

**GLI-IKBKE REQUIREMENT IN KRAS-INDUCED
PANCREATIC TUMORIGENESIS**

A Dissertation Presented

By

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To My Grandfather

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC), one of the most aggressive human malignancies, is thought to be initiated by *KRAS* activation. Here, we find that transcriptional activation mediated by the GLI family of transcription factors, although dispensable for pancreatic development, is required for *KRAS* induced pancreatic transformation. Inhibition of GLI using a dominant-negative repressor (Gli3T) inhibits formation of precursor Pancreatic Intraepithelial Neoplasia (PanIN) lesions in mice, and significantly extends survival in a mouse model of PDAC. Further, ectopic activation of the GLI1/2 transcription factors in mouse pancreas accelerates *KRAS* driven tumor formation and reduces survival, underscoring the importance of GLI transcription factors in pancreatic tumorigenesis. Interestingly, we find that although canonical GLI activity is regulated by the Hedgehog ligands, in the context of PDAC, GLI transcription factors initiate a unique ligand-independent transcriptional program downstream of *KRAS*, that involves regulation of the RAS, PI3K/AKT, and NF- κ B pathways.

We identify I-kappa-B kinase epsilon (*IKBKE*) as a PDAC specific target of GLI, that can also regulate GLI transcriptional activity via positive feedback mechanism involving regulation of GLI subcellular localization. Using human PDAC cells, and an *in vivo* model of pancreatic neoplasia, we establish *IKBKE* as a novel regulator of pancreatic tumorigenesis that acts as an effector of *KRAS*/GLI, and mediates pancreatic transformation. We show that genetic knockout of *Ikbke* leads to a dramatic inhibition of initiation and progression of pancreatic intraepithelial

neoplasia (PanIN) lesions in mice carrying pancreas specific activation of oncogenic *Kras*. Furthermore, we find that although IKBKE is a known NF- κ B activator, it only modestly regulates NF- κ B activity in PDAC. Instead, we find that IKBKE strongly promotes AKT phosphorylation in PDAC *in vitro* and *in vivo*, and that IKBKE mediates reactivation of AKT post-inhibition of mTOR. We also show that while mTOR inhibition alone does not significantly affect pancreatic tumorigenesis, combined inhibition of IKBKE and mTOR has a synergistic effect leading to significant decrease tumorigenicity of PDAC cells.

Together, our findings identify GLI/IKBKE signaling as an important oncogenic effector pathway of KRAS in PDAC that regulates tumorigenicity, cell proliferation, and apoptosis via regulation of AKT and NF- κ B signaling. We provide proof of concept for therapeutic targeting of GLI/IKBKE in PDAC, and support the evaluation of IKBKE as a therapeutic target in treatment of pancreatic cancer, and IKBKE inhibition as a strategy to improve efficacy of mTOR inhibitors in the clinic.

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LIST OF SYMBOLS AND ABBREVIATIONS

PDAC	Pancreatic Ductal Adenocarcinoma
PNET	Pancreatic Neuroendocrine Tumor
MEK	MAPK/Erk Kinase
MAPK	Mitogen Activated Protein Kinase
PI3K	Phosphatidylinositol-3 Kinase
mTOR	Mammalian Target of Rapamycin
S6K	S6 Kinase
Gli	Glioma associated oncogene homolog
Ptch1	Patched 1
FoxA2	Forkhead box protein A2
Smo	Smoothed
Hh	Hedgehog
NF- κ B	Nuclear Factor kappa light chain enhancer of activated B cells
I κ B	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
IKBKE	Inhibitor of nuclear factor kappa-B kinase subunit epsilon
TBK1	Tank Binding Kinase 1
NEMO	NF-kappa-B essential modulator
NIK	NF- κ B Inducing Kinase
RelA	v-rel avian reticuloendotheliosis viral oncogene homolog A
TGF- β	Transforming Growth Factor β
H&E	Hematoxylin & Eosin
IHC	Immunohistochemistry
NSG	NOD.Cg- <i>Prkdc</i> ^{scid} <i>Il2rg</i> ^{tm1Wjl} /SzJ

Chapter I: INTRODUCTION

Pancreatic Ductal Adenocarcinoma (PDAC) is the most common type of pancreatic cancer, accounting for over 90% of all pancreatic malignancies¹. PDAC is among the most aggressive malignancies, with a 5 year survival rate of less than 5%². PDAC accounts for over 40,000 deaths in the US every year, and by 2020, it is predicted to become the second leading cause of cancer deaths³. Surgery remains the only potentially curative option for PDAC, however, a majority of the patients present with unresectable, and metastatic disease at the time of diagnosis⁴. PDAC is also highly refractory to conventional chemotherapy, and the lack of effective treatment options results in a dismal survival rate⁵.

PDAC treatment and prognosis

Surgery is the most important treatment option for PDAC, and is known to significantly improve patient survival. Pancreaticoduodenectomy (Whipple procedure), the core surgical treatment for patients with pancreatic cancer can increase 5 year survival rates to over 20%, and median survival to over 24 months in PDAC patients^{6,7}. However, only 15-20% of patients are considered eligible for surgery⁸. Even after surgical resection, prognosis is poor due to localized recurrence of the tumors, and also distant metastases due to microscopic disease⁹.

A majority of the PDAC patients, who are not eligible for surgery, are treated with cytotoxic chemotherapy. The nucleoside analogue Gemcitabine has been used as

the standard chemotherapeutic agent in treatment of PDAC. Prior to Gemcitabine, 5'-Fluorouracil (5'-FU) was used in treating patients, however, Gemcitabine was shown to have a modest survival benefit when compared to 5'-FU (median survival 5.6 vs 4.4 months) in patients with advanced PDAC¹⁰. Gemcitabine treatment also resulted in a significantly improved 1 year survival rate of 18%, compared to 2% in patients treated with 5'-FU. Furthermore, the median time to progressive disease in patients treated with Gemcitabine was 9 weeks, compared to 4 weeks in patients treated with 5'-FU. Patients treated with Gemcitabine also showed a significant decrease in pain intensity, with 23.8% patients experiencing decreased pain compared to 4.8% of patients treated with 5'-FU.

Recently, a combination therapy of Oxaliplatin, Irinotecan, Leucovorin, and 5'-FU (FOLFIRINOX) was shown to have a significant survival benefit compared to Gemcitabine (median survival 11.1 months vs 6.8 months with Gemcitabine) in patients with metastatic pancreatic cancer¹¹. However, due to higher toxicity and side effects compared to Gemcitabine, only patients with good performance status (ECOG 0-1) are eligible for FOLFIRINOX treatment. Combining Gemcitabine with Nab-Paclitaxel has recently been shown to improve survival (median survival of 8.5 months vs 6.7 months with Gemcitabine alone), and is a standard treatment for patients with advanced PDAC¹². Paclitaxel is a taxane that interferes with microtubule assembly, and acts as an inhibitor of mitosis¹³. Nab-Paclitaxel (Nano particle Albumin-bound Paclitaxel) is an albumin-stabilized paclitaxel formulation that enables delivery of the hydrophobic Paclitaxel, and was developed to reduce

cytotoxicity associated with other solvents used for Paclitaxel delivery¹⁴. Data comparing FOLFIRINOX treatment with Gemcitabine/Nab-Paclitaxel has not been published, and this clinical trial is currently underway (NCT01488552). Currently, both FOLFIRINOX, and Gemcitabine/Nab-Paclitaxel are considered standard treatment options for patients with advanced PDAC.

Chemotherapeutic agents are also used in adjuvant therapy for patients with resectable PDAC. In patients treated with surgery, Gemcitabine treatment post-resection was shown to significantly improve prognosis (median disease free survival of 13.4 months vs 6.9 months in the control arm)¹⁵. A clinical trial evaluating the benefit of FOLFIRINOX as adjuvant therapy in resectable PDAC is currently underway (NCT01660711). The potential for pre-operative neo-adjuvant chemotherapy in resectable PDAC is also being evaluated. A previous study has shown that patients treated with Gemcitabine in combination with Cisplatin before surgery had higher rates of resection (70%) compared to patients treated with Gemcitabine alone (38%), and patients treated with combination therapy also showed a higher rate of 1 year survival (62%) compared to patients treated with Gemcitabine alone (42%)¹⁶. A recent study has also shown that patients treated with pre-operative Gemcitabine based chemoradiotherapy showed increased median survival (45 months) compared to patients treated with surgery alone (23.5 months)¹⁷. Treatment with 5'-FU or Gemcitabine based chemoradiation prior to surgery has also shown to improve median survival as well as increased resectability, and decreased lymph node metastasis¹⁸. Further clinical trials

evaluating the efficacy of neoadjuvant chemotherapy in PDAC are currently underway (NCT01900327, NCT01521702, NCT01771146, NCT02148549).

Targeted therapies for PDAC have also been evaluated. Combination of Gemcitabine with the VEGF inhibitor Bevacizumab failed to improve patient outcome compared with treatment with Gemcitabine alone¹⁹. The farnesyl transferase inhibitor Tipifarnib, when used in combination with Gemcitabine failed to show benefit in extending patient survival²⁰. Combination of Gemcitabine with the Hedgehog pathway inhibitor IPI-296 was found to be less beneficial compared with the Gemcitabine plus placebo arm, and the trial was discontinued (NCT01130142). The HER2 inhibitor Herceptin, when tested in combination with Gemcitabine in metastatic PDAC with HER2 overexpression failed to provide clinical benefit²¹. mTOR inhibitors Everolimus, and Temsirolimus have also failed to show efficacy in PDAC^{22,23}. The EGFR inhibitor Erlotinib, when combined with Gemcitabine, showed a modest, but statistically significant survival benefit compared to Gemcitabine treatment alone (6.24 months vs 5.91)²⁴. Experiments in mouse models have shown that while EGFR inhibition using monoclonal antibodies, Erlotinib, or genetic knockout can impair acinar-to-ductal metaplasia, and initiation of tumorigenesis, this requirement can be bypassed by inactivation of p53²⁵. Additional studies in mice have shown that EGFR signaling may be mediated by PI3K and STAT3, and tumor explants lacking p53 and EGFR were sensitive to PI3K and STAT3 combined inhibition, thus highlighting potential benefit in combined inhibition of EGFR, PI3K, and STAT3²⁶. Currently, Erlotinib is

the only targeted therapy agent approved for treatment of PDAC, however the use of Erlotinib in combination with other targeted therapy agents needs to be further evaluated. Clinical trials testing efficacy of targeted therapeutics against other candidates such as PARP (NCT01585805), CCR2 (NCT01413022), AKT (NCT01783171), Aurora A Kinase (NCT01924260), and CTLA4 (NCT02311361) are currently underway.

While cytotoxic chemotherapy, and targeted therapy using Erlotinib has been shown to improve prognosis, and quality of life in PDAC patients, the overall survival benefit accorded by these treatments is modest, and a majority of the patients succumb to the disease within 1 year of diagnosis, even with treatment. In order to reduce the high rate of mortality associated with PDAC, there is a need to develop additional treatment options, and gain better insight into the mechanisms underlying the initiation, progression, and metastasis of PDAC.

Pathology of Pancreatic Cancer

PDAC arises through early precursor lesions called pancreatic intraepithelial neoplasia (PanIN). Histologically, these lesions are graded as PanIN1, PanIN2, or PanIN3 with increasing order of severity respectively²⁷. PanIN1 lesions have a flat or papillary mucinous epithelium without cellular atypia. PanIN2 lesions have cellular atypia with predominantly papillary architecture. PanIN3 lesions resemble carcinoma *in situ*, and show increased cellular and nuclear atypia, as well luminal invasion²⁸. In genetically modified mouse models, it has been shown that PanIN

lesions can arise from multiple cell types in the pancreas including ductal cells, islets, and acini²⁹. In the presence of oncogenic mutations in *KRAS*, and chronic inflammation, non-ductal cells such as islets, and acini have been shown to undergo reprogramming into a “duct-like” cell type before initiating PanIN lesion formation in mouse models (Figure 1.1)³⁰. PanIN lesions subsequently progress to full blown adenocarcinoma, and metastatic cancer, with the most common sites of metastasis being the liver and lungs².

In addition to the epithelial component, the tumor microenvironment, consisting of stromal and immune cells is a significant component of PDAC tumors. One feature of PDAC is a high degree of desmoplastic reaction surrounding the tumor cells³¹. Major components of the stroma in PDAC are the pancreatic stellate cells, fibroblasts, and immune cells³². Paracrine signals from the epithelium are known to activate the desmoplastic reaction and promote stromal proliferation^{33,34}. Stromal proliferation has been shown to be promoted by Sonic Hedgehog ligand secretion by the epithelium³⁵. The stromal microenvironment surrounding the epithelium in PDAC plays an important role in tumor growth and response to therapy³⁶. In transplantation models, it has been shown that stromal depletion using inhibitors of the Hedgehog pathway activator Smoothened can lead to decrease in tumor growth, and ectopic expression of Sonic Hedgehog in pancreatic epithelial cells can promote stromal proliferation^{37,35}. In another study, it was shown that treatment with the Smoothened inhibitor Cyclopamine led to a statistically significant increase in survival of mice with PDAC (67 days with

treatment vs 61 days in vehicle³⁸. In a mouse model of PDAC with oncogenic KRAS activation and p53 loss, it was shown that stromal depletion using a Smoothed inhibitor can lead to increased vasculature in the tumors, and improve delivery of chemotherapeutic agents, which leads to improved survival in the mice³⁹.

Although pancreatic desmoplasia was previously thought to promote tumor growth, it has recently been shown that depletion of the stroma in PDAC mouse models leads to more aggressive tumors. In a genetically modified mouse model of PDAC, it was shown that deletion of Sonic Hedgehog can accelerate tumor formation and decrease survival⁴⁰. In this study, it was also shown that inhibition of Smoothed using a small molecule inhibitor IPI-926 resulted in decreased desmoplasia, and acceleration of tumor formation and decreased survival, which was consistent with earlier findings about Smoothed inhibition in the clinic. The tumors with depleted stroma were shown to have increased vasculature and poor differentiation. These findings indicate that the stroma may play a role in restricting tumor growth by maintaining differentiation of the tumors, and blocking angiogenesis. Additional mouse experiments have shown that genetic depletion of myofibroblasts can lead to accelerated tumor growth, however, in this model it was shown that depletion of the stroma leads to decreased angiogenesis in the tumors, which is in contrast to the findings with Sonic Hedgehog deletion⁴¹. In this model, it was shown that regulatory T-cell (Treg) infiltration is increased in tumors with myofibroblast depletion, thus leading to increased immunosuppression, and the

acceleration of tumor growth, which was rescued by treatment with a monoclonal antibody targeting CTLA4. However, the two models discussed above utilize different genetic approaches to develop PDAC tumors, with the Sonic Hedgehog depletion study using *Trp53* deletion as a strategy, and the myofibroblast study utilizing TGF- β depletion to achieve PDAC formation. Further experiments are warranted to elucidate the role of the stroma in PDAC and the underlying mechanisms.

PDAC tumors have a high infiltration of immune cells, which contribute to inflammation. Inflammation, and immune cell infiltration play a tumor promoting in PDAC⁴². In the initial stages of transformation of pancreatic cells, inflammation in the context of KRAS activation can accelerate PanIN lesion formation⁴³. Also, NF- κ B mediated inflammation in PDAC has been shown to amplify KRAS activity through positive feedback mechanisms in a mouse model of PDAC⁴⁴. Cytokine secretion by immune cells is known to promote cell survival and apoptosis evasion by tumor cells⁴⁵. Another mechanism by which immune cell infiltration promotes PDAC growth is via suppression of immune surveillance. In many types of solid tumors, infiltration of tumors by effector T cells is often associated with improved clinical outcomes. However, in pancreatic cancer, there is evidence to suggest that KRAS activation in the epithelium leads to preferential recruitment of immunosuppressive macrophages, myeloid cells, and regulatory T-cells, that in turn suppress effector T-cell function in the tumor microenvironment⁴⁶. These findings are consistent with data that suggests that reversion of

immunosuppression using CD40 agonists produced significant clinical response in patients⁴⁷. Also, inhibition of Regulatory T-cells in PDAC using an anti-CTLA4 monoclonal antibody has been shown to cause significant tumor reduction and increased survival in a mouse model of PDAC⁴⁸. Together these findings highlight a key role played by immune cell infiltration, and inflammation in the PDAC tumor microenvironment.

Genetics of PDAC

A number of genetic alterations and signaling events have been identified, that mediate initiation of the PanIN lesions, and their progression to full blown adenocarcinoma through regulation of the associated cell survival, apoptosis evasion, and desmoplastic and inflammatory response (Figure 1.1).

PDAC is known to be hereditary in a minority of cases. Genetic disorders such as Peutz-Jeghers syndrome, melanoma pancreatic cancer syndrome, hereditary breast-ovarian cancer syndrome, and familial adenomatous polyposis syndrome are associated with an increased risk of pancreatic cancer⁴⁹. Inherited mutations in genes such as *BRCA1*, *BRCA2*, *PALB2*, *ATM*, and *CKDNA2A*, which are associated with genetic disorders are also known to increase the risk of pancreatic cancer. Although certain genetic mutations can predispose patients to PDAC, in a majority of cases, PDAC is thought to arise sporadically through somatic mutations. Oncogenic activation of KRAS is a universal feature in PDAC, and *KRAS* is mutated in >90% of PDAC cases⁵⁰. The most common mutations are

located at codon G12 (>98% cases), which inhibit the intrinsic GTPase activity of the protein, and cause constitutive activation of KRAS^{51,52}. KRAS activation is considered to be an initiating event for PDAC, as somatic mutations in *KRAS* are detected even in early neoplastic lesions, and targeting of an oncogenic allele of *Kras* to the pancreas in mouse models has been shown to be sufficient in initiating PanIN neoplasms that phenocopy the pancreatic transformation and tumorigenesis in human patients^{53,54}. KRAS is also required for progression and maintenance of PDAC tumorigenesis, as it has been shown in a mouse model that turning off expression of an inducible oncogenic allele of *Kras* in mice with established pancreatic neoplasms led to regression of the neoplasia⁵⁵.

While oncogenic activation of KRAS leads to initiation of PanIN lesions and pancreatic transformation, loss of tumor suppressors is required for progression to full blown adenocarcinoma and metastasis. A number of tumor suppressors have been shown to be inactivated in PDAC patients. The most common loss of function alterations are found in the tumor suppressors *TP53*, *INK4A/ARF*, and *SMAD4*⁵⁶. *TP53* missense mutations are found in ~50% of PDAC cases⁵⁷. *TP53* missense mutations can act in a dominant-negative manner, and accumulation of the mutant p53 protein contributes to evasion of growth arrest in cells and causes metastasis in a mouse model of PDAC⁵⁸.

Genetic alterations in the tumor suppressor *INK4A/ARF* are identified in 80-95% of PDAC cases, and homozygous deletions in the locus are detected in ~40% of cases^{59,60}. The *INK4A/ARF* locus encodes tumor suppressors p16 and p14. P16

acts as an inhibitor of the Cyclin dependent kinases CDK4 and CDK6, which mediate G1 to S phase transition via phosphorylation and inhibition of RB. p14 inhibits MDM2 mediated degradation of p53, thus leading to increased p53 levels. INK4A/ARF can induce senescence through activation of RB and p53⁶¹. Homozygous deletion of the *Ink4a/Arf* locus in combination with oncogenic *Kras* has been shown to significantly accelerate tumor formation, and decrease survival in a mouse model of PDAC⁶². *Ink4a/Arf* deletion can also promote metastasis, and recently it has been suggested that this loss of tumor suppressor may also contribute to EMT⁶³. *Ink4a/Arf* loss with oncogenic KRAS has also been shown to activate Notch and NF- κ B signaling in PDAC⁶⁴.

Genetic alterations in *SMAD4*, a critical component of the TGF- β pathway, are identified in ~50% of PDAC cases⁶⁵. The TGF- β pathway is known to play a tumor suppressor role. Earlier studies have shown that a majority of PDAC cases carry deletions at chromosome 18q21.1, which encodes the *SMAD4* gene, and cases with intact 18q21.1 carry mutations in the *SMAD4* gene, thus implicating *SMAD4* as a candidate tumor suppressor⁶⁶. The *SMAD4* protein acts as a positive regulator of the TGF- β pathway, and inactivation of *SMAD4* may promote tumorigenesis by inhibiting TGF- β activity. In a mouse model with oncogenic KRAS activation, *Smad4* homozygous deletion has been shown to dramatically accelerate tumor formation and mortality⁶⁷.

A recent study involving large scale analysis of human PDAC samples, as well as functional characterization using the Sleeping Beauty transposon system in mice,

and shRNA knockdown in cell lines has led to the identification of previously uncharacterized genomic alterations in PDAC, that may contribute towards tumorigenicity⁶⁸. Alterations in a number of genes involved in axon guidance were identified in this study along with alterations in genes involved in chromatin modification, and DNA damage response. Subsequent studies have also implicated the SLIT-ROBO axon guidance pathway in migration and metastasis in PDAC⁶⁹. Further analysis of these newly identified genomic alterations in PDAC is necessary in order to obtain insight into their role in tumorigenesis.

The oncogenic activation of KRAS, along with subsequent loss of tumor suppressors cooperates in progression of PanIN precursor lesions to full blown adenocarcinoma, and metastatic PDAC. While KRAS oncogenic mutation, and the loss of tumor suppressors is essential for pancreatic tumorigenesis, a number of downstream signaling events mediate the process of pancreatic transformation and tumorigenesis.

Signaling pathways in PDAC

In addition to genetic alterations in oncogenes and tumor suppressors, another feature of PDAC is the aberrant activation and misregulation of signaling pathways. Among these are pathways such as RAS, NF- κ B, RAF/MEK/ERK, PI3K/AKT/mTOR, and Hedgehog/GLI signaling, that are known to play a role in transformation, apoptosis evasion, inflammation, and development.

RAS signaling

RAS proteins are small GTPases that play a central role in signal transduction. The three mammalian RAS proteins HRAS, NRAS, and KRAS share a high degree of homology, and are frequently mutated in cancers⁷⁰. As discussed previously, *KRAS* is the most frequently mutated gene in pancreatic cancer, and activating mutations in KRAS, specifically the KRAS^{G12D} mutation, are found in a majority of PDAC cases. Normal KRAS protein cycles between the GTP-bound active form and the GDP-bound inactive form. Transition to the active GTP-bound form is mediated by guanine exchange factors (GEFs), and the switch to GDP-bound is mediated by the intrinsic GTPase activity of the KRAS protein. Mutations found in PDAC at Glycine-12 inhibit the intrinsic GTPase activity of KRAS, thus leading to constitutive activation of the oncogene⁵⁰.

Under normal conditions, RAS proteins drive a signaling cascade that recruits a number of effector pathways such as RAF/MEK/ERK PI3K/AKT, RALGDS, RASSF, RIN, and PLC ϵ ⁷¹. These pathways connect RAS to biological processes such as cell proliferation, differentiation, apoptosis, and senescence⁷². Constitutive activation of KRAS leads to misregulation of these signaling pathways, and the related biological processes, which can cause oncogenic transformation. In addition to its role in cell proliferation, and survival, oncogenic KRAS has recently been shown to also regulate inflammation, and metabolic rewiring in PDAC^{73,74}. KRAS initiated metabolic rewiring enables cancer cells to utilize an alternative pathway to metabolize glutamine, which is required for cell proliferation.

The RAS effector pathways RAF/MEK/ERK, and PI3K/AKT are known to play an important role in mediating pancreatic transformation. In addition to the normal RAS effector pathways, oncogenic KRAS can also regulate other signaling pathways that are not normally associated with RAS signaling such as NF- κ B, and Hedgehog/GLI. Together, these effector pathways mediate RAS signaling, and oncogenic transformation in pancreatic cancer. KRAS can be considered a master regulator of pancreatic cancer due to its requirement in initiation, and progression of the disease, as well as its central role in regulating the signaling cascade that drives tumorigenicity. Although conceptually KRAS is an attractive therapeutic target for PDAC treatment, efforts made to target KRAS with small molecules have been largely unsuccessful. One reason for this is that the RAS proteins lack well defined binding pockets for small molecules⁷⁵. Recently, there have been some developments in small molecule based RAS inhibition. Molecules that can bind to RAS and disrupt its association with the RAS-GEF SOS have been identified⁷⁶. Although these molecules have been shown to successfully inhibit RAS activity in biochemical assays, and in cell culture, the dosage required to achieve this effect is high, which is a challenge for their use in *in vivo* models. Recently, small molecules that can preferentially bind to the oncogenic mutant form of KRAS (G12C) have also been reported⁷⁷. These molecules were shown to inhibit KRAS activity and induce apoptosis and decrease cell viability in lung cancer cell lines. While this is a significant development towards RAS targeted therapy, the molecules identified in the study are shown to specifically bind to the Cysteine

residue at codon 12 in the mutant KRAS, which may limit their potential for use in treatment of PDAC, as the KRAS^{G12C} mutation is observed in only ~2% of PDAC cases⁷⁸. The potential for these inhibitors in the treatment of this subset of PDAC cases may still be evaluated, and the MiaPaca-2 human PDAC cell line, which carries the KRAS^{G12C} mutation, may be used as a preliminary model for this purpose. Another approach that has been evaluated is the inhibition of farnesyl transferase inhibitors (FTIs), that inhibit the association KRAS with the cell membrane⁷⁹. However, these inhibitors have been unsuccessful, as KRAS can utilize an alternative mechanism dependent on geranylgeranylation to localize to the membrane⁸⁰. An alternative approach in targeted therapy for PDAC is to identify, and target effector pathways that are essential for KRAS mediated pancreatic transformation.

RAF/MEK/ERK

The RAF/MEK/ERK pathway, is a key downstream mediator of EGFR and RAS signaling that has been implicated in many cancers⁸¹. RAF activation is initiated by the association of the N-terminal RAF binding domain (RBD) of the protein with the active GTP-bound form of RAS⁸². There are three RAF isoforms: ARAF, BRAF, and CRAF. RAF proteins are kinases that phosphorylate and activate MEK1/2, which in turn can activate ERK1/2 via phosphorylation⁸³. Under normal circumstances, RAF/MEK/ERK couples signals from the cell surface to transcription factors through a process that is tightly regulated⁸⁴. Deregulation of the RAF/MEK/ERK pathway can occur either through somatic mutations of

pathway components, or through abnormal activation of upstream signaling components such as EGFR or RAS⁸⁵. Aberrant activation of the RAF/MEK/ERK pathway is known to drive cell proliferation, cell survival, and metastasis during oncogenic transformation⁸⁶. The RAF/MEK/ERK pathway has been successfully targeted in melanoma, and pathway inhibitors have been approved for use in the clinic^{87,88}. Oncogenic BRAF mutations have been identified in melanoma, lung cancer, and thyroid cancer⁸⁹. While activating mutations in BRAF (V599E) have been identified in a small subset of *KRAS* wild type PDAC cases, mutations in the RAF/MEK/ERK pathway are rare in PDAC, and the primary mechanism of pathway activation is upstream regulation by *KRAS*^{90,91}.

In a mouse model of PDAC, constitutive BRAF activation has been shown to phenocopy *KRAS* oncogenic activation⁹². Inhibition of the RAF/MEK/ERK pathway using small molecules can also induce growth arrest in pancreatic cancer cells⁹³. Inhibition of this pathway was also shown to sensitize tumors to Gemcitabine in a xenograft model of PDAC⁹⁴. Recently, treatment with a MEK inhibitor was shown to decrease tumor growth in a patient derived xenograft model of pancreatic cancer⁹⁵. Due to the importance of the RAF/MEK/ERK pathway in *KRAS* mediated transformation, and the success of pathway inhibitors in the clinic, further studies are warranted in order to evaluate the efficacy of inhibition of this pathway in pancreatic cancer.

PI3K/AKT/mTOR

The PI3K/AKT/mTOR pathway is commonly deregulated in cancer, and plays an important role in regulating cellular processes such as cell proliferation, senescence, apoptosis, and metabolism^{96,97}. PI3 Kinases are lipid kinases that phosphorylate the inositol 3'-OH group in inositol phospholipids. There are three classes of PI3Ks, of which Class I is most commonly implicated in cancer. Class I PI3Ks consist of a regulatory subunit, and a catalytic subunit. Three mammalian genes, *PIK3R1*, *PIK3R2*, and *PIK3R3*, encode p85 α , p85 β , and p55 γ regulatory subunits, respectively. The genes *PIK3CA*, *PIK3CB*, and *PIK3CD* encode the catalytic isoforms p110 α , p110 β , and p110 δ ⁹⁸. Class I PI3Ks phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP2) to generate the secondary messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3)⁹⁹. PI3Ks are known to be activated by receptor tyrosine kinases (RTKs) in response to extracellular signals¹⁰⁰. PI3Ks can also be activated by RAS independently of RTKs¹⁰¹.

PI3K mediated conversion of PIP2 to PIP3 can lead to the activation of various downstream signaling cascades. The most well characterized effector of PI3K signaling is the Serine/Threonine kinase AKT, which has been implicated in many cancers and plays an important role in apoptosis evasion and metabolism¹⁰². PIP3 can bind to the pleckstrin homology (PH) domains of AKT and the kinase PDK1, and PDK1 in turn activates AKT via phosphorylation at Threonine-308¹⁰³. The activity of PI3K can be countered by the tumor suppressor PTEN. PTEN is a phosphatase that converts PIP3 to PIP2, thus preventing AKT phosphorylation¹⁰⁴.

Under normal conditions, the activation of AKT by PI3K is a tightly controlled process, which is regulated by upstream signals and also negative regulators such as PTEN¹⁰⁵. In cancer, genetic alterations in PI3K, RAS, and PTEN commonly result in abnormal activation of AKT¹⁰⁶. AKT2 is also amplified in a minority of PDAC cases¹⁰⁷.

AKT is phosphorylated at the Serine-473 residue in the hydrophobic motif of the C-terminal tail by the mTOR kinase. Phosphorylation at Serine-473 is believed to precede phosphorylation at Threonine-308, and contribute to AKT activation¹⁰⁸. The mTOR kinase is a component of two functionally distinct protein complexes: mTORC1, and mTORC2. The mTORC1 complex plays an important role in ribosomal biogenesis and protein synthesis. The mTORC2 complex is involved in regulation of cell proliferation, survival, apoptosis, and metabolism¹⁰⁹. Activation of AKT is the most well characterized function of the mTORC2 complex, and is known to play a central role in cancer.

Therapeutic inhibition of mTOR is currently being evaluated as targeted therapy in treatment of various malignancies. The mTOR inhibitors Temsirolimus and Everolimus have shown significant benefit in treatment of Renal Cell Carcinoma and have been approved by the FDA¹¹⁰. mTOR inhibitors have also been successful in combination therapy for HER2 negative breast cancer, and also for treating subependymal giant cell astrocytoma (SEGA), and have received FDA approval^{111,112}. In a mouse model of PDAC, the mTOR inhibitor Rapamycin was shown to induce tumor regression in the background of oncogenic *Kras* combined

with *Pten* loss, but not *Trp53* loss¹¹³. Recently, Everolimus was also approved for treatment of Pancreatic Neuroendocrine Tumors (PNET) following clinical trials that showed a significant survival benefit¹¹⁴. PNET, a less aggressive tumor which makes up a small minority of all pancreatic malignancies, arises from the islet cells and is distinct from PDAC at the pathological, and molecular level. While Everolimus showed efficacy in treatment of PNET, one consequence of treatment with the drug was upregulation of AKT activation in the tumors¹¹⁵. Everolimus specifically inhibits the mTORC1 complex, but not the mTORC2 complex. Inhibition of the mTORC1 complex leads to activation of the receptor tyrosine kinase (RTK) IGF-1 as a consequence of relieved negative feedback, which in turn causes AKT activation¹¹⁶. Since AKT activity plays an important role in PDAC, specific mTORC1 inhibition may not be a good strategy in targeting mTOR signaling. One potential solution to this problem may be to use dual PI3K/mTOR inhibitors, as RTK activation of AKT is mediated by PI3K. However, in order to consider this approach, feedback activation of AKT in PDAC, and the potential role of RTKs/PI3K in this process in the context of PDAC needs to be further analyzed. Second generation mTOR kinase inhibitors that act as ATP competitive inhibitors, and target both mTORC1 and mTORC2 are currently being evaluated in clinical trials¹¹⁷. Inhibition of both mTORC1 and mTORC2 using mTOR kinase inhibitors may be beneficial in preventing feedback activation of AKT. Due to their potential in targeting both AKT activation and also other significant biological processes,

and their success in treating PDAC in preclinical mouse models, further research into the efficacy of mTOR inhibitors in treatment of PDAC is warranted.

NF- κ B

The NF- κ B signaling pathway plays an important role in the regulation of cell survival, proliferation and apoptosis, and is a critical mediator of the immune response, and inflammation. The NF- κ B family of transcription factors consists of p65 (RELA), RELB, C-REL, p50/p105, and p52/p100¹¹⁸. In the unstimulated state, the NF- κ B transcription factors bind to I κ B proteins, which sequester the transcription factors in the cytoplasm, thus inactivating them¹¹⁹. There are two NF- κ B signaling pathways in cells: the canonical NF- κ B signaling pathway, and the non-canonical NF- κ B signaling pathway. Canonical NF- κ B activation is mediated primarily by the I κ B Kinases (IKK) IKK α and IKK β . In response to activation of upstream receptors, adaptor proteins such as TRAFs and RIPs recruit an IKK complex consisting of IKK α , IKK β , and NEMO (IKK γ). IKK α and IKK β are activated by NEMO, and in turn phosphorylate the I κ B repressor at two Serine residues, which in turn leads to K48-ubiquitination mediated proteosomal degradation of I κ B. Degradation of I κ B leads to nuclear translocation of the NF- κ B transcription factors, which in turn drive expression of the NF- κ B target genes¹²⁰. In the non-canonical NF- κ B pathway, upstream signals such as cytokines lead to activation of the NF- κ B Inducing Kinase (NIK). NIK phosphorylates and activates homodimers of IKK α , which in turn phosphorylate p100 leading to its partial proteolysis and release of the p52/RELB complex which drives transcription of NF- κ B target genes¹²⁰. The

NF- κ B pathway can also be activated by atypical I κ B kinases IKK ϵ (IKBKE) and TBK1. IKBKE and TBK1, which share a high degree of homology with each other, but not with the other IKK kinases, are activated in a NEMO independent manner. Both IKBKE and TBK1 can phosphorylate I κ B, and also play an important role in interferon signaling. IKBKE can also directly phosphorylate and activate the transcription factor RELA (p65)¹²¹.

Aberrant NF- κ B signaling has been implicated in a number of malignancies. The oncogenic properties of NF- κ B signaling derive from its ability to regulate processes involved in cancer such as cell proliferation, and apoptosis. NF- κ B mediated inflammation has also been shown to contribute to the development of cancer¹²². Canonical NF- κ B signaling has been shown to be activated in response to oncogenic KRAS activation in PDAC. In a mouse model, it was shown that Caerulin induced chronic inflammation can lead to significant upregulation of the activity of mutant KRAS in the pancreas⁴⁴. In this study, the activity of the NF- κ B activator IKK β was found to be essential for inflammation induced pancreatic transformation in the presence of oncogenic KRAS. Furthermore, it was found that treatment with an inhibitor of COX2, an NF- κ B target gene, led to loss of inflammation induced KRAS activation, thus highlighting a role for NF- κ B mediated feedback activation of KRAS. In a mouse model of PDAC with *Kras* activation and *Ink4a/Arf* deletion, genetic ablation of IKK β was shown to significantly extend survival and impair tumorigenesis¹²³. IKK β deletion resulted in significant reduction of pancreatic neoplasia initiation, and decrease in expression of pro-inflammatory

cytokines, as well as lymphocyte infiltration. In the same study, it was shown that KRAS oncogenic activation could regulate activity of the AP-1 transcription factors, which in turn drove expression of the cytokine IL1 α , and p62 which results in upstream activation of IKK β . Pancreatitis is known to be a risk factor in PDAC¹²⁴. These findings highlight a potential mechanism through which inflammation may lead to feedback activation of oncogenic KRAS, and how pro-inflammatory stimuli and NF- κ B pathway activity may promote a positive feedback loop which leads to sustained NF- κ B signaling. Also, these findings provide proof of concept for therapeutic targeting of the canonical NF- κ B signaling pathway in PDAC.

Recent studies have also indicated that non-canonical NF- κ B signaling mediated by constitutive NIK activation may regulate cell proliferation in PDAC cells¹²⁵. Chronic inflammation, which is a feature of PDAC, can upregulate NF- κ B signaling in PDAC cells in the presence of oncogenic KRAS, which in turn leads to further cytokine production by the epithelial cells, and immune cell infiltration in the tumors¹²⁶. In human PDAC cells, KRAS activation is known to upregulate the expression of the kinase GSK3 α , which activates the canonical NF- κ B pathway by promoting association of the TAK1 kinase with TAB1, which leads to activation of IKK. GSK3 α has also been shown to activate the non-canonical NF- κ B pathway downstream of KRAS by promoting processing of p100, and nuclear accumulation of p52. Inhibition of GSK3 α can inhibit growth of human PDAC explants¹²⁷. Since GSK3 α has been shown to activate both the canonical as well as non-canonical NF- κ B in the context of oncogenic KRAS, small molecule based GSK3 α should be

further evaluated as a therapeutic strategy for targeting NF- κ B signaling and tumorigenicity in PDAC.

Hedgehog/GLI

The Hedgehog/GLI Signaling pathway is a crucial developmental pathway that regulates multiple processes during embryonic development, and adult tissue homeostasis¹²⁸. Components of this pathway in mammals include the ligands Sonic Hedgehog (SHH), Indian Hedgehog (IHH), and Desert Hedgehog (DHH). Secreted Hedgehog ligands bind to their cellular receptor Patched1 (PTCH1), which is a 12 transmembrane domain protein. The binding of Hedgehog to Patched1 leads to loss of repression of Smoothened (SMO), a 7 pass transmembrane surface protein, by Patched1¹²⁹. Activated Smoothened initiates an intracellular signaling cascade that leads to the activation and nuclear translocation of the GLI family of transcription factors, which in turn drive the expression of Hedgehog target genes. The GLI family of transcription factors includes the zinc finger proteins GLI1, GLI2, and GLI3. The GLI proteins possess context dependent repressor and activator functions. GLI1 and GLI2 function mostly as transcriptional activators, whereas GLI3 functions mostly as a transcriptional repressor (Figure 1.2)¹³⁰.

Aberrant activation of the Hedgehog signaling pathway was first identified in Basal Cell Carcinoma (BCC). Inactivating mutations in Patched1, which lead to a loss of repression of Smoothened and constitutive activation of the pathway, have been identified in ~90% of the cases of Basal Cell Carcinoma¹³¹. Activating mutations in

Smoothed have also been identified in BCC. The role of aberrant Hedgehog signaling in multiple human malignancies became apparent with the discovery that Gorlin Syndrome, a rare genetic condition in which the patients develop multiple BCCs during their lifetimes, and are also predisposed to other cancers such as Medulloblastoma, and Rhabdomyosarcoma, is characterized by a loss of function mutation in Patched1¹³². One third of all human medulloblastoma cases involve upregulation of hedgehog signaling due to mutations in the pathway components¹³³. These findings in human patients have been confirmed by genetically engineered mouse models in which ectopic expression of SHH, GLI1, or GLI2, and constitutively active Smoothed led to the development of BCC^{134,135}. Apart from the classical Hedgehog associated cancers, aberrant Hedgehog Signaling has been found to play a role in a variety of other malignancies such as breast cancer, prostate cancer, melanoma, lung cancer, and pancreatic cancer¹³⁶.

Hedgehog/GLI Signaling in Pancreatic Tumorigenesis

While Indian Hedgehog is expressed in the developing pancreas, Sonic Hedgehog expression is undetectable. SHH plays a role in restricting pancreatic growth, as knockout of SHH leads to expansion of pancreatic mass, and overgrowth of endocrine cells¹³⁷. In contrast, ectopic expression of SHH in the pancreas under the control of the *Pdx1* promoter leads to disruption of normal development, and conversion of pancreatic mesoderm to intestinal mesenchyme¹³⁸. While Sonic Hedgehog (SHH) ligand is normally not expressed in adult pancreas, it is

expressed in PanIN lesions in mice carrying oncogenic KRAS, and increased SHH expression correlates with increasing histological grades of the lesions (PanIN 1-3), and also in full blown adenocarcinoma¹³⁹. During development, in the gastrointestinal tract, Hedgehog ligand secreted by the epithelium signals to the mesenchyme in a paracrine manner and drives mesenchymal proliferation¹⁴⁰. Non-canonical GLI signaling, in which GLI transcription factors act independently of upstream Hedgehog/Smoothed activity in a cell autonomous manner has also been reported in other contexts¹⁴¹. In pancreatic cancer Hedgehog/GLI activity has been reported to be regulated through paracrine, autocrine, as well as non-canonical mechanisms.

Ligand dependent Hedgehog/GLI signaling:

The paracrine model for Hedgehog signaling in pancreatic tumorigenesis proposes that Hedgehog ligands secreted by the pancreatic tumor epithelium signal to the mesenchyme, and drive expression of Hedgehog responsive genes in the mesenchyme (Figure 1.3). It has been shown that in xenograft mouse models, tumor cells obtained from human patients were capable of driving Hedgehog target gene expression in the mouse stroma and that expression of these target genes in the stroma correlated with SHH and IHH secretion by the tumor cells³⁷. Genetic ablation or chemical antagonist mediated inhibition of Smoothed in the mouse stroma also resulted in inhibition of tumor growth in the xenograft models. Also, Hedgehog target gene expression has been reported in the mesenchyme, and

activation of smoothened in the pancreatic epithelium was not sufficient to induce neoplasia.

According to the autocrine model, Hedgehog ligands secreted by the tumor epithelium signal to the epithelial cells and drive hedgehog pathway activation in the epithelium to promote tumorigenesis (Figure 1.4). It has been shown that treatment of PDAC cell lines with Cyclopamine results in increased apoptosis, and decreased proliferation in cells in culture as well as in xenograft models¹³⁹. This evidence is corroborated by other studies which show that ectopic expression of SHH in Pancreatic Duct Epithelial Cells (PDECs) drives proliferation in the cells, and protects them from apoptosis. Transplantation of the SHH expressing PDEC cells in immunocompromised mice can also lead to the formation of PDAC precursor lesions. Also, SHH and KRAS expression have a synergistic effect on the tumorigenicity of the PDECs when transplanted into immunocompromised mice¹⁴².

Non-Canonical GLI signaling:

The non-canonical model for Hedgehog signaling in pancreatic malignancies suggests that GLI transcriptional activity can be driven in the absence of the Hedgehog ligand and Smoothened activation during pancreatic tumorigenesis (Figure 1.5). This model is supported by the findings which show that expression of Hedgehog target genes in the epithelium of PanIN mice is sustained even in the absence of Smoothened, the canonical bottleneck for Hedgehog signaling¹⁴³. In this study, the authors also present evidence that GLI1 cooperates with KRAS *in*

vitro to enhance proliferation in human PDAC cell lines, and that GLI1 can be regulated by KRAS, and TGF- β in PDAC cells. These findings support a model in which GLI transcriptional activity is maintained in PDAC tumor epithelial cells in the absence of canonical Hedgehog signal transduction, and is instead regulated by oncogenic KRAS. While GLI1 acts downstream of KRAS in PDAC cells, ectopic expression of a dominant active allele of *Gli2* (*Cleg2*) in the pancreas has been shown to drive formation of undifferentiated tumors, in the absence of upstream Hedgehog pathway activation or KRAS mutation¹³⁹. These undifferentiated tumors did not arise through PanIN lesions as is the case in human PDAC formation. In contrast, when the *Cleg2* allele was expressed in combination with oncogenic *Kras*, it led to the formation of accelerated PanIN lesions. Together, these findings highlight a potentially critical role of GLI transcription factors in PDAC, and cooperation between GLI and oncogenic KRAS in pancreatic tumorigenesis.

While the studies described above point to a significant involvement of Hedgehog/GLI in pancreatic tumorigenesis, the mechanism of signaling as well as the implications of Hedgehog/GLI activity in the epithelial and stromal compartments of PDAC tumors are less clear. Recently published data about paracrine Hedgehog signaling and the tumor suppressive role of the stroma in PDAC are in conflict with an earlier report which indicates that stromal ablation may be beneficial in treating tumors. Part of the difference in these findings can be attributed to the differences in design of the two experiments. In the 2009 study involving treatment of mice bearing PDAC tumors with the Smoothed inhibitor

IPI-926³⁹, the authors failed to detect an effect on tumor growth after treatment with the Smoothed antagonist alone, however, a statistically significant improvement in survival was observed with combined treatment of IPI-926 with Gemcitabine. However, as this experiment involved only 3 weeks of treatment, the duration of the study may not be sufficient to study long term effects of stromal depletion. In the 2014 study involving stromal depletion⁴⁰, the mice were treated for a longer duration using a similar treatment protocol, which led to decreased survival in response to combined IPI-926/Gemcitabine compared to treatment with IPI-926 alone. These findings are consistent with the results of IPI-926 treatment for PDAC in the clinic. Significant improvement in vasculature, as well stromal depletion in response to Smoothed inhibition was observed in both the studies. This suggests that the difference in survival outcome in response to IPI-926 in the two studies may be attributed to the differential effect of short term vs chronic Smoothed inhibition and stromal depletion, rather than a difference in the underlying mechanism of activity of IPI-926.

Findings about Smoothed independent non-canonical GLI activity in the PDAC epithelium are also in conflict with earlier reports about autocrine Hedgehog signaling in PDAC. Although it was shown earlier that treatment with the Smoothed antagonist Cyclopamine can lead to decreased survival in human PDAC cells *in vitro*, and in xenograft models *in vivo*, Cyclopamine is known to have non-specific effects at dosages used in this experiment^{144,145,37}, which may imply that the effect of the inhibitor on the PDAC cells is Hedgehog/Smoothed

independent. Also, the *in vivo* experiment in this study does not distinguish between the effect of Cyclopamine on the stroma vs the epithelium, and therefore does not distinguish between autocrine and paracrine Hedgehog signaling. Also, while it has been shown in a previous study that Sonic Hedgehog may protect pancreatic epithelial cells from apoptosis, and promote survival via autocrine mechanisms, and also drive tumorigenicity in mouse models¹⁴², this study utilizes a gain of function approach in which Sonic Hedgehog is ectopically expressed. While Sonic Hedgehog ectopic expression may promote pancreatic tumorigenesis via autocrine mechanisms under certain conditions, the available evidence is not sufficient to conclude a role for autocrine Hedgehog signaling in PDAC.

Although published data implicates Hedgehog/GLI activity in pancreatic tumorigenesis, a requirement per se for GLI transcriptional activity for tumor initiation and progression has not been demonstrated *in vivo*. In this study, we use a mouse model to test the requirement of GLI transcription factors in initiation of pancreatic neoplasia, as well as progression to full blown adenocarcinoma. We also test the role played by individual GLI transcription factors in pancreatic tumorigenesis, and identify a novel oncogenic transcriptional program regulated by GLI in pancreatic cancer.

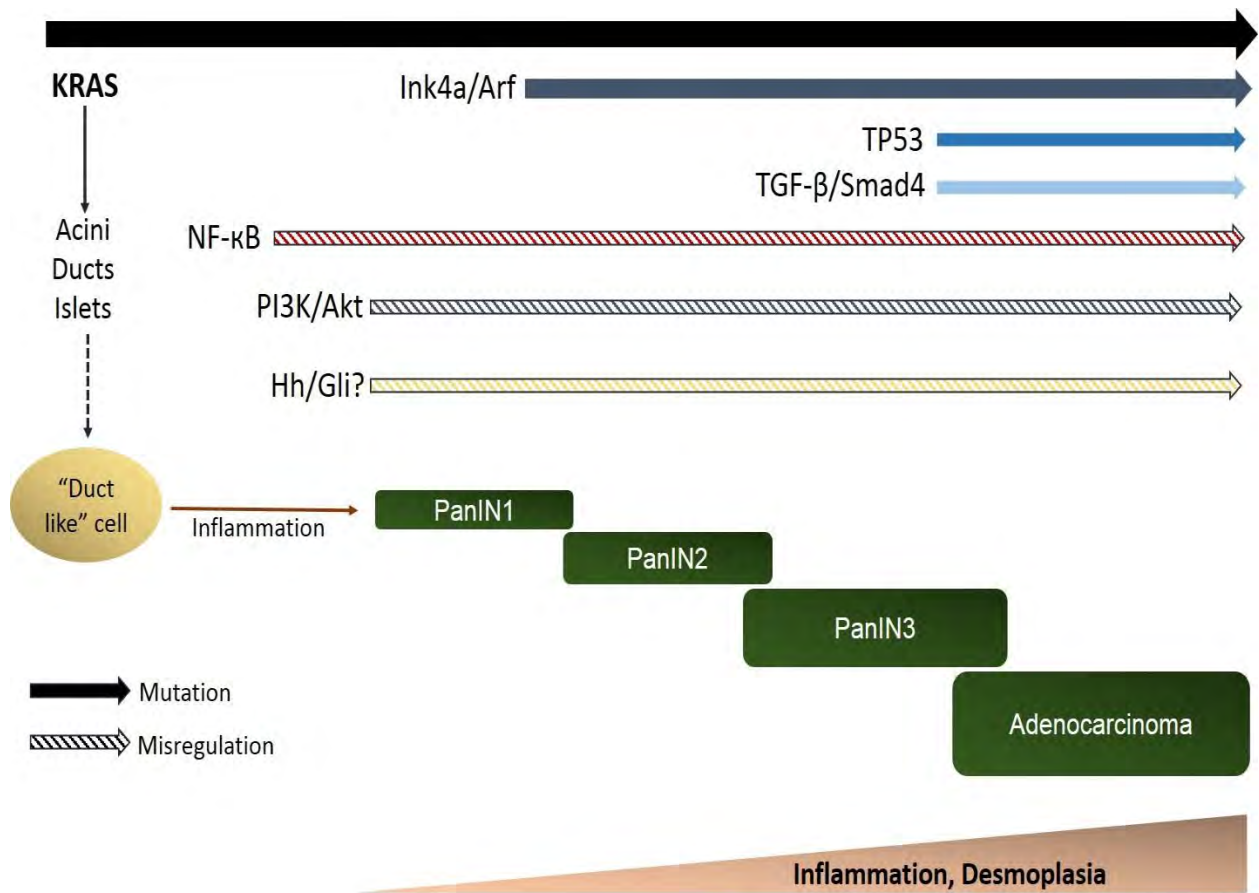


Figure 1.1: Initiation and progression of Pancreatic Ductal Adenocarcinoma (PDAC)

Adapted from Bardeesy et al 2002, Morris et al 2010, and Eser et al 2013 ^{30,56,146}.

Oncogenic mutations in *KRAS* are the initiating event in pancreatic transformation. In mouse models, *KRAS* activation in acinar, ductal, as well as islet cells can lead to transformation to a duct-like cell, which in the presence of chronic inflammation can give rise to PanIN lesions. PanIN lesions can progress in intensity to full blown adenocarcinoma. In human cases, loss of function genetic alterations are acquired in tumor suppressors during progression to adenocarcinoma. Downstream signaling pathways such as NF-κB, PI3K/AKT, and Hedgehog/GLI are misregulated during transformation, and increased desmoplasia and inflammation is observed.

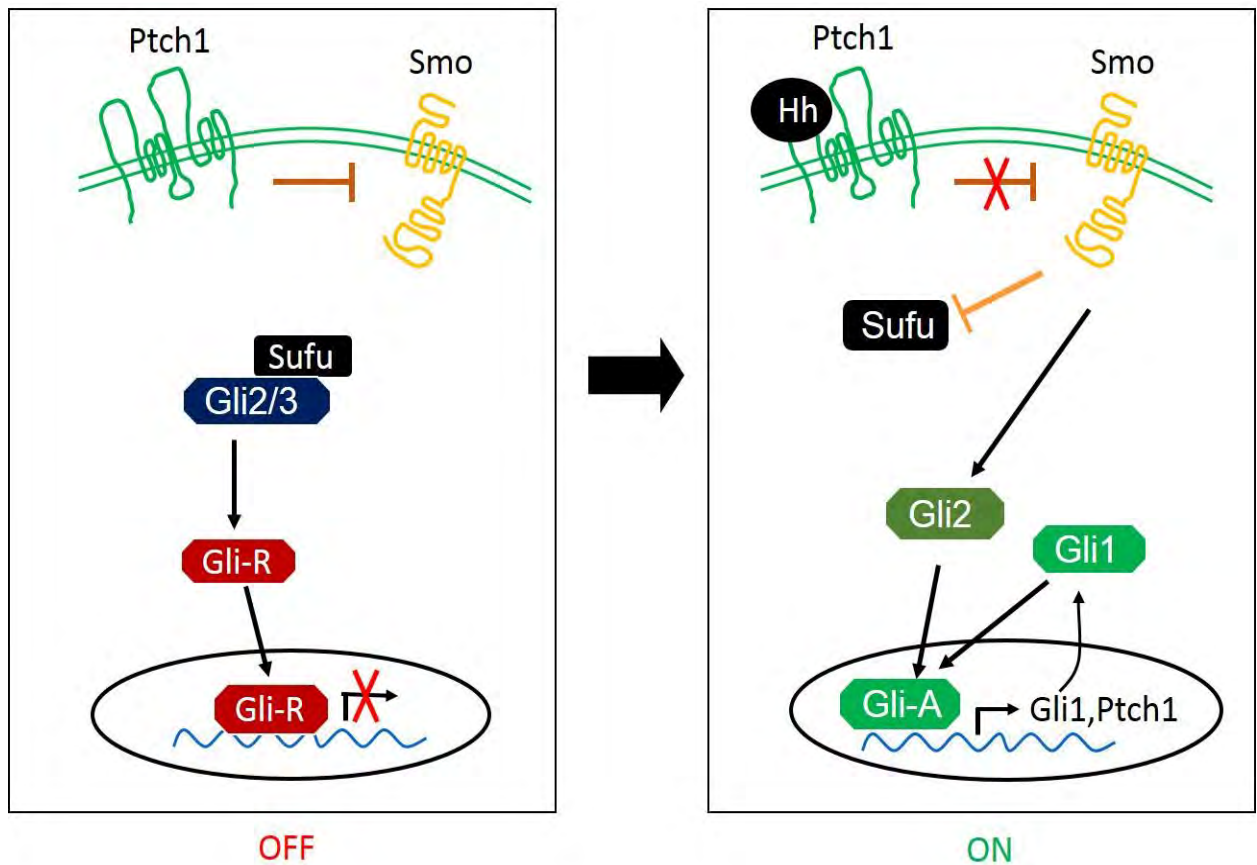


Figure 1.2: Mammalian Hedgehog Signaling Pathway

Adapted from Ruel et al 2009¹⁴⁷.

A) In the absence of Hedgehog ligands, Patched1 inhibits Smoothed, and Suppressor of Fused (SUFU) sequesters the GLI transcription factors in the cytoplasm

B) When Hedgehog ligands bind to Patched1, Smoothed is activated, and SUFU inhibition of Gli transcription factors is relieved, leading to transcriptional activation.

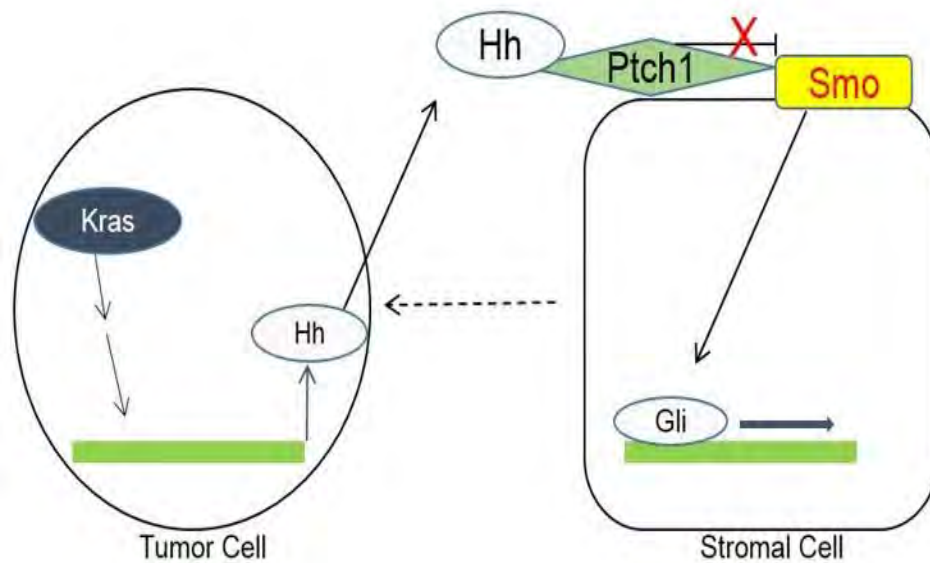


Figure 1.3: Paracrine Hedgehog/GLI Signaling in PDAC

Adapted from Theunissen et al 2009³⁴

Hedgehog ligands are secreted by the pancreatic tumor epithelium in response to oncogenic KRAS activation. The ligands inhibit Patched1 on the stromal cells and drive downstream pathway activation and GLI transcriptional activity in the stroma via Smoothened. Stromal cells may engage in reciprocal signaling with the tumor epithelium.

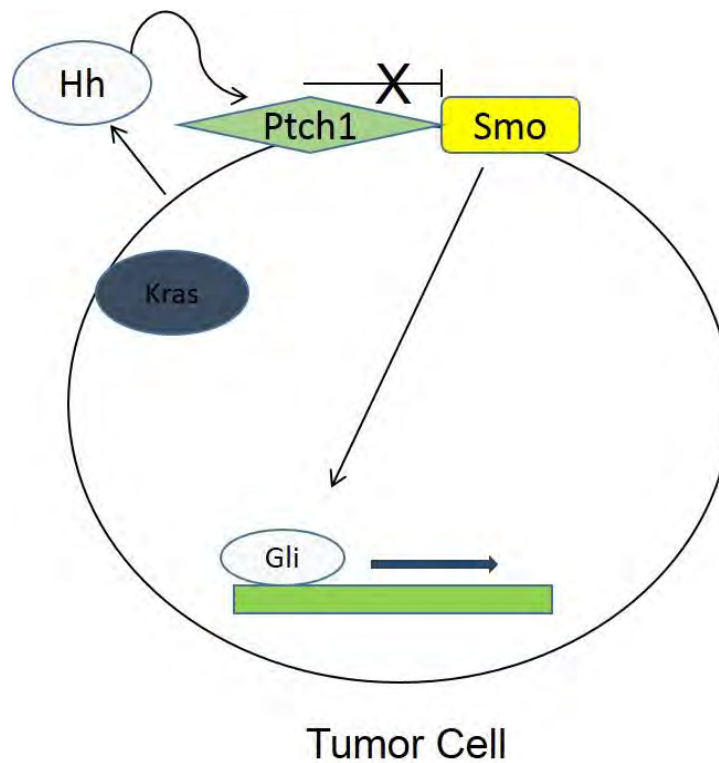


Figure 1.4: Autocrine Hedgehog/GLI signaling in PDAC

Hedgehog ligand is secreted by the pancreatic epithelium, and signals to Patched1 in the epithelium, which leads to downstream pathway activation via Smoothened, and GLI transcriptional upregulation.

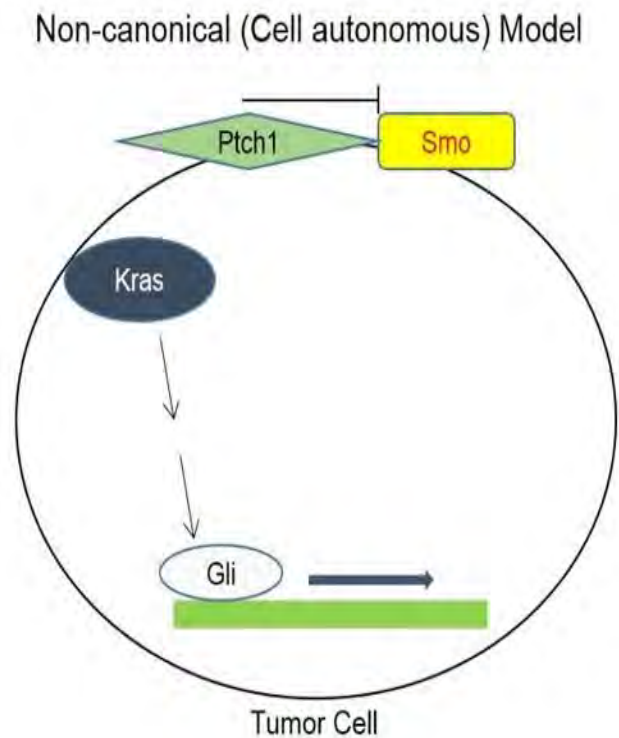


Figure 1.5: Non-canonical GLI signaling in PDAC

GLI transcription is activated in the pancreatic tumor epithelium independently of Hedgehog/Smoothed by oncogenic KRAS, and drives target gene expression in the tumor epithelium.

Chapter II: GLI requirement in KRAS induced initiation and progression of PDAC

Figure Contributions

Data for this chapter has been published in Rajurkar et al 2012, PNAS¹⁴⁸.

Figure 2.5 was provided by Dr. Wilfredo DeJesus Monge and has been published in Rajurkar et al 2012.

Data for gene expression in mouse BCC, medulloblastoma, and rhabdomyosarcoma (Figure 2.9B) was obtained from Mao et al 2006¹⁴⁹

RESULTS

GLI transcriptional activity is dispensable for pancreatic development

To investigate the role of GLI transcriptional activity in pancreatic development, we used a *Rosa26* knock-in allele of Gli3T (*R26-Gli3T*), which allows ectopic expression of the Gli3T protein from the ubiquitously expressed *Rosa26* locus following Cre-mediated recombination (Figure 2.1E). Gli3T is a C-terminally truncated form of GLI3, which acts as a dominant repressor of GLI transcription¹⁵⁰. We found that over-expression of Gli3T specifically inhibits GLI1 and GLI2 dependent gene transcription, but not lymphoid enhancer-binding factor-1 (LEF1) or serum-response-factor (SRF) mediated gene transcription in cultured NIH3T3 cells (Figure 2.2A-C). These results show the specificity and effectiveness of the Gli3T allele in blocking Hedgehog/GLI transcriptional activation.

We crossed *R26-Gli3T* mice to *P48Cre* (*Ptf1a-Cre*) transgenic mice that direct Cre recombinase expression to the epithelial lineages of the mouse pancreas¹⁵¹. *P48Cre;R26-Gli3T* mice were born at the expected frequency, and their pancreata showed normal parenchymal architecture and cytodifferentiation up to the age of 12 months, the longest time examined (n = 10) (Figure 2.2E and J). In the *R26-Gli3T* allele, a 3XFlag tag and IRES-Venus unit are also inserted at the C-terminus of Gli3T to facilitate identification of transgene expression¹⁵⁰. Therefore, we used detection of the Flag-tag with immunoblotting (Figure 2.2D), and Venus fluorescence on cryosections (Figure 2.2N) to confirm Cre-mediated expression of

Gli3T in pancreatic tissue from *P48Cre;R26-Gli3T* mice. We analyzed the expression of the endocrine markers Insulin and Glucagon, and the acinar cell marker Amylase, and found no significant differences between Gli3T-expressing, and control pancreata (Figure 2.2F, G, H, K, L and M). This data shows that GLI inhibition does not affect differentiation of the pancreatic epithelial lineages, and suggests that cell-autonomous Hedgehog/GLI activity is largely dispensable for the proper development of mouse pancreas.

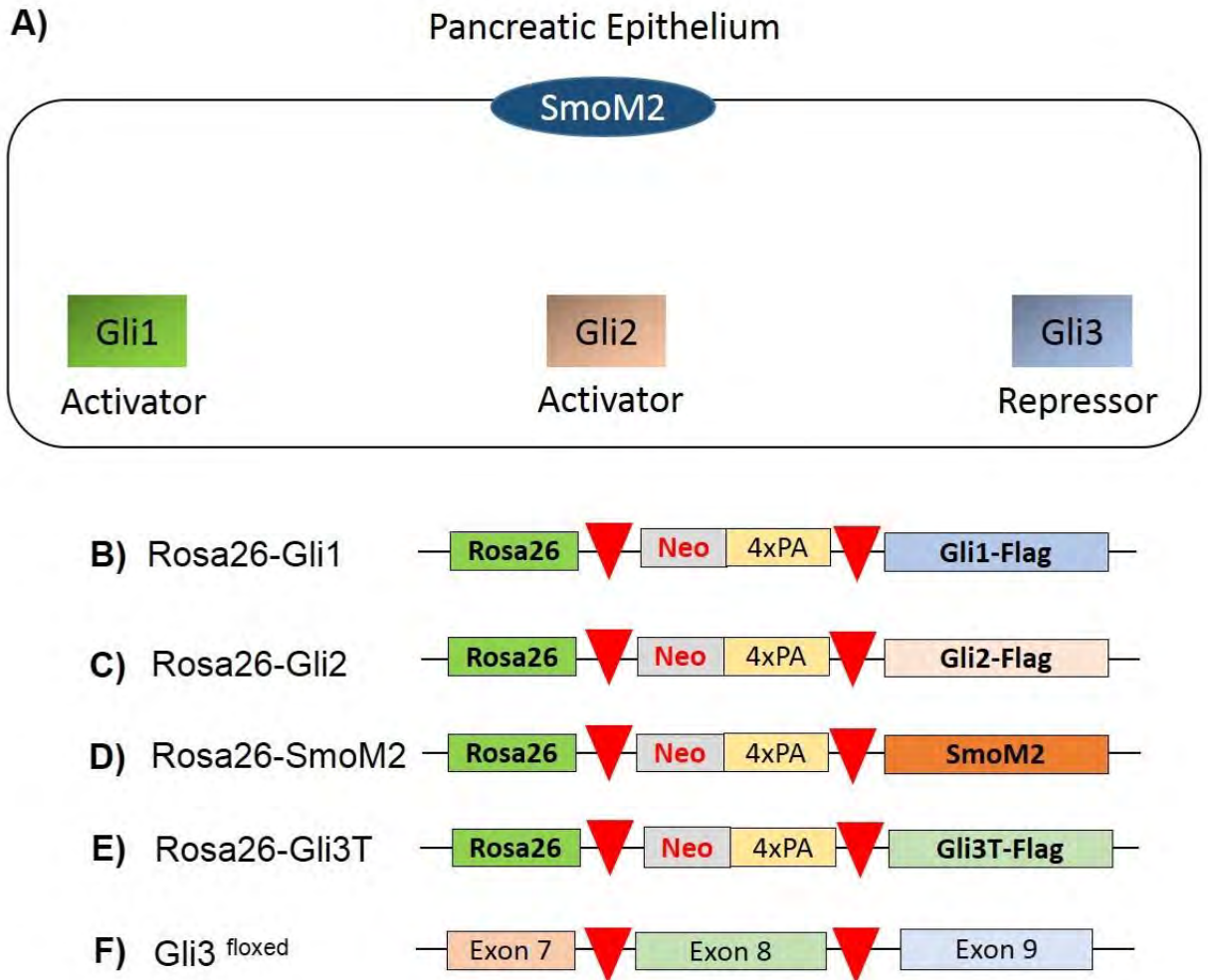


Figure 2.1: Schematic representation of Hedgehog/GLI alleles

A) SmoM2 is a constitutively active mutant of Smoothed that acts as an upstream activator of GLI transcription factors. GLI1, and GLI2 are transcriptional activators, and GLI3 is predominantly a transcriptional repressor. (B-E) *Gli1*, *Gli2*, *SmoM2*, and *Gli3T* alleles are knocked into the *Rosa26* locus which is ubiquitously expressed in mice. Expression is dependent on Cre-mediated recombination. Gli3T is a C-terminal truncated version of GLI3 that is capable of inhibiting transcription by all the GLI transcription factors. (F) *Gli3* floxed allele carries loxP sites flanking exon 8 of the endogenous *Gli3*. Cre-mediated recombination enables excision of exon 8 thereby abrogating *Gli3* expression.

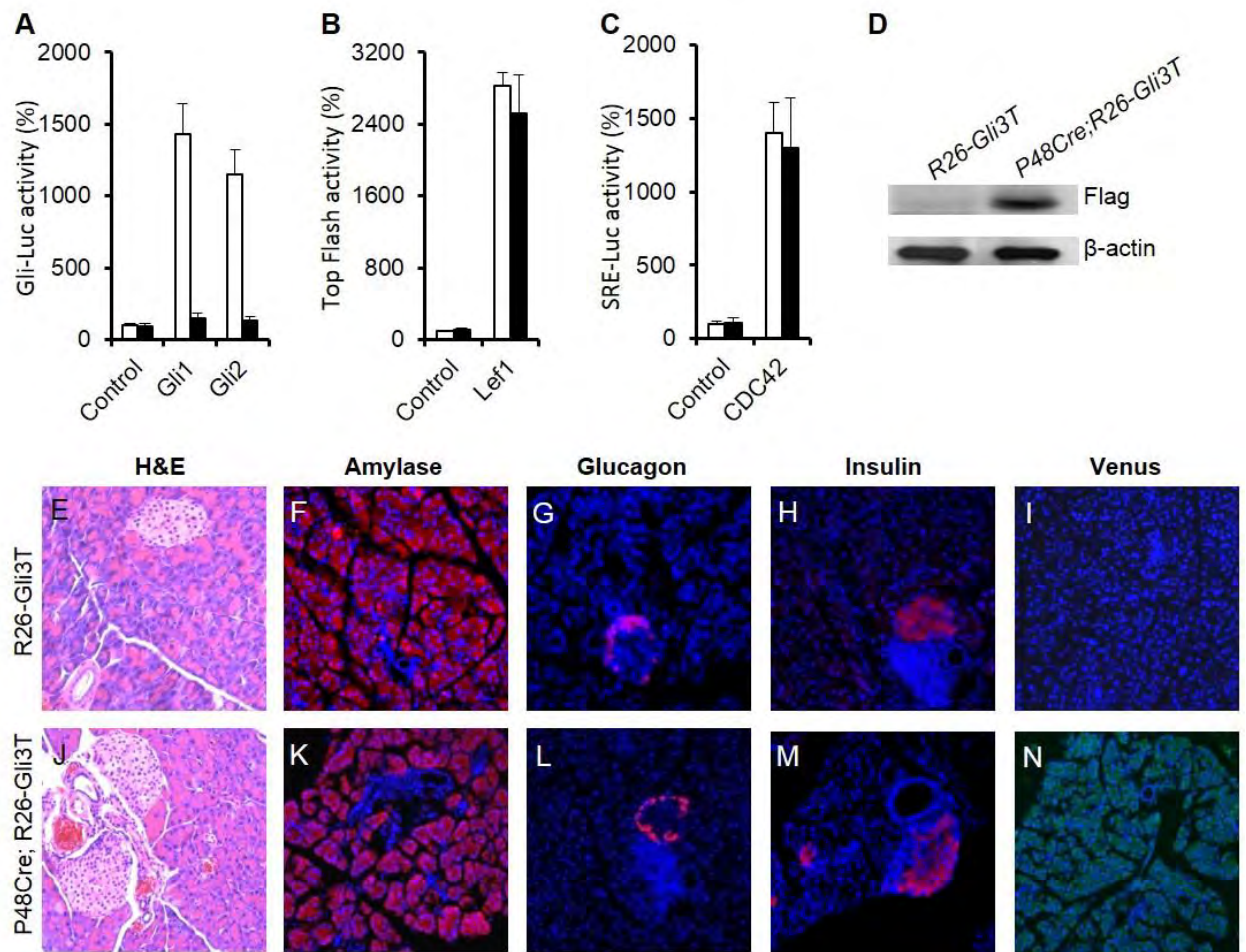


Figure 2.2: GLI activity is dispensable for pancreatic development

(A–C) Gli3T blocks Gli-dependent transcriptional activity. Gli3T inhibited Gli-luciferase reporter activity induced by GLI1 and GLI2 in transfected NIH3T3 cells (A). Gli3T does not inhibit TCF4-induced TopFlash reporter activity (B) or serum-response factor–dependent SRE-luc reporter activity induced by the CDC42 GTPase (C). Open bars represent control cells; black bars represent Gli3T-expressing cells. Data shown are expressed as mean \pm standard deviation. (D) Western blot analysis of Gli3T–Flag expression in pancreatic lysates from 6-month-old *R26-Gli3T* and *P48Cre;R26-Gli3T* mice. (E–N) H&E staining (E and J), immunofluorescence staining (red) of Amylase (F and K), Glucagon (G and L), and Insulin (H and M), and Venus fluorescence (I and N) in pancreata derived from 12 month old *R26-Gli3T* (E–I) and *P48Cre;R26-Gli3T* (J–N) mice.

GLI activation does not affect pancreatic development

To further test the role of individual GLI transcription factors in pancreatic development, we also utilized *Rosa26* knock-in alleles of *Gli1* and *Gli2* which act as transcriptional activators (Figure 2.1B and C)^{152,153}. In addition, we used a conditional knockout allele of the repressor *Gli3* (*Gli3^{fl/fl}*) to specifically delete *Gli3* in the pancreatic epithelium (Figure 2.1 F)¹⁵⁴. We bred the *R26-Gli1*, *R26-Gli2*, and *Gli3^{fl/fl}* mice with *P48Cre* mice to generate *P48Cre;R26-Gli1*, *P48Cre;R26-Gli2*, and *P48Cre;Gli3^{fl/fl}* mice (N=8). We found that pancreas of these mice had largely normal architecture compared to wild type mice at 12 months of age (Figure 2.3A-D).

We also tested expression of the endocrine markers Insulin and Glucagon, and acinar cell marker Amylase in the pancreas of *P48Cre;R26-Gli1* mice using immunofluorescence. We found that *P48Cre;R26-Gli1* mice had normal expression of the markers when compared to the control mice (Figure 2.3 E-K), thus indicating that GLI activation does not affect differentiation of the pancreatic lineages. This data suggests that cell autonomous GLI1/2 activation or GLI3 removal does not affect development and differentiation of the pancreas.

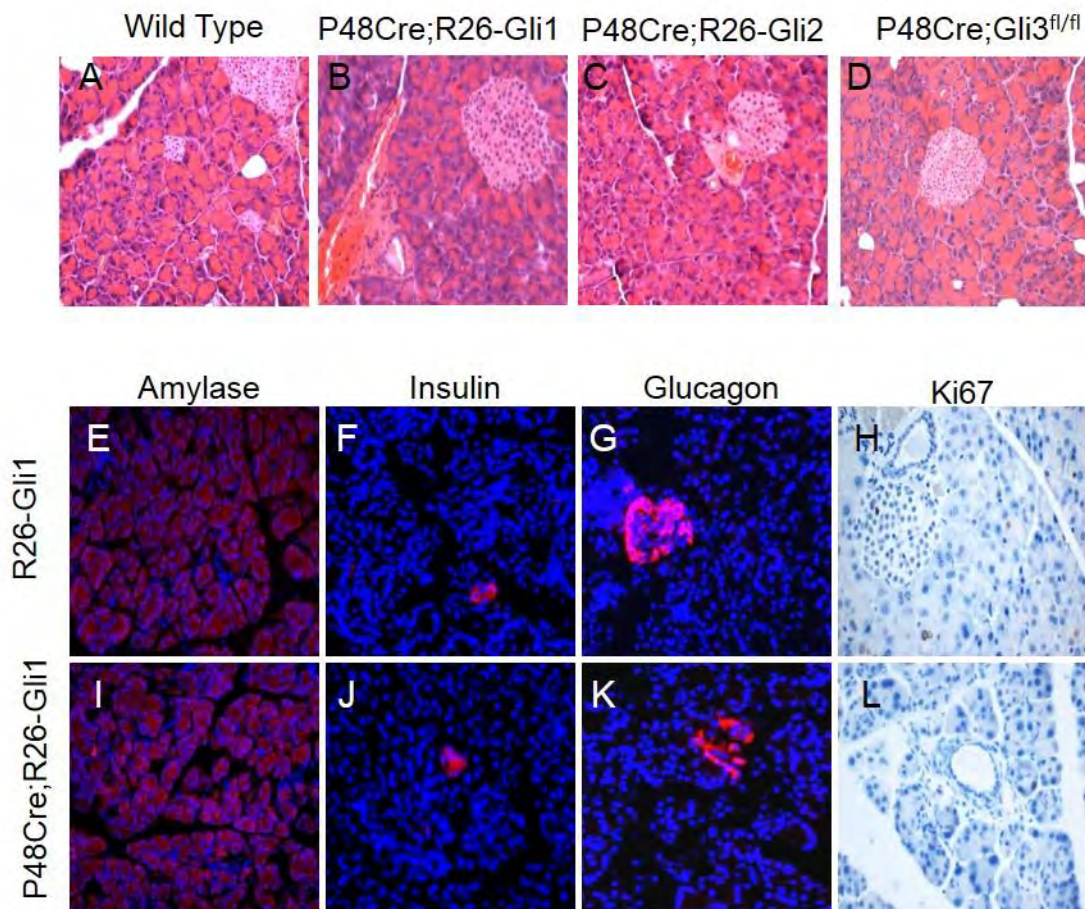


Figure 2.3: GLI activation does not affect pancreatic development

(A-D) H&E stained sections of pancreata from 12 month old wild type (A), *P48Cre;R26-Gli1* (B), *P48Cre;R26-Gli2*, *P48Cre;Gli3^{fl/fl}* mice. (E-L) Immunofluorescence staining (red) for Amylase (E and I), Insulin (E and J), and Glucagon for pancreata from 12 month old *R26-Gli1* (E-G) and *P48Cre;R26-Gli1* (I-K) mice, and immunohistochemistry for Ki67 for pancreata from 12 month old *R26-Gli1* (H), and *P48Cre;R26-Gli1* (L) mice.

GLI activity is required for KRAS induced PanIN lesions

We next investigated the specific role for GLI transcription in regulating KRAS initiated pancreatic tumor development *in vivo*. For this purpose, we generated a mouse model in which simultaneous activation of KRAS and inhibition of GLI transcription was achieved by breeding mice harboring a conditionally activated *Kras* allele (*LSL-Kras^{G12D}*)¹⁵⁵ with *P48Cre;R26-Gli3T* mice. As expected, Cre-mediated activation of the *LSL-Kras^{G12D}* allele in the mouse pancreas resulted in the development of slowly progressive pancreatic intraepithelial neoplasia lesions (PanINs)⁵⁴. At 6 months of age, *P48Cre;LSL-Kras^{G12D}* mice developed early PanIN lesions (Figure 2.4A). These lesions showed evidence of epithelial transformation, with associated mucin accumulation as detected by Alcian blue staining, and a high proliferation index, demonstrated by Ki67 immunohistochemistry (IHC) (Figure 2.4E and I). By 12 months of age, the pancreata of the *P48Cre;LSL-Kras^{G12D}* mice displayed evidence of advanced lesions, including PanIN2 and PanIN3 grade lesions (Figure 2.4C).

In contrast, inhibition of GLI activity resulted in a dramatic reduction in KRAS driven tumorigenesis. *P48Cre;LSL-Kras^{G12D};R26-Gli3T* mice (n=15) examined at 6 months and 12 months of age showed a largely normal parenchymal architecture in the pancreas, with little evidence of neoplasia (Figure 2.4B and 2D). There was no evidence of epithelial transformation, as assayed by Alcian blue staining, and most of the cells in the pancreas were non-proliferating as determined by Ki67 staining (Figure 2.4F and 2J). There was also no evidence of reactive stroma,

which is a hallmark of pancreatic transformation. These findings suggest a critical requirement for GLI transcriptional activation in KRAS induced PanIN lesion formation *in vivo*.

Interestingly, we did detect a few rare PanIN1 lesions in three *P48Cre;LSL-Kras^{G12D};R26-Gli3T* mice (Figure 2.4G and 2H). This observation suggests the possibility that the GLI requirement could eventually be overcome, or that the lesions that developed failed to express Gli3T. To differentiate between these possibilities, we assayed for Gli3T transgene expression in these lesions. Because of the high background in detecting the Gli3T C-terminal Flag-tag with immunofluorescence staining or IHC, we took advantage of the IRES-Venus unit inserted in the *R26-Gli3T* allele that allows detection of transgene expression by Venus fluorescence on cryosections. We found that the lesions from the *P48Cre;LSL-Kras^{G12D};R26-Gli3T* mice were Venus negative compared to the adjacent normal appearing islet and acinar tissues (Figure 2.4G and H), thus indicating that the Gli3T transgene was not expressed in these lesions. These results suggest that the development of rare lesions in animals bearing the *R26-Gli3T* allele is due to inefficient Cre recombination, and the failure to express Gli3T.

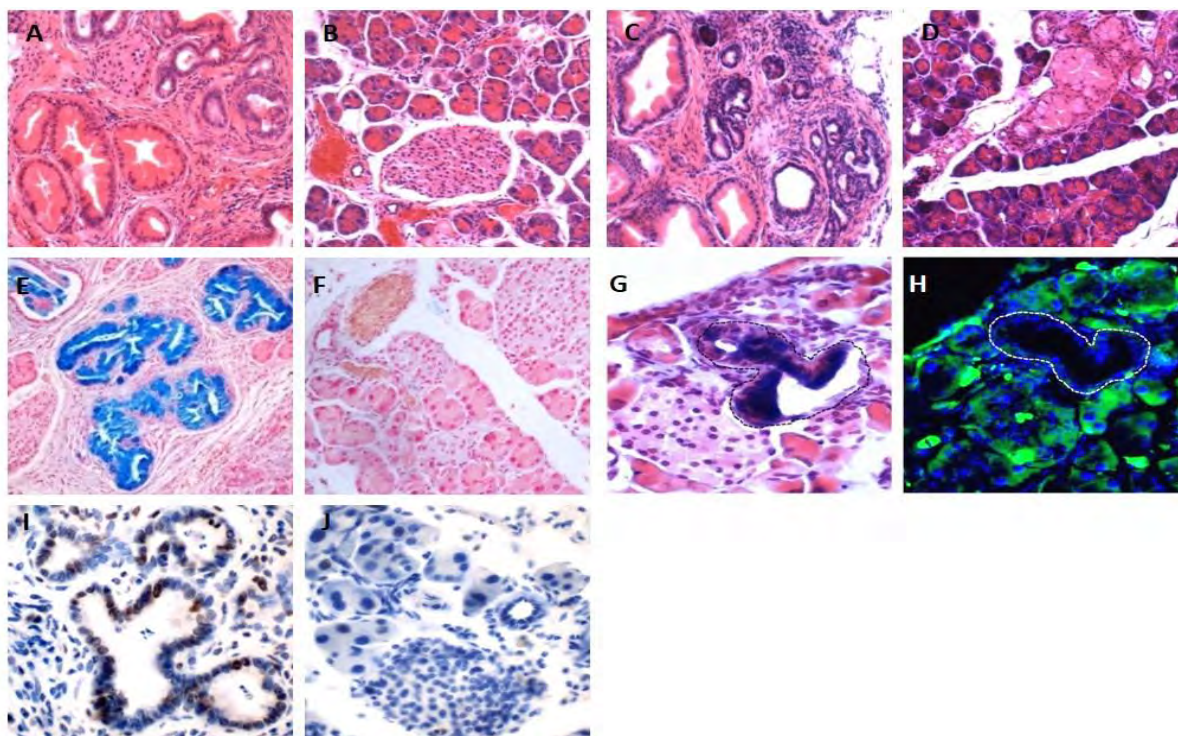


Figure 2.4: GLI transcriptional activity is required for KRAS induced pancreatic neoplasia

(A–D) Representative H&E stained sections from pancreas of 6 month old (A–B), and 12 month old (C–D) *P48Cre;LSL-Kras^{G12D}* (A and C), and *P48Cre;LSL-Kras^{G12D};R26-Gli3T* (B and D) mice. H&E staining shows widespread advanced PanIN formation in *P48Cre;LSL-Kras^{G12D}* pancreata, but only rare PanIN1 lesions were detected in a 12-mo-old and *P48Cre;LSL-Kras^{G12D};R26-Gli3T* sections. (E–F) Alcian blue stained paraffin sections from 6 month old *P48Cre;LSL-Kras^{G12D}* (E), and *P48Cre;LSL-Kras^{G12D};R26-Gli3T* (F) mice. Alcian blue staining indicates widespread neoplasms in 12 month old *P48Cre;LSL-Kras^{G12D}* mice, but no evidence of neoplasia in *P48Cre;LSL-Kras^{G12D};R26-Gli3T* mice. (G–H) H&E staining (G), and Venus fluorescence on a PanIN lesion from a *P48Cre;LSL-Kras^{G12D};R26-Gli3T* mouse. Note robust Venus fluorescence in adjacent acinar and endocrine cells indicating expression of Gli3T, but lack of fluorescence within the lesion. The boundary of the lesions from the adjacent slides is delineated by dashed black (G) and white (H) lines. (I–J) Ki67 immunohistochemistry on sections from pancreas of 6 month old *P48Cre;LSL-Kras^{G12D}* (I), and *P48Cre;LSL-Kras^{G12D};R26-Gli3T* (J) mice. High number of proliferative cells are seen in *P48Cre;LSL-Kras^{G12D}* but not *P48Cre;LSL-Kras^{G12D};R26-Gli3T* mouse pancreas.

GLI activation is required for Kras-dependent PDAC formation

Our data suggests that GLI transcriptional activity is required for KRAS initiated formation of precursor PanIN lesions. To test whether GLI activity is required for progression of these lesions to full blown adenocarcinoma, we generated compound mice bearing a single conditional knockout *Trp53* allele¹⁵⁶, in addition to the *LSL-Kras^{G12D}*, *R26-Gli3T*, and *P48Cre* alleles. Mice negative for the *R26-Gli3T* allele rapidly developed pancreatic adenocarcinoma, with a median latency of 111 days (range 85-164 days) (Figure 2.5A). These tumors were predominantly moderate to poorly-differentiated ductal adenocarcinomas that were frequently invasive and metastatic, with dissemination to lymph nodes, the adjacent intestine, liver, peritoneal cavity and lungs (Figure 2.5B, panels i-v). By contrast, mice bearing the *R26-Gli3T* allele developed carcinomas with a longer latency, with a median age of 193 days (range 115-270 days) (Figure 2.5A). In spite of delayed kinetics, the tumors that developed in the *R26-Gli3T* positive animals were histologically indistinguishable from those in *R26-Gli3T* negative mice, and were also commonly metastatic (Figure 2.5B, panels vi-ix).

The eventual formation of pancreatic tumors in animals bearing the *R26-Gli3T* allele again raises the question whether the tumors that developed failed to express Gli3T, or whether *Trp53* deletion obviates the need for GLI activity, as suggested by a previous study¹⁵⁷. Thus, we assayed for the presence of the Gli3T protein in lysates from tumors, and cell lines derived from these tumors, by immunoblotting with an anti-Flag antibody. While Gli3T protein could be readily

detected in 293T cells transfected with a Gli3T expression construct (and pancreas tissue from *P48Cre;R26-Gli3T* mice, see Figure 2.2), Gli3T could not be detected in any of the tumor (Figure 2.5C, top panel) or cell line (Figure 2.5C, lower panel) lysates. To determine whether these tumors were derived from cells that failed to undergo recombination, we analyzed DNA extracted from tumors and cell lines with PCR primers that differentiate between unrecombined and recombined *R26-Gli3T* alleles. The unrecombined allele was readily detected in all samples isolated from mice carrying the *R26-Gli3T* allele, whereas only one tumor sample tested positive for the presence of the recombined allele (Figure 2.5D). Interestingly, a cell line derived from the tumor that tested positive for recombination was negative for the presence of the recombined *R26-Gli3T* allele, suggesting that the cells in the tumor carrying the recombined allele represented a minority of the sample, or were normal epithelial cells entrapped within the mass of the tumor. Collectively, our data demonstrate that GLI transcriptional activity is required for pancreatic adenocarcinoma formation *in vivo*.

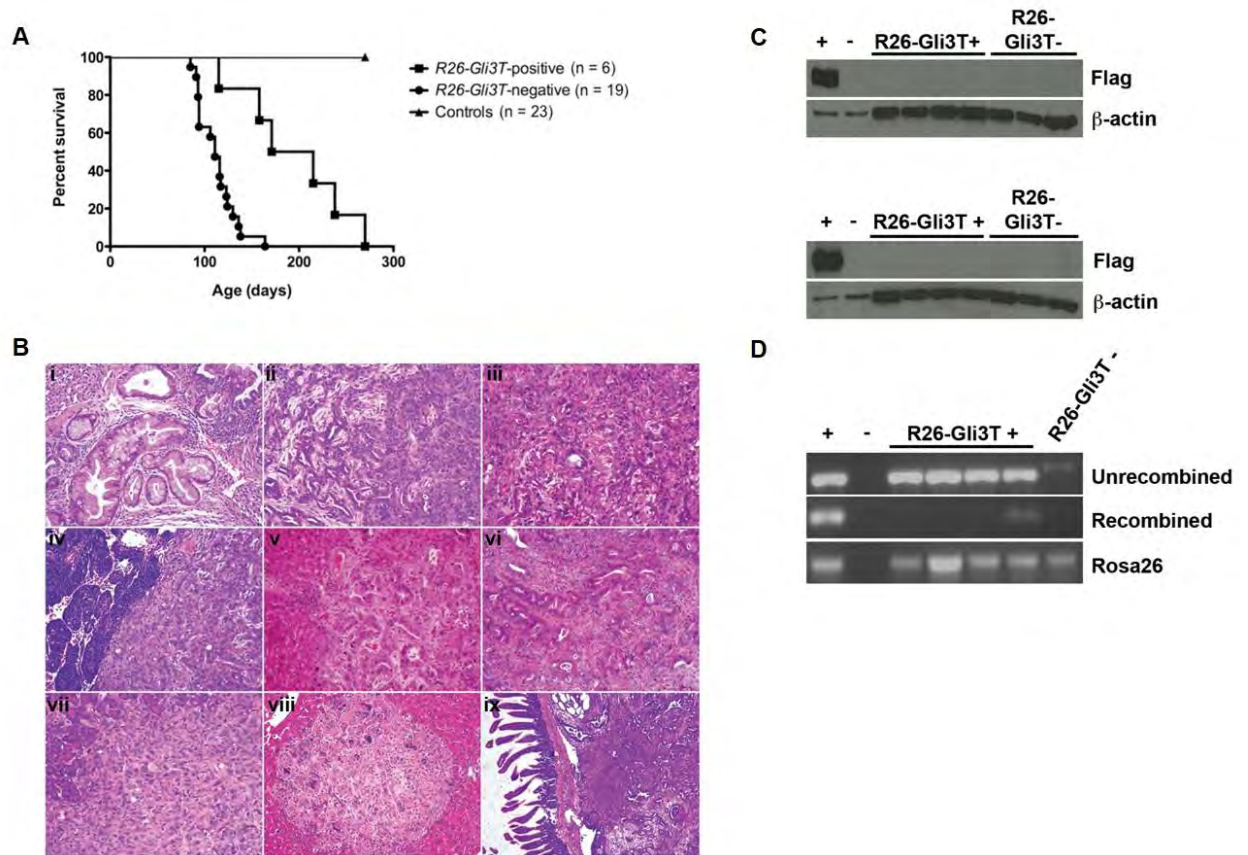


Figure 2.5: GLI inhibition prolongs survival in a mouse model of PDAC

(A) Kaplan–Meier survival curve for *P48Cre;LSL-Kras^{G12D};Trp53^{flox/wt}* mice, *P48Cre;LSL-Kras^{G12D};Trp53^{flox/wt};R26-Gli3T* mice, and *P48Cre* and *LSL-Kras^{G12D}* negative littermate controls. $P < 0.001$ for comparison between R26-Gli3T–positive and –negative animals. (B) Representative H&E-stained histological sections from lesions arising in *P48Cre;LSL-Kras^{G12D};Trp53^{flox/wt}* (i–v) and *P48Cre;LSL-Kras^{G12D};Trp53^{flox/wt};R26-Gli3T* (vi–ix) mice. PanIN lesions (i), glandular PDAC (ii), undifferentiated carcinoma (iii), lymph node metastasis (iv), and liver metastasis (v) were identified in *P48Cre;LSL-Kras^{G12D};Trp53^{flox/wt}* mice. Glandular PDAC (vi), undifferentiated carcinoma (vii), liver metastasis (viii), and intestinal invasion (ix) were observed in *P48Cre;LSL-Kras^{G12D};Trp53^{flox/wt};R26-Gli3T* mice. (C) Immunoblotting for Gli3T protein (using an anti-Flag antibody) in lysates from *R26-Gli3T* positive (+) and –negative (–) tumors (Upper) and cell lines derived from these tumors (Lower). (D) PCR of genomic DNA to detect the recombination status of the *R26-Gli3T* allele in *R26-Gli3T* positive tumor samples. Amplification of the native *Rosa26* locus was used as a control.

GLI, but not Smoothed activation accelerates KRAS initiated pancreatic tumorigenesis

Prior work has suggested that KRAS regulates *Gli1* expression in PDAC cells, and GLI1 activity is critical for PDAC cell survival and transformation *in vitro*¹⁴³. Reports have also suggested that GLI2 ectopic expression in the absence of oncogenic KRAS can give rise to undifferentiated tumors in the pancreas¹³⁹. In order to test whether GLI transcriptional activity can cooperate with KRAS in driving tumorigenesis, we used the *Rosa26* knock-in alleles of *Gli1* and *Gli2*, and the *Gli3* floxed allele to generate *P48Cre;LSL-Kras^{G12D};R26-Gli1*, *P48Cre;LSL-Kras^{G12D};R26-Gli2*, and *P48Cre;LSL-Kras^{G12D};R26-Gli3^{fl/fl}* mice.

Previous data has suggested that conditional knockout of the GLI upstream activator Smoothed in the context of oncogenic KRAS does not affect pancreatic tumorigenesis¹⁴³. In order to test whether activation of Smoothed can cooperate with oncogenic KRAS, we also used a previously described oncogenic allele of Smoothed (*SmoM2*) that was knocked in to the *Rosa26* locus (*R26-SmoM2*)¹⁴⁹.

P48Cre;LSL-Kras^{G12D};R26-Gli1, *P48Cre;LSL-Kras^{G12D};R26-Gli2*, *P48Cre;LSL-Kras^{G12D};R26-SmoM2*, and *P48Cre;LSL-Kras^{G12D};R26-Gli3^{fl/fl}* mice were generated and monitored for over 12 months. We found that *P48Cre;LSL-Kras^{G12D};R26-Gli1*, and *P48Cre;LSL-Kras^{G12D};R26-Gli2* showed dramatically accelerated tumorigenesis compared to *P48Cre;LSL-Kras^{G12D}* mice. At 2 months age, when normal architecture is preserved in most of the pancreas of

P48Cre;LSL-Kras^{G12D} mice, with normal islets, acini, and ducts making up majority of the pancreas, and only a small number of low grade PanIN1 lesions (Figure 2.6A). In contrast to this, *P48Cre;LSL-Kras^{G12D};R26-Gli1*, and *P48Cre;LSL-Kras^{G12D};R26-Gli2* mice presented with high grade PanIN3 lesions (Figure 2.6B-C) that showed signs of severe nuclear atypia, and luminal invasion. Normal pancreatic architecture was almost completely destroyed in these mice.

While KRAS activation in the pancreas was sufficient to initiate transformation, the *P48Cre;LSL-Kras^{G12D}* mice did not show significantly accelerated mortality compared to wild type mice, and most *P48Cre;LSL-Kras^{G12D}* mice lived over 12 months. However, we found that *P48Cre;LSL-Kras^{G12D};R26-Gli1*, and *P48Cre;LSL-Kras^{G12D};R26-Gli2* had a dramatically reduced survival, with most mice not living past 2 months age (Figure 2.6F). This data suggests that GLI1 and GLI2 can cooperate with oncogenic KRAS to accelerate pancreatic tumorigenesis.

In contrast to the GLI1/2 activation, we found that the rate of pancreatic transformation in *P48Cre;LSL-Kras^{G12D};R26-SmoM2*, and *P48Cre;LSL-Kras^{G12D};R26-Gli3^{fl/fl}* was not significantly different from *P48Cre;LSL-Kras^{G12D}* mice. *SmoM2* ectopic expression, and *Gli3* deletion did not affect survival of the mice, with most mice living past 12 months (Figure 2.6D-F). Our findings suggest that while GLI1, or GLI2 ectopic expression can dramatically accelerate pancreatic tumorigenesis in cooperation with KRAS, oncogenic activation of Smoothed, and deletion of *Gli3* does not affect pancreatic tumorigenesis.

Molecular analysis of the *P48Cre;LSL-Kras^{G12D};R26-Gli1* advanced lesions revealed that cell proliferation, as measured by Ki67 staining, was significantly increased compared to the in the *P48Cre;LSL-Kras^{G12D}* mice (Figure 2.7A-C). Consistent with a previous report⁵⁸, we also detected senescence-associated β -galactosidase expression in early PanIN lesions from two-month-old *P48Cre;LSL-Kras^{G12D}* mice (Fig. 2.7D), while high-grade PanIN lesions from age-matched *P48Cre;LSL-Kras^{G12D};R26-Gli1* mice did not exhibit detectable senescence-associated β -galactosidase staining (Figure 2.7E), suggesting escape from KRAS induced growth arrest/senescence. Immunofluorescence for Cytokeratin-8 conducted on *P48Cre;LSL-Kras^{G12D};R26-Gli1* confirmed the epithelial nature of the PanIN lesions, and we also found evidence of a high degree of desmoplasia in the pancreas as evidenced by Smooth Muscle Actin staining (Fig. 2.7I).

In spite of dramatically accelerated PanIN lesion formation and mortality, we did not detect invasive PDAC in the *P48Cre;LSL-Kras^{G12D};R26-Gli1*, and *P48Cre;LSL-Kras^{G12D};R26-Gli2* mice, and metastasis was not observed in any of the mice we examined. Interestingly, we detected large multilocular cystic lesions resembling mucinous cystic neoplasms (Figure 2.7G) in two of the *P48Cre;LSL-Kras^{G12D};R26-Gli1* mice, in addition to the PanIN lesions we described above. The cysts from the *P48Cre;LSL-Kras^{G12D};R26-Gli1* mice were as large as 2 cm in diameter and lined by columnar epithelial cells, where abundant mucin production was demonstrated by reaction with Alcian blue (Figure 2.7H). Together, our results suggest that GLI

activation is not sufficient to initiate PDAC, but synergizes with KRAS to promote pancreatic tumor formation *in vivo*.

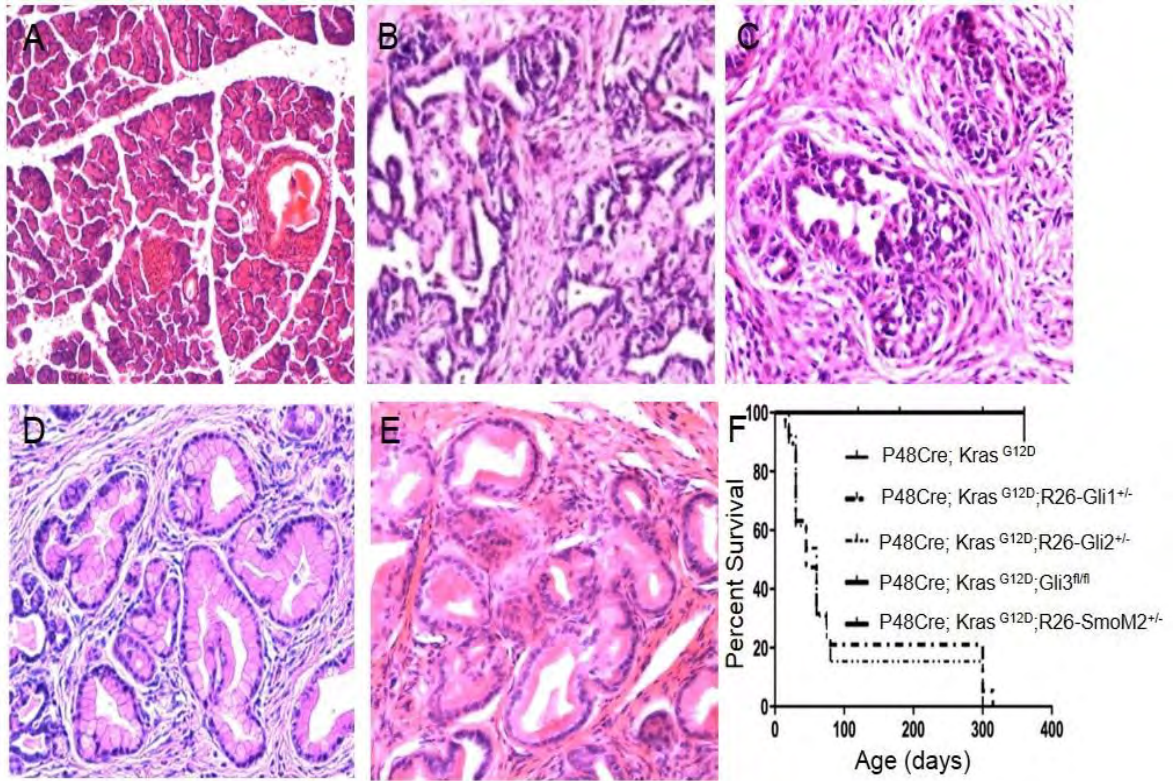


Figure 2.6: Activation of GLI accelerates KRAS induced pancreatic tumorigenesis

(A-C) H&E staining on pancreas sections of 2 month old *P48Cre;LSL-Kras^{G12D}* (A), *P48Cre;LSL-Kras^{G12D};R26-Gli1* (B), and *P48Cre;LSL-Kras^{G12D};R26-Gli2* (C) mice. Mice with GLI1 or GLI2 ectopic expression in combination with *KRAS^{G12D}* show formation of advanced PanIN lesions whereas mice with *KRAS^{G12D}* show rare instances of early PanIN lesions. (D-E) H&E staining on pancreas sections of 6 month old *P48Cre;LSL-Kras^{G12D}* (D), and H&E staining on pancreas sections of 2 month old *P48Cre;LSL-Kras^{G12D};Gli3^{fl/fl}* (E) mice. Abrogation of GLI3 expression does not significantly affect PanIN lesion formation. (F) Kaplan-meier survival curve for *P48Cre;LSL-Kras^{G12D}*, *P48Cre;LSL-Kras^{G12D};R26-Gli1*, *P48Cre;LSL-Kras^{G12D};R26-Gli2*, *P48Cre;LSL-Kras^{G12D};R26-SmoM2*, and *P48Cre;LSL-Kras^{G12D};R26-Gli3^{fl/fl}* mice. GLI1/2 activation leads to a dramatic decrease in survival, while ectopic expression of *SmoM2* and deletion of *Gli3* does not affect survival.

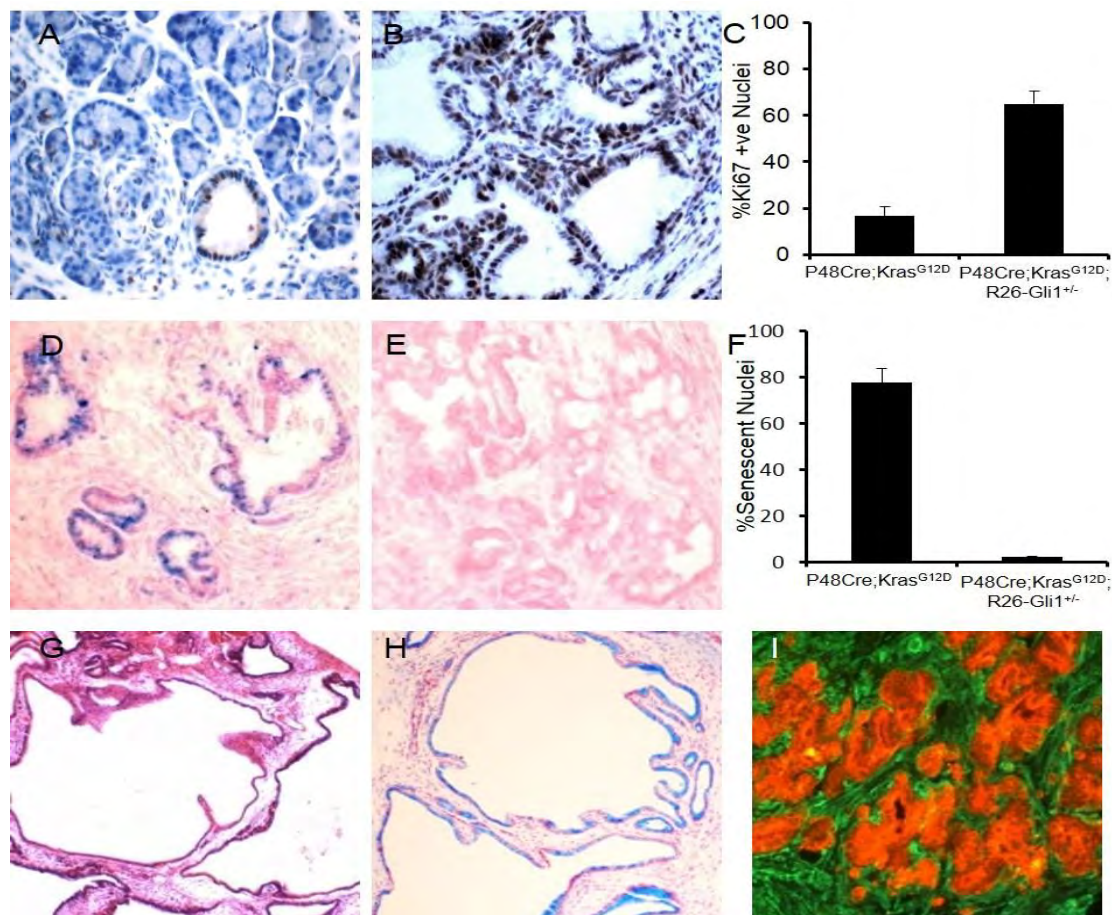


Figure 2.7: GLI1 drives cell proliferation, evasion of senescence and desmoplasia in pancreatic neoplasms

(A-C) Immunohistochemistry for Ki67 on pancreata sections from 2 month old *P48Cre;LSL-Kras^{G12D}* (A), and *P48Cre;LSL-Kras^{G12D};R26-Gli1* (B) mice. Quantification of Ki67 positive nuclei shows significantly higher proliferation in *P48Cre;LSL-Kras^{G12D};R26-Gli1* mouse PanIN lesions (C). (D-E) β -Galactosidase staining for senescence on sections from 12 month old *P48Cre;LSL-Kras^{G12D}* (D), and *P48Cre;LSL-Kras^{G12D};R26-Gli1* (E) mice. Quantification of β -Galactosidase positive cells shows significantly higher senescence in *P48Cre;LSL-Kras^{G12D};R26-Gli1* mouse PanIN lesions (F). (G) H&E staining of pancreatic cystic lesions from a 1 month old *P48Cre;LSL-Kras^{G12D};R26-Gli1* mouse. (H) Alcian blue staining revealing abundant mucin production in epithelial cells in the cysts. (I) Immunofluorescence for Cytokeratine 8 (red), and Smooth muscle actin (green) on sections from a *P48Cre;LSL-Kras^{G12D};R26-Gli1* mouse showing strong desmoplastic reaction.

A GLI dependent transcriptional program in PDAC cells

The results from our loss-of-function and gain-of-function genetic analyses underscore the functional importance of cell autonomous GLI activation in pancreatic epithelial transformation. However, the GLI mediated transcriptional program in pancreatic cancer remains largely unexplored.

Panc1 and MiaPaCa2 are human PDAC cell lines that contain activating mutations in *KRAS*, and are a commonly used model to study pancreatic cancer¹⁴³. We found that inhibition of GLI activity in these cell lines by ectopic expression of Gli3T led to a significant decrease in cell proliferation, increase in apoptosis, and decrease in tumorigenicity measured by soft agar colony formation (Figure 2.8 A-C). Knockdown of *GLI1* in these cells using two different shRNAs also led to a dramatic decrease in cell proliferation, increase in apoptosis, and decrease in tumorigenicity (Figure 2.8D-F), and these findings could also be replicated by inhibition of GLI activity using a small molecule inhibitor of GLI, Gant61¹⁵⁸ (Figure 2.8G-I). This data highlights the importance of GLI transcriptional activity in maintaining the oncogenic properties of the *KRAS* mutant human PDAC cell lines. We used the Panc-1 cell line as a model to further analyze the transcriptional program regulated by GLI in PDAC.

To identify GLI dependent downstream target genes, we performed gene expression profiling on Gli3T expressing Panc-1 cells, and vector controls. We transfected Panc-1 cells with a Gli3T-IRES-nuclear GFP expression construct, and

24 hours post-transfection we isolated GFP-positive cells by flow cytometry (FACS), and performed expression profiling using Affymetrix chips. As expected, we detected upregulation of *GLI3*, that likely reflects the expression of the ectopic Gli3T transgene. We identified 265 genes that were significantly downregulated by Gli3T (refer to Rajurkar et al 2012 for data set¹⁴⁸); among them, *PTCH1* and *FOXA2* are known transcriptional targets of the Hedgehog/GLI pathway (Figure 2.9A). Interestingly, we found that the majority of the genes identified in our array were not known GLI target genes, and were not previously associated with Hedgehog/GLI signaling. When we compared the genes identified in our screen to the Hedgehog target genes expressed in Hh induced BCC, medulloblastoma, and rhabdomyosarcoma, we found only few overlapping genes (Figure 2.9B), thus indicating that Gli regulates a unique transcriptional program in the context of pancreatic cancer.

We identified several genes involved in regulating RAS intracellular signal transduction, including *SOS2* (RasGEF), *RASA1* (RasGAP), *RIN2* and *RASSF4/5* (Figure 2.9A), suggesting possible feedback regulation of Kras signaling in cancer cells influenced by GLI activity. The PI3K/AKT and MEK/Erk pathways are KRAS stimulated signaling pathways that have been implicated in tumorigenesis¹⁵⁹. In our transcriptional profiling, we found that two subunits of PI3K, *PIK3R1* and *PIK3C2B*, were among the genes whose expression was significantly downregulated by Gli3T (Figure 2.9A), indicating a possible interaction between GLI and PI3K/AKT signaling. We also detected an enrichment of genes associated

with the NF- κ B pathway, including IKBKE (IKK ϵ), TRAF1, TRAF3IP2 and NFKBIE, indicating that GLI may play a role in regulating the NF- κ B pathway in PDAC (Figure 2.9A). Together, these findings point to a potential previously unidentified role played by GLI in regulating the KRAS, PI3K/AKT and NF- κ B pathways in PDAC.

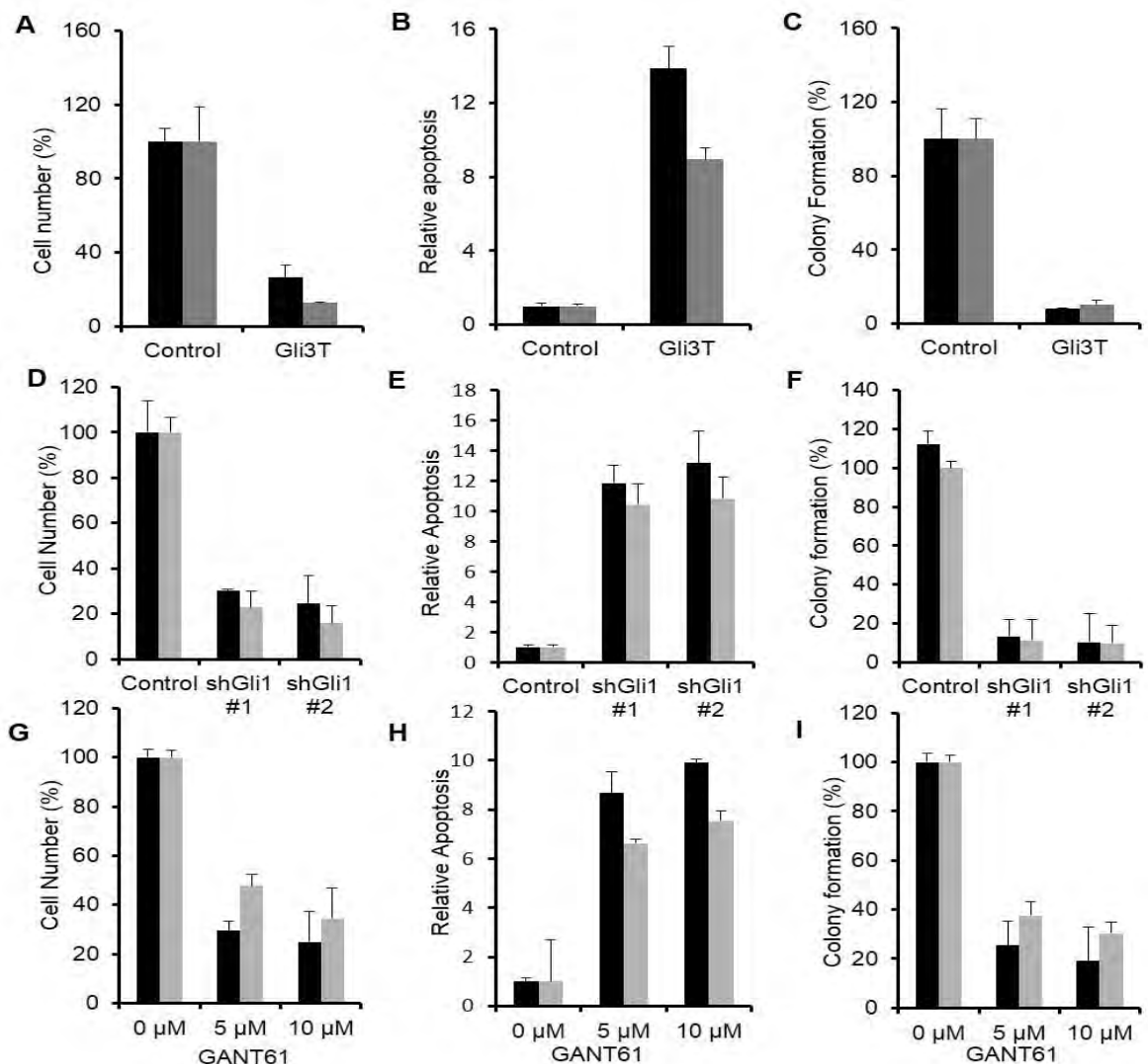


Figure 2.8: GLI transcriptional activity is required for human PDAC cell survival

Significant decrease in cell proliferation is seen as measured by MTT assay in response to inhibition by Gli3T (A), shRNA mediated knockdown of *GLI1* (D), and treatment with small molecule GLI inhibitor Gant61 (G) in Panc-1 (black bars), and MiaPaca2 (grey bars) cells. Apoptosis levels are significantly increased in response to Gli3T repression (B), shRNA knockdown of *GLI1* (E), and treatment with Gant61 (H) in Panc-1 and MiaPaca2 cells as measured by immunofluorescence for cleaved-Caspase-3. Tumorigenicity of Panc-1 and MiaPaca2 cells is significantly decreased in response to Gli3T repression (C), *GLI1* knockdown (F), and Gant61 treatment (I). Error bars represent standard deviation.

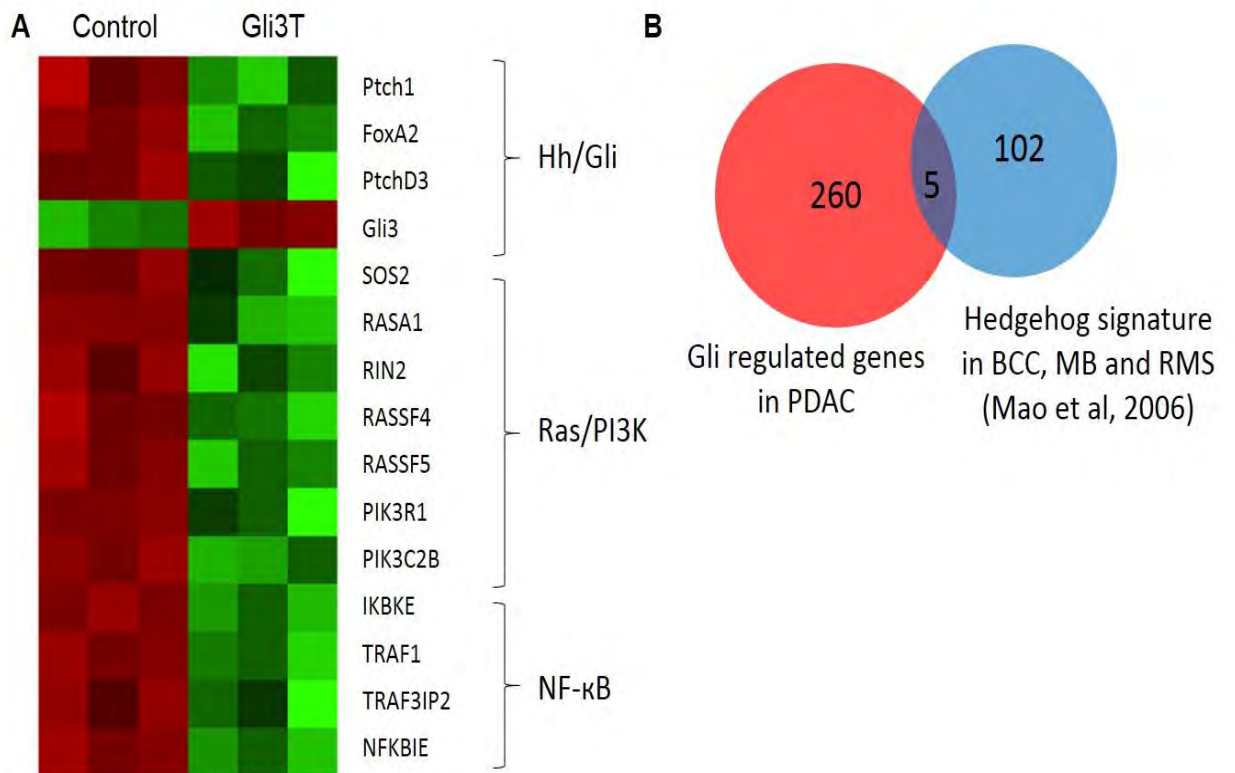


Figure 2.9: A GLI mediated transcriptional program in PDAC

(A) Heat map illustrating mRNA expression of selected genes in Gli3T-expressing and control Panc1 cells. Red, high expression; green, low expression. (B) Schematic showing number of Gli regulated genes in PDAC, and BCC, medulloblastoma (MB) and rhabdomyosarcoma (RMS).

GLI regulates AKT activation in PDAC:

As discussed previously, the PI3K/AKT pathway is an important effector of KRAS in pancreatic transformation. While KRAS is known to directly regulate PI3K activity at the cell membrane, our findings implicate GLI as a potential transcriptional regulator of the PI3K pathway. In order to further test the role of GLI in PI3K/AKT signaling, we inhibited GLI transcriptional activity in Panc-1 cells using the Gli3T repressor. We found that GLI inhibition led to a significant decrease in AKT phosphorylation at Serine-473. In contrast, we did not detect any significant difference in phosphorylation of ERK, which is another important oncogenic effector of KRAS that acts downstream of RAF/MEK in PDAC (Figure 2.10A). Also, we found that inhibition of GLI activity using the small molecule inhibitor Gant61 led to a dosage dependent decrease in AKT phosphorylation at Serine-473 (Figure 2.10B). We decided to further explore the phosphorylation status of both AKT and ERK *in vivo* in *P48Cre;LSL-Kras^{G12D}* and *P48Cre;LSL-Kras^{G12D};R26-Gli1* mouse pancreas. Immunohistochemical staining revealed that while AKT phosphorylation at Serine-473 was markedly upregulated in the PanIN lesions of *P48Cre;LSL-Kras^{G12D};R26-Gli1* mice compared to *P48Cre;LSL-Kras^{G12D}* mice (Figure 2.10 C-D), there was no significant difference in the phosphorylation levels of ERK (Figure 2.10 E-F). Our data suggests that GLI plays a role in regulating the PI3K/AKT pathway in pancreatic transformation. These findings also point to an additional layer of complexity in KRAS regulation of PI3K/AKT signaling.

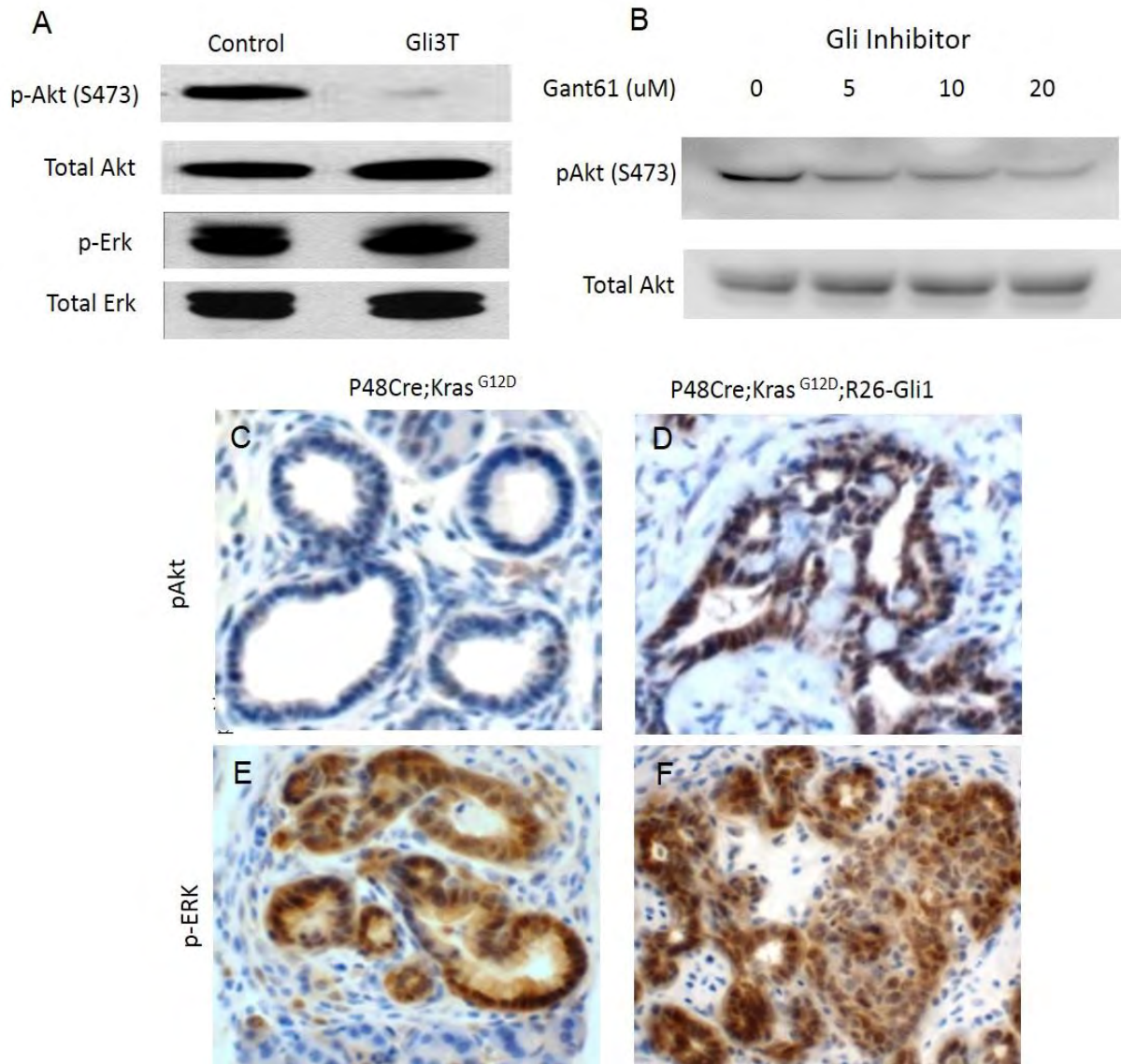


Figure 2.10: GLI mediates AKT phosphorylation in PDAC

(A) Immunoblot analysis of levels of AKT phosphorylation (p-AKT S473), total AKT, ERK phosphorylation (p-ERK), and total ERK, in isolated Gli3T-expressing and control Panc1 cells. (B) Immunoblot showing levels of phospho-AKT and total AKT in response to varying doses of Gant61 48 hours after treatment with inhibitor. (C-F) IHC for p-AKT (C and D) and p-ERK (E and F) in PanIN from 2 month old *P48Cre;LSL-Kras^{G12D}* (C and E) and *P48Cre;LSL-Kras^{G12D};R26-Gli1* (D and F) mice.

GLI mediated regulation of and NF- κ B activity in PDAC

As our gene expression profiling data suggested a possible regulation of the NF- κ B pathway by GLI, we further explored the relationship between NF- κ B and GLI signaling using human PDAC cells, and *in vivo*. We first examined whether NF- κ B transcriptional activity is regulated by GLI. Panc-1 cells expressing Gli3T exhibited a significantly lower level of NF- κ B activity compared to cells expressing a control plasmid, as assayed by measuring the activity of a synthetic NF- κ B luciferase reporter gene (Figure 2.11A). Next we tested whether NF- κ B target gene expression is regulated by GLI in PDAC. When treated with Gli3T, NF- κ B target gene expression was significantly downregulated in Panc-1 cells as measured by quantitative RT-PCR (Figure 2.11B), suggesting regulation of NF- κ B activity by GLI. In order to test regulation of NF- κ B by GLI *in vivo*, we conducted immunohistochemical staining for RELA (p65) on pancreata of 2 month old *P48Cre;LSL-Kras^{G12D}* and *P48Cre;LSL-Kras^{G12D};R26-Gli1* mice, as well as human PDAC samples. As described previously, p65 is a member of the NF- κ B family, and nuclear accumulation of p65 indicates activation of the classical NF- κ B pathway. We found that, compared to those in *P48Cre;LSL-Kras^{G12D}* mice, epithelial cells in *P48Cre;LSL-Kras^{G12D};R26-Gli1* mice exhibited significantly higher levels of p65 in the nucleus (Figure 2.11C-D). The high levels of nuclear p65 were comparable to the p65 nuclear translocation in the human PDAC samples. In summary, these observations demonstrate regulation of NF- κ B

signaling by GLI proteins in PDAC cells, and highlight a potential mechanism of regulation of NF- κ B activity by oncogenic KRAS in PDAC formation.

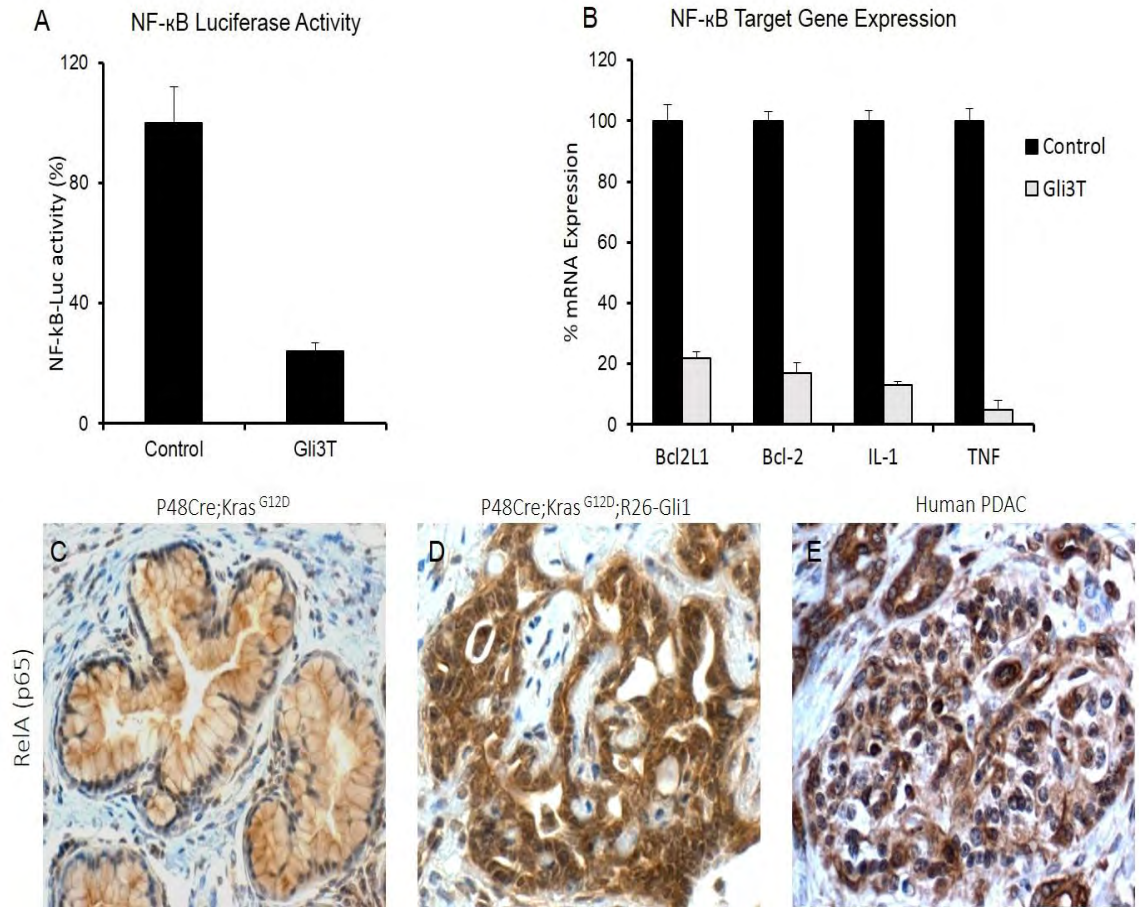


Figure 2.11: GLI regulates NF-κB signaling in PDAC

(A) Relative expression of a p65 (RELA) luciferase reporter in Panc-1 cells expressing Gli3T, and a control vector. Significant decrease in p65 luciferase activity is seen in response to Gli3T mediated repression. (B) Relative NF-κB target gene expression normalized to GAPDH in control, and Gli3T expressing Panc-1 cells. Significant decrease in NF-κB target gene expression is seen in response to Gli3T mediated repression. Error bars represent standard deviation. (C-E) Immunohistochemistry for p65 in 2 month old *P48Cre;LSL-Kras^{G12D}* (C), *P48Cre;LSL-Kras^{G12D};R26-Gli1* (D) mouse pancreas sections, and a section of human PDAC sample (E). High levels of nuclear p65 indicating NF-κB pathway activation are seen in *P48Cre;LSL-Kras^{G12D};R26-Gli1* sections and human PDAC samples, but not in *P48Cre;LSL-Kras^{G12D}* mouse pancreas.

MATERIALS AND METHODS

Mouse strains

P48Cre, *LSL-Kras^{G12D}*, *Trp53^{flox}*, *R26-Gli3T*, *Rosa-26Gli1*, *Rosa26-Gli2*, *Rosa26-SmoM2*, and *Gli3^{fl/fl}* mice have been described before^{54,160,150,161,154}. *P48Cre;LSL-Kras^{G12D};R26-Gli3T*, *P48Cre;LSL-Kras^{G12D};R26-Gli1*, *P48Cre;LSL-Kras^{G12D};R26-Gli2*, *P48Cre;LSL-Kras^{G12D}*, and *P48Cre;LSL-Kras^{G12D};R26-SmoM2* were obtained by interbreeding *P48Cre* mice with *LSL-Kras^{G12D};R26-Gli3T*, *LSL-Kras^{G12D};R26-Gli1*, *LSL-Kras^{G12D};R26-Gli2*, and *LSL-Kras^{G12D};R26-SmoM2* mice respectively. *P48Cre;LSL-Kras^{G12D}; Gli3^{fl/fl}* mice were obtained by crossing *P48Cre* mice to *LSL-Kras^{G12D}; Gli3^{fl/fl}* mice. Offspring from the cross of *LSL-Kras^{G12D};R26-Gli3T* to *P48cre;Trp53^{flox/flox}* mice were followed longitudinally for tumor development for 270 days. All mouse experiments were performed according to the guidelines of IACUC at University of Massachusetts Medical School.

Tissue collection and histology

Upon euthanasia, primary pancreatic tissues and metastatic lesions were separated in pieces and fixed in 4% (wt/vol) paraformaldehyde. For paraffin sections, tissues were dehydrated, embedded in paraffin blocks, and cut at a thickness of 6 μ m. For frozen sections, tissues were dehydrated in 30% (wt/vol) sucrose and embedded in Optimal Cutting Temperature compound (OCT; Sakura Finetek), and sections were cut at a thickness of 12 μ m. For RNA and protein

analysis, tissues were flash frozen in liquid nitrogen. Tissue sections (6 μ m) were stained with H&E using standard reagents and protocols.

Generation of cell lines from PDAC tumors

Pancreatic tumors were dissected and minced in cold DMEM containing 10% (vol/vol) FBS. The tumor tissue was then plated in a 10-cm tissue culture dish and allowed to adhere for 2 days. Media was then changed and tumor pieces removed. The epithelial character of the isolated cells was confirmed by staining for pancreas epithelial markers such as cytokeratins 8 and 19.

Immunohistochemistry, immunofluorescence and immunoblotting

For immunohistochemistry, high-temperature antigen retrieval was conducted in Sodium Citrate solution (pH 6.0) on paraffin sections for 30 minutes. Sections were blocked in a buffer containing 5% (wt/vol) BSA and 0.1% (vol/vol) Triton X-100 in PBS and then were incubated overnight at 4 °C in primary antibodies diluted in blocking buffer. Primary antibodies used were: Ki67 (1:500, Abcam), phospho-AKT S473 (1:50, Cell Signaling), phospho-ERK (1:500, Cell Signaling), and p65 (1:50, Santa Cruz). Signal detection was accomplished with biotinylated secondary antibodies in the Vectastain ABC kit (Vector Labs).

For immunofluorescence, OCT sections were washed with PBS and incubated in blocking buffer containing 5% (vol/vol) sheep serum, 1% (vol/vol) FBS, and 0.1% (vol/vol) Triton X-100 in PBS. Sections then were incubated overnight at 4 °C in primary antibodies diluted in blocking buffer. Primary antibodies used were

Glucagon (1:3,000; gift of Andrew Leiter, University of Massachusetts Medical School, Worcester, MA); Amylase (1:800; Sigma); Insulin (1:100; Abcam); smooth muscle actin (1:500; Sigma); and cytokeratin-8 (1:100; Developmental Studies Hybridoma Bank). Alexa Fluor fluorescent conjugated secondary antibodies (Invitrogen) were used for detection at a concentration of 1:500 diluted in blocking buffer. Slides then were mounted in mounting medium containing DAPI.

For immunoblotting, primary antibodies used were: FlagM2-HRP (1:1000, Sigma), β -Actin (1:1000, Sigma), phospho-AKT S473 (1:1000, Cell Signaling), phospho-ERK (1:1000, Cell Signaling), total AKT, (1:1000, Cell Signaling), total ERK (1:1000, Cell Signaling). HRP conjugated secondary antibodies used for detection were obtained from Jackson Laboratories.

Alcian blue staining and senescence-associated β -galactosidase staining

For Alcian blue staining, paraffin sections were hydrated and stained for 30 minutes at room temperature using Alcian blue reagent (IHC World). Sections were counterstained with Nuclear Fast Red. For senescence-associated β -Galactosidase staining, frozen sections were washed in PBS and stained overnight using Senescence β -Galactosidase staining solution (Cell Signaling Technology). Sections were counterstained using eosin.

Cell proliferation, apoptosis and soft-agar assays

Gli3T-expressing GFP-positive Panc1 or MiaPaCa2 cells were isolated 24 h after transfection using flow cytometry. For shRNA knockdown, Panc-1 or MiaPaca2

cells were infected with pLKO-based lentiviruses expressing shRNAs and were selected for 4 days using puromycin. For Gant61 inhibition, Panc-1 or MiaPaca-2 cells were treated with varying doses of Gant61 dissolved in DMSO. Cell proliferation assays were performed in triplicate.

For the MTT-based cell-proliferation assay, the cells were seeded at a density of 3,000 cells per well in a 96-well plate, treated with 5 mg/mL MTT 5 days after seeding, and lysed in DMSO 4 hours later. Absorbance was measured at 595 nm. The assay was performed in triplicate.

For the apoptosis assay, cells were plated in chamber slides after cell sorting, drug selection, or Gant61 treatment. Cells were fixed in 4% paraformaldehyde. Immunostaining with a polyclonal antibody against cleaved caspase-3 (1:400; Cell Signaling) was used as a marker for apoptosis. Apoptotic cells were counted in three wells, and relative apoptosis was measured by comparing the number of apoptotic cells with the control sample. Standard deviation was used to calculate error bars.

For the anchorage-independent growth assay using soft-agar, cells were seeded at a density of 6,000 cells per well in a six-well plate of 0.3% agarose in DMEM containing 10% (vol/vol) FBS. Colonies from 12 fields of view were counted 14 days later. Assays were performed in triplicate.

Luciferase reporter analysis

Cells were co-transfected with luciferase reporter constructs, GliBS-Luc (gift of Dr. H. Kondoh, Osaka University), TOPflash (Addgene), SRE-luc (gift of Dr. D. Wu, Yale University), NF- κ B-Luc (gift of Dr. F. Chan, UMMS), and expression vectors for Renilla luciferase, Gli3T, Gli1, Gli2, Lef1 and CDC42. Luciferase assays were conducted 48 hours after transfection using the dual-luciferase reporter kit (Promega).

Detection of recombination at the R26-Gli3T locus

Polymerase chain reaction was performed on isolated genomic DNA using the primer pair (Forward: 5'- GTAGTCCAGGGTTTCCTTGATG-3', Reverse: 5'- TGCTACTTCCATTTGTCACGTC-3') for detection of the unrecombined *R26-Gli3T* allele, and (Forward: 5'-GTAGTCCAGGGTTTCCTTGATG-3', Reverse: 5'- GGACTTTCATCCTCATTGGAAG-3') for detection of the recombined allele. Primers to the native *Rosa26* locus were used as a control (Forward: 5'- GGAGCGGGAGAAATGGATATG-3', Reverse: 5'- AAAGTCGCTCTGAGTTGTTAT-3').

Quantitative RT-PCR

cDNA synthesis was conducted using Invitrogen SuperScript II kit. Primers used for qRT-PCR were human *GAPDH* (forward: 5'-ATGGGGAAGGTGAAGGTCG-3'; reverse: 5'-GGGGTCATTGATGGCAACAATA-3'); mouse *Gapdh* (forward: 5'- AGGCCGGTGCTGAGTATGTC-3'; reverse: 5'-TGCCTGCTTCACCACCTTCT-3'); β -actin (forward: 5'-TGACAGGATGCAGAAGGAGA-3'; reverse: 5'-

CTGGAAGGTGGACAGTGAGG-3'); human *GLI1* (forward: 5'-
 CCAGCGCCCAGACAGAG-3'; reverse: 5'-GGCTCGCCATAGCTACTGAT-3');
 mouse *Gli1* (forward: 5'-GTCGGAAGTCCTATTCACGC-3'; reverse: 5'-
 CAGTCTGCTCTCTTCCCTGC-3'); human *PTCH1* (forward: 5'-
 CCACAGAAGCGCTCCTACA-3'; reverse: 5'-CTGTAATTTGCCCCCTTCC-3');
 mouse *Ptc1* (forward: 5'-AACAAAAATTCAACCAAACCTC-3'; reverse: 5'-
 TGTCTTCATTCCAGTTGATGTG-3'); human *IL1A* (forward:
 ATCATGTAAGCTATGGCCCACT; reverse: CCTTCCCGTTGGTTGCTACTA),
 mouse *Il1a* (forward: 5'- TCTATGATGCAAGCTATGGCTCA-3'; reverse: 5'-
 CGGCTCTCCTTGAAGGTGA-3'); human *TNFA* (forward:
 CCTCTCTCTAATCAGCCCTCTG; reverse: GAGGACCTGGGAGTAGATGAG),
 mouse *tnf* (forward: 5'- CAGGCGGTGCCTATGTCTC-3'; reverse: 5'-
 CGATCACCCCGAAGTTCAGTAG-3'); human *BCL2L1* (forward:
 CTGCTGCATTGTTCCCATAG-3'; reverse: 5'-TTCAGTGACCTGACATCCCA-3'),
 mouse *Bcl2l1* (forward: 5'- ACATCCCAGCTTCACATAACCC-3'; reverse: 5'-
 CCATCCCGAAAGAGTTCATTCAC-3'); human *BCL2* (forward: 5'-
 ATGTGTGTGGAGAGCGTCAA-3'; reverse: 5'-CGTACAGTTCCACAAAGGCA-
 3'); and mouse *Bcl2* (forward: 5'-GCTACCGTCGTGACTTCGC-3'; reverse: 5'-
 CCCCACCGAACTCAAAGAAGG-3'). All qPCR assays were conducted in
 triplicate.

shRNA mediated knockdown

Panc1 or MiaPaCa2 cells were infected with pLKO-based lentiviruses encoding shRNAs targeting human Gli1 (shGLI1#1: CATCCATCACAGATCGCATTT; shGLI1#2: GCTCAGCTTGTGTGTAATTAT). Infected cells were selected in 5 µg/mL puromycin for 4 days.

Affymetrix gene chip analysis

Panc1 cells were transfected with a GFP-expressing vector carrying Gli3T (pCIG-Gli3T) or a GFP-expressing empty vector (pCIG). GFP-positive cells were isolated 24 h after transfection using flow cytometry. RNA was isolated, labeled, and hybridized to mouse GeneST1.0 chips (Affymetrix) according to Affymetrix protocols. Three independent biological samples were used for chip analysis. Statistical analyses were performed using R, a system for statistical computation and graphics (<http://www.r-project.org>). Genes with adjusted *P* value <0.05 and absolute fold change ≥ 1.5 were considered potential targets for further investigation.

Chapter III: IKBKE signaling in pancreatic neoplasia

Figure Contributions

Data for Figure 3.6B was contributed by Dr. Martin Fernandez-Zapico of Mayo Clinic, Rochester, MN

Results

IKBKE is a regulated by GLI in pancreatic cancer, and is required for transformation

In our gene expression analysis in human PDAC cells, we identified a number of genes that were regulated in a GLI dependent manner that were previously not associated with Hedgehog/GLI signaling. The I κ B Kinase Epsilon (IKBKE/IKK ϵ) was a candidate GLI target gene identified in our screen. IKBKE is an atypical I κ B kinase that is known to play a role in regulation of the NF- κ B pathway¹¹⁸. We found that inhibition of GLI using the dominant-negative repressor Gli3T led to a significant decrease in *IKBKE* mRNA and protein levels in Panc-1 cells (Figure 3.1A and D). Similarly, shRNA mediated knockdown using two different shRNAs targeting *GLI1*, and treatment with a small molecule inhibitor of GLI (Gant61) led to a significant decrease in IKBKE mRNA expression levels in Panc-1 cells, suggesting that IKBKE expression is regulated by GLI (Figure 3.1B-C). In our *in vivo* model, we found that IKBKE protein levels were markedly upregulated in PanIN lesions in *P48Cre;LSL-Kras^{G12D};R26-Gli1* mice compared to *P48Cre;LSL-Kras^{G12D}* mice, as assayed by immunohistochemical staining (Figure 3.1E-F). These findings implicate IKBKE as a novel GLI target gene in PDAC.

Because of its role in transformation of breast cancer cells¹⁶², we hypothesized that IKBKE may also promote tumorigenesis in the pancreas. We utilized two different shRNAs targeting *IKBKE* and treated two different human PDAC cell lines Panc-1 and MiaPaca2 with these shRNAs to achieve significant *IKBKE* knockdown (Figure 3.1G, and 3.2A). In response to *IKBKE* knockdown, we detected a significant decrease in cell proliferation as detected by MTT assay in both Panc-1 (Figure 3.1H), and MiaPaca2 cells (Figure 3.2B), and this decrease was partially rescued by the expression of a non-targetable mouse IKBKE (mIKBKE) construct. The mIKBKE construct carried a c-terminal myc-tag to enable detection of the protein (Figure 3.1K, and 3.2E). A considerable increase in the level of apoptotic cells, measured by cleaved-Caspase-3 immunofluorescence, was also detected in response to *IKBKE* knockdown in both Panc-1 and MiaPaca2 cells (Figure 3.1I, and 3.2C), which could also be rescued by expression of the non-targetable mIKBKE. We also tested the role of IKBKE in tumorigenicity of the Panc-1 and MiaPaca2 cells, as assayed by soft agar colony formation assay. We found that *IKBKE* knockdown led to a significant decrease in soft agar colony formation in both the cell lines, and this phenotype could be partially reversed by expression of the mIKBKE (Figure 3.1J, and 3.2D). Together our findings suggest that IKBKE is regulated by GLI in PDAC, and is essential for the survival, and tumorigenicity of human PDAC cells *in vitro*.

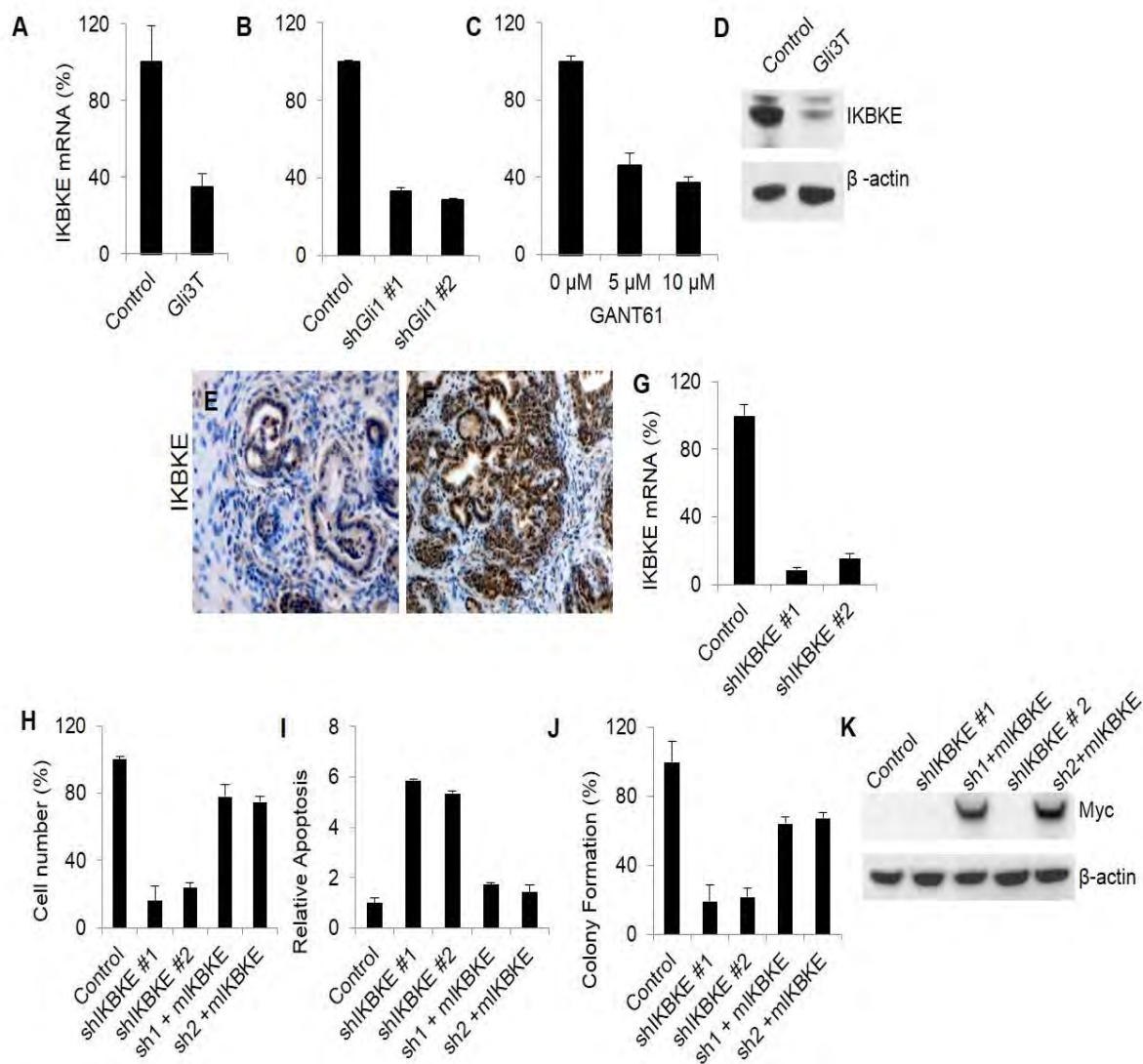


Figure 3.1: IKBKE is regulated by GLI and is required for PDAC cell transformation

(A-C) *IKBKE* mRNA levels in Panc-1 cells in response to repression by Gli3T (A), shRNA mediated knockdown of Gli1 (B), and treatment with Gant61 (C). (D) Western blot showing IKBKE and β -Actin levels in response to repression by Gli3T in Panc-1 cells. Significant decrease in *IKBKE* mRNA and protein levels is seen in response to inhibition of GLI transcriptional activity, suggesting GLI dependent expression of *IKBKE*. (E-F) Immunohistochemistry for IKBKE in 2 month old

P48Cre;LSL-Kras^{G12D} (E), and *P48Cre;LSL-Kras^{G12D};R26-Gli1* mouse pancreas sections. PanIN lesions from *P48Cre;LSL-Kras^{G12D};R26-Gli1* mice express significantly higher levels of IKBKE suggesting possible GLI dependent regulation of IKBKE expression *in vivo*. (G) Quantitative RT-PCR for *IKBKE* mRNA levels in response to knockdown of IKBKE using two different shRNAs in Panc-1 cells. Significant IKBKE knockdown is seen. (H-K) Significant decrease in cell proliferation (H), increase in apoptosis (I), and decrease in tumorigenicity (K) is seen as measured by MTT assay, cleaved-Caspase-3 staining, and soft agar assay respectively, in response to knockdown of *IKBKE*. This phenotype can be partially rescued by expression of a non-targetable version of IKBKE (mIKBKE). (H) Expression of mIKBKE can be detected by C-terminal myc-tag on the protein.

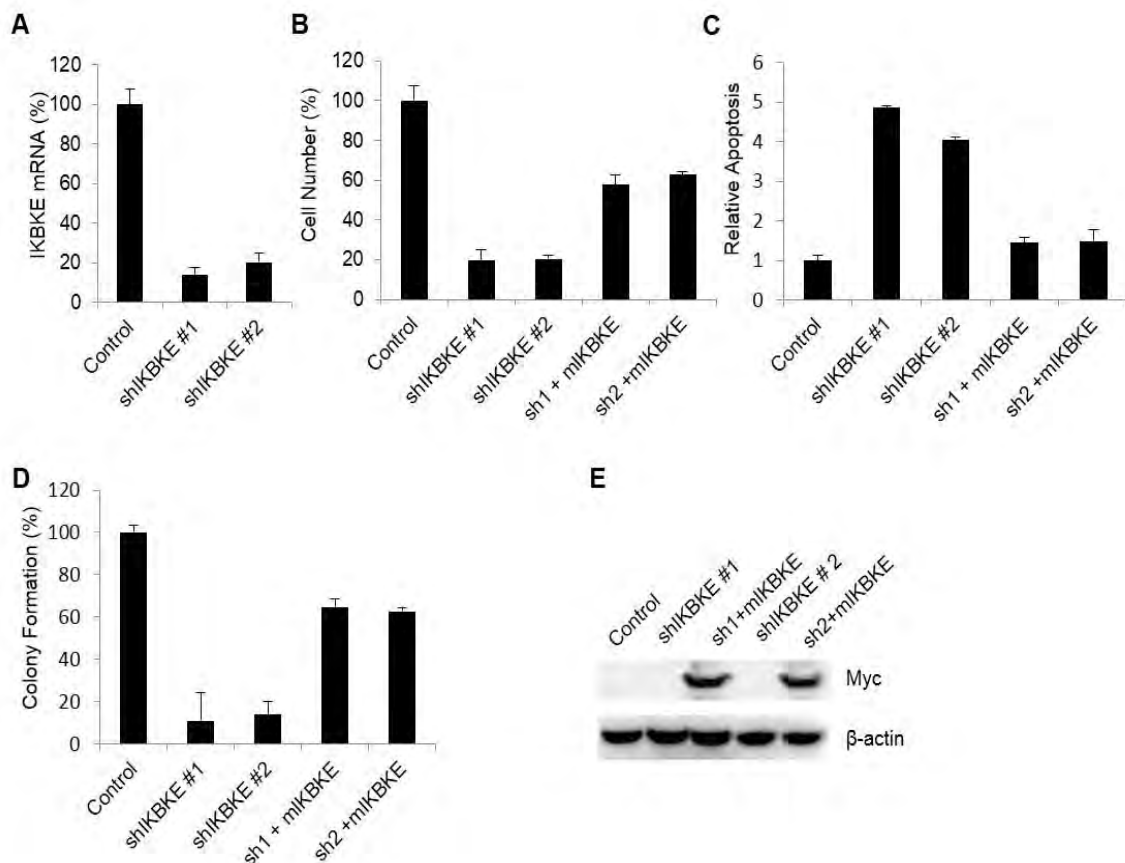


Figure 3.2: IKBKE is required for survival of MiaPaca2 cells *in vitro*

(A) Quantitative RT-PCR for *IKBKE* mRNA levels in MiaPaca2 cells in response to shRNA mediated knockdown of *IKBKE* with two different shRNAs. (B-D)

Significant decrease in cell proliferation (B), increase in apoptosis (C), and decrease in tumorigenicity (D) is seen as measured by MTT assay, cleaved-Caspase-3 staining, and soft agar assay respectively, in response to knockdown of *IKBKE*. This phenotype can be partially rescued by expression of a non-targetable version of IKBKE (mIKBKE). (E) Expression of mIKBKE can be detected by C-terminal myc-tag on the protein.

IKBKE, but not TBK1 acts downstream of KRAS in PDAC

We have established that GLI regulates an oncogenic program downstream of KRAS in PDAC, and IKBKE may act as an oncogenic effector of GLI in PDAC cells. IKBKE and the closely related I κ B kinase TBK1 share a high degree of homology, and are known to have overlapping functions in other contexts¹⁶³. Although TBK1 was not identified in our gene expression analysis, it is known to play a role in various malignancies¹⁶⁴. In KRAS driven lung adenocarcinoma, dual inhibition of IKBKE and TBK1 using a small molecule inhibitor has been shown to reduce KRAS downstream signaling activity¹⁶⁵. However, the possible role of TBK1 in pancreatic cancer has not been explored, and a functional connection between KRAS and IKBKE/TBK1 in pancreatic cancer has not been established.

We first examined the expression of both proteins in pancreatic cancer patients. We conducted immunohistochemical staining for IKBKE and TBK1 in a Tissue Microarray of human pancreatic cancer samples (N=62) and found that while IKBKE was highly expressed in a majority of PDAC samples, TBK1 expression was minimal (Figure 3.3 A-C). To identify whether IKBKE or TBK1 acts downstream of KRAS in PDAC, we conducted shRNA mediated knockdown of *KRAS* in Panc-1 cells. Knockdown of *KRAS* led to a significant decrease in expression of IKBKE, but not TBK1 in Panc-1 cells (Figure 3.3D-E) as measured by immunoblotting, and quantitative RT-PCR. To test the requirement of IKBKE/TBK1 in PDAC cell transformation, we used two different shRNAs targeting either of the proteins and measured the effect of the knockdown on apoptosis, cell

viability, and soft agar colony formation. We found that while knockdown of *IKBKE* resulted in a significant increase in apoptosis as measured by relative cleaved-Caspase-3 staining, *TBK1* knockdown did not affect apoptosis (Figure 3.3F). *IKBKE* knockdown also resulted in a significant decrease in cell viability as measured by MTT assay in Panc-1 cells, while *TBK1* knockdown had little or no effect (Figure 3.3G). Similarly, we found that while *IKBKE* knockdown led to a significant decrease in tumorigenicity as measured by soft agar assay in Panc-1 cells, knockdown of *TBK1* did not affect tumorigenicity (Figure 3.3H). Our data suggests that in the context of PDAC, *IKBKE* but not *TBK1* is the major oncogenic effector of KRAS.

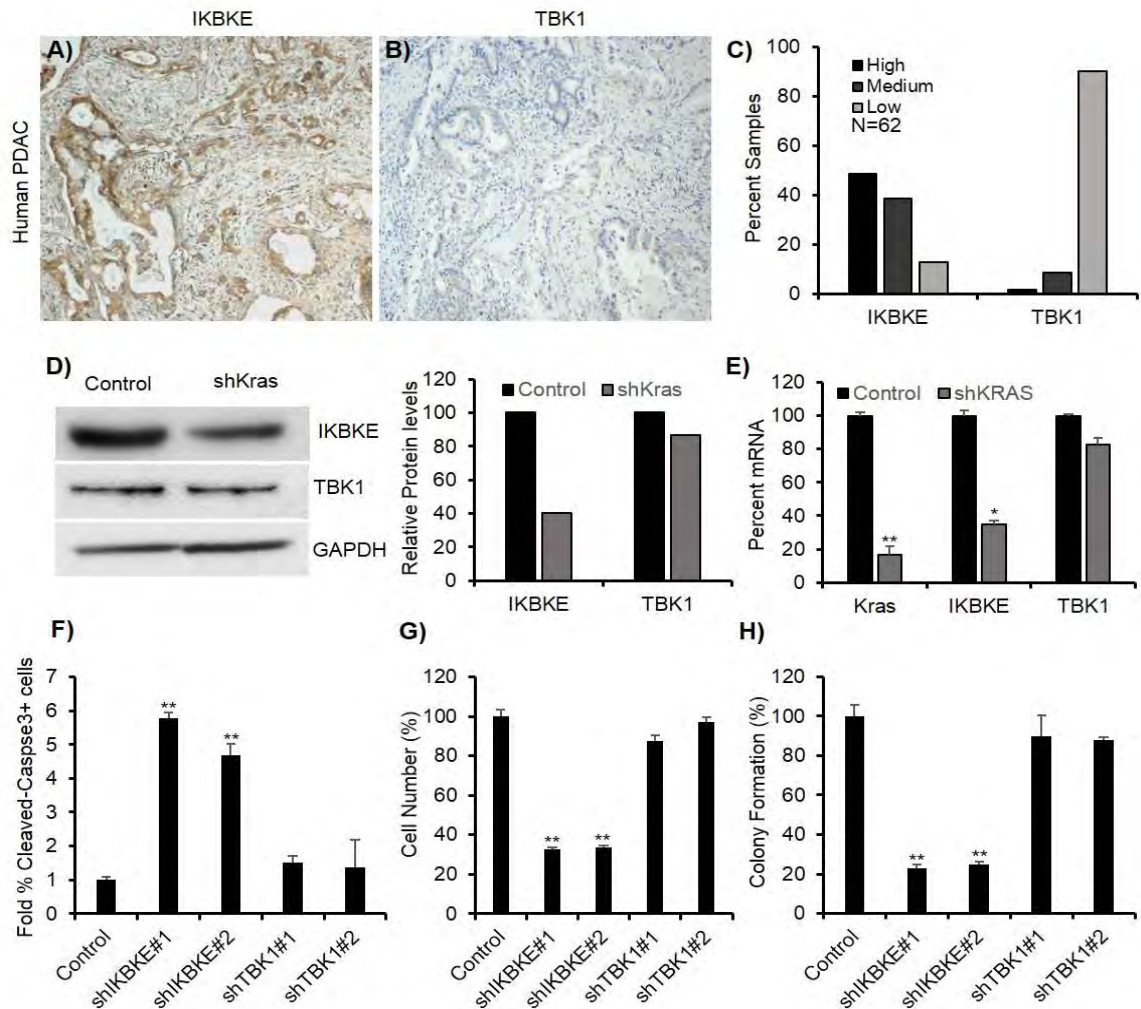


Figure 3.3: IKBKE but not TBK1 acts downstream of KRAS in pancreatic ductal adenocarcinoma

(A-C) Immunohistochemistry staining of IKBKE (A), and TBK1 (B) in human PDAC samples. (C) Quantification of IKBKE and TBK1 IHC in 62 human PDAC samples shows that IKBKE but not TBK1 is highly expressed in a majority of human PDAC cases. D) Western blot analysis in Panc-1 cells indicates that IKBKE protein levels are downregulated in response to *KRAS* knockdown. TBK1 protein levels are relatively unchanged. E) mRNA analysis using quantitative RT-PCR shows that *IKBKE* mRNA expression is downregulated in response to *KRAS* knockdown in Panc-1 cells. *TBK1* mRNA levels remain relatively unchanged. (F-H) shRNA mediated knockdown of *IKBKE* or *TBK1* in Panc-1 cells indicates an increase in fold apoptosis measured by cleaved Caspase-3 positive cells (F), decrease in cell

viability measured by MTT assay (G), and decrease in tumorigenicity measured by soft agar colony formation (H) in response to IKBKE but not TBK1 knockdown. Error bars represent Standard Deviation. Statistical significance was determined using a Student's two-tailed t-test. * $P < 0.05$; ** $P < 0.01$

IKBKE is required for pancreatic neoplasia formation and progression *in vivo*

In order to test the requirement of IKBKE in pancreatic transformation *in vivo*, we utilized a mouse model carrying whole body knockout of *Ikbke*¹⁶⁶. We first analyzed the requirement of IKBKE in pancreatic development, and found that *Ikbke*^{-/-} mice had histologically normal pancreatic architecture at 12 months of age (N=6) as compared to wild type mice (Figure 3.4A-B), with normal development of islets, acini, and ducts. We conducted immunohistochemical staining to analyze expression of the acinar cell marker amylase, and islet cell marker insulin and found no significant difference between the expression of these markers in *Ikbke*^{-/-} vs wild type mice (Figure 3.4C-F), thus indicating normal pancreatic differentiation. These results indicate that IKBKE is not required for development of pancreas.

To test the role played by IKBKE in pancreatic neoplasia, we utilized the mouse model described in Chapter II, in which an oncogenic allele of *Kras* (G12D) is targeted to the endogenous locus and expressed specifically in the pancreatic epithelium using Cre-recombinase expressed under the *P48* (*Ptf1a*) promoter⁵⁴. As expected, the *P48Cre;LSL-Kras*^{G12D} mice developed pancreatic intraepithelial neoplasia (PanIN) lesions of histological grades ranging from PanIN1-3, depending on the age of the mice, with some 12 month old mice displaying full blown adenocarcinoma. Using immunohistochemical staining, we found that IKBKE expression in the PanIN lesions correlated with the histological grade of the

lesions, with increased expression in higher grade lesions, and very high expression in the case of adenocarcinoma (Figure 3.5A).

To achieve simultaneous KRAS activation and IKBKE loss in the pancreas, we crossed the *P48Cre* mice with *Kras^{G12D};Ikbke^{-/-}* mice, and analyzed pancreas from each of the genotypes at ages 3 months, 6 months, and 12 months. We found that the *P48Cre;LSL-Kras^{G12D}* (N=15) mice developed PanIN grade 1 and instances of grade 2 lesions at the age of 3 months, with increased number of PanIN 2 and 3 grade lesions at 6 months (Figure 3.5C-E). The normal pancreatic architecture was lost by 12 month age, with advanced grade lesions covering majority of the pancreas, and instances of adenocarcinoma in some mice. In contrast to this, we found that pancreas of *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mice (N=24) were largely normal at all the 3 time points, with some low grade PanIN lesions (Figure 3.5F-H). Quantification of PanIN lesions from H&E stained sections obtained from all the mouse pancreas samples showed significantly delayed onset, and reduced intensity of pancreatic neoplasms in *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mice (Figure 3.5I-K).

We conducted Ki67 staining on stage matched lesions from *P48Cre;LSL-Kras^{G12D}* and *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mice to evaluate differences in proliferation. PanIN lesions from *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mice had less Ki67 positive cells compared to similar staged lesions from *P48Cre;LSL-Kras^{G12D}* mice (Figure 3.5L). Quantification of Ki67 positive cells from the lesions indicated significantly less proliferation in the lesions from *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mice (Figure 3.5M).

This data indicates that IKBKE loss may impair cell proliferation, and therefore delay progression of the PanIN lesions, which is consistent with our finding that IKBKE loss leads to loss of cell proliferation in human PDAC cell lines. Our findings suggest a critical requirement of IKBKE in the process of KRAS induced pancreatic transformation *in vivo*, and progression of pancreatic neoplasms to adenocarcinoma.

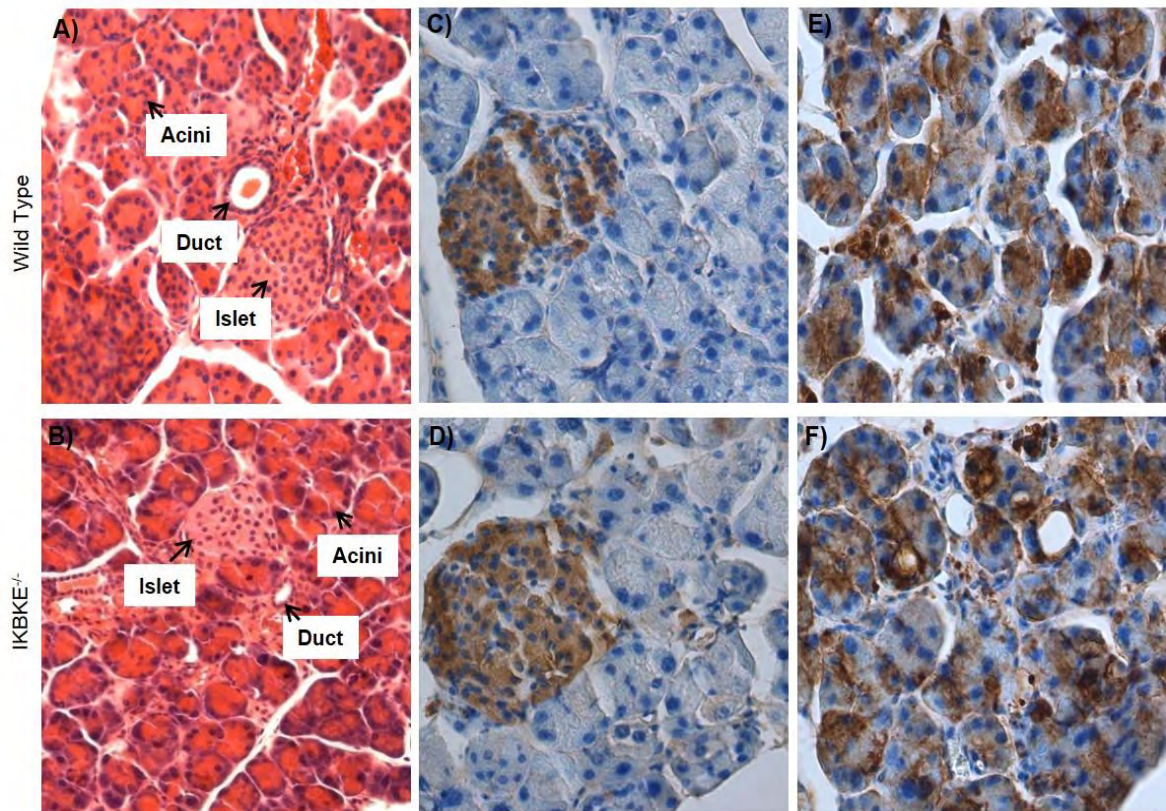


Figure 3.4: IKBKE is dispensable for pancreatic development

(A-B) Representative images showing H&E staining of 12 month old wild type (A), and *Ikbke*^{-/-} (B) mouse pancreas. Arrows indicate Acini, Ducts, and Islets. *Ikbke*^{-/-} mice have normal pancreatic architecture and loss of IKBKE does not affect development of pancreas. (C-D) Immunohistochemical staining on 12 month old pancreas sections for Insulin in wild type (C), and *Ikbke*^{-/-} (D) mouse pancreas indicates normal islet function. (E-F) IHC for Amylase in 12 month old pancreas sections from wild type (E), and *Ikbke*^{-/-} (F) shows normal acinar function

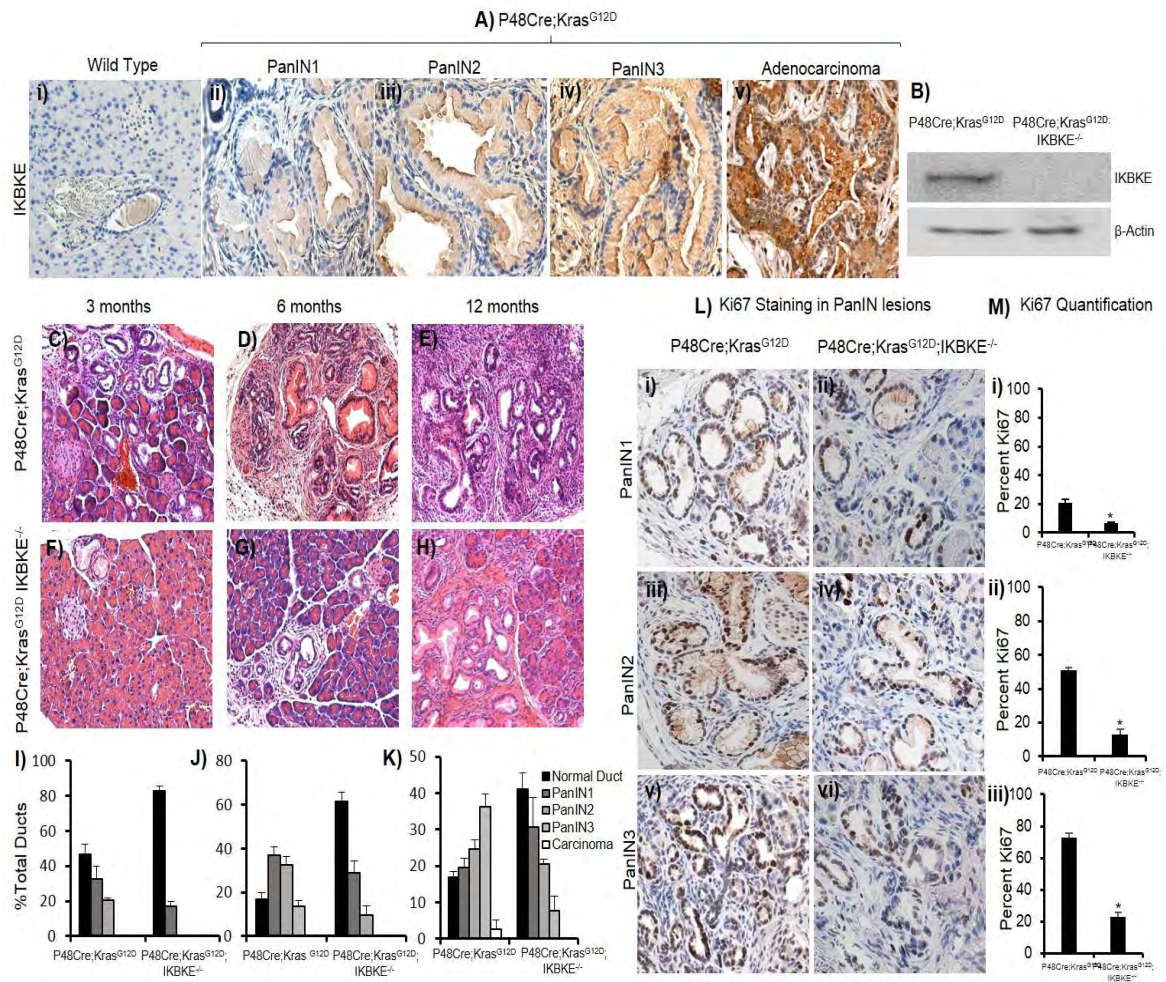


Figure 3.5: IKBKE requirement in KRAS induced Pancreatic Tumorigenesis

(A) Immunohistochemistry for IKBKE protein levels in wild type mouse pancreas (i), and *P48Cre;LSL-Kras^{G12D}* mouse pancreas in PanIN1 (ii), PanIN2 (iii), PanIN3 (iv) stage precursor lesions, and full blown Adenocarcinoma (v). IKBKE is not expressed in wild type pancreas but is expressed at increasing levels with increasing severity of pancreatic neoplasms. (B) Western blot showing IKBKE levels in pancreas of 12 month old *P48Cre;LSL-Kras^{G12D}* and *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mice. IKBKE is not expressed in pancreas from *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mice but is expressed in *P48Cre;LSL-Kras^{G12D}* mice. (C-E) Representative H&E staining images of pancreas of 3 month old (C), 6 month old (D), and 12 month old (E) *P48Cre;LSL-Kras^{G12D}* mice. (F-H) Representative H&E staining images of pancreas of 3 month old (F), 6 month old (G), and 12 month old (H) *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mice. (I-K) Quantification of grade of PanIN

lesions in age matched *P48Cre;LSL-Kras^{G12D}* and *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mouse pancreas at ages 3 months (I), 6 months (J), and 12 months (K). Comparison of age matched pancreas indicates significantly delayed initiation and progression of pancreatic neoplasms in *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mice compared to *P48Cre;LSL-Kras^{G12D}* mice. (L) Immunohistochemistry for Ki67 in stage matched pancreas of *P48Cre;LSL-Kras^{G12D}* (i, iii, v), and *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* (ii, iv, vi) mice. (M) Quantification of Ki67 positive cells in PanIN1 (i), PanIN 2 (ii), and PanIN3 (iii) lesions of *P48Cre;LSL-Kras^{G12D}* and *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mouse pancreas. Significantly less number of Ki67 positive cells in PanIN lesions of *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mice compared to *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mice indicates decreased number of proliferative cells. Error bars represent Standard Deviation. Statistical significance was determined using a Student's two-tailed t-test. * $P < 0.05$; ** $P < 0.01$

IKBKE is a direct transcriptional target of GLI, and mediates feedback regulation of GLI activity

As we initially identified *IKBKE* as a potential GLI target gene in a PDAC cell microarray, we decided to further explore the relationship between GLI and *IKBKE*. Consistent with our gene expression data, inhibition of GLI transcriptional activity using either the Gli3T repressor, or shRNA mediated knockdown of *GLI1* in Panc-1 cells led to a significant decrease in *IKBKE* but not *TBK1* mRNA levels (Figure 3.6A), thus implicating *IKBKE* but not *TBK1* as a GLI target gene. Furthermore, when we compared mRNA expression levels of *IKBKE* vs *GLI1* in human PDAC patient samples, we found a very strong correlation ($R = 0.79$, $P < 0.0001$) between the expression of the two genes (Figure 3.6B).

In order to test whether *IKBKE* was a direct transcriptional target of GLI, we conducted chromatin immunoprecipitation on Panc-1 cells expressing a Flag-tagged version of Gli3T. We found significant enrichment of the *IKBKE* promoter region in the ChIPed samples, as well as enrichment of the promoter region of *PTCH1*, which is a known target gene (Figure 3.6C). Sequence analysis of the *IKBKE* promoter region revealed the existence of a candidate GLI binding site (GACTTCCCA), which carries a 2 base-pair mismatch to the GLI consensus sequence GACCACCCA, 130 bp upstream of the transcriptional start site in the *IKBKE* promoter. In order to test whether GLI regulates transcription of *IKBKE* by binding to its promoter, we cloned the *IKBKE* promoter region ~300 bp upstream of the start site, in a PGL3 luciferase reporter construct. In order to test the

specificity of the candidate GLI binding site, we also generated a mutant version of the construct in which the candidate GLI binding site was mutated to “GGCGCGCC”. We then conducted a luciferase assay in Panc-1 cells with both versions of the *IKBKE*-promoter luciferase, as well as Gli-BS luciferase, which carries an 8x GLI binding site upstream of a luciferase reporter. We found that in response to the Gli3T repressor, relative expression of the Gli-BS luciferase, and luciferase with the *IKBKE* promoter was significantly downregulated. However, no significant difference was seen in expression of the *IKBKE* promoter luciferase carrying the mutant GLI binding site, in response to the Gli3T repressor (Figure 3.6D). These findings indicate that GLI can regulate transcription of *IKBKE* by binding to its promoter region, and that mutation of the GLI binding site ablates GLI regulation of *IKBKE* transcription.

Although GLI transcriptional activity has been shown to be important in pancreatic cancer, the upstream regulation of GLI activity in PDAC has not been well characterized. In the context of PDAC, it is known that GLI acts independently of its canonical upstream regulators Hedgehog, PTCH1, and SMO¹⁴³, however, regulation of GLI at the post-translational level is not well studied, although both *GLI1* activity and localization has been shown to be regulated in other cellular contexts^{167,168,169}. Here, we further explored the potential role of *IKBKE* in the regulation of GLI. We found that in Panc-1 cells, shRNA mediated knockdown of *IKBKE* led to a significant decrease in mRNA levels of the GLI target genes *GLI1*, *FOXA2*, and *PTCH1* (Figure 3.6E). Pancreas of *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mice

showed considerably lower levels of mRNA of the GLI target genes compared to *P48Cre;LSL-Kras^{G12D}* mice (Figure 3.6F). These findings suggest that IKBKE plays a role in regulation of GLI transcriptional activity. As GLI1 is a shuttling protein, and the activity of GLI1 is regulated by the subcellular localization of the protein¹⁶⁷, we tested the role played by IKBKE in regulation of subcellular localization. We ectopically expressed a GFP-fusion version of GLI1 in 293T cells to visualize the localization of the protein. We found that when expressed ectopically, the Gli1-GFP was localized mostly to the cytoplasm in punctate structures (Figure 3.6G-i,iv). However, when we ectopically expressed wild type IKBKE, but not a kinase-dead version of IKBKE (K38A), the Gli1-GFP proteins were translocated to the nucleus (Figure 3G-ii, iii, v, vi). This data shows that IKBKE kinase activity promotes nuclear translocation of GLI1. To further test the role played by IKBKE in regulating GLI activity and localization, we utilized a mutant version of GLI1 (Gli1-AHA) that is constitutively localized to the nucleus¹⁶⁷. In 293T cells, we found that co-expression of IKBKE, but not IKBKE-K38A led to a synergistic increase in the transcriptional activity of GLI1, as measured by Gli-BS luciferase expression (Figure 3.6H). However, co-expression of IKBKE or IKBKE-K38A did not significantly affect the activity of Gli1-AHA. As Gli1-AHA is constitutively localized to the nucleus, unlike wild type GLI1 which shuttles between the nucleus and cytoplasm, our results indicate that IKBKE regulates GLI activity primarily by regulating nuclear localization of the protein. Taken together,

our findings point to a potential reciprocal interaction between IKBKE and GLI downstream of KRAS in PDAC cells.

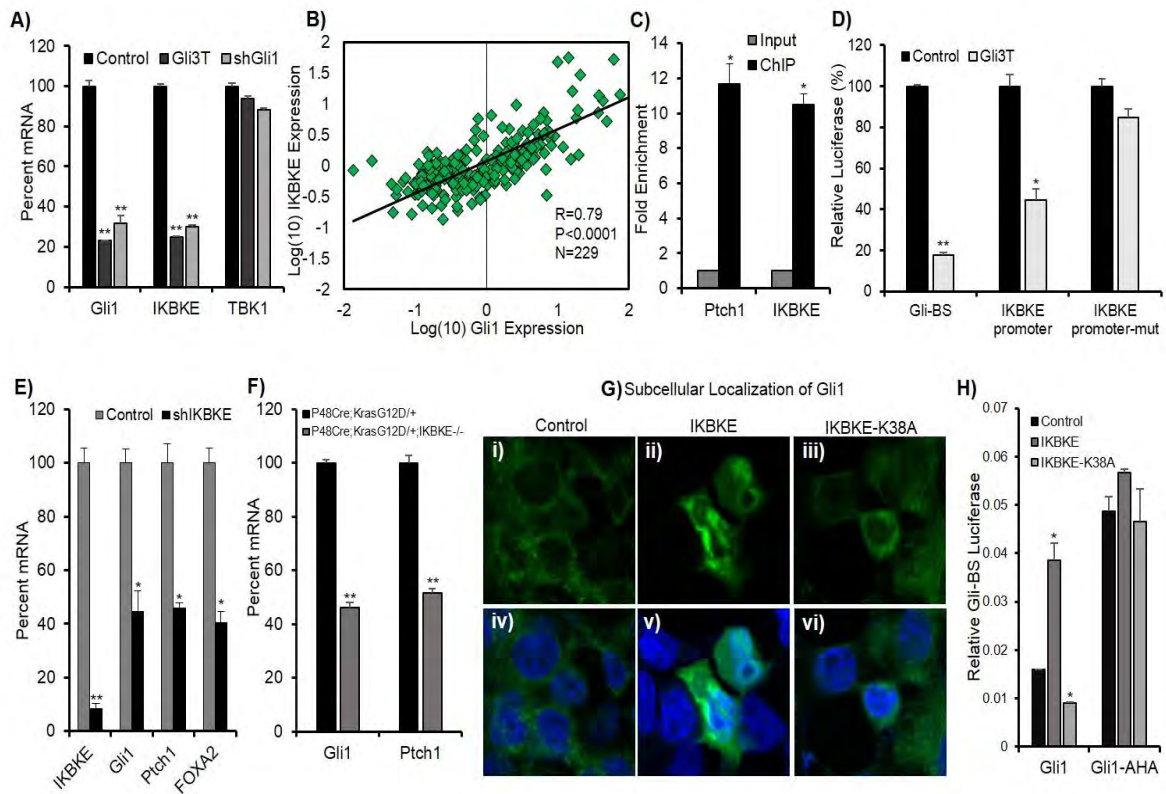


Figure 3.6: Reciprocal GLI-IKBKE Signaling in Pancreatic Ductal Adenocarcinoma

(A) Quantitative RT-PCR in Panc-1 cells showing *GLI1*, *IKBKE*, and *TBK1* mRNA expression after inhibition of GLI activity using a dominant negative GLI (Gli3T), or an shRNA targeting *GLI1*. While *GLI1* and *IKBKE* expression is decreased upon inhibition of Gli activity, *TBK1* expression is unchanged. (B) Correlation of *GLI1* and *IKBKE* mRNA expression in 229 human PDAC tissue samples. Pearson coefficient R value of 0.79 indicates high degree of correlation between *GLI1*, and *IKBKE* expression in the tumors. (C) Quantitative PCR of DNA enriched using ChIP against Flag-tag in Panc-1 cells infected with Gli3T-Flag indicates significant enrichment of *IKBKE* promoter region as well as the promoter of a known GLI target gene (*PTCH1*). (D) Relative luciferase activity in Panc-1 cells of Gli-BS Luciferase, *IKBKE* promoter luciferase containing the GLI binding site in *IKBKE* promoter cloned in a luciferase reporter, and *IKBKE* promoter carrying a mutated GLI binding site cloned in a luciferase reporter. While activity of Gli-BS luciferase, and *IKBKE* promoter luciferase is significantly decreased in response to inhibition using dominant-negative Gli3T, the activity of *IKBKE* promoter mutant luciferase is

relatively unchanged. (E) Quantitative RT-PCR indicates that GLI target gene expression in Panc-1 cells is significantly decreased in response to shRNA mediated knockdown of *IKBKE*. (F) Quantitative RT-PCR in tissue samples indicates significantly reduced GLI target gene expression in 6 month old *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mouse pancreas compared to age-matched *P48Cre;LSL-Kras^{G12D}* pancreas. (G) Subcellular localization of a Gli1-GFP fusion gene transfected in 293T cells with (i-iii) and without (iv-vi) over lay with DAPI. While Gli1 is localized mainly in the cytoplasm without IKBKE expression (i, iv), IKBKE expression drives nuclear localization of GLI1 (ii, v). Expression of a kinase-dead version of IKBKE (K38A) causes cytoplasmic retention of GLI1 (iii, vi). (H) Co-expression of IKBKE with GLI1 synergistically increases GLI transcriptional activity as measured by Gli-BS Luciferase expression in 293 cells. IKBKE expression has no effect on the activity of Gli1-AHA, a mutant form of Gli1 that is constitutively localized to the nucleus. Kinase-dead version of IKBKE can inhibit transcriptional activity of GLI1 but not Gli1-AHA. Error bars represent Standard Deviation. Statistical significance was determined using a Student's two-tailed t-test. * P<0.05; ** P<0.01

Regulation of NF- κ B signaling by IKBKE

IKBKE was initially identified as an I κ B kinase involved in regulation of NF- κ B signaling¹⁷⁰. As NF- κ B signaling has been implicated in pancreatic cancer^{44,73}, and as the oncogenic activity of IKBKE has been linked to its ability to activate NF- κ B in other cancers^{162,165}, we tested the role played by IKBKE in NF- κ B activation in the context of PDAC. We found that while in a p65 luciferase assay in 293T cells IKBKE can strongly activate NF- κ B signaling in an I κ B dependent manner (Figure 3.7A), shRNA mediated knockdown of *IKBKE* in Panc-1 PDAC cells leads to only a modest downregulation of known NF- κ B target genes (Figure 3.7B). Also, we do not see a significant downregulation of NF- κ B target gene mRNA levels in pancreas of *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mice compared to *P48Cre;LSL-Kras^{G12D}* mice (Figure 3.7C).

Nuclear localization of the NF- κ B subunit p65 is a marker for NF- κ B pathway activation, and IKBKE is known to drive p65 nuclear translocation through inactivation of I κ B. To test NF- κ B pathway activation, we used immunoblotting to compare the levels of p65 in nuclear and cytoplasmic fractions of Panc-1 cells after shRNA mediated knockdown of *IKBKE*. We found that while *IKBKE* knockdown led to a modest decrease in levels of p65 in the nuclear fraction (Figure 3.7D), significant amounts of p65 was still present in the nucleus, which may account for the sustained NF- κ B target gene expression after *IKBKE* knockdown. In order to study the relationship between NF- κ B pathway activation and IKBKE protein levels in patients, we conducted immunohistochemistry for p65, and IKBKE on a tissue

microarray of human PDAC samples. p65 localization in the tumor samples was classified either as predominantly nuclear, or predominantly cytoplasmic. Staining intensity for IKBKE protein in tumors was graded as either low, medium, or high. Analysis of p65 localization vs IKBKE protein levels was conducted for each sample and a Pearson correlation was obtained between the two. Analysis revealed a lack of significant correlation between p65 localization and IKBKE protein levels ($R=0.16$, $P>0.05$, $N=62$) in the tumor samples (Figure 3.7E-F).

Furthermore, we compared subcellular localization of p65 in stage-matched PanIN lesions of *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* and *P48Cre;LSL-Kras^{G12D}* mice using immunohistochemistry. We found that even with *Ikbke* knockout, nuclear p65 was present in the PanIN lesions and there was no significant difference in nuclear localization of p65 between lesions of *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* and *P48Cre;LSL-Kras^{G12D}* mice (Figure 3.7G-I). Our findings indicate that while IKBKE is capable of regulating the NF- κ B pathway, in the context of PDAC it contributes only modestly to NF- κ B regulation, since significant amount of NF- κ B pathway activity is still maintained upon IKBKE loss. This also indicates that IKBKE oncogenic activity in the context of pancreatic cancer may be mediated by NF- κ B independent mechanisms.

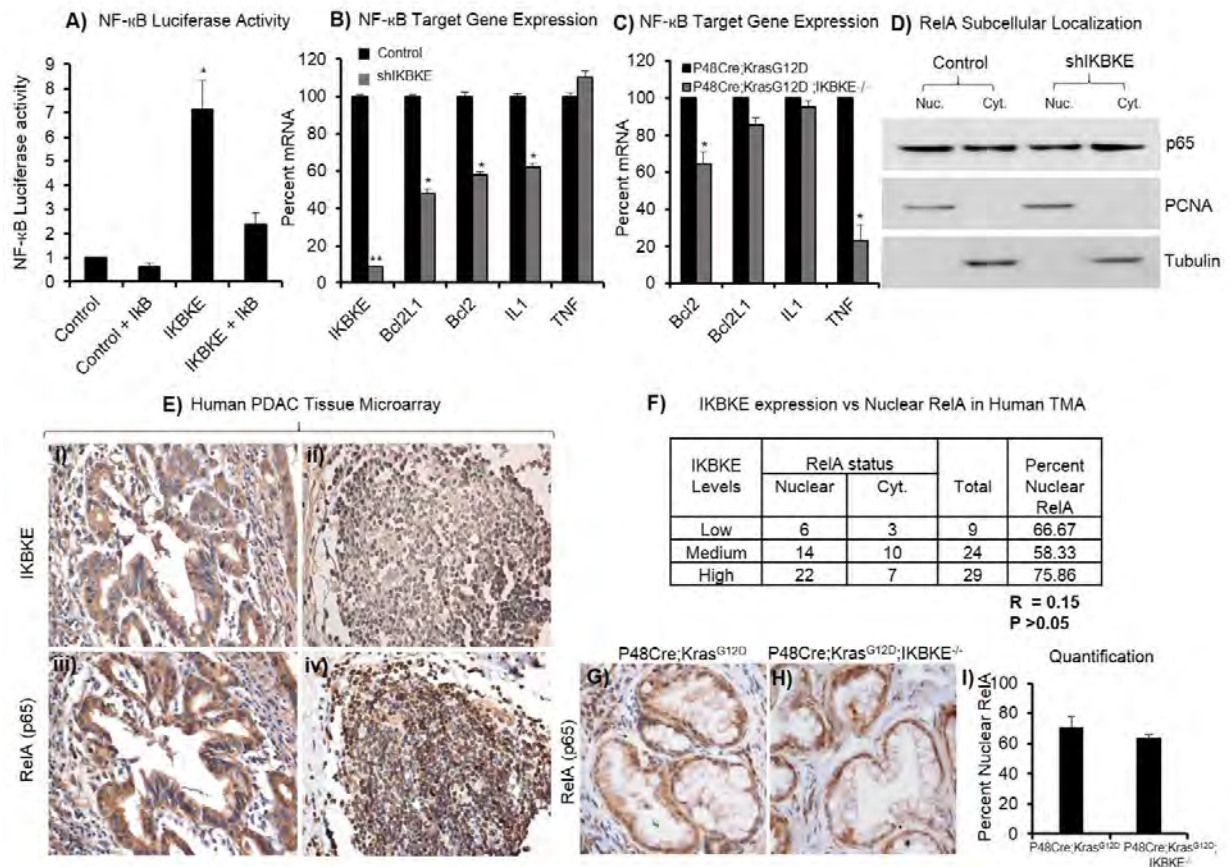


Figure 3.7: Regulation of NF- κ B activity by IKBKE

(A) NF- κ B luciferase activity in 293T cells is significantly increased in response to IKBKE ectopic expression. IKBKE activation of NF- κ B activity can be inhibited by a dominant negative version of I κ B suppressor. (B) Quantitative RT-PCR shows that NF- κ B target gene mRNA expression in Panc-1 cells is modestly downregulated in response to shRNA mediated knockdown of *IKBKE*. (C) Quantitative RT-PCR of NF- κ B target gene mRNA expression in pancreas of 6 month old *P48Cre;LSL-Kras^{G12D}*, and *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mice. No significant decrease in NF- κ B target gene expression is seen in response to knockout of *Ikbke* in mice. (D) Western blot showing RelA (p65) in nuclear and cytoplasmic fractions of Panc-1 cells with and without shRNA knockdown of *IKBKE*. PCNA and Tubulin are used as controls for nuclear and cytoplasmic fractions respectively. No significant difference is seen in p65 subcellular localization in response to *IKBKE* knockdown. (E) Representative images comparing immunohistochemistry of IKBKE (i,ii) and p65 (iii, iv) in matched human PDAC tissue samples. (F) Correlation between subcellular localization of p65 and

IKBKE protein levels in 62 human PDAC samples. Pearson coefficient $R = 0.15$ does not indicate a significant correlation ($P > 0.05$) between IKBKE protein levels and subcellular localization of p65. (G-I) Immunohistochemistry for p65 in stage matched PanIN lesions in *P48Cre;LSL-Kras^{G12D}* (G), and *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* (J) mouse pancreas. Quantification of p65 subcellular localization (I) indicates no significant differences in nuclear vs cytoplasmic p65 in *P48Cre;LSL-Kras^{G12D}* and *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mouse PanIN lesions. Error bars represent Standard Deviation. Statistical significance was determined using a Student's two-tailed t-test. * $P < 0.05$; ** $P < 0.01$

IKBKE regulates AKT activation in PDAC

As we found that NF- κ B regulation may not account for IKBKE tumorigenicity in the context of PDAC, we decided to evaluate alternative mechanisms through which IKBKE may promote tumorigenesis. The PI3K/AKT signaling pathway is known to act downstream of KRAS in PDAC, and play a critical role in transformation, apoptosis evasion, and chemoresistance^{146,171}. We have also found that GLI can regulate phosphorylation of AKT in pancreatic cancer cells¹⁴⁸. Also, IKBKE is known to phosphorylate AKT *in vitro* and in cancer cells¹⁷². Here, we decided to test whether IKBKE plays a role in regulation of AKT phosphorylation in PDAC. We found that *IKBKE* knockdown in Panc-1 cells led to a significant decrease in the phosphorylation of AKT but not ERK (Figure 3.8A), and phospho-AKT levels were significantly decreased in the stage-matched PanIN lesions of *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mouse pancreas compared to *P48Cre;LSL-Kras^{G12D}* mice (Figure 3.8C-F).

Normally, the mTORC2 complex is known to phosphorylate AKT at Serine-473 and mediate AKT activation¹⁰⁸. However, we found that in Panc-1 cells, basal level of AKT phosphorylation at both Serine-473, and Threonine-308 was maintained even after mTOR inhibition using the small molecule inhibitor Torin1 (Figure 3.8B). Furthermore, this basal phosphorylation of AKT was significantly reduced following knockdown of IKBKE in combination with mTOR inhibition. In order to test whether the relationship between IKBKE expression and AKT phosphorylation in patients, we conducted immunohistochemistry for IKBKE and phospho-AKT Serine-473 on

a tissue microarray of human PDAC samples. We found a significant correlation between IKBKE protein levels and AKT phosphorylation at Serine-473 in the tumor samples ($R=0.606$, $P<0.0001$, $N=62$) (Figure 3.8G-H). Our findings suggest that IKBKE may regulate AKT phosphorylation in PDAC both *in vitro* and *in vivo*.

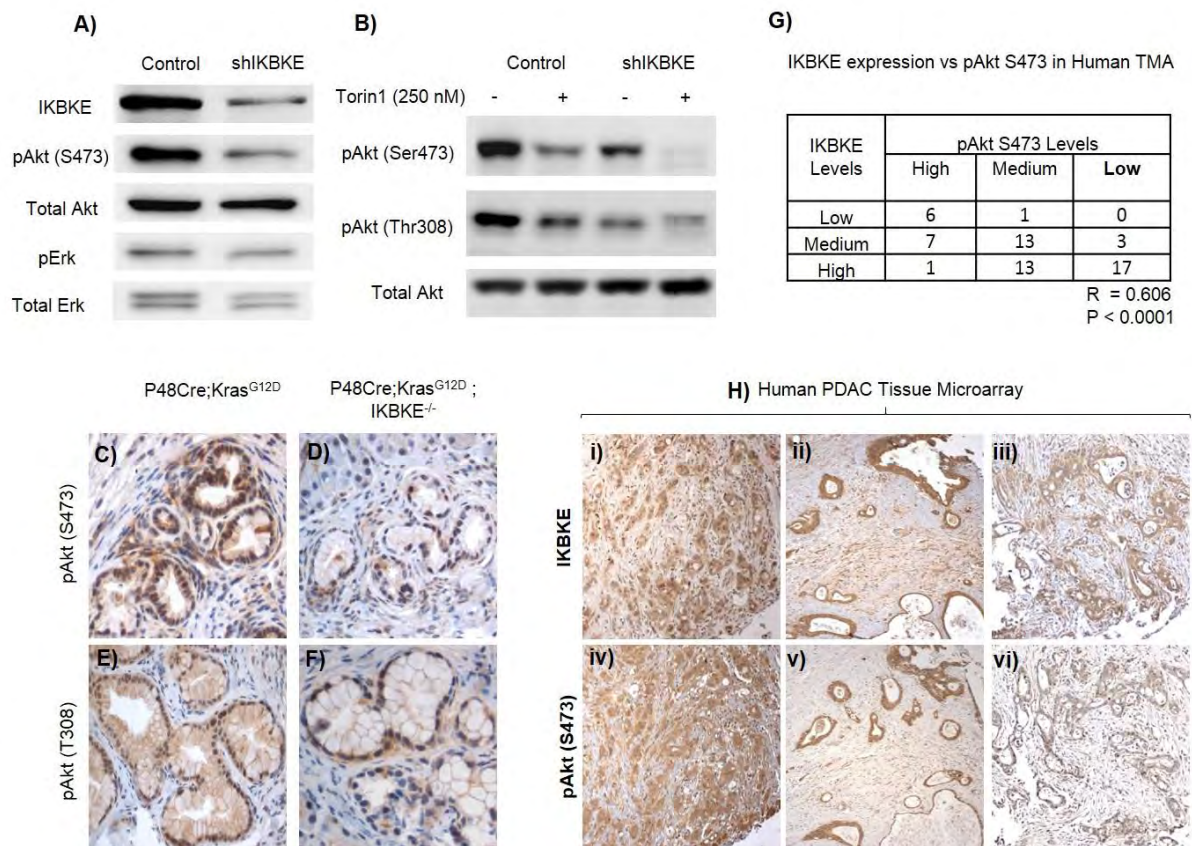


Figure 3.8: IKBKE activates AKT in Pancreatic Ductal Adenocarcinoma

(A) Western blot showing phosphorylation of AKT and ERK in serum starved Panc-1 cells in response to shRNA mediated *IKBKE* knockdown. *IKBKE* knockdown leads to decrease in phosphorylation of AKT at Serine-473, but not ERK phosphorylation. (B) Western blot of Panc-1 cells with or without inhibition of mTOR using Torin1, and shRNA mediated knockdown of *IKBKE*. Inhibition of mTOR or *IKBKE* individually leads to decrease in phosphorylation of AKT at Serine-473 and Threonine-308. Combined inhibition of mTOR and *IKBKE* leads to synergistic inhibition of AKT phosphorylation at Serine-473 and Threonine-308. (C-F) Immunohistochemistry for AKT phosphorylation at Serine-473 (C, D), and Threonine-308 (E, F) in stage matched PanIN lesions of *P48Cre;LSL-Kras^{G12D}* (C, E), and *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* (D,F) mouse pancreas. *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* PanIN lesions have significantly reduced Akt phosphorylation compared to *P48Cre;LSL-Kras^{G12D}* lesions. (G) Correlation of IKBKE protein levels and AKT Serine-473 phosphorylation levels in human PDAC tissue microarray. Pearson coefficient of R=0.606 shows significant correlation (P<0.0001) between

IKBKE protein levels and AKT phosphorylation. (H) Representative images of immunohistochemistry for IKBKE (i-iii) and phospho-AKT Serine-473 in matched human PDAC tissue samples. Significant correlation is seen between relative IKBKE expression and AKT phosphorylation.

IKBKE regulates AKT reactivation post-mTOR inhibition

While mTOR inhibitors have been approved for treatment of certain malignancies^{173,114,174}, they have not been effective in the treatment of pancreatic adenocarcinoma²². One of the problems concerning the use of mTOR inhibitors in the clinic is the reactivation of AKT post-inhibition of mTOR^{175,176}. While compensatory activation of upstream RTK signaling, and subsequent phosphorylation of AKT at Threonine-308 has been shown to be a mechanism of resistance to mTOR inhibition in breast cancer¹⁷⁷ the underlying mechanism of resistance to mTOR inhibition in PDAC is not known.

When we treated Panc-1 cells with the mTOR inhibitor Torin1, we found that while AKT phosphorylation at Serine-473 and Threonine-308 was inhibited 6 hours post-treatment, phosphorylation at both the sites was subsequently restored in the presence of serum 12 hours after treatment with the inhibitor (Figure 3.9A). Phosphorylation of other mTOR targets S6K and 4EBP1 continued to be inhibited even 24 hours after treatment, thus indicating that the reactivation of AKT phosphorylation was mTOR-independent. These findings were in contrast to the earlier findings in breast cancer cells¹⁷⁷ which showed reactivation of AKT phosphorylation only at Threonine-308 but not Serine-473, thus suggesting that the AKT reactivation in pancreatic cancer cells is mediated by a different underlying mechanism. As we have found that IKBKE can phosphorylate at both Serine-473, and Threonine-308, and maintain basal level of AKT phosphorylation in the absence of mTOR, we decided to test the role of IKBKE in AKT reactivation.

We found that shRNA mediated knockdown of IKBKE ablated the reactivation of AKT post-inhibition of mTOR in Panc-1 cells (Figure 3.9B). Furthermore, we found that while treatment with the mTOR inhibitor alone did not affect survival of PDAC cells, knockdown of IKBKE sensitized Panc-1 cells to the mTOR inhibitor, and IKBKE knockdown combined with mTOR inhibition led to a synergistic decrease in cell viability (Figure 3.9C). Combined mTOR inhibition with IKBKE knockdown also led to a significant increase in apoptosis as measured by cleaved-Caspase-3 staining (Figure 3.9D), and a decrease in tumorigenicity in Panc-1 cells as measured by soft agar assay (Figure 6E).

As we have previously seen that GLI can regulate AKT phosphorylation in PDAC, and we have found IKBKE to be a downstream mediator of GLI activity, we decided to test the role played by GLI in AKT reactivation. We found that similar to the effect of IKBKE knockdown, inhibition of GLI using a small molecule inhibitor Gant61 in Panc-1 cells prevented reactivation of AKT post-inhibition of mTOR (Figure 3.10A). As Gant61 treatment leads to decrease in *IKBKE* mRNA levels along with those of other Gli target genes (Figure 3.10B), the effect of Gant61 on AKT reactivation can be attributed to its inhibition of GLI, and the subsequent transcriptional inhibition of IKBKE. We also found that Gant61 treatment phenocopies the effect of combined IKBKE and mTOR inhibition by sensitizing Panc-1 cells to Torin1, and combined Gant61 and Torin1 treatment synergistically increases apoptosis, and decreases tumorigenicity of the cells (Figure 3.10C-E). Our findings implicate combined GLI/IKBKE signaling as playing a crucial role in reactivation of AKT post-mTOR

inhibition. This AKT reactivation IKBKE may be responsible for resistance towards mTOR small molecule inhibitors in PDAC.

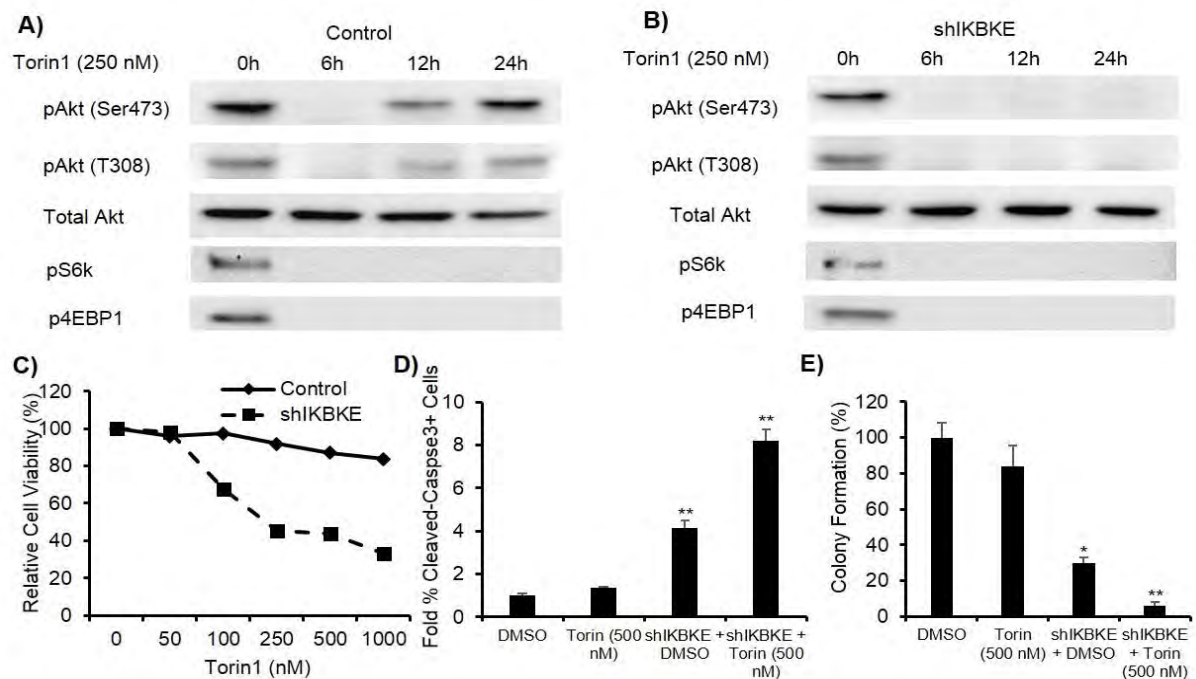


Figure 3.9: IKBKE mediates AKT reactivation post-mTOR inhibition

(A) Western blot showing phosphorylation of AKT and other mTOR substrates in response to treatment with Torin1 in Panc-1 cells. AKT phosphorylation at Serine-473 and Threonine-308, which is inhibited immediately after mTOR inhibition by Torin1, is subsequently recovered. Sustained inhibition phosphorylation of mTOR substrates S6K and 4EBP1 suggests that mTOR activity is inhibited and AKT re-phosphorylation is independent of mTOR activity. (B) Western blot of AKT and other mTOR substrates in response to treatment of Torin1 in Panc-1 cells with shRNA mediated knockdown of IKBKE. AKT phosphorylation is not recovered after inhibition of mTOR suggesting IKBKE plays a role in AKT re-phosphorylation post-mTOR inhibition. (C) Relative cell viability of Panc-1 cells measured by MTT assay 5 days after treatment with increasing dosage of the mTOR inhibitor Torin1 in the presence and absence of *IKBKE* knockdown. While mTOR inhibition alone is not sufficient to significantly affect cell viability, *IKBKE* knockdown sensitizes cells to mTOR inhibition and leads to dosage dependent decrease in cell viability. (D) Cleaved-Caspase-3 staining in Panc-1 cells indicates that while mTOR inhibition alone using Torin1 does not lead to a significant increase in apoptosis, combined inhibition of IKBKE and mTOR leads to a significant increase in apoptosis. (E) Soft agar colony formation assay in Panc-1 cells indicates that while mTOR inhibition alone does not affect tumorigenicity of the cells, combined

inhibition of mTOR and IKBKE leads to significant decrease in tumorigenicity. Error bars represent Standard Deviation. Statistical significance was determined using a Student's two-tailed t-test. * $P < 0.05$; ** $P < 0.01$

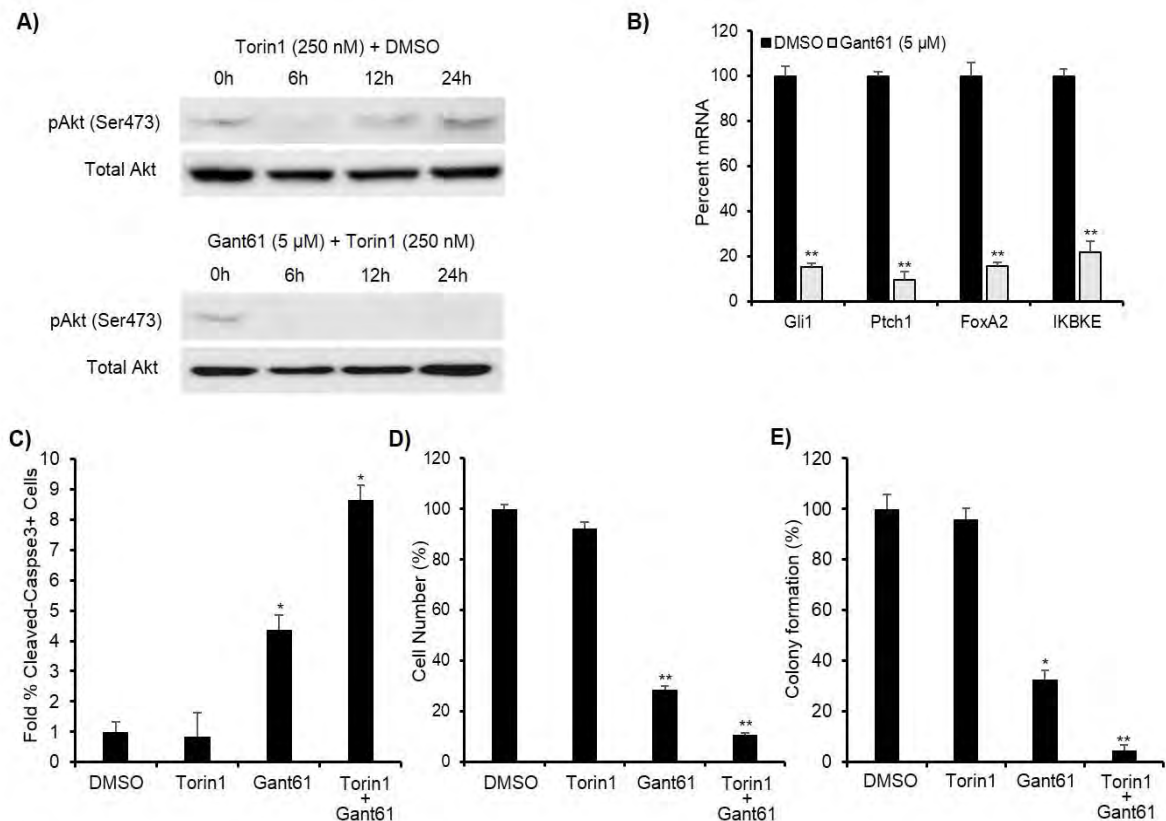


Figure 3.10: GLI regulation of AKT reactivation post-mTOR inhibition

(A) AKT phosphorylation at Serine-473 is inhibited in Panc-1 cells 6 hours after treatment with mTOR inhibitor Torin1. AKT phosphorylation is restored after 12 hours of treatment with Torin1. In the presence of GLI inhibitor Gant61, restoration of AKT phosphorylation is inhibited. (B) Quantitative RT-PCR shows that treatment of Panc-1 cells with Gant61 inhibits mRNA expression of GLI target genes including *IKBKE*. (C) Apoptosis in Panc-1 cells measured by cleaved Caspase-3 staining. Torin1 treatment alone does not significantly affect apoptosis. Treatment with Gant61 causes an increase in apoptosis in Panc-1 cells. Combined treatment with Torin1 and Gant61 synergistically increases apoptosis in Panc-1 cells. (D) Viability of Panc-1 cells as measured by MTT assay after 5 days of treatment. Treatment with Torin1 does not affect cell viability. Treatment with Gant61 significantly decreases cell viability. Combined treatment with Torin1 and Gant61 synergistically decreases viability of Panc-1 cells. (E) Tumorigenicity of Panc-1 cells measured by soft agar colony formation. Treatment with Torin1 does not affect soft agar colony formation by Panc-1 cells. Treatment with Gant61 leads to a significant decrease in soft agar colony formation by Panc-1 cells. Combined

treatment with Torin1 and Gant61 leads to a synergistic decrease in soft agar colony formation by Panc-1 cells. Error bars represent Standard Deviation. Statistical significance was determined using a Student's two-tailed t-test. * $P < 0.05$; ** $P < 0.01$

Materials and Methods

Mouse strains

P48Cre, and *LSL-Kras^{G12D}* mouse strains have been described in Chapter II. *Ikbke^{-/-}* mice¹⁶⁶ were obtained from Jackson Laboratories. *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mice were obtained via interbreeding *P48Cre* mice with *LSL-Kras^{G12D};Ikbke^{-/-}* mice. All mouse experiments were performed according to the guidelines of IACUC at University of Massachusetts Medical School.

Tissue collection and histology:

Upon euthanasia, pancreatic tissue was fixed in 4% (wt/vol) paraformaldehyde for 24 hours. For paraffin sections, tissue was dehydrated and embedded in paraffin blocks and cut at a thickness of 6 μ m. Paraffin sections were stained with hematoxylin and eosin (H&E) using standard reagents and protocols. Human PDAC tissue microarray was obtained from Shanghai Outdo Biotech.

Immunohistochemistry, immunofluorescence, and immunoblotting:

For immunohistochemistry, high-temperature antigen retrieval was conducted in Sodium Citrate solution (pH 6.0) on paraffin sections for 30 minutes. Sections were blocked in a buffer containing 5% (wt/vol) BSA and 0.1% (vol/vol) Triton X-100 in PBS and then were incubated overnight at 4 °C in primary antibodies diluted in blocking buffer. Primary antibodies used were: Ki67 (1:500, Abcam), phospho-AKT (1:50, Cell Signaling), IKBKE (1:50, Santa Cruz) for mouse sections, IKBKE (1:100, Sigma) for human sections, TBK1 (1:100, Cell Signaling), p65 (1:50, Cell

Signaling), Amylase (1:800, Sigma), and Insulin (1:100, Abcam). Signal detection was accomplished with biotinylated secondary antibodies in the Vectastain ABC kit (Vector Labs). For immunofluorescence, primary antibodies used were: Glucagon (1:3000, gift of Dr. Andrew Leiter, UMMS), Amylase (1:800, Sigma), Insulin (1:100, Abcam).

For immunofluorescence, cells grown in 8 well chamber slides were fixed for 5 minutes in 4% paraformaldehyde, blocked in a buffer containing 5% (vol/vol) sheep serum, 1% (vol/vol) FBS, and 0.1% (vol/vol) Triton X-100 in PBS for 1 hour, and then incubated with primary antibody overnight. Cleaved-Caspase-3 antibody (1:400, Cell signaling) was used for apoptosis assay. Alexa Fluor fluorescent conjugated secondary antibody (Invitrogen) was used for detection at a concentration of 1:500 diluted in blocking buffer. Slides then were mounted in mounting medium containing DAPI.

For immunoblotting, the primary antibodies used were FlagM2-HRP (1:1,000, Sigma); β -Actin (1:1,000, Sigma); phospho-AKT S473 (1:1,000, Cell Signaling), phospho-Akt T308 (1:1000, Cell Signaling), phospho-ERK (1:1,000, Cell Signaling); total AKT (1:1,000, Cell Signaling); Total ERK (1:1,000, Cell Signaling); IKBKE (1:1,000; Sigma), TBK1 (1:1000, Cell Signaling), phospho-S6K (1:1000, Cell Signaling), phospho-4EBP1 (1:1000 Cell Signaling), p65 (1:1000 Cell Signaling), PCNA (1:1000 Abcam), β -Tubulin (1:1000 Cell Signaling), and Myc (1:1,000; Developmental Studies Hybridoma Bank). HRP-conjugated secondary antibodies used for detection were obtained from Jackson Laboratories.

Cell proliferation, apoptosis and soft-agar assays:

Cell proliferation, apoptosis, and soft-agar assays were conducted as described in Chapter II.

Lentiviral shRNA knockdown and rescue experiments:

Cells were infected with pLKO-based lentiviruses encoding shRNAs targeting human Gli1 (sh*GLI1*#1: CATCCATCACAGATCGCATTT; sh*GLI1*#2: GCTCAGCTTGTGTGTAATTAT), human KRAS (sh*KRAS*: GAGGGCTTTCTTTGTGTATTT) human *TBK1* (sh*TBK1*#1: GCAGAACGTAGATTAGCTTAT; sh*TBK1*#2: GCGGCAGAGTTAGGTGAAATT) and human *IKBKE* (sh*IKBKE*#1: TGGGCAGGAGCTAATGTTTCG; sh*IKBKE*#2: GAGCATTGGAGTGACCTTGTA). Infected cells were selected in 5 µg/mL puromycin for 4 days prior to conducting assays.

Luciferase reporter analysis:

GliBS-Luc was described in Chapter II. NF-κB luciferase (p65-Luc) was a gift from Dr. Francis Chan (University of Massachusetts Medical School, Worcester, MA). Reporters were co-transfected with Renilla expression plasmids, and expression vectors for Gli3T, IKBKE, IKBKE K38A, IκB or Gli1-AHA using lipofectamine. For Gli1 knockdown, the cells were cotransfected with shRNAs targeting GLI1 along with NF-κB luciferase and Renilla expression plasmids. For Gant61 treatment, the Panc1 cells were co-transfected with NF-κB reporter and Renilla Luciferase and

then were treated with 5 μ M or 10 μ M Gant61 6 hours after transfection. *IKBKE* promoter luciferase was generated by cloning a 300 bp region upstream of the human *IKBKE* transcription start site into a PGL3 luciferase vector. Luciferase assays were conducted 48 hours after transfection using the dual-luciferase reporter kit (Promega). Assays were conducted in triplicate.

Quantitative RT-PCR:

cDNA synthesis was conducted using Invitrogen SuperScript II kit. Primers used for qRT-PCR were human *IKBKE* (forward: 5'-TGCGTGCAGAAGTATCAAGC-3'; reverse: 5'-TACAGGCAGCCACAGAACAG-3'); mouse *Ikbke* (forward: 5'-GCGGAGGCTGAATCACCAG-3'; human *GAPDH* (forward: 5'-ATGGGGAAGGTGAAGGTCG-3'; reverse: 5'-GGGGTCATTGATGGCAACAATA-3'); mouse *Gapdh* (forward: 5'-AGGCCGGTGCTGAGTATGTC-3'; reverse: 5'-TGCCTGCTTCACCACCTTCT-3'); human *GLI1* (forward: 5'-CCAGCGCCCAGACAGAG-3'; reverse: 5'-GGCTCGCCATAGCTACTGAT-3'); mouse *Gli1* (forward: 5'-GTCGGAAGTCCTATTCACGC-3'; reverse: 5'-CAGTCTGCTCTCTTCCCTGC-3'); human *PTCH1* (forward: 5'-CCACAGAAGCGCTCCTACA-3'; reverse: 5'-CTGTAATTTGCCCCCTTCC-3'); mouse *Ptc1* (forward: 5'-AACAAAAATTCAACCAAACCTC-3 'reverse: 5'-TGTCTTCATTCCAGTTGATGTG-3'); human *IL1A* (forward: ATCATGTAAGCTATGGCCCACT; reverse: CCTTCCCGTTGGTTGCTACTA), mouse *Il1a* (forward: 5'- TCTATGATGCAAGCTATGGCTCA-3'; reverse: 5'-CGGCTCTCCTTGAAGGTGA-3'); human *TNFA* (forward:

CCTCTCTCTAATCAGCCCTCTG; reverse: GAGGACCTGGGAGTAGATGAG), mouse *Tnf* (forward: 5'- CAGGCGGTGCCTATGTCTC-3'; reverse: 5'- CGATCACCCCGAAGTTCAGTAG-3'); human *BCL2L1* (forward: CTGCTGCATTGTTCCCATAG-3'; reverse: 5'-TTCAGTGACCTGACATCCCA-3'), mouse *Bcl2l1* (forward: 5'- ACATCCCAGCTTCACATAACCC-3'; reverse: 5'- CCATCCCGAAAGAGTTCATTAC-3'); human *BCL2* (forward: 5'- ATGTGTGTGGAGAGCGTCAA-3'; reverse: 5'-CGTACAGTTCCACAAAGGCA-3'); and mouse *Bcl2* (forward: 5'-GCTACCGTCGTGACTTCGC-3'; reverse: 5'- CCCCACCGAACTCAAAGAAGG-3'). All qPCR assays were conducted in triplicate.

Nuclear and cytoplasmic fractionation

For nuclear and cytoplasmic fractionation, Panc-1 cells were infected either with sh*IKBKE*#1, or with an shRNA targeting GFP, and selected with puromycin for 4 days. Nuclear and cytoplasmic fractions were separated using a kit from G Biosciences according to manufacturer's protocol.

Gli1 subcellular localization

293T cells seeded in an 8 well chamber slide were co-transfected with a human GLI1 expression vector fused to a C-terminal GFP-tag, along with either an IKBKE expression vector, IKBKE-K38A expression vector, or a control vector. The cells were fixed in 4% paraformaldehyde and mounted with a mounting medium containing DAPI 48 hours after transfection.

Chapter IV: DISCUSSION

GLI requirement in KRAS induced pancreatic tumorigenesis

Although significance of known effectors of KRAS such as PI3K/AKT, and RAF/MEK/ERK has been well characterized in PDAC⁵⁰, effectors of KRAS downstream signaling at the transcriptional level remain elusive. Crosstalk between the Sonic Hedgehog pathway, and KRAS signaling has been reported^{142,139}. While previous reports had implicated the transcription factor GLI1 as a potential effector of KRAS in human cell lines, an *in vivo* requirement for GLI transcriptional activity in PDAC had not been established¹⁴³.

Here, using a dominant repressor allele of *GLI3* (Gli3T), that inhibits all GLI mediated transcriptional activation, we demonstrate for the first time that GLI transcriptional activity in the epithelium is specifically required for pancreatic tumor formation *in vivo*, although it is dispensable for normal pancreatic development. To achieve this, we used a mouse model of pancreatic transformation with oncogenic KRAS activation in the pancreas, that models pancreatic neoplasia initiation and progression, as well as a model of pancreatic adenocarcinoma with p53 inactivation in combination with oncogenic KRAS (KPC model). We find that GLI inhibition prevents formation of early PanIN lesions even in the presence of oncogenic KRAS, thus indicating that GLI transcriptional activity is required for pancreatic tumor initiation. Our *in vivo* findings are further supported by *in vitro* experiments which show that Gli3T expression can prevent oncogenic KRAS induced proliferation in pancreatic ductal epithelial cells (PDECs), and also abrogate KRAS mediated survival of these cells in response to Cyclohexamide

treatment¹⁴⁸. Together, these data suggest that GLI transcriptional activity plays an essential role in mediating transformation of the pancreatic epithelium.

Furthermore, we find that GLI inhibition significantly extends survival in a mouse model of pancreatic adenocarcinoma (KPC model). Although mice carrying the *Gli3T* transgene still developed tumors, isolation of cells from tumors that arise from the mice carrying the *Gli3T* allele showed that the cells failed to undergo recombination and do not express Gli3T. This indicates that the tumors that were developed with delayed latency in these mice arose from a population of cells that was deficient in Gli3T, thus highlighting the importance of GLI transcriptional activity in pancreatic tumorigenesis.

Our findings implicate GLI as essential not only for the initiation of pancreatic transformation, but also for progression to full blown Adenocarcinoma. In addition to the *in vivo* mouse models, we also show that GLI transcriptional activity is required for tumorigenicity of human PDAC cell lines *in vitro*. Together, our data implicates GLI as an oncogenic effector of KRAS required for pancreatic tumorigenesis, and provides proof of concept for therapeutic targeting of GLI in PDAC.

A caveat of our loss of function model was the use of a dominant-negative repressor allele of GLI to inhibit transcriptional activity, instead of genetic ablation of the GLI proteins. GLI1 and GLI2 are known to have overlapping functions in various contexts¹³⁰, and *Gli1^{-/-};Gli2^{-/-}* mice have severe developmental defects¹⁷⁸,

which necessitated the use of a conditional dominant-negative repressor in our model. While the possibility of the Gli3T repressor having some off-target effects cannot be excluded, our *in vitro* studies indicate that repression of GLI activity by Gli3T is specific (Fig. 2.1), and we find that the *in vitro* effect of Gli3T induced repression can be replicated by shRNA mediated knockdown of *GLI1* and also treatment with the GLI small molecule inhibitor Gant61, which supports our conclusions. However, another more robust approach to test our findings further would be to develop a conditional knockout allele of *Gli1*, and combine it with *Gli2* conditional knockout to specifically delete both *Gli1* and *Gli2* in the pancreatic epithelium in the background of oncogenic *Kras*.

GLI1/2/3 in pancreatic transformation:

While our loss of function model establishes the requirement for GLI transcriptional activity in pancreatic tumorigenesis, the Gli3T allele acts as a dominant negative repressor for all the GLI transcription factors, and hence does not distinguish between the role played by individual GLI proteins. In order to study the role of individual GLI transcription factors in PDAC, we utilized an allele of wild type *Gli1*, and an N-terminal truncated allele of *Gli2* (Gli2ΔN) knocked into the *Rosa26* locus. The N-terminal truncated version of GLI2 was used because unlike GLI1, GLI2 carries a repressor domain at the N-terminal which suppresses GLI2 activator function. We also utilized a conditional knockout allele of *Gli3* (*Gli3^{fl/fl}*) to test the role played by the Gli3 repressor in pancreatic transformation, which has previously not been studied.

Our findings suggest that the activation of GLI1 or GLI2 alone, in the absence of oncogenic KRAS is not sufficient to initiate pancreatic transformation, and mice with GLI1/2 activation in the pancreatic epithelium have largely normal pancreatic architecture and differentiation. These findings are in contrast to an earlier report that showed that GLI2 activation alone was sufficient to drive formation of undifferentiated tumors in the pancreas¹³⁹. However, this difference in phenotype may be explained by a difference in the design of the two studies. In our experiment, the *Ptf1a* (*P48*) promoter was used to drive expression of Cre, which is expressed later on in the pancreatic progenitor cells compared to the *Pdx1* promoter used in the other study^{179,180}. Also, the *Gli2* transgene used in our study was expressed from the *Rosa26* locus, whereas the *Gli2* allele used in the other study (*Cleg2*) was expressed under the control of the CAGGS promoter, a highly active hybrid CMV/ β -Actin promoter. Differences in expression levels of GLI2 may account for the difference in phenotype seen in our studies. This possibility can be tested by comparing expression levels of GLI2 in the two different models.

While GLI1/2 activation alone is not sufficient to drive pancreatic tumorigenesis, we find that both GLI1 and GLI2 can cooperate with oncogenic KRAS in pancreatic transformation. Ectopic expression of either GLI1 or GLI2 Δ N in the pancreatic epithelium led to the accelerated formation of advanced PanIN lesions in mice. While mice with oncogenic KRAS expression in pancreas develop PanIN lesions, at the age of 2 months most of the pancreas display normal architecture with only a few low grade PanIN lesions. In contrast, we found that GLI1 or GLI2 Δ N ectopic

expression in combination with oncogenic KRAS led to the formation of advanced PanIN3 grade lesions at the age of 2 months. Most of these mice did not survive past 2 months of age, in contrast to mice with oncogenic KRAS activation alone, which lived past 12 months, thus indicating a dramatically reduced survival.

We also observed a high degree of desmoplasia in response to the GLI1/2 activation, as well as evidence of ascites, both of which are features of advanced pancreatic malignancy. However, we failed to detect evidence of full blown invasive adenocarcinoma, and metastasis even in mice that survived up to 10 months of age. There could be multiple explanations for this. One possibility is that while GLI1/2 can cooperate with oncogenic KRAS in driving pancreatic transformation, additional genetic alterations such as loss of tumor suppressors are required for progression to full blown adenocarcinoma, and activation of GLI1/2 alone does not bypass these requirements. Another possibility is that a majority of the mice with aggressive GLI1/2 PanIN lesions die due to pancreatic dysfunction before developing adenocarcinoma. A third intriguing possibility is that the strong desmoplastic reaction, and massive stromal proliferation induced by GLI1/2 may act to restrain further epithelial tumor formation by inhibiting angiogenesis, as proposed by some recent studies⁴⁰.

Unlike GLI1/2 activation, the deletion of the transcriptional repressor GLI3 from the pancreatic epithelium had no effect on the development of the pancreas. Also, *Gli3* deletion did not affect the initiation and progression of PanIN lesions in response to oncogenic KRAS activation. The pancreas of *P48Cre;LSL-Kras^{G12D};Gli3^{fl/fl}* mice

were histologically similar to those of the *P48Cre;LSL-Kras^{G12D}* mice. As discussed previously, GLI3 is primarily a transcriptional repressor. Loss of function mutations in Gli3 are known to lead to deregulation of the Hedgehog signaling pathway, and are implicated in developmental disorders such as Greig cephalopolysyndactyly syndrome and Pallister Hall Syndrome^{181,182}. *GLI3* is also known to be mutated in a minority of PDAC cases, and Gli3 expression is detected in PDAC cells^{91,143}. Our findings imply that GLI3 does not play a significant role in PDAC, and *Gli3* deletion does not affect pancreatic transformation by *KRAS*. While our *in vivo* data suggests that unlike GLI1 and GLI2, GLI3 does not play a significant role in pancreatic transformation, the difference in the design of the *Gli1*, *Gli2*, and *Gli3* genetic experiments may also affect interpretation of our data. Firstly, GLI1 and GLI2 are expressed ectopically in our mouse model at levels that are likely higher than the physiological levels. Whether the effect of GLI1/2 mediated acceleration of PanIN lesions is due to higher levels of expression of the proteins, or due to differential transcriptional/post-transcriptional regulation of the ectopically expressed transgenes cannot be determined from our experiments. Experiments involving genetic ablation of endogenous GLI1 and GLI2 proteins need to be conducted in order to further evaluate the role of the individual proteins in pancreatic transformation. Also, expression levels of GLI target genes in the pancreas of mice with oncogenic *KRAS*, and oncogenic *KRAS* with GLI1/2 activation or GLI3 deletion need to be compared to test whether the lack of phenotype with GLI3 deletion is due to the lack of difference in GLI transcriptional

activity. If this is the case, then it may imply either that the GLI3 proteins are not functional during pancreatic transformation, or that the activity of these proteins is not sufficient to counter transcriptional activation by GLI1/2. Another possibility could be that while GLI3 may be expressed in PanIN lesions, they may be inactive due to lack of post-translational regulation in the pancreatic context.

Interestingly, bioinformatics analysis using the Oncomine database indicates that while GLI1 and GLI3 are upregulated in human PDAC samples compared to normal pancreas, GLI2 expression is relatively unchanged. Previous findings in human PDAC cells have indicated that GLI2 is undetectable in human PDAC cells, and KRAS knockdown leads to a downregulation of GLI1 but not GLI3 in PDAC cells¹⁴³. Although GLI2 may not be expressed at sufficient levels, or in a KRAS dependent manner in pancreatic cancer, our data clearly shows that GLI2 is capable of playing an important role in promoting pancreatic neoplasia in combination with KRAS. Furthermore, our findings that shRNA mediated knockdown of *GLI1* is sufficient to impair survival of PDAC cells indicates that while GLI2 is capable of promoting pancreatic transformation upon ectopic expression, GLI1 may be the primary GLI downstream effector of Kras in PDAC.

Cell-autonomous (non-canonical) GLI activity in PDAC

As discussed previously, there are multiple possible mechanisms of Hedgehog/GLI signaling in pancreatic tumorigenesis. Our study provides strong evidence for Hedgehog ligand independent, non-canonical GLI activity in PDAC.

Our finding that inhibition of GLI transcription is sufficient to halt KRAS induced transformation in pancreas, along with previous reports that the ablation of the upstream GLI regulator Smoothed does not affect GLI activity¹⁴³ suggest cell-autonomous regulation of GLI in the pancreatic epithelium. Also, while Hedgehog/GLI activity in the pancreatic stroma has previously been shown to be important, stromal GLI activity is regulated by Hedgehog ligands secreted by the epithelium¹⁸³. This is in contrast to GLI activity in the epithelium, which is regulated by oncogenic KRAS. Together, these findings point to differential regulation of GLI transcriptional activity in the epithelium and the stroma of the pancreas.

Our findings that GLI1/2 can accelerate pancreatic neoplasia in the presence of oncogenic KRAS, but are not sufficient to initiate tumorigenesis in the absence of KRAS activation, suggest a potentially important role in post-translational regulation of these transcription factors by KRAS. An earlier report which shows that Kras may prevent Gli1 protein degradation in PDAC cells via the RAF/MEK/ERK pathway supports this hypothesis¹⁸⁴. Furthermore, the inability of oncogenic Smoothed (*SmoM2*) activation to accelerate tumorigenesis suggests that canonical Hedgehog signaling upstream of GLI does not play a role in regulation of these transcription factors in the pancreatic epithelium. This is in contrast to the stroma, where smoothed inhibition can inhibit activity of the GLI transcription factors¹⁸⁵. These findings highlight context dependent differences in regulation of GLI activity in the pancreatic epithelium and the stroma. However, while our findings highlight the requirement of GLI in the pancreatic epithelium in

tumorigenesis, they do not disprove the existence of paracrine Hedgehog signaling between the epithelium and the stroma. As discussed previously, there is significant evidence that shows paracrine Hedgehog signaling in pancreatic cancer, and ligand dependent GLI activity in the stroma. Instead, our findings suggest that while Hedgehog dependent GLI activity in the stroma may play a role in pancreatic cancer, inhibition of Smoothened and other Hedgehog pathway components upstream of GLI may not be sufficient to inhibit tumorigenesis, due to the cell autonomous nature of GLI activity in the pancreatic epithelium, and the important role played by non-canonical GLI signaling in pancreatic cancer. Our conclusion is supported by recent findings which show that treatment with a Smoothened inhibitor IPI-296 may not only fail to inhibit pancreatic cancer growth, but may also marginally accelerate it, and the failure of Smoothened inhibitors in the clinic in treatment of PDAC^{185,186}.

Unique pancreas specific transcriptional program in PDAC

In classic Hedgehog/Smoothened dependent cancers, such as BCC, medulloblastoma, rhabdomyosarcoma, the GLI proteins are known to drive a transcriptional program that controls cell cycle and proliferation, through regulation of genes such as *CyclinD1*, and *Myc*^{187,188}. However, in the context of PDAC, we found that a majority of the genes regulated by GLI were not associated with canonical Hedgehog signaling, and that *Cyclin D1* and *Myc* expression was not significantly altered upon GLI inhibition, although in our microarray analysis we identified several known generic GLI target genes such as *GLI1*, *PTCH1*, and

FOXA2. These findings suggest that GLI regulates a unique transcriptional program in the context of PDAC. Interestingly, we identified a number of genes involved in regulation of RAS signaling such as *SOS2*, *RASA1*, *RIN2*, *RASSF4*, and *RASSF5* that were downregulated in response to GLI repression. *SOS2* is a positive regulator of RAS signaling, while *RASA1*, and *RIN2* are negative regulators, and *RASSF4* and *RASSF5* are effectors of RAS induced tumor suppression. This suggests the existence of a possible feedback loop between RAS and GLI in PDAC. Further, we identified genes involved in regulation of PI3K/AKT signaling such as *PIK3R1*, and *PIK3C2B*. While our subsequent experiments revealed transcriptional regulation of *IKBKE* by GLI as a mechanism of GLI regulation of AKT activity, we have found that regulation of Akt phosphorylation by *IKBKE* is modest compared to AKT regulation by GLI. The regulation of *PIK3R1*, and *PIK3C2B* may constitute an additional mechanism through which Gli may regulate AKT activation. This hypothesis can be tested further by conducting shRNA mediated knockdown of these genes and testing its effect on AKT phosphorylation. We also identified a number of genes involved in regulation of the NF- κ B pathway such as *IKBKE*, *TRAF1*, *TRAF3IP2*, *MAP3K14*, and *NFKBIE*. As discussed previously, the PI3K/AKT and NF- κ B signaling pathways play an important role in pancreatic tumorigenesis, and are known to act downstream of KRAS in regulating processes such as apoptosis, cell proliferation, and inflammation. Our findings implicate transcriptional regulation by GLI as a potential mechanism through which these pathways are regulated by KRAS in

PDAC. Our data also highlights a novel downstream oncogenic program involving RAS/PI3K/NF- κ B signaling mediating GLI tumorigenic activity. The importance of the newly identified candidate Gli target genes can be further evaluated using shRNA mediated knockdown in human PDAC cells.

PI3K/Akt regulation by Gli

The PI3K/AKT pathway is a major effector of RAS signaling that has been implicated in cell transformation¹⁴⁶. The mechanisms through which RAS regulates PI3K, and subsequently AKT activation have been relatively well characterized¹⁰¹. Here, we identified an additional level of mechanistic control of AKT activation in KAS dependent PDAC cells. We find that inhibition of GLI using the Gli3T repressor in human PDAC cells leads to a significant decrease in AKT phosphorylation. Furthermore, we find that treatment with a small molecule inhibitor of GLI (Gant61) can lead to a dosage specific decrease in AKT phosphorylation in PDAC cells. We also find that in our mouse model, ectopic expression of GLI1 in combination with oncogenic KRAS can significantly increase AKT phosphorylation. Interestingly, we find that ectopic expression of GLI1 can help bypass KRAS induced senescence in PanIN lesions. Activation of the KRAS oncogene is known to induce senescence in normal cells, a process which is mainly regulated by the MAPK/ERK pathway¹⁸⁹, but can be relieved by activation of the PI3K/AKT pathway¹⁹⁰. Evasion of senescence in response to GLI1 ectopic expression may be mediated by the PI3K/AKT pathway in the PanIN lesions. This can be tested by conducting staining for β -galactosidase, and

immunohistochemistry for AKT phosphorylation on adjacent sections of pancreas from *P48Cre;Kras^{G12D};R26-Gli1* mice, and analyzing correlation between senescent cells and relative AKT phosphorylation between different PanIN lesions. Alternatively, GLI1 can be ectopically expressed, or repressed using shRNA mediated knockdown in HPNE cells, which are immortalized human pancreatic ductal cells, in combination with oncogenic KRAS to test whether GLI1 enables bypass of oncogene induced senescence.

Regulation of NF-κB signaling by GLI

As discussed previously, deregulation of NF-κB signaling and its associated inflammatory response are the hallmarks of PDAC and have been shown to be essential for pancreatic transformation. KRAS oncogenic activation is known to drive NF-κB activation in the context of pancreatic cancer. It has been shown that KRAS can drive secretion of IL-1 and canonical NF-κB pathway activation through transcriptional regulation of AP-1⁴⁴, and non-canonical NF-κB pathway activation via regulation of GSK3α¹²⁷. However, as the NF-κB pathway can be regulated at multiple levels¹⁹¹, the link between KRAS and NF-κB signaling in PDAC needs to be further explored.

In our gene expression analysis, we found that the expression of a number of regulators of the NF-κB pathway was downregulated in response to repression by Gli3T. Furthermore, we found that the expression of an artificial luciferase construct carrying p65 binding sites (p65-luciferase) was downregulated by Gli3T

expression in PDAC cells. We also found that repression by Gli3T led to a significant downregulation in expression of NF- κ B target genes and cytokines such as BCL2L1, IL-1, and TNF- α . This data suggests that GLI may regulate the NF- κ B pathway in the context of PDAC. The GLI-NF- κ B connection is further supported by our analysis of *Kras-Gli* pancreatic tumor models, as well as human PDAC samples, although the mechanisms through which GLI may regulate NF- κ B remain unclear and further studies are clearly needed. Interestingly, we identified GLI dependent expression of a number of known NF- κ B pathway regulators such as the TNF receptor regulator *TRAF1*, *TRAF3IP2*, non-canonical NF- κ B kinase *MAP3K14*, *IKBKE*, and also the TAK1 ubiquitinator *TRIM8*. Alternatively, it is also possible that GLI may regulate expression of cytokines, which in turn activate NF- κ B via autocrine signaling. Another possibility is that GLI may regulate expression of NF- κ B target genes via transcriptional co-activation, therefore functioning as a crucial link connecting KRAS signaling, NF- κ B pathway activation, and inflammatory response during PDAC pathogenesis.

GLI mediated inflammation in PDAC

As discussed previously, inflammation has been established to be a critical mediator of pancreatic transformation, and chronic pancreatitis is known to be a major risk factor in pancreatic cancer¹²⁴. While previous studies on inflammation in PDAC have focused on known regulators of the NF- κ B pathway, in our study, we demonstrate for the first time, a potential role of GLI signaling in regulation of inflammation in PDAC. In our study, we identified GLI dependent expression of a

number of pro-inflammatory cytokines such as *IL1A*, *TNFA*, *CX3CL1*, as well as other regulators of inflammation such as *IKBKE*, *MAP3K14*, *IRF1*, *TRAF3IP2*, and *TRIM8*. Other studies have also shown that GLI1 may regulate IL-6 expression in the stroma, which activates STAT3 in the pancreatic epithelium through reciprocal signaling¹⁹². STAT3 activation is known to be an important event in inflammation induced pancreatic transformation¹⁹³. Although previous studies have implicated IKK β , COX2, IL-1 α , and p62 in KRAS induced inflammatory response in PDAC, GLI transcriptional activation of pro-inflammatory cytokines, and regulators of inflammatory response may represent an additional level of regulation of inflammation by KRAS in PDAC. Although GLI can activate inflammation in PDAC, the link between the downstream effectors of GLI and inflammatory response needs to be further explored.

Therapeutic implications of non-canonical GLI activity in PDAC

We have demonstrated that in PDAC, cell autonomous activity of Gli transcription factors in the epithelium is required for KRAS induced oncogenic transformation. We have also established GLI as a critical oncogenic effector of KRAS mediated pancreatic transformation and demonstrated regulation of the PI3K/AKT and NF- κ B pathways. Our studies provide proof of concept that therapeutic inhibition of GLI should be further evaluated as in development of targeted therapy for PDAC.

The activity of GLI in the pancreatic epithelium is independent of the upstream Hedgehog/Smoothed signaling. Currently, the only Hedgehog pathway

inhibitors that have been successful in the clinic are Smoothed inhibitors. The Smoothed inhibitor Vismodegib has been approved for treatment of Basal Cell Carcinoma, and Smoothed inhibitors are being evaluated for treatment of other malignancies¹⁹⁴. However, since the GLI activity in the pancreatic tumor epithelium is independent of Smoothed, PDAC tumors, which arise from the epithelium are refractory to Smoothed inhibition. Unlike the epithelium, the stromal component, which makes up a significant mass in PDAC tumors, is sensitive to Smoothed inhibition, as the GLI activity in the stroma is regulated by upstream Hedgehog/Smoothed signaling¹⁸³. Hence, in PDAC tumors, treatment with Smoothed inhibitors would serve to deplete the stroma, but not the epithelial component of the tumors. However, recent studies have shown that stromal depletion alone may accelerate tumorigenesis, as the stroma serves to restrict angiogenesis⁴⁰. Hence, targeting Hedgehog signaling in the stroma alone may not be beneficial in treatment of PDAC. A better therapeutic strategy may be to target the GLI transcription factors instead, as the PDAC epithelium is dependent on the activity of these proteins. Another approach may be to identify and target downstream effectors of GLI in the tumor epithelium, such as IKBKE, that are required for tumorigenicity.

IKBKE requirement in pancreatic transformation

IKBKE was first identified as an oncogene in the context of breast cancer where it was shown to activate the NF- κ B pathway during transformation¹⁶². Here, we show that IKBKE but not TBK1 is a critical oncogenic effector of KRAS in pancreatic

ductal adenocarcinoma both *in vitro* and *in vivo*. The identification of IKBKE as a novel regulator of pancreatic tumorigenesis opens up new avenues for development of targeted therapy for PDAC, as efforts to develop small molecule inhibitors of IKBKE are already underway, some of which have shown efficacy *in vivo*¹⁶³. In this study, we have identified an *in vivo* requirement for IKBKE signaling in the initiation and progression of pancreatic neoplasia. The *P48Cre;Kras^{G12D}* model used in our system mimics initiation of pancreatic neoplasms but not progression to adenocarcinoma. While our *in vitro* studies with human PDAC cells highlight a critical requirement for IKBKE in formation of adenocarcinoma, this needs to be further evaluated using *in vivo* models. To achieve this, IKBKE knockout can be combined with the “KPC model” of pancreatic adenocarcinoma in which KRAS is activated in the pancreatic epithelium along with knockout of *TP53*. Analysis of survival of mice as well as kinetics of PDAC formation can be used as an indicator for the involvement of IKBKE in PDAC tumors.

An alternative mouse model in which PTEN loss is combined with oncogenic KRAS can also be used. This would particularly be significant because of an earlier report that *PTEN* deficient PDAC tumors, but not *TP53* deficient PDAC tumors are vulnerable to small molecule based mTOR inhibition in mouse models¹¹³. Differences in the role of IKBKE/mTOR in regulation of AKT in the background of *PTEN* loss vs *TP53* loss can be evaluated using these two models. The human PDAC cell lines used in our study carry mutations in *TP53*, and we have shown these cell lines to be refractory to mTOR inhibition, which can be overcome by

IKBKE knockdown. One possibility that needs to be evaluated is that *TP53* mutant PDAC tumors may be dependent on IKBKE for AKT activation, hence refractory to mTOR inhibitors, whereas *PTEN* deficient tumors may be sensitive to mTOR inhibition due to lack of IKBKE dependence. The two mouse models described above may be used to test this hypothesis.

In our *in vivo* loss of IKBKE function model, we use a whole body knockout allele in which IKBKE expression is lost not only in the pancreatic epithelium, but also in the tumor microenvironment and in all other tissue types. This is distinct from our GLI loss of function model in which we specifically inhibit GLI activity in the epithelium. There are various implications of using a whole body knockout instead of an epithelial specific knockout. Firstly, a whole body knockout model more closely mimics drug based inhibition of IKBKE, as the function of IKBKE is lost not in a specific tissue compartment but in all tissues types. In contrast to a whole body knockout, an epithelial specific knockout model does not take into account the possibility that the particular gene may play a potentially tumor suppressive role when expressed in other tissue types, which would have significant implications for drug based therapy. Also, the IKBKE knockout mice used in our study are healthy, which implies that specific IKBKE inhibition may not be associated with severe side effects.

A limitation of our *in vivo* model is that it does not distinguish between the requirement of IKBKE in the epithelium vs the tumor microenvironment. As discussed previously, the tumor microenvironment plays an important regulatory

role in PDAC initiation, progression, and response to therapy, and immune cell infiltration is a key feature of PDAC tumors. IKBKE is known to play an important role in Type I Interferon response via phosphorylation of IRF3, and IRF7, and is known to activate the NF- κ B pathway in leukocytes¹²¹. The possibility that at least part of the antitumor activity of IKBKE inhibition may be due to the role of IKBKE in the tumor microenvironment cannot be excluded. To test the significance of IKBKE activation in the tumor epithelium, a xenograft model in which IKBKE is specifically knockdown using an inducible shRNA in PDAC cell line derived tumors can be utilized. An alternative approach using the highly immunocompromised *NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ* mice (NSG)¹⁹⁵ can also be used. These mice have severe defects in the innate immune system, and lack functional T-cells, B cells, and Natural Killer cells. Hematopoietic Stem Cells (HSCs) isolated from either *Ikbke* knockout or wild type mice can be transplanted into the NSG mice to generate IKBKE deficient or IKBKE wild type immune cells. Human PDAC cell lines can then be used to generate PDAC tumors in these mice via orthotopic transplantation. The differences in tumor growth and pathology between mice carrying IKBKE ablation and mice with wild type IKBKE can be evaluated to study the stromal contribution of IKBKE in tumorigenesis.

IKBKE-GLI reciprocal interaction in PDAC

Our studies have clearly established IKBKE as a GLI target gene downstream of KRAS in PDAC cells. Interestingly, we also found evidence to suggest that IKBKE can engage in feedback regulation of GLI likely via regulation of GLI1 nuclear

localization. GLI1 transcriptional activity can be regulated by phosphorylation by different kinases such as S6K1, PKA, and PKC-gamma in both developmental and tumorigenic contexts. In addition, PKA phosphorylation has been shown to regulate cytoplasmic-nuclear shuttling of GLI1. It is possible that IKBKE may regulate subcellular localization of GLI1 via direct. Bioinformatic analysis of the GLI1 protein sequence using the Kinexus software reveals candidate IKBKE phosphorylation sites at Serine-480, Serine-521, and Serine-538 on GLI1. These residues are located in close proximity to the Nuclear Export Signal (NES) of the GLI1 protein. Phosphorylation of GLI1 and subsequent inhibition of nuclear export has been previously established as a mechanism of GLI1 regulation¹⁶⁷. Hence, it is possible that IKBKE may activate GLI1 post-translationally by preventing its nuclear export, however, further experiments are needed to test this hypothesis. IKBKE regulation of GLI1 phosphorylation can be tested preliminarily by testing whether co-expression of the two proteins in 293 cells results in differential phosphorylation of GLI1 using phos-tag western blot. Furthermore, although immuno-precipitation of GLI1 has been challenging due to lack of a specific high quality antibody, recent technological advances such as the CRISPR/Cas9 system can be utilized to tag endogenous GLI1 in PDAC cells with a Flag-tag followed by immuno-precipitation, and mass spectrometric analysis to test differential phosphorylation at various sites on GLI1 in response to IKBKE knockdown. This technique can also be used to test subcellular localization of endogenous GLI1 in response to IKBKE knockdown or activation. Finally, it is important to consider the

possibility that IKBKE regulation of GLI1 may be an indirect event. To test this, an *in vitro* kinase assay can be conducted to test whether IKBKE is capable of phosphorylating GLI1 directly. Together, these experiments may provide better insight into the mechanism of regulation of GLI1 by IKBKE.

Signaling pathways regulated by IKBKE in PDAC

Although IKBKE is a Serine/Threonine kinase that was first identified as an I κ B kinase, and an activator of NF- κ B signaling, it shares only ~30% structural homology with the canonical I κ B kinases IKK α and IKK β ¹⁹⁶. In addition to phosphorylating I κ B, IKBKE is known to play an important role in activating IRF3 (interferon regulatory factor 3), and IRF7 (interferon regulatory factor 7) in activation of Type I Interferon Response, and IKBKE is also known to directly phosphorylate and activate the NF- κ B transcription factor c-Rel¹⁹⁷. In breast cancer, the oncogenic activity of IKBKE can be attenuated by inhibition of NF- κ B signaling, thus suggesting that IKBKE induced transformation is mainly mediated by NF- κ B. However, surprisingly, we found that, unlike in breast cancer, the oncogenic activity of IKBKE in PDAC is not dependent on its ability to activate NF- κ B signaling, and is likely mediated by alternate mechanisms. In our human PDAC cell lines we see only modest regulation of nuclear localization of the NF- κ B transcription factor p65 by IKBKE. In our *in vitro* and *in vivo* systems, we use p65 nuclear localization in the epithelium as a marker for NF- κ B pathway activation. While based on our findings it is apparent that IKBKE regulation of NF- κ B pathway activation is modest, another possibility is that IKBKE may act through a p65

independent manner via regulation of C-REL as previously described¹⁹⁷. This possibility can be further tested by measuring nuclear vs cytoplasmic localization of c-REL in response to IKBKE knockdown in PDAC cells. In addition to the NF- κ B pathway components, IKBKE can phosphorylate other substrates such as CYLD, FOXO3a, TRAF2, and AKT^{198,199,200,172}. In particular, IKBKE has been shown to directly phosphorylate AKT at both Threonine-308 and Serine-473 in a PI3K/mTOR independent manner in biochemical assays; however, its physiological and clinical relevance remains unclear. Our data provide a strong evidence that IKBKE is critical for maintaining AKT activity in PDAC. AKT phosphorylation at Threonine-308 is normally regulated by PDK1 in response to PI3K activation, and Serine-473 phosphorylation is regulated by the mTORC2 complex. We find that treatment with the mTOR kinase inhibitor Torin1 leads to a decrease in AKT phosphorylation at both Threonine-308, and Serine-473 in serum starved Panc-1 cells. Although mTORC2 complex does not directly phosphorylate AKT at Threonine-308, dephosphorylation of the Serine-473 residue has been proposed to cause transient inhibition of phosphorylation at Threonine-308²⁰¹. This effect has been attributed to the ability of the phosphorylated Serine-473 residue to provide a docking site for PDK1²⁰². Our findings are consistent with this hypothesis as we see loss of AKT phosphorylation at Threonine-308 with mTOR inhibition. Although AKT phosphorylation is relatively inhibited by mTOR inhibition, we find that a basal level of phosphorylation is maintained even while mTOR is inhibited. We find that mTOR inhibition, when combined with shRNA-mediated

knockdown of IKBKE in Panc-1 cells leads to a synergistic decrease in phosphorylation of AKT at both Serine-473, and Threonine-308. These findings suggest that while mTOR certainly plays a role in phosphorylating AKT in PDAC, IKBKE is a major contributor to AKT phosphorylation at both Serine-473 and Threonine-308, and combined inhibition of mTOR and IKBKE is necessary to inhibit AKT activation. Thus, activation of AKT is likely the primary mechanism of IKBKE mediated pancreatic tumorigenesis underlying KRAS/GLI activation.

Combined IKBKE/mTOR inhibition in PDAC

Although they have been successfully used in the treatment of certain malignancies such as Renal Cell Carcinoma, mTOR inhibitors have not been effective in treatment of PDAC in the clinic. Resistance to mTOR inhibitors can be mediated by multiple mechanisms. Until recently, majority of the mTOR inhibitors tested in clinical trials are “Rapalogs” which are analogs of Rapamycin. Rapalogs are known to preferentially inhibit mTORC1 complex but are not very effective in inhibition of mTORC2, which mediates AKT phosphorylation²⁰³. Thus, Rapalogs fail to inhibit one of the crucial functions of mTOR which is important in oncogenic transformation. Inhibition of mTORC1 can also increase AKT phosphorylation at Threonine-308, as S6K, an mTORC1 target normally acts as a negative regulator of IGF-1 signaling, and IGF-1 can drive PI3K activation through the activity of IRS1²⁰⁴. Hence, Rapalogs are not an ideal choice of treatment in cancers that are dependent on AKT activity such as PDAC. mTOR kinase inhibitors, which are second generation mTOR inhibitors are considered to be more effective than

Rapalogs because they can target both mTORC1, and mTORC2. However, in a model of breast cancer, it was recently shown that even mTOR kinase inhibitors were unable to suppress AKT reactivation which, in the context of breast cancer, was mediated by increased HER2 kinase mediated RTK activation, and independent of S6K activity¹⁷⁷. In this study, it was shown that combined inhibition of mTOR with HER2 led to a decrease in tumor formation *in vivo*. Hence, in order to effectively utilize mTOR inhibitors for treatment of cancer, it is essential to identify underlying causes of resistance to the inhibitors, of which there are multiple possibilities.

In our study, we found that mTOR inhibition alone had no effect on the survival of human PDAC cell lines, which is consistent with earlier findings in patients. Furthermore, we found that although mTOR inhibition in PDAC temporarily led to ablation of AKT phosphorylation at both Serine-473 and Threonine-308, phosphorylation at both these sites was soon restored in the presence of serum. Earlier studies in non-PDAC cell lines have shown that in case of mTOR independent AKT reactivation, only phosphorylation at Threonine-308 is restored, whereas Serine-473 phosphorylation remains inhibited. Hence, reactivation of AKT phosphorylation at both the sites indicates a potentially different, previously undiscovered mechanism underlying resistance to mTOR inhibition. Since we have found that IKBKE plays a crucial role in AKT regulation in PDAC, we evaluated the possibility of IKBKE mediating mTOR-independent AKT reactivation.

We found that after shRNA mediated knockdown of IKBKE, AKT rephosphorylation at both Threonine-308 and Serine-473 was inhibited.

Our findings suggest that in the context of PDAC, IKBKE may mediate resistance to mTOR inhibition by driving mTOR-independent AKT reactivation. Consistent with these results, we also found that IKBKE knockdown led to sensitization of Panc-1 cells to mTOR inhibition, and significantly increased apoptosis and decreased tumorigenicity in the cells. Our results provide proof of concept that combined IKBKE/mTOR inhibition may be beneficial in treatment of PDAC by overcoming reactivation of AKT.

Further *in vivo* experiments are necessary to test whether combined IKBKE/mTOR targeting can lead to inhibition of PDAC tumors in mice. We propose the following two approaches:

- 1) Utilize the previously described “KPC model”²⁰⁵ with Cre-dependent expression of oncogenic KRAS, along with a dominant negative allele of *Trp53* in the mouse pancreas to mimic human PDAC tumor formation *in vivo*. Once the tumors are established, divide the mice randomly into four treatment arms: i) Vehicle alone, ii) ATP competitive mTOR kinase inhibitor AZD8055²⁰⁶, iii) IKBKE small molecule inhibitor Amlexanox¹⁶³, iv) AZD8055 in combination with Amlexanox. Survival of the mice in different treatment arms can be compared to determine potential benefit of treatment

2) Generate patient derived xenograft (PDX) models of PDAC via subcutaneous engraftation of human PDAC tumors in immunodeficient NSG mice^{207,208}. Once PDAC tumors are engrafted, randomly assign the mice into four treatment arms, and treat with the IKBKE/mTOR inhibitors as described above. Tumor volume measurement can be used as an indicator of efficacy of the inhibitors in PDAC treatment.

Data from the pre-clinical *in vivo* experiments described above may provide robust evaluation of the potential for combined IKBKE/mTOR targeted therapy in treatment of PDAC in the clinic.

Summary (Fig. 4.1)

In our study, we have identified a critical *in vivo* requirement for cell autonomous GLI transcriptional activity in the pancreatic epithelium for initiation of neoplasia, and progression to adenocarcinoma. Using a well established model of pancreatic adenocarcinoma, we have provided proof of concept that inhibition of GLI transcription factors may be beneficial in treatment of PDAC. Furthermore, we have identified a novel PDAC specific transcriptional program regulated by GLI that involves activation of the NF- κ B and PI3K/AKT signaling pathways, and we have established GLI as a mediator of KRAS induced NF- κ B and AKT activation.

We have also identified IKBKE as a transcriptional target of GLI, which plays an important role in mediating pancreatic transformation downstream of KRAS. We have established that IKBKE engages in feedback regulation of the GLI pathway

via regulation of nuclear localization of GLI1. We have found IKBKE to play a modest role in activating NF- κ B in PDAC, and established IKBKE as a regulator of AKT phosphorylation, which plays an important role in mediating AKT reactivation post-mTOR inhibition. Furthermore, we have established that IKBKE mediates resistance to mTOR inhibition in the context of PDAC and provided proof of concept for combined targeting of IKBKE and mTOR in pancreatic cancer.

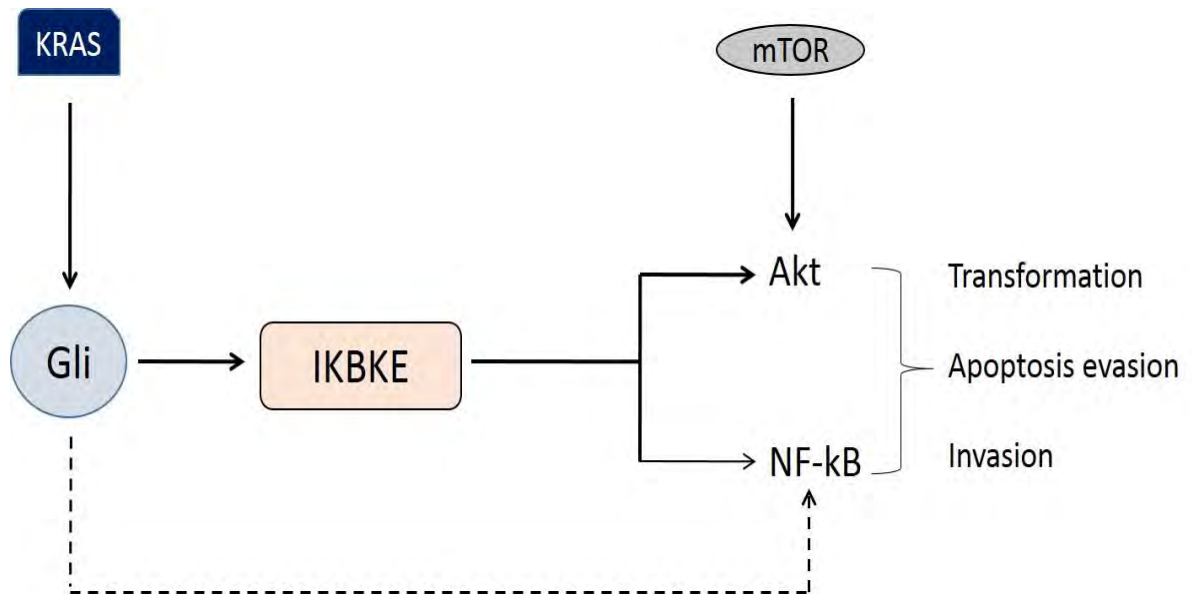


Figure 4.1: Gli-IKBKE signaling in pancreatic adenocarcinoma

GLI is activated downstream of oncogenic KRAS in the pancreatic tumor epithelium, and drives a transcriptional program that leads to AKT and NF-κB activation. IKBKE mediates GLI mediated activation of AKT, and partially mediates NF-κB activation downstream of Gli. IKBKE and mTOR play a crucial role in AKT activation, and IKBKE mediates AKT reactivation post-mTOR inhibition.

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