ONCOGENIC KRAS, GSK-3, NF-κB AND TBK1: THE INTERPLAY AND CONSEQUENCES IN PROMOTING PANCREATIC AND LUNG CANCER

Deepali Bang

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Approved by:

Albert Baldwin

Channing Der

Jenny Ting

Mohanish Deshmukh

Blossom Damania

Ben Major

ABSTRACT

Deepali Bang: Oncogenic KRAS, GSK-3, NF-κB and TBK1: the interplay and consequences in promoting pancreatic and lung cancer
(Under the direction of Dr. Albert Baldwin)

The development of KRAS targeted therapy has evolved into targeting the complex signaling pathways activated downstream of oncogenic KRAS and associated with disease progression. Recent focus has been made on targeting the constitutive activation of the transcription factor, NF-κB. However, advancement of this therapeutic strategy is dependent on fully understanding the elusive mechanisms underlying constitutive NF-κB activity in KRAS driven cancers. Glycogen synthase kinase-3 has previously been implicated in regulating pro-survival NF-κB signaling in pancreatic cancer cells through IκB kinase (IKK), but a distinction between roles of the individual isoforms, GSK-3α and GSK-β has not been done. Moreover, TGF-β activated kinase 1 (TAK1), an upstream regulator of IKK activity, was recently shown to regulate survival in KRAS positive colorectal cancers. Here, we characterize mutant KRAS dependent GSK-3α regulation of NF-κB in pancreatic cancer cells. Our data suggests that mutant KRAS upregulates GSK-3α, which promotes pro-survival canonical NF-κB via stabilization of TAK-TAB complex. We also provide initial evidence of GSK-3α dependent regulation of pro-survival non-canonical NF-κB in pancreatic cancer cells. Importantly, we show that pharmacological inhibition of GSK-3 suppresses growth of human pancreatic tumor explants with a concomitant loss of oncogenic gene expression like c-MYC and TERT. Collectively, we propose that GSK-3α regulates multiple arms of

NF-κB pathway in KRAS positive pancreatic cancer cells, identifying GSK-3α as promising therapeutic target. Recently, Tank Binding Kinase-1 (TBK1), an IKK-related kinase, has been implicated in survival of KRAS positive cancer cells. Here, we use a conditional TBK1 knockout mouse model with simultaneous activation of KRAS and inactivation of p53 in lung epithelial cells to provide initial evidence of a role of TBK1 in KRAS driven lung tumorigenesis. These data broadens our understanding of NF-κB regulation in KRAS malignancies and implicates GSK-3α, TAK1 and TBK1 as emerging therapeutic targets.

This work is dedicated to all my family- my father, who has been my mentor all along, my mother who has supported me all along, and my husband who has been my mentor, my friend and my support all along.

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LIST OF ABBREVIATIONS

ATP	Adenosine-5-‘triphosphate
Cre	Cre recombinase enzyme
DMSO	Dimetheyl sulphur oxide
EGFR	Epidermal growth factor receptor
FTI	Farnesyltransferase inhibitors
GAP	GTPase activating protein
GDP	Guanosine-5’diphosphate
GEF	Guanine nucleotide exchange factor
GEMM	Genetically engineered mouse models
GSK-3	Glycogen synthase kinase-3
GTP	Guanosine -5’triphosphate
ICMT	Isoprenylcysteine carboxylmethyltransferase
IKK	IκB kinase
IκB	Inhibitor of κB
MAP3K	Mitogen activated protein kinase kinase kinase
NAK	NFκB activating kinase
NF-κB	Nuclear kactor kappa B
NSCLC	Non-small cell lung cancer
PanIN	Pancreatic intraepithelial neoplasia
PDAC	Pancreatic ductal adenocarcinoma
PI3K	Phosphatidylinositol 3-kinase (PI3K)

RALGDS	Ral guanine nucleotide-disassociation stimulator
RCE1	RAS converting enzyme 1
TAB	TGFβ activated kinase binding protein
TAK1	TGFβ activated kinase 1
TANK	TRAF-associated NF-κB activator
TBK1	Tank binding kinase 1
TGF-β	Transforming growth factor beta
TNFR	TNF receptor
TNFα	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

1.1 Summary

KRAS is frequently mutated and drives oncogenesis in a variety of human cancers (1,2). Unsuccessful attempts at direct targeting of KRAS has led investigators to consider targeting the downstream effector pathways of KRAS. The constitutive activation of the transcription factor NF-κB is among the many deregulated signaling pathways downstream of oncogenic KRAS that has been associated with multiple malignancies, including pancreatic cancer and lung cancer. Consequently, there has been focus on targeting constitutive NF-κB activity in these cancers (3,4) (5). Advancement of this therapeutic strategy is dependent on fully understanding the complex signaling events that drive NF-κB activity. Recently Tank binding kinase-1 (TBK1), an IκB kinase (IKK) related kinase, which regulates NF-κB signaling, was shown to be important for KRAS-driven cancers (6,7). Here I provide new insight into elusive signaling events downstream of oncogenic KRAS that drive tumorigenesis in pancreatic and lung cancer and discuss glycogen synthase kinase-3α (GSK-3), and TGF-β activated kinase-1 (TAK1), and TBK1 as potential therapeutic targets for these cancers.

The following will provide a general overview of KRAS signaling, NF-κB signaling in cancer and evidence of regulation by GSK-3 and TAK1, and TBK1

signaling. Furthermore, I will review the functional significance of constitutive KRAS activity during oncogenesis and discuss the relevance to target this pathway in pancreatic and lung cancer.

1.2 RAS proteins

RAS proteins are proto-oncogenes that are frequently mutated in human cancers. The mammalian genome encodes three RAS genes that give rise to four ubiquitously expressed highly homologous proteins: HRAS (homologous to the oncogene of the Harvey murine sarcoma virus), KRAS- (homologous to oncogene of Kirsten murine sarcoma virus; K-RAS 4A and K-RAS 4B are splice variants of single gene) and NRAS (which does not have a retroviral homolog and was first isolated from a neuroblastoma cell lines) (8).

The ~21kDa RAS proteins cycle between inactive (GDP)-bound and active (GTP)-bound conformations and function as molecular switches that couple cell surface receptor activation to intracellular signaling pathways. (1). The activation of RAS proteins, that is, the exchange of GDP with GTP, is an intrinsically slow process and is catalyzed by guanine nucleotide exchange factors (GEFs). However, this exchange is reversible. The inactivation of RAS involves the hydrolysis of the γ-phosphate of GTP to GDP catalyzed by GTPase-activating proteins (GAPs) and is irreversible. This inactivation of RAS activity by GAPs is the predominant target of the most of the oncogenic mutations of RAS. The resulting oncogenic versions of RAS proteins are

resistant to GAP-mediated GTP hydrolysis, which renders them constitutively active. GTP- bound RAS can interact productively with more than 20 effectors, including Raf, phosphatidylinositol 3-kinase (PI3K) and Ral guanine nucleotide-dissociation stimulator (RALGDS), to regulate various cellular responses including proliferation, survival and differentiation (Figure 1.1).

Newly synthesized RAS is a cytosolic protein that undergoes posttranslational modifications, which helps localize RAS to the correct subcellular compartment — principally the inner face of the plasma membrane. These modifications include the covalent attachment of a non-sterol isoprenoid from farnesyl pyrophosphate (FPP) to a cysteine residue that is close to the carboxyl terminus by prenylation. Farnesyltransferase inhibitors (FTIs) block this farnesylation, so RAS remains in the cytosol and is unable to function. After prenylation an endopeptidase, named RAS converting enzyme 1(RCE1), removes the end three amino acids from the carboxyl terminus of the protein. The new carboxyl terminus is then methylated by isoprenylcysteine carboxylmethyltransferase (ICMT), followed by palmitoylation and transfer to the plasma membrane. KRAS4B attaches to the plasma membrane without palmitoylation(1).

The following section will review roles of RAS signaling in cancer with a focus on pancreatic and lung cancer.

1.3 Role of RAS in Cancer

Oncogenic roles of RAS family of GTPases are well established. Mutational activation of RAS occurs in over 30% of human cancer with pancreatic (90%), colon

(50%), thyroid (50%), lung (30%) and melanoma (25%) having the highest prevalence(8). Different tumor types have specific associations with individual RAS isoforms. For example KRAS, the most frequently mutated isoform in most cancers, is associated with pancreatic cancer, non-small cell lung cancer (NSCLC), colorectal cancer and seminoma. In contrast HRAS mutations are more strongly associated with tumors of the skin and of the head and neck and NRAS mutations are common in hematopoietic malignancies (9). All these mutations occur most frequently in codons 12, 13, or 61 (Figure 1.2) and stabilize RAS in a constitutively active GTP bound conformation (2). Moreover, several other human cancers harbor alteration in factors that lie upstream of RAS, leading to over expression or mutational activation of growth factor receptor tyrosine kinase, such as epidermal growth factor (EGFR and ERBB2). Alternatively factors downstream of RAS are also commonly mutated, such as mutation of B-RAF in melanoma and colon cancer (1).

Activated mutant RAS in cells can promote several of the characteristics of malignant transformation. These include increased proliferation due to upregulation of several transcription factors that are required for cell cycle entry and progression, including FOS, JUN, ELK1, ATF2 and NF-κB. These factors in lead to induction of cell cycle regulators such as cyclin D1, which leads to inactivation of the retinoblastoma (RB) pathway, and suppression of cell- cycle inhibitors such as KIP1 (9). As such, cyclin D1-deficient mice are resistant to developing mammary cancers and squamous tumors that are induced by the HRAS oncogene (10). In addition, RAS transformed cells become desensitized to apoptosis through PI3K and RAF signaling. Activated PI3K leads to downregulation of the pro-apoptotic protein BAK1, and augments IAP levels through the

activation of NF-κB (11). Oncogenic RAS-induced effector pathways also impinge on metabolic reprogramming of cancer cells, a known hallmark of cancer (12). The effect on cellular metabolism is exerted through concurrent activation of MAP3K and PI3K leading to upregulation of HIF1α (13), and through upregulation of autophagy(14,15), both of which stimulate a glycolytic shift. RAS effector pathway also lead to the induction of angiogenesis, mainly by means of ERK mediated transcriptional upregulation of angiogenic factors (like VEGF), matrix metallo proteinases (16). Oncogenic RAS can also subvert antitumor immunity by down-regulating expression of antigen-presenting major histocompatibility complexes (MHC) on cancer cells(17), and through the recruitment of immunosuppressive regulatory T cells (TRegs) and myeloid-derived suppressor cells (MDSCs) to the tumor site(18,19). Finally RAS -dependent signaling pathways including the RAS–MAPK, RAS–PI3K, RAS–RAL GTPase and RAS–RHO GTPase have been demonstrated to have an essential role in metastatic progression (9). Targeting RAS and its effector pathway could, therefore, have a potential impact on almost all aspects of malignancy.

The next two sections review the role of KRAS in driving two of the most difficult to treat cancers: Pancreatic and lung cancer.

1.3.1 Role of KRAS in pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is the most common malignancy of the pancreas and is the fourth leading cause of cancer-related deaths in the United States (20). *KRAS*, *is* invariantly among the most frequently mutated genes associated with human pancreatic cancers. Annually, the number of victims of the disease approaches the

number of new diagnoses. Approximately 46,420 new cases of pancreatic cancer will be expected in 2014, while 39,590 patients are estimated to die from this disease(20). The nucleoside analog, gemcitabine, is among the most active single-agent chemotherapy currently used to treat advanced stages of pancreatic cancer and is considered to be the first-line therapy for patients (21). Lack of efficient early-detection methods, aggressive metastatic potential, and chemotherapy resistance all account for the dismal mortality rate (a 5-year survival rate of 6% is associated with pancreatic cancer (20). Therefore, it is critical to advance our understanding of the molecular pathogenesis of pancreatic cancer in order to develop effective targeted chemotherapies to treat this disease.

Extensive histological and genetic studies have characterized the pathogenesis of pancreatic cancer into three early stages of pre-invasive lesions known as pancreatic intraepithelial neoplasia (PanIN) (Fig. 1.3) (22). Recent tumor genome sequencing studies have established the prevalence of mutant KRAS in PanINs, (23), and in pancreatic cancer(24,25) with increased precision. The most common mutation in *KRAS* is one amino-acid substitution in position 12 of the KRAS protein, leading to a glycine (G) to aspartic acid (D) substitution, although other variants, such as G to V are also common (Table 1.1) (26-29). Malignant progression from PanINs to invasive and metastatic disease is accompanied by the early acquisition of activating mutations in the KRAS oncogene, which occurs in >90% of cases, and subsequent loss of tumor suppressors including INK4A/ARF, TP53, and SMAD4 (30). Genetically engineered mouse models (GEMM) have provided evidence for oncogenic KRAS (KRASG12D) as a major driver in PDAC initiation and maintenance, with the aforementioned tumor suppressor genes constraining progression (31-33) (34,35).

1.3.2 Role of KRAS in lung cancer

Lung cancer is the leading cause of cancer-related deaths worldwide (20). The two major forms of lung cancer are non-small cell lung cancer (about 85% of all lung cancers) and small-cell lung cancer (about 15%). Non-small cell lung cancer (NSCLC) has amongst the worst prognoses of all human malignancies. Non-small cell lung cancer can be divided into three major histologic subtypes: squamous-cell carcinoma, adenocarcinoma, and large-cell lung cancer; adenocarcinoma is the most common type accounting for >40% of all lung cancers (36). Analysis of resected tumors has revealed several frequent molecular changes: KRAS is mutated to an activated form in 30% of NSCLCs, EGFR is up-regulated in 10%–40%, the p53 tumor suppressor gene is mutated or deleted in 50%, and the INK4A/ARF locus is often deleted or hypermethylated (Table 2).

KRAS mutations are also observed in a substantial number of sporadic and chemically induced lung adenocarcinomas in mice (37-39). Further evidence for the role of KRAS in oncogenesis of lung cancer comes from mouse studies where expression of mutant KRAS is sufficient to cause transformation and development of adenocarcinoma (40,41) .

Most KRAS mutations in lung adenocarcinoma are G→T transversions (substitutions of a purine for a pyrimidine) and affect exon 12 (in 90% of patients) or exon 13. As seen in pancreatic cancers, KRAS mutations appear to be an early event in lung cancers (e.g., detectable in the preinvasive lesions of atypical adenomatous hyperplasia and bronchoalveolar carcinoma) that precedes lung adenocarcinoma.

Importantly, activating point mutations in KRAS are associated with poor prognosis and therapy resistance (42,43).

Taken together, the role of oncogenic KRAS in initiating and driving pancreatic and lung adenocarcinomas is well established. Thus targeting RAS and its effector pathway has been a focus for cancer biologists seeking to develop rational anti-cancer drugs as explained in the next section.

1.4 Targeting RAS as a therapeutic approach to cancer

For RAS as a therapeutic target, post-translational modifications, known to be required for RAS biological activity has been a very attractive target(44). In this regard, Farnesyl transferase that catalyse attachment of farnesyl isoprenoid group to RAS proteins were an obvious target. Number of highly effective Farnesyl transferase inhibitors (FTIs) have been identified that inhibit cell growth of a large variety of cancer cell lines *in vitro* and *in vivo* as tumor xenografts (45,46). However, KRAS, the most frequently mutated RAS oncogene in human cancers, can undergo alternative prenylation by GGTase I in FTI-treated cells, resulting in a persistent membrane localization of KRAS and concomitant upregulation of downstream signaling. Inhibition of the two post prenylated enzymes RCE1 and ICMT has also met little success (47,48). While blocking RCEI activity in tumor cells or mouse models has shown only moderate effects, inhibition of ICMT affects multiple pathways besides RAS leading to toxic effects like atherosclerotic vascular injury (8,46).

Taken together, strategies that compromise RAS post-translational processing

have, for the most part, not been rewarding. The idea of preventing RAS expression by antisense or RNA interference seems promising, but the successful application of this technology is currently limited by lack of efficient delivery, uptake, and gene silencing. Additionally the observation that tumor cells expressing mesenchymal markers are relatively independent on the presence of RAS oncogene has raised questions on the potential of elimination of RAS expression as an effective cancer therapy (49).

Collectively, developing therapeutic agents to directly block oncogenic RAS activity has been a challenging and unsuccessful endeavor, for reasons discussed above. Therefore, a great deal of effort has been applied to developing therapies that target effector pathways downstream of RAS (Figure 1.1). Understanding which effector pathways are required for RAS-driven oncogenesis is critical for determining which pathways should be targeted for therapeutic purposes. As a result, new insight has emerged in comprehending the network of deregulated signal transduction pathways downstream of oncogenic KRAS. Most RAS effector pathways are comprised of kinase cascades, providing multiple nodes for potential therapeutic intervention. Several RAS effectors have been identified and comprehensively described, including RAF-MEK-ERK and PI3K signaling (1,8,46). In the case of lung and pancreatic cancers, constitutive signaling from PI3K/AKT, EGFR, and RAS/RAF/MAPK pathways have all been implicated to play key roles in the development and progression of these cancers (42) (50). Interestingly, these deregulated pathways utilize molecular crosstalk with the transcription factor NF-κB (50). Thus, focus has been made on targeting constitutive NF-κB signaling as an alternative therapeutic strategy to treat these adenocarcinomas (4,5,51-

53). However, advancement of this strategy is dependent on fully understanding the signaling events that drive constitutive NF-κB activation.

1.5 Constitutive NF-κB in Cancer

NF-κB was originally discovered over 25 years ago as a nuclear factor that binds the immunoglobulin κ enhancer sequence in B-cells(54). NF-κB has since been characterized as an inducible family of conserved dimeric transcription factors consisting of: RelA/p65, RelB, c-Rel, p52 (p100 precursor), and p50 (p105 precursor) (Fig. 1.4). The N-terminal region of NF-κB consists of a highly conserved, 300 amino-acid, Rel homology domain (RHD) which promotes dimerization, nuclear localization, and DNA binding. A C-terminal transactivational domain (TAD) is located within RelA/p65, RelB, and c-Rel and facilitates NF-κB transcriptional activity upon inducible posttranslational modifications. The C-terminal region of the precursor NF-κB members (p100 and p105) contains an inhibitor ankryin repeat domain which can undergo proteolytic cleavage, to generate the active p52 and p50 subunits. Although processing of p105 to p50 is known to be constitutive, p100 to p52 processing is tightly regulated (55).

The inducible activity of NF-κB plays a central role in the regulation of diverse biological processes such as inflammation, immunity, development, cell proliferation, and survival (56). The NF-κB target genes that drive these biological processes include cytokines (i.e. IL-6, IL-12 TNF-α, and LTβ), chemokines (i.e. MCP1, IL-8, RANTES, and MIP-1α), and adhesion molecules (i.e. ICAM and VCAM)(56).

In resting cells, NF-κB is rendered inactive and sequestered in the cytoplasm through interaction with an inhibitor protein called IκB. NF-κB is typically activated through one of two distinct pathways. Canonical NF-κB activity can be triggered by various stimuli including inflammatory cytokines, microbial infection, DNA damage, and mitogens(57). These stimuli promote the activation of the IκB kinase (IKK) complex which consists of a regulatory subunit (IKKγ) and two catalytic, serine/ threonine kinase subunits (IKKα and IKKβ). Once this complex is activated, IKKβ phosphorylates NF-κB-bound IκB proteins which leads to its rapid ubiquitination and subsequent proteosomal degradation (58,59). Consequently, NF-κB dimers can undergo nuclear translocation and bind to κB consensus sequences within target gene promoters (Fig. 1.5).

In the non-canonical NF-κB signal transduction, diverse stimuli such as B-cell activating factor (BAFF) and CD40 ligand, trigger a signaling cascade which activates the NF-κB inducing kinase (NIK) (55). NIK drives the activation of IKKα homodimers which subsequently phosphorylates p100, leading to its proteolytic processing and the generation of p52 (Fig. 1.5) (60). As a result, activated RelB/p52 heterodimers can translocate to the nucleus to drive NF-κB-dependent gene transcription.

Deregulated NF-κB activity has been linked to chronic inflammatory diseases (i.e. rheumatoid arthritis and lupus) and tumorgenesis (61). In cancer NF-κB is known to promote angiogenesis, metastasis, cell survival, cell cycle progression, and insensitivity to growth inhibition- which are all well described hallmarks of cancer (12).Constitutive NF-κB activation can be observed in a wide range of malignancies including those of

breast, colon, lung cancer, melanoma, multiple myeloma, esophageal adenocarcinma, and pancreas (62) (63). NF-κB is often thought to serve as the bridge between inflammation and cancer (63). In colitis-associated cancer, deletion of IKKβ inhibited transcription of proinflammatory cytokines and reduced tumor formation in vivo (64).

Due to the role of NF-κB in tumorigenesis, NF-κB is an attractive target for cancer therapeutics. Several IKKβ inhibitors are available to determine the efficacy of IKK/NF-κB inhibition in various types of cancer. For example, treatment of lung tumor cells ex vivo with an IKKβ inhibitor reduced NF-κB activation (5), and treatment of animals with mutant KRAS-driven lung tumors resulted in tumor regression and prolonged survival (65). However, IKKβ inhibition also leads to lymphocyte toxicity (66) and granulocytosis (67) in healthy animals. Further studies are required to determine the effects of IKKβ inhibition in other *in vivo* cancer models, and whether the benefits outweigh the side effects.

1.5.1 RAS and NF-κB

Studies from our group revealed that oncogenic H-RAS induces cellular transformation in fibroblasts by activating NF-κB (68), and NF-κB activation is required to suppress oncogenic H-RAS–induced apoptosis(69). Other studies have shown a correlation between increased NF-κB activity and expression of oncogenic K-RAS (70,71). Duran and colleagues (72) showed a mechanistic link between K-RAS, IKK, and NF-κB through the signaling adaptor p62. Meylan et al. (53) demonstrated that inhibition of NF-κB signaling led to an apoptotic response in p53-null lung cancer cell lines, while inhibition of the pathway *in vivo* in the context of $KRAS^{G12D}$–driven lung tumorigenesis

showed reduced tumor development both at the time of tumor initiation and after tumor progression. Additionally, our group (5) showed that deletion of NF-κB subunit p65/RelA reduced the number of KRAS– induced lung tumors both in the presence and absence of p53, and the tumors that emerged in the absence of p65/RelA showed a higher number of apoptotic cells, reduced spread, and showed lower grade. In KRAS-driven pancreatic cancers, both canonical and non-canonical NF-κB have been found to be constitutively active and promote survival and chemoresistance (51,73-78). Recently knockout of IKKβ was shown to suppress tumor growth/progression in a *KRAS/INK4a* null animal model of pancreatic cancer (79). Oncogenic RAS also induces inflammatory cytokines that activate NF-κB and STAT3. For example, KRAS-driven non-small cell lung cancers (NSCLC) (7) and pancreatic ductal adenocarcinomas (PDAC) (8) engage cell autonomous IL-1 signaling. Recently IKK related kinases, TBK1 and IKKe have also been implicated in promoting survival of KRAS-driven tumors (6,80).

Collectively, these reports and others underscore the need to target IKK and NF-κB as a chemotherapeutic strategy in KRAS-driven cancers. The following sections will discuss the regulation of NF-κB by two versatile serine theronine kinases, transforming growth factor β-activated kinase 1 (TAK1) and glycogen synthase kinse-3 (GSK-3).

1.6 TAK1-dependent regulation of NF-κB

TAK1 belongs to the evolutionarily conserved mitogen-activated protein kinase kinase kinase (MAP3K) family and was originally discovered to function within the transforming growth factor-β (TGF-β) signaling pathway(81). In addition to the TGF-

β ligand family, this versatile serine/theronine kinase can be activated by a diverse set of stimuli such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and lipopolysaccharide (LPS) (82,83). Upon receptor activation, TAK1 can initiate individual downstream signaling cascades culminating in the activation of IKK and MAPKs (JNK and p38) (83,84). TAK1 plays a central role in NF-κB signaling through direct phosphorylation of IKKβ (85), and promotes expression of various cellular stress and inflammatory-response genes.

TAK1 activity is dependent on various phosphorylation events and interaction with essential signaling adapters called TAK1 binding proteins (TAB1, TAB2, TAB3 and TAB4) (Fig. 1.6) which facilitate autophoshorylation at Thr178, Thr184 and Ser 192 following stimulation (86-88). Notably, TAB proteins have been demonstrated to play a crucial role in facilitating TNF-α and IL-1β-dependent activation of NF-κB and MAPK pathways (87,89).

Deregulated TAK1 signal transduction has been associated with constitutive NF-κB activity in human cancer cell lines (90,91). TAK1 was shown to promote cell proliferation in human head and neck squamous cell carcinnoma (HNSCC) cell lines by mediating constitutive NF-κB activity (90). Additionally, TLR2 overexpression was reported to promotes constitutive TAK1/ NF-κB signaling and cell invasion in MDA-MB-231 cells (91). Recently TAK1 has been shown to be important for survival of KRAS-dependent colorectal cancers and to play an important role in the chemoresistance of pancreatic cancers (92,93).Taken together, TAK1 plays a key role in cross regulating the complex pathways leading to NF-κB activity in human cancers, and could thus, be considered a novel therapeutic target.

1.7 GSK-3-dependent NF-κB Signal Transduction

Glycogen synthase kinase-3 (GSK-3) was discovered 25 years ago as a serine/threonine kinase involved in the downregulation of glycogen biosynthesis through the phosphorylation and inactivation of glycogen synthase (94). There are two mammalian isoforms, GSK-3α and GSK-3β, that are encoded by two independent genes and share 85% overall sequence identity and 93% similarity within the catalytic domain (95,96) (97). A significant difference between GSK-3 isoforms is observed in the N-terminal region where GSK-3α possesses an extended glycine-rich tail (Fig. 1.7). Although purified GSK-3 isoforms exhibit similar substrate properties (96), evidence from GSK-3α and GSK-3β deficient mouse models suggest non-overlapping substrate specificities10. GSK-3α deficient mice exhibit enhance glucose and insulin sensitivity(98), while GSK-3β deficiency is embryonic lethal at day E13.5 due to liver degeneration (99).

Unlike most protein kinases, GSK-3 is constitutively active in resting cells and is primarily regulated by various upstream signaling events that lead to the phosphorylation and inactivation of GSK-3 (Fig. 1.8). AKT promotes the serine phosphorylation (S9/21) and inactivation of both GSK-3 isoforms downstream of PI3K (100). GSK-3 is most recognized for its role in Wnt/β-catening pathway where its leads to β-catenin phosphorylation and subsequent proteosomal degradation. Canonical Wnt/ β-catenin signaling is an evolutionarily conserved pathway which plays a key role during development by regulating cell growth, differentiation, and polarity (97).

GSK-3 is a multi-tasking kinase that plays an intricate role in diverse signaling pathways. As a consequence, GSK-3 is known to have an expanded range of substrate specificities (Fig. 1.8). The consensus motif for GSK-3 substrates is Ser/Thr-X-X-X-Ser/Thr (X represents any amino acid). GSK-3 has the unique ability of targeting substrates that have previously phosphorylated (primed) at the C-terminal Ser/Thr residues of the consensus motif (101). Interestingly, there has been an increasing body of literature implicating the role GSK-3 plays in cross regulating the activity of NF-κB.

The first evidence of GSK-3-dependent regulation of NF-κB was provided by James Woodgett and collegues (99). Interestingly, embryonic lethality was observed in GSK-3β homozygous null mice between E13.5 and E14.5 days due to TNF-α-dependent hepatocytes apoptosis. Notably, this phenotype is consistent with the homozygous deletion of the p65 subunit of NF-κB (102) and IKKβ (103). To demonstrate GSK-3β-dependent NF-κB regulation, mouse embryonic fibroblasts from GSK-3β-deficient mice were also shown to be defective in TNF-α-induced NF-κB activation (99).

The exact mechanism of how GSK-3β regulates inducible NF-κB activity remains controversial. Reports have implicated GSK-3β to either regulate early stages of NF-κB activation (IKK complex activation and IκB degradation) or later stages (NF-κB transcription activity at target gene promoters). Bharat Aggarwal and colleagues reported that GSK-3β null MEFs are defective in inducing IKK activity and IκBα phosphorylation/ degradation in response to TNF-α, IL-1β, and lipopolysaccharide (104). In contrast with this report, work from our lab and others have suggested that GSK-3β deficiency, as well as, pharmacological GSK-3 inhibition diminishes cytokine-induced NF-κB activity at the transcriptional complex level (99,105,106). We also reported the

role of GSK-3 in regulating IKK-driven NF-κB activity in pancreatic cancer cells (75). Further evidence showed that GSK-3β can directly target the phosphorylation of p65 (amino acids 354-551), thus promoting NF-κB transactivation (106). Overall, GSK-3β plays an essential role in cross regulating cytokine-induced NF-κB activation through either an IKK-dependent or independent mechanism. Due to the known pro-survival functions of NF-κB, studies have also focused on the potential role GSK-3 may play during carcinogenesis.

1.7.1 GSK-3 Inhibitors as a Targeted Therapy for Cancer

GSK-3 has been linked to deregulated signaling pathways leading to the pathogenesis of various human diseases. Consequently, GSK-3 has emerged over the years as a therapeutic target for Alzheimer's disease, bipolar disorder, dementia, and noninsulin-dependent diabetes mellitus (NIDDM) (107). Moreover, there is increasing evidence linking aberrant GSK-3 signaling with the development of cancer. GSK-3 is generally considered a tumor suppressor due to its role in suppressing the oncogenic effects of the Wnt/ β-catenin pathway in colorectal cancer (108). However, this paradigm has been challenged by recent reports that suggest GSK-3 plays an oncogenic role by facilitating cell growth, survival, and constitutive NF-κB activity. Advancement of these studies was made possible by the development of pharmacological GSK-3 inhibitors.

Lithium was the first tool discovered used to inhibit GSK-3 activity (109). However, the broad kinase specificity range of lithium has prompted pharmaceutical companies to develop small molecule inhibitors with increased specificity for GSK-

3(110). SB 216763 (ATP-competitive, IC_{50} = 34.3 nM), SB 415286 (ATP-competitive, IC_{50} = 77.5 nM), AR-A014418 (ATP-competitive, IC_{50} = 104 nM), and TDZD-8 (non-ATP-competitive, IC_{50} = 2μM) are among the most recently developed GSK-3 inhibitors that have been implicated to have therapeutic potential in human cancers (111-113). Notably, these inhibitors do not provide significant selectivity between GSK-3α and GSK-3β isoforms due to sequence identity within the kinase domain.

Studies within the past five years have implicated GSK-3 inhibitors as a potential targeted therapy for prostate cancer, multiple myeloma, leukemia, and pancreatic cancer(114-117). Although the mechanism of action remains unclear, GSK-3 inhibitors may function partially by suppressing constitutive, pro-survival signaling to NF-κB. Our lab and others have provided evidence of GSK-3-dependent constitutive pro-survival IKK-NF-κB activity in pancreatic cancer cell lines (75,118,119). These reports demonstrated that GSK-3 is active in pancreatic cancer cells, and that GSK-3 inhibition suppresses constitutive NF-κB reporter activation and target gene expression. Furthermore, the suppression of constitutive NF-κB activity following GSK-3 inhibition correlated with decreased cell growth/ survival in both *in-vitro* (75,118) and *in-vivo* models (119). All the above studies either focused on GSK-3β or did not distinguish between the two isoforms, GSK-3α and GSK-3β, however, a recent study implicated GSK-3α in promoting acute myelogenous leukemia (120). Taken together, these reports establish GSK-3 inhibition as a potential therapeutic strategy for cancer but needs further investigation to dissect the contribution of the individual isoforms in various malignancies.

1.8 Role of non-canonical IKK, TBK1 in cancer

In addition to the conventional IKKs, a related pair of non-canonical kinases, IKKε (IKKi, encoded by *IKBKE*) and TBK1 (NAK), have been identified as important mediators of both inflammatory and oncogenic signaling. TBK1 was identified as an interaction partner with the scaffolding molecule, TRAF-associated NF-κB activator (TANK) (121). It comprises of an N-terminal kinase domain, an ubiquitin- like domain, a C-terminal LZ and a HLH motif (Fig. 1.8) and is a critical inducer of interferon signaling in response to viral infection (80). TBK1 can be activated in a TLR dependent or independent manner by viral components or ds RNA/DNA respectively, leading to phosphorylation of interferon regulatory factors (IRFs) 3, 5 and 7. This allows for heterodimerization and nuclear translocation of the IRFs and induction of pro-inflammatory and antiviral genes, *IFNα/β.*

TBK1 is highly expressed in lung, breast and colon cancers and a TBK1 mutation, P675L, was recently identified in lung adenocarcinoma (6). In regards to the role of TBK1 downstream of oncogenic KRAS, the RAS-like RalB mediated activation of TBK1 was shown to promote TBK1 assembly with the exocyst complex, where it facilitates transformation through the phosphorylation of the secretory protein Sec5. Importantly, suppression of either TBK1 or Sec5 induced apoptosis in RAS-transformed cells, and expression of oncogenic alleles of KRAS induced cell death in TBK1-deficient murine embryonic fibroblasts (122). Additionally TBK1 was found to be important for AKT signaling, an important downstream effector of KRAS, through direct phosphorylation of AKT (123). TBK1 has been shown to sustain KRAS-dependent cancer cell viability through regulation of NF-κB subunit c-Rel (6)and promote tumor survival in murine lung

cancer by activating autocrine cytokine signaling of CCL5 and IL-6 (7). Moreover, TBK-1 dependent non-canonical NF-κB signaling has been observed to contribute to autophagy addiction in KRAS driven NSCLC (124).

To sum it all, the above results identify TBK1 as a novel and effective target downstream of oncogenic RAS highlighting the need to further investigate the functions of TBK1.

1.8.1 Mouse Models of TBK1

Studies of TBK1 function have been hindered by the fact that homozygous deletion of TBK1 is lethal *in utero* at approximately embryonic day 14.5 as a result of massive liver degeneration and apoptosis, a phenotype similar to knockout of p65(125). This embryonic lethality can be rescued by breeding these animals onto a $TNF^{-/-}$ or $TNFR1^{-/-}$ background and thus most studies in which TBK1 deficient mice were used, such animals were $TBK1^{-/-}$ $TNF^{-/-}$ or $TBK1^{-/-}$ $TNFR1^{-/-}$ double knockouts. Recently a conditional $TBK1^{fl/fl}$ model was crossed with B-cell specific Cre recombinase to generate *TBK1*-BKO mice heterozygous for the Cre gene, and with B-cell specific ablation of TBK1(126). Using these mice, TBK1 was shown to negatively regulate non-canonical NF-κB signaling by phosphorylating NIK leading to its degradation. In another study, a conditional $TBK1^{fl/fl}$ was crossed with a line of transgenic mice that carry the Prml-Cre recombinase transgene. This transgene caused conversion of the $TBK1^{fl}$ allele to $TBK1^{\Delta}$ in the sperm of male mice by Cre-mediated deletion of exon 2. $TBK1^{\Delta}$ allele expressed low amounts of an inactive and much smaller in size TBK1 protein and mice homozygous for this allele ($TBK1^{\Delta/\Delta}$) were found to be viable on a 129S5 background

but not C57BL6 (127). Thus a true TBK1 deficient mouse model is still not available to establish the role of TBK1 in KRAS driven oncogenesis *in vivo*.

1.9 Conclusions

Despite continuous research efforts, tumors harboring KRAS mutations, such as pancreatic and non-small cell lung adenocarcinomas, remain the most difficult to treat and lack any effective targeted therapies. The evolution of pancreatic and lung cancer chemotherapy is dependent on the development of agents that specifically target deregulated molecular pathways downstream of mutations associated with disease progression (128). Pancreatic and lung cancers are among many human malignancies that utilize constitutive NF-κB signaling. GSK-3 has been demonstrated to regulate constitutive IKK and the subsequent pro-survival NF-κB activity in pancreatic cancer cells, while the IKK related kinase TBK1 has been implicated in the survival of lung cancer cells through c-Rel. Therefore, chemotherapeutic agents that target GSK-3 and TBK1 activity may be efficacious in the treatment of these malignancies. Advancement of this therapeutic strategy requires full understanding of the complex molecular mechanisms that regulate constitutive GSK-3-IKK-NF-κB activity in pancreatic cancer cell and TBK1-cRel activity in lung cancer cells.

This chapter reviewed the critical roles GSK-3, TAK1 and TBK1 play in promoting constitutive NF-κB activity in human cancers. Importantly, GSK-3 (75,118,119) have been reported to drive constitutive NF-κB activity and cell growth/ survival in pancreatic cancer. Moreover, deregulated TAK1 signaling has been

associated with oncogenic KRAS in colorectal cancer (92) and may also be linked to constitutive IKK and NF-κB activity and chemoresistance in pancreatic cancer (93). Despite the evidence of GSK-3-dependent NF-κB activation in pancreatic cancer, the mechanism of regulation and a clear distinction between the role of two isoforms, GSK-3α and GSK-3β remains elusive. Similarly, though TBK1 has been implicated in lung cancer, the effect of TBK1 deletion on KRAS driven lung cancers *in situ* is undetermined.

The remaining chapters will characterize the requirements of GSK-3 isoforms (GSK-3α and GSK-3β), and TAK1 for maintaining constitutive NF-κB activity and cell growth/ survival in pancreatic cancer cells. Furthermore, I will investigate the potential link between these signaling components, and propose that constitutive canonical and non-canonical NF-κB activity is driven by a unique GSK-3α- signaling cascade. Collectively, these studies emphasize the significance of NF-κB signaling in pancreatic cancer, and highlight the potential of GSK-3α and TAK1 as novel therapeutic targets for this disease.

Finally, I will the first true TBK1 conditional knockout mouse and use it to investigate and propose the negative effect of TBK1 inhibition on the development of lung adenocarcinomas in mice.

FIGURES

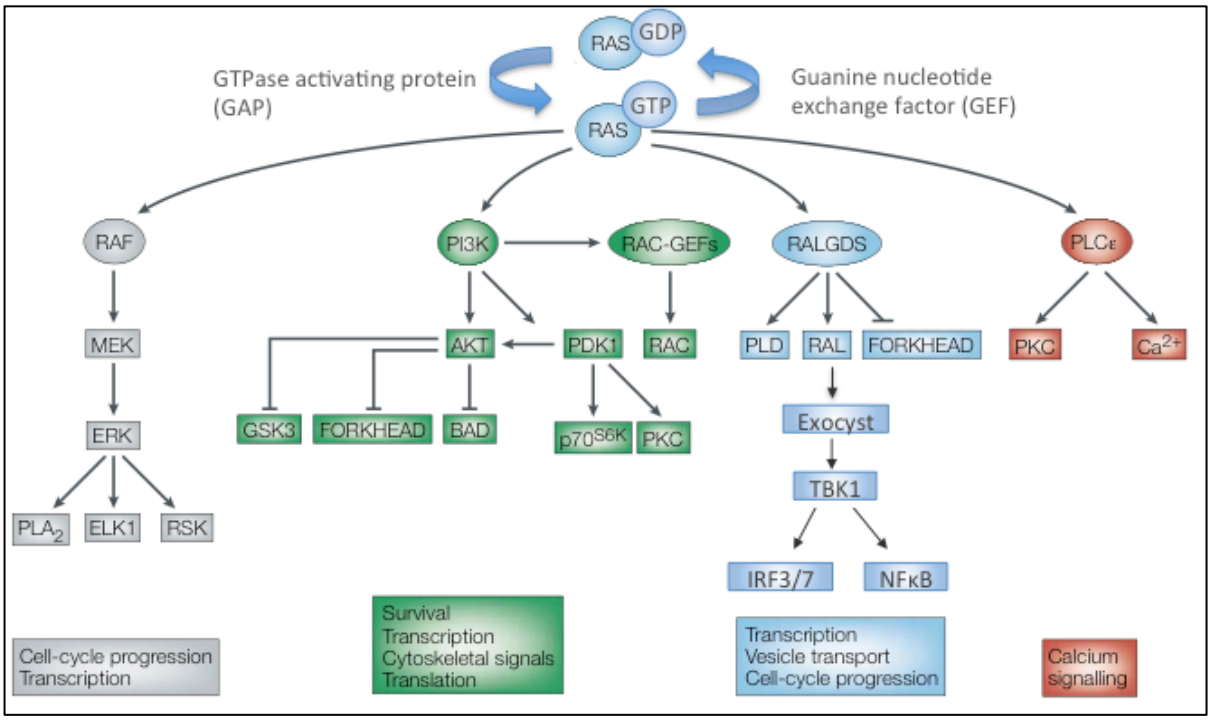

Figure 1.1 RAS signaling in an active GTP bound state, RAS interacts with several families of effector protein. RAS activate Raf protein kinase that initiate MAP kinase cascade which leads to ERK activation. ERK has numerous substrate like ELK1 that regulate cell cycle progression. RAS also activates PI3Ks which activate numerous target protein including cell survival signaling kinase. PI3K also activates the Rac-Rho pathway. Another pathway is RAS related protein, which activates the cell cycle progression. Phospholipase Cε is activated by RAS which leads to activation of Ca signalling pathway. Adapted from Downward J, Targeting Ras signaling pathways in cancer therapy, Nature Reviews (3) 2003:11-22

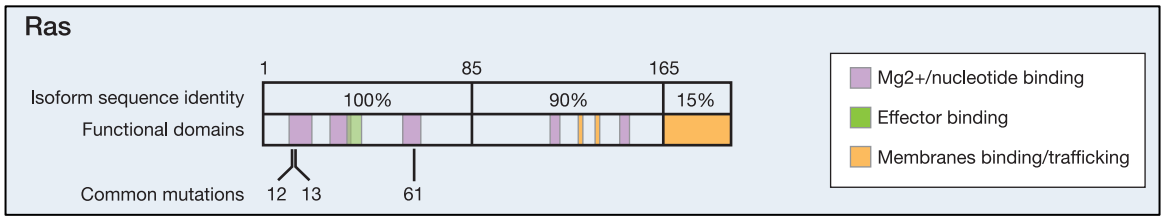

Figure 1.2 Oncogenic mutations of RAS: The key oncogenic mutations are in the region that is identical among the 3 isoforms. Of the forty-four separate point mutations characterized for RAS isoforms, 99.2% occur at codons 12, 13 and 61. (2)

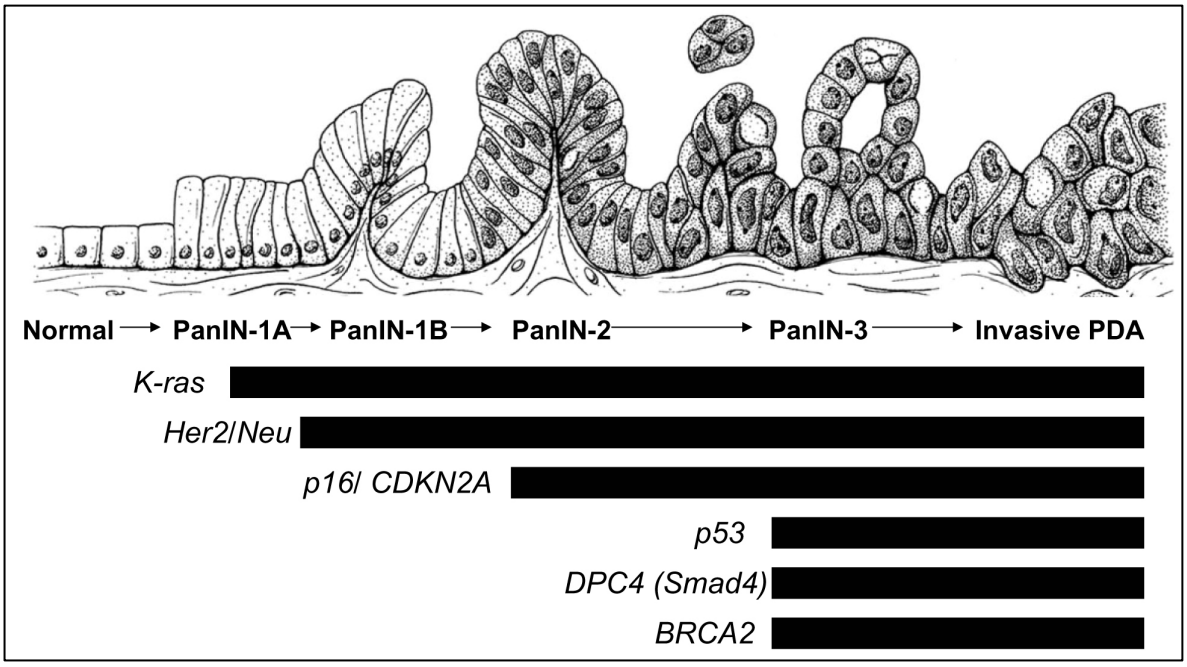

Figure 1.3. Accumulation of genetic mutations during pancreatic carcinogenesis. Pancreatic cancer progression is characterized by three stages of pancreatic intraepithelial neoplasia (PanIN-1A/B, PanIN-2, and PanIN-3) followed by invasive pancreatic ductal adenocarcinoma (PDA). The above "PanINgram" illustrates increasing degree of cytoplasmic and nuclei atypia used to classify each PanIN lesion (Maitra, Adsay et al. 2003). As indicated, genetic alterations in onocogenes (K-RAS and Her2/ Neu) and tumor suppressors (p16, p53, DPC4, and BRCA2) arise during distinct stages during PanIN progression. (Adapted from Wilson, W, The Regulation of constitutive NF-κB activity by GSK-3 in pancreatic cancer cells, 2008) Adapted with permission.

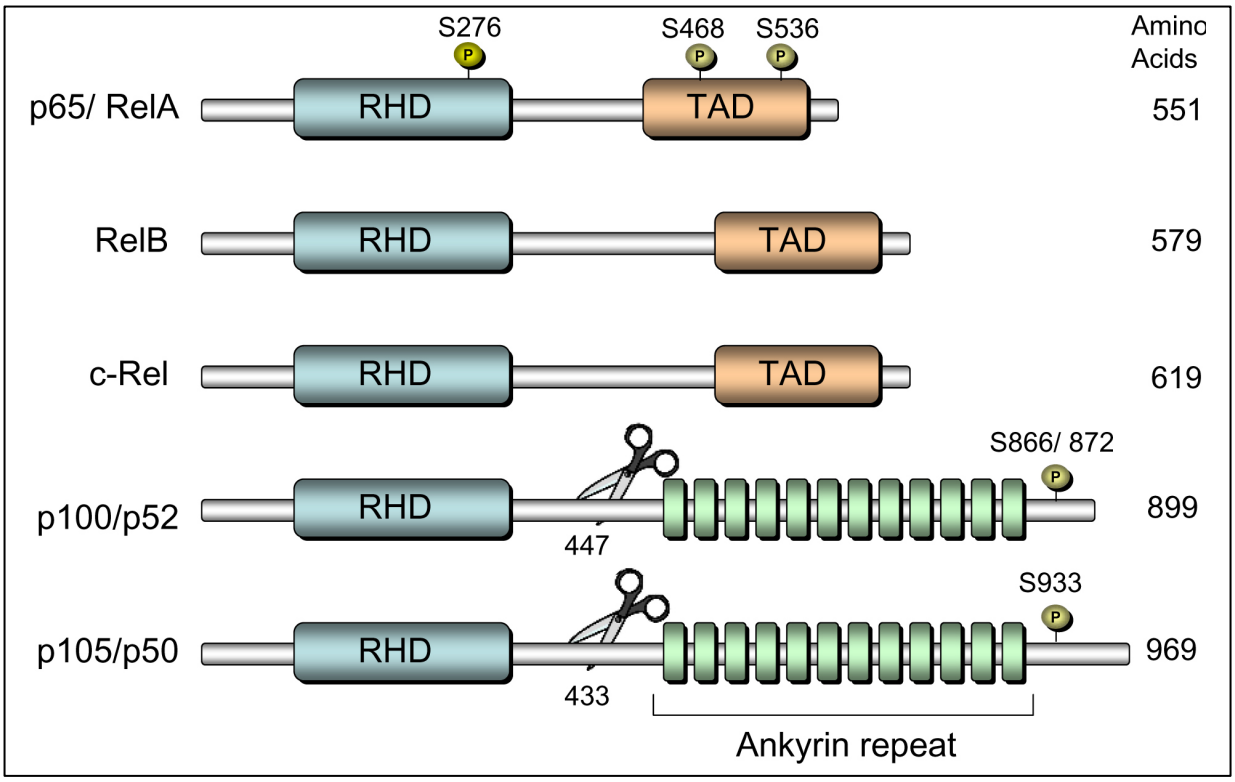

Figure 1.4. Domain organization of NF-κB family members. The evolutionarily conserved NF-κB family members indicated above exists as homo- or heterodimers. The N-terminal rel homology domain (RHD) mediates DNA binding and coupling with NF-κB family members. NF-κB transcriptional activity is facilitated by the C-terminal transactivational domain (TAD). The phosphorylation of p65 at the indicated sites has been shown to promote transcriptional activity. p100 and p105 exist as precursors consisting of an inhibitory ankyrin repeat domain which masks nuclear localization sequence within the RHD of p65, RelB, and c-Rel. Upon phosphorylation, p100 and p105 undergo proteolytic cleavage giving rise to mature p52 and p50 respectively. (Adapted from Wilson, W, The Regulation of constitutive NF-κB activity by GSK-3 in pancreatic cancer cells, 2008) Adapted with permission.

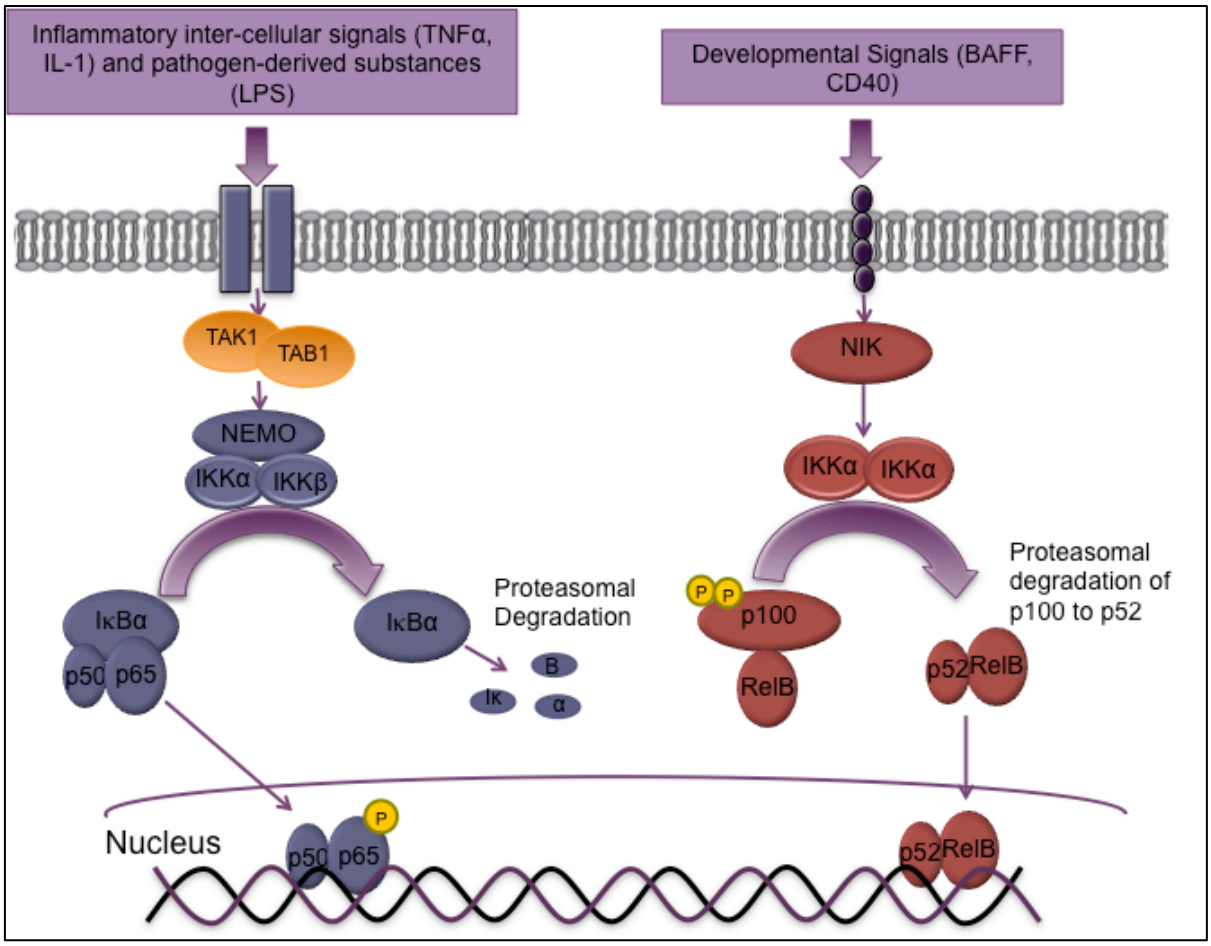

Figure 1.5. Canonical versus non-canonical NF-κB signal transduction. (Left) Canonical NF-κB signaling can be triggered by the indicated stimuli which converge on the activation of the IKK complex (IKKα, IKKβ, and IKKγ). Upon activation, IKK phosphorylates NF-κB-bound, IκBα at serines 32/ 36. Consequently, IκBα undergoes rapid ubiquitination and subsequent proteosomal degradation. Liberated NF-κB can undergo nuclear translocation and activate target gene expression. (Right) Non-canonical NF-κB signaling can be initiated by the indicated stimuli which activate downstream NIK and subsequent IKKα homodimers. IKKα phosphorylates p100/ RelB heterodimers at serines 866/ 872. As a result, p100 undergoes proteolytic cleavage giving rise to an active p52/ RelB heterodimer.

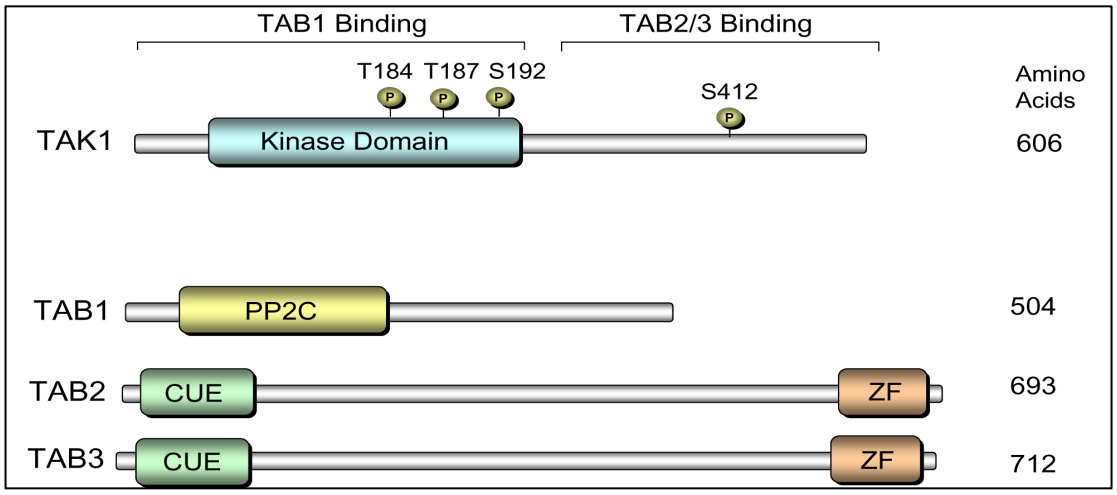

Figure 1.6. Domain organization of TAK1 and TAB proteins. TAK1 consists of an N-terminal kinase domain which undergoes autophosphorylation at the indicated serine/ threonine residues upon activation. TAK1 exist in complex with TAK1-binding proteins (TAB1, TAB2, TAB3). TAB1 interacts with N-terminal region of TAK1, while TAB2/3 interacts at the C-terminal region. TAB1 consists of an uncharacterized, N-terminal protein phosphatase 2C (PP2C) domain. TAB2/3 contains CUE and zinc-finger domains which facilitates interaction with polyubiquitin chains. (Adapted from Wilson, W, The Regulation of constitutive NF-κB activity by GSK-3 in pancreatic cancer cells, 2008) Adapted with permission.

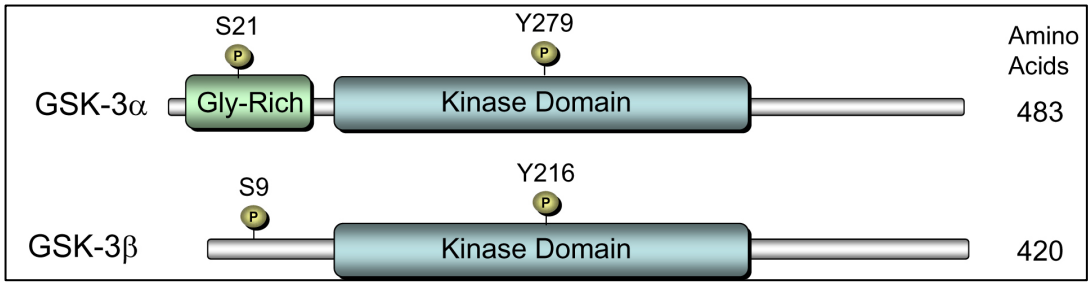

Figure 1.7. Glycogen synthase kinase-3 isoforms. Glycogen synthase kinase-3 exist a two, closely related mammalian isoforms (GSK-3α and GSK-3β). Gly-Rich indicates the N-terminal glycine-rich region located in GSK-3α. GSK-3 activity is mediated by autophosphorylation within the kinase domain at tyrosine residues 279 and 216 in GSK-3α and GSK-3β respectively. GSK-3 activity undergoes inhibitory phosphorylation at N-terminal serine residues 21 and 9 in GSK-3α and GSK-3β respectively. . (Adapted from Wilson, W, The Regulation of constitutive NF-κB activity by GSK-3 in pancreatic cancer cells, 2008) Adapted with permission.

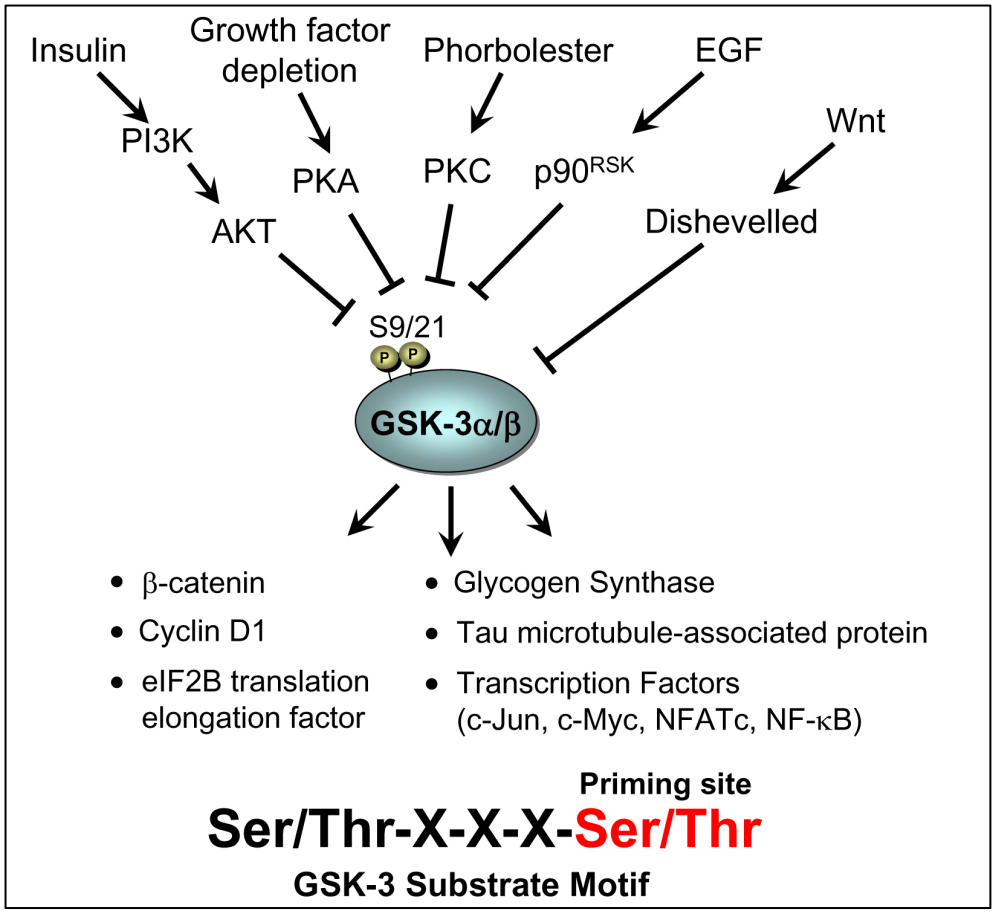

Figure 1.8. GSK-3 regulatory pathways and substrate targets. GSK-3 can be negatively regulated by a variety of upstream signaling events through the phosphorylation at serines 9 and 21 in GSK-3β and GSK-3α respectively. GSK-3 phosphorylates serine/ threonine residues in substrates that encompass the indicated substrate motif. Select substrates require a priming phosphorylation event by a secondary kinase to enhance GSK-3 substrate phosphorylation. The various substrates recognized by GSK-3 are indicated above. (Adapted from Wilson, W, The Regulation of constitutive NF-κB activity by GSK-3 in pancreatic cancer cells, 2008) Adapted with permission.

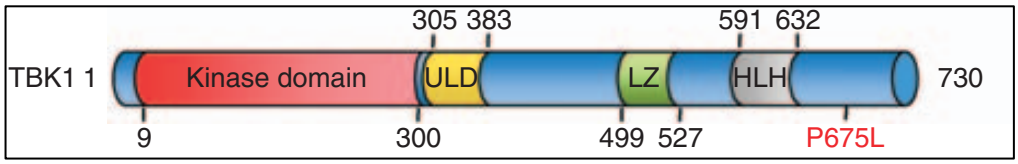

Figure 1.9. TBK1 domain organization The major domains of each TBK1 are depicted with amino-acid numbers that correspond to the human proteins. Somatic mutations of TBK1 identified in lung adenocarcinomas are marked in red. ULD, Ubiquitin-like domain; LZ, leucine zipper; HLH, helix-loop-helix; NBD, NEMO-binding domain (adapted from Shen, RR and Hahn, WC Oncogene, 2010)

Gene	Mutation	Frequency (%)
Oncogenes		
K-RAS	Point mutation	>95
HER2/ neu	Overexpression	90
AKT2	Gene amplification	10-20
Tumor Suppressors		
p16 ^{INK4a}	Point mutation Homozygous deletion Promoter hypermethylation	90
p53	Point mutation Homozygous deletion Loss-of-heterozygosity	75
SMAD4	Point mutation Deletion	50
BRCA2	Point mutation Deletion	5-10

Table 1.1 Frequency of genetic mutations associated with pancreatic cancer.
(Adapted from Wilson, W, The Regulation of constitutive NF-κB activity by GSK-3 in pancreatic cancer cells, 2008) Adapted with permission

Abnormality	Non–Small-Cell Lung Cancer		Small-Cell Lung Cancer
	Squamous-Cell Carcinoma	Adenocarcinoma	
Precursor			
Lesion	Known (dysplasia)	Probable (atypical adenomatous hyperplasia)	Possible (neuroendocrine field)†
Genetic change	*p53* mutation	*KRAS* mutation (atypical adenomatous hyperplasia in smokers), *EGFR* kinase domain mutation (in nonsmokers)	Overexpression of c-MET
Cancer			
KRAS mutation	Very rare	10 to 30%‡	Very rare
BRAF mutation	3%	2%	Very rare
EGFR			
Kinase domain mutation	Very rare	10 to 40%‡	Very rare
Amplification§	30%	15%	Very rare
Variant III mutation	5%¶	Very rare	Very rare
HER2			
Kinase domain mutation	Very rare	4%	Very rare
Amplification	2%	6%	Not known
ALK fusion‖	Very rare	7%	Not known
MET			
Mutation	12%	14%	13%
Amplification	21%	20%	Not known
TITF-1 amplification	15%	15%	Very rare
p53 mutation	60 to 70%	50 to 70%‡	75%
LKB1 mutation	19%	34%	Very rare
PIK3CA			
Mutation	2%	2%	Very rare
Amplification	33%	6%	4%

* Non–small-cell lung cancer includes squamous-cell carcinoma and adenocarcinoma.
† Neuroendocrine fields have been detected only in tissue surrounding tumors and have been characterized by extremely high rates of allelic loss and by c-MET overexpression (Salgia R: personal communication).
‡ Variations are based in part on smoking profiles.
§ The percentages include increased gene copy numbers from amplification or polysomy and represent percentages from resected cancers. The percentages are higher in primary tumors from patients with metastatic disease. Increased copy numbers have been reported in squamous dysplastic lesions but not in adenocarcinoma precursors.
¶ Genomic *EGFR* variant III mutations have been detected only in lung squamous-cell carcinoma, and these tumors are sensitive preclinically to irreversible EGFR tyrosine kinase inhibitors. The incidence of 5% is substantially lower than that of 30 to 40% for the detection in squamous-cell carcinoma or adenocarcinoma by immunohistochemical analysis or other techniques.
‖ The anaplastic lymphoma kinase *(ALK*) fusion gene (involving chromosome 2p), consisting of parts of *EML4* and *ALK,* is transforming in fibroblasts and occurs in adenocarcinoma but not in other types of non–small-cell lung cancer or other nonlung cancers.

Table 1.2 Genetic abnormalities in specific lung cancers
(Adapted from Herbst, R et al., Lung Cancer, The New England Journal of Medicine, 2008)

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CHAPTER II[1]

GSK-3α PROMOTES ONCOGENIC KRAS FUNCTION IN PANCREATIC CANCER VIA TAK1-TAB STABILIZATION AND REGULATION OF NON-CANONICAL NF-κB

2.1 Summary

Mutations in KRAS drive the oncogenic phenotype in a variety of tumors of epithelial origin. The NF-κB transcription factor pathway is important for oncogenic RAS to transform cells and to drive tumorigenesis in animal models. Recently TAK1, an upstream regulator of IKK, which controls canonical NF-κB, was shown to be important for chemoresistance in pancreatic cancer and for regulating $KRAS^+$ colorectal cancer cell growth and survival. Here we show that $KRAS^+$ upregulates GSK-3α leading to its interaction with TAK1 to stabilize the TAK1/TAB complex to promote IKK activity. Additionally, GSK-3α is required for promoting critical non-canonical NF-κB signaling in pancreatic cancer cells. Pharmacologic inhibition of GSK-3 suppresses growth of human pancreatic tumor explants, consistent with the loss of expression of oncogenic genes such as c-myc and TERT. These data identify GSK-3α as a key downstream effector of oncogenic KRAS via its ability to coordinately regulate distinct NF-κB signaling pathways.

[1] This chapter has been adapted from: Bang, D et al. GSK-3α Promotes Oncogenic KRAS Function in Pancreatic Cancer via TAK1-TAB Stabilization and Regulation of Non-Canonical NF-κB. Cancer Discovery 2013. 3: 690-703.

2.2 Introduction

Numerous epithelial-derived cancers express mutated/activated KRAS, and many of these cancers depend upon KRAS-induced signaling for regulation of growth, survival and metabolism (1,26). For example, the great majority of pancreatic ductal adenocarcinomas (PDAC) exhibit mutations in KRAS. However, specific targeting of mutant oncogenic KRAS has been a therapeutic challenge (87,88). Thus, targeting downstream effectors has emerged as an alternative approach to inhibiting oncogenic KRAS functions. Examples of downstream signaling proteins that are important in KRAS-dependent growth and survival are the kinases TAK1 and GSK-3, in the context of colorectal (TAK1) and pancreatic cancers (TAK1, GSK-3) (68,69) (89). Interestingly, TAK1 and GSK-3 have roles in promoting the activity of components of the NF-κB transcription factor family, which is known to be important for KRAS-driven oncogenesis.

NF-κB represents a family of evolutionary conserved transcription factors consisting of RelA (p65), c-Rel, RelB, p50 (precursor p105) or p52 (precursor p100) subunits (44,90-92). Dimers of these subunits regulate transcription of genes associated with inflammation, proliferation, and regulation of cell death. NF-κB complexes are inhibited through interaction with IκB proteins. Two distinct pathways have been characterized that lead to activation of NF-κB. The first, and most intensively studied, is the canonical NF-κB pathway defined by activation of the p65-p50 dimer. This pathway is dependent upon the activation of the IκB kinase complex (IKK) which consists of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ) (90). The activated IKK

complex phosphorylates IκB leading to its ubiquitination and subsequent proteasome-dependent degradation, allowing NF-κB to accumulate in the nucleus and facilitate transcription of target genes. The second is the non-canonical NF-κB signaling pathway, which is dependent upon activation of NIK (NF-κB inducing kinase). Activated NIK leads to activation of IKKα homodimers, which phosphorylate p100, leading to its proteolytic processing and generation of the active p52 subunit. Consequently RelB-p52 heterodimers accumulate in the nucleus to drive transcription of target genes (93).

NF-κB activity has been linked to the progression of multiple human cancers where it suppresses cell death and promotes cell proliferation, angiogenesis and invasion (44,91). We initially showed the importance of NF-κB in RAS-induced cell transformation (51). We and others have confirmed this in animal models of RAS-driven cancer (3,41,42,94). Consistent with this, NF-κB is constitutively active in the great majority of pancreatic cancer cell lines and tumors (95). Previous work has demonstrated that constitutive NF-κB activity is dependent upon IKK in pancreatic cancer cell lines (55-58) and knockout of IKKβ suppresses tumor growth/progression in a KRAS/INK4a null animal model of pancreatic cancer (61). Moscat and colleagues showed the importance of p62 in coordinating TRAF6 to regulate IKK downstream of oncogenic KRAS-induced signaling (54). Elucidating additional signaling components in the canonical NF-κB pathway as well as understanding events associated with non-canonical NF-κB activation induced by KRAS is important in understanding KRAS-induced transformation.

Transforming growth factor-beta activated kinase 1(TAK1) is a mitogen activated protein kinase kinase kinase (MAP3K) that initiates downstream NF-κB and MAPK signaling in response to cytokines (63). TAK1 activity is dependent upon its association with TAK1-binding partners (TAB1, TAB2, TAB3 and TAB4) which facilitate auto-phosphorylation of TAK1 (Thr178, Thr184, Ser192) within the kinase activation loop. (64,96). Upon activation, TAK1 promotes the activity of p38 and JNK MAPKs (97). TAK1 plays a central role in NF-κB activation through direct phosphorylation of IKKβ (63). As described above (68,69), TAK1 has been shown to be important for survival of KRAS-dependent colorectal cancers and to play an important role in the chemoresistance of pancreatic cancers.

GSK-3 is a multifunctional serine/threonine kinase that exists as closely related isoforms (GSK-3α and GSK-3β) (72). It is a key enzyme involved in diverse biological processes such as cell cycle progression, differentiation and apoptosis (98). Moreover, GSK-3 has been implicated in as playing an oncogenic role in various human malignancies including pancreatic cancer (76,78,98). While most studies have focused on GSK-3β and its involvement in regulating the WNT/β-catenin pathway, a recent study implicated GSK-3α in promoting AML (80). We and others have demonstrated that GSK-3 regulates growth and survival in pancreatic cancer cell lines by driving constitutive IKK and subsequent NF-κB DNA binding and activity (57,79). However, the mechanism of how GSK-3 activates IKK and a clear distinction, if any, between the roles of the two isoforms GSK-3α and GSK-3β remains elusive.

Here we investigate the roles of GSK-3α, GSK-3β, and TAK1 downstream of mutant KRAS in driving constitutive NF-κB signaling, proliferation and survival in pancreatic cancer cells. We establish a regulatory link between GSK-3α and TAK1 and propose that constitutive canonical NF-κB activity is driven by a unique GSK-3α-TAK1-IKK signaling cascade. We also provide evidence that GSK-3α regulates non-canonical NF-κB activity in pancreatic cancer cells, independent of GSK-3β, which contributes to growth/survival of pancreatic cancer cells. Moreover, we show that acute inhibition of GSK-3 in human pancreatic tumor explants suppresses tumor growth and identify a comprehensive transcriptional profile that is changed upon GSK-3 inhibition. Collectively, these data provide new insight into constitutive NF-κB regulation in oncogenic KRAS-induced pancreatic cancer, and establish GSK-3α and TAK1 as potential therapeutic targets for this disease.

2.3 Results

2.3.1 GSK-3α/β promote proliferation/survival of pancreatic cancer cells.

Consistent with previous reports (57,78), we observed a decrease in proliferation of two well characterized $KRAS^{+}$ pancreatic cancer cell lines, Panc-1 and MiaPaCa-2, upon treatment with the selective GSK-3 inhibitor, AR-A014418 in a dose dependent manner (Fig. 1A, Supplementary S1). To exclude potential off-target effects of the drug and to determine the individual requirements for GSK-3α and GSK-3β for cell survival/proliferation, RNA interference was utilized to knock-down individual isoforms. Significant reduction in the cell index of MiaPaCa-2 cells was observed following GSK-

3α RNA interference as compared with non-targeting control and GSK-3β siRNA or both GSK-3α/β siRNA (Fig. 1B).

To further explore the effects of GSK-3 inhibition on pancreatic cancer cell function, we analyzed the effect of GSK-3α/β knockdown on the colony formation of pancreatic cancer cells in soft agar. GSK-3α RNA interference inhibited soft agar growth of MiaPaCa-2 cells significantly as compared to non-targeting control or GSK-3β siRNA (Fig. 1C). Importantly the size of the colonies formed by GSK-3α depleted cells was significantly smaller than that of control or GSK-3β depleted cells. To determine if this abrogated soft agar growth correlated with induction of apoptosis, we measured PARP cleavage after the knockdown of GSK-3α and GKS-3β. Depletion of either GSK-3α or GSK-3β led to a modest increase in PARP cleavage (Supplementary S1 and see below).

Consistent with previous reports (57,78), GSK-3 inhibition suppressed phosphorylation of IKK and of RelA/p65 (Fig. 1D), markers of canonical NF-κB signaling. Interestingly GSK-3 inhibition also decreased the levels of TAK1, upstream IKK kinase (63). This led us to hypothesize and investigate a link between GSK-3 and TAK1 in regulating NF-κB activity in pancreatic cancer cells.

2.3.2 Oncogenic KRAS promotes TAK1-TAB interaction to drive canonical NF-κB activity.

The activity of TAK1 is dependent upon its association with TAK-1 binding proteins (TAB1, TAB2, TAB3) (64). To determine whether TAK1 is active in pancreatic

cancer cell lines, we examined the status of TAK1- TAB1 binding in pancreatic cancer cells and questioned if mutant KRAS affects TAK1-TAB1 interaction. TAK1-TAB1 interaction was analyzed by immunoprecipitating TAK1 from an immortalized, human pancreatic ductal epithelial cell line (HPDE6), and in the derivative $KRAS^{G12V}$-transformed HPDE6 cells (HPDEKR+) and from MiaPaCa-2 cells. Relative to KRAS+ MiaPaCa-2 cells and HPDE6KR+ cells, a significant (34%) decrease in TAK1-TAB1 binding was found in HPDE cells (Fig. 2A) indicating that mutant KRAS promotes TAK1-TAB1 interaction. Consistent with this hypothesis, knockdown of KRAS in HPDEKR+ cells strongly reduced TAK1 protein levels and TAK1-TAB interaction in HPDEKR+ cells. (Supplementary S2). These data demonstrate that mutant KRAS drives constitutive TAK1 activity in pancreatic cancer cell lines via stabilizing TAK1.

To determine whether TAK1 activity is required for NF-κB activity in pancreatic cancer cells, the effects of transient TAK1 depletion were measured relative to canonical NF-κB activity. siRNA mediated TAK1 knockdown resulted in diminished phosphorylation of IκBα (Serine 32/36), stabilization of total IκBα levels and reduced phosphorylation of p65/RelA, indicating reduced canonical NF-κB activation (Fig. 2B). Taken together, our data indicate that TAK1 plays a key role in regulating NF-κB activity in pancreatic cancer cells, consistent with the study by Melisi et al (69), and that oncogenic KRAS promotes TAK1 activity by promoting TAK1 stabilization and hence the TAK1-TAB interaction.

To determine the functional significance of TAK1-mediated NF-κB activity, we examined the effect of TAK1 depletion on pancreatic cancer cell proliferation/survival. siRNA mediated TAK1 depletion significantly reduced the cell index of MiaPaCa-2 cells relative to the non-targeting control throughout the time-course in an MTS assay (Fig. 2C). This reduced cell index correlated with a weak PARP cleavage response (Supplementary S1). Importantly, this effect of TAK1 depletion was specific to cells harboring mutant KRAS, as knockdown of TAK1 did not decrease cell proliferation in HPDE6 cells unlike HPDEKR+ (Fig. 2D). Notably, the effect of TAK1 depletion on cell index was significantly weaker than that seen with pharmacologic inhibition of GSK-3, possibly because of incomplete knockdown of TAK1.

To achieve a greater inhibition of TAK1 kinase activity, we analyzed the effect of a selective and potent TAK1 kinase inhibitor, 5Z-7-oxozaenol, on cell survival/proliferation of pancreatic cancer cells (68). Consistent with our hypothesis, TAK1 inhibitor treatment resulted in a greater decrease in cell proliferation of MiaPaCa-2 compared to TAK1 knockdown (Fig. 2E). Again the inhibitory effect of TAK1 inhibitor on cell proliferation was specific to mutant KRAS harboring cells. (Supplementary S3). The decrease in phosphorylation of p38 (TAK1 substrate) confirms the inhibition of TAK1 activity upon 5Z-7-oxozaenol treatment (Supplementary S2).

A modest increase in PARP cleavage upon TAK1 and GSK-3 inhibition (Supplementary S2) argues against the induction of apoptosis as a major mechanism resulting in reduced cell index. We thus, examined the effect of TAK1 inhibition on cell

cycle distribution. Panc-1 cells were treated with 5Z-7-oxozaenol and stained with PI. An increase in the number of cells in G2/M phase was observed with increasing concentration of the TAK1 inhibitor (Supplementary S4), suggesting that TAK1 inhibition leads to a G2/M arrest in pancreatic cancer cells leading to decreased proliferation. As shown below, the effect of GSK-3 inhibition on cell proliferation may be via TAK1 and thus cell cycle deregulation. Overall, these data suggest that TAK1 is active downstream of mutant KRAS in pancreatic cancer cells and mediates constitutive NF-κB signaling to regulate proliferation by altering cell cycle progression.

2.3.3 GSK-3α facilitates constitutive TAK1 activity by maintaining TAK1-TAB1 interaction.

We and others previously demonstrated that GSK-3 drives constitutive IKK and NF-κB activity in pancreatic cancer cell lines (57). In Fig. 1C we observed a decrease in TAK1 levels with GSK-3 inhibition in Panc-1 and MiaPaCa-2 cells. To further analyze a role for GSK-3 in regulating TAK1-dependent NF-κB signaling, Panc-1 and MiaPaCa-2 cells were treated with GSK-3 inhibitor over a 24-hour time course. Results from this experiment revealed a time-dependent decrease in levels of TAK1, its binding partners TAB1 and TAB2, and NF-κB p65 phosphorylation (Fig. 3A). Though the kinetics of decrease in TAK1 and TABs levels were different in Panc-1 and MiaPaCa-2 cells, the levels of TAK1 were always consistent with the loss of GSK-3 activity, as seen by suppression of phospho-glycogen synthase, a substrate of GSK-3. Moreover, we also saw a decrease in TAK1 substrate phosphorylation (phospho-p38) with GSK-3 inhibition (Supplementary S5). To determine if the loss in TAK1 protein levels is due to

proteasome-dependent degradation, Panc-1 and MiaPaCa-2 cells were treated with AR-A014418 in the presence of proteasome inhibitor (MG-132). The results demonstrated that MG-132 treatment restored reduced TAK1 levels in Panc-1 cells but not in MiaPaCa-2 cells (Supplementary S6). To demonstrate that the decrease in TAK1 levels was not an off-target effect of AR-A01448, siRNA was used to transiently knockdown GSK-3α and/or GSK-3β in Panc-1 and MiaPaCa-2 cells. 48 hours after siRNA transfection, a decrease in TAK1 levels was observed with knockdown of GSK-3α but not of GSK-3β (Fig. 3B).

To further analyze the mechanism of GSK-3-TAK1 regulation, we investigated whether GSK-3 isoforms can physically interact with TAK1. Endogenous TAK1 was immunoprecipitated from MiaPaCa-2 cells and blotted for GSK-3α and GSK-3β. We observed GSK-3α co-precipitating with TAK1 in KRAS transformed pancreatic cancer cells (MiaPaCa-2 and HPDE-KR+) as well as non-transformed HPDE (Fig. 3C), although the level of interaction was higher in the KRAS+ cells. Consistent with previous studies (99), expression of oncogenic KRAS led to the upregulation of GSK-3 isoforms (Fig. 3C). Since GSK-3α-TAK1 co-immunoprecipitated in non-transformed HPDE's as well as transformed pancreatic cancer cells, we sought to explore this interaction in other cells like 293T cells. These cells have very low levels of GSK-3 and TAK1 as compared to pancreatic cell lines, prompting us to overexpress these proteins to detect any interaction. Expression of V5-tagged GSK-3α with TAK1 in 293T cells led to an interaction as determined by co-immunoprecipitation (Fig. 3D). No interaction between over-expressed GSK-3β and TAK1 was observed indicating specificity to GSK-3α (data not shown).

To determine if GSK-3 kinase activity is required for maintaining the TAK1-TAB interaction (and not just TAK1 and TAB levels), we evaluated the effect of GSK-3 inhibition on TAK1-TAB1 complex in MiaPaCa-2 cells. Since AR-A014418 treatment affects TAK1 levels, we transiently over-expressed TAK1 in MiaPaCa-2 cells, before treating the cells with AR-A014418. As is evident in Fig. 3E, GSK-3 inhibition suppressed the co-precipitation between TAK1 and TAB1, even under conditions where TAK1 and TAB1 levels are maintained. Thus GSK-3 catalytic activity appears to be important in maintaining the TAK1-TAB1 complex. Overall, these data and those described above demonstrate that mutant KRAS induces TAK1-TAB1 interaction that is stabilized by GSK-3α leading to higher TAK1-dependent NF-κB activity in pancreatic cancer cells.

2.3.4 GSK-3α facilitates constitutive non-canonical signaling in pancreatic cancer cells.

Non-canonical NF-κB pathway leading to processing of NF-κB2/p100 to p52, has been shown to be constitutively active in pancreatic cancer cells (59,60). To this point, the data links GSK-3 to constitutive canonical NF-κB signaling via TAK1. However, the effect of GSK-3 inhibition on cell survival/proliferation was much greater than that observed with depletion of TAK1, suggesting that another component regulated by GSK-3 affects pancreatic cell survival/proliferation. Thus, we asked if GSK-3 regulates the non-canonical arm of NF-κB in pancreatic cancer cells. Processing of p100 to p52 in Panc-1 and MiaPaCa-2 cells was suppressed when these cells were treated with the GSK-

3 inhibitor, AR-A014418 (Fig. 4A). To account for potential off-target effects of the GSK-3 inhibitor, p100-p52 processing was analyzed upon GSK-3α/β depletion by siRNA. Depletion of GSK-3α but not GSK-3β led to a decrease in p100-p52 processing in Panc-1 and MiaPaCa-2 cells (Fig. 4B), again highlighting differences between GSK-3α and GSK-3β. Upon processing of p100, p52 accumulates in the nucleus to promote transcription of its target genes. We examined the effect of GSK-3α knockdown on the nuclear localization of p52. Surprisingly we saw that GSK-3α depletion suppressed p52 levels only in the nuclear fraction and not in the cytoplasmic extract (Fig. 4C). This suggests that either GSK-3α promotes nuclear accumulation of p52 or that GSK-3α regulates the processing of p100 in the nuclear fraction.

The functional importance of the non-canonical NF-κB pathway was measured via the effect of p100 knockdown on the cell index of pancreatic cancer cells. As compared to the non-targeting control, p100 knockdown suppressed the cell index of Panc-1 cells in a cell impedance assay (Fig. 4D). This suppression of cell index was greater than that observed with TAK1 depletion. Knockdown of p100 modestly increased PARP cleavage in MiaPaCa-2 but not in Panc-1 cells (Supplementary S7). Overall, these data provide a novel link between GSK-3α and the non-canonical NF-κB pathway in pancreatic cancer cells. These results may explain why knockdown of GSK-3α (that affects both canonical and non-canonical NF-κB) leads to a greater suppression of cell growth as compared to knockdown of GSK-3β or TAK1 (Fig. 1 C, D).

2.3.5 GSK-3 inhibition suppresses growth of patient-derived pancreatic tumor explants.

Our data and previous reports suggest that GSK-3 inhibition may be a potential target for pancreatic cancer treatment because of its effect on NF-κB activity. However, the effect of GSK-3 inhibition on human pancreatic tumors has never been evaluated. To analyze the effect of GSK-3 inhibition on tumor growth *in vivo,* we explanted replicates of a human pancreatic tumor in nude mice. Two weeks later, GSK-3 inhibitor, AR-A014418, was administered intraperitoneally at 120 mg/kg twice a day for two days. The growth of the tumor was then monitored over a course of 28 days. Consistent with our hypothesis and data, GSK-3 inhibition suppressed tumor growth in mice by approximately 50% (Fig. 5A). To analyze the effect of acute GSK-3 inhibition on the TAK1/NF-κB signaling in mice, we repeated the AR-A014418 treatment study on pancreatic tumor explants for shorter time courses (1h, 2h, 6h and 8h). We observed a time-dependent decrease in TAK1 and TAB levels in these tumors, consistent with our studies in pancreatic cancer cell lines (Fig. 5B). Overall these data indicate that GSK-3 inhibition suppresses pancreatic tumor growth with a concomitant decrease in TAK1-TAB activity.

2.3.6 Effect of GSK-3 inhibition on gene expression in human patient-derived pancreatic tumor explants.

We next sought to determine the effect of GSK-3 inhibition on gene expression in the human pancreatic tumor explants in mice. Gene expression microarrays were utilized

to identify global changes in gene expression that occurred in these tumors when treated with GSK-3 inhibitor. Supervised gene expression analyses were conducted to quantify gene expression changes in the tumors, after 2 or 8 hours of AR-A014418 treatment (Fig. 6A). GSK-3 inhibition led to a statistically significant change in expression (based on SAM analysis) for 470 genes, of which 155 changed more than 2 fold (Fig. 6A). We further analyzed known or suspected to be NF-κB target genes changed by GSK-3 inhibition. In this group, GSK-3 inhibition led to a statistically significant down-regulation of 17 genes after 8h and 22 genes after 2 hours of treatment (Fig. 6B). 15 out of these genes were downregulated more than 1.5 fold, with greater downregulation occurring at the 8h time point. As shown in Fig. 6C, most of these genes have been shown to have a pro-oncogenic role in pancreatic cancer. For example, we observe a decrease in the expression of pro-proliferative genes like c-Myc, TERT, and cIAP2 with GSK-3 inhibition.

2.4 Discussion

Mutant KRAS is expressed in virtually all pancreatic cancers as well as in other epithelial-derived cancers where it serves as a key oncogenic factor, promoting proliferation and survival (1,26). Despite extensive research, less than 4% of patients diagnosed with pancreatic cancer are expected to survive past 5 years (100). Since no Ras inhibitors have been effective (26,87), current research efforts are focusing on targeting deregulated signaling pathways downstream of $KRAS^{+}$. The advancement of this therapeutic strategy is dependent on a detailed understanding of the complex molecular mechanisms underlying signaling events that regulate disease progression.

NF-κB is known to be constitutively active in majority of the pancreatic tumors and pancreatic cancer cell lines, where it regulates proliferation, survival, metastasis and invasion (95). NF-κB has also been shown to be activated downstream of oncogenic Ras and promote the oncogenic phenotype (42,94). Thus, the signaling cascades activating NF-κB pathway have become attractive targets for novel chemotherapeutic approaches in pancreatic and other cancers (95). There is evidence that multiple of arms of NF-κB are activated in various cancers (44), thus the ability to block NF-κB activity broadly may require multiple inhibitors unless a factor can be identified that regulates multiple NF-κB -relevant signaling pathways.

Previous studies from our lab and others have shown that GSK-3 inhibition reduces pancreatic cancer cell viability *in vitro* and suppresses tumor cancer cell line growth *in vivo* at least partly through the downregulation of NF-κB activity (57,78,79,101).

However the mechanism by which GSK-3 regulates NF-κB and the distinct roles of the two isoforms, GSK-3α and GSK-3β, are not well characterized. In this report, we provide the first evidence for a role of GSK-3α in regulating proliferation and anchorage-independent growth of pancreatic cancer cells independent of GSK-3β. We previously reported that GSK-3 signals through IKK to mediate canonical NF-κB signaling in pancreatic cancer cells (57). Here, we propose that GSK-3 and IKK are functionally linked through GSK-3α-dependent control of TAK1-TAB1 complex stability/activity, and provide the first evidence for regulation of non-canonical NF-κB by GSK-3α.

TAK1 is a central regulator of NF-κB signaling in diverse physiological processes including development, immune responses and survival (63). Its binding partner, TAB1 was recently shown to play an essential role in the maintenance of TAK1 activity in epithelial tissues (64). Here, we show that the TAK1-TAB1 complex is active in KRAS+ cancer cells and is stabilized via a GSK-3α-dependent mechanism (Fig. 2). TAK1 was recently identified as a pro-survival mediator in $KRAS^{+}$-dependent colon cancer (68). While Singh et al argue against the pro-survival role of TAK1 in pancreatic cancer cells, another group showed that inhibition of TAK1 leads to a proapoptotic phenotype in pancreatic cancer cells, by suppressing NF-κB (68,69) Our results supports aspects of each of these studies, as we observe a reduction in NF-κB activity and cell proliferation with inhibition of TAK1 in pancreatic cancer cells (Fig. 2), however we do not see a strong corresponding increase in apoptosis as measured by cleavage of caspase 3 or PARP under these conditions (Supplementary S1,S2). However, inhibition of TAK1

leads to G2/M arrest in pancreatic cancer cells (Supplementary S4), which presumably causes a decrease in cell proliferation.

A mechanism to describe how TAK1 is activated downstream from oncogenic KRAS has not been described. Previously, Moscat and colleagues (54) showed the involvement of p62 in regulating TRAF6 ubiquitination downstream of oncogenic KRAS to promote IKK activity. Here, we provide the first evidence of a regulatory link between GSK-3α and TAK1. We show that pharmacological inhibition of GSK-3 in pancreatic cancer cells leads to a decrease in levels of TAK1 and its binding partners TAB1 and TAB2 (Fig 3A,B, Supplementary S8). The decrease in TAK1 and TAB levels were detected as early as 1h following GSK-3 inhibitor treatment and were maintained at low levels as long as GSK-3 activity was inhibited. The *in vitro* results were reproduced *in vivo* when human pancreatic tumor explants were treated with AR-A014418 (Fig. 5). These results are corroborated by siRNA knockdown of GSK-3α in pancreatic cancer cells. The interaction between GSK-3α and TAK1 was found in non-transformed HPDE cells, suggesting that GSK-3α can interact with TAK1 irrespective of presence or absence of mutant KRAS. However, mutant KRAS upregulates the expression of GSK-3 (99) (Supplementary S9), which then promotes the stabilization of the active TAK1-TAB1 complex. TAK1 can drive IKK-dependent NF-κB signaling leading to increased proliferation of $KRAS^{+}$ transformed cells. The mechanistic link between GSK-3α and TAK1 is unclear since there is no consensus substrate motif of GSK-3 (S/T-XXX-S/T) in TAK1. However, Taelman et al have shown that TAB1 and TAB2 contain multiple GSK-3 consensus motifs (102). Thus, there is a possibility that GSK-3 phosphorylates TABs to regulate the

TAK1-TAB1 complex and thus the stability of TAK1. This hypothesis will be addressed in future studies.

Although we demonstrate that GSK-3 regulates IKK through TAK1, the effect on cell proliferation with GSK-3 inhibition is much stronger as compared to TAK1 inhibition. These results suggested that additional regulatory events downstream of GSK-3 promote pancreatic cancer cell growth/survival. Non-canonical NF-κB pathway has been shown to be constitutively active and to be contributing to proliferation/survival in pancreatic cancer cells (59,60). Thus we explored the potential involvement of GSK-3 in regulating p100/NF-κB2 processing. GSK-3 inhibition, or knockdown of GSK-3α (but not GSK-3β) leads to significantly reduced p100 processing in pancreatic cancer cells (Fig. 4A,B, Supplementary S8). Importantly, we demonstrate a significant reduction in cell proliferation of pancreatic cancer cells, upon knocking down p100 subunit (Fig. 4D). We also saw a decrease in p100 and p52 levels upon TAK1 inhibition, which is consistent with p100 being a known transcriptional target of canonical NF-κB (data not shown). Interestingly we observed a decrease in nuclear accumulation of p52 subunit upon GSK-3α knockdown while, the cytoplasmic levels of p100 and p52 remained unchanged (Fig. 4C). This indicates a distinct effect of GSK-3α on nuclear p52 different from that derived via inhibition of TAK1-regulated canonical NF-κB. GSK-3 has been earlier shown to be accumulated in the nucleus in pancreatic cancers (79) which raises the potential that the GSK-3 effect on non-canonical NF-κB is via processing of nuclear p100. It is also possible that GSK-3α affects the nuclear transport of p52 subunit. We thus hypothesize that GSK-3α regulates both canonical and non-canonical NF-κB pathway in pancreatic cancer cells. Notably our results on the regulation of p100 processing by GSK-3α are

likely not to relate to the recent work demonstrating the role of GSK-3β and Fbxw7α-mediated degradation of p100 in multiple myeloma (103) as we do not see p100 accumulation upon GSK-3α knockdown. However, our study does support the pro-survival role of p100 – p52 processing in a way similar to that described by Sangfelt and colleagues (104).

The human tumor explant study demonstrates a 50% inhibition of KRAS-mutant tumor growth upon a 2-day treatment with GSK-3 inhibitor (Fig. 5). This study was performed with a commercially available GSK-3 inhibitor, indicating the need for more extensive studies on a broader group of pancreatic tumors using a pharmaceutical grade inhibitor. To analyze the effect of GSK-3 inhibition on NF-κB target gene expression, we compared the gene expression profile of tumors, before and after treatment with AR-A014418. We observed a down-regulation of several established NF-κB target genes such as c-myc and TERT, which are known to be upregulated in pancreatic cancer (Fig. 6C). It is noteworthy that we saw a selective effect on NF-κB dependent gene expression. These results are consistent with previous studies that GSK-3 and Ras induce only a selective arm of the NF-κB pathway (73,105), which is different from the well characterized NF-κB regulated downstream of cytokine-induced signaling. NF-κB may also be regulated by upstream activators other than GSK-3 in these tumors, thus inhibiting GSK-3 alone will not shut down the entire NF-κB signaling.

Interestingly, significant changes were observed in long non-coding RNAs upon GSK-3 inhibition, which are now emerging as key regulators of oncogenesis (106). We

understand that GSK-3 plays important roles in other signaling pathways, including Wnt, Notch, Hedgehog, which have been implicated in pancreatic cancers (107) and which may account for the rest of the observed changes in gene expression. Implications of GSK-3 inhibition on the β-catenin stabilization and Wnt pathway needs consideration (107), however our encouraging results on tumor growth inhibition and previous studies strongly support the therapeutic potential of GSK-3 inhibitors.

Collectively, this report provides the first evidence of GSK-3α in promoting oncogenic RAS function through regulation of TAK1-TAB activity, upstream of IKK and canonical NF-κB, and via control of non-canonical NF-κB activity in pancreatic cancer cells (Fig.7). Importantly, this data provides evidence for different roles of GSK-3α and GSK-3β in regulating NF-κB signaling in pancreatic cancer cells, and highlights the need for the development and testing of GSK-3α specific drugs.

2.5 Materials and Methods

Cell culture and reagents

Panc-1, MiaPaCa-2 were purchased from American Type Culture Collection and used for no longer than 6 months before being replaced. Human pancreatic ductal epithelial cell line (HPDE6) and KRAS4B^{G12V}–transfected HPDE (HPDEKR+) were generous gifts from Dr. Channing Der (UNC-Chapel Hill) and were maintained in keratinocyte serum-free growth medium. All cell culture reagents were obtained from Invitrogen (Carlsbad, CA). The following antibodies were purchased from Cell Signaling Techonology: phospho-Glycogen synthase (Ser^{641}), Glycogen Synthase phospho-p65

(Ser^{536}), p-65, TAB1, TAB2, Histone H3, Cleaved PARP (Asp^{214}), PARP, phospho-p38 (Thr^{180}/Tyr^{182}), p38; Santa Cruz Biotechnology: GSK-3α/β, TAK1, GAPDH, β-tubulin; Millipore: p100/p52 and Sigma Aldrich: Anti-Flag (M2). GSK-3 inhibitor (AR-A014418) was purchased from Sigma-Aldrich. TAK1 inhibitor (5Z-7-oxozaenol) was purchased from Tocris Bioscience.

siRNA interference

The following human siRNA (siGenome SMARTpool) was purchased from Dharmacon as a pool of four annealed dsRNA oligonucleotides: MAP3K7 (M-003790-06), GSK-3α(M-003009-01), GSK-3β (M-003010-03), NF-κB2 (M-003918-02), and non-targeting control#3 (D001201-03). Dharmafect transfection reagent 1 was used to transfect 100nM siRNA according to manufacturer's instruction and cells were harvested 48 or 72 hours after transfection, as stated.

Western blot analysis

Cytoplasmic, nuclear and whole cell extracts were prepared as described previously (57). Tumor lysates were prepared by homogenizing ~1mm piece of tumor in lysis buffer (50mM Tris (pH7.6), 150mM HCL, 2mM EDTA). NP-40 was added at 1% v/v and incubated on a rocking platform for 30 minutes in cold room and cellular debris precipitated using centrifugation. Protein extracts were quantified by Bradford assay (Bio-rad Laboratories) and analyzed by SDS-PAGE (57).

MTS Assay

Cells were seeded in a 96-well plate and allowed to incubate for 24 hours. The cells were then treated with the indicated concentrations of AR-A014418 for indicated

times and cellular proliferation measured using the CellTiter 96 Aqueous solution, in accordance to the manufacturer's protocol (Promega). For siRNA experiments, the cells were seeded in the plate 48 hours after siRNA transfection.

Cell impedance assay/ real time cell analysis (RTCA)

Background impedance signal was measured with 50μL cell culture medium per well of an E-Plate 16. The final volume in a single well was adjusted to 100μL cell culture medium by adding additional 50 μL medium containing 1000 cells. For siRNA experiments, cells were seeded 48 hours after transfection. For inhibitor studies, 5Z-7-oxozaenol was added one day after seeding. After plating, impedance was routinely recorded in 2-hour intervals using the xCELLigence RTCA MP instrument (Roche).

Soft Agar assay.

MiaPaCa-2 cells were transiently transfected with indicated siRNA as above. 48 hours after transfection, 8000 cells were suspended in a 0.4% bacto-agar/DMEM layer (1ml) and plated over a 0.6% bacto-agar/DMEM layer (2ml) in 6-well plates. 0.5ml media was added next day and every 3-4 days thereafter for 2 weeks. After one week colonies were stained with 0.5ml of MTT (2mg/ml, Sigma Aldrich). Images were acquired and colonies counted using ImageJ 1.46 software.

Microarray analysis

Total RNA was purified using RNeasy plus RNA isolation kit (Qiagen), reverse-transcribed, labeled, and hybridized to an Agilent v2 8X60K whole human DNA microarray. Microarrays were scanned using an Agilent DNA microarray scanner and features were extracted using Agilent Feature Extraction software version 10.7.3.1. Data

were uploaded to the University of North Carolina Microarray Database (UMD). Gene expression data were extracted from the UMD for each sample as $\log_2$ Cy5/Cy3 ratios, filtering for probes with Lowess normalized intensity values greater than 10 in both channels and for probes with data on greater than 70% of the microarrays. Hierarchical clustering analyses was carried out using Gene Cluster 3.0 (108), data were viewed using Java TreeView version 1.1.5r2 (109). Expression changes were determined using a linear model with terms for treatment time points (110). This model was fit to each probe, and genes corresponding to treatment effects with a q-value ≤ 0.05 (111) were considered statistically significant. The data are publicly available in Gene Expression Omnibus database (accession number GSE42559)

Tumor explant study

Tiny fragments (1.5-2mm) of a resected human pancreatic tumor were implanted subcutaneously into the flanks of nude mice. Two weeks later, GSK-3 inhibitor, AR-A014418 or vehicle control, DMSO was given intraperitonealy at 120mg/kg twice a day for two days. The growth of the tumor was measured every alternate day for 28 days. For shorter treatments, tumor was harvested immediately after the indicated time periods.

FIGURES

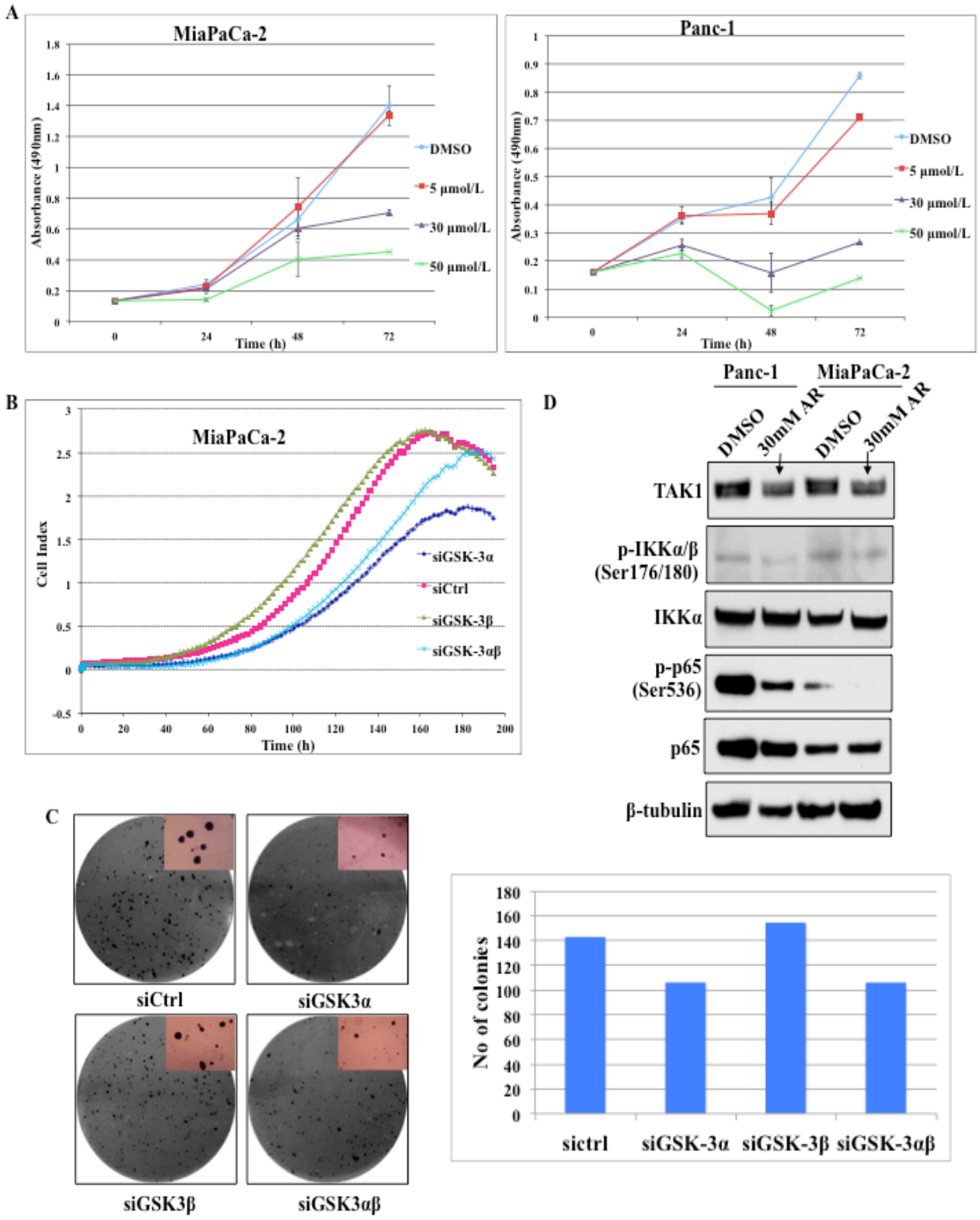


Figure 2.1. GSK-3α and GSK-3β regulate growth and NF-κB activity in pancreatic cancer cells. A, MiaPaCa-2 and Panc-1 cells were treated with dimethyl sulfoxide (DMSO) or the indicated concentrations of the GSK-3 inhibitor AR-A014418 for 24, 48, and 72 hours. Cell proliferation was measured in triplicate at each time point using a

colorimetric MTS tetrazolium assay. B, MiaPaCa-2 cells were transiently transfected with GSK-3a, GSK-3b, both GSK-3a and GSK-3b, or nontargeting siRNA. Forty-eight hours after transfection, cell growth was measured in triplicate at each time point using a cell impedance assay. C, MiaPaCa-2 cells were transiently transfected with the indicated siRNA as above. Forty-eight hours after transfection, cells were suspended in bacto-agar growth medium, and 7 days later, plates were examined for colony formation. The data shown are representative of 2 independent experiments, each carried out in triplicate. D, Panc-1 and MiaPaCa-2 cells were treated with DMSO or 30 μmol/L of GSK-3 inhibitor AR-A014418 for 24 hours. Whole-cell extracts were immunoblotted with specified antibodies

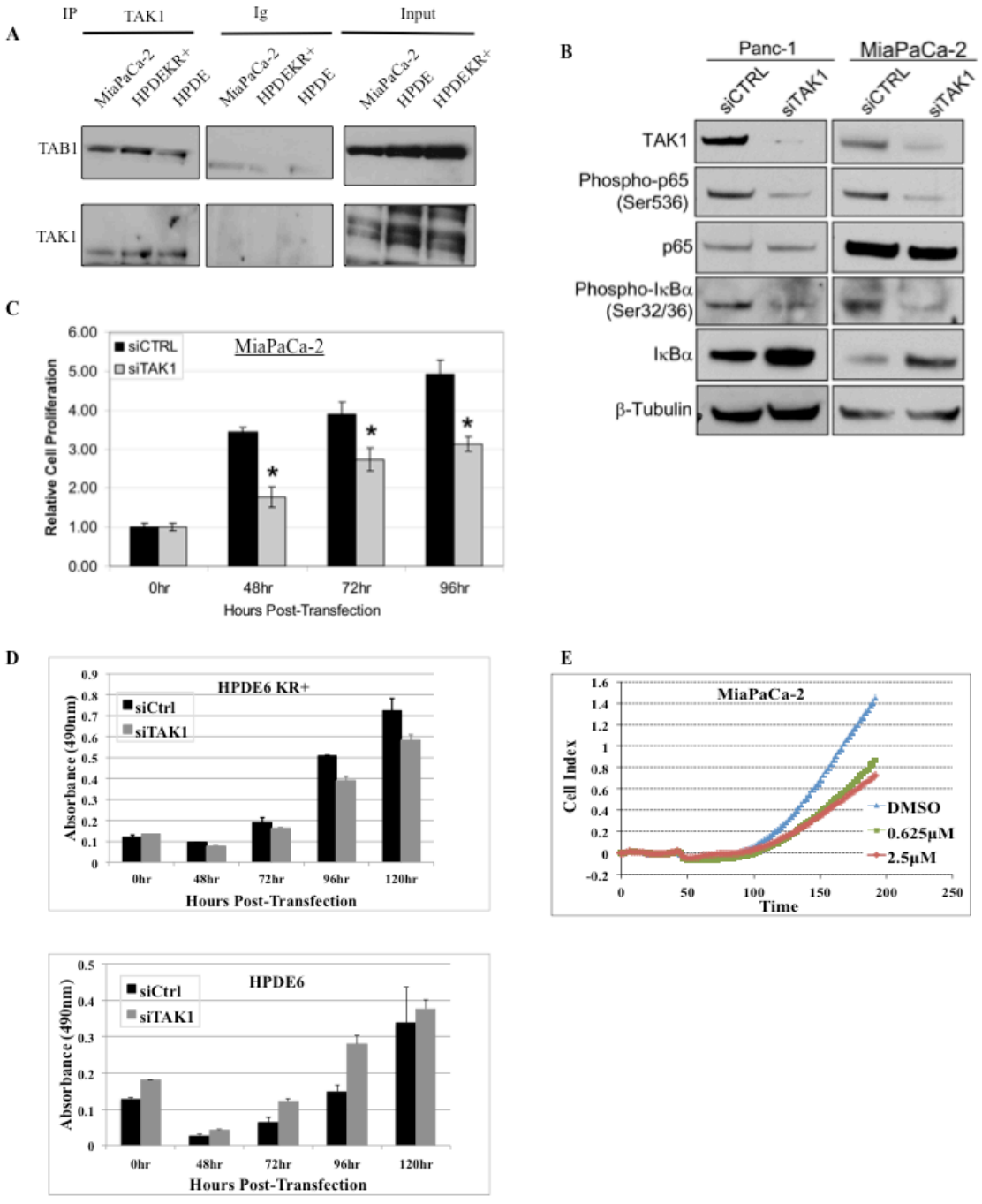


Figure 2.2 TAK1 is constitutively active and regulates NF-κB activity in pancreatic cancer cells. (A) Endogenous TAK1 was immunoprecipitated from whole cell lysates of the indicated cell lines and blotted for TAB1. (B) Panc-1 and MiaPaCa-2 cells were transiently transfected with non-targeting or TAK1 siRNA for 48 hours and immunoblot

performed using indicated antibodies. (C) MiaPaCa-2 were transfected with siRNA as indicated above and cell viability was measured at the indicated times post-transfection. Samples were measured in triplicates and normalized to untransfected cells. (D) HPDE6 and HPDEKR+ cells were transfected with siRNA targeted against TAK1 and cell viability measured as above. (E) MiaPaCa-2 cells were treated with DMSO or the TAK1 inhibitor, 5Z-7-oxozaenol (OZ) and cell impedance measured in triplicate every 2 hours.

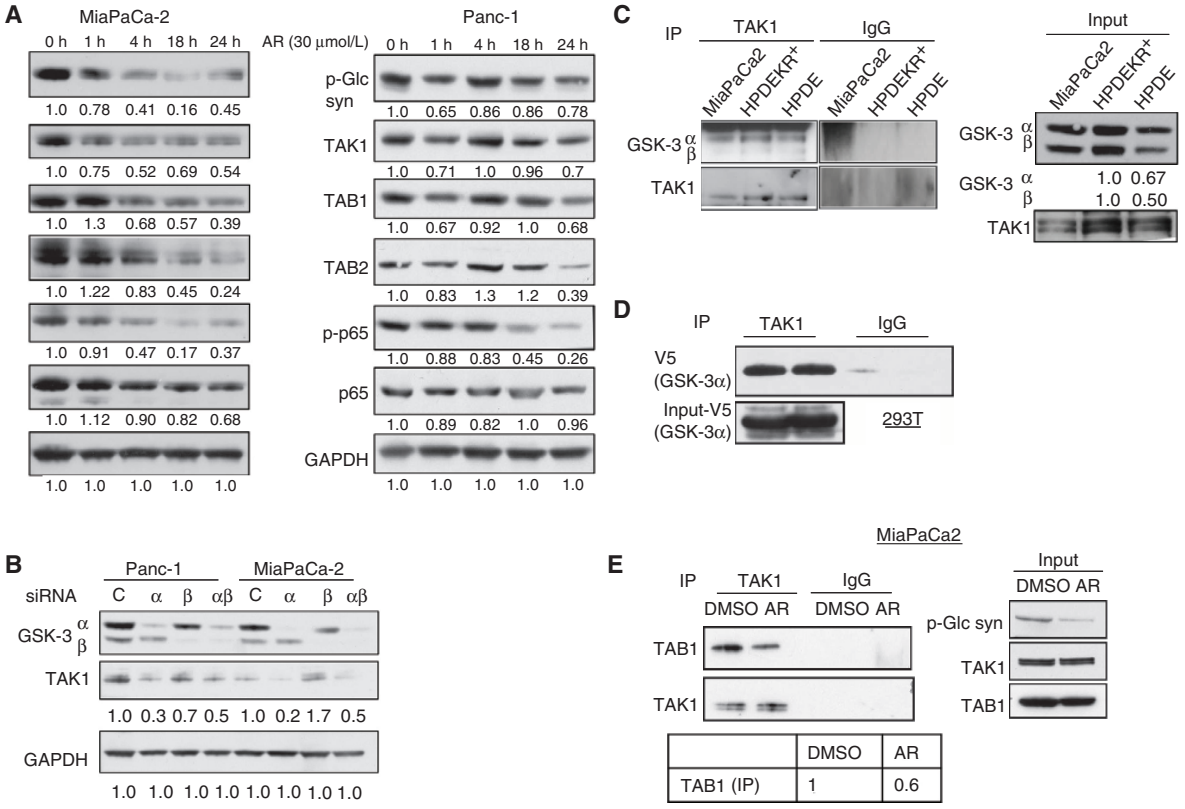


Figure 2.3. GSK-3 inhibition suppresses TAK1 levels. (A) Indicated cell lines were treated with GSK-3 inhibitor (AR-A014418) for indicated time periods and immunoblotting performed on whole cell lysates. Data is representative of 3 independent experiments. (B) Panc-1 and MiaPaCa-2 cells were transiently transfected with indicated siRNA for 48 hours, whole cell lysates harvested and immunoblotted against indicated antibodies. (C) Endogenous TAK1 was immunoprecipitated from the indicated cell lines and immunoblotted for GSK-3α and GSK-3β. (D) TAK1 and V5-tagged GSK-3α were transiently expressed in 293T cells for 24h. TAK1 was immunoprecipitated and blotted for V5. (E) MiaPaCa-2 cells were transiently transfected with TAK1 plasmid. 24h later they were treated with AR-A014418 for 24h. TAK1 was immunoprecipitated and blotted for TAB1.

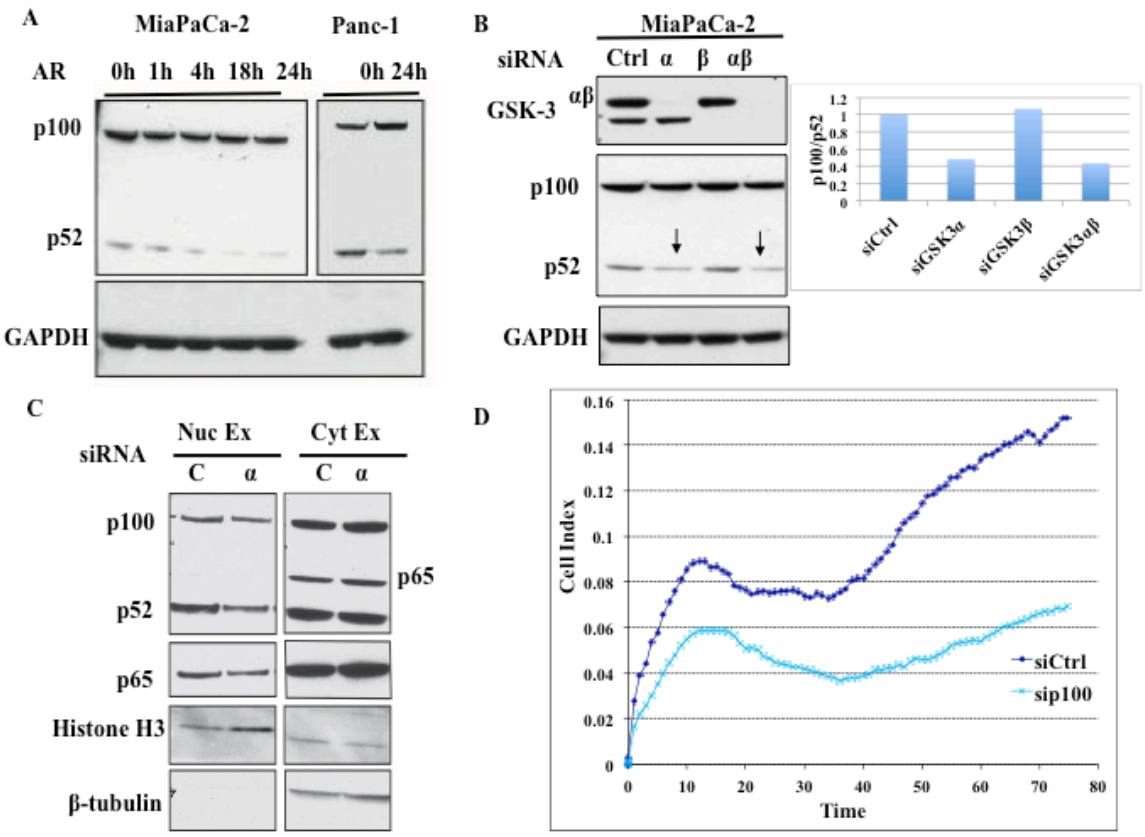


Figure 2.4. GSK-3α regulates the non-canonical NF-κB pathway in pancreatic cancer cells. (A) Panc-1 and MiaPaCa-2 cells were treated with DMSO or AR-A014418 (30μM) for indicated time periods and whole cell lysates immunoblotted with the indicated antibodies. (B) MiaPaCa-2 cells were transiently transfected with indicated siRNA for 72 hours and whole cell lysates immunoblotted for indicated antibodies. On right is quantitation of the western blot. (C) Panc-1 cells were transiently transfected with indicated siRNA for 72 hours, nuclear and cytoplasmic fractions harvested and immunoblotted for indicated antibodies. (D) Panc-1 cells were transiently transfected with indicated siRNA for 48 hours and cell impedance measured as above.

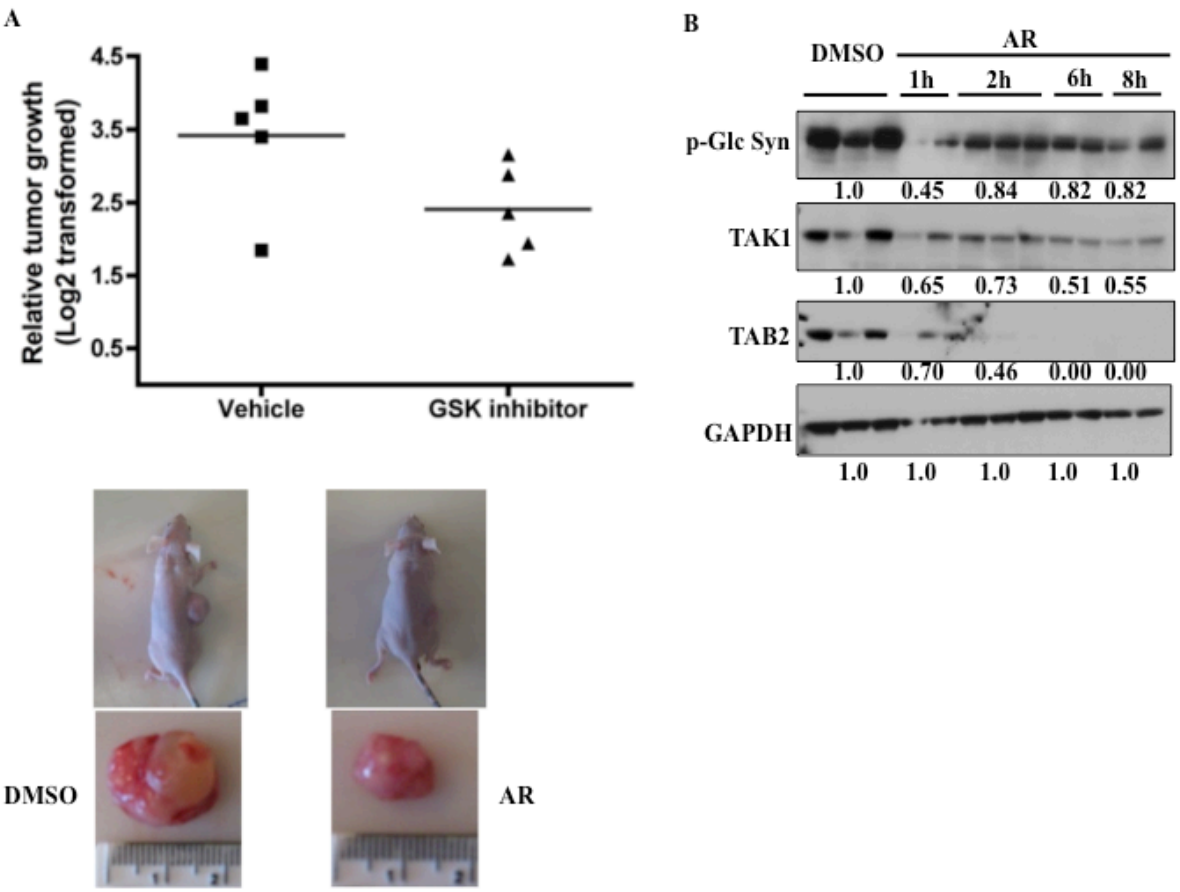
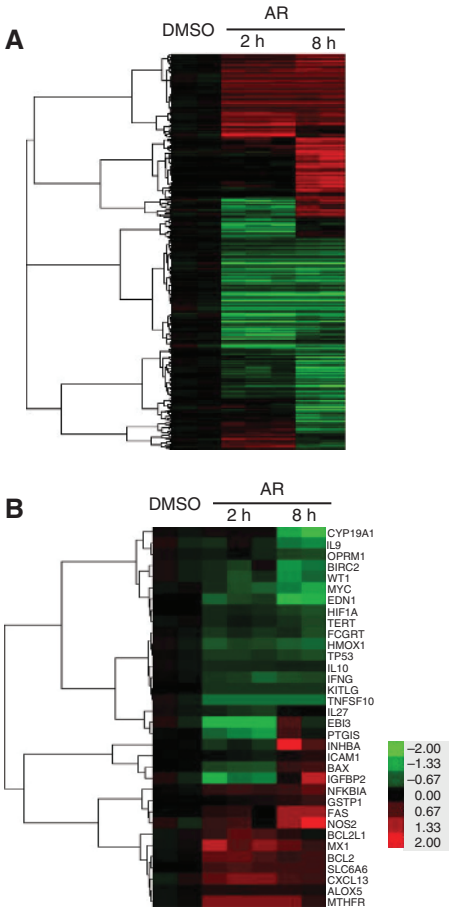


Figure 2.5. GSK-3 inhibition suppresses tumor growth in mice. (A) Nude mice were explanted with replicates of human pancreatic tumor and two weeks later treated with the GSK-3 inhibitor (AR-A014418) at 120mg/kg twice a day for two days (A) or the indicated time periods (B). (A). Tumor volume was measured over 28 days and is represented as the fold change in tumor volume compared to treatment start. n (vehicle)= 4, n (treatment) = 5. (lower panel) Representative photograph of mice at the end of 28 days. (B) Tumors were harvested after indicated time periods and immunoblotted with the indicated antibodies.



C

	Genes	Status in pancreatic cancer	Fold	Function
Txn factors and their modulators	*TP53*	Mutated in 50%–75% pancreatic cancer	1.43	Cell growth, apoptosis
	MYC	Overexpressed	2.22	Cell growth, apoptosis
	WT1	Overexpressed	1.92	Cellular development and survival
	HIF-1α	Overexpressed	1.5	Adaptive response to hypoxia
Regulators of apoptosis	*BIRC2 (cIAP2)*	High expression associated with shorter survival	2	Inhibitor of apoptosis
Cytokines/ Chemokines	*TNFSF10*	Pancreatic cancer resistant to Trail	1.9	Proapoptotic
	IL-9	NF-κB dependent transactivation	2	Antiapoptotic
	IFN-γ	Expressed	1.5	Innate and adaptive immunity
Enzymes	*TERT*	Highly expressed	1.36	Antiapoptotic
	HMOX1	Inhibition sensitizes to chemotherapy	1.7	Heme metabolism, O_2 sensor
	CYP19A1	Variants associated with KRAS tumors	2.8	Aromatase, biosynthesis of estrogen
Growth factors, ligands, and modulators	*KITLG*	Regulates growth in normal pancreas	1.3	Stem cell factor
Immunoreceptor	*FCGRT*		1.4	Receptor for Fc regions of IgG
Miscellaneous	*EDN1*	Mediates ischemia in acute pancreatitis	2.8	Vasoconstrictor peptide
	OPRM1		1.5	GPCR. Activates MAPK, PI3K, NF-κB, PKC

Figure 2.6. GSK-3 inhibition leads to changes in NF-κB target gene expression. Total RNA was isolated from human pancreatic tumor explants treated with AR-A014418 for 2h or 8h and evaluated by microarray. Gene changes that were statistically significant by SAM analysis are shown in the heat map ($p \leq 0.01$). Color key is for $\log_2$ ratio. (B) Statistically significant changes in known NF-κB target genes are shown in the heat map ($p \leq 0.05$). (C) NF-κB target genes that were downregulated >1.5 fold are listed with their known function in pancreatic cancer ($p \leq 0.05$).

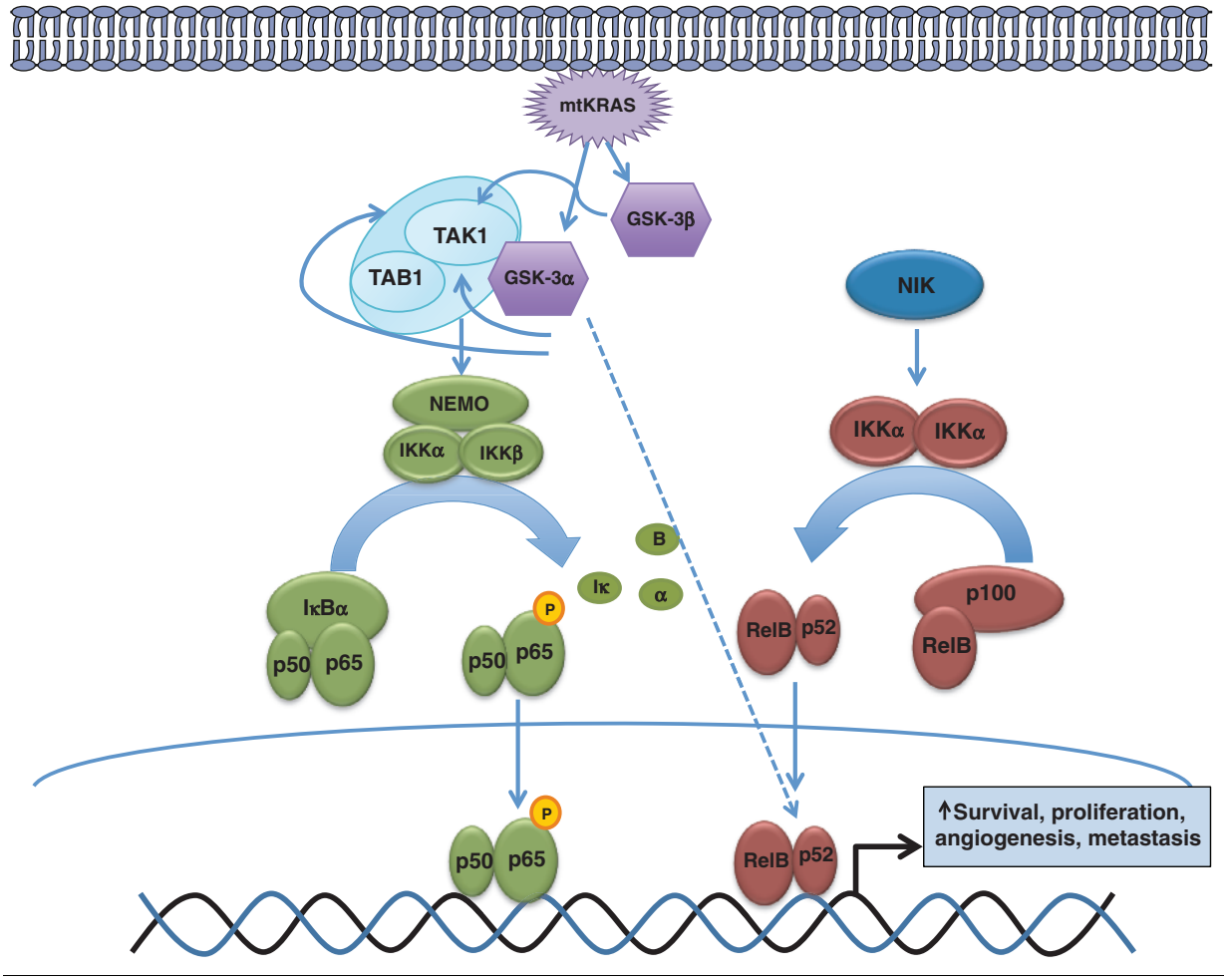


Figure 2.7. Model of GSK-3-NF-κB pathway downstream of mutant KRAS in pancreatic cancer. In a normal cell, TAK1-TAB1 complex is minimally active and NF-κB pathway is dormant. However, mutant KRAS leads to transcriptional upregulation of GSK-3α and GSK-3β, that can stabilize TAK1, TAB1 and TAK1-TAB1 complex leading to constitutive canonical NF-κB activation. GSK-3α also drives the non-canonical NF-κB pathway by promoting/stabilizing nuclear p52, thus leading to constitutive non-canonical NF-κB activation. NF-κB drives transcription of genes involved in survival, proliferation, metastasis that contributes to an aggressive pancreatic phenotype.

Supplementary Data - Additional Materials and Methods

Densitomentric analyses and statistics of immunoblots

Densitometric analyses was performed using adobe photoshop elements 9. The signal intensities were normalized to the respective loading controls and then compared to the respective control of the experiment. The differences were evaluated using Students two-tailed t test and P value <0.05 were considered significant.

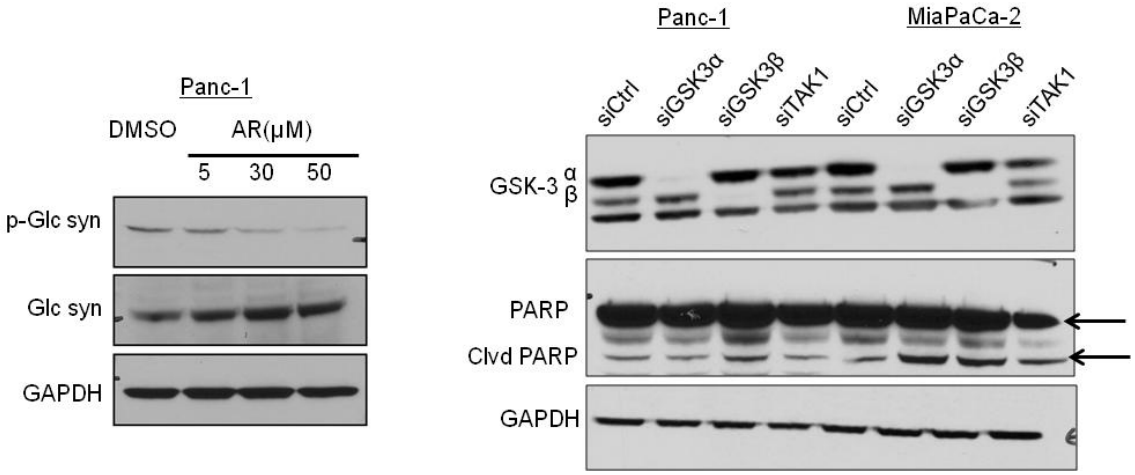
Cell cycle analysis

Panc-1 was seeded at 40,000 cells/6cm plates and treated for 24h with TAK1 inhibitor, 5Z-7-oxozaenol at the indicated concentrations. For FACS-based cell cycle analysis cells were fixed with 70% Ethanol after detachment, followed by centrifugation and resuspension in 100µl staining buffer (2% FBS, 1X PBS and 340 µg/ml RNAse) for 30 minutes at 37°C. Cells were then stained with 100µl Propidium Iodide labeling solution (50 µg/ml Propidium Iodide ,BD Biosciences) and incubated for 30 min at room temperature in the dark. Finally cells were analyzed by flow cytometry using a Beckman Coulter CyAn**.**

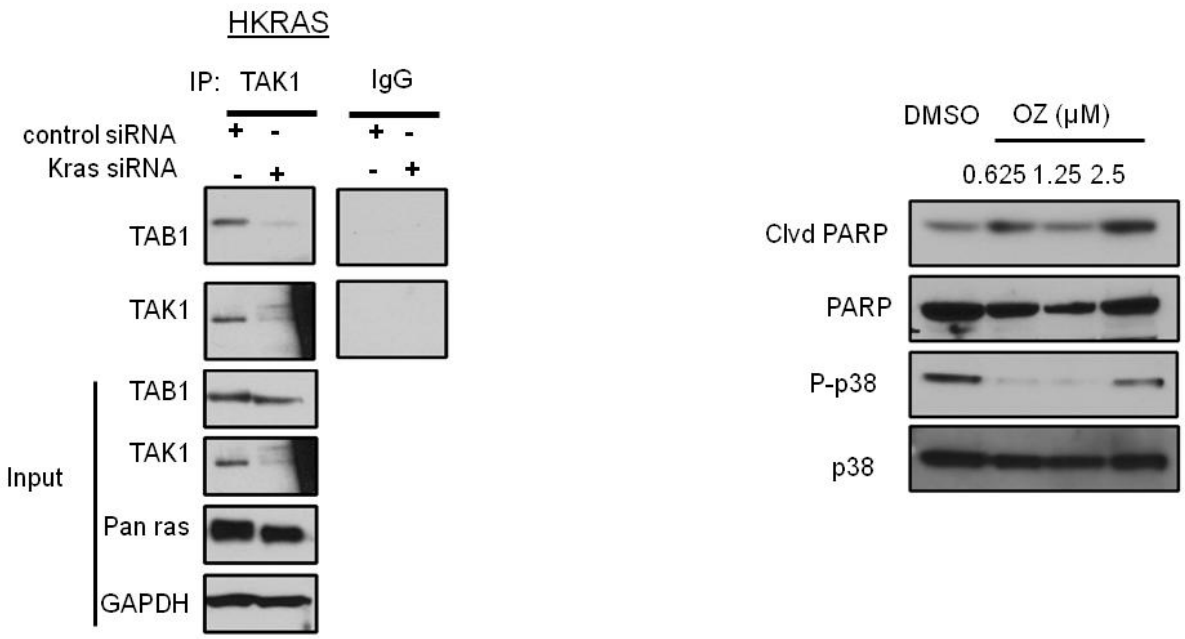
Real time RT-PCR

Total RNA was isolated using RNeasy Plus RNA Isolation kit (Qiagen , Germany) according to the manufacturer's protocol. Reverse transcription was conducted using 1 µg of total RNA using MMLV reverse transcriptase (Invitrogen, Carlsbad, CA)

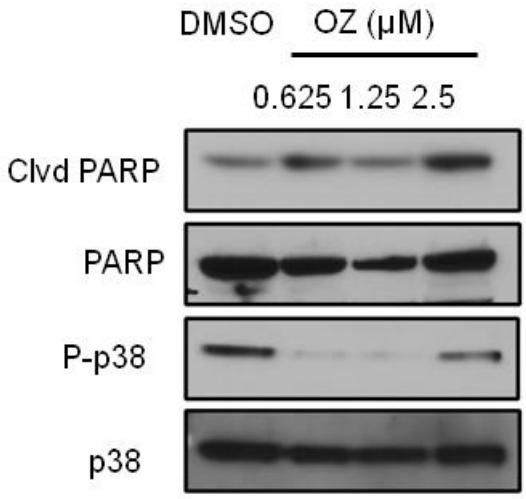
according to the manufacturer's protocol. Real time RT-PCR was then performed using TaqMan gene expression assays for Kras, GSK-3α and GusB (Applied Biosystems Inc., Forest City, CA) on an ABI 7500 real time PCR system (Applied Biosystems, Inc., Forest City, CA). GusB was used as endogenous control and siKras values were normalized to siCtrl.



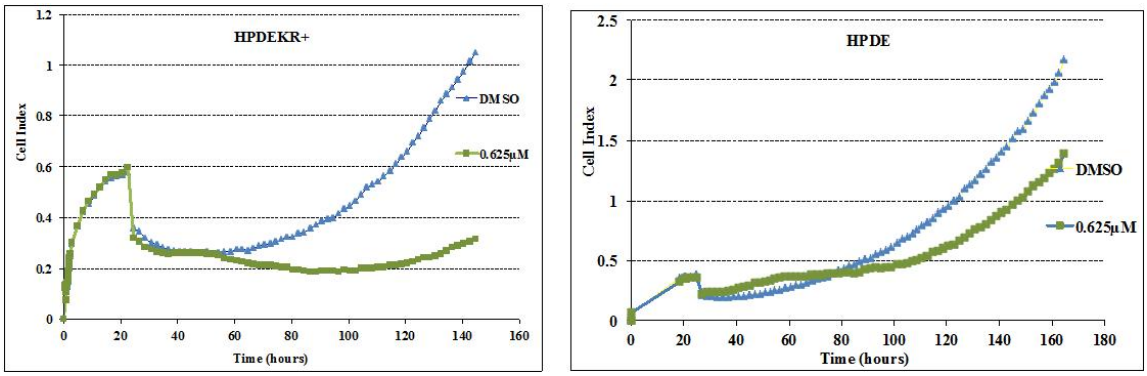
Supplemental Figure 2.1 Left: **AR-A014418 inhibits GSK-3 activity in a dose dependent manner**. Panc-1 cells were treated with increasing concentrations of GSK-3 inhibitor, AR-A014418, like in Fig.1A. Whole cells extracts were prepared and immunoblotted for specified antibodies. p-Glycogen synthase is a downstream substrate of GSK-3 and thus an indicator of its kinase activity.
Right: **GSK-3 regulates apoptosis in MiaPaCa-2 cells**. Panc-1 and MiaPaCa-2 cells were transiently transfected with indicated siRNAs for 72 hours. Whole cell extracts were immunoblotted for specified antibodies.



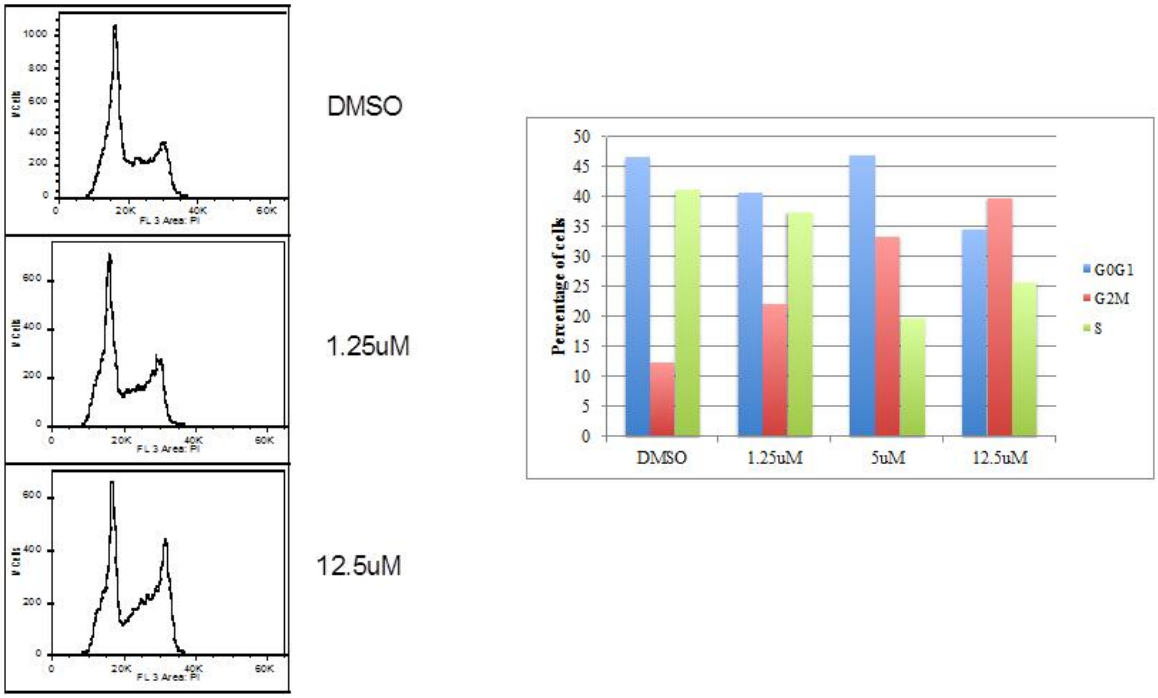
Supplemental Figure 2.2A Left: **KRAS regulates TAK1 levels**. HKRAS cells were transiently transfected with non-targeting or KRAS siRNA for 72 hours. TAK1 was immunoprecitated from whole cell extracts and immunoblotted for specified antibodies. Right: **TAK1 inhibition induces apoptosis**. MiaPaCa-2 cells were treated with the TAK1 inhibitor, 5Z-7-oxozaenol at indicated concentrations for 24 hours. Whole cells extracts were immunoblotted with specified antibodies.



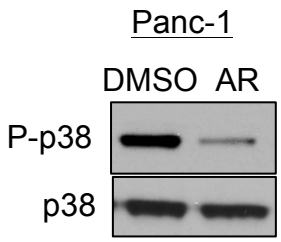
Supplemental Figure 2.2B. TAK1 inhibition induces apoptosis. MiaPaCa-2 cells were treated with the TAK1 inhibitor, 5Z-7-oxozaenol at indicated concentrations for 24 hours. Whole cells extracts were immunoblotted with specified antibodies.



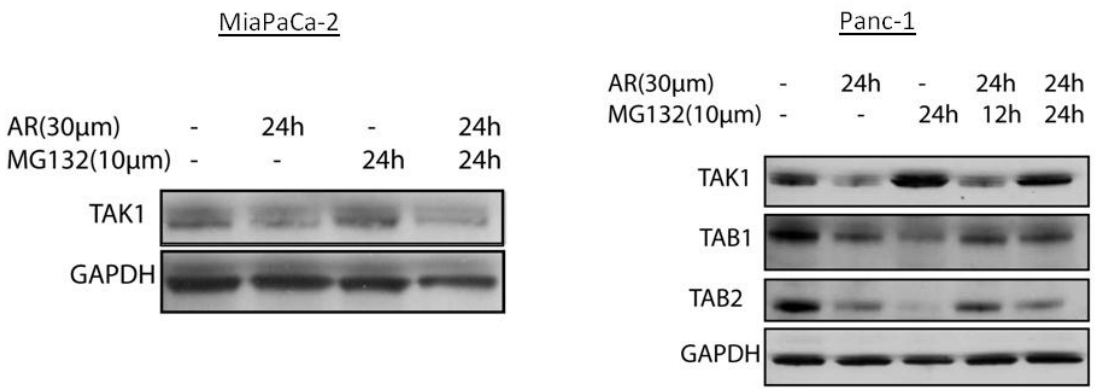
Supplemental Figure 2.3 TAK1 inhibition decreases cell proliferation in KRAS^{+} cells. Indicated cell lines were seeded in E-plate-16 (Roche) at 3000 cells/well/100µl. 24hours after plating, cells were treated with TAK1 inhibitor 5Z-7-oxozaenol (0.625µM), or vehicle control, DMSO. Cell impedance was measured every 2 hours for the entire course of experiment using RTCA.



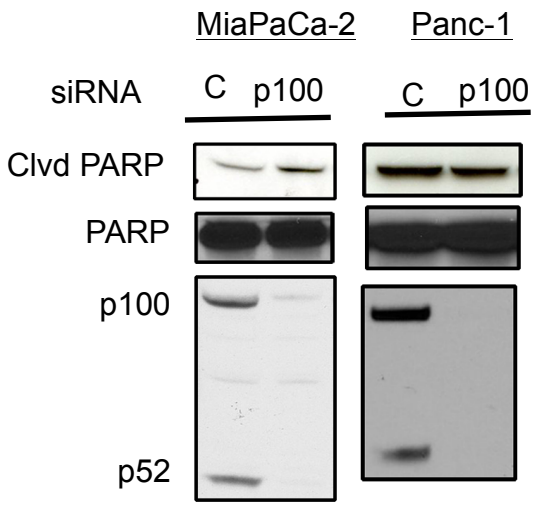
Supplemental Figure 2.4 TAK1 inhibition alters cell cycle regulation. Panc-1 cells were treated with TAK1 inhibitor, 5Z-7-oxozaenol or vehicle control, DMSO for 24 hours. Cells were fixed and stained with Propidium Iodide and analyzed by Flow cytometry.



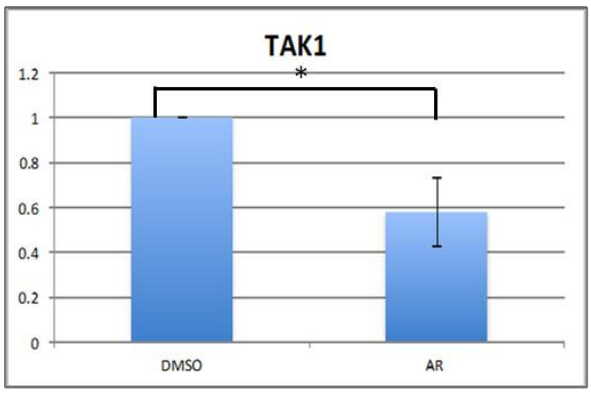
Supplemental Figure 2.5 GSK-3 inhibition inhibits TAK1 mediated signaling. Panc-1 cells were treated with GSK-3 inhibitor, AR-A014418 (30μM) for 24 hours and whole cell lysates immunoblotted for indicated antibodies.

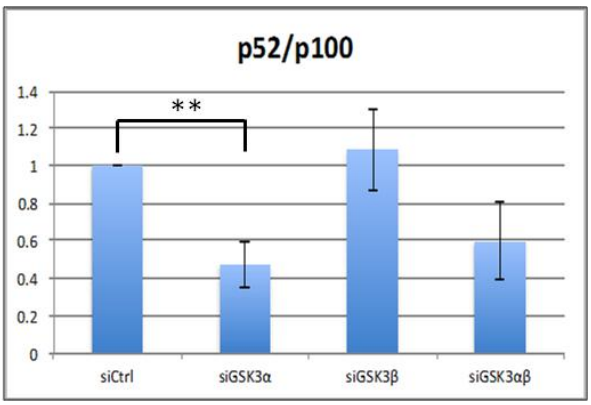


Supplemental Figure 2.6 GSK-3 inhibition leads to a decrease in TAK1 protein
Panc-1 and MiaPaCa-2 cells were treated with GSK-3 inhibitor, AR-A014418 or vehicle control, DMSO in the presence or absence of MG132 for 24 hours. Whole cell extracts were immunoblotted for indicated antibodies.

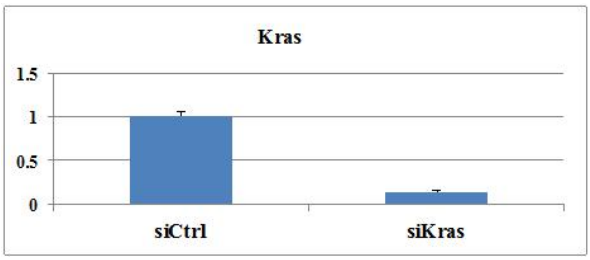


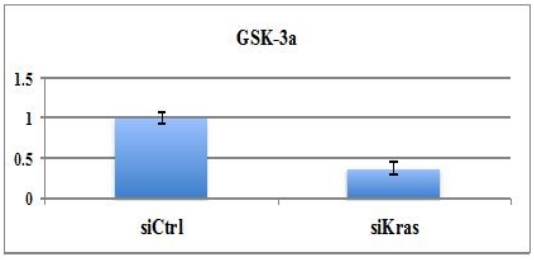
Supplemental Figure 2.7 NF-κB2 depletion leads to increase in PARP cleavage. Indicated cell lines were transiently transfected with specified siRNAs, and 72 hours later whole cell extracts immunoblotted for cleaved PARP.





Supplemental Figure 2.8 GSK-3 inhibition leads to a decrease in TAK1 levels and p100-p52 processing in a statistically significant manner. All data obtained with independent experiments with Panc-1 and MiaPaCa-2, treated with GSK-3 inhibitor for 24 hours (right panel) or transiently transfected with indicated siRNA's for 72 hours (left panel) were quantified by densitometric analyses and is expressed as mean±SD. Statistical evaluation of the differences were calculated using students two-tailed t test and P value < 0.05 was considered significant.





Supplemental Figure 2.9 KRAS regulates GSK-3α mRNA levels. HKRAS cells were transiently transfected with non-targeting control or KRAS siRNA. RNA was isolated, reverse transcribed to cDNA and real time RT-PCR performed for KRAS and GSK-3α. Gus B was used as endogenous control.

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CHAPTER III

A REQUIREMENT FOR TBK1 IN KRAS-INDUCED LUNG CANCER

3.1 Summary

KRAS dependent lung cancer cells have been proposed to be addicted to the presence of Tank Binding Kinase 1 (TBK1) via regulation of c-Rel activity and basal autophagy. However the study of TBK1 function *in vivo* has been deterred by the embryonic lethal phenotype of TBK1 deficient mice. To determine if TBK1 is an important regulator of lung tumorigenesis *in vivo*, we generated a mouse model where activation of oncogenic K-RAS in lung cells was coupled with the inactivation of TBK1. Using this model system, we show that deletion of TBK1 reduces the number of K-RAS–induced lung tumors in the absence of the tumor suppressor p53. Loss of TBK1 in the neoplastic lesions also led to a reduction in p-AKT, a known regulator of pro-survival signaling in oncogensis. These studies demonstrate the importance TBK1 in KRAS-induced lung tumorigenesis and identify it as a potential therapeutic target for this disease.

3.2 Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide (1). Non-small-cell lung cancer (NSCLC) accounts for 85% of all lung cancer cases, with

adenocarcinoma being the major subtype. NSCLC development is associated with frequent mutations in a few well-defined oncogenes and tumor suppressor genes. Oncogenic mutations in KRAS are found in ~30% of NSCLCs, c-MYC is up-regulated in 20%–30%, whereas the tumor suppressor gene p53 is mutated or deleted in ~50%, and the INK4A/ARF locus is often deleted or hypermethylated (2,3). Furthermore, KRAS mutations are found in >90% of spontaneous and chemically induced mouse lung tumors (2). Such diverse mutations invariably affect responsiveness to standard therapies and increase the complexity of effective targeting.

Effective inhibition of KRAS activity has been shown to revert malignant cells to a non-malignant phenotype, however cancer therapies aimed at direct inhibition of KRAS activity have so far been unsuccessful (4). An alternative strategy to target KRAS-induced tumors is to identify and target specific molecular vulnerabilities conferred by the genetic background of oncogenic KRAS mutations. In this regard, Tank Binding Kinase 1 (TBK1) has been identified as a critical regulator of the survival and/or proliferation of KRAS mutated NSCLC cells (5-7).

TBK1 was originally described as an NF-κB activating kinase (8) based on the fact that mice deficient for *TBK1* died from massive liver apoptosis *in utero* (9), a phenotype similar to the mice deficient for *p65, IKK-β or NEMO* (10). Subsequent studies disclosed a crucial role for TBK1 in the innate immune response where it activates interferon regulatory factor 3 (IRF3), in response to Toll-like receptor activation (10). Increasing evidence now indicates that aberrant activation of TBK1 is associated

with development of human cancers downstream of oncogenic KRAS. In a systematic RNAi screening study Barbie et al., demonstrated that TBK1 is specifically required for survival of lung cancer cells harboring oncogenic KRAS mutations (5). The observation that TBK1-null mouse embryonic fibroblasts (MEF) displayed increased cell death, and decreased cell transformation upon oncogenic RAS expression, further underscores the role of TBK1 in tumorigenesis (11). Furthermore, TBK1 has been shown to promote pro-survival AKT signaling (12,13), to regulate basal autophagy (6) and to control autocrine CCL5 and IL-6 signaling in lung cancer cells (7). Collectively, these studies highlight the importance of TBK1 in oncogenesis. However, because of the embryonic death of mice with global *TBK1* deficiency, the *in vivo* biological functions of TBK1 have largely remained unknown. Recently two conditional TBK1 deficient mouse models were generated to study the role of TBK1 in innate immunity but its role in oncogenesis *in vivo* has not been investigated.

Here, I provide the first description of the role of TBK1 in lung cancer *in vivo.* Preliminary data shows that genetic deletion of *TBK1* in KRAS-induced lung cancer mouse model reduces lung tumorigenesis in the absence of the tumor suppressor p53. The loss of TBK1 led to a reduction in both tumor size and number. Consistent with the previous reports of TBK1's role in promoting AKT signaling, we saw a decrease in oncogenic KRAS dependent p-AKT in the transformed lung epithelial cells of TBK1 deficient mice. Overall, these results show TBK1 as an important KRAS oncogenic effector in lung cancer making TBK1 an attractive drug target to approach KRAS related malignancies.

3.3 Materials and Methods

Generation of *TBK1*$^{fl/fl}$ C58BL/6 mice

TBK1 was targeted in E14Tg2a.4 ES cells (derived from the 129P2/Ola mouse strain) by a Cre/lox conditional deletion strategy at TransViragen (UNC-Chapel Hill, USA) using the *TBK1*^{fl} construct depicted in **Fig. 1A**. This *TBK1*^{fl} construct contains LoxP sites flanking *TBK1* exon 6 and a *NEO*^{R} selection cassette. Appropriately targeted ES cells were identified by Southern analysis using 5' external, 3' external and neomycin probes. The 5' external probe, produced using primer-1, 5'-CACGCCCCAACTTTGTTTCC-3', and primer-2, 5'-TGGAAGTCCATACGCATTGG-3', was hybridized to BamHI-digested genomic DNA, with a 13.1 kb band detected for the WT allele and a 9.3 kb band detected for the targeted allele. The 3' external , produced using primer-1, 5'-CTGTTGTTGCTGTGACAGCC-3', and primer-2, 5'-CCAGCTTGTTACCCTCTTGC-3', was hybridized to EcoRV-digested genomic DNA, with a 19.7 kb band detected for the WT allele and a 16.5 kb band detected for the targeted allele. With the neo probe, produced using primer-1, 5'-TGACAATTAATCATCGGCATAGTATATCGG-3', and primer-2, 5'-AAGAACTCGTCAAGAAGGCGATAGA-3', targeted, unexcised DNA produced a 12.6kb band and no band was produced with the WT DNA. ES cells carrying the *TBK1*^{fl} allele were treated with Flp to remove the neo cassette and clones that had neo deleted were injected into C57BL/6 blastocysts to generate chimeras that subsequently transmitted the *TBK1*^{fl} allele without the Flp to the F1 generation in crosses with C57BL/6 mice. These mice were heterozygous for Flpe and were backcrossed with

C57BL/6 to obtain mice carrying *TBK1*^{fl} allele without Flpe (confirmed using Flp genotyping assays as below). To distinguish between WT, targeted (with NEO cassette), conditional (without Ne cassette) and deleted *TBK1* allele, mice were genotyped by PCR of tail genomic DNA using the primers and genotyping assays shown in Table 1. The location of each primer with respect to TBK1 allele is shown in Fig.1B. The mice were backcrossed to the F6 generation in crosses with C57BL/6.

Generation of *KRAS*$^{LSL\text{-}G12D/WT}$*p53*$^{flox/flox}$ *TBK1*$^{fl/fl}$ C57BL/6 mice

The *TBK1*$^{fl/fl}$ were crossed with *KRAS*$^{G12D/+}$ *p53*$^{fl/fl}$ (KF*)* mice to generate *TBK1*$^{fl/fl}$ *KRAS*$^{G12D/+}$ *p53*$^{fl/fl}$ (TKF) mice. Mice were genotyped by PCR of tail genomic DNA using the primers and genotyping assays shown in Table1.

Animal husbandry and Cre-expressing adenovirus administration

KRAS$^{LSLG12D/WT}$ *p53*$^{flox/flox}$ mice (14) (2), and mice generated by interstrain crossings were housed in pathogen-free conditions according to the protocols approved by the University of North Carolina Institutional Animal Care and Use Committee. Lung tumor induction was performed by intranasal administration of 1×10^7 plaque-forming units of Cre-expressing adenovirus (adenocre; Gene Transfer Vector Core, University of Iowa, Iowa City, IA) in selected animals at 8 weeks of age, as described (14).

Western blotting and immunohistochemistry

Western blotting and immunohistochemistry were performed as described (14). The antibodies used were as follows: anti–phospho-AKT- Ser^{473} and GAPDH from

Santa Cruz; TBK1 and IKKε from Cell Signaling and F4/80 from Abcam. The immunohistochemistry slides were scanned and scored using aperio cytoplasmic algorithm. This tool scores the intensity of staining in the cytoplasm/nuclei in terms of a H-score. H score is calculated using formula: H-Score = (% at 0) * 0 + (% at 1+) * 1 + (% at 2+) * 2 + (% at 3+) * 3. This score produces a continuous variable that ranges from 0 to 300.

Histopathologic analysis

Histopathological analysis was done as described (14). Briefly, mice were euthanized by intraperitoneal administration of 250 mg/kg Avertin followed by surgical resection of the portal vein. Lungs were perfused with saline and inflation fixed overnight with 10% formalin. Fixed tissues were embedded in paraffin, sectioned at 5-μm thickness, and stained with H&E.

Tumor number and grade analysis

Tumor slides from each lung lobe (same orientation and level section used for each lobe) were scored for number and size blindly using aperio imagescope.

Detection of Cre-mediated recombination

Cre-mediated recombination of the TBK1 conditional allele was detected by PCR amplification with primers flanking the deleted site (sequences given in table 1). Amplification of the wild- type (WT) allele results in a ~2.5kb product, while the amplification of the deleted allele results in a 146-bp product . Amplification was performed by denaturation at 95°C for 1 minute, followed by 35 cycles of amplification at 98°C for 10 seconds, 52°C for 30 seconds, and 72°C for 90 seconds, with a final

extension step of 10 minutes at 72°C.

RNA isolation and real-time PCR analysis

Total RNA was purified using RNeasy Plus RNA Isolation Kit (Qiagen) as previously described (15). Real-time PCR analysis was performed in an ABI 7000 Sequence Detection System as previously described (16) using Taqman Gene Expression Assay primer-probe sets (all from Applied Biosystems) for TBK1 (exon6-7; Mm01156588_m1), TBK1 (exon 4-5 Mm00451150_m1), Relative quantitation was determined by the $\Delta\Delta C_t$ method using GAPDH (Mm99999915_g1) as the endogenous control.

3.4 Results

Generation of TBK1$^{fl/fl}$ mice

Conventional TBK1-/- mice die at embryonic day 14.5 due to massive liver degeneration (9). We, thus, used a conditional deletion strategy, detailed in Fig. 1A to create a TBK1-deficient mouse model. 129P2ES cells were generated that harbor a TBK1 conditional knockout allele (TBK1^{fl}) with LoxP sites flanking exon 6 and a NEO^R targeting cassette. The NEO^R targeting cassette was flanked by FRT recombination sites to allow removal by FLPe recombinase. Exon 6 encodes a part of activation loop in the kinase domain and upon removal causes a frame shift, leading to complete gene inactivation. Appropriately targeted ES cells were electroporated with FLPe recombinase to remove the NEO^R cassette (17). These ES cells were injected into C57BL/6 blastocysts

to generate chimeric mice. Chimeric mice heterozygous for the *TBK1*^{fl} allele and FLPe without the NEO^{R} cassette were mated with a line of transgenic C57BL/6 mice to remove FLPe. Progenies of this mating that carry the *TBK1*^{fl} allele without FLPe and NEO^{R} cassette were interbred to produce *TBK1*$^{fl/fl}$ mice. The resulting *TBK1*$^{fl/fl}$ mice were infected by intranasal administration of adenocre to delete TBK1 specifically in lung epithelial cells. To confirm that recombination of the conditional TBK1 allele occurred in the lungs of TBK1$^{fl/fl}$ mice, we measured excision by PCR in DNA isolated from whole lungs or tails of mice 1 week post-infection (Fig. 2A). A 419-bp product was predicted assuming the correct splicing of exon 5 to exon 7 and it was only observed in the DNA from lungs of adenocre infected TBK1$^{fl/fl}$ mice (Fig. 2A). However, no change in the levels of TBK1 protein was observed by immunoblotting on extracts prepared from whole lungs 3 days, 1 week or 2 week post-infection with adenocre (Fig. 3B). This can be attributed to the inefficient adenocre infection of lung epithelial cells. RT-PCR performed was performed on RNA from the above lungs using primers spanning exons 4/5 or exons 6/7. The latter revealed a reduction in TBK1-specific transcripts post-infection with adenocre (Fig. 2C).

Generation of a TBK1 deficient model of KRAS- induced lung cancer

To examine the role of TBK1 activity in KRAS- induced lung cancer, we generated a mouse model of KRAS and p53– induced lung cancer with loss of *TBK1*. To generate this model, we used oncogenic KRAS–inducible *KRAS*$^{LSL\text{-}G12D/WT}$ *p53*$^{flox/flox}$ mice (referred to as KF mice), where expression of oncogenic *KRAS*^{G12D} is triggered

by Cre recombinase-mediated removal of the LSL element with concomitant loss of p53 floxed allele (2,14,18). Activation of oncogenic $KRAS^{G12D}$ induces lung tumor formation(2) and the loss of tumor suppressor p53 leads to generation of lung tumors with a shorter latency and advanced histopathology (19,20) and does not affect the ability of NF-κB to potentiate KRAS-induced lung tumorigenesis (14,21). The $KRAS^{LSL\text{-}G12D/WT}$ $p53^{flox/flox}$ mice were then bred to $TBK1^{fl/fl}$ mice to generate $TBK1^{fl/fl}$ $KRAS^{LSL\text{-}G12D/WT}$ $p53^{flox/flox}$ (referred to as TKF mice). TKF and KF mice were infected by intranasal administration of adenocre to activate oncogenic $KRAS^{G12D}$, inactivate p53 and TBK1 specifically in lung epithelial cells and induce lung tumor formation. It is important to note that only one mouse each for TKF and KF group was used in the present study.

Deletion of TBK1 in the mouse lung decreases KRAS-induced lung tumorigenesis

Given that TBK1 has been reported to support KRAS-driven oncogenesis especially in the context of lung cancer (5,7,11), we asked if genetic deletion of the TBK1 gene in the KF mouse model affects KRAS induced oncogenesis. Lungs of KF and TKF mice from above, were infected with adenocre to activate K-Ras^{G12D} and inactivate p53 and TBK1 simultaneously ($TBK1^{\Delta}/KRAS^{G12D}/p53^{\Delta}$ mice).

As evaluated by pathologic examination of dissected lungs at 13 weeks postinfection, $TBK1^{\Delta}/KRAS^{G12D}/p53^{\Delta}$ mouse displays a reduced number of tumors than $TBK1^{WT}/KRAS^{G12D}/p53^{\Delta}$ mouse (Fig. 3A). Interestingly, loss of TBK1 affected not only the number of lesions formed but reduced their size and thus the percentage of lungs

covered with tumors (Fig. 3B,C). As revealed by immunohistochemical studies, the neoplastic lung lesions found in *TBK1*$^{\Delta}$/*KRAS*^{G12D}/*p53*$^{\Delta}$ mouse lacked TBK1 expression (Fig. 3D,E) confirming that recombination of the conditional TBK1 allele occurred in the lungs of *TBK1*$^{\Delta}$/*KRAS*^{G12D}/*p53*$^{\Delta}$ mouse. As these results indicate, although not absolutely required for tumor formation, TBK1 greatly potentiates KRAS–induced transformation in the lung *in situ*.

Deletion of TBK1 in the mouse lung does not affect the levels of IKKε

The non canonical IκB kinaseε (IKKε) is the closest TBK1 homolog and has been implicated to play an important role in RAS-induced oncogenesis (22,23). Thus, we asked if TBK1 loss led to a compensatory increase in IKKε. As seen in Fig. 4, no difference was observed in IKKε neoplastic lesions or the whole tissue of *KRAS*^{G12D}/*p53*$^{\Delta}$ *and TBK1*$^{\Delta}$/*KRAS*^{G12D}/*p53*$^{\Delta}$ mice.

Deletion of TBK1 in the mouse lung decreases p-AKT in KRAS induced lung tumors

AKT signaling is a well established pro-survival pathway downstream of oncogenic RAS (3,4). Consistent to activation of KRAS, *KRAS*^{G12D} mice have been associated with increased levels of phospho-AKT (14). Recently studies have identified AKT as a novel substrate of TBK1 that mediates TBK1's prosurvival role in oncogenesis (12,13). To gain insight into the regulation of AKT by TBK1 in KRAS-induced lung tumorigenesis, we investigated if the loss of TBK1 in this mouse model affects p-AKT levels. Consistent with previous findings, p-AKT was high in the *KRAS*^{G12D}/*p53*$^{\Delta}$

neoplastic lesions, whereas reduced p-AKT staining was observed in the *TBK1*$^{\Delta}$/*KRAS*^{G12D}/*p53*$^{\Delta}$ lesions (Fig. 5).

3.5 Discussion and Future Directions

Mutant KRAS occurs frequently in lung cancers where it serves as a key oncogenic factor, promoting survival and chemoresistance (3,24). Despite the presence of several targeted therapeutics (EFGR and VEGF inhibitors) lung cancer remains the leading cause of cancer related deaths worldwide (1). Because direct inhibition of KRAS has been unsuccessful, current research focuses on oncogenic effectors downstream of mutant KRAS, (25-27), (7,21,28).

TBK1, has been implicated in KRAS driven oncogenesis where it has been shown to be required for transformation by oncogenic KRAS(11) , sustain KRAS-dependent cancer cell viability (5), and regulate autocrine cytokine signaling (7) and basal autophagy (6). Thus TBK1 has become an attractive target for novel chemotherapeutic approach in lung cancer and other KRAS driven malignancies. However, studies of TBK1 function *in vivo* have been hindered because of the embryonic death of mice with global *TBK1* deficiency. Here, we provide the first description of the effect of *TBK1* deletion in a KRAS-induced mouse model of lung cancer.

Consistent with the previous studies, we show that Cre-generated deletion of *TBK1* in parallel with *KRAS*^{G12D} activation and *p53* deletion in the lung epithelial cells, led to reduced transformation as analyzed by tumor number and size (Fig 2). Importantly, the loss of *TBK1* only reduces, but does not eliminate tumorigenesis

downstream of oncogenic KRAS expression. This can be attributed to the incomplete deletion of TBK1 gene in the lung, due to the inefficient Cre infection of lung epithelial cells. Alternatively, IKKε, the closest homolog of TBK1 may play a compensatory role in driving the oncogenesis. IKKε has been shown to be important in oncogenic process downstream of KRAS in ways very similar to that described for TBK1 (7,13,29). We thus hypothesized that TBK1 loss may lead to an increase in IKKε levels, driving the oncogenic signaling in TKF mice. However, we did not observe any difference in IKKε levels between $KRAS^{G12D}/p53^{\Delta}$ and $TBK1^{\Delta}/KRAS^{G12D}/p53^{\Delta}$ mice as measured by immunohistochemistry (Fig. 4) suggesting that basal levels of IKKε signaling or other pathways activated downstream of KRAS are sufficient to promote lung tumorigenesis in the absence of TBK1.

It is important to note that the KRAS induced lung cancer mouse model used in the present study also had a deletion of the tumor suppressor p53. Loss of p53 is known to generate tumors faster and of a higher grade but it does not affect the number of lesions formed(14,19). Since we observed a reduction in the number of tumors in the absence of TBK1 (Fig. 2A), it suggests that TBK1 may play role in KRAS-driven tumorigenesis independent of the status of the tumor suppressor p53. However, future studies in p53 wild type mice are required to ascertain the effect of TBK1 loss on the development of KRAS-induced lung cancer in the presence of p53.

TBK1 loss also led to a decrease in p-AKT in the neoplastic lesions (Fig. 5), in conjunction with the previously described role of TBK1 in promoting AKT signaling (12,13). This suggests that downstream of KRAS, TBK1 may be utilizing the pro-

survival AKT signaling to promote tumorigenesis but will require further studies in more animals for confirmation.

Apart from TBK1, IKKε and AKT, oncogenic mutations in KRAS drive tumor growth by engaging multiple downstream pathways, including RAF-MAPK (30), RAL-GEF (26,27) and inflammatory signals such as NF-κB. As mentioned above, TBK1 was originally described as NF-κB activating kinase, and is also known to activate IKKβ (10). Work from our lab and others have previously shown that NF-κB subunits, p65 and c-Rel are both important in the survival of KRAS- transformed cells (21,and 28,31). Barbie et al., implicated the significance of c-Rel downstream of TBK1 in survival of KRAS transformed cells (5). Furthermore, activated NF-κB (p65) has been shown to promotes oncogenic process in $KRAS^{G12D}/p53^{\Delta}$ mouse model (14,21). These studies are consistent with a model where both NF-κB subunits would be activated by KRAS to promote oncogenesis. Thus it would be important to analyze and compare the activation of NF-κB subunits, p65 and c-Rel in the lung epithelial cells of TKF and KF mice post-infection with adenocre.

Collectively, this study provides the first evidence of the role of TBK1 in promoting KRAS-driven lung cancer *in vivo*. Although concurrent MAPK/PI3K pathway inhibition is under clinical evaluation, multiple approaches are likely necessary to identify effective KRAS targeted therapy. In this regard TBK1 seems to be a promising drug target to approach KRAS-induced lung cancer because the loss of TBK1 seemingly impairs lung tumor formation. This is in agreement with the previous studies implicating TBK1 importance downstream of oncogenic KRAS (5-7,11,32). However, due to the

limited number of animals used in the study (only 1 per test and control group) further studies with more animals need to be done to obtain a statistically significant result. Additionally, further studies looking at the activation of NF-κB, cytokine signaling, apoptosis and mitotic rate will be required to ascertain the mechanisms employed by TBK1 to potentiate transformation.

FIGURES

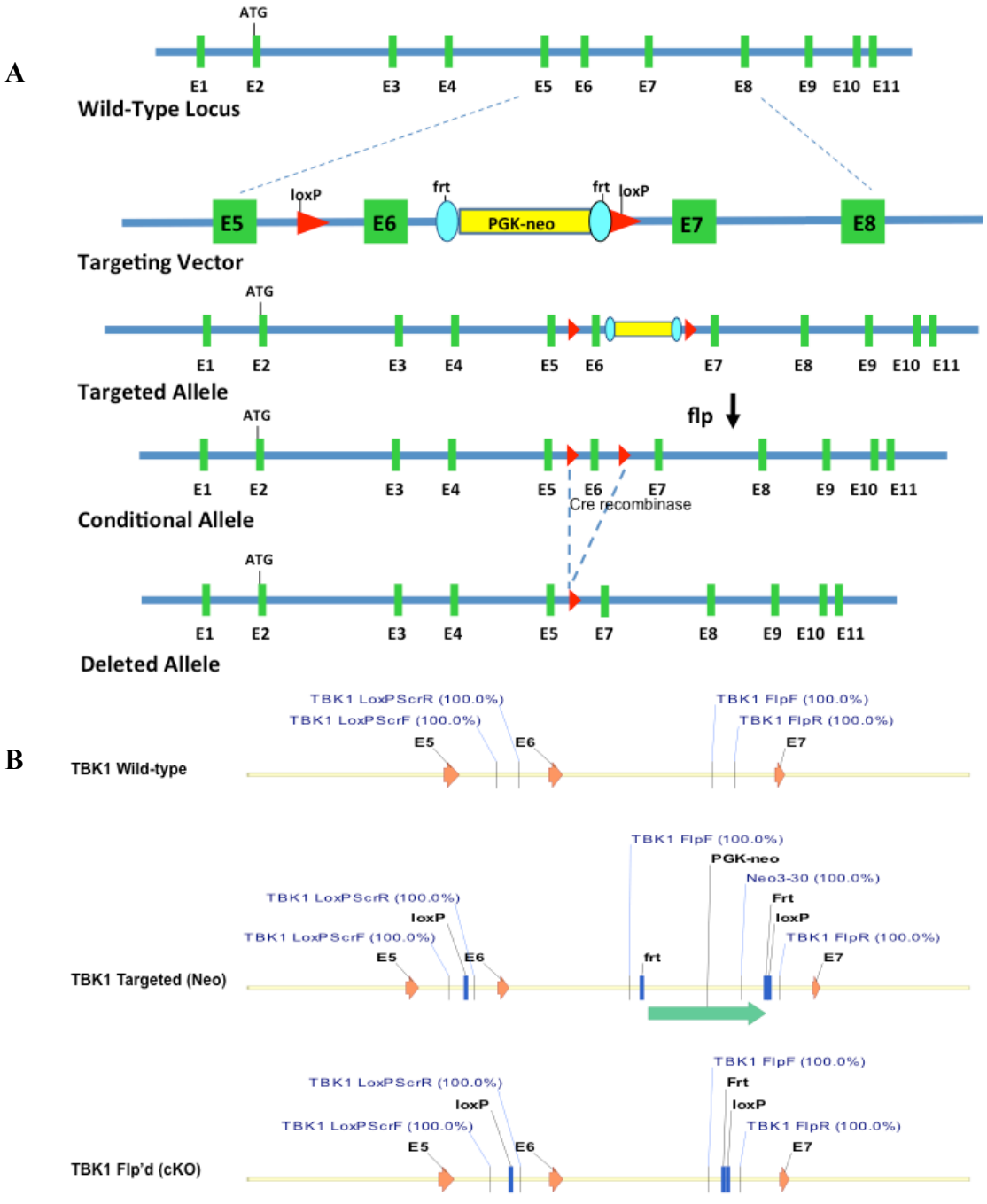


Figure 3.1 Generation of TBK1$^{fl/fl}$ mice. (A)The structure of the *TBK1* gene, the targeting vector, the *TBK1^{fl}* allele, and the deleted TBK1 allele is shown. Yellow box indicates the neomycin-resistance cassette which is flanked by FRT recombination sites. (B) The location of primers used for genotyping *TBK1$^{fl/fl}$* mice with the presence or absence of *NEO^{R}* and *FLPe*

#	Primer	Sequence
1	Neo3-30	5′-CGCATCGCCTTCTATCGCCTTCTTGACGAG-3′
2	TBK1 LoxPF	5′-TTATGTGCATTGATGTTTTACCTGC-3′
3	TBK1 LoxPR	5′-ATTTGCTGCATGCCATCCAA-3′
4	TBK1 FlpF	5′-CTCCATGGAGGAAGAATTAG-3′
5	TBK1 FlpR	5′-AGATGCTCTACGACTATCCA-3′
6	FlpeF	5′-CACTGATATTGTAAGTAGTTTGC-3′
7	FlpeR	5′-CTAGTGCGAAGTAGTGATCAGG-3′
8	Flpe wtF	5′-TGTTTTGGAGGCAGGAAGCACTTG-3′
9	Flpe wtR	5′-AAATACTCCGAGGCGGATCACAAG-3′

Flpe Genotyping Assays	WT *TBK1* *allele*	cKO *TBK1* allele
FlpeF + FlpeR	-	725
Flpe wtF + Flpe wtR	500	-

TBK1 Genotyping Assays	WT *TBK1* allele	Targeted *TBK1* allele	cKO *TBK1* allele	Deleted *TBK1* allele
Neo3-30 + TBK1 FlpR	-	575	-	-
TBK1 LoxPF + TBK1 LoxPR	291	388	388	-
TBK1 FlpF + TBK1 FlpR	285	-	401	-
TBK1 LoxPF + TBK1 FlpR	-	-	-	419

Table 3.1 Primers used for genotyping *TBK1$^{fl/fl}$* mice. The sequence of the primers used for genotyping *TBK1$^{fl/fl}$* mice with and without *NEO^{R}* and *FLPe* and the genotyping assays are indicated.

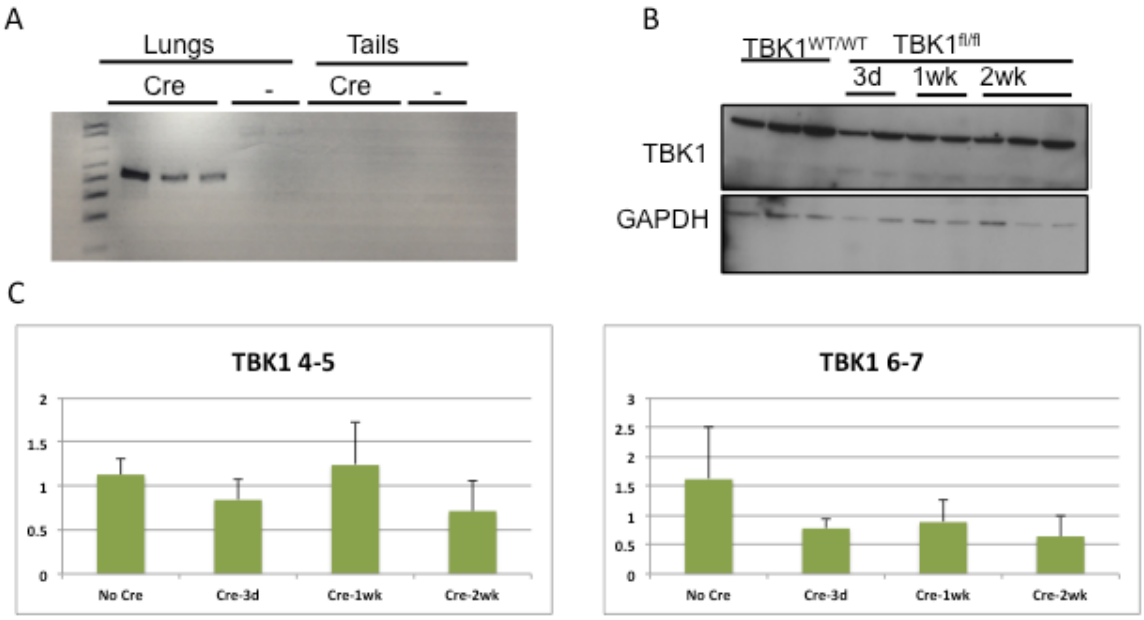
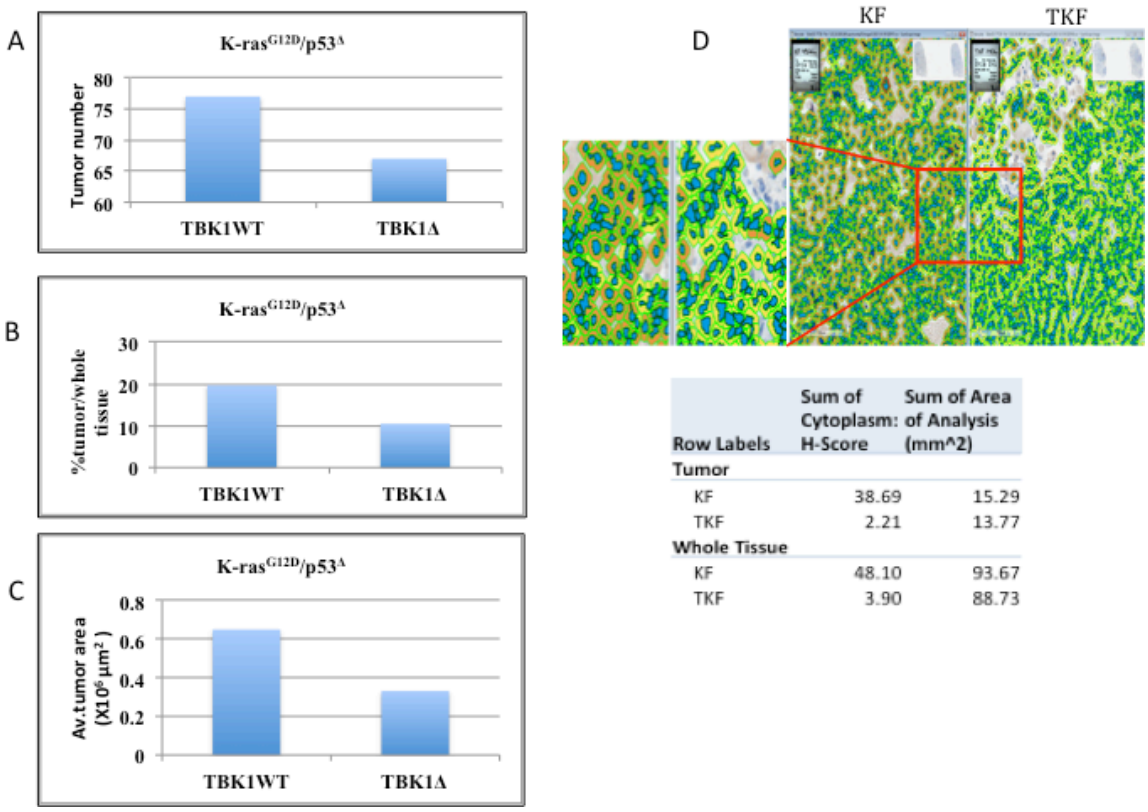
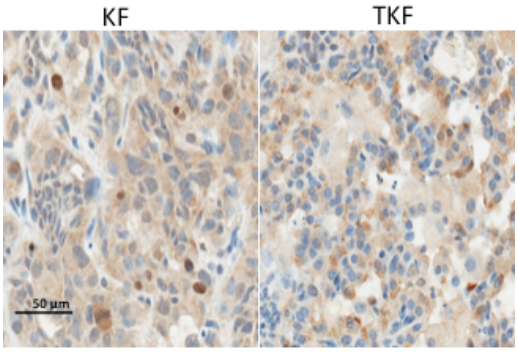


Figure 3.2 Characterization of *TBK1*$^{\Delta/\Delta}$ mice. (A) *TBK1*$^{fl/fl}$ were either infected or not infected with adenocre. 1 week post-infection lung and tail genomic DNA was collected and subjected to PCR using primers that anneal to exon 6. (B) *TBK1*$^{fl/fl}$ and *TBK1*$^{WT/WT}$ mice were infected with adenocre and lung protein lysates were collected at indicated time points post-infection and immunoblotted with specified antibodies. (C) *TBK1*$^{fl/fl}$ mice were either infected or not infected with adenocre and RNA collected from lungs at indicated time points post-infection was subjected to RT-PCR with primers annealing to either exon 4-5 (left panel) or exon6-7 (right panel) of TBK1 gene.



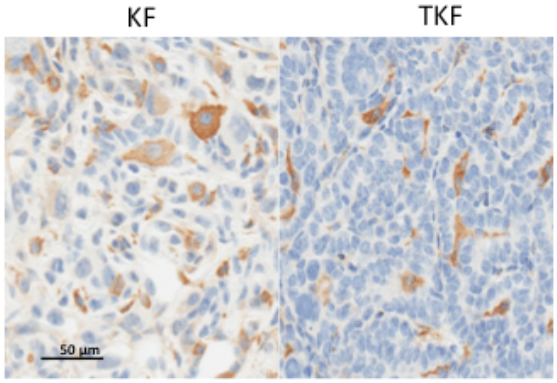
Row Labels	Sum of Cytoplasm: H-Score	Sum of Area of Analysis (mm^2)
Tumor		
KF	38.69	15.29
TKF	2.21	13.77
Whole Tissue		
KF	48.10	93.67
TKF	3.90	88.73

Figure 3.3 Deletion of TBK1 in the mouse lung decreases KRAS-induced lung tumorigenesis *TBK1$^{\Delta}$/KRAS^{G12D}/p53$^{\Delta}$* (TKF)and *KRAS^{G12D}/p53$^{\Delta}$* (KF) mice were analyzed 13 weeks post-infection. (A) number of K-Ras–induced neoplastic lesions was determined by counting lesions in H&E-stained lung sections as described (see Materials and Methods). (B) Total tumor area was divided by the whole tissue area to determine the tumor spread over the tissue. (C)Average size of the neoplastic lesions was calculated as described (see Materials and Methods) (D) immunohistochemistry for TBK1 (positive cells are shown brown) and its quantification (lower panel).



Mouse	Sum of Score (0-300)		Sum of Percent Total Positive	
	Tumor	WT	Tumor	WT
KF	20.2742	34.5579	18.325	28.5147
TKF	18.9888	40.2495	16.5865	32.4014

Figure 3.4. Deletion of TBK1 in the mouse lung does not affect the levels of IKKε *TBK1*$^{\Delta}$/*KRAS*^{G12D}/*p53*$^{\Delta}$ (TKF) and *KRAS*^{G12D}/*p53*$^{\Delta}$ (KF) mice were analyzed 13 weeks post-infection by immunohistochemistry for IKKε (positive cells are shown brown) and the data was quantified as described (see Materials and Methods)



Mouse	Sum of Score (0-300)		Sum of Percent Total Positive	
	Tumor	WT	Tumor	WT
KF	15.506	7.35985	11.5368	6.16068
TKF	8.2918	11.9069	6.89518	10.6395

Figure 3.5. Deletion of TBK1 in the mouse lung decreases p-AKT in KRAS induced lung tumors *TBK1*$^{\Delta}$/*KRAS*^{G12D}/*p53*$^{\Delta}$ (TKF) and *KRAS*^{G12D}/*p53*$^{\Delta}$ (KF) mice were analyzed 13 weeks post-infection by immunohistochemistry for p-AKT (positive cells are shown brown) and the data was quantified as described (see Materials and Methods)

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CHAPTER IV

CONCLUSIONS & FUTURE DIRECTIONS

The goal of this research has been to investigate novel and effective drug targets in KRAS-driven malignancies by understanding the complex signaling pathways downstream of KRAS that promote disease progression.

KRAS is the most frequently mutated oncogene in human cancers and has thus been a "target" for cancer biologists since its discovery 30 years ago. Yet in 2014, there are no drugs that target KRAS protein directly or act on KRAS induced tumors. Indeed, KRAS-driven tumors remain the most difficult to treat, for example lung and pancreatic cancer, which are the foremost and fourth leading causes of cancer related deaths in the United States, respectively (1). Previously, we and others have demonstrated the importance of NF-κB in promoting oncogenesis downstream of mutant RAS (2-6). Furthermore, NF-κB has been observed to be active in tumor biopsies from KRAS induced lung cancer patients, (7), in KRAS positive prostrate epithelial cells (8) and in both pancreatic cancer cells and melanoma in a RAS dependent manner (9,10). Several other candidates have emerged as important mediators of the transforming effects of oncogenic RAS, including the kinases TGF-β activated kinase 1 (TAK1) (11,12), glycogen synthase kinase 3 (GSK-3) (13,14) and Tank Binding Kinase 1 (TBK1) (15-17). Interestingly the transcription factor, NF-κB is known to be regulated by all the above-mentioned kinases but the mechanisms are not clear in the context of specific

malignancies.

Chapter two underscores the oncogenic role GSK-3α plays in pancreatic cancer. Consistent with previous reports (18,19), our data indicates that GSK-3 drives constitutive, pro-survival NF-κB activity in pancreatic cancer. The primary focus of this project was to dissect the individual roles of the two isoforms- GSK-3α, GSK-3β in regulating NF-κB activity in pancreatic cancer cells. Our data provides the first evidence for a role of GSK-3α in regulating pro-survival NF-κB activity in pancreatic cancer cells (see Figure 2.1). Our data suggests that TAK1 is constitutively activity in pancreatic cancer cells and plays a role in maintaining constitutive NF-κB signaling (see Figure 2.2). Importantly, we propose that GSK-3α may be linked to constitutive IKK and NF-κB through the stabilization of TAK-TAB complex (see Figure 2.3). We observed a reduction in TAK/TAB levels following GSK-3 inhibition in both pancreatic cancer cell lines as well as human pancreatic tumor explants. However, further studies are needed to establish the mechanistic link between GSK-3α and TAK1. One possible hypothesis is that GSK-3α phosphorylates TAB1/TAB2 that leads to the activation/stabilization of TAK/TAB complex in KRAS positive pancreatic cancer cells. This hypothesis is based on the finding that TAB1 and TAB2 contain multiple GSK-3 consensus motifs (20) and should be addressed in future studies. Thus our *in vitro* studies identify TAK1 and GSK-3α as potential drug targets in pancreatic cancer. Future studies evaluating the effects of TAK1 inhibitor and GSK-3α specific inhibitor on the development/maintenance of pancreatic tumor *in vivo* would lay the path for the development of these drugs for clinical use.

Previous studies looking at GSK-3 and NF-κB activity have invariably focused on the canonical NF-κB pathway (18,21). However, our data provides the first description for the role of GSK-3α in regulating pro-survival non-canonical NF-κB in pancreatic cancer cells and pancreatic tumor explants. Further studies are required to address the mechanism of how GSK-3α affects processing of the non-canonical NF-κB subunit, p100 to its active form p52. Since we observed a distinct effect of GSK-3α on nuclear p52 (See Figure 2.4) and since GSK-3 is known to accumulate in the nucleus of pancreatic cancer cells (14), two possible explanations exists: (1) nuclear GSK-3α regulates nuclear p100 processing or (2) GSK-3α affects the nuclear transport of p52. At the time of these studies, a GSK-3α specific drug was unavailable commercially, and thus we performed our human tumor explant study with a pan-GSK-3 inhibitor. We observed a 50% tumor growth inhibition in the presence of a GSK-3 inhibitor with a concomitant decrease in expression of several NF-κB target genes (see Figure 2.5, 2.6). However in the light of the above study, there is a definite need to develop a GSK-3α specific drug and determine its effect on pancreatic cancer *in vivo*. We used a tumor explant xenograft model for our studies but it would be interesting to compare these results in a genetic model of pancreatic cancer in mice.

Collectively, the studies in Chapter 2 identify two novel drug targets in pancreatic cancer- TAK1 and GSK-3α. TAK1 was recently identified as an important mediator of cell survival in KRAS dependent colon cancer (11) and of chemoresistance in pancreatic cancers (12). Our study not only confirms their findings but builds on to it by adding GSK-3α as an important kinase upstream of TAK1. Thus it is possible that the

chemoresistance conferred by TAK1 in pancreatic cancer cells or the regulation of cell survival by TAK1 in colon cancer cells is controlled by GSK-3α. The testing of this hypothesis will need to be done in future studies and may potentially find a universal mediator of KRAS oncogenic roles in GSK-3α.

Another kinase which has recently been implicated in KRAS-driven oncogenesis is TBK1 (15,16,22). TBK1 was originally identified as an NF-κB activating kinase which functions as a key regulator of innate immunity. We provide here the first description of the oncogenic role of TBK1 *in vivo* using a conditional TBK1 knockout mouse model. A KRAS/p53 driven lung cancer mouse model (2,3) was used to induce lung tumors with a concomitant loss of TBK1 in the lung epithelial cells. We observed a reduction in tumor size and numbers upon the loss of TBK1 in the lungs (Figure 3. 3) identifying TBK1 as a promising drug target in KRAS positive lung cancers. The major caveat of this study was the number of animals used (only 1) and thus it is essential to expand the number of animals to ascertain a statistically significant role of TBK1 in promoting lung tumor number and size. Furthermore the mechanisms employed by TBK1 to promote lung tumors need further investigation. In this regard we can investigate the effect of TBK1 loss on mitotic and apoptotic rate, NF-κB and cytokine signaling in KRAS/p53 and KRAS driven lung tumors.

Our studies used a KRAS/p53 driven lung caner mouse model but it is possible that the role of TBK1 in lung cancer may be dependent on the p53 status. Previous studies have shown that p53 leads to development of tumors fasters and of a higher grade (23). NF-κB has been shown to be important in lung tumorigenesis irrespective of the

p53 status (2) but whether TBK1's role in lung tumorigenesis is dependent on the presence/absence of p53 needs to be tested as this will be a very important in designing personalized therapy for patients. Thus, future studies looking at the effect of knocking out TBK1 and activating KRAS mutation on lung tumorigenesis in the lungs of mice with wildtype p53 are required.

Our preliminary data suggest that TBK1 is important in initiation and development of KRAS positive lung tumors. However, whether TBK1 is required for maintenance of these lung tumors is a different question and needs to be addressed for any therapeutic application. To address this question we can use TBK1 inhibitors in a KRAS or KRAS/p53 driven lung cancer mouse model. Once the tumors reach a measurable size in these mice models, the mice can be treated with TBK1 inhibitors and monitored for the growth of the tumor over time. Simultaneously the effect of TBK1 inhibitor needs to be tested *in vitro* in KRAS mutant lung cancer cell lines and cell lines derived from the above induced tumors. If the data from above studies supports the hypothesis that TBK1 is important in development and/or maintenance of KRAS mutant lung tumors, it may be worth while to develop a knock-in mouse model of kinase dead TBK1. This inactive TBK1 mouse model can be used to assess the initiation/development of tumors in a KRAS driven lung cancer model. The absence or decrease in lung tumors in the presence of kinase dead TBK1 will confirm the importance of TBK1 kinase activity in lung tumor development. These results will definitely support the development of specific and potent TBK1 inhibitors for pre-clinical and clinical studies.

Chapter two uncovered a role of GSK-3α in regulating two arms of pro-survival NF-κB (canonical and non-canonical) while chapter 3 investigated the oncogenic role of

another NF-κB activating kinase, TBK1. Additionally there is some preliminary data that suggests a link between GSK-3α and TBK1- leading us to hypothesize that GSK-3α sits on top of multiple of NF-κB signaling downstream of mutant KRAS and thus blocking GSK-3α can affect oncogenesis via inhibiting multiple downstream pro-survival pathways. This hypothesis is currently being tested *in vitro* using TBK1 null MEFs and multiple KRAS mutant cancer cells. The availability of TBK1 conditional knockout mice will allow to study this question *in vivo* by looking at the effect of TBK1 knock-out on GSK-3α expression, or effect of GSK-3 inhibitors on development of lung cancers on TBK1 null/KRAS mutant background.

Apart from the cancer-related implications, our study also has implications in the realm of immunology. TBK1 is known to be important in an anti-viral response but the absence of a TBK1-null mouse has been a hindrance in evaluating its role *in vivo*. Availability of a conditional TBK1 knockout mouse will help address this question. Additionally the phenotype of TBK1 null mice (embryonically lethal due to liver apoptosis (24) is not clearly understood. Initially the effect was attributed to impaired NF-κB signaling in TBK1 null mice. However it has been shown that TBK1 is not essential for NF-κB signaling (25). Thus further studies are needed to determine the mechanisms behind the embryonic death of TBK1 null mice, which can now be conducted in the TBK1 conditional knockout mouse.

Taken together, the previous chapters identified and proposed several novel drug targets for KRAS- driven malignancies- including, TAK1, GSK-3α and TBK1. Further investigation into the effects of specific inhibitors of these on KRAS induced tumors *in*

vivo is required to determine the clinical potential of these targets. KRAS driven tumors are one of the most therapy-resistant tumor with high mortality rates and thus there's need to identify and develop novel therapeutics. Although concurrent MAPK/PI3K pathway inhibition is under clinical evaluation, multiple approaches (like those investigated in this project) are likely necessary to identify effective KRAS targeted therapy. The implications of these results go way beyond pancreatic and/or lung cancers and extend to other KRAS mutant cancers, including colorectal cancer, breast cancer, seminoma among others. Thus future studies focusing on other KRAS tumors may identify TAK1, GSK-3α and TBK1 as important mediators of KRAS oncogenic signaling in all KRAS tumors. It will be interesting to observe and compare the effects of TAK1/GSK-3α/TBK1 kinase inhibitors alone and in combination in various KRAS mutant tumors and cell lines. These studies will answer questions like 1) Do TAK1/GSK3/TBK1 act in a same pathway to control cell survival or do their inhibitors have an additive effect? 2) Are GSK3/TAK1/TBK1 inhibitors are tumor suppressive in all KRAS mutant tumors or only specific ones? 3) Do GSK-3/TAK1/TBK1 inhibitors have a synergistic effect with current chemotherapeutic agents?

In conclusion these results have laid the foundation for development of multiple drug targets and future investigations into the effects of TAK1/GSK-3α/TBK1 targeting on KRAS driven malignancies are required to determine if the benefits outweigh the costs of these novel therapeutics.

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