THE ROLE OF CLP36 IN PANCREATIC CANCER CELLS DURING MIGRATION AND IN CELL SHAPE MORPHOLOGY

by
Eleana Parajón

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Abstract

Principles that govern cell shape change, govern tissue development and regeneration and provide a model for understanding diseases including cancer. Due to the overexpression of CLP36 and ACTN4 in other cancers, I hypothesized that the interaction between ACTN4 and adaptor protein CLP36 in pancreatic cancer cells has a role in migration and cell shape morphology. Cell migration assays, including scratch wound and transwell assays, were used to assess the migration of metastatically derived pancreatic cancer cells; depletion of *clp36* decreases cell migration and invasion *in vitro*. Immunofluorescence was used to assess the localization of CLP36 at the cell edges. CLP36 localizes in actin protrusions in metastatically derived pancreatic cancer cells. These results suggest that CLP36 is an important regulator of pancreatic cancer cell migration. This study provides information on CLP36 in cell migration, CLP36 expression and its possible contribution to the progression of pancreatic cancer. Also, this approach provides information on the power of upregulation and downregulation of proteins in pancreatic cancer suggests strategies for manipulating the proteins to treat disease in the future.

Advisor: Douglas Robinson, Ph.D.

Preface

This thesis is the product of my Masters research initiated in June 2017 and finished in April 2018. The research was done in the Johns Hopkins University in Baltimore, Maryland. I was introduced to cell behavior, the cytoskeleton, and data analysis by Dr. Dustin G. Thomas who guided me throughout my experiments and for the helpful discussions and mentorship to do my study. After learning about different cell assays and using them to test aspects of cell migration, I was intrigued by cell behavior and morphology in pancreatic cancer cells.

I would like to thank Dr. Douglas Robinson, who provided me with the funding, guidance and mentorship to do my study. Also, I would like to thank my lab mates especially Yinan Liu, Jennifer Nguyen, Kathleen DiNapoli, Eric Schiffhauer, and Priyanka Kothari for helping me and providing moral support. Additionally, I would like to thank Dr. Kathryn Tifft Oshinnaiye for the constant support throughout my project.

Finally, I would like to thank my family and friends for their continuous support throughout my research.

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Chapter 1: Cell Behavior and the Cytoskeleton

Cells and tissues derive their functions from their shapes, adhesion and motile characteristics, and structural integrity. These morphological features are derived from a highly integrated network of structural and signaling proteins. Signaling proteins direct the structural proteins, but in turn, the structural proteins sense mechanical inputs and feed this information back onto the signaling pathways. In the sections below, the key elements discussed are proteins in the cytoskeleton and cell behavior that leads to cell invasion and migration. Cell migration can lead to cancer cell metastasis or invasion into healthy host tissue. Learning about the cellular and molecular basis of migration and invasion in cancer cells will help us understand how cancer cells spread and can lead to new treatment strategies.

1.1.1 Actin

Actin is a protein that assembles into microfilaments, one of the components of the cytoskeleton. Actin is present as a free monomer, G-actin, or as a linear polymer, F-actin. The polymeric form is generally the functional form, but pools of G-actin are necessary to build polymers when and where the cell requires them. Dynamic actin filaments are essential for many cellular functions, such as processes like muscle contraction, cell motility, signaling, cell division and cell shape. The epithelial-mesenchymal transition in metastatic cancer cells is regulated by the remodeling of actin [3]. Actin remodeling is a process that allows for alterations of cellular organization. Actin-binding proteins aid in the alteration of actin filaments. Actin monomers join the positive end of the filament in the ATP state, while ADP-actin monomers dissociate from

the negative end in a process called treadmilling. Actin polymerization and de-polymerization are dependent on the conversion of G-actin to F-actin and hydrolysis of the ATP. The concentration of G-actin and F-actin continuously fluctuate and the assembly and disassembly of F-actin will undergo treadmilling [4]. Superimposed upon the intrinsic dynamics of actin polymer assembly and disassembly, the cell uses many actin binding proteins, which further modulate the actin cytoskeleton dynamics, increasing the richness and control [5]. Actin binding proteins have many different roles including sequestration of actin monomers and actin filament capping, breaking, and cross-linking.

1.1.2 Alpha-actinins

Alpha-actinins are necessary for the cross-linking of filamentous actin in the cytoskeleton to protect cells from mechanical stress and control cell movement. The interactions between alpha-actinin and proteins regulate cell adhesion and motility. Alpha-actinin is a family of cross-linking proteins that includes four paralogous family members: alpha-actinin-1 (ACTN1), alpha-actinin-2 (ACTN2), alpha-actinin-3 (ACTN3) and alpha-actinin-4 (ACTN4). The muscle-associated actinins are ACTN 2 and ACTN 3, while non-muscle isoforms are ACTN1 and ACTN4. The muscle isoforms are found in skeletal and smooth muscle and organize actin filaments into sarcomeric structures, which constitute the minimal contractile unit. The non-muscle paralogs organize actin filaments into the networks that contribute to non-muscle cell structure, migration and adhesion. ACTN4 has been linked to metastasis in cancer through cellular features and cellular misbehavior [6-9].

ACTN4 regulation and interactions have been shown to have a role in cancer cells and metastasis. ACTN4 has been studied in the context of lung cancer metastasis to the brain. ACTN4 expression is prominent in metastatic brain tumors, but not primary lung cancer tissue [10]. Lung cancer metastasis to the brain is due to the interaction of ACTN4 with actin reorganization in the cytoskeleton, cell motility and focal adhesion [10]. Patients with an increase in the copy number of ACTN4 have metastatic phenotypes leading to lower prognosis, like salivary gland carcinoma [11]. Studies with animal models have shown ACTN4 as an important regulator of cell motility. Specifically, in murine lung fibroblasts, ACTN4 is significant in cell behavior like migration, spreading, adhesion and proliferation [12]. Knocking down ACTN4 impairs fibroblasts in cell migration, spreading adhesion and proliferation, suggesting that ACTN4 is necessary for normal cell morphology and motility [12]. ACTN4 enhances cancer cell motility, invasion and metastasis. Focal adhesions are dynamic protein complexes through which the cell's cytoskeleton connects to the extracellular matrix. In colorectal cancer cells, ACTN4 provokes immature focal adhesions which leads to cell motility and invasion, while ACTN1 does not [13]. Findings have suggested that alpha-actinins are linkers between cytoskeleton and integrin's and participate in force transmission at adhesion sites to facilitate adhesion growth [60]. ACTN4 works with cytoskeleton proteins and overexpression of ACTN4 functions to promote breast cancer tumorigenesis through cell motility [14].

1.2 CLP36/PDLIM1/CLIM1

Due to the association of alterations between actin and alpha actinins in cancer, another protein called CLP36 is of interest in cancer cells because it interacts with alpha actinins. CLP36 has been shown to associate with actin filaments during shape change, migration and contraction of endothelial cells. As discussed before, cytoskeletal proteins are important regulators of cell migration in metastatic cancer. CLP36 (a.k.a. CLIM1, PDLIM1, or Elfin) is a 38 kD protein that has an N-terminal PDZ domain and a C-terminal LIM domain. CLP36 is expressed in the heart, lungs, liver, spleen, colon, vasculature, and blood and is found in epithelial and endothelial cells. During cell shape change events, including spreading, migration and contraction of these cells, CLP36 associates with actin filaments and stress fibers [1, 2]. CLP36 binds to two paralogous actin crosslinking proteins, non-muscle alpha-actinin-1 and alpha-actinin-4, which associate with actin structures involved in key cellular processes, like cell shape change, migration, polarity, and cytokinesis. High expression of alpha-actinin 4 in the colon and CLP36 have been found to specifically interact in colon epithelial cells through an ACTN4-CLP36 complex. The complex extends to the actin stress fibers of the non-muscle cells [2]. The association of CLP36 and ACTN4 in actin stress fibers is relevant to cancer cell migration and metastasis.

1.3 LIM domain proteins

LIM domain proteins regulate gene expression, organization of cytoskeleton proteins, determination of cell fate, and formation of tumors. LIM domain proteins act as adaptors to support the association of protein complexes. LIM domains are 50-60 amino

acids long and contain two conserved zinc motifs. The two zinc fingers contain eight cysteine and histidine residues [15].

LIM domains are found in other proteins related to nuclear and cytoplasmic proteins. The proteins that are localized to the nucleus contain two LIM domains and are named LIM only proteins (LMO). LMOs are common in genes that are activated by translocations that can occur in hematopoietic cancers and mesenchymal tumors [16]. Also, LIM domains are found in kinases like Lmk1 and Lmk2 [17, 18]. PDZ-LIM proteins like CLP36 have been suggested to act as adapters between kinases and the cytoskeleton. The LIM domain containing proteins have important roles in biological processes like cell fate decisions and organ development [19]. The interaction between ACTN4, CLP36, and actin can lead to changes in cell behavior and migration which can promotes cancer.

1.4 Cell Behavior

1.4.1 Cell adhesion

Cells have a variety of properties including the ability to adhere to surfaces or to neighboring cells. Cells have special proteins called cell adhesion molecules that allow cells to stick to each other (*e.g.* E-cadherin) and their surroundings (*e.g*. integrins). Adhesion proteins are organized into cellular adhesion structures such as focal adhesions, which mediate adhesion to surfaces, and adherens junctions, which mediate adhesion to neighboring cells [20]. An additional role of focal adhesions and adherens junctions is to detect forces (including resistive tensile forces) that emanate from the extracellular matrix [21] or that are exerted by surrounding cells [22]. The active components of tensile forces are generated by the cell's cytoskeleton, including contractile myosin II

motors and actin polymer remodeling, and are regulated in part by signaling cascades [23].

The disturbance of epithelial cells results in pathologies like fibrosis and cancer [24]. Defects in cell adhesion can lead to carcinogenic processes such as epithelial-to-mesenchymal (EMT) transitions [24]. EMT is the disturbance of epithelial cell polarity and reduction in adhesion, providing cells with increased ability to migrate and invade. Signaling pathways that generate EMT induce E-cadherin (major cell-cell adhesion protein) repressors like the *Snail* genes [25, 26]. Changes in the cell increase the ability to migrate through a range of environments with varying stiffnesses [27, 28]. Tissue stiffness variations are associated with cancer and often increase due to changes in the extracellular matrix composition during tumor growth, further disrupting the forces between epithelial cells and the extracellular matrix and promoting metastatic migration [29, 30]. Loss of cell adhesion complexes may lead to spreading of cancer due to loss of contact and anchoring to the extracellular matrix.

1.4.2 Cell division

Cell division in mammalian cells occurs through activation and deactivation of proteins that regulate the phases of the cell cycle. Here, I provide a concise summary of the process. The cell cycle contains five phases: G0 (non-cycling quiescent phase), G1 (growth phase 1), S (synthesis), G2 (growth phase 2), and M (mitosis). The first four phases are called interphase. In G0, the cells are in a resting state and some cells maintain this state for their lifespan. Growth factors stimulate cells to enter G1 and then into S phase where DNA is replicated. In the S phase, DNA replication is largely completed,

and the cell prepares to undergo mitosis. During mitosis, the chromosomes are separated, and the cell divides itself into two daughter cells during cytokinesis. Mitosis is broken down into five sub-phases: prophase, prometaphase, metaphase, anaphase, and telophase. Throughout the cell cycle, cellular surveillance systems ensure that only healthy cells multiply. When an error is found, a checkpoint control is activated, which prevents the cell from progressing until the error is corrected [31]. Importantly, loss of cell cycle control can lead to human cancer due to check point errors accumulating, resulting in proliferation and instability in the genome. Cancer cells have mutations that can lead to cyclin-dependent kinase (CDK) hyper-activation. About 30 cyclin-dependent kinase inhibitors have been developed to target these kinases [31]. Inhibitors of CDKs, can induce cell-cycle arrest. There is extraordinary interest in targeting the cell cycle through inhibition, including the CDKs, for the development of anti-cancer drugs.

1.4.3 Cell Invasion and Migration

Cell invasion is a result of the ability of cells to be motile and navigate through the extracellular matrix. Cells demonstrate a variety of types of movement, including migration of cells on a surface or tissue, migration of cells into a wound during wound healing, and cell division. Another example of cell movement is contraction which is responsible for the function of muscle cells and the separation of daughter cells during cell division. Motors pull membrane tubes along cytoskeletal filaments which allows for contraction. During migration, rapid polymerization of the cytoskeleton by adding subunits to the end allows for cell movement. As seen in the schematic drawing in figure 1, the protrusion of a lamellipodium is controlled by the polymerization of actin filaments

at the leading edge, allowing for protrusions of the front end of migrating cells where actin (seen in red) is accumulated. The membrane is extended forward by the pushing of the actin filaments, thus allowing for cell body movement. Once the membrane has extended the membrane becomes attached to the surface and a new adhesion is formed. After forward extension of the front of the cell, the cell body is moved forward and de-adhesion (focal adhesions are broken) occurs at the rear of the cell which completes the forward movement of the cell (Fig. 1).

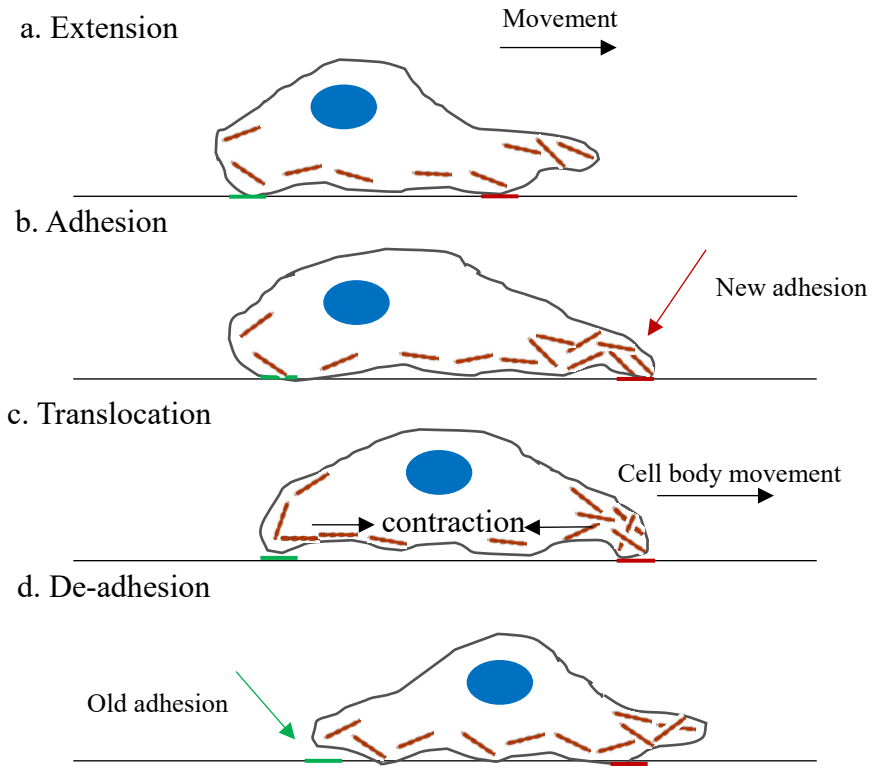


Figure 1. Model of Cell Movement
This schematic drawing shows cell movement primarily driven by actin. A) Extension: motility is initiated by actin protrusions at the cell's leading edge. B) Adhesion: protrusion of the leading edge will allow for a new adhesion. C) Translocation: the cell body contracts which causes retraction of the rear and forward movement of the cell body. D) De-adhesion: the cell will detach from the surface to which it was adhering. This repeated cycle of extension, adhesion, translocation, and de-adhesion allows the cell to migrate.

1.4.4 Cancer Metastasis

1.4.4.1 Endothelial-mesenchymal transition (EMT)

In metastasis, cancer cells travel through the blood or lymph system and form new tumors in other parts of the body. Steps that cancer cells undergo to metastasize are: breaking away from original tumor, traveling to other parts of the body, growing in the new location and avoiding attacks from the immune system. Movement of cancer cells and seeding of new tumor cells in tissues is a process called invasion-metastasis cascade [32, 33]. Several types of carcinoma cells can obtain secondary tumor capability through epithelial-mesenchymal transition including pancreatic [34], prostate [35], breast [36], ovarian [37], and renal [38]. Many cancers result from epithelial tissues where abnormalities in cell-cell and cell-matrix interactions occur during tumor development. The epithelial-mesenchymal transition schematic drawing (Fig. 2) derived from Jean Paul Thiery's model of epithelial-mesenchymal transition represents the process of how cells undergo EMT to mesenchymal-epithelial (MET) [39].

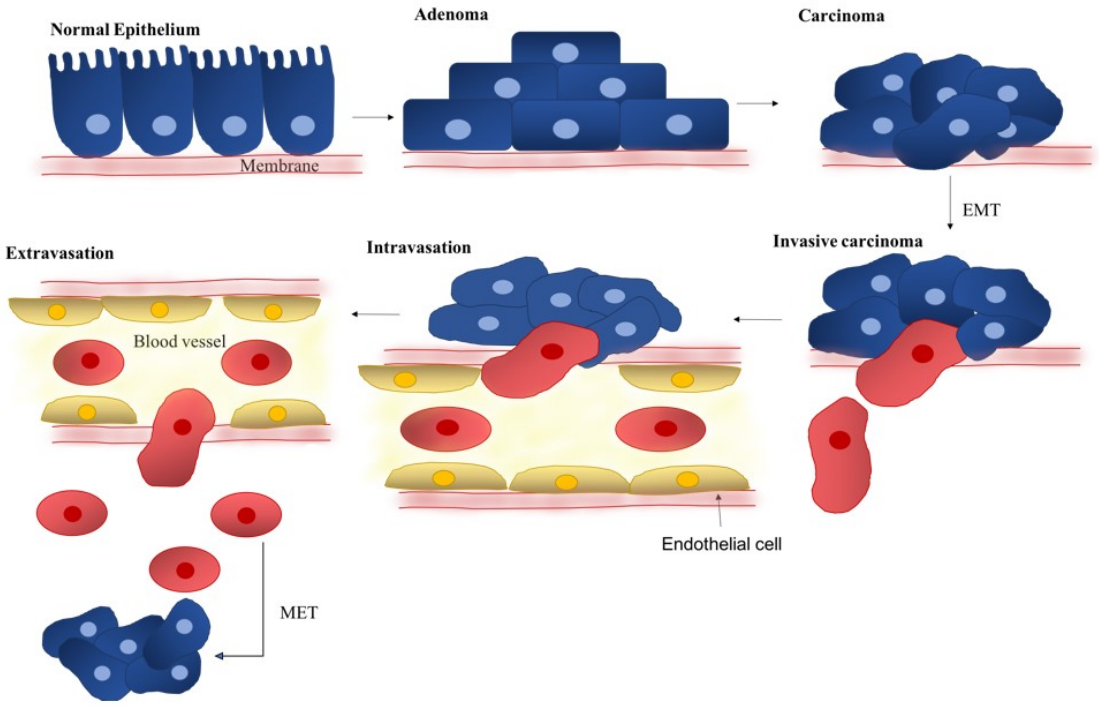


Figure 2. Epithelial-Mesenchymal Transition
Schematic drawing of normal epithelial cells showing loss of cell adhesion (carcinoma) and cell polarity, allowing the cells to gain migratory properties. The cells undergo EMT and become invasive and can travel through the blood vessel through processes of intravasation and extravasation. After, the cells transition into epithelial properties which involves the transition from the motile cells to the formation of polarized cells (MET).

Differentiated carcinomas tend to grow and spread more slowly than poorly differentiated cancer cells and often re-differentiate by mesenchymal-epithelial re-transition (MET). The EMT-MET switches suggest a motivating force of metastasis [40]. Invasive cancer cells arrested in growth phase and proliferation occurred in re-differentiated metastasis, suggesting that EMT needs to be reversed for the cell to grow and colonize [40], supported by EMT inducing transcription factors that can prevent proliferation [41]. EMT-MET switches in metastasis have been suggested that this transition is important for secondary tumor growth [42, 43]. There is strong evidence for

EMT-MET switches in embryonic systems, but in human cancer, the MET event is still unclear [39].

Understanding EMT-MET switches can lead to further understanding of the progression of cancer. Studying metastatic cancers can pave the way to stop or prevent the metastasis of primary cancer cells. Current treatments and drugs are focused on preventing or relieving symptoms. Since every patient is different, it is important for ongoing research to study cell morphology, behavior, and proteins associated with cancer to then apply this knowledge to diseases so that it paves the way for patient-specific targeted therapy.

Cancer is an illness in which characteristics of cell behavior are lost or disturbed. Uncontrolled cell proliferation, migration, invasion, and cell morphology are common features of cancers. Actin, alpha-actinins and CLP36 are key players that reinforce these cellular processes. Abnormal expression of cytoskeletal associated proteins play an important role in the ability of cancer cells to metastasize and resist drug therapies, understanding this role will help with future drug therapies and prognosis.

Chapter 2: The Role of CLP36 in Pancreatic Cancer

1.1 Background

Pancreatic cancer is a deadly disease that affects about 55,000 people in the United States and is usually not detected until later stages (poor prognosis). The best chance of successful treatment is when the cancer is detected early. Research is focused on approaches that may help to detect earlier stages before it spreads. Also, research is focused on identifying mutated genes and proteins that may be causing abnormal changes in the cells. This information will be useful in developing new drugs that target abnormal changes, as well as a possibility to screen for the cancer in individuals that are at high risk for developing the disease. Thus, *in vitro* studies like cell migration assays are important because they provide the foundation that leads to *in vivo* studies in animal models that can lead to targeted therapy.

1.1.1 CLP36, alpha actinins and PDZ-LIM interactions

Studies have tried to identify proteins that induce macromolecules that recognize antibodies in patients with pancreatic cancer because the identification of tumor antigens can potentially provide a detection for early diagnosis. CLP36 is a protein that is recognized by the immune system in patients with pancreatic adenocarcinomas [46]. Additionally, CLP36 suppresses epithelial to mesenchymal transition in colorectal cancer cells by stabilizing beta-catenin [47]. CLP36 and PDLIM4 (PDZ and LIM domain protein 4) both are actinin-associated LIM proteins that have roles in forming stress fibers in fibroblasts by recruiting alpha-actinin-1 [48].

CLP36 has a PDZ (N-terminal) domain and LIM (C-terminal) domain. PDZ-LIM proteins act as adapters for cytoskeleton proteins and kinases [49, 50]. Through the PDZ and LIM motifs, proteins can associate with cytoskeletal and signaling proteins [51]. More specifically, PDZ domain interacts with the cytoskeleton [49, 50, 52, 53] and the LIM domain interacts with kinases [50, 54, 55]. Most of these studies have been performed in muscle due to high expression of PDZ-LIM proteins in the muscle. ACTN2 and ACTN3 are muscle specific, which anchor actin thin filaments at the Z lines in striated muscle and dense bodies in smooth muscle [56]. On the other hand, there are two non-muscle actinins, apha-actinin-4 and alpha-actinin-1, which are less concentrated at stress fibers and detected in focal adhesions. Expression of alpha-actinin-4 is prompted in migrating cells [8]. CLP36 is expressed in non-muscle tissues with low expression levels in the pancreas [57]. In addition, CLP36 is highly expressed in epithelial cells, localizes to actin stress fibers through the PDZ domain, and interacts with alpha-actinin-1 and alpha-actinin-4 [2].

1.1.2 Pancreatic cancer

Pancreatic cancer arises when cells in the pancreas begin to multiply uncontrollably and form a mass and then metastasize to other parts of the body. There are different types of pancreatic cancer. Pancreatic adenocarcinoma starts within the epithelial lining of the pancreatic ducts. Non-adenocarcinoma, another type of pancreatic cancer arises from hormone-producing cells of the pancreas. Non-adenocarcinoma is less aggressive than pancreatic adenocarcinoma. Patients with pancreatic adenocarcinoma usually show no symptoms during the early stages, and symptoms do not develop until

the disease reaches a stage where it spreads to other parts of the body [44], including nearby lymph nodes, liver, peritoneal cavity, large intestine or lungs [45]. Therefore, pancreatic adenocarcinoma is one of the most lethal diseases with only ~6-7% of patients surviving to 5 years.

CLP36 may be an adaptor between actin stress fibers and LIM-binding proteins. The low expression level of CLP36 in the pancreas and that it interaction with alpha-actinins it led me to my initial question: Is CLP36 overexpressed in pancreatic cancer cells? Preliminary data suggests that CLP36 is highly expressed in human metastatic pancreatic cancer cells (AsPC-1). Then, I asked if CLP36 overexpression is associated with the migration of cells that lead to metastasis. Recent findings demonstrate that high levels of CLP36 in breast cancer is associated with cell migration and metastasis [58]. Is CLP36 necessary to drive migration behavior in AsPC-1 pancreatic cancer cells? Is overexpressed CLP36 sufficient to drive migration behavior in cancer cells? I hypothesized that if CLP36 interacts with alpha-actinin-4, then knock down of CLP36 may affect actin, leading to a decrease in migration.

Methods

Cells and Cell Culture

Human ascites derived pancreatic cancer (AsPC-1) were purchased from ATCC and grown in RPMI 1640, L-Glutamine media (Gibco), supplemented with 1% penicillin and streptomycin, 10% FBS (ATLAS Bio), and insulin (4mg/mL). Cells were detached using 0.5% trypsin (Life Technology).

Human primary tumor-derived cells (Panc10.05), Human pancreatic ductal epithelial cells (HPDE) were obtained from Dr. Ming-Sound Tsao (University of Toronto, Ontario Canada), human primary tumor-derived cells (Panc10.05) and human metastatically-derived cells (AsPC-1) were purchased from ATCC.

Engineered cell lines

Lentiviral knockdown cell lines were generated in AsPC-1 parental strains. For lentiviral knockdown, the hairpins used (Sigma Mission shRNA) were:
(A) 3UTR1238S21C1, (B) 3UTR1238S1C1, (C) 3UTR1183S1C1, (D) CDS869S1C1, (E) CDS669S1C1, (Vector) pLK0.1 as control

Target plasmids were co-transfected with envelope plasmid such as pCMV-VSV-G and pCM-dR8.2, via Transit 20/20 (Mirus) transfection reagent into Lenti-X HEK293t cells. Sixteen hours post transfection, the media was changed to fresh DMEM (10% FBS/1% penicillin streptomycin). Media containing virus was harvested after 24 hrs lentiviral infection to infect AsPC-1 cells. Positively infected AsPC-1 cells were then selected for with 5 mg/ml puromycin for 3 days by kill-curve analysis. Knockdown of CLP36 was confirmed with western analysis and Coomassie staining. AsPC-1 cells with

CLP36 knockdown were fixed for confirmation through cell imaging. Transfection efficiency of CLP36 was optimized in AsPC-1 with a 3:1 ratio using Mirus Transit IT 2020 reagent in reduced serum media- OptiMEM (GIBCO).

Cell Lysis and Western Blot Analysis

Cell extracts were lysed in RIPA buffer containing 1:100 Protease inhibitor cocktail, 1:100 RNase, 1:100 phenylmethylsulfonyl fluoride (PMSF), 1:1000 Aprotinin. Concentration of proteins was determined using Bio-Rad Bradford assay. Proteins were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membrane (Bio-Rad). After incubation with 5% milk in phosphate buffered saline with Tween-20 (PBST), the blots were probed with primary antibodies. The antibodies used were rabbit polyclonal PDLIM1/CLP36 1:5000 (Proteintech) and GAPDH mouse monoclonal 1:1000 (Proteintech). Fluorescently labeled secondary antibodies used were 680 nm Goat-anti-mouse (1:10,000), 800 nm Goat-anti-rabbit (1:10,000) (Licor). Signals were detected and quantified on the LI-COR Infrared Imaging System.

PCR

Total RNA of ASPC cells was isolated with a TRIzol reagent. Reverse transcriptase-GoScript Reverse Transcription system (PROMEGA) was used to create a cDNA library.

PCR cloning was performed on the cDNA library using a ViiA7 Realtime PCR System (Applied Biosystems). Primer sequences used here:
5'-AAAAAAGTCGACATGACCACCCAGCAG-3' (mCherry N1),

5'-AAAAAAACCGGTCGCTTGGGGAACACAG-3' (mCherry N1)

PCR purification was done using QIAquick PCR purification kit (QIAGEN), PCR product ran on an agarose gel to confirm and extract bands using glass milk or QIAquick gel extraction kit (QIAGEN).

Constructs

Restriction digest was performed on vectors pmCherry N-1 and PCR product (CLP36). Ligation was performed using 10X T4 DNA Ligase Buffer and T4 DNA Ligase. Transformation was performed with in-house competent STBL2 *E. coli* cells, and cells were plated on kanamycin plates (50 mg/mL) followed by overnight incubation at 30 °C. Minipreps were performed using glass milk and QIAgen DNA purification kit (QIAGEN). Constructs were sequenced at the Synthesis and Sequencing Facility at JHMI using Sanger Sequencing with the PCR primers. Additional primers were used along with the PCR primers to confirm that the DNA sequences in the constructs are correct.

Forward: 5'-CCAGAGGAGACCAGAC-3'

Reverse: 5'-TGCTCAGAAGTTGCCTATG-3'

After confirmation of correct construct, maxi-prep was performed using glass milk to obtain high concentration of construct.

Cell assays

2D random migration/Scratch wound assay

AsPC-1 cells were plated at a sub-confluent concentration in a 24-well tissue culture plate. Cells were serum starved for 24 hours. After starvation, cells were

scratched using a micropipette tip (3 scratches per well) and stimulated by changing to PANC media. For 16 hours following scratching, cells were tracked using the High content imager (IXM) at the Microscope facility at JHMI.

Transwell assays

AsPC-1 cells were plated in 6.5-mm PET membrane transwell inserts with 8-µm pores (Costar #3464) in a 24-well plate with 5,000 cells per well. Cells were allowed to adhere overnight in PANC media. Cells were washed with PBS, then cell media was changed to serum-free RPMI 1640 to starve cells for 24 hrs (top chamber and well). After starvation, cells were stimulated by changing media to PANC media in the well (bottom part of chamber). Cells were incubated for 24 hrs at 37 degrees C/5% CO_2 and then fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained with 1 µg/ml DAPI. Before imaging, the top chamber was swabbed with a cotton-tip swab and washed to remove cells that did not translocate. A total of five random fields per transwell insert were imaged using a 10X objective (NA), and number of nuclei were counted and averaged.

Immunofluorescence

Cells used were AsPC-1 parental control, knockdowns (A and B) and vector control. Cells were seeded onto collagen coated slides (rat tail collagen 3 mg/mL). Primary antibodies used were 1:1000 Clp36 rabbit 1:1000 (Proteintech), ACTN4 mouse 1:500 (SANTA-CRUZ). Secondary antibodies used were Alexa Fluor 488 goat-anti-mouse (green) (1:500), Alexa Fluor 568 goat anti-rabbit (red) (1:500), Alexa Fluor 647 goat-

anti-mouse (far-red) (1:500). Stained the cells nuclei with DAPI (1:1000) and fluorescein isothiocyanate- (FITC) phalloidin stain for actin (1:500) using the LEICA spinning disc and Zeiss 510 laser-scanning microscopes.

Live-cell microscopy

Cells were isolated and plated onto a collagen-coated 35-mm dish and cultured for 24 hrs before imaging. Time-lapse images were acquired with a 3I spinning disk microscope equipped with a heated chamber and CO_2 controlling unit. Time-lapse sequences fluorescence images were collected at three stage-position sites every 10 min for 16 hr.

Statistical analysis

Statistical analysis was performed using KaleidaGraph (Synergy Software). Significance of differences were identified using ANOVA with a Fisher's least significant difference (LSD) post-test.

Results

To determine what cell line to use for my project, western analysis was performed to assess CLP36 expression in different cell lines. The human-derived lines included HPDE (immortalized Human pancreatic ductal epithelial cells), Panc10.05 (stage II pancreatic adenocarcinoma-derived), AsPC-1 (stage IV ascites-metastasis-derived) and HTC116 (colorectal carcinoma). The results suggest that CLP36 is highly expressed in AsPC-P cells and less expressed in HPDE, Panc10.05 and HTC116 cells (Fig. 3).

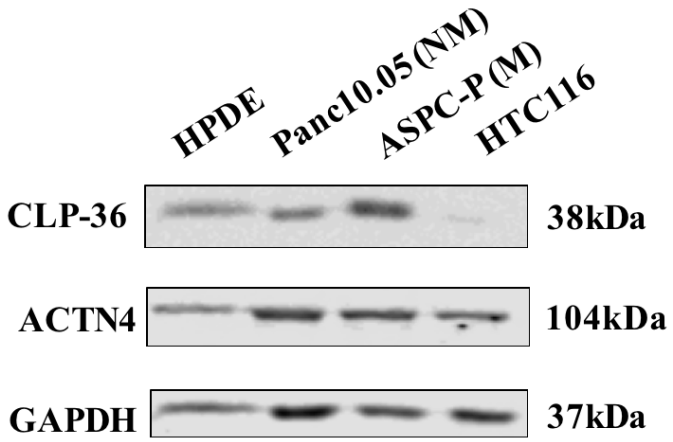


Figure 3. Expression of CLP-36 in Pancreatic Cell Lines
Western analysis was performed with antibodies against CLP36, ACTN4 and GAPDH in different cell lines. GAPDH was used as a loading control. The pancreatic cell lines used were (HPDE-human pancreatic duct epithelial cell line, Panc10.05-non-metastatic pancreatic adenocarcinoma, AsPC-P-metastatic pancreatic adenocarcinoma) and HTC116-colorectal carcinoma. (NM: non-metastatic, M: metastatic)

To test CLP36 as an important regulator of cell migration, I performed immunofluorescence on AsPC-1 cells to determine the localization of endogenous CLP36 when the cells are seeded on collagen for higher adhesion to the cell culture surface. CLP36 localizes to cell edges, while actin appears all over the cell and cell edges. Actin shows a stronger fluorescence at the areas where CLP36 is also enriched (Fig. 4).

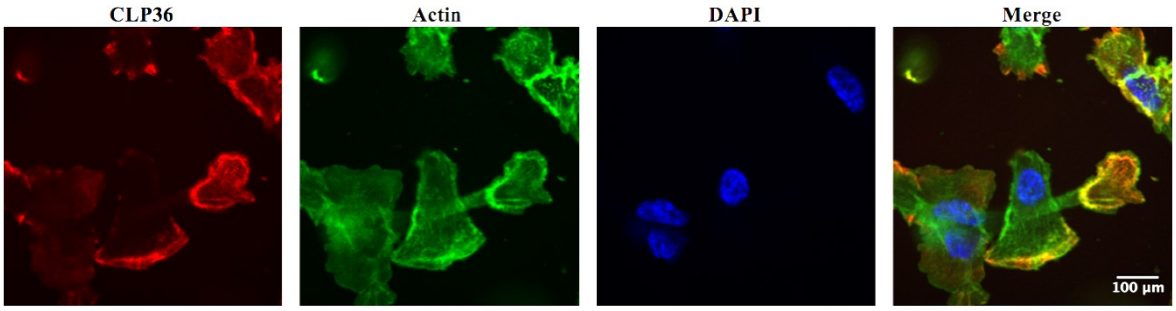


Figure 4. Localization of Endogenous CLP36
Immunofluorescence microscopy of ASPC cells fixed on collagen and labeled with CLP36 (red), FITC phalloidin for actin (green) and the nuclei (blue) were labeled with DAPI.

CLP36 was knocked down using shRNA in AsPC-1 cells to test whether CLP36 is necessary for cell migration. Western analysis showed that shRNA knockdown was accomplished by shRNAs "A", "B", "D" and "E" (refer to methods) (Fig. 5).

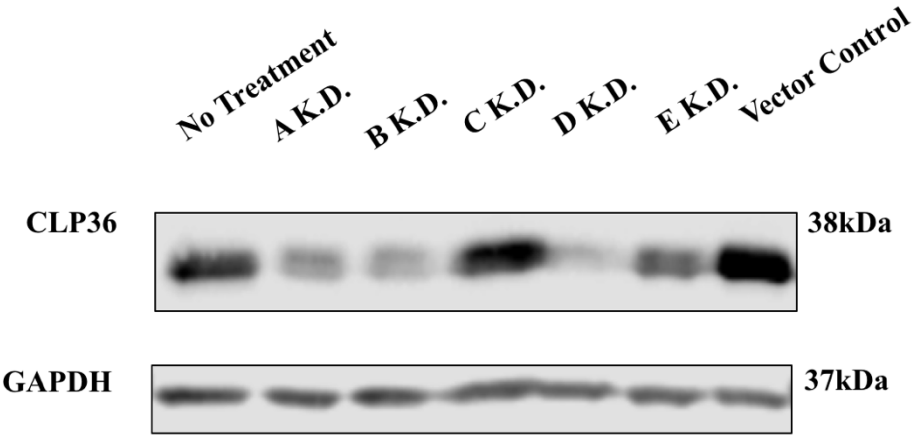


Figure 5. Assessing efficiency of knockdown
Western analysis using antibodies against CLP36 to evaluate knockdown of CLP36 by several shRNAs (A-E). GAPDH was used as a loading control.

The shRNA constructs "A" and "B" were selected for future experiments because they effectively knocked down CLP36 and are the untranslated region (UTR) and the coding region (CDS), respectively. The untranslated region of mRNA is involved in gene expression and can contribute to mRNA stability and protein translation. However, the

coding region, as the name implies, directly encodes the proteins. Thus, while both are useful for studying the loss of function phenotypes, shRNAs that target the UTR can then be used for rescue experiments with plasmids that can encode the protein. Such future rescue experiments are invaluable to ensure that the observed effect is due to knockdown of CLP36 and not due to off-target effects. Further confirmation of knockdown was assessed by immunofluorescence staining of CLP36. Actin and the nucleus were also stained. Cells treated with shRNAs "A" and "B" both have less fluorescence intensity for CLP36 as compared to AsPC control (no treatment) and the negative/vector control (Fig. 6).

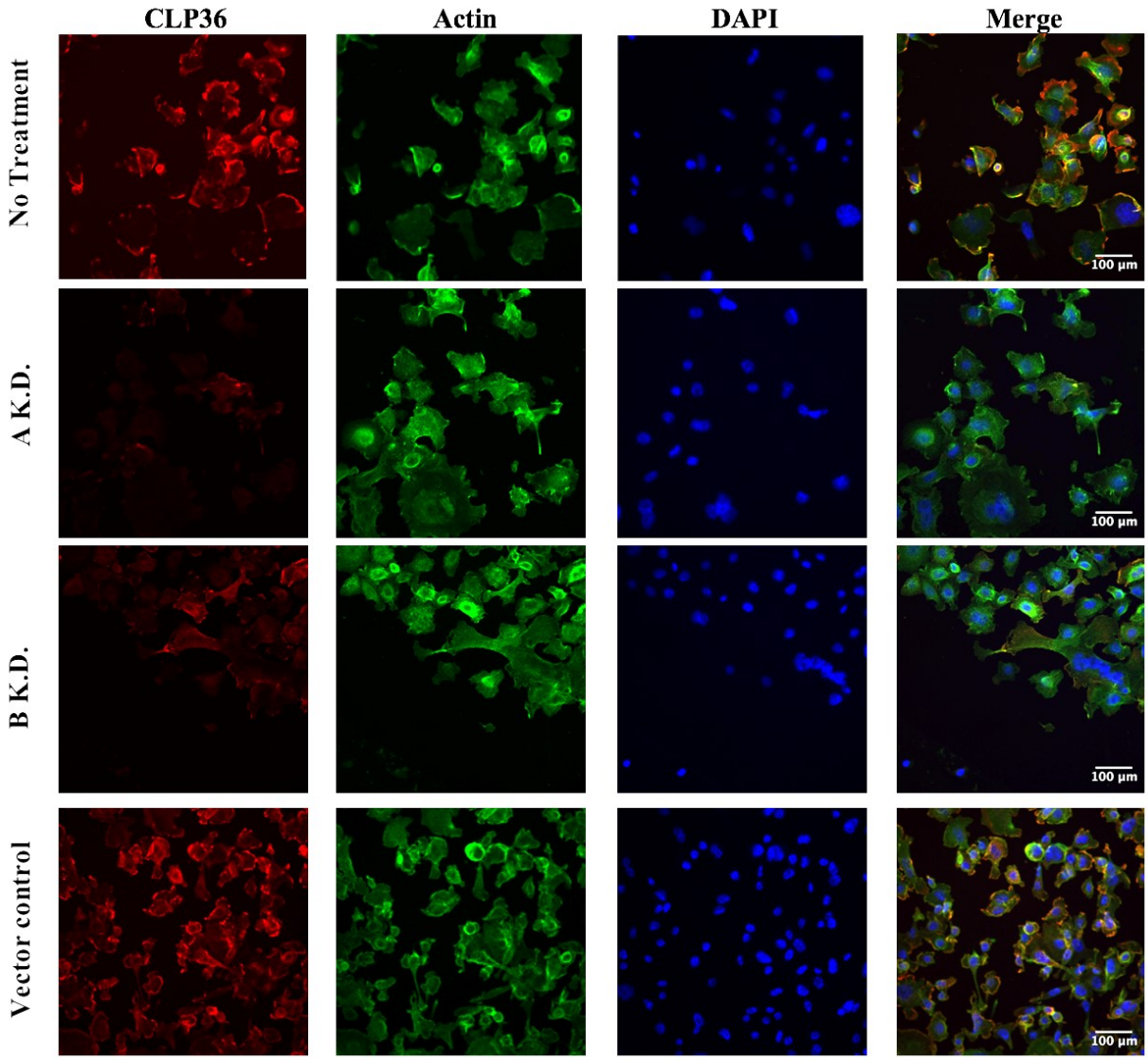
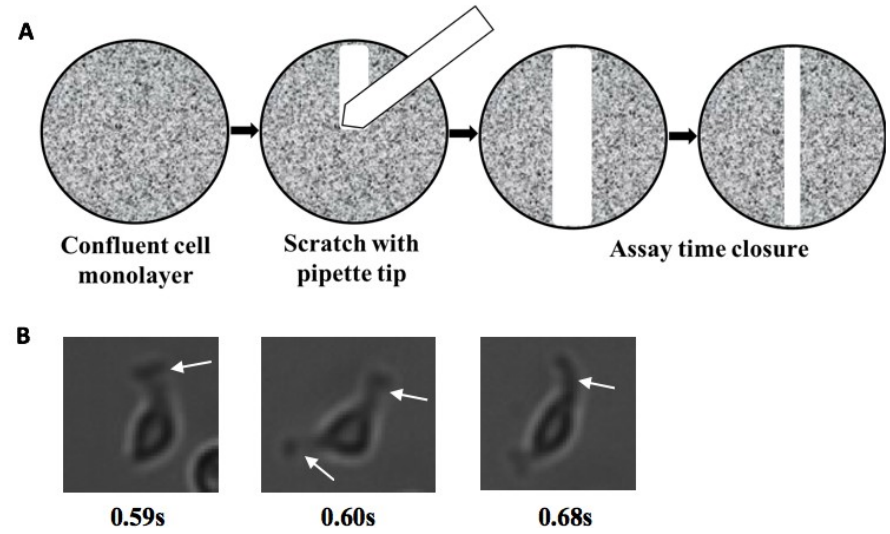


Figure 6. Knockdown of CLP36 in AsPC cells
Immunofluorescence of fixed cells on collagen stained with CLP36 (red). Fluorescence of actin (green) and DAPI (blue).

To test whether knockdown of CLP36 affects migration in AsPC cells, scratch wound assays were performed (Fig. 7a), and cellular morphology, such as the formation of lamellipodia, and the ability of the cells to migrate, was analyzed (Fig. 7b). Both knockdowns "A" and "B" showed significantly decreased average distance traveled by the cells (Fig. 7c). Average distance traveled by shRNA A treated cells compared to

control was decreased (p<0.0002). ShRNA B also traveled less than the control (p<0.0005). When shRNAs A and B were compared to the vector control, the distance traveled decreased (p=0.005, p=0.0095) (Fig. 7c).



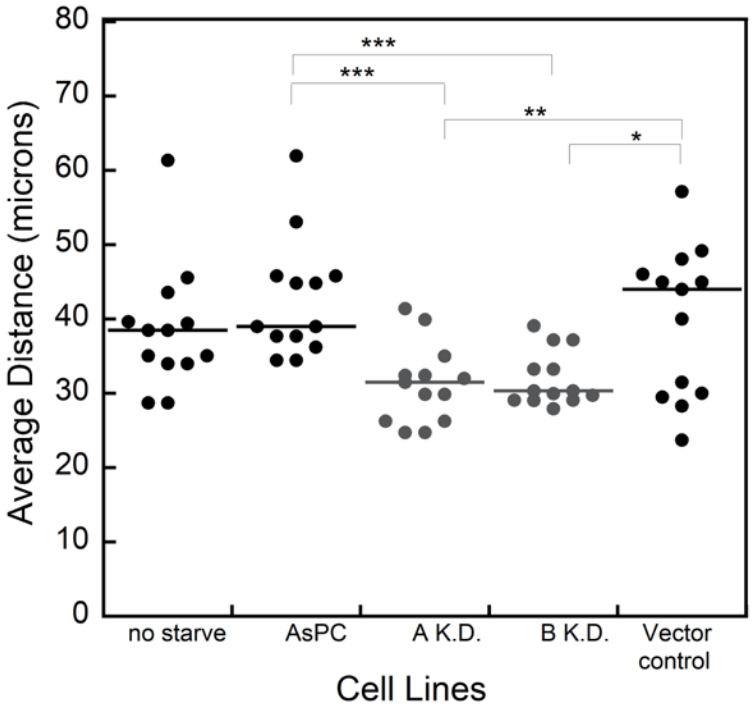


Figure 7. Scratch Wound Assay: Average Distance of CLP36 Knockdowns

(A) Diagram representing scratch wound assay. (B) Morphology of migrating cells from the time-lapse microscopy of the scratch wound assay (zoomed in picture showing cells protrusions), the white arrows are showing lamellipodia. (C) Dot plot showing the average distance moved of AsPC cells in different conditions, each dot represents the average distance of 5 cells randomly selected per well (n=13). ANOVA with Fisher's Least Significant Difference post hoc test: * p=0.0095, **p=0.005, ***p=0.0005, ***p=0.0002

In the transwell assays, the cells with shRNA B traveled less through the pores as compared to the vector control (p=0.0228) (Fig. 8b). CLP36 cells migrated more than shRNA B, which was not quite significant, but the assay still showed that less cells migrating through the pore in the shRNA A (p=0.0522). Although the p value is not as significant, it is still close to p<0.05 and ultimately the cells with CLP36 knockdown traveled less through the pores (Fig. 8b).

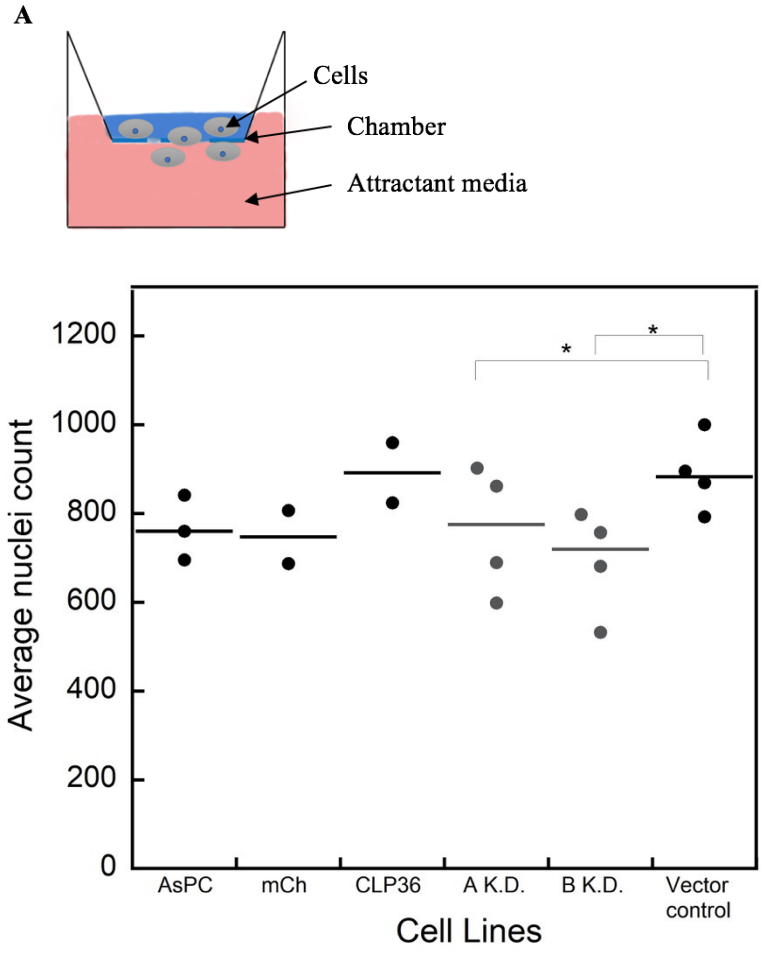


Figure 8. Transwell Nuclei Count
(A) Diagram of the transwell insert apparatus used to measure cell invasion. (B) Dot plot representing transwell assay cell migration. Five areas in the membrane chamber were selected per chamber (n=4). Cells were stained with DAPI to count the nuclei. AsPC is a control, mCherry N1 was selected as a vector control and CLP36-mCherry was transfected into AsPC cells. "A" and "B" are the knockdowns, while "F" is the control. *p=0.0522, p=0.0228

To further study the localization of alpha actinin 4 and CLP36, live-cell imaging was performed. CLP36 and ACTN4 both localized at the same cell edges when the cell was migrating. CLP36 had higher expression in the cytosol than ACTN4. As the cells traveled on collagen, they made protrusions where both CLP36 and ACTN4 accumulated at the cell edge (Fig. 9a). The fluorescence was quantified by placing a yellow line across the cell. The intensity of fluorescence increased on the cell edges. Less fluorescence was observed where the cell is flatter and in the nucleus (Fig. 9b).

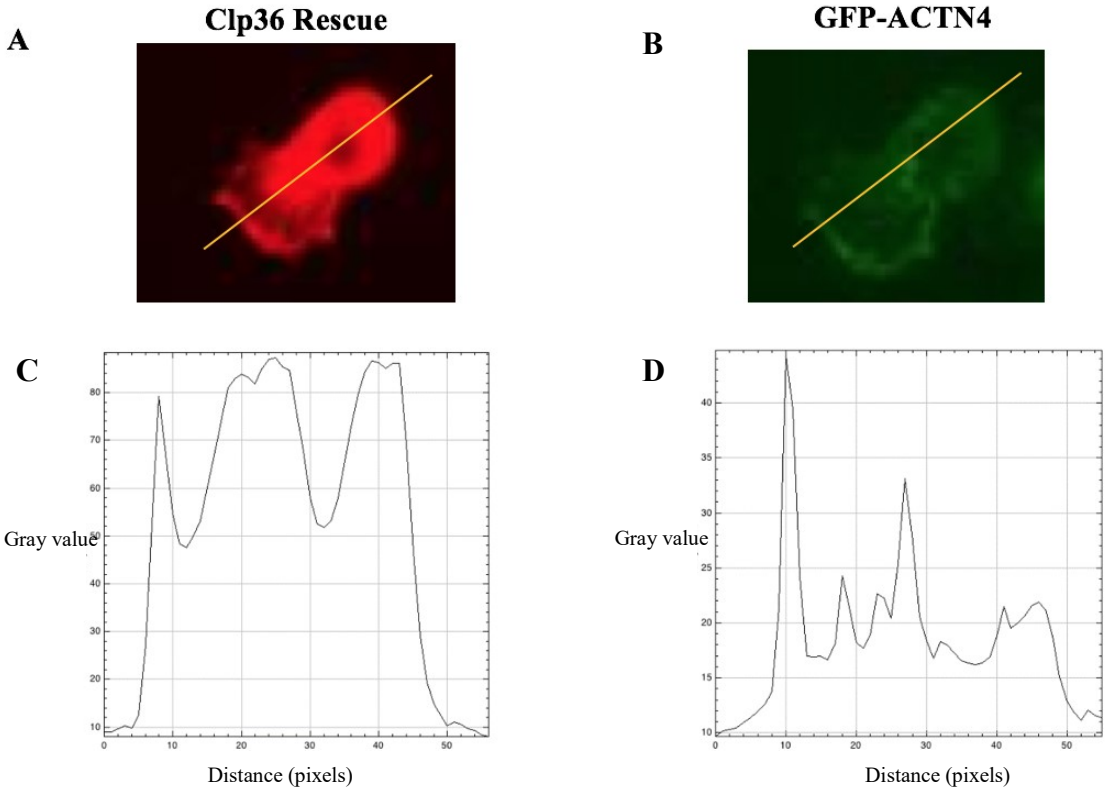


Figure 9. Live Imaging
(A) CLP36 knockdown cells were transfected with mCherry-N1-CLP36 and (B) GFP-ACTN4 and imaged. Cell is shown at 1.10 seconds. (C, D) Quantification of intensity of the fluorescence.

Discussion

Metastatic spread of cancer cells is one of the leading cause of mortality in patients with pancreatic cancer. Cell migration is of huge interest in different cancer cells, and recent studies have shown that CLP36 expression was increased in breast cancer progression [59]. CLP36 in breast cancer progression lead me to want to study CLP36 in metastatically derived pancreatic cancer cells.

Here I show that CLP36 is overexpressed in AsPC-1 cells as compared to non-metastatic pancreatic cancer cell lines, and CLP36 affects cell migration. These findings suggest that the adaptor protein CLP36 is necessary to promote cell migration. Furthermore, the observations imply that targeting proteins that are overexpressed can have significant effects on cell migration which can affect metastatic load and ultimately patient survival. Additionally, other studies have shown that CLP36 binds alpha-actinin-4 [2], a protein that has been associated with cellular motility and cancers [8]. Although there are interactions between CLP36 and alpha-actinin 4, it is unknown if the increase of CLP36 expression is involved in the progression of pancreatic cancer. The results demonstrate that CLP36 is an important influence for some pancreatic cancer cell migration *in vitro*.

The scratch wound assay is one of the simplest ways to study cell migration, as it was useful in determining migration ability of cells or as whole cell masses. In this case, it was used to observe individual cell morphological characteristics and migration. The analysis of the scratch wound assay results shows that AsPC-1 cells migrate towards each other as if to close an open area. When CLP36 is knocked down, migration decreased, indicating that CLP36 helps the cells migrate. Measuring the distance migrated by cells

with CLP36 knockdown and compared to the controls, revealed specific migration changes and migratory phenotypes. The phenotypes observed in control cells were lamellipodia and cell protrusions, while in knockdowns the cells did not have protrusions and stayed in a sphere-like shape, not allowing the cells to migrate as much when compared to control cells. While the control cells formed lamellipodia where the protein actin was projected on the leading edge, it allowed the whole cell to propel and migrate against the culture environment. The cells with CLP36 knocked down appeared to protrude less and stayed more as a sphere. Given that higher expression of alpha-actinin 4 functions in pancreatic cancer cell migration, it will be interesting to determine whether increased level of alpha-actinin 4 is affected by lower protein levels of CLP36.

The transwell migration and invasion assays helped to assess the ability of single cells to migrate when CLP36 is knocked down. Fewer cells traveled through the porous membrane when CLP36 was knocked down, suggesting that CLP36 has a role in cell migration. The control (shControl) cells were unaffected in their ability to migrate through the pores. Since knocking down CLP36 reduced cell migration through the porous membrane, I hypothesize that a decrease in cell migration is happening because of the interaction between CLP36, actin, ACTN4 or another unknown protein interaction. One interesting open question is “does CLP36 interact directly with actin or other cytoskeleton proteins?”

In confocal images, CLP36 and actin were concentrated and co-localized in the leading edges of the pancreatic cancer cells (Fig. 4), suggesting that CLP36 has a role in cell polarization. For the cells to move with a specific direction, they must be organizing

their cytoskeleton with the interaction of CLP36 to generate protrusions in the front and retraction in the rear.

Future Directions

Further studies should focus on protein interactions between proteins involved in cell migration, providing light onto cell migration and how cell migration can lead to cell metastasis. CLP36-mCherry C1 should be created to assess if the N1 tag position affects the protein itself and then compare both constructs to assess if there are any differences in localization, expression, and if it affects endogenous CLP36. Other cell lines like non-metastatic pancreatic cells should be tested to test whether CLP36 promotes cell migration more generally. mCherry-N1-CLP36 should be transfected into other cell lines, specifically in non-metastatic cells to determine if overexpression of CLP36 is sufficient to promote migration in non-metastatic cells. All the migration assays should be performed in different culture environments, including 3D. Additionally, the knockdowns should be rescued by expressing the CLP36 to see if migration is restored, thereby helping to confirm that the defect is directly due to loss of CLP36. The ability to rescue the knockdown will also allow for mutated versions of CLP36 to be tested. Possible mutants to test could include mutations that disrupt the alpha-actinin binding sites in CLP36 and perhaps a deletion of the PDZ domain. These mutants can then be used to test for their ability to interact with alpha-actinin 4 in pancreatic cancer cells. If the deletion of alpha-actinin 4 binding domain affects the ability of CLP36 to promote pancreatic cancer cell migration, this result would suggest that the binding of alpha-actinin 4 to CLP36 is essential for the protein's function.

Cell migration and invasion are important for metastasis in pancreatic cancer. To test whether CLP36 influences metastasis *in vivo*, short hairpin RNA lentiviral vectors that knockdown CLP36 can be used to create stable knockdown of CLP36 expression on pancreatic cancer cells (*e.g*. AsPC-1s or a mouse-derived pancreatic cancer cell line). The metastatic potential of the pancreatic cancer cells expressing different levels of CLP36 could be analyzed for their ability to form metastases using mouse metastatic models. Such models include the hemisplenectomy assay, which tests the ability of the cells to form liver metastases, a primary site for pancreatic cancer metastasis formation. In the future, these studies may suggest possible therapeutic approaches based on targeting CLP36 and alpha-actinin 4 that may be an effective intervention against pancreatic cancer.

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ELEANA PARAJON
786-541-4992 eparajo1@jhu.edu
3215 North Charles Street Apt. 506 Baltimore Maryland 21218
Birth Place: Pinar Del Rio, Cuba

EDUCATION

Johns Hopkins University May 2018
Bachelor's Degree in Biology
Master's in Molecular and Cell Biology May 2018

Miami Dade Honors College May 2015
Highest Honors and Distinction: Associate in Arts (Biology), GPA: 3.89

Westland Hialeah Senior High School May 2013
Superintendent's Diploma of Distinction (Cum Laude), GPA: 4.5

WORK EXPERIENCE

Graduate Researcher: Robinson Lab at Johns Hopkins Medical Institute Oct'16-current
- Pancreatic cancer research
- Test aspects of the biophysical properties of the cytoskeleton and the relationship between the cell's structural and regulatory machinery

Pediatric Intern/Research: Johns Hopkins Medical Institute: Neonatal Intensive Care Unit May'16-July'16
- Summer Internship Program 2016
- Research project funded by HCOP
- Full time position for 10 weeks
- Poster presentation "Leptin in Newborn Rat Pups Increases Hypercapnic Ventilation and Activates Respiratory Related Neurons in the Brain Stem"
- Leptin hormone in neonates and respiratory ventilation (hypercapnia)

Research Intern: United States Department of Agriculture (USDA) June'15 – Aug'15
- Bio control on invasive air potato plant in Florida
- Prepared a poster that was later presented at Miami Dade College Symposium

Tutor: STEP UP, Miami Dade College North Campus Jan'15 - Aug'15
- Tutored students in Calculus and Chemistry courses
- Met with faculty mentors to discuss classroom lectures and coursework

Student Assistant: Miami Dade College North Campus May '14 - Dec '14
- Prepared necessary solutions and reagents for the laboratory class
- Work with Professors to prepare laboratory class for the students and maintain a safe environment for class

EXTRACURRICULAR EXPERIENCE

Member: Tri Beta Biological Honor Society Dec '17
- Rho Phi chapter
- Honor and professional society for students of the biological sciences. Activities are designed to stimulate interest, scholarly attainment, and investigation in the biological sciences, and to promote the dissemination of information and new interpretations among students of the life sciences.

Mentor/Intern: MERIT Health Leadership Academy Feb '17
- Ultimate goal is to eliminate health care disparities by transforming underrepresented high school students into health care leaders

Member: The Visionaries April '16

Member: American Red Cross Corp at Hopkins April '16
- Active volunteer organization that works under the broader American Red Cross Central Maryland Chapter and the Center for Social Concern at the Johns Hopkins University.

Member: Eclectics Aug' 2015
- Co-ed student-run dance group that works with a wide variety of dance styles

Member: Florida-Caribbean Consortium for Agriculture Education & Hispanic Workforce Development Sept'14-15
- Took horticulture class, farmed for experience and provided produce to local homeless shelters
- Research opportunities with USDA agencies and participated in a convention related to the field
- Member of Minorities in Agriculture, Natural Resources, and Related Sciences (MANRRS)

Director (Honors in Action): Phi Theta Kappa-Mu Epsilon Chapter — June'14 – May '15
- Created an action project and discussed with members to make a positive impact on the environment
- Performed cleaning drive in Miami City

Secretary: Pre-Medical and Pre-Pharmacy Club — Aug'14 – July'15
- Provided students a forum for finding opportunities in the field

Senator: Student Government Association — Aug'14 – May'15
- Helped create a stronger environment through civic engagement

STEM Ambassador — Aug'13-Jun'2015
- Teach young students about Science, Technology, Engineering, Mathematics

Varsity Cheerleading — June'11-June'13
- Main back spot for stunts
- Contributed to choreography

Dance — June'11-June'13
- Performances all year-round in high school
- Won second place in high school talent show

Varsity Volleyball — May' 10-May'13
- Main hitter, Most improved player award

AWARDS

Dean's List	Johns Hopkins University	Fall 2017
Gail J. McGovern Scholarship	Johns Hopkins University	Fall 2016
Dean's List	Johns Hopkins University	Fall 2015
Dean's List	Miami Dade College	Fall '13-Summer '15
Scholarship Fund	Hispanic Scholar	June 2015
Transfer Scholar	Jack Kent Cooke Foundation	May 2015
Presidential volunteer service award	Miami Dade College	July 2014
Florida Medallion Scholarship	Miami Dade College	Aug'13-May' 15
American Dream	Miami Dade College	Spring 2014

CERTIFICATIONS

- Certificate Cardio Pulmonary Resuscitation /AED — Nov '16-18
  - Training Center: The Johns Hopkins Hospital
- Certified Nursing Assistant — May 2013
- Certified Medical Administrative Assistant — April 2012

VOLUNTEER EXPERIENCE

- Hopkids: Children Center (David M. Rubenstein Child Health Building) — Sept '16-Dec '16
  - Pediatric Specialty Clinic
  - Harriet Lane Clinic
  - 18 hours
- Barclay Elementary School 1st grade — Jan'-April 2016
  - 35 hours
- Miami Dade College — Aug' 2013-15
  - North Campus Biology Lab General Prep: 20 hours
  - North Campus Single Stop: 20 hours
  - North Campus Letter Writing Workshop: 17 hours
  - North Campus Peer Tutoring: 67.5 hours
  - North Campus Lake Rejuvenation Project: 12 hours
  - North Campus the Palmetum: An Outdoor Learning Environment: 52 hours
- Day Care: 150 hours — 2011-2013

LANGUAGES

- English (Fluent)
- Spanish (Fluent)