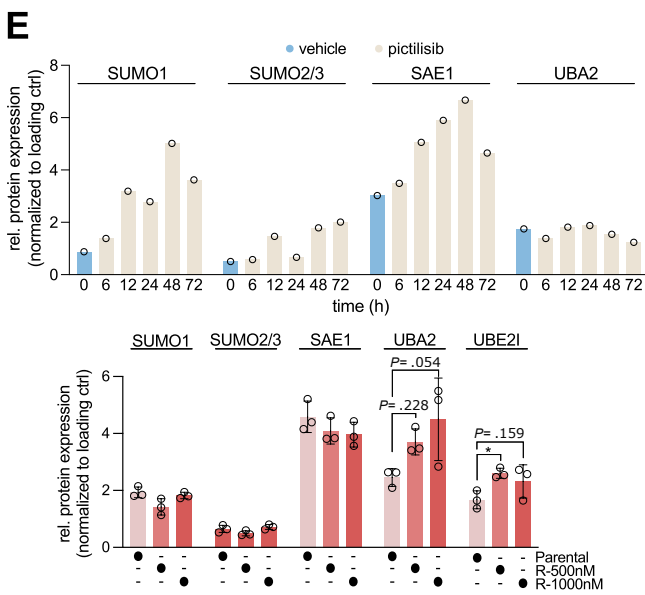
**B.** Complete blood count from the *C57BL6/J* mice treated with the indicated regimens reveals normal counts (LYM: Lymphocytes, MON: Monocytes, GRA: Granulocytes, EOS: Eosinophils, RBC: Red Blood Cell, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Hemoglobin, MCHC: Mean Corpuscular Hemoglobin Concentration, RDW: Red Cell Distribution Width, PLT: Platelets, MPV: Mean Platelet Volume). Significance determined with one-way ANOVA with Tukey's post hoc test.

**C.** Blood serum and liver enzyme analysis from the *C57BL6/J* mice treated with the indicated regimens does not show any sign of toxicity (LDH: Lactate Dehydrogenase, ALP: Alkaline Phosphatase, GPT: Glutamine Pyruvate Transaminase, ALT: Alanine Aminotransferase, GOT: Glutamate Oxaloacetate Transaminase, AST: Aspartate Aminotransferase, Calcium, Inorganic phosphate, Albumin, Urea, total protein, Bilirubin). Significance determined with one-way ANOVA with Tukey's post hoc test.

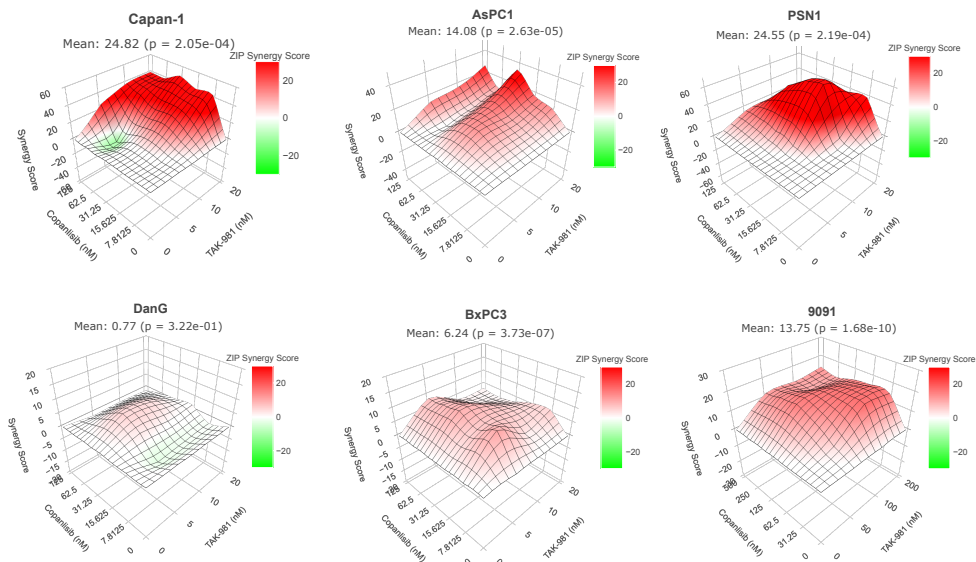


Supplemental Figure 1

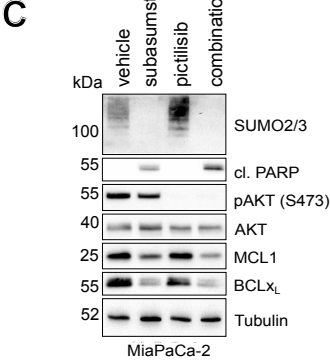
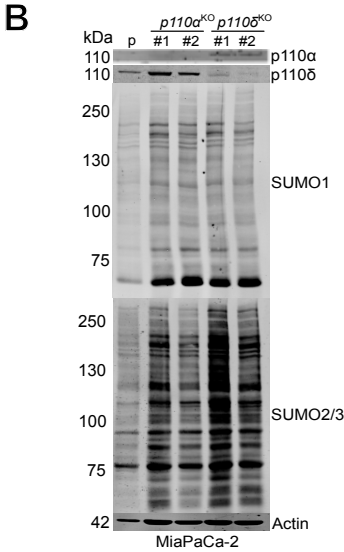
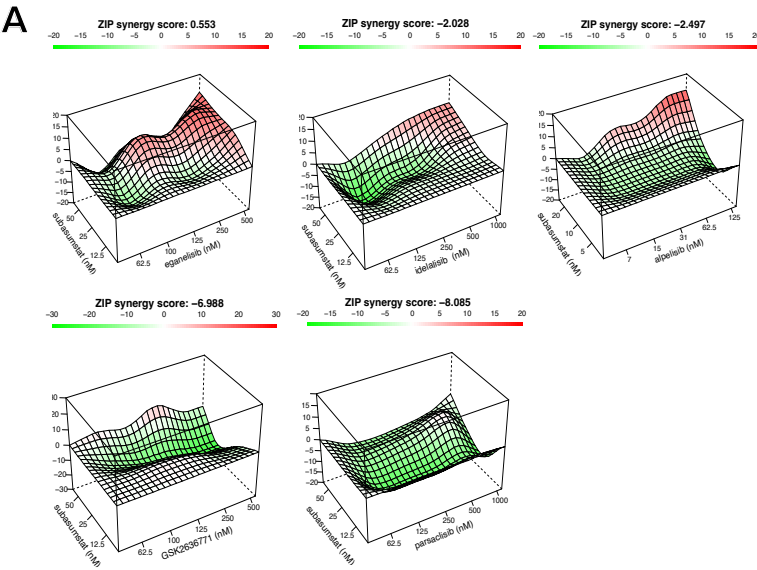




# Supplemental Figure 2

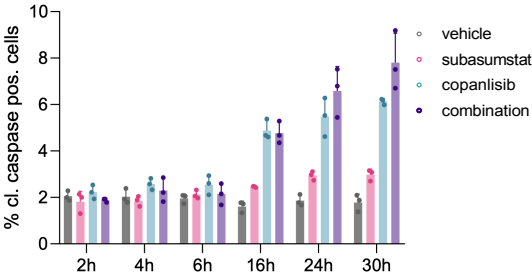
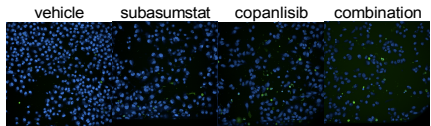


Supplemental Figure 3

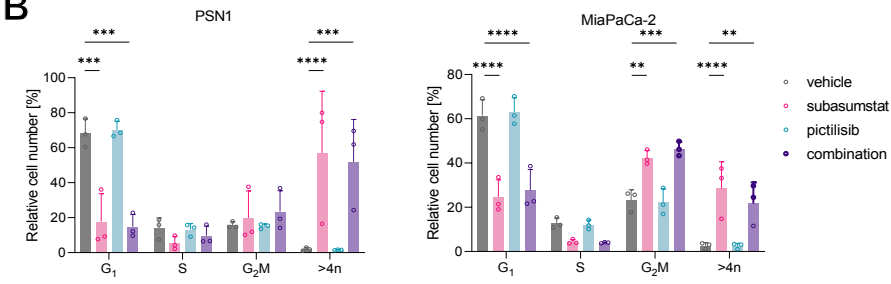


# Supplemental Figure 4

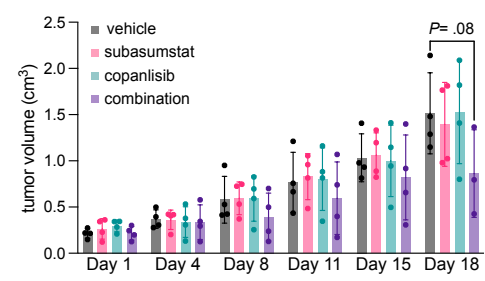
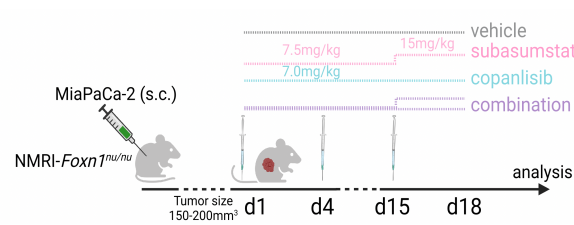
A



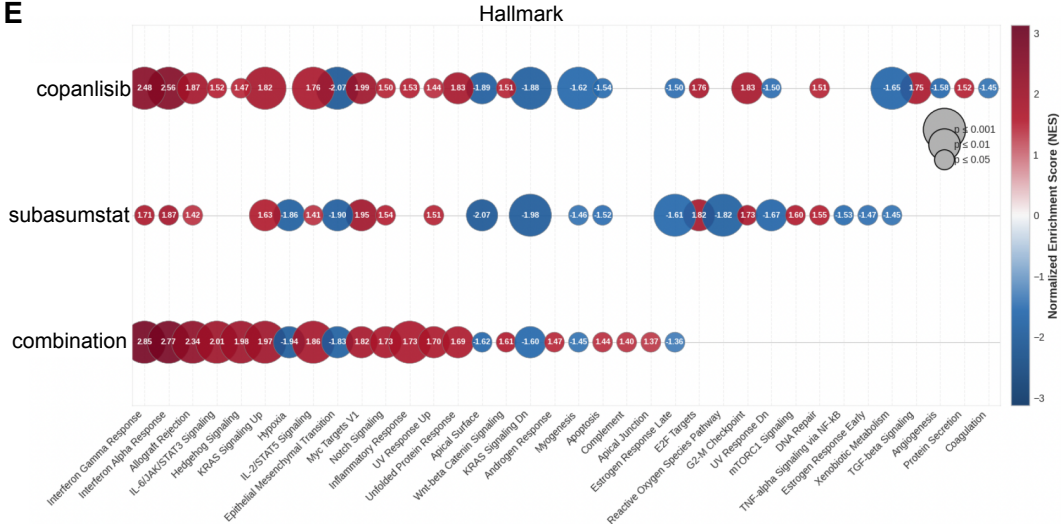
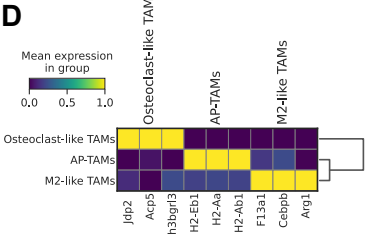
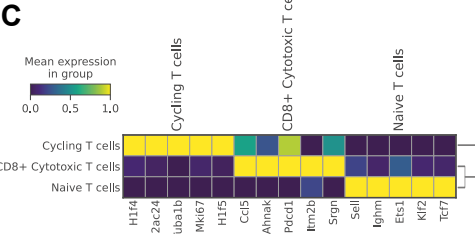
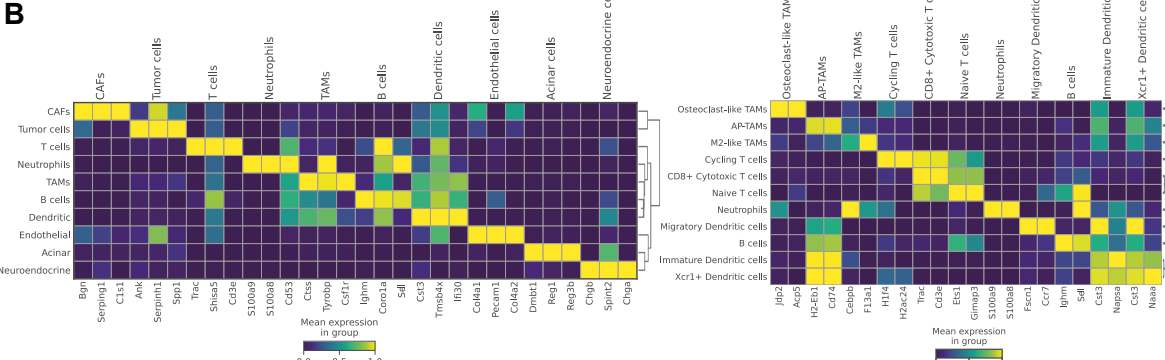
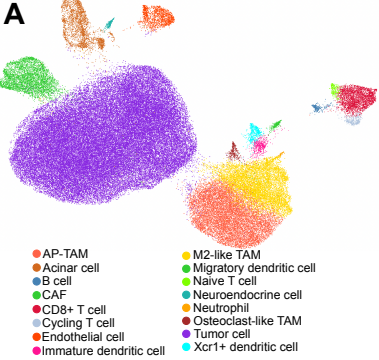
B



Supplemental Figure 5

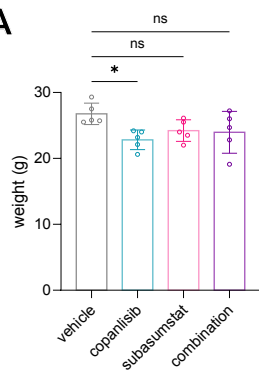


Supplementary Figure 6

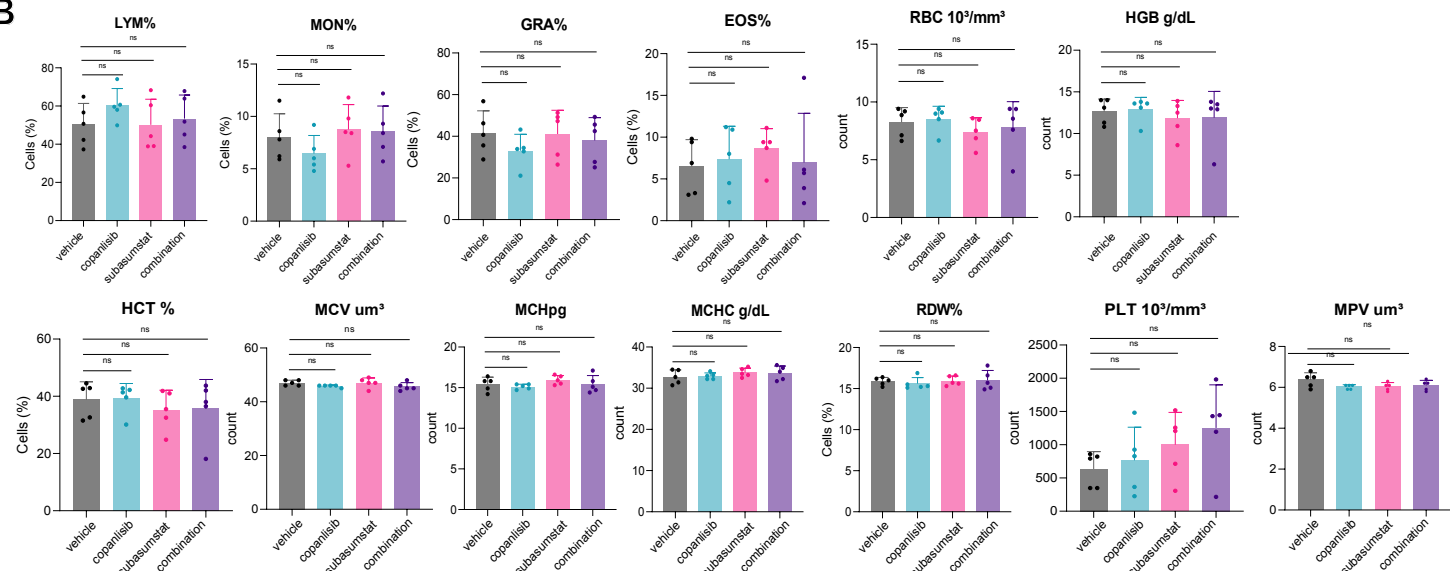


## A

A



# B



C

