Elucidating Plectin Specific Pathways to Develop Novel Pancreatic Cancer Therapies

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease with a 5-year survival rate of 13%. Despite decades of research, there have been no significant improvements in overall prognosis for PDAC patients in over 50 years, accentuating the need for novel therapies. Plectin, a cytoskeletal linker protein, has been identified as a novel cancer-specific target as it is mislocalized to the cell surface (herein described as CSP) in cancer. Loss-of-function studies have implicated CSP as a pro-tumorigenic regulator of cancer cell proliferation, migration, and invasion in PDAC. While CSP has been identified as a tumorigenic factor, the mechanism of plectin-induced cancer remains elusive. The primary goal of this project was to understand the molecular mechanism of plectin and its role in tumorigenesis as well as potential resistance mechanisms to plectin targeted drugs. We utilized CRISPR/Cas9 to develop a tetracycline-inducible complete knockdown of plectin in human PDAC cell lines (L3.6pl, Panc-1, and MIA PaCa-2) to examine real-time mechanisms across stages of plectin knockdown. PDAC cell lines contained a negative control group (HRPT knockdown) and a plectin knockdown group. Short hairpin RNA (shRNA) was used in preliminary experiments to knockdown HPRT and plectin in PDAC cell lines. These lysates were analyzed by western blot to assess the expression of key regulators of the cell cycle and signaling pathways, p21, cyclin D1, and phosphorylated ERK1/2. Initial western blots analyzing the CRISPR/Cas9 knockdown cell lines suggested refinement for tetracycline treatment and gave preliminary insight into the mechanism of plectin-mediated proliferation.

Keywords: PDAC, plectin, inducible CRISPR/Cas9

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease with a 5-year survival rate of 13%[1]. Despite decades of research and numerous clinical trials, there have been no significant improvements in overall prognosis for PDAC patients in over 50 years - highlighting the urgent need for innovative therapeutic strategies targeting both primary and metastatic PDAC.

In 2008, *Kelly et al.* pioneered a phage-display-based functional proteomic approach to identify novel cancer-specific targets on the cell surface of cancer cells. Among these was plectin, normally a 500 kDa protein known for its contribution to cytoskeletal structure and dynamics in normal homeostatic conditions. Plectin is comprised of multiple domains including a variable N terminus, a classical actin-binding domain (ABD) and a plakin domain, a central helical rod domain, and a long C-terminal domain (**Figure 1**). Alternative splicing of the first exon in the plectin gene enables about a dozen isoforms differing from each other by their N-terminal head domains[2].

While plectin traditionally functions intracellularly in maintaining cytoskeletal integrity under homeostatic conditions, it is aberrantly expressed on the surface of malignant PDAC cells as cell surface plectin (CSP)[3]. This unique cell-surface localization makes CSP a compelling therapeutic target and a biomarker to distinguish between normal and malignant tissues. Loss-of-function studies have implicated plectin as a pro-tumorigenic regulator of cancer cell proliferation, migration, and invasion in PDAC, ovarian cancer, prostate cancer, lung cancer, head and neck squamous cell carcinoma (HNSCC), and breast cancer (**Figure 2**)[3–9]. Studies by *Soo Shin* et al. show that plectin promotes tumor growth *in vivo*. Plectin specific isoforms 1a and 1f are upregulated in PDAC where the inhibition of these isoforms leads to decreased proliferation (**Figure 3**)[10]. Furthermore, early clinical evidence from a phase 1 trial using anti-CSP monoclonal antibodies has demonstrated the safety of targeting CSP, with patient responses marked by disease stabilization[11]. These findings suggest that CSP is a clinically relevant driver of tumor progression and an attractive therapeutic target.

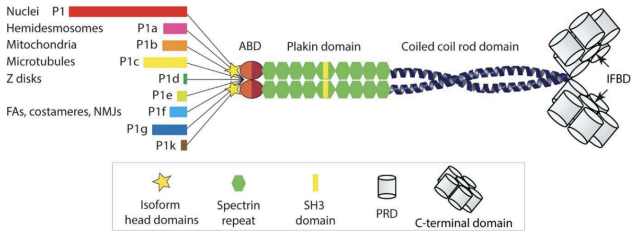


Figure 1. Schematic representation of a plectin dimer[2]. The N-terminal domain contains two actin-binding domains (ABDs) and two plakin domains (PD) where the C-terminal domains each contain six plectin repeat domains (PRDs), which harbor IF-binding domain (IFBD). Stars at the N-terminal domain stand for the different isoform-specific head domains of which there are nine (color-coded bars). Note: the figure is not drawn to scale, while the length of the bars is proportional to the size of the head domains.

Despite these advances, the molecular mechanisms by which CSP contributes to tumorigenesis remain poorly understood, as do the pathways that enable cancer cells to develop resistance to CSP-targeted therapies. This gap in knowledge limits our ability to optimize patient selection and develop more effective treatments. Addressing these critical questions provide mechanistic insights into CSP-mediated signaling and resistance mechanisms in PDAC.

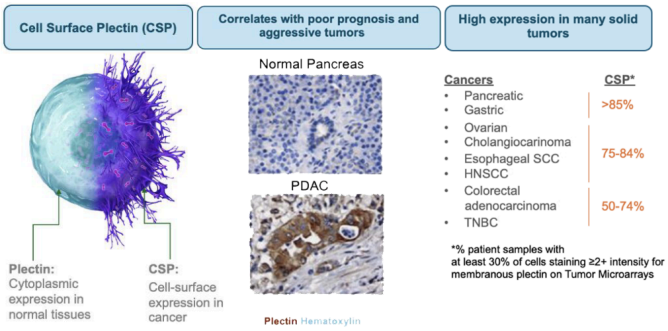


Figure 2. CSP is highly expressed in multiple cancers . Plectin, a cytolinker protein normally found internal to cells has been identified to be mislocalized and highly expressed in cancer subtypes (left panel). This cell surface plectin (CSP) is a beneficial marker for several cancers including PDAC, Gastric, and HNSCC, and correlates with a poor prognosis and aggressive tumors in PDAC, as well as other cancers (middle and right panel).

In this paper, we utilized CRISPR-Cas9 to develop a tetracycline-inducible knockdown of plectin and sought to optimize the expression of Cas9 with varied concentrations of tetracycline for varied durations. By leveraging inducible CRISPR/Cas9 systems to interrogate the role of CSP in tumor biology, our study introduced a novel and dynamic method to explore mechanisms of tumor progression and resistance. Unlike static genetic knockdowns or conventional overexpression systems, inducible systems provide temporal control, enabling us to study the real-time consequences of plectin depletion across multiple stages of cancer cell adaptation; this made the approach uniquely suited to uncovering dynamic and transient processes that static models may miss. Our focus on understanding how CSP drives tumor proliferation was also innovative, as it went beyond correlational studies to dissect the specific signaling pathways that mediate plectin's pro-tumorigenic effects. Exploring CSP-associated signaling pathways helped to uncover downstream effectors critical to tumor proliferation. Specifically, we evaluated cell cycle proteins in the presence and absence of CSP. We focused on cell cycle promoting and repressing proteins including phosphorylated ERK and cyclin-D1 as well as p21, respectively.

We hypothesized that after the generation of an inducible CRISPR/Cas9 system in PDAC cell lines, western blots will reveal the downregulation of cell cycle promoting proteins and the upregulation of cell cycle repressing proteins.

The outcomes of this research have the potential to transform our understanding of plectin's role in PDAC and could extend to other malignancies where CSP plays a role in tumor progression. Ultimately, this work provided a strong foundation for the development of novel CSP-targeted therapies, addressing an urgent clinical need and improving outcomes for patients with this devastating disease.

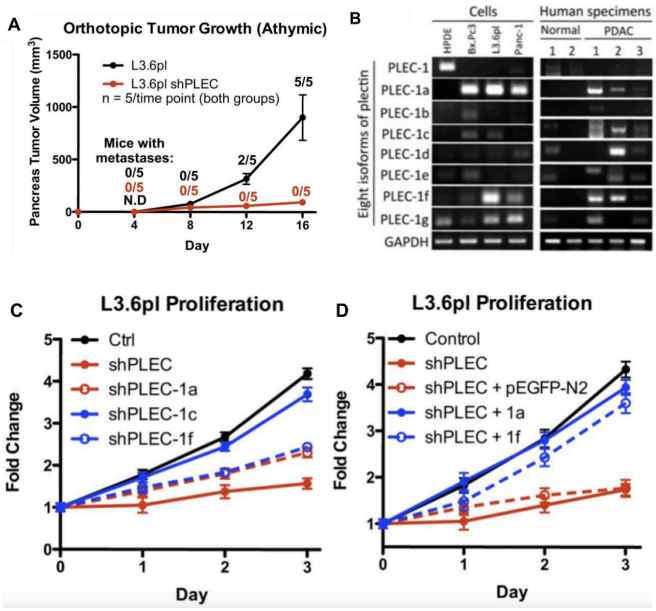
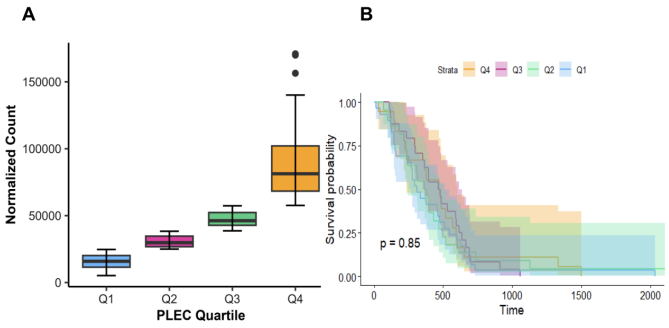


Figure 3. Plectin promotes tumor growth *in vivo* and specific isoform inhibition leads to decreased proliferation in PDAC. A. Orthotopic mouse model with L3.6pl PLEC positive and L3.6pl shPLEC from *Shin, S et al.*[10] reveals PLEC is necessary for tumor growth *in vivo*. N.D = not detectable/measurable via caliper; #/5 indicates the number of mice with metastases. **B.** PCR analysis from *Shin, S et al.*[10] shows mRNAs of plectin isoforms in PDAC cell lines, and in human PDAC tissue samples, further showing 1a and 1f are upregulated in PDAC. **C-D** Proliferation graphs from *Shin, S et al.*[10] **C.** Knockdown of PLEC isoforms 1a and 1f show decreased proliferation *in vitro*. **D.** Subsequent restoration of PLEC 1a and 1f in shPLEC cells leads to rescued proliferation.

Results

High Plectin Expression Leads to Worse Survival in PDAC

Data from TCGA-PAAD database was imported into R, then stratified into quartiles based on normalized PLEC counts. A patient survival analysis using the Kaplan–Meier (KM) plotter database, which integrates data from TCGA-PAAD database, revealed that for this subset of patients, high plectin mRNA expression was highly correlated with worse overall survival (**Figure 4**). Further analysis showed expression levels of plectin isoforms 1a and 1f were significantly greater in PDAC tumors compared to normal controls and other isoforms (**Figure 4**). These results suggest plectin isoforms 1a and 1f play an important role in tumorigenesis and lead to worse patient prognosis.



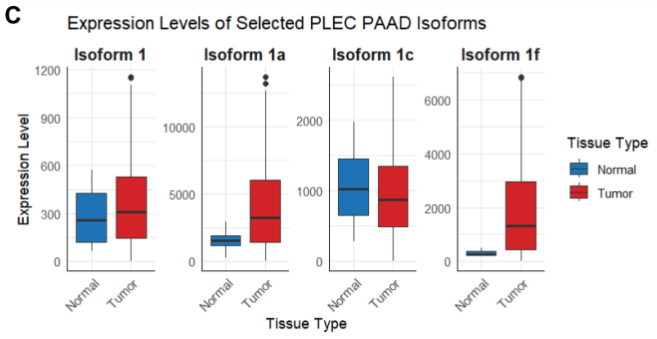


Figure 4. High plectin leads to worse survival in PDAC. A. Quartiles were generated using data from the TCGA-PAAD database based on plectin expression levels where Q1 is low plectin expression and Q4 is high plectin expression. **B.** Kaplan-Meyer survival plots from TCGA-PAAD databases show that patients with high plectin have an overall worse survival probability than patients with low plectin. **C.** Expression levels of plectin isoforms via the TCGA-PAAD database show variable isoform expression in PDAC tissue compared to normal controls with a significantly greater expression of plectin isoforms 1a and 1f found in tumors.

Generation and Characterization of Inducible CRISPR/Cas9 System

Given the precision and versatility of the CRISPR/Cas9 for targeted genome editing, we engineered a tetracycline inducible system to enable real time control over Cas9 expression in PDAC cell lines, L3.6pl, Panc-1, and MIA PaCa-2. The system comprises of two key components: (1) a single guide RNA (sgRNA) targeting either plectin or the control gene HPRT, and (2) the Cas9 endonuclease, which forms a ribonucleoprotein complex with the sgRNA to direct site-specific DNA cleavage. To ensure tight regulation of Cas9 activity, we employed a tetracycline-responsive promoter system. In the absence of tetracycline, the tetracycline-controlled transcriptional silencer (tTS) binds to the tetracycline-responsive element (TRE) promoter, suppressing Cas9 transcription. Upon the introduction of tetracycline, the reverse tetracycline-controlled transactivator (rtTA) activates Cas9 expression, enabling gene editing. Lentiviral vectors were used to deliver the system into PDAC cells. When introduced to the cells, the lentiviral particles bound and fused to the cell membranes and then released their vector of interest (**Figure 5**) into the cytoplasm which is converted to DNA, integrated into the host DNA, and finally transcribed.

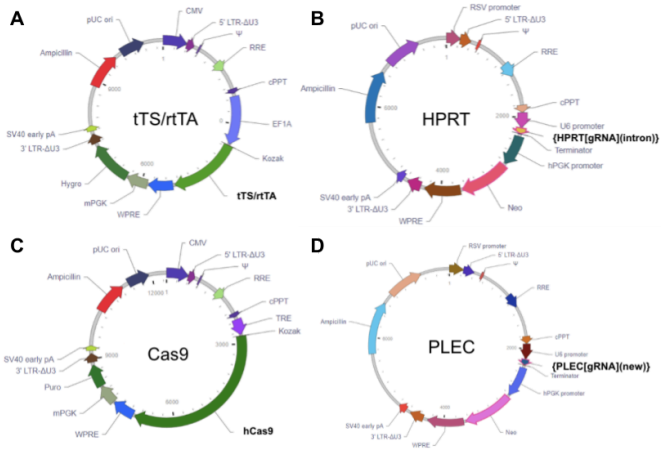


Figure 5. Lentiviral vectors contain components of the tetracycline-inducible CRISPR/Cas9 system. A-D. The lentiviral particles contained the following vectors, obtained from *Vectorbuilder*: **A.** the genes for tTS and rtTA, **B.** the Cas9 gene, **C.** the sgRNA for HPRT (sgRNA sequence sourced from *GenScript*), and **D.** the sgRNA for PLEC, creating a premature stop codon in plectin gene exon 8 (sgRNA sequence sourced from *Wenta, T. et al.*[12]).

To validate the tetracycline (tet)-inducible CRISPR/Cas9 system, we first confirmed the successful assembly and delivery of all required components. Lentiviral particles were generated to deliver four key vectors (**Figure 5**): (1) a construct encoding the tTS and rtTA, (2) the Cas9 endonuclease gene under a tetracycline-responsive promoter, (3) an sgRNA targeting the control gene HPRT, and (4) an sgRNA targeting PLEC exon 8, designed to introduce a premature stop codon. DNA electrophoresis confirmed successful transduction of PDAC cells with the PLEC- or HPRT-targeting sgRNA vectors (**Figure 6**). To assess the functionality of the system, L3.6 inducible Cas9 (iCas9) cells were treated with tetracycline (1 µg/mL) for 2, 4, and 6 days to induce Cas9 expression and subsequent PLEC knockdown. However, Western blot analysis revealed suboptimal Cas9 induction and PLEC knockdown under these conditions (**Figure 6**).

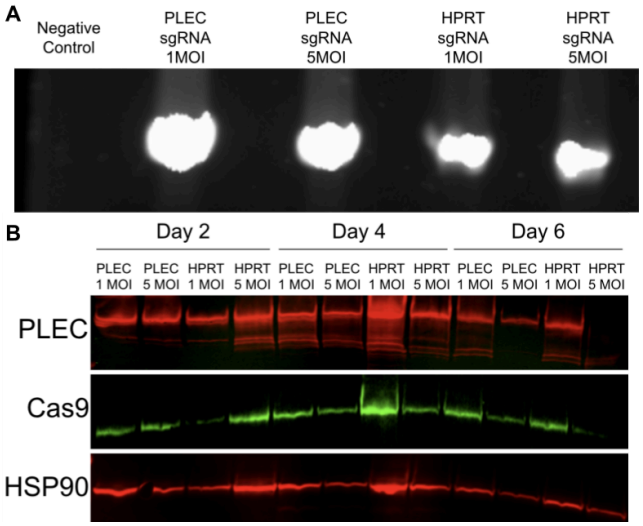


Figure 6. Validation of CRISPR/Cas9 inducible knockdown cell lines. A. DNA electrophoresis gel confirms successful transduction of PDAC cell line with sgRNA PLEC or HPRT vectors (rtTS and Cas9 vectors were previously integrated; data not shown). **B.** L3.6 inducible Cas9 (iCas9) cells were treated with tet (1ug/mL) for 2, 4, and 6 days to induce Cas9 expression and subsequent PLEC gene knockdown. Analysis via western blotting revealed poor response to tet activation given current treatment. Further optimization of tet treatment including tet concentration and days post-tet induction is needed.

Plectin contributes to increased PDAC cell proliferation

Because the inducible-CRISPR/Cas9 knockdown cell lines need to be optimized further, short hairpin RNA (shRNA) plectin knockdown cell lines were used to evaluate cell cycle proteins in the presence and absence of CSP. Cell lysates from shRNA plectin knockout and HPRT knockout (control) were collected for western blots analyses (**Figure 7**). These analyses revealed no expression differences of p21, cyclin D1, and phosphorylated ERK1/2 between control and plectin knockdown.

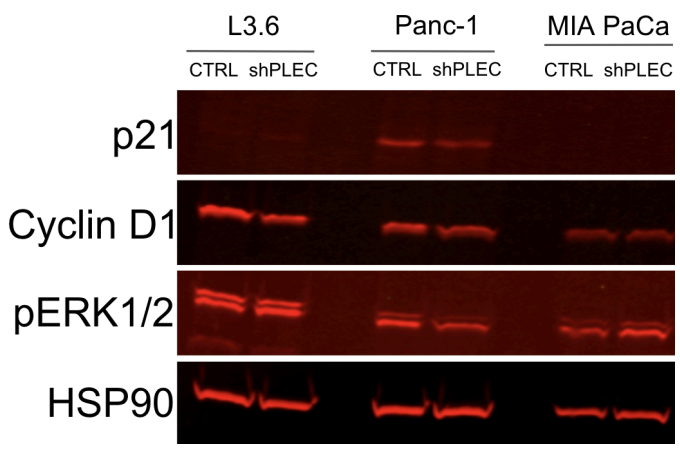


Figure 7. Plectin has downstream effects on cell cycle proteins. Whole cell lysates of PDAC cell lines L3.6pl, Panc-1, and MIA-PaCa-2 CTRL shRNA and PLEC shRNA and were analyzed by western blot to assess the expression of key regulators of the cell cycle and signaling pathways. Blots were probed for p21, cyclin D1, and phosphorylated ERK1/2). HSP90 was used as a loading control. These results provide initial insight into cell cycle status and pathway activity under experimental conditions.

Discussion

CSP has emerged as a critical regulator of pancreatic cancer progression, yet the molecular mechanisms underlying its tumorigenic effects remain poorly understood. In this study, we developed a tetracycline-inducible CRISPR/Cas9 system to achieve knockdown of plectin in PDAC cell lines and to uncover CSP's role in tumorigenesis. Strikingly, our bioinformatic analysis demonstrated that high plectin expression correlates with significantly worse overall survival in PDAC patients. Studies have implicated CSP in multiple aggressive cancer phenotypes across various malignancies, with isoform-specific roles becoming increasingly apparent[3-10]. Our work builds upon these foundations by demonstrating that plectin isoforms 1a and 1f are particularly critical in PDAC, consistent with their elevated expression in human tumors compared to normal tissue. Western blot analysis of these shRNA knockdown cells revealed no significant differences in p21, cyclin D1, or phosphorylated ERK1/2 expression compared to control cells (**Figure 7**).

Previous studies have implicated CSP in promoting aggressive cancer phenotypes across multiple malignancies, with emerging evidence of isoform-specific roles. Our findings align with this literature, as we observed elevated expression of plectin isoforms 1a and 1f in human PDAC tumors compared to normal tissue. Although our shRNA knockdown did not yield significant changes in cyclin D1 or phosphorylated ERK1/2, previous reports and preliminary data from our inducible CRISPR system suggest that CSP may still contribute to proliferative signaling through the MAPK pathway. The lack of detectable changes in these proteins in the shRNA model could reflect incomplete knockdown or compensatory mechanisms, highlighting the need for further investigation using optimized CRISPR-mediated depletion.

Beyond PDAC, our findings have important implications for other CSP-positive malignancies. The survival correlation we observed in PDAC mirrors similar prognostic associations reported for lung adenocarcinoma and head and neck squamous cell carcinoma, suggesting conserved oncogenic functions across tumor types[8-10]. The inducible CRISPR system we developed could be readily adapted to study CSP biology in these other contexts, potentially revealing common vulnerabilities that could be targeted with pan-cancer CSP-directed therapies. Future studies should explore CSP's role in the tumor microenvironment and its potential as a target for combination therapies with existing treatments.

Materials and Methods

Cell Culture and Reagents

Human PDAC cell lines, L3.6pl, Panc-1, and MIA PaCa-2, were obtained from the ATCC. All cell lines were maintained in DMEM supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. MIA PaCa-2 cells were additionally supplemented with 2% horse serum. All cells were maintained at 37 °C in 5% CO_2.

Western Blot Analysis

Cells were rinsed with HBSS prior to lysis in RIPA buffer supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). Total protein concentrations were determined using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). Equivalent protein amounts were resolved on 4–15% Tris-Glycine eXtended precast polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes (Thermo Fisher Scientific). Membranes were blocked with Odyssey Blocking Buffer (LI-COR, Lincoln, NE, USA) and incubated overnight at 4 °C with the appropriate primary antibodies. Following washing steps, membranes were incubated with infrared dye-conjugated secondary antibodies (LI-COR) and imaged using a LI-COR Odyssey Infrared Imaging System. Densitometry measurements were calculated using Image Studio software. Primary antibodies used include Cyclin D1 (ab134175) and p21 (ab109520) from Abcam (Cambridge, UK). Phosphorylated Erk1/2$^{Thr202/Tyr204}$ (#4370) from Cell Signaling Technology (Danvers, MA, USA).

Bioinformatic Analysis

Publicly available gene expression data from 178 PDAC tumor samples were obtained from The Genomic Data Commons and analyzed in R. Patients were stratified into quartiles based on normalized plectin expression levels. Kaplan–Meier survival analysis was performed using this data to evaluate overall survival across plectin expression quartiles. This data was also used to evaluate expression levels of plectin isoforms.

DNA Isolation and Gel Electrophoresis

DNA was extracted using the DNeasy Blood & Tissue Kit (#69504) from Qiagen (Hilden, Germany) according to the manufacturer's protocol. Briefly, cells were lysed using Proteinase K and Buffer ATL, followed by binding of DNA to a silica membrane in the presence of Buffer AL and ethanol. The bound DNA was washed with Buffers AW1 and AW2 and eluted in nuclease-free water. DNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Isolated DNA samples were separated by electrophoresis on 4–15% Tris-Glycine eXtended (TGX) precast polyacrylamide gels from Bio-Rad. Gels were run at a constant voltage in Tris-Glycine running buffer, and DNA was visualized using SYBR Safe DNA Gel Stain from (Thermo Fisher Scientific) and imaged on a gel documentation system.

Lentiviral Transduction

Lentiviral particles were produced to deliver a tetracycline-inducible CRISPR/Cas9 system targeting plectin or a non-targeting control (HPRT) into PDAC cells. Synthetic sgRNAs targeting plectin or HPRT were obtained from GenScript (EasyEdit sgRNA). Ribonucleoprotein complexes were assembled by incubating Ultra NLS Cas9 nuclease (GenScript, #Z03621) with sgRNAs in nuclease-free water and TE buffer according to the manufacturer's instructions. RNPs were delivered into cells using Lipofectamine CRISPRMAX reagent (Invitrogen, #CMAX00001) and Opti-MEM media (Gibco, #31985062) following the manufacturer's protocol. Cells were transduced with Cas9 endonuclease at a MOI of 1, along with sgRNA at either 1 or 5 MOI.

End Matter

Acknowledgments

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