UNIVERSIDADE DE SÃO PAULO
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The role of dual-specificity phosphatase 6 (DUSP6) in metastasis and metabolism of pancreatic cancer cells

O papel da fosfatase de dupla especificidade 6 na metástase e metabolismo de células de carcinoma pancreático

Ribeirão Preto – SP
2022

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Tese de Doutorado apresentada à Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo, para obtenção do título de Doutora em Ciências, área de concentração em Genética.

Orientadora: Profa. Dra. Vanessa da Silva Silveira

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Ruckert, Mariana Tannús

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Keywords: pancreatic cancer; DUSP6; metastasis; metabolism; glycolysis.
Palavras-chave: câncer de pâncreas; DUSP6; metástase; metabolismo; glicólise.

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"É duro tanto ter que caminhar
E dar muito mais do que receber
E ter que demonstrar sua coragem
À margem do que possa parecer
E ver que toda essa engrenagem
Já sente a ferrugem lhe comer"

Zé Ramalho, Admirável Gado Novo

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LIST OF ABBREVIATIONS

2-DG – 2-Deoxy-D-glucose

ADM – Acinar-to-ductal metaplasia

ANOVA – Analysis of variance

ATCC – American Type Culture Collection

ATP – Adenosine triphosphate

BCA – Bicinchoninic acid

BSA – Bovine serum albumin

CAF – Cancer associated fibroblasts

CCLE – Cancer Cell Line Encyclopedia

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl sulfoxide

DUSP – Dual-specificity phosphatase

ECM – Extracellular matrix

EdU – Ethynyldeoxyuridine

EMT – Epithelial-mesenchymal transition

ESCC - Esophageal squamous cell carcinoma

FBS – Fetal bovine serum

FOLFIRINOX – Combination of drugs that includes leucovorin calcium, fluorouracil, irinotecan hydrochloride, and oxaliplatin.

GDP/GTP - Guanine diphosphate / Guanine triphosphate

GEF – Guanine nucleotide exchange factor

GEO – Gene Expression Omnibus

GEPIA – Gene Expression Profiling Interactive Analysis

GSEA – Gene set enrichment analysis

GTex – The Genotype-Tissue Expression

HBP – Hesoxamine biosynthesis pathway

INCA – Brazilian National Institute of Cancer

IPMN – Intraductal papillary mucinous neoplasia

HCC – Hepatocellular carcinoma

MKP – MAP kinase phosphatases

MUT - Mutant

NSCLC – Non-small cell lung cancer

PanIN – Pancreatic intraepithelial neoplasm

PBS – Phosphate buffered saline

PEB – Protein extraction buffer

PI – Propidium iodide

PPP – Pentose phosphate pathway

PSMF – Phenylmethanesulfonyl fluoride

PTK – Protein tyrosine kinases

PTP – Protein tyrosine phosphatases

RIPA – Radioimmunoprecipitation assay buffer

ROS – Reactive oxygen species

RPMI – Roswell Park Memorial Institute Medium

SBT – Serous borderline tumors

SCA – Serous carcinomas

TAD – Transcriptional activation domain

TCGA – The Cancer Genome Atlas

TME – Tumor microenvironment

UPC – Universal exPress Codes

WT – Wild type

ABSTRACT

The role of dual-specificity phosphatase 6 (DUSP6) in metastasis and metabolism of pancreatic cancer cells

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive tumor and is majorly caused by the constitutive activation of mutant KRAS – found in more than 90% of PDAC cases. The undruggability of KRAS mutations has led to efforts of finding new therapeutic targets that focus on downstream molecules in the MAPK pathways. The regulation of these kinase activities is orchestrated by a negative feedback network played by a series of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), which in turn activate and inhibit the phosphatase function. DUSP6 is a dual-specificity phosphatase that regulates ERK1/2 phosphorylation and, therefore, RAS pathway activation. Data from different PDAC datasets revealed that *DUSP6* is overexpressed in metastatic tumor samples compared to primary tumor samples and to non-tumoral pancreatic tissue. Overall survival analysis indicated that patients with high *DUSP6* expression have a worse prognosis than patients with low *DUSP6* expression, reaffirming its clinical relevance. Moreover, we observed that *DUSP6* is overexpressed in the quasi-mesenchymal/stromal subtype, which was previously described to be correlated with the glycolytic phenotype and the worst prognosis among all the other described PDAC subtypes. Considering the aforementioned, we hypothesized that DUSP6 could play a role in metabolism reprogramming in PDAC and, therefore, induce a more aggressive phenotype, leading to metastasis development. To investigate DUSP6 role in metastasis development and progression we developed DUSP6 stable knockdown in PDAC cells lines and performed genotypic and phenotypic analysis to evaluate metastatic and metabolic behaviors. Surprisingly, we observed different phenotypes among the cell lines used, which we believe is derived from the different genetic backgrounds and metabolic

subtypes involved. Overall, results indicate that DUSP6 play a role in the metastatic process in PDAC, modifying phenotypes that are closely related to the cells capacity to survive and thrive in an unfamiliar environment. Also, DUSP6 plays a role in the metabolic reprogramming of these cells, as we observe that its knockdown induces glycolysis in these cells under blockage of the mitochondrial respiration. Nevertheless, the mechanism behind these changes remains to be further investigated.

Keywords: pancreatic cancer; DUSP6; metastasis; metabolism; glycolysis.

RESUMO

O papel da fosfatase de dupla especificidade 6 na metástase e metabolismo de células de carcinoma pancreático

O adenocarcinoma ductal pancreático (ADP) é um tumor altamente agressivo e majoritariamente causado pela ativação constitutiva de KRAS mutante – encontrado em mais de 90% dos casos de ADP. Os desafios em desenvolver um inibidor para o oncogene KRAS tem levado a esforços para encontrar novos alvos terapêuticos em moléculas a jusante das vias MAPK. A regulação da atividade dessas quinases é orquestrada por uma retroalimentação negativa que envolve uma série de proteínas tirosinas quinases e proteínas tirosina fosfatases que, por sua vez, ativam e inibem a função das fosfatases. DUSP6 é uma fosfatase de dupla especificidade que regula a fosforilação de ERK1/2 e, dessa forma, a ativação da via RAS. RNA-seq de diferentes bancos de dados demonstram que *DUSP6* está hiperexpressa em amostras metastáticas em comparação a amostras de tumor primário e de tecido pancreático não-tumoral. Uma análise de sobrevida global indica que pacientes com alta expressão de *DUSP6* apresentam pior prognóstico que os pacientes com baixa expressão de *DUSP6*, reafirmando a importância clínica desse alvo. Ainda, observamos que *DUSP6* está hiperexpressa no subtipo quasi-mesenquimal/estromal, que foi previamente correlacionado com o fenótipo glicolítico e com o pior prognóstico entre todos os demais subtipos descritos. Considerando o descrito, a hipótese deste trabalho é de que DUSP6 pode desempenhar um papel na reprogramação metabólica do ADP, contribuindo assim para um fenótipo mais agressivo e, consequentemente, o desenvolvimento de metástase. Visando investigar o papel de DUSP6 no desenvolvimento e progressão tumoral, linhagens de ADP com inibição estável de DUSP6 foram utilizadas em ensaios funcionais *in vitro* para avaliar a capacidade de migração, invasão e o comportamento metabólico dessas células.

Surpreendentemente, observamos diferentes fenótipos entre as linhagens celulares utilizadas, o que acreditamos estar relacionado ao fato de que tais linhagens possuem alterações genéticas distintas e subtipos metabólicos específicos. Em suma, os resultados obtidos indicam que DUSP6 pode impactar o processo metastático do ADP, por meio da modulação da capacidade migratória e invasiva das células. Ainda, DUSP6 pode controlar a reprogramação metabólica, uma vez que observamos que a inibição dessa fosfatase promove o aumento da glicólise diante do bloqueio da respiração mitocondrial. No entanto, os mecanismos que permeiam essas mudanças ainda precisam ser esclarecidos.

Palavras-chave: câncer de pâncreas; DUSP6; metástase; metabolismo; glicólise.

GENERAL INTRODUCTION

1.1. The pancreatic ductal adenocarcinoma

The pancreas is an organ that belongs to the gastrointestinal system, being characterized as a mixed gland because it has endocrine and exocrine functions [1]. Its exocrine portion, consisting of acinar and ductal cells, produces and secretes digestive enzymes into the intestine; its endocrine portion – the islets of Langerhans – produces and secretes hormones that act directly on energy metabolism. The alpha and beta cells regulate cellular glucose uptake by producing glucagon and insulin, respectively. This process provides the initial substrate for energy production by oxidative phosphorylation or anaerobic glycolysis [1, 2].

Because it is a complex organ with multiple cell types, the pancreas can also harbor several types of tumors. Pancreatic ductal adenocarcinoma (PDAC) accounts for more than 90% of cases of pancreatic tumors and is named for its histological similarity with ductal cells [3, 4]. PDAC is one of the most lethal cancers, accounting for the seventh leading cause of cancer-related deaths around the world. In 2016 it surpassed breast cancer, becoming the third leading cause of cancer-related deaths in the United States [5-7]. According to the National Cancer Institute (INCA), PDAC is responsible for approximately 2% of cancers cases diagnosed in Brazil and 4% of total cancer-related deaths in the country (National Cancer Institute, 2021).

Compared to other solid tumors, PDAC has one of the worst prognoses. Despite showing a small improvement over the last few years, the overall 5-year survival is only 9% [7, 8]. The main factors involved in the poor survival of PDAC patients include the lack of identifiable symptoms and/or early diagnosis markers, which prevents early detection and culminates in advanced stage diagnosis [1].

Complete surgical resection is the only available therapeutic option that offers a significant increase in survival time. However, only 30 to 40% of patients have resectable

tumors at the time of diagnosis because the others are diagnosed when the tumor has already compromised the adjacent artery or the tumor has invaded into adjacent organs [9]. In these cases, the overall 5-year survival is 15-25% [10]. Neo-adjuvant therapy – in which chemotherapy is administered prior to surgery – can be used to shrink the tumor and make it more resectable by improving the clearance from the adjacent artery/tissue [11]. Alternatively, adjuvant therapy with gemcitabine was established in 1997, when a randomized trial showed that patients treated with gemcitabine had a 1-year survival increase of 18% compared with fluorouracil (2%) [12]. Currently, Gemcitabine/nab-paclitaxel (Gem-abraxane) or FOLFIRINOX is the standard therapy for metastatic PDAC [13], yet increase in overall patient survival is very low, thus demonstrating the need for investment in finding new therapeutic targets for the tumor [14].

Risk factors related to the development of PDAC can be divided into modifiable and non-modifiable risks. Modifiable risks are associated to lifestyle, such as eating and drinking habits, smoking, obesity, pancreatitis and other variants such as socioeconomic status and health insurance access. Among modifiable risk factors, smoking is the main one, with doubled risk of developing PDAC between smokers compared to non-smokers [15]. Non-modifiable risks comprise age, gender, ethnicity, diabetes, genetic mutations and family history of PDAC [16]. The disease mainly affects black men in advanced age, rarely affects individuals under 40 years-old, and in individuals older than 80 the risk is 40 times higher [1, 14]. Regarding genetic factors, several studies indicate that family history of PDAC increases the risk of developing the disease – individuals with family members affected by PDAC are 9 times more likely to develop the disease [17]. It is estimated that 10% of the cases are related to familial genetic predisposition [6, 18].

PDAC tumor progression is slow and it is estimated that 10 to 30 years elapse between the onset of the initial lesion until the patient's eventual death [19]. PDAC

tumorigenesis can be divided into three main moments: T1, which occurs between the formation of lesions such as pancreatic intraepithelial neoplasms (PanINs) and infiltrating carcinoma formation; T2, which is the period between the end of T1 and formation of the metastatic subclone; and T3, which comprises the period between the end of T2 and patient's eventual death (Figure 1) [19].

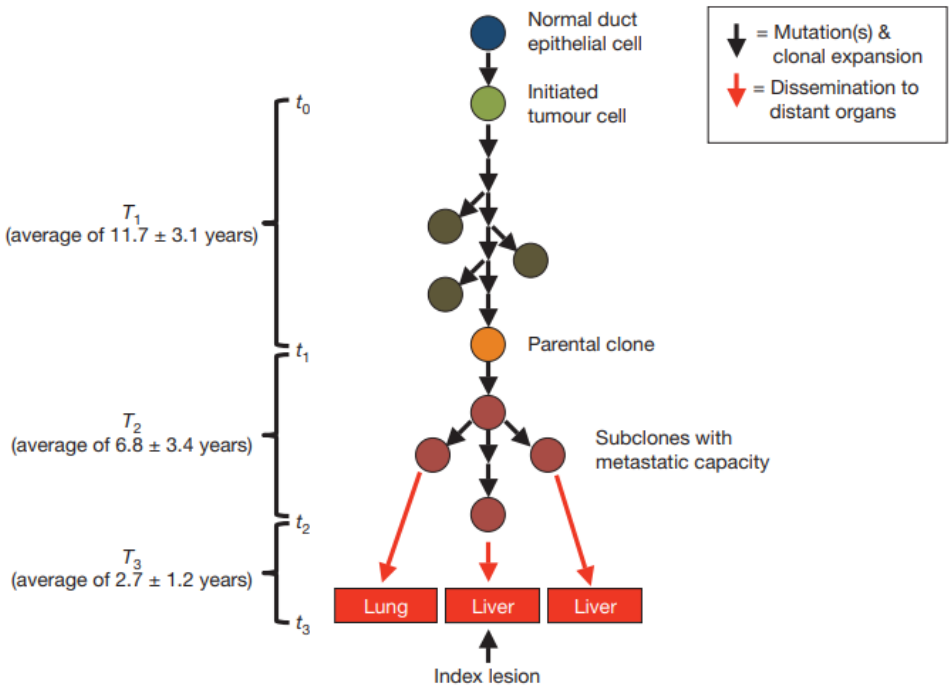

Figure 1 – PDAC tumorigenesis cascade. The first event to occur in PDAC tumorigenesis is a driver mutation – usually in KRAS – in a normal ductal cell. This mutation will then start an uncontrolled clonal proliferation, leading to accumulation of secondary mutations. This event allows the emergence of pancreatic intraepithelial neoplasias (PanINs), which characterizes the end of T1. Next, one founder cell in a PanIN lesion will start to progress to an infiltrating carcinoma, characterizing the end of T2. Later, subclones with metastatic capacity will give rise to lesions in organs such as the lungs, the liver and the peritoneum, which corresponds to T3. [20]

Although some PDAC cases occur due to family predisposition, the vast majority occur sporadically [21]. Like many other tumor types, PDAC tumorigenesis results from an accumulation of acquired mutations in oncogenes, tumor suppressor genes, and maintenance genes [22]. Despite the fact that the first lesions related to tumor

development do not usually present genetic alterations, the first mutation detected in PDAC tumorigenesis is the *KRAS* oncogene activation. Mutations in this gene are found in more than 90% of these tumors and are, therefore, considered driver mutations [23, 24]. In addition to *KRAS* activation, loss of function of tumor suppressor genes such as *TP53*, *CDKN2*, *INK4A*, *SMAD4* and *BRCA2* are also closely related to PDAC progression (Figure 2) [1, 14, 22].

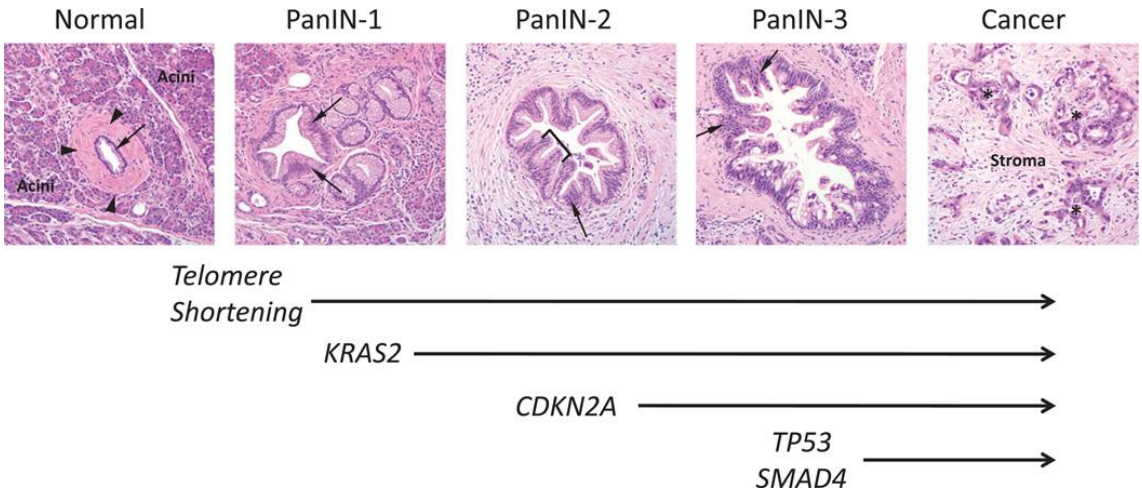

Figure 2 – PDAC morphological evolution and genetic mutational landscape. A normal pancreatic duct is characterized by low cuboidal epithelium (indicated by arrows), surrounded by a fibrotic cuff (indicated by arrowheads) and normal acini. When PanIN-1 lesions arise, one can observe the mucinous hyperplasia of the ductal cells (indicated by arrows). Evolution to PanIN-2 brings characteristics such as cytological atypia, nuclear enlargement, crowding of the cells (indicated by the arrow) and, most evidently, the papillary folding of the epithelium (indicated by the bracket). In PanIN-3 lesions, the previously cited are events are accompanied by a complete loss of cell polarity (indicated by arrows), the presence of mitotic figures and growth of the neoplastic epithelium. PanIN-3 is the last stage preceding the infiltrating carcinoma (indicated by asterisks). Last image shows the abundant desmoplastic stroma, typical component of the PDAC microenvironment [25].

1.2. The RAS signaling pathway

The RAS family consists of three different genes (*HRAS*, *NRAS* and *KRAS*) that encode four highly homologous 21 kDa proteins, differing only in the C-terminal region

sequence [26]. All are widely expressed in the body, although they have differences in expression between tissues. HRAS is expressed mostly in the skin and skeletal muscle; NRAS in the testicles and thymus; and KRAS, in turn, in the intestine and thymus [27]. The RAS protein is a family of small GTPases, which are proteins capable of catalyzing the conversion of guanine triphosphate (GTP) to guanine diphosphate (GDP). In their inactive state, RAS family proteins are bound to GDP molecules. When a guanine nucleotide exchange factor (GEF) binds to the RAS protein, it releases GDP and, consequently, binds a GTP. The binding of GTP to RAS induces GEF dissociation and induces a change in protein conformation, thereby increasing its affinity for its downstream effectors, such as Raf [28].

Among the RAS family genes, mutations in the *KRAS* oncogene are the most commonly found in PDAC. The most common KRAS mutation is the result from the substitution of a glycine by aspartic acid at codon 12 (G12D) – other substitutions are rarer in this type of tumor, including G12V and G12C [22, 29]. Oncogenic mutations like $KRAS^{G12D}$ result in constitutive activation of the protein since GTP has weaker dissociation [30]. This activation results in alteration of a number of downstream signaling pathways – such as the extracellular signal-regulated mitogen/kinases (MAPK/ERK) pathway – and culminates in uncontrolled increase in cell proliferation as well as other tumorigenic events, such as increased cell survival, malignant differentiation and energy metabolism reprogramming [27, 31-33].

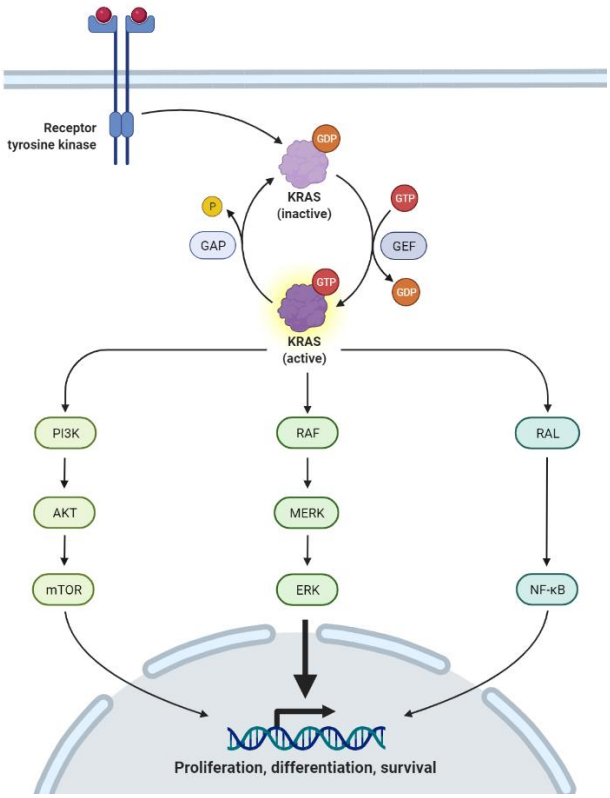

Figure 3 – Mutated KRAS signaling pathway. Oncogenic mutations in the KRAS molecule result in constitutive activation of the protein because GTP is permanently bound. Permanent activation leads to alteration of downstream signaling pathways – such as MAPK/ERK, PI3K/AKT and NF-κB pathways – and culminates increased cell proliferation, malignant differentiation and survival. Adapted from "KRAS signaling pathways", created with BioRender.com (2021).

1.3. Metastasis in PDAC

As previously stated, PDAC has one of the worst prognosis among solid tumors. This is related to the fact that early PDAC stages are mostly asymptomatic, and this condition leads to a very difficult early diagnosis. Even when the patient starts to present symptoms, they are highly unspecific, such as abdominal pain, jaundice, weight loss and fatigue [34]. Therefore, the vast majority of patients (80 – 90%) bare an unresectable tumor – locally advanced or metastatic stage – at the moment of diagnosis [35, 36].

Distant PDAC metastasis are commonly found in the liver, the peritoneum and the lungs, although it has been reported in other less common sites [37-41]. One of the main features linked to PDAC aggressiveness is the fact that even small primary tumors

such as 2 cm diameter are associated with metastasis occurrence [37]. Therefore, even if detected early patients are likely to already have metastases or disseminated tumor cells. It has also been reported that among patients that underwent resection of their infiltrative tumors, 73% presented local recurrence and 66% had metastatic disease [42].

Studies by Campbell, Yachida and colleagues comparing primary PDAC samples to metastatic ones suggested that at least 7 years are necessary for the formation of the metastatic subclone after the primary tumor is established, and 2 to 3 more years as necessary for it to colonize other sites, leading to patient's eventual obit [20, 43]. If this is true, it can be assumed that PDAC progression is a linear series of events and that metastasis is a late stage event in this timeline.

Although tumor progression is well described, the metastatic process for PDAC is not yet fully understood. Previous studies have demonstrated that the majority of circulating tumor cells are unable to form secondary tumors [44], and others have demonstrated that there is a considerable variance between patients regarding metastasis occurrence [42]. Considering that, it is intuitive to assume that circulating cells that successfully colonize other sites gain some kind of advantage that is most likely related to genetic or epigenetic alterations [44]. Rapid tumor growth and increased proliferation rates require an enormous amount of energy and, therefore, metabolic adaptations to sustain this demand. Primary PDAC tumors are highly adapted to a nutrient-deprived environment due to poor oxygenation, low vascularity and intense fibrous stroma; but when the cells migrate and seed in distant organs, as in the hepatic parenchyma, they find a nutrient-full soil and need to re-adapt to metabolize excessive amounts of glucose [45]. Therefore, although the genetic mutations panel is largely shared between primary and metastatic PDAC cases [20, 46], it is clear that the cells need to acquire a whole different

behavior to successfully establish metastatic sites and that metabolism reprogramming is an important key to achieve this goal.

1.4. Metabolic reprogramming in PDAC

Constitutive activation of pathways such as MAPK/ERK requires the tumor cell to adapt its energy metabolism to sustain the anabolic demand imposed by uncontrolled cell proliferation [47]. Under physiological aerobic conditions, cells capture glucose from the extracellular medium, first processing it into pyruvate via the glycolytic pathway and subsequently producing ATP through the electron chain in the mitochondrial membrane. Under anaerobic conditions, energy production in mitochondria is reduced, prioritizing anaerobic glycolysis with lactate production [48]. Warburg and colleagues observed a different metabolic behavior in tumor cells: even under aerobic conditions these cells preferentially use glycolysis as their primary energy source – thus, performing an aerobic glycolysis process [49]. This phenomenon is known as the "Warburg effect" and is currently considered one of the hallmarks of cancer [48]. Besides reprogramming its internal metabolism, tumor cells also interact with other components from the tumor microenvironment (TME), such as non-malignant cells and the extracellular matrix (ECM) to support its increased energy demand [50].

Interestingly, many published studies have already demonstrated that metabolic changes in PDAC highly correlate with metastasis [51], chemoresistance [52] and immunosuppression [53]. Daemen and colleagues have shown that two main metabolic subtypes can be identified according to tumor phenotype, where: epithelial (classical) phenotype showed lipogenic behavior, while mesenchymal phenotype showed glycolytic behavior [54]. Later, other studies have proposed that PDAC metabolic profiles were divided in 4 different groups – glycolytic, cholesterogenic, quiescent and mixed. In this case, tumors with higher glycolytic score and lower cholesterogenic score, as well as in

Daemen's classification, are associated with worst overall survival and chemoresistance [55].

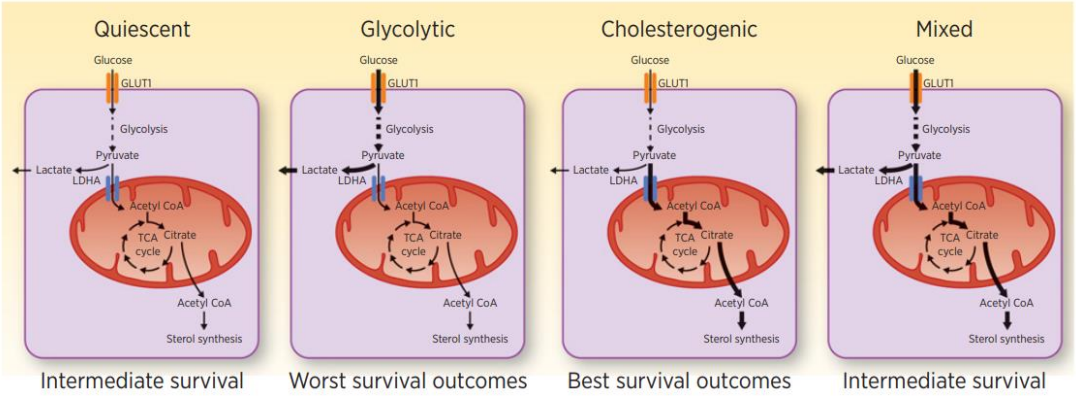

Figure 4 – PDAC metabolic subtypes. Karasinska and colleagues proposed that PDAC can be categorized into four different metabolic subtypes, based on mRNA expression profiles. Samples presenting a high score of genes correlated to the glycolytic pathway fall into the glycolytic cohort and have these patients have the worst prognosis of all groups. On the opposite, samples with high score of sterol biosynthesis correlated genes fall into the cholesterogenic cohort and the patients have the best prognosis. Samples that present a low score of both gene profiles fall into the quiescent cohort, whereas samples with high score of both profiles fall into the mixed cohort. Patients in these two last cohorts present intermediate survival [56].

Several published studies provide sufficient evidence that oncogenes and tumor suppressor genes regulate tumor metabolism reprogramming [57], and it has been shown that oncogene *KRAS* is capable of promoting glycolysis [58, 59]. Aiming to understand the participation of KRAS in PDAC metabolic reprogramming, Ying and colleagues developed mice with the $KRAS^{G12D}$ inducible mutation exclusively in pancreatic tissue and found that the oncogene is essential for tumor maintenance *in vivo* and that the phenotype is partly due to changes in energy metabolism [32]. Induction of *KRAS* expression increased glucose uptake through *Glut1* overexpression, and promoted a shift of intermediates from the glycolytic pathway to the hexosamine biosynthesis pathway (HBP) and to the pentose phosphate pathway (PPP). The study also demonstrated that the observed metabolic phenotype was mediated by activation of the MAPK/Erk pathway through Myc activity and regulation of genes encoding glucose metabolism-related

enzymes such as hexokinase 1 and 2 (*Hk1* and *Hk2*), phosphofrutokinase-1 *(Pfk1*) and lactate dehydrogenase A (*Ldha*) [32].

Son and colleagues later investigated the role of oncogene *KRAS* in glutamine metabolism in the same mouse models developed by Ying *et al*. (2012). In this context, it was observed that the induction of *KRAS* expression lead to an increase in the expression of *GOT1*, a gene encoding the glutamic oxaloacetic transaminase enzyme, with a concomitant reduction in the expression of *GLUD1*, which encodes glutamate dehydrogenase. These results demonstrate that the *KRAS* oncogene promotes activation of a non-canonical glutamine pathway in PDAC [60].

Subsequently, the same group used as a model *KRAS*-dependent (responsive to apoptosis-inducing *KRAS* silencing) and *KRAS*-independent (non-responsive to silencing) cell lines to evaluate how oncogene dependence impacts the metabolic reprogramming of these cells. Inactivation of MAPK/ERK was observed to significantly impact nucleotide production in *KRAS*-dependent strains in comparison to *KRAS*-independent strains. The study further identified that nucleotide metabolism is the major element in resistance to MEK inhibitors in PDAC cells, also suggesting that PPP and pyrimidine biosynthesis pathways may be interesting targets for sensitization of these cells [33]. I has also been described that the inhibition of the RAS/ERK signaling pathway upregulates autophagy in PDAC, which is linked to a possible compensation axis to the glycolic and mitochondrial function suppression [61]. Autophagy is a key process in PDAC and plays a critical role regulating reactive oxygen species (ROS) production and maintaining oxidative phosphorylation [62, 63] (Figure 5).

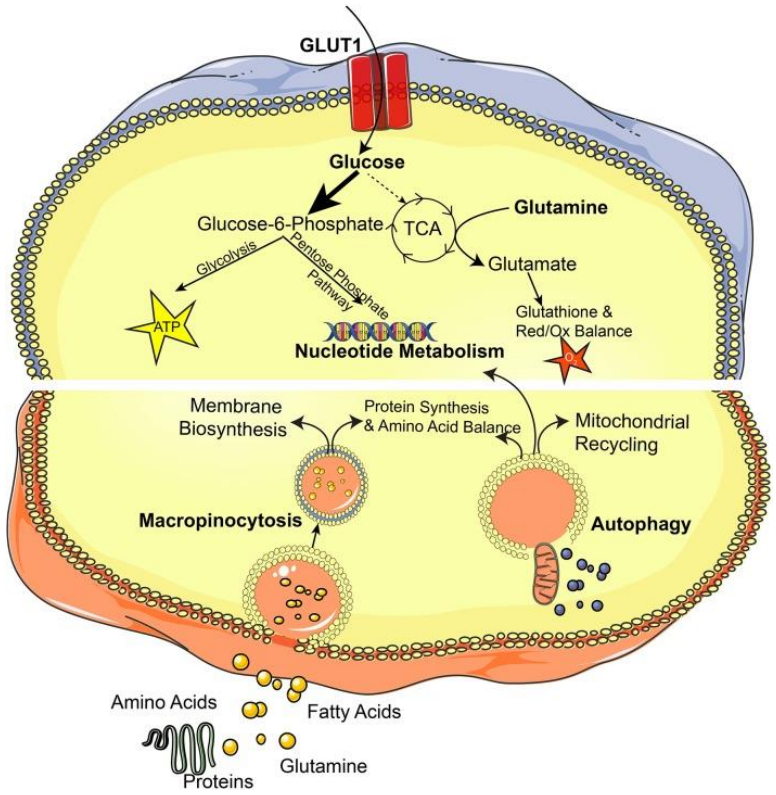

Figure 5 – RAS and metabolism. Metabolic pathways altered by the oncogenic RAS in RAS-driven cancer cells [64].

Interestingly, recent studies provide sufficient evidence that the glycolysis upregulation in PDAC cells is closely related to the metastatic process [51, 65]. Dovmark and colleagues reported that the lactate produced by the tumor cells can be transported by both monocarboxylate transporters (MCT) and connexin-43 channels to the extracellular environment, serving as a signaling molecule to promote invasion and metastasis [66, 67].

Collectively, these studies demonstrate the complexity of KRAS's role in PDAC metabolic reprogramming and how elucidating these mechanisms is essential for a thorough understanding of tumor biology.

1.5. Scientific Challenge: Dual-Specificity Phosphatases (DUSPs)

Although the *KRAS* oncogene is known to be involved in a variety of tumorigenic processes and inhibition of its expression reduces cell proliferation in PDAC strains, transposition to the clinics has not been successful and still represents a major challenge

(Figure 6) [68, 69]. Recently, a small molecule known as Adagrasibe (MRTX849) showed to be effective in specifically inhibiting $KRAS^{G12C}$ mutant in non-small cell lung cancer (NSCLC) in a Phase III clinical trial [70]. Nevertheless, although very prevalent in NSCLC, $KRAS^{G12C}$ mutations represent a very small part of the PDAC patients (~1%), whereas $KRAS^{G12D}$ represent de vast majority of PDAC cases and a challenge to inhibitor's development [29]. Thus, it is necessary to identify new targets for efficient modulation of MAPK/ERK pathway activity.

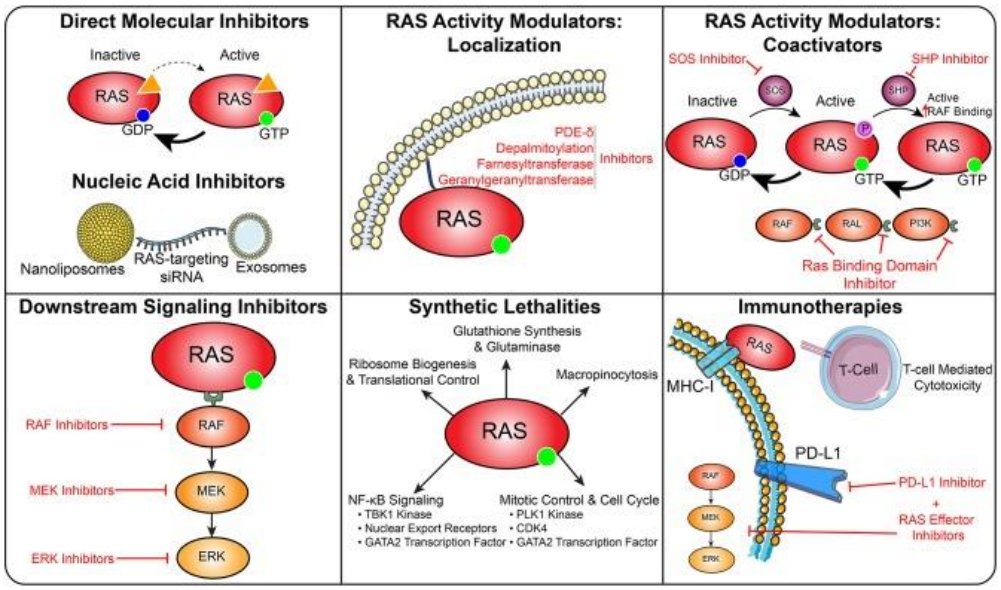

Figure 6 – RAS inhibition strategies. A summary of the therapeutic strategies for targeting RAS. Many approaches have been tried so far, with little success along the way [64].

In this context, it is already well described in literature that the regulation of these pathways is orchestrated by a fine balance between phosphorylation events (that adds phosphate groups to proteins) – and dephosphorylation events (removal of phosphates) – mediated by protein tyrosine phosphatases (PTP) [71]. Given that PTPs act on dephosphorylation of their targets, it is intuitive to imagine that these proteins may play a tumor suppressor role by blocking the activation of oncogenic kinases. However, several evidences show that PTPs are overexpressed in a variety of tumors in which,

counterintuitively, they positively regulate these signaling pathways, promoting tumor progression and thus also acting as oncogenic proteins [72].

The PTPs constitute a family of 125 members subdivided into classes and groups according to their structure and function [73]. Class I members are grouped for sharing a cysteine residue as a catalytic domain. Within this class, two groups concentrate the largest portion of these PTPs: the classic PTPs group and the VH1-Like/DUSPs. Dual-specificity phosphatases (DUSPs) receive this name because they have various substrates such as tyrosine, serine and threonine, as well as phosphoinositides, complex carbohydrates and even RNA [73, 74]. The group can be further divided into 6 smaller subgroups: myotubularins, slingshots, CDC14s, PRLs, PTENs, atypical DUSPs, and MAPK phosphatases (MKPs) [74]. MKP subgroup comprises ten DUSPs and all of them contain a site in the N-terminal region that can recognize and regulate MAPK specifically [75]. These ten DUSPs can be further subdivided into three groups according to sequence homology, cell sublocation and substrate specificity. The first group comprises DUSP1/MKP-1, DUSP2 (PAC1), DUSP4/MKP-2 and DUSP5, which are nuclear, mitogen- and stress-inducible phosphatases; the second group comprises DUSP6/MKP-3, DUSP7/MKP-X and DUSP9/MKP-4, which are cytoplasmic and ERK specific phosphatases; and the third group comprises DUSP8 (M3/6), DUSP10/MKP-5 and DUSP16/MKP-7 which are found in both nucleus and cytoplasm, and are specific to JNK and p38 [75, 76].

Among ERK1/2-specific MKPs, DUSP6 stands out for being described in a wide variety of tumors [77, 78]. The levels of DUSP6 appears to reflect stage of disease as Furukawa and colleagues observed that DUSP6 is overexpressed in dysplastic pancreatic carcinoma/*in situ* but downregulated in invasive carcinoma, especially in poorly differentiated carcinoma [79]. Downregulation in advanced PDAC correlated with

epigenetic control mechanisms, such as hypermethylation at the promoter region of this gene [80]. Several studies have shown that reintroduction of *DUSP6* expression in *in vitro* PDAC cell lines reduces cell proliferation and promotes apoptosis, and, additionally, suppresses the MAPK/ERK pathway [79, 81, 82].

Recently, Hsu and colleagues described that DUSP6 mediates activation-induced metabolic commitment towards glycolysis in T cells [83]. This is the first time that DUSP6 is directly related to glycolysis induction. Considering that DUSP6 is negative regulator of the ERK/MAPK pathway, which is directly correlated to glycolysis induction in PDAC; appears to differentially expressed in different disease stages; and correlates with metabolic commitment, it can be suggested that DUSP6 might play a role in metabolic reprogramming in PDAC.

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HYPOTHESIS

HYPOTHESIS

The predominant KRAS mutations in pancreatic ductal adenocarcinoma (PDAC) pose challenge when it comes to developing specific drugs to target it, so we sought to investigate downstream regulators for the ERK/MAPK pathway. DUSP6 came out *in silico* analysis as an overexpressed gene in tumor samples and, more specifically, metastatic samples. This observation leads us to the first hypothesis of this work: DUSP6 plays a role in the metastatic process in PDAC.

The second hypothesis also derives from *in silico* analysis, which suggested that DUSP6 expression in PDAC samples is correlated to glycolysis. Therefore, we hypothesized that DUSP6 plays a role in metabolism reprogramming in PDAC, one of the hallmarks of cancer.

Knowing that glycolysis is associated with a worse outcome in patients due to a more aggressive tumor phenotype, we reached our third and final hypothesis for this work: DUSP6 plays a role in the metastatic process in PDAC through regulation of cell metabolism.

OBJECTIVES

OBJECTIVES

The overall aim of this work is to better understand DUSP6's role in pancreatic adenocarcinoma cells in late stages of tumor progression, when the cells need to reprogram their behavior to promote metastasis.

The thesis was divided in three major objectives. The first aim to conduct a comprehensive review in the literature about the role of phosphatases in cancer, specifically pancreatic cancer. The results of this first aim are presented in Chapter I, in form of a manuscript published in *Cellular and Molecular Life Sciences* (IF: 9.261).

The second aim was to establish DUSP6's role in the metastatic process in PDAC. For this we performed *in silico* expression and survival analysis in publicly available datasets. Then, we generated DUSP6 knockdown cell lines and performed proliferation, migratory and invasive capacity assays. These results are described in Chapter II of this thesis, in form of a manuscript in preparation to be submitted.

The third and last aim was to investigate DUSP6's role in metabolism and determine if both phenotypes were correlated. We wanted to understand if metabolism reprogramming mediated by DUSP6 activity would be beneficial for tumor progression and, consequently, to metastasis. For this we performed gene set enrichment analysis in different datasets and stablished a correlation between DUSP6 and glycolysis. Then we took advantage of DUSP6 knockdown and overexpression cell lines to perform glycolysis rate, lactate secretion and expression array analysis. To determine the correlation between metabolic and metastatic phenotype, we repeated proliferation and migratory capacity assays under glycolysis inhibition. The results are described in Chapter III of this thesis, in form of a manuscript in preparation to be submitted.

SPECIFIC AIMS

Chapter I

- Provide a detailed description of the human phosphatases and their classification;
- Describe their role in tumorigenesis as oncogenic or tumor suppressive molecules;
- Describe their role and clinical implications specifically in pancreatic ductal adenocarcinoma (PDAC);
- Summarize the phosphatase inhibitors described in literature so far.

Chapter II

- Determine DUSP6 expression patterns in human PDAC samples and its correlation to overall patient survival;
- Assess DUSP6 role in PDAC cell lines and correlation to metastatic potential in *in vitro* models.

Chapter III

- Investigate DUSP6's correlation to cell metabolism in PDAC;
- Functionally assess DUSP6 role in PDAC cell lines and evaluate the correlation to cell metabolism;
- Determine the possible correlation between metabolic reprogramming and metastasis in PDAC upon DUSP6 modulation *in vitro*.

CHAPTER I

Protein Tyrosine Phosphatases: Promising Targets in Pancreatic Ductal Adenocarcinoma

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Protein Tyrosine Phosphatases: Promising Targets in Pancreatic Ductal Adenocarcinoma

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Running title: PTPs as targets in pancreatic cancer

Keywords: Dual-specificity phosphatases; Molecular targets; Pancreatic cancer; Tyrosine phosphatases.

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer that is associated with a very poor prognosis, representing the fourth leading cause of cancer related deaths. KRAS driver mutations occur in approximately 95% of PDAC cases and cause the activation of several signaling pathways such as mitogen-activated protein kinase (MAPK) pathways. The regulation of these kinase activities is orchestrated by a negative feedback network played by a series of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), which in turn activate and inhibit the phosphatase function. The human PTPome comprises 125 members, and these proteins are classified into three distinct families according to their structure. For a long time, PTPs were thought to act only as tumor suppressor proteins; however, presently, it has become clear that PTPs have both inhibitory and stimulatory effects on cancer-associated signaling processes and that deregulation of PTP function is associated with tumorigenesis. Several PTPs have displayed either tumor suppressor or oncogenic characteristics in the development and progression of PDAC. In this sense, PTPs have been presented as promising candidates for the treatment of human pancreatic cancer, and many PTP inhibitors have been developed since these proteins were first associated with cancer. Nevertheless, some challenges persist regarding the development of effective and safe methods to target these molecules and deliver these drugs. In this review, we discuss the role of PTPs in tumorigenesis as tumor suppressor and oncogenic proteins. We have especially focused on the differential expression of these proteins in PDAC, as well as their clinical implications and possible targeting for pharmacological inhibition in cancer therapy.

1 Pancreatic Ductal Adenocarcinoma Overview

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer that develops from ductal cells of the exocrine portion of the organ [1]. Currently, it is the fourth largest cause of cancer-related death in developed countries, and it is foreseen that the disease will become the second leading cause of cancer death until 2030 [2].

PDAC is associated with an extremely poor prognosis mainly because, in 80% of cases, patients are diagnosed in advanced stages of the disease [3]. The symptoms are nonspecific, or in some cases, there are no symptom manifestations, and the lack of specific and sensitive tumor markers contributes to the late diagnosis [4]. Besides, another important feature of PDAC is the aggressiveness, with vascular local growth and early distant metastases that preclude curative surgical resection in most patients [5].

The poor prognosis is also explained by low drug penetration in the dense pancreatic stroma and by low tumor vascularization, leading to a high level of chemoresistance by the tumor cells [6]. Together, these factors contribute to an overall survival rate in 5-years of only 5% which classifies PDAC as one of the most aggressive malignancies worldwide [7]. The development of invasive PDAC is preceded by acinar-ductal metaplasia (ADM) or by neoplastic precursor lesions such as pancreatic intraepithelial neoplasia (PanINs), mucinous cystic neoplasia, intraductal papillary mucinous neoplasia (IPMN) and atypical flat lesions [8]. The most common precursor lesions, PanINs, comprise a spectrum of neoplastic lesions with morphological and genetic alterations that represent the progressive stages of PDAC development [9]. Among cumulative genetic changes that result in PanINs advancement, oncogenic activation of KRAS represents the primary genetic mutation in nearly ninety-five percent of PDAC cases [10].

PanINs of higher degrees also present genetic mutations in CDKN2A, TP53 and SMAD4, in addition to an increasing number of diverse gene mutations and alterations such as telomere loss [11]. CDKN2A and TP53 are the most frequently mutated tumor suppressor genes in PDAC. Disruption in CDKN2A, an essential cell cycle regulator, occurs in nearly 90% of PDAC cases and somatic mutations, leading to TP53 inactivation in more than 50% of the cases. Inactivation of both tumor suppressor genes is a relatively late event in the development of pancreatic cancer and occurs only in severely dysplastic lesions (PanIN-3) or invasive carcinoma [12].

Despite the constantly rising number of genetic alterations in PDAC, KRAS activation is a very early genetic mutation found in low-grade PanIN 1A lesions; most importantly, mutant KRAS is sufficient to initiate different precursor lesions and direct their progress to a latent PDAC development [10]. KRAS is a GTPase that belongs to the RAS family, which is responsible for signaling pathway activation through interaction between membrane receptors and information transmission from the cell membrane to the nucleus [13]. Oncogenic KRAS results from point mutations at codons G12, G13 or Q61 – 95% of PDAC cases harbor a KRASG12D mutation [14] – impairing KRAS GTPase function. Consequently, mutant KRAS is constantly connected to a GTP molecule and, therefore, activated, leading to constitutive stimulation of the downstream signaling pathways that promote several cancer-related events [15].

In PDAC, two of the main networks driven by KRAS are the PI3K/AKT and mitogen-activated protein kinase (MAPK) signaling pathways. MAPK pathways are grouped into three families—extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK) and stress-activated protein kinases (SAPK/p38-MAPK)—and their activation occurs by the phosphorylation of tyrosine and threonine residues by MAP2K. When activated, they can phosphorylate transcription factors and, thus, play a

critical role in the regulation of events such as cell proliferation, survival, metabolism, differentiation and apoptosis [16]. In general, the regulation of the activity of these kinases is orchestrated by a negative feedback network played by a series of protein tyrosine kinases (PTKs) and proteins tyrosine phosphatases (PTPs) that controls the duration and strength of signal activation [17].

2 The Protein Tyrosine Phosphatase Superfamily

Protein phosphorylation and dephosphorylation is a well-known regulatory mechanism in the cells of higher eukaryotes [18]. Emerging in evolution later than serine (Ser) and threonine (Thr) phosphorylation, tyrosine (Tyr) phosphorylation has been described by its role in cell-to-cell and transmembrane signaling, enabling the establishment of multicellular organisms [19]. Posttranslational Tyr phosphorylation is regulated by two types of enzymes: PTKs, which insert a phosphate group in a Tyr residue, and PTPs, which can reverse this phosphorylation process [20]. These PTP enzymes are clustered into the human PTPome with 125 members and are classified according to their structure [21]. To be considered a PTP, a PTP domain and a conserved catalytic site must be present, characterized by a C-xxxxx-R loop, where C is a cysteine (Cys) residue, x is any amino acid and R is arginine (Arg) [22]. The remaining members of the PTPome either present a structurally defined PTP domain, a C-xxxxx-R domain with no PTP domain, high similarity with a recognized PTP member's sequence, or experimentally validated Tyr phosphatase activity [21].

2.1. Mechanism of Action of Protein Tyrosine Phosphatases

The reaction performed to dephosphorylate a substrate was first characterized in 1994 [23, 24]. The main core of the hydrolysis reaction is the amino acid that integrates the catalytic domain—Cys, aspartic acid (Asp) or histidine (His)—according to each

family of PTPs [25]. The process occurs in two steps, being initiated by a nucleophilic attack on the phosphate group, mediated by the catalytic domain residue. At this point, the dephosphorylated substrate leaves the intermediate compound, and a PTP's Asp residue (WPD loop) donates a proton to the now unoccupied tyrosyl group [26]. In the second and final step of this process, the PTP is regenerated with the release of the phosphate group. This phosphate release, on the other hand, is mediated by a water molecule, which was first deprotonated by the same Asp that donated a proton to substrate in the first step [25].

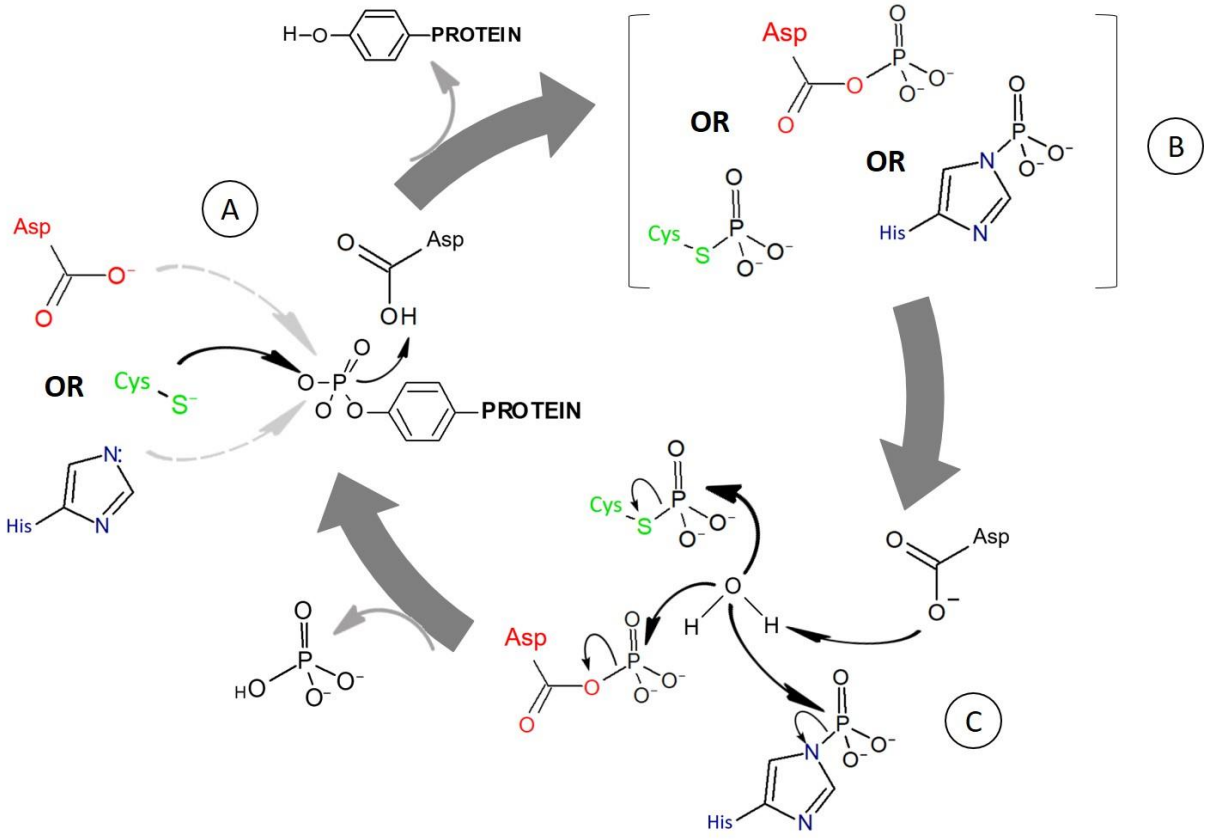

Figure 1 – The two-step mechanism of action of PTP. The scheme shows a pTyr protein that acts as the substrate for PTPs with a Cys-, Asp- or His- catalytic residue. The PTP catalytic domain performs a nucleophilic attack on the phosphate group forming a covalent bond, while an Asp residue from the WPD loop donates a proton to the unoccupied tyrosyl group, releasing the dephosphorylated substrate (A). The PTP and phosphate group form a transient phospho-enzyme intermediate – according to the family of PTPs acting in the reaction – (B), which is later restored

when the Asp residue deprotonates a water molecule, finally, mediating the release of the phosphate group from the phosphatase (C). Gray-dashed arrows in (A) indicate that nucleophilic attack can be alternatively performed by Asp- or His-PTPs. Adapted from Alonso and colleagues (2016).

2.2. Classification of Protein Tyrosine Phosphatases

The members of the human PTPome are classified into three distinct families according to the core amino acid present in their catalytic domain: Cys-based phosphatases, which are subdivided into Class I, II and III; Asp-based phosphatases; and His-based phosphatases [25, 27] (Figure 2). Cys-based PTPs represent most of the members (116 proteins). Therefore, we will be focusing on them in the current review.

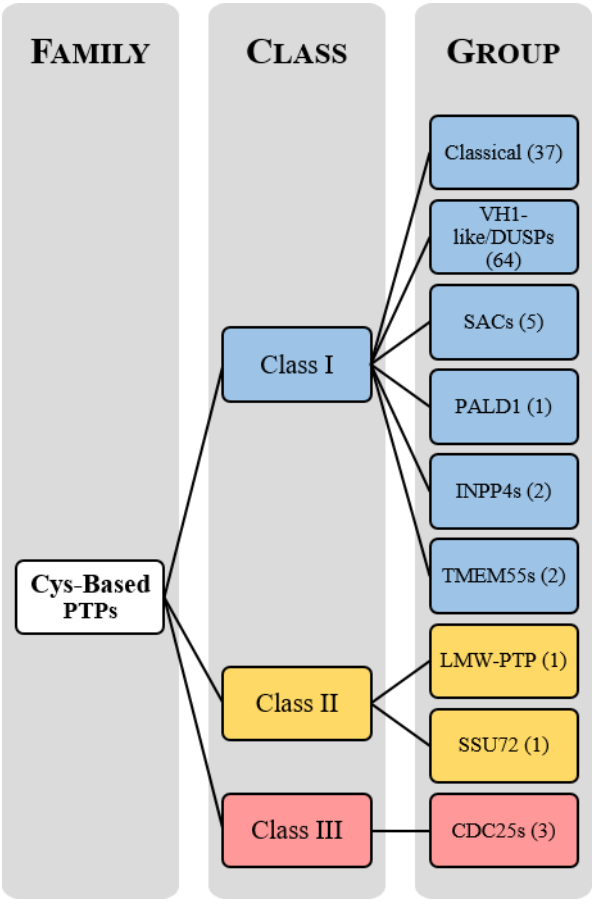

Figure 2 – Classification of Human Cys-Based PTPs. Tree-structured diagram showing the human Cys-based PTPs family subdivided into classes and groups. The numbers in parentheses indicate how many members belong to the group. This diagram follows the classification proposed by Alonso et al. (2016).

2.1.1. Class I Cys-Based Phosphatases

The class I Cys-based PTPs account for more than 100 members of the superfamily, being considered the most extensive group [26]. All of its members have evolved from the same ancestor [27, 28], and the latest reviews [25] subclassify these into six smaller groups: classical PTPs, VH1-like/dual-specificity phosphatases (DUSPs), SAC phosphatases, Paladin, INPP4s and TMEM55s.

Classical PTPs are so called because of their specificity for pTyr. The deep structure of the catalytic site can discriminate between pTyr residues and pSer/pThr residues, which are shorter [25, 26]. This group can be further divided by the presence or absence of a transmembrane region, which defines its members as receptor-like or non-receptor PTPs [27, 29]. In addition to having a transmembrane region, 13 out of 20 receptor-like PTPs also exhibit two distinct PTP domains and an extracellular domain, a finding that correlates with studies that show that cell-to-cell or cell-to-matrix adhesion processes are important functions of these proteins [27-30]. In addition to the absence of the transmembrane region, non-receptor PTPs exhibit a regulatory and a targeting domain that differ from the receptor-like PTPs [25].

Besides classical PTPs, class I Cys-based phosphatases are also composed of VH1-like/DUSPs, which are named because the first member of this group was identified in the vaccinia poxvirus H1-open read frame [30]. DUSP stands for dual-specificity phosphatases, and these proteins are so called because, different from classical PTPs, this group presents various substrates such as phosphor-Tyr, phosphor-Ser and phosphor-Thr, as well as signaling lipids, such as phosphoinositides, complex carbohydrates and even RNA [25, 27]. Among these proteins, there are the mitogen-activated protein kinase phosphatases (MKPs), which represent 10 of the 64 members in this group and are strongly related to the regulation of events such as proliferation, survival and

differentiation [31, 32]. In addition to MKPs, another important component of this group is the phosphatase and tensin homolog deleted on chromosome ten (PTEN) tumor suppressor, which is among the most important tumor suppressor genes that have been described in carcinogenesis [33].

PTEN is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase that exerts its functions in three different ways: lipid phosphatase-dependent, protein phosphatase-dependent or scaffold-dependent. PTEN exhibits five structural distinct functional domains: a C2 lipid/membrane-binding domain; a N-terminal phosphatidylinositol (PtdIns)(4,5)P2-binding domain (PBD); a C-terminal tail showing Pro, Glu, Ser and Thr (PEST) sequences, which is related to its ability to target protein for proteasomal degradation; a class I PDZ-biding motif (PDZ-BD), acting in protein-protein interaction site; and a catalytic phosphatase domain [34].

Despite its activity as a tyrosine, serine and threonine phosphatase, PTEN differs from the most protein tyrosine phosphatases, as this protein preferentially dephosphorylates phosphoinositide substrates [34]. This phosphatase acts by dephosphorylating the lipid second messenger phosphatidylinositol(3,4,5)-trisphosphate (PIP3) into phosphatidylinositol(4,5)biphosphate (PIP2), preventing its recruitment to the membrane region and, therefore, inhibiting AKT activation [35]. Nevertheless, PTEN is able to exert functions independently of PIP3 by acting as a scaffold protein both in the cytoplasm and nucleus [36]. This observation revealed that PTEN can also work in a non-enzymatic manner, which is acknowledged as a non-canonical function [34].

The third group in class I is the SACs PTPs, which are involved in the dephosphorylation of phosphoinositides (PIs). To date, 5 members of this group have been described, and they are involved in processes such as PI homeostasis, membrane trafficking and cell signaling [25, 37].

Paladin (also known as PALD1) is the fourth member of class I PTPs. PALD1 is a protein that exhibits two recognized PTP domains, with a C-xxxxx-R structure [25]. Thus far, no phosphatase activity has been reported for this protein. However, it was described as a negative regulator of insulin signaling [38].

The fifth and sixth groups are INPP4s and TMEM55s, which are the most recently described members of class I PTPs, integrated by two members each, all of them exhibiting the C-xxxxx-R domain [39, 40]. INPP4s and TMEM55s are involved in the dephosphorylation of inositol polyphosphates and PIs, respectively [21, 41]. INPP4B has been described to negatively regulate the PI3K/AKT signaling pathway [42], while TMEM55B was shown to stabilize p53 and induce apoptosis [43].

2.1.2. Class II Cys-Based Phosphatases

The class II Cys-Based PTPs differ from class I for presenting an Asp residue, which is involved in the catalysis reaction, more than 100 amino acids downstream from the C-xxxxx-R domain; in class I, this residue precedes the catalytic site's sequence [25, 26]. Two proteins integrate this class: LMW-PTP (ACP1) and the recently described Ssu72, which will not be further discussed in this review because it specifically dephosphorylates Ser residues [44, 45]. The function of LMW-PTP remains poorly understood, but some studies have indicated that this protein might play a role in the immune response and oncogenesis [46, 47].

2.1.3. Class III Cys-Based Phosphatases

The final class of Cys-Based PTPs, class III, comprises three members: CDC25A, CDC25B and CDC25C. These proteins act on the activation of CDKs by dephosphorylating them on their inhibitory site; therefore, they play a role in cell cycle progression and the response to DNA damage [48]. This class of PTPs differentiates from

the two others by its absence of the WPD loop. It has been proposed that a conserved Glu, adjacent to the catalytic domain, might play the role of Asp in the catalytic reaction [49, 50].

3 Regulatory Role of Protein Tyrosine Phosphatases in Cancer: Friend or Foe?

Signal transduction is a complex process that effectively transmits extracellular signals through a cascade of molecular events involving protein phosphorylation—by PTKs—and dephosphorylation—by PTPs—ultimately resulting in a cellular response [51]. The regulation of these signaling cascades controls the biological outcome of important processes, such as cell proliferation, growth, survival, metabolism, differentiation and cell death. The loss or disruption of the signal strength/duration can result in altered cellular processes such as uncontrolled cell growth and defective apoptosis, contributing to disease development such as tumorigenesis [51].

For a long time, PTPs were thought to act only as tumor suppressor proteins by blocking the signal response through oncogenic kinase dephosphorylation. However, it has now become clear that a significant number of PTPs is overexpressed in human cancers, and they do not suppress tumor growth; instead, they positively regulate signaling pathways and promote tumor development and progression. Currently, they can play a role as tumor suppressor PTPs, oncogenic PTPs or even both, as their function are highly dependent on cellular context [51]. In the following sections this review will discuss the most relevant PTPs.

3.1. Protein Tyrosine Phosphatases as Tumor Suppressors

Impaired tumor suppressor PTP function, often observed in cancer, is characterized by the loss of gene function, which can occur because of gene deletions, inactivating mutations, and epigenetic alterations, such as promoter methylation [52].

Several tumor suppressor PTPs have been identified whose loss has been shown to promote tumorigenesis, growth, and metastasis in *in vitro* and *in vivo* models [52]. However, most PTPs'substrates are still-to-be-determined, which limits the understanding of PTPs function in tumorigenesis.

Classical PTPs

Considering the relevance of PTP superfamily several approaches have been performed to identify mutations in other PTPs. One of the first tyrosine phosphatome analysis has been performed in colorectal cancer and has identified loss-of-function mutations in both receptor-like PTPs (such as PTPRF, PTPRG and PTPRT) and non-receptor PTPs (such as PTPN3, PTPN13 and PTPN14) [53]. In most of them, nonsense, missense, frameshift mutations or splice-site alterations mainly resulted in truncated proteins lacking phosphatase activity. In the same study, these findings have also been reported for lung and gastric cancers [53]. On the other hand, in genome analysis of colorectal tumors, stratified according to genetic instability, despite the high frequency of mutations in PTP genes (frameshift mutations was described in *PTPRA*, *PTPRS*, *PTPN5*, *PTPN13*, *PTPN21* and *PTPN23*), they did not seem to play a common role in microsatellite instability-mediated tumorigenesis [54].

Several other receptor-like PTPs have also been identified as tumor suppressor phosphatases. For example, PTPRK can regulate cell adhesion, migration and proliferation in breast cancer cells through EGFR and HER2 dephosphorylation [55, 56]. Furthermore, the loss of PTPRK can lead to AKT signaling hyperactivation and promotion of tumorigenesis, as reported in colon cancer development [57]. These reports illustrate a few among several substrates of PTPRK.

Inactivating mutations in conserved domains of *PTPRD* and *PTPRT* genes, two important JAK/STAT signaling regulators, have also been frequently related to human

cancers (mainly neck squamous cell carcinomas, melanomas, lung cancers and glioblastomas) [58, 59]. Interestingly, these PTPs seems to play an important role also in drug response, as deleterious alterations in *PTPRT* and *PTPRD* have recently been associated to bevacizumab resistance in patients with metastatic colorectal cancer [60].

In addition to gene deletions and inactivating mutations, loss of expression by epigenetic modifications in the promoter region may also be responsible for the loss of tumor suppressor PTP function [51]. Inhibition of gene expression of receptor-like PTPs (such as PTRG, PTRD, PTPRF, PTPRM, PTPRK and PTPRO) and non-receptor-type PTP, PTPN13 has been reported to occur due to hypermethylation of CpG islands within the promoter region of these genes in several tumors such as acute lymphoblastic leukemia (ALL), lung cancer samples, adenocarcinomas, glioblastomas and squamous cell carcinomas [58, 61-64]. For instance, *PTPRO* promoter methylation has been described in about 50% of human lung cancer [65].

Accordingly, the reestablishment of PTPRK and PTPRO has shown increased apoptosis susceptibility in ALL and reduced anchorage-independent growth, proliferation, resistance to apoptosis and more sensitivity to cytotoxic chemotherapy in lung cancer (respectively), suggesting indeed that these phosphatases act as tumor suppressors [65, 66].

Hypermethylation of the non-receptor PTPN6 gene promoter region has also been reported in leukemias and lymphomas. PTPN6 (also named SHP1) is an antagonist of growth factor signaling in epithelial and haematopoietic cells and the loss of this phosphatase activity has been closely associated with hematological malignancies pathogenesis mediated by Janus kinase 3 (JAK3) and STAT3 (signal transducer-activator of transcription 3) tyrosine phosphorylation levels [67-69].

Dual-specificity phosphatases

One of the most important intracellular signaling pathways, the PI3K/AKT pathway, plays an essential role in cell cycle and apoptosis control, and its dysregulation is frequently implicated in PDAC and other human cancers [70]. PI3K activation is negatively regulated by PTEN which is one of the most studied tumor suppressor phosphatases [71]. PTEN activity on PIP3 inhibits PI3K pathway and hinder cellular processes mediated by the AKT/mTOR axis such as cell cycle progression, induction of cell death and angiogenesis promotion [34]. Deletions and mutations within the *PTEN* gene are frequently observed in several primary tumors, including pancreatic cancer, which result in activation of the PI3K/ AKT pathway and reestablishes growth promotion, survival and tumor development [72-74].

Regarding the other DUSPs, in the last few years the literature has shown an increase number of reports assessing different member of DUSPs family. For example, DUSP2 phosphatase, also known as phosphatase of activated cells 1 (PAC1), is a transcription target of p53 and E2F1 and it is a key regulator of apoptosis and growth suppression through MAPK pathways [75]. DUSP2 downregulation has been reported in many human cancers and its expression level is inversely correlated with HIF-1α [76]. In this report, DUSP2 suppression mediated through hypoxia was able to prolong ERK activation and consequently promoted tumor progression and chemoresistance, suggesting a key role of DUSP2 as a downstream regulator of HIF-1α [76]. These studies have demonstrated that DUSP2 may be a novel target for drug therapy.

DUSP6 (also named MKP-3) have been described in many types of tumors where it can play variable roles. This phosphatase is mainly described in literature for dephosphorilation of ERK1/2, acting in a negative feedback for the activation of RAS/ERK signaling pathway. Therefore, it was classified as a tumor-suppressor PTP in

a variety of tumors, such as pancreatic, lung, ovarian, esophageal squamous cell and nasopharyngeal cancers [77-80]. Nevertheless, reports in literature have also shown that DUSP6 can act as an oncogenic PTP. Messina and colleagues observed that DUSP6 overexpression in human glioblastoma cultures lead to tumor-promoting phenotype through increase of clonogenic capacity and chemoresistance to cisplatin [81]. Similarly, Song and colleagues observed an impairment of cell proliferation, migration and invasion capacity, as well as cell cycle arrest after DUSP6 inhibition in triple-negative breast cancer cells [82]. In addition, oncogenic role has also been reported in thyroid cancer and acute myeloid leukemia (AML) with FLT3-ITD [83, 84]. In malignant melanoma, DUSP6 has a very interesting role: its function is highly dependent on the histological subtype [85]. Both oncogenic and tumor-suppressor roles have been described in melanoma cells with different genetic background. These observations underlie the complexity of DUSP6's interaction with tumorigenic processes.

As well as DUSP6, DUSP9 (also named MKP-4), is also known for its preference for dephosphorilation of ERK1/2, although, differently from DUSP6, DUSP9 also exhibits activity against p38 and JNK [86, 87]. Despite not being as extensively studied as DUSP6 in the tumorigenesis context, some studies have linked DUSP9 to important functions in cancer cells. In an Affymetrix GeneChip analysis, Liu and colleagues observed downregulation of DUSP9 in a non-Ras model of epithelial carcinogenesis. Loss of DUSP9 was also observed in tumors independently generated by the application of chemical or physical carcinogens in mice. Restoration of DUSP9 expression increased levels of tumor cell death through activation of p38 and JNK in addition to inactivation of ERK [88]. Overexpression of DUSP9 in tumorigenic cells led to almost total suppression of tumor formation following sub-cutaneous injection into BALB/c neonatal mice when compared with untransduced cells [88]. Later, Wu and colleagues observed

that DUSP9 is silenced by the promoter region hypermethylation in gastric cancer samples when compared to tumor-free samples. They also observed that overexpression of DUSP9 in gastric cancer cell lines impaired cell proliferation *in vitro*, besides leading to cell cycle arrest [89]. Taken together these results are suggestive of a tumor suppressor function for DUSP9.

These findings illustrate how PTPs role in tumorigenesis can be diverse and mostly dependent on context (cellular, gene expression or protein activity).

3.2. Protein Tyrosine Phosphatases as Oncogenes

In contrast to tumor suppressor genes, oncogene PTPs are characterized by a gain of function as a result of gene amplification, activating mutations, and translocations, which could lead to aberrant expression, tumor formation, growth and metastasis [52]. Some oncogenic PTPs are present in various tumors; therefore, they are the most studied ones.

Classical receptor-like PTPs

Regarding receptor-like PTPs, several phosphatases have been related to cancer development with oncogenic function. In this section, the most relevant will be briefly presented.

PTPs with oncogenic function seem to participate in tumorigenesis mediating the activation of Src-signaling cascades. Src family members are involved in multiple signal transduction events and cellular functions such as mitogenesis and cell cycle progression [90]. The most important regulatory phosphorylation site in Src is Tyr527 (chicken Src numbering) and when phosphorylated Tyr527 binds to the inactive Src SH2 and SH3 domain and impairs accessibility for binding. For example, PTPRA is overexpressed in breast and colon tumors, and promotes tumorigenesis through activation of Src family

kinases (SFKs) *in vitro* and *in vivo* [90]. Overexpression of PTPRA dephosphorylates pTyr527 consequently activating Src domains for binding and therefore stimulating neoplastic transformation [90].

Interestingly, depletion of PTPRA reduced the number of ER+ ErbB2 (HER2)-positive breast tumors per mouse in xenograft models by a reduction in Akt phosphorylation, highlighting the impact of PTPRA also on the PI3K pathway, being involved in the tumor initiation process. However, no differences in tumor latency, tumor volume or metastasis were observed suggesting that compensatory pathways may overcome the effect of PTPA knockdown. On the contrary, depletion of PTPRA by RNAi induces apoptosis in ER− breast and colon cancer cells but not in ER+ breast cancer cell lines tested [91, 92].

PTPRE displays the highest degree of sequence and structural similarity to PTPRA and it is possible that it may share some of the transforming abilities of PTPRA and that upregulation of PTPRE in mammary tumors is indeed linked to the transformation process. PTPRE overexpression appears to be mammary tumor-specific, being highly expressed in murine mammary tumors initiated by HER2/*Neu* and *RAS activation,* but not in mammary tumors initiated by c-*Myc* or *Int-2* [93-95]. Complementary studies showed that HER2/Neu induces phosphorylation of PTPRE and that this phosphorylation is required for activation of Src by PTPRE in mammary tumors [93-95].

Similar to PTPRA and PTPRE, PTPRJ (also named DEP-1) overexpression has also been observed in breast cancer, where its expression correlates with activation of Src in highly invasive breast cancer cells compared to less invasive or untransformed cell lines tested *in vitro* [96]. Analysis of clinical data sets indicated that intermediate expression of PTPRJ in invasive breast cancers correlated with faster relapse and the

decreased survival of patients. Active PTPRJ and the downstream activation of the Src pathway are linked to the promotion of a proinvasive and prometastatic phenotype in breast cancer cells [96].

PTPRJ has also been pointed as an essential driver of VEGF-dependent permeability, angiogenesis, and metastasis, thus cooperating for cancer invasiveness mainly mediated through Src signaling pathway [97]. Altogether, these new reports suggest that PTPRJ could also mediate the development of Src family kinases driven tumors and consequently represent a novel therapeutic route to cancer treatment [96]. Interestingly, PTPRJ has also been reported as an antiproliferative regulator strongly downregulated in several human cancers, once again illustrating the paradoxal role of PTPs in cancer. [98-102].

Receptor-like PTPs also participate in the activation of other cancer-related pathways. For instance, PTPRZ-B isoform knockdown resulted in reduced migration and proliferation of glioma cells *in vitro* and also inhibited tumor growth *in vivo* [103]. Interestingly, expression of only the PTPRZ-B extracellular segment was sufficient to rescue the *in vitro* migratory and proliferative phenotype that resulted from PTPRZ-B knockdown. PTPRZ have also been implicated in neuronal migration during central nervous system development [103-105]. A tumor-promoting role of PTPRZ has been also observed in ovarian cancer through ERK 1/2 signaling activation [106].

Several other receptor-like PTPs have been assessed as the emerging role of the PTPs in regulating cancer development has increased the interest in studies regarding this topic. The role of other receptor-like PTPs in cancer are summarized in Table I.

Classical non-receptor PTPs

Src-homology 2 (SH2) domain-containing protein tyrosine phosphatase 2 (SHP2), a non-receptor PTP also known as PTPN11, was the first oncogenic PTP to be reported.

It has been described to be overexpressed in human leukemia and breast cancer [107, 108]. SHP2 positively regulates cell signaling pathways that promote cell growth, motility, differentiation and survival, such as RAS/ERK [51]. Activated SHP2 induces the RAS/ERK pathway by dephosphorylating RAS-GAP or Sprouty proteins, two important RAS inhibitors. Although its activating mutations found in leukemia may link this PTP to an oncogenic function, there have been reports that indicate that it might have a tumor-suppressor role in other types of cancer. Bard-Chapeau and colleagues have shown that tissue-specific deletion of SHP2 in mice hepatocytes promoted inflammatory responses through Stat3 pathway, leading to inflammation, necrosis and tumor development [109]. Same group has previously shown that the tissue-specific SHP2 deletion suppresses ERK1/2 signaling as well as liver regeneration following partial hepatectomy [110].

Other studies have also demonstrated similar results, in which SHP2 inhibition reduces growth and invasiveness in cancer cells by inhibiting RAS/ERK and PI3K/AKT signaling pathways [111-113]. Bard-Chapeau and colleagues also observed a dramatically decreased expression of SHP2 in 12 of 104 human patient samples of hepatocellular carcinoma (HCC) compared to adjacent tissue, suggesting that downregulation of SHP2 might be related to tumor initiation and/or progression in a subset of human HCCs [109]. This finding is supported by Jiang and colleagues, which performed a microarray analysis of 333 HCC patient samples and their adjacent non-tumor tissue, and used immunohistochemistry to determine SHP2 expression. They observed a significantly reduced expression of SHP2 in tumor tissues compared to their non-tumor pairs. Survival analysis showed that low expression of SHP2 has an association with poorer overall survival and a multivariate analysis showed that this variable is an independent prognosis marker [114]. Another interesting finding about

SHP2 in HCC is that this PTP acts together with PTEN suppressing tumorigenesis [115], in contrast to what has been previously described in leukemogenesis [116].

Qi and colleagues when comparing protein expression of SHP2 in esophageal squamous cell carcinoma (ESCC) samples with adjacent normal tissues observed that this protein phosphatase is overexpressed in normal samples. The low expression of SHP2 in ESCC promoted increased clonogenic capacity *in vitro* and cell proliferation *in vitro* and *in vivo*. The expression of SHP2 results in the negative regulation of STAT3 (signal transducer and activator of transcription 3) by acting on its dephosphorylation which characterizes SHP2 as a tumor suppressor [117].

In prostate stem cells it was observed that SHP2 activity may be related to the action of chondroitin 4-sulfate (C4S). The interaction of SHP2 with C4S may favor the inactivity of SHP2 which prevents interaction with targets of dephosphorylation such as phosphorylated RAS and results in phosphorylation of pathway substrates. Increased phosphorylation of, for example, phospho-ERK1/2, may lead to increased cell signaling with transcriptional effect. Taken together, these data indicate that SHP2 may play a double role in tumorigenesis [118].

Another classical non-receptor PTP that may play a dual role in tumorigenesis is PTP1B. Also known as PTPN1, this classical PTP regulates the activity of many signaling pathways in cancer, such as cell proliferation, apoptosis and metastasis, mainly through the modulation of RAS/ERK and PI3K/AKT signaling pathways [119]. Several reports on PTP1B function have described a tumor suppressor role by dephosphorylation of substrates that could drive malignant transformation through constitutively active PTKs. Indeed, a few studies have shown that up-regulation of PTP1B expression promotes cell differentiation and prevents malignant transformation in diverse human cancers [119].

More recently, however, PTP1B was presented as an oncogenic molecule. In these studies, a strong overexpression pattern has been observed in several human cancers such as gastric, prostate and breast cancers, and was frequently associated with a poor prognosis [120-122]. In gastric cancer, PTP1B promotes cell proliferation, survival and invasiveness through Src-related signaling pathways, such as RAS/MAPK and PI3K/AKT pathway [121].

For example, in prostate cancer, *PTPN1* gene is often amplified in metastatic tumors and has been pointed as a direct target of the androgen receptor. PTP1B knockdown has decreased cell proliferation and has abrogated androgen-induced migration and invasion *in vitro* [123]. In a prostate cancer model, knockdown of PTP1B has also provoked reduced cell growth, apoptosis induction, and cell cycle arrest, and impairs migration and invasion by reversing the epithelial-mesenchymal transition (EMT) process, through dephosphorylation of the cadherin-catenin complex [124].

PTP1B function as a tumor promoter was also demonstrated in for breast cancer cells. In another study, genetic deletion of PTP1B had delayed tumor progression induced by activated ErbB-2, evidencing that PTP1B is critical in breast tumor development and that anti-ErbB2 therapies combined with pharmacological inhibition of PTP1B activity may pose as potential therapeutic targets [122]. Although several studies present controversial results regarding PTP1B function, a growing number of robust evidences confirm its oncoprotein activity in several contexts, indicating that this phosphatase may represent an important therapy target approach.

PTPRN, also known as IA-2, is widely expressed in neuroendocrine and neuronal cells and participates in secretion of hormones and neurotransmitters [125]. In small cell lung cancers (neuroendocrine origin tumor), knockdown of PTPRN mediated by miR-342 reduced tumor growth rates through suppression of autocrine secretion [126],

suggesting that PTPRN may be a potential therapy target for the treatment of autocrine-induced tumors.

Another important non-receptor phosphatase has been widely reported for their role in breast cancer. First, expression of an immature form of PTPRN2, known as proPTPRN2, is associated with lymph node-positive breast cancer patients that presents poor prognosis. In breast cancer experimental models, proPTPRN2 was capable to promote tumor growth in both *in vivo* and *in vitro* approaches. This effect is mediated by an interaction between PTPRN2 and TRAF2, which suppresses apoptosis of the tumor cells [127]. In other recent study, PTPRN2 was shown to collaborate with PLCβ1 to hydrolyze PI(4,5)P2 in the plasma membrane of human breast cancer cells, releasing membrane-bound cofilin, increasing actin remodeling and consequently promoting metastatic migration [128]. Patients with metastatic breast cancer that show high expression level of PTPRN2 had presented a worse metastasis-free survival [128]. Altogether these findings demonstrate the key role of PTPRN2 in breast cancer cell migration and metastasis promotion and point to a possible new drug therapy target.

Several other non-receptor phosphatases have also been widely described for their pro-tumorigenic function and association with chemoresistance and cancer prognosis. The most relevant non-receptor PTPs with oncogenic features are also summarized in Table I.

Dual-specificity phosphatases

Regarding phosphatases among the class I Cys-based phosphatases composed of VH1-like/DUSPs are involved in the regulation of MAPK activities during processes of initiation and development of human cancer [129]. The overexpression of DUSP1 (also named MKP-1) was observed in a range of human epithelial tumors including prostate, colon and bladder. Interestingly, elevated levels was seen only in the early phases of

disease with levels of DUSP1 expression falling progressively in tumors of higher histological grade and in metastases [130]. DUSP1 is upregulated in response to a variety of cellular stress conditions including oxidative stress, hypoxia and DNA damaging agents, suggesting that it may play a key role in the regulation of MAPK activities within the tumor microenvironment [131, 132].

Upregulation of DUSP1 is inversely correlated with both JNK activity and apoptosis biomarkers, indicating that this phosphatase might have an anti-apoptotic role in these tumors via its activity towards JNK [133, 134]. DUSP1 levels have also been studied in primary ovarian tumors where moderate to strong expression of DUSP1was detected, being considered as a prognostic marker for shorter progression-free survival [135]. In different experimental approaches to inhibit DUSP1 in ovarian cancer cells, it has been observed that DUSP1 inhibition caused JNK activation and significantly enhanced cells sensitivity to several cytotoxic drugs such as alkylating agents (mechlorethamine), anthracyclines (doxorubicin) and microtubule inhibitors (paclitaxel) [135]. Xenograft experiments in nude mice using siRNA-expressing cell lines (against DUSP1) have shown a decrease in tumor growth rates and increased cellular response to cisplatin in a non-small lung cancer model [136-138]. The same outcome has been reported in gastric cancer model in which DUSP1 mediate apatinib chemoresistance [139]. Taken together, these studies indicate that DUSP1 overexpression may mediate chemoresistance in different cancer types.

Other DUSPs are involved in the malignancy of tumors, DUSP4 (also named MKP-2) is overexpressed in serous borderline tumors (SBT) of the ovary when compared with serous carcinomas (SCAs). SBTs present a more benign phenotype with a lack of stromal invasion suggesting that DUSP4 might play some role in the more benign behavior of SBT via the suppression of ERK-dependent events associated with

degradation of the extracellular matrix [140]. [141]. And a study showed that DUSP7 mRNA and protein were highly expressed in leukocytes obtained from AML and acute lymphoblastic leukemia (ALL) patients [142].

The final subgroup of VH1/DUSP PTPs are the phosphatases of regenerating liver (PRLs). This subgroup comprises three members: PRL1, PRL2 and PRL3. PRL3 (also known as PTP4A3) has been described to play an important role in cell proliferation and metastasis through aberrant Src activation and consequently hyperactivation of the RAS/ERK and p38 signaling pathways [143-146]. High expression of PTP4A3 also promotes cell motility and invasion through the activation of Rho GTPase. PTP4A3 overexpression has been already associated with breast, colon, gastric and liver cancers [144]. Corroborating its function, PTP4A3 knockdown can suppress cell growth *in vivo* by reducing proliferation marker (Ki67), phospho-ERK1/2, and p38 levels while increasing apoptotic marker levels [143].

Taken together, the findings discussed so far indicate a promising role of oncogene phosphatases as potential therapeutic targets. The most frequently observed tumor suppressor PTPs and oncogene PTPs are described in Table I.

Table I. Frequently observed protein tyrosine phosphatases and their role in cancer

PTP	Function	Signaling Pathway	Cancer	Reference
Classical Phosphatases: Receptor-like PTPs				
PTPRK	Suppressor Oncogene	PI3K/AKT RAS/JNK	Breast Prostrate	[56] [147]
PTPRJ (DEP1)	Suppressor Oncogene	RAS/ERK Src	Breast, colon, lung, thyroid, pancreatic and myeloid leukemia Breast	[100-102] [96]
PTPRA and PTPRE	Suppressor Oncogene	- Src and PI3K/AKT	Colorectal Breast and colon	[53, 54] [90-92, 148, 149] [93-95, 150]
PTPRD	Suppressor	-	Head and neck squamous cell	[58, 59, 151]

			carcinoma, melanoma, lung and glioblastoma	
PTPRK	Suppressor	PI3K/AKT	Breast, colon, lung, ALL and thyroid	[58, 59, 151] [152-154]
PTPRT	Suppressor	-	Colon and pancreatic	[53, 155]
PTPRF and PTPRG	Suppressor		Colorectal, breast, gastric, ALL, lung, adenocarcinomas, glioblastomas and squamous cell carcinomas	[152-154] [93-95, 150]
PTPRF	Oncogene	-	Breast	[156]
PTPRO	Suppressor Oncogene	- Src	Gastric, lung, colon and ALL B-CCL	[58, 59, 151] [157]
PTPRZ	Oncogene	RAS/ERK	Glioma and ovarian	[103-106, 158]
PTPRH	Oncogene	-	Colon and non-small cell lung	[159, 160]
PTPRM	Oncogene	-	Glioblastoma	[161-163]
PTPRU	Oncogene	-	Gastric and glioma	[164, 165]
PTPRC	Oncogene	JAK/STAT	Head and neck	[166]
Classical Phosphatases: Non-receptor PTPs				
PTPN1 (PTP1B)	Oncogene	RAS/ERK, PI3K/AKT, Src and JAK/STAT	Gastric, prostate, breast, lung, colorectal, hepatocellular, NSCLC, colon, thyroid, Hodgkin's lymphoma and chronic myeloid leukemia (CML)	[120-124, 167-174]
PTPN2	Suppressor Oncogene	- -	T-ALL B-cell lymphoma, SCLC and breast	[175] [126-128] [176]
PTPN3	Suppressor Oncogene	 RAS/p38	Colorectal, lung, breast, and gastric Colon, breast, intrahepatic cholangiocarcinoma and gliomas	[152-154] [177-180]
PTPN5	Suppressor	-	Colorectal	[152-154]
PTPN6 (SHP1)	Suppressor	JAK/STAT	Leukemia and lymphoma	[67-69]
PTPN11 (SHP2)	Oncogene	RAS/ERK	Leukemia, breast and pancreatic	[107, 108, 111-113, 181-183]
PTPN13	Suppressor Oncogene	- -	Colorectal, lung, breast, and gastric	[152-154] [152-154]

			Astrocytoma, ovarian and Ewing's sarcoma	
PTPN14	Suppressor	-	Colorectal, lung, breast, and gastric	[152-154]
PTPN21	Suppressor Oncogene	- -	Colorectal Bladder and cervical	[152-154] [184, 185]
PTPN23	Suppressor	-	Colorectal	[152-154]
VH1-like/DUSPs PTPs				
PTEN	Suppressor	PI3K/AKT	Several, including breast, prostate, ovarian and pancreatic	[71-74, 186]
DUSP1 (MKP-1)	Oncogene	RAS/JNK	Prostrate, colon, bladder, ovarian and NSCLC	[130-138]
DUSP2 (PAC1)	Oncogene	-	Ovarian	[141]
DUSP4 (MKP-2)	Oncogene	RAS/ERK	Serous borderline tumors (SBT) of the ovary	[140]
DUSP6 (MKP-3)	Suppressor Oncogene	RAS/ERK	Pancreatic, lung, ovarian, esophageal squamous cell, nasopharyngeal cancers and some types of melanoma Glioblastoma, thyroid Carcinoma, breast, AML (FLT3-ITD) and some types of melanoma	[77-80, 85] [81-85]
DUSP7	Oncogene	-	ALL and AML	[142]
DUSP9	Suppressor	RAS/p38, RAS/JNK and RAS/ERK	Epithelial carcinogenisis	[88]
PTP4A3 (PRL3)	Oncogene	RAS/ERK and RAS/p38	Breast, colon, gastric and liver	[143-146]

4 Protein Tyrosine Phosphatase in PDAC: Regulatory Role and Clinical Implications

Classical PTPs

Clearly, PTPs have both inhibitory and stimulatory effects on cancer-associated signaling processes, and dysregulation of PTP function is associated with tumorigenesis. As aforementioned, several PTPs display either tumor suppressor or oncogenic characteristics in the development and progression of many cancers, including PDAC.

Considering the entire Cys-Based PTP family, the most prominent findings thus far are mainly related to the classical PTP and DUSP subgroups.

Some of the classical PTPs and DUSPs have already been described for their role in PDAC tumorigenesis, growth and metastasis processes. In general, inhibition of tumor suppressor phosphatases or the activation of oncogenic phosphatases in PDAC context, may contribute to an even worse prognosis, often leading to a decrease in patients overall survival [51]. Among classical PTPs a few candidates with a relevant role in PDAC can be highlighted, such as SHP2, PTPN14, PTPRJ and PRLs, as PTP4A variants.

The transcriptional levels of *SHP2* have been recently analyzed in The Cancer Genome Atlas (TCGA) RNA-seq data from KRAS-mutant PDAC patients and showed no association with the patients' overall survival rates [187]. By contrast, SHP2 seems to play a relevant role in the overall survival rate as stated by Zheng and colleagues. In this study, PDAC patients with higher SHP2 expression presented significantly lower survival rates, suggesting that SHP2 activation, rather than the gene expression levels, are involved in PDAC carcinogenesis [181]. SHP2 genetic deletion and pharmacological inhibition have been reported to impair PanIN progression, desmoplasia and disrupt the mesenchymal epithelial transition and acinar transdifferentiation, suggesting that SHP2 activation is indeed required for mutant KRAS-driven PDAC [182]. Supporting this statement, SHP2 suppression could abrogate MEK inhibitor adaptive resistance in mutant KRAS-driven cancers, including PDAC, where combined therapy of SHP2 and MEK inhibitors suppresses tumor growth, suggesting a new perspective on MEK inhibitor therapy for PDAC [188].

Another important phosphatase in pancreatic cancer development, PTPN14, has been recently reported as an important p53 target gene for tumor suppression. In this study, mice expressing p53 transcriptional activation domain (TAD) mutants were

analyzed, and a p53 mutant in the second TAD (p5353,54) showed an enhanced capacity to suppress pancreatic cancer progression. This p53 mutant has been called as a "super-tumor suppressor" due to its ability to transactivate specific p53 target genes. Among these targets, the major player in PDAC tumor suppression is PTPN14, which interacts with Yap, modulates the Hippo regulatory pathway and plays an essential role in p53-mediated tumor suppression in PDAC [155].

In another transcriptome-based study, Stephens and colleagues analyzed a large subset of pancreatic cancer cell lines and PDAC primary samples to address the PRL phosphatase expression pattern and reported that both PRL1 and PRL2 are significantly upregulated compared with those in normal pancreas tissue. Functional assays performed in pancreatic cells also confirmed the role played by PRL1 and PRL2 in cell growth and survival, thus supporting the hypothesis that PRL phosphatases regulate key pathways such as the AKT and ERK signaling pathways and are directly involved in PDAC tumorigenesis and metastasis [189].

PTPRJ is also downregulated in pancreatic cancer and it is drastically reduced in several pancreatic carcinoma cell lines when compared with normal pancreatic tissue. Restoration of PTPRJ activity inhibits proliferation, reduces colony formation, disrupts the cell cycle and leads to apoptosis. The growth of the PSN1 pancreatic cell line in xenograft tumors was blocked by the intratumoral injection of a recombinant adeno-associated virus carrying PTPRJ [100].

Taken together, the results showed that these tumor suppressor phosphatases illustrate the relevance of the role of PTPs in PDAC progression and the drug response.

Dual-specificity phosphatases

As a KRAS-driven cancer, PDAC is strikingly dependent on MAPK signaling pathways, which include ERK1/2, JNK and p38, to regulate cell proliferation, survival,

the stress response and differentiation [190]. Constitutive activation of MAPKs occurs in various human tumors mostly because the intense proliferative cell capacity is closely related to elevated levels of MAPK activity [190, 191].

MAPK inactivation is mediated, in part, by the dephosphorylation of both serine/threonine and tyrosine residues by DUSPs, which represent the major negative regulators of MAPK activities. Because MAPK signaling pathways are a feature in tumor development and progression, by regulating their activation, through signal extent and duration, the DUSPs may dictate the differentiation or proliferation fate within a developing cell, as well as various cellular responses during carcinogenesis, such as metabolic reprogramming and the drug response [192].

In PDAC cells, the first reports on the role of DUSPs relied on ERK inhibitors. For example, DUSP6 (also named MKP-3), the major ERK inhibitor, is upregulated in pancreatic dysplastic ductal cells and corresponds to precancerous lesions or early neoplastic changes; however, it is downregulated in primary and invasive pancreatic carcinoma, especially in the poorly differentiated type, leading to uncontrolled cell growth and malignancy promotion [78]. Hypermethylation of the *DUSP6* promoter has been related to the abrogation of DUSP6 function and reestablishment of this phosphatase (adenovirus-mediated expression) in cultured pancreatic cancer cells has promoted strong reduction of ERK phosphorylation. DUSP6 mediated ERK inhibition significantly suppress cell growth and promoted apoptosis, showing that DUSP6 exerts apparent tumor-suppressive effects *in vitro* [78, 193].

By contrast, investigating another well-known ERK negative regulator, Liao et al. demonstrated that DUSP1 (also named MKP-1) is overexpressed in PDAC samples compared with those in normal pancreatic tissues. Activation of DUSP1 expression has been associated with increased proliferation of pancreatic ductal cells, and DUSP1

downregulation has been shown to attenuate tumor growth *in vivo* and *in vitro*, suggesting that deregulation of the DUSP1 function is associated with enhanced tumorigenicity in PDAC cells [194]. This discrepancy between the roles of the two ERK negative regulators in PDAC carcinogenesis reveals the complexity of the signaling loops mediated by DUSPs. It is noteworthy that the substrate specificity, subcellular localization and spatiotemporal regulation of DUSPs play extremely relevant roles in this scenario, highlighting the importance of a very extensive investigation of these mechanisms. Regarding this topic, several works have provided relevant insights into the role of DUPSs in PDAC tumorigenesis.

For example, Lee and coworkers have analyzed the expression profile of DUSP28 using Universal exPress Codes (UPCs) with Gene Expression Omnibus (GEO) databases and observed significant upregulation among pancreatic cancer samples compared with normal pancreatic samples [195]. DUSP28 was also highly expressed in many pancreatic cancer cell lines with known resistance to anti-cancer drugs as analyzed in the Cancer Cell Line Encyclopedia (CCLE) database. To validate this finding, *in vitro* and *in vivo* assays have been performed and, in both situations, DUSP28 overexpression was intrinsically related to gemcitabine and doxorubicin chemo-resistance mediated by apoptosis inhibition and induced migration activity [195].

Interestingly, the drug-resistance in pancreatic cancer cells occurred mainly through ERK1/2 pathway modulation, different from previous findings on DUSP28 function, in hepatocellular carcinoma cells, where its role on p38 activity regulation has been reported [196]. In more recent studies from the same group, Lee et al. have also demonstrated that DUSP28 plays a functional role in the cell migration and survival of pancreatic cancer cells through key molecules such as MUC5B and MUC16 and PDGF-A (an important growth factor-regulated by MUC1) [197, 198]. In this case, DUSP28

interacts with PDGF-A to form an autocrine loop to provoke signal induction that specifically exacerbates the malignancy of pancreatic cancer cells mediated by ERK1/2 and p38 intracellular signaling [197]. Taken together, these findings indicate that DUSP28 might be a target molecule to inhibit pancreatic cancer.

Another promising target for PDAC has recently emerged. Hijiya and collaborators have studied genetic abnormalities that could drive the transformation of pancreatic carcinoma *in situ* (CIS) to invasive cancer by genomic profiling approach. They have reported that in comparison to noninvasive pancreatic carcinoma, invasive tumors have higher frequency of genomic copy-number aberrations and, specifically, the loss of 8p11.22-ter was more often associated with invasive tissues [199]. Analyzing the expression profile of pancreatic cancer cells lacking 8p11.22, several candidate genes have emerged, and, among them, the MAPK phosphatase DUSP4 was the only one related to carcinoma invasiveness [199]. To elucidate its functional role, restoration of DUSP4 was induced *in vitro* and could confirm the suppression of pancreatic cell invasiveness. Accordingly, restoration of DUSP4 expression in orthotopic xenograft models led to the abolishment of tumor growth, invasiveness and, consequently, extended survival.

These events, modulated by DUSP4, were mediated by ERK inactivation, corroborating once again, the already well-known essential role of ERK in PDAC progression and the drug response. It is also crucial to emphasize that the results presented thus far based on numerous clinical trials of several malignancies, including PDAC, clarify how challenging it is to achieve molecular targeted therapy using MEK inhibitors alone [199]. In this scenario, elucidating DUSPs function specifically on PDAC progression could contribute essential molecular and biological information on key molecules that could be therapeutically exploited through manipulation of ERK signaling.

Finally, as one of the most studied PTPs in the tumorigenesis context, as previously mentioned, PTEN has also been extensively related to PDAC development. Downregulation of PTEN expression has been observed in PDAC cell lines [71] and samples from patients with PDAC [186]. Foo and colleagues analyzed 133 samples from patients with stage II PDAC, and their results showed that the loss of PTEN favors a worse prognosis. In addition, PTEN loss is also correlated with tumor recurrence, metastasis and a shorter patient survival, supporting the idea that PTEN is an important tumor suppressor in PDAC [186]. In a murine pancreatic KRASG12D model, PTEN heterozygous deletion has been shown to accelerate acinar-to-ductal metaplasia and PanINs and was directly implicated in both PDAC initiation and progression, reinforcing its essential contribution to tumorigenesis in a dosage-dependent condition [200]. Zeleniak and colleagues, for example, demonstrated that PTEN stabilizes and protects metastasis suppressor protein 1 (MTSS1) from proteasomal degradation.

Therefore, PTEN protein loss results in the decreased expression of MTSS1 and, consequently, increased metastatic capacity [201]. In accordance with these studies, Wartenberg and colleagues showed that severe PTEN loss occurs in later stages, after the establishment of the invasive tumor, thus improving the metastatic spreadability of PDAC tumoral cells [202]. Taken together, these findings demonstrate that, although all the mechanisms underlying these interactions are not fully understood, PTEN plays an extremely important role in PDAC.

Collectively, all the information presented thus far shows the essential role of PTPs in tumorigenesis (Figure 3). Despite this great improvement, the specific role of PTPs in PDAC remains elusive, and further investigation in the context of pancreatic cancer progression could bring new insights into the role of these phosphatases and clarify

their rising potential as promising candidates for the treatment of human pancreatic cancer.

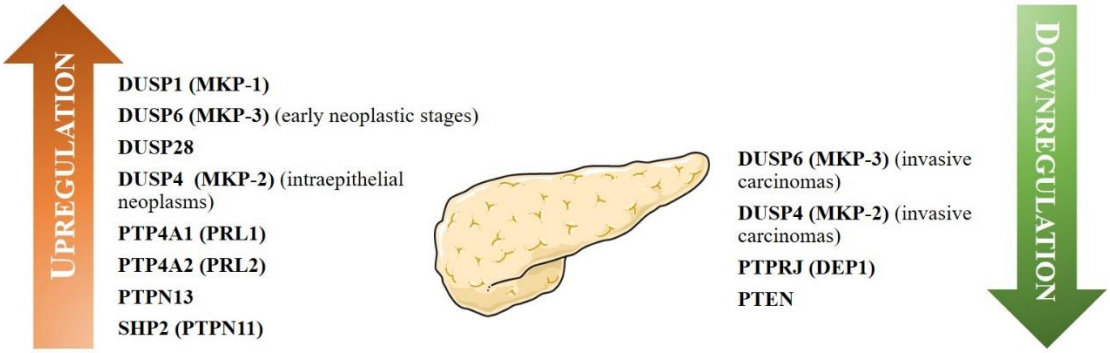

Figure 3 – Protein Tyrosine Phosphatases Expression in Pancreatic Cancers. Differentially expressed PTPs in pancreatic cancers, where oncogenic PTPs are upregulated and tumor suppressor PTPs are downregulated. Some PTPs, such as DUSP6 and DUSP4, may show different expression patterns according to the tumor stage.

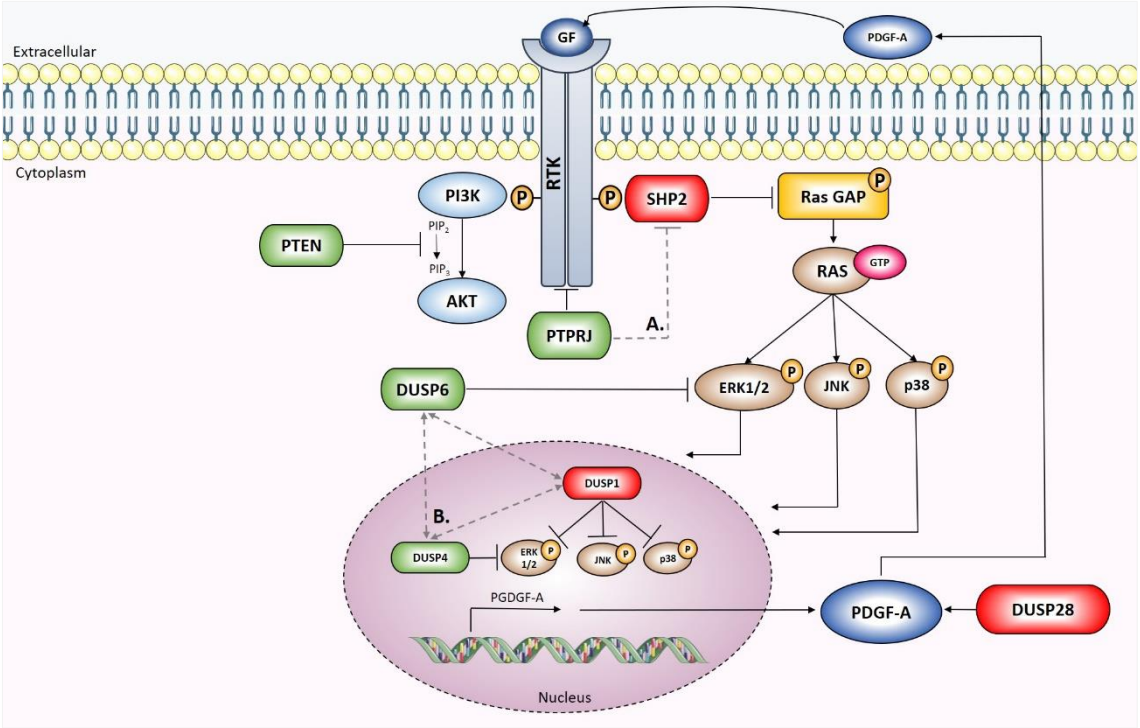

Figure 4 – Main phosphatases acting on PDAC related signaling pathways and a possible crosstalk between them. A. PTPRJ and SHP2. Inhibition of receptor tyrosine kinases (RTK) phosphorylation, mediated by PTPRJ, could cause an impact in activation of SHP2, which would result in the activation of its direct target RAS GAP and, consequently, to the inactivation of RAS and downregulation of its downstream signaling pathways. In pancreatic cancer, PTPRJ is

downregulated allowing phosphorylation of RTKs, activation of SHP2, inhibition of RAS GAP and constitutive activation of RAS. **B. DUSP6, DUSP4 and DUSP1.** These three phosphatases have ERK1/2 as the main common target. However, in the context of PDAC, these proteins present a differential regulation, whereas DUSP6 and DUSP4 are expressed in different levels according to the tumor stage and DUSP1 is constantly upregulated, which may inhibit ERK1/2 activity in the nucleus. Thus, it is possible that there is a feedback loop between these DUSPs, but these inferences are not yet described in the literature. To validate these two proposals, more studies are needed to evaluate these possible interactions.

5 Protein Tyrosine Phosphatase Inhibitors

The oncogenic behavior of some PTPs revealed the idea that inhibitors for these molecules could be beneficial for cancer therapy. Many PTP inhibitors have been developed since these proteins were first associated with cancer (Table II) [203-205]. However, there were many challenges in this process. The first obstacle in developing PTP inhibitors is the highly conserved residues in the binding and hydrolysis sites among the PTPs [206]. This feature makes it difficult to develop specific inhibitors without disturbing the activity of other PTPs. The second obstacle concerns the ionic feature of the PTP active site. Given that the PTP active site is positively charged, screening programs usually identify negatively charged molecules as potential inhibitors [206]. Nevertheless, these molecules have difficulty penetrating the cell membrane because of their negative charge, which is not an ideal feature for a developing drug.

Although there are difficulties in the process, some strategies have been proposed to overcome this, such as the design of molecules that bind to regions external to the active site [51]. In this sense, in 2003, Xie and colleagues reported the first sensitive and potent inhibitor to a well-known oncogenic PTP, the PTP1B [199]. Xie and colleagues developed bidentate ligands using a nonconserved aryl-phosphate binding site, which is adjacent to the PTP1B active site. Therefore, these molecules

can bind to both the active and adjacent sites. Although this compound was reported to have high efficacy in inhibiting PTP1B, no effect was seen in tumor cells due to poor cellular uptake [199]. Given this issue, the compound was later modified to increase cell membrane permeability, leading to a final version that overcame the problem and showed efficient inhibition of PTP1B [207]. Nevertheless, several other compounds for PTP1B inhibition have been developed and are being tested in clinical trials. Some of them have been discontinued because of high toxicity [208], but others are currently in phase II clinical trials for type II diabetes [209] and phase I clinical trials for metastatic breast cancer (NCT02524951) [51]. Considering that PTP1B is regulated by reactive oxygen species, Haque and colleagues have developed conformation-sensing antibodies which stabilizes the protein oxidized form and, therefore, inhibits PTP1B function [210]. This discovery has brought a new strategy to overcome the therapeutic challenges to develop inhibitors that successfully acts on PTP-active sites.

In addition to PTP1B, SHP2 is another PTP whose constitutive activation is highly related to various tumors [211, 212]. Therefore, this protein has also been a target for inhibitor development, with similar issues as the ones found for PTP1B. In this case, the principal obstacle is that SHP2 has high homology with SHP1 which, on the other hand, has been found to be a tumor suppressor PTP [51]. Zeng and colleagues developed an indole salicylic acid inhibitor that binds to its active site and a nearby peripheral site that is exclusively found in this PTP [213]. Additionally, compound 11a-1, as it is named, displays a more than 5-fold preference for SHP2 over the other 20 PTPs and a 7-fold preference for SHP2 over SHP1 [213]. This compound showed efficient blockade of ERK1/2 and Akt activation and, therefore, antiproliferative activity in cancer cell lines [213, 214]; additionally, it also

suppressed tumor growth in xenograft melanoma models [214]. The latest developed inhibitor took advantage of a SHP2 activation feature – this phosphatase is activated by phosphorylated proteins that exhibit a properly spaced phosphor-tyrosine residue which binds to the terminal regions of SHP2 domains. This biding releases the auto-inhibitory interface and allows substrate recognition and dephosphorilation [215, 216]. Noticing that, Chen and colleagues, from Novartis Institutes for Biomedical Research, have used screening strategies to seek a molecule that could work with this mechanism, by locking SHP2 in an auto-inhibitory conformation. SHP099 was found in this study and showed to be specific to SHP2 by having no detectable activity against a panel of 21 phosphatases and, more importantly, against SHP1 [217]. Nevertheless, SHP009 leads to suppression of RAS-ERK signaling pathway and, consequently, reduced proliferation of human tumor cells *in vitro*, as well as in mouse tumor xenograft models [217].

The PRL subgroup is also a known oncogenic group of PTPs, and its inhibition has become a tool for cancer research [143-146]. To date, several compounds have been described as inhibitors to these molecules, such as thienopyridone [218] and analog 3 [219]. Nevertheless, given the importance of PRL member trimerization, disruption of trimer formation has presented itself as an interesting approach to escape the specificity issues found when developing these compounds [220-222]. In this sense, Cmpd-43 was described as a binder of the PRL1 trimer interface, which blocks PRL1 trimerization, and, therefore, suppresses PRL1-mediated cell proliferation and migration through the attenuation of ERK1/2 and Akt activation [223]. This compound was not only efficient *in vitro* but also in xenograft melanoma models, suggesting that trimerization is essential to PRL activation and might be an interesting therapeutic approach for tumors overexpressing these PTPs [223].

DUSP1 is another PTP found to be overexpressed in various solid tumors [136, 191, 194, 224, 225]. Since then, it has become another plausible target for inhibitor development. As well as PTP1B and SHP2, DUSP1 inhibitor development has also faced specificity problems [226]. In 2009, Molina and colleagues identified a small molecule that blocked Dusp6 activity in zebrafish embryos [227]. The same group showed that the compound, named BCI, also exhibited activity against human DUSP6, and, surprisingly, it also showed efficacy to inhibit human DUSP1[227]. They suggested that the response in both MKPs is due to their overlapping substrate specificity, while DUSP6 is specific for ERK, DUSP1 dephosphorylates ERK, JKN and p38 [226]. Recent studies have suggested that DUSP6 inhibition by BCI increases the sensitivity to cisplatin in gastric cancer cells *in vitro*, as well as in cell-based xenografts and patient-derived xenografts *in vivo* [195]. Additionally, studies have shown that BCI can induce apoptosis and suppress the proliferation of lung cancer cell lines *in vitro* [228].

Korotchenko and colleagues identified a BCI analog, named BCI-215, which showed no toxicity to zebrafish embryos or to an endothelial cell line, although it maintained the capacity to inhibit both MKPs [229]. Interestingly, BCI-215 markedly reduced the survival and motility of a human breast cancer cell line, but not of normal hepatocytes, showing the selective tumor cell cytotoxicity that researchers restlessly seek [230]. Therefore, these compounds have shown promising alternatives for DUSPs targeting cancers that overexpress these proteins.

Table II. Features of cancer related protein tyrosine phosphatase inhibitors

Inhibitor	Target	Type	Positive features	Negative features	Reference
Ertiprotafib	PTP1B	-	Potent and selective PTP1B inhibitor	Unsatisfactory Phase II clinical outcome; dose-limiting side effects.	[231, 232]
"Compound II"	PTP1B	Reversible bidentate inhibitor	High efficacy in inhibiting PTP1B	No effect was seen in tumor cells due to poor cellular uptake.	[199]
"Prodrug 1b"	PTP1B	Difluoromethyl phosphonate prodrug	Excellent solution to the drug delivery.	-	[207]
Trodusquemine (MSI-1436)	PTP1B	Reversible noncompetitive allosteric inhibitor	Potent and selective PTP1B inhibitor; Phase I clinical trial for breast cancer and Phase II for type II diabetes.	-	[209, 233]
scFv45	PTP1B	Allosteric oxidation-stabilizing inhibitor	Selectivity for PTP1B-OX over oxidized PTPN1 and PTPN2; potential to be applied in other PTPs affected by reversible oxidation.	-	[210, 234]
Compound 11a-1	SHP2	Reversible bidentate noncompetitive inhibitor	More than 5-fold preference for SHP2 over the other 20 PTPs and a 7-fold preference for SHP2 over SHP1; tumor growth suppression in xenograft melanoma models	-	[213, 214]
SHP099	SHP2	Allosteric inhibitor	Specific to SHP2: no detectable activity against a	-	[217]

			panel of 21 phosphatases including SHP1; Efficacy in cell-based assays, oral bioavailability and tumor growth suppression.		
Cmpd-43	PRL	Allosteric oligomerization inhibitor	Tumor growth suppression in melanoma xenograft models.	-	[223]
BCI	DUSP6/DUSP1	Allosteric inhibitor	Inhibits DUSP6 and DUSP1, but not DUSP5; induced cell death in patient-derived pre-B ALL cells; increases sensitivity to cisplatin in gastric cancer cells *in vitro*; induces apoptosis and suppresses proliferation of lung cancer cell *in vitro*.	Not specific for DUSP6; high toxicity.	[195, 227, 228, 235]
BCI-215	DUSP6/DUSP1	Allosteric inhibitor	Similar potency and reduced toxicity compared to BCI; selective tumor cell cytotoxicity.	-	[230]

6 Future Directions on Phosphatase Inhibitors in PDAC Therapy

PTPs are genetically altered in various human cancers, providing compelling evidence that they also play critical roles in tumorigenesis. It is interesting that some of

the PTPs are mutated in a tissue-specific manner, exhibiting growth suppressor or oncogenic characteristics.

Advances in phosphatase investigation in the cancer field have already been made, demonstrating that phosphatases are critical growth-regulatory molecules that are potentially "druggable". As reviewed here, the inhibition of oncogenic phosphatases can suppress the growth of human cancers. However, the most important issue for future studies includes the identification of the crucial signaling mechanisms that are perturbed by PTP inactivation and activation, and the associated signaling pathways will provide an essential understanding of the oncogenic process. Underlying those biological processes will be important for the development of more effective PTP inhibitors for use in cancer diagnostics and therapies because several studies have demonstrated that oncogenic PTPs are attractive candidates for the development of targeted therapy.

It will also be a challenge for the pharmaceutical communities to discover effective and safe methods to target these cancer-promoting molecules and deliver the targeting drugs. Once all these challenges are overcome, a whole new class of drugs for cancer treatment will become available to treat many life-threatening cancers. It is a matter of time before this gene family moves from a hopeful vision in oncology management to a target for new sets of drugs and therapies.

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CHAPTER II

DUSP6 modulates metastatic phenotype of pancreatic cancer cells

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DUSP6 modulates metastatic phenotype of pancreatic cancer cells

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive tumor and is majorly caused by the constitutive activation of mutant KRAS – found in approximately 90% of PDAC cases. The undruggability of genetic KRAS mutations has led to efforts of finding new therapeutic targets that focus on downstream molecules in this pathway. DUSP6 is a dual-specificity phosphatase that regulates ERK1/2 phosphorylation and, therefore, RAS pathway activation. *In silico* analysis in different datasets revealed that *DUSP6* is overexpressed in primary tumor samples compared to non-tumoral pancreatic tissue. Also, DUSP6 overexpression is confined to the tumor cells/epithelial compartment, shown by *in silico* and confirmed by RNA-ISH analysis. Further *in silico* analysis revealed that *DUSP6* is overexpressed in metastatic samples compared to correlated to primary tumor samples and that its overexpression correlates with the quasi-mesenchymal/squamous molecular subtype. Overall survival analysis indicated that patients with high *DUSP6* expression have a worse prognosis than patients with low *DUSP6* expression, reaffirming its clinical relevance. To investigate DUSP6 role in metastasis development and progression we developed DUSP6 stable knockdown in PDAC cells lines and took advantage of BCI – a pharmacological inhibitor. We performed cell proliferation, migration and invasion assays *in vitro*. Surprisingly, we observed different phenotypes among the cell lines used, which we believe is derived from the different genetic backgrounds involved. Overall, results indicate that DUSP6 play a role in the metastatic process in PDAC, modifying phenotypes that are closely related to the cells capacity to survive and thrive in an unfamiliar environment. Nevertheless, the mechanism behind these changes remains to be further investigated.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer representing more than 90% of the cases [1, 2]. PDAC is one of the most lethal invasive cancers and accounts for the seventh leading cause of cancer-related deaths around the world [3]. Although overall survival has greatly increased in the last decades, the current rate of 9% is still abysmal [3, 4]. PDAC is usually detected in late stages of tumorigenesis, mainly due to unspecific symptoms, lack of early diagnosis markers and refractoriness to chemotherapy [5]. Currently, the only curative option for this tumor is complete resection, which is unavailable in locally advanced and metastatic cases – found in 80 to 90% of patients at diagnosis [6, 7].

PDAC metastasis are usually found in the liver, peritoneum and the lungs [8-12] and although largely studied it is a process not yet fully understood. Studies have demonstrated that not all the circulating tumor cells successfully colonize new environments. Therefore, this leads to the hypothesis that cells need to gain some kind of advantage that allows them to form secondary tumors – most likely genetic and epigenetic changes [13]. Many mutations such as KRAS are commonly found in both primary tumors and metastatic cases [10, 12], suggesting that they are not required for metastasis establishment in novel sites. Nevertheless, some alterations are found specifically in metastatic cells. For instance, Wilentz and colleagues reported that *DPC4* (*SMAD4*) loss' frequency is higher in metastatic cases compared to early stages of tumorigenesis and is closely related to tumor cells' invasiveness [14]. Gene expression alterations can also be accessed in this context, nevertheless, most of the studies have been focusing in addressing changes between non-neoplastic pancreatic tissue and tumor tissue. Using *in silico* analysis and public available datasets, our group has identified the dual-specificity phosphatase 6 (*DUSP6*) as a differentially expressed gene between primary and

metastatic PDAC tumor samples. *DUSP6* was found to be upregulated in PDAC in metastatic samples compared to both primary tumor samples and normal pancreatic tissue.

DUSP6 is a dual-specificity phosphatase known to specifically bind and dephosphorylate ERK1/2 and, therefore, downregulate ERK/MAPK pathway [15, 16]. This phosphatase is described in literature in a variety of tumors, playing both oncogenic and tumor suppressing role; therefore, showing to act in a context-dependent manner [16]. In PDAC, DUSP6 was previously reported by Furukawa and colleagues to be upregulated in early stages of tumorigenesis, such as in PanIN lesions, and downregulated in the invasive carcinoma, particularly in the poorly differentiated subtype [17]. This phenomenon is reported to occur due to hypermethylation of *DUSP6*'s promoter, leading to abrogation of gene expression and increased activity of the ERK/MAPK pathway [18].

These observations raise a question regarding DUSP6's role in PDAC: if this phosphatase acts as a tumor suppressor molecule, why is DUSP6 upregulated in metastatic samples? This work attempts to elucidate the role of DUSP6 in PDAC's metastatic process. Using BCI – a pharmacological inhibitor for DUSP6 [19] – and gene silencing, we show an increase in ERK/MAPK activation, with consequences in cell proliferation, migration and invasion capacity; nevertheless, different phenotypes were observed in different genetic backgrounds. Our results suggest that DUSP6 plays a role in metastasis development and establishment, but the mechanism behind this modulation needs further investigation.

MATERIALS & METHODS

Cell lines and culture

MIA PaCa-2, PANC-1, AsPC-1, BxPC-3, Capan-2 and SW1990 were purchased from ATCC (Manassas, VA, USA). MIA PaCa-2 and PANC-1 are primary human tumor-derived PDAC cell lines expressing mutant KRAS, mutant p53, and deletion of CDKN2A. AsPC-1 and SW1990 are metastatic human PDAC cell lines expressing mutant KRAS, mutant p53, and deletion of CDKN2A. Capan-2 is a human primary tumor-derived cell line containing a $KRAS^{G12V}$ mutation. BxPC-3 is a human primary tumor-derived cell line expressing mutant p53 and deletion of CDNK2A. K8484 and DT8082 cells isolated from the KPC (and LSL-$Kras^{G12D/+}$; $Tp53^{R172H/+}$; Pdx1-Cre) mouse were acquired from David Tuveson's Lab (Cold Spring Harbor Laboratory) [20]. P4313 were isolated from KC (LSL-$Kras^{G12D/+}$; Pdx1-Cre) and acquired from Andy Lowy (UC San Diego) [21]. PKT62, KPC and PKT CAFs cells lines were isolated at the VanSaun Lab and validated with western blot (Figure S1). Capan-2, AsPC-1 and SW1990 were cultured in high glucose (4.5 g/L) RPMI-1640 medium (ThermoFisher Scientific, Waltham, MA USA cat# A1049101) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals/RD Systems, Atlanta, GA USA) and antibiotic-antimycotic (Thermo Fisher Scientific cat# 15240062). All the other K8484 cell line was cultured in high glucose (4.5 g/L) Dulbecco's Modified Eagle Media (ThermoFisher Scientific cat# 11-995-073) supplemented with 5% heat-inactivated FBS (R&D Systems-Atlanta Biologicals) and antibiotic-antimycotic (ThermoFisher Scientific). All the other cell lines were maintained in high glucose DMEM, 10% FBS, and antibiotic-antimycotic. Cells were kept in culture conditions (37°C, 5% CO_2 and humid atmosphere) as recommended by ATCC.

Plasmids and gene knockdown

To generate DUSP6 knockdown cells, short hairpin RNA (shRNA) constructs targeting three independent regions of DUSP6 mRNA (#1 5'-CTGTGGTGTCTTGGTACATTG-3'; #2 5'-TCTAATCCAAAGGGTATATTT-3', #3 5'-ATTCGGCATCAAGTACATCTT-3'), were obtained from Millipore Sigma (Burlington, MA, USA). Empty vector pLKO.1 puro (Addgene) was used as a scrambled control. Packaged lentiviruses were generated by transfecting HEK293T cells with respective plasmids and the Trans-Lentiviral™ Packaging Mix (Open Biosystems) to produce viral supernatants which were subsequently used to transduce pancreatic cancer cell lines. Successfully transfected cells were selected with Puromycin treatment (1-2,5µg/mL) until complete cell death was achieved in the non-transduced cells.

RNA isolation and RT-qPCR

Briefly, 10^6 cells were seeded in 6-well plates and left overnight in incubator. In the next day, cells were washed with 1X PBS and harvested in QIAzol Lysis Reagent (Qiagen, GmbH, cat #79306). Samples were kept in -80°C until total RNA was isolated using the Direct-zol RNA Miniprep kit (Zymo Research Corporation) according to manufacturer's protocols. RNA quantification and quality analysis were performed using NanoDrop 2000 (Thermo Fisher Scientific Inc.). For cDNA, 1.5-2µg of RNA were used with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, cat #4368814) following manufacturer's instructions and further diluted 1:10 before storage in -20°C. Real-time PCR analysis was carried out with the 2X SYBR Green qPCR Master Mix (ApexBio Technology, cat #K1070) on a ViiA 7 Real-Time PCR System (Applied Biosystems) using the following primers: human DUSP6 (Fwd: ATGGTAGTCCGCTGTCCAAC / Rev: ACGTCCAAGTTGGTGGAGTC); mouse Dusp6 (Fwd: TGTTTGAGAATGCGGGCGAGTT / Rev: ACAGTTTTTGCCTCGGGCTTCA); human

ACTB (Fwd: TCGTGATGGACTCCGGTGAC / Rev: CGTGGTGGTGAAGCTGTAG); mouse Actb (Fwd: GTGACGTTGACATCCGTAAAGA / Rev: GCCGGACTCATCGTACTCC). Each sample was run in triplicate using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) and fold-change was evaluated relative to normal samples and determined using *ACTB* levels as a reference.

Drugs and treatments

(E/Z)-BCI hydrochloride (Sigma-Aldrich, USA, cat #B4313) was primarily dissolved in DMSO in a final concentration of 5mM. Aliquots were maintained at 4°C. For treatments, the drug was dissolved in media, never exceeding 0.5% of DMSO. Human recombinant TGF-α (Peprotech Inc., USA, cat #100-16A) was primarily reconstituted in sterile water (1mg/mL), then diluted in 1X PBS with 0.1% BSA to a final concentration of 50µg/mL. Aliquots were kept at -20°C.

Western blotting

Briefly, 10^6 cells were plated in 6-well plates, placed in the incubator overnight and collected the next day for protein isolation. Cells were washed with 1X PBS, lysed in ice-cold RIPA buffer (1X RIPA; 10mM NaF; 1mM PMSF), sonicated and incubated on ice for 10 minutes. Then, cells were centrifuged at 16,000G for 10 minutes at 4°C. Protein quantification was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific) following manufacturer's instructions. For western blotting assays, 10µg of total proteins were loaded in each lane of 10% polyacrylamide gels and submitted to electrophoresis. Proteins were transferred to nitrocellulose blots using the semi-dry method in a Trans-Blot Turbo Transfer System (Bio-Rad). Specific proteins were detected using the following primary antibodies: P-ERK1/2 (p44/42 MAPK T202/Y204, cat # 4370), ERK1/2 (Total p44/42 MAPK, cat #4695), E-cadherin (cat #3195), N-cadherin (cat #1316), β-actin (cat #12262) purchased at Cell Signaling; and MKP-3 (Cat #sc-137246), from Santa Cruz Biotechologies. Secondary

antibodies were Peroxidase AffiniPure F(ab')$_2$ Fragment Donkey Anti-Mouse IgG (H+L) and Donkey Anti-Rabbit IgG (H+L) from Jackson ImmunoResearch Laboratories Inc. (cat. #715-036-151 and #711-036-152, respectively). Images were obtained using a FluorChem M (Bio-Techne) and analyzed with ImageJ (NIH, Bethesda, MD).

RNA *in situ* hybridization

RNA in situ hybridization was performed using the RNAscope® technology (Advanced Cell Diagnostics, Inc). Briefly, tumors were collected from mice, sectioned and immediately fixed in 10% buffered formalin for 24 hours. Then, tumor sections were preserved in 70% ethanol, embedded in paraffin blocks, cut in 5µm sections and placed in pre-treated slides. Sections were freshly cut for the assay and dried in room temperature to preserve RNA integrity. Sections were baked for 1 hour at 60°C, then deparaffinized using Histo-clear (Electron Microscopy Science; cat. #6411004) and 100% ethanol. Next, sections were treated with RNAscope® Hydrogen Peroxide for 10 min in room temperature, followed by target retrieval step with RNAscope® 1X Target Retrieval Reagent for 15 minutes and, finally, RNAscope® Protease Plus treatment for 30 minutes at 40°C. Finally, we proceeded to the RNAscope® 2.5 HD Duplex Reagent Kit, following manufecterer's protocol. Specific probes utilized in this assay were: Mm-Dusp6 (cat. #429321), Mm-Krt19-C2 (cat. #402941-C2) and Mm-Pdgfrb-C2 (cat. #411381-C2). Sections were imaged under 20x magnification using a EVOS™ M5000 Imaging System (Thermo Fisher Scientific).

Proliferation assay

Cell proliferation was accessed using ethynyldeoxyuridine (EdU) incorporation method. Briefly, 2.5-5x10^4 cells were seeded in 24-well plates in complete media and incubated overnight. In the next day, media was replaced by the appropriate treatments and incubated for 18 hours. At this point, EdU was added to the wells in a final concentration of 10mM and cells were incubated again for 6 hours. At the end of this timepoint, cells were

trypsinized, harvested and fixed with 10% buffered formalin overnight at 4°C with gentle agitation. In the next day, cells were washed with PEB-T (1x PBS, 2mM EDTA, 1% BSA, 0.5% Triton-X) and subsequently with PB (1x PBS, 1% BSA). Next, cells were ressuspended in the Click-It Reaction (H_2O, 100mM Tris pH 8.5, 1mM $CuSO_4$, 1µM azide-dye) and 100mM ascorbic acid. Then, cells were incubated with 0.5µg/mL propidium iodide (PI), washed twice and ressuspended in PEB (1x PBS, 2mM EDTA, 1% BSA) for flow cytometry analysis. Cells were analyzed for 530 nm and 695 nm emission in an Attune NxT Flow Cytometer (Thermo Fisher Scientific). FCS files were analyzed with FlowJo™ v10.8 Software (BD Biosciences) and percentage of EdU-positive cells was taken from the PI-positive single cells.

Migration assay

First, 2-4x10^5 cells were seeded in 24-wells plates and cultured until full confluence. Then, a scratch was made at the center of the plate using a 200µL pipette tip. Wells were washed twice with 1X PBS to discard all the debris and media with appropriate treatments was added. Plates were photographed at 0 and 24 hours using a using a EVOS™ M5000 Imaging System (Thermo Fisher Scientific). Scratch area was calculated using the Wound Healing Coherency Tool (Montpellier Ressources Imagerie) on ImageJ (NIH, Bethesda, MD) and results are expressed as healing percentage – the difference in scratch area between time 0 and time 24.

Invasion assay

Inserts were placed in 24-wells plates, 100µL of Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Gibco; cat. #A1413202) was added on top of each membrane and plates were taken to the incubator for 1 hour for the matrix to solidify. Then, 0.5-1x10^5 cells were seeded in each insert in 500µL of serum-free media and 500µL of complete media with 10% FBS was added to the bottom of the 24-wells plate, for FBS to

serve as a chemoattractant. Plates were incubated for 24 hours at 37°C with 5% CO_2. At the end of the experiment, the cells that remained at the top part of the membrane, which represents non-migratory cells, were removed with a cotton swab. Cells that have moved through the pores and to the bottom of the membrane were fixed with formalin and then stained with Hoechst (1ug/ml) for 5 minutes. Finally, cells were washed with 1X PBS to remove extra dye and imaged under fluorescent microscope EVOS™ M5000 Imaging System (Thermo Fisher Scientific). Each membrane was photographed in 5 random fields and the sum of counted cells with the Cell Counter plug-in on ImageJ (NIH, Bethesda, MD) was considered the total for this replicate.

In silico analysis

DUSP6 expression analysis was performed using 4 independent datasets obtained from The Cancer Genome Atlas (TGCA_PAAD), The Genotype-Tissue Expression (GTEx, normal pancreatic tissue) and the Gene Expression Omnibus (GEO, GSE62452; GSE28735; GSE15471). Overall survival analysis was performed on Gene Expression Profiling Interactive Analysis (GEPIA) using UCSC Xena project (http://xena.ucsc.edu) datasets. Differential expression of DUSP6 in epithelial and stromal compartments was performed using the GSE93326 with the R2 Genomics Analysis and Visualization Platform. Finally, DUSP6 expression correlation to Metastatic Potential (MetMap 500) was performed at the DepMap Portal using the Cancer Cell Line Encyclopedia (CCLE). We specifically filtered the cell lines for Exocrine Adenocarcinoma, which correspond to PDAC cells lines.

Statistical analysis

Samples sizes were chosen to achieve a minimum of triplicates for all experiments. For assessment of statistical significance, ordinary one-way ANOVA with Šidák's multiple-comparison test or Welch's unpaired 2-way t-test was used when appropriate and indicated. All statistical tests were performed using GraphPad Prism 9 software and results were

considered significant if $\alpha = 0.05$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

RESULTS

DUSP6 expression is upregulated in the tumor cells but not in stromal cells

To assess *DUSP6* expression in PDAC samples we took advantage of 4 distinct *in silico* datasets available in public platforms (GEO, TCGA and GTEx). We observed that *DUSP6* is upregulated in primary tumor samples compared to non-tumoral pancreatic tissue in all the analyzed datasets (Figure 1A-D). Taken this into consideration, we decided to check for DUSP6 expression *in silico* in different tumor compartments. We took advantage of GSE93326 and assessed gene expression in tumor *versus* stromal cells. In this regard, we observed that *DUSP6* is upregulated in tumor cells compared to stromal cells ($P = 1.33x10^{-8}$; Figure 1E).

We validated this analysis by checking for DUSP6 protein levels in a panel of 6 human PDAC cell lines, 4 mice PDAC cells lines and 2 mice CAFs cell lines. In human cell lines we see a variation of DUSP6 levels that might be explained by the different genetic mutation backgrounds (Table S1). On the hand, mice cell lines show more consistent levels of the protein: DT8082 and K8484 show the highest DUSP6 levels, whilst P4313 presents intermediate level and PKT62 presents the lowest level (Figure 1F, Table S1).

Next, we sought to investigate *Dusp6* expression in mice tumor tissue by using the RNA-ISH technology, RNAscope. We checked for *Dusp6* mRNA levels in tumor sections from LSL-$Kras^{G12D/+}$; Pdx1-Cre (KC) and LSL-$Kras^{G12D/+}$; $Tp53^{R172H/+}$; Pdx1-Cre (KPC), combined with *Krt19* (tumor marker) and *Pdgfrb* (stromal marker). As we observe in Figure 1G, *Dusp6* (green dots) is strongly co-expressed with *Krt19* (red dots, upper images) but does not co-localize with *Pdgfrb* (red dots, lower images) in both genetic backgrounds (Figure 1G). In accordance with the protein levels evaluated by

western blot, it is clear that KC and KPC mice overexpress *Dusp6* in the malignant cells, but not in the stromal compartment.

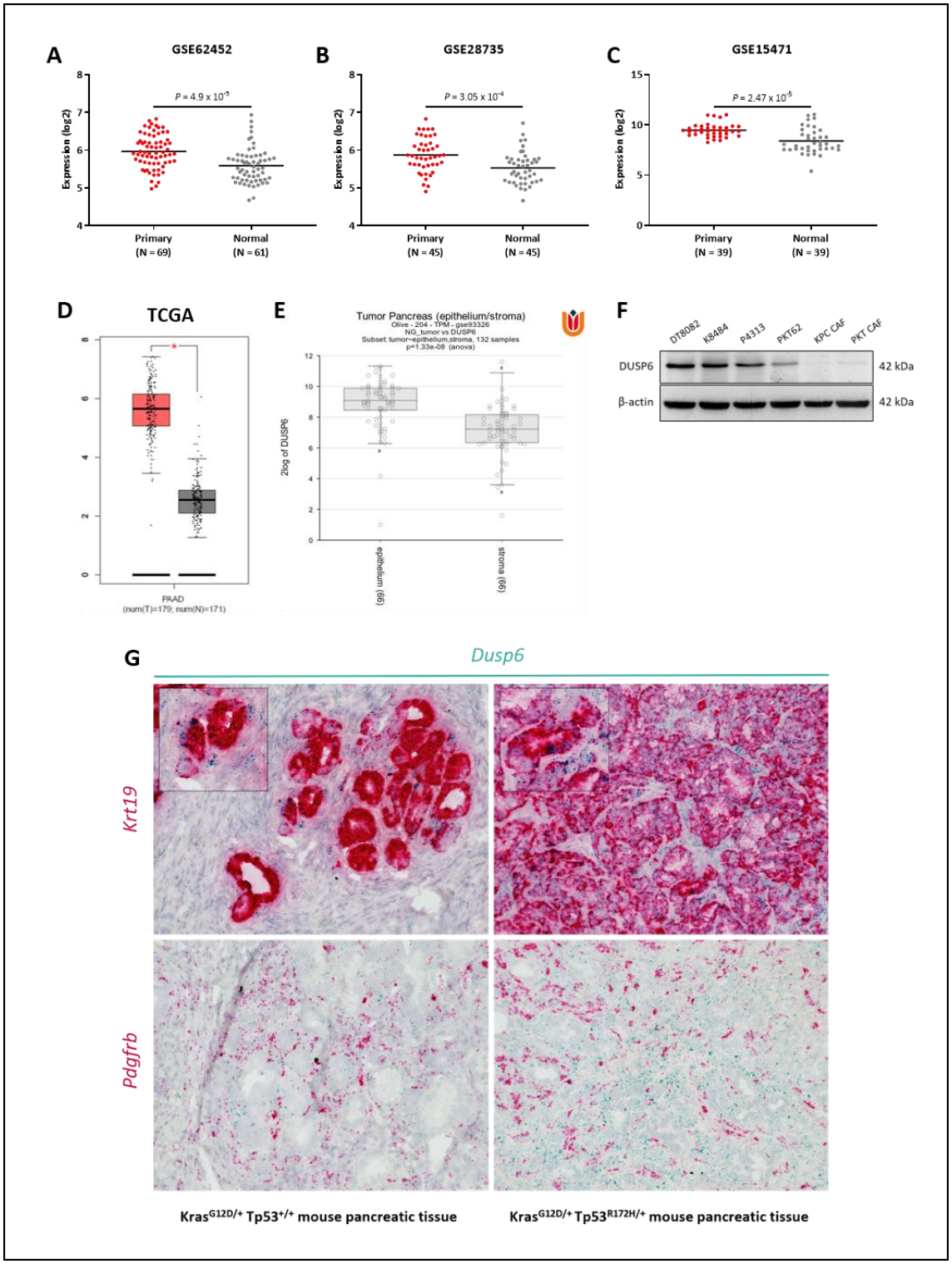

Figure 1 – *DUSP6* is overexpressed in tumor tissue compared to normal pancreatic tissue. *In silico* datasets GSE62452 (**A**), GSE28735 (**B**), GSE15471 (**C**) and TCGA (**D**), assessing DUSP6's differential expression between non-tumoral pancreatic tissue and primary tumor tissue ($P < 0.0001$). Analysis performed on GSE93326 (**E**) shows that DUSP6 is significantly

overexpressed in epithelial tissue (tumor) compared to stromal tissue ($P < 0.0001$). Results were confirmed by western blotting (**F**) comparing DUSP6 protein levels in mouse tumor cell lines and cancer associated fibroblasts (CAF) cell lines. Lastly, we assessed *Dusp6* expression in tumor sections derived from KC and KPC mice using the RNAscope technology and observed that *Dusp6* (green) was strongly overexpressed in tumorigenic lesions, and largely co-localized with *Krt19* (red, upper images) expression. *Dusp6* did not co-localize with *Pdgfrb* (red, lower images), a stroma/fibroblasts marker (**G**).

DUSP6 is overexpressed in metastatic tumor samples and correlates with patients' prognosis

Considering that DUSP6 is overexpressed in primary tumor samples, we argued about its expression in metastatic cases. To evaluate that, we took advantage of the *in silico* dataset GSE71729 and compared *DUSP6*'s expression in a cohort of 145 primary tumor samples and 61 metastatic samples derived from different secondary sites. We observed that *DUSP6* is overexpressed in metastatic samples compared to both primary tumor samples ($P = 9.87 \times 10^{-12}$) and non-tumoral pancreatic tissue ($P = 2.73 \times 10^{-15}$; N = 46; Figure 2A). Likewise, in an analysis performed on the TCGA dataset, we observed that high *DUSP6* expression in PDAC patients correlates with worse overall survival ($P = 0.039$; Figure 2B).

Although *DUSP6* overexpression in metastatic samples is counterintuitive, considering that it negatively regulates the ERK/MAPK pathway, these data are supported by further *in silico* analysis we performed utilizing Collisson's [22] and Bailey's [23] PDAC molecular subtyping. We took advantage of the datasets GSE15471 and FI335684 and analyzed *DUSP6* expression in the distinct PDAC subtypes. In GSE15471 dataset (Figure 2C), we observed that *DUSP6* is overexpressed in the quasi-mesenchymal subtype compared to non-tumoral pancreatic ($P < 0.0001$) tissue and exocrine-like subtype ($P < 0.05$). Likewise, in FI335684 dataset (Figure 2D), we observed *DUSP6* overexpression in the squamous subtype compared to the three other subtypes.

Both quasi-mesenchymal and squamous subtype share similar molecular signature and worse prognosis among the other PDAC subtypes, therefore, reinforce the idea that *DUSP6* might play an important role in advanced stages of the disease.

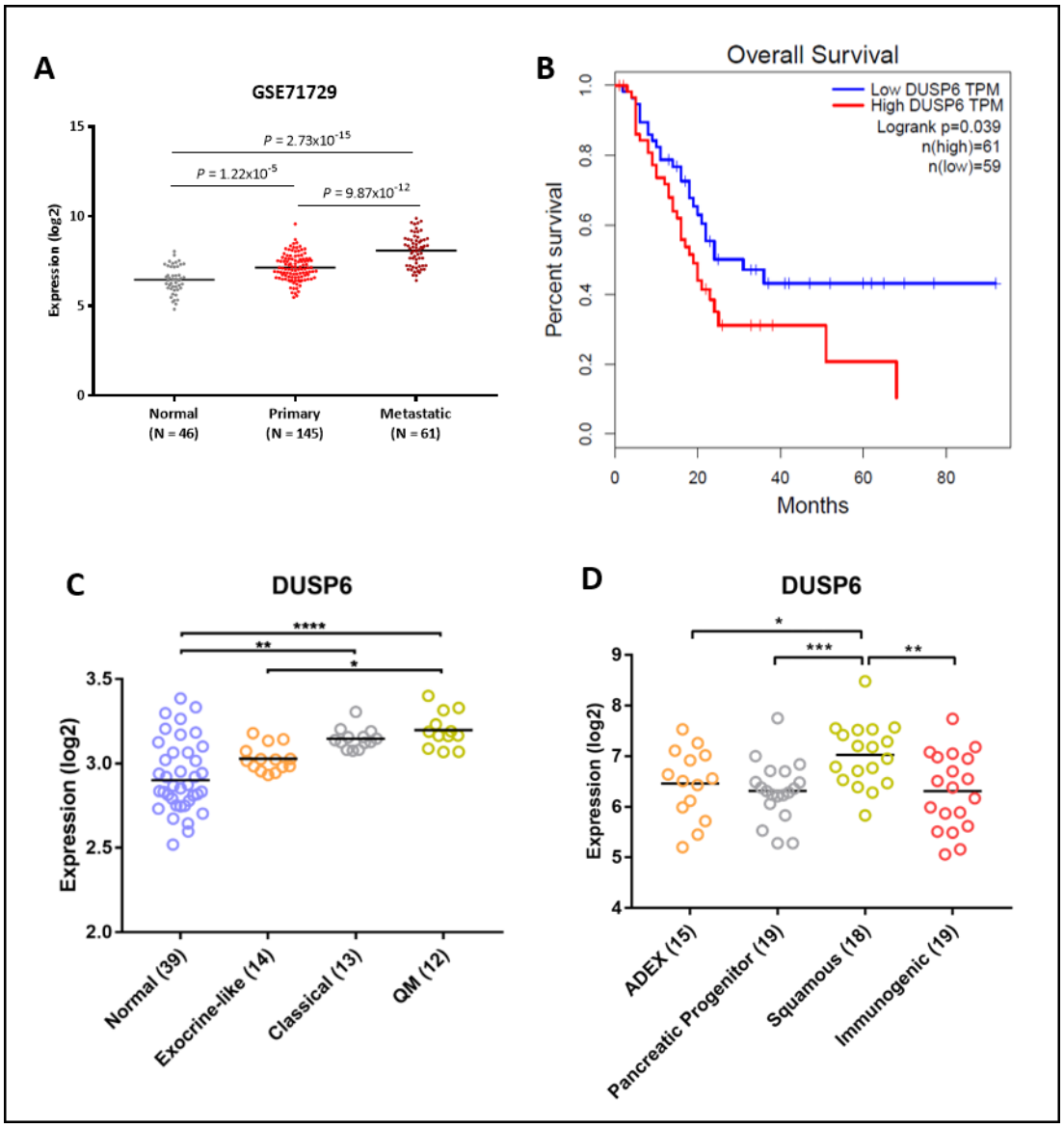

Figure 2 – *DUSP6* is overexpressed in metastatic tumor samples and correlates with patients' prognosis. *In silico* dataset GSE71729 (**A**) assessing DUSP6's differential expression between non-tumoral pancreatic tissue, primary and metastatic tumor tissue ($P < 0.0001$). **B)** Correlation analysis between DUSP6 expression and patients' overall survival using the TCGA dataset, in which high DUSP6 expression correlates with worse overall survival ($P = 0.039$). DUSP6 expression was assessed among tumor subtypes proposed by Collisson *et al.* (**C**; $P < 0.0001$) and Bailey *et al.* (**D**; $P < 0.001$) and we observe an overexpression in quasi-mesenchymal and squamous subtypes, which are also correlated molecularly. QM: quasi-mesenchymal. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

DUSP6 inhibition affects proliferation, migration and invasion in PDAC cell lines *in vitro*

Considering that DUSP6 expression seems to be required for PDAC metastasis, we decided to knockdown DUSP6 in 3 PDAC cell lines – AsPC-1, BxPC-3 and K8484 – that presented the highest levels of the protein among the cell lines tested in this study (Figure 1F – S2). To confirm DUSP6 knockdown, we performed gene expression and protein level analysis (Figure S3). Next, we analyzed cell proliferation upon DUSP6 knockdown by EdU incorporation in full serum and no-serum conditions, aiming to stimulate and repress proliferation, respectively. AsPC-1 demonstrated impairment in proliferation upon DUSP6 knockdown in both conditions, and the phenotype was severely aggravated under serum starvation ($P < 0.001$; Figure 3A). Similarly, in BxPC-3, we observe a comparable phenotype of proliferation impairment ($P < 0.0001$). Nonetheless, in this case, serum starvation does not seem to affect these cells as it affects AsPC-1 ($P < 0.001$; Figure 3B). K8484, on the opposite, are slightly affect by serum starvation, but do not respond to DUSP6 knockdown (Figure 3C).

Next, we sought to analyze the effects of DUSP6 knockdown on cell migratory capacity. Migration *in vitro* was evaluated within 24 hours after the scratch (time 0). In this context, cells presented completely different phenotypes. AsPC-1 showed a significant reduction of their migratory capacity with DUSP6 knockdown ($P < 0.0001$; Figure 3D), while BxPC-3 suffered no impact at all (Figure 3E). K8484, on the other hand, showed a significant increase in their migratory capacity with DUSP6 knockdown (Figure 3F).

We decided to inhibit DUSP6 using a pharmacological inhibitor, BCI, to confirm the migratory phenotype previously observed using gene knockdown. First, we performed western blot analysis to confirm ERK1/2 phosphorylation upon BCI treatment, since

DUSP6 is known to specifically dephosphorylate this MAPK. BCI successfully induce ERK1/2 activation in AsPC-1 and BxPC-3 (Figure S4), but not in K8484 (not shown). Then, we constructed a viability curve to define an optimal work concentration for the drug in these two cell lines (Figure S4). Treatment with BCI recapitulated DUSP6 knockdown in BxPC-3 and did not change migratory capacity in these cells. Surprisingly, upon BCI treatment, AsPC-1 showed an increase in migratory capacity ($P < 0.0001$), the opposite phenotype compared to DUSP6 knockdown in these cells (Figure S4). Nevertheless, it was previously reported that BCI can also inhibit the activity of DUSP1 due to similarity in the phosphatases' catalytic site [19]. Therefore, it cannot be disregarded that a possible off-target inhibition might be confounding the results we observe in this case.

Lastly, we assessed invasive capacity by analyzing the cells ability to penetrate a Matrigel layer in 24 hours, simulating the extracellular matrix. We observed that AsPC-1 had an impairment in their invasive capacity upon DUSP6 knockdown, in accordance to the migratory capacity reduction we previously observed. BxPC-3, on the opposite, showed an increased invasive capacity when lacking DUSP6, although no significant change was seen regarding migratory capacity. K8484 showed no significant differences in this context, despite of their increased migratory capacity previously observed (Figure 3G).

An *in silico* correlation analysis performed with 30 PDAC cell lines from the Cancer Cell Lines Encyclopedia (CCLE) and the Metastasis Map (MetMap 500) revealed a moderate positive correlation for *DUSP6* expression and the metastatic potential from these cell lines ($R = 0.611$; $P = 0.0003$; Figure 3H). Furthermore, this correlation analysis shows that *DUSP6* gene expression is the eighth strongest positive correlation for metastatic potential in this PDAC cell line cohort among a list of 1000 genes (Figure 3I).

Finally, we conducted a gene expression analysis using the CCLE in which we separated PDAC cells in two groups of cell lines derived from primary tumors and cell lines derived from metastatic sites. Then, we checked for *DUSP6* expression between the two groups and observed that cell lines derived from metastatic sites significantly overexpress *DUSP6* in comparison to cell lines derived from primary tumors ($P = 0.029$; Figure 3J), confirming our previous observations. Altogether, these results suggest that DUSP6 is involved with the metastatic process, but it might play different roles depending on the genetic background.

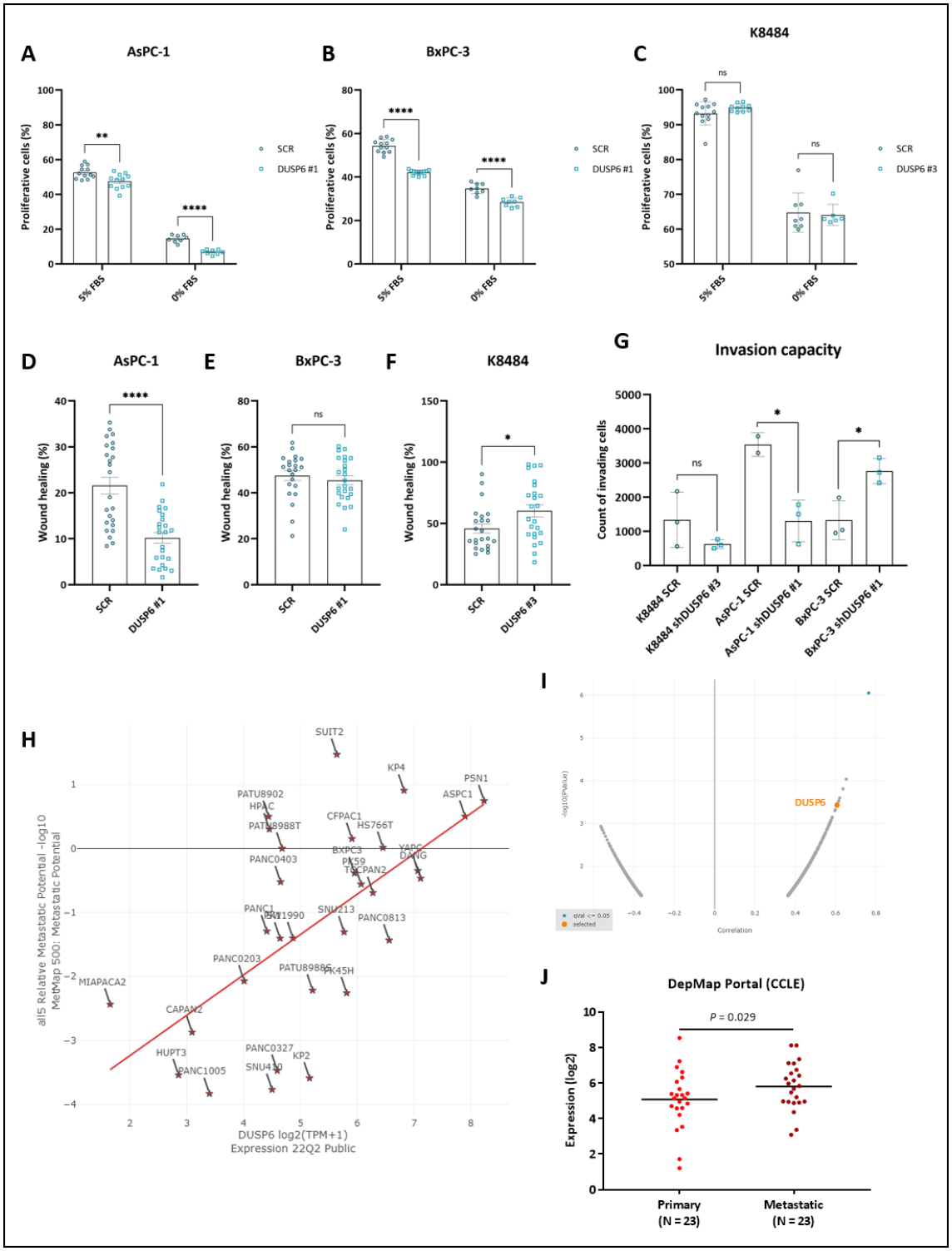

Figure 3 – DUSP6 inhibition affects proliferation, migration and invasion in PDAC cell lines *in vitro*. We evaluated proliferation capacity on PDAC cell lines upon DUSP6 knockdown and observed that AsPC-1 (**A**) and BxPC-3 (**B**) with DUSP6 knockdown show a significant decrease in proliferation in comparison to control, both in complete media and serum starvation conditions. K8484 (**C**), on the opposite, show no significant differences in proliferation. Regarding migratory capacity, we observe different behaviors among the cell lines: AsPC-1 (**D**) show a significant decrease while K8484 (**F**) show a significant increase in comparison to control. BxPC-3 (**E**), show

no significant differences in migratory capacity. **G)** Finally, invasive capacity was assessed and, similarly to the migratory opposing phenotype, AsPC-1 showed a decreased invasive potential, while BxPC-3 showed an increased invasive potential. K8484 did not reach statistical significance. **H)** Using the DepMap portal we assessed correlation between DUSP6 expression in PDAC cell lines from CCLE and their metastatic potential, observing a moderate correlation ($R = 0.611$; $P = 0.0003$). Nevertheless, in the same dataset, *DUSP6* showed to be the eighth highly positively correlated gene (**I**). Finally, DUSP6 was found to be overexpressed in metastatic PDAC cell lines in comparison to primary tumor cell lines in CCLE dataset (**J**). Proliferation and migration assays: Graphs show 3 independent experiments with 4-6 replicates each. Results represent mean ± SEM; Invasion assay: Graph shows 1 independent experiments with 3 replicates. Results represent mean ± SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

DISCUSSION

The phosphatase DUSP6 has already been described for being differentially expressed during the phases of PDAC tumorigenesis [17]. Nevertheless, little is known about this phosphatase's role in metastasis development and establishment. Our group observed that *DUSP6* is overexpressed in primary tumor samples when compared to non-tumoral pancreatic tissue in *in silico* datasets. Considering that DUSP6 is a negative regulator for the ERK/MAPK signaling pathway, it is not expected that this phosphatase's overexpression would be correlated to tumor development. Aiming to unravel this counterintuitive overexpression, we performed gene knockdown with specific short hairpins in PDAC cell lines and then performed phenotypical analysis for proliferation, migration and invasion.

First, we wanted to understand if *DUSP6* overexpression was associated to the tumor or stromal cells. *In silico* analysis showed that DUSP6 is overexpressed in primary tumor samples compared to non-tumoral pancreatic tissue and that this expression in confined to the epithelial compartment of the tumor, rather than the stromal compartment. RNA *in situ* hybridization shows that *Dusp6* overexpression in KC and KPC mice co-localizes with *Krt19* expression, but not with *Pdgfrb* expression. Kidger and colleagues recently showed similar RNA *in situ* hybridization results using KC mice, in which is clear that *DUSP6* expression is found in ductal cells in PanINs, but not in stromal cells [24]. These results confirm that PDAC tumor cells overexpress *DUSP6*, suggesting that this phosphatase might have an important role in tumor development.

Next, we evaluated DUSP6 expression in late-stage PDAC samples *in silico*. We observed that *DUSP6* is overexpressed in metastatic samples compared to primary tumor samples. Besides, high DUSP6 expression correlates with lower overall survival from PDAC patients in the TCGA cohort. Finally, when we analyzed *DUSP6* expression

among different PDAC molecular subtypes [22, 23], *DUSP6* appears overexpressed exactly in quasi-mesenchymal/squamous subtype, which are described to be associated with worse prognosis [25]. These data combined support the hypothesis that *DUSP6* is overexpressed in cells that confer a more aggressive phenotype to the tumor, impacting aspects such as metastasis development and worse overall survival in PDAC.

Many previous studies in different tumors have already linked high *DUSP6* expression to worse prognosis and metastasis [26-28]. Nevertheless, in PDAC, it has largely been described as a tumor suppressor gene, since Furukawa and colleagues reported downregulation of *DUSP6* via hypermethylation of its promoter over PDAC tumor progression [17, 18]. Kidger and colleagues also proposed a tumor suppressor role for DUSP6 since they observed that KCD6$^{-/-}$ animals developed a higher number of poorly differentiated tumors and liver metastasis [24]. Almost 20 years ago, when Furukawa and colleagues first published about DUSP6 in PDAC, the study was developed using a relatively small cohort of patients who derived from the same healthcare institution and, back then, little was known about molecular subtyping or the many differences that can be found among patients who apparently share the same disease. In Kidger and colleagues work, although the models used for the study all share the same genetic background, they have a Dusp6$^{-/-}$ from the beginning of tumorigenesis, which does not mimic the phenotype that was initially proposed for this phosphatase. In their model, Dusp6 is constitutively absent, which leads to a permanent state of ERK1/2 activation. In this sense, it is expected that mice will develop more aggressive tumors and more metastatic focuses. What we currently argue is if DUSP6 might actually be downregulated at some point in primary tumors – or at least in a subset of PDAC tumors –, but its expression is required for metastatic cells to re-establish in a new environment.

To answer this question, we subjected PDAC cell lines to DUSP6 stable knockdown and evaluated cell proliferation, migration and invasion capacity. Surprisingly, we did not find a consensus in the phenotypes we observed. Regarding proliferation, all the significant changes observed were towards an impairment in this capacity. On the contrary, migration and invasion phenotypes were completely diverse among the cells lines, confusing a possible conclusion on the topic. Primarily, we can argue that the knockdown levels in K8484 are substantially lower than in AsPC-1 and BxPC-3, which can possible have led to less expressive phenotypes in this cell line. We hypothesize that this might be due to innate or inherited cell resistance to the selection antibiotic, or to a natural compensation of expression due to dependence from this signaling pathway. Considering BxPC-3, we point out that this cell line does not harbor a KRAS mutation and, therefore, its tumorigenic behavior does not rely on this condition. Therefore, the phenotype we observe is this cell line might be happening through different mechanisms than we primarily hypothesized. Lastly, AsPC-1, a metastatic PDAC cell line, which harbors a KRAS mutation, mimics more closely the genetic background we sought to investigate. What we observed with this cell line was a significant impairment of migration and invasion capacity upon DUSP6 knockdown and increased ERK1/2 activation. Unfortunately, we were not able to recapitulate this phenotype by treating the cells with BCI, but it is worthy restate that BCI is not specific for DUSP6 and acts in DUSP1 too. DUSP1 is a phosphatase that dephosphorylates SAPK/JNK and p38 MAPK, but also acts on ERK1/2 [29]. Both SAPK/JNK and p38 MAPK orchestrate a series of events that are extremely relevant for tumorigenesis [30-32] and, therefore, a possible DUSP1 inhibition as an off-target effect can confound the results creating misleading conclusions. Ideally, for further investigation, AsPC-1 should be injected in nude mice to

finally observe *in vivo* how DUSP6 knockdown can affect metastasis establishment in this context.

CONCLUSIONS

In summary, the results suggest that DUSP6 plays an important role in the metastatic capacity of PDAC cells, since its knockdown clearly has an effect in cell migration and invasion capacity. Nevertheless, this role is context-dependent and DUSP6 might act as a tumor suppressor or an oncogene protein according to the cells' mutational background and behavior.

Further investigation on the mechanisms behind this phenotype is strongly encouraged with transcriptional and phosphoproteomics profiling to better understand how this phosphatase can control such important events in PDAC tumorigenesis.

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AUTHOR'S CONTRIBUTIONS

MTR: methodology, data collection, formal analysis, visualization, paper writing, and editing; RMW, BBB, VSS & AEE: data collection, review, and editing RMW: data collection and data analysis; MNV: supervision, methodology, data analysis, paper

writing, editing; VSS: funding acquisition, project administration, conceptualization, supervision, methodology, data analysis, paper writing, editing.

COMPLIANCE WITH ETHICAL STANDARDS

This study does not require ethical approval.

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SUPPLEMENTARY INFORMATION

Table S1 – Genetic mutational background of the PDAC cell lines used in this study.

Cell line	KRAS	p53	Species
MIA PaCa-2	G12C	MUT	Human
PANC-1	G12D	MUT	Human
AsPC-1	G12D	MUT	Human
BXPC-3	WT	MUT	Human
Capan-2	G12V	MUT	Human
SW1990	G12D	WT	Human
DT8082	G12D	MUT	Mouse
K8484	G12D	MUT	Mouse
P4313	G12D	WT	Mouse
PKT62	G12D	WT	Mouse

WT = wild type; MUT = mutant

Figure S1 – Validation of PKT62, KPC and PKT CAFs cell lines established at the VanSaun Lab.

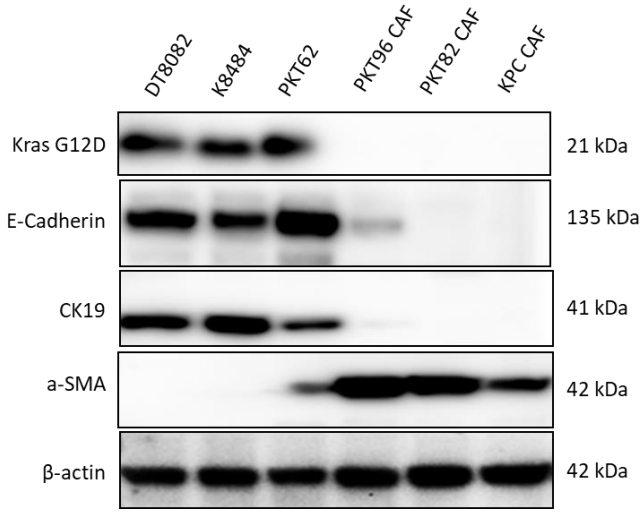


Figure S2 – DUSP6 protein levels in a panel of human PDAC cell lines.

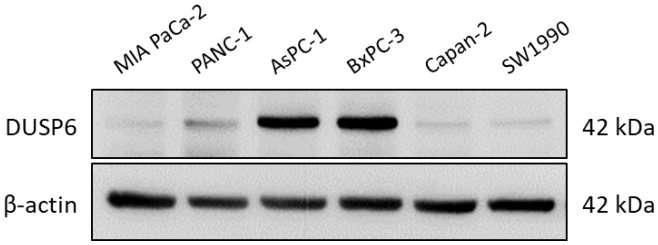


Figure S3 – Validation of DUSP6 knockdown in PDAC cell lines.

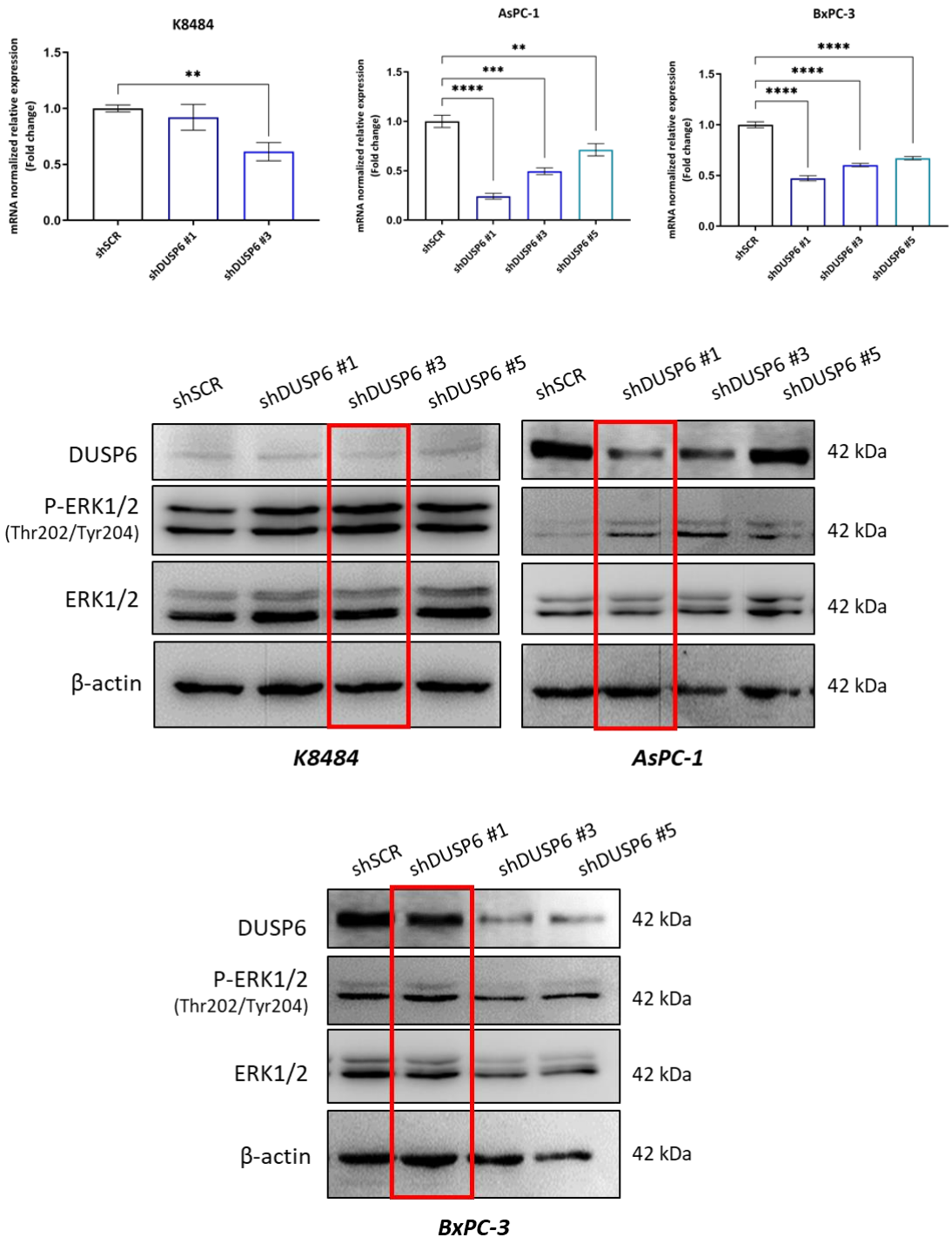
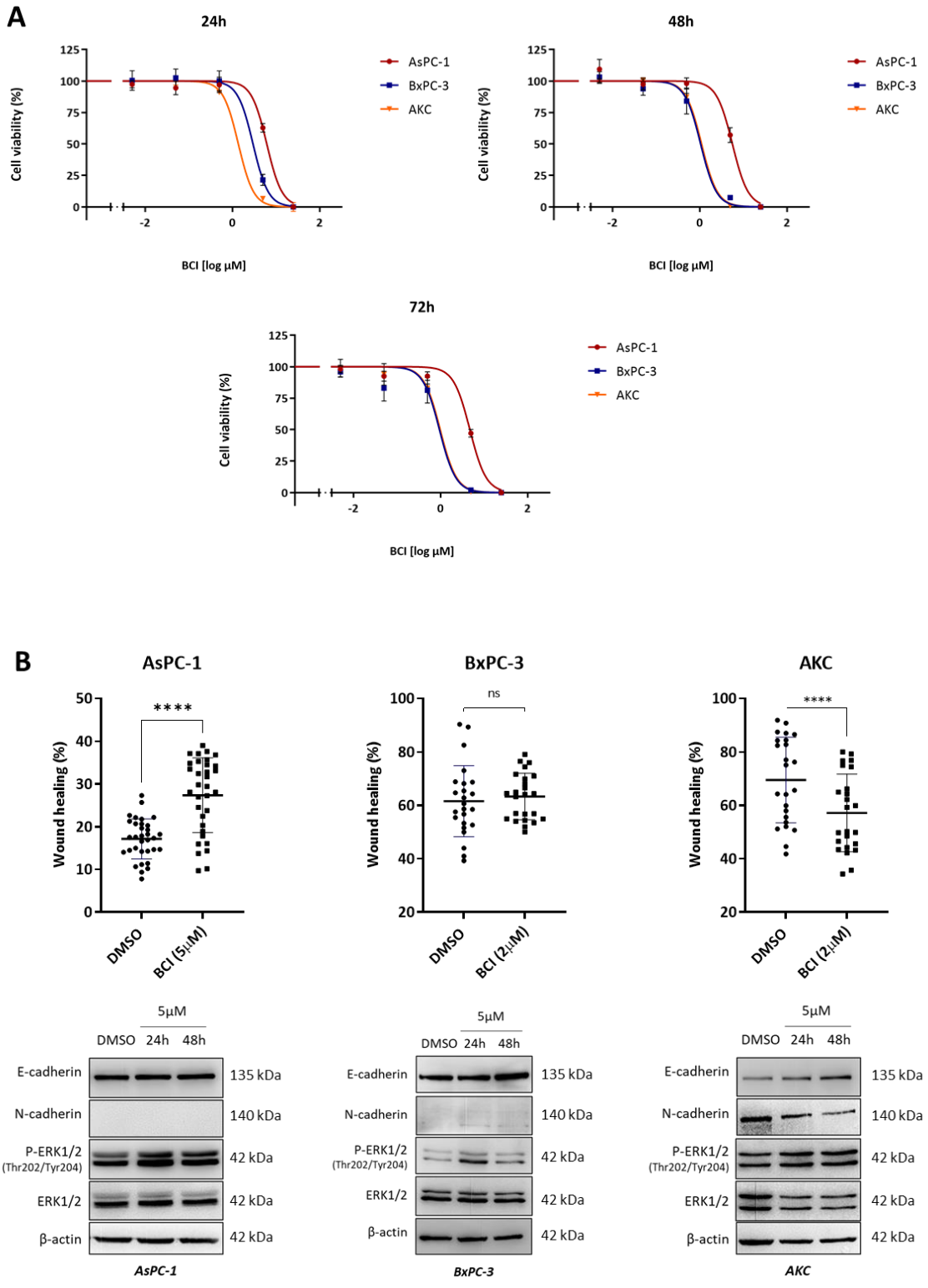


Figure S4 – BCI treatment for DUSP6 pharmacological inhibition in PDAC cell lines. **A)** MTT assay for definition of BCI's IC50 in PDAC cell lines. **B)** BCI's effect on migratory capacity in PDAC cell lines.



CHAPTER III

DUSP6 modulates glycolysis in pancreatic cancer cell lines

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DUSP6 modulates glycolysis in pancreatic cancer cell lines

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ABSTRACT

Pancreatic ductal adenocarcinoma is a highly lethal solid tumor that is, in the vast majority of cases, initiated by an oncogenic mutation in KRAS. These mutations, among other events, are recognized for acting as a non-canonical pathway in metabolism reprogramming, inducing the cells to overexpress glucose receptors and to utilize this fuel in glycolysis, in detriment of oxidative phosphorylation. DUSP6 is a phosphatase known to specific bind and dephosphorylate ERK1/2, being previously described in the literature as a tumor-suppressive molecule in PDAC. Nevertheless, *in silico* analysis indicate that DUSP6 is overexpressed in PDAC tumor samples compared to normal pancreatic tissue. More precisely, we observed that *DUSP6* is overexpressed in the quasi-mesenchymal/stromal subtype, which was previously described to be correlated with the glycolytic phenotype and worst prognosis among all the other described PDAC subtypes. Considering the aforementioned, we hypothesized that DUSP6 could play a role in metabolism reprogramming in PDAC and, therefore, induce a more aggressive phenotype, leading to metastasis development. To confirm this, we took advantage of *in silico* PDAC datasets and performed a gene set enrichment analysis, which pointed to a correlation between *DUSP6* expression and glycolysis. A gene set enrichment analysis with the differentially expresses genes pointed to a regulation of the tricarboxylic acid (TCA) cycle. Next, we evaluated the glycolytic rates of PDAC cell lines with DUSP6 knockdown and observed that lack of DUSP6 induces glycolysis utilization upon oxidative phosphorylation inhibition. Then, we performed proliferation and migratory capacity assays in the same cells upon glycolysis inhibition, observing diverging results among the cell lines. Finally, we performed a gene expression array to assess the mechanisms behind DUSP6 changes in metabolism. Taken together, these results suggest

that DUSP6 plays a role in metabolism reprogramming in PDAC but were not yet able to establish a connection with the metastatic process in this tumor.

INTRODUCTION

Metabolism reprogramming is highly complex and was described by Hanahan and Weinberg in 2011 as one of the hallmarks of cancer [1]. Conditions such as the increased proliferation that leads to high energy demand and the adaptation to the hostile microenvironment induce the cells to reprogram their metabolism, activating non-canonical metabolic pathways [2]. Pancreatic adenocarcinoma (PDAC), for instance, is characterized by an acidic microenvironment, due to high lactate secretion, and oxygen depletion, caused by the fibrotic stroma and low vascularity [2]. Recently, several studies have categorized PDAC cells' metabolic subtypes according to gene expression profile and patients prognosis, reinforcing the importance of better comprehending this tumor feature [3-5].

From these studies, it is well established that PDAC tumors with a glycolytic profile are highly aggressive compared to the remaining metabolic subtypes [4, 5]. Daemen and colleagues utilized a 42-genes panel to compare the molecular and metabolic PDAC subtypes and concluded that the glycolytic subtype highly correlates with the quasi-mesenchymal subtype [3]. This molecular subtype has been previously described as the one with the worst prognosis, presenting high proliferation rates, metastasis occurrence and drug resistance [6]. Later, Karasinska and colleagues showed that besides glycolysis signature activation, the glycolytic subtype cells also presented *KRAS* and *MYC* amplification, implying that they rely on these mutations for metabolic reprogramming. Oncogenic KRAS was shown to induce glycolysis in a myriad of ways, such as increasing *GLUT1, HK1/2* and *LDHA* expression [7]. Furthermore, in accordance to the study previously published by Daemen, the glycolytic subtype also correlates with the basal-like [8] and squamous [9] molecular subtypes, which represent subtypes with the worst prognosis within their respective classifications [4]. Therefore, disrupting

glucose metabolism in this context becomes an interesting strategy to target the most aggressive PDAC subtype.

Recently, our group identified that the dual-specificity phosphatase 6 (DUSP6) is overexpressed in the quasi-mesenchymal and the squamous subtypes (Chapter II). DUSP6 is a negative regulator of the ERK/MAPK pathway and specifically dephosphorylates ERK1/2, inactivating the protein [10, 11]. Besides that, DUSP6 also overexpressed in metastatic samples compared to primary tumor samples in publicly available datasets and correlated with worse patient overall survival in the TCGA dataset (Chapter II), reinforcing the hypothesis of its correlation with tumor aggressiveness. Considering our recent findings on DUSP6 and the non-canonical oncogenic KRAS role in regulating glycolysis in PDAC cells, we hypothesized that DUSP6 might exert a role in PDAC metastasis through metabolic reprogramming, with special impact in the glycolysis pathway.

To test this hypothesis, firstly we performed gene set enrichment analysis in 3 independent datasets and observed a consistent DUSP6 correlation with glycolysis. Next, we induced DUSP6 overexpression in MIA PaCa-2 – which does not express the phosphatase – and performed a gene expression array with glucose-related metabolism genes. Eight from 92 genes were differentially expressed upon DUSP6 overexpression, reinforcing its role in metabolism reprogramming. Then, we knocked down DUSP6 in PDAC cell lines and performed live glycolysis rate assay, in which we observed increased glycolysis in 2 out of 3 cell lines. Changes in glycolysis rate were accompanied by increased lactate secretion. Nevertheless, when we treated the cells with 2-DG to inhibit the glycolytic pathway we did not observe any significant changes in cell migratory capacity. Taken together, our results suggest that DUSP6 modulates metabolism in PDAC cells, but these changes do not seem to affect their migratory potential.

MATERIALS & METHODS

Cell lines and culture

MIA PaCa-2, AsPC-1 and BxPC-3 were purchased from ATCC (Manassas, VA, USA). MIA PaCa-2 is a primary human tumor-derived PDAC cell lines expressing mutant KRAS, mutant p53, and deletion of CDKN2A. AsPC-1 is a metastatic human PDAC cell lines expressing mutant KRAS, mutant p53, and deletion of CDKN2A. BxPC-3 is a human primary tumor-derived cell line expressing mutant p53 and deletion of CDNK2A. K8484 cells isolated from the KPC ($KRas^{G12D/+}$; $p53^{R172H/+}$) mouse were acquired from David Tuveson's Lab (Cold Spring Harbor Laboratory) [12]. AsPC-1 was cultured in high glucose (4.5 g/L) RPMI-1640 medium (ThermoFisher Scientific, Waltham, MA USA cat #A1049101) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals/RD Systems, Atlanta, GA USA) and antibiotic-antimycotic (Thermo Fisher Scientific cat# 15240062). K8484 cell line was cultured in high glucose (4.5 g/L) Dulbecco's Modified Eagle Media (ThermoFisher Scientific cat# 11-995-073) supplemented with 5% heat-inactivated FBS (R&D Systems-Atlanta Biologicals) and antibiotic-antimycotic (ThermoFisher Scientific). All the other cell lines were maintained in high glucose DMEM, 10% FBS, and antibiotic-antimycotic. Cells were kept in culture conditions (37°C, 5% CO_2 and humid atmosphere) as recommended by ATCC.

Plasmids and gene knockdown

To generate DUSP6 knockdown cells, short hairpin RNA (shRNA) constructs targeting three independent regions of DUSP6 mRNA (#1 5'-CTGTGGTGTCTTGGTACATTG-3'; #2 5'-TCTAATCCAAAGGGTATATTT-3', #3 5'-ATTCGGCATCAAGTACATCTT-3'), were obtained from Millipore Sigma (Burlington, MA, USA). Empty vector pLKO.1 puro (Addgene) was used as a scrambled control. For DUSP6 overexpression, we purchased

plasmids R777-E043 Hs.DUSP6, containing DUSP6 mRNA sequence, and pLX304, the empty lentiviral vector used as negative control, from Addgene. For cloning, we utilized Clonase™ Gateway™ LR Clonase II Enzyme Mix (Invitrogen) following manufacturer's instructions. Coning efficiency was validated with automated Sanger sequencing using specific primers as following: pLX-ORF F 5'- CACCAAAATCAACGGGACTT-3'; pLX-ORF R 5'-CAACACCACGGAATTGTCAG-3'. Packaged lentiviruses were generated by transfecting HEK293T cells with respective plasmids and the Trans-Lentiviral™ Packaging Mix (Open Biosystems) to produce viral supernatants which were subsequently used to transduce pancreatic cancer cell lines. Successfully transfected cells were selected with Puromycin treatment (1-2,5µg/mL) until complete cell death was achieved in the non-transduced cells.

RNA isolation and RT-qPCR

Briefly, 10^6 cells were seeded in 6-well plates and left overnight in incubator. In the next day, cells were washed with 1X PBS and harvested in QIAzol Lysis Reagent (Qiagen, GmbH, cat #79306). Samples were kept in -80°C until total RNA was isolated using the Direct-zol RNA Miniprep kit (Zymo Research Corporation) according to manufacturer's protocols. RNA quantification and quality analysis were performed using NanoDrop 2000 (Thermo Fisher Scientific Inc.). For cDNA, 1.5-2µg of RNA were used with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, cat #4368814) following manufacturer's instructions and further diluted 1:10 before storage in -20°C. Real-time PCR analysis was carried out with the 2X SYBR Green qPCR Master Mix (ApexBio Technology, cat #K1070) on a ViiA 7 Real-Time PCR System (Applied Biosystems) using the following primers: human DUSP6 (Fwd: ATGGTAGTCCGCTGTCCAAC / Rev: ACGTCCAAGTTGGTGGAGTC); mouse Dusp6 (Fwd: TGTTTGAGAATGCGGGCGAGTT / Rev: ACAGTTTTTGCCTCGGGCTTCA); human

ACTB (Fwd: TCGTGATGGACTCCGGTGAC / Rev: CGTGGTGGTGAAGCTGTAG); mouse Actb (Fwd: GTGACGTTGACATCCGTAAAGA / Rev: GCCGGACTCATCGTACTCC). Each sample was run in triplicate using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) and fold-change was evaluated relative to normal samples and determined using *ACTB* levels as a reference.

In silico analysis

For gene set enrichment analysis we utilized the publicly available datasets TCGA_PAAD and CPTAC_PDAC, obtained from LinkedOmics Platform, and GSE15741 obtained from GEO Databases. To search for co-expressed genes with the same DUSP6 gene signature, Pearson's correlation was used with a cutoff of $|R| > 0.6$ through the basic packages of R. Finally, functional gene enrichment analysis (GSEA) for Panther Pathways of genes correlated with DUSP6 was performed using the LinkedOmics Platform. For enrichment analysis of the differentially expressed genes in the glucose metabolism array, we utilized the Enrichr platform based on the BioPlanet 2019 enrichment set.

Human Glucose Metabolism Array

High-throughput genotypic screening was performed by RT-qPCR, using personalized plates from TaqMan Array (ThermoFisher Scientific, Invitrogen) with a panel of 92 metabolic genes (Figure S1). Quantitative PCR was carried out on a QuantStudio™ 7 Pro Real-Time PCR System (Thermo Fisher Scientific). For data analysis, we took advantage of the package HTqPCR [13], defining $\alpha = 0.05$ as a threshold for differentially expressed genes.

Glycolytic rate assay

The glycolysis rate was obtained through the Seahorse XF Glycolytic Rate Assay Kit (Agilent, Santa Clara, USA), following manufacturer's protocol. Briefly, 2.5-4x10^4 cells were seeded in a 96-well plate and incubated overnight. In the next day, cells were washed twice with Seahorse XF DMEM medium with pH 7.4 (1 mM pyruvate, 2 mM glutamine and 10 mM glucose) and a final volume of 180µL of the same was added to each well. Proton efflux rate (PER) was measured (pmol/min) at 11 time points with sequential injections of 0.5µM Rotenone/Antimycin A and 50mM 2-Deoxy-D-glucose (Thermo Scientific, cat. #AC111980010). For nuclei staining, 5µg/mL Hoechst was added to Rotenone/Antimycin A solution. PER values were calculated and normalized to the cell count number using an automated imaging and cell counting workflow. For normalization, PER value per 10,000 cells was set and the Agilent Seahorse XF system automatically calculated and generated the normalized results.

Lactate assay

For lactate measurement, 2.5x10^4 cells were seeded in 96-well plates and allowed to sit down overnight in the incubator. The next day, complete media in the plate was replaced with new media containing 0.5% FBS and cells were incubated for 24 hours. At the end of the experiment, culture plates were centrifuged at 450 G for 5 minutes and 10µL of media were collected from each well for the subsequent assay. For lactate quantification, we utilized the Glycolysis Cell-Based Assay Kit (Cayman Chemical Company, Ann Arbor, MI, cat #600450) and followed the manufacturer's protocol. Absorbance was read in 490nm in a Synergy H1 Multimode microplate reader (Bio-Tek/Agilent Technologies).

Proliferation assay

Cell proliferation was accessed using ethynyldeoxyuridine (EdU) incorporation method. Briefly, 2.5-5x10^4 cells were seeded in 24-well plates in complete media and incubated overnight. In the next day, media was replaced by the appropriate treatments and incubated for 18 hours. At this point, EdU was added to the wells in a final concentration of 10mM and cells were incubated again for 6 hours. At the end of this timepoint, cells were trypsinized, harvested and fixed with 10% buffered formalin overnight at 4°C with gentle agitation. In the next day, cells were washed with PEB-T (1x PBS, 2mM EDTA, 1% BSA, 0.5% Triton-X) and subsequently with PB (1x PBS, 1% BSA). Next, cells were resuspended in the Click-It Reaction (H2O, 100mM Tris pH 8.5, 1mM CuSO4, 1µM azide-dye) and 100mM ascorbic acid. Then, cells were incubated with 0.5µg/mL propidium iodide (PI), washed twice and resuspended in PEB (1x PBS, 2mM EDTA, 1% BSA) for flow cytometry analysis. Cells were analyzed for 530 nm and 695 nm emission in an Attune NxT Flow Cytometer (Thermo Fisher Scientific). FCS files were analyzed with FlowJo™ v10.8 Software (BD Biosciences) and percentage of EdU-positive cells was taken from the PI-positive single cells.

Migration assay

First, 2-4x10^5 cells were seeded in 24-wells plates and cultured until full confluence. Then, a scratch was made at the center of the plate using a 200µL pipette tip. Wells were washed twice with 1X PBS to discard all the debris and media with appropriate treatments was added. Plates were photographed at 0 and 24 hours using a EVOS™ M5000 Imaging System (Thermo Fisher Scientific). Scratch area was calculated using the Wound Healing Coherency Tool (Montpellier Ressources Imagerie) on ImageJ (NIH, Bethesda, MD) and results are expressed as healing percentage – the difference in scratch area between time 0 and time 24.

Statistical analysis

Samples sizes were chosen to achieve a minimum of triplicates for all experiments. For assessment of statistical significance, ordinary one-way ANOVA with Šidák's multiple-comparison test or Welch's unpaired 2-way t-test was used when appropriate and indicated. All statistical tests were performed using GraphPad Prism 9 software and results were considered significant if $\alpha = 0.05$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

RESULTS

DUSP6 expression in PDAC datasets correlates with the glycolysis pathway

To further comprehend DUSP6 overexpression in PDAC samples, we wanted to unravel the pathways in which it is involved. To do so, we took advantage of publicly available *in silico* datasets to run a gene set enrichment analysis. We ran the Panther Pathway enrichment analysis in 3 independent datasets and we observed that Glycolysis (P00024) came out as one of the strongest correlated pathways to DUSP6 in PDAC in all of the datasets (Figure 1A-C). These data suggest that DUSP6 overexpression might be important for the tumor cells to reprogram their metabolism.

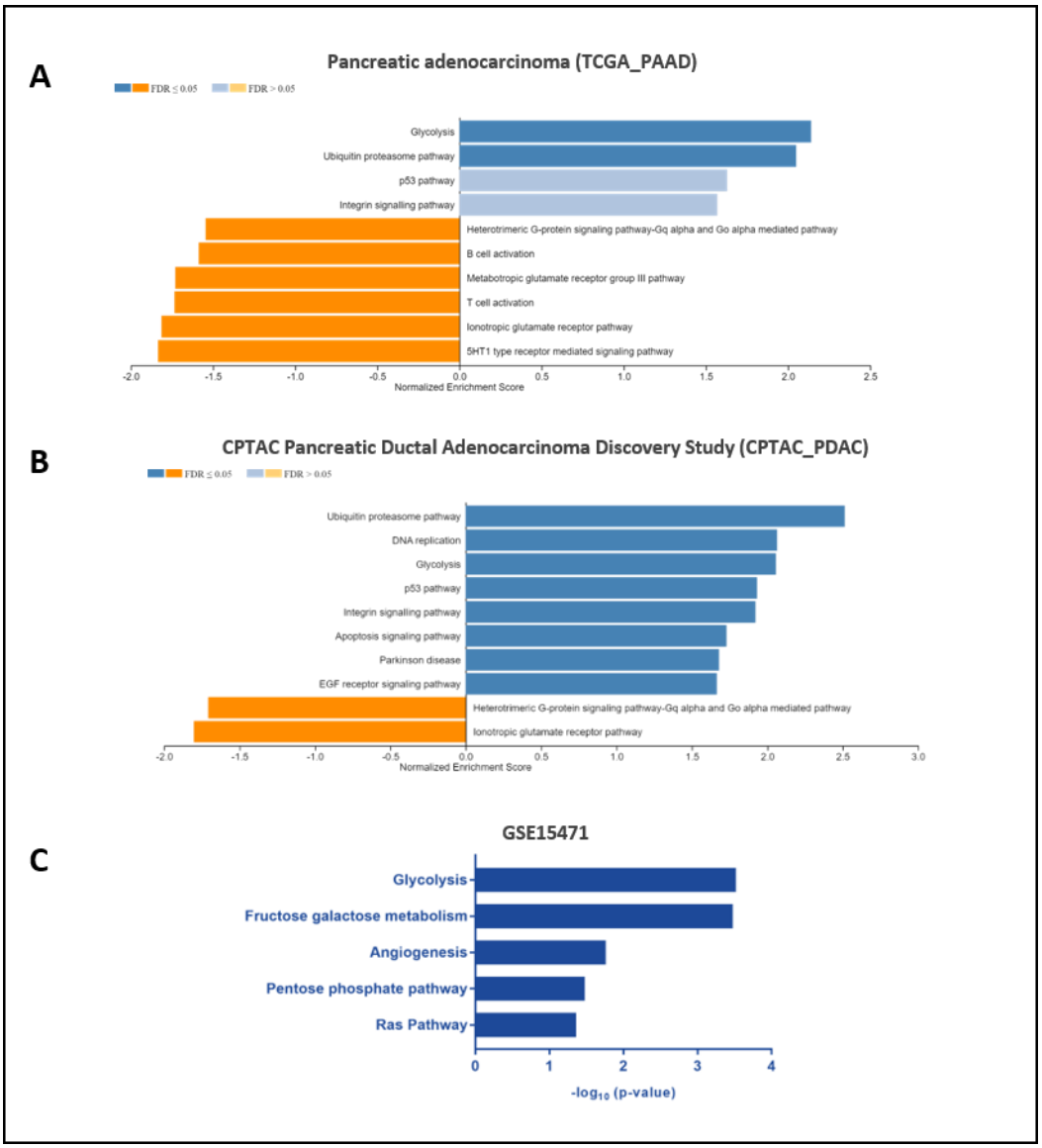

Figure 1 – DUSP6 expression in PDAC datasets correlates with the glycolysis pathway. Gene set enrichment analysis (GSEA) performed in 3 independent datasets (TCGA_PAAD (**A**), CPTAC_PDAC (**B**) and GSE15471(**C**)) show that DUSP6 is consistently correlated to glycolysis using the Panther classification system.

DUSP6 expression significantly changes glucose-related genes' expression profile

Aiming to determine how DUSP6 promotes metabolic changes in PDAC cells, we took advantage of the MIA PaCa-2 cell line, which does not express DUSP6 due to a hypermethylation in the gene promoter [14]. Then, we induced DUSP6 overexpression (Figure S3) in these cells and performed a glucose metabolism-related gene expression array. We observed that among the 92 genes included in the panel, 8 genes were found to be differentially expressed upon DUSP6 overexpression (Figure 2A). From these eight, 3 genes – *RBKS*, *SUCLG2* and *GFPT1* – were downregulated and 5 genes – *SDHA*, *GSK3A*, *PDK2*, *MDH1* and *PDK4* were upregulated.

Following the gene expression array analysis, we used the generated gene list to perform an enrichment analysis aiming to understand which metabolic pathways were most affected. Surprisingly, we observed that the differentially expressed genes following DUSP6 overexpression were mostly correlated with the tricarboxylic acid (TCA) cycle (Figure 2B). Although results clearly indicate that DUSP6 plays a role in metabolism regulation, it is not yet enough to determine how this phosphatase acts in these pathways, since the number of differentially expressed genes is a limiting factor for the reliability of the analysis.

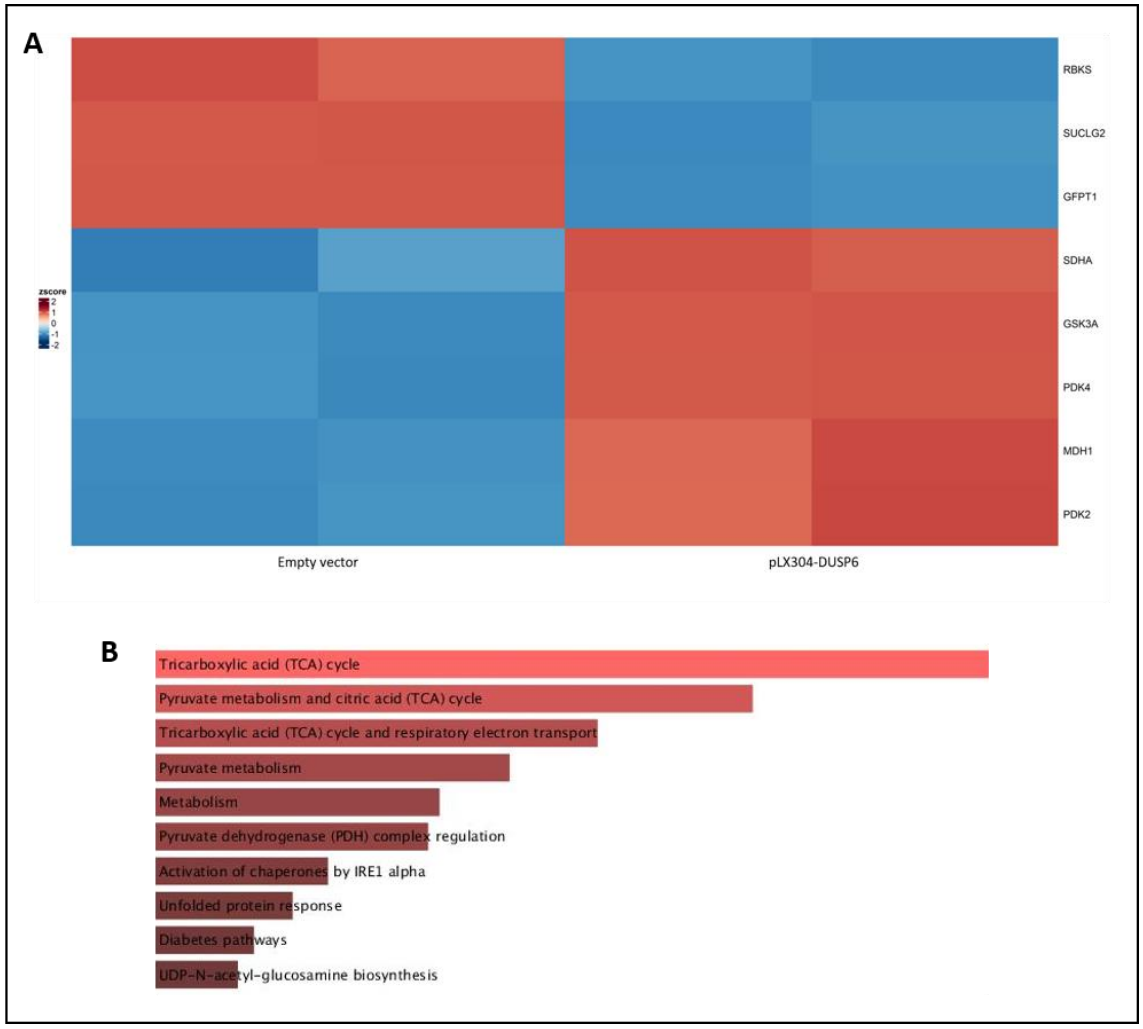

Figure 2 – DUSP6 expression significantly changes glucose-related genes' expression profile. We induced DUSP6 overexpression in MIA PaCa-2 and performed a glucose metabolism related-gene expression array to investigate the mechanisms by which DUSP6 modulates glycolysis in PDAC cell lines. Eight out of 92 genes showed to be differentially expressed upon DUSP6 overexpression. (**A**). We then took this list of 8 genes and performed an *in silico* gene set enrichment analysis (GSEA) using the Enrichr platform to try to elucidate how they were correlate to each other. To our surprise, results indicate an enrichment in the BioPlanet 2019 for the tricarboxylic acid (TCA) cycle, suggesting that in this cell line, this pathway might be affect by DUSP6 exogenous activity (**B**). Heatmap is representative for the mean of 2 independent experiments.

DUSP6 downregulation upregulates glycolysis in PDAC cell lines

To determine how DUSP6 impacts cell metabolism, we knocked down DUSP6 in 3 PDAC cell lines – AsPC-1, BxPC-3 and K8484 and performed a live metabolic assay to measure glycolytic rates upon DUSP6 absence. Here is important to mention that

according to Daemen and colleagues' metabolic subtypes for PDAC human cell lines, AsPC-1 is a slow proliferating cell line while BxPC-3 is a lipogenic cell line [3]. K8484 were not included in their study since it is a mouse cell line. Nevertheless, in this assay, we observed that K8484 control cells have the highest basal glycolytic rate of the 3 cell lines (Figure 3A). Besides that, K8484 show a 62% of glycoPER, demonstrating that most of its energy source is glucose catabolism (Figure S2). AsPC-1 and BxPC-3 control cells, expectedly, showed basal glycolytic rates close to zero (Figure 3A). Upon DUSP6 knockdown, K8484 and AsPC-1 showed no significant changes in basal glycolysis, while BxPC-3 already had a significant increase in glucose consumption ($P < 0.001$; Figure 3A). Next, following treatment with Rotenone and Antimycin A, inhibitors of the respiratory complex I and III, respectively, we observe a significant increase in glycolysis in AsPC-1 and BxPC-3 with DUSP6 knockdown in comparison to the control ($P < 0.0001$). K8484, on the other hand, show an increase in glycolysis compared to basal glycolytic levels, but no significant difference between the control and DUSP6 knockdown (Figure 3B). After 2-DG addition to the assay, glycolysis was significantly inhibited in all the conditions, although some post 2-DG acidification was still present in AsPC-1 and BxPC-3 with DUSP6 knockdown in comparison to the control, probably due to residual glycolysis (Figure 3C).

Lastly, we assessed basal lactate secretion in the extracellular media upon DUSP6 knockdown using a colorimetric assay. In accordance with what we observed in the glycolytic rate assay, there was a significant increase in BxPC-3 lactate secretion compared to control ($P < 0.0001$). K8484 and AsPC-1, in accordance to the basal glycolytic rate observed in the Seahorse assay, showed no significant difference compared to control in basal lactate secretion (Figure 3D). Altogether, these data suggest that DUSP6 has a role in glycolysis pathway activation, specially under deprivation of

other energy sources like the mitochondrial respiratory chain. Nevertheless, this phenotype is not positively correlated to DUSP6 expression as we anticipated, but rather negatively correlated, since its knockdown is increasing glycolysis in these cells.

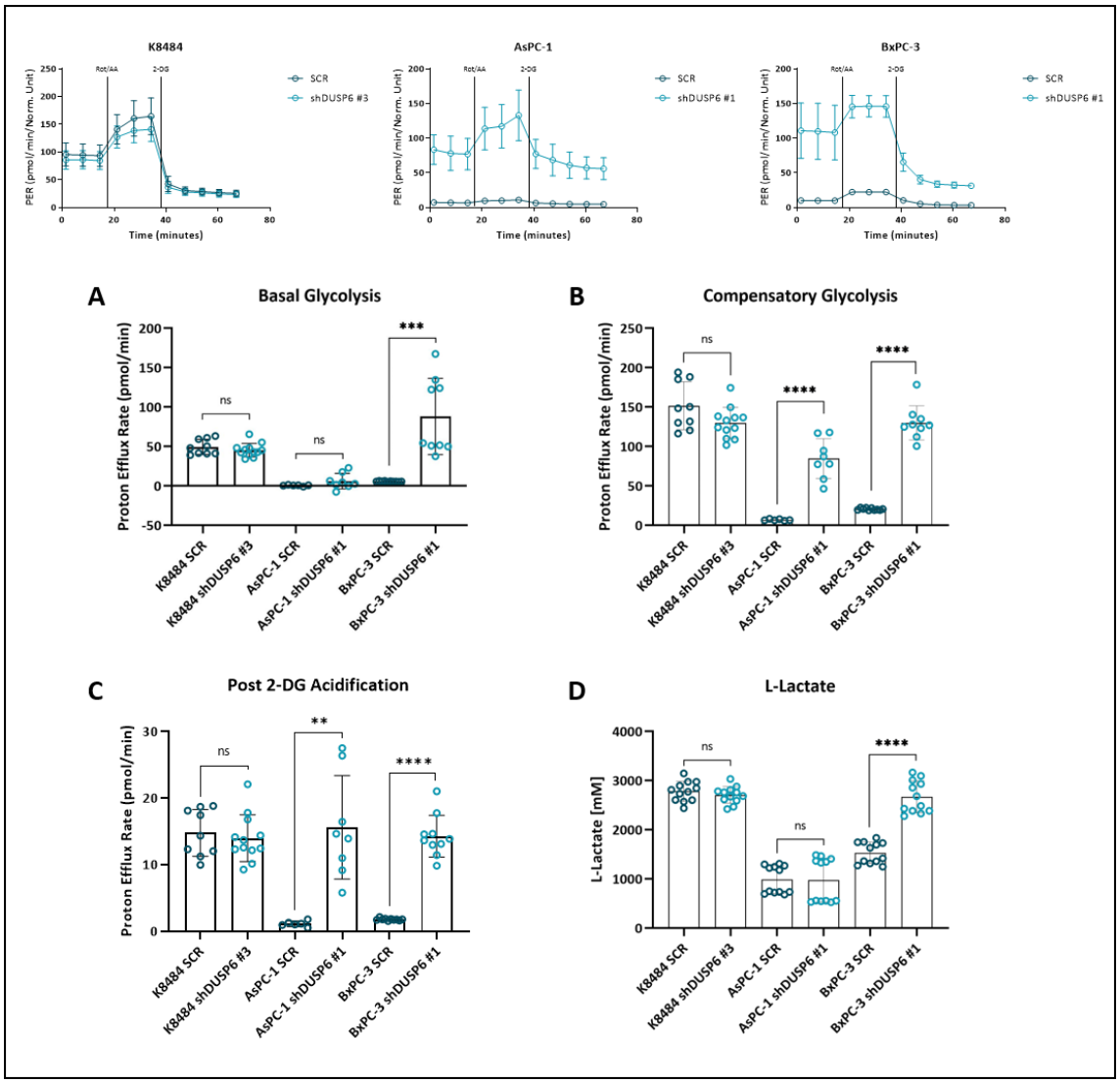

Figure 3 – DUSP6 downregulation upregulates glycolysis in PDAC cell lines. Glycolysis rate assay was performed to access glycolytic profile of the cell lines upon DUSP6 knockdown. **A)** Basal glycolysis refers to glycolysis before the injection of any compounds to cells. From the 3 cell lines, only BxPC-3 show increased basal glycolysis ($P < 0.001$) upon DUSP6 knockdown. **B)** Compensatory glycolysis is measured after inhibition of complexes I and III of the mitochondrial respiratory chain and shows that both AsPC-1 and BxPC-3, upon DUSP6 knockdown, are able to utilize glycolysis as energy source ($P < 0.0001$), while K8484 show no significant difference. **C)** Post 2-DG acidification show acidification that remains after glycolysis irreversible inhibition. Results observed for AsPC-1 ($P < 0.01$) and BxPC-3 ($P < 0.0001$) are likely residual glycolysis due to the increased activation on the previous step. **D)** L-Lactate assay

measures basal lactate levels in the extracellular media upon DUSP6 knockdown. In accordance with the previously observed in **A**, only BxPC-3 show increased basal lactate secretion ($P < 0.0001$). Seahorse Glycolysis Rate Assay was performed in 12 replicates. L-Lactate assay was performed in 2 independent experiments with 6 replicates each. Results represent mean ± SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

DUSP6 downregulation does not impact cell migratory capacity upon glycolysis inhibition with 2-DG

To better understand how glycolysis is affecting these cell lines' upon DUSP6 knockdown, we started performing a cell proliferation assay. We reduced fetal bovine serum in the media to decrease its effect in metabolism and assessed cell proliferation with and without 2-DG addition. As we previously reported, both AsPC-1 and BxPC-3 show a significant reduction in proliferation upon DUSP6 knockdown compared to their controls, but no K8484 ($P < 0.0001$; Figure 4A-C). After 2-DG treatment, K8484 show a decrease in proliferation both in the control and DUSP6 knockdown, but reduction in DUSP6 knockdown cells is significantly higher than in the control ($P < 0.01$; Figure 3A). BxPC-3 also showed a significant reduction in proliferation in both conditions after 2-DG treatment, but this time reduction was more significant in the control cells ($P < 0.0001$; Figure 4C). Finally, AsPC-1 cells show no visible effect in proliferation compared to the same condition without 2-DG (Figure 4B).

To further investigate if DUSP6's role in glycolysis impacts the tumor cells' metastatic potential, we performed a migratory capacity assay combined with 2-DG treatment. We had previously reported that DUSP6 knockdown increases K8484 migratory capacity ($P < 0.05$), while in AsPC-1 we observed a significant decrease ($P < 0.0001$; not published). Regarding BxPC-3, no change was observed in this context. Contrary to what we expected, treatment with 2-DG had no significant impact in previously observed migratory phenotype in AsPC-1 or BxPC-3 (Figure 4E-F). On the

opposite, K8484, which previously had an advantage on migratory capacity upon DUSP6 knockdown, showed a complete impairment in migration after 2-DG treatment ($P < 0.0001$; Figure 4D). These results suggest that glycolysis is essential for K8484 to migrate, but if DUSP6 plays a role in this process remains to be established. AsPC-1 and BxPC-3, on the other hand, seem to utilize another source of energy for cell migration.

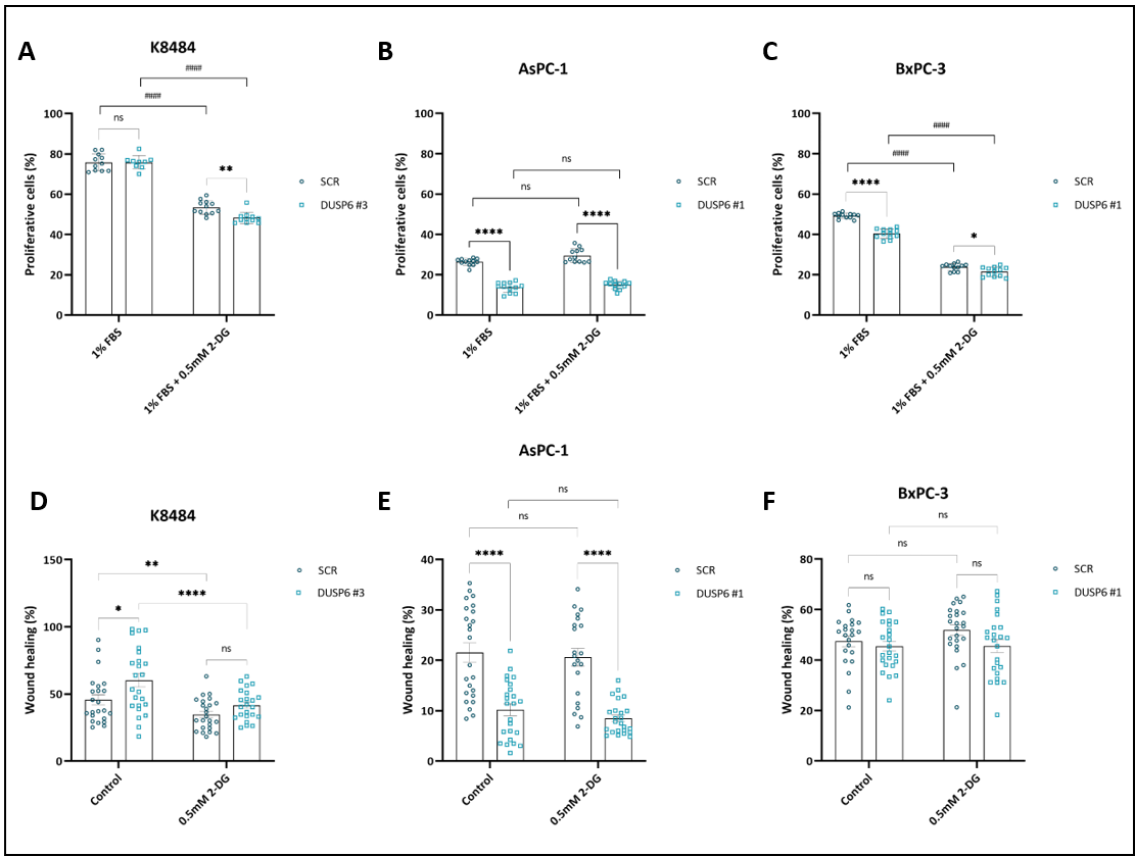

Figure 4 – DUSP6 downregulation does not impact cell migratory capacity upon glycolysis inhibition with 2-DG. We evaluated proliferation capacity on PDAC cell lines upon DUSP6 knockdown and observed that AsPC-1 (**A**) and BxPC-3 (**B**) with DUSP6 knockdown show a significant decrease in proliferation in comparison to control, both in the presence or absence of 2-DG. Nevertheless, AsPC-1 are not affected by 2-DG addition, while BxPC-3 are significantly impacted ($P < 0.0001$), showing they are more dependent on glycolysis for proliferation than AsPC-1. K8484 (**C**), on the opposite, show no significant differences in proliferation in the absence of 2-DG, but are significantly impacted by its addition ($P < 0.0001$), which seems to be more significant for the DUSP6 knockdown cells ($P < 0.01$). Regarding migratory capacity, we observe different behaviors among the cell lines: K8484 (**D**) show a significant increase in comparison to control in the absence of 2-DG ($P < 0.05$). Nevertheless, with 2-DG addition, this advantage is lost ($P < 0.0001$). AsPC-1 (**E**) show a significant decrease in migratory capacity

compared to control regardless of the presence or absence of 2-DG ($P < 0.0001$). Finally, BxPC-3 (**F**), show no significant differences in migratory capacity. Graphs show 3 independent experiments with 4-6 replicates each. Results represent mean ± SEM; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; #### $P < 0.0001$.

DISCUSSION

Metabolism reprogramming was defined as one of the hallmarks of cancer by Hanahan and Weinberg more than a decade ago [1]. And although many discoveries have been done in the past 11 years, many aspects of tumor metabolism are still a challenge for scientists. In PDAC, metabolism reprogramming is essential for progression and survival, since the dense and desmoplastic stroma represents around 90% of the tumor volume, depriving the tumor cells from properly receiving oxygen [15].

It is well established that oncogenic KRAS induces glycolysis in PDAC cells through increased glucose uptake, upregulation of *GLUT1* and other rate-limiting glycolytic enzymes [7]. Taking in consideration that DUSP6 is a negative regulator for the ERK/MAPK pathway and is upregulated in PDAC samples in *silico*, we hypothesized that DUSP6 might be involved in metabolism reprogramming in this tumor. Then, other *in silico* gene set enrichment analysis performed by our group in 3 independent datasets showed that DUSP6 expression in PDAC samples is correlated to glycolysis. DUSP6's role in regulating glycolysis through the ERK/MAPK has been previously described by Tong and colleagues in an ovarian cancer model. In their study, they describe DUSP6 as a target for the E3 ubiquitin-ligase TRIM59, overexpressed in this tumor. In this case, TRIM59 ubiquitinates DUSP6, targeting it for proteassomal degradation and, therefore, preventing the negative regulation of the axis ERK1/2/c-Myc/LDHA/Glycolysis [16].

We performed a glucose metabolism-related gene expression array to assess changes in metabolic genes upon DUSP6 overexpression in MIA PaCa-2, a cell line that originally does not express DUSP6. We observed that 8 genes were differentially expressed, being 3 significantly downregulated and 5 upregulated. We took this list of genes and performed a gene set enrichment analysis which, surprisingly, pointed to a correlation with the tricarboxylic acid (TCA) cycle. MIA PaCa-2 was defined by Daemen

and colleagues as a highly glycolytic cell line [3], and Xu and colleagues have shown that these cells lack DUSP6 expression due to hypermethylation in its gene promoter [14]. Therefore, it is intuitive to believe that glycolysis in these cells is regulated by different key molecules, and DUSP6 sudden overexpression might induce these cells to reprogram their metabolism in ways we did not anticipated. Consequently, it is important that we expand this transcriptional investigation to other cell lines to further understand molecularly how DUSP6 affects the glycolytic pathway in PDAC.

To investigate DUSP6's role in glycolysis in PDAC cells, we knocked down DUSP6 in 3 PDAC cell lines and performed glycolysis rate assay and lactate secretion measurements. Similarly, to what Tong and colleagues reported, we observed a significant increase in the glycolytic pathway upon DUSP6 knockdown in two out of three cell lines, accompanied by an increased basal lactate secretion in the extracellular media in one of the two. Although in accordance to what was previously published in other studies and intuitively expected considering ERK/MAPK role in glycolysis, surprisingly, these results go against what we anticipated with the *in silico* analysis. Nevertheless, we must consider that publicly available datasets do not discriminate between mutational backgrounds, tumor subtypes and/or metabolic subtypes. Nowadays we have available datasets that allow us to separate samples according to the molecular subtypes [6, 9], but unfortunately the number of samples comprised in each group is not enough to perform a robust enrichment analysis between groups.

Next, we sought to evaluate how this glycolysis induction by DUSP6 knockdown would affect proliferation and migratory capacity in PDAC cells. We observed that both cell lines in which glycolysis is increased, there is also a significant reduction in proliferation upon DUSP6 knockdown. This could be explained by the fact that AsPC-1 is a slow proliferating cell line and BxPC-3 is lipogenic [3] and, might not benefit from

increased glycolytic activity. Besides that, is also important to consider that ERK1/2 hyperactivation via DUSP6's suppressed activity was previously reported to exceed a favorable threshold in lung adenocarcinoma models eliciting responses such as senescence, vacuolization and cell death [17]. Similar phenotype was observed by our group when inducing DUSP6 overexpression in MIA PaCa-2 and, consequently diminishing oncogenic KRAS activity (data not shown), and is corroborated by Unni and colleagues in an event called "oncogene addiction" [17]. Therefore, it is plausible to conclude that tumor cells need to fine-tune ERK/MAPK activation to sustain their proliferation rate and avoid induced toxicity, a job that is partially done by DUSP6 among other negative regulators.

Regarding migratory capacity, we observed that only K8484 benefited from DUSP6 knockdown to increase migratory capacity. AsPC-1 showed a decreased migratory phenotype, while BxPC-3 showed no significant changes. Observing these results from the metabolic perspective, glycolysis induction did not seem to benefit migratory capacity in AsPC-1 and BxPC-3; on the other hand, it actually decreased it in AsPC-1, suggesting that these cells might rely on a different energy source to metastasize. On the opposite, K8484, which showed to be already more glycolytic than the other cells lines, did not show an increase in glycolysis usage, although had an increased migratory capacity. We can hypothesize that these cells are already using the maximum of their glycolytic capacity and, therefore, were not affected in this aspect. Nevertheless, treatment with 2-DG made clear that glycolysis is essential for these cells to migrate once it completely extinguished migratory advantage of the DUSP6 knockdown cells over the control cells. In that sense, it remains to be established by which mechanisms DUSP6 knockdown favors migratory capacity on K8484. Lastly, AsPC-1 and BxPC-3 treatment

with 2-DG did not elicit any changes on their migratory phenotype, once again reinforcing that these cells might rely on different energy sources to metastasize.

CONCLUSIONS

In summary, the data compiled in this work shows that DUSP6 knockdown has an impact in metabolic reprogramming in PDAC cells through stimulation of ERK1/2 activation, increasing they glycolytic pathway usage. Nevertheless, these changes seem to affect differently each cell line in the study, presumably due to their mutational background and their contrasting metabolic behaviors. Unfortunately, we were not yet able to establish a link between these metabolic changes and their metastatic phenotype, although there is strong evidence in literature that these events are linked.

Further investigation on this matter should include phosphoproteomics profiling in cell lines with different metabolic subtypes upon DUSP6 knockdown/overexpression, to deeply comprehend how this phosphate affects their behavior and who are its potential targets – since right now DUSP6 is known to be specific for ERK1/2. Also, it is imperative to conduct a targeted metabolomics analysis to identify specifically which metabolites are being affected by DUSP6 activity.

Widening this investigation could bring new insights into this phosphatase's role in tumor metabolism, which has not yet, to our knowledge, been described. Also, it could point out new targets for DUSP6 in metabolic pathways and open new research questions regarding its importance in PDAC.

ACKNOLEDGEMENTS

We would like to acknowledge Dr. David Tuveson for supplying the murine K8484 cell line used in these studies. We acknowledge the Flow Cytometry Core Laboratory, which is sponsored, in part, by the NIH/NIGMS COBRE grant P30 GM103326 and the NIH/NCI Cancer Center grant P30 CA168524. We would also like

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AUTHOR'S CONTRIBUTIONS

MTR: methodology, data collection, formal analysis, visualization, paper writing, and editing; RMW, BBB, VSS & AEE: data collection, review, and editing RMW: data collection and data analysis; MNV: supervision, methodology, data analysis, paper writing, editing; VSS: funding acquisition, project administration, conceptualization, supervision, methodology, data analysis, paper writing, editing.

COMPLIANCE WITH ETHICAL STANDARDS

This study does not require ethical approval.

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SUPPLEMENTARY INFORMATION

Figure S1 – Human Glucose Metabolism (Thermo Fisher Scientific, Invitrogen™) array plate configuration.

ACLY	ACO1	ACO2	ALDOA	ALDOC	BPGM	CS	DLAT	DLD	DLST	ENO1	ENO2
FBP1	FBP2	FH	G6PC	G6PC3	G6PD	GALM	GBE1	GCK	GPI	GSK3A	GSK3B
GYS1	GYS2	H6PD	HK2	IDH1	IDH2	IDH3A	IDH3B	IDH3G	MDH1	MDH1B	MDH2
OGDH	PC	PCK1	PCK2	PDHA1	PDHB	PDK1	PDK2	PDK3	PDK4	PDP2	PDPR
PFKL	PGAM2	PGK1	PGK2	PGLS	PGM1	PGM2	PGM3	PHKA1	PHKB	PHKG1	PHKG2
PKLR	PRPS1	PRPS1L1	PRPS2	PYGL	RBKS	RPE	RPIA	SDHA	SDHB	SDHC	SDHD
SUCLA2	SUCLG1	SUCLG2	TALDO1	TKT	TPI1	UGP2	PKM2	LDHA	LDHB	SCL2A1	GOT1
GOT2	GLUD1	GFPT1	ASCT2	GLS	MYCN	SLC16A1	SLC16A4	18S	GAPDH	GUSB	ACTB

Figure S2 – Percentage Proton Efflux Rate derived from Glycolysis (%glycoPER) extracted from Seahorse Glycolysis Rate Assay.

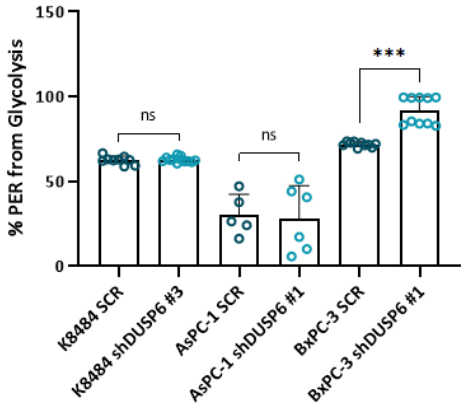
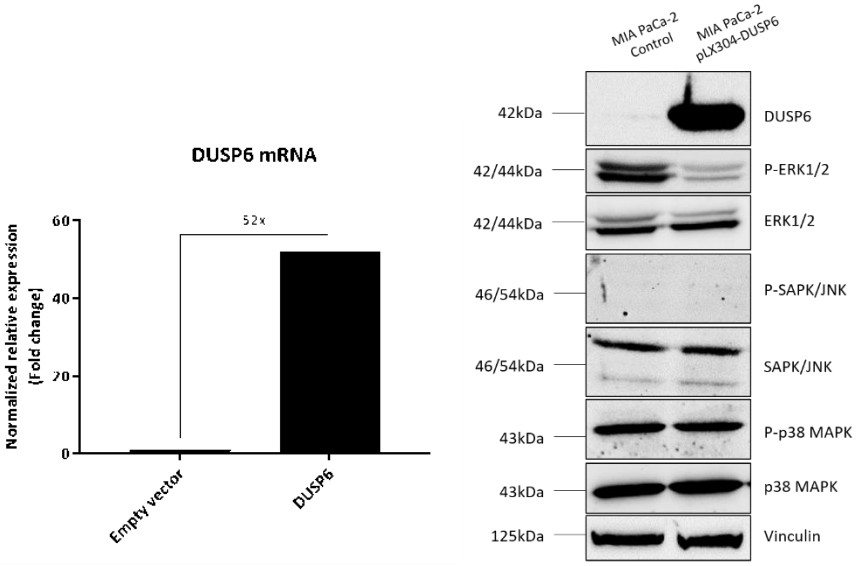


Figure S3 – Validation of DUSP6 overexpression in MIA PaCa-2.



GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

Overall, the results presented in this thesis summarize the relevant role that phosphatases play in tumorigenesis. In Chapter I, we provided a detailed description of the human phosphatases and how they act as regulators of other proteins activation. Then we focused on their role and clinical implications in pancreatic ductal adenocarcinoma (PDAC), and lastly on the development of inhibitors for these molecules.

Next, in Chapters II and III, we focused on DUSP6's role in PDAC and how this phosphatase acts in late stages of the disease, such as metastasis development. Altogether, the results obtained in Chapter II suggest that DUSP6 is overexpressed in primary tumor samples – specifically in the tumor, and not stromal cells – in comparison to non-tumoral pancreatic tissue. Moreover, this phosphatase is overexpressed in metastatic tumor samples in comparison to primary tumor samples. Consistently, patients with high DUSP6 expression show worse overall survival than patients with low DUSP6 expression. Nevertheless, *in vitro* analysis upon DUSP6 knockdown reveal that the migratory and invasive phenotypes can greatly vary among the cell lines and, therefore, different contexts. Taken together, results indicate that DUSP6 regulates phenotypes related to metastasis in PDAC, but the genetic landscape of the cells has great impact on the phenotype observed.

Finally, in Chapter III, we focused on DUSP6's role in metabolism reprogramming and a possible correlation to the metastatic phenotype. The results we obtained in a gene set enrichment analysis suggest that DUSP6 expression in PDAC is correlated to glycolysis. In a deeper molecular investigation using a gene expression array, the results suggest that DUSP6 overexpression in a cell line that previously lacked this phosphatase largely correlates with the tricarboxylic acid (TCA) cycle. This lead into a molecular mechanism for DUSP6 role in metabolism needs further investigation to

work up to a solid conclusion. Then, in a live metabolism assay, we observed that cells lacking DUSP6 greatly increase their glycolysis response to mitochondrial respiration inhibition, although basal glycolysis levels were only altered in one of the cell lines. Finally, regarding the correlation between metabolic changes and metastatic phenotype, although we observed significant changes in proliferation upon glycolysis inhibition, we did not observe suggestive changes in the migratory phenotype of the cell lines that showed a great increase in glycolysis usage. Taken together, these results suggest that DUSP6 plays a role in metabolism reprogramming, mainly favoring glycolysis in its absence, since it causes an increased ERK1/2 activation. Unfortunately, with the data we obtained until the conclusion of this thesis, we were not yet able to establish a connection between metabolic and metastatic phenotypes impacted by DUSP6.

For further development of this project, our group is currently working in a transcriptional analysis performed upon DUSP6 knockdown using RNA-seq aiming to determine a group of genes that are correlated with DUSP6. Therefore, it will help to unravel the molecular mechanisms and signaling pathways involved in each context. For a more comprehensive approach, we are also working in coordinate the RNA-seq with a phosphoproteomics analysis. That will allow us to assess which proteins are differentially phosphorylated and maybe identify novel DUSP6's targets. Furthermore, we believe that a metabolomics assay would also greatly contribute to better understand DUSP6 role in metabolism and exactly how its absence enables the cells to become more glycolytic, specially under mitochondrial respiration inhibition.

APPENDIX

AWARDS RECEIVED

1. Doctoral Dissertation Research Award (Fulbright Brazil)

FULBRIGHT Brasil

TERMS of AWARD

MARIANA TANNUS RUCKERT, Universidade de São Paulo, Department of Genetics, marianaruckert@gmail.com, has been awarded a grant by the Fulbright Commission in Brazil, established by Presidential Decree n. 7.176 on May 12, 2010, under the Mutual Educational Exchange Act, sponsored by the United States of America Department of State, Bureau of Educational and Cultural Affairs and the Government of Brazil. Please note that Fulbright awards are contingent upon receipt of medical clearance, placement/affiliation, and the availability of funds.

The grantee will be required to complete the Fulbright grant in the academic program and at the host institution specified in these Terms of Award. Grant dates and funding amounts stated in this document are subject to change, contingent upon the institution's operating status, availability of the academic program, changes in the institution's academic program start date, travel availability, and evolving travel or health advisories. The grant is activated only if the grantee can obtain a passport, J-1 visa and enter the United States to begin the academic program outlined in these Terms of Award at the host institution, and is dependent on the availability of funds.

Project Title:
"Unraveling dual-specificity phosphatases role in PDAC metabolic reprogramming using tumor organoid models."
Grant Category: Doctoral Dissertation Research Award
Period of Grant:
March 15, 2021 - December 14, 2021
Total Length:
9 months
Host Institution: University of Kansas Medical Center
Department of Cancer Biology
Kansas City, KS
Academic Advisor: Assistant Professor Michael VanSaun
Phone: (913) 945-6334
Dependents accompanying the grantee: NO

Grant benefits: For the period of grant stated above the grantee will receive the following benefits.

Benefits paid by the Fulbright Commission through wire-transfer to the grantee's bank account in the U.S.
International air ticket (upon presentation of boarding pass and US bank account info. International travel must be with U.S flag carrier.): USD 2.000,00
Settling-in Allowance (upon presentation of boarding pass and US bank account info. International travel must be with U.S flag carrier.): USD 2.000,00
Events allocation (up to this amount in one time reimbursement, upon prior approval of travel plan): USD 2.000,00
Stipend (MAR/2021): USD 6.600,00
Stipend (AUG/2021): USD 5.280,00
Total: USD 17.880,00
English Training (if applicable)
Limited health care benefit plan (ASPE)
Mandatory health insurance (if applicable)

Dr. Luiz Valcov Loureiro
Executive Director
Brasília, December 02, 2020

AGREEMENT

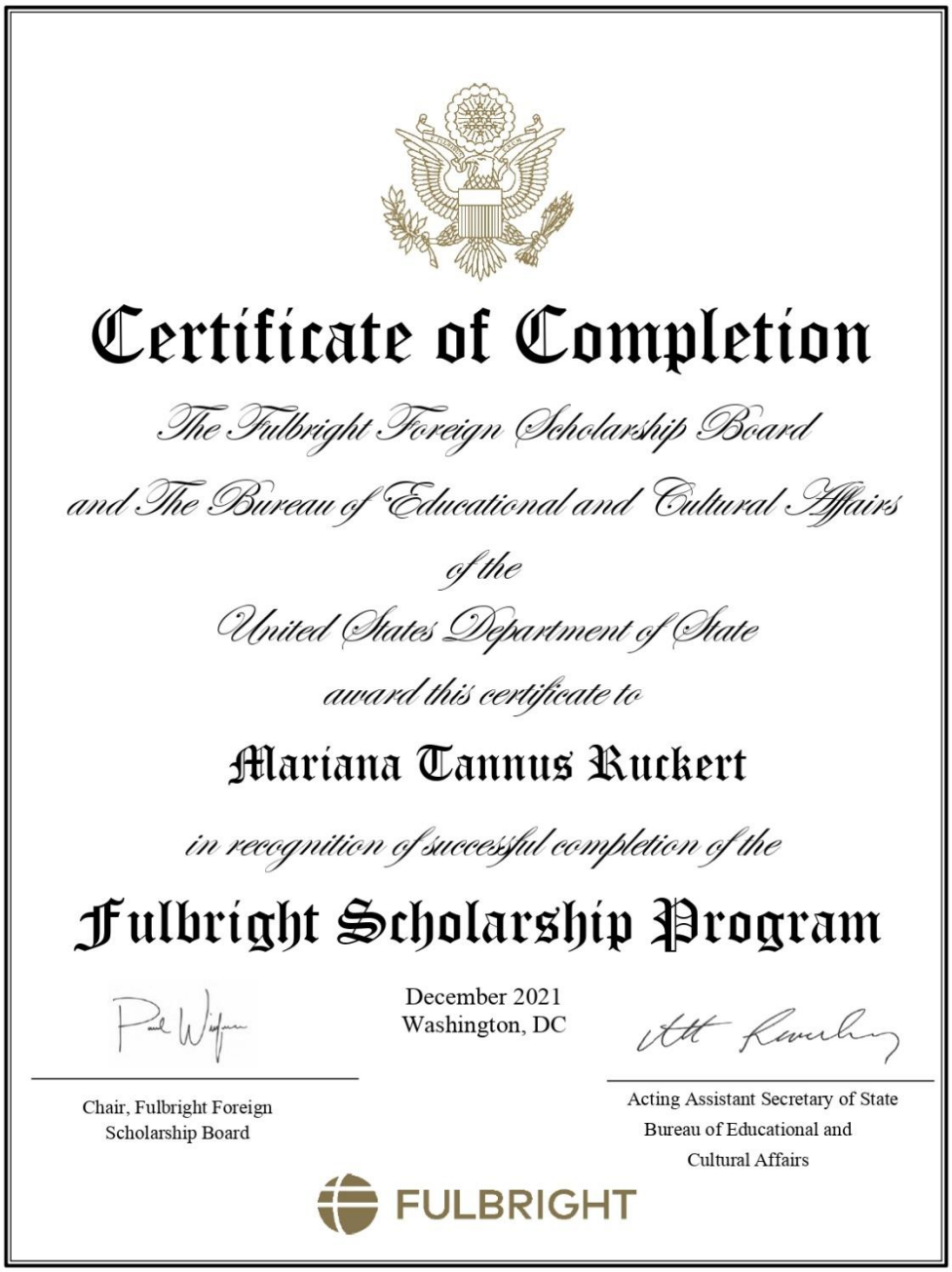
(Please, select one of the options)

[X] I have read the additional information and I hereby accept the grant. [ ] I decline the grant and I am returning all materials promptly to the Comission.

Mariana T. Ruckert
Grantee's signature

12/02/2020
Date (mm/dd/yyyy)

Comissão Fulbright Brasil



Certificate of Completion

The Fulbright Foreign Scholarship Board

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December 2021
Washington, DC

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2. Helmsley Fellowship (2022 Cold Spring Harbor Laboratory Course on Pancreatic Cancer)

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April 18, 2022

Congratulations! You have been awarded a **Helmsley Fellowship** to help defray the cost of your participation in the 2022 CSHL course on Pancreatic Cancer. The amount of your financial aid is reflected in your invoice as *less amount waived*.

The Helmsley Charitable Trust (http://helmsleytrust.org) is interested in supporting interdisciplinary science as well as scientists who cross-train to become more interdisciplinary in their research. As part of its generous contributions to the CSHL Meetings & Courses program, the Trust underwrites fellowships for course trainees who make significant disciplinary changes in part by taking a CSHL course (e.g., from a non-biology field like physics or engineering into biology). We are pleased to announce that you have been selected to receive one of these fellowships in recognition of the cross-disciplinary nature of your academic and scientific career to date.

Upon accepting this award and participating in the course, you will be contacted by CSHL twice: once at the end of this calendar year and once at the end of next year. The purpose will be to help assess the overall impact the course had on your research and scientific career. You will be directed to brief, anonymous surveys, and your responses will be aggregated with those of other Helmsley awardees for program tracking, evaluation, and reporting purposes. We would appreciate your participation in these surveys so that we may continue to award Helmsley Fellowships to future course trainees.

Once again, congratulations! Please feel free to contact me or the Course Registrar if you have any questions or concerns.

Sincerely,

David Stewart, Ph.D.
Executive Director, Meetings & Courses
Phone: 516-367-8346
Email: stewart@cshl.edu

SCIENTIFIC PRODUCTION

Papers in peer-reviewed journals

Ruckert, M.T., de Andrade, P.V., Santos, V.S., Silveira, V.S. Protein tyrosine phosphatases: promising targets in pancreatic ductal adenocarcinoma. *Cell. Mol. Life Sci*. 76, 2571–2592 (2019). https://doi.org/10.1007/s00018-019-03095-4;

Manley, S.J., Olou, A.A., Jack, J.L., **Ruckert, M.T.**, Walsh, R. M., Eades, A. E., Bye, B. B., Ambrose, J., Messaggio, F., Anant, S., VanSaun, M. N. Synthetic adiponectin-receptor agonist, AdipoRon, induces glycolytic dependence in pancreatic cancer cells. *Cell Death Dis* 13, 114 (2022). https://doi.org/10.1038/s41419-022-04572-8;

Olou, A.A., Ambrose, J., Jack, J.L., Walsh, R. M., **Ruckert, M.T.**, Eades, A. E., Bye, B. B., VanSaun, M. N. Inhibition of SHP2 implicates a crucial role for PDHA1 in adipocyte maintenance and pancreatic cancer cell crosstalk. (Submitted and under review at *Journal of Cell Communication and Signaling*);

Santos, V. S., Vieira, G. M., de Andrade, P. V., Nagano, L. F., **Ruckert, M. T**., Silveira, V. S. Atypical phosphatase DUSP11 enhances gemcitabine induced cell death in pancreatic adenocarcinoma by suppressing nc886-mediated NF-kB activation. (In preparation; to be submitted at *Cellular and Molecular Life Sciences*)

Abstracts published

Andrade, P. V., **Ruckert, M. T.**, Biagi Júnior, C. A. O., Silveira, V. S. Abstract C51: Targeted inhibition of DUSP1 and DUSP6 suppresses pancreatic adenocarcinoma

cells' growth and glucose metabolism via SAPK/JNK pathway activation. *Cancer Res* 15, 79 (2019). https://doi.org/10.1158/1538-7445.PANCA19-C51;

Ruckert, M. T., Biagi Júnior, C. A. O., Silveira, V. S. DUSP6 impacts expression pattern of glucose metabolism-related genes in pancreatic cancer cells. 65º Brazilian Congress of Genetics E-Book (2019);

Gomes, I. M. O., Andrade, P. V., **Ruckert, M. T.**, Cruzeiro, G. A. V., Borges, K. S., SILVEIRA, V. S. Interaction between DUSP1 and YAP1 in pancreatic ductal adenocarcinoma. 65º Brazilian Congress of Genetics E-Book (2019);

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Ruckert, M.T., Bye, B. B., VanSaun, M. N., Silveira, V. S. Abstract 157: DUSP6 modulates migration and glycolysis in PDAC cells. *Cancer Res* 15, 82 (2022). https://doi.org/10.1158/1538-7445.AM2022-157;

Protein tyrosine phosphatases: promising targets in pancreatic ductal adenocarcinoma

Cellular and Molecular Life Sciences
https://doi.org/10.1007/s00018-019-03095-4

REVIEW

Protein tyrosine phosphatases: promising targets in pancreatic ductal adenocarcinoma

Mariana Tannús Ruckert[1] · Pamela Viani de Andrade[1] · Verena Silva Santos[1] · Vanessa Silva Silveira[1]

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Abstract
Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer. It is the fourth leading cause of cancer-related death and is associated with a very poor prognosis. KRAS driver mutations occur in approximately 95% of PDAC cases and cause the activation of several signaling pathways such as mitogen-activated protein kinase (MAPK) pathways. Regulation of these signaling pathways is orchestrated by feedback loops mediated by the balance between protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), leading to activation or inhibition of its downstream targets. The human PTPome comprises 125 members, and these proteins are classified into three distinct families according to their structure. Since PTP activity description, it has become clear that they have both inhibitory and stimulatory effects on cancer-associated signaling processes and that deregulation of PTP function is closely associated with tumorigenesis. Several PTPs have displayed either tumor suppressor or oncogenic characteristics during the development and progression of PDAC. In this sense, PTPs have been presented as promising candidates for the treatment of human pancreatic cancer, and many PTP inhibitors have been developed since these proteins were first associated with cancer. Nevertheless, some challenges persist regarding the development of effective and safe methods to target these molecules and deliver these drugs. In this review, we discuss the role of PTPs in tumorigenesis as tumor suppressor and oncogenic proteins. We have focused on the differential expression of these proteins in PDAC, as well as their clinical implications and possible targeting for pharmacological inhibition in cancer therapy.

Keywords Pancreatic cancer · Tyrosine phosphatases · Dual-specificity phosphatases · Molecular targets

Pancreatic ductal adenocarcinoma overview

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer; it develops from ductal cells of the exocrine portion of the organ [1]. Currently, it is the fourth leading cause of cancer-related death in developed countries, and this disease is predicted to become the second leading cause of cancer death by 2030 [2].

PDAC is associated with an extremely poor prognosis mainly because, in 80% of cases, patients are diagnosed during the advanced stages of the disease [3]. The symptoms are nonspecific, or in some cases, there are no symptom manifestations, and the lack of specific and sensitive tumor markers contributes to the late diagnosis [4]. Therefore, only 20% of patients can undergo surgery, because most cases present a metastatic diagnosis, making surgical resection, which is the most effective treatment strategy for PDAC, unfeasible [5].

The poor prognosis is also explained by reduced drug penetration in the dense pancreatic stroma and by low levels of tumor vascularization, leading to a high level of chemoresistance in tumor cells [6]. Together, these factors contribute to the abysmally poor global 5-year survival rate of only 5%. This characterizes PDAC as one of the most aggressive malignancies, and annually, it is estimated that approximately 340,000 people die of the disease worldwide [7]. The development of invasive PDAC is preceded by acinar–ductal metaplasia (ADM) or by neoplastic precursor lesions such as pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasia, intraductal papillary mucinous neoplasia (IPMN) and atypical flat lesions [8]. The most common precursor lesions, PanINs, comprise a spectrum of neoplastic lesions with morphological

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Synthetic adiponectin-receptor agonist, AdipoRon, induces glycolytic dependence in pancreatic cancer cells

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ARTICLE OPEN

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Synthetic adiponectin-receptor agonist, AdipoRon, induces glycolytic dependence in pancreatic cancer cells

Sharon J. Manley[1,3], Appolinaire A. Olou[1,3], Jarrid L. Jack[1], Mariana T. Ruckert[1], R. McKinnon Walsh[1], Austin E. Eades[1], Bailey A. Bye[1], Joe Ambrose[1], Fanuel Messaggio[2], Shrikant Anant[1] and Michael N. VanSaun[1]✉

Obesity creates a localized inflammatory reaction in the adipose, altering secretion of adipocyte-derived factors that contribute to pathologies including cancer. We have previously shown that adiponectin inhibits pancreatic cancer by antagonizing leptin-induced STAT3 activation. Yet, the effects of adiponectin on pancreatic cancer cell metabolism have not been addressed. In these studies, we have uncovered a novel metabolic function for the synthetic adiponectin-receptor agonist, AdipoRon. Treatment of PDAC cells with AdipoRon led to mitochondrial uncoupling and loss of ATP production. Concomitantly, AdipoRon-treated cells increased glucose uptake and utilization. This metabolic switch further correlated with AMPK mediated inhibition of the prolipogenic factor acetyl coenzyme A carboxylase 1 (ACC1), which is known to initiate fatty acid catabolism. Yet, measurements of fatty acid oxidation failed to detect any alteration in response to AdipoRon treatment, suggesting a deficiency for compensation. Additional disruption of glycolytic dependence, using either a glycolysis inhibitor or low-glucose conditions, demonstrated an impairment of growth and survival of all pancreatic cancer cell lines tested. Collectively, these studies provide evidence that pancreatic cancer cells utilize metabolic plasticity to upregulate glycolysis in order to adapt to suppression of oxidative phosphorylation in the presence of AdipoRon.

Cell Death and Disease (2022)13:114; https://doi.org/10.1038/s41419-022-04572-8

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer death with a dismal five year survival rate of 9% [1]. Around 90% of pancreatic cancer is the more lethal pancreatic ductal adenocarcinoma (PDAC) [2]. The current verified risk factors for pancreatic cancer include smoking, alcohol consumption, aging, pancreatitis, and obesity [3, 4]. Of these, the rising rate of obesity is positively correlated with the increasing incidence of pancreatic cancer [5–8]. While obesity harbors dysregulation of multiple physiological systems, we have demonstrated that dysregulation of adipose-derived cytokines, termed adipokines [9], directly influences pancreatic cancer proliferation, migration, and tumor growth [10–12]. Other studies have demonstrated that adipokines have additional pleiotropic roles in cancer progression, including acting as inflammation mediators, growth factors, and angiogenic factors [9, 11, 13, 14].

Adiponectin is an adipokine secreted at high levels from adipose tissue of the lean population [15–18]. Cellular signaling in response to binding of adiponectin to its receptors, AdipoR1 and AdipoR2 [19–22], is mediated by APPL1 (adaptor protein-containing pleckstrin homology domain, phosphotyrosine-binding domain, and leukine zipper motif), an adaptor protein known to link adiponectin receptors to downstream adiponectin-signaling pathways [23]. Key downstream adiponectin mediators include the energy-sensing protein AMP kinase (AMPK) and the peroxisome proliferator-activated receptor (PPAR) [24]. As an inhibitor of anabolic processes and an activator of catabolic processes such as lipid breakdown, adiponectin can suppress proliferation and induce an anticancer response. Accordingly, clinically low plasma adiponectin levels are associated with increased cancer risk [25–27] and adiponectin is considered to be antitumorigenic from studies that showed it reduced tumorigenic activity in breast [28–31], endometrial [32–34], colorectal [35–37], prostate [38–41], and pancreatic cancers [25]. The mechanism by which adiponectin exerts its antitumorigenic effects is not clear, but while adiponectin-mediated AMPK signaling has been implicated in certain cancers [41], alternate mechanisms through JAK-STAT suppression have been shown [42, 43]. Additionally, adiponectin may also directly affect the mitogen-activated protein-kinase (MAPK) pathway via activation of the stress-induced p38 MAPK or suppression of pERK [43].

AdipoRon is a synthetic analog that mimics the actions of adiponectin by stimulating both AdipoR1 and AdipoR2 [22] to subsequently activate an increase in p-AMPK and PPAR similar to endogenous adiponectin [22]. Animal studies using AdipoRon found that, just like adiponectin, it ameliorates insulin resistance, diabetes, and inflammation with a maximal circulating value of 11.8 μM [22]. Furthermore, AdipoRon also has antiproliferative and anticancer properties [44]. With regard to pancreatic cancer, we demonstrated that the effects of adiponectin and AdipoRon on cancer cells were partially due to suppression of the STAT3 signaling pathway as well

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Inhibition of SHP2 implicates a crucial role for PDHA1 in adipocyte maintenance and pancreatic cancer cell crosstalk

Appolinaire A. Olou[1], Joe Ambrose1, Jarrid L. Jack[1], McKinnon Walsh[1], **Mariana T. Ruckert[1]**, Austin E. Eades[1], Bailey A. Bye[1], and Michael N. VanSaun[1]

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Running Title: SHP2 promotes ROS-driven adipogenesis
Keywords: SHP2, PDHA1, ROS, adipocytes, cancer
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Abstract: Adipocytes are the most abundant cell type in the adipose tissue, and their dysfunction is a significant driver of obesity-related pathologies, such as cancer. The mechanisms that 1) drive the maintenance and secretory activity of adipocytes and 2) mediate the cancer cellular response to the adipocyte-derived factors are not fully understood. To address that gap of knowledge, we investigated how alterations in Src homology region 2-containing protein (SHP2) would alter adipocyte function and tumor crosstalk. We found that phospho-SHP2 levels are elevated in adipose tissue of obese mice, obese patients, and differentiating adipocytes. *In vitro* studies demonstrated that SHP2 partially co-localized with PDHA1, positively associated with PDHA1 expression and promoted a reactive oxygen species (ROS)-driven adipogenic program. Accordingly, this SHP2-PDHA1-ROS regulatory axis was crucial for adipocyte maintenance and secretion of interleukin-6 (IL-6), a key cancer-promoting cytokine. Mature adipocytes treated with an inhibitor for SHP2, PDHA1, or ROS exhibited an increased level of pro-lipolytic and thermogenic proteins, corresponding to an increased glycerol release, but a suppression of secreted IL-6. A functional analysis of adipocyte-cancer cell crosstalk demonstrated a reduced growth of pancreatic cancer cells exposed to conditioned media (CM) from mature adipocytes previously treated with inhibitors for SHP2/PDHA1/ROS. Importantly, PDAC cell growth stimulation in response to adipocyte CM correlated with PDHA1 induction and was suppressed by a PDHA1 inhibitor. The data point to a novel role for 1) SHP2-PDHA-ROS in adipocyte maintenance and secretory activity and 2) PDHA1 as a regulator of the pancreatic cancer cells response to adipocyte-derived factors.

Atypical phosphatase DUSP11 enhances gemcitabine induced cell death in pancreatic adenocarcinoma by suppressing nc886-mediated NF-kB activation

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Keywords: DUSP11, atypical phosphatase, gemcitabine response, pancreatic cancer.
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Abstract: Pancreatic ductal adenocarcinoma (PDAC) represents one of the deadliest cancers among all solid tumors. First-line treatment relies on gemcitabine (Gem) and despite treatment improvements, refractoriness remains a universal challenge. Attempts to decipher how feedback-loops control signaling pathways towards drug resistance have gained attention in recent years. In this study, a CRISPR/Cas9-based phenotypic screen was performed to identify targets from the dual-specificity phosphatases (DUSP) family acting in Gem response in PDAC cells. The approach revealed the atypical RNA phosphatase DUSP11 as a potential target, whose inhibition creates vulnerability of PDAC cells to Gem. DUSP11 genetic inhibition impaired cell survival and promoted apoptosis, synergistically enhancing Gem cytotoxicity. In silico transcriptome analysis of RNA-seq data from PDAC human samples identified NF-κB signaling pathway highly correlated with DUSP11 upregulation. *In vitro*, Gem-induced NF-κB phosphorylation was blocked upon DUSP11 inhibition. Lastly, we attempted to identify which molecules would be targeted by DUSP11 to mediate NF-κB regulation and observed a significant increase in vault RNAs expression in DUSP11-/- cells. Particularly, we observed a sustained expression of nc886 (aka vtRNA 2-1) after Gem exposure in DUSP11-/- cells indicating that this non-coding RNA could mediate DUSP11 function to modulate NF-κB signaling cascade. In conclusion, this study provides new insights on DUSP11 role in RNA biology and Gem response in PDAC cells.

EXTRACURRICULAR ACTIVITIES

Organizing committee member of the XXIV Genetics Summer Course. Ribeirão Preto Medical School, University of São Paulo. Ribeirão Preto, São Paulo, Brazil. 2019.

Participation in the 2nd High Content Screening Brazilian Meeting. Ribeirão Preto, São Paulo, Brazil. July 15 to July 19, 2019.

Participation in the 65th Brazilian Congress of Genetics. Águas de Lindóia, São Paulo, Brazil. September 17 to September 20, 2019. Poster presentation entitled "DUSP6 impacts expression pattern of glucose metabolism-related genes in pancreatic cancer cells".

Organizing committee member of the XXV Genetics Summer Course. Ribeirão Preto Medical School, University of São Paulo. Ribeirão Preto, São Paulo, Brazil. 2020.

Participation in the Cancer Biology Research Program Meeting. Olathe, Kansas, EUA. September 1st, 2021. Poster presentation entitled "Pharmacological Inhibition of DUSP6 Reveals a Potential Role in PDAC Metastasis".

Participation in the American Pancreatic Association Annual Meeting. Miami, Florida, EUA. November 3rd to November 6, 2021. Poster presentation entitled "Pharmacological Inhibition of DUSP6 Reveals a Potential Role in PDAC Metastasis".

Participation in the 2nd Annual KUCC Research Week. Kansas City, Kansas, EUA. November 15 to November 19, 2021. Poster presentation entitled "DUSP6 modulates PDAC metastatic process through glycolysis regulation". Prize of US$200 for Cancer Biology Highest Ranked Abstracts.

Participation in the American Association for Cancer Research Annual Meeting. New Orleans, Louisiana, EUA. April 9 to April 13, 2022. Poster presentation entitled "DUSP6 modulates migration and glycolysis in PDAC cells".

Participation in the Cold Spring Harbor Course on Pancreatic Cancer 2022. Lloyd Harbor, New York, EUA. June 15 to June 23, 2022. Participation partially funded by The Helmsley Charitable Trust.