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, TGF-β-activated kinase 1 (TAK1), an upstream regulator of IκB kinase (IKK), which controls canonical NF-κB signaling, was shown to be important for chemoresistance in pancreatic cancer and for regulating KRAS-mutant colorectal cancer cell growth and survival. Here, we show that mutant KRAS upregulates glycogen synthase kinase 3α (GSK-3α), leading to its interaction with TAK1 to stabilize the TAK1-TAB complex to promote IKK activity. In addition, GSK-3α is required for promoting critical noncanonical 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.

**SIGNIFICANCE:** GSK-3α functions to promote IKK/NF-κB activity downstream of oncogenic KRAS via stabilization and activation of the TAK1-TAB complex and to promote noncanonical NF-κB activity via control of nuclear levels of NF-κB2. Inhibition of GSK-3 strongly suppresses growth of human pancreatic tumor explants with downregulation of certain oncogenic NF-κB target genes such as c-myc and TERT. Cancer Discov; 3(6): 1–14. © 2013 AACR.
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, 2). For example, the great majority of pancreatic ductal adenocarcinomas exhibit mutations in KRAS. However, specific targeting of mutant oncogenic KRAS has been a therapeutic challenge (3, 4). 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 TGF-β-activated kinase 1 (TAK1) and glycogen synthase kinase 3 (GSK-3), in the context of colorectal (TAK1) and pancreatic cancers (TAK1, GSK-3; refs. 5–7). 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 evolutionarily conserved transcription factors consisting of RelA (p65), c-Rel, RelB, p50 (precursor p105), or p52 (precursor p100) subunits (8–11). 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 (IKK), which consists of 2 catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ; ref. 8). 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 noncanonical NF-κB signaling pathway, which is dependent upon activation of NF-κB–inducing kinase (NIK). 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 (12).

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 (9, 10). We initially showed the importance of NF-κB in RAS-induced cell transformation (13). We and others have confirmed this animal models of RAS-driven cancer (14–17). Consistent with this, NF-κB is constitutively active in the great majority of pancreatic cancer cell lines and tumors (18). Previous work has shown that constitutive NF-κB activity is dependent upon IKK in pancreatic cancer cell lines (19–22), and knockout of IKKβ suppresses tumor growth/progression in a KRAS/INK4a null animal model of pancreatic cancer (23). Duran and colleagues (24) showed the importance of p62 in coordinating TRAF6 to regulate IKK downstream of oncogenic KRAS-induced signaling. Elucidating additional signaling
components in the canonical NF-κB pathway, as well as understanding events associated with noncanonical NF-κB activation induced by KRAS, is important in understanding KRAS-induced transformation.

TAK1 is a mitogen-activated protein kinase kinase kinase (MAP3K) that initiates downstream NF-κB and mitogen-activated protein kinase (MAPK) signaling in response to cytokines (25). TAK1 activity is dependent upon its association with TAK1-binding partners (TAB1, TAB2, TAB3, and TAB4), which facilitate auto-phosphorylation of TAK1 (Thr178, Thr184, and Ser192) within the kinase activation loop (26, 27). Upon activation, TAK1 promotes the activation of p38 and c-Jun N-terminal kinase (JNK) MAPKs (28). TAK1 plays a central role in NF-κB activation through direct phosphorylation of IKK (25). As described earlier (5, 6), 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; ref. 29). It is a key enzyme involved in diverse biologic processes such as cell-cycle progression, differentiation, and apoptosis (30). Moreover, GSK-3 has been implicated as playing an oncogenic role in various human malignancies, including pancreatic cancer (30–32). Although 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 acute myelogenous leukemia (33). We and others have shown that GSK-3 regulates growth and survival in pancreatic cancer cell lines by driving constitutive IKK and subsequent NF-κB DNA binding and activity (21, 34). However, the mechanism of how GSK-3 activates IKK and a clear distinction, if any, between the roles of the 2 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 noncanonical 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 oncopgenic KRAS-induced pancreatic cancer, and establish GSK-3α and TAK1 as potential therapeutic targets for this disease.

RESULTS

GSK-3α/β Promote Proliferation/Survival of Pancreatic Cancer Cells

Consistent with previous reports (21, 31), we observed a decrease in proliferation of 2 well-characterized KRAS-mutant 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 and Supplementary Fig. 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 used to knockdown 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 with nontargeting 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 GSK-3β. Depletion of either GSK-3α or GSK-3β led to a modest increase in PARP cleavage (Supplementary Fig. S1 and below).

Consistent with previous reports (21, 31), 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, the upstream IKK kinase (25). This led us to hypothesize and investigate a link between GSK-3 and TAK1 in regulating NF-κB activity in pancreatic cancer cells.

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, and TAB3; ref. 27). 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-mutant 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–TAB1 interaction in HPDEKR+ cells (Supplementary Fig. S2). These data show that mutant KRAS drives constitutive TAK1 activity in pancreatic cancer cell lines via stabilization of 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 (Ser 32/36),
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Figure 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-3α, GSK-3β, both GSK-3α and GSK-3β, 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.

stabilization of total IκBα levels, and reduced phosphorylation of p65/RelA, indicating reduced canonical NF-kB activation (Fig. 2B). Taken together, our data indicate that TAK1 plays a key role in regulating NF-kB activity in pancreatic cancer cells, consistent with the study by Melisi and colleagues (6), 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-kB 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 nontargeting control throughout the time-course in an MTS assay (Fig. 2C). This reduced cell index correlated with a weak PARP cleavage response (Supplementary Fig. 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 (5). Consistent with our hypothesis, TAK1 inhibitor treatment resulted in a greater decrease in
the proliferation of MiaPaCa-2 cells compared with TAK1 knockdown (Fig. 2E). Again, the inhibitory effect of the TAK1 inhibitor on cell proliferation was specific to mutant KRAS-harboring cells (Supplementary Fig. S3). The decrease in phosphorylation of p38 (a TAK1 substrate) confirms the inhibition of TAK1 activity upon 5Z-7-oxozaenol treatment (Supplementary Fig. S2).

A modest increase in PARP cleavage upon TAK1 and GSK-3 inhibition (Supplementary Fig. 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 propidium iodide. An increase in the number of cells in G2–M phase was observed with increasing concentrations of the TAK1 inhibitor (Supplementary Fig. 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.

**GSK-3α Facilitates Constitutive TAK1 Activity by Maintaining TAK1–TAB1 Interaction**

We and others previously showed that GSK-3 drives constitutive IKK and NF-κB activity in pancreatic cancer cell lines (21). 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). Although the kinetics of decrease in TAK1 and TAB 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 phosphoglycogen synthase (p-Glc syn), a substrate of GSK-3. Moreover, we also saw a decrease in TAK1 substrate phosphorylation...
(phospho-p38) with GSK-3 inhibition (Supplementary Fig. 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 showed that MG-132 treatment restored reduced TAK1 levels in Panc-1 cells but not in MiaPaCa-2 cells (Supplementary Fig. S6). To show 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. Forty-eight 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 nontransformed HPDE (Fig. 3C), although the level of interaction was higher in the KRAS-mutant cells. Consistent with previous studies (35), expression of oncogenic KRAS led to the upregulation of GSK-3 isoforms (Fig. 3C). Because GSK-3α and TAK1 co-immunoprecipitated in nontransformed HPDEs as well as transformed pancreatic cancer cells, we sought to explore this interaction in other cells such as 293T cells. These cells have very low levels of GSK-3 and TAK1 as compared with pancreatic cancer cells, 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 coimmunoprecipitation (Fig. 3D). No interaction between overexpressed 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 the TAK1–TAB1 complex in MiaPaCa-2 cells. Because AR-A014418 treatment affects TAK1 levels, we transiently overexpressed TAK1 in MiaPaCa-2 cells before treating the cells with AR-A014418. As is evident in Fig. 3E, GSK-3 inhibition suppressed the coprecipitation between TAK1 and TAB1, even under conditions where
TAK1 and TAB1 levels are maintained. Thus, GSK-3 catalytic activity seems to be important in maintaining the TAK1–TAB1 complex. Overall, these data and those described earlier show 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.

**GSK-3α Facilitates Constitutive Noncanonical Signaling in Pancreatic Cancer Cells**

The noncanonical NF-κB pathway leads to processing of NF-κB2/p100 to p52 and has been shown to be constitutively active in pancreatic cancer cells (36, 37). To this point, the data link 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 cancer cell survival/proliferation. Thus, we asked if GSK-3 regulates the noncanonical 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 knockdown of GSK-3α. 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 either that 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 noncanonical NF-κB pathway was measured via the effect of p100 knockdown on the cell index of pancreatic cancer cells. As compared with the nontargeting 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...
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Figure 5. GSK-3 inhibition suppresses tumor growth in mice. A, nude mice were explanted with replicates of human pancreatic tumors and 2 weeks later treated with the GSK-3 inhibitor AR-A014418 at 120 mg/kg twice a day for 2 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 with treatment start. n (vehicle) = 4, n (treatment) = 5. Bottom, representative photograph of mice at the end of 28 days. B, tumors were harvested after the indicated time periods and immunoblotted with the indicated antibodies. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

PARP cleavage in MiaPaCa-2 but not in Panc-1 cells (Supplementary Fig. S7). Overall, these data provide a novel link between GSK-3α and the noncanonical NF-κB pathway in pancreatic cancer cells. These results may explain why knockdown of GSK-3α (that affects both canonical and noncanonical NF-κB) leads to a greater suppression of cell growth as compared with knockdown of GSK-3β or TAK1 (Fig. 1C and D).

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 2 days. The growth of the tumor was then monitored over the 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 TAK1/NF-κB signaling in mice, we repeated the AR-A014418 treatment study on pancreatic tumor explants for shorter time courses (1, 2, 6, and 8 hours). 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.

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 used 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 Significance of analysis Microarray (SAM)]
analysis] for 470 genes, of which 155 changed more than 2-fold (Fig. 6A). We further analyzed known or suspected NF-kB target genes changed by GSK-3 inhibition. In this group, GSK-3 inhibition leads to changes in NF-kB target gene expression. Total RNA was isolated from human pancreatic tumor explants treated with AR-A014418 for 2 or 8 hours and evaluated by microarray. Gene changes that were statistically significant by SAM analysis are shown in the heatmap (P ≤ 0.05). In B, statistically significant changes in known NF-kB target genes are shown in the heatmap (P ≤ 0.05). C, NF-kB target genes that were downregulated more than 1.5-fold are listed with their known function in pancreatic cancer (P ≤ 0.05). PI3K, phosphoinositide 3-kinase.

Fig 6. GSK-3 inhibition leads to changes in NF-kB target gene expression. Total RNA was isolated from human pancreatic tumor explants treated with AR-A014418 for 2 or 8 hours and evaluated by microarray. Gene changes that were statistically significant by SAM analysis are shown in the heatmap (P ≤ 0.01). Color key is for log ratio. A, statistically significant changes in known NF-kB target genes are shown in the heatmap (P ≤ 0.05). B, NF-kB target genes that were downregulated more than 1.5-fold are listed with their known function in pancreatic cancer (P ≤ 0.05). PI3K, phosphoinositide 3-kinase.

**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, 2). Despite extensive research, less than 4% of patients diagnosed with pancreatic cancer are expected to survive past 5 years (38). Because no RAS inhibitors have been effective (2, 3), current research efforts are focusing on targeting deregulated signaling pathways downstream of mutant 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-kB is known to be constitutively active in a majority of pancreatic tumors and pancreatic cancer cell lines, where it regulates proliferation, survival, metastasis, and invasion (18). NF-kB has also been shown to be activated downstream of oncogenic RAS and to promote the oncogenic phenotype (14, 15). Thus, the signaling cascades activating the NF-kB pathway have become attractive targets for novel chemotherapeutic approaches in pancreatic and other cancers (18). There is evidence that multiple of arms of NF-kB signaling are activated in various cancers (10), thus the ability to block NF-kB activity broadly may require multiple inhibitors unless a factor can be identified that regulates multiple NF-kB–relevant signaling pathways.

Previous studies from our laboratory 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 (21, 31, 34, 39). However, the mechanism by which GSK-3 regulates NF-κB and the distinct roles of the 2 isoforms, GSK-3α and GSK-3β, are not well characterized. In this report, we provide the first evidence for a role of GSK-3 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 (21). 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 noncanonical NF-κB by GSK-3α.

TAK1 is a central regulator of NF-κB signaling in diverse physiologic processes, including development, immune responses, and survival (25). Its binding partner, TAB1 was recently shown to play an essential role in the maintenance of TAK1 activity in epithelial tissues (27). Here, we show that the TAK1–TAB1 complex is active in KRAS-mutant cancer cells and is stabilized via a GSK-3α-dependent mechanism (Fig. 2). TAK1 was recently identified as a prosurvival mediator in mutant KRAS-dependent colon cancer (5). Although Singh and colleagues (5, 6) argue against the prosurvival 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. Our results support aspects of each of these studies, as we observed a reduction in NF-κB activity and cell proliferation with inhibition of TAK1 in pancreatic cancer cells (Fig. 2), but we did not see a strong corresponding increase in apoptosis as measured by cleavage of caspase-3 or PARP under these conditions (Supplementary Figs. S1 and S2). However, inhibition of TAK1 led to G1–M arrest in pancreatic cancer cells (Supplementary Fig. S4), which presumably caused a decrease in cell proliferation.

A mechanism for how TAK1 is activated downstream from oncogenic KRAS has not been described. Previously, Duran and colleagues (24) 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 pharmacologic inhibition of GSK-3 in pancreatic cancer cells led to a decrease in levels of TAK1 and its binding partners TAB1 and TAB2 (Fig. 3A and B and Supplementary Fig. S8). The decrease in TAK1 and TAB levels was detected as early as 1 hour following GSK-3 inhibitor treatment and was 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 nontransformed HPDE cells, suggesting that GSK-3α can interact with TAK1 irrespective of the presence or absence of mutant KRAS. However, mutant KRAS upregulates the expression of GSK-3 (ref. 35; Supplementary Fig. 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 mutant KRAS-transformed cells. The mechanistic link between GSK-3α and TAK1 is unclear, as there is no consensus substrate motif of GSK-3 (S/T-XXX-S/T) in TAK1. However, Taelman and colleagues (40) have shown that TAB1 and TAB2 contain multiple GSK-3 consensus motifs. Thus, there is a possibility that GSK-3 phosphorlates TABs to regulate the TAK1–TAB1 complex, and thus the stability of TAK1. This hypothesis will be addressed in future studies.

Although we showed that GSK-3 regulates IKK through TAK1, the effect on cell proliferation with GSK-3 inhibition was much stronger as compared with TAK1 inhibition. These results suggested that additional regulatory events downstream of GSK-3 promote pancreatic cancer cell growth/survival. The noncanonical NF-κB pathway has been shown to be constitutively active and to contribute to proliferation/survival in pancreatic cancer cells (36, 37). 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), led to significantly reduced p100 processing in pancreatic cancer cells (Fig. 4A and B and Supplementary Fig. S8). Importantly, we show a significant reduction in cell proliferation of pancreatic cancer cells upon knocking down the 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 signaling (data not shown). Interestingly, we observed a decrease in nuclear accumulation of the p52 subunit upon GSK-3α knockdown, whereas 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 earlier been shown to accumulate in the nucleus in pancreatic cancers (34), which raises the potential that the GSK-3 effect on noncanonical NF-κB signaling is via processing of nuclear p100. It is also possible that GSK-3α affects the nuclear transport of the p52 subunit. We thus hypothesize that GSK-3α regulates both canonical and noncanonical NF-κB pathways 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 showing the role of GSK-3β and Fbxw7α-mediated degradation of p100 in multiple myeloma (41), as we did not see p100 accumulation upon GSK-3α knockdown. However, our study does support the prosurvival role of p100–p52 processing in a way similar to that described by Arabi and colleagues (42).

The human tumor explant study showed a 50% inhibition of KRAS-mutant tumor growth upon a 2-day treatment with GSK-3 inhibitor (Fig. 5). This study was conducted 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 downregulation 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
Figure 7. Model of the GSK-3–NF-κB pathway downstream of mutant KRAS in pancreatic cancer. In a normal cell, the 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 the TAK1–TAB1 complex, leading to constitutive canonical NF-κB activation. GSK-3α also drives the noncanonical NF-κB pathway by promoting/stabilizing nuclear p52, thus leading to constitutive noncanonical NF-κB activation. NF-κB drives transcription of genes involved in survival, proliferation, and metastasis that contributes to an aggressive pancreatic cancer phenotype.

RAS induce only a selective arm of the NF-κB pathway (43, 44), which is different from the well-characterized NF-κB-regulated pathway 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 pathway.

Interestingly, significant changes were observed in long noncoding RNAs upon GSK-3 inhibition, which are now emerging as key regulators of oncogenesis (45). We understand that GSK-3 plays important roles in other signaling pathways, including Wnt, Notch, and Hedgehog, which have been implicated in pancreatic cancers (46) and which may account for the rest of the observed changes in gene expression. The implications of GSK-3 inhibition on β-catenin stabilization and the Wnt pathway need to be considered (46); 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 the role of GSK-3α in promoting oncogenic RAS function through the regulation of TAK1–TAB activity, upstream of IKK and canonical NF-κB pathway, and via control of noncanonical NF-κB activity in pancreatic cancer cells (Fig. 7). Importantly, these data provide evidence for different roles of GSK-3α and GSK-3β in regulating NF-κB signaling in pancreatic cancer cells, and highlight the need for the development and testing of GSK-3α-specific drugs.

METHODS

Cell Culture and Reagents

Panc-1 and MiaPaCa-2 were purchased from American Type Culture Collection and used for no longer than 6 months before being replaced. HPDE cell line (HPDE6) and KRAS4B G12V–transfected HPDE (HPDEKR+) were generous gifts from Dr. Channing Der (University of North Carolina-Chapel Hill, Chapel Hill, NC) and were maintained in keratinocyte serum-free growth medium. All cell culture reagents were obtained from Invitrogen. The following antibodies were purchased from Cell Signaling Technology: phosphoglycogen synthase (Ser641), glycogen synthase phospho-p65 (Ser536), p-65, TAB1, TAB2, histone H3, cleaved PARP (Asp214), PARP,
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phospho-p38 (Thr180/Tyr182), p38, from Santa Cruz Biotechnology; GSK-3α/b, TAK1, GAPDH, β-tubulin; from Millipore; p100/p52; and from 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 4 annealed double-stranded RNA (dsRNA) oligonucleotides: MAP3K7 (M-003790-06), GSK-3α (M-003009-01), GSK-3β (M-003010-03), NF-κB2 (M-003918-02), and nontargeting control#3 (D001201-03). Dharmafect transfection reagent 1 was used to transfect 100 mmol/L siRNA according to manufacturer’s instructions, 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 (21). Tumor lysates were prepared by homogenizing approximately 1 mm piece of tumor in lysis buffer [50 mmol/L Tris (pH 7.6), 150 mmol/L HCl, and 2 mmol/L 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 (21).

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 the indicated times and cellular proliferation measured using the CellTiter 96 AQueous solution, in accordance with 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

Background impedance signal was measured with 50-μL cell culture medium per well of an E-Plate 16. The final volume in a single well was adjusted to 100 μL cell culture medium by adding additional 50 μL medium containing 1,000 cells. For siRNA experiments, cells were seeded 48 hours after transfection. For inhibitor studies, 5Z-7-oxozaenol was added 1 day after seeding. After plating, impedance was routinely recorded in 2-hour intervals using the xCELLigence real-time cell analysis MP instrument (Roche).

Soft Agar Assay

MiaPaCa-2 cells were transiently transfected with the indicated siRNAs as above. Forty-eight hours after transfection, 8,000 cells were suspended in a 0.4% bacto-agar/Dulbecco’s Modified Eagle Medium (DMEM) layer (1 mL) and plated over a 0.6% bacto-agar/DMEM layer (2 mL) in 6-well plates. Of note, 0.5 mL media was added the next day and every 3 to 4 days thereafter for 2 weeks. After 2 days, the growth of the tumor was measured every alternate day for 28 days. For short term treatments, the tumor was harvested immediately after the indicated time periods.

Microarray Analysis

Total RNA was purified using RNeasy Plus RNA Isolation Kit (Qiagen), reverse-transcribed, labeled, and hybridized to an Agilent v2 8 x 60 K 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 more than 10 in both channels and for probes with data on more than 70% of the microarrays. Hierarchical clustering analyses were conducted using Gene Cluster 3.0 (47), data were visualized using Java TreeView version 1.1.5r2 (48). Expression changes were determined using a linear model with terms for treatment time points (49). This model was fit to each probe, and genes corresponding to treatment effects with a q value 0.05 or less (50) were considered statistically significant. The data are publicly available in Gene Expression Omnibus database (accession number GSE42559).

Tumor Explant Study

Tiny fragments (1.5–2 mm) 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 dimethyl sulfoxide was given intraperitoneally at 120 mg/kg twice a day for 2 days. The growth of the tumor was measured every alternate day for 28 days. For short term treatments, the tumor was harvested immediately after the indicated time periods.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: D. Bang, W. Wilson, A.S. Baldwin
Development of methodology: D. Bang, W. Wilson
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Bang, W. Wilson, M. Ryan, J.J. Yeh
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Bang, M. Ryan, J.J. Yeh, A.S. Baldwin
Writing, review, and/or revision of the manuscript: D. Bang, W. Wilson, J.J. Yeh, A.S. Baldwin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Bang, A.S. Baldwin
Study supervision: A.S. Baldwin

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