

SUPPLEMENTAL METHODS

Cell culture

MiaPaCa2 and PaTu-8988T cell lines were cultured in DMEM (Gibco/Life Technologies, Darmstadt, Germany) supplemented with 10% fetal calf serum (FCS) (Merck Millipore/Biochrom, Berlin, Germany). DanG, BxPc3, PSN1, IMIM-PC1, HUPT4, PaTu-8988S, ASPC1, SW1990, MZ1-PC, SU8686, PANC0203, PANC0504, HPAC, HUPT3 and HPAFII cells were cultured in RPMI 1640 medium (Gibco/Life Technologies™, Darmstadt, Germany) supplemented with 10% FCS. HCT116 cells were cultured in McCoy's 5A medium supplemented with 10% FCS. All cell culture media were supplemented with 1% (w/v) penicillin/streptomycin (ThermoFisherScientific/Life technologies, Waltham, MA, USA). Establishment of murine PDAC cell lines has been described previously [1]. The used lines were established from murine *Kras*^{G12D}-driven or *PI3K/p110*^{H1047R}-driven murine PDACs [2, 3]. Murine PDACs cell lines were cultured in DMEM medium (Gibco/Life Technologies) supplemented with 10% FCS and 1% (w/v) penicillin/streptomycin. Identity of the murine PDAC cell lines was verified using genotyping PCR. Human cell lines were authenticated by Single Nucleotide Polymorphism (SNP)-Profiling conducted by Multiplexion (Multiplexion GmbH, Heidelberg, Germany) or short tandem repeat (STR) profiling (Mycrosynth, Balgach, Switzerland). Cell lines were tested for Mycoplasma contamination by a PCR-based method [4] or externally tested by Multiplexion (Multiplexion GmbH, Heidelberg, Germany).

Generation of patient-derived PDAC organoids and primary-dispersed cell lines

Primary patient-derived PDAC 3D organoids were generated from primary resected human pancreatic adenocarcinoma surgical specimen according to the Tuveson protocol described in [5] and [6]. Diagnosis of PDAC was confirmed by pathological examination. The primary human PDAC 3D organoid models and primary-dispersed cell lines were established and analyzed in accordance with the declaration of Helsinki, were approved by the local ethical committee (Project 207/15, 1946/07, and 330/19), and written informed consent from the patients for research use was obtained prior to the investigation. All organoids used have a

documented *KRAS* mutation: B25: G12V, B48: G12D, B54: G12D, B61: G12D. Due to continuous experimentation with these lines and re-thaw failures, some of these lines are not available for further experimentation. For drug screening purposes, organoids were dispensed through enzymatic (TrypLE Reagents, Thermo Scientific) and mechanical force. Cell-Matrigel suspensions were placed into 96-well plates and ML-93 treatment was initiated 24 hours after plating. Viability of cultures was measured 5 days after drug addition via CellTiter-Glo 3D Cell Viability Assay (Promega, Mannheim, Germany) using a luminescence microplate reader (FLUOstar OPTIMA).

Primary-dispersed human PDAC cells (HuPDAC3, HuPDAC7, HuPDAC17) were isolated from surgically-resected (HuPDAC3, HuPDAC17) or PdX-derived (HuPDAC7) human PDAC as described [7]. These cells were cultured in RPMI 1640 Medium (Gibco/Life Technologies™, Darmstadt, Germany) supplemented with 20% FCS and 1% Penicillin-Streptomycin (Merck, Sigma-Aldrich, Darmstadt, Germany). The cells were used in-between passage 10-20 in all experiments. All primary-dispersed human PDAC cell lines harbor a *KRAS*^{G12D} mutation.

Retroviral transduction

Human PDAC cell line IMIM-PC1 was engineered to stably express the ecotropic receptor via transduction with MSCV-rtTA-IRES-EcoReceptor-PGK-puro followed by selection with puromycin as described [8]. The IMIM-PC1 RIEP cell line (expressing the ecotropic receptor) was infected to stably express the MYC-estrogen receptor (MYC^{ER}) fusion protein via retroviral transduction with the MSCV MYC^{ER}-IRES-GFP plasmid [9]. The cells were then FACS-sorted for GFP expression (FACS Aria, Becton Dickinson). To generate murine MYC^{ER} cells, pBabepuro-MYC^{ER} plasmid (Addgene # 19128, provided by Dr. Wafik El-Deiry) [10] was used. The empty vector was used as control. pBabepuro-MYC^{ER} plasmid or empty vector was transfected into “Phoenix” retroviral packaging cells with TransIT®-LT1 Transfection Reagent (Mirus Bio, Madison, WI, USA). Primary murine PDAC target cells (PPT-5671, PPT-536361, PPT-8024, PPT-S559) all generated from murine *Ptf1a*^{Cre/+}, *LSL-Kras*^{G12D} PDACs, were transduced with retroviral particles with 8 µg/ml Polybrene (Merck Millipore, Darmstadt, Germany) and selected with 3 µg/mL Puromycin (Santa Cruz Biotechnology, Dallas, TX, USA).

Expression of MYC^{ER} was confirmed by immunoblotting. Cells stably expressing the MYC-ER fusion protein were treated with 500nM (human cells) or 600nM (murine cells) 4-OHT for time points indicated to activate MYC.

Murine PDAC cell lines (Kras^{G12D}-derived) PPT-5671 and PPT-53631 were genetically engineered to stably express MYC by retroviral transduction using the MSCV-MYC-IRES-GFP vector (Addgene # 18770, provided by Dr. Scott Lowe) [11]. Corresponding control cells were established using the MSCV-IRES-GFP empty vector (Addgene # 27490, provided by Dr. Warren Pear) [12]. The respective plasmids were transfected into “Phoenix Eco” retroviral packaging cells using Lipofectamine 2000 reagent (Thermo Fisher Scientific). The cells were then FACS-sorted for GFP expression (FACS Aria, Becton Dickinson). Expression of MYC and GFP was confirmed by immunoblotting.

For lentiviral production of shRNAs, HEK 293T cells were transfected with lentiviral packaging plasmids (Addgene plasmid #12260 and #12259, both provided by Dr. Didier Trono) and plasmids containing murine *Ube2i* targeting shRNAs (#54: TRCN0000040839 and #56: TRCN0000040841)(Mission library, Sigma-Aldrich) or empty vector (Addgene plasmid #10878, provided by Dr. Bob Weinberg [13]). A knock-down was only detected with the shRNA #54: TRCN0000040839. The puromycin resistance has been subcloned to GFP, to allow FACS sorting. Lentivirus has been harvested in DMEM with 10% FCS and transductions were performed in the presence of 8µg/ml polybrene (Sigma-Aldrich).

Competitive repopulation assay

Murine PDAC cell lines PPT-5671 and PPT-53631 were retrovirally transduced to overexpress the MYC oncoprotein and the GFP reporter gene using the MSCV_MYC_IRES_GFP vector. Empty vector control lines, expressing GFP, were generated using the MSCV_IRES_GFP vector. For competitive repopulation experiments, PPT-5671 and PPT-53631 cells expressing the MYC oncogene together with GFP were mixed in a 20:80 ratio with parental cells (MYC+WT) and cultured for 5 days at 500nM ML-93 or a DMSO control in 6-well plates (total amount of cells: 5 x 10⁵ cells per well). In analogy, GFP expressing control cells were mixed in a 20:80 ratio with wild type cells (control+WT) and cultured for 5 days and treated with

500nM ML-93 and DMSO accordingly. Cells were split 1:2 on d1 and d4. Medium, inhibitor and the DMSO control was replaced at each splitting. On day 5, FACS analysis (Beckman Coulter CyAn ADP LX) was performed to assess the fraction of GFP positive cells. The fold change in GFP positive cells was calculated as the ratio of GFP positive cells on d5 vs. d0 for both the MYC+WT and the control+WT setting. The fold change in GFP positive cells under SUMO inhibition with ML-93 for 5 days was normalized using the DMSO control for both MYC+WT and EV+WT and represented as normalized relative fold change in the manuscript.

Western blotting

Whole cell suspensions were lysed using specific lysis buffers with the final concentration: 50 mM Hepes, 150mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 20 mM NEM, and 0.1 % Tween or RIPA buffer (150mM NaCl, NP-40 1% v/v, Sodium-deoxycholate 0.5%, SDS 0.1%, 25 mM Tris). For protein lysis, 900µl of the above described buffer were supplemented with 10µl PMSF (stock 100mM), 40µl suspension of 1:1ml diluted Roche Mini-Complete tablet, 2.5µl NaF (stock 0.4M) and 1µl of NaVO₄ (stock 100mM) as protease inhibitors. Protein concentrations were assessed using Bradford reagent. Protein lysates were fractioned on SDS PAGE gels, transferred to Immobilon-P or Nitrocellulose (both from Millipore) membranes and incubated with specific primary antibodies. Primary and secondary antibodies and the thioester blots (Fig. S3B) are described below. Western blots were visualized by the Odyssey Infrared Imaging System (Licor, Bad Homburg, Germany) or the OPTIMAX X-Ray Film Processor (PROTEC, Oberstenfeld, Germany). For ECL measurement, western blots were incubated with HRP-linked secondary antibodies (GE Healthcare, USA) and SuperSignal™ West (ThermoFisher, USA) was used as HRP substrate. For ECL visualization CL-Xposure Film (ThermoFisher, USA) was used. Western blots were quantified using Odyssey software.

Immunoblotting antibodies

Protein	Company	Clone, Product #
SUMO1	Cell signaling, rabbit (1:1000)	21C7
SUMO2/3	Cell signaling, rabbit (1:1000)	8A2

c-MYC	Cell signaling	9402S
beta-Actin	SigmaAldrich	A5316, AC-15
RanGap1	Abcam	ab92360
alpha-tubulin	SigmaAldrich	T5168 B-5-1-2
beta-tubulin	Developmental Studies Hybridoma Bank	E7
GAPDH	Acris	ACR001PS
Secondary mouse IgG HRP Linked Whole Ab (1:10,000)	GE Healthcare	NA931V
Secondary rabbit IgG HRP Linked Whole Ab (1:10,000)	GE Healthcare	NA934V
Secondary Anti-rabbit IgG (H+L) (DyLight® 800 Conjugate)	Cell Signaling Technology	#5151
Secondary Anti-Mouse IgG (H+L) (DyLight® 700 Conjugate)	Cell Signaling Technology	#5470

Immunohistochemistry antibodies and conditions

	Immunohistochemistry protocols including machine; pretreatment condition; primary antibody; dilution; Detection Kit		
Protein	MYC	Sumo1	Sumo2/3
Clinical PDAC cohort	BXT; CC1; abcam, ab32072; 1:50; Ultraview Detection Kit	Bond; ER2(40); DHSB Sumo1 76-86; 1:200; Polymer Refine Detection Kit	Bond; ER2(40); DHSB Sumo2 8A2; 1:200; Polymer Refine Detection Kit
Organoids			
Xenograft tissues	Bond; ER1(30); abcam, ab32072; 1:50; Polymer Refine Detection Kit without post primary		

For the IHC detection of Ki67 and cleaved Caspase3 of xenografted tissue the following antibodies were used:

Anti-Ki67 antibody [SP6] (ab16667), Abcam, Dilution 1:50

Cleaved Caspase-3 (Asp175) Antibody #9661, Cell Signaling, Dilution 1:150

RNA isolation and expression analysis

Expression levels were assessed with quantitative PCR as described [8]. In brief, RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) or the Maxwell[®]16 Total RNA Purification Kit (Promega, Mannheim, Germany) and transcribed into cDNA with the Omniscript RT kit (Qiagen). qPCR was performed using a TaqMan cyclor (Applied Biosystems, Applied Biosystems Inc., Carland, CA; USA) and the Platinum SYBR Green qPCR SuperMix-UDG kit (ThermoFisherScientific). Expression analysis was performed applying the $\Delta\Delta C_t$ method.

qPCR Primers

Human: ODC1: Forward: 5`T C C T G G A G A G T T G C C T T T G T G A G A 3`; Reverse: 5`T C G A G G A A G T G G C A G T C A A A C T C T 3`; *CAD*: Forward: 5`T A G T C C T T G G C T C T G G C G T C T A 3`; Reverse: 5`T A G T C G G T G C T G A C T G T C T C T G 3`; *GAPDH*: Forward: 5`A A T C C C A T C A C C A T C T T C C A 3`; Reverse: 5`T G G A C T C C A C G A C G T A C T C A 3`; *SUMO1*: Forward: 5` T T C A A C T G A G G A C T T G G G G 3` ; Reverse: 5`T G G A A C A C C C T G T C T T T G A C 3`; *SUMO2*: Forward: 5`G C C G A C G A A A G C C C A A G G 3`; Reverse: 5` T G A C A A T C C C T G T C G T T C A C A A 3`; *MYC*: Forward: 5` T C A G A G T C T G G A T C A C C T T C T G C T 3`; Reverse: 5` T G C G T A G T T G T G C T G A T G T G T G G A 3`; *HSPE1*: Forward: 5` C A T C A T G T T G A T G C C A T T T C A 3`; Reverse: 5` T G G A G G C A C C A A A G T A G T T C T 3`; *SAE1*: Forward: 5`A C T G G A G C A G T G A G A A A G C A 3`; Reverse: 5` G C A G G T C A G G A C T A A T A C C C A 3`; *SAE2*: Forward: 5` A A C C T C C A G T T C C G T T G G A C 3`; Reverse: 5` T C C T G A G G T T T G C A G C A G A G 3`; *UBE2I*: Forward: 5` C C C A T T T G G T T T C G T G G C T G 3`, Reverse: 5` A C A T T T T G G T G G C G A A G A T G G 3`; *SUMO3*: Forward: 5` C C C A A G G A G G G T G T G A A G A C 3`; Reverse: 5` A T T G A C A A G C C C T G C C T C T C 3`.

Murine: Myc: Forward: 5' TTCCTTTGGGCGTTGGAAAC3'; Reverse: 5'GCTGTACGGAGTCGTAGTCG3'; *Cad*: Forward: 5'CTGCCCGGATTGATTGATGTC3'; Reverse: 5'GGTATTAGGCATAGCACAAACCA3'; *Odc1*: Forward: 5'ACATCCAAAGGCCAAAGTTGG3'; Reverse: 5'AGCCTGCTGGTTTTGAGTGT3'; *Gapdh*: Forward: 5'GGGTTCTATAAATACGGACTGC3'; Reverse: 5'TACGGCCAAATCCGTTCAACA3'. *Ube2i*: Forward: 5'GGAAGCCTGGAGGAAGGAC3'; Reverse: 5'GATGAAACAGTGGGGGCTCA3'; *Ubiquitin*: Forward: 5'GCAAGTGGCTAGAGTGCAGAGTAA3'; Reverse: 5'TGGCTATTAATTATTCTGGTCTGCAT3'; *Sae1*: Forward: 5'GCCCTGTAAAGAGGCGCTA3'; Reverse: 5'TGATGCCCAGGGAGTCAAC3'; *Uba2/Sae2*: Forward: 5'CGCAAGAGGAAACCTCCAGT3'; Reverse: 5'TCTCCGCTAAATGGACTCG3'.

UBE2I Thioester Western Blots

For thioester blots (SFig. 4), nonreducing SDS–PAGE was performed as recently described [14] and following antibodies were used: UBC9 (Epitomics, 2426-1), SUMO2/3 (monoclonal rabbit antibody generated by Takeda), UBCH10 (Boston Biochem, A650), and UBC12 (monoclonal mouse antibody generated by Takeda) used at a 1:1,000 dilution. The secondary Alexa 680–labeled antibody to rabbit/mouse IgG (1:5,000) were purchased from Invitrogen (A-21076, A-21058). Blots were imaged with a LI-COR Odyssey Infrared Imaging System. The ML93 concentration producing a half-maximal response (EC_{50}) were calculated using intensity values from LI-COR Immunoblot scans which were normalized to an α -tubulin loading control as described [14].

Biochemical and cellular assays of ML-93 activity

The ATP-inorganic pyrophosphate (PPi) exchange assay was carried out as described [14, 15]. Reactions were run using 2 nM SAE incubated with 1 μ M SUMO2 and 100 μ M PPi

(containing 50 c.p.m./pmol [³²P]PPi) in the presence of 1000 μM ATP. For assessment of cellular activity, HCT-116 cells were treated for 4h with increasing concentrations of ML-93, and 1 μM of ML-792 as a positive control, and assayed by Western blot hybridization for inhibition of formation of UBC9-SUMO thioester conjugates, UBC12-NEDD8 thioester conjugates, and UBC10-Ub thioester conjugates, as well as inhibition of global SUMOylation, as described [14].

Clinical PDAC patient cohort

Tissue microarrays of primary tumors in a primary resected human PDAC cohort were used to evaluate the protein expression in human tumor tissues. This cohort was investigated previously [16] and consists of 262 individuals that received partial pancreatoduodenectomy for PDAC between 1991 and 2006 at the Charité University Hospital, Berlin, Germany. Grading and staging followed the WHO recommendations at the time of cohort generation (TNM-classification of the 7th edition). The use of this tumor cohort for biomarker analysis has been approved by the Charité University ethics committee (EA1/06/2004). The tissue microarrays were generated as described [17, 18]. In short, three tumor cores (diameter 1.5 mm) of representative tumor areas selected by a board-certified pathologist on H&E stained slides were punched out of formalin-fixed paraffin embedded (FFPE) tissue blocks and arranged in a newly generated paraffin block.

Histological analysis and immunohistochemistry

Serial 2μm-thin sections prepared from paraffin blocks of embedded tissue and TMAs with a rotary microtome (HM355S, ThermoFisher Scientific, Waltham, USA) were collected and subjected to histological and immunohistochemical analysis. Hematoxylin-Eosin (H.-E.) staining was performed on deparaffinized sections with Eosin and Mayer's Haemalaun according to a standard protocol.

Immunohistochemistry was performed on automated staining systems (Ventana Benchmark XT (BXT), Ventana, Tucson, USA or Leica Bond Rxm (Bond), Leica, Wetzlar, Germany) with

different protocols (see Table in SM&M). Counterstaining was done with hematoxylin and a positive reaction, visible as a dark brown precipitate, was scored in a semiquantitative manner by two experienced comparative pathologists (AM and KS).

Clonogenic Assay

Human and murine PDAC cells were plated in medium containing ML-93 in 24-well plates for 5-7 days. Afterwards the medium was carefully removed from the wells and washed 3 times with PBS. The colonies were stained with 0.2% Crystal Violet solution (Sigma by Life Technologies TM, Darmstadt, Germany) for 20 minutes on a shaker at room temperature. To remove background staining, the wells were washed 3 times with tap water, dried and scanned. Afterwards Crystal Violet dye was solubilized in 1% SDS solution (Serva Electrophoresis GmbH, Heidelberg, Germany) and the absorbance at 570 nm was determined with a microplate reader (CLARIOstar, BMG Labtech, Ortenberg Germany). OD of vehicle treated controls was arbitrarily set to 1 and the therapeutic effect is depicted as relative colony formation.

Viability Assays and SUMO inhibitor treatment

PDAC cells were plated and after 24 hours treated with ML-93/ML-792. After 72 hours, viability of 2D culture cells was measured with an MTT assay in a 96-well format as described [6]. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromid was purchased from Sigma (Munich, Germany) (10 mg/ml). In brief, 10 μ L of the MTT dye was added per well followed by an incubation for 4 hours at 37°C. After media removal formazan crystals were dissolved in 200 μ L DMSO:EtOH (v/v). Cell viability was determined by measuring the absorption at 595 nm in a Thermo/LabSystem Multiskan RC Microplate Reader (Artisan Technology Group, Champaign, IL, USA). In addition to MTT assay, cellular viability was measured by CellTiter-Glo ATP Viability Assay. In short, 25 μ L CellTiter-Glo® Reagent (Promega) was added to each well of a 96-well plate after 72 hours of drug treatment. After 15 minutes of incubation on a shaker at room temperature, luminescence was measured on a FLUOstar OPTIMA microplate reader (BMG Labtech). Cellular viability of human PDAC organoids was determined using the

CellTiter-Glo® 3D ATP Viability Assay according to the protocol of the manufacturer (Promega). Viability was determined by measuring luminescence on a FLUOstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany). Viability was measured 3 days (2D culture) or 5 days (organoid culture) after the addition of the drug. The OD or luminescence of vehicle-treated controls was arbitrary set to 1 and the dose-response is depicted as relative viability. To determine the ML-792 and ML-93 dose response curves a seven-point drug dilution was used.

Annexin V-, Cell Cycle-FACS, Viability analysis by FACS

Induction of apoptosis via SUMO inhibition was assessed by either Annexin V/propidium iodide (PI) or Annexin V/4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) flow cytometric analysis depending on the cell lines investigated. Briefly, ML-93 and DMSO treated cells were stained with APC Annexin-V (Biolegend®, Cat: 640941) or PI (Sigma-Aldrich). Transgenic cell lines expressing the GFP reporter gene were stained with APC Annexin-V (Biolegend®, Cat: 640941) and DAPI (Thermo Fisher Scientific) to minimize spectral overlap. The apoptotic fraction was defined as Annexin V-positive/propidium iodide-negative cells. For cell lines expressing the GFP reporter gene, the apoptotic fraction was defined as the Annexin V-positive/DAPI-negative cells, respectively. Propidium iodide-positive and DAPI-positive cells were deemed to be necrotic cells regardless of their Annexin V staining properties. Annexin-V-negative/propidium iodide-negative as well as annexin-V-negative/DAPI-negative cells were classified as viable cells. PDAC cells were treated with ML-93 or DMSO control for the indicated time point, fixed in ice-cold ethanol (70%) and resuspended in propidium iodide and RNase A (Qiagen, Hilden, Germany) in phosphate-buffered saline. The proportion of cells in each cell cycle phase was determined using flow cytometric assessment of DNA content (*CyAn ADP Lx*, Becton Dickinson, San Jose, CA, USA). Analysis of data were performed using FlowJo™ (FlowJo, LLC Ashland, OR, USA) software.

Generation of in vivo xenografts and SUMO inhibitor toxicity

All animal experiments were performed in accordance with regional Gothenburg University animal ethics committee approval 100/16 and 5.8.18-01949/2018 and approval of Regierung von Oberbayern ROB-55.2-2532.Vet_02-17-230. The tumor cells were suspended in RPMI, mixed 1:1 with Matrigel (BD Biosciences) and transplanted subcutaneously onto the flanks of immunocompromised, non-obese severe combined immune deficient interleukin-2 chain receptor γ knockout mice (NOG mice; Taconic, Denmark) (PaTu-8988T, PSN1, BxPc3, and IMIM-PC1 lines) or NOD.CB17-Prkdcscid/NCrCrl mice (NOD SCID mice; Charles river, Italy) (HuPDAC7 cell line). 1×10^6 cells were used for PaTu-8988T, PSN1, BxPc3, and IMIM-PC1 lines, 2×10^6 cells were used for the HuPDAC7 line. Mice were weighted and tumors measured using calipers twice a week. The metric tumor volume (V) was calculated by measurements of length (L) and width (W) by applying the following equation: $V = 0.5 \times (L \times W^2)$. Treatments were started when the tumors were actively growing, judged by increasing volumes on repeated caliper measurements. ML-93 was dissolved in beta hydroxypropyl cyclodextrin and mice were dosed intravenously with 50mg/kg body weight per dose. Dosing regimen for intravenous delivery were two consecutive days per week. Tumor size was measured until best response, or until no further effects could be expected. Mice were sacrificed before or when tumors reached ethical size limit. For *in vivo* testing of ML-93 toxicity, female C57Bl6/J mice were treated with 50 mg/kg ML-93 or vehicle control on day 1 and 2. On day 8 blood samples were analysed on a blood counter (scil Animal Blood Counter, USA) and single cell suspensions from spleens were generated (100 μ M cell strainer). Following red blood cell lysis (ACK Lysing Buffer, GIBCO, Thermo Fisher Scientific), splenocytes were snap frozen for consecutive western blot analysis and processed following the described protein lysis protocol.

Proteome analysis by mass spectrometry

Sample preparation

Human PDAC cell lines PATU-8988T and PSN1 were treated with 500nM of SUMOi for 48h in triplicates. Cells were lysed in 2% SDS lysis buffer, shortly heated to 95 °C, then sonicated and centrifuged at 16000 g for 5 minutes. In the following, protein content was determined

using the DC Protein Assay Kit from BioRad. For in-solution digest, 20 µg of each sample was precipitated using 4 volumes of acetone for 1 hour at -20 °C. After centrifugation a wash step with 90% acetone was included. The precipitated pellet was shortly dried at room temperature and then resuspended in 6M urea/2M thiourea. Proteins were reduced with DTT, following an alkylation step using chloroacetamide. Digestion was performed in only 2M urea with the endopeptidase Lys-C (Wako) in combination with trypsin (sequence grade, Promega) overnight at 37 °C. Digestion was stopped by acidifying. Finally, peptides were desalted and concentrated by the STAGE tipping technique (Stop and Go Extraction) described by Rappsilber et al. [19].

Liquid chromatography and mass spectrometry

LC-MS/MS analysis was performed utilizing an Easy-nLC II via a nano-electrospray ionization source to the Orbitrap Elite mass spectrometer. Peptides were separated according to their hydrophobicity on an in-house packed 17 cm long 75µm ID column with 3 µm C18 beads (Dr Maisch GmbH). The binary buffer system used consisted of solution A: 0.1% formic acid and solution B: 80% acetonitrile, 0.1% formic acid. For proteome analysis, a linear gradient of 120 minutes was used (0-120 min, 33% B). Then the concentration of solution B was increased to 50% in 5 minutes and finally increased to 95% in 5 minutes.

Orbitrap Elite settings: MS spectra were acquired with a maximal injection time of 100 ms, a resolution of 120000 at 200 m/z and 1×10^6 as an AGC target. MS/MS spectra of the top 20 most intense peaks were obtained by collision-induced dissociation (CID) in the ion trap. The maximal injection time was set to 25 ms, with an AGC target of 5×10^3 and a rapid scan mode.

Data Analysis

The acquired raw files were processed in one single run using the MaxQuant software (version 1.5.8.0) and its implemented Andromeda search engine [20, 21]. Assignment of proteins was achieved by correlation of electrospray ionization-tandem mass spectrometry (ESI-MS/MS) fragmentation spectra with the Uniprot human database (version 2017), additionally including

a list of common contaminants. All searches were performed using default settings for mass tolerances for MS and MS/MS spectra. Tryptic specifications were chosen. Carbamidomethyl at cysteine residues was set as fixed modification whereas oxidation at methionine and acetylation at the N-terminus were chosen as variable modifications. Further, the false discovery rate for proteins and peptide-spectrum matches was set to 1% as default and the minimal peptide length was defined to be seven amino acids. Proteins were quantified using the integrated MaxLFQ algorithm [22], allowing only unique peptides for quantification and retaining unmodified counterpart peptides. The minimum LFQ ratio count was set to 2, FastLFQ was enabled and the number of minimal unique peptides was set to 1 for identification. Furthermore, the match-between-run feature was used with a time window set to 0.7 minutes.

The Perseus software (version 1.5.8.5) was used for downstream analysis of the data. Using the filter option, contaminants, reverse entries and proteins only identified by a modified peptide were removed. In the following, LFQ intensities were logarithmized and normal distribution of the LFQ values was ensured by visual histogram analysis. Correlation of triplicates was checked by multiscatter plot analysis. For statistical analysis, triplicates were grouped into one group and the significant difference of two sample groups was tested using the Student's t-test as a two-sample test.

RNAseq analysis

For RNA-seq of 4-OHT treated IMIM-PC1 cells, an Illumina TruSeq Stranded RNA Library Prep Kit was used and further analyzed on an Illumina HiSeq2000 system (DKFZ Heidelberg NGS core facility). Resulting Fastq files were obtained from DKFZ Heidelberg NGS core facility (approximately 25M reads/sample (single-end reads)) and further processed and analyzed using the Galaxy Project platform [23]. First, adapters were removed from Fastq files using TrimGalore! (Galaxy version 0.4.3.1), afterwards sequencing-reads were mapped to the human reference genome hg19 (GRCh37) using Bowtie2 (Galaxy version 2.3.2.2) [24] and annotated with the hg19 GTF annotation file, obtained from the UCSC genome browser

database [25]. Differential expression of count data (htseq-count 0.6.1galaxy3) was determined by DESeq2 (Galaxy version 2.11.39) [26, 27].

Human BxPC3, Pa-Tu-8988T, PSN1 with and without ML-93 treatment were analyzed in triplicates. Murine 53631PPT cells retroviral transduced with a hMYC-cDNA expression vector and the respective control with and without ML-93 treatment were analyzed in quintuplicates. To verify positive integration of pDNA hMYC-cDNA, IRES and GFP a respective fasta file has been generated and been mapped to all murine samples using bowtie2 [17] and visualized by IGV [28]. Library preparation for bulk 3'-sequencing of poly(A)-RNA was done as described previously [29]. Briefly, barcoded cDNA of each sample was generated with a Maxima RT polymerase (Thermo Fisher) using oligo-dT primer containing barcodes, unique molecular identifiers (UMIs) and an adapter. 5' ends of the cDNAs were extended by a template switch oligo (TSO) and full-length cDNA was amplified with primers binding to the TSO-site and the adapter. cDNA was tagged with the Nextera XT kit (Illumina) and 3'-end-fragments finally amplified using primers with Illumina P5 and P7 overhangs. In comparison to Parekh et al. the P5 and P7 sites were exchanged to allow sequencing of the cDNA in read1 and barcodes and UMIs in read2 to achieve a better cluster recognition. The library was sequenced on a NextSeq 500 (Illumina) with 75 cycles for the cDNA in read1 and 16 cycles for the barcodes and UMIs in read2. Data was processed using the published Drop-seq pipeline (v1.0) to generate sample- and gene-wise UMI tables [30]. Reference genomes (GRCm38, murine; GRCh37, human) were used for alignment. Transcript and gene definitions were used according to the ENSEMBL annotation release 75. Accession numbers: GSE119423, PRJNA489233 and PRJEB34637. In addition, we used a RNA-seq dataset of 38 murine PDAC cancer cell lines that was recently described [3] and can be accessed via ENA: PRJEB23787. mRNA expression profiles of conventional human PDAC cell lines were from the Cancer Cell Line Encyclopedia [31] and downloaded via the cBioPortal platform (<http://www.cbioportal.org>) [32].

Gene expression profiling, gene set enrichment analysis, transcriptomics and genomics data analysis

Normalized gene expression and clinical data, corresponding to Fig. 2F, were obtained from Bailey et al. (nature16965-s2) [33]. Gene expression values were transformed into z-scores (indicating the deviation from the population mean in units of standard deviation) for each gene and sample in comparison to all the other samples. For a clearer representation in the heat map, the range of z-scores is split into six intervals, each corresponding to a distinct color. For different classes of tumor subtypes and degrees of differentiation, we used Fisher's Exact Test to test for their respective enrichment. For gene set enrichment analysis (GSEA), we used GeneTrail2 1.6 [34]. A detailed description of the unweighted GSEA performed by GeneTrail2 1.6 can be found below. In addition to GeneTrail2, we accessed the GSEAsoftware v.3.0 via the Broad Institute (www.broadinstitute.org) to perform gene set enrichment analysis [35]. Statistical values (nominal p-value, FDR q-value) are indicated. TCGA PAAD mRNA expression data and clinical data sets were accessed via UCSC cancer genomics browser [36]. The 75th and 25th percentile, were defined as thresholds for "high" and "low" expression. TCGA PDAC survival data for UBE2I, SUMO1, SUMO2, and SUMO3 were accessed and plotted via the OncoLnc webpage (<http://www.oncolnc.org/>) [37]. Genomics data for CNA analysis was assessed using the cBioPortal online platform [32, 38]. Genes regulated by ML-93 in human PDAC lines (log FC +/- 0.58, FDR<0.05) were analyzed using the Hallmark gene sets of the MSigDB. Pearson correlation of ML-792 and ML-93 GI₅₀ to mRNA expression is described in SM&M. The Pearson correlation coefficient was used as a rank to run a pre-ranked GSEA with the GSEA 4.0.1 software.

Unweighted GSEA by GeneTrail2

To assess altered biological pathways and processes in the SUMO^{high} group in comparison to the SUMO^{low} group, scores of differential expression were computed using Independent Shrinkage t-Test [39] and an unweighted Gene Set Enrichment Analysis was performed on a variety of functional categories using the GeneTrail2 web service [34].

GeneTrail2 is a comprehensive web service providing access to different tools for the statistical analysis of molecular signatures with a focus on enrichment analyses. These include the well-known weighted gene set enrichment analysis (GSEA), which has been developed by Subramanian et al. [35] (Broad Institute, www.broadinstitute.org), as well as an unweighted version of GSEA. In the classical (weighted) GSEA, p-values are typically computed using permutation-based approaches, which are limited to p-values as small as $1/(\text{number of permutations})$. In contrast to this, the unweighted GSEA allows for exact p-value computation based on a dynamic programming algorithm [40]. Besides lower runtimes, the exact p-value assessment has the major advantage that extremely significant results can be better distinguished from marginally significant ones.

The main difference between the weighted and the unweighted GSEA lies in the computation of the running sum statistic, which in the former case additionally assigns a weight to each gene, mirroring its correlation with the phenotype. This distinction is also reflected in the corresponding running sum plots.

As a multitude of gene sets are tested simultaneously in exploratory enrichment analyses, the obtained p-values need to be corrected for multiple-hypothesis testing to prevent the accumulation of type-1 error. As correction for multiple hypothesis testing, we used the method by Benjamini and Yekutieli [41] as provided by GeneTrail2, resulting in the indicated q-values.

Correlation of GI₅₀ values with mRNA expression

The sensitivity to SUMO inhibitors ML792 and ML793 across murine pancreatic cancer cell lines ($n=38$, only *Kras*^{G12D}-driven lines) was correlated with gene expression obtained by RNA-seq [3]. GI₅₀ values were used as a measure of drug sensitivity and log₂-counts per million computed using the edgeR-limma pipeline from the Bioconductor Project [42, 43, 44] served as a measure of gene expression. Correlation coefficients were calculated using the Pearson method.

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