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THE ROLE OF PHLPP IN PANCREATIC CANCER

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THE ROLE OF PHLPP IN PANCREATIC CANCER

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By

Alena Jeanne Smith
Lexington, Kentucky

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2015

ABSTRACT OF DISSERTATION

THE ROLE OF PHLPP IN PANCREATIC CANCER

Medicine has come a long way in recent years with reliable treatments for many cancers. Pancreatic ductal adenocarcinoma (PDAC) has very few treatment options available. PDAC has a dismal 5 year survival rate of 4% and a median survival span of 6 months from point of diagnosis; with a high rate of chemotherapy and radiation resistance. A better understanding of the molecular events leading to cancer progression is needed in order to improve the treatment and prognosis of PDAC patients. We begin to elucidate the functional importance of PHLPP on suppressing progression and metastasis of PDAC. PHLPP belongs to a novel family of Ser/Thr protein phosphatases. Our previously published studies have demonstrated that PHLPP plays a tumor suppressor role in colon cancer by negatively regulating Akt and inhibiting cell proliferation. To determine the effect of PHLPP on cell migration and invasion, stable cells were generated to knock down or overexpress PHLPP in PDAC cells. The ability of cells to migrate and invade was examined using Transwell assays. We found that increased PHLPP expression significantly reduced the rate of migration and invasion in PDAC cells whereas knockdown of PHLPP had the opposite effect. To begin to elucidate the molecular mechanism underlying PHLPP-mediated inhibition of migration and invasion in PDAC cells, we discovered that the expression level of β4 Integrin was decreased in PHLPP overexpressing cells and increased in PHLPP knockdown cells. The increased expression of β4 Integrin has been shown to promote PDAC development and metastasis, although the mechanism leading to β4 Integrin upregulation is less clear. Interestingly, we found that the expression of β4 Integrin was highly sensitive to PI3K/Akt/mTOR activity in cells in which inhibition of PI3K/Akt/mTOR signaling significantly decreased the expression of β4 Integrin. Moreover, the quantitative real-time RT-PCR analysis revealed that the mRNA expression of β4 Integrin was not altered by changes in PHLPP expression or PI3K/Akt/mTOR activity,

thus suggesting a post-transcriptional mechanism. Taken together, these results identify a tumor suppressor role of PHLPP in PDAC. Mechanistically, PHLPP suppresses PDAC cell migration and invasion by negatively controlling β4 Integrin expression through its ability to inhibit PI3K/Akt/mTOR signaling.

KEYWORDS: pancreatic cancer, PHLPP, phosphatase, Akt, integrin

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THE ROLE OF PHLPP IN PANCREATIC CANCER

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This doctoral dissertation is dedicated to the two most precious little beings in my life: Selena & Dorian. May you embrace dignity, live with respect, thirst for knowledge, and always seek truth!

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Chapter 1. Introduction

The Pancreas

The pancreas is a unique organ that is physically situated behind the stomach. The word pancreas itself is derived from the Greek roots “pan” meaning “all” and “creas” translated to “flesh” [1]. It is considered part of both the exocrine and the endocrine system. As a glandular organ the pancreas is responsible for actions within the digestive system as well as regulating sugar homeostasis in the blood.

The organ itself is around 15-25 cm in length and weighs approximately 70-150 grams [1, 2]. The head of the organ is “nestled” into the duodenum while the body lays behind the stomach and the tail of the pancreas is near the spleen. The main pancreatic duct runs the length of the pancreas, combines with the common bile duct, and empties its contents into the duodenum through the Ampulla of Vater (**Figure 1.1**) [2].

Histology of the pancreas

The pancreas as explained earlier is a unique organ that is both part of the exocrine and endocrine system. In order to achieve this multi-functional and duality role for the pancreas the specialized cells of the pancreas must be arranged in such a way that it allows for separation of function and compartmentalization. Anatomy of both the exocrine and the endocrine portion of the pancreas will be discussed below.

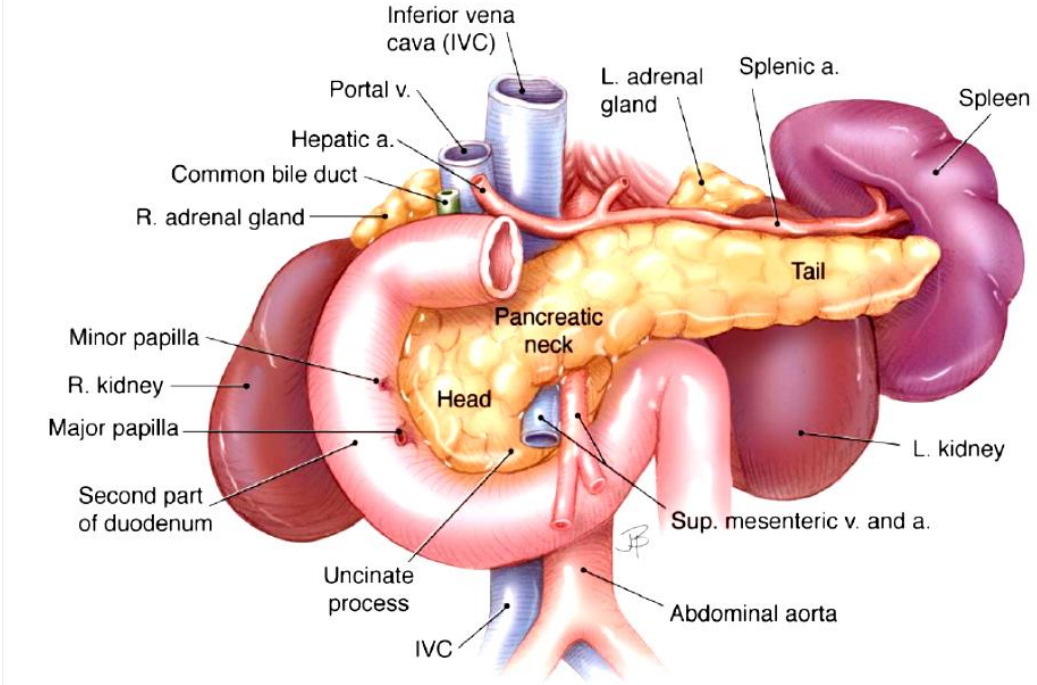

Inferior vena cava (IVC)
Portal v.
Hepatic a.
Common bile duct
R. adrenal gland
L. adrenal gland
Splenic a.
Spleen
Tail
Pancreatic neck
Head
Minor papilla
R. kidney
Major papilla
Second part of duodenum
Uncinate process
IVC
Sup. mesenteric v. and a.
Abdominal aorta
L. kidney

Figure 1.1. Anatomy of the pancreas

The head of the pancreas is nestled into the duodenum while the tail extends towards the spleen (adapted from [2]).

Exocrine Structure

The exocrine portion of the pancreas is responsible for aiding in food digestion. In order for the pancreas to complete this task it must release digestive enzymes into the duodenum. Without the exocrine function then individuals would find themselves in a state of malnutrition [2, 3]. Not only does the pancreas secrete digestive enzymes, but also releases ions and water into the duodenum as well. This is important for a few major reasons. The water helps transport the enzymes as well as the food on into the digestive tract and the ions help to neutralize the pH of the gastric chyme. The alkalinity of the pancreatic exocrine juices is due to the high concentration (up to 140 mM) of $NaHCO_3$ within the secretion. If the gastric chyme was allowed to maintain an acidic pH then the digestive enzymes would lose activity [3].

The exocrine pancreas consists of the acinus and the draining ducts. The acinar cells drain into the acinus ducts which lead to the interlobular ducts. The interlobular ducts then make way to the main pancreatic ductal system. The acini can be of multiple forms, including spherical, tubular, or an in between form. They are tasked with synthesizing, storing, and secreting digestive enzymes. The basolateral side houses receptors for both hormones and neurotransmitters whereas the apical side contains the zymogen granules with the digestive enzymes inside of them [3].

The centroacinar cell is a unique cell within the pancreas in that it acts as a progenitor for different types of cells [3, 4]. Recently, a new cell type called the pancreatic stellate cell was discovered in the pancreas. They are star-shaped

cells believed to be responsible for laying down the basement membrane. These stellate cells are currently being investigated for their role in pancreatic cancer development [3, 5]. Then we have the ductal epithelial cells, which are responsible for the formation of ductal passages within the pancreas as well as where the most common form of pancreatic cancer arises from [3].

The digestive enzymes found within the pancreas are actually capable of digesting the very organ that produces them. Therefore, in order to prevent these harmful events mechanisms such as storage in acidic granules have evolved [3, 6]. Another such prevention is the synthesis and storage of some of these enzymes as precursors. They are found as proenzymes in the pancreas but quickly activate within the duodenum. This activation occurs when trypsin is activated through an enzyme called enterokinase, which removes the N-terminal hexapeptide fragment on trypsin. Trypsin is then free to exert its activity onto the proenzymes. An inhibitor of trypsin called PSTI (Pancreatic Secretory Trypsin Inhibitor) is also synthesized in the secretory pathway of the digestive enzymes as well as the zymogen granules [3, 6].

Amyase is an enzyme that is secreted in both the pancreas and the salivary glands. However, the salivary gland secreted form has a different molecular weight and electrophoretic mobility. This may be attributed to the difference in its carbohydrate content. For both isoforms the enzymatic activity remains the same in that they digest both starch and glycogen. The optimum pH for activity is neutral pH but you will find it active in the stomach acid [3, 7].

Another active enzyme called lipase is primarily secreted by the pancreas. It is capable of hydrolyzing a triglyceride molecule to two fatty acid molecules from carbons 1 and 3. This hydrolyzing reaction also leads to a monoglyceride released with a fatty acid esterified to a glycerol at carbon 2. The interesting part is how the lipase is able to perform its reaction in a water environment when oil and water do not mix. The function of lipase is facilitated by bile acid and colipase. They aid in the binding of lipase to the oil/water interface of triglyceride droplets, where lipase exerts its activity [3, 8]. In addition, phospholipases hydrolyze fatty acid ester linkage at carbon 2 of phosphatidylcholine [3, 8].

Proteases are enzymes that aide in the breakdown of proteins from consumed food. There are two major types of proteases: endopeptidases and exopeptidases. Regardless of type of proteases, all are stored as the proenzyme form inside of the zymogen granules. This keeps them inactive until they are deposited in the duodenum where they are activated by trypsin. Interesting enough trypsin is an example of endopeptidase as well as chymotrypsin. Endopeptidases cleave peptide bonds of proteins internally at specific sites. Exopeptidases however cleave 1 amino acid at a time either from the NH_2 or COOH terminal ends of the protein. Further digestion of oligopeptides and free amino acids occur through the brush-border enzymes. From there they may be transported across the transluminal surface of the small intestine by Na^+-H^+ coupled transporters [3, 9].

Some of the inorganic compounds found in the pancreatic secretions into the duodenum aren’t just water, but contains many ions: sodium, potassium,

chloride, and bicarbonate. They are just as important in the function of the exocrine pancreas as the enzymes themselves. They provide the flow of the enzymes from the acinus to the duodenum as well as allowing for the change of the pH of the gastric chyme from acidic to neutral [3, 10, 11].

In summary, the exocrine pancreas is a beautiful conglomerate of cooperative enzymes and compounds that work in cohesive unity to provide nourishment for a large and extremely diverse organism.

Endocrine Structure

The endocrine system is dispersed throughout the exocrine system and tissue within the pancreas. These hormone secreting cells are grouped together in little areas referred to as islets, which can be translated to as a small island or in anatomy as a distinct portion of tissue surrounded by entirely different tissue. Langerhans believed in 1869 that he was seeing intrapancreatic lymph nodes [12]. This was later disproven however not only are hormone secreting cells present but so are nerve cells as well as the area being highly vascularized [13].

The islets of Langerhans is the distinct tissue that house the hormone secreting cells of the endocrine system within the pancreas. There are around a million islets found in the average pancreas of a healthy adult [13, 14]. Each islet measuring around 0.2 mm in diameter is surrounded by a capsule made of fibrous connective tissue. There are five major cell types found in the islets: alpha, beta, delta, PP, and epsilon [13-15]. The alpha and beta cells in the islet of Langerhans play the principle role in regulating blood glucose levels by the

coordinated secretion of glucagon and insulin, respectively. The alpha and beta cells work in opposition to each other. The activity of alpha cells is stimulated during times of hypoglycaemic conditions. Alpha cells secrete glucagon which exerts a catabolic effect through activation of glycogenolysis and gluconeogenesis within the liver. Compared to the vast knowledge of beta cell structure and function little is known about the alpha cells. This lack of knowledge is primarily due to the low concentration of alpha cells within the islets and the lack of techniques available for isolation and characterization. What is known is that at low levels of glucose ion channels generate action potentials through Na^+ and Ca^{2+}. This electrical activity triggers the release of glucagon. When the concentration of glucose rises these events are inhibited. There are other mechanisms that glucagon release could be controlled by such as through fatty acid exposure but very little is known about these different regulations [13, 15, 16].

The beta cells are the most studied and well-characterized cells that reside in the islet. They typically make up the majority of the cell population as well. These hormone producing cells secrete insulin. In contrast to glucagon, insulin activates glycogenesis or the production of glycogen within the liver. [Kelly] [Eleyat] [Korc] In beta cells the ATP-sensitive K^+ ion channels are open while the voltage gated Ca^{2+} ion channels are closed during resting conditions. In response to an increase in glucose concentration, glucose is diffused across the membrane into the cell through GLUT2 transporters via facilitated diffusion. Glucose undergoes glucose metabolism leads to increased production of ATP.

The ATP/ADP ratio changes and as ATP concentration increases it closes the K^+ ion channels inhibiting the transport of K^+ outside of the cell. As the potassium levels rise inside the cell the voltage gated Ca^{2+} channels are activated allowing the calcium ions to cross the cell membrane into the cell [13, 15, 17]. Once inside they bind to their respective receptors allowing the insulin containing vesicles within the cell to bind to the inside of the cell membrane allowing for the release of the insulin through exocytosis [13, 15, 18] (**Figure 1.2**).

The delta cells found in the islet of Langerhans in the pancreas produce a hormone called somatostatin. One interesting note about somatostatin is that it inhibits the function of both the alpha cells and the beta cells. [[19] Delta cells are thought to only affect the adjacent alpha and beta cells. [20] It is believed that somatostatin is either diffused through the interstitium or transported via the microvasculature of the islet itself[21] PP cells are also referred to as the Gamma cells and make up an even less population of the islets than the delta cells. PP cells secrete a protein called pancreatic polypeptide or PP. The highest concentration of PP can be found towards the head of the pancreas. [22] PP is believed to regulate appetite and food intake. It has been found to be of lower concentrations after eating but significantly expressed in anorexia nervosa patients. [23]The least abundant cell in the islets is known as the epsilon cell. Epsilon cells produce a hormone called ghrelin, which inhibits the release of insulin from beta cells within the islets. It is considered a local regulator of insulin just like PP produced by the gamma cells. Ghrelin is being considered as a possible target for diabetes treatment. [24, 25]

The information presented above is a brief summary of a simplistic overview of the function of the pancreas and it is easy to see how pancreatic cancer is so very catastrophic to the individual.

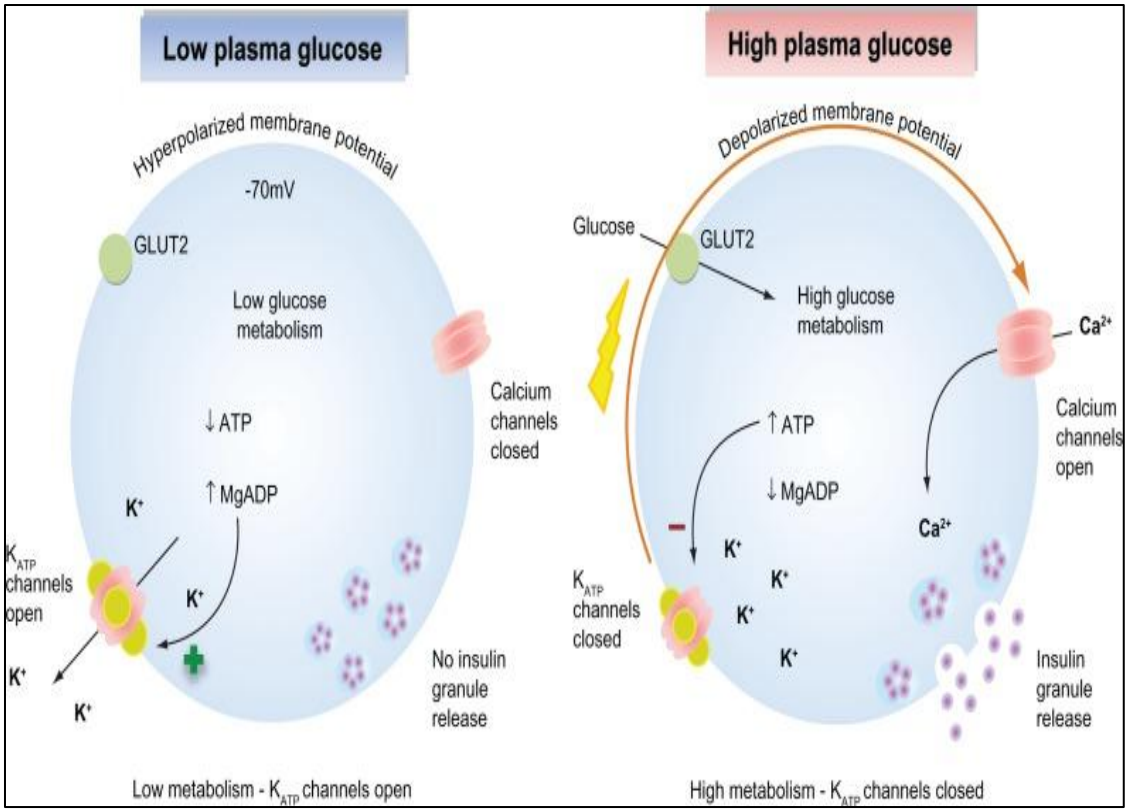

Low plasma glucose
Hyperpolarized membrane potential
-70mV
GLUT2
Low glucose metabolism
↓ATP
↑MgADP
K+
K+
K+
K+
K+ channels open
Calcium channels closed
No insulin granule release
Low metabolism - K ATP channels open
High plasma glucose
Depolarized membrane potential
Glucose
GLUT2
High glucose metabolism
↑ATP
↓MgADP
K+
K+
K+
K+
K+ channels closed
Ca2+
Ca2+
Calcium channels open
Insulin granule release
High metabolism - K ATP channels closed

Figure 1.2. Insulin secretion in beta cells of the pancreas.

While in the normal resting state (low glucose) the K^+ channel is open allowing for the K^+ ions to cross the membrane and travel to outside the cell. As glucose concentration increases it is transported through facilitated diffusion by the GLUT2 transporter into the beta cell of the pancreas. From there it goes through glucose metabolism which gives the final product of ATP. As ATP concentration increases it binds to the K^+ ion channels causing them to close. Eventually this membrane depolarization allows for the Ca^{2+} channels to open which gives an influx of Ca^{2+} ions. This leads to the vesicles containing insulin to bind to the cell membrane releasing insulin through exocytosis (adapted from [18]).

Risk Factors and Pancreatic cancer

Before we get to the heart of pancreatic cancer we need to look at some risk factors. Three major risk factors have the capacity to affect a large majority of the American population in various ways: smoking, diabetes, and pancreatitis.

Looking at the 2013 statistics for smoking in the United States roughly 18 out of 100 adults smoked which translates to 42.1 million adults who smoke. This preventable epidemic kills around 480,000 Americans each year [26]. Since the act of smoking doubles the risk of pancreatic cancer and around 20-25% of all pancreatic cancers are caused by smoking, it is imperative that the link between the two be understood [27]. Other studies have demonstrated that smoking increases the expression of oncogenes in specific tissues, such as the lung. This includes mutation in the KRAS gene. As we will see later KRAS is found activated in 90% of all pancreatic cancers. Understanding the mechanism on activation of KRAS is also of the utmost importance and it may be linked to smoking [28]. However, it should be noted that the majority of studies linking smoking to pancreatic cancer has epidemiological [27, 29]. There has been a few interesting studies to note that in pancreatic cancer patients who were smokers not only did they harbor more mutations but also a larger variety of point mutations [29].

Diabetes mellitus or diabetes is a collection of disorders that give enhanced blood sugar levels for a sustained time period [30]. There are three major types of diabetes: Type I, Type II, and gestational. Type I diabetes is due to beta cell loss in the pancreas, typically an immune destruction [31]. Type II

diabetes is the inability of cells to respond to insulin [32]. Gestational diabetes is an interesting case where pregnant women with no previous history of diabetes develop high blood sugar [33]. Through a large meta analysis it was discovered that 75% of pancreatic cancer patients also carried diabetes mellitus [34]. The risk factors for developing pancreatic cancer through diabetes are most likely through prolonged insulin exposure. Insulin can regulate cell proliferation as a growth hormone. While in diabetes the cells may become resistant to insulin as far as being able to reduce glucose in the blood, the other effects of insulin may still be prevalent. Another action of insulin is to decrease the insulin-like growth factor binding protein thereby increasing insulin-like growth factor or IGF. IGF is an even stronger oncogene than insulin [35]. It is quite easy to understand how preventing diabetes or treating it successfully could have an impact on pancreatic cancer statistics.

Pancreatitis is a disorder characterized by inflammation of the pancreatic organ. One interesting aspect of the relationship between pancreatitis and pancreatic cancer is the genetic mutations found in pancreatitis is also found in pancreatic cancer [36, 37]. Morphological and functional changes within the pancreas can be found in chronic pancreatitis patients. There are some common cytokines that are found in both. These include IL-6, TNF-alpha, IL-8, PDGF, and TGF-beta [38]. Another interesting finding is that macrophages which are regulators of inflammation and secrete pro-inflammatory cytokines are found throughout histological samples of pancreatic cancer as well as pancreatitis [39].

Repeated tissue damage through inflammatory reactions during chronic pancreatitis leads to pancreatic cancer formation [39].

Pancreatic Intraepithelial Neoplasia (PanIN)

Pancreatic Intraepithelial Neoplasia or commonly referred to as PanIN is the step-wise characterization of pancreatic lesions leading into carcinoma. PanINs are found to be more common in pancreata histological sections of pancreatic cancer patients than in chronic pancreatitis; however, they are more prevalent in chronic pancreatitis patients than typical patients [40]. It is important to note that there are two other types of lesions found in pancreatic cancer patients; however, they are significantly less profound and the PanIN lesions are the most common. The other two lesions are called mucinous cystic neoplasm [41] and intraductal papillary mucinous neoplasm (IPMN). Due to the prevalence of PanIN lesions we will focus on those [40].

PanIN development at each stage has been extensively studied. Common gene mutations as well as morphological phenotypical changes have characterized and associated with each stage (**Figure 1.3**) [42, 43]. There are 4 known stages of PanIN: PanIN-1A, PanIN-1B, PanIN-2, and PanIN-3. PanIN-1A and PanIN-1B are considered low-grade lesions that are flat (PanIN-1A) or papillary (PanIN-1B) in nature with typical nuclear phenotypes. PanIN-1A is considered to have tall columnar cells and can be indistinguishable from non-neoplastic lesions. PanIN-1B is basically indistinguishable from PanIN-1A except that it is papillary in nature. PanIN-2 lesions are much more complex with

atypical nuclear characteristics including loss of nuclear polarity, nuclear crowding, pleomorphism, nuclear pseudostratification, and nuclear hyperchromasia. PanIN-3 lesions are considered carcinoma-in-situ with loss of polarity, nuclear atypical, and mitosis. However, PanIN-3 lesions are still found at the basement membrane.

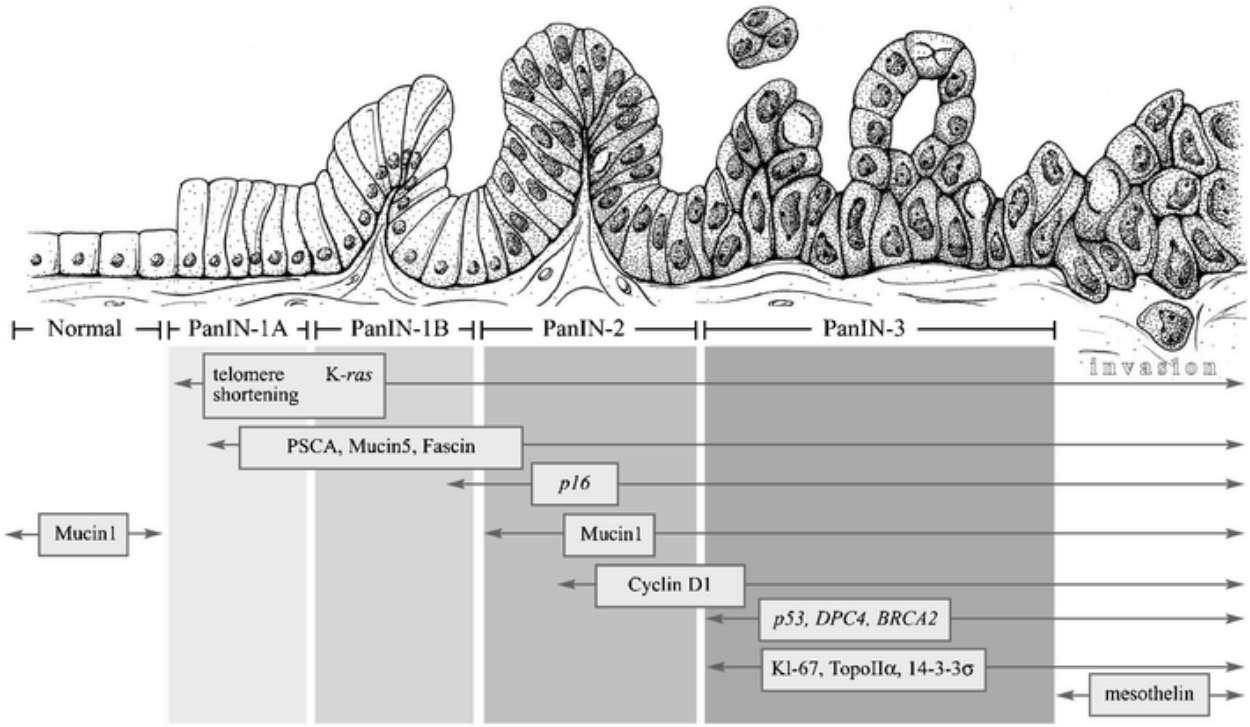

Normal
PanIN-1A
PanIN-1B
PanIN-2
PanIN-3
invasion
telomere shortening
K-ras
PSCA, Mucin5, Fascin
p16
Mucin1
Mucin1
Cyclin D1
p53, DPC4, BRCA2
Kl-67, TopoIIα, 14-3-3σ
mesothelin

Figure 1.3. PanIN development.

There are 4 stages of PanIN: PanIN-1A, PanIN-1B, PanIN-2, and PanIN-3. Each PanIN stage is characterized by known mutations within the pancreatic ductal cells as well as phenotypic changes. This figure is adapted from Koorstra et al. [42].

Many times you can see budding off of small cluster cells, some necrosis, and goblet cell irregularities [42-45]. From this point carcinoma develops where there is invasion and loss of basement membrane localization. Activation of oncogenes such as KRAS and inactivation of tumor suppressors such as SMAD4 and CDKN2A/INK4A allow tumors to progress from PanIN to carcinoma [46, 47].

There have been some recently discovered morphological evidence that gives detail as to how these lesions can cause further damage to the pancreas. Recently, PanIN lesions have been noted to have lobulocentric atrophy. This type of atrophy would be able to decrease acinar cell flow of the secretions raising the possibility of a localized inflammatory reaction. Interesting to note that KRAS mutations were found in these samples [48].

As will be further explained below there have been characterization of the genetic changes that occur during PanIN development. Although a specific order has not been determined it does seem that some mutations occur before others, such as the activating KRAS mutation is set before the p53 mutation [40]. Telomere shortening was recently discovered to be present in PanIN-1 lesions. This could possibly be used as a histological technique to identify patients with chronic pancreatitis who are at risk of developing pancreatic cancer [40].

As we have seen genetic alterations that are found in pancreatitis, PanIN development and pancreatic cancer protein expression profiles are significantly similar between both PanIN lesions and pancreatic cancer. These changes of protein expression range from enhanced expression of survivin and adhesion molecules. Mucins are another protein that is expressed in both PanIN type

lesions and pancreatic cancer but not other types of pancreata lesions. Mucins can be used to distinguish between the different types of lesions and signal to the treating physician that the patient has a heightened possibility of developing pancreatic cancer [40, 47, 49].

Genetic alterations in pancreatic cancer

There are many risk factors currently known that increase the risk of developing PDAC and this includes is but not limited to age, smoking, obesity, diabetes, and pancreatitis [50]. An increased risk for developing PDAC can also be seen in familial clustering indicating that hereditary gene mutations contribute to PDAC development [51, 52]. The Cancer Genome Atlas project has identified a number of genetic alterations in human pancreatic cancer. Table 1.1 shows the most common mutations that are found in pancreatic cancer. The functional contribution of these genetic mutations as well as other genetic alterations in pancreatic cancer are discussed in more details below and followed by a separate section on KRAS.

Gene	Chromosome Location	Percentage of cancers with a mutation	Type of Mutation
KRAS	12p13	>90%	Gain of Function
P16INK4/CDKN2/MTSI	9p21	80%	Loss of Function
TP53	17p13	50%-75%	Loss of Function
DPC4/SMAD4	18q21	~50%	Loss of Function

Table 1.1. Common genetic mutations found in pancreatic cancer.

Pancreatic cancer carries some unique common genetic mutations. These interesting mutations may present a variable chemotherapy treatment target that is unique for pancreatic cancer.

P16INK4/CDKN2/MTSI genetic variations in pancreatic cancer

Cyclins, have no intrinsic activity, bind to cyclin-dependent kinases or CDKs and target them to subcellular locations as well as help activate them. When bound with their respective CDKs the complex is called maturation-promoting factor [53]. CDKs are serine threonine kinases that regulate the cell cycle [54]. Taking it from there inhibitors of CDKs are called cyclin-dependent kinase inhibitor proteins or CDKIs or CKIs. There are two families of CKIs: Inhibitors of CDK4 (INK4) and the KIP family. The INK4 family of CKIs house 4 members which include p16 (INK4a), p15 (INK4b), p18 (INK4c), p19 (INK4d). These 4 members are considered tumor suppressors [55]. The gene for p16INK4 is referred to as CDKN2. At another point the protein was also called multiple tumor suppressor gene 1 [41].

INK4a, found on chromosome 9, can undergo a homozygous deletion, an intragenic mutation, and hypermethylation of the promoter [42, 49, 56-58]. The INK4a gene arrests the cell between the G1-S phase through binding to CDK4/6 and inhibiting its action. CDK4/6 binds to cyclin D and becomes an active complex that phosphorylates retinoblastoma protein (pRB). Once phosphorylated, pRB is able to disassociate from the transcription factor E2F1. When E2F1 is freed from pRB it is able to enter into the nucleus and promote transcription of genes that promote cell cycle progression. [59] Therefore, inactivation of p16 leads to activation of CDK4 and CDK6 and subsequent cell cycle progression without the proper checks and balances [55]. It seems that the other INK4 proteins (INK4b,c,d) are not involved in the etiology of cancer

according to current research [55]. Homozygous deletions of INK4a have been found in lymphomas and sarcomas as well as childhood leukemias [60, 61]. Hypermethylation of the promoter of INK4a have also been seen in cancers as well [62].

Mutation of p16 is found in virtually all pancreatic cancers, but also seen in the PanINs: 30% PanIN-1A and 1B, 50% PanIN-2, and 71% in PanIN-3 [42, 63]. It was recently shown that KRAS activation and loss of INK4a locus combined can give rise to ductal adenocarcinoma through progression of PanINs [64]. They are considered coordinating pathways that promote carcinogensis. The coordinating link may be through the relationship of INK4a and constitutively active $KRAS^{G12V}$. The $KRAS^{G12V}$ is most likely the initiating mutation for pancreatic cancer development; however, subsequent deletion of INK4a would confer sustained activity of KRASV12 without interference of any attenuating factors [65]. Attri et al found that around 80% of pancreatic tumors had some sort of INK4a inactivation change. These changes include the intragenic mutation, hypermethylation and homozygous deletion [66]. Future investigations should revolve around INK4a and its possible activity and influence on KRAS as well as how the different types of INK4a mutations could affect the outcomes of pancreatic cancer. Samples from pancreatitis patients could be evaluated for INK4a mutations. If changes in INK4a were present it would give validation that these patients need to be carefully followed for development of pancreatic carcinoma.

TP53 genetic variations in pancreatic cancer

The tumor suppressor p53 (encoded by the TP53 gene) has many roles within the cell, but the major role that is exploited in cancer is its role in the arrest of the cell cycle at the G1/S phase. Through binding of p53 to a specific p53 binding site on the promoter of *gadd45*, it is able to induce transcription. Once GADD45 is transcribed it can interact with PCNA to initiate nucleotide excision repair.[67] There are also some other mechanisms in which p53 activates DNA repair, but those are quite intensive and not necessary for understanding the general role of p53. Apoptosis control is another area of active research concerning p53 [68].

One of the most common genetic mutations found in cancer is actually in p53 [69]. Mutations of p53 at an intragenic site coupled with loss of second allele occurs in greater than 50% of pancreatic cancers [49]. The typical mutation is within the DNA-binding region that contacts the DNA. These type of mutations result in loss of ability for p53 to bind to the DNA and affect transcription of target genes [70]. While it is true that mutant p53 is a loss of function events, there is a caveat that these mutations may cause p53 to act like a dominant negative and exert a type of gain of function. This was shown when sustained expression of p53 lead to increase activation of PDGFRβ (Platelet Derived Groth Factor Receptor beta) [71]. The p53 loss of function mutation tends to occur during PanIN-3, and again correlates it with a late genetic event [72, 73]. It is interesting to note that most of the commonly used pancreatic cell lines all carry p53 mutations [74]. The focus of studying p53 mutations in pancreatic cancer is to

uncover the possibility of new or adjuvant treatments. While these new treatments would most likely require early detection that is why investigation into PanIN development from associated with risk factors would provide so useful.

DPC4/SMAD4 genetic variations in pancreatic cancer

SMAD4 a tumor suppressor and downstream signaling event of the transforming growth factor β (TGF-β) family, is found mutated in ~50% of pancreatic cancers and is a late genetic event like p53 [49, 74]. SMAD4 is involved as a co-SMAD that binds to other members of the SMAD family like SMAD2. Together the complex is able to bind to DNA sequences and affect transcription of targeted genes. The most common mutation is homozygous deletion, loss of an allele coupled with intragenic mutation. SMAD4 accumulates in the nucleus after TGFβ is stimulated. SMAD4 functions to check cell cycle arrest, and to regulate apoptosis [42]. Patients who had resective surgery did significantly better if their tumors expressed wild type SMAD4 [75]. The 5 year survival advantage of patients who expressed wild type SMAD4 was determined to be approximately 20% whereas only 13% survived to 5 years without SMAD4 expression. Although it is currently not known which of SMAD4s activities are biologically advantageous what is known that patients have a poorer outcome without SMAD4 expression warranting further studies in its activities [75].

Genetic alterations in pancreatic cancer could be used as a histological reference in patients who are at risk for developing PDAC. These high risk patients would include pancreatitis patients, diabetic patients, and smokers. Going forward in this study we decided to look a little deeper into oncogenic

aberrations in pancreatic cancer, specifically events that lead to changes in the PI3K/Akt and Raf/MEK/ERK pathways.

EGFR and HER2/neu amplification in pancreatic cancer

The Epidermal Growth Factor Receptor (EGFR) and Her2/neu (ERBB2) are transmembrane receptors belonging to the ERBB receptor tyrosine kinase family [76]. EGFR and Her2 can function as heterodimers in cells. The ligands for EGFR include EGF, TGF-β, amphiregulin, and epiregulin [77]. After ligand binding, EGFR activates two well known oncogenic pathways the PI3K/Akt pathway and the Raf/MEK/ERK pathway. EGFR-mediated signaling is upregulated in many malignancies. This upregulation can come from gene amplification or upregulation of EGFRs respective ligands [78]. An activating mutation in the kinase domain leads to unchecked activity of EGFR in the absence of ligands [79]. The expression of EGFR in the pancreas is typically limited to the ductal cells or the Islet of Langerhans. Overexpression of EGFR is found in 40% to 70% of pancreatic cancers and HER2 overexpression in a smaller subset of cases [80]. Association of EGFR in pancreatic cancer is found in high-grade tumors especially. Therapeutic strategies aimed at targeting both EGFR and Her2 receptors are being developed to treat pancreatic cancer [81-83]. These therapeutic strategies may prove to be non useful as greater than 90% of all advanced pancreatic cancer carries the activating KRAS mutation. KRAS works downstream of EGFR and other receptor tyrosine kinases. Inhibiting EGFR would have little effect on prognosis of advanced patients; however, there is always hope as adjuvant treatment [64, 82].

Hedgehog signaling in pancreatic cancer

It was recently discovered that in approximately 75% of PDAC tumors Hedgehog (Hh) signaling is dysregulated, and specifically there is aberrant Hh ligand expression [84]. Hh signaling is found in embryonic development where different parts of the embryo have different expression of Hh ligands allowing for polarity to occur. There are three secreted ligands: sonic hedgehog (SHH), Indian hedgehog (IHH), and Desert Hedgehog (DHH). These Hh ligands signal through binding to a receptor called Patched or (PTC); however, without Hh ligands PTC binds to Smoothened or SMO and represses its activity. When Hh binds to PTC the repression of SMO is released allowing for the Gli nuclear family of transcription factors to accumulate. Gli transcription factors activate factors that allow for proliferation such as cyclin D and evasion of apoptosis such as BCL2 [44, 85]. Activation of Hh signaling promotes PDAC via two different mechanisms: i) Hh ligands are over-expressed in the tumor microenvironment, which leads to hyperactivation of Hh signaling in the pancreatic tumor cells; and ii) there is ligand-independent activation in the tumor epithelium as the result of mutations of the signaling molecules (such as PTC) in the Hh pathway. Aberrant Hh activity is found throughout the entire PanIN progression and into carcinoma [84].

Wnt/β-catenin signaling in pancreatic cancer

The Wnt/β-catenin pathway is used in embryonic development similar to Hh signaling. Briefly, Wnt ligands bind to Frizzled receptors which induce inactivation of specific cytosolic proteins such as APC that promote β-catenin

proteosomal degradation. Without degradation, β-catenin accumulates in the cytosol then translocates to the nucleus where it promotes transcription of proteins through binding to TCF/LEF factors [44]. Both cytosolic and nuclear accumulation of β-catenin has been observed in pancreatic cancer. Inhibiting β-catenin through RNAi severely limits proliferative ability of PDAC. β-catenin accumulation in pancreatic cancer may be due to over-expression of Wnt ligands, but at this time the mechanism remains poorly understood and it isn't clear which β-catenins targets are important forpancreatic cancer development [44, 86]. It is interesting to note is that Wnt signaling appears to be tissue specific for example, in melanoma, Wnt inhibits Raf/MEK/ERK signaling, but in both colorectal and pancreatic cancer it activates the oncogenic pathway [86].

Oncogenic signaling mediated by KRAS

KRAS (Kristen-RAS), a member of the RAS family GTP-binding proteins (GTP-ase), is the most commonly mutated oncogene in PDAC. The RAS family is known to mediate a large number of important cellular processes such as proliferation, cell survival and differentiation. RAS proteins are not very efficient at converting GTP to GDP; therefore, they require a GAP (GTPase Activating Protein) such as RasGAP (RASA1) to convert GTP to GDP quickly. After GTP is then hydrolyzed, the nucleotide must be released from KRAS. A guanine nucleotide exchange factor or GEF like Son of Sevenless (SOS) takes over this responsibility releasing the GDP to the cytosol and freeing KRAS to bind to another GTP.

The most common mutations in KRAS are activating point mutations within codon 12. These point mutations are found in around 30% of early PDAC lesions but are present at 95% to 99% of advanced cases of PDAC [50, 87]. The activating mutations desensitize KRAS to GAPs or prevent GTP hydrolysis resulting in a constitutively active KRAS [87-89]. Activating KRAS mutations initiate tumorigenesis by promoting the formation of PanIN lesions, and recently murine studies have shown that sustained activation of KRAS is required for continued progression of PDAC [90].

Activation of KRas leads to sustained activation of key signaling pathways within the ductal cells including but not limited to the RAF/ERK/MEK pathway and the PI3K/Akt pathway (**Figure 1.4**). These mitogenic cellular pathways aid in crucial oncogenic adaptation such as migratory ability, survival, and increased proliferation rate [87]. It has been shown recently that activated KRAS enhances the Warburg effect by elevating pancreatic cancers dependence on aerobic glycolysis. This metabolic reprogramming is most likely through the activation of PI3K/Akt pathway and its effector proteins such as HK1, Glut1, and Pfk1 [91]. Changes in the microenvironment in pancreatic cancer are directly linked to activated oncogenic KRAS as well. Continued inflammatory onslaught to the pancreas and the immune system activation induces fibrotic and inflammatory remodeling of the pancreas, which leads to prolonged activation of KRAS [92]. To treat KRAS mutant tumors, combinatorial treatment that inhibits both PI3K/Akt and MEK/ERK would likely be needed [90].

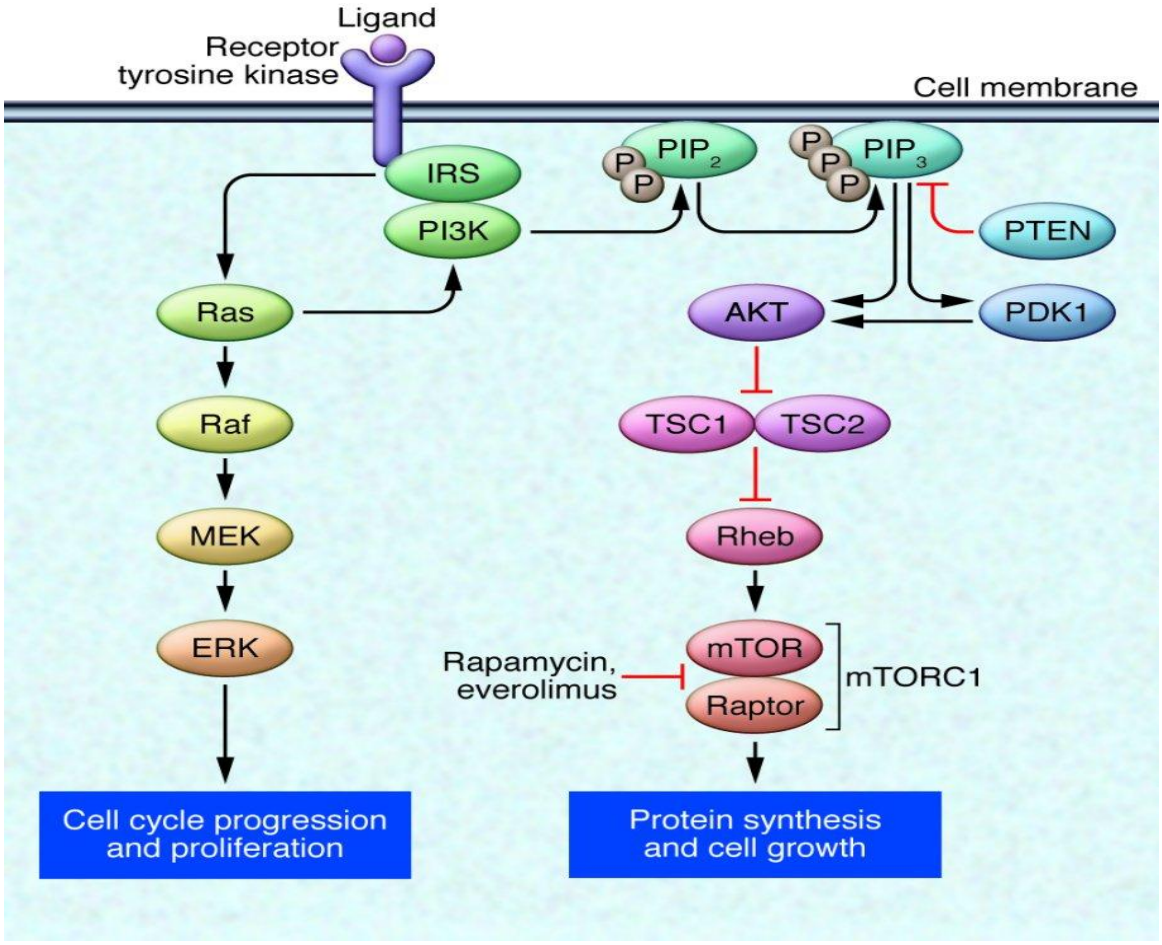

Ligand
Receptor tyrosine kinase
Cell membrane
IRS
PI3K
PIP2
PIP3
PTEN
Ras
AKT
PDK1
Raf
TSC1
TSC2
MEK
Rheb
ERK
Rapamycin, everolimus
mTOR
Raptor
mTORC1
Cell cycle progression and proliferation
Protein synthesis and cell growth

Figure 1.4. RAS-mediated activation of both Raf/MEK/ERK and PI3K/Akt pathway.

This illustration shows the ability of RAS activate both Raf and PI3K two major oncogenic pathways in pancreatic cancer. Activating KRas mutations allows for sustained activation of both pathways. This figure is adapted from Mohseni et al. [93].

The role of integrin in pancreatic cancer

In order for cells, regardless of type, to migrate they must sustain a transient interaction between themselves and the substrate to which the cells adhere to in order to undergo migration. Even transients interaction between the cell and the substrate is the workings of a consensus of proteins and protein complexes [94] One of the proteins involved are the Integrins. Integrins are transmembrane receptors consisting of α and β heterodimers that are capable of binding the extracellular matrix (ECM) and linking to the intracellular actin network of the cell [95]. Examples of ECM proteins include laminin, fibronectin, and collagen. Integrins are found distributed in migrating cells in a polarized manner, in that increased integrin clustering is found at the leading edge of the cell where PIP_3 levels rise as PI3K activity increases leading to adhesion formation [94].

It was recently discovered that α6β4-integrin expression is markedly increased in PDAC, with a step-wise increase in expression through PanIN and on into carcinoma [96-98]. Integrins not only function as adhesion molecules that promote migration, but also activate both PI3K/Akt and Raf/MEK/ERK oncogenic pathways [99, 100]. Integrins activate FAK and SFK proteins upon outside in activation. This is achieved upon binding of integrins to one of its ligands found in the ECM (**Figure 1.5**). The activation of FAK leads to activation of PI3K which in turn promotes phosphorylation and activation of Akt. SFK proteins activate SHC which leads to downstream activation of Raf/MEK/ERK and proliferation signals [101].

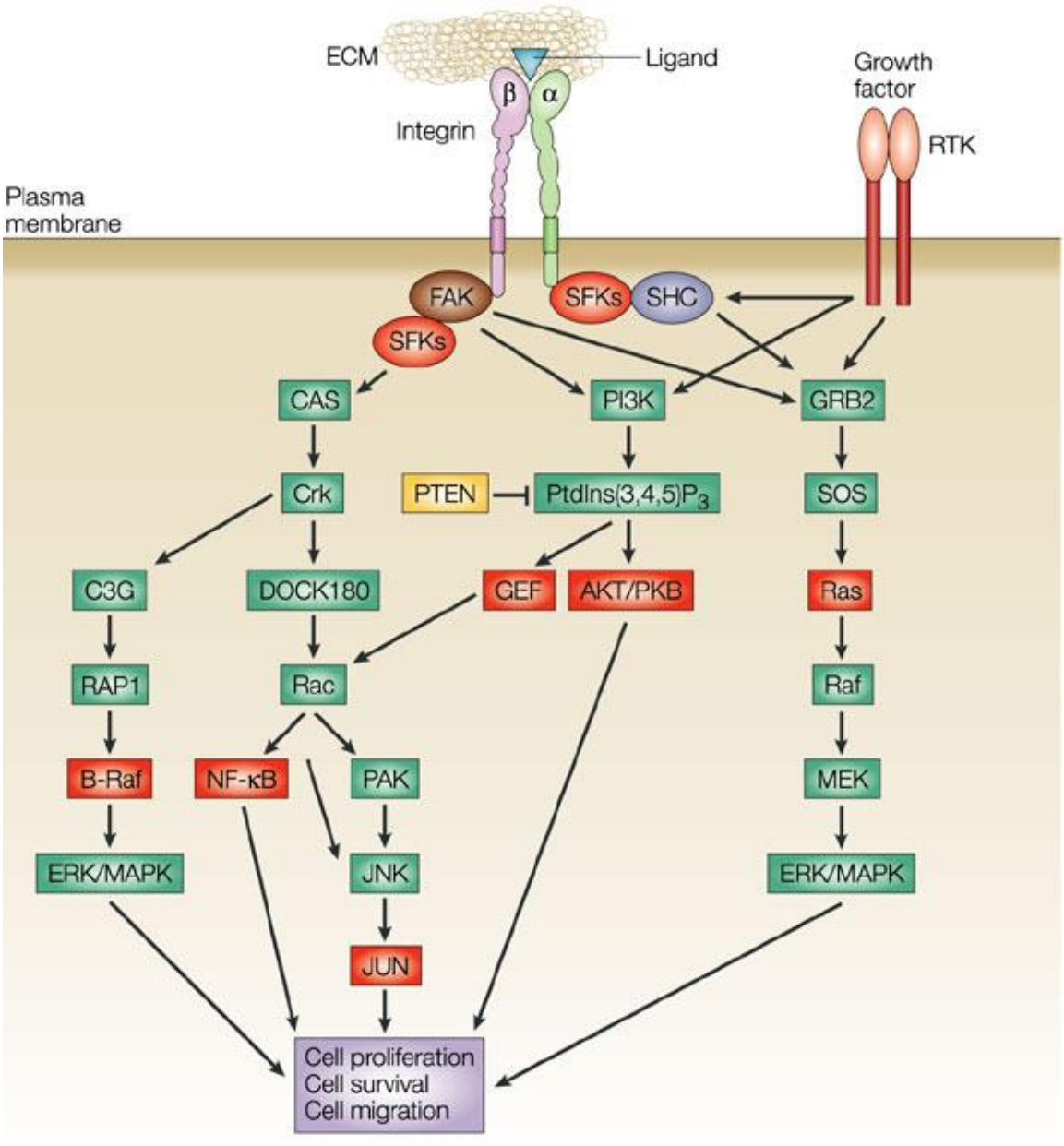

ECM
Ligand
Growth factor
β
α
Integrin
RTK
Plasma membrane
FAK
SFKs
SFKs
SHC
CAS
PI3K
GRB2
Crk
PTEN
PtdIns(3,4,5)P3
SOS
C3G
DOCK180
GEF
AKT/PKB
Ras
RAP1
Rac
Raf
B-Raf
NF-κB
PAK
MEK
ERK/MAPK
JNK
ERK/MAPK
JUN
Cell proliferation
Cell survival
Cell migration

Figure 1.5. Integrin activation leads to activation of PI3K/Akt and Raf/MEK/ERK pathways.

Integrin activation leads to cell proliferation, survival, and migration through activation of multiple oncogenic pathways. This figure is adapted from Guo et al. [101].

The tumor suppressor role of PHLPP

PHLPP (PH domain leucine-rich-repeats protein phosphatase) belongs to a novel family of Ser/Thr protein phosphatases that consists of PHLPP1 and PHLPP2 isoforms. Two splice variants of PHLPP1, PHLPP1α and PHLPP1β, have been reported, of which PHLPP1β contains an in-frame extension at the N-terminus of PHLPP1α (**Figure 1.6**) [102, 103]. PHLPP1 has first been proposed as a regulator of circadium rhythm as the mRNA expression of PHLPP1 in the rat brain suprachiasmatic nucleus region varies in a circadian-dependent manner. Thus, it was initially named SCOP for suprachiasmatic nucleus circadian oscillatory protein [104]. Overexpression of PHLPP1β in mouse forebrain impairs memory formation in transgenic mice by inhibiting ERK signaling [105]. Importantly, Gao et al. identified PHLPP as the novel protein phosphatase that directly dephosphorylates Akt and turns off Akt-mediated growth and survival signaling (**Figure 1.7**) [106]. PHLPP is then renamed based on its domain structure as it contains a PH domain, a leucine rich repeat region, a phosphatase domain, and a PDZ ligand motif (**Figure 1.6**) [106, 107].

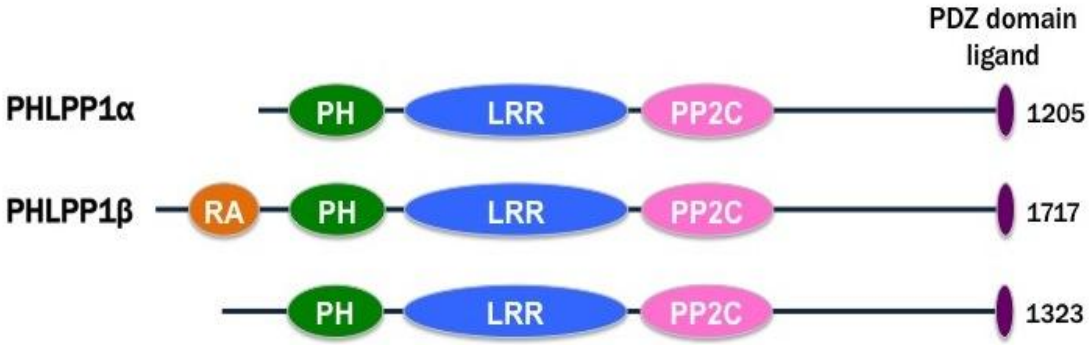

PDZ domain ligand
PHLPP1α
PH
LRR
PP2C
1205
PHLPP1β
RA
PH
LRR
PP2C
1717
PH
LRR
PP2C
1323

Figure 1.6. Domain structures of PHLPP1 and PHLPP2 isoforms.

Both PHLPP isoforms contain a PH domain, a leucine rich repeat region (LRR), a phosphatase domain (PP2C-like phosphatase domain), and a PDZ ligand motif. Total numbers of amino acids are labeled for each isoform.

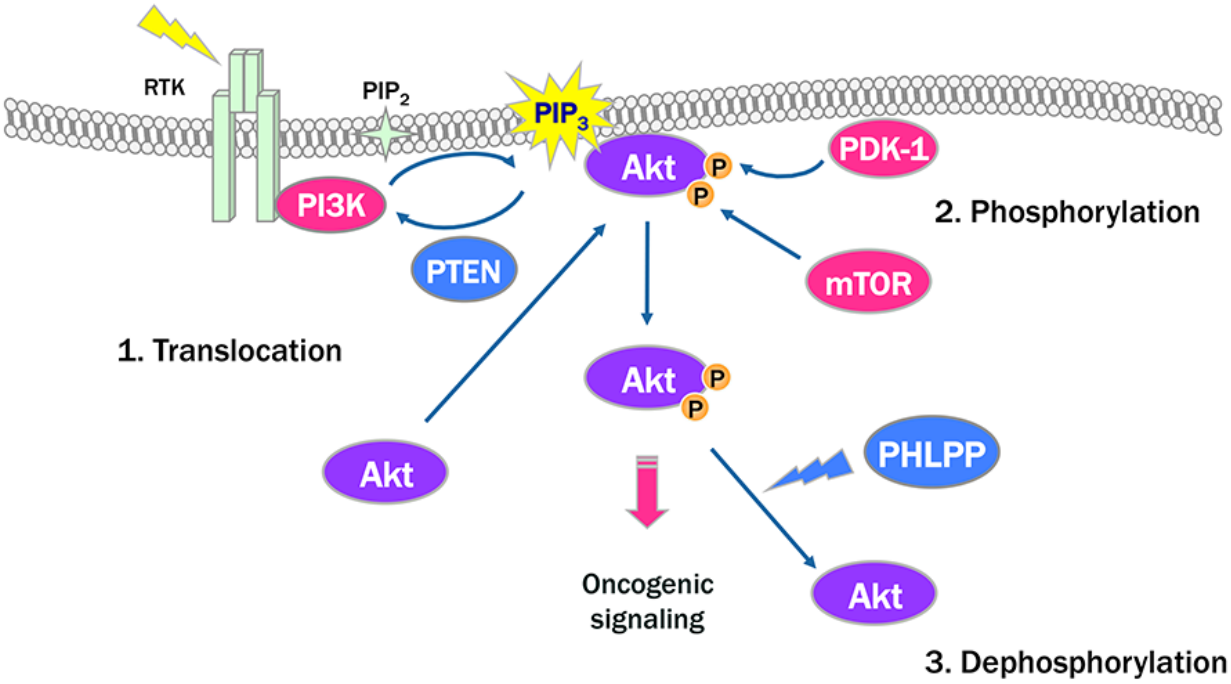

RTK
PIP2
PIP3
Akt
P
P
PDK-1
2. Phosphorylation
PI3K
PTEN
mTOR
1. Translocation
Akt
P
P
Akt
PHLPP
Oncogenic
signaling
Akt
3. Dephosphorylation

Figure 1.7. PHLPP-mediated dephosphorylation and inactivation of Akt.

Activation of Akt requires membrane translocation and phosphorylation at both T308 and S473 sites. This PI3K-dependent activation of Akt can be prevented by PTEN, a lipid phosphatase, which removes the activation signal. PHLPP inactivates Akt by directly dephosphorylating Akt protein.

Increasing evidence has indicated that both PHLPP isoforms serve as tumor suppressors in different types of cancer, which include colorectal, lung, liver and pancreatic cancer [108-111]. It has been shown that PHLPP negatively regulates multiple oncogenic pathways by directly dephosphorylating and inactivating key signaling molecules, such as Akt, S6K, and RAF1 [106-108, 112-114]. Moreover, PHLPP promotes the degradation of conventional PKCs by dephosphorylating the hydrophobic motif of PKC [115]. One interesting caveat is that Akt is part of this feedback loop with S6K, in which S6K-mediated phosphorylation of IRS at Ser-302 prevents the association of IRS with IGFR and downregulates IRS protein, thus, preventing activation of Akt following IGFR activation [116]. PHLPP also regulates the negative feedback loop of Akt by dephosphorylating S6K and decreasing its activity [113].

PHLPP is frequently downregulated in colorectal cancer patients and reintroduction of PHLPP into colon cancer cells inhibits cell proliferation, *in vitro,* and tumorigenesis in nude mice [108]. Recently, Li et al. demonstrated that PHLPP is a negative regulator of the RAS/RAF pathway by directly dephosphorylating RAF1 and inhibiting its kinase activity. Knockdown of PHLPP enhances signaling through the RAF/MEK/ERK cascade, induces EMT, and increases cell migration and invasion [117]. Genetic deletion of PHLPP1 promotes the development of invasive intestinal adenocarcinoma and decreases survival in Apc^{Min} mice [117]. In pancreatic cancer, PHLPP expression is reduced in PanIN lesions compared to normal controls, and is almost entirely lost in PDAC tissues [118].

This correlates with increasing phosphorylation of Akt through different PanIN stages to PDAC [118]. Collectively, PHLPP provides a balance in both Raf/MEK/ERK and PI3K/Akt pathways; and loss of PHLPP leads to aberrant activation of these pathways giving rise to cells that are able to proliferate unchecked while having an increase in ability to migrate (**Figure 1.8**).

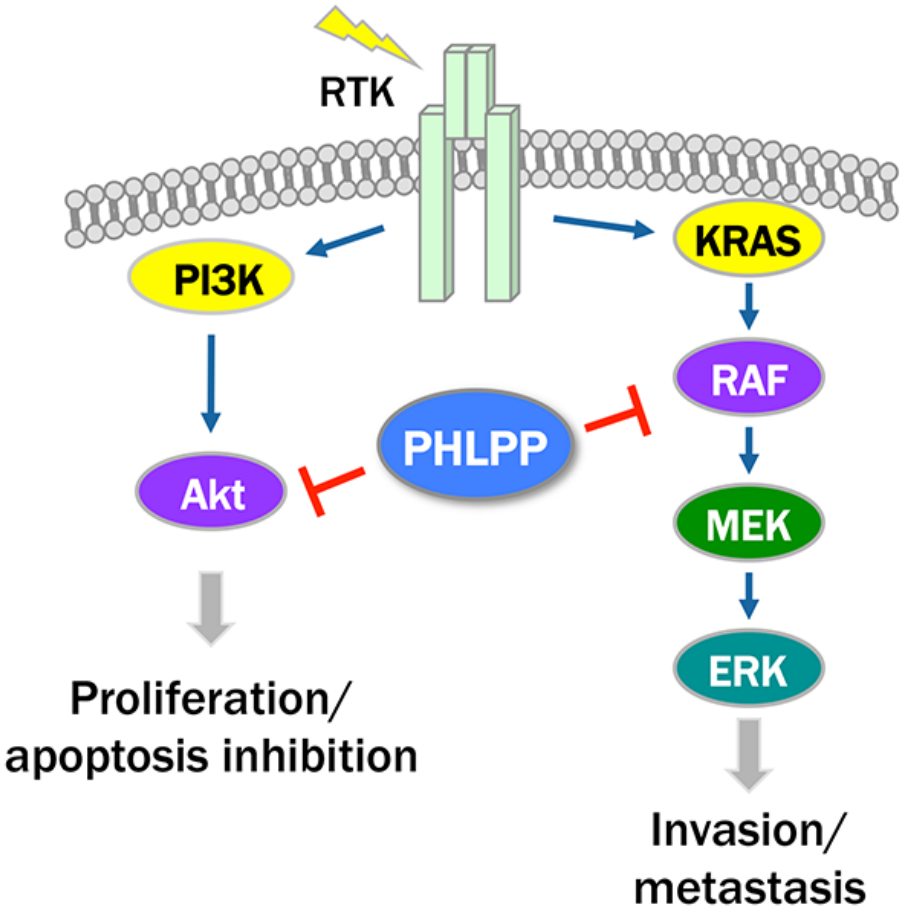

RTK
PI3K
KRAS
RAF
PHLPP
Akt
MEK
ERK
Proliferation/
apoptosis inhibition
Invasion/
metastasis

Figure 1.8. PHLPP plays a pivotal role in controlling the signaling balance of both PI3K/Akt and RAS/RAF pathways.

These two oncogenic pathways, PI3K/Akt and Raf/MEK/ERK pathways, are exploited during turmorigenesis and metastasis. PHLPP is a converging point that regulates both.

Significance of this study

Increasing numbers of studies have indicated that PHLPP plays an important role in turning off major oncogenic signaling pathways [119]. The majority of these studies have focused on colorectal cancer and revolved around the oncogenic kinase Akt. Recent studies show PHLPP not only regulates the activity of the PI3K/Akt pathway, but also has the ability to inhibit the Raf/MEK/ERK pathway by dephosphorylating RAF1. These two pathways play central roles in PanIN lesion development and especially PDAC formation, and close to 95% of PDAC metastatic tumors are found to have active KRAS mutations. Given the extremely low survival rates of pancreatic cancer, new diagnostic and treatment strategies are urgently needed in order to improve patient survival in pancreatic cancer.

In this study, we investigated how the change in expression of PHLPP regulated PI3K/Akt and RAF/MEK/ERK signaling in PDAC cells. We discovered that PHLPP negatively regulates the migratory ability of PDAC. After differing PHLPP levels of expression in pancreatic cancer cells we noticed that α4β6 integrin expression was significantly altered. This may be through PHLP regulating the lysosomal mediated degradation pathway of integrins. Our study establishes a novel link between PHLPP, Akt, and integrin. Better understanding of how PHLPP functions as a tumor suppressor and the underlying molecular mechanism may help to identify new therapeutic targets in pancreatic cancer.

Chapter 2. Methods and Materials

Cells and reagents

The following pancreatic ductal adenocarcinoma cell lines, ASPC-1, Panc-1, and Suit-2, were kindly provided by Dr. Kathleen O'Connor (University of Kentucky). The Panc-1 cells used in this study were sorted for their high integrin expression as described previously [120]. Panc-1 cells were cultured in DMEM whereas ASPC-1 and Suit-2 cells were cultured in RPMI-1640. All media were supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin/streptomycin.

The shRNAs for human PHLPP1, PHLPP2, AKT1 and AKT2 genes used in this study were constructed in pLKO.1-puro vector and purchased from Sigma-Aldrich [121]. The targeting sequences are as the following: PHLPP1, 5'-CGAGGTCTTTCCCGAAGTTAT-3', PHLPP2, 5'-GCCTGAACTTGTCCCATAATA-3', AKT1, 5'-GGACAAGGACGGGCACATTAA-3' and AKT2, 5'- CCCTTAAACAACTTCTCCGTA-3'. A plasmid carrying a non-targeting sequence was used to create the control cells. For virus packaging, the control or gene-specific shRNA constructs were co-transfected with psPAX2 and pMD2.G packing plasmids (Addgene) into 293T cells using polyethylenimine (PEI). The virus containing media were collected, filtered, and overlaid onto pancreatic cells in the presence of polybrene (8 μg/ml). The infected cells were then subjected to selection with puromycin (1 μg/ml). To overexpress human PHLPP1 or PHLPP2, the retroviral expression plasmids, including pBabe-puro-HA-PHLPP1β and pBabe-puro-HA-PHLPP2, were described previously [108,

114]. The stable PHLPP overexpressing cells were generated by infecting cells with retrovirus encoding HA-PHLPP1β or HA-PHLPP2 and selecting with puromycin (1 μg/ml).

PHLPP1 and PHLPP2 antibodies were obtained from Proteintech and Bethyl Laboratories, respectively. The phospho-Akt (p-Akt for the Ser473 site), phospho-ERK1/2 (p-ERK for Thr202/Tyr204), phospho-MEK1/2 (p-MEK for Ser217/221), EGFR and E-cadherin antibodies were from Cell Signaling. The vimentin antibody was from BD Biosciences whereas the γ-tubulin antibody was from Sigma-Aldrich. The following integrin antibodies were used in this study: for Western blotting, anti-β1 rabbit mAb (D2E5, Cell Signaling) and anti-β4 mouse mAb (clone 7, BD Biosciences); and for blocking ECM binding, anti-β1 mouse mAb (clone 6S6, Millipore) and anti-β4 mouse mAb (clone ASC-8, Millipore). The following chemicals, including PI3K inhibitor LY290042, rapamycin, PP242 and Akt inhibitor VIII, were purchased from EMD/CalBiochem. The MEK1/2 inhibitor PD0325901 was purchased from Selleck Chemicals, and lysosome inhibitors bafilomycin A1 and cycloheximide were from Sigma-Aldrich.

Immunoblotting

Cultured cells were harvested and lysed in Lysis Buffer (50 mM Na_2HPO_4, 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1 mM DTT, 200 mM benzamidine, 40 mg ml^{-1} leupeptin, 200 mM PMSF) and the detergent-solublized cell lysates were obtained after centrifugation for 5 minutes at 16,000 g at 4 ^{o}C. Equal amounts of cell lysates as determined by

Bradford assays were resolved by SDS-PAGE and subjected to immunoblotting analysis. The density of ECL signals was obtained and quantified using a FluoChem digital imaging system (Alpha Innotech).

Wound Healing Assay

Cultured cells were plated in a 6 well plate in their respective media. Using a 200uL pipette tip a wound was made through the center of each well. The cells were washed with cold 1XPBS three times to rinse away any non-attached cell. The plate was placed in the incubator for 24 hours. Pictures were taken at time 0 and at 24 hours. The diameter of the wound was measured at time 0 and time 24 hours. The percent wound healed was calculated by migration of the cells closing the gap or wound.

Transwell migration assays

Serum starved pancreatic cancer cells were seeded into the upper chamber of Transwell inserts with an 8-μm pore size membrane (Corning). For migration assays, the bottom of the inserts were coated with collagen (15 μg/ml) or laminin (25 μg/ml), and the cells were allowed to migrate towards media containing either HGF (20 ng/ml) or IGF-1 (20 ng/ml) in the lower chamber for 2-5 hours. At the end of the incubation period, Transwell inserts were fixed in methanol and stained with 0.5% crystal violet. The numbers of cells that were migrated to the lower chamber of the Transwell were counted using an inverted microscope at 20X magnification.

Time-lapse live cell imaging and analysis

Stable control and PHLPP overexpressing Panc-1 cells and PHLPP knockdown ASPC-1 cells were serum starved for 2 hours and seeded as single cells onto collagen (15 μg/ml) or laminin (25 μg/ml) coated glass bottom culture dishes (MatTek). The migration of live cells was recorded in media containing HGF (20 ng/ml) using Nikon BioStation IM equipped with CO_2 incubation chamber. Time-lapse phase images were taken either every 5 or 10 minutes for 6 hours with a 20X objective. The movement of cells was tracked and analyzed using Nikon Element AR software.

3D cell culture

Single cell suspension of pancreatic cancer cells was prepared in serum free media and embedded into collagen/Matrigel (1:1) mixture. The cells were overlaid with 2% Matrigel in 2% FBS containing media. The cells were allowed to grow into cyst-like structures in the 3D matrix for 1-2 weeks. The size and morphology of the cysts were examined by phase-contrast microscopy using a Nikon Ti-E inverted microscope. For immunofluorescence staining, the cysts were fixed in 4% paraformaldehyde and permeabilized using 1% Triton X-100 in PBS. Actin was stained using Alexa 488-conjugated phalloidin while the nuclei of the cells were stained with DAPI-containing mounting medium. Cells were visualized using an Olympus FlowView FV1000 confocal laser-scanning microscope.

Immunofluorescence (IF) staining

IF staining was performed following procedures described previously [122]. Briefly, stable control and PHLPP overexpressing Panc-1 cells and PHLPP knockdown ASPC-1 cells were serum starved for 2 hours and seeded as single cells onto collagen (15 μg/ml) coated coverslips and allowed to attach for 20 minutes. The cells were then fixed in 4% paraformaldehyde and followed by incubating with labeling buffer (1% bovine serum albumin in phosphate-buffered saline, pH 7.4) to block nonspecific binding. In labeling buffer the antibody specific for anti-β4-integrin antibody from BD biosciences was diluted and added to the cells for 2 hour incubation. The cells were then washed 3 times for 5 minutes each wash with 1XPBS. Alexa 488-conjugated phalloidin and Alexa 594 fluorescent dye were diluted in labeling buffer and incubated with the cells for 1 hour. The basolateral distributions of F-actin were visualized using a Nikon laser TIRF (Total Internal Reflection Fluorescence) microscope.

Real-time PCR

Total RNA was isolated with RNeasy kit (Qiagen) from different stable pancreatic cancer cell lines. Equal amounts of RNA were used as templates for the synthesis of cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosysems). Real-time PCR reaction was performed using the StepOne Real-Time PCR system (Applied Biosysems) with β1- and β4-specific probes. All values were normalized to the level of β-actin.

Statistical Analysis

The results from the experiments to assess the rate of migration, levels of protein and mRNA expression, cell growth in 3D, and velocity and distance migrated by single cells were summarized using mean ± SEM or mean ± SD. All experiments were performed in triplicate and pairwise comparisons were carried out using two-sample t-tests. Statistical significance of values among different groups was set at $p < 0.05$.

Primer:	Designation:	Sequence:
CD29 (Igβ1) Forward	β1-Integrin Primer	TGACCATCTCTTCTCCTCTCCAC
CD29 (Igβ1) Reverse	β1-Integrin Primer	GGAGTCACACTGGTCAACAGAG
CD104 (Igβ4) Forward	B4-Integrin Primer	AAAAAGGATTGAGTTCCTTGCAAAAA
CD104 (Igβ4) Reverse	B4-Integrin Primer	CCCAGAGTCTTGTGGTTCTCTAAC

Table 2.1: Primers used for qRT-PCR

Primers for both β4 integrin and β1 integrin were used in qRT-PCR studies.

Chapter 3

Results

Introduction

Accumulation of genetic mutations in oncogenes and tumor suppressors lead to the development of pre-malignant lesions which eventually through genetic instability give rise to carcinoma within the pancreatic ductal epithelium [123]. Thus, a better understanding of the molecular events leading to cancer progression is needed in order to improve the treatment and prognosis of PDAC patients. Key mutations in oncogenes such as *KRAS* with deactivation of tumor suppressor genes *SMAD4* and *CDKN2A/INK4A* have been implicated in the development and progression of pancreatic cancer [47, 124]. In addition, it has been shown that overexpression of integrin α6β4 promotes migration and invasion of pancreatic cancer cells and is associated with the progression of PDAC [97, 120]. Integrins are known to contribute to tumor progression and metastasis by directly activating a number of oncogenic signaling pathways, including PI3K/Akt and RAS/RAF pathways, in various types of cancer [99, 100]. However, the molecular mechanism by which the expression of integrin proteins is regulated remains elusive in pancreatic cancer cells.

PHLPP (PH domain leucine-rich repeat protein phosphatase) belongs to a novel family of Ser/Thr protein phosphatases. There are two isoforms, PHLPP1 and PHLPP2, identified in this family [102, 103, 106, 107]. Both PHLPP isoforms were first discovered as the phosphatases for Akt that directly dephosphorylate

the hydrophobic motif Ser473 site and inactivate the kinase [106, 107]. Moreover, it has been shown that PHLPP dephosphorylates Ser338, a key activation site on RAF1, and inhibits the downstream signaling through RAF/MEK/ERK in colon cancer cells [114]. Therefore, PHLPP may exert its tumor suppressor function by negatively regulating both the PI3K/Akt and RAS/RAF pathways. Recently, Nitsche et al discovered that there is a stage-dependent downregulation of PHLPP in pancreatic cancer patient specimens, thus suggesting a tumor suppressor role of PHLPP in pancreatic cancer [111]. However, it remains unknown whether loss of PHLPP expression promotes cancer cell migration in pancreatic cancer.

In this study, we investigated the effect PHLPP expression had on cell migration and motility in pancreatic cancer cells.. We identified a novel connection between PHLPP expression and integrin function. Results from our study revealed that PHLPP-loss increases cell motility by upregulating integrin expression and inducing EMT. Furthermore, this regulation of integrin expression mediated by PHLPP may be through lysosomal degradation of integrin, specifically β4 and β3 integrin.

PHLPP negatively regulates the activity of Akt and MEK/ERK in pancreatic cells

To determine if PHLPP negative regulates both the PI3K/Akt and Raf/MEK/ERK pathway in human pancreatic cancer, we established stable cell lines overexpressing PHLPP1 or PHLPP2 in Panc-1 cells, which express very

low levels of endogenous PHLPPs. The PHLPP1 gene potentially encodes two differentially spliced variants, PHLPP1α and PHLPP1β [102]. Since the longer transcript of PHLPP1, PHLPP1β, is the predominant form expressed endogenously in all pancreatic cell lines examined, we used PHLPP1β in our study. We first determined the effect of PHLPP overexpression on cell signaling. As shown in **Figure 3.1**, both Akt and MEK/ERK activity were downregulated in PHLPP overexpressing cells compared to control cells as indicated by decreased phosphorylation of Akt, MEK, and ERK. Next, to determine the effect of endogenous PHLPP on inhibiting Akt and MEK/ERK signaling, PHLPP was silenced in ASPC-1 cells, which express relatively higher levels of endogenous PHLPPs, using lentiviral-mediated RNAi. Immunoblotting results revealed that phosphorylation of Akt, MEK, and ERK was significantly elevated when PHLPP expression was knocked down (**Figure 3.2**). Consistent with previous reports on the tumor suppressor function of PHLPP in other cancer types [108, 114, 125-127], our results here provide the initial evidence that PHLPP is capable of inhibiting both Akt and MEK/ERK signaling in pancreatic cancer cells.

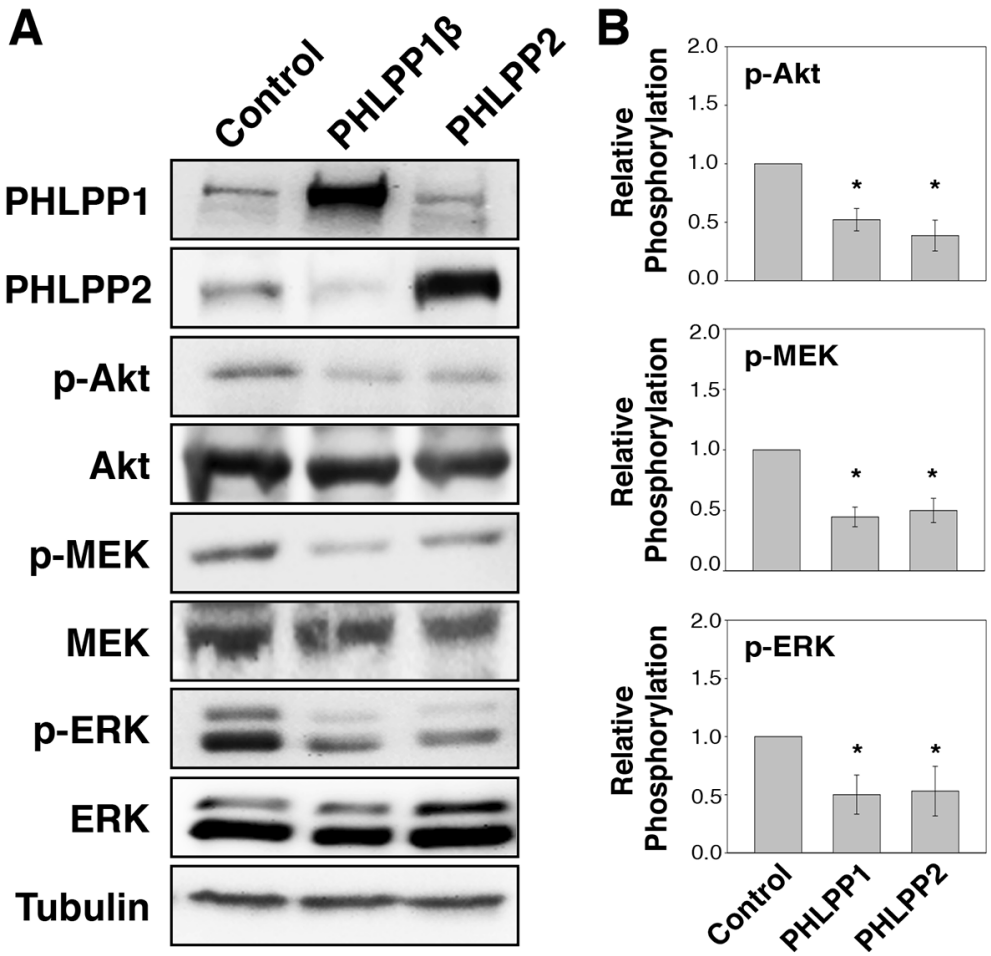

A
Control
PHLPP1β
PHLPP2
PHLPP1
PHLPP2
p-Akt
Akt
p-MEK
MEK
p-ERK
ERK
Tubulin
B
p-Akt
p-MEK
p-ERK
Relative Phosphorylation
2.0
1.5
1.0
0.5
0.0
*
*
Control
PHLPP1
PHLPP2

Figure 3.1. Overexpression of PHLPP isoforms inhibits PI3K/Akt and MEK/ERK signaling.

(A) Stable control, HA-PHLPP1β or HA-PHLPP2 overexpressing Panc-1 cells were generated using retrovirus-mediated infection. The cell lysates were prepared and analyzed for phosphorylation and total protein expression by immunoblotting. (B) Relative phosphorylation for p-Akt, p-MEK, p-ERK were calculated and normalized to those of total Akt, MEK and ERK, respectively. The level in control cells was set to 1. Data represent the mean ± SEM (n=3, * $p<0.05$ by two-sample t-tests).

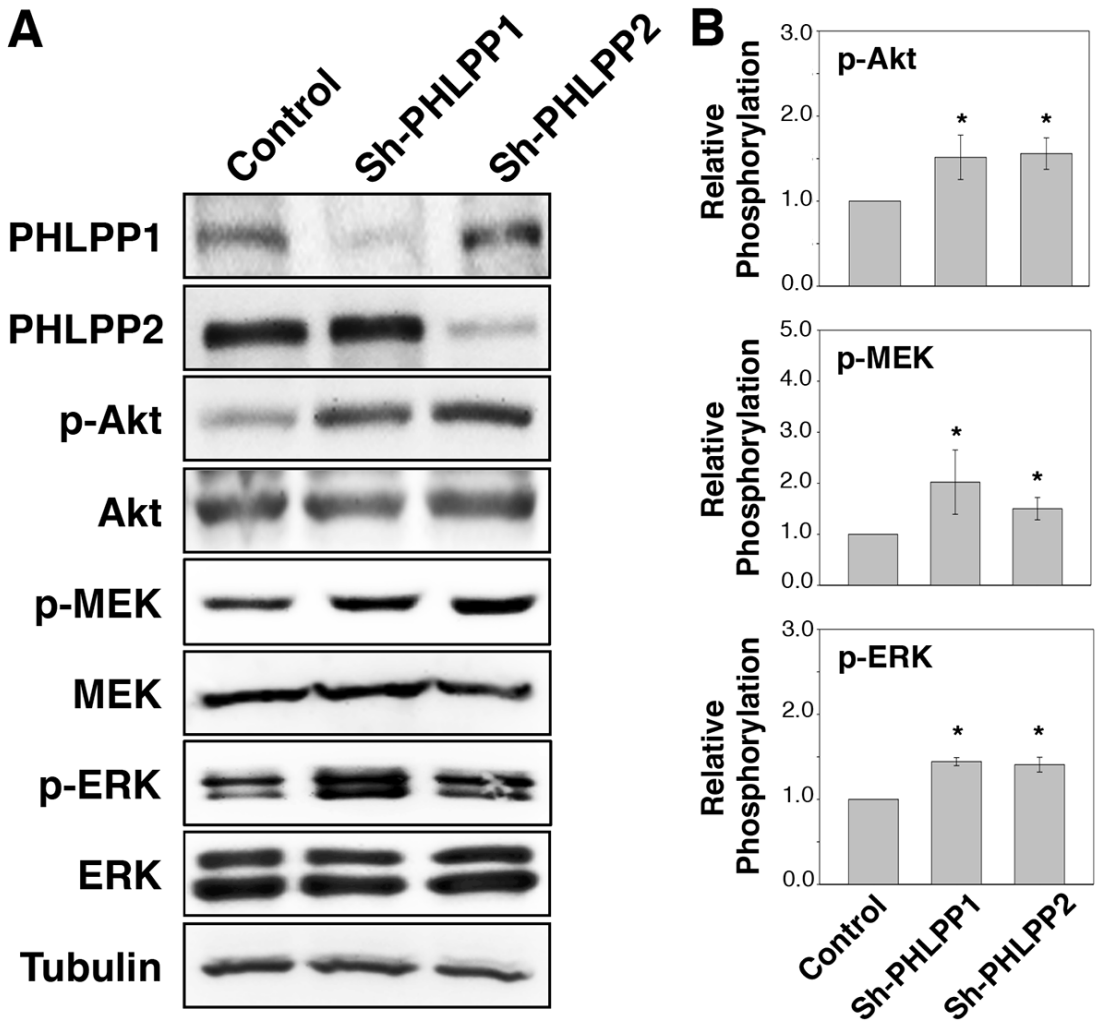

A
Control
Sh-PHLPP1
Sh-PHLPP2
PHLPP1
PHLPP2
p-Akt
Akt
p-MEK
MEK
p-ERK
ERK
Tubulin
B
p-Akt
p-MEK
p-ERK
Relative Phosphorylation
3.0
2.0
1.0
0.0
5.0
4.0
*
Control
Sh-PHLPP1
Sh-PHLPP2

Figure 3.2. Loss of PHLPP expression enhances PI3K/Akt and RAF/MEK/ERK signaling.

(A) Stable control (sh-Con) and PHLPP knockdown (sh-PHLPP1 and sh-PHLPP2) ASPC-1 cells were generated using lentivirus-mediated RNAi. The cell lysates were analyzed for phosphorylation and total protein expression by immunoblotting. (B) Relative phosphorylation for p-Akt, p-MEK, p-ERK were calculated and normalized to those of total Akt, MEK and ERK, respectively. The level in control cells was set to 1. Data represent the mean ± SEM (n=3, * $p<0.05$ by two-sample t-tests).

PHLPP negatively regulates cell migration in pancreatic cancer cells

Previous studies of PHLPP expression in colorectal cancer cells showed an enhanced ability for PHLPP knockdown cells to migrate and transition into EMT [114]. To test if PHLPP regulates cell motility in pancreatic cancer cells, we first examined the expression of EMT markers in PHLPP overexpressing Panc-1 and PHLPP knockdown ASPC-1 cells. As shown in **Figure 3.3**, PHLPP overexpression markedly increased the expression of E-cadherin, an epithelial cell marker, and decreased the expression of vimentin, a mesenchymal cell marker. Conversely, knockdown of either PHLPP isoform decreased E-cadherin whereas increased vimentin expression (**Figure 3.3**), suggesting that PHLPP downregualtion promotes EMT in pancreatic cancer cells.

To begin the initial study of migration in pancreatic cancer we investigated the ability of PHLPLP overexpressing Panc-1 cells to heal a wound introduced into the cultured cells. **Figure 3.4** shows the significant difference in ability of the PHLPP overexpressing cells to close the gap after the wound was introduced compared to the control cells. By 24 hours post scratch the control pancreatic cancer Panc-1 cells had healed the wound by 90% but the PHLPP overexpressing cell lines had about a 40-45% gap closure. This was the first hint that PHLPP could influence the migratory ability of pancreatic cancer cells.

Furthermore, to determine if PHLPP regulates pancreatic cancer cell migration. PHLPP overexpressing Panc-1 cells and PHLPP knockdown ASPC-1 cells were subjected to Transwell migration assay. We found that PHLPP overexpressing cells migrated significantly slower than the control cells as

determined by Transwell migration assays (**Figure 3.5**). In contrast, knockdown of PHLPP significantly enhanced the ability of pancreatic cancer cells to migrate in response to HGF. Similar results were obtained when using IGF-1 and collagen were used as chemoattractants (**Figure 3.6**). Taken together, we have identified a role of PHLPP in inhibiting EMT and cell motility in pancreatic cancer cells.

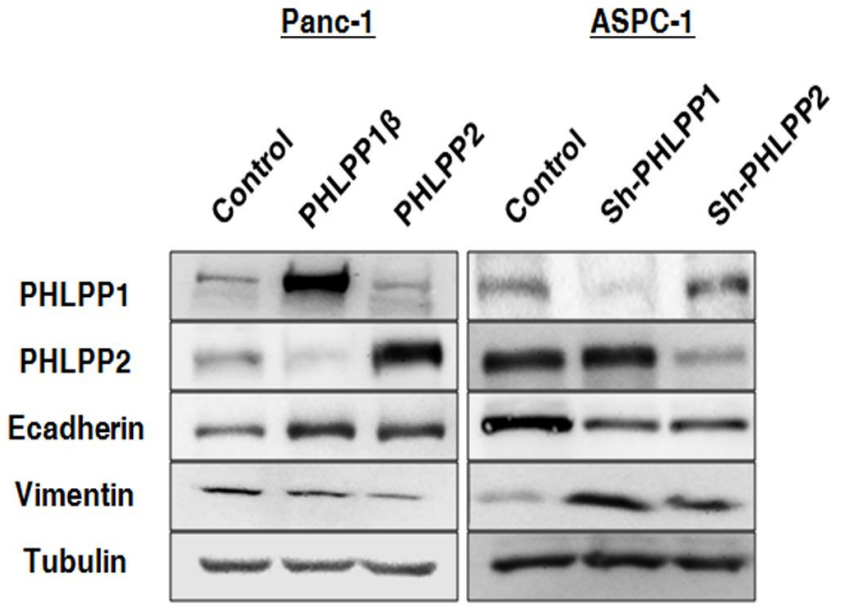
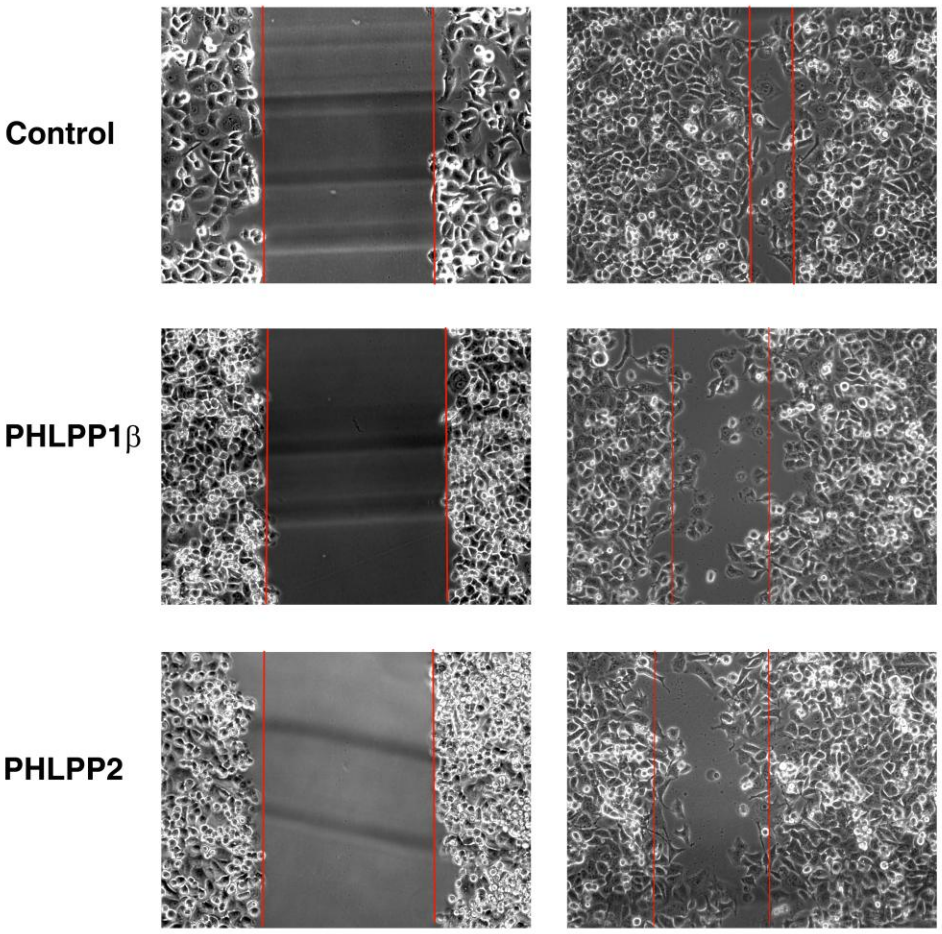

Panc-1
ASPC-1
Control
PHLPP1β
PHLPP2
Control
Sh-PHLPP1
Sh-PHLPP2
PHLPP1
PHLPP2
Ecadherin
Vimentin
Tubulin

Figure 3.3. Knockdown of PHLPP expression induces EMT phenotype in pancreatic cancer cells.

Cell lysates were prepared from stable control and PHLPP overexpressing Panc-1 as well as PHLPP knockdown ASPC-1 cells. The expression levels of E-cadherin and vimentin were analyzed using immunoblotting.

Panc-1


Control
PHLPP1β
PHLPP2

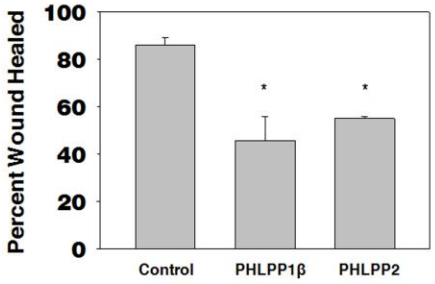

Percent Wound Healed
100
80
60
40
20
0
*
*
Control
PHLPP1β
PHLPP2

Figure 3.4. PHLPP inhibits the ability of pancreatic cancer cells to heal a wound.

Panc-1 cells were plated in a 6-well plate, a wound was introduced by scratching the cells off using a 200uL pipette tip. The diameter of the wound was measured at time 0 and 24 hour. Each experiment was done in duplicates and three independent experiments were averaged and expressed as mean ± SEM (* $p<0.05$ by two-sample t-tests compared to the control cells).

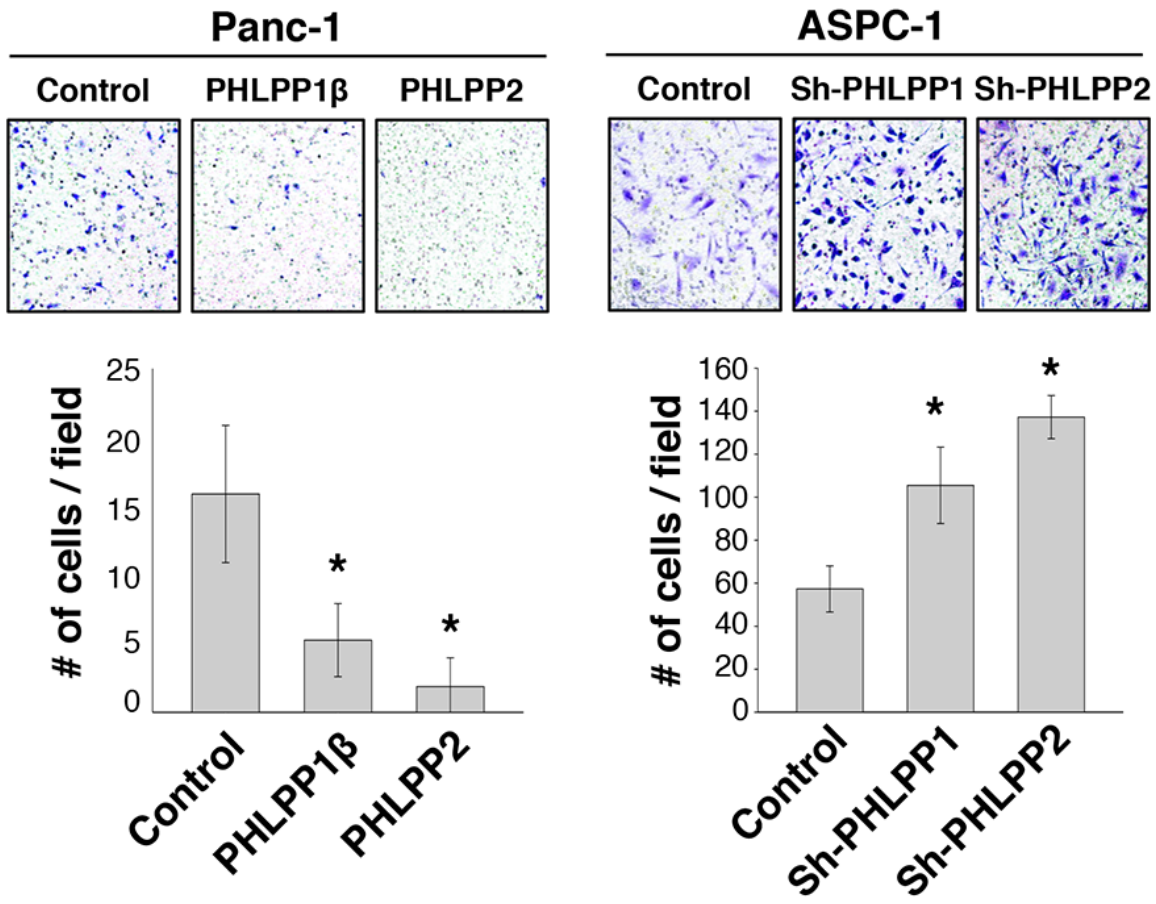

Panc-1
Control
PHLPP1β
PHLPP2
ASPC-1
Control
Sh-PHLPP1
Sh-PHLPP2
# of cells / field
25
20
15
10
5
0
*
*
Control
PHLPP1β
PHLPP2
# of cells / field
160
140
120
100
80
60
40
20
0
*
*
Control
Sh-PHLPP1
Sh-PHLPP2

Figure 3.5. PHLPP negatively regulates pancreatic cancer cell migration.

Stable control and PHLPP overexpressing Panc-1 cells and stable control and PHLPP knockdown ASPC-1 cells were subjected to Transwell migration assays using HGF and laminin as chemoattractants. The inserts shown were representative images of cells migrated in the Transwell assays. Each experiment was done in duplicate and three independent experiments were averaged and expressed as mean ± SEM (* $p<0.05$ by two-sample t-tests compared to the control cells).

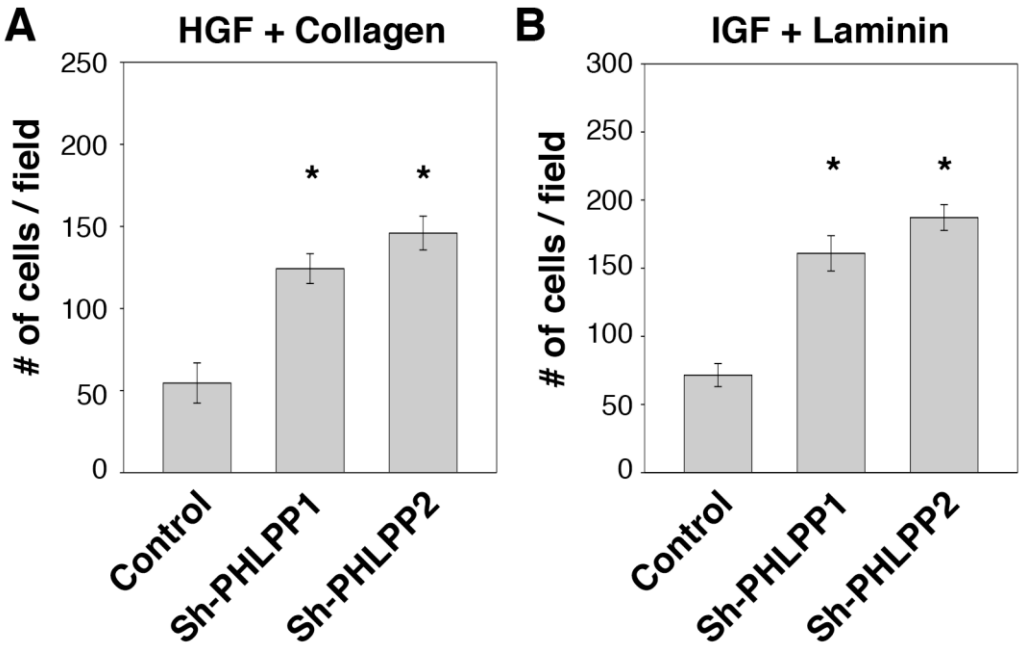

A
HGF + Collagen
# of cells / field
250
200
150
100
50
0
*
*
Control
Sh-PHLPP1
Sh-PHLPP2
B
IGF + Laminin
# of cells / field
300
250
200
150
100
50
0
*
*
Control
Sh-PHLPP1
Sh-PHLPP2

Figure 3.6. Migration of pancreatic cancer cells is amplified when PHLPP expression is lost.

Stable control and PHLPP knockdown ASPC-1 cells were subjected to Transwell migration assays using either HGF and collagen (A) or IGF-1 (20 ng/ml) and laminin (B) as chemoattractants. Each experiment was done in duplicate and three independent experiments were averaged and expressed as mean ± SD (* $p<0.05$ by two-sample t-tests compared to the control cells).

We next determined the effect of PHLPP on modulating cell motility at the single cell level. To this end, PHLPP overexpressing Panc-1 and PHLPP knockdown ASPC-1 cells were subjected to cell tracking experiments to monitor the cell movement in real time. The Biostation allows for pictures to be taken at specific intervals, which then can be used to track each individual cells migration. Stable control and PHLPP overexpressing or knockdown cells were seeded on glass-bottom plates coated with laminin, starved, then stimulated with HGF. We found that overexpression of PHLPP resulted in a significant decrease in cell motility when compared to control cells (**Fig. 3.7A**). The velocity and average distances traveled were significantly decreased in PHLPP overexpressing cells as well (**Fig. 3.7B-C**). To determine the effect of silencing the endogenous PHLPP expression at the single cell level, similar experiments were performed using control and PHLPP knockdown ASPC-1 cells. We found that PHLPP knockdown cells were considerably more motile (**Fig. 3.8**), and the velocity and average distances traveled were significantly increased in PHLPP knockdown cells (**Fig. 3.8B-C**). Taken together with results obtained in Transwell migration assays, we showed that PHLPP expression negatively impacts single cell migration and velocity. Since increased motility is often associated with increased aggressiveness in order for pancreatic cancer cells to metastasize, our data provide evidence supporting the tumor suppressor role of PHLPP.

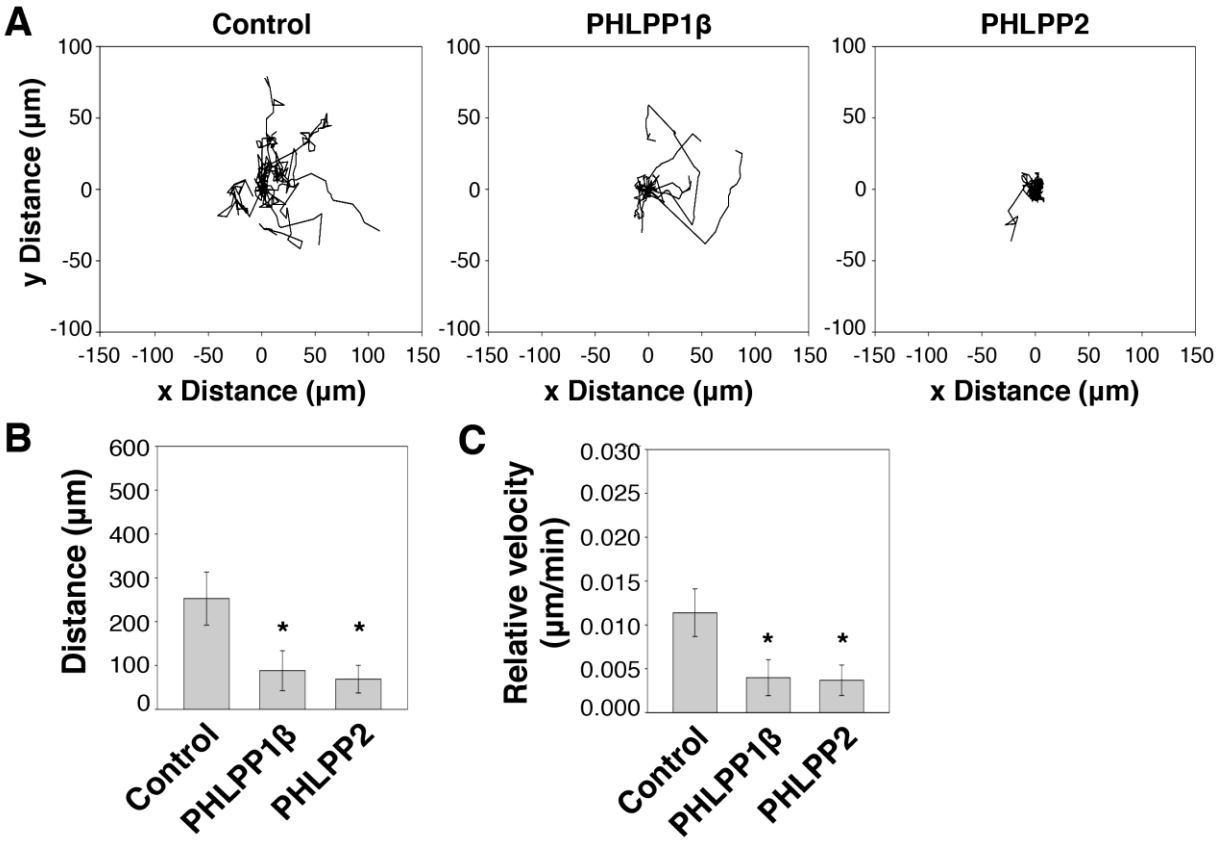

A
Control
PHLPP1β
PHLPP2
y Distance (μm)
x Distance (μm)
x Distance (μm)
x Distance (μm)
B
Distance (μm)
Control
PHLPP1β
PHLPP2
C
Relative velocity (μm/min)
Control
PHLPP1β
PHLPP2

Figure 3.7. PHLPP inhibits cell motility at the single cell level.

(A) Migration patterns of stable control and PHLPP overexpressing Panc-1 cells on laminin coated plates using HGF as the chemoattractant. The trajectories of 12 randomly chosen cells for each cell line were plotted in the graphs. (B-C) The average distance traveled (B) and the relative velocity (C) of 12 stable control and PHLPP overexpressing Panc-1 cells during 6 hours of migration. Data represent mean ± SD (n = 12 cells/line, * $p<0.05$ by two-sample t-tests).

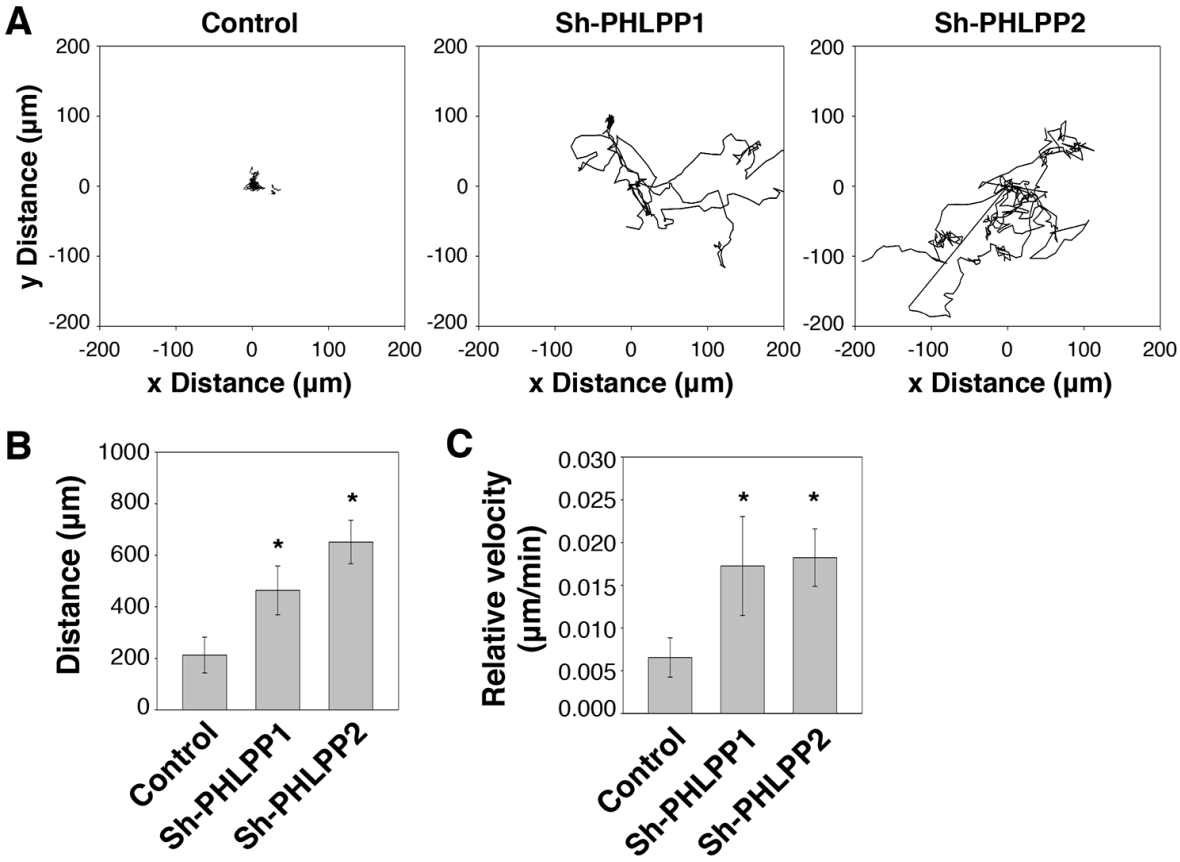

A
Control
Sh-PHLPP1
Sh-PHLPP2
y Distance (μm)
x Distance (μm)
x Distance (μm)
x Distance (μm)
200
100
0
-100
-200
-200
-100
0
100
200
B
Distance (μm)
1000
800
600
400
200
0
*
*
Control
Sh-PHLPP1
Sh-PHLPP2
C
Relative velocity (μm/min)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
*
*
Control
Sh-PHLPP1
Sh-PHLPP2

Figure 3.8. Knockdown of PHLPP increases cell motility at the single cell level.

(A) Migration patterns of stable control and PHLPP knockdown ASPC-1 cells on laminin coated plates using HGF as the chemoattractant. The trajectories of 12 randomly chosen cells for each cell line were plotted in the graphs. (B-C) The average distance traveled (B) and the relative velocity (C) of 12 stable control and PHLPP knockdown ASPC-1 cells during 6 hours of migration are shown. Data represent mean ± SEM (n = 12 cells/line, * $p<0.05$ by two-sample t-tests).

PHLPP inhibits the invasive growth of pancreatic cancer cells in 3D culture

EMT requires the process of cancer cells sending extensions away from the cell body in order to migrate. To further determine the effect of PHLPP on invasive growth of pancreatic cancer cells, we used 3D cell culture systems as they closely mimic the cancer microenvironment and they provide a better opportunity to explore the effects of oncogenes and tumor suppressors on modulating morphogenesis as well as invasive growth [128, 129]. Both PHLPP overexpressing Panc-1 and PHLPP knockdown ASPC-1 cells were seeded into Matrigel and allowed to grow for up to 7 days. The control Panc-1 cells grew into large multi-globular clusters with spikes of membrane protrusions extending into Matrigel (**Figure 3.9A-B**). However, the formation of membrane protrusions was completed blocked in PHLPP overexpressing cells and the size of cell clusters was significantly decreased. (**Figure 3.9C**)

Although no clear membrane protrusions were observed in ASPC-1 cells grown in Matrigel, PHLPP knockdown cells formed significantly larger globular clusters compared to the control cells, suggesting that PHLPP-loss increases the invasive growth ability of pancreatic cancer cells (**Figure 3.10**)

In addition, TIRF (Total Internal Reflection Fluorescence) imaging was incorporated into our studies to look at formation of stress fibers, extensions, and integrin expression. Control Panc-1 cells showed the formation of both stress fibers and long filamentous extensions at the basolateral membrane surrounding the entire cells whereas very few actin stress fibers were observed in PHLPP

overexpressing cells (**Figure 3.11**). Integrin staining in PHLPP overexpressing cells seems to be located more centrally to the cell possibly closer to the nucleus of the cell instead of at the extensions compared to control. The reduction of integrin expression at the extensions and protrusions could possibly negatively affect migration and cell motility. However, when ASPC-1 cells were stained for both actin and integrin it is apparent that there are major filamentous extensions, large protrusions from the basolateral membrane surrounding the cells and the most interesting aspect is the large amount of integrin staining at the basolateral membrane and protrusions (**Figure 3.12**). ASPC-1 control cells have nice actin stress ring formation and integrin staining inside of the stress ring, but still in punctate formation (**Figure 3.12**), unlike the Panc-1 overexpressing cells where the integrin staining is more diffuse and less punctate (**Figure 3.11**). In the ASPC-1 PHLPP knockdown cells integrin staining is most noticeable at the protrusions and filamentous extensions seen coming from the basolateral membrane. The β4-integrin staining is punctate in pattern and seems to be deposited outside of the cell membrane giving a unique arrangement (**Figure 3.12**). Collectively, these results showed that decreased PHLPP expression alters actin cytoskeleton rearrangement and increases invasive growth of pancreatic cancer cells.

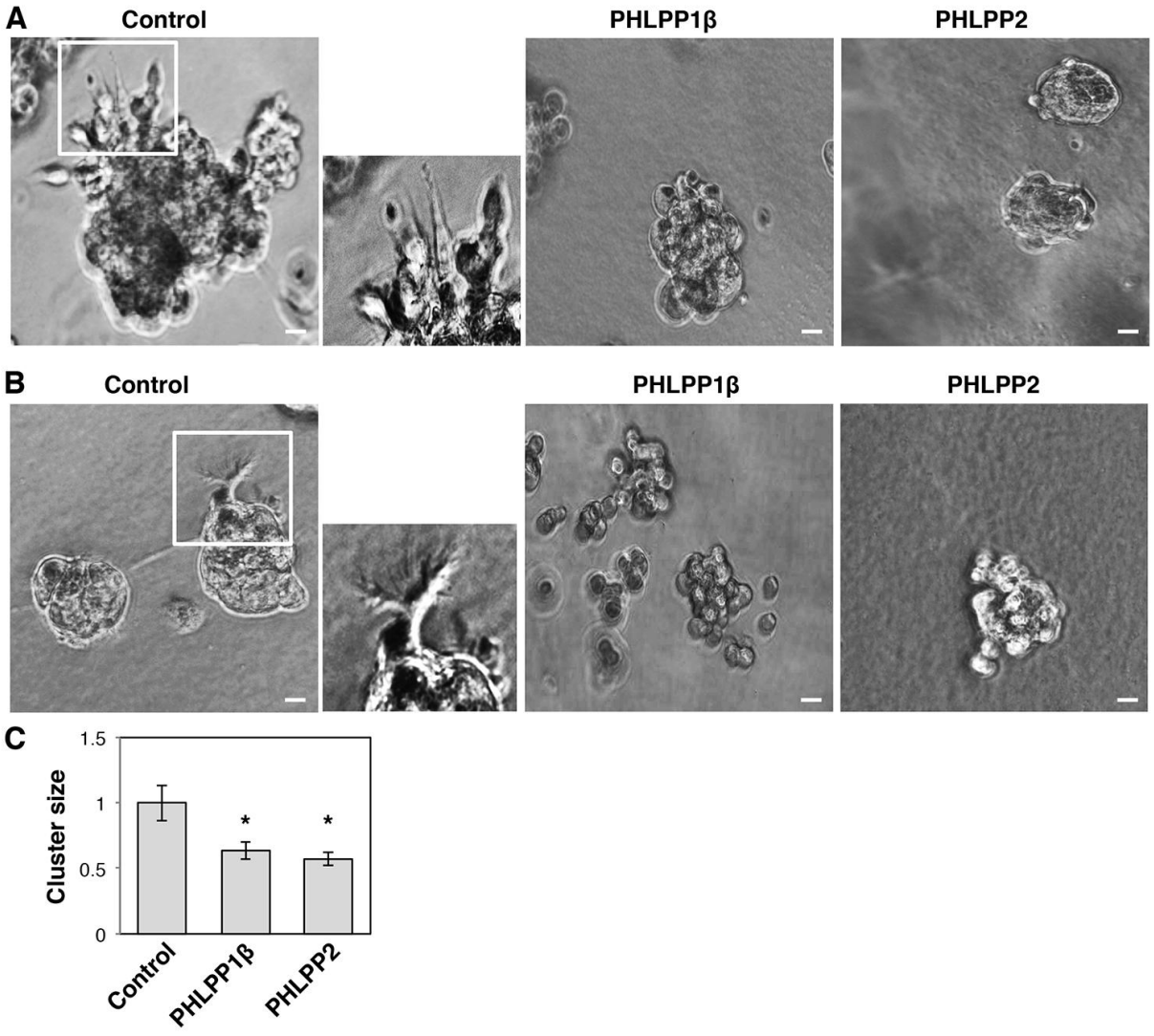

A
Control
PHLPP1β
PHLPP2
B
Control
PHLPP1β
PHLPP2
C
Cluster size
1.5
1
0.5
0
*
*
Control
PHLPP1β
PHLPP2

Figure 3.9. PHLPP inhibits the invasive phenotype of pancreatic cancer cells grown in 3D culture.

Stable control and PHLPP overexpressing Panc-1 cells were seeded in Matrigel and allowed to grow for 7 days. On the 3rd day (Top panel) and the 7th day (Bottom panel) after seeding phase contrast images were taken with the 10X objective on using an inverted Nikon microscope. The boxed regions were enlarged and shown next to the original images. Scale bars, 100 μm. (C) The size of cell clusters at day 7 was measured and analyzed using Nikon Element AR software. Data represent mean ± SEM (n = 18 cells/line, * $p < 0.05$ by two-sample t-tests).

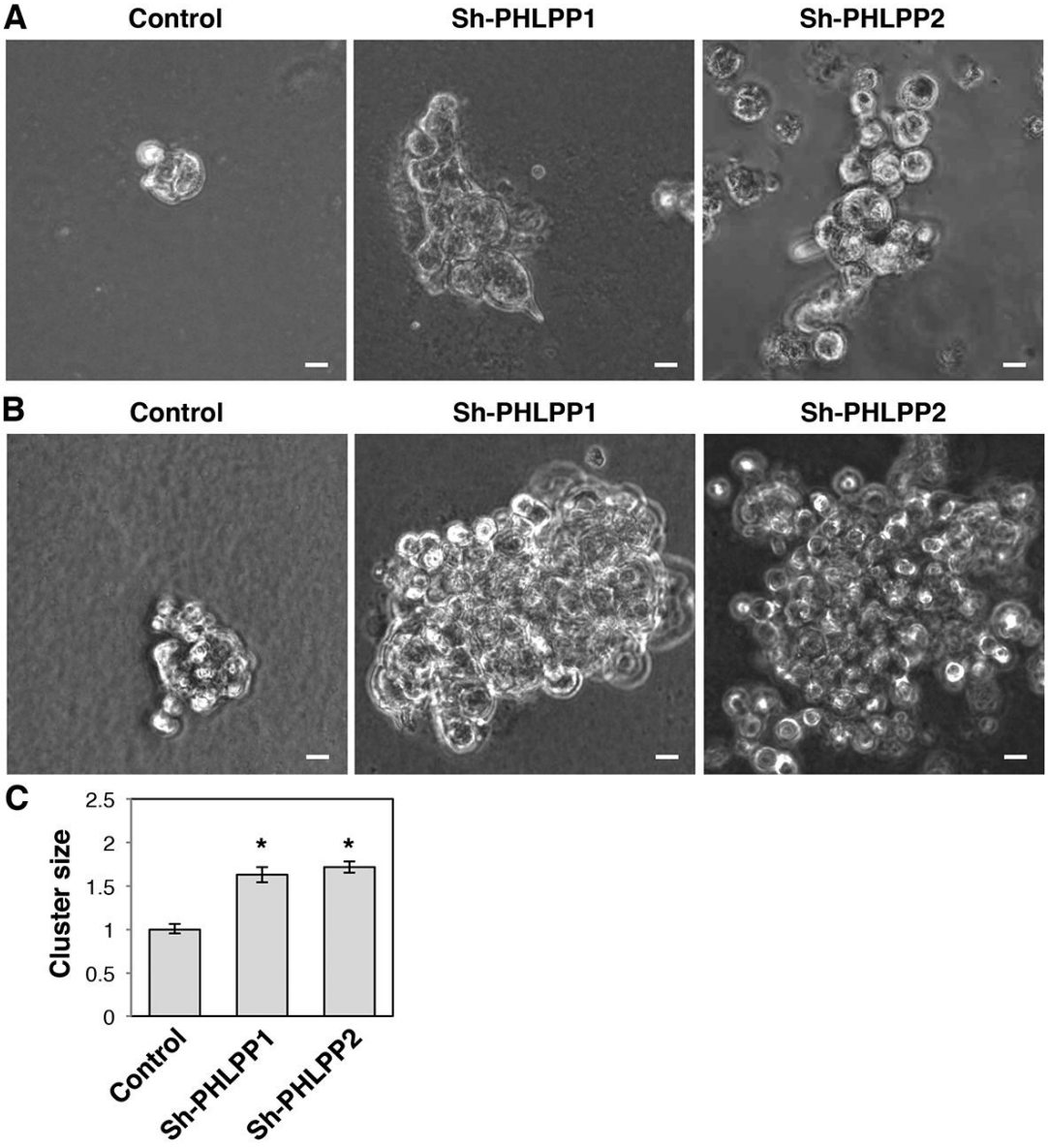

A
Control
Sh-PHLPP1
Sh-PHLPP2
B
Control
Sh-PHLPP1
Sh-PHLPP2
C
Cluster size
2.5
2
1.5
1
0.5
0
*
*
Control
Sh-PHLPP1
Sh-PHLPP2

Figure 3.10. Knockdown of PHLPP promotes the invasive growth of pancreatic cancer cells in 3D culture.

Stable control and PHLPP knockdown ASPC-1 cells were seeded in Matrigel and allowed to grow for 7 days. On the 5th day (A) and the 7th day (B) after seeding phasecontrast images were taken with the 10X objective on using an inverted Nikon microscope. Scale bars, 100 μm. (C) The size of cell clusters at day 7 was measured and analyzed using Nikon Element AR software. Data represent mean ± SEM (n = 18 cells/line, * $p < 0.05$ by two-sample t-tests).

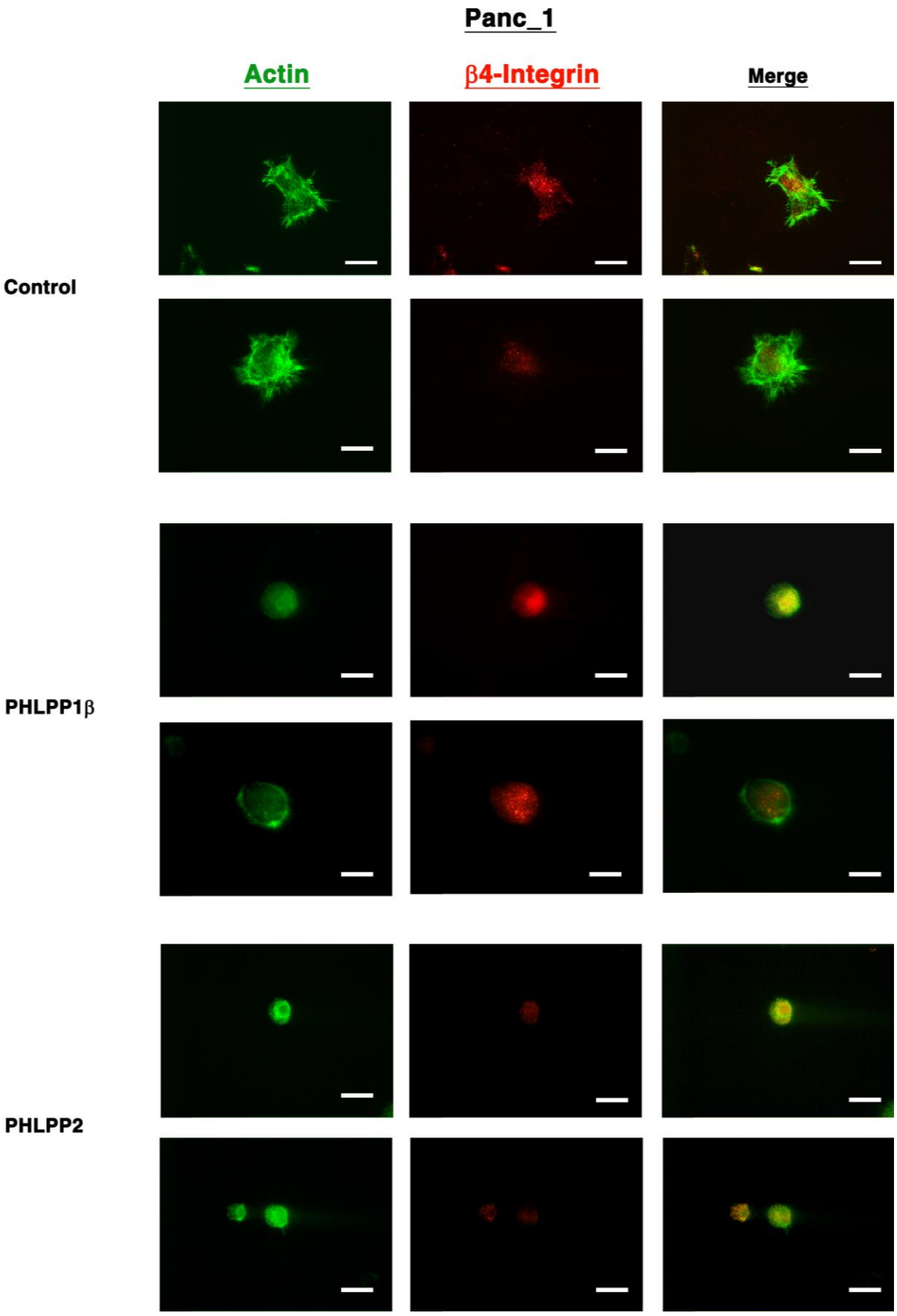

Panc_1
Actin
β4-Integrin
Merge
Control
PHLPP1β
PHLPP2

Figure 3.11. PHLPP affects actin cytoskeleton dynamic in pancreatic cancer cells.

Stable control and PHLPP overexpressing Panc-1 cells were seeded onto collagen coated cover slips in serum-free media. After 20 minutes of incubation at 37^{o}C the cells were fixed with 4% paraformaldehyde, blocked with anti-goat serum, incubated with β4-integrin antibody, and stained with Alexa488-phalloidin and Alexa 594 anti-mouse dye. TIRF images were from the basolateral membrane of the cells and two representative images are shown for each cell line (scale bars, 20 μm).

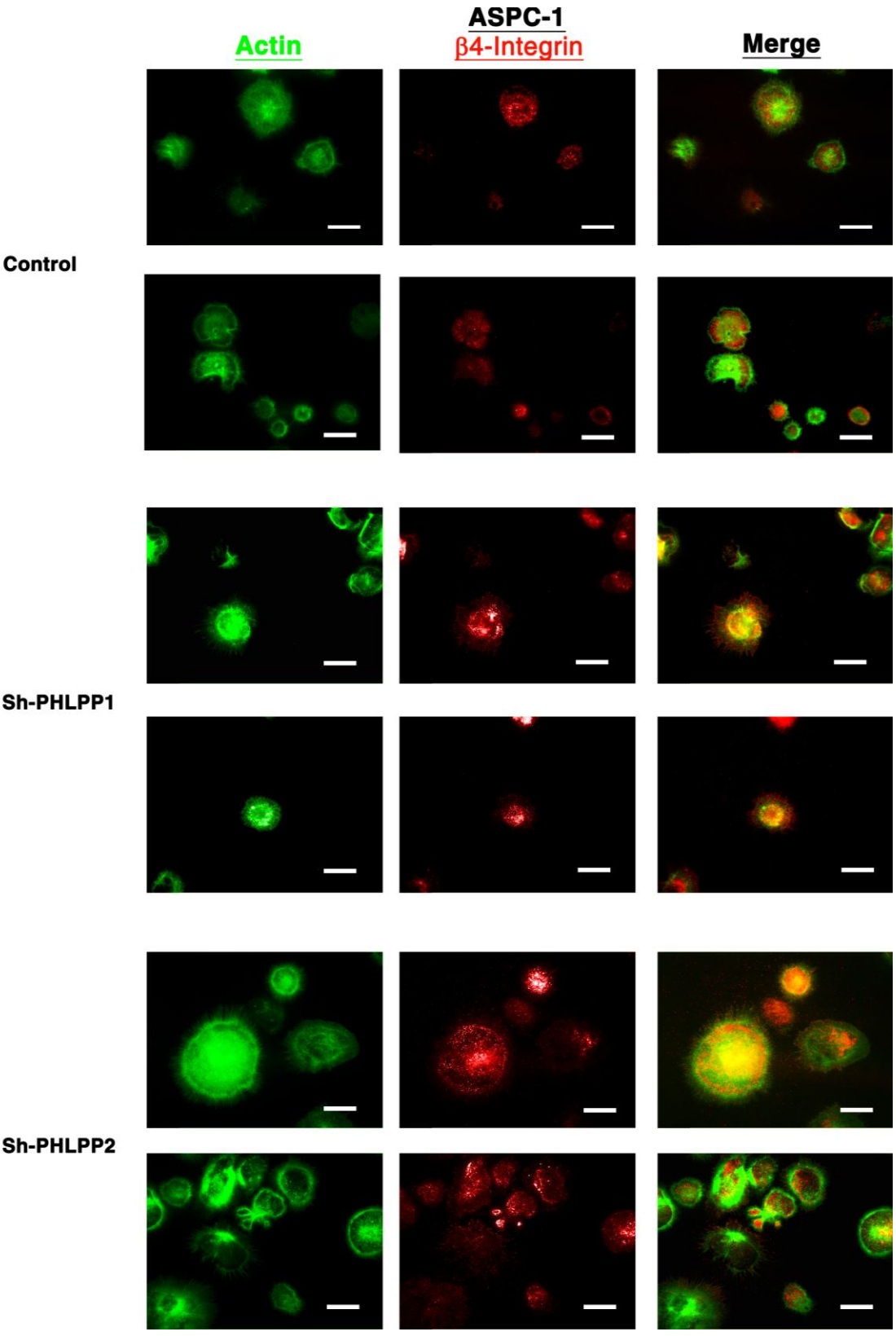

ASPC-1
Actin
β4-Integrin
Merge
Control
Sh-PHLPP1
Sh-PHLPP2

Figure 3.12. Knockdown of PHLPP affects integrin localization in pancreatic cancer cells.

Stable control and PHLPP overexpressing Panc-1 cells were seeded onto collagen coated cover slips in serum-free media. After 20 minutes of incubation at 37^{o}C the cells were fixed with 4% paraformaldehyde, blocked with anti-goat serum, incubated with β4-integrin antibody, and stained with Alexa 488-phalloidin and Alexa 594 anti-mouse dye. TIRF images were from the basolateral membrane of the cells and two representative images are shown for each cell line (scale bars, 20 μm).

PHLPP regulates the expression of integrin in pancreatic cancer cells

Previous studies have shown that increased β4 integrin expression is associated with pancreatic cancer progression [97]. Interestingly, when examining the expression of β integrin using Western blotting analysis, we found that the expression of both β4 and β1 integrin was significantly reduced in PHLPP overexpressing Panc-1 cells, suggesting that increased expression of PHLPP suppresses integrin expression. On the other hand, knockdown of PHLPP in ASPC-1 cells results in a significant increase in the expression of both β4 and β1 integrin (**Figure 3.13**).

To further determine if PHLPP-mediated regulation of β4 and β1 integrin expression is controlled at the transcriptional level, total RNAs were isolated from PHLPP overexpressing Panc-1 and PHLPP knockdown ASPC-1 cells, and quantitative real-time PCR (qRT-PCR) analysis was performed using probes specific for the β4 or β1 integrin gene. The results revealed that the mRNA levels of β4 and β1 integrin were not significantly altered by changes in PHLPP expression (**Figure 3.14**). Interestingly, the mRNA expression of β1 integrin was increased in PHLPP1β overexpressing cells. However, since the expression of β1 integrin protein was decreased, this increase in mRNA expression unlikely contributes to PHLPP induced downregulation of β1 integrin. Collectively, these results indicated that PHLPP negatively regulates integrin expression via a post-translational mechanism in pancreatic cancer cells.

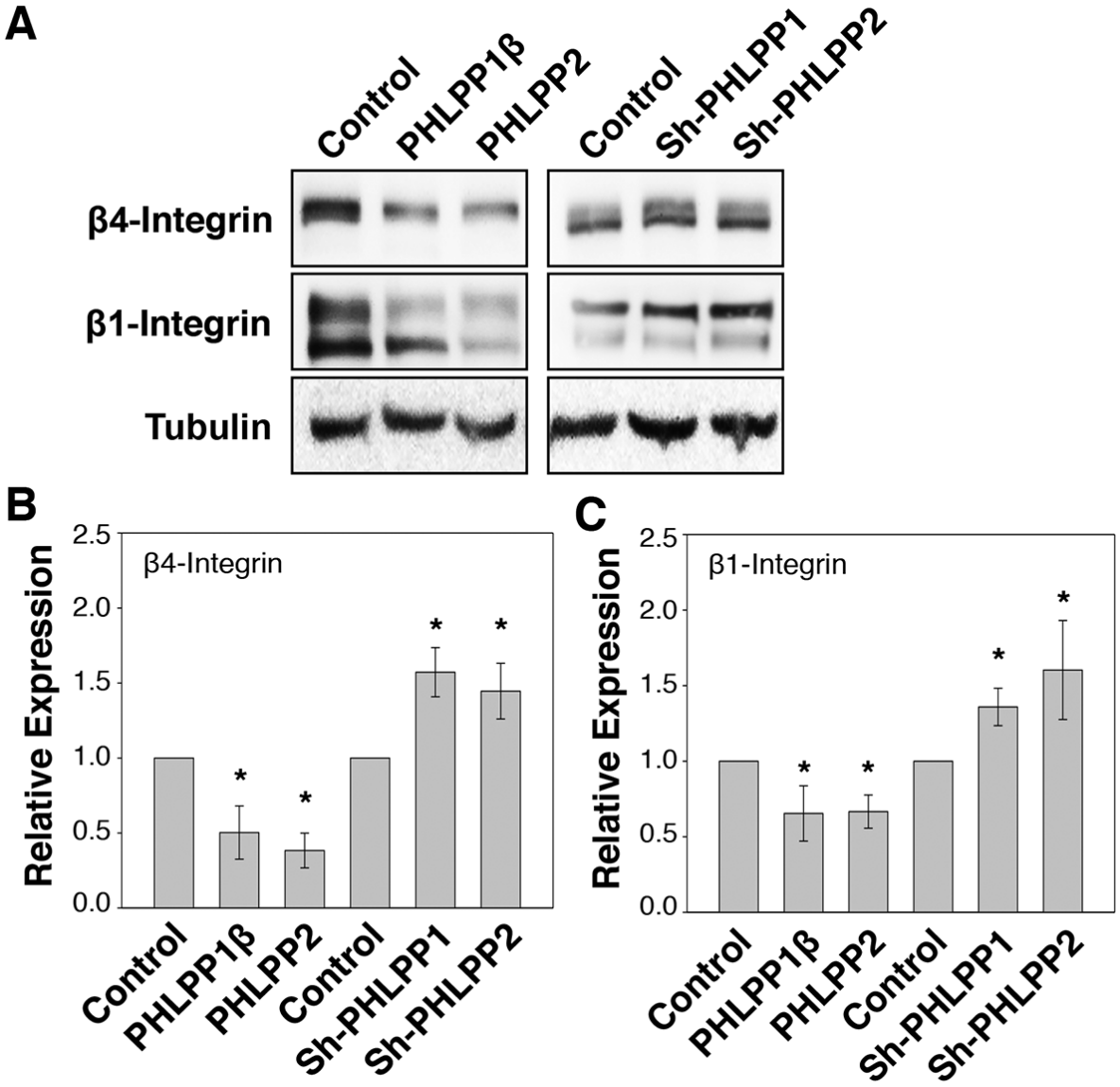

A
Control
PHLPP1β
PHLPP2
Control
Sh-PHLPP1
Sh-PHLPP2
β4-Integrin
β1-Integrin
Tubulin
B
β4-Integrin
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
Control
PHLPP1β
PHLPP2
Control
Sh-PHLPP1
Sh-PHLPP2
C
β1-Integrin
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
Control
PHLPP1β
PHLPP2
Control
Sh-PHLPP1
Sh-PHLPP2

Figure 3.13. Integrin protein expression inversely changes in regard to PHLPP expression.

(A) Cell lysates prepared from PHLPP overexpressing Panc-1 and PHLPP knockdown ASPC-1 cells were analyzed for the expression of β1 and β4 integrin and tubulin using immunoblotting. (B-C) Relative expression of β1 (B) and β4 integrin (C) was calculated and normalized to tubulin. The level in control cells was set to 1. Data represent the mean ± SEM (n=3, * $p<0.05$ by two-sample t-tests).

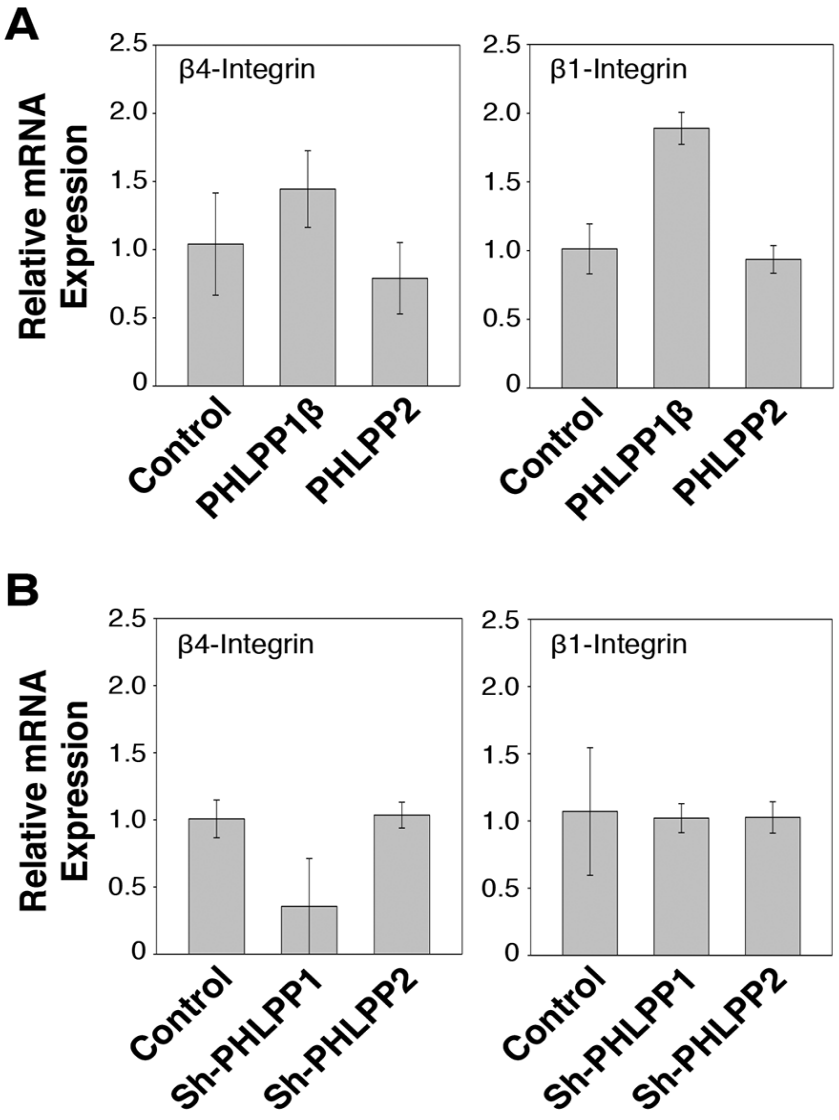

A
Relative mRNA Expression
β4-Integrin
β1-Integrin
2.5
2.0
1.5
1.0
0.5
0
Control
PHLPP1β
PHLPP2
B
Relative mRNA Expression
β4-Integrin
β1-Integrin
Control
Sh-PHLPP1
Sh-PHLPP2

Figure 3.14. Integrin mRNA expression is not altered relative to PHLPP expression.

Total RNAs were isolated from PHLPP overexpressing Panc-1 cells (A) and PHLPP knockdown ASPC-1 cells (B). Quantitative real-time PCR analysis was performed using probes specific for human β1 and β4 integrin genes. Each experimental point was done in triplicates, and the graphs represent the mean ± SD (n=3).

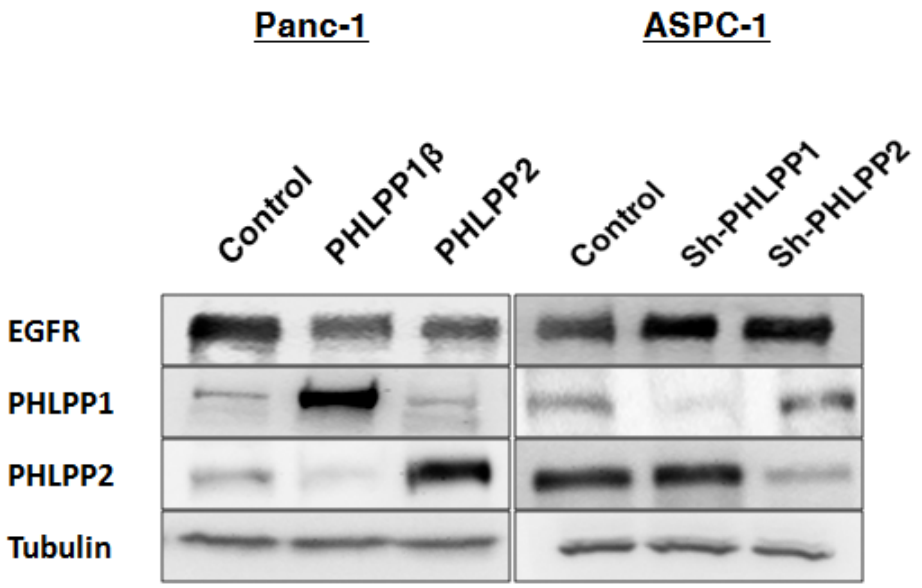

Panc-1
ASPC-1
Control
PHLPP1β
PHLPP2
Control
Sh-PHLPP1
Sh-PHLPP2
EGFR
PHLPP1
PHLPP2
Tubulin

Figure 3.15. EGFR protein expression mirrors integrin protein expression in response to PHLPP activity.

Cell lysates prepared from PHLPP overexpressing Panc-1 and PHLPP knockdown ASPC-1 cells were analyzed for the expression of EGFR, PHLPP1, PHLPP2 and tubulin using immunoblotting.

Interestingly, we found that not only was integrin expression altered when PHLPP expression was changed so was EGFR (**Figure 3.15**). EGFR is an oncogene that is commonly activated in many cancers including pancreatic cancer [77, 79, 81-83]. Activation of EGFR in pancreatic cancer can also be achieved via increased secretion of EGFR ligands by the tumor cells themselves [83]. Co-localization of EGFR with integrin has been reported in the lipid rafts suggesting common mechanisms may control the protein expression of both EGFR and integrins [130].

To further investigate the mechanism by which PHLPP regulates integrin expression, we tested if the PI3K/Akt or the MEK/ERK pathway is involved in controlling the expression of integrin proteins. Inhibiting the PI3K/Akt pathway using a PI3K inhibitor, LY294002, in ASPC-1 cells resulted in an approximately 40% reduction in both β4 and β1 integrin expression over time (**Figure 3.16A-B**). PP242, an inhibitor of PI3K and mTOR, is able to reduce integrin expression in ASPC-1 cells. However, this was not seen when rapamycin, an mTOR inhibitor, was used to treat the pancreatic cancer cells (**Figure 3.16 C**). Similar decrease of integrin expression was seen in cells treated with an Akt inhibitor, Akt-VIII (**Figure 3.16D**). In contrast, treating cells with a MEK inhibitor, PD325901, did not alter the expression of integrin (**Figure 3.17A**), suggesting that PHLPP-induced downregulation of integrin is likely mediated through inhibition of Akt signaling. As a control, we found that the mRNA levels of integrin were not significantly changed in cells treated with LY294002 (**Figure 3.17B**).

To further validate the influence that PI3K/Akt pathway has over integrin expression, we stably knocked down either Akt1 or Akt2 in ASPC-1 cells. In Akt knockdown cells both β4 and β1 integrin expression was greatly reduced compared to control (**Figure 3.18**). Just as seen previously in PHLPP overexpressing cells (**Figure 3.15**), EGFR protein expression was markedly reduced in Akt knockdown pancreatic cancer cells. Interestingly, knockdown of Akt induced mesenchymal-epithelial transition (MET), the reverse process of EMT, in ASPC-1 cells as indicated by increased E-cadherin and decreased vimentin expression. Similar results were observed in PHLPP overexpressing cells (**Figure 3.3**). Taken together, our findings suggested that PHLPP negatively regulates β4 and β1 integrin expression likely through inhibition of Akt activity in pancreatic cancer cells.

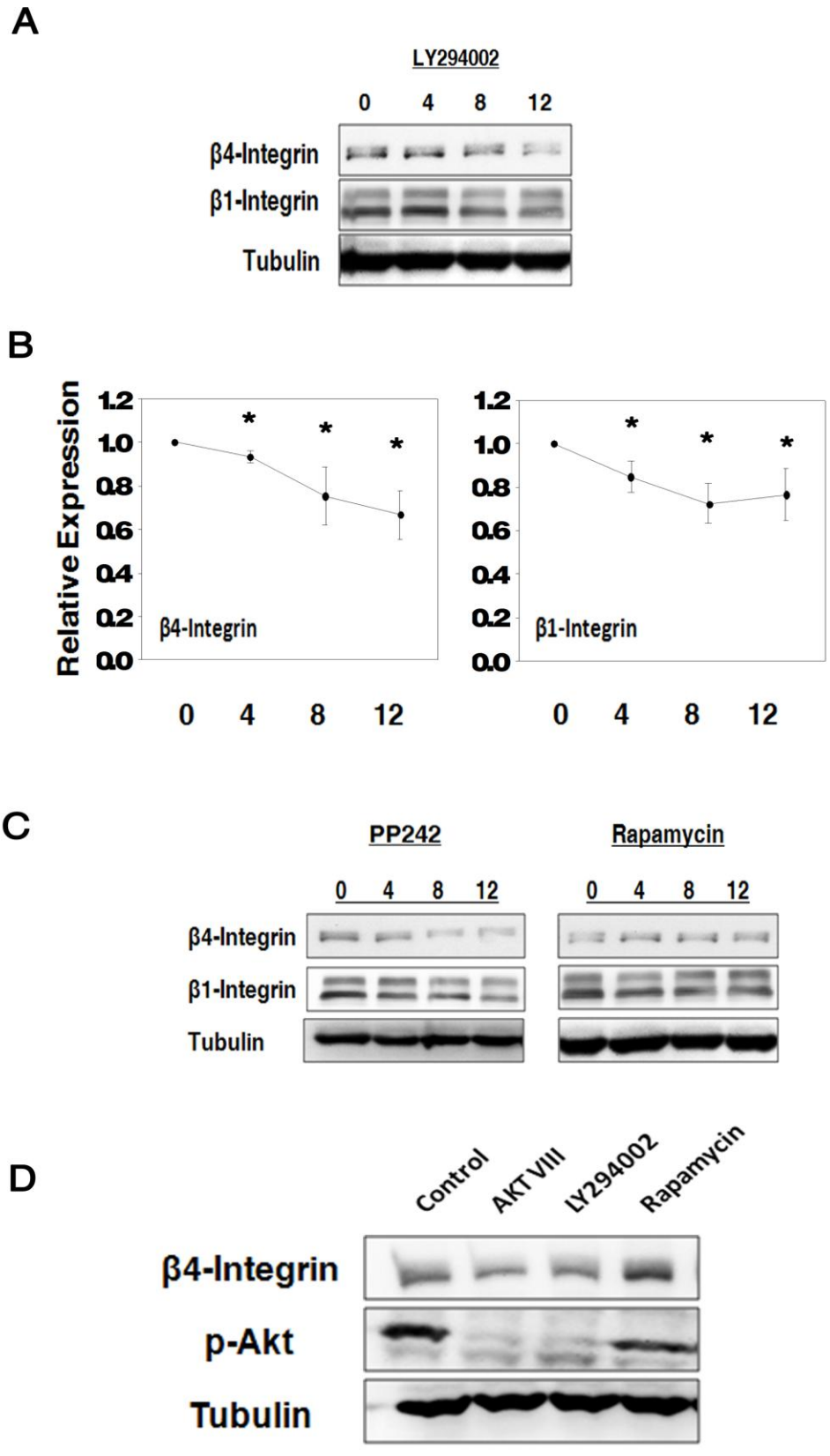

A
LY294002
0 4 8 12
β4-Integrin
β1-Integrin
Tubulin
B
Relative Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
β4-Integrin
β1-Integrin
0 4 8 12
C
PP242
Rapamycin
0 4 8 12
β4-Integrin
β1-Integrin
Tubulin
D
Control
AKT VIII
LY294002
Rapamycin
β4-Integrin
p-Akt
Tubulin

Figure 3.16. Inhibition of PI3K/Akt pathway decreases integrin expression.

(A) ASPC-1 cells were incubated with LY294002 for 0-12 hours. At indicated time points, cell lysates were prepared and analyzed for the expression of β1 and β4 integrin and tubulin using immunoblotting (B) Relative expression of β1 and β4 integrin at different time points following LY294002 treatment in ASPC-1 cells was calculated and normalized to tubulin. The level in untreated cells was set to 1. Data represent the mean ± SEM (n=3, * $p < 0.05$ by two-sample t-tests). (C) ASPC-1 cells were incubated with PP242 or rapamycin for 0-12 hours. At indicated time points, cell lysates were prepared and analyzed for the expression of β1 and β4 integrin and tubulin using immunoblotting. (D) ASPC-1 cells were incubated with AKT VIII, LY294002, and Rapamycin for 8 hours. Cell lysates were prepared and analyzed for the expression of β4 integrin, p-AKt, and tubulin using immunoblotting.

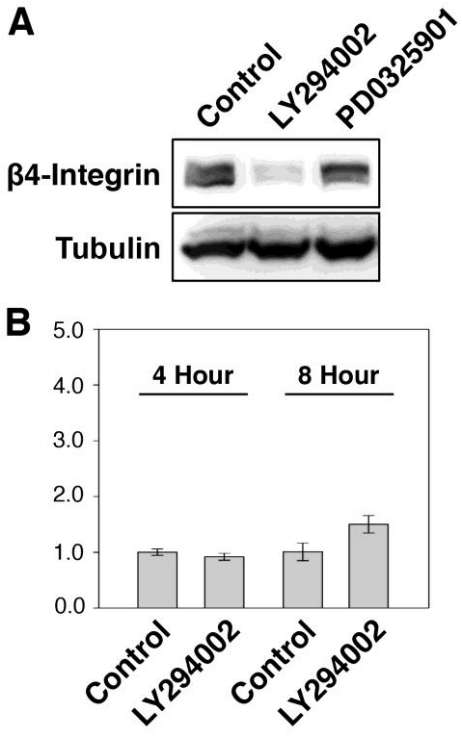

A
Control
LY294002
PD0325901
β4-Integrin
Tubulin
B
5.0
4.0
3.0
2.0
1.0
0.0
4 Hour
8 Hour
Control
LY294002
Control
LY294002

Figure 3.17. The effect of inhibiting PI3K/Akt and MEK/ERK signaling on the expression of integrins in pancreatic cancer cells.

(A) ASPC-1 cells were treated with DMSO, LY294002 or PD0325901 for 16 hours. Cell lysates were prepared and analyzed for the expression of β4 integrin and tubulin using immunoblotting. (B) Panc-1 cells were treated with LY204002 (20 nM) for 4 or 8 hours and total RNAs were extracted. Real-time PCR analysis was performed using probes specific for human β4 integrin gene. Each experimental point was done in triplicates, and the graphs represent the mean ± SD (n=3).

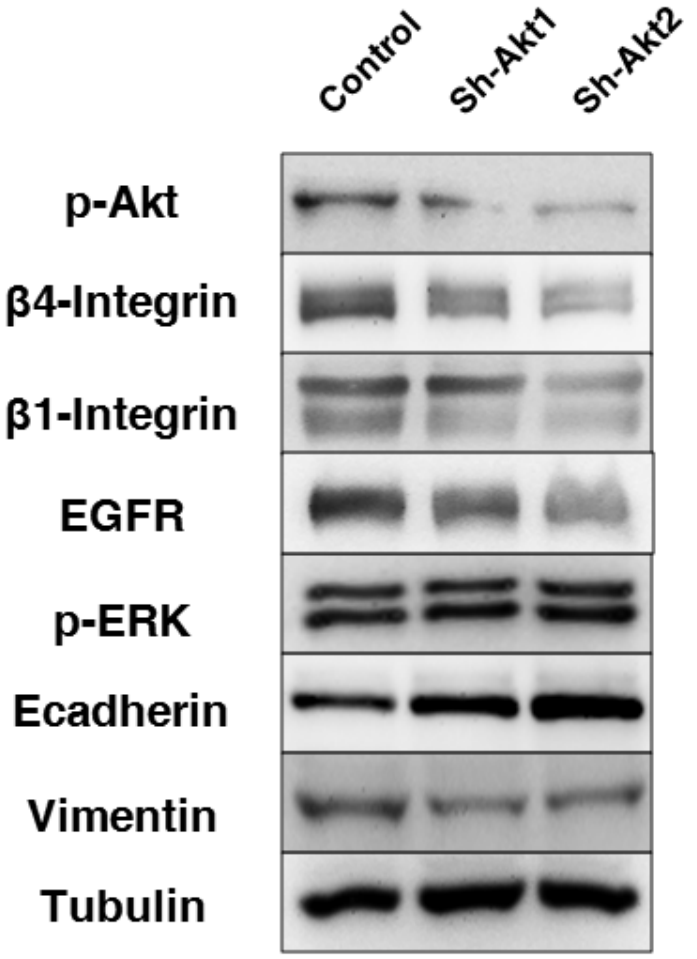

Control
Sh-Akt1
Sh-Akt2
p-Akt
β4-Integrin
β1-Integrin
EGFR
p-ERK
Ecadherin
Vimentin
Tubulin

Figure 3.18. Knockdown of Akt decreases the expression of integrin in pancreatic cancer cells.

Cell lysates prepared from sh-control, sh-Akt1 and sh-Akt2 ASPC-1 cells were analyzed for the expression of β1 and β4 integrin. The expression of EGFR, pERK, E-cadherin, vimentin and tubulin was also analyzed using immunoblotting.

Blocking integrin activity inhibits PHLPP-loss induced increase in cell motility

To determine the functional impact of PHLPP-mediated inhibition of integrin expression, we examined the rate of cell migration in control and PHLPP knockdown ASPC-1 cells in the presence of blocking antibodies against β4 or β1 integrin. As shown in **Figure 3.19A**, incubating cells with either β4 or β1 integrin blocking antibodies significantly reduced cell migration in both control and PHLPP knockdown cells as measured by Transwell assays. Moreover, inhibiting integrin activation impaired cell motility at the single cell level as the relative velocity in the control and PHLPP knockdown cells became indistinguishable after the blocking antibody treatment (**Figure 3.19B**). These data suggested that increased integrin expression in PHLPP knockdown cells is responsible for increased cell migration.

We then assessed the ability of Akt knockdown ASPC-1 cells to migrate in response to HGF. Surprisingly, Akt knockdown cells had enhanced migratory ability when compared to control cells (**Figure 3.20**). This paradoxical effect of Akt on cell migration has been reported in breast cancer cells in which Akt-mediated inhibition of NFAT activity has been indicated as the underlying molecular mechanism [131, 132]. In addition, when treating Akt knockdown cells with β1-integrin blocking antibody, the ability of control cells to migrate was significantly inhibited as described in **Figure 3.19**. However, the β1-integrin blocking antibody was unable to block migration of Akt knockdown cells (**Figure 3.20**). Interestingly, we found the activation of ERK was increased in Akt

knockdown cells (**Figure 3.20**). This activation of ERK may mediate the integrin-independent effect of silencing Akt on cell migration in pancreatic cancer.

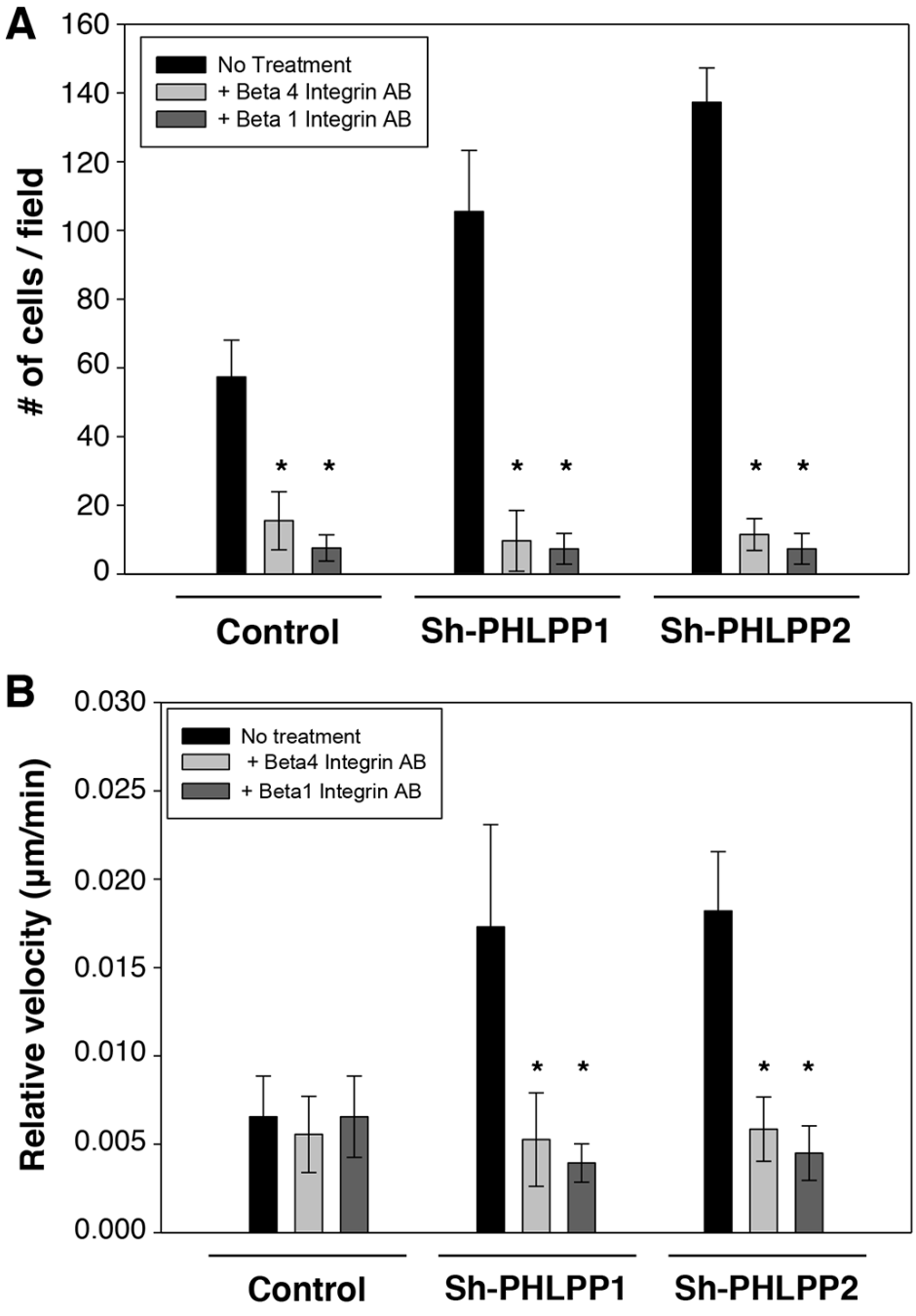

A
160
140
120
100
80
60
40
20
0
# of cells / field
No Treatment
+ Beta 4 Integrin AB
+ Beta 1 Integrin AB
*
*
*
*
*
*
Control
Sh-PHLPP1
Sh-PHLPP2
B
0.030
0.025
0.020
0.015
0.010
0.005
0.000
Relative velocity (µm/min)
No treatment
+ Beta4 Integrin AB
+ Beta1 Integrin AB
*
*
*
*
Control
Sh-PHLPP1
Sh-PHLPP2

Figure 3.19. The PHLPP-mediated regulation of cell migration relies on its ability to control integrin expression.

(A) Stable control and PHLPP knockdown ASPC-1 cells were subjected to Transwell migration analysis using HGF as the chemoattractant. To block integrin activation, ASPC-1 cell suspensions were incubated with blocking antibodies of β1 or β4 integrin in serum-free media containing 1% BSA for 20 minutes at 4 °C. The cells were then seeded onto the collagen coated Transwells in the presence of the blocking antibodies and allowed to migrate for 4 hours at 37°C. Each experiment was done in duplicate and three independent experiments were averaged and expressed as mean ± SEM (* $p<0.01$ by two-sample t-tests compared to the control cells). (B) Cells as treated as above with blocking antibodies were monitored for single cell motility using time-lapse imaging. The cells were allowed to migrate in response to HGF for 6 hours. The average velocities of 12 cells from each group are shown. Data represent mean ± SEM (n = 12 cells/line, * $p<0.05$ by two-sample t-tests).

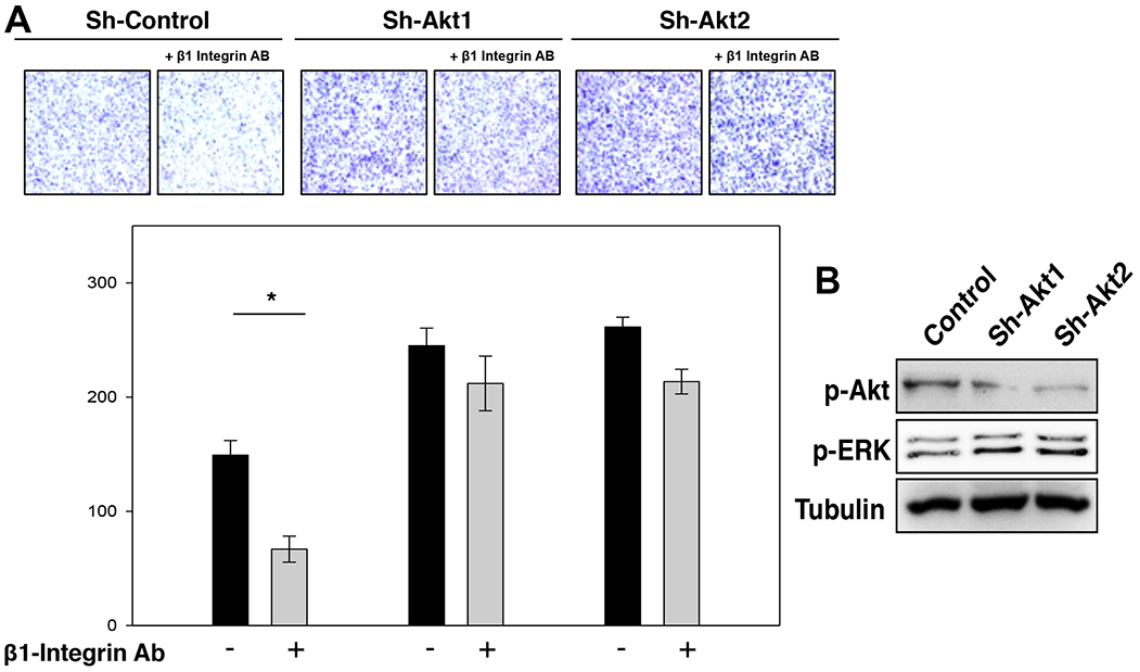

A
Sh-Control
Sh-Akt1
Sh-Akt2
+ β1 Integrin AB
+ β1 Integrin AB
+ β1 Integrin AB
300
200
100
0
*
β1-Integrin Ab
-
+
-
+
-
+
B
Control
Sh-Akt1
Sh-Akt2
p-Akt
p-ERK
Tubulin

Figure 3.20. The effect of Akt knockdown on cell migration in pancreatic cancer cells.

(A) Stable control and Akt knockdown ASPC-1 cells were subjected to Transwell migration assays using HGF as chemoattractant. To block integrin activation, ASPC-1 cell suspensions were incubated with the β1 integrin blocking antibody in serum-free media containing 1% BSA for 20 minutes at 4 °C. The cells were then seeded onto the collagen coated Transwells in the presence of the blocking antibodies and allowed to migrate for 4 hours at 37°C. Each experiment was done in duplicate and three independent experiments were averaged and expressed as mean ± SEM (* $p<0.01$ by two-sample t-tests compared to the control cells). (B) Cell lysates prepared from control and Akt knockdown ASPC-1 cells were analyzed using immunoblotting.

Integrin expression is controlled through lysosomal degradation in pancreatic cancer cells

Recent studies have suggested that integrins can be internalized upon binding to extracellular matrix (ECM) ligands and trafficked to lysosome for degradation [133-136]. To determine if decreased integrin expression seen in PHLPP overexpressing cells is due to increased lysosomal degradation, we incubated control and PHLPP overexpressing Panc-1 cells in NH_4Cl containing media. The presence of NH_4Cl, a lysosomotropic alkalinizing agent, inhibits lysosomal protein degradation. Indeed, both β4 and β1 integrin expression increased in response to lysosome inhibition with NH_4Cl (**Figure 3.21**). At the end of 8-hour treatment, the expression of β4 and β1 integrin in PHLPP overexpressing cells was similar as that of the control cells (**Figure 3.21**). Similar results were obtained in cells treated with different lysosome inhibitors, Bafilomycin A1 and chloroquine (**Figure 3.21**). Taken together, we showed that inhibition of lysosome-mediated protein degradation rescues the integrin expression in PHLPP overexpressing pancreatic cancer cells.

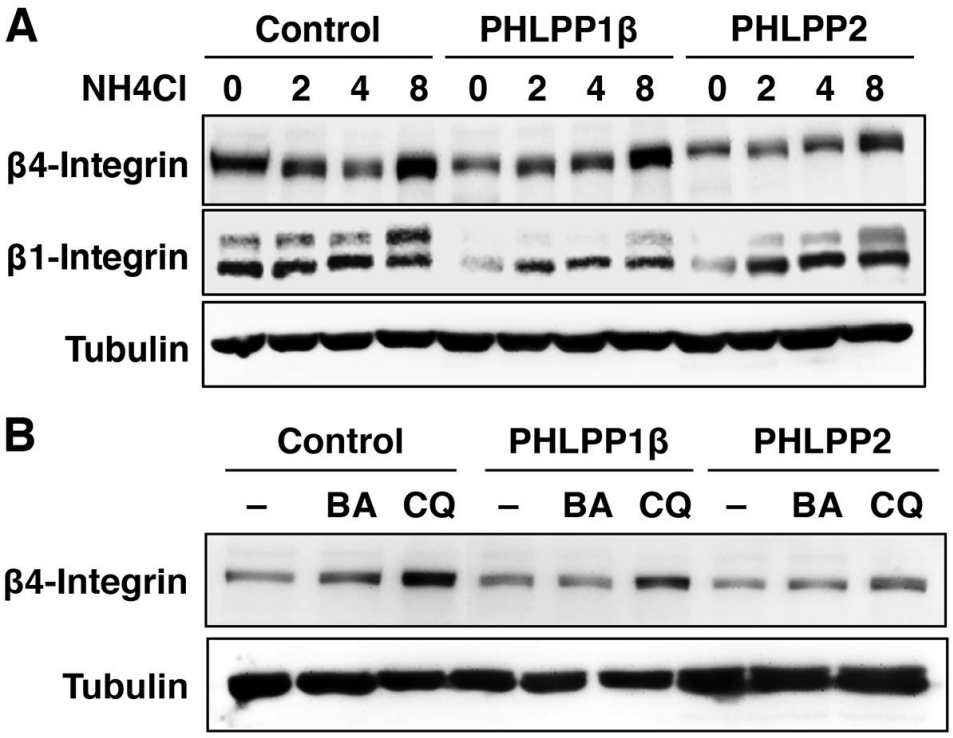

A
Control
PHLPP1β
PHLPP2
NH4Cl
0 2 4 8 0 2 4 8 0 2 4 8
β4-Integrin
β1-Integrin
Tubulin
B
Control
PHLPP1β
PHLPP2
– BA CQ – BA CQ – BA CQ
β4-Integrin
Tubulin

Figure 3.21. Inhibiting lysosomal degradation pathway gives enhanced expression of integrin.

(A) Stable control and PHLPP overexpressing Panc-1 cells were treated with NH4Cl (5 mM) for 2, 4, and 8 hours. Cell lysates were prepared and analyzed by immunoblotting for integrin expression. (B) Stable control and PHLPP overexpressing Panc-1 cells were treated with Bafilomycin A (BA) or Chloroquine (CQ) for 4 hours. Cell lysates were prepared and analyzed by immunoblotting for β4 integrin expression.

Chapter 4. Discussion and Future Directions

PHLPP functions as a negative regulator of cell migration in pancreatic cancer.

A number of recent studies have provided strong evidence suggesting that PHLPP serves as an important tumor suppressor in various cancer types [103, 106-108, 125, 126, 137]. For example, it has been shown that PHLPP is downregulated in colon cancer patients and decreased PHLPP expression promotes tumor growth as the result of increased activation of both PI3K/Akt and RAS/RAF oncogenic signaling [103, 108, 114]. Similarly, reduced PHLPP expression has been reported in pancreatic cancer patient specimens and re-expression of PHLPP1 in pancreatic cancer cells induces cell death and inhibits tumorigenesis [111]. However, the role of PHLPP in regulating cell migration has not been determined in pancreatic cancer. Here we show that PHLPP plays an important role in influencing cell motility. Specifically, overexpression of either PHLPP isoform decreases cell migration whereas knockdown of endogenous PHLPPs significantly enhances cell motility in pancreatic cancer cells. The role of PHLPP in pancreatic cancer has been supported by the results obtained from wound healing assay, transwell assay, and single cell motility assays. Moreover, the level of PHLPP expression regulates the ability of pancreatic cancer cells to grow in 3D culture. This effect can be seen in both the knockdown and overexpressing pancreatic cells. Knockdown PHLPP ASPC-1 cells have significantly larger cluster formation; whereas, overexpressing PHLPP in Panc-1 cells significantly inhibits the formation of large clusters. Instead these Panc-1

overexpressing PHLPP pancreatic cancer cells have a much reduced cluster size compared to control as well as loss of filamentous extensions and protrusions from the cell and cluster. This PHLPP-mediated inhibition of cell growth and migration coincides with inhibition of both Akt and MEK/ERK activation. In our study, the effect of PHLPP on regulating cell motility in pancreatic cancer cells is not isoform specific, with either PHLPP1 or PHLPP2 overexpression is able to inhibit cell migration whereas knockdown of either isoform results in a similar increase in cell motility. This lack of isoform specificity is also seen in regarding to the changes in EMT phenotype of pancreatic cancer cells. In Panc-1 cells overexpressing either isoform results in enhanced expression of E-cadherin and loss of vimentin. E-cadherin is an epithelial cell marker whereas vimentin is considered a mesenchymal cell marker. Loss of E-cadherin and gain of vimentin are the phenotypes associated with EMT and increased migratory potential. The enhanced EMT phenotype is shown in stable PHLPP knockdown ASPC-1 cells when compared to control. Pancreatic cancer is extremely aggressive and can quickly become metastatic. It is important to find new chemotherapy targets that are unique to pancreatic cancer. PHLPP's ability to influence EMT and migration of pancreatic cancer is likely related to its regulation of integrin degradation. Reduced integrin expression at the cell membrane may impair lamellopodia extension and adhesion to the ECM that are required for cell migration. Taken together, our findings establish a novel link connecting PHLPP downregulation with enhanced integration function in pancreatic cancer.

Regulation and functional implication of integrin trafficking

In this study, we show that PHLPP plays a novel role in altering integrin expression. Since varying PHLPP expression, meaning overexpression of PHLPP in Panc-1 cells or treating cells with PI3K/Akt inhibitors such as AktVIII and LY294002, has no effect on decreasing the mRNA levels of integrins, PHLPP may regulate integrin expression via a post-translational mechanism. Our findings that PHLPP overexpression and treating cells with PI3K/Akt inhibitors result in a similar decrease in β1 and β4 integrin expression suggest that reduced Akt activity is likely the molecular mechanism underlying PHLPP-mediated downregulation of integrins. Indeed, knockdown of either Akt1 or Akt2 significantly decreases both β1 and β4 integrin expression. Furthermore, using lysosome inhibitors we find that the expression of both β1 and β4 integrins increase significantly, rescuing the effects of PHLPP overexpression in Panc-1 cells. Whereas, treating cells with a proteasome inhibitor MG-132 has no effect on rescuing β1 and β4 integrin expression in PHLPP overexpressing cells. This gives the first hint that the point of influence for PHLPP may be in allowing for integrin degradation instead of endosomal recycling. Another interesting finding is that EGFR protein expression is altered in response to changes of PHLPP expression levels to a similar extent as the integrin protein expression. When PHLPP is overexpressed, EGFR protein expression is reduced or inhibited compared to control; however, when PHLPP is lost through a stable knockdown mechanism EGFR protein expression is increased. When lysosome inhibitors are used to treat pancreatic cancer cells there is an enhanced expression of integrin

compared to untreated cells, and lysosomal inhibitors are able to rescue the loss of integrin expression in PHLPP overexpressing cells. These results suggest that PHLPP may regulate integrin expression by promoting lysosomal-mediated degradation of integrin proteins. Previous studies have supported the notion that of lysosome plays a major role in mediating degradation of integrin [133, 134, 138, 139]. If PHLPP can enhance integrin lysosomal degradation then cell motility may be halted therefore providing a new possible therapeutic target to prevent metastasis of pancreatic cancer.

The cell surface delivery and endocytosis of integrins are dynamically regulated processes and are fundamental for cell migration in wound healing and during cancer cell metastasis [138, 140]. Like many cell surface receptors, integrins are known to undergo endo/exocytic cycles, in which they are internalized, trafficked through endosome, and then recycled back to the plasma membrane [100, 139-141]. This recycle process occurs rather efficiently as the degradation rates of integrins are generally very slow [136, 142]. However, recent studies reveal that ligand-bound integrins can be ubiquitinated and sorted to lysosome for degradation by the ESCRT pathway [136, 143]. Preventing lysosome-mediated degradation of integrins increases recycling of integrins to the plasma membrane and enhances integrin-mediated cancer cell migration and invasion [139, 141]. It was recently discovered that sorting nexin 17 binds to the free cytoplasmic tail of β1-integrin while in the endosome preventing its degradation and allowing for endosomal recycling [144]. Endosomal recycling of cell surface receptors and integrins is a major process that may confer EMT and

aggressiveness of cancer. The ability of cancer cells to recycle receptor tyrosine kinases (RTKs) and integrins allow them to effectively and efficiently produce extensions that allow for migration [138]. It has been suggested that oncogenic mutations in EGFR may allow for the receptors to evade lysosomal degradation and promote tumor progression [138]. Accepted beliefs in EGFR signaling include that the majority of signaling occurred at the plasma membrane and is attenuated through endosomal internalization of the receptor. However, this was recently challenged when it was demonstrated that signaling can still occur after internalization from the actual endosomes [145]. Oncogenic mutations in EGFR lead to sustained activation even when there is a large deletion of the extracellular domain. This gives a constitutively active protein with less ability to be silenced through internalization [145]. It is a possibility that when EGFR and integrin reside in the same lipid raft that these types of mutations in EGFR could lead to reduction of internalization of integrin and allowing for sustained signaling through integrin itself by keeping it in the active conformation [146]. In addition, there is evidence supporting a physical interaction between EGFR and integrins, which would provide another mechanism for coordinated control of EGFR and integrin protein expression [147].

Promotion of lysosome-dependent degradation of integrins, has been shown previously, by activating PI3K/Akt signaling which enhances integrin recycling to the plasma membrane in a GSK-3β-dependent manner. GSK-3β phosphorylates kinesin light chain and inhibits the activity of kinesin. This leads to inhibition of endosomes trafficking through the microtubules and subsequent

maturation into late endosomes and merging with the lysosomes [148]. One assay that could be performed to determine the potential involvement of GSK-3β is to determine the phosphorylation of GSK-3β in PHLPP overexpressing Panc-1 cells and PHLPP knockdown ASPC-1 cells. It has been shown recently that GSK-3β and Akt are associated with Appl endosomes, a subpopulation of early endosomes [149]. It is possible that the close association of Akt and GSK-3β allows for Akt-mediated phosphorylation and subsequently inactivation of GSK-3β. In pancreatic cancer, activation of Akt as the result of PHLPP-loss would lead to inactivation of GSK-3β and allow integrins to bypass lysosomal-mediated degradation and remain membrane bound. Using integrin recycling assays, we would be able to look at integrin recycling under various settings, such as PHLPP overexpressing cells, PHLPP knockdown cells, Akt knockdown cells, activation of the PI3K/Akt pathway via stimulation with growth factor receptor ligands, and inhibition of the PI3K/Akt pathway using inhibitors. This assay will allow us to directly assess how integrin expression at the plasma membrane is regulated by PHLPP and PI3K/Akt signaling.

At this moment our data suggests the involvement of PI3K/Akt pathway on regulating integrin expression, but our data is limited to using the inhibitor approach. Since the pancreatic cancer cells used in our study contain a genetic background of activated mutant KRas which allows for sustained and prolonged activation of both the PI3K/Akt pathway and the Raf/MEK/ERK pathway, it is a possibility of using dominant negative proteins downstream of KRas that would provide a mechanism to tease out the role that each pathway plays. We are able

to silence Akt and we can use shRNA to knockdown function of Raf/MEK/ERK or we can overexpress a dominant negative form of Raf1 which would help in terminating the Raf/MEK/ERK signaling pathway [150]. A dominant negative form of ERK could also be used to create a situation where the pathway is terminated further down the cell signaling cascade. This was investigated in human pancreatic cancer cell lines and it was discovered that expression of dominant negative ERK lead to reduced survival of pancreatic cancer cells in colony formation studies [151]. Using these approaches we could further elucidate either the PI3K/Akt pathway or the Raf/MEK/ERK pathway is responsible for altering integrin protein expression. These additional studies are needed in order to provide a complete picture for future therapeutic targets.

Another role Akt may play in integrin expression is through direct phosphorylation. It has been shown that Akt phosphorylates both β3 and β1 integrins tail thereby allowing adhesion molecules to interact with their tails keeping them forcefully anchored within the cell membrane [152, 153]. It is interesting that β4 integrin carries the longest tail out of all of the integrins and would allow for direct Akt phosphorylation. Future experiments can be performed to replace endogenous β4 integrin with a mutant β4 integrin that lacks the cytoplasmic tail. Comparing the effect of PHLPP on regulating the expression of mutant integrin with the wild-type integrin will allow for further elucidation of the molecular mechanism by which PHLPP controls the expression of integrins. Using TIRF microscopy as shown in **Figure 3.11-3.12** we can determine if mutant β4 and β1 integrin are better retained at the basolateral membrane. As in

Figure 3.12 the staining of integrin is punctate and may coincide with recycling endosomes. This can also be viewed through TIRF imaging by looking at co-localization of integrin with recycling endosomal marker Rab11 [154]. There is evidence suggesting that PHLPP is associated with the plasma membrane or the endosomal membrane [126, 155]. Using TIRF we could demonstrate the possibility of a physical interaction between PHLPP and integrin at the plasma membrane or the endosomal level. PHLPP has a PH domain that has been shown to bind PIP_3. PIP_3 is also found on endosomes therefore providing another possible link between PHLPP and integrin [156].

In a previous study an ARF GAP called ACAP1 was shown to be targeted to recycling endosomes in order to sort cargo leading either to lysosomal degradation or allowed to recycle to the plasma membrane. Li et al demonstrated that ACAP1 is required for integrin endosomal recycling and co-localizes at recycling endosomes. When cells are stimulated, most likely this would be when integrin binds to its respective ligand or there's stimulation through an RTK, integrins (such as β1 integrin) are sent to be recycled through recycling endosomes. ACAP1 associates with β1 integrin at the recycling endosomes interface. Akt associates with ACAP1 and phosphorylates its Ser554 residue. This allows ACAP1 to sort the internalized endosomes cargo such as integrin into recycling endosomes so it can be re-associated with the plasma membrane [157]. This provides another mechanism linking Akt activation to integrin recycling. It is possible that when PHLPP is downregulated, activated Akt leads to phosphorylation of ACAP1 which allows the internalized integrins to be

sorted into the recycling endosome instead of continuing its degradation path to the lysosome. Future studies can determine the phosphorylation of ACAP in PHLPP overexpressing and knockdown cells. In addition, ACAP1 can be silenced using RNAi in PHLPP knockdown where Akt is continuously active to evaluate its involvement. Co-localization of ACAP1, Akt, and integrin in pancreatic cancer cells with the endosomal recycling marker Rab11 can also be performed. These studies will provide additional mechanistic insight into integrin recycling.

This PHLPP-mediated regulation of integrin recycling process likely has a broader implication in the trafficking of membrane receptors. One interesting observation in our study is that not only is integrin expression changed in response to PHLPP expression, but also is EGFR a known RTK that is degraded through the lysosomal pathway. It would not be surprising if EGFR and integrin as well as other growth factor receptors and adhesion molecules use some of the same mechanisms in order to regulate their lysosomal degradation and endosomal recycling pathways. Using some of the same machinery would be efficient and provide a unique way to regulate very intricate multi-step processes. Thus, PHLPP represents a novel regulator of cell motility by possibly controlling the balance of integrin recycling and degradation, as well as other RTKs, in pancreatic cancer cells.

Integrin and invadosomes

Integrins are believed to be found in the “invadosomes” when it comes to migration or adhesion signaling especially in cancer [158]. Invadosomes are structures in invasive cells that are similar to podosomes in structure and function. It has been known for some time that integrins have two different conformations, low and high affinity conformations, which may allow them to sense the outside environment or particular ECM [159]. Essentially the interaction between integrin and ECM proteins connects the motility of the cell to the environment both internal and external [158]. Although a number of studies have indicated β3 integrin as the major regulator of invadosome formation because of its co-localization with the invadosome structures [160, 161], β3 integrin deficient cells are still capable of forming invadosomes [162]. Additional studies show that β1 but not β3 integrins are required for invadosome formation and stabilization; and the activation state of β1 has been linked to the integrin-mediated degradation of ECM [162, 163]. It has been suggested that actin bundles are grouped together with integrins and the ECM substrates to create the invadosomes (**Figure 4.1**) [162].

There are some novel concepts that allow for these invadosomes to become predominantly explored in the future of cancer and especially pancreatic cancer research. One such interesting concept is that metallomatrix proteins are used by the invadosomes to promote degradation of the ECM to allow for the invadopodia and invadosome formation in cancer cells [164]. In addition, it has been shown not only pancreatic cancer cells but the cancer-associated

fibroblasts (CAFs) can form invadosomes as well. One study demonstrates that high expression of palladin in CAFs promotes the formation of invadosomes and invadopodia, which are responsible for creating channels in the ECM for pancreatic cancer cells to invade and metastasize through tumor stroma [165].

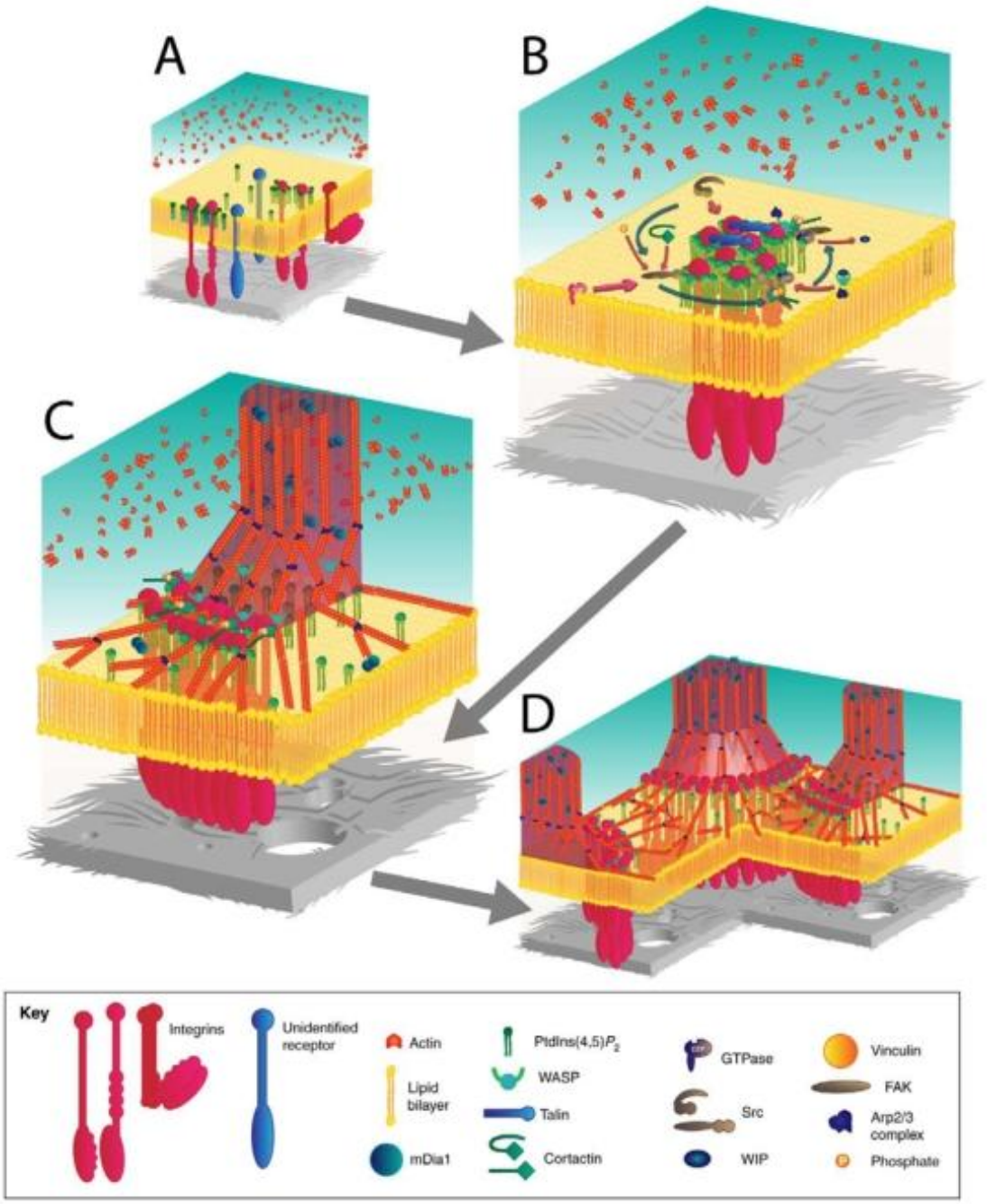

A
B
C
D
Key
Integrins
Unidentified receptor
Actin
Lipid bilayer
mDia1
PtdIns(4,5)P_2
WASP
Talin
Cortactin
GTPase
Src
WIP
Vinculin
FAK
Arp2/3 complex
Phosphate

Figure 4.1. The invadosome and its components.

The invadosome is comprised of many components with the major players being integrins, receptors, the lipid membrane, actin, talin, and other proteins and structures of the cell. This figure is adapted from Albiges-Rizo et al. [166].

Interestingly, Neel et al. have discovered that KRAS mutation by itself was sufficient to induce invadopodia formation in pancreatic cancer cells. However, activation of the RAF/MEK/ERK pathway is not required to form the invadopodia whereas treating cells with inhibitors of the PI3K/Akt pathway blocks the formation of invadopodia [167]. This finding supports our results that PHLPP-dependent downregulation of integrin expression is mediated by Akt inactivation and inhibition of the PI3K/Akt pathway in pancreatic cancer cells. Enhanced activation of Akt in PHLPP knockdown cells may sustain the expression of integrin via continual recycling of integrins to the membrane. Given the role of integrin in promoting invadosome formation, loss of PHLPP may enhance the invasive growth of pancreatic cancer cells through invadosomes and invadopodia formation. To support this hypothesis, we find that the expression of integrin is mainly localized at the cell extensions in PHLPP knockdown ASPC-1 cells as detected by TIRF microscopy (**Figure 3.11-3.12**). The vesicle-like staining patterns of integrins may reflect the recycling of integrins that are associated with invadosome through the endosomes along the actin bundles. Future studies are needed to further test the hypothesis that Akt activity is involved in regulating the trafficking and recycling of integrins and to elucidate how integrins are internalized from the invadosomes through the endocytic pathway and sorted for lysosomal degradation.

Conclusions

While our study provides the first glimpse of PHLPP as a tumor suppressor in pancreatic cancer as a regulator of migration through possible control of integrin expression and degradation, more studies are needed to extend our findings. To determine the functional significance of PHLPP *in vivo*, we can take advantage of PHLPP1 and PHLPP2 knockout mice. It will be important to investigate if PHLPP-loss promotes pancreatic carcinoma formation and progression in vivo using mouse models. Future studies can be performed to cross PHLPP1 or PHLPP2 knockout mice with KRas activating mutant mice in order to develop a working mouse model where the effect of PHLPP-loss in pancreatic will be monitored on the background of KRas activation. Since only KRAS mutation is needed to begin the process of PanIN development, it is feasible that elimination of tumor suppressor PHLPP would accelerate pancreatic tumorigenesis in these mouse models. Additional studies on signaling activation as the result of PHLPP-loss will help to identify potential therapeutic targets in pancreatic cancer.

As previously stated, late stage diagnosis and the aggressiveness of pancreatic carcinoma, PDAC patients have a very low survival rate [124, 168]. The treatment options for PDAC are few and surgical resection is nearly impossible for the average patient [169]. Understanding the molecular mechanism leading to EMT and progression of PDAC potentially has a long lasting benefit in creating new treatment strategies, therapeutic targets, and even gene editing techniques. In this study, we provide the first strong evidence

supporting a tumor suppressor role of PHLPP in pancreatic cancer. Moreover, our finding that decreased PHLPP expression promotes EMT and cell motility through the possibility of inhibiting lysosome degradation of integrin proteins identifies a novel mechanism by which PHLPP suppresses oncogenic signaling. Further studies are warranted to further elucidate how PHLPP controls the protein expression of integrin through Akt activity. The ramifications of future studies would not only benefit pancreatic cancer but possibly contribute to carcinoma knowledge across all cancer types as well as auto-immune disorders, aging related diseases, diabetes, obesity, and other PI3K/Akt and Raf/MEK/ERK pathway related diseases. Taken together, our study highlights the importance of future studies to further explore the possibility of using PHLPP as a diagnostic marker and a chemotherapeutic target in pancreatic cancer.

Appendix

List of Abbreviations

BA: Bafilomycin A

CAF: Cancer-associated fibroblast

CQ: Chloroquine

DHH: Desert hedgehog

ECM: Extracellular matrix

EGFR: Epidermal growth factor receptor

EMT: Epithelial-mesenchymal transition

FBS: Fetal bovine serum

GSK-3β Glycogen synthease kinase 3β

HGF: Hepatocyte growth factor

Hh: Hedgehog

IF: Immunofluorescence

IGF-1: Insulin-like growth factor 1

IHH: Indian hedgehog

IRS: Insulin receptor substrate

MET: Mesenchymal-epithelial transition

PanIN: Pancreatic intraepithelial neoplasia

PDAC: Pancreatic ductal adenocarcinoma

PEI: Polyethylenimine

PHLPP: PH domain leucine-rich-repeats protein phosphatase

PI3K: Phosphatidylinositol-3-kinases

PKC: Protein kinase C

PTC: Patched

qRT-PCR: Quantitative real-time PCR

RTK: Receptor tyrosine kinase

SCOP: Suprachiasmatic nucleus oscillatory protein

SHH: Sonic hedgehog

SMO: Smoothened

TGF-β: Transforming growth factor β

TIRF: Total internal reflection fluorescence

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Vita

Alena Smith

Education:

PhD (Defense September 15th, 2015) in Molecular and Cellular Biochemistry, The University of Kentucky College of Medicine.

Master of Science in Biology, The University of Arkansas at Little Rock,

Bachelor of Science in Molecular Biology/Biotechnology, double major in Molecular Biology and Biotechnology with a minor Chemistry, Salem International University, May 2002

Research Experience

Graduate Research

Department of Molecular and Cellular Biochemistry, University of Kentucky College of Medicine, Research Advisor: Dr. Tianyan Gao

Molecular studies of PHLPP1/2 expression in pancreatic cancer

PHLPP1/2 possible role in the promotion of cancer formation in the pancreas through loss of expression

Integrin dependent migration in pancreatic cancer

Department of Biology, University of Arkansas at Little Rock, fall 2006 – fall 2010, Research Advisor: Dr. Stephen Grace

Biochemical analysis of the antioxidant properties of the Green Tea polyphenol EGCG (Epigallocatechin Gallate) when exposed to the oxidant peroxynitrite (PN)

Ex vivo analysis of apoptotic effects and DNA protection by EGCG when tissue culture is exposed to PN

Undergraduate Research

Department of Molecular Biology, Salem International University, fall 1998-spring 2002, Research Advisor: Dr. Patrick K. Lai

Molecular analysis of p10 and p40 Borna Disease Virus proteins

Professional Experience

Teaching Assistant,

Department of Molecular and Cellular Biochemistry, The University of Kentucky College of Medicine, August 2011 - 2014

Teaching Assistant,

Department of Biology, The University of Arkansas at Little Rock, August 2008-December 2008 Course: Environmental Biology (Lab) (for entering Biology majors). Held office hours, attended classes, corrected papers and exams, and independently lectured to 25 students per instructional class.

Teaching Assistant,

Department of Biology, The University of Arkansas at Little Rock, August 2005-May 2009 Course: Anatomy & Physiology I (Lab) (for entering Nursing Students, General Studies, and Biology students). Held office hours, attended classes, corrected papers and exams, held dissections, and independently lectured to 60-90 students approximately 22-25 at a time.

Teaching Assistant,

Department of Biology, The University of Arkansas at Little Rock, August 2005-May 2009 Course: Anatomy & Physiology II (Lab) (for entering Nursing Students, General Studies, and Biology students). Held office hours, attended classes, corrected papers and exams, held dissections, taught various medical techniques like blood pressure readings, and independently lectured to 60-90 students approximately 22-25 at a time.

Teaching Assistant,

Department of Biology, The University of Arkansas at Little Rock, August 2005-May 2009 Course: Biometry (graduate students for all departments) Corrected and graded papers and written work along with biostatistical data sets for approximately 20 students.

Technical Experience

Computer Program Skills

I have evolved technical skills in diverse and varied computer programs: SAS programs along with other statistical programs using analysis such as ANOVA, Sigma Plot, Microsoft Office Suite (PowerPoint, Word, and Excel), specialty software for specific instruments (HPLC-MS, HPLC, EPR, microplate spectrophotometer, fluorescent microscope), and specialty software for analysis of data output for assays such as apoptosis, comet cell assay, and gel analysis.

Assay Techniques

I have obtained a diverse and intimate knowledge of various assays from biochemistry to molecular biology techniques including but not limited to: HPLC, HPLC-MS (including direct inject), EPR, microplate spectrophotometer, PCR, PCR primer design, RTPCR, QRTPCR, RNA (microRNA) isolation, DNA sequencing, recombinant cloning (bacterial), plasmid work (restriction enzyme usage, amplification, cloning, and extraction), protein amplification and extraction (use of His-Ni columns), bacterial transformation, DNA nicking assay, tissue culture (including growing, splitting, seeding, and freezing the cells), protein knockdown studies, apoptosis assays, comet cell assay, SDS-PAGE, Western, Southern, Northern, micro RNA studies and assays using agarose gels. Murine husbandry and multiple experiments dealing with extracting and analyzing various mouse tissues from brain to pancreas. I am experienced in 3D culture, Transwells, migration assays, immunofluorescence techniques, TIRF microscopy, biostation migration assays, and many other numerous techniques.

Collaboration Skills

In this day and age of science funding, crowd source funding, and collaborative efforts, it is imperative that laboratories specializing in various skills work together to configure a future that includes both monetary incentive and scientific gain. With available funding dwindling, learning new skills and listening to other's ideas is a technique that has now been put into play in both small and large lab settings. I believe being trained at a NCI designated cancer center has given me skills that I can take anywhere from being able to discuss new research techniques with non-cancer labs to building a bridge between scientists and physicians to take research from bench to bedside.

Scientific Editing

Novartis Drug Representative Module for Psoriasis (June 2014) (see attached)

Design

Content coordinator

Reference coordinator

Editing medical and scientific content

Module design

Knowledge design

IL-17 module design and editing

Medical review of content

Pfizer Drug Representative Module for Treatment of Menopausal Symptoms, (May 2014)

Editing medical and scientific content

Validating references

Awards & Honors

2012 ASCB Grant Conference Funding

2012 ASBMB Child Care Grant

Member of Phi Beta Kappa: joined during undergraduate

Member of Scientists and Engineers of America

Dean's List: Salem International University & University of Arkansas at Little Rock

Conferences

NASA_EPSCORE 2009 conference, Arkansas

NASA_EPSCORE 2010 Conference, Arkansas

ASBMB Conference 2012, San Diego, CA**.** **"**Loss of PHLPP Expression in Pancreatic Cancer"

ASCB Conference Chair 2012 Appalachian Biology Conferences, Charleston, WV

AACR 1013 Attendee

ASCB Conference Chair/Director October 2013, Appalachian Biology Conference, Charleston, WV

AACR Pancreatic Cancer Conference April 2014, New Orleans, Louisiana

GRANTS

ASCB Conference Funding Request Appalachian Regional Cell Conference 2012

Funded

ASCB Conference Funding Request Appalachian Regional Cell Conference 2013

Funded

ASCB Conference Funding Request Appalachian Regional Cell Conference 2014

Funded

Positions Held

CEO LenaJeanne Cosmetics (2013-present)

Board of Directors (2011- present)

Appalachian Regional Cell Conference

Manuscripts in Revision

"PHLPP negatively regulates cell motility through inhibition of Akt activity and integrin expression in pancreatic cancer cells"

References will be given upon request.