Protein Kinase D1 mediates anchorage-dependent and independent growth of tumor cells via the zinc-finger transcription factor Snail1

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Running title: Snail1 mediates PKD1-induced growth of cancer cells

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**Background:** The Protein kinase D (PKD) family is involved in the control of cell motility and proliferation.

**Results:** PKD1 controls growth of cancer cells through phosphorylation of Snail1 at S11.

**Conclusion:** Only PKD1, but not PKD2 mediates isoform-specific control of pancreatic cancer cell proliferation through Snail1.

**Significance:** We demonstrate for the first time isoform-specific control of pancreatic cancer growth by a single phosphorylation of a substrate.

**SUMMARY**

We here identify Protein Kinase D1 (PKD1) as a major regulator of anchorage-dependent and independent growth of cancer cells, controlled via the transcription factor Snail1. Using FRET, we demonstrate that PKD1, but not PKD2, efficiently interacts with Snail1 in nuclei. PKD1 phosphorylates Snail1 at Ser11. There was no change in the nucleo-cytoplasmic distribution of Snail1 using wild type Snail1 and Ser11-phospho-site mutants in different tumor cells. Regardless of its phosphorylation status or following co-expression of constitutively active PKD, Snail1 was predominantly localized to cell nuclei. We also identify a novel mechanism of PKD1-mediated regulation of Snail1 transcriptional activity in tumor cells. The interaction of the co-repressors HDAC1/2 as well as lysyl-oxidase-like protein LOXL3 with Snail1 is impaired when Snail1 is not phosphorylated at S11 which leads to reduced Snail1-associated HDAC activity. Additionally, LOXL3 expression is up-regulated by ectopic PKD1 expression, implying a synergistic regulation of Snail1-driven transcription. Ectopic expression of PKD1 also up-regulated proliferation markers such as Cyclin D1 and Ajuba. Accordingly, Snail1 and its phosphorylation at Ser11 were required and sufficient to control PKD1-mediated anchorage-independent growth and anchorage-dependent proliferation of different tumor cells. In conclusion our data show that PKD1 is crucial to support growth of tumor cells via Snail1.

**INTRODUCTION**

The Protein Kinase D (PKD) family of serine/threonine kinases consists of three members PKD1 (PKCµ), PKD2 and PKD3. They share similar structural features and often phosphorylate the same substrates (1-7). The protein kinase D family has been implicated in the regulation of proliferation of different cells including pancreatic cancer cells (6-11). We have previously identified protein kinase D as a major regulator of cancer cell motility and invasion (2-5). However, it is as yet unclear whether these functions are regulated by all PKD isoforms in a similar fashion and via the same PKD targets or substrates. Therefore we investigated how PKD1 and PKD2, two PKD isoforms that mediate vital functions in pancreatic tumor growth and angiogenesis are involved in the regulation of pancreatic cancer cell growth (12-14). We initiated a bioinformatics screening approach using Scan Site (15) to identify putative PKD phosphorylation consensus motifs in potentially relevant PKD substrates and identified in accordance with Du et al. (16) Snail1 as a putative PKD substrate. Snail1 is an important zinc-finger transcription factor controlling epithelial-mesenchymal transition (EMT) and tumor growth (17,18). Snail1 transcriptional activity can be mediated by regulation of protein stability via lysyl-oxidase-like proteins (LOXL) (19,20). LOXL isoforms 2 and 3 interact with Snail to modify critical lysine residues and thereby stabilize the protein (19). Snail1 repressor activity is also modulated by phosphorylation of 6 residues via Glycogen synthase kinase 3 beta (GSK3beta) inducing nuclear export and beta-Trcp controlled Ubiquitin-dependent-degradation (20,21). Snail1 transcriptional repression is mediated by recruitment of a Sin3A/Histone deacetylase 1 and 2 (HDAC1/HDAC2) complex. This interaction is critical for Snail1 repressor function and dependent on the N-terminal SNAG-domain of Snail1 (22), which is adjacent to the PKD phosphorylation consensus in the protein.

Thus, this study aimed at identifying how phosphorylation of Snail1 by PKD regulates Snail1 activity, tumor cell growth and invasive features and to determine whether Snail1 phosphorylation by PKDs is isoform specific.
EXPERIMENTAL PROCEDURES

Cell culture—Panc89 (PDAC), Panc1 (PDAC), HEK293T and HeLa cells were maintained in RPMI media supplemented with 10% FCS and Pen/Strep. Panc1 cells were transfected using Turbofect (Fermentas), siRNAs were transfected using Oligofectamine (Invitrogen). Experiments in HeLa cells were performed using HeLa Monster reagent (Mirus). Panc1, HEK293T and HeLa cells were acquired from ATCC. Stable Panc89 cells used in this study were described previously (4,5). For production of lentiviruses 6Mio HEK293T cells were transfected using Lipofectamine 2000 (Invitrogen). Virus supernatants were harvested after 48h and used for transduction of stable Panc89 cell lines. Cells were subsequently subjected to pyromycin selection to generate semi-stable cell lines used in assays.

Plasmids, antibodies and dye-reagents—GFP-tagged expression constructs for PKD1, PKD1KD (K612W), PKD2-GFP and PKD2KD-GFP have been described previously (5,23). Snail1-FLAG and Snail1-GFP constructs (21) were acquired from Addgene. Snail1-FLAG S11A/S11E and Snail1-GFP S11A/S11E mutants were generated by site-directed mutagenesis and verified by sequencing (Quick Change II Kit, Stratagene). Snail1S11A forward: 5´CTC-GTC-AGG-AAG-CCC-GCC-GAC-CCT-GG-AAG, Snail1S11A reverse: 5´CTT-CCG-ATT-GGG-GTC-GGC-GGG-CTT-CCT-GAC-GAG, Snail1S11E forward: 5´CTC-GTC-AGG-AAG-CCC-GAG-GAC-CCT-GG-AAG, Snail1S11E reverse: 5´CTT-CCG-ATT-GGG-GTC-CTC-GGG-CTT-CCT-GAC-GAG. Short hairpin RNAs against lacz, PKD1 and PKD2 were described previously (4). Anti-E-Cadherin antibody was purchased from BD Bioscience. Ajuba, Snail1 and Cyclin D1 antibodies were acquired from Cell Signaling. Anti-FLAG M2, anti-Actin AC40 and anti-Tubulin were from Sigma Aldrich. LOXL3 (Lysyl-oxidase-like) antibodies were purchased from Abnova and Sigma Aldrich, respectively. Anti-GFP antibody was acquired from Roche. HDAC1 and HDAC2 antibodies were from Abcam. qPCR primers were obtained from Qiagen. PKD1 C20 antibody was acquired from Santa Cruz. PKD2 antibody was obtained from Calbiochem. Non-target shRNA control (scramble, shc002), sh_Snail1 #1 (NM_005985.2-136s1c1) as well as sh_Snail1 #2 (NM_005985.2-504s1c1) were from Sigma-Aldrich. Immunofluorescence secondary antibodies were purchased from Invitrogen. pMotif antibody was a gift of Peter Storz (Mayo Clinic, USA).

Total cell lysates and co-immunoprecipitation—Total cell lysates and co-immunoprecipitations were performed as described previously (3,5,24). In brief, total cell lysates were either prepared by solubilizing cells in RIPA buffer [50mM Tris, pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP40, 0.25% deoxycholinat, 0.1% SDS, plus complete protease and PhosStop inhibitors (Roche)] or 2% SDS lysis buffer [10 mM Hepes, 150mM NaCl, 1mM EDTA; pH 6.8 plus inhibitors]. Lysates were clarified by centrifugation at 13.000g for 10 minutes. For immunoprecipitation equal amounts of proteins were incubated with specific antibodies for 1.5 hours at 4°C. Immune complexes were collected with protein G-Sepharose (GE Healthcare) for 30 min at 4°C and washed three times with lysis buffer [20 mM Tris pH 7.4, 5mg MgCl2, 150mM NaCl, 1% Triton-X100]. Precipitated proteins were released by boiling in sample buffer and subjected to SDS-PAGE. The proteins were blotted onto nitrocellulose membranes (Pall, Germany). After blocking with 2% BSA in TBST blots were probed with specific antibodies. Proteins were visualized by HRP-coupled secondary antibodies using ECL (Thermo Fisher). Quantitative analysis of western blots was done by measuring integrated band density using NIH ImageJ. Values shown represent fold change in respect to control.

Quantitative real-time PCR (qPCR)—Quantitative real-time PCRs were performed in a BioRad iQ5 cycler with SYBR-Green. Total RNA was isolated using a RNeasy Mini Kit (Qiagen). We used 400 ng of total RNA for cDNA synthesis. Quantitative real-time PCR analysis was performed in 3 replica and at least three independent experiments using qPCR primers for GAPDH (control), LOXL1-3 and Cyclin D2 (Qiagen). Results were calculated using the ∆∆Ct method normalized to GAPDH and vector control cells.

3D BME cell culture—3D BME culture was performed by seeding 10.000 cells of stable Panc89 cell lines (4,5) in BME (growth factor
reduced, phenol red-free, Cultrex, R&D, Trevigen). Tumor cell clusters were documented after 16 days at 10x magnification (Fig.7A), or 32 days (Fig.7G) at 8x magnification using a Keyence microscope. Diameters of tumor clusters in images were quantified in perpendicular directions for each cluster using spacial calibration of images (NIH ImageJ). For statistical analysis, conditions were compared using frequency distribution histograms. Statistical significance was calculated using two-tailed unpaired students t-test.

Immunofluorescence confocal microscopy and acceptor photo-bleach FRET-HeLa cells were transfected with HeLa Monster and seeded at a density of 150,000 cells per well on glass cover slips. After adhesion over night cells were fixed with 4% formaldehyde at room temperature (RT) for 20 min, washed, quenched with 0.1M glycerine and then permeabilized with 0.1% Triton-X100. Samples were blocked and stained in PBS supplemented with 5% FCS/0.05% Tween-20. Primary as well as secondary Alexa-dye antibodies (Invitrogen) were incubated for 2h, respectively. Samples were mounted after extensive washing in FluoromountG (Southern Biotechnology) and analyzed by a Confocal Laser Scanning Microscope TCS SP5 (Leica) equipped with respective 63x Plan Apo oil immersion objectives. Images were acquired in sequential scan mode and processing was done using NIH ImageJ. The scale bar represents 10 μm. Acceptor-photobleach FRET experiments were performed in transiently transfected HeLa cells processed as stated previously. FRET measurements were performed by acquiring pre- and post bleach images of donor and acceptor using Leica Acceptor-photobleach FRET macro. Thresholded percent FRET values were depicted using a 7 color-LUT. Quantitative FRET analysis was performed by calculating mean FRET efficiency and SEM for n=18 cells and two independent conditions (PKD1 vs. PKD2). Statistical significance (p<0.0001****) was calculated using two-tailed unpaired students t-test.

Cell proliferation assays-Cell proliferation assays were performed with transiently transfected HeLa cells. After 24h 5000 cells were seeded in 100μl standard growth media in triplicate replica per condition in 96-well culture plates for time points T0, T24, T48. After adhesion over night, T0 cells were fixed and stained with crystal violet (0.5% in H2O/ 20% v/v Methanol) for 20 min at RT. After extensive washing plates were dried and additional plates were processed after 24h and 48h in the same manner. To quantify cell density, crystal violet was dissolved in 100μl Methanol per well and adsorption was measured at 550nm using a Tecan1000 plate reader. Doubling time was calculated using linear regression (Prism Software). Cell densities in graphs are shown for mean OD 550 values in triplicate replica ± SEM.

HDAC activity assays-HDAC activity assays were performed using a fluorometric kit (Cayman Chemical Company). 3 Mio HeLa cells were seeded in 10cm dishes, with two dishes per condition. Cells were lysed after 48h according to the manufacturer’s description. Assays were performed in black 96-well plates in triplicate replicas per condition. In order to measure HDAC activity 10μl of crude nuclear extract were used after normalization of protein content by a BCA kit. Deacetylation of a specific HDAC substrate was measured at 455 nm (excitation 360 nm) using a Tecan M1000 plate reader. Assays were further normalized for GFP transgene expression in crude nuclear extracts (Snail1-GFP and Snail1S11A-GFP) by measuring GFP fluorescence at 535 nm (excitation 475 nm).
Statistical analysis was performed using one-way Anova with Bonferroni multiple comparison post-testing.

Statistical analysis—Statistical analysis was performed using Prism software version 5.00 for Windows, GraphPad Software, San Diego California USA.

RESULTS

Following a bioinformatics screen we identified in accordance with (16) Snail1 as a putative PKD substrate and mapped the respective phosphorylation site to Ser11.

Mapping of PKD1 phosphorylation sites in Snail1—Figure 1A depicts a structural overview of Snail1, with the putative PKD phosphorylation site located at Ser11 directly adjacent to its snag-domain (amino acids 1-9). The potential phosphorylation site LVRKPS* matches the published PKD phosphorylation consensus sequence LXRXXS* and also partially the PKD p-substrate antibody recognition sequence (p-Motif, LXR(Q/K/E/M)(M/L/K/E/Q/A)S*) (25,26). Using anti-p-Motif antibody we investigated Snail1 in vivo phosphorylation by PKD1 (Fig. 1B). Active PKD1 enhanced phosphorylation of Snail1, whereas Snail1 phosphorylation was barely detectable in cells expressing catalytically inactive PKD1KD. In addition, phosphorylation of Snail1 was absent when Ser11 was replaced by Ala (S11A), even in the presence of active PKD1. Thus, in accordance with data published by Du and colleagues (16) Ser11 is a PKD phosphorylation site in vivo and it is the only PKD phosphorylation site in Snail1 (Fig.1B). Next we wanted to assess the upstream regulation of Snail1 by PKD isoforms 1 and 2. To determine whether both isoforms would interact with Snail1 in intact cells we performed co-localization and fluorescence resonance energy transfer (FRET) studies.

Only PKD1 interacts efficiently with Snail1 in the nuclei of HeLa cells—For co-localization and FRET studies we used transiently transfected HeLa cells ectopically expressing Snail1-FLAG together with PKD1-GFP or PKD2-GFP, respectively. Both PKD 1 and -2 were localized to nuclei of HeLa cells and co-localized with Flag-tagged Snail1 (Figs. 2A and B). To further characterize this co-localization and to determine a potential interaction, we performed acceptor photobleach FRET studies. Figure 2A displays a representative FRET experiment for PKD1-GFP and Snail1-FLAG. Images (A') and (B') depict donor pre- and post-bleach states, whereas (D') and (E') show acceptor pre- and post-bleach images, respectively. The relative increase in donor fluorescence intensity is marked by arrow heads in post-bleach images (B'). Percent FRET values indicating interaction of the two proteins are shown in (F) depicted by a seven color LUT (Fig.2A, A’-F’). Similar experiments were performed for PKD2-GFP and Snail1-FLAG (Fig.2B, A’-F’). Active PKD2 is known to phosphorylate nuclear substrates (27). However, interaction of wild type PKD2 and Snail1 was barely detectable. Figure 2C displays the statistical analysis of mean FRET efficiency and SEM for PKD1-GFP (n=18) and for PKD2-GFP (n=17) cells. Mean FRET efficiency dropped markedly by 3.38 fold for PKD2 (3.8 % ± 1.1) as compared to PKD1 (12.9 % ± 1.1) with some cells displaying no interaction at all (Fig.2B). FRET efficiency values for all experiments are shown in supplemental table 2). These data indicate that PKD1 preferably interacts with Snail1 and suggests that interaction between PKDs and Snail1 is isoform specific. We further verified these findings by co-precipitation experiments with endogenous snail1 and PKD1 (Fig.2D).

Du et al. (16) reported that phosphorylation of Snail1 at Ser11 by PKDs regulates its nuclear export by interaction with 14-3-3 sigma proteins in epithelial cell lines including C4-2 tumor cells. Yet, the authors conceded that tumor cells may have different mechanisms for regulating Snail1 transcriptional activity. We investigated how subcellular localization of Snail1 was altered when Ser11 was phosphorylated by PKD1 in two epithelial cancer cell lines, Panc1 human pancreatic cancer cells and. HeLa cervical cancer cells.

Sub-cellular localization of Snail1, Snail1S11A and Snail1S11E is not changed—To first investigate how Snail1 phosphorylation would impact its subcellular localization we performed localization studies with Snail1, the S11A as well as the S11E phospho-site mutants in HeLa and Panc1 cells, respectively. There was no detectable change in subcellular localization using the phospho-site mutants compared to Snail1 wild type (wt) in HeLa.
cells (Fig.3A) and Panc1 cells (data not shown). We quantified subcellular distribution in 3 experiments using HeLa cells and found Snail1 predominantly localized in nuclei independent of its phosphorylation status in more than 99% of at least 1000 cells quantified per condition Fig.3A (A’-O’). We also did not observe any change in the subcellular localization of wild type Snail1 upon co-expression with constitutively active PKD1 in both HeLa (Fig.3B) and Panc1 cells (data not shown). Similar data were obtained with endogenous Snail1 in both cell lines expressing active PKD1 (supplemental Fig.1A). In addition, there was no change in the subcellular localization of predominantly cytoplasmatic endogenous Snail1 in non-transformed, immortalized HEK293T cells upon expression of active PKD1 or kinase-inactive PKD1KD (supplemental Fig.1B).

According to Du et al. (16) Snail1 should have exhibited nuclear localization in cells expressing PKD1KD in this setting. Thus, in the cell lines examined in this paper 14-3-3 binding to a consensus surrounding Ser11 does not seem to be the relevant mechanism for Snail1 subcellular localization. This prompted us to investigate further molecular mechanisms to explain the function of Snail1 phosphorylation by PKD1.

Regulation of Snail1-mediated transcriptional activity by lysyl-oxidase-like family members 2 and 3-In addition to HDACs 1 and -2 (22), lysyl-oxidase-like family members LOXL2 and -3 are known Snail1 interaction partners, enhancing Snail1 protein stability and Snail1-dependent regulation of marker genes (19). To investigate their role in the regulation of Snail1 transcriptional activity downstream of PKD1, we screened initially a panel of pancreatic cancer cell lines including Panc89 cells stably expressing GFP-vector or PKD1-GFP (4,5) as well as HeLa cells for the presence of Snail1, PKD1 and the LOXL3 isoform (Fig.4A). Snail1 and LOXL3 proteins were present in HeLa, Panc1, MiaPaca, Panc89 and the stable Panc89 cell lines. PKD1 was expressed at different levels in all cell lines. Snail1 was strongly expressed in HeLa, Panc1 and both stable Panc89 cell lines prompting us to use these cells for further analyses. Using quantitative real-time PCR (qPCR) we tested additionally which LOXL isoforms were present in these stable Panc89 GFP-vector or PKD1-GFP cells and whether a further upstream regulation by PKD1 may be involved. Both, LOXL2 and -3 isoforms were expressed in Panc89 cells. To our surprise, the expression of LOXL3, but not of LOXL2, was significantly up-regulated by 5.5 fold ± 0.36 in cells expressing PKD1 (Fig. 4B, C) suggesting a PKD1-dependent synergistic regulation of Snail1 activity via LOXL3. We investigated next how phosphorylation at S11 would impact co-regulation by HDACs. Thus we initially performed co-localization studies of (wt) Snail1 and Snail1-S11A with the published co-repressor HDAC1 (22) in HeLa cells. Both Snail1-FLAG and Snail1S11A-FLAG co-localized with the endogenous co-repressor HDAC1 in the nuclei (Fig.4D). We were further able to demonstrate interaction of Snail1-FLAG with HDAC1 in the nuclei by acceptor photo-bleach FRET (data not shown).

Binding of HDAC1, HDAC2 and LOXL3 is impaired by Snail1S11A reducing HDAC activity–In order to study the molecular impact on HDAC binding following phosphorylation of S11 we performed co-immunoprecipitation studies with phospho-site mutants. The snail co-repressors HDAC1 and -2 interact with Snail1 in transcriptional complexes to regulate the expression of target genes (22). LOXL2 and 3 isoforms are also acting as co-regulators, modifying Snail1-mediated transcriptional regulation by enhancing its stability. The interaction of LOXL2 with Snail1 has been shown to be dependent on the N-terminal part of Snail1, which contains the snag-domain (amino acids 1-9) adjacent to the S11 phosphorylation site and this part is also essential for interaction with HDAC transcriptional co-repressors (19,28). Thus we performed co-immunoprecipitation experiments in HeLa cells following co-expression of FLAG-tagged HDACs with Snail1 phospho-site mutants as well as with endogenous HDACs 1 and -2 (22). Strikingly, the interaction of both HDAC1 (data not shown) and -2 with Snail1 was decreased upon expression of the Snail1S11A mutant but not upon expression of Snail1S11E (Fig.4E). Fig. 4F depicts the result of 3 independent co-immunoprecipitation experiments for co-expressed HDAC2. HDAC2 binding was significantly reduced with the S11A-mutant and almost returned to wild type level with S11E. HDAC1 demonstrated the same overall pattern of regulation (data not shown). We also investigated the interaction of endogenous
HDACs with wild type Snail1 as well as its 11A- and 11E-mutant proteins. **Figures 4G** and H show that binding of endogenous HDAC1 and HDAC2 to Snail1S11A was reduced as compared to wild type Snail1 from 1 to 0.53 times integrated band density for HDAC1 and to 0.58 times for HDAC2, whereas it was increased for the S11E-mutant to 1.29 for HDAC1 and 1.24 times for HDAC2. Additional co-immunoprecipitation experiments with endogenous HDACs 1 and 2 also using other tags may be found in supplemental Fig.2A-D. Thus, phosphorylation of Snail1-S11 by PKD1 is likely to be required for the stable interaction with its co-repressors (Fig.4E-H). In accordance with these data, LOXL3 interaction with Snail1S11A was also decreased, as observed in co-immunoprecipitation experiments. Integrated band densities were reduced from 1 for wt to 0.3 times for the Snail1S11A mutant (supplemental Fig.2E). We next assessed how the phosphorylation-dependent interaction of Snail1 with its co-regulators modulates HDAC transcriptional regulatory activity.

**Snail1-dependent HDAC activity and regulation of proliferation markers**—We performed HDAC activity assays measuring (wt) Snail1 as well as Snail1S11A-associated histone deacetylation to verify results of co-immunoprecipitation experiments. GFP-vector, Snail1-GFP or Snail1S11A-GFP constructs were ectopically expressed in HeLa cells for 48h and crude nuclear extracts were prepared using a HDAC activity assay kit (Cayman Chemical Company). Equal amounts of extract were used in assays and results were further normalized to GFP-Snail1 transgene expression present in nuclear lysates. In line with interaction studies, statistical analysis of three independent HDAC assays demonstrated reduced activity in cells expressing the Snail1S11A mutant by 21.37% compared to wt Snail1 (Fig.5A). Expression of Snail1 transgene controls in crude nuclear lysates is shown in supplemental Fig. 3A. Since Snail1-associated HDAC activity contributes only partially to the total HDAC activity, as demonstrated by inhibition with the HDAC inhibitor trichostatin (Fig. 5A), the extent of activity reduction by the 11A mutant is remarkable and also matches the markedly reduced HDAC1/-2 as well as LOXL3 binding (Fig.4, E-H, supplemental 2E). Since we were interested in the role of Snail1 in the control of pancreatic cancer growth we investigated next, whether HDAC activity also translated into expression of marker proteins known to be involved in proliferation (Fig.5B, C). Thus we investigated proliferation markers regulated downstream of Snail1 and PKD1 in Panc1 and the stable Panc89 cell lines. Panc1 cells were transiently transfected with GFP-vector, Snail1-GFP or Snail1S11A-GFP and changes in Cyclin D1 expression levels were observed. (29-31). Cyclin D1 was markedly up-regulated by Snail1-GFP, whereas expression of Snail1S11A reduced Cyclin D1 expression (vector: 1, Snail1-GFP: 2.7, Snail1S11A-GFP: 1.48, fold integrated band density, Fig. 4B). We further tested the effects of the phospho-mimetic Snail1S11E mutant on Cyclin D1 expression. Indeed, Cyclin D1 was markedly up-regulated by Snail1S11E (supplemental Fig.3B). In order to substantiate our findings we additionally investigated a second proliferation marker, Ajuba. Interestingly, we find Ajuba to be a downstream target of Snail1. Ajuba is known to regulate cell cycle progression and G2/M transition by enhancing Aurora A kinase activity through direct interaction (32-34). Thus Ajuba is involved in mitotic checkpoint control (34). Additionally, Aurora A and -B kinases are over-expressed in cancer tissues and potentially tumorigenic (32). Ajuba protein levels were up-regulated by (wt) Snail1 whereas its expression was reduced by Snail1-S11A (vector: 1, Snail1-GFP: 2.22, Snail1S11A-GFP: 1.78, fold integrated band density, Fig.5B). To further validate our results, we assessed the regulation of the same markers in Panc89 cells stably expressing either vector, PKD1-GFP or kinase-inactive PKD1KD-GFP (Fig.5C). In line with Fig.5B Cyclin D1 was up-regulated 3.9 fold by PKD1-GFP, while its expression dropped to 2.74 fold upon expression of PKD1KD-GFP. The expression of Ajuba was up-regulated by PKD1-GFP to 2.9 fold compared to only 1.6-fold by PKD1KD-GFP. Thus, PKD1-mediated regulation of proliferation markers is similar to that of Snail1 and its phospho-site mutants. However, PKD1KD-GFP was not capable to act fully as a dominant negative construct in these experiments. This may be explained in line with literature (23) by a prominent localization of PKD1KD-GFP at the Trans-Golgi network (TGN) as evidenced by strong
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co-localization with TGN marker TGN46 (supplemental Fig. 3C).

In order to further assess whether the regulation of downstream targets by PKDs was indeed isoform specific we examined the regulation of another Snail target, Cyclin D2 (18) by qPCR in Panc89 cells expressing PKD1- or PKD2-GFP, respectively. In line with our previous findings, Cyclin D2 expression was up-regulated 1329-fold by wild type PKD1, whereas its expression was only up-regulated 90.8-fold by wild type PKD2, only 7% of the effect of PKD1. These data confirm the selective regulation of Cyclin D2 by PKD1, as compared to PKD2 (Fig.5D).

We next investigated whether these biochemical data would translate into biological readouts. At first, soft agar assays were performed to identify changes in anchorage-independent growth, mediated by PKD1 and -2 isoforms or the respective kinase-inactive proteins.

**PKD1, but not PKD2, enhances anchorage-independent growth in Panc89 cells**—Figure 6A shows the statistical analysis of 5 independent soft agar experiments performed in Panc89 cells stably expressing GFP-vector, PKD1-GFP, kinase-inactive PKD1 (PKD1KD-GFP), PKD2-GFP or kinase-inactive PKD2 (PKD2KD-GFP), respectively (4,5). In line with our FRET studies and the biochemical data shown above, only wild type PKD1 significantly increased the average number of colonies per visual field by 31.4 times as compared to GFP-vector cells. PKD1KD reduced the number of colonies as compared to PKD1-GFP by 3.6 times, but still had a minor effect on anchorage independent growth of Panc89 cells which correlates well with the data on proliferation marker expression (Fig. 5B). In contrast, PKD2 had no effect on anchorage-independent growth (Fig.6 A, B, D', E'). Fig. 6B, A'-E' shows representative colonies documented for quantification. In addition to the significant increase in colony number, colony size was also markedly increased upon expression of PKD1 (B'). Thus, our results again indicate a PKD1-isoform specific regulation of anchorage-independent proliferation in pancreatic cancer cells. We additionally performed soft agar experiments in transiently transfected Panc1 cells expressing GFP-vector, (wt) Snail1-GFP and the S11A mutant construct (Fig.6C). In line with previous experiments performed with PKD1 anchorage-independent growth in Panc1 cells was significantly enhanced by (wt) Snail1 (3.65 times) as compared to vector control (p<0.0001****) and reduced by 44% upon expression of the S11A mutant compared to (wt) Snail1 (p<0.0001****) (Fig.6C). Colonies documented for the respective conditions are depicted in supplemental Fig. 4A, (A'-C'), transgene expression of total cell lysates is shown in supplemental Fig.4B.

Snail1 is required to mediate PKD1-regulated effects on anchorage-independent growth—In order to demonstrate that PKD1-mediated Snail1 phosphorylation is required for PKD1-induced anchorage-independent growth of pancreatic cancer cells, we performed soft agar assays in Panc89 cells stably expressing vector or PKD1-GFP, with two different shRNAs against Snail1, as well as a non-targeting scrambled control. Fig.7A displays the summarized statistical analysis of three soft agar assays in Panc89 cells. PKD1 expression increased anchorage-independent growth as compared to vector cells by 84.6%. In vector cells, knockdown of Snail1 reduced the average number of colonies per visual field by 60.4% for sh Snail1 #1 and 53.6% for sh Snail1 #2. For PKD1 expressing cells, Snail1 knockdown reduced the number of colonies by 68.5% for sh Snail1 #1 and 82.8% for sh Snail1 #2, (Table 1). We also quantified colony size. PKD1 expression enhanced colony size and this was reduced by knockdown of Snail1 (data not shown). Examples of images used for quantification of colony numbers at 4x magnification are shown in Fig.7B for all conditions (A'-F'). The respective knockdown controls for Snail1 in the stable cell lines are shown in Fig. 7C. In conclusion, these data indicate that Snail1 as a downstream target of PKD1 is required to regulate anchorage-independent growth of pancreatic cancer cells by Ser11 phosphorylation.

**PKD1 enhances, whereas PKD1KD inhibits Panc89 tumor cluster growth in 3D BME culture**—To investigate a PKD1-dependent regulation of anchorage-dependent tumor cluster growth and proliferation we performed 3D basement membrane extract (BME) culture using stable Panc89 cells expressing PKD1- and PKD1KD-GFP. Vector, PKD1-GFP and PKD1KD-GFP cells were seeded in BME
extract and documented after 16 days of growth. Fig.8A displays representative examples of tumor cell clusters used for the assessment of 3D growth (diameter). In line with the soft agar assays, the average size of tumor cell clusters with stable ectopic expression of PKD1-GFP was significantly increased by 10.1% as compared to GFP-vector expressing cells (p<0.0005***) (Fig. 8A, B). PKD1KD-GFP significantly reduced the average cluster diameters by 10.3% when compared to vector controls (p<0.0001****) (Fig.8B), indicating that PKD1 is also involved in the regulation of anchorage-dependent growth of pancreatic tumor cell clusters. Fig.8C and D depict the respective frequency distribution histograms of tumor cluster diameters for PKD1-GFP and PKD1KD compared to GFP control cells. These data demonstrate that PKD1 expression resulted in a higher percentage of larger clusters, whereas PKD1KD expressing cells formed smaller colonies. Corroborating these data, we performed proliferation assays in HeLa cells to investigate a general regulation of anchorage-dependent proliferation by Snail1-S11 phosphorylation. GFP-vector, (wt) Snail1 and the Snail1S11A mutants were transiently expressed in HeLa cells and proliferation was quantified by measuring OD 550 values of crystal violet stained cells at time points T0, T24 and T48h. 48h after transfection (wt) Snail1 markedly decreased doubling times from 56.25 to 35.65 h (vector versus Snail1-GFP) enhancing proliferation, whereas expression of Snail1S11A had virtually no effect on the doubling time (52.17 h) (Fig.8E) Transgene expression is shown in supplemental Fig.5. Thus, PKD1-dependent phosphorylation of Snail1 at S11 is involved in controlling anchorage-dependent and -independent growth and proliferation in 2D and 3D environments. In order to further validate our data on the regulation of proliferation by PKD1, we performed lentiviral-mediated knockdown experiments in GFP-vector cells followed by 3D BME culture (Fig.8 F-H). Clusters were documented after 32 days (Fig.8G). In line with all previous data, knockdown of PKD1 resulted in drastically reduced cluster sizes (diameters) of 38.3% in PKD1 knockdown cells (Fig.8H). Specific knockdown of PKD1 and not PKD2 was verified by isoform-specific antibodies (Fig.8F). Frequency distribution histograms show shift to smaller cluster diameters following knockdown of PKD1 (Fig.8I). Taken together, these findings indicate that PKD1 enhances proliferation and anchorage-dependent growth of tumor cell clusters in 3D culture. By contrast, PKD1KD-GFP or knockdown of PKD1 significantly inhibits proliferation and this is mediated by phosphorylation of Snail1 at S11.

**DISCUSSION**

PKDs have been involved in the regulation of important cellular features such as proliferation (10,11,13,35-37) motility and invasiveness (2-5) of different tumor types. However, specific and detailed functions for distinct PKD isoforms have not been addressed so far. Previous work by Ochi et al. (38) has proposed a function for PKD1 in the regulation of anchorage dependent growth. However, the properties of distinct PKD isoforms were not directly compared or addressed by inhibitors that are not isoform specific. Thus it is as yet unclear whether PKD isoforms act in a redundant, or specific fashion in tumors.

Our findings indicate that PKD1, as opposed to PKD2, regulates the expression of marker proteins involved in a hyper-proliferative phenotype such as Cyclin D1 and -D2 (29,31) as well as Ajuba (33,34) via phosphorylation of Snail1 at serine 11 in pancreatic cancer cells. Our data also suggest, that phosphorylation at this site is necessary for efficient binding of vital co-repressors to Snail1, such as HDAC2, modulating Snail1-dependent HDAC activity. In contrast to Du and colleagues (16) Snail1 phosphorylation at S11 did not affect nucleo-cytoplasmic shuttling of the protein. This may be explained by 14-3-3σ down-regulation in many tumor cells by different mechanisms (39), including promotor methylation or inhibition downstream of p53 mutations, thereby facilitating cancer formation by many routes (40). Indeed, PKD1KD was not able to induce nuclear localization of primarily cytoplasmatic Snail1 in non-transformed, immortalized HEK293T cells (supplemental Fig.1B). Here we propose a different mechanism for the regulation of Snail1 function by PKD1 in tumor cells, the phosphorylation-dependent binding of co-repressors such as HDAC2 to Snail1. In addition to regulation of HDAC activity we have identified another regulatory mechanism induced by PKD1 that affects...
Snail1 mediates PKD1-induced growth of cancer cells  
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Snail1 function: PKD1 is required for up-regulation of LOXL3, that can stabilize the Snail1 protein (Fig.4A, B, C, supplemental Fig.2E).

In conclusion our data demonstrate that PKD1 enhances proliferation (10,36) of pancreatic and other cancer cells and this regulation is mediated by Snail1 via phosphorylation at Ser11. Snail1 is therefore required and sufficient for PKD1-driven proliferation and anchorage-independent growth of different tumor cells.

An overview on PKD1-mediated Snail1 regulation and control of biological effects is depicted in Fig.9.

Thus, PKD1 expression could be relevant for primary tumors to drive proliferation and initiate EMT, preparing cells for the dissemination phase. At later stages, however, when cells are invading in the surrounding matrix, or tumor stroma, loss of PKD1 activity could even be beneficial, since loss of PKD1 enables cells to acquire a high-motility phenotype via the regulation of Actin regulatory proteins such as Cortactin and S

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Figure 1: Mapping of PKD phosphorylation sites in Snail1 in vivo. (A) Structural overview of Snail1. N-terminal snag-domain, serine-proline-rich region, destruction box, NES (nuclear export sequence) and C2H2 zinc fingers. The putative PKD phosphorylation consensus motif of Snail1 S11 and the consensus sequence of the pPKD substrate Motif antibody (pMotif) are shown below the graph. (B) Mapping of Snail1 phosphorylation at Ser11 in vivo. Blots depict immunoprecipitates of FLAG-Snail1 from HeLa cells co-expressing Snail1-FLAG, Snail1S11A-FLAG constructs with active (CA) and
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kinase-inactive (KD) PKD1. Control blots on the right-hand side display transgene expression. Phosphorylation of Snail1 at S11 was probed using pMotif antibody and re-probed with anti-FLAG M2.

Figure 2: PKD1- and PKD2-GFP co-localize with Snail1-FLAG in nuclei of HeLa cells. Only PKD1-GFP is capable of efficiently interacting with Snail1-FLAG in nuclei, whereas interaction efficiency is significantly reduced by 3.38 times for PKD2. (A) Panels A'-F', acceptor-photobleach FRET experiment in HeLa cells co-expressing PKD1-GFP and Snail1-FLAG labeled with anti-FLAG-M2 and Alexa 546 antibodies. Images (A'-B') depict donor pre- and post-bleach, whereas (D'-E') display acceptor pre- and post-bleach states, respectively. Bleach-ROIs are shown in (C'). Percent FRET values are depicted in (F') and FRET is represented by a thresholded 7 color-LUT. (B) Acceptor-photobleach FRET experiment in HeLa cells co-expressing PKD2-GFP and Snail1-FLAG. Images (A'-B') depict donor pre- and post-bleach, whereas (D'-E') display acceptor pre- and post-bleach states respectively. Bleach-ROIs are shown in (C'). Percent FRET values are depicted in (F'). Images shown are single confocal section. The scale bar represents 10µm. (C) Statistical analysis of acceptor-photobleach FRET experiments displayed in (A) and (B). The graph depicts mean FRET efficiency and SEM for PKD1 (n=18 cells) and PKD2-GFP (n=17 cells) experiments. FRET efficiency values for all experiments are shown in supplemental table 2. Statistical significance (p<0.0001****) was calculated using two-tailed unpaired students t-test. (D) Endogenous snail1 and PKD1 interact. Anti-PKD1 and non-specific IgGs were used for immunoprecipitation from Panc89 vector cells. IPs were subsequently probed for the presence of endogenous snail1 using specific antibodies.

Figure 3: Phosphorylation of S11 by PKD does not alter subcellular distribution of Snail1. (A) Snail1-GFP (A'-E'), Snail1S11A-GFP (F' -J'), Snail1S11E-GFP (K -O) are predominantly localized to the nucleus independent of Snail1S11-mutation status. (B) Co-expression of constitutively active PKD1.CA-GFP (A) with wild type Snail1-FLAG (B, E) does not alter subcellular localization of Snail1 (A-E). Nuclei were stained with DAPI. Images depict single confocal sections. The scale bar represents 10µm.

Figure 4: (A) Snail1, LOXL3 and PKD1 are expressed in a subset of pancreatic cancer cell lines, HeLa cells and stable Panc89 cells expressing GFP-vector as well as PKD1-GFP. 200µg of total cell lysates were probed with specific antibodies. (B) Expression and upstream regulation of the Snail1 co-regulator lysyl-oxidase-like proteins 2 and 3 in stable Panc89 cell lines. LOXL3 but LOXL2 is up-regulated by ectopic PKD1. The graph displays fold change in regulation relative to vector controls. qPCR for LOXL2 and LOXL3 was performed on RNA isolated from stable Panc89 cells expressing GFP and PKD1-GFP. Four independent experiments were quantified in triplicate replica. Results were normalized to GAPDH and calculated according to the ΔΔCT-method. Statistical significance was calculated using one-way Anova with Dunnett's multiple comparison post-testing (p<0.05***). (C) LOXL3 expression is up-regulated in stable PKD1-GFP Panc89 cells. 250µg of total cell lysates were probed for LOXL3 using specific antibodies. (D) Regulation of Snail1 activity by phosphorylation at S11. Co-localization of Snail1-FLAG (A'-C') and Snail1S11A-GFP (D'-E') with their endogenous co-repressor HDAC1 in HeLa nuclei. Images depict single confocal sections. The scale bar represents 10µm. (E) Mutation of Snail1S11A impairs interaction of Snail1 with co-expressed FLAG-HDAC2 whereas binding is reconstituted with snail1S11E. Proteins were probed with respective specific antibodies in Western blots. (F) Statistical analysis of 3 independent co-precipitation experiments (E). Fold change in HDAC2 co-precipitation with Snail1 and mutants was calculated from integrated band densities of western blots. Significance was calculated using students t-test. (G) Co-immunoprecipitation (IP) of endogenous HDAC1 with Snail1-, Snail1S11A- and Snail1S11E-GFP from HeLa total cell lysates. Endogenous HDAC1 was probed with specific antibodies and IPs were re-probed for Snail1 expression by anti-Snail1 antibody. (H) Co-immunoprecipitation of endogenous HDAC2 with Snail1-, Snail1S11A- and Snail1S11E-GFP in HeLa total cell lysates. Endogenous HDAC2 was probed with specific antibodies and IPs were re-probed for Snail1 expression.

Figure 5: Snail1-dependent Histone-deacetylase activity and regulation of proliferation markers (A) Snail1S11A reduces Snail1-associated HDAC activity, as compared to wild type Snail1. HDAC activity was measured using a fluorometric assay kit. Crude nuclear extracts from 10 Mio HeLa nuclei
were normalized for protein expression and HDAC activity was measured in triplicate wells per condition in 96 well plates (Tecan infinity M1000) for GFP-vector, Snail1-GFP as well as Snail1S11A-GFP. For Snail1-GFP and Snail1S11A-GFP results were further normalized to GFP-transgene expression levels in crude lysates. The graph depicts the combined statistical analysis of 3 experiments. Statistical significance was calculated using one-way Anova with Bonferroni multiple comparison post-testing. Expression of transgenes in HeLa crude nuclear extracts and loading controls are shown in supplemental Fig. 3C. (B) Snail1S11A impairs Snail1-mediated proliferation marker protein expression in Panc1 cells. Panc1 cells were transfected with GFP, Snail1-GFP and Snail1S11A-GFP. Cyclin D1 and Ajuba markers involved in the regulation of proliferation, were probed in 60µg of total cell lysates with specific antibodies. Transgenes were probed with anti-Snail antibody. Actin was used as a loading control. (C) PKD1 and PKD1KD-GFP regulate proliferation marker protein levels in a similar pattern to phospho-site mutants. The expression levels of Cyclin D1 and Ajuba were probed with respective antibodies in 60µg of total cell lysates of stable Panc89 cells. Transgenes were detected with anti-GFP antibody. Tubulin was used as a loading control.

Figure 6: (A) PKD1, as opposed to PKD2, enhances anchorage independent growth in soft agar experiments. We seeded 10.000 cells of stable Panc89 cell lines expressing GFP, PKD1-GFP, PKD1KD-GFP, PKD2-GFP and PKD2KD-GFP in triplicate wells in 0.5% soft agar and documented assays after 13 days. (A) The graph depicts the average number of colonies and SEM per visual field documented at 10x magnification for 5 independent experiments. Statistical significance (p<0.0001 ****) was calculated using two-tailed unpaired students t-test. (B) Panel A’-D’, example of soft agar colonies documented for quantification. The scale bar represents 100µm. (C) Snail1 expression enhances anchorage-independent growth of Panc1 cells as compared to vector control, whereas Snail1S11A reduces the number of colonies in respect to wild type Snail1. We transiently transfected 50.000 Panc1 cells and subsequently seeded cells in triplicate wells per assay and in 3 experiments in 0.5% soft agar. Assays were documented after 6 days. The graph depicts the average number of colonies and SEM per well at 10x magnification. Statistical significance (p<0.0001****) was calculated using one-way Anova with Bonferroni multiple comparison post-testing. Representative transgene expression and images of colonies are shown in supplemental Fig. 4A, B.

Figure 7: Snail1 is a necessary and sufficient mediator of PKD1-regulated anchorage-independent growth and proliferation in pancreatic cancer cells. Stable Panc89 cells expressing GFP-vector and PKD1-GFP were transduced with lentiviruses expressing non-target shRNA (scramble, Sigma-Aldrich), sh_Snail1 #1 (NM_005985.2-136s1c1, Sigma-Aldrich) as well as sh_Snail1 #2 (NM_005985.2-504s1c1, Sigma-Aldrich) and subjected to antibiotic selection. Then we used 10.000 cells of stable cell lines expressing the respective constructs and shRNAs and seeded cells in triplicate wells in 0.5% soft agar. Assays were documented after 10 days at 4x magnification for colony counting (A) The graph depicts the combined average number of colonies per visual field of 3 experiments, with 6 images at 4x magnification per well and 3 replica wells per experiment. (B) Exemplary images (A’-F’) used for quantification of colony numbers at 4x magnification. The scale bar represents 100µm. Table1 displays average differences [%] in colony number between conditions. Statistical significance (p<0.0001****) was calculated using one-way Anova with Bonferroni multiple comparison post-testing. (C) Control blots for knockdown efficacy of endogenous Snail1 with sh_Snail1 #1 and #2 in stable Panc89 cells. Snail1 expression levels were probed in 60 µg of total cell lysates using anti-snail1 antibody. Tubulin was used as loading control.

Figure 8: 3D-growth in BME extract. (A) A’-C’ We seeded 10.000 single cells of stable Panc89 cell lines expressing GFP, PKD1-GFP and PKD1KD-GFP in BME gel and documented assays after 16 days. The scale bar represents 100µm. (B) PKD1 significantly enhances clusters growth, whereas PKD1KD decreases cluster size. The average diameter of tumor cell clusters was quantified in perpendicular directions for each cluster using spacial calibration of images, vector (n=150), PKD1-GFP (n=161) and PKD1KD-GFP (n=181). The graph depicts average diameters and SEM of 3 experiments. (C) Frequency distribution histograms of structure diameters for vector versus PKD1-GFP. (D) Frequency distribution histogram of structure diameters for vector versus PKD1KD-GFP. (E) Snail1 enhances, while S11A mutation inhibits proliferation in HeLa cells after 48h. The combined analysis of 3 independent proliferation assays was performed in transiently transfected cells expressing vector, Snail1-GFP and Snail1S11A-GFP. Cells were seeded after 24h at a density of 5000
cells per well in triplicate replica in 96 well plates. Cell density was quantified by measuring OD 550 of crystal violet stained cells dissolved in Methanol at time points T0, 24, 48h. The graph depicts the relative mean intensities for the respective cell lines after 24 and 48h, respectively. Statistical significance was calculated using unpaired students t-test. Doubling times were calculated using linear regression (Graph Pad, Prism). Representative transgene expression is shown in supplemental Fig.5 (F) Panc89 GFP-vector cells were transduced with lentiviruses expressing scramble control shRNA (Sigma-Aldrich) and shRNA_PKD1 (NM_002742.x-2978s1c1, Sigma-Aldrich). A PKD1 knockdown was probed using a specific anti-PKD1 antibody in semi-stable cell lines following selection. Blots were re-probed for PKD2 expression and Actin was used as loading control. (G) Semi-stable Panc89 vector sh_scramble and sh_PKD1 expressing cells were seeded at 10,000 single cells in BME gel and documented after 32 days. The scale bar represents 100µm. (H) Knockdown of PKD1 significantly reduces clusters growth (diameter). The average diameter of tumor cell clusters was quantified in perpendicular directions for sh_scramble (n=45) and sh_PKD1 (n=84). The graph depicts average diameters and SEM of 3 experiments. Numbers in the graph denote fold change in percent. (I) Frequency distribution histogram for knockdown of PKD1 versus scramble sh_RNA control. Knockdown of PKD1 significantly reduces cluster sizes in the BME matrix.

Figure 9: Overview of PKD1-mediated Snail1 regulation. PKD1 phosphorylation of Snail1 Ser11 is necessary for efficient binding of co-repressors HDAC1 and 2 as well as LOXL3. Expression of LOXL3, acting as a functional transcriptional co-activator, is also up-regulated by PKD1, implying a positive synergistic activation of Snail1. Snail1 phosphorylation at Ser11 by PKD1 enhances Snail1 marker protein expression involved in proliferation and anchorage-independent growth. This regulation is necessary as well as sufficient to modulate hyper-proliferation in Panc89 PDAC cells and other cell lines.

Table 1: Regulation of anchorage-independent growth by PKD1 and Snail1 shRNAs. Relative differences [%] in colony numbers are indicated by positive and negative values, respectively.
PKD consensus for S11 in Snail1: 

\[-5 -3 \text{ LVRKPS}^*\]

**pMotif consensus sequence:** 

\[-5 -3 \text{ LXR(Q/K/E/M)(M/L/K/E/Q/A)S}^*\]

---

**Figure 1**

A. PKD domain

B. PKD ectopic

PKD endogen
Figure 2

A

A' Donor-Pre-Bleach

PKD1-GFP

B' Donor-Post-Bleach

Bleach ROIs

C' Acceptor-Post-Bleach

D' Acceptor-Pre-Bleach

Snail1-FLAG-546

E' Acceptor-Post-Bleach

F' %-FRET (P color7 LUT)
Figure 2

B

A' Donor-Pre-Bleach

PKD2-GFP

B' Donor-Post-Bleach

C'

Bleach ROIs

D' Acceptor-Pre-Bleach

Snail1-FLAG-546

E' Acceptor-Post-Bleach

F'

%-FRET (IP color7 LUT)

C

0.1292 ± 0.01117
N=18

0.03828 ± 0.01064
N=17

PKD1-GFP Snail1-FLAG-546
PKD2-GFP Snail1-FLAG-546

D

IP non-specific IgG

IP PKD1

110 kDa

anti-PDK1

PKD1

30 kDa

anti-Snail1

Snail1

IgG
Figure 3

A
Figure 3

B

A'  PKD1.CA-GFP
B'  Snail1-FLAG
C'  DAPI
D'  Merge
E'  overexposed

Snail1-FLAG
Figure 4

A

- HeLa
- Panc1
- MinPca
- BAFc
- Capan1
- Panc90
- stable Panc90 GFP
- stable Panc90 PKD1-GFP

- 30kDa
- anti-Snail

- 80kDa
- anti-LOXL3

- 130kDa
- 105kDa
- anti-PKD1

- 43kDa
- anti-Actin

B

- fold expression

C

- Vector
- PKD1-GFP

- 80kDa
- anti-LOXL3

- 43kDa
- anti-Actin
Figure 4

D

A' Snail1-FLAG

B' HDAC-1

C' Merge

D' Snail1S11A-FLAG

E' HDAC-1

F' Merge
Figure 4

E

HDAC2-FLAG

Vector   +   +   +   +

IP anti-GFP, probe: anti-HDAC2

HDAC2

65 kDa

re-probe: anti-Snail1

Snail1-GFP

60 kDa

Control blots:

IP anti-GFP, probe: anti-HDAC2

65 kDa

anti-HDAC2

60 kDa

anti-Actin

43 kDa

anti-Actin

F

p=0.002 **

p=0.0496 *

HDAC2

Snail1-GFP

Fold change

H

Vector   Snail1-GFP   Snail1S1A-GFP   Snail1S1E-GFP

IP anti-GFP, probe: anti-HDAC1

65 kDa

0.53

1.29

IP anti-GFP, reprobe: anti-Snail1

Snail1-GFP

60 kDa

H

Vector   Snail1-GFP   Snail1S1A-GFP   Snail1S1E-GFP

IP anti-GFP, probe: anti-HDAC2

65 kDa

0.58

1.24

IP anti-GFP, reprobe: anti-Snail1

Snail1-GFP

60 kDa
Figure 5

A

[Bar chart comparing signal intensity with various treatments: GFP-Vector, Snail1-GFP, Snail1SH1A-GFP, and Snail1SH2A-GFP. The chart shows a significant decrease in signal intensity with Snail1SH1A-GFP compared to the control (Vector).]

B

[Western blot analysis with the following bands:
- 35 kDa: Cyclin D1 (anti-Cyclin D1)
- 60 kDa: Ajuba (anti-Ajuba)
- 43 kDa: Snail1-GFP (anti-Snail1)
- Actin (anti-Actin)]

C

[Western blot analysis with the following bands:
- 35 kDa: Cyclin D1 (anti-cyclin D1)
- 60 kDa: Ajuba (anti-Ajuba)
- 135 kDa: PKD1-GFP (anti-GFP)
- 55 kDa: Tubulin (anti-Tubulin)]

D

[Bar graph showing fold expression of PKD1 in control and treated samples: Vector C0-6hDE, PKD1C0-6hDE, and PKD1C0-6hDE with PKD1-GFP. The graph indicates a significant decrease in PKD1 expression in the treated samples compared to the control.]
Figure 7

A

![Bar chart showing average number of cells per field for different conditions.]

B

![Images showing cell morphology and density for different conditions.]

C

![Western blot showing protein expression levels for different conditions.]
Figure 8

Doubling times [h]:

- GFP-Vector: 56.25
- Snail1-GFP: 35.65
- Snail1S11A-GFP: 52.17

Average cluster diameters [m]:

- GFP-Vector: 124.8 ± 2.372 (N=161)
- Snail1-GFP: 113.4 ± 2.161 (N=150)
- Snail1S11A-GFP: 101.7 ± 1.480 (N=181)

Histograms showing the distribution of average cluster diameters and relative mean intensity.
Figure 8

F

| sh scramble | sh PKD1 |
|-------------|---------|
| PKD1        | 0.52    |
| anti-PKD1   |         |
| PKD2        |         |
| reprobe: anti-PKD2 |     |
| Actin       |         |
| anti-Actin  |         |

G

A'

GFP-Vector_sh scramble

B'

GFP-Vector_sh PKD1

H

** ** **
- 38.3%

261.6 ± 11.88 N=45

161.3 ± 3.977 N=84

I

![Graph showing bin center and average cluster diameter in micrometers.](http://www.jbc.org)
Figure 9

Proliferation markers
Cyclin D1/2, Ajuba-Aurora A

Anchorage-dependent and independent growth (2D and 3D BME culture)

Proliferation

PKD1

up-regulation

LOXL 3
positive regulatory activity

PKD1

Snail1 transcriptional regulatory complex

S11

Snail1

PKD1

HDAC1/2

necessary and sufficient

Proliferation markers
Cyclin D1/2, Ajuba-Aurora A

by guest on March 21, 2020http://www.jbc.org/Downloaded from
Table 1: Regulation of anchorage-independent growth by PKD1 and snail1 shRNAs. Relative differences [%] in colony numbers are indicated by positive and negative values, respectively.

| Average number of colonies | +/- [% change] |
|----------------------------|----------------|
| Vector_sh scramble to PKD1-GFP_sh scramble | +84.6 |
| Vector_sh scramble to Vetcor_sh snail1 #1 | -60.4 |
| Vector_sh scramble to Vetcor_sh snail1 #2 | -53.6 |
| PKD1-GFP_sh scramble to PKD1-GFP_sh snail1 #1 | -68.5 |
| PKD1-GFP_sh scramble to PKD1-GFP_sh snail1 #2 | -82.8 |
Protein Kinase D1 mediates anchorage-dependent and independent growth of tumor cells via the zinc-finger transcription factor Snail1

Tim Eiseler, Conny Koehler, Subbaiah Chary Nimmagadda, Arisia Jamali, Nancy Funk, Golsa Joodi, Peter Storz and Thomas Seufferlein

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