KLF6 Mediates de novo Ornithine Synthesis and Polyamine Production in Pancreatic Ductal Adenocarcinoma

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KLF6 Mediates *de novo* Ornithine Synthesis and Polyamine Production

in Pancreatic Ductal Adenocarcinoma

A dissertation presented

by

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I have reviewed this thesis. It represents work done by the author under my supervision.

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OVERVIEW

Pancreatic ductal adenocarcinoma (PDAC) is a malignancy that has one of the lowest 5-year survival rates amongst all cancers due to its aggressiveness and low progress in elucidating new therapies for this disease. A recent discovery from our lab is the reliance of PDAC on glutamine for the production of polyamines within the tumor microenvironment, a process that will support cancer cell proliferation and tumor growth. The transcriptional factor KLF6, which is highly expressed in PDAC, was identified to be essential for *de novo* ornithine synthesis (DNS), an unconventional pathway that PDAC cells employ to use glutamine, instead of arginine, for polyamine synthesis and to promote cell survival. However, the full mechanism that leads to KLF6 activation and its complete function in DNS and polyamine synthesis remain to be elucidated. In this study, we aim to investigate the role of KLF6 in polyamine synthesis and PDAC tumorigenesis. Initially, we observed that KLF6 is strongly expressed in murine tumor pancreatic tissue in comparison to normal pancreatic tissue. We then explored the mutational frequency of KLF6 in human and murine PDAC cell lines that could lead to its active state, but no mutation was found. We optimized antibody recognition of both murine and human KLF6 protein and immunoprecipitation conditions, however, no phosphorylation of KLF6 was observed upon modulation of EGF signaling via immunoblot analysis. Upon pharmacological inhibition of MEK, we detected no significant change in KLF6 protein expression in human PDAC cell lines, suggesting that KRAS downstream signaling is influencing KLF6 activity via post-translational modifications. Additionally, we optimized *in vitro* and immunoprecipitation conditions for Coomassie staining identification of KLF6 and mass spectrometry analysis. This work sheds a new light for potential therapeutic targets of PDAC and prepares the field for future work investigating KLF6 and tumorigenesis.

Keywords: KLF6, DNS, PDAC

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LIST OF ABBREVIATIONS

DNA   deoxyribonucleic acid

DNS   *de novo* ornithine synthesis

EGF   epidermal growth factor

ERK   extracellular signal-regulated kinase

IHC   immunohistochemistry

IP   immunoprecipitation

KLF6   kruppel-like factor 6

KRAS   kirsten rat sarcoma virus

LC-MS   liquid chromatography-mass spectrometry

MAPK   mitogen-activated protein kinase

MEK   mitogen-activated protein kinase

mRNA   messenger ribonucleic acid

p53   transformation-related protein 53

PanIN   pancreatic intraepithelial neoplasia

PDAC   pancreatic ductal adenocarcinoma

PI3K   phosphatidylinositol 3-kinase

PTM   post-translational modification

RT-PCR   real-time quantitative polymerase chain reaction

shRNA   small hairpin ribonucleic acid

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1 INTRODUCTION

1.1 Tumor microenvironment

The fundamental biological features of cancerous cells, which can happen in any tissue, are the capacity to indiscriminately proliferate, infiltrate, and evade apoptosis (1). However, the cellular components of a tumor is not only based on cancer cells, but also on endothelial cells and pericytes, immune cells, and cancer-associated fibroblasts (2). As the interaction between these different cell populations in the tumor microenvironment (TME) are the subject of ongoing research, better understanding of their roles could prove necessary to reveal tumor resistance to currently available therapeutic drugs (3,4). It is well known that tumoral components and its pathophysiological mechanisms can be more complex than that of healthy tissues (1). Divergent metabolic behavior between these different cell populations is equally intriguing. For example, the limited availability of nutrients in the TME for consumption between these cells and how excretory products are addressed as well as the mechanisms underlying cancer cell recruitment of pro-tumorigenic cells and their evasion of cytotoxic immune cells are currently being studied in detail.

Non-cancerous cells within the TME are regarded as not having mutations in the DNA, or in other words, are genetically stable, but they have a role and can influence both cancer cells and the tumor metabolic behavior (5). The immune cells present in the TME are considered one of the most complex due to heterogeneity in their response. A wide variety of T cells (e.g., helper, regulatory, cytotoxic, and memory T cells) as well as macrophages (e.g., M1 and M2) can be found in the TME, where they can have divergent impacts on clinical outcomes. M1 macrophages are typically considered to have anti-tumor properties, whereas M2 macrophages, also known as tumor-associated macrophages (TAMs), are known to promote various pro-tumorigenic effects in cancer. These effects can include regulation of

angiogenesis and lymph angiogenesis, suppression of the immune system, induction of hypoxia, promotion of tumor cell proliferation and facilitation of metastasis (6,7).

Fibroblasts can constitute a significant portion of the tumor depending on the tissue. In the context of stress or tissue injury, fibroblasts phenotypically transform into myofibroblasts, which are also known as cancer-associated fibroblasts (CAFs), leading to a dense desmoplasia and an increase in the likelihood of cancer formation. However, CAFs can also originate from other cell types such as endothelial, smooth muscle and stem cells (2). In the case of pancreatic tissue, there are reports of CAFs originating from pancreatic stellate cells (PSCs), which are myofibroblast-like cells located in the surrounding areas of the exocrine functional units of the pancreas (8). Additionally, CAFs are able to secrete cytokines that attract cells to form appropriate conditions for tumor growth and evade immune response, as observed in mouse models of cancer and in studies that suggest cancer cells utilizing fibrotic components towards tumorigenesis (2,9–11). Collectively, these findings indicate that the TME changes as the tumor grows, allowing each cell group to contribute significantly to tumor maintenance. Moreover, increased attention has been recently given to changes in cellular metabolism in cancer. The metabolic switch that occurs in tumorigenesis is thought to be a broad concept, despite the tissue of origin, as it is also considered to be an emerging characteristic of cancer (1). Typically, changes in the consumption of glucose and glutamine occur in the TME as the regulation of rate limiting enzymes and certain reactions are altered upon tumor growth. Nonetheless, the importance of other molecules, such as lactate, ammonia and lipids has also been revealed (12–14).

1.2 Cancer metabolism

The observation that cancer cells have altered metabolic pathways can be traced back to the early 20th century, when German biochemist Otto Warburg discovered that cancer cells have a unique way of generating energy (15). Warburg observed that cancer cells have a

propensity to undergo glycolysis, a process that transforms glucose into pyruvate to generate energy, even in the presence of sufficient oxygen (aerobic glycolysis). On the other hand, healthy cells undergo oxidative phosphorylation, a process that generates more energy by consuming the same amount of glucose, or in other words, it is less efficient. Therefore, Warburg hypothesized that the altered metabolism that occurs in cancer cells is due to a defect in the mitochondria, where oxidative phosphorylation occurs (16). However, this hypothesis has been challenged by more recent research indicating that some cancer cells are able to generate ATP through oxidative phosphorylation as well as glycolysis, with the exception of mutations in the succinate dehydrogenase (SDH) and fumarate hydratase (FH) genes or in subunits of the oxidative phosphorylation complex, which can halt its ability to undergo oxidative phosphorylation (17). Despite that, this preference for glycolysis was later named "Warburg Effect" and is considered a characteristic of cancer metabolism, where an enhanced consumption of glucose is facilitated by a group of glucose transporters (GLUTs), such as GLUT1 (18,19). Since then, other studies have noted other changes in cancer cell metabolism, such as in the pentose phosphate pathway, in the fatty acid metabolism and in the glutamine metabolism, revealing potential therapeutic targets (1,20,21). Interestingly, cancer cells consume more glutamine to meet their anabolic requirements (22). Previous research has revealed that the oncogene MYC drives this enhancement in glutamine consumption, where deficiency in glutamine leads to selective apoptosis in cells expressing Myc, highlighting the crucial role of glutamine for cancer cell survival (23,24). Previous research aimed at investigating the role of Myc in glutamine metabolism from multiple mouse models. It was revealed that Myc can control the activity of glutamine synthetase (GS) and glutaminase (GLS), suggesting that its role in tumorigenesis is not only caused by the catabolism of glutamine.

The tumor suppressor p53 was also shown the ability to control energy production and antioxidant response in cells via regulation of Glutaminase 2 (Gls2), glycolysis, reactive oxygen species synthesis and apoptosis (25,26). Moreover, mutant Ras has been found to have a role in autophagy and induce macropinocytosis by consuming nutrients from the surrounding environment as well as inducing glucose use and lactate synthesis (12,21,27).

It is well known that the development of pancreatic ductal adenocarcinoma (PDAC) and of non-small cell lung cancer (NSCLC) are dependent on KRAS mutation and p53 loss. In murine mouse models, the G12D mutation in Kras induce a preferential consumption of glucose by the tumors, a metabolic activity that is not affected by inhibition of glutaminase. However, the cells cultured *in vitro* were affected by such inhibition and demonstrated an increased usage of glutamine to replenish depleted metabolic intermediates (28). For these reasons, further understanding the glutamine metabolic changes that happen in PDAC is crucial to revealing potential therapeutic targets. This highlights the importance for *in vivo* research of the tumor as whole with involvement of the TME to further elucidate metabolic intricacies and nutrient consumption. The combination of stable isotope tracing with mass spectrometry in conjunction with gas or liquid chromatography (GC-MS, LC-MS) has further advanced the cancer metabolism field by facilitating the investigation of carbon and nitrogen sources contribution towards metabolic pathways and assessment of its metabolic dynamics, leading to an increased scientific attention in the role of metabolism in cancer cell behavior (29–31).

1.3 Polyamine Metabolism

The native polyamines, putrescine, spermidine, and spermine, are small polycationic alkylamines that are found within cells at millimolar concentrations. With the ability to interact with macromolecules such as RNA, DNA and proteins, they play key roles in cellular processes associated with proliferation, migration and survival, such as translation, transcription,

chromatin remodeling, autophagy, metastasis, tumorigenesis, and immune system function (32–40).

Polyamine homeostasis is a process that is tightly regulated by a balance between biosynthesis, catabolism and transport. In specific cell types, the transportation of polyamines into secretory vesicles contributes to the presence of polyamines within the surrounding microenvironment (41). In fact, changes in polyamine homeostasis can happen in response to microenvironmental factors, including hypoxia, the microbiota and diet (42–45). For cancer cells to sustain persistent proliferation, it is imperative to maintain elevated intracellular levels of polyamines (46). This is achieved through a combination of augmented biosynthesis, enhanced transport, and reduced catabolism that is facilitated by oncogenes, such as KRAS, MYC, BRAF, FOS, and JUN (47–52). One essential aspect of polyamine metabolism involves modifying the translation initiation factor eIF5A through a process known as hypusination (53).

Various cancers exhibit heightened polyamine levels compared to normal cells, particularly pancreatic cancer (54). In fact, even the basal levels of spermidine in normal pancreatic tissue surpass those found in any other mammalian tissue (55,56). Previous studies have indicated that pancreatic cancers heavily rely on polyamines (57). Both KRAS (a gene mutated in over 90% of PDAC) and MYC (a gene that has a copy number gain in over 50% of human PDAC cell lines) act as upstream regulators that contribute to the activation of polyamine metabolism. It is well-established that modifications in these genes lead to elevated intracellular polyamine levels, ultimately fueling tumor growth (32,47,50). Interestingly, MYC directly regulates the expression of key genes involved in polyamine biosynthesis, namely, ornithine decarboxylase (ODC; encoded by ODC1) and S-adenosylmethionine decarboxylase (AMD1). These two enzymes serve as the rate-limiting steps of polyamine synthesis (47,58). This regulation by oncogenes implies that targeting polyamine metabolism could serve as a promising therapeutic approach for treating cancer. Although there is substantial research on

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the impact of manipulating polyamine homeostasis in tumor cells, there is limited understanding of how polyamine-modulating agents affect non-tumor cells within the TME (53,59–61). This includes their influence on immune cell function and cancer-associated immunity. Nevertheless, the evidence indicating that polyamines possess anti-inflammatory and immunosuppressive properties suggests that strategies aimed at reducing polyamine levels may enhance the antitumor immune response (46).

1.4 Pancreatic Ductal Adenocarcinoma

Within the last few years substantial progress has been made in detecting and treating cancer, leading to a considerable increase in survival rates for many cancer patients. Despite recent advances, pancreatic cancer has one of the lowest 5-year survival at about 11%, which makes it the 3rd cause of cancer mortality when gender-specific breast and prostate cancers are excluded (62). In the United States there are 64,050 estimated new cases and 50,550 estimated deaths for 2023, making pancreatic cancer one of the deadliest cancers (62). While only representing a small fraction of all cancer diagnoses, it is set to become the second leading cause of cancer deaths by 2040 (63). The poor prognosis and aggressiveness of PDAC is strongly associated with its late diagnosis, microenvironmental factors, and cancer cell intrinsic alterations (64).

PDAC commonly originates in the head of the pancreas with surrounding tissues including spleen, peritoneal cavity and lymphatics being infiltrated, and most often with metastasis to the lungs and liver. The disease is characterized by the presence of a dense stroma of fibroblasts and inflammatory cells, called desmoplasia, which makes it difficult to identify different cell populations, as the tumor is mainly composed of stroma (65). Histopathologic and clinical studies have characterized three PDAC precursor lesions: pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasm (MCN), and intraductal papillary mucinous neoplasm (IPMN) (66,67). The majority of PDAC cases comes from PanINs, which are

microscopic lesions of dysplasia (66). Pancreas specimens from autopsy studies and surgical resection cases have suggested that PanINs are common in older adults. An increased incidence of PanINs in patients with PDAC initially suggested their biologic relationship. PanINs displays a range of divergent morphological alterations relative to normal ducts that represent graded stages of increasingly dysplastic growth (66,68). A variety of molecular profiling studies have subsequently reinforced the PanIN-to-PDAC progression model through identification of an increasing number of genetic modifications in higher grade PanINs (68–76). The heterogeneity of the tumor, genetic complexity and low cellular tumor composition allow it to become resistant to therapies, which have mostly been unsuccessful in clinical trials (77,78). So, it is critical to fully understand the metabolic mechanisms of this disease and elucidate potential therapeutic targets to inform the future development of an effective therapy.

Molecular events have been linked with defined histopathologic stages of PDAC progression, driven by accumulation of genetic alterations and development of a distinctive microenvironment (79). The driving oncogene in PDAC arises from an activating mutation in the G12 residue of KRAS (KRAS^{G12D}) which is found in more than 90% of tumors (80). This results in abnormal downstream signaling through pathways such as mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) that are responsible for fundamental physiological cellular functions, such as survival and growth (81). KRAS protein has long been considerable to be chemically intractable or undruggable due to its small size, relatively smooth and shallow surface and lack of drug binding pockets (82). Recently, drugs have been developed that target KRAS G12C (83,84), which is common in lung cancer, as well as G12D (85,86), which is frequent in PDAC. These drugs are entering the clinic, including alternative therapeutic methods such as a pan-KRAS drug (87). Nonetheless, from what was observed in animal studies and lung cancer clinical trials, primary and secondary resistance are expected to arise (88,89).

KRAS mutation occurs in low grade PanIN lesions (PanIN-1A) and is an early event in malignant transformation into PDAC. Whole exome sequencing of pancreatic tumors revealed alterations in TP53, SMAD4, and CDK2NA, characterized as genetically complex with chromosomal rearrangements which contributes to treatment resistance of the disease (77). Consistent with this finding, mouse models of pancreatic cancer driven by a pancreas-specific Cre and a latent knock-in allele of oncogenic Kras (LSL-KrasG12D; PdxCre) have a long latency or require the loss of tumor suppressor genes (TP53, CDKN2A, SMAD4) to be able to develop a tumor (90,91). While many of these genetic alterations have been validated in PDAC pathogenesis, major lingering questions center on how these mutations contribute to the tumor biological features of the neoplasms.

Our previous work demonstrates that PDAC tumors enhance transcriptional pathways regulating nitrogen metabolism with an increased urea cycle flux for the disposal of excess nitrogen (92). Therefore, a new approach for targeting the metabolic dependencies of this tumor and the development of an effective therapy for this disease is urgently needed. Our group has robust data demonstrating that PDAC tumor microenvironment is deficient in arginine, leading to glutamine-derived production of polyamines, independent of the urea cycle, via *de novo* ornithine synthesis (DNS), a reaction that is largely restricted to early infancy in humans (93). Maintenance of polyamine synthesis, such as putrescine, spermidine and spermine, allows for cell survival and tumor growth, as polyamines are involved in chromatin modification and nucleic acid synthesis (46). This early data contrasts the established role of arginine as the main nitrogen contributor for polyamine synthesis and displays the metabolic rewiring occurring at TME level. Most importantly, our group has recently identified Krüppel-like Factor 6 (KLF6) to be a downstream effector of cellular signaling that is induced by KRAS[G12D] (94), possibly through post-translational modifications (PTMs), however, the mechanisms and the types of PTMs that lead to KLF6 activation is unknown.

1.5 Kruppel-like Factor 6

The Krüppel-like factors (KLFs) are a family of zinc finger DNA-binding proteins encoded by the KLF genes in humans (95). The C-terminal region of all human KLF proteins consists of three Cys2-His2 (C2H2) repeats, which form a highly conserved zinc finger domain involved in DNA binding. These repeats exhibit a preference for binding to specific motifs such as the "GC-box" or "CACCC", typically found in promoter regions (96). Conversely, the N-terminal domain facilitates interactions with various other proteins, including coactivators, corepressors, transcriptional factors, and chromatin-modifying enzymes (96). Consequently, the functional diversity of KLFs is determined by the characteristics and interactions mediated by their N-terminal domain. The KLF proteins play crucial roles in regulating various cellular processes, including cell differentiation, metabolism, proliferation, injury responses and cell survival (95). Therefore, any disruption in KLF function can lead to disturbances in cellular homeostasis, potentially contributing to the development of pathological conditions (95). KLF6 is a remarkable example of that by functioning as a transcriptional activator, where its expression is modulated depending on the disease, primarily due to alternative splicing events that generate isoforms with specialized functions (97). The human KLF6 gene consists of four exons located on the short arm of chromosome 10 (10p15) and can be transcribed into seven distinct transcripts. However, only three of these transcripts are believed to be translated into proteins.

The full-length KLF6 is designated as the primary transcript, which generates a protein consisting of 283 amino acids of ~32 kDa in molecular weight. Initially discovered in the placenta as a core promoter-binding protein (CPBP) involved in regulating the expression of pregnancy-related genes, it is also known by alternative names such as ZF9, core promoter element-binding protein (COPEB), B-cell-derived protein 1 (BCD1) and the suppressor of tumorigenicity 12 (ST12) (98–100). A single nucleotide polymorphism (SNP) at intervening

sequence 1-27 (G > A) generates novel splicing sites, resulting in the formation of three spliced variants: SV1, SV2, and SV3 (97). These KLF6 spliced variants exhibit variations in both length and structure as a result of utilization of distinct splicing donor and splicing acceptor sites. The full-length KLF6, guided by the nuclear localization sequences (NLS) found at the end of exon 2, localizes within the nucleus, supporting its role as a transcriptional regulator. However, in the case of KLF6-SV1 and KLF6-SV2, the NLS domain is lost due to alternative splicing. Consequently, these variants are unable to be transported into the nucleus and, instead, remain in the cytoplasm, leading to an inability to regulate the gene transcription process (97). On the other hand, KLF6-SV3 still retains the NLS domain but loses the entire exon 3. Among these three spliced variants, KLF6-SV1 has received extensive research attention, particularly in cancer, while the functions of the other two isoforms are still not fully understood (97,101). Analysis of the recent The Cancer Genome Atlas (TCGA) RNA-Seq data showed that KLF6 was not a common target for genetic alterations in cancer cases, and its deregulation in cancer could be caused by other mechanisms, potentially at the level of transcriptional or translational regulations (102,103). The central domain of KLF6 is notably rich in Ser and Thr residues, making it susceptible to PTMs, particularly phosphorylation. PTMs involve the addition of covalent moieties to amino acid residues, serving to alter, refine, and expand the intrinsic biological functions of a protein. Previous study confirmed the constitutive phosphorylation of KLF6 *in vivo* (98). Lang et al. observed increased KLF6 phosphorylation by glycogen kinase 3 beta (GSK3ß) through *in vitro* and *in vivo* 32P incorporation assays (104). Additionally, ribosomal protein S6 kinase beta-1 (S6K1) has been shown to phosphorylate KLF6 and upregulate transcriptional levels of TGFB gene (105). Several kinases have been shown to enhance the level of KLF6 phosphorylation, and, to date, a total of six phosphorylation sites have been identified via mass spectrometry-based proteomics analyses (106–112). Among these, five sites, namely Thr147-p, Ser150-p, Ser151-p, Ser171-p, and Ser192-p, are situated

within the central domain rich in Ser and Thr residues. Notably, the Ser233-p phosphorylation site is located outside of this Ser- and Thr-rich central domain (113,114). However, the exact functional implications for each site have not been comprehensively examined or systematically explored. Furthermore, four lysine acetylation sites have been reported for KLF6: Lys-209-Ac, Lys213-Ac, Lys218-Ac and Lys228-Ac, but to date, only a single ubiquitylation site has been documented, Lys66-Ub (113,115–118).

In contrast to its commonly recognized role as a tumor suppressor (119–127), multiple studies have reported the growth-promoting functions of KLF6 in cancer (128–132). For instance, in acute myeloid leukemia (AML), the pro-oncogenic fusion protein RUNX1-ETO was found to upregulate KLF6 expression (128). In AML, where KLF6 is highly expressed, it synergistically collaborates with RUNX1-ETO to enhance the expression of this fusion protein, consequently promoting leukemia development (128). In hepatocellular carcinoma (HCC), two independent studies revealed that KLF6 protected HCC cells from apoptosis, thereby facilitating tumor progression (129,130). Moreover, in ductal breast carcinoma, KLF6 was observed to colocalize with the ERBB2 oncoprotein in the nucleus, and its expression positively correlated with estrogen receptor alpha expression (131). Targeting KLF6 in breast cancer cell lines resulted in lower proliferation, highlighting its pro-oncogenic roles in breast cancer (131). Remarkably, in clear cell renal cell carcinoma (ccRCC), KLF6 expression was driven by a super-enhancer, and its perturbation through CRISPR reduced cancer cell growth both *in vitro* and *in vivo* by disrupting the mTORC1 signaling pathway and lipid homeostasis (132). KLF6 transcriptional expression is known to be upregulated in PDAC tumors in comparison to normal pancreatic tissue. Therefore, we hypothesize that KRAS-dependent post-translational modification of KLF6 is fundamental to induce DNS-mediated synthesis of polyamines and PDAC tumor growth. This could be possible via 3 mechanisms: protein stabilization, activation and nuclear localization. In this study, we will explore the role of KLF6

in KRAS-dependent *de novo* polyamine synthesis and PDAC tumor growth, as its functional activity has not been fully elucidated in PDAC. More specifically, through our innovative experimental models, we aim to optimize KLF6 protein detection in our assays and elucidate the functional roles pertaining its sites of post-translational modification occurring in human PDAC cell lines.

2 MATERIALS AND METHODS

2.1 Reagents

KLF6 antibodies used for this study were 14716-1-AP (ProteinTech), PA5-79560 (Thermo Fisher Scientific), 39-6900 (9A2, Thermo Fisher Scientific) and sc-365633 (Santa Cruz Biotechnology). Other primary antibodies used were: AKT (4691, Cell Signaling Technology), AKT pS473 (4058, Cell Signaling Technology), AKT pT308 (4056, Cell Signaling Technology), ERK1/2 (4695, Cell Signaling Technology), ERK1/2 pT202/Y204 (4376, Cell Signaling Technology) and ß-actin (sc-47778 HRP, Santa Cruz Biotechnology) for western blotting; Fluorescent secondary antibodies were anti-mouse (A-11029, Invitrogen), anti-rabbit (F-2765, A-21429, A-11037, A-21442, Invitrogen) and anti-goat (A-32849, Invitrogen). Horseradish peroxidase-conjugated secondary antibodies were anti-mouse, anti-rabbit (7076 and 7074, respectively, Cell Signaling Technology) and anti-goat (P044901, Dako). Species-matched IgG controls were MAB002 (mouse, R&D Systems), sc-2027 (rabbit, Santa-Cruz Biotechnology) and AB-108-C (goat, R&D Systems). Normal chicken serum (S-3000, Vector Laboratories) and DAPI mounting media (H-1200, Vector Laboratories) were used. For species-matched IgG control, a rabbit IgG antibody was used (2729, Cell Signaling Technology). DMSO (R&D, 240-B-010), and MEK inhibitor AZD6244 (Selleckchem) were also used for *in vitro* studies. All other reagents were purchased from Sigma Chemical.

2.2 Cell Culture

Human PDAC cell lines (AsPC-1, MIA PaCa-2, HPAC, PANC-1, BxPC-3 and SW1990) were acquired from the American Type Culture Collection (ATCC) with an exception of SUIT-2 cells which were derived from the Japanese Collection of Research Bioresources; PA-TU-8988T and PA-TU-8902 were collected from the German Collection of Microorganisms and Cell Cultures. Cells were authenticated by STR profiling at ATCC and were grown in RPMI-1640 media (Sigma) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco). HEK293T cells obtained from ATCC were grown in DMEM media containing 10% FBS and 1% penicillin-streptomycin. Every cell line tested negative for mycoplasma using LookOut Mycoplasma PCR Kit (Sigma, MP0035). All cells were maintained at 37 °C in a humidified incubator with 5% CO_2 and were used from passages number 10 in experiments.

2.3 *In vitro* Pharmacological Treatment and Inhibitors

Cells were grown with 10% FBS, then plated in 10cm dishes to 65% confluence and allowed for 24h recovery. The cells were then treated with EGF (50 ng/mL, PeproTech, #100-15) for 15min or 1h. MEK inhibitor (50nM, Selleckchem, AZD6244) was applied for either 72h or 30min after 6h or 18h starvation (RPMI-1640 or DMEM media containing 0% FBS). After treatment, the cells were harvested for protein or RNA.

2.4 Generation of Recombinant Lentivirus

HEK293T cells were used for lentivirus packaging containing psPAX2 (Addgene) and pMD2.G (Addgene) using Lipofectamine2000 Transfection Reagent (Invitrogen, 11668500). For generation of human PDAC cells or murine cells with stable KLF6 or control (Scramble) knockdown, lentivirus were packed with pLKO plasmids containing either human KLF6 shRNA #1 (TRCN0000013711 - target sequence: ACTCAGATGTCAGCAGCGAAT) targeting all KLF6 isoforms, #2 (TRCN0000013710 – target sequence:

GCTCCCACTGTGACAGGTGTT) targeting isoforms A, B and D, #3 (target sequence: CGGCTGCAGGAAAGTTTAC) targeting isoforms A and C, and #4 (target sequence: GGAGAAAAGCCTTACAGAT) targeting isoforms A, B and D, or murine Klf6 shRNA #1 (TRCN0000085632 – target sequence: TGATGAGTTGACCAGACACTT) #3 (TRCN0000218960 – target sequence: GATCAGCTCCAGCTTTAATTA) or Scramble shRNA (Addgene plasmid #26701 – target sequence: CCTAAGGTTAAGTCGCCCTCG) used as a control. Lentiviral supernatant was harvested after 48h and then applied to human PDAC cell lines for 24h for stable transduction. Puromycin (2 ug mL^{-1}, Sigma-Aldrich, P8833) or Blasticidin (10 mg mL^{-1}, Invitrogen, A11139-03) were used to select only the transduced cell lines. pMD2.G was a gift from Didier Trono (Addgene plasmid #12259), and psPAX2 was a gift from Didier Trono (Addgene plasmid #12260).

2.5 Western Blotting Analysis

Cell monolayers were rinsed with ice-cold PBS and lysed on ice using lysis buffer containing EDTA-free protease inhibitors (Roche), 50 mM HEPES KOH, pH 7.4, 40mM NaCl, 2 mM EDTA, 1.5 mM orthovanadate, 50 mM NaF, 10 mM pyrophosphate, 10 mM glycerophosphate and 1% Triton X-100. The proteins were resolved via 8-12% SDS-PAGE and were transferred to a nitrocellulose membrane or polyvinylidene difluoride (PVDF, Amersham). For the development phase, the membranes were first blocked with 5% skim milk, incubated with a respective primary antibody at 4°C overnight, and after being washed with PBS+Tween (Sigma Aldrich), the membranes were incubated with HRP anti-mouse or anti-rabbit antibodies (Cell Signaling Techlonogy) for 1h at room temperature. Signal was released using ECL luminescence detection kit (Amersham) and exposed to photographic film in a dark room. Normalised densitometry was performed on ImageJ (NIH, USA). All antibodies were used at 1:1000 dilution and ß-Actin was used as a loading control.

2.6 RNA isolation and quantitative RT-PCR analysis

Cells were lysed in RNA STAT-60 and the total mRNA was isolated according to the manufacturer's instructions, then treated with DNase I (RNase-free) and reverse-transcribed into cDNA using the SuperScript II First-Strand Synthesis System (Invitrogen, 18064071). As previously described, qPCR reactions were performed using Applied Biosystems ViiA 7 Real-Time PCR system and melt curves were checked for product specificity.(133) Reactions contained cDNA from reverse transcription of 25 ng total RNA, 150 nM of each primer and 5 uL of 2X-Jump Start SYBR Green PCR Mix (Invitrogen). All reactions were done in 10 uL volume in triplicates. Individual mRNA relative expression was normalized to Cyclophilin and calculated using the comparative CT method. PCR amplification consisted of 5 min of an initial denaturation step at 95°C, followed by 46 cycles of PCR at 95°C for 15s, 60°C for 30 s and 10 min of an initial denaturation step at 95°C. The primers for the genes investigated written 5'-3' were: human KLF6, forward TCTGGAGGAGTACTGGCAA and reverse CAGATCTTCCTGGCTGTCAA; murine Klf6, forward TCCCAATGTGTAGCATCTTCC and reverse AAGATAGCGTTCCAACTCCAG; human ODC1, forward CTGGGCGCTCTGAGATTGTC and reverse CCAGCTTCTCACAAAGGCAAC; murine Odc1, forward TTTAGCCACCTCAGATGCTTC and reverse TTACTGCTAAGGACATTCTGGAC; human OAT, forward GGTGCGCTCTCACAACCTTT and reverse GAGGTGCTCTCAAATCCTTCG; Cyclophilin, forward GGAGATGGCACAGGAGGAA and reverse GCCCGTAGTGCTTCAGCTT;

2.7 Immunoprecipitation

The entire procedure was performed under refrigerated conditions at 4°C or on wet ice. Cells grown in 10cm dishes were lysed in 700 uL of lysis buffer while kept on ice, and subsequently scraped into 1.5 mL Eppendorf tubes. The lysates were then centrifuged at

15,000g for 10 minutes at 4°C. The resulting supernatants were carefully transferred to new tubes and subjected to a second centrifugation at 15,000g for 5 minutes at 4°C. The cleared supernatants were transferred to another new tube. To ensure equal protein concentrations, normalization was performed, and the samples were brought up to a minimum volume of 1 mL using the lysis buffer. A fraction of 50 uL of the lysate was preserved for immunoblotting purposes. The remaining lysate was incubated at 4°C overnight while gently rocking with 1 to 8ug of the immunoprecipitation antibody. The immunoprecipitation antibodies were bound to protein A/G agarose beads (Thermo Fisher Scientific, 20421). Protein G agarose beads (15920-010, Invitrogen) were washed with lysis buffer, and then resuspended to result in a 1:1 slurry. Then, 80 uL of the resuspended beads were added to each immunoprecipitation sample and incubated for an additional 2h at 4°C while gently rocking. Three washes were performed using 1 mL of lysis buffer, and the beads were either centrifuged at 2,500g, followed by removal of all of the supernatant and resuspension in 1X sample buffer. The samples were then heated at 95°C for 5 minutes and stored at -80°C.

2.8 Immunohistochemistry

Paraffin-embedded tissue sections of mice pancreata were deparaffinized in xylene. The tissue sections underwent incubation with 2% hydrogen peroxide in methanol bath to prevent the activity of naturally occurring tissue peroxidases. Subsequently, the sections were rehydrated through a series of decreasing ethanol baths (100%, 95%, 70%), and underwent antigen retrieval for 10 minutes in citrate buffer solution (pH 6.0, 10 mM sodium citrate, 0.05% Tween-20). Sections were incubated with blocking buffer (5% BSA in TBS-Tween) for 1h at room temperature, and then stained with primary antibody (KLF6, 1:50 dilution, Santa Cruz) overnight at 4°C. After washing, sections were incubated with HRP-linked secondary antibodies for 1h at room temperature, and the staining was then revealed using mounting

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medium (Vector). Slides were analyzed under an Olympus microscope (Olympus, Tokyo, Japan) and representative photomicrographs were taken.

2.9 Statistical Analysis

Data are presented as means ± SEM. Paired t-tests were used for comparison between two groups, and one-way analysis of variance (ANOVA) for comparing three or more groups in GraphPad Prism 8 (GraphPad Software, San Diego, CA). Values of $P < 0.05$ were considered as statistically significant.

3 RESULTS

3.1 KLF6 is upregulated in tumor pancreatic tissue

To investigate the prevalence of KLF6 expression in PDAC tumors, we performed immunohistochemistry analysis in the pancreata of wild-type and iKRAS mice. A stronger expression of KLF6 was identified in the tumor pancreas of iKRAS mouse model in comparison to normal mouse pancreata. The higher expression of KLF6 was observed in multiple cell types and tissue regions, particularly in the nuclei of transformed ductal cells (Figure 1). These data suggest that KLF6 is upregulated in response to KRAS oncogenic signaling as a mediator for PDAC pathology and tumorigenesis.

3.2 There is no mutation in the region of interest of KLF6 in PDAC cell lines

Table 1 displays the results for sequencing a region of DNA of KLF6 that is rich in serine and threonine residues in human (SUIT-2, AsPC-1, MIA PaCa-2, BxPC-3, PA-TU-8988T and SW1990) and murine (iKRAS7017, iKRAS196, KPC7916, KPC3693) PDAC cell lines, in order to reveal any mutation that could be involved in the induction of the active state of KLF6 via PTMs. However, after optimizing the best primers for amplifying and sequencing our KLF6 genomic region of interest, no mutation was found in any of the cell lines analyzed, which suggests that, within the region rich in sites susceptible to phosphorylation by putative

kinases, there is no mutation occurring that would promote the activation of KLF6 or such mutation are rare.

3.3 Optimization of KLF6 antibody in human and murine cell lines

As KLF6 protein expression levels upon KRAS^{G12D} or upon pharmacological inhibition of MEK pathway in human and murine PDAC cell lines have not yet been uncovered, we aimed at identifying the best antibody for detection of both endogenous and exogenous KLF6 protein expression, in both human and murine cell lines. To do this, we acquired different shRNAs that, in combination, target all KLF6 isoforms, as well as qPCR primers that detect all three isoforms through binding to a conserved region. We also obtained commercially available polyclonal antibodies that detect all three KLF6 variants that are translated into proteins. We tested four anti-KLF6 antibodies for western blotting upon *in vitro* conditions that overexpress and knockdown KLF6. Figure 2A reveals inconsistent and unreliable western blotting results from two different batches of the same mouse monoclonal antibody (Thermo Fisher Scientific) against human and murine KLF6. The polyclonal rabbit antibody (Thermo Fisher Scientific) detects overexpression of human KLF6 protein in HEK293T cells in addition to multiple non-specific bands, but it does not detect reduced protein expression upon knockdown of Klf6 in the murine cell line analyzed (Figures 2B and 2C). Detection of endogenous human KLF6 was not reliable using this antibody (data not shown), which suggests that it is only able to accurately detect exogenous overexpression of human KLF6 (Figures 2B and 2C). To address this issue, we tested another polyclonal antibody (ProteinTech), which could identify both endogenous human protein being reduced upon knockdown of KLF6 and exogenous overexpression of human KLF6 (Figure 2D). However, it did not detect a reduction of murine KLF6 (Figure 2D), meaning that it specifically detects human protein. Finally, we tested a monoclonal antibody (Santa Cruz) aiming to accurately detect murine Klf6 protein. Figure 2E reveals specific detection of a reduced murine Klf6 protein expression upon knockdown in

comparison to scramble control, whereas no human protein was identified, which means that this monoclonal antibody is capable of detecting endogenous Klf6 protein expression in a murine cell line. We then conducted transient and stable overexpression of KLF6 in a PDAC cell line (SUIT-2) and transient overexpression in HEK293T to compare both polyclonal antibodies aiming to identify the most specific one (Figure 2F). We could observe that the ProteinTech antibody is the most specific one, that transient overexpression in HEK293T cells is the most efficient in comparison to other PDAC cell lines, and that stable overexpression of KLF6 in SUIT-2 and MIA PaCa-2 cell lines are the most efficient in comparison to stable overexpression in ASPC-1 cells. Overall, this data reveals that by testing different antibodies, we were able to identify one that accurately detects both endogenous and exogenous human KLF6 protein and endogenous murine Klf6 protein for our experiments.

3.4 Optimization of EGF signaling modulation

Stimulation of EGF signaling is crucial for inducing RAS and ERK signaling, and consequently induce KLF6 activation. In order to optimize signaling modulation *in vitro*, we first cultured HEK293T cells in 10% FBS or starved them with 0% FBS for 2h or 6h or 18h, or starved with 0% FBS for 2h or 6h or 18h and then applied EGF for 1h to assess how the KRAS downstream signaling would operate under such conditions. Figure 3 shows that p-AKT and p-ERK1/2 expressions are reduced the most at 6h of starvation in comparison to 2h, and that EGF stimulation for 1h yields a stronger rebound expression of pERK and pAKT after 6h of starvation, in comparison to the other groups.

3.5 No evident phosphorylation of KLF6 was found via western blotting

Immunoprecipitation of KLF6 followed by immunoblotting of pSer/Thr proteins was performed to identify phosphorylation of KLF6 upon EGF signaling stimulation, and a reduced phosphorylation level upon cell starvation. We transduced SUIT-2 cells with either GFP as a control or KLF6 and divided in three groups: control (cultured in 10% FBS), starved (0% FBS

starvation for 6h) and EGF stimulated (EGF stimulation for 1h post 6h starvation). Then, we performed immunoprecipitation with KLF6 polyclonal antibody in both transient and stable transduction of KLF6 (Figures 4A and 4B). The same experiment was replicated by performing immunoprecipitation with pan p-Ser/Thr antibody instead (Figures 4C and 4D). In both cases, the cell signaling manipulation worked as expected with a reduction in the levels of p-ERK1/2 and p-AKT (S473) upon 6h starvation; interestingly, KLF6 overexpression led to increased p-ERK1/2 levels, suggesting feed-forward regulation of signaling (Figures 4A, 4B, 4C and 4D). Furthermore, we observed an increase in the levels of both p-AKT and p-ERK1/2 upon EGF stimulation, which goes in line with our signaling optimization results. However, no specific pattern was found in the expression of immunoprecipitated bands at the level of 37 kDa upon signaling manipulation, which suggests that the bands displayed are not phosphorylated KLF6, but a smear of the heavy chain IgG. Moreover, no pattern was found with the use of a light-chain specific secondary antibody, to exclude the heavy-chain IgG from influencing the protein signal on the blot, since they are of similar sizes (~ 32-37 kDa) (data not shown). To be certain that the KLF6 immunoprecipitation has effectively worked, the immunoblots revealed an elevated increase of immunoprecipitated KLF6 protein levels in comparison to the whole-cell lysate (Figures 4A and 4B).

3.6 KLF6 transcriptional expression is not altered upon MEK Inhibition

Previous data in our laboratory demonstrated that when mutated KRAS is turned off *in vivo* or silenced in human PDAC cell lines, there is a decrease in expression levels of enzymes involved in DNS (e.g., OAT, ODC1, SRM and SMS). Furthermore, it was elucidated that both mRNA and protein levels of these enzymes are also downregulated with pharmacological MEK inhibition, so is the case when *Klf6* is knocked down *in vivo*. However, when Kras is turned off *in vivo*, only a slight reduction of Klf6 transcriptional level is observed, which strongly suggests that mutated KRAS possibly leads to MEK-mediated activation of KLF6 via PTMs.

Therefore, we analyzed whether KLF6 expression is reduced in PDAC cell lines (HPAC, PA-TU-8988T, SW1990, PANC-1, AsPC1, BxPC3, MIA PaCa-2, PATU-8902 and SUIT-2) at both transcriptional and translational levels with pharmacological *in vitro* inhibition of the MEK pathway. Effective inhibition of the MEK pathway was confirmed with a drastic reduction in phosphorylation levels of ERK1/2 (Figure 5A). We observed that cells maintained only the transcriptional expression levels of KLF6 upon MEK inhibition (Figures 5A and 5B), whereas ODC1 transcriptional level has decreased with MEK inhibition in some cell lines (Figure 5C).

3.7 Optimization of EGF cell signaling with MEK inhibition for mass spectrometry

MEK pathway is suggested to activate KLF6 via phosphorylation, therefore, optimization of its signaling modulation *in vitro* is necessary. To evaluate the optimal conditions of modulation of the suggested upstream pathway of KLF6 activation *in vitro*, MEK pathway was pharmacologically inhibited in starved SUIT-2 cells via 30-minute incubation with AZD6244 or DMSO (control group) after 1h or 15-minute stimulation with EGF. A 30-minute inhibition was sufficient to completely abrogate ERK phosphorylation (Figure 6A). A higher phosphorylation of ERK is observed after only 15 minutes of EGF incubation in comparison to 1h (Figure 6B). This is likely due to known recycling of the EGF receptor post-stimulation, which restricts the extent and duration of downstream signaling activation, making a 15-minute EGF stimulation the best condition for activation of the MEK signaling pathway signaling and, potentially, KLF6 activation. To better evaluate the transfection efficiency of KLF6 with Lipofectamine in two PDAC cell lines (AsPC-1 and SUIT-2), we performed analysis of KLF6 protein expression by using different ratios of Lipofectamine and DNA. In AsPC-1 cells, there was no significant difference in KLF6 expression with a higher concentration of Lipofectamine or DNA (Figure 6C), making the ratio of 1:1 (DNA:Lipofectamine) the most cost-effective condition. In SUIT-2 cells, there was a sizeable

change in KLF6 expression by both doubling the Lipofectamine concentration and DNA (Figure 6C), revealing the importance of using a higher ratio of both reagents for a better transient transfection efficiency in this cell line.

3.8 Optimization of protein detection via Coomassie staining for mass spectrometry

To be able to analyze which KLF6 sites are being post-translationally modified via mass spectrometry, we first need to identify a specific band corresponding to KLF6 in a gel through Coomassie staining, so that the protein can be purified for analysis. After transducing KLF6 plasmid or empty vector (EV) for 24h into SUIT-2 cells and using 10 mg of protein lysate for KLF6 immunoprecipitation, we could detect a faint band above 37 kDa that is slightly stronger in the KLF6 group in comparison to the EV group and that suggests it is KLF6 protein (Figure 7A). However, when we transduced both groups with KLF6 and used an increased protein amount of 62.5 mg for immunoprecipitation with IgG control and KLF6, we could not observe any difference in the expression of the bands right above 37 kDa by comparing the two groups (Figure 7B). In addition, no other bands around the level of 37 kDa was detected (Figure 7B), suggesting that KLF6 is, in fact, not being detected. Nevertheless, the same experiment was performed using AsPC-1 (Figure 7C) and HEK293T cells (Figure 7D), and we were able to identify two distinct bands (around 37 kDa) that were stained on the KLF6 group but not on the IgG, which suggests the presence of full-length KLF6 and one of its isoforms via Coomassie staining. To confirm that KLF6 was successfully transfected into the cells, we immunoblotted both immunoprecipitation and whole-cell lysate samples for KLF6. In both AsPC-1 and HEK293T cells, we detected a strong KLF6 protein expression in either immunoprecipitation or whole-cell lysate samples, with an exception for the samples that were used for immunoprecipitation with IgG control (Figures 7E and 7F). In order to identify the PTMs induced by the KRAS-MEK axis, we applied the optimal signaling modulation conditions of the suggested upstream pathway of KLF6 activation *in vitro*. We

pharmacologically inhibited the MEK signaling pathway in starved HEK293T cells via 30-minute incubation with AZD6244 or DMSO after a 15-minute stimulation with EGF (Figures 7G and 7H). However, only one band below the level of 37 kDa was more strongly stained in all the other groups in comparison to the IgG group (Figures 7G and 7H), potentially suggesting the detection of KLF6.

4 SUMMARY

In summary, we observed a higher expression of KLF6 in iKRAS PDAC compared to normal Kras[WT] pancreas. No mutation that could induce activation of KLF6 in the human and murine PDAC cell lines analyzed was detected, we identified the best commercially available antibody for detection of both human and murine KLF6 in western blotting and for immunoprecipitation purposes, and we optimized the best *in vitro* conditions for EGF stimulation and MEK signaling inhibition in PDAC cell lines and in HEK293T cells. Additionally, to measure KLF6 activity, we observed that the transcriptional and translational expression levels of downstream targets of KLF6, but not the transcriptional expression levels of KLF6, are changed upon MEK pharmacological inhibition, indicating that KLF6 activity is dependent on MEK-ERK signaling via PTMs. We also reached the step where we could optimize the best conditions for yielding KLF6 protein for future mass spectrometry analysis of PTMs dependent on the KRAS-MEK-ERK signaling pathway.

5 DISCUSSION AND FUTURE PERSPECTIVES

PDAC is an extremely aggressive malignancy which accounts for over 90% of pancreatic cancer cases (134). Despite advancements in therapeutic approaches, surgical resection at very early stages is the only curative option as metastatic disease is generally resistant to therapy. Therefore, there is a pressing need to explore novel therapeutic avenues and enhance our comprehension of PDAC's underlying biology. Thus far, endeavors to target

the metabolic pathways of PDAC for therapeutic purposes have not yielded favorable outcomes.

Mutations associated with PDAC drive multiple transformations in the phenotype of pancreatic cells, enabling adaptation to the challenging TME, and a rapid rate of proliferation by activating survival signaling pathways. The capacity of these cancer cells to thrive in such conditions largely stems from the rewiring of cellular metabolism, ensuring sustained proliferation even in nutrient-depleted surroundings.

Polyamines have been associated with a broad spectrum of biological pathways, encompassing differentiation, immune response, and the regulation of the cell cycle. Putrescine, spermidine, and spermine are essential polyamines in mammalian organisms. They execute key functions in nearly all forms of life, and their production is frequently inceased in various cancer subtypes. Despite our current knowledge about these small polycationic molecules, we have only scratched the surface when it comes to unraveling their significance to human pathology. The widespread elevation of polyamine metabolism detected in different cancer types is likely influenced by prominent cancer-associated genes (e.g., MYC and KRAS) that regulate polyamine synthesis. These genes known to drive tumorigenesis are able to modulate gene expression required for production of polyamines at transcriptional level (135). Similar to many other cancer types, PDAC also relies on increased levels of polyamines to maintain tumor growth. Interestingly, there is a negative correlation between expression of polyamine genes and survival rates in PDAC (136).

These findings make polyamine synthesis an interesting target for cancer therapy. Promising results have emerged from animal studies that aim to explore therapeutic effectiveness by combining polyamine synthesis inhibitor with the blockade of polyamine transport, as the effectiveness of a promising polyamine inhibitor as a standalone anticancer agent has been modest. However, the optimization of such an approach is a challenge due to

our limited comprehension of polyamine transport mechanisms. Hence, acquiring a deeper understanding of polyamine metabolism and transport in cancer cells, as well as fully identifying the role these molecules play in tumor growth, holds significant translational potential.

Previous work in our laboratory has provided insights into the involvement of polyamine metabolism in pancreatic cancer cells. The initial step of polyamine synthesis involves the conversion of the amino acid ornithine to putrescine through the action of the enzyme ODC1. Cells have multiple ways available for generating ornithine, with arginine serving as the primary source for ornithine and subsequent putrescine production. Metabolic tracing assays conducted in animal models of PDAC have shown an atypical mechanism employed by PDAC tumors to induce ornithine and polyamine biosynthesis, where PDAC cells heavily depend on the amino acid glutamine as a crucial fuel source for ornithine and polyamine biosynthesis. The conversion of glutamine into ornithine is a reversible pathway regulated by the enzyme ornithine aminotransferase (OAT). However, this reaction typically has a direction from ornithine to glutamine, making PDAC one of the exceptions alongside a specific subset of immune cells, early infancy and the fasting intestine, where the production of ornithine and polyamines primarily relies on glutamine consumption. Prior work in the laboratory has demonstrated that selectively inhibiting the conversion of glutamine to ornithine by genetic ablation of OAT effectively leads to a depletion of polyamines in PDAC cells, being as efficient in preventing polyamine production as loss of ODC1, a rate-limiting enzyme for the production of polyamines. Notably, both genetic and pharmacological interventions targeting OAT and polyamine synthesis resulted in a reduction in tumor growth *in vivo* and lower cancer cell proliferation *in vitro*. In the absence of OAT, there is a lack of capacity to redirect arginine towards ornithine as a means to maintain the production of ornithine and polyamines, even when PDAC cells are cultured in arginine-abundant medium, possibly due

to epigenetic effects, as recent research has highlighted the influential role of the polyamine-elF5A-hypusine axis in regulating epigenetic processes (60).

Previous studies implicate that KRAS is a key regulator of polyamine metabolism in cancer, which is further validated by our previous work that shows KRAS mutation increasing transcriptional levels of OAT and driving polyamine synthesis. Deletion of mutant KRAS in human and mouse PDAC cells resulted in reduced expression of OAT, ODC1, and polyamine synthases, and suppressed the synthesis of glutamine-derived ornithine and putrescine, while the synthesis from arginine remained unaffected, providing further evidence of KRAS influence on glutamine-derived polyamine production. These findings were replicated using pharmacological inhibitors targeting MEK, a substrate of KRAS. Additionally, KLF6 was identified as a downstream mediator of the KRAS signaling that plays a role in this regulatory mechanism. However, KRAS deletion does not significantly affect transcriptional levels of KLF6, which implies that PTMs may be regulating KLF6 in PDAC. This current work further explores the existence and role of a KRAS-MEK-KLF6 axis that governs glutamine-derived polyamine synthesis and metabolism in PDAC.

Our work with collaborators (137) identified a series of putative kinases (CDK, p38, ERK1/2 and JNK) that score favorably for phosphorylating the Ser171 and Ser192 sites of KLF6. A stretch of Ser/Thr-Pro sites, including Ser84, Ser92, Ser120, Thr147, and Ser151, are potential sites for phosphorylation by GSK3, as previously reported (104). Additionally, a Pro170Ser mutation, as displayed by the COSMIC database in pancreatic cancer, would create a new phosphorylation site for the activin family receptor kinases (ALKs) and CDC7, once Ser171 is phosphorylated. However, the most frequent point mutation in KLF6 occurs within its zinc finger domain at R252, near Thr255, which is the target of a kinase (PBK) that inhibits zinc finger proteins during mitosis (138). A mutation in R252 is predicted to maintain the active state of KLF6 by reducing PBK regulation. In an attempt to further understand that and

elucidate whether there was any KLF6 mutation driving its activation in PDAC cells, we sequenced human and murine PDAC cell lines, but no mutation was found, in accordance with the data available at cBioPortal that identified low levels of genetic mutation frequency occurring in KLF6 within the pancreatic tissue (102,103). This means that, in these cell lines that we tested, there is no mutation that would directly create new sites of phosphorylation or that would drive phosphorylation via adjacent Ser/Thr sites, as in the case of prostate cancer that displays a Leu169Pro mutation in KLF6 and makes Ser171 an excellent target for ERK and p38.

By evaluating how much protein levels of KLF6 are expressed upon KRAS[G12D] mutation in PDAC cell lines and how much of it is influenced by KRAS downstream signaling, an optimization of the best KLF6 antibody commercially available was necessary. We were not only able to accurately identify one good polyclonal antibody that detects endogenous and exogenous human KLF6 (and potentially its isoforms) on the western blotting and that specifically binds to human KLF6 when performing immunoprecipitation in multiple cell lines, but also a good monoclonal antibody that accurately detects endogenous murine Klf6.

To be able to identify how KLF6 phosphorylation is influenced by EGF signaling, we have observed that starving cells for 6h is the optimal length to reduce growth signaling and yield a stronger rebound of EGF signaling activation upon 1h stimulation with EGF *in vitro*. However, when immunoprecipitation of phosphorylated Ser/Thr proteins or KLF6 was performed under optimal conditions, no evident phosphorylation of KLF6 was found, even with the use of a light-chain specific antibody, which excludes heavy-chain IgG expression from the blot.

When modulating the ERK pathway via pharmacological inhibition of MEK in PDAC cell lines, we observed that KLF6 expression is not significantly altered at transcriptional level, however, its downstream targets are affected, in accordance with a previous work conducted

in our laboratory (94). As previously suggested, by confirming that KLF6 mRNA expression is not significantly altered upon MEK inhibition, we are able to infer that PTMs are involved not only in the activity regulation of KLF6, but also in its stability, as shifts in weight and protein expression levels amongst its isoforms could be detected via immunoblot upon MEK inhibition. The extent of such involvement may vary by cell line, dependent on specific intracellular processes. KLF6-SV1, for example, is known to have a dominant-negative role on full-length KLF6 (139), indicating that MEK inhibition could potentially influence other KLF6 isoforms.

Understanding the intricacies of KLF6 activation is key to fully establishing the mechanisms towards polyamine synthesis and tumorigenesis in PDAC. With the use of mass spectrometry technology, we hope to identify the specific sites of phosphorylation in KLF6 that are dependent on the KRAS-MEK signaling axis. To do this, we first optimized MEK and EGF signaling modulation in PDAC cell lines, where we identified a 30-minute inhibition on MEK to be sufficient to abrogate ERK signaling. Additionally, we noted that a 15-minute stimulation with EGF yielded the highest protein expression level of ERK phosphorylation, in accordance with established knowledge of a negative feedback in EGFR activity with prolonged stimulation (140). The transient transfection method of KLF6 *in vitro* was also established to be the most efficient in yielding the highest protein expression via western blotting analysis. When staining with Coomassie, we could suggest that bands representing full-length KLF6 and one of its isoforms were detected in lysates from AsPC-1 and HEK293T cells. However, it still needs to be validated via mass spectrometry analysis, including the sites of PTMs dependent on ERK signaling. Alternatively, we could have used MG132 to further increase protein yield for Coomassie staining detection and analyze KLF6 protein stability upon MEK inhibition.

One of the limitations of overexpressing KLF6 in cells is that it could cause disruption of physiological cellular processes, as it can lead to an imbalance in metabolic pathways, altering the cell's behavior. For example, if there is a positive feedback involving KLF6, its overexpression could allow for even further activation of the KRAS-MEK axis. On the other hand, if KLF6 is required to be part of a protein complex to exert its effects, it could lead to less KLF6 activation, as the production of a co-factor or another protein member of the complex would not follow the same amount of available KLF6. Besides all that, the method of protein transfection also affects the cell viability, with transient transfection being better accepted by the cells we used, when compared to stable transfection. Further validation work is also necessary to establish whether the polyclonal antibodies we used distinctly detect all KLF6 isoforms with the usage of different shRNAs targeting distinct isoforms. Moreover, we have not looked in detail about the nuclear localization of KLF6 via immunofluorescence or nuclear/cytoplasmic protein fractionation. It could suggest how much KLF6 is being activated via KRAS/MEK-dependent PTMs, since transcriptional factors, in general, upon activation, undergo specific changes that enable them to translocate into the nucleus. However, it would still not provide the full picture, as some of the KLF6 expressed within the nucleus could be in an inactive state.

Evidence has demonstrated that KLF6 is highly expressed in PDAC and serves key roles in the malignant progression of many cancer types, including glioblastoma multiforme, clear cell and papillary kidney cancer, acute myeloid leukemia and PDAC. Recently, KLF6-related targeted therapy has made considerable progress under the unremitting exploration of scientists.

I hypothesize that the siRNA-mediated loss or CRISPR knockout of KLF6 in pancreatic tissue could halt tumor growth, lead to spontaneous apoptosis of cancer cells as well as inhibit invasion, migration and metastasis of tumor cells via different mechanisms. As it happens to

other cancers (e.g., lung adenocarcinoma) by targeting KLF6-SV1 with siRNA, downregulation of KLF6 in PDAC tumors could restore the sensitivity of cancer cells to chemotherapy (e.g., cisplatin and gemcitabine) via control of downstream apoptosis pathways. Therefore, if siRNA-mediated inhibition of KLF6 and chemotherapy are used in combination, the superimposed therapeutic effect of apoptosis recovery induced by them can be significantly higher than that with either agent alone, bringing novel therapeutic opportunities for these patients. Previous studies have highlighted the potential of siRNA/RNAi-based therapies in malignant solid tumors (141–144). However, considering PDAC tumor is distinct from other solid tumors on the basis of its composition, where it is mostly composed of stroma with a slight proportion of cancer cells, it may be a substantial obstacle for this type of therapy to be incorporated by the cancer cells, a well-known problem with previous PDAC therapies. Therefore, further research and development of more effective delivery systems for RNAi and CRISPR therapy that specifically targets the gene of interest may be required. Alternatively, if targeting KLF6 is not feasible or effective by currently available methods, targeting downstream genes or molecules that are activated by KLF6, or even upstream targets of KLF6, would be deemed a reasonable approach with similar expected results.

Overall, there is still a vast amount of work that needs to be done in studying the role of KLF6 and its splice variants in PDAC, and in understanding how to use it for therapeutic benefit for the patients. In the end, there is a crucial need to further explore the mechanisms through which polyamines affect gene expression and epigenetics in PDAC, including potential interactions with DNA and histone proteins, the indirect effects mediated by hypusinated eIF5A, and the distinct roles of individual polyamines and KLF6 PTMs towards this process.

Further work following identification of PTMs by mass spectrometry will involve specific site mutation/s to inactivate KLF6 within the cell and evaluate their effects on PDAC cell growth *in vitro*, PDAC tumor growth *in vivo* and on polyamine metabolism via metabolic

tracing assays. Moreover, it is important to determine in future studies if these same mechanisms are also involved in polyamine synthesis and tumor growth in other cancer types.

Ultimately, as KLF6 is strongly upregulated in PDAC holding a crucial role in the transcriptional and metabolic regulation of cancer cells, it acts as a potential therapeutic target for PDAC, particularly in combination with chemotherapy or other therapies in development that aim to target different tumor-promoting pathways.

6 CONCLUSION

In conclusion, we were able to optimize the main assay that determines whether KRAS-dependent activation of KLF6 via PTMs induces polyamine synthesis in PDAC, by identifying a suitable antibody that detects and immunoprecipitate KLF6, by optimizing *in vitro* conditions for MEK pathway inhibition and KLF6 protein detection via Coomassie staining for mass spectrometry analysis of PTMs. We established the framework for future work aimed at analyzing the role of KLF6 PTMs in PDAC tumorigenesis. Finally, this work sheds a new light on the field for translational medicine and drug development.

TABLES

Cell Type	Cell Line		# of Mutations	Mutations in DNA	AA
Human	AsPC-1	1st Trial	1	576 - CCC to CCT	Silent - Pro to Pro
		Repeated Seq.	0		
	BxPC3		0		
	MiaPaCa-2		0		
	PaTu-8988	1st Trial	10	567 – GAT to T_T	Asn to Tyr
				582 – GGC to CGC	Ala to Arg
				597 – TTT to ATT	Phe to Ile
				603- GGC to GGA	Silent – Gly to Gly
				606- TCG to AGC	Silent – Ser to Ser
				639 – AGC to AGA	Ser to Arg
				663- CAG to CAC	Gln to His
				666 – CGG to ACG	Arg to Thr
				699 – TGG to CGG	Trp to Arg
				753 – CGA to ACA	Arg to Thr
		Repeated Seq.	0		
	SUIT-2		0		
	SW 1990		0		
Mouse	iKras	NK-7017	0		
	iKras	AK-196	0		
	KPC	NK-7916	0		
	KPC	NK-3693	0		

Table 1: No mutation detected in the KLF6 region of interest analyzed in PDAC cells.

FIGURES AND FIGURE LEGENDS

Healthy   PDAC
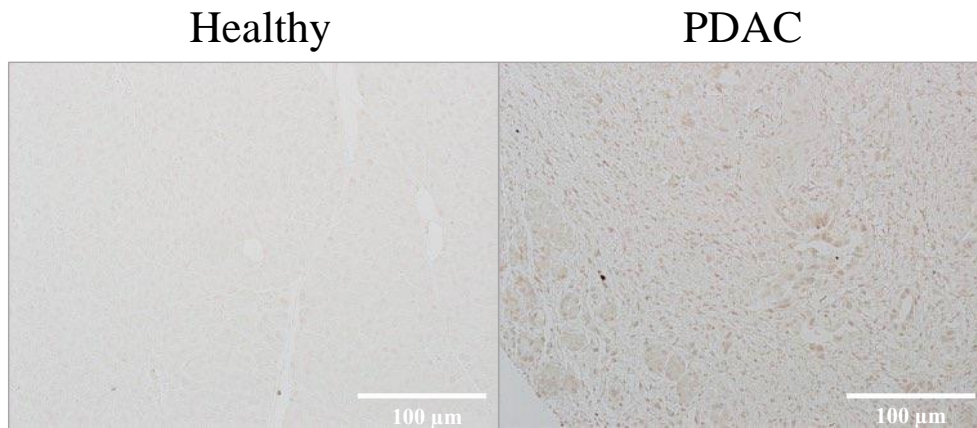

Figure 1: KLF6 is upregulated in tumor pancreatic tissue. Representative immunohistochemical staining of KLF6 in PDAC tumors and normal pancreas of tumor-bearing iKrasG12D or non-tumor-bearing iKrasWT mice treated with Doxycycline (2g per 1L of drinking water) for 3 weeks. Scale bar: 100 μm.
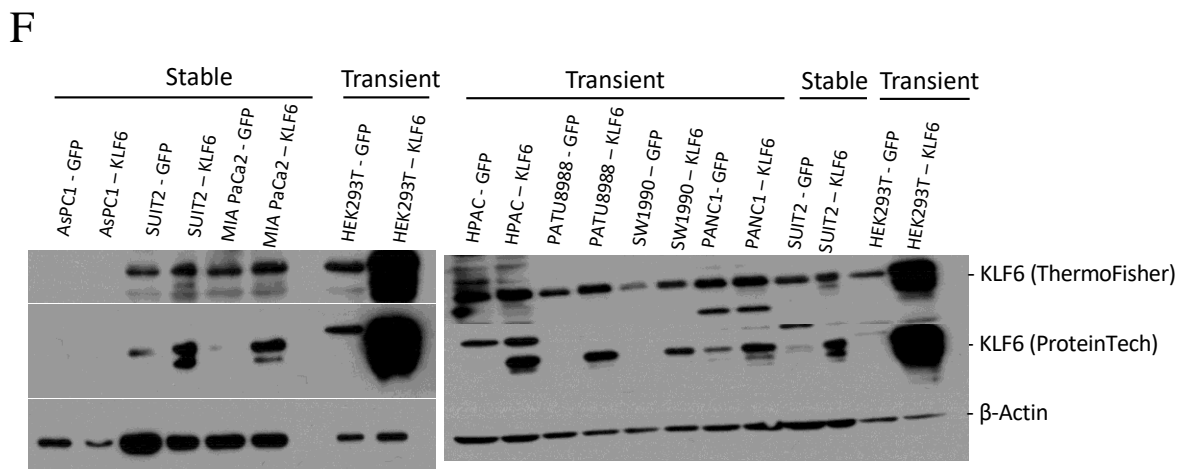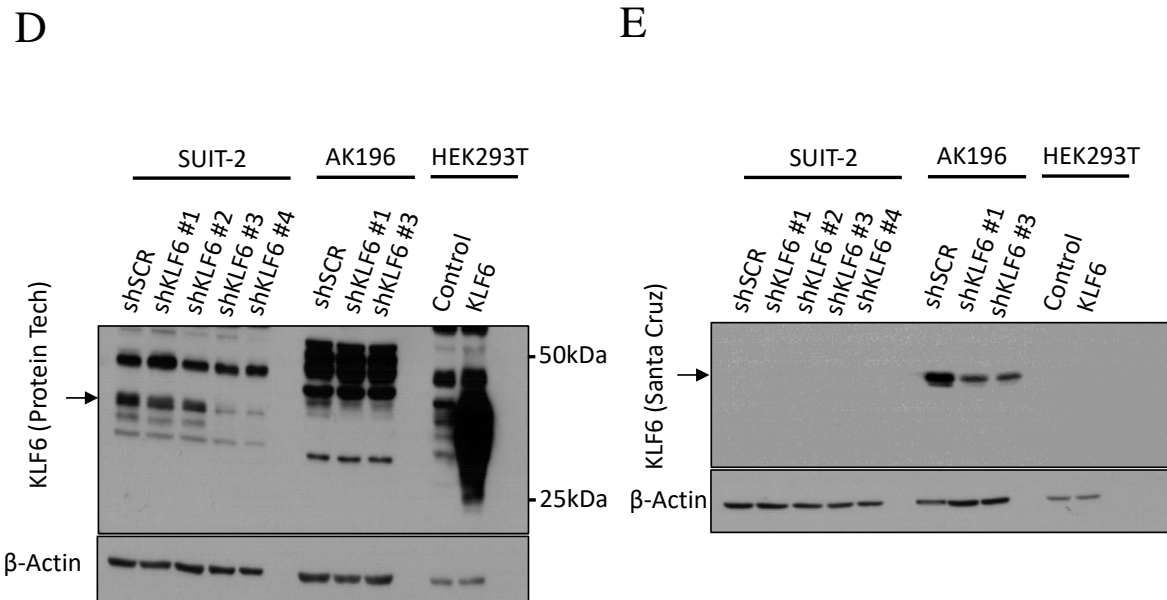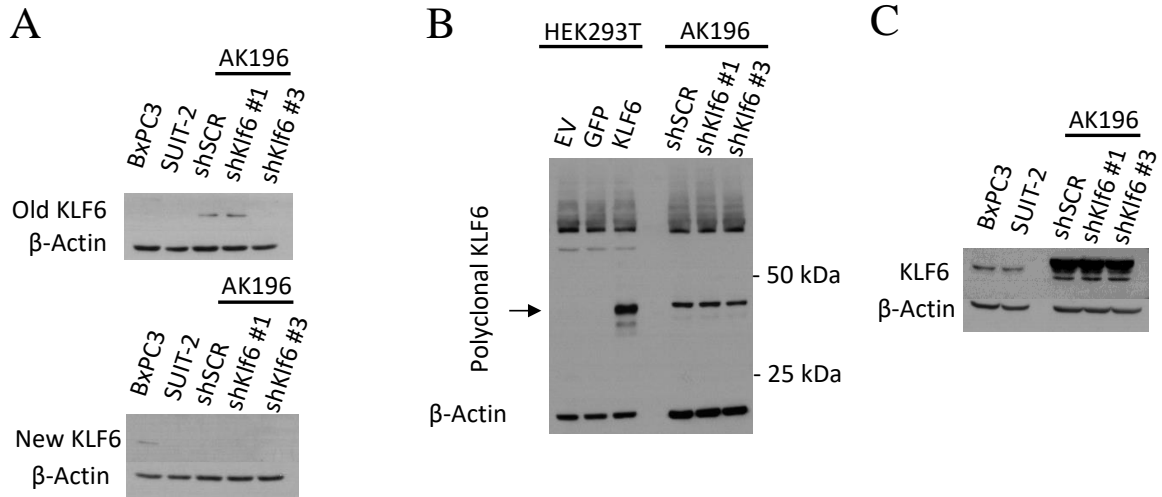
Figure 2: Optimization of KLF6 antibody in human and murine cell lines. (A) Western blot analysis of endogenous KLF6 expression using two different batches of a monoclonal KLF6 antibody (9A2, Thermo Fisher Scientific) in human (BxPC-3 and SUIT-2) PDAC cell lines, and mouse (iKRAS, AK196) PDAC cell line upon knockdown of Klf6. (B) Western blot representation of exogenous KLF6 expression in HEK293T cells and endogenous murine KLF6 expression in a mouse (AK196) cell line using a polyclonal KLF6 antibody (PA5-79560, Thermo Fisher Scientific). (C) Western blot analysis of endogenous KLF6 expression using a polyclonal KLF6 antibody (PA5-79560, Thermo Fisher Scientific) in human (BxPC-3 and SUIT-2) PDAC cell lines, and mouse (iKRAS, AK196) PDAC cell line upon knockdown of Klf6. (D) Western blot analysis of endogenous KLF6 expression using a polyclonal KLF6 antibody (14716-1-AP, Protein Tech) in human (SUIT-2) and mouse (iKRAS, AK196) PDAC cell lines upon knockdown of KLF6, in addition to endogenous and exogenous detection of human KLF6 in HEK 293T cells. (E) Western blot representation of endogenous Klf6 expression using a monoclonal KLF6 antibody (sc-365633, Santa Cruz Biotechnology) in human (SUIT-2) and mouse (iKRAS, AK196) PDAC cell lines upon knockdown of KLF6, in addition to HEK 293T cells. (F) Western blot analysis of endogenous and exogenous human KLF6 expression by comparing two polyclonal KLF6 antibodies (14716-1-AP, Protein Tech, and PA5-79560, Thermo Fisher Scientific) in multiple human PDAC cell lines and HEK293T cells via stable or transient transfection of GFP or KLF6. ß-Actin was used as a loading control for normalization.
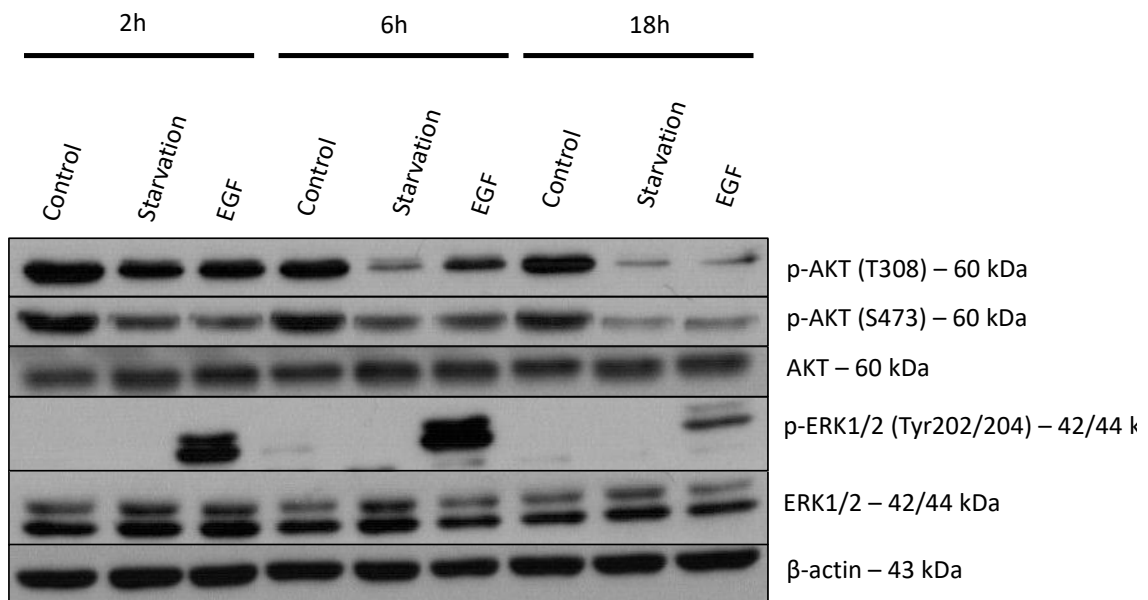
Figure 3: Optimization of EGF signaling modulation. Western blotting analysis of p-AKT (T308), p-AKT (S473), AKT, p-ERK1/2 (Thr202/Tyr204) and ERK1/2 in HEK293T cells treated for 2h, 6h, or 18h with media

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containing 10% FBS (control group), 0% FBS (starvation group) or 0% FBS followed by 1h EGF stimulation (EGF group). ß-Actin was used as a loading control for normalization.
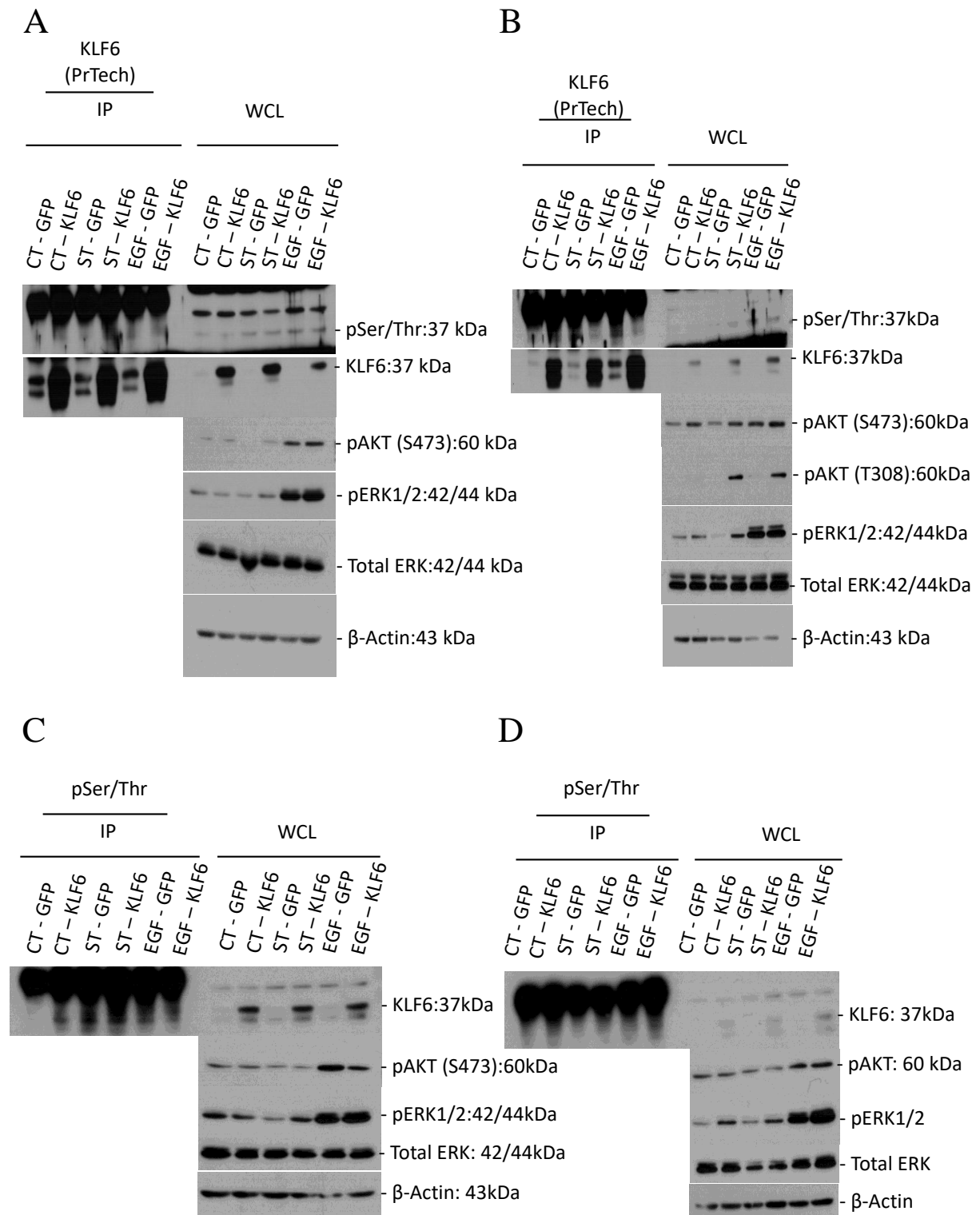
Figure 4: No KLF6 phosphorylation was observed via immunoprecipitation and western blotting. (A) Western blotting analysis of KLF6, p-Ser/Thr, p-AKT (S473), p-ERK1/2 (Thr202/Tyr204) and ERK1/2 in whole-

cell lysates (WCL) and immunoprecipitation (IP) samples of SUIT-2 cells that were stable transfected with lentiviruses containing exogenous human KLF6 or GFP followed by immunoprecipitation with a polyclonal KLF6 antibody (Protein Tech). (B) Western blotting analysis of KLF6, p-Ser/Thr, p-AKT (S473), p-AKT (T308), p-ERK1/2 (Thr202/Tyr204) and ERK1/2 in WCL and IP samples of SUIT-2 cells that were transient transfected with exogenous human KLF6 or GFP followed by immunoprecipitation with a polyclonal KLF6 antibody (Protein Tech). (C) Western blotting representation of KLF6, p-AKT (S473), p-ERK1/2 (Thr202/Tyr204) and ERK1/2 in WCL and IP samples of SUIT-2 cells that were stable transfected with lentiviruses containing exogenous human KLF6 or GFP followed by immunoprecipitation with a p-Ser/Thr antibody. (D) Western blotting representation of KLF6, p-AKT (S473), p-ERK1/2 (Thr202/Tyr204) and ERK1/2 in WCL and IP samples of SUIT-2 cells that were transient transfected with exogenous human KLF6 or GFP followed by immunoprecipitation with a p-Ser/Thr antibody. ß-Actin was used as a loading control for normalization.
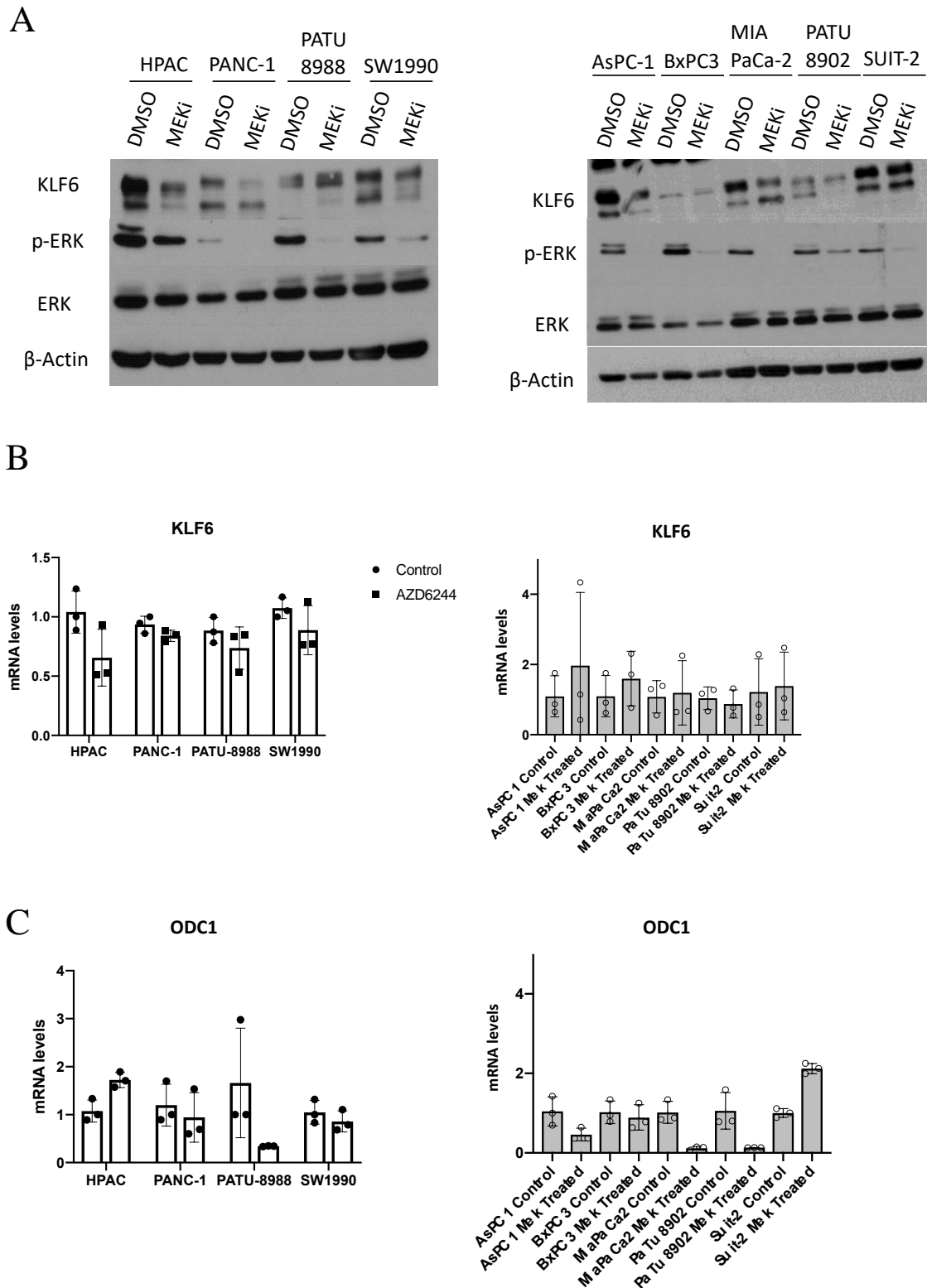
Figure 5: KLF6 activity, rather than expression, is altered upon MEK inhibition. (A) Western blotting analysis of KLF6 and a KRAS downstream pathway (ERK1/2 and p-ERK1/2) protein expression in nine human PDAC cell lines treated with 72h of DMSO or AZD6244. (B) Gene expression assessment of KLF6 mRNA levels in nine human PDAC cell lines treated with 72h of DMSO or MEK inhibitor (AZD6244, 50 nM). (C) Quantitative

RT-PCR measurement of ODC1 mRNA levels in nine human PDAC cell lines treated with 72h of DMSO or MEK inhibitor (AZD6244, 50 nM). ß-Actin was used as a loading control for western blotting normalization. n = 3 biological replicates, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p < 0.0001.
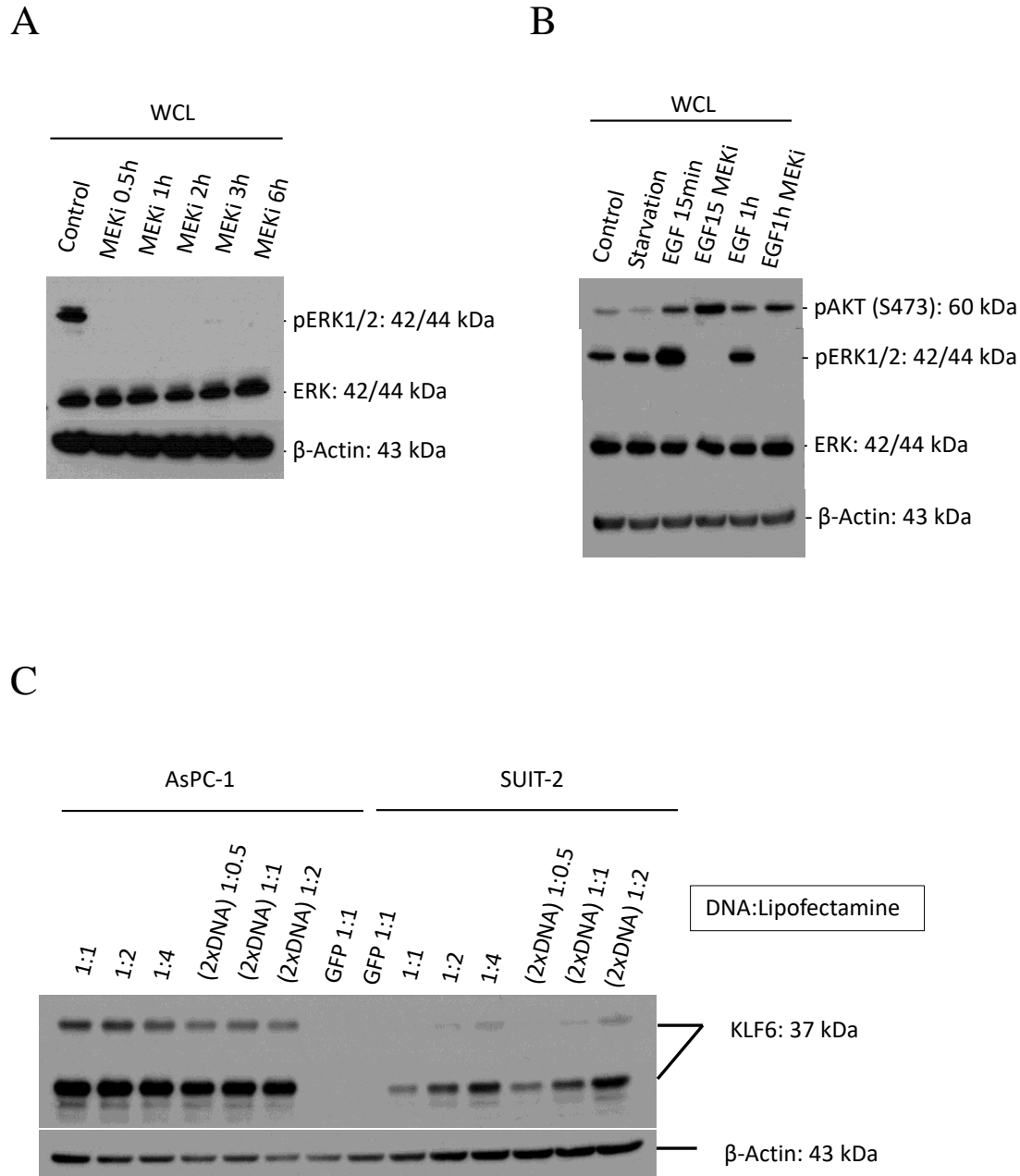
Figure 6: Optimization of transfection efficiency and EGF cell signaling with MEK inhibition for mass spectrometry analysis. (A) Western blotting analysis of ERK1/2 phosphorylation levels in whole-cell lysate (WCL) of SUIT-2 cells treated with 30 min or 1h or 2h or 3h or 6h of MEK inhibitor (AZD6244, 50 nM) or DMSO (control group). (B) Western blotting analysis of p-AKT (S473) and ERK1/2 phosphorylation levels in whole-cell lysate (WCL) of SUIT-2 cells treated for 6h with media containing 10% FBS (Control group) or 0%

FBS (Starvation group) followed by addition of EGF (50 ng/mL) for 15 min (EGF 15min group) or 1h (EGF 1h group) prior to MEK inhibition (AZD6244, 50 nM) or DMSO treatment for 30 min. (C) Western blotting analysis of KLF6 protein expression in whole-cell lysate (WCL) of AsPC-1 and SUIT-2 cells transduced with increasing ratios of DNA (KLF6) and Lipofectamine 2000. ß-Actin was used as a loading control for western blotting normalization.
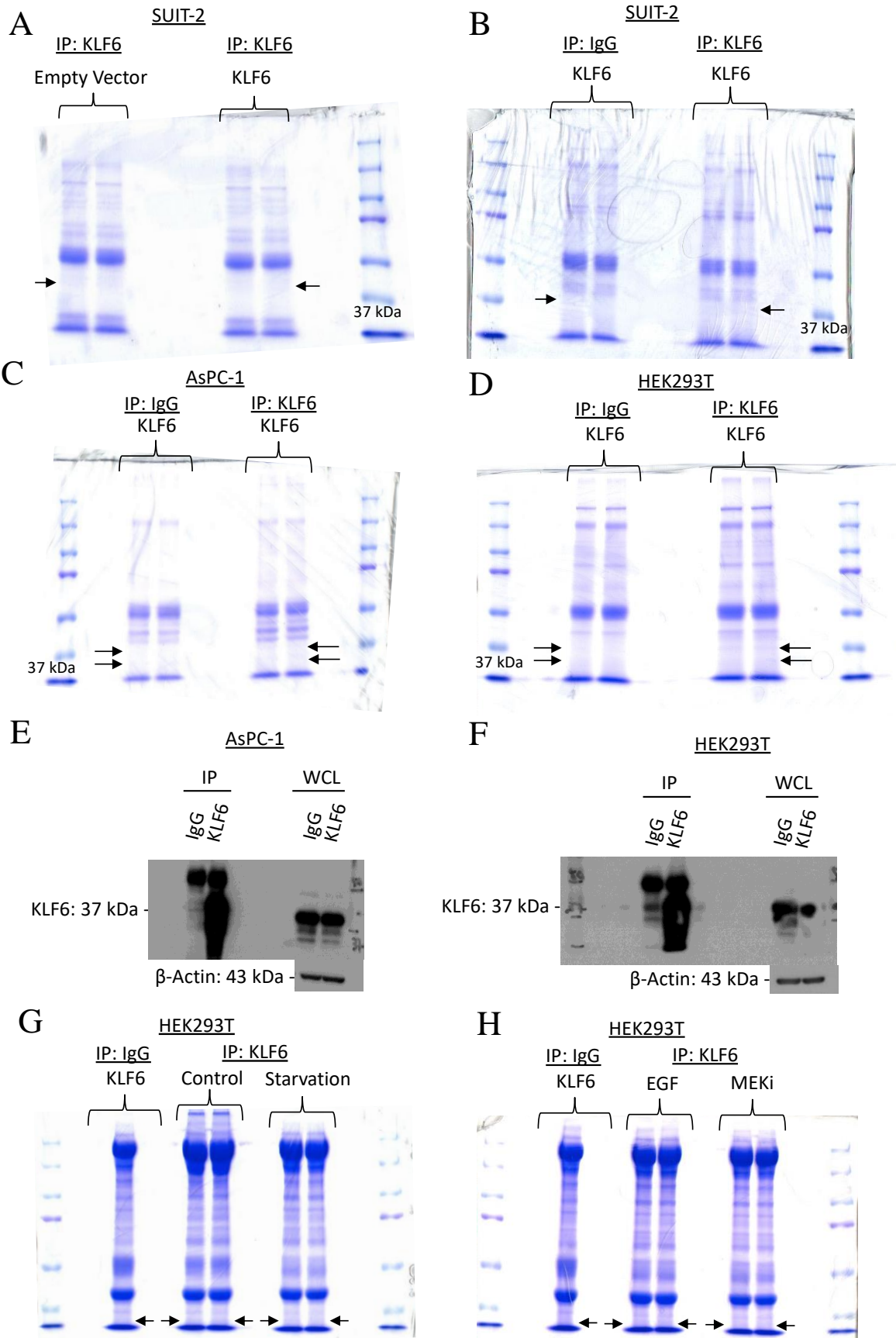
Figure 7: Optimization of KLF6 detection via Coomassie staining for mass spectrometry analysis. (A)

Coomassie staining of 8% SDS-PAGE gel containing two lanes of 10 mg of protein extract derived from SUIT-2

cells transduced with Empty Vector or human exogenous KLF6 DNA followed by immunoprecipitation with a polyclonal KLF6 antibody from Protein Tech. (B) Coomassie staining of 8% SDS-PAGE gel containing two lanes of 62.5 mg of protein extract derived from SUIT-2 cells transduced with human exogenous KLF6 DNA followed by immunoprecipitation with a polyclonal KLF6 antibody from Protein Tech (KLF6 group) or IgG control (IgG group). (C) Coomassie staining of 8% SDS-PAGE gel containing two lanes of 63 mg of protein extract derived from AsPC-1 cells transduced with human exogenous KLF6 DNA followed by immunoprecipitation with a polyclonal KLF6 antibody from Protein Tech (KLF6 group) or IgG control (IgG group). (D) Coomassie staining of 8% SDS-PAGE gel containing two lanes of 75 mg of protein extract derived from HEK293T cells transduced with human exogenous KLF6 DNA followed by immunoprecipitation with a polyclonal KLF6 antibody from Protein Tech (KLF6 group) or IgG control (IgG group). (E) Western blotting analysis of KLF6 protein expression in whole-cell lysates (WCL) and immunoprecipitation (IP) samples of AsPC-1 cells that were transient transfected with human exogenous KLF6 followed by immunoprecipitation with a polyclonal KLF6 antibody from Protein Tech (KLF6 group) or IgG control (IgG group). (F) Western blotting analysis of KLF6 protein expression in whole-cell lysates (WCL) and immunoprecipitation (IP) samples of HEK293T cells that were transient transfected with human exogenous KLF6 followed by immunoprecipitation with a polyclonal KLF6 antibody from Protein Tech (KLF6 group) or IgG control (IgG group). (G) Coomassie staining of 8% SDS-PAGE gel containing lanes of 66 mg of protein extracts that underwent immunoprecipitation with a polyclonal KLF6 antibody from Protein Tech (KLF6 groups) or IgG control (IgG group), derived from HEK293T cells transduced with human exogenous KLF6 DNA and treated for 18h with media containing 10% FBS (Control group) or 0% FBS (Starvation group) followed by DMSO treatment for 30 min. (H) Coomassie staining of 8% SDS-PAGE gel containing lanes of 66 mg of protein extracts that underwent immunoprecipitation with a polyclonal KLF6 antibody from Protein Tech (KLF6 groups) or IgG control (IgG group), derived from HEK293T cells transduced with human exogenous KLF6 DNA and treated for 18h with media containing 10% FBS or 0% FBS followed by addition of EGF (50 ng/mL) for 15 min (EGF group) prior to MEK inhibition (AZD6244, 50 nM, MEKi group) or DMSO treatment for 30 min. ß-Actin was used as a loading control for western blotting normalization.

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