Synergistic targeting and resistance to PARP inhibition in DNA damage repair-deficient pancreatic cancer

SUPPLEMENTARY METHODS

Ethics statement

All animal care and procedures followed German or Spanish legal regulations and were previously approved by the respective governmental review board of the state of Baden-Württemberg (Permission no. 1369, 1273, O.195-7, and 1347), of the state of Nordrhein-Westfalen (Permission no. 8.87-50.10.32.09.018), the state of Lower Saxony (Permission no. 33.19-42502-04-17/2407), the Universidad Autónoma de Madrid Ethics Committee (CEI 60-1057-A068 and CEI 103-1958-A337), and La Comunidad de Madrid (PROEX 335/14 and PROEX 294/19). All mouse work aspects were carried out following strict guidelines to insure careful, consistent and ethical handling of mice. The biomaterial used was provided by the biobank of the University Hospital of Ulm following the regulations of the Biobank and the vote of the Ethics Committee of the University of Ulm (PDAC Liver Metastasis): 72/2019 (human tissue and blood), by the Ruhr University Bochum following the regulations and the vote of the Ethics Committee of the Ruhr University Bochum (Permission no. 3534-9 and 3841-10), by the University Medical Center Göttingen (Approval no. 11/5/17), and by the NYU Langone Health following the regulations and the guidelines of the NYU Institutional Review Board (Permission no. S16-00122). Some PDAC patient-derived xenografts were obtained from Dr. Manuel Hidalgo under a Material Transfer Agreement with the Spanish National Cancer Centre (CNIO), Madrid, Spain (Reference no. I409181220BSMH and I405271505PHMH).

Mice

Atm$^{fl/fl}$, *Trp53*$^{fl/fl}$, *LSL-Kras*$^{G12D/+}$ and *Ptf1a*$^{Cre/+}$ were previously described [1, 2, 3, 4]. Eight-week-old female Hsd:Athymic Nude-*Foxn1*^{nu} mice were purchased from Envigo. Mice were housed and bred in a conventional health status-controlled animal facility. All animal care and procedures followed German legal regulations and were previously approved by the governmental review board of the state of Baden-Württemberg. All the aspects of the mouse work were carried out following strict guidelines to insure careful, consistent and ethical handling of mice.

Cell culture

LSL-Kras$^{G12D/+}$; *Ptf1a*$^{Cre/+}$ (KC) and *Atm*$^{fl/fl}$; *LSL-Kras*$^{G12D/+}$; *Ptf1a*$^{Cre/+}$ (AKC) cells were respectively isolated from *LSL-Kras*$^{G12D/+}$; *Ptf1a*$^{Cre/+}$ and *Atm*$^{fl/fl}$; *LSL-Kras*$^{G12D/+}$; *Ptf1a*$^{Cre/+}$ mice and immortalized as described previously [5]. Cells were cultured in DMEM, containing 10% FBS and P/S (100 IU/mL penicillin and 100 µg/mL

streptomycin sulfate). MIA PaCa-2 (ATCC CRL-1420) and PANC-1 (ATCC CRL-1469) cells were purchased from the ATCC and were cultured in DMEM, containing 10% FBS and P/S.

Organoids were isolated as previously described [6]. Briefly, cryopreserved tumors from patients were digested with accutase (Sigma-Aldrich) for 30 min at 37°C. Cells were then filtered through an 100 µm filter and seeded on a Matrigel GFR (Corning)-coated plate in organoid culture medium containing WNT-3/RSPOI-conditioned medium [7] and 5% Matrigel GFR.

To generate PARPi-resistant AKC (R-AKC) cell lines, AKC cells at 70% confluence were treated with olaparib, respectively at their half maximal inhibitory concentration (IC_{50}). Cells were passaged when they reached 70-90% of confluence. Upon every passage, cells were then treated with incremental increasing doses of olaparib, until cells were able to grow in the presence of olaparib at a concentration of 5 times the IC_{50} (18.5 µM).

All cells were propagated at 37°C under 5% (v/v) CO_2 atmosphere. All experiments were performed between passage 5 and 15. Mycoplasma tests were regularly performed using the Mycoprobe mycoplasma detection kit (R&D Systems).

5-fluorouracil (NSC 19893), adavosertib (MK-1775), AZD0156, AZD7762, carboplatin (JM-8), CC-115, clarithromycin (A-56268), cisplatin, dabrafenib (GSK2118436), decitabine, doxorubicin (adriamycin), elacridar (GW120918), etoposide, everolimus (RAD001), gemcitabine (LY188011), irinotecan (CPT-11), KU-60019, lapatinib (GSK572016), mirin, mitomycin C, N-acetylcystein, niraparib (MK-4827), NU7026 (LY293646), oxaliplatin (L-OHP), paclitaxel (NSC 125973), palbociclib (PD-0332991), pemetrexed (LY-231514), olaparib (AZD2281, KU-00594), PD0325901, PD-1/PD-L1 inhibitor 1, pevonedistat (MLN4924), SCR7, selumetinib (AZD6244), sorafenib (BAY 43-9006), sunitinib (SU11248), tivantinib (ARQ 197), trametinib (GSK1120212), VE-821, VE-822 (VX970), venetoclax (ABT-199), vinorelbine, vorinostat (SAHA) and wortmannin were purchased from Selleckchem. Dorsomorphin, LDN193189, L67, and metformin were purchased from Sigma-Aldrich. Erlotinib was purchased from LC Laboratories. JAK inhibitor I was purchased from EMD Millipore. PI-103 was purchased from Tocris. SB431542 was purchased from Axon.

Cytotoxicity assay

Prior to seeding, patient-derived organoids were dissociated into single cells using collagenase/dispase and accutase. Organoids were then seeded in 96 well-plates (2,000 cells per well) for four days before treatment. After four additional days of treatment, cell viability was analyzed with the CytoTox-GloTM Cytotoxicity Assay (Promega) according to the manufacturer's protocol. Luminescence was measured using a Tecan Infinite M200 Pro. Cell death ratio (CDR) was determined using this formula: CDR = (S1:S2)Drug/(S1:S2)Vehicle. Percentage of survival was determined using this formula: (S2-S1)Drug/(S2-S1)Vehicle. Data are represented as mean ± SD of two independent experiments.

Colony formation assay

Cells were seeded in 12-well plates (1,000 cells per well). Twenty-four hours after seeding, cells were treated for three days. After further culturing for seven days, cells were fixed with methanol and stained with 5% Giemsa.

Migration assays

For transwell migration assay, 2×10^5 cells were seeded in serum-free medium in the upper chamber of transwells with 8 µm pore size membrane (24-well format, Falcon) and complete medium in the lower chamber. After 24 h, cells were fixed with cold 4% formaldehyde and stained with DAPI and 5% Giemsa. Cells migrating to the membrane lower side were counted using ImageJ software (National Institutes of Health).

qPCR

Total RNAs were extracted using the RNeasy Mini Kit (Qiagen). First-strand cDNAs were prepared using 250 ng of RNA and SuperScript II Reverse Transcriptase in the presence of random primers (Thermo Fisher Scientific) according to the manufacturer's protocol. Quantitative PCR were performed using an Applied Biosystems QuantStudio 3 System (annealing temperature 60°C) and PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). As described previously [8], all the real-time values were averaged and compared using the threshold cycle (CT) method, where the amount of target RNA ($2^{-\Delta\Delta CT}$) was normalized to the endogenous expression of *18S* or *HPRT* (ΔCT). The amount of target mRNA in control cells was set as the calibrator at 1.0. Primers used for quantitative RT-PCR were purchased from Biomers, Qiagen and Sigma-Aldrich and listed in Supplementary Table 2.

Immunostaining

Cells were grown on glass coverslips, fixed in 4% formaldehyde at 4°C, and permeabilized with 0.05% Tween20 for 15 min before immunofluorescence experiments. F-actin was stained with phalloidin-Atto565 (1:500; Sigma-Aldrich). DNA was stained with DAPI (1 µg/mL; Sigma-Aldrich). Primary and secondary antibodies are listed in Table 2. Images were obtained at ambient temperature using an oil immersion objective (Plan-Neofluar 100×/1.3 oil M27; Carl Zeiss) mounted on a Zeiss Axio Imager Z1 fluorescence microscope (Carl Zeiss) equipped with an AxioCam MRm camera and AxioVision LE imaging software (Carl Zeiss).

For nuclear aberration (micronucleus and multinucleated cell) quantification, a minimum of 200 cells per condition were counted. For anaphase bridge quantification, a minimum of 20 anaphases per condition were counted. Concerning the quantification of H2AX p-S139 foci-positive cells, a minimum of 400 cells per condition were counted. All cell countings were performed on at least 20 random pictures per condition. Data are represented as mean ± SD of two independent experiments performed in two independent KC cell lines and two independent AKC cell lines.

All histological experiments were performed as previously described [8]. Primary and secondary antibodies are listed in Table 3. Bright-field images were obtained using a HCX PL APO 40×/0.85 (Leica) objective mounted on a Leica DM5500 B microscope equipped with a Leica DFC420C camera and Leica Application Suite software (Leica). Acquired pictures were subsequently analyzed using ImageJ software. Cleaved caspase-3-positive areas were normalized to total surface to calculate the respective percentage occupied by positive staining per field. For H2AX p-S139-positive cells quantification, a minimum of 5,000 cells per condition were counted. All quantifications were performed on at least three random pictures of all subcutaneous grafts (n ≥ 4 per condition) included in the study and on at least three random pictures of ≥ 3 patient-derived xenografts. All analyzed pictures were carefully checked by eye to exclude artifacts and false positive areas. Data are represented as mean ± SEM.

For RAD51 immunofluorescence staining evaluation, KC and AKC cells were treated for 48 h with PAD (PARPi, 1 µM olaparib; ATRi, 20 nM VE-822; DNA-PKi, 30 nM CC-115). Pre-extraction for 1 min was followed by 10 min fixation with 3.7% formaldehyde at RT. Before the staining, cells were permeabilized with 0.5% Triton for 12 min and blocked with 5% goat serum for 1 h at room temperature. Cells were then incubated 1 h at 37°C with primary antibodies: anti-RAD51 (1:500, Santa Cruz Biotechnology, clone H-92, sc-8349) and anti-Cyclin A2 (1:100, Abcam, clone E67.1, ab39). Finally, the cells were stained with secondary antibodies either conjugated with AlexaFluor555 (1:1,000, anti-rabbit, Invitrogen) and AlexaFluor488 (1:2,000, anti-mouse, Invitrogen). Images were acquired with the Keyence BZ 9000 microscope (Keyence) with 100× oil immersion objective. Foci quantification was carried out with a Keyence BZ II Analyzer software. Data are represented as mean ± SEM of two independent experiments evaluating 182-372 nuclei in each experiment, whereby RAD51 foci scores were obtained in two independent KC cell lines and two independent AKC cell lines.

Double-strand break repair assay

Assay conditions were performed as previously described [9] using the repair substrates developed in [10, 11]. Recombination frequencies were calculated by dividing the green fluorescent cell counts per life cell count by the corresponding transfection efficiency. Data are represented as mean ± SEM and correspond to six measurements from two independent experiments each, whereby measurements were performed in two independent KC cell lines and two independent AKC cell lines.

Metaphase spreads

Cells were prepared as previously described [12]. For each condition, a minimum of 30 metaphases were evaluated regarding chromosome number, ring chromosome, chromatid break, gap and fragment, and chromatid monocentric and dicentric fusions.

Complete blood count

Blood was collected from the facial vein in EDTA-coated microvette tubes (Sarstedt) and analyzed in a Hemavet 950FS (Drew Scientific) whole blood counter. For each group, ≥ 3 mice were enrolled. Data are represented as mean ± SEM.

Flow cytometry

For cell cycle analysis by flow cytometry, cells were fixed in cold 70% ethanol at 4°C for 30 min, washed with PBS and then resuspended in a solution containing RNase A (2 mg/mL) and DAPI (40 µg/mL).

After harvesting cells by trypsinization, DNA damage analyses by flow cytometry were performed using the Apoptosis, DNA damage and Cell Proliferation kit (BD Pharmingen) according to the manufacturer's instructions. Samples were subjected to flow cytometry (2×10^4 cells were acquired) on a Becton Dickinson LSR II equipped with BD FACSDIVA (BD Biosciences) and analyzed with FlowJo (FlowJo, LLC) software. Data are represented as mean ± SEM of three independent experiments performed in two independent KC cell lines and two independent AKC cell lines.

Bone marrow was flushed from femurs of treated mice with ice-cold Hank's balanced salt solution (HBSS, Thermo Fisher Scientific) using a 23-gauge needle, followed by passage through a 70 µm sterile nylon cell strainer (Sigma-Aldrich). Blood was collected as described above. For the removal of red blood cells, blood and bone marrow preparations were incubated in 10 mL red cell lysis buffer (144 mM NH_4Cl, 17 mM Tris, pH 7.2) for 5 min, at room temperature. Staining of cells was performed in FACS buffer (PBS, 0.3% (w/v) BSA and 0.1% (w/v) NaN_3). Non-specific binding of antibodies to Fc-receptors was blocked by pre-incubating cells with mAb 2.4G2 (BD Biosciences) directed against the FcγRIII/II CD16/CD32 (0.5 µg mAb/10^6 cells/100 µL). Marker combinations used for the different cell types are shown in Supplementary Table 4 and used antibodies are listed in Supplementary Table 5. Frequencies of living single cells grouping to the respective subpopulations were determined by flow cytometry using a BD LSR-II Flow Cytometer (BD Biosciences) and FCS Express V3 (DeNovo) software. For each group, ≥ 3 mice were enrolled. Data are represented as mean ± SEM.

Western blotting

Total protein extracts were prepared using RIPA lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and commercial protease and phosphatase inhibitor cocktail tablets (Roche)) and were subjected to electrophoresis on SDS/PAGE. Chromatin-bound protein extracts were prepared as previously described [13]. Protein extracts (40 µg) were subjected to electrophoresis on SDS/PAGE. The separated proteins were transferred onto PVDF membranes (Millipore) by electroblotting. Western blots were revealed using the SuperSignal West Dura Extended Duration Substrate (Pierce). Primary and secondary antibodies are listed in Table 3.

Chicken chorioallantoic membrane assays

Chicken chorioallantoic membrane (CAM) assays were performed as previously described [14]. Briefly, 1 × 10^6 KC and AKC cells in 1:1 serum-free DMEM:Matrigel GFR were xenografted onto chicken CAM 8 days post-fertilization (within 5-mm silicon rings), and irinotecan 50 mg/kg or vehicle was applied topically on days 9 and 10. Three days after treatment initiation, xenografts were explanted, fixed in 5% PFA, and processed for histopathological examination. Tumor surface (mm^2) was evaluated using ImageJ software and normalized to the area of the silicon ring. For each group, ≥ 9 xenografts were implanted. Data are represented as mean ± SD.

Subcutaneous assay

Cells were implanted by subcutaneous injection of 1 × 10^6 cells in 100 µL of 1:1 serum-free DMEM:Matrigel GFR into the flank of eight-week-old (24-26 g body weight) female Hsd:Athymic Nude-*Foxn1*^{nu} mice. Tumor take was > 85% for all cell lines. Tumor size was measured thrice a week with a caliper, and the tumor volumes were determined with the following equation: $v = (l \times w^2) \times \pi / 6$ (where v is volume, l is length and w is width). For each group, ≥ 8 allografts were implanted. Data are represented as mean ± SEM. Tumors were randomized when the volume reached 80 to 150 mm^3 before starting treatment.

For KC and AKC allograft experiment, a solution of olaparib, VE-822 and CC-115 (PAD: PARP inhibitor, ATR inhibitor and DNA-PK inhibitor; respectively, 50.0 mg/kg, 20.0 mg/kg and 2.5 mg/kg Q2D×5) or irinotecan (50 mg/kg Q3D×3) were administered by i.p. injection. For MIA PaCa-2 xenograft experiment, a solution of PAD (olaparib 50.0 mg/kg, VE-822 20.0 mg/kg and CC-115 2.5 mg/kg Q2D×13 for *ATM*$^{+/\Delta}$ and Q2D×12 for *ATM*$^{+/+}$) and a solution of AZD0156 (ATM inhibitor; 2.25 mg/kg Q6D×4) were administered by i.p. injection. Body weight progression data are represented as mean ± SEM.

Measurements and injections were performed blindly. Mice were euthanized after 8 (KC and AKC cells) and 25 (MIA PaCa-2 cells) days of therapy or when tumors reached a maximal volume of 1,000 mm^3. Tumors were then resected and fixed in cold 4% formaldehyde for 24 h and embedded in paraffin for histological analysis.

Patient-derived xenografts

For subcutaneous implantation, 4 mm^3 pieces of patient-derived xenografts (patient-derived *in vivo* expanded pancreatic tumors), were embedded in ECM Gel from Engelbreth-Holm-Swarm murine sarcoma (Sigma-Aldrich) and transplanted into the flank of eight- to twelve-week-old (24-26 g body weight) female Hsd:Athymic Nude-*Foxn1*^{nu} mice. Tumor take was ≥ 80% for all patient-derived xenografts. Tumor size was measured thrice a week with a caliper, and the tumor volumes were determined with the following equation: $v = (l \times w^2) \times \pi / 6$ (where v is volume, l is length and w is width). For each group, ≥ 5 patient-derived xenografts were implanted. Data are represented as mean ± SEM. Tumors were randomized when the volume reached 40 to 60 mm^3 before starting treatment. A solution of PAD (olaparib 50.0 mg/kg, VE-822 20.0 mg/kg and CC-115 2.5 mg/kg, every second day) and a solution of AZD0156 (ATM inhibitor; 2.25 mg/kg, every sixth day) were administered by i.p. injection. Measurements and injections were performed blindly. Mice were

euthanized when tumors reached a maximal volume of 400 mm^3. Tumors were then resected and fixed in cold 4% formaldehyde for 24 h and embedded in paraffin for histological analysis.

Analysis of publicly available databases

We conducted analyzes of DDR gene alteration frequency in human primary pancreatic adenocarcinoma (PDAC) using the cBioPortal platform (http://www.cbioportal.org/) [15, 16]. For this analysis, we queried Pancreatic Adenocarcinoma (QCMG, Nature 2016) [17], Pancreatic Adenocarcinoma (TCGA, Provisional) [18], and Pancreatic Cancer (UTSW, Nat Commun 2015) [19] sequencing data sets (751 tumor samples). Of note, patient TCGA-IB-7651 was excluded from the analysis due to artificial high mutation load (1,432 single nucleotides variants). *ATM* alterations and their localizations were analyzed with cBioPortal on the same datasets. Analyzes were performed with publicly available data sets on the 7/23/2019.

To conduct analysis of DDR gene alterations on a PanCancer study, we queried sequencing data set from MSK-IMPACT Clinical Sequencing Cohort (MSKCC, Nat Med 2017) (10,945 tumor samples) using the cBioPortal platform. This analysis was performed with the publicly available data set on the 4/6/2019.

RNA-sequencing

Library preparation for bulk 3'-sequencing of poly(A)-RNA was done as described previously [20]. Briefly, barcoded cDNA of each sample was generated with a Maxima RT polymerase (Thermo Fisher Scientific) 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); after pooling of all samples, full-length cDNA was amplified with primers binding to the TSO-site and the adapter. cDNA was tagmented 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. [20], 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. Gencode gene annotations version M18 and the mouse reference genome major release GRCm38 were derived from the Gencode homepage (https://www.gencodegenes.org/). Dropseq tools v1.12 [21] was used for mapping the raw sequencing data to the reference genome. The resulting UMI filtered countmatrix was imported into R v3.4.4. Prior differential expression analysis with DESeq2 1.18.1 [22], dispersion of the data was estimated with a parametric fit including the treatment the cell lines underwent as explanatory variable in the model. The Wald test was used for determining differentially regulated genes between the treated and non-treated group and shrunken log_2 fold changes were calculated afterward, with setting the type argument of the lfcShrink function to normal. A gene was determined to be differentially regulated if the absolute log_2 fold change was greater than 0.848 or lower than -0.848 and the adjusted *p*-value was below 0.05.

Exome sequencing

Genomic DNA isolation was isolated from cell pellets and tail samples using the DNeasy Blood & Tissue Kit (Qiagen), according to the manufacturer's instructions. DNA concentration was fluorimetrically determined using the Qubit 3.0 system (Thermo Fisher Scientific). Library for Whole Exome Sequencing were prepared using the SureSelect^{XT} Mouse All Exon kit (Agilent), following the manufacturer's instructions. Samples were sequenced on an Illumina NovaSeq 6000 sequencer, resulting in a median targeted coverage of ~80. The GATK Best Practice suggestions were followed for alignment and mutation calling. After read trimming using Trimmomatic 0.38 (LEADING:25 TRAILING:25 MINLEN:50), BWA-MEM 0.7.17 was used to align reads to the mouse reference genome (GRCh38.p6). Picard 2.18.26 and GATK 4.1.0.0 were used for postprocessing (CleanSam, MarkDuplicates, BaseRecalibrator) using default settings. Somatic mutations were called using MuTect2 v4.1.0.0. Mutations with at least two reads supporting the alternate allele and a base coverage of at least 10 in the tumor and germline were kept. Single nucleotide variants (SNVs) and insertions/deletions (Indels) ≤ 10 base pairs were annotated using SnpEff 4.3t, based on ENSEMBL 92. Copywriter 2.6.1.2 was used for the detection of copy number variations.

Gene term enrichment analysis

Gene ontology (GO) enrichment analysis and pathway enrichment analysis in DEGs were conducted using the annotation tool DAVID v6.8 (http://david.abcc.ncifcrf.gov) [23, 24] and the hallmark data sets from the Molecular signatures database v6.2 (MSigDB, Broad Institute; http://software.broadinstitute.org/gsea/msigdb). Significant enrichments are defined with FDR < 0.10 and $p < 0.05$.

Statistical analysis

GraphPad Prism software was used for statistical analysis. For cell viability assays, cytology and histology quantifications, migration assays, flow cytometry experiments, chorioallantoic membrane assays, allograft, xenograft, and patient-derived xenograft subcutaneous experiments in *Nude* mice, qRT-PCR and, western blot quantifications, statistical significances were tested using Student's *t*-test. For DNA fiber assay and RAD51 foci evaluation, statistically significant differences were calculated using Dunn's multiple comparison test. For double-strand break repair assay, statistical significances were tested using Mann-Whitney U test. For flow cytometry analysis of hematopoietic progenitors and immune cell subtypes, the statistical significance of differences in the mean cell frequencies between treated groups and vehicle-treated control groups was determined using unpaired Student's *t*-test. Statistical significance in contingency tables were tested using Fisher's exact test. All tests were considered to be statistically significant when $p < 0.05$.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. ATM-deficiency opens vulnerabilities in pancreatic ductal adenocarcinoma.

A, Frequency of DNA repair gene alterations (n=60) in primary pancreatic ductal adenocarcinomas (PDAC) (n=751) from three pancreatic cancer sequencing data sets [17, 18, 19]. Only altered samples are represented. **B**, Localization and types of *ATM* mutations (n=27) in primary PDAC as in **A**, and **C**, contingency table comparing *ATM* mutation type and localization. **D**, Frequency of *ATM* alterations in various cancer types (n=10,945) from MSK-IMPACT clinical sequencing cohort. Only cancer types presenting *ATM* alterations are represented. *ATM* is the first mutated DNA damage response gene (from the same gene panel as in **A**) in cancer types written in red.

Supplementary Figure S2. ATM deficiency does not sensitize cells to chemotherapies.

A, **C**, **E**, **G**, **I**, **K**, Viability assay and **B**, **D**, **F**, **H**, **J**, **L**, colony formation assay on $Atm^{+/+}$; $LSL\text{-}Kras^{G12D/+}$; $Ptf1a^{Cre/+}$ (KC) and $Atm^{fl/fl}$; $LSL\text{-}Kras^{G12D/+}$; $Ptf1a^{Cre/+}$ (AKC) cells treated with increasing concentrations of oxaliplatin (**A** and **B**), cisplatin (**C** and **D**), paclitaxel (**E** and **F**), trametinib (**G** and **H**), AZD7762 (**I** and **J**), and CC-115 (**K** and **L**).

Supplementary Figure S3. ATM deficiency sensitizes cells to topoisomerase inhibition.

A, **C**, Viability assay and **B**, **D**, colony formation assay on $Atm^{+/+}$; $LSL\text{-}Kras^{G12D/+}$; $Ptf1a^{Cre/+}$ (KC) and $Atm^{fl/fl}$; $LSL\text{-}Kras^{G12D/+}$; $Ptf1a^{Cre/+}$ (AKC) cells treated with increasing concentrations of irinotecan (**A** and **B**) and etoposide (**C** and **D**). **E**, Schematic representation of the chorioallantoic membrane assay shown in (**F**) with treatment administration schedule. **F**, Chick chorioallantoic membrane assay performed with KC and AKC cells treated or not with irinotecan (Iri, 2 µM) and **G**, quantification of tumor surface of resected tumors. Scale bars represent 5 mm. **H**, Schematic representation of the subcutaneous assay shown in (**I**) with treatment administration schedule. **I**, Time-dependent development (over the course of 17 days) of subcutaneously engrafted tumors arising either from KC and AKC cells (respectively, black circle and red square) treated or not (respectively, dashed lines and solid lines) with irinotecan (50 mg/kg). S.c., subcutaneous. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$; a, $p < 0.05$ when compared to irinotecan-treated KC allografts.

Supplementary Figure S4. Additional synergy interaction analyses.

A, **C**, **E**, **G**, Viability assay and **B**, **D**, **F**, **H**, drug synergism analysis using zero interaction potency (ZIP) model on $Atm^{+/+}$; $LSL\text{-}Kras^{G12D/+}$; $Ptf1a^{Cre/+}$ (KC) and $Atm^{fl/fl}$; $LSL\text{-}Kras^{G12D/+}$; $Ptf1a^{Cre/+}$ (AKC) cells treated with varying combinations of olaparib (PARPi) and AZD7762 (CHEK1/CHEK2i) (**A** and **B**), trametinib (MEK1/2i) (**C** and **D**), irinotecan (TOP1i) (**E** and **F**) or etoposide (TOP2i) (**G** and **H**). **I**, **J**, **K**, Drug synergism analysis using ZIP model of viability assays respectively shown in Figure 1H (**I**), Figure 1K (**J**), and Figure 1N (**K**). Black squares represent an area synergy δ score and a cell viability below 70%.

Supplementary Figure S5. PAD cocktail induces DNA damage and chromosome aberrations.

A, Immunofluorescence staining for H2AX p-S139 (green) and direct fluorescence staining of cortical actin by phalloidin-Atto565 (red) and **B**, quantification of H2AX p-S139 foci-positive cells in *Atm*$^{+/+}$; *LSL-Kras*$^{G12D/+}$; *Ptf1a*$^{Cre/+}$ (KC) and *Atm*$^{fl/fl}$; *LSL-Kras*$^{G12D/+}$; *Ptf1a*$^{Cre/+}$ (AKC) cells treated or not with olaparib (PARPi, 1 µM), VE-822 (ATRi, 20 nM) and CC-115 (DNA-PKi, 30 nM) in combination for 48 h (PAD, PARPi/ATRi/DNA-PKi). Cells were counterstained with DAPI (blue). Scale bars represent 10 µm. **C**, Direct fluorescence staining of DNA by DAPI (white) and **D**, quantification of chromosome aberrations and **E**, chromatid fusions in metaphase spreads of KC and AKC cells treated or not with olaparib, VE-822 and CC-115 as single agents or in combination for 48 h (as in **A**). White arrows show chromosome aberrations (**C**). Scale bars represent 10 µm in left panels and 3 µm in vertical right panels. **F**, Direct fluorescence staining of DNA by DAPI (white) and **G**, quantification of micronuclei in KC and AKC cells treated or not with olaparib, VE-822 and CC-115 as single agents or in combination for 48 h (as in **A**). White arrows show micronuclei (**F**). Scale bars represent 10 µm. D, chromatid dicentric fusion; Fr, chromatid fragment; G, chromatid gap; M, chromatid monocentric fusion. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Supplementary Figure S6. Genetic or chemical targeting of ATM sensitizes human PDAC cells to DNA damaging drugs.

A, Schematic representation of CRISPR/Cas9 strategy to generate *ATM*$^{+/\Delta}$ and *ATM*$^{\Delta/\Delta}$ human PDAC cells. **B**, Qualitative PCR analysis of CRISPRed MIA PaCa-2 clones. **C**, DNA sequence of wild-type *ATM* and CRISPRed *ATM*$^{+/\Delta}$ MIA PaCa-2 clones at *ATM* exons 3-4. **D**, qRT-PCR analysis of *ATM* expression in *ATM*$^{+/+}$, *ATM*$^{+/\Delta}$ and, *ATM*$^{\Delta/\Delta}$ MIA PaCa-2 cells. **E**, Viability assay analysis of olaparib treatment in CRISPRed *ATM*$^{+/+}$ and *ATM*$^{+/\Delta}$ PANC-1 cells. **F**, **G**, Drug synergism analysis using ZIP model of viability assays respectively shown in Figure 5C (**F**) and Figure 5J (**G**). Black squares represent an area synergy δ score and a cell viability below 70%. **H**, Viability assay on CRISPRed *ATM*$^{+/+}$ and *ATM*$^{+/\Delta}$ PANC-1 cells treated with varying combinations of VE-822 (ATRi) and CC-115 (DNA-PKi) and a fixed dose of olaparib (PARPi) plus an ATM inhibitor (AZD0156). Veh, vehicle. ***, $p < 0.001$; ****, $p < 0.0001$.

Supplementary Figure S7. ATM inhibition sensitizes PDAC patient-derived organoids to PAD therapy.

A, Schematic representation of cytotoxicity assay shown in (**C**). **B**, Representative images of patient-derived organoids (PDOs) isolated from patient-derived xenografts. Scale bars represent 400 µm. **C**, Cytotoxicity assay on PDOs treated or not with an ATM inhibitor (AZD0156, 2 µM) and with olaparib (PARPi, 25 µM), VE-822 (ATRi, 20 nM) and CC-115 (DNA-PKi, 30 nM) in combination for 96 h (PAD, PARPi/ATRi/DNA-PKi).

Supplementary Figure S8. ATM inhibition sensitizes PDAC patient-derived xenografts to PAD therapy.

Time-dependent development (over the course of 17 days (Gö14, Bo62, Bo61, Bo59), 16 days (Panc185 and Panc420), or 12 days (Gö8 and Gö9)) of subcutaneously engrafted tumors arising from ten PDXs treated or

not (respectively, dashed lines and solid lines) with a combination of AZD0156 and PAD as in Figure **7D** and **7H**, with representative macroscopic images. Scale bars represent 5 mm.

Supplementary Figure S9. PAD plus ATM inhibitor therapy results in tolerable adverse effects in immunocompetent C57BL/6J mice.

A, Graphic representation showing results of flow cytometry analyses of common lymphoid progenitor (CLP), common myeloid progenitor (CMP), megakaryocyte/erythrocyte progenitor (MEP), and granulocyte/macrophage progenitor (GMP) populations in peripheral blood of C57BL/6J mice enrolled in the toxicity assay and treated with AZD0156 (2.25 mg/kg) and PAD (olaparib (50 mg/kg), VE-822 (20 mg/kg) and, CC-115 (2.5 mg/kg)) as shown in Figure **7L**. **B**, Graphic representation showing results of flow cytometry analyses of natural killer (NK) cell, B cell, CD3+ T cell, macrophage, neutrophil, myeloid dendritic cell (mDC), and plasmacytoid dendritic cell (pDC) populations in bone marrow and in peripheral blood of C57BL/6J mice enrolled in the toxicity assay and treated with AZD0156 and PAD as in (**A**). **C**, **D**, Immunohistochemistry staining for H2AX p-S139 and cleaved caspase-3 (CC3) in resected gut (**C**) and liver (**D**) from toxicity assay shown in Figure **7L**. Scale bars represent 150 µm. BM, bone marrow; Veh, vehicle. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Supplementary Figure S10. Resistance to PARP inhibition in ATM-deficient PDAC cells involves drug efflux and induces an epithelial-mesenchymal transition.

A, Volcano plot displaying differential expressed genes between PARP inhibition-resistant *Atm*$^{fl/fl}$; *LSL-Kras*$^{G12D/+}$; *Ptf1a*$^{Cre/+}$ (R-AKC) and *Atm*$^{fl/fl}$; *LSL-Kras*$^{G12D/+}$; *Ptf1a*$^{Cre/+}$ (AKC) cells. Red dots represent the upregulated expressed transcripts and blue dots represent the downregulated expressed transcripts. **B**, DAVID-based and **C**, Molecular Signature Database-based gene ontology biological process term enrichment analyses in R-AKC *vs.* AKC cells. **D**, qRT-PCR analysis of drug efflux transporters gene expression in AKC and R-AKC cells. **E**, Gene set enrichment analysis in R-AKC *vs.* AKC cells. NES, normalized enrichment score; Nom. *p*-value, nominal *p*-value; FDR, false discovery rate. ****, $p < 0.0001$.

Supplementary Figure S11. Clonal evolution during acquisition of PARP inhibition resistance in ATM-deficient PDAC cells.

Whole exome sequencing-based clonogenicity analysis of *Atm*$^{fl/fl}$; *LSL-Kras*$^{G12D/+}$; *Ptf1a*$^{Cre/+}$ (AKC) and PARP inhibition-resistant *Atm*$^{fl/fl}$; *LSL-Kras*$^{G12D/+}$; *Ptf1a*$^{Cre/+}$ (R-AKC) cell lines.

Supplementary Figure S12. Resistance to PARP inhibition in ATM-deficient cells involves alternative DNA damage repair pathway.

A, qRT-PCR analysis of *Fancd2*, *Mgmt*, and *Paxip1* gene expression in *Atm*$^{fl/fl}$; *LSL-Kras*$^{G12D/+}$; *Ptf1a*$^{Cre/+}$ (AKC) and PARP inhibition-resistant *Atm*$^{fl/fl}$; *LSL-Kras*$^{G12D/+}$; *Ptf1a*$^{Cre/+}$ (R-AKC) cells. **B**, Viability assay on AKC and R-

AKC cells treated or not with increasing doses of gamma radiation. **C**, qRT-PCR analysis of *Lig1* gene expression in *shScramble*- (shScr) and *shLig1*-harboring R-AKC cells. LD_{50}, lethal dose 50%. ***, $p < 0.001$; ****, $p < 0.0001$.

SUPPLEMENTARY FIGURES

Supplementary Figure S1. ATM-deficiency opens vulnerabilities in pancreatic ductal adenocarcinoma.

Supplementary Figure S2. ATM deficiency does not sensitize cells to chemotherapies.

Supplementary Figure S3. ATM deficiency sensitizes cells to topoisomerase inhibition.

Supplementary Figure S4. Additional synergy interaction analyses.

Supplementary Figure S5. PAD cocktail induces DNA damage and chromosome aberrations.

Supplementary Figure S6. Genetic or chemical targeting of ATM sensitizes human PDAC cells to DNA damaging drugs.

Supplementary Figure S7. ATM inhibition sensitizes PDAC patient-derived organoids to PAD therapy.

Supplementary Figure S8. ATM inhibition sensitizes PDAC patient-derived xenografts to PAD therapy.

Supplementary Figure S9. PAD plus ATM inhibitor therapy results in tolerable adverse effects in immunocompetent C57BL/6J mice.

Supplementary Figure S10. Resistance to PARP inhibition in ATM-deficient PDAC cells involves drug efflux and induces an epithelial-mesenchymal transition.

Supplementary Figure S11. Clonal evolution during acquisition of PARP inhibition resistance in ATM-deficient PDAC cells.

Supplementary Figure S12. Resistance to PARP inhibition in ATM-deficient cells involves alternative DNA damage repair pathway.

SUPPLEMENTARY TABLES

Supplementary Table 1. Germline mutation frequency of DNA damage response genes in human primary pancreatic adenocarcinoma (PDAC). For that, we screened and analyzed germline mutations data from three unselected PDAC cohorts [25, 26, 27] (1,441 tumor samples).

Supplementary Table 2. qPCR oligonucleotide sequences used in this study.

Supplementary Table 3. Primary and secondary antibodies used in this study.

Supplementary Table 4. Antibody combinations used in this study for the detection of hematopoietic progenitors and immune cell subtypes by flow cytometry.

Supplementary Table 5. Antibodies used in this study for the detection of hematopoietic progenitors and immune cell subtypes by flow cytometry.

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