# Effects of cingulin and SPTBN1 protein knockout on pancreatic ductal adenocarcinoma tumor cell polarity and subtype

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## Abstract

This project aims to discover how knocking out the cell polarity proteins cingulin (CGN) and spectrin beta, non erythrocytic 1 (SPTBN1) affects PDAC tumor cell polarity and growth. Inducible exogenous CGN and SPTBN1 constructs will be transfected into PDAC organoids, and afterwards CGN and SPTBN1 will be knocked out with CRISPR. The tumor organoids will then be viewed with immunofluorescence microscopy to assess cell polarity and subjected to a CellTiter-Glo growth assay to assess cell viability. To study the effect of CGN and SPTBN1 on cell viability, a Bravo Pipetting protocol was developed to plate efficient organoid growth assays. The CGN exogenous construct was created and validated, SPTBN1 was sequence validated and initial immunofluorescence staining of CGN and SPTBN1 in a classical organoid line before knockout was negative. It is possible that the inability to stain for the proteins is due to non-specific primary antibodies, requiring optimization for staining organoids or a need for better control lines.

## Introduction

- Pancreatic Ductal Adenocarcinoma (PDAC) comprises 90% of pancreatic cancers, has a 5-year survival rate of only 9% (American Cancer Society, 2020) and is the 4th leading cause of cancer deaths worldwide.[1]
- PDAC has a classical and a basal subtype with different gene expression profiles. The basal subtype has significantly worse prognosis and response to current treatments.[2]
- Classical tumors have a more organized structure with defined ducts compared to basal. These observations imply that classical tumors also have more clearly established cell polarities as compared to basal tumors.
- Proteomic analysis revealed that several cell polarity proteins, including CGN and SPTBN1, are much more phosphorylated in classical compared to basal tumors. Decreased cell polarity has been found to play a role in promoting cancer metastasis and tumor growth.[3]

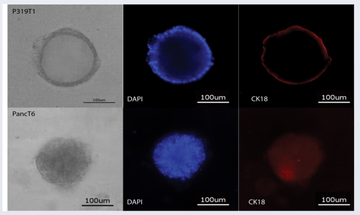



**Figure 1:** Immunofluorescence imaging of a classical (top) and basal (bottom) organoid line. Classical line shows more defined organization with a hollow center, while the basal line shows less organization and cell polarity.[4]

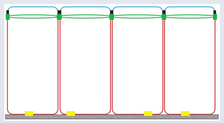
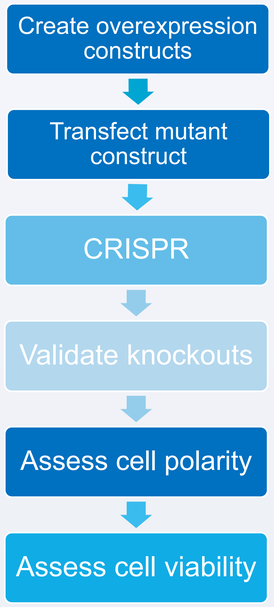


**Figure 2:** Diagram of epithelial cells showing cell polarity with an apical side (blue), tight junctions (black) and basolateral side (red).[5] Source: Andreas Wodarz and Inke Näthke

- Reduced SPTBN1 was correlated with shorter survival of pancreatic cancer patients. This could suggest that SPTBN1 aids in pancreatic tumor suppression, which it has already been shown to do in the GI tract.[6]
- Organoids are 3d clusters of cells that model organs and allow for better analysis of cell polarity.
- Goal: Discover whether CGN and SPTBN1 proteins have a role in determining PDAC tumor subtype, structure and rate of tumor growth.
- Hypothesis: Knocking out CGN and SPTBN1 will cause classical tumors to increase basal-like features such as decreased cell polarity and higher growth because CGN and SPTBN1 are involved in maintaining cell polarity and architecture.
- Cell polarity in cancer is a relatively new area of research and CGN and SPTBN1 have not been studied extensively in PDAC. By discovering more about the effects of cell polarity and specific cell polarity proteins such as CGN and SPTBN1 on cancer, more effective and targeted treatments can be developed in the future.

## Methods

### Overall Project Overview



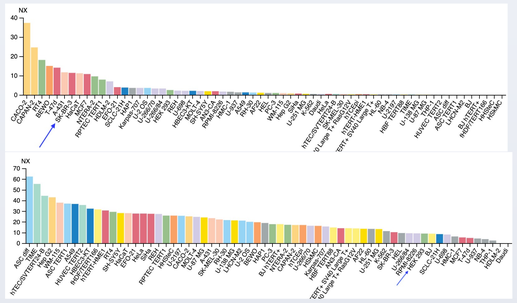




**Figure 3**[7]**:** Positive control 2D cell lines were found for CGN (top) and SPTBN1 (bottom) using the Human Protein Atlas database. SK-Br-3 and HEK 293T cells were chosen for CGN and SPTBN1 respectively based on expression.

### Immunofluorescent staining pre-knockout

1. **PDX Organoid establishment:**

Patient derived xenografts Human tumors were transplanted into immunocompromised mice to create patient derived xenograft (PDX) tumors. The PDX tumors were then harvested and cultured into organoids.

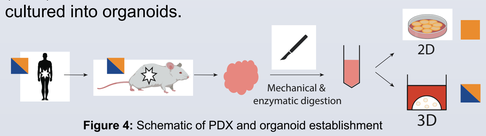



**Figure 4:** Schematic of PDX and organoid establishment

2. **Immunofluorescent staining:**

Organoids were plated on Matrigel domes. The organoids were then fixed, permeabilized, blocked and incubated with primary and secondary antibody and DAPI. Primary antibody only and secondary antibody only negative controls were used for each staining test, along with a positive cell line control.

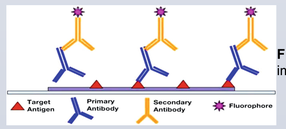



**Figure 5:** Schematic of indirect immunofluorescence.[8]

3. **Immunofluorescent Imaging**

Images were taken with Keyence microscope in brightfield, DAPI (blue fluorescence that stains for DNA) and either mCherry (red) or GFP (green). mCherry and GFP exposure was adjusted in test lines according to negative controls.

## Results

### CGN and SPTBN1 not visible in wild-type Classical organoids

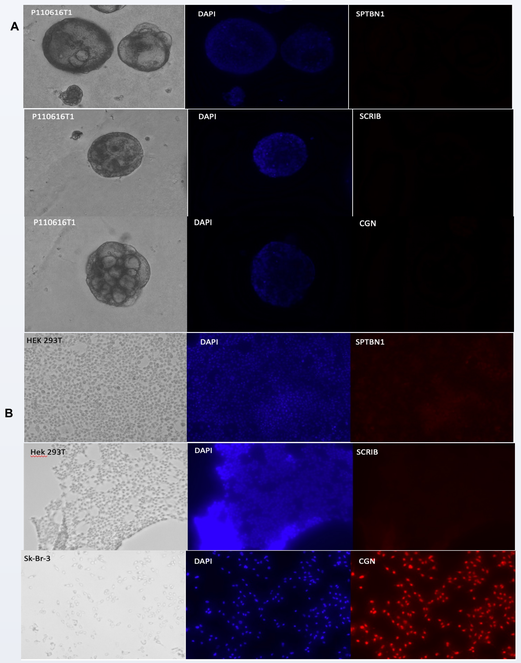



**Figure 6: (A)** P110616T1 classical organoid stained with mCherry for either SPTBN1, SCRIB or CGN. **(B)** Hek 293T and Sk-Br-3 2D positive control cells stained for SPTBN1, SCRIB (a basolateral membrane marker) or CGN. Positive control cell lines showed fluorescence for each protein. CGN showed high expression. SPTBN1 and SCRIB both had fluorescence but at lower levels than expected compared to the Abcam website.

### Programming Bravo Automated Liquid Handling Platform to Plate Organoid Growth Curve Assays

**Purpose:**

Achieve quick, automated, consistent plated organoid domes for tumor cell growth assays and drug dose-response assays. Vworks software and Javascript protocol developed to handle high viscosity and low temperature requirement of Matrigel.

**Troubleshooting:**

- Optimize flow rate for viscous Matrigel to eliminate foaming during pipetting.
- Adjust pipette depth to pick up tips and dispense domes completely in each well.
- Design and implement plating pattern using JavaScript for efficiency and minimal use of expensive Matrigel.
- Maximize speed of protocol to accommodate Matrigel low temperature requirement.
- Matrigel needs to be mixed frequently so that each well can have an even mixture of organoids.

**Figure 7:** Bravo Automated Liquid Handling Platform[9]

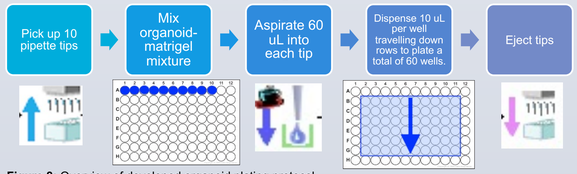



**Figure 8:** Overview of developed organoid plating protocol.

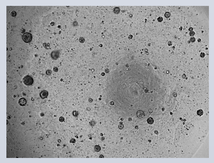


**Figure 9:** Brightfield image of organoid dome plated by robot on day of plating

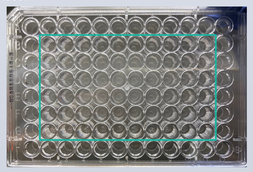


**Figure 10:** 96-well plate plated with organoids in Matrigel in a 6x10 configuration using programmed Bravo Pipettor Robot

## Results

### SPTBN1 plasmid validated

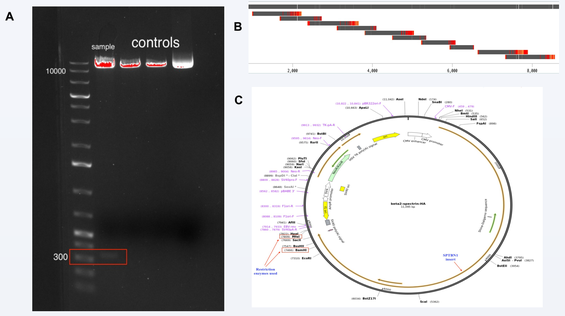



**Figure 11: (A)** Restriction enzyme digest of SPTBN1 insert in pEGFP-N1 vector with high fidelity BamHI /MfeI shows bands at a little above 300 and 10,000 bp. If correct, there should be bands at 343bp/10752bp **(B)** Benchling alignment of Sanger sequencing results with the NCBI SPTBN1 gene sequence matched. Red mismatches due to imprecise sequencing at the ends are validated by other primers. **(C)** Plasmid map of SPTBN1 insert in pEGFP-N1 with BanHI/MfeI labelled.[10]

## Conclusion

- SPTBN1 and SCRIB had low signal in (+) HEK293T control, but none in organoid.
- CGN had strong 2d cell line (+) control signal but none in organoid.
- Possible solutions: Choose better +/- control lines for SPTBN1, and SCRIB. Use CGN controls in Matrigel. Do an siRNA knockdown as a negative control.
- Developed Bravo Robot protocol that can plate organoid domes for growth assays.
- We ordered and have received the 3-point mutated exogenous construct in lentiviral vector of cingulin and are waiting for the SPTBN1 construct.

## Future Studies and Directions

- Roles of other cell polarity proteins in PDAC.
- Stain with better SPTBN1 positive and negative controls.
- Stain with different organoid lines and types and concentrations of primary antibody
- Perform growth assays on CGN and SPTBN1 wildtype and knockout organoids

## References

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