# Extended materials and methods

## Commercial cell lines

The pancreatic cancer cell lines MIA PaCa-2, HPAF-II and BxPC-3 were purchased from ATCC (Manassas, VA, USA). MIA PaCa-2 and HPAF-II were grown at 37°C in presence of 5% CO2 and in Dulbecco’s modified Eagle’s medium/F-12 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 2.5% horse serum, 2 mM L-glutamine and 1x antibiotic–antimycotic (Gibco, Thermo Fisher Scientific). BxPC-3 cells were grown at 37 °C in presence of 5% CO2 and in RPMI 1640 (Invitrogen, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1x antibiotic–antimycotic (Gibco, Thermo Fisher Scientific). Cells were routinely tested every month for the absence of mycoplasma contamination using the MycoAlertTM Mycoplasma detection kit (Lonza). Cell lines were not authenticated in-house and were not passaged more than 30 times.

## Inhibitors

The Mek1/2 inhibitor MEK162 was provided as powder by Novartis (Basel, Switzerland). For in vitro experiments, MEK162 was diluted in DMSO (ATCC, Manassas, VA, USA) to a final concentration of 10 mM. For in vivo administration, a suspension of MEK162 in 10% Tween-80 was incubated overnight at 4 °C before diluting 20-fold with 1% Na-carboxymethylcellulose and 0.5% methylcellulose, respectively. The final concentration of active MEK162 was 1.5 mg/ml, which was administrated orally by gavage to 15 mg/kg morning and evening.

Trametinib was purchased from MedChemExpress (Monmouth Junction, NJ, USA) and diluted in DMSO for in vitro use to a final concentration of 10mM. The Erk1/2 inhibitor SCH772984 was purchased from Selleckchem (Houston, TX, USA) and diluted in DMSO for in vitro use to a final concentration of 10mM. Erk5 inhibitor XMD8-92 was purchased from Selleckchem (Houston, TX, USA) and diluted in DMSO for *in vitro* use. The Mek5 inhibitor BIX02189 was purchased by MedChem Express (Monmouth Junction, NJ, USA). The K-RasG12C inhibitor AMG-510 was purchased from MedChemExpress and diluted in DMSO for in vitro use.

dTAG-13 (Tocris Bioscience, Bristol, UK, cat no. 6605) was used to trigger the proteasome degradation of Slug-FKBPF36V. For in vitro use, dTAG-13 was diluted in DMSO to a final concentration of 10 mM. For in vivo administration, dTAG-13 was resuspended in a 5% DMSO and 20% solutol saline solution and administered by intraperitoneal injection. For assessing in vitrodegradation, we incubated the cells 24 hours whereas for assessing degradation in vivo, tumors were collected 6 hours after the last treatment.

## Generation of cells resistant to MEK162

In the first approach (R1), cells were treated after attachment with 1.2 μM MEK162, which corresponded to ~10 x IC50 of MEK162 on MIA PaCa-2 cells. After 2 days, cell culture was replenished with drug-free medium until cells were confluent. In the second strategy (R2), MIA PaCa-2 cells were treated with increasing concentrations of MEK162, starting from 0.25 µM (~2 x IC50) and increasing over time, until reaching 1.2 μM in the last round of treatment. In this strategy, MEK162 was maintained in the medium until confluence. Resistant cells from P-PDX #57 were obtained following the second strategy, with increasing concentrations of MEK162 (started with 45 nM and finished with 5800 nM). As control, we cultured MIA PaCa-2 and P-PDX #57 parental cells in the presence of vehicle alone during the same period and under the same conditions.

**Generation of cells resistant to AMG-510**

To generate resistance, MIA PaCa-2 cells were treated after attachment with increasing concentrations of AMG-510, starting from 5.5 nM and increasing over time, until reaching 60 nM in the last round of treatment. AMG-510 was maintained in the medium until confluence.

## Proliferation assays

To determine cell proliferation, two wells of a 96-well plate (corresponding to times 0 and 72h) were seeded with 1100 cells in 85 μl. One well was seeded with 85 μl of medium without cells to provide a background reference value.

After 24 h, the cells were fixed in the first plate (time 0) by adding 80 μl 10% glutaraldehyde to each well followed by 30-min incubation. After washing wells with water, this plate was kept a 4 °C and later stained together with the other plates. In the rest of the plates, 85 μl of medium with or without different concentrations of drug was added to all wells and then returned to the cell incubator for another 72 h. After 3 days, cells were fixed. The wells were washed in water, dried for 5 min upside down on paper and then stained with 70 μl of 0.1% crystal violet solution. Finally, the wells were washed, dried overnight and assayed with 10% acetic acid and 560 nm absorbance measurements. For the pancreatic cancer-derived primary cultures, 2400 cells were seeded in 85 μl per well and cells were grown for 6 days.

## Western Blot, immunohistochemistry and antibodies

For western blot, protein extracts were isolated by lysing the cells in Radioimmunoprecipitation Assay Buffer (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS and 50 mM Tris pH 7.4) supplemented with 5 μM β-glycerophosphate, 5 μM sodium fluoride, 1 μM sodium orthovanadate and cOmplete™, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, 1 tablet per 50 ml lysis buffer).

Protein lysates were resolved by SDS PAGE and then transferred to a 0.45 μm nitrocellulose membrane (GE Healthcare Biociences, Chicago, IL, USA). 20-30 μg protein lysate were loaded per experiment. Membranes were incubated with 5% BSA (Sigma-Aldrich) or 5% milk in 1X Tris-buffered saline (TBS) with 1% tween (TBS-T, Sigma-Aldrich). After blocking, membranes were incubated overnight with primary antibodies.

After washing three times with TBS-T, membranes were incubated with horseradish peroxidase-conjugated antibodies (GE Healthcare) for 1 hour. Membranes were developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore) and protein bands were visualized in AmershamTM Imager 600 (GE Life Sciences).

Antibodies used were: ERK1/2 (1:1000 from stock; #9102, Cell Signaling Technology (CST)), p-ERK1/2 (1:1000; #9101, CST), Tubulin (1:1000; #T9026, Sigma-Aldrich), GAPDH (1:5000; #ab128915, Abcam), Slug (1:1000, #9585, CST), Cyclin D1 (1:1000, # 55506, CST), ERK5 (1:1000, #3372, CST), p-ERK5 (1:1000, #3371, CST), Fibronectin (1:1000, #ab299, Abcam), Vimentin (1:1000, #5741, CST), AKT (1:1000 from stock; #9272, CST), p-AKT (Ser473) (1:1000; #9271, CST), p-4E-BP1 (Thr70) (1:1000; #9455, CST), Snail (1:1000; #3879, CST), SNAI3 (1:1000; #PA5-95254, Invitrogen), TBX2 (1:500; #sc-514291, Santa Cruz), Twist2 (1:1000; #ab66031, Abcam), ZEB1 (1:1000; #3396, CST).

For immunohistochemistry, fixed tissue samples embedded in paraffin were sectioned at 4 μm thickness. Sections were heated at 60oC, deparaffinized with xylene and hydrated with two steps of incubation with different dilutions of ethanol. Antigen retrieval was performed by boiling the samples for 20 minutes in citrate buffer pH 6. Endogenous peroxidase was blocked by incubating the slides in the presence of 3% peroxide hydrogen (Merck Millipore) diluted in absolute methanol. Slides were also blocked with 3% BSA (Sigma-Aldrich) in 1x PBS for 10 minutes. Then, samples were incubated with primary antibodies diluted in EnVision FLEX Antibody Diluent (Agilent technologies) overnight. Primary antibodies used were as follows: p-ERK1/2 (1:100; #9101, CST), Slug (1:500; #MA5-26385, Thermo Fisher Scientific), p-ERK5 (1:100; #3371, CST), Ki-67 (1:100; #M7240, Agilent) and Caspase-3 (1:100; #9661, CST) . Next, the slides were incubated in EnVision System HRP-Labelled Polymer Secondary antibody. The samples were finally stained with DAB substrate chromogen (Agilent technologies) for 1-4 minutes and counterstained with Harris Hematoxylin for 2 minutes, followed by dehydration with ethanol and xylene, and finally mounted in DPX.

## Human phospho-kinase antibody array

The relative levels of phosphorylation of 43 kinase phosphorylation sites and two related total proteins were assessed by using a commercial human phospho-kinase antibody array (R&D systems, Minneapolis, MN, USA).

Whole cell lysates were prepared according to manufacturer’s protocol. 300 μg protein were diluted in array buffer and loaded into the corresponding membranes containing the spotted capture antibodies overnight. Protein expression was detected by biotinylated phospho-specific detection antibodies and then visualized by chemiluminescence according to the manufacturer’s protocol.

## Exome sequencing

DNA was isolated from fresh cell pellets washed in 1x PBS, using the QIAmp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA libraries were prepared using the Agilent (Santa Clara, CA, USA) SureSelect XT Library Prep Kit according to the manufacturer's protocol. Target enrichment was performed using the Agilent SureSelect XT Human All Exon v5 capture set. Sequencing with 100 base paired end reads of targeted enrichment libraries was performed on the HiSeq 2500 sequencer.
A quality check of the raw data was performed using the FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were filtered first by quality using FASTX-Toolkit (v. 0.0.14, http://hannonlab.cshl.edu/fastx\_toolkit/index.html) and then by length using HOMER (La Jolla, San Diego, CA, USA) (v. 4.7). The remaining reads were mapped to the Sanger human reference (hg19) by bwa (v. 0.6.2) with default settings. The resulting binary alignment map files were processed using SAMtools (San Francisco, CA, USA) (v. 0.1.19) and the Genome Analysis ToolKit (GATK) release 3.2.0. (Cambridge, MA, USA). In brief, binary alignment map files were binary compressed, sorted, and indexed by SAMtools (samtools view, sort, and index tools), duplicated reads were then removed by the SAMtools function rmdup, and base quality score recalibration and local realignment around indels followed the recommended workflow of the GATK toolkit (RealignerTargetCreator, IndelRealigner, BaseRecalibrator and PrintReads). Variants were called by VarScan (v2.3.7) with the following parameters: minimum variant allele frequency of 5%, a minimum coverage of 10 reads, at least 7 reads that confirm the mutation and a P-value below 0.05. Annotation of the vcf files was performed with ANNOtate VARiation. Variants were filtered: variant positions must not be listed as a single-nucleotide polymorphism in the 1000 genome project; variant position must be annotated as exonic by RefSeq (Release 45); and synonymous/nonsynonymous calls were made and the synonymous excluded from further analysis. All filtering was performed using in house parsers.

## RNA-seq preparation and data analysis

Three biological replicates of parental MIA PaCa-2 cells and one replicate of each resistant clone were sequenced. RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s recommendations. RNA quantity and purity were measured with the NanoDrop spectrophotometer (Thermo Fisher Scientific). TruSeq Stranded Total RNA kit protocol (Illumina Inc.) was used to prepare the RNA-Seq libraries: Briefly, Ribosomal RNA (rRNA) was depleted from 1.0 ug of total RNA using the RiboZero Magnetic Gold Kit (Illumina Inc). rRNA-depleted samples were fragmented, cDNA was synthesized and converted into libraries. The size and quality of the libraries were assessed with a High Sensitivity DNA Bioanalyzer assay (Agilent Technologies Inc., Santa Clara, CA).

Libraries were sequenced in a HiSeq2000 instrument, with a read length of 2x100bp. On average, 84 million paired-end reads were generated per sample. Images analysis, base calling and quality scoring of the run were processed using the manufacturer’s software Real Time Analysis (RTA 1.18.64) and followed by generation of FASTQ sequence files by CASAVA. The quality of the reads was checked using the FastQC tool (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and RNA-SeQC (32). Low-quality reads were discarded by fastx toolkit (v. 0.0.14) (http://hannonlab.cshl.edu/fastx\_toolkit/). TopHat (18) was used to map the RNA-Seq reads to the GRCh37/hg19 genome allowing a maximum of three mismatches. The resulting bam files were sorted, indexed using the picard tool (v. 1.79) (http://broadinstitute.github.io/picard/index.htm/)and were used to calculate the numbers of the reads which were mapped to the exons of a gene based on Cufflinks (18). Data were normalized to reads per kilo-base of the exon model per million mapped reads (RPKM), which is the representation of the expression values of the genes. Finally, through Cuffdiff in Cufflinks, Differentially Expressed Genes (DEGs) were obtained with the cutoff of |log2(Ratio)| more than 1 and an FDR-adjusted-pvalue less than 0.05.

The RNAseq data in this study have been deposited in Sequence Read Archive (SRA) database and are accessible through the SRA Bioproject (PRJNA719991).

## Gene Set Enrichment Analysis (GSEA)

Pathway enrichment was assessed through the pre-ranked version of GSEA, and we used gene sets derived from the HALLMARK database (19). Only statistical gene sets are represented in Figure 2C (NOM p-val<0.05)). Normalized Enrichment Score is defined in <https://www.gsea-msigdb.org/gsea/doc/GSEAUserGuideTEXT.htm#_Enrichment_Score_(ES)>

## RNA isolation and qRT-PCR

Total RNA was isolated from adherent cells by using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. RNA was eluted from silica-membrane RNeasy spin columns in RNase-free water and quantified using NanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific).

cDNA was prepared from 2 μg template RNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to manufacturer’s protocol.

Real-time quantification of transcript abundance was determined by qRT-PCR using the Taqman Gene Expression probes and TaqMan Universal Master Mix II, with UNG (Thermo Fisher Scientific) following the manufacturer’s protocol, in 384-well plates in 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific). The following TaqMan probes (Thermo Fisher Scientific) were used: SNAI1 (Hs00195591\_m1), SNAI2 (Hs00161904\_m1), SNAI3 (Hs01018996\_m1), TBX2 (Hs00911929\_m1), TWIST2 (Hs03986784\_s1), ZEB1 (Hs00611024\_m1), GAPDH (Hs02758991\_g1). Data was analyzed with SDS, RQ Manager, and DataAssist software (Applied Biosystems), using the 2-ΔCT method. *GAPDH* was used as an endogenous control.

## *In vitro* adhesion, migration and invasion assays.

For assessing cell adhesion, cells were labeled with 5 μM of Cell Tracker Green reagent (Invitrogen), following the manufacturer’s protocol, and kept overnight in serum-free medium. 24 hours later, 5 × 104 cells were seeded in triplicates in 24-well plates previously coated with 300 μg/ml Corning Matrigel matrix (Corning) in 1x DMEM F-12. Two hours after seeding, cells were washed carefully with 1x PBS and fixed with 4% formalin. Fixed cells were visualized and counted using the Fiji software. A total of 5 pictures per well were taken. Normalized number of cells are shown, and two-sided t-test was used to analyze statistical differences between groups.

Migration assay. Cells were labeled with 5 μM of Cell Tracker Green reagent (ThermoFisher), following the manufacturer’s protocol, and kept overnight in serum-free medium. 24 hours later, 5 × 104 cells were seeded in triplicates in 10 μg/ml fibronectin (Sigma-Aldrich) coated Fluoroblok BD Biocoat Cell Culture Inserts (Corning) in serum-free medium, while the wells were loaded with complete growth medium. Eight hours after seeding, cells were washed with 1x PBS and fixed with 4% formalin. Migration to the basolateral side was visualized and counted using the Fiji software. A total of 5 pictures per well were taken. Normalized number of cells are shown, and two-sided t-test was used to analyze statistical differences between groups.

Invasion assay. Cells were labeled with 5 μM of Cell Tracker Green reagent, following the manufacturer’s protocol, and kept overnight in serum-free medium. Next day, 5 × 104 cells were seeded in triplicates on 300 μg/ml matrigel-coated Fluoroblok BD Biocoat Cell Culture Inserts in serum-free medium, while the wells were loaded with complete medium. Eight hours after seeding, cells were washed with PBS and fixed with 4% formalin. Migration to the basolateral side was visualized and counted using the Fiji software. A total of 5 pictures per well were taken. Normalized number of cells are shown, and two-sided t-test was used to analyze statistical differences between groups.

## 3D growth in matrigel

To assess the ability to form 3D spheroids, cells were mixed in growth-reduced factor matrigel (BD Bioscience, cat no. 354234) in a concentration of 1,000 cells per 25 μl of matrigel. Each drop was dispensed in the center of the well from adherent 48-well plate and incubated for 15 min at room temperature. After matrigel was solidified, 250 μl of 3D culture media (DMEM F-12 supplemented with 1% glutamine, 1X B-27 supplement, 20 ng/ ml hFGF and 20 ng/ml hEGF and 1X Anti-Anti) were added to each well. Media was replaced twice a week and 3D structure formation was assessed after 15 days.

## Orthotopic mouse model of pancreatic cancer

Animal work was performed according to protocols approved by the Ethical Committee for the Use of Experimental Animals at the Vall d’Hebron Institute of Oncology. MIA PaCa-2, R1-16 and R2-39 luciferase cells were suspended in Matrigel to 80.000 cells/μl. Using a Hamilton needle, 10 μl cell suspension was injected into the tail of the pancreas of 5-week-old female BALB-c Nude mice. In order to see the efficiency of injection, cells were monitored at day 0 with the IVIS-200 imaging system from Xenogen (PerkinElmer, Waltham, MA, USA). The rate of tumor growth was monitored weekly. Mice were killed after 40-60 days, and all major organs were resected in order to determine the existence of distant metastasis by means of luciferase signal by IVIS. Metastasis was considered positive if luciferase signal was greater than the background.

## Primary cell cultures

All PDXs have been established at VHIO following institutional guidelines. The IRBs at Vall d’Hebron Hospital provided approval for this study in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients who provided tissue samples. To obtain primary cell cultures from pancreatic cancer PDXs, tumor pieces of approximately 750-1000 mm3 were minced with scalpels and digested in digestion solution: 300 U/ml collagenase IA (Sigma-Aldrich, St Louis, MO, USA) in 10 ml pancreatic medium for 1 hour at 37oC in 15 ml conical tubes (Corning Life Sciences, New York, NY, USA). The digested tissue was centrifuged at 300 RCF for 5 minutes. The pellet of digested tissue was resuspended in 13 ml pancreatic medium. 15-20 seconds were allowed for the undigested tissue to precipitate at the bottom of the conical tube. The supernatant containing the digested tissue was seeded in a 10 cm cell culture dish (Corning Life Sciences, New York, NY, USA). Once several colonies of epithelial cells were attached, the medium was replaced twice a week until confluence and then cell cultures were expanded. All PC-PDXs were previously sequenced by amplicon sequencing.

The samples used for the generation of PC-PDX6, 10, 21 and 27 were classified as classic ductal adenocarcinomas during their pathological evaluation. The samples that produced PC-PDX30, 37, 57 and 61 originated from needle biopsies of liver metastases in patients with advanced PC.

## Virus production, transduction and plasmids

For lentivirus production, HEK293T cells were transfected with the pMD2.G (#12259, Addgene, Watertown, MA, USA) envelope expressing plasmid and the psPAX2 (#12260, Addgene) lentiviral packaging vector, using polyethylenimine (PEI, Polysciences, Warrington, PA, USA) as transfection agent. 24 hours after transfection, the growth medium was replaced with medium containing 5mM sodium butyrate. 48 hours later, viral particles-containing supernatant was harvested and filtered with 0.45 μm con PVDF filters. For infections, approximately 35% confluent target cells were incubated with the viral supernatants (diluted 1:5 in growth medium) and 8 μg/ml polybrene overnight. Infected cells were selected with 1 μg/ml puromycin, starting 2 days after infection, and subsequently maintained with 0.5 μg/ml puromycin in the growth media.

For retrovirus production, HEK239T cells were transfected with pCMV-VSV-G (#8454, Addgene) envelope plasmid and pUMVC packaging vector (#8449, Addgene), using PEI as transfection agent. The rest of the steps proceeded as described above.

For expressing luciferase, the Lentiviral Dual Reporter CMV-GFP-T2A-Luciferase plasmid (system biosciences, Palo Alto, CA, USA) was used. Retroviral pPGS-hSLUG.fl.flag was used for overexpressing Slug (#25696, Addgene). For Slug silencing, the plasmids were obtained from the lentiviral MISSION® shRNA Library (Clones TRCN0000271239, TRCN0000271298, TRCN0000271300, TRCN0000271362 and TRCN0000271389, Sigma).

To generate Slug-FKBPF36V fusion protein, Gateway recombination technology (Invitrogen) was used to recombine pENTR223 plasmid (clone HsCD00508549; DNASU plasmid repository, Tempe, AZ, USA) containing a SNAI2 sequence lacking the STOP codon with the pLEX\_305-C-dTAG destination vector (a gift from James Bradner; Addgene plasmid #91798), according to manufacturer’s protocol.

To generate FKBPF36V-Slug fusion protein, Gateway recombination technology (Invitrogen) was used to recombine pEntr1A donor vector containing human Slug cDNA sequence with the pLEX\_305-N-dTAG destination vector (a gift from James Bradner; Addgene plasmid #91797), according to manufacturer’s protocol. Slug cDNA was cloned into the Gateway™ pENTR™ 1A Dual Selection Vector (Invitrogen). Primers used to amplify Slug were CCTGACTAGGGATCCTAAGCCGCCACCATGCCGCGCTCCTTC (Forward) and GTAGTCCTACTCGAGTCAGTGTGCTACACAGCA (Reverse).

## Subcutaneous injection of MIA PaCa-2 and resistant cells

0.8x106 MIA PaCa-2 and MEK162-resistant cells were resuspended in 50% Matrigel:PBS and injected subcutaneously into BALB-c Nude mice. When tumors reached 100-200 mm3, mice were treated with MEK162 5 times a week. In the case of metastasis assessment from subcutaneous injection, luciferase-expressing cells were injected, and tumors were resected when they were above 1000 mm3. Lung metastases were measured after 94 days.

**Statistical analysis**

## For in vitro and in vivo experiments, comparisons between two groups were made by two-tailed Student’s *t* test.