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Supplemental Information

Circadian Dysregulation of the TGFβ/SMAD4 Pathway Modulates Metastatic Properties and Cell Fate Decisions in Pancreatic Cancer Cells

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Supplemental Data Items

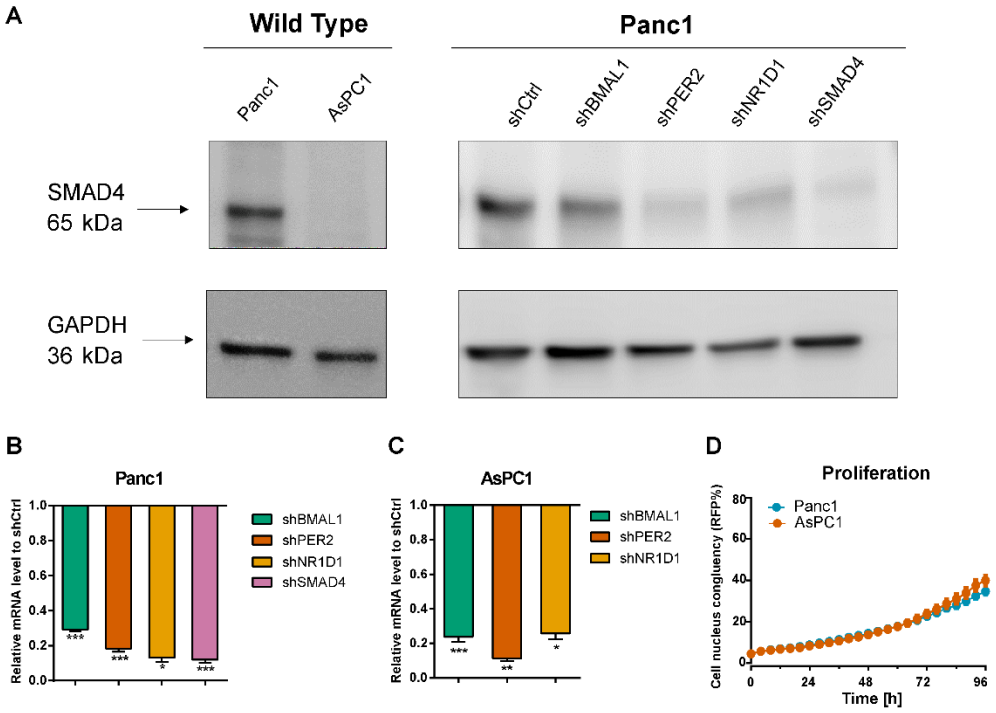

Figure S1. Western blot analysis of SMAD4 protein expression for KDs and OE cells. Related to Figure 1. (A) Western blot analysis of SMAD4 (65kDa) for wild type PDA cells and Panc1 KD cells (*shCtrl*, *shBMAL1*, *shPER2*, *shNR1D1* and *shSMAD4*). Depicted is one representative replicate, GAPDH is provided as loading control. (B - C) KD efficiency for sh*BMAL1*, sh*PER2*, sh*NR1D1* and sh*SMAD4*. Gene expression analysis of corresponding KD genes in Panc1 (B) and AsPC1 (C) cells. Relative gene expression is shown compared to the corresponding *shCtrl* (pLKO.1) (mean ± SEM, n = 3, t-test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001). KD efficiency in Panc1 *shBMAL1*: 70.8% (0.29 ± 0.01), *shPER2*: 81.7% (0.18 ± 0.02), *shNR1D1*: 86.1% (0.13 ± 0.02) and *shSMAD4*: 88.5% (0.12 ± 0.02). KD efficiency in AsPC1 *shBMAL1*: 76.2% (0.24 ± 0.03), *shPER2*: 88.7% (0.11 ± 0.02) and *shNR1D1*: 74.2% (0.26 ± 0.03). (D) Proliferation assays for pancreatic cell lines (Panc1 and AsPC1) were performed using NucLight Rapid Red Reagent for the IncuCyte S3 Live Cell System Analysis (mean ± SEM, n = 8).

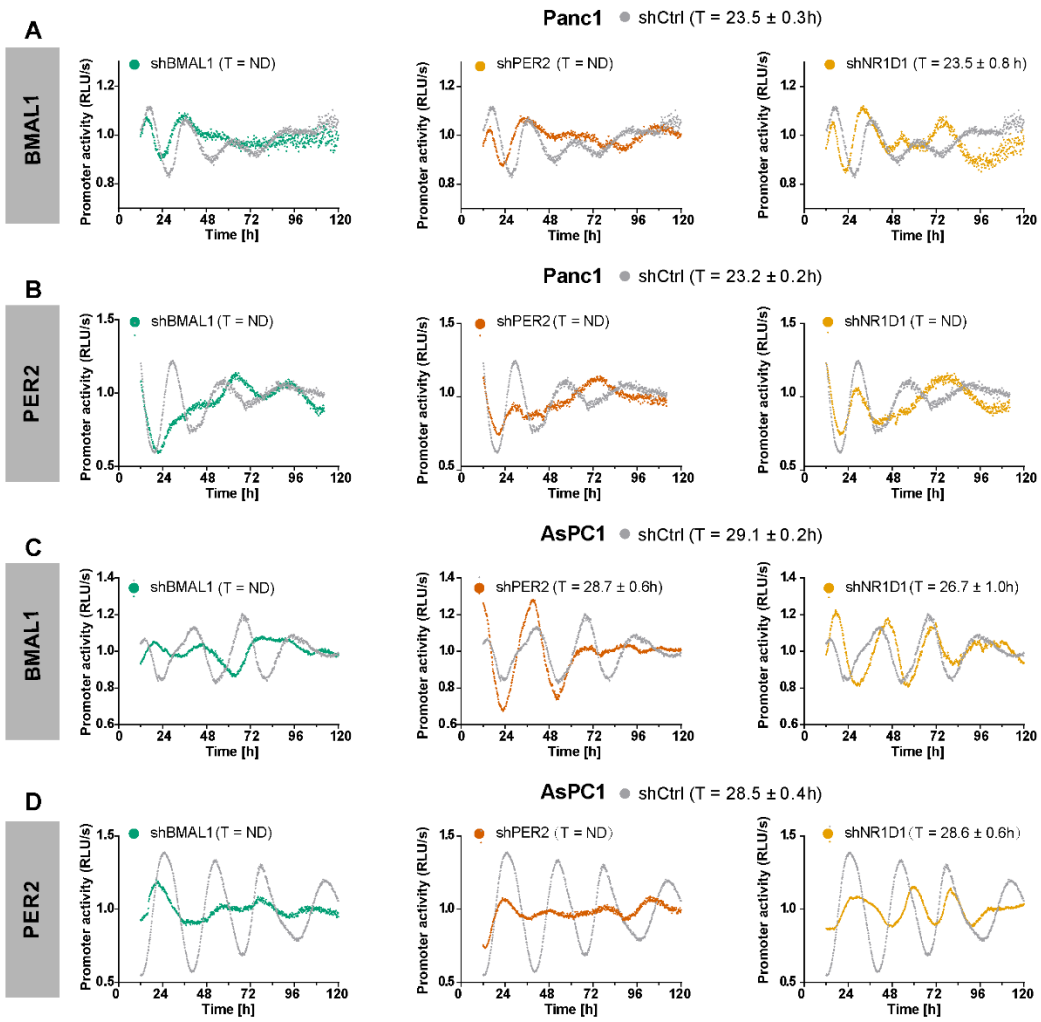

Figure S2. The promoter activity of BMAL1 and PER2 after clock genes KD. Related to Figure 2. Bioluminescence recordings for the promoter activity of *BMAL1* and *PER2* for *shCtrl* (pLKO.1) and KDs (*shBMAL1*, *shPER2* and *shNR1D1)* in Panc1 (A - B) and AsPC1 (C - D). Depicted is one representative replicate (mean ± SEM, n = 3).

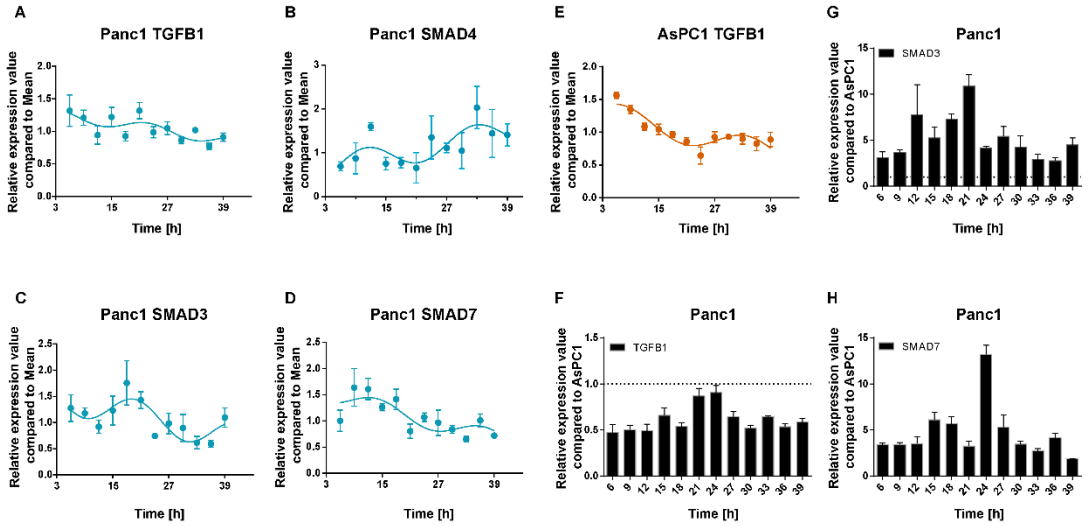

Figure S3. Panc1 (SMAD4 proficient) cells show circadian oscillations in expression for elements of the TGFβ pathway. Related to Figure 2. (A - E) 33h time-course gene expression analysis for *TGFβ1*, *SMAD4*, *SMAD3* and *SMAD7* in Panc1 and AsPC1 wild type cells (n = 3, mean ± SEM, a cosine curve was fitted to all data sets, Table. 3). (F - H) Comparisons of gene expression (*SMAD3*, *SMAD7* and *TGFβ1*) for each time point over 33 h (mean ± SEM, n = 3).

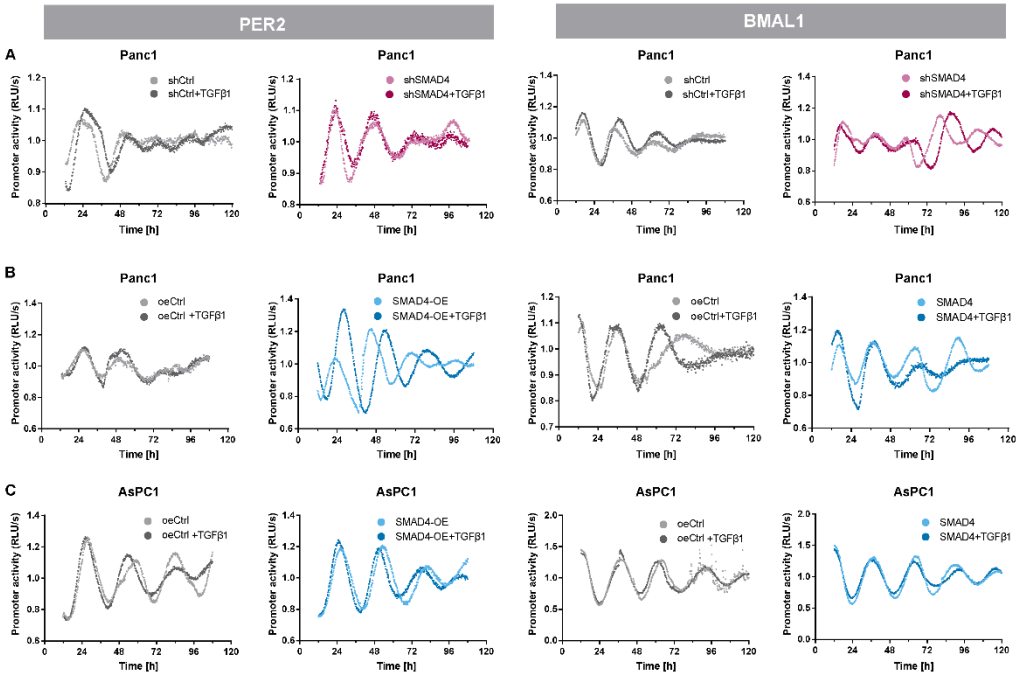

Figure S4. The real-time bioluminescence recording of a *BMAL1* and *PER2* promoter activity for activation or inactivation of the canonical TGFβ pathway. Related to Figure 2.

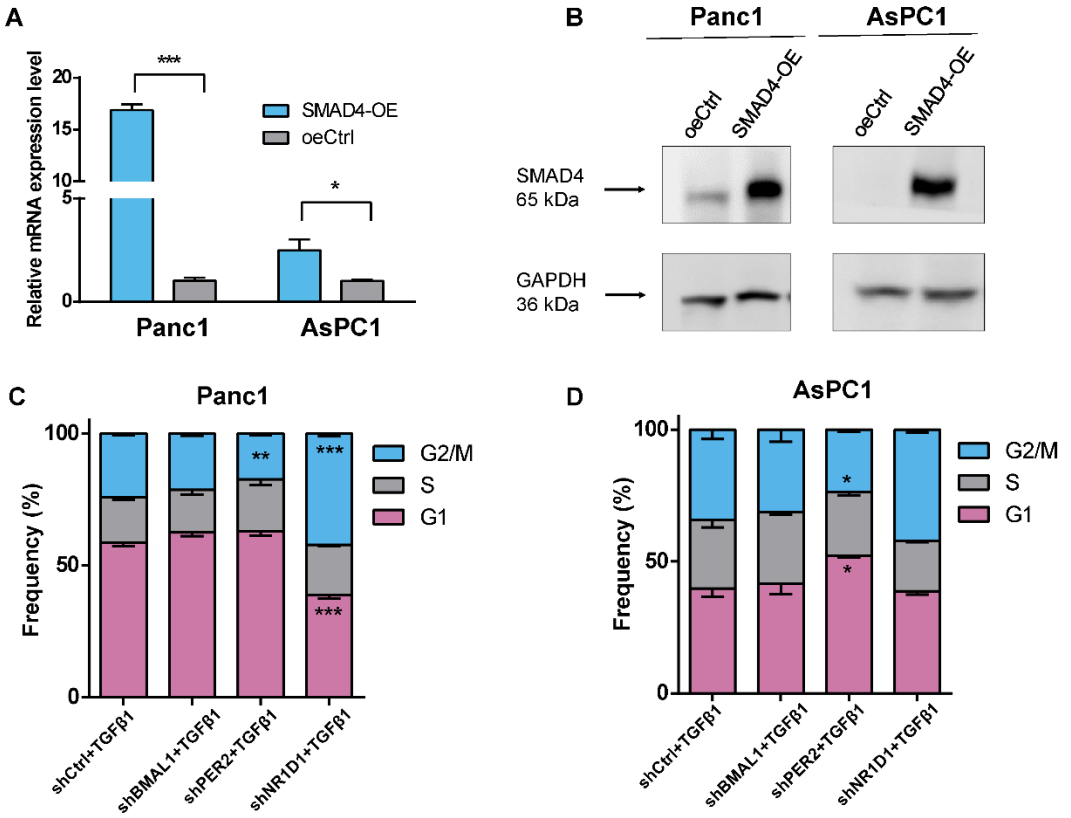

Figure S5. Clock disruption stimulated with TGFβ impacts on cell cycle in PDA cells. Related to Figures 3 and 4. (A) *SMAD4* overexpressing efficiency after γ-retroviruses transduction. Gene expression analysis of *SMAD4* in Panc1 and AsPC1 cells. Relative gene expression value is shown compared to the corresponding empty vector oeCtrl (Flag-puro). (B) Shown is one representative replicate for the Western blot analysis of SMAD4 (65kDa) for PDA cells containing overexpression construct and respective control (*SMAD4*-OE and oeCtrl). GAPDH was d as loading control. (mean ± SEM, n = 3). (C-D) Cell cycle measurements after KDs of BMAL1, PER2 and NR1D1 with or without a 24h-TGFβ1 stimulation. Cell cycle phase distributions were compared with their respective control conditions (n = 3, mean ± SEM, two-way ANOVA, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

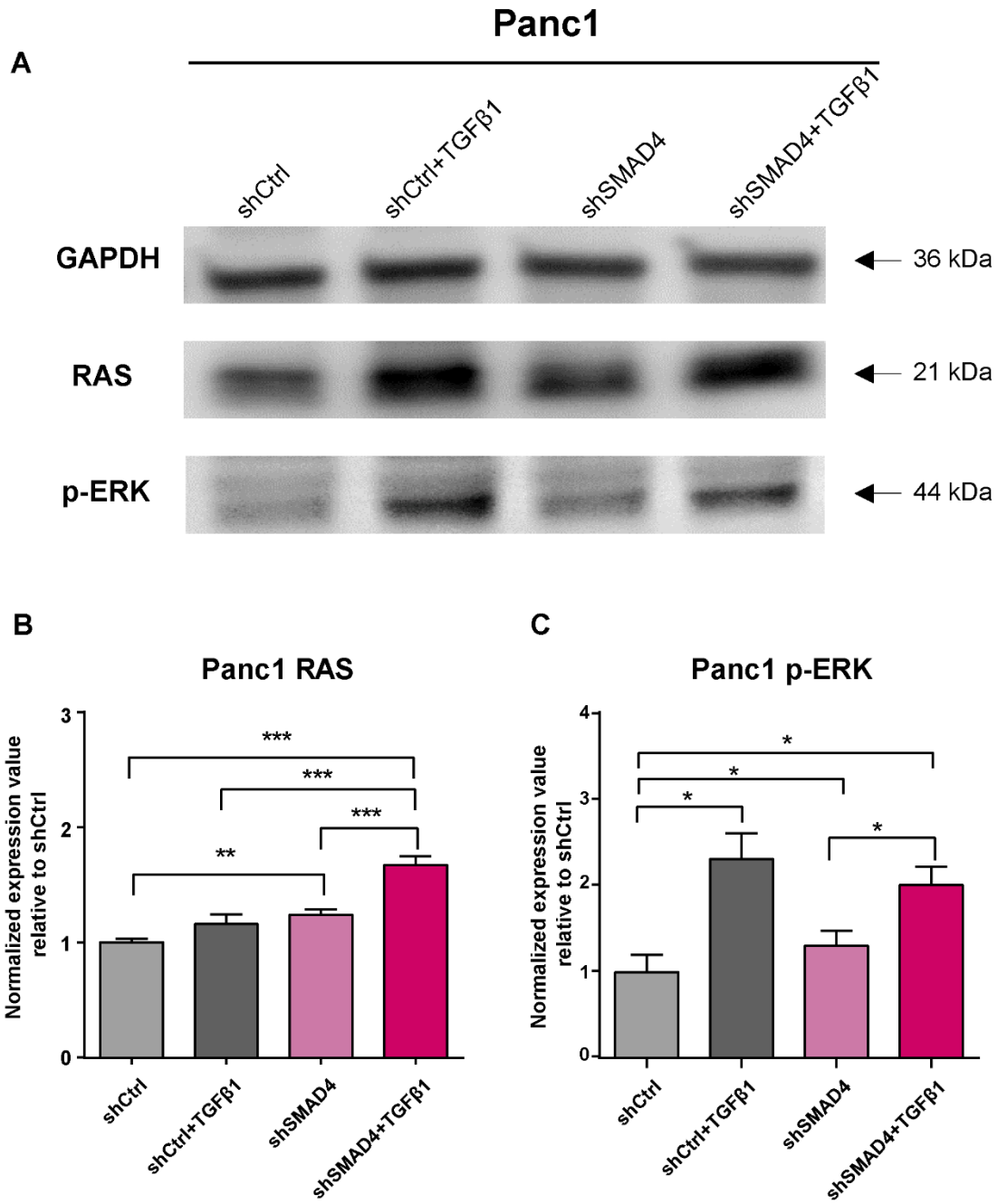

Figure S6. Loss of SMAD4 and TGFβ stimulation promote the overexpression of p-ERK and RAS. Related to Figures 3 and 4. (A) Shown is one representative replicate for the Western blot analysis of RAS (21kDa) and phosphorylated-ERK (p-ERK, 44.42kDa) for Panc1 cells containing *shSMAD4* construct and respective control (*SMAD4*-KD and *shCtrl*) with or without a 24h-TGFβ-stimulation. GAPDH (36kDa) was used as loading control. (B-C) Relative protein expression level of RAS, p-ERK for each condition as compared to *shCtrl* (one-way ANOVA, mean ± SEM, n ≥ 3, **p* < 0.05, ***p* < 0.01, ****p* < 0.001).

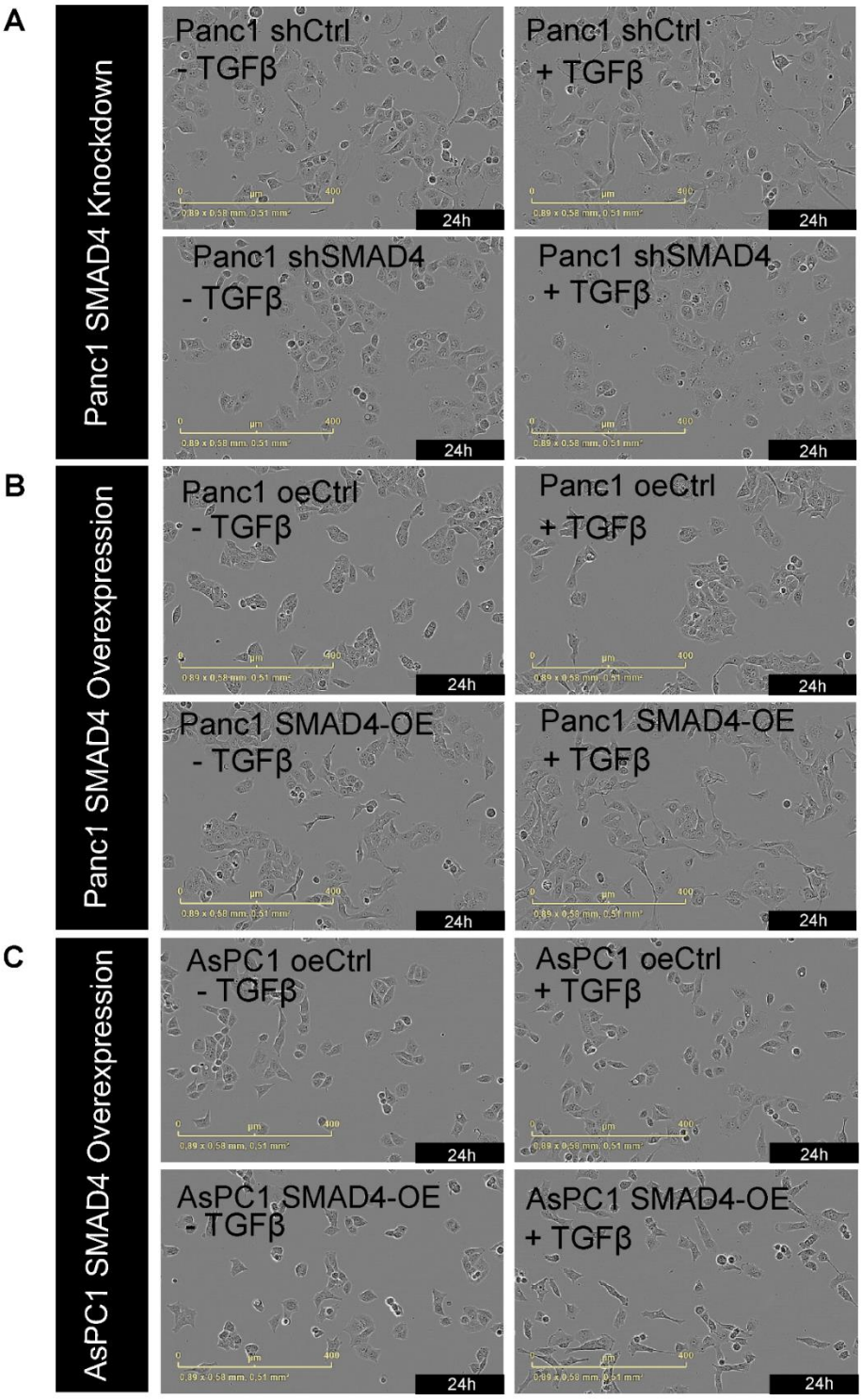

Figure S7. Morphology changes after activation and inactivation of the canonical TGFβ pathway. Related to Figure 5. *SMAD4*-KD and -OE cells were stimulated with 10ng/ml TGFβ1 or the corresponding solvent. Changes in cell morphology were observed 24h after stimulation (20x objective, scale bar = 400 µm).

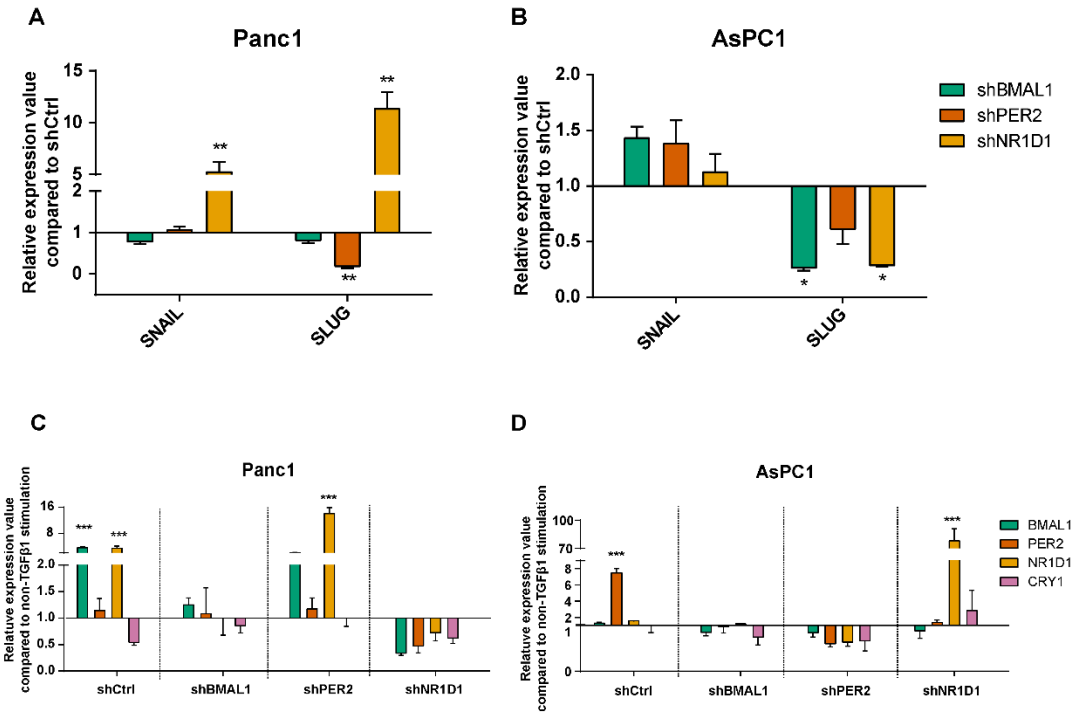

Figure S8. TGFβ stimulation impacts the clock gene expression differentially in SMAD4-proficient and -deficient PDA cells. Related to Figure 6. (A - B) mRNA levels of EMT markers (*SNAIL and SLUG*) in PDA cells were altered after knockdown of core-clock genes (*shBMAL1*, *shPER2* and *shNR1D1*). Depicted are RT-qPCR results for KD PDA cells compared to the *shCtrl* (mean ± SEM, n = 3, t-test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001). (C - D) The comparison of clock genes (*BMAL1*, *PER2*, *NR1D1* and *CRY1*) expression with stimulation of TGFβ1 (10ng/ml) or its solvent. Data shown as comparison to the non-TGFβ1 stimulation (mean ± SEM, n = 3, t-test **p* < 0.05, ***p* < 0.01, ****p* < 0.001).

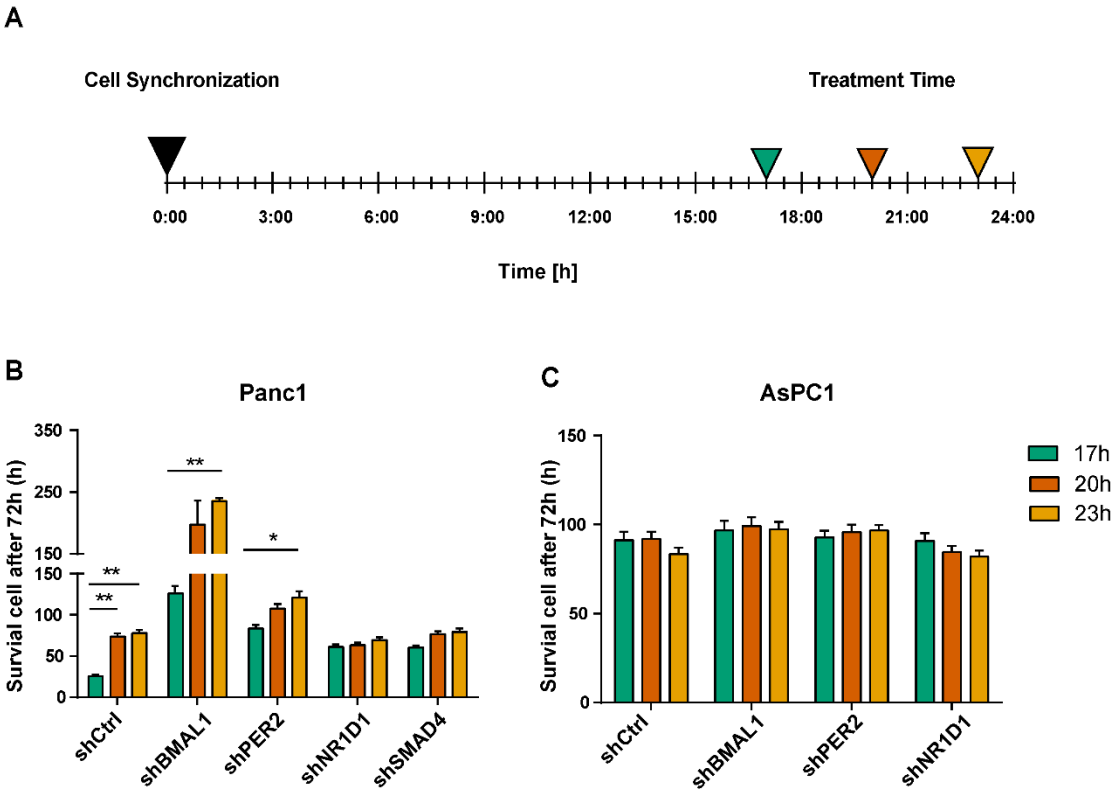

Figure S9. Cytotoxicity assays were performed using NucLight Rapid Red Reagent for the IncuCyte S3 Live Cell System Analysis. Related to Figure 7. (A) At 17h, 20h, 23h after cell synchronization respectively, gemcitabine was added to the cell culture medium. (B - C) 72h after treatment, the number of living cells per well was quantified using the IncuCyte S3 Live-Cell Analysis System. Depicted are the comparisons to the 17h time point. (means ± SEM, n = 6, two-way ANOVA, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Table S1. Primer sequences.

	Primer sequence	
Gene	**Forward**	**Reverse**
SMAD3	F:5'-CCCCAGAGCAATATTCCAGA-3'	R:5'-GGCTCGCAGTAGGTAACTGG-3'
SMAD4	F:5'-TGTGCCTGGTTTGATGGTAA-3'	R:5'-GCCATTTTCCCAATCTGCTA-3'
SMAD7	F:5'-TACCGTGCAGATCAGCTTTG-3'	R:5'-AGTTTGAAGTGTGGCCTGCT-3'
DEC1	F:5′-GTCTGTGAGTCACTCTTCAG-3	R:5′-GAGTCTAGTTCTGTTTGAAGG-3′
DEC2	F:5′-CACCTTTGACGTCTTTGGAG-3′	R:5′-GAGAGTGGGAATAGATGCAC-3′
E-cadherin	F:5'-ATTGCAAATTCCTGCCATTC-3'	R:5'-CTCTTCTCCGCCTCCTTCTT-3'
N-cadherin	F:5'-CTCCTATGAGTGGAACAGGAACG-3′	R:5'-TTGGATCAATGTCATAATCAAGTGCTGTA-3'
Vimentin	F:5'-GGGAGAAATTGCAGGAGGAG-3'	R:5'-ATTCCACTTTGCGTTCAAGG-3'
CD133	F:5'-CCCCAGGAAATTTGAGGAAC-3'	R:5'-TCCAACAATCCATTCCCTGT-3'
SNAIL	F: 5′-GAGGCGGTGGCAGACTAG-3′	R: 5′-GACACATCGGTCAGACCAG-3′
SLUG	F: 5′-CATGCCTGTCATACCACAAC-3′	R: 5′-GGTGTCAGATGGAGGAGGG-3′

Transparent Methods

Cell culture

The PDA cell lines Panc1 and AsPC1 were used as an *in vitro* model system for our study. Panc1 (ATCC: CRL-1469) was derived from the primary tumor of a male patient with a doubling time of 52h (Lieber et al., 1975). AsPC1 (ATCC: CRL-1682) was established from ascites of a female patient with similar doubling time as Panc1 (Watanabe et al., 2012). Both cell lines were maintained in RPMI 1640 (Gibco, CA, USA) supplemented with 1% penicillin-streptomycin (Gibco) and 10% fetal bovine serum (Gibco). For lumicycle measurements and IncuCyte S3 analysis, RPMI 1640 (Gibco) was supplemented with 10µM HEPEs (Gibco) to avoid pH variation. All cells were incubated at 37°C in a humidified atmosphere with 5% CO2.

Lentivirus production of *Bmal1*: Luc, *Per2*: Luc reporters and shRNA-mediated knockdown

Lentiviral elements containing a *BMAL1*-promoter-driven luciferase (BLH) or a *PER2*-promoter-driven-Luciferase (PLB) were generated as previously described (Brown et al., 2005). For the knockdown of *BMAL1*, *PER2*, *NR1D1* and *SMAD4*, a TRC lentiviral shRNA glycerol set (Dharmacon Inc., Lafayette, CO, USA) specific for each gene was used consisting of 5 - 6 individual shRNAs. The construct with best knockdown efficiency was determined by RT-qPCR or Western-blot analysis and used for further experiments.

For lentiviral production, HEK293T (ATCC: CRL-11268) cells were seeded in a 75 cm^2 cell culture flask and co-transfected with 4.2 µg packaging plasmid psPAX, 2.5 µg envelope plasmid pMD2G and 5.8 µg expression plasmid (Bmal1:luc-hygromycin, PER2:luc-Blasticidin, pLKO.1 empty vector or specific knockdown plasmids) using the CalPhos mammalian transfection kit (Clontech Fremont, CA, USA) according to the manufacturer's instruction. Virus particles were harvested and centrifuged at 4000xg for 15 min to remove cell debris. The supernatant was filtered (0.45 µm filter, Sarstedt, Nümbrecht, DE) and used for lentiviral transduction.

Retrovirus production for *SMAD4* overexpression

pBabe-puro-Smad4-Flag was a gift from Sam Thiagalingam (Addgene plasmid # 37041; http://n2t.net/addgene:37041; RRID: Addgene_37041). To generate retrovirus, HEK293T (human, kidney, ATCC: CRL-11268) cells were seeded in 75 cm^2 culture flasks and co-transfected by 6µg pBabe-puro empty vector or pBabe-Smad4-Flag along with 0.3 µg PMD2.G envelope and 2.7µg pUMVC packaging plasmids using FuGENE HD Transfection Reagent (Promega, WI, USA) according to manufacturer's introduction. The supernatant was replaced after 12 h, subsequently, retroviral particles were collected at 24h and 36h after incubation. The retroviral particles were centrifuged at 4100xg for 15 min to remove cell debris filtered (45 µm filter, Sarstedt, Nümbrecht, DE), and stored at -80°C for further usage.

Lentivirus and retrovirus transduction

Cells were transduced with 1.5 ml virus filtrate including 8 µg/µl protamine sulphate (Sigma, MO, USA) and 4 µg/µl polybrene (Sigma) in 6-well plates. To enhance transduction efficiency, plates were centrifuged at 800xg for 90 minutes at 35°C. Subsequently, the supernatant was replaced after 6 - 8 hours. Stably-transduced cells were selected using the corresponding antibiotics (BMAL1: Luc hygromycin, PER2: Luc blasticidin; *shBMAL1*, *shPER2*, *shNR1D1*, *shSMAD4* and pLKO.1 empty vector puromycin; pBabe-puro empty vector and pBabe-Smad4-Flag puromycin). Untraduced cells were used as control. pLKO.1 empty vector and pBabe-puro empty vector are referred as *shCtrl* and oeCtrl respectively.

Cell synchronisation

For all experiments, cells were synchronized by medium change. For the cytotoxicity analysis, time point 0 h is defined as the time point of medium change. Untreated control cells were prepared in the same way, but treated with the corresponding vehicle control (H2O for gemcitabine).

Bioluminescence measurement

For live-cell bioluminescence measurements, cells were seeded onto 35mm dishes (Thermo scientific MA, USA) and maintained in phenol red-free RPMI1640 (Gibco) containing 10% FBS, 1% penicillin-streptomycin, 10µM HEPES supplemented with 250 µM D-Luciferin (PJK, Kleinblittersdorf, DE). Prior to the measurement, cells were synchronized by medium change and washed with 1xPBS twice to avoid the influence of phenol red. Subsequently, the live-cell bioluminescence was recorded by a LumiCycle instrument (Actimetrics, Wilmette, IL, USA) for five consecutive days. For live-cell bioluminescence measurements of *SMAD4* knockdown or overexpression with TGFβ1 stimulation, cells were treated and maintained with 10ng/ml TGFβ1 (Stem Cell Technologies, Vancouver, CA) for five consecutive days. The concentration of TGFβ1 was retrieved from a previous publication (Ellenrieder et al., 2001). Chronostar software was used for data analysis (Sporl et al., 2011). The data of the first 12 hours was automatically excluded to avoid the influence of intrinsic noise of the device. Bioluminescence measurements were performed at least three times, as indicated.

RNA extraction and gene expression analysis by RT-qPCR

Total RNA was isolated with the plus RNeasy Mini kit (Qiagen, Venlo, NL) following the manufacturer's instructions. For a single time-point RT-qPCR, cell medium was replaced by fresh medium 3 hours before RNA extraction. Subsequently, the medium was discarded and cell pellets were washed twice with 1xPBS. To digest genomic DNA, homogenized cell lysates were passed through gDNA eliminator spin column with a 30s centrifugation at 17000xg. RNA was eluted in 20-30 µl Rnase-free water. The final RNA concentration was measured using a Nanodrop 1000 (Thermo Fisher Scientific) and stored at -80 °C for further usage. For RT-qPCR analysis, the extracted RNA was reverse transcribed into cDNA by using random hexamers

(Eurofins MWG Operon) and Reverse Transcriptase (Life technologies). RT-qPCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) in 96-well plates. For the detection of human gene expression, primers were either custom designed (**Table. S1**) or a human Quantitect Primer assay (Qiagen) was used. Gene expression levels were normalised by Gapdh mRNA. The qPCR was performed in a CFX Connect Real-Time PCR Detection System (Biorad). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Mean and SEM were calculated including biological and technical replicates.

Proliferation assays

For the proliferation assay, 5000 cells/ well of each condition were seeded in a 96-well plate (Sarstedt). Cells were allowed to adhere. Subsequently, the medium was replaced with fresh medium containing TGFβ1 or its solvent. Plates were measured in the IncuCyte® S3 Live Cell System Analysis (Sartorius, Göttingen, Germany). Four images per well were recorded every 2 hours. The analysis was performed using the IncuCyte S3 Software (Sartorius).

For TGFβ1 stimulation, stimulated and control cells were treated with and maintained in 10ng/ml TGFβ1 (Stem Cell Technologies, Vancouver, CA) or its solvent (0.1% BSA, Sigma) for five consecutive days.

Apoptosis assays

Cells were seeded in a 96-well plate (Sarstedt) at a concentration of 5000 cells/ 100 µL RPMI medium and incubated overnight at 37 °C with 5% CO2. After incubation, cell media were replaced with fresh medium containing caspase 3/7 reagent (Sartorius, 1:2000) and TGFβ1 or its solvent. Cell apoptosis was measured using the IncuCyte® S3 Live Cell System Analysis (Sartorius). Cells were scanned every 2 hours with a 20x objective and by using the phase and green image channels.

Migration assays

60000 cells/ well of each condition were seeded in a 96-well Essen Image Lock TM microplate (Essen BioScience, Michigan, USA) and incubated overnight at 37 °C, 5% CO2. In the next day, the WoundMakerTM (Essen BioScience) was used to create precise and reproducible wounds. Subsequently, the medium was replaced with a fresh medium containing TGFβ1 (10 ng/mL) or its solvent and the plate was placed in the IncuCyte® S3 Live Cell System Analysis (Essen BioScience). Image acquisition was performed by setting the “scan type” to Scratch Wound and Wide Mode, using the 10x objective. The plate was scanned every 2 hours. The analysis was performed in the IncuCyte S3 Software (Sartorius), the wound width for each well were exported and the migration speed were calculated using either the time of wound closed or the cut-off of 48 hours, which is shorter than the doubling-times of Panc1 and AsPC1 (48 - 52h). Biological triplicates and 8 technical replicates were carried out for each experiment.

Invasion assays

5000 cells/ well of each condition were mixed with 20µl 5mg/ml Basement Membrane Matrix (Trevigen Gaithersburg, MD, USA) seeded in the inner chamber of a 96-well Incucyte Chemotaxis cell invasion clear view plates (Sartorius). Subsequently, the plate was centrifuged at 50x G to avoid the formation of bubbles. Basement membrane matrix was allowed to polymerize at 37°C for 45 minutes to form a reconstituted basement membrane. Cell invasion was measured using the IncuCyte® S3 Live Cell System Analysis. Cells were scanned every 2 h with a 20x objective and phase image channels. Biological triplicates and 8 technical replicates were carried out for each experiment.

Cytotoxicity assays

5000 cells/well were seeded in a 96-well plate (Sarstedt) containing 100 µL RPMI medium and incubated overnight at 37 °C with 5% CO2. After incubation, the supernatant was replaced with fresh medium containing IncuCyte® Cytotox Reagents (Sartorius, (Red)) and appropriate concentrations of gemcitabine (Panc1, 9.5µM; AsPC1, 23.9µM (Awasthi et al., 2013)). For the time-dependent treatment assay, cells were synchronized by a medium change. At specific time points after synchronization (17h, 20h and 23h). Gemcitabine in a solution containing IncuCyte® Cytotox Reagents (Red) was added into each well with a micro-pipette. Cell cytotoxicity was measured using the IncuCyte® S3 Live Cell System Analysis (Sartorius). Cells were scanned every 2 hours with a 20x objective and using the phase and red image channels. Biological triplicates and 8 technical replicates were carried out for each experiment.

Cytotoxicity assays with live-cell nuclear labelling

Cells were labelled with IncuCyte® NucLight Rapid Red Reagent (Sartorius) 30 min before measurements. Cell survival after treatment was quantified using the IncuCyte® S3 Live Cell System Analysis (Sartorius). The plates were scanned every 2 hours with a 20x objective and using the phase and red image channels.

Treatment with Gemcitabine

Treatment concentrations for each PDA cell line were determined based on the experimentally determined IC50 value in a previous study (Awasthi et al., 2013).

Western blot

Cells were synchronized by medium change, gently detached from the dish at 3h after synchronization, sedimented by low-speed centrifugation and resuspended in lysis buffer. Aliquots containing 20 mg of proteins from each cell lysate were subjected to SDS polyacrylamide gel electrophoresis and transferred to a Nitrocellulose Membranes (GE Healthcare Amersham™) using Trans-Blot Turbo Transfer System. Membranes were probed with the following primary antibodies: SMAD4 (1:5000; ab40759, Abcam, Cambridge, UK); GAPDH (1:20000; ab9485, Abcam); RAS (1:5000; ab52939, Abcam); p-ERK (1:2000, Cell

Signaling Technology, Frankfurt am Main, Germany). After incubation with corresponding secondary antibody (1:2000; ab205718, Abcam), signals were detected using the Amersham ECL Select Western Blotting Detection Reagent (GE Health care, Chicago, US), acquired by Image Quant LAS 4000 series (GE Health care). Data was analysed by image J v1.8 (developed by National Institutes of Health).

Cell cycle analysis

$1x10^6$ cells under the logarithmic growth phase were collected, washed with 1xPBS (Gibco) and fixed with ice cold 80% ethanol. Cells were maintained in medium containing 10ng/ml TGFβ1 stimulation (or the solvent) for 24 hours before the measurement. Subsequently, samples were washed with PBS and incubated in a 200ul of 1xPBS solution containing 0.5% Tween20 (Sigma), 1% BSA (Sigma), 2 N HCl/Triton x-100 (Sigma) and 10 mg/mL of Rnase (AppliChem, Cat. No. A2760) for 30 minutes at room temperature. For PI staining, supernatant was removed, the fixed cell pellets were resuspended and stained in 500 µL of 1xPBS containing 50 µM PI (Sigma) for 30 minutes at 37°C. Subsequently, supernatant containing PI solution was removed and the stained cells were resuspended in cold 500 µL 1xPBS and read in FACS Cabilur (Becton Dickinson, NJ, USA). Cell cycle analysis was conducted by fitting a univariate cell cycle model using the Watson pragmatic algorithm as implemented in FlowJo v10.2 (FlowJo LLC).

Statistical Analysis

Experiments were carried out with at least three biological replicates per condition. Data are provided as mean ± SEM. *p*-value < 0.05 was considered as statistically significant. The significance of differences between groups (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) was analysed by one-way or two-way ANOVA followed by Tukey`s multiple comparisons test and the unpaired two-tailed t-test or multiple t-tests using the Prism software (version 6.0; GraphPad Prism).

Cosinor Analysis

To detect significant ($p < 0.05$) circadian rhythmic expression, the cosinor analysis was performed. The *p*-value was calculated using a QUICK CALCS (GraphPad, https://www.graphpad.com/quickcalcs/pValue1/). The oscillating transcripts were estimated by GrapdPad Prism. 6 software and fitting a non-linear equation as following:

$$Y(t) = B + A \cdot (\cos\frac{2\pi \cdot t}{P} + \varphi) + S \cdot t$$

t = Time, B = baseline, A = amplitude, S = slope, φ = acrophase, P = period.

Analysis of pancreatic adenocarcinoma data from a cohort of patients retrieved from the TCGA data base

Clinical information for pancreatic adenocarcinoma with overall patient survival was retrieved from The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov). Mutational frequencies in the TCGA PDA patient population (184 pancreatic adenocarcinoma samples from PanCancer Atlas) were plotted using cBio Cancer Genomics Portal (http://cbioportal.org) (Cerami et al., 2012; Gao et al., 2013). OncoPrint functionality was used for graphical representation of mutation frequency for 10 candidate genes retrieved from our results. Survival curves were plotted using a Cox model that includes coxph (Surv (times, died) ~ gene + grade1 + grade2 + grade3 + age) via OncoLnc (http://www.oncolnc.org), each tumour grade is represented as a separate term as 1 or 0 (Anaya, 2016). The survival data includes clinical data for patients with complete clinical information needed for the analysis and based on a follow up or days until death greater than zero. Previous studies revealed contribution of additional clinical parameters to the benefit provided by chronotherapy (i.e., age). Therefore, in our cox survival analysis we included patient age as an additional clinical parameter. The PDA cohort was divided into two equal groups (N1=87, N2=87 patients). based on the mean expression value of the candidate gene, the change in gene expression levels were categorized as high or low for equal number of patients in the cohort (N = 87 for high expression group and N = 87 for low expression group).

Supplemental References

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