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IKBKE is required during KRAS-induced pancreatic tumorigenesis

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Abstract

Pancreatic Ductal Adenocarcinoma (PDAC) is one of the deadliest malignancies lacking effective therapeutic strategies. Here we show that the non-canonical I κ B-related kinase, IKBKE, is a critical oncogenic effector during KRAS-induced pancreatic transformation. Loss of IKBKE inhibits the initiation and progression of pancreatic tumors in mice carrying pancreatic specific KRAS activation. Mechanistically, we demonstrate that this pro-tumoral effect of IKBKE involves the activation of GLI1 and AKT signaling, and independent of the levels of activity of the NF κ B pathway. Further analysis reveals that IKBKE regulates GLI1 nuclear translocation, and promotes the reactivation of AKT post-inhibition of mTOR in PDAC cells. Interestingly, combined inhibition of IKBKE and mTOR synergistically blocks pancreatic tumor growth. Together, our findings highlight the functional importance of IKBKE in pancreatic cancer, support the evaluation of IKBKE as a therapeutic target in PDAC, and suggest IKBKE inhibition as a strategy to improve efficacy of mTOR inhibitors in the clinic.

Keywords

IKBKE; KRAS; AKT; mTOR; pancreatic cancer

INTRODUCTION

Pancreatic Ductal Adenocarcinoma (PDAC) is the most common and aggressive type of pancreatic cancer (1, 2). *KRAS* mutation, detected in >90% of PDAC cases, is a critical oncogenic event during PDAC initiation and progression (3). Mutant *KRAS* is known to activate multiple signaling pathways to promote its oncogenic activity, including MAPK, PI3K, RAL-A/B, and NF- κ B pathways (4–10); however, the stimulation of these cascades does not explain the pleiotropic effects of mutant *KRAS*. The identification of additional

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downstream pathways is critical to define the mechanism underlying KRAS-induced pancreatic tumorigenesis, and targeting these pathways may enable a more effective therapeutic strategy for PDAC treatment.

We previously demonstrated that Hedgehog ligand independent GLI activity is critical for KRAS-induced pancreatic transformation *in vivo* (11), and identified IKBKE as a downstream target of GLI1 in PDAC cells (11). IKBKE and its closely related kinase, TBK1, were originally identified as the non-canonical I κ B kinases involved in regulation of NF- κ B signaling (12, 13). The oncogenic activity of IKBKE/TBK1 has been linked to NF- κ B activation in several tumorigenic contexts (14–18). TBK1 has also been reported to be activated by KRAS via a RALB-dependent mechanism to promote tumor cell survival (19). Despite their potential importance, the genetic requirement of these kinases in tumorigenesis, including KRAS-induced PDAC formation, has not been demonstrated, and it is not clear which pathways play major roles downstream of IKBKE *in vivo*.

Here, we show that IKBKE function is critical for KRAS^{G12D} dependent pancreatic transformation *in vivo*. Surprisingly we find that IKBKE is not essential for cytokine/NF- κ B activation during pancreatic tumorigenesis; however, it engages in reciprocal regulation of GLI, regulates mTOR-independent AKT activity, and promotes AKT re-activation upon mTOR inhibition in PDAC cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Mouse strains

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

Tissue collection and histology

Upon euthanasia, pancreatic tissue was fixed in 4% paraformaldehyde for 24 hours. For paraffin sections, tissue was dehydrated and embedded in paraffin blocks. Paraffin sections were stained with hematoxylin and eosin (H&E) using standard reagents and protocols. Human PDAC tissue microarray was obtained from UMass Tissue Bank and Genvelop.

Immunohistochemistry and immunoblotting

For immunohistochemistry, antigen retrieval was conducted in Sodium Citrate solution (pH 6.0) for 30 minutes. Sections were blocked in a buffer containing 5% BSA and 0.1% Triton X-100 in PBS and incubated overnight at 4 °C in primary antibodies diluted in blocking buffer. Primary antibodies used were: Ki67 (1:500, Abcam), phospho-AKT (1:50, Cell Signaling), IKBKE (1:50, Santa Cruz) for mouse sections, IKBKE (1:100, Sigma) for human sections, TBK1 (1:100, Cell Signaling), p65 (1:50, Cell Signaling), Amylase (1:800, Sigma), and Insulin (1:100, Abcam). Signal detection was accomplished with biotinylated secondary antibodies in the Vectastain ABC kit (Vector Labs).

For immunoblotting, the primary antibodies used were Flag-HRP (1:1,000 Sigma); β -Actin (1:1,000, Sigma); phospho-AKT S473 (1:1,000, Cell Signaling), phospho-Akt T308 (1:1000, Cell Signaling), phospho-ERK (1:1,000, Cell Signaling); total AKT (1:1,000, Cell Signaling); total ERK (1:1,000, Cell Signaling); IKBKE (1:1,000, Sigma), TBK1 (1:1000, Cell Signaling), phospho-S6K (1:1000, Cell Signaling), phospho-4EBP1 (1:1000, Cell Signaling), p65 (1:1000, Cell Signaling), PCNA (1:1000, Abcam), β -Tubulin (1:1000, Cell Signaling), Cleaved PARP Asp 214 (1:1000, Cell Signaling), and GLI1 (1:1000, Cell Signaling). HRP-conjugated secondary antibodies used for detection were obtained from Jackson Laboratories.

Cell lines

293T (CRL-3216), Panc-1 (CRL-1469), and MiaPaca-2 (CRL-1420) cell lines were obtained from the ATCC repository. Cell line characterization by ATCC is conducted by STR analysis. Cell lines were expanded, and cryogenically frozen upon acquisition to establish stocks, and stored in liquid nitrogen until use. Cell lines were cultured for a maximum of 3 months before experimentation. Mycoplasma testing was conducted using the Universal Mycoplasma Detection kit (ATCC), and cell lines were also analyzed for morphology, and proliferation before experimentation.

Cell proliferation, apoptosis and soft-agar assays

Cell proliferation, apoptosis, and soft-gar assays were conducted as previously described(11).

Lentiviral shRNA knockdown experiments

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

Luciferase reporter analysis

NF- κ B luciferase (p65-Luc) was a gift from Dr. Francis Chan (University of Massachusetts Medical School, Worcester, MA). Reporters were co-transfected with the expression vectors for Gli3T, IKBKE, IKBKE K38A, Gli1-AHA using lipofectamine 2000. *IKBKE* promoter luciferase was generated by cloning a 300bp region upstream of the human *IKBKE* transcription start site into a PGL3 luciferase vector. Luciferase assays were conducted 48 hours after transfection using the dual-luciferase reporter kit (Promega). Assays were conducted in triplicate.

Quantitative RT-PCR

cDNA synthesis was conducted using Invitrogen SuperScript II kit. Primers used for qRT-PCR were human *IKBKE* (forward: 5'-TGCCTGCAGAAGTATCAAGC-3'; reverse: 5'-TACAGGCAGCCACAGAACAG-3'); mouse *Ikbke* (forward: 5'-

GCGGAGGCTGAATCACCAG-3'; human *GAPDH* (forward: 5'-ATGGGGAAGGTGAAGGTCG-3'; reverse: 5'-GGGGTCATTGATGGCAACAATA-3'); mouse *Gapdh* (forward: 5'-AGGCCGGTGCTGAGTATGTC-3'; reverse: 5'-TGCCTGCTTCACCACCTTCT-3'); human *GLI1* (forward: 5'-CCAGCGCCAGACAGAG-3'; reverse: 5'-GGCTCGCCATAGCTACTGAT-3'); mouse *Gli1* (forward: 5'-GTCGGAAGTCCTATTCACGC-3'; reverse: 5'-CAGTCTGCTCTCTTCCCTGC-3'); human *PTCH1* (forward: 5'-CCACAGAAGCGCTCCTACA-3'; reverse: 5'-CTGTAATTTCCGCCCTTCC-3'); mouse *Ptc1* (forward: 5'-AACAAAAATTCAACCAAACCTC-3' reverse: 5'-TGTCTTCATTCCAGTTGATGTG-3'); human *IL1A* (forward: ATCATGTAAGCTATGGCCCACT; reverse: CCTTCCCGTTGGTTGCTACTA), mouse *Il1a* (forward: 5'-TCTATGATGCAAGCTATGGCTCA-3'; reverse: 5'-CGGCTCTCCTTGAAGGTGA-3'); human *TNFA* (forward: CCTCTCTCTAATCAGCCCTCTG; reverse: GAGGACCTGGGAGTAGATGAG), mouse *Tnf* (forward: 5'-CAGGCGGTGCCTATGTCTC-3'; reverse: 5'-CGATCACCCCGAAGTTCAGTAG-3'); human *BCL2L1* (forward: CTGCTGCATTGTTCCCATAG-3'; reverse: 5'-TTCAGTGACCTGACATCCCA-3'), mouse *Bcl2l1* (forward: 5'-ACATCCCAGCTTCACATAACCC-3'; reverse: 5'-CCATCCCGAAAGAGTTCATTAC-3'); human *BCL2* (forward: 5'-ATGTGTGTGGAGAGCGTCAA-3'; reverse: 5'-CGTACAGTTCCACAAAGGCA-3'); and mouse *Bcl2* (forward: 5'-GCTACCGTCGTGACTTCGC-3'; reverse: 5'-CCCCACCGAACTCAAAGAAGG-3'). All qPCR assays were conducted in triplicate.

Nuclear and cytoplasmic fractionation

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

Xenograft mouse models

MiaPaca-2 PDAC cells were stably infected either with the doxycycline inducible lentiviral vectors pTRIPZ and ptet-pLKO (Addgene) expressing shRNAs targeting either IKBKE or mTOR. These stable cell lines were then injected mice at a concentration of 1×10^7 cells in a volume of 100 μ l in 1:1 ratio with matrigel in subcutaneously in the flanks of NOD-SCID mice (Jackson Laboratory). Tumors were allowed to grow to a volume of ~ 100 mm³ after which Doxycycline was administered in drinking water at a concentration of 100 μ g/ml. Tumors were allowed to grow for 30 days and measured every 3 days using calipers. Tumor volume was calculated using the formula $w^2 \times l$. Tumors were dissected and whole tumors were imaged after 30 days of Doxycycline treatment.

RESULTS

IKBKE acts downstream of KRAS to promote PDAC initiation

Immunohistochemistry (IHC) analysis of IKBKE expression in a Tissue Microarray of human pancreatic cancer samples (N=105) showed IKBKE expression is high in the

majority of human PDAC samples, but minimal in normal human pancreas (Figure 1A–B, S1A–D, I). The closely related non-canonical I κ B-related kinase, TBK1, was also expressed in human PDAC, although in a less degree (Figure S1E–I). To further explore their connection to KRAS in PDAC, we conducted shRNA mediated knockdown of KRAS in human PDAC cells carrying oncogenic mutant KRAS. Knockdown of this GTPase led to a significant decrease in expression of IKBKE, but not TBK1 in Panc-1 and MiaPaca2 PDAC cells (Figures S2A–D) as measured by the immunoblotting and quantitative RT-PCR analyses. In addition, we showed that knockdown of IKBKE in human PDAC cells resulted in significant increase in apoptosis, marked decrease in cell proliferation, as well as transformation as measured by the soft agar colony formation assay (Figure S2E–J). Together, our data suggests that IKBKE is a downstream target of KRAS that may mediate its oncogenic effect in PDAC.

IKBKE is required for pancreatic neoplastic transformation *in vivo*

To further test the biological role of IKBKE *in vivo*, we utilized a mouse model carrying whole body knockout of *Ikbke* (21). The *Ikbke*^{−/−} mice had histologically normal pancreatic architecture at 12 months of age (N=6) as compared to wild type mice (Figure S3A–B). IHC staining of the acinar cell marker amylase, and islet cell marker insulin also showed normal pancreatic differentiation (Figure S3C–F). These results suggest that IKBKE is not required for development of pancreas.

To examine whether IKBKE function is specifically required during KRAS-induced pancreatic transformation, we utilized the mouse model in which an oncogenic allele of *Kras* (KRAS^{G12D}) is targeted to the endogenous locus and expressed specifically in the pancreatic epithelium using Cre-recombinase expressed under the *P48* (*Ptf1a*) promoter (22). As expected, the *P48Cre;LSL-Kras*^{G12D} mice developed pancreatic intraepithelial neoplasia (PanIN) lesions of histological grades ranging from PanIN1–3, depending on the age of the mice, with some 12 month old mice displaying full blown PDAC (Figure 1G–I). Consistent with our IHC analysis in human PDAC, we found that IKBKE expression was also highly upregulated in mouse PanIN lesions as well as in PDAC compared to wild type mouse pancreas (Figure 1C–E).

To achieve simultaneous KRAS activation and IKBKE loss in the pancreas (Figure 1F), we generated *P48Cre;Kras*^{G12D};*Ikbke*^{−/−} mice, and analyzed pancreas at ages 3 months, 6 months, and 12 months. We found that the *P48Cre;LSL-Kras*^{G12D} (N=15) mice developed PanIN grade 1 and instances of grade 2 lesions at the age of 3 months, with increased number of PanIN 2 and 3 grade lesions at 6 months (Figure 1G, H). The normal pancreatic architecture was lost by 12 month age, with advanced grade lesions covering majority of the pancreas (Figure 1I), and instances of adenocarcinoma in some mice. In contrast, pancreas of *P48Cre;LSL-Kras*^{G12D};*Ikbke*^{−/−} mice (N=24) were relatively normal at these 3 time points, with some low grade PanIN lesions at 6 and 12 month time points (Figure 2J–L). Quantification of PanIN lesions from H&E stained sections obtained from the mouse pancreas samples also showed significantly inhibition of initiation and delayed onset of pancreatic neoplastic transformation in *P48Cre;LSL-Kras*^{G12D};*Ikbke*^{−/−} mice (Figure 2O). We also conducted Ki67 staining on stage matched lesions from *P48Cre;LSL-Kras*^{G12D} and

P48Cre;LSL-Kras^{G12D};Ikbke^{-/-} mice. PanIN lesions from *P48Cre;LSL-Kras^{G12D};Ikbke^{-/-}* mice had less Ki67 positive cells compared to similar staged lesions from *P48Cre;LSL-Kras^{G12D}* mice (Figure 2M, N, P, and Figure S4). This data indicates that IKBKE loss may impair cell proliferation, and therefore delay progression of the PanIN lesions. Together, our findings suggest a critical requirement of IKBKE in both KRAS-induced initiation and progression of pancreatic transformation.

NF- κ B activity in PDAC cells is independent of IKBKE

IKBKE was initially identified as an I κ B kinase involved in regulation of NF- κ B signaling (12). The cytokine-NF- κ B axis has been shown to play an important role in pancreatic tumorigenesis (9, 10, 29). Thus, we examined the possible role of IKBKE in NF- κ B activation in PDAC. We found that, although IKBKE overexpression could activate the NF- κ B luciferase reporter activity in an I κ B dependent manner (Figure 3A), IKBKE knockdown in PDAC cells did not significantly affect the expression of several known NF- κ B target genes and cytokines (Figure 3B). In addition, we did not detect significant downregulation of NF- κ B target gene expression in the pancreata of *P48Cre;Kras^{G12D};Ikbke^{-/-}* mice compared to *P48Cre;Kras^{G12D}* mice (Figure 3C).

Nuclear localization of the NF- κ B subunit p65, a hallmark for NF- κ B pathway activation, was not affected by *IKBKE* knockdown in PANC1 cells (Figure 3D). Furthermore, we compared subcellular localization of p65 in stage-matched PanIN lesions of *P48Cre;Kras^{G12D};Ikbke^{-/-}* and *P48Cre;Kras^{G12D}* mice using immunohistochemistry. We found that nuclear p65 was present in the PanIN lesions and there was no significant difference in nuclear localization of p65 between lesions of *P48Cre;Kras^{G12D};Ikbke^{-/-}* and *P48Cre;Kras^{G12D}* mice (Figure 3E-F). Our findings indicate that IKBKE does not appear to play a major role in NF- κ B activation in KRAS-induced pancreatic tumorigenesis, and that IKBKE oncogenic activity in the context of PDAC is mediated by NF- κ B independent mechanisms.

IKBKE is a direct target of GLI1 in PDAC cells

Our expression profiling analysis in human PDAC cells identified IKBKE as a candidate GLI1 downstream target gene (11). Consistent with this idea, we found that inhibition of GLI transcriptional activity by either the dominant-negative Gli3T repressor (11), or shRNA mediated knockdown of *GLI1* in Panc-1 cells decreased *IKBKE* but not *TBK1* mRNA levels (Figure 2A), suggesting that TBK1 does not act downstream of GLI1. In addition, chromatin immunoprecipitation in Gli3T-expressing Panc-1 cells showed significant enrichment of GLI protein in the *IKBKE* promoter region 130 bp upstream of the transcriptional start site, as well as the promoter region of the known GLI target gene, *PTCH1* (Figure 2B). Sequence analysis of the *IKBKE* promoter region revealed the existence of a candidate GLI binding site (GACTTCCCA) (Figure 2C). We generated IKBKE promoter-driven luciferase reporter constructs with a wild-type (IKBKE-Luc) or mutated GLI binding site (IKBKE-m-Luc) (Figure 2C). We showed that Gli3T was able to inhibit the luciferase activity of IKBKE-Luc, as well as Gli-BS-Luc, a luciferase reporter carrying 8 consecutive GLI canonical binding sites (24). However, it failed to block IKBKE-m-Luc activity in Panc1 cells (Figure 2D).

Together, these findings suggest that *IKBKE* is a direct transcriptional target of GLI in PDAC cells.

We next compared mRNA expression levels of *IKBKE* vs *GLI1* in human PDAC patient samples, and found a strong correlation ($R = 0.79$, $P < 0.0001$) between the expression of the two genes in human PDAC samples (Figure 2E). This tight correlation led us to further explore the *IKBKE*-*GLI1* interaction. Despite the importance of the Hh ligand-independent *GLI1* activity in PDAC (11, 25), the upstream mechanism regulating this non-canonical *GLI1* activation is not well understood. Interestingly, we found that *IKBKE* knockdown in Panc-1 cells led to a significant decrease in mRNA levels of the GLI target genes *GLI1*, *FOXA2*, and *PTCH1* (Figure 2F). Pancreas tissue from *P48Cre;Kras^{G12D};IKBKE^{-/-}* mice also exhibited significantly lower levels of mRNA of the *GLI1* target genes compared to *P48Cre;Kras^{G12D}* mice (Figure 2G), suggesting that *IKBKE* may be involved in regulating GLI activity in pancreatic tumor cells.

We and others have shown that regulation of the intracellular localization of *GLI1*, a nuclear-cytoplasmic shuttling protein, is important for its transcriptional activity (26–28). We found that, when *IKBKE* and GFP-fused version of *GLI1* were co-expressed, *IKBKE* promotes the nuclear translocation of *GLI1* (Figure 2H, J), measured by immunofluorescence staining and western blot analysis. This *IKBKE* effect was dependent on its kinase activity, as a kinase-dead *IKBKE* mutant (K38A), was not able to promote *GLI1* translocation (Figure 2H, J). To further test *IKBKE* and GLI interaction, we utilized a mutant version of *GLI1* (*Gli1*-AHA) that is constitutively localized to the nucleus (26). We found that co-expression of *IKBKE*, but not *IKBKE*-K38A, led to a synergistic increase in the activation of wild type *GLI1* (Figure 2I). However, co-expression of *IKBKE* or *IKBKE*-K38A did not significantly affect the activity of *Gli1*-AHA (Figure 2I). More importantly, when endogenous *IKBKE* was knocked down in MiaPaCa2 PDAC cells by shRNA, it markedly increased the cytoplasmic fraction of *Gli1* protein (Figure 2K). Taken together, these data uncover a previously unknown *IKBKE*-mediated Gli regulation in PDAC cells, and suggest that *IKBKE* modulates GLI activity by controlling its nuclear localization.

IKBKE promotes AKT activation in PDAC

Further analysis of the mechanism found that levels of phosphorylated AKT, a substrate of *IKBKE* at both Serine-473 and Threonine-308 sites (**insert ref**) were markedly decreased in the stage-matched PanIN lesions of *P48Cre;Kras^{G12D};Ikbke^{-/-}* mouse pancreas compared to *P48Cre;Kras^{G12D}* mice (Figure 4A–B). Furthermore, the phospho-AKT levels in a tissue microarray of human PDAC samples (N=62) significantly correlated between AKT phosphorylation and expression levels of *IKBKE* in the samples as indicated by a Pearson coefficient of $R = 0.606$ ($P < 0.0001$) (Figure 4C). In addition, we showed that *IKBKE* knockdown in Panc-1 cells led to a decrease in the phosphorylation of AKT but not ERK (Figure 4D), thus suggesting *IKBKE*-dependent phosphorylation of AKT in PDAC.

The mTORC2 complex is known to phosphorylate AKT at Serine-473 and thereby mediate AKT activation (33). In Panc-1 cells treated with the mTOR kinase inhibitor, Torin-1, AKT phosphorylation was down-regulated (Figure 4E). However, AKT phosphorylation was further reduced in cells with both mTOR inhibition and *IKBKE* knockdown (Figure 4E),

suggesting that IKBKE mediated AKT phosphorylation is likely mTOR independent, and that both pathways are involved in AKT activation in PDAC cells. mTOR inhibitors have been approved for treatment of certain malignancies (34, 35). However, they appear to be ineffective in treating PDAC (36). One of the problems concerning the clinical utility of mTOR inhibitors is the reactivation of AKT post-inhibition of mTOR (37, 38). It has been reported that in breast cancer cells, mTOR kinase inhibition resulted in sustained inhibition of AKT phosphorylation at Serine-473; however, the compensatory activation of upstream receptor tyrosine kinase (RTK) and subsequent re-phosphorylation of AKT at Threonine-308 leads to AKT re-activation and resistance to mTOR inhibition (38). Interestingly, we found that, in Panc-1 cells treated with Torin-1, AKT phosphorylation at Serine-473 and Threonine-308 was initially inhibited 6 hours post-treatment; however, phosphorylation at both sites was restored as early as 12 hours after Torin-1 treatment (Figure 5A). Phosphorylation of other mTOR targets S6K and 4EBP1 continued to be inhibited even 24 hours after treatment (Figure 5A), suggesting that reactivation of AKT is mTOR-independent and may be mediated by an RTK-independent mechanism.

Because of the IKBKE-AKT connection, we decided to test whether IKBKE is involved in AKT reactivation in PDAC cells. We found that in Panc-1 cells with shRNA mediated *IKBKE* knockdown, the reactivation of AKT post-inhibition of mTOR was ablated (Figure 5B). Furthermore, although mTOR inhibition alone did not significantly affect the survival of Panc1 and MiaPaCa2 cells (Figure 5C, S5A–D), knockdown of IKBKE sensitized these cells to the mTOR inhibitor. *IKBKE* knockdown combined with mTOR inhibition led to a synergistic decrease in cell viability, significant increase in apoptosis (Figure 5D), and inhibition of transformation of PDAC cells (Figure 5C–E, S5B–D). To test the combined IKBKE and mTOR inhibition as a potential PDAC therapeutic strategy *in vivo*, we generated human MiaPaCa2 cell lines stably expressing doxycycline inducible shRNAs targeting *IKBKE*, *mTOR*, or both (Figure S5). We subcutaneously injected 1×10^7 cells from each of the stable cell lines into the flanks of NOD-SCID mice. The tumors (N=6 per cell line) were allowed to grow to a size of $\sim 100 \text{ mm}^3$ before doxycycline treatment. Tumor volume was measured every 3 days for the next 30 days before the animals were sacrificed. We found that *IKBKE* knockdown decreased tumor growth in the mice, and *mTOR* knockdown did not significantly affect tumor formation. However, combined knockdown of *mTOR* and *IKBKE* resulted in a synergistic inhibition of xenograft tumor growth *in vivo* (Figure 5F, G). To evaluate the molecular characteristics of tumor growth inhibition by *mTOR* and *IKBKE* knockdown, we conducted immunohistochemical staining for phospho-AKT and phospho-S6K. S6K phosphorylation was blocked in tumors derived from sh*mTOR* and sh*IKBKE*/sh*mTOR* cell lines (Figure S6D, J) but maintained in the tumors derived from control and sh*IKBKE* cell lines (Figure S6A, G). We found that in spite of mTOR knockdown, AKT phosphorylation at Serine-473 in the tumors was maintained at high levels (Figure 5H, I), while combined knockdown of IKBKE and mTOR led to obliteration of AKT phosphorylation (Figure 5J, K, S6K). Together, our findings implicate a critical role for IKBKE in AKT activation in PDAC cells both *in vitro* and *in vivo*.

DISCUSSION

The nearly universal presence of activating mutations in the KRAS oncoprotein in PDAC suggest that inhibiting key downstream signaling nodes may be an effective therapeutic strategy in this disease. However, the identities of the key downstream signaling nodes remain poorly understood. Indeed, targeting of well-characterized signaling molecules such as MEK, PI3K and mTOR have all been ineffective thus far in PDAC. Here, we demonstrate that inhibition of the atypical I κ B kinase IKBKE may be an important component of combinatorial therapeutic strategies in PDAC.

The I κ B kinase-related kinases, IKBKE and TBK1, were originally identified by their ability to act as I κ B kinases to regulate NF- κ B signaling (12, 13). Later on it was reported that these versatile kinases engage multiple different substrates, including CYLD, TRAF2, FOXO3, and AKT (16, 30–32, 39, 40). However, the context-dependent downstream regulation by IKBKE/TBK1 in diseases and cancers is not well understood. Our study highlights the *in vivo* importance of IKBKE in Kras-induced pancreatic tumorigenesis, and suggests that IKBKE, but not TBK1, is likely the major I κ B kinase-related kinase involved in PDAC. Interestingly, previously published studies indicated that TBK1 inhibition represented a synthetic lethal interaction with mutationally activated KRAS in colorectal, lung and breast cancer cells (19). The differential requirement for IKBKE and TBK1 may reflect their different expression levels in PDAC specimens. In addition, TBK1 is known to be activated downstream of the monomeric GTPase RALB, itself a downstream effector of KRAS (41). Yet, Lim et al demonstrated that RALA facilitates, while RALB impedes, KRAS-induced transformation in PDAC cells (42). Thus, it is intriguing that the selective dependence of IKBKE in PDAC may be the consequence of differential functional requirement of RALA/B in this tumorigenic context.

Critically, our results also suggest that IKBKE is largely dispensable for NF- κ B activation in PDAC, but functions as one of the key regulators for AKT activity. Moreover, our studies also reveal a novel mechanism underlying AKT reactivation upon mTOR inhibition. In contrast to RTK-mediated AKT reactivation at T308 in breast cancer cells (38), IKBKE dependent AKT reactivation in PDAC cells involves both S473 and T308 phosphorylation, highlighting the complex signaling mechanisms contributing to AKT reactivation and resistance to mTOR inhibition in different tumors. Of note, concurrent inhibition of mTOR and IKBKE kinases profoundly impaired the growth of pancreatic cancer xenografts. While additional *in vivo* studies are required to validate the efficacy of this therapeutic approach, our observations provide a rationale for testing combination of IKBKE and mTOR inhibitors that are currently in clinical development for treating Kras-driven PDAC.

Our study also identified GLI1 nuclear translocation and transcriptional activity signaling as a novel downstream pathway regulated by IKBKE. It remains to be determined whether IKBKE mediated promotion of Gli1 nuclear localization is via direct phosphorylation or indirect regulation. Nonetheless, given our finding that non-canonical activation of GLI transcription factors stimulates IKBKE gene expression (11), these results indicate the presence of an oncogenic feed forward mechanism downstream of mutant KRAS with potential therapeutic implications. Additional studies directed toward the identification and

characterization of other critical pathways downstream of IKBKE and GLI may therefore shed further light on the mechanisms underlying PDAC development and therapeutic resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. IKBKE requirement in Kras-induced pancreatic tumorigenesis

Immunohistochemistry staining of IKBKE in human (A–B), and mouse pancreas (C–E). IKBKE protein levels are significantly higher in human PDAC samples (B) compared to wild type pancreas (A). PanIN lesions (D), and PDAC tissue (E) derived from P48Cre;KRAS^{G12D} mice have significantly higher IKBKE compared to wild type pancreas (C). (F) Western blot showing IKBKE levels in pancreas of 12 month old P48Cre;Kras^{G12D} and P48Cre;Kras^{G12D};IKBKE^{-/-} mice. IKBKE is not expressed in pancreas from P48Cre;Kras^{G12D};IKBKE^{-/-} mice but is expressed in P48Cre;Kras^{G12D} mice. (G–I) Representative H&E staining images of pancreas of 3 month old (G), 6 month old (H), and 12 month old (I) P48Cre;Kras^{G12D} mice. (J–L) Representative H&E staining images of pancreas of 3 month old (J), 6 month old (K), and 12 month old (L) P48Cre;Kras^{G12D};IKBKE^{-/-} mice. (M) Quantification of grade of PanIN lesions in age matched P48Cre;Kras^{G12D} and P48Cre;Kras^{G12D};IKBKE^{-/-} mouse pancreas at ages 3 months, 6 months, and 12 months. Comparison of age matched pancreas indicates significantly delayed initiation and progression of pancreatic neoplasms in P48Cre;Kras^{G12D};IKBKE^{-/-} mice compared to P48Cre;Kras^{G12D} mice. (N–O) Immunohistochemistry for Ki67 in 6 month old P48Cre;Kras^{G12D} (N), and P48Cre;Kras^{G12D};IKBKE^{-/-} (O) mice indicates decreased number of proliferative cells (P). Error bars represent Standard Deviation.

Figure 2. Reciprocal IKBKE-Gli1 interaction in PDAC cells

(A) qPCR analysis in Panc-1 cells showing *GLI1*, *IKBKE*, and *TBK1* mRNA expression after inhibition of GLI activity using a dominant negative GLI (Gli3T), or shRNA targeting *GLI1*. (B) Quantitative PCR of DNA enriched using ChIP against Flag-tag in Panc-1 cells infected with Gli3T-Flag indicates significant enrichment of IKBKE promoter region as well as the promoter of a known GLI target gene (PTCH1), but not control ACTB promoter region. (C) Schematic showing location of GLI binding site and mutated GLI binding site upstream of luciferase reporters. (D) Relative luciferase activity in Panc-1 cells expressing Gli-BS luciferase reporter (Gli-BS-Luc), *IKBKE* luciferase containing the wild type GLI binding site (IKBKE-Luc), and *IKBKE* luciferase carrying a mutated GLI binding site (IKBKE-m-Luc), with or without Gli3T ectopic expression. (E) Correlation of GLI1 and IKBKE mRNA expression in human PDAC tissue samples (n=229). Pearson coefficient of $R = 0.79$ indicates high degree of correlation between GLI1, and IKBKE expression in the tumors. (F) Quantitative RT-PCR analysis of transcription of IKBKE and the known GLI target genes, GLI1, PTCH1, and FOXA2, in Panc-1 cells with *IKBKE* knockdown. (G) Quantitative RT-PCR in tissue samples indicates significantly reduced expression of GLI target genes, *Gli1* and *Ptch1*, in 6 month old *P48Cre;Kras^{G12D};Ikbke^{-/-}* mouse pancreas

compared to age-matched *P48Cre;Kras;^{G12D}* pancreas. (H) Subcellular localization of Gli1-GFP fusion protein in transfected 293T cells with (i–iii) and without (iv–vi) overlay with DAPI. While GLI1 is localized mainly in the cytoplasm without IKBKE expression (i, iv), IKBKE expression drives nuclear localization of GLI1 (ii, v). Expression of a kinase-dead version of IKBKE (K38A) causes cytoplasmic retention of GLI1 (iii, vi). (I) Co-expression of IKBKE, but not the kinases-dead version of IKBKE, with GLI1 synergistically increases GLI transcriptional activity as measured by Gli-BS Luciferase activity in 293T cells. IKBKE expression has no effect on the activity of Gli1-AHA, a mutant form of Gli1 that is constitutively localized to the nucleus. Error bars represent Standard Deviation. (J) Western blot analysis of nuclear and cytoplasmic fractions indicates that ectopic coexpression with wild type but not catalytically inactive (K38A) IKBKE leads to significant nuclear localization of GLI1. PCNA, and Tubulin are used as controls for nuclear (N) and cytoplasmic fractions (C) respectively. (K) Western blot analysis of nuclear and cytoplasmic fractions in Panc-1 cells indicates decrease in endogenous GLI1 nuclear localization after shRNA mediated knockdown of IKBKE. Statistical significance was determined using a Student's two-tailed t-test. * $P < 0.05$; ** $P < 0.01$.

Figure 3. IKBKE does not play a significant role in regulating NF- κ B activity in PDAC
 (A) NF- κ B luciferase activity in 293T cells is significantly increased in response to IKBKE ectopic expression. IKBKE activation of NF- κ B activity can be inhibited by a dominant negative version of I κ B suppressor. (B) qPCR analysis of mRNA levels of NF- κ B target genes, *BCL2L1*, *BCL2*, *IL1* and *TNFA*, in Panc-1 cells with and without *IKBKE* knockdown. (C) Quantitative RT-PCR analysis of mRNA levels of NF- κ B target genes, *Bcl2L1*, *Bcl2*, *Il1* and *Tnf*, in pancreas of 6 month old *P48Cre;Kras^{G12D}*, and *P48Cre;Kras^{G12D};Ikbke^{-/-}* mice. (D) Western blot showing RELA (p65) in nuclear and cytoplasmic fractions of Panc-1 cells with and without shRNA knockdown of *IKBKE*. PCNA and Tubulin are used as controls for nuclear and cytoplasmic fractions respectively. (E–F) Immunohistochemistry for p65 in stage matched PanIN lesions in *P48Cre;Kras^{G12D}* (E), and *P48Cre;Kras^{G12D};Ikbke^{-/-}* (F) mouse pancreas. Error bars represent Standard Deviation. Statistical significance was determined using a Student's two-tailed t-test. * $P < 0.05$; ** $P < 0.01$

Figure 4. IKBKE regulates AKT activation in PDAC cells in vivo and in vitro
(A–B) Immunohistochemistry for AKT phosphorylation at Serine-473 in stage matched PanIN lesions of *P48Cre;Kras^{G12D}* (A), and *P48Cre;Kras^{G12D};IKBKE^{-/-}* (B) mouse pancreas. *P48Cre;Kras^{G12D};IKBKE^{-/-}* PanIN lesions have significantly reduced AKT phosphorylation compared to *P48Cre;Kras^{G12D}* lesions. (C) Correlation of IKBKE protein levels and AKT Serine-473 phosphorylation levels in human PDAC tissue microarray. Representative images of immunohistochemistry for IKBKE (i–iii) and phospho-AKT Serine-473 (iv–vi) in matched human PDAC tissue samples. (D) Western blot showing phosphorylation of AKT and ERK in serum starved Panc-1 cells in response to shRNA mediated *IKBKE* knockdown. *IKBKE* knockdown leads to decrease in phosphorylation of AKT at Serine-473, but not ERK phosphorylation. (E) Western blot of Panc-1 cells with or without inhibition of mTOR using Torin-1, and shRNA mediated knockdown of *IKBKE*. Inhibition of *IKBKE* and mTOR leads to decrease in phosphorylation of AKT at Serine-473 and Threonine-308.

Figure 5. IKBKE mediates AKT reactivation post-mTOR inhibition

(A–B) Western blot showing phosphorylation of Akt and other mTOR substrates, S6K and 4EBP1, in control Panc-1 cells (A) and Panc-1 cells with IKBKE knockdown (B) in response to treatment with Torin-1 up to 24 hours. (C) Relative cell viability of Panc-1 cells measured by MTT assay 5 days after treatment with increasing dosage of the mTOR inhibitor Torin-1 in the presence and absence of *IKBKE* knockdown. (D) Cleaved-Caspase 3 staining in Panc-1 cells indicates that while mTOR inhibition alone using Torin-1 does not lead to a significant increase in apoptosis, combined inhibition of IKBKE and mTOR leads to a significant increase in apoptosis. (E) Soft agar colony formation assay in Panc-1 cells indicates that while mTOR inhibition alone does not affect tumorigenicity of the cells, combined inhibition of mTOR and IKBKE leads to significant decrease in tumorigenicity. (F) Tumor volume measurement indicates significant decrease in growth of MiaPaca-2 cell line derived xenograft tumors (n = 6) in response to inducible *IKBKE* knockdown, and synergistic effect with combined *IKBKE* and *mTOR* knockdown while *mTOR* knockdown has no effect. (G) Representative images of xenograft tumors 4 weeks after induction of shRNA indicate significant reduction in tumor volume in response to *IKBKE* knockdown and synergistic reduction with combined *IKBKE/mTOR* knockdown. (H–K) Representative IHC images showing phosphorylation of AKT at Serine-473 in xenograft tumors derived from Control (H), *shmTOR* (I), *shIKBKE* (J), and *shIKBKE/shmTOR* (K) MiaPaca-2 cell lines. Error bars represent Standard Deviation. Statistical significance was determined using a Student's two-tailed t-test. * P<0.05; ** P<0.01