Maintenance of Constitutive IκB Kinase Activity by Glycogen Synthase Kinase-3α/β in Pancreatic Cancer

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Abstract

Constitutive nuclear factor κB (NF-κB) activation is among the many deregulated signaling pathways that are proposed to drive pancreatic cancer cell growth and survival. Recent reports suggest that glycogen synthase kinase-3β (GSK-3β) plays a key role in maintaining basal NF-κB target gene expression and cell survival in pancreatic cancer cell lines. However, the mechanism by which GSK-3β facilitates constitutive NF-κB signaling in pancreatic cancer remains unclear. In this report, we analyze the contributions of both GSK-3 isoforms (GSK-3α and GSK-3β) in regulating NF-κB activation and cell proliferation in pancreatic cancer cell lines (Panc-1 and MiaPaCa-2). We show that GSK-3 isoforms are differentially required to maintain basal NF-κB DNA binding activity, transcriptional activity, and cell proliferation in Panc-1 and MiaPaCa-2 cells. Our data also indicate that IκB kinase (IKK) subunits are not equally required to regulate pancreatic cancer–associated NF-κB activity and cell growth. Importantly, we provide the first evidence that GSK-3 maintains constitutive NF-κB signaling in pancreatic cancer by regulating IKK activity. These data provide new insight into GSK-3–dependent NF-κB regulation and further establish GSK-3 and IKK as potential therapeutic targets for pancreatic cancer. [Cancer Res 2008;68(19):8156–63]

Introduction

Pancreatic cancer represents the fourth leading cause of cancer-related death in the United States, with a 5-year patient survival rate of 5% (1). Moreover, an estimated 33,370 patients were expected to die from this disease in 2007 (1). The dismal mortality rate from pancreatic cancer stems from its aggressive metastatic nature and its ability to resist conventional chemotherapies (2). Therefore, expanding our knowledge of the complex molecular pathways responsible for the development of pancreatic cancer is critical for the discovery of new therapeutic strategies. Deregulated nuclear factor κB (NF-κB) and glycogen synthase kinase-3 (GSK-3) signaling are among the many pathways that have been implicated in the pathogenesis of pancreatic cancer.

NF-κB represents a family of evolutionarily conserved transcription factors consisting of five members: c-Rel, RelA (p65), RelB, p50 (NF-κB1/p105 precursor), and p52 (NF-κB2/p100 precursor; ref. 3). The most studied NF-κB complex consists of the p65/p50 heterodimer. In resting cells, NF-κB is rendered inactive within the cytoplasm through association with inhibitory IκB proteins. Various inflammatory stimuli can trigger the activation of the IκB kinase (IKK) complex, which consists of a regulatory subunit (IKKγ) and two catalytic subunits (IKKα and IKKβ; ref. 4). On IKK activation, IκB is phosphorylated and subsequently targeted for rapid proteosomal degradation, thus liberating NF-κB for nuclear translocation, enhanced DNA binding, and transcriptional regulation (5).

Constitutive activation of NF-κB has been characterized in numerous human cancers and is associated with regulating genes that control cell survival, proliferation, metastasis, and angiogenesis (6). Importantly, constitutive NF-κB activation has been observed in 70% of human pancreatic cancers as well as in human pancreatic cancer cell lines and animal models (7–9). Studies have also shown constitutive IKK activity to play a key role in regulating cell survival and cell cycle progression in multiple *in vitro* pancreatic cancer models (8, 10). Thus, there has been growing interest in using IKK as a chemotherapeutic target for pancreatic cancer.

GSK-3 is a serine/theronine kinase that exists as two highly similar mammalian isoforms (GSK-3α and GSK-3β; refs. 11, 12). GSK-3 is recognized for its role in down-regulating β-catenin, thus suppressing the transcriptional activity of T-cell–specific transcription factor/lymphoid enhancer factor complexes within the Wnt/β-catenin pathway (13). Numerous reports have subsequently shown the involvement of this multifunctional kinase in regulating a variety of transcription factors involved in cancer progression, including NF-κB (13–18). A *gsk-3β*–deficient mouse model provided the first evidence of GSK-3–dependent NF-κB regulation (19). These data show that the loss of GSK-3β results in defective NF-κB signaling in response to tumor necrosis factor-α (TNF-α). Furthermore, we previously reported that GSK-3β specifies promoter-specific recruitment of p65/RelA to NF-κB–dependent genes in response to TNF-α (20). A previous report has also implicated GSK-3β in playing a critical role in regulating constitutive NF-κB reporter activity and target gene expression within *in vitro* pancreatic cancer models (21). However, the mechanism by which GSK-3β drives constitutive or inducible NF-κB has not been characterized.

Despite the structural similarity between GSK-3α and GSK-3β, evidence suggests that these isoforms are not functionally redundant in regulating NF-κB (19, 22). In this report, we characterize the individual roles that GSK-3 isoforms play in maintaining constitutive NF-κB activity and cell proliferation in pancreatic cancer cell lines (Panc-1 and MiaPaCa-2). We show that both GSK-3 isoforms can function to regulate basal NF-κB DNA binding and transcriptional activity, whereas GSK-3α predominantly controls cell growth and survival. Our data also show that IKKα and IKKβ exhibit different requirements to drive constitutive

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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NF-κB activity in a pancreatic cancer cell type dependent manner. Additionally, we provide the first evidence that links GSK-3 to constitutive IKK activity in pancreatic cancer cells.

Materials and Methods

Cell cultures and reagents. Panc-1 (CRL-1496) and MiaPaCa-2 (CRL-1420) pancreatic cancer cell lines were obtained from the American Type Culture Collection. Panc-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin. MiaPaCa-2 cells were maintained in DMEM supplemented with 10% FBS and 2.5% horse serum. Cells were cultured in DMEM supplemented with 0.5% FBS for 24 h before experimentation. All cell culture reagents were obtained from Invitrogen. The following antibodies were obtained from Santa Cruz Biotechnology: p65, p50, GSK-3α/β, β-tubulin, and glutathione *S*-transferase (GST). IKKα clone 14A231 and IKKβ clone10AG2 antibodies were purchased from Upstate Biotechnology. The following antibodies were obtained from Cell Signaling Technology: phospho-p65 (Ser^{536}), phospho-glycogen synthase (Ser^{641}), glycogen synthase, cleaved caspase-3 (Asp^{175}), and caspase-3. TNF-α was purchased from Promega. GSK-3 inhibitors (AR-A014418 and SB216763) were obtained from Sigma-Aldrich. The IKKβ inhibitor (compound A) was provided by Bayer Healthcare.

Small RNA interference. The following small interfering RNAs (siRNA; siGenome SMARTpool) were obtained from Dharmacon as a pool of four annealed double-stranded RNA oligonucleotides: IKKα (M-003473-02), IKKβ (M-003503-03), GSK-3α (M-003009-01), and GSK-3β (M-003010-03), plus nontargeting control #3 (D001201-03). In brief, cells were cultured to 70% confluency in six-well plates. Dharmafect 1 transfection reagent was used to transfect 100 nmol/L siRNA according to the manufacturer's instruction.

Electrophoretic mobility shift assay and NF-κB DNA binding ELISA. Electrophoretic mobility shift assay (EMSA) and NF-κB supershift analysis were done on nuclear extracts as previously described (20) using ^{32}P-labeled oligonucleotide probe corresponding to an NF-κB site within the MHC class I promoter region. Relative p65 DNA binding activity was quantified using the TransAM NF-κB p65 transcription factor assay kit (Active Motif) according to manufacturer's instructions. DNA binding activity was measured in triplicate at 450-nm wavelength on a Versamax Microplate Reader (Molecular Devices Corp.).

Western blot analysis. Whole cell lysates were prepared on ice using Mammalian Protein Extraction Reagent (Pierce Biotechnology) according to the manufacturer's instructions. Cytoplasmic extracts were prepared as previously described (23). Protein extracts were quantified by Bradford assay (Bio-Rad Laboratories) and analyzed by SDS-PAGE as previously described (20).

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium cellular proliferation assay. Cells were seeded in triplicate at 3×10^3 per well (96-well plate) and cultured in the presence or absence of GSK-3 or IKKβ inhibitors at the indicated time course. Alternatively, cells were transiently transfected with appropriate siRNA and cultured at the indicated time points posttransfection. At the end of each time point, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) compound (Promega) was added and absorbance was read at 490 nm on a Versamax Microplate Reader (Molecular Devices).

Dual-luciferase reporter assay. Cells were seeded in triplicate at 2×10^4 per well (24-well plate), transfected with the appropriate siRNA as described above, and cultured for 24 h. After siRNA transfection, cells were cotransfected with 200 ng of luciferase reporter construct containing tandem NF-κB binding sites from the MHC class I promoter region and 5 ng of pRL-TK Renilla luciferase construct (Promega) using Fugene6 transfection reagent (Roche Applied Science). Cells were cultured for an additional 24 h, harvested in passive lysis buffer, and analyzed according to Dual-Luciferase Assay System protocol (Promega). Relative light units were measured on an Lmax Microplate Luminometer (Molecular Devices) and normalized to pRL-TK Renilla luciferase light units.

Real-time PCR. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Two micrograms of RNA were reverse transcribed into cDNA using Superscript II Reverse Transcriptase (Invitrogen). Real-time PCR was done and analyzed as previously described (20) using TaqMan Gene Expression Assay primer-probe sets (Applied Biosystems) for *IκBα* (Hs00153283_m1) and *Bcl-xL* (Hs00236329_m1).

IKK kinase assay. Whole cell lysates were prepared on ice for 45 min in lysis buffer containing 20 mmol/L Tris (pH 8.0), 500 mmol/L NaCl, 0.25% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1× protease inhibitor (Roche Applied Science), and 1× phosphatase inhibitor cocktail 1 (Sigma-Aldrich). IKK complexes were immunoprecipitated from 500-μg protein extract using an IKKα antibody (Upstate Biotechnology). An *in vitro* kinase assay was done and analyzed as previously described (20). GST-IκBα substrate phosphorylation was visualized by autoradiography and quantitated using Image Quant version 5.2 software (Molecular Dynamics).

Luminescence-based caspase-3/7 activity assay. Cells were plated in triplicate at 2×10^3 per well in white-walled 96-well plates (Becton Dickinson). Cells were transiently transfected with siRNA as described above. Caspase-3/7 activity was measured at 48, 72, and 96 h posttransfection using the Caspase-Glo 3/7 assay (Promega) according to the manufacturer's instructions. Caspase-Glo 3/7 assay uses a caspase-3/7 tetrapeptide DEVD substrate that produces a luminescent signal on cleavage. Relative light units were measured on an Lmax Microplate Luminometer (Molecular Devices).

Statistical analysis. Prism software (GraphPad Software, Inc.) was used for statistical analysis of data. An unpaired *t* test was used to evaluate differences between group means. $P < 0.05$ was considered to be statistically significant.

Results

GSK-3 isoforms regulate constitutive NF-κB activity in pancreatic cancer cells. A recent study emphasized the role that GSK-3β plays in maintaining constitutive NF-κB reporter activity and target gene expression in pancreatic cancer cells (21). However, this report did not address the individual requirements of both GSK-3 isoforms in regulating NF-κB. Furthermore, the mechanism by which GSK-3 regulates NF-κB activity requires understanding. Here we have analyzed constitutive NF-κB DNA binding and transcriptional activity in two well-known *in vitro* pancreatic carcinoma models (Panc-1 and MiaPaCa-2). As shown in Fig. 1*A*, constitutive NF-κB DNA binding activity is detected in Panc-1 cells through EMSA analysis of nuclear extracts. This form of NF-κB activity is shown to be the p65/p50 heterodimer through supershift analysis (Fig. 1*A*). EMSA analysis of MiaPaca-2 cells showed constitutive DNA binding of p65/p50 heterodimers as well as p50 homodimers (Fig. 1*A*). Cells were treated with two structurally distinct pharmacologic inhibitors of both GSK-3 isoforms (AR-A014418 and SB-216763) to determine a potential requirement of GSK-3 in controlling constitutive NF-κB DNA binding activity. Previously reported treatment duration and inhibitor concentrations (21) were used in our studies. Treatment with either GSK-3 inhibitor for 24 h at 15 and 25 μmol/L reduces p65/50 DNA binding in both cell lines (Fig. 1*B*). DNA binding activity of p50 homodimers was largely unaffected by GSK-3 inhibition in MiaPaCa-2 cells. To ask whether direct inhibition of GSK-3 isoforms would affect NF-κB constitutive DNA binding activity in pancreatic cancer cells, RNA interference of GSK-3α and GSK-3β was used. Western blot analysis confirms that GSK-3 RNA interference was isoform specific and significantly reduced GSK-3 protein levels relative to nontargeting siRNA control (Fig. 1*C*). Loss of either GSK-3 isoform reduced constitutive NF-κB DNA binding in both cell lines (Fig. 1*D*). Moreover, an ELISA-based DNA binding assay showed a significant

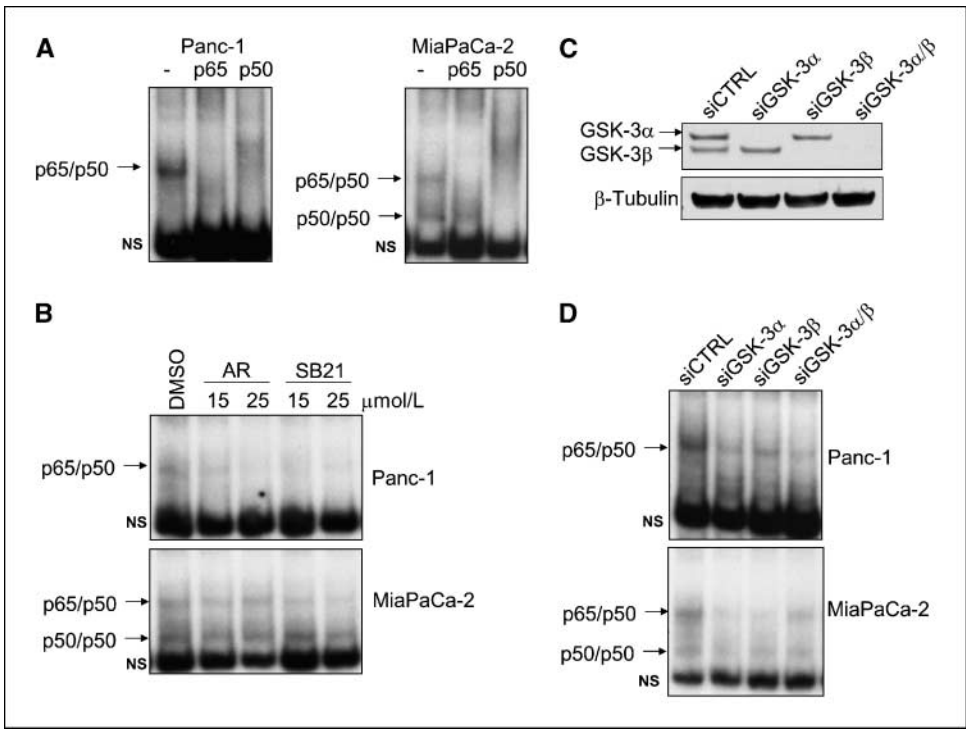

Figure 1. GSK-3 is required for constitutive NF-κB DNA binding. EMSA was done on Panc-1 and MiaPaCa-2 nuclear extracts using ^{32}P-labeled NF-κB–specific probe. *A,* supershift analysis was done using antibodies against p65 and p50. *Arrows,* p65/50 and p50/p50 NF-κB complexes. *NS,* nonspecific binding. *B,* cells were treated with vehicle control (DMSO) or GSK-3 inhibitors (AR-A014418 and SB216763) at 15 and 25 μmol/L for 24 h. *C,* Panc-1 cells were transiently transfected with 100 nmol/L siRNA targeted against GSK-3α, GSK-3β, combined GSK-3α/β, and nontargeting control (*siCTRL*) for 48 h. Cytoplasmic extracts were harvested and separated by SDS-PAGE. Immunoblots were done with the specified antibodies. *D,* nuclear extracts were harvested from Panc-1 and MiaPaCa-2 cells transfected with siRNA as described above and EMSA was done.

reduction of p65 binding activity following the knockdown of either GSK-3 isoform (Supplementary Fig. S1).

To determine whether GSK-3 isoforms play roles in regulating NF-κB transcriptional activity, we measured the effect of GSK-3 RNA interference on basal NF-κB-luciferase reporter activity. Our data show that knockdown of either GSK-3 isoform resulted in a significant reduction of constitutive NF-κB-luciferase reporter activity in Panc-1 and MiaPaCa-2 cells (Fig. 2*A*). Next, we examined whether GSK-3–dependent regulation of reporter activity corresponded with the expression of two NF-κB–regulated genes (*IκBα* and *Bcl-xL*). Loss of GSK-3β alone and both GSK-3α/β significantly suppressed expression of *Bcl-xL* (Fig. 2*B*). Furthermore, knockdown of either GSK-3 isoform alone or combined GSK-3α/β resulted in reduced expression of *IκBα* (Fig. 2*B*). Ser^{536} phosphorylation of

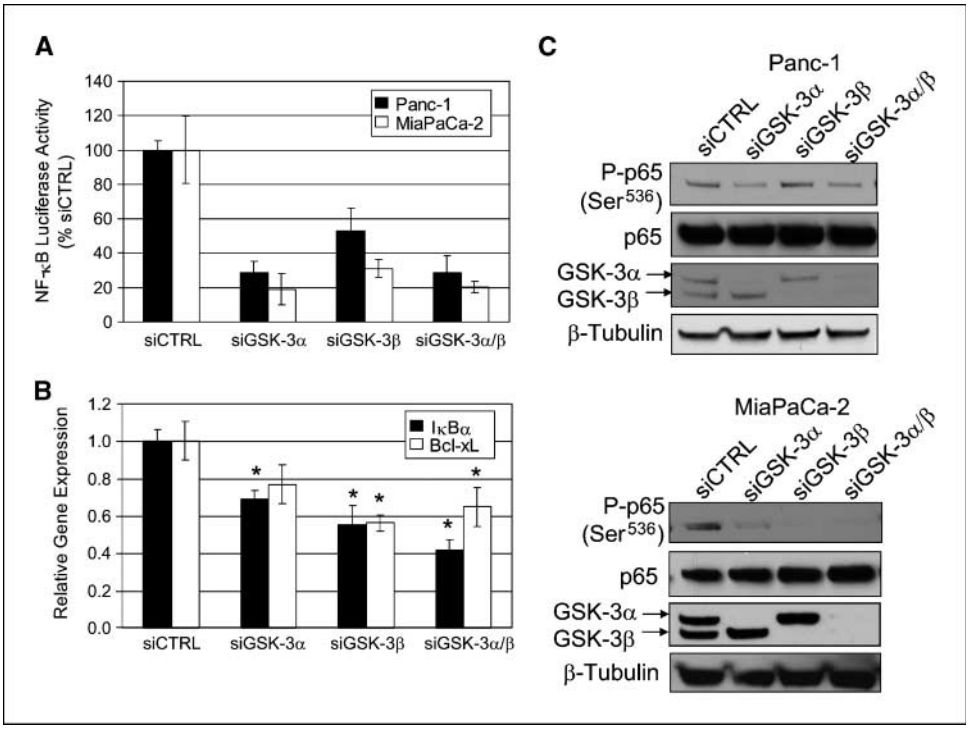

Figure 2. GSK-3 regulates NF-κB transcriptional activity. *A,* Panc-1 and MiaPaCa-2 cells were transiently transfected with 100 nmol/L siRNA targeted against GSK-3α, GSK-3β, combined GSK-3α/β, and nontargeting control for 24 h. Cells were subsequently transfected with 3X-NF-κB and Renilla luciferase reporter constructs for 24 h. Luciferase activity was measured in triplicate and indicated as percent activity relative to nontargeting control. Reporter activities from cells transfected with GSK-3 siRNA were all found to be significantly different relative to nontargeting control ($P < 0.05$). *B,* Panc-1 cells were transfected with siRNA as described above for 48 h. RNA was harvested, and expression of *IκBα* and *Bcl-xL* was analyzed by real-time PCR in triplicate. *, $P < 0.05$, relative to nontargeting control. *C,* Panc-1 and MiaPaCa-2 were transiently transfected with siRNA as described above for 48 h. Whole cell extracts were harvested and separated by SDS-PAGE. Immunoblots were done with the specified antibodies.

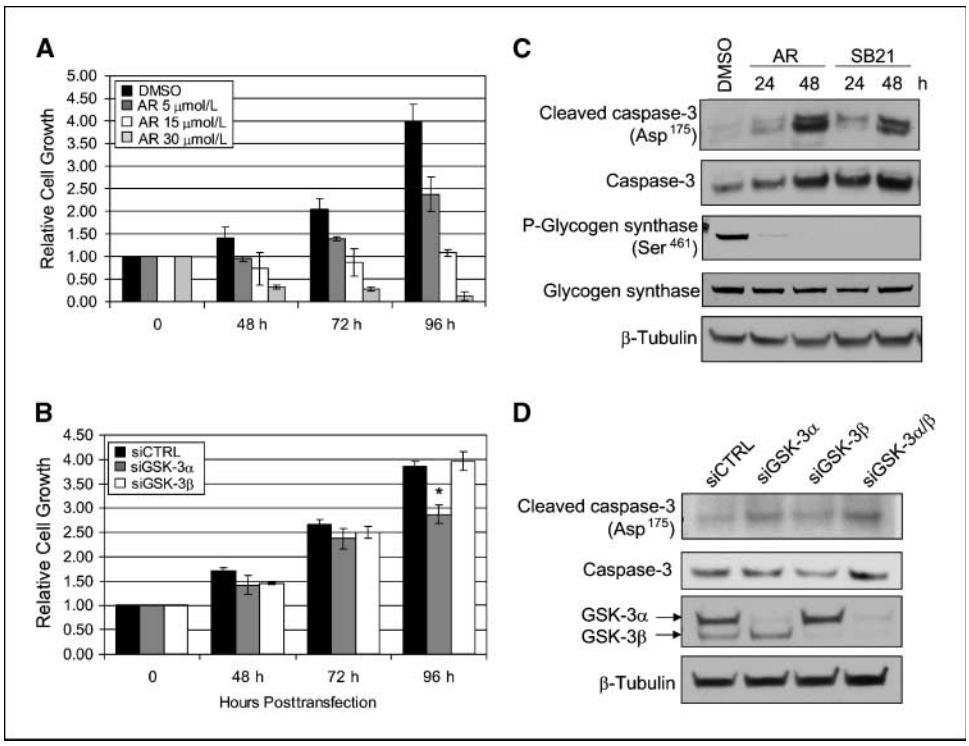

Figure 3. Blockade of GSK-3 suppresses cell proliferation. *A,* Panc-1 cells were treated with DMSO or 5, 15, and 30 μmol/L of the GSK-3 inhibitor (AR-A014418) for 48, 72, and 96 h. Cell growth was measured in triplicate at each time point using a colorimetric MTS tetrazolium assay. *B,* Panc-1 cells were transiently transfected with siRNA targeted against GSK-3α, GSK-3β, and nontargeting control. Cell growth was measured as described above at 48, 72, and 96 h posttransfection. Data were normalized to the initial cell density before GSK-3 inhibitor treatment or siRNA transfection. *, $P < 0.05$, relative to nontargeting control. *C,* Panc-1 cells were treated with DMSO or 30 μmol/L of GSK-3 inhibitors (AR-A014418 and SB216764) for 24 and 48 h. Whole cell extracts were harvested and separated by SDS-PAGE. *D,* Panc-1 cells were transiently transfected with 100 nmol/L siRNA targeted against GSK-3α, GSK-3β, combined GSK-3α/β, and nontargeting control for 48 h. Whole cell extracts were harvested and separated by SDS-PAGE. Immunoblots were done with the specified antibodies.

the NF-κB subunit, p65/RelA, correlates with its transcriptional activity (24). To determine whether the GSK-3 isoforms regulate p65 phosphorylation, RNA interference was used. Extracts were prepared from cells treated with control siRNA or siRNA for either GSK-3 isoform. Consistent with the elevated NF-κB activity in Panc-1 in MiaPaCa-2 cells, we also observe constitutive Ser^{536} phosphorylation of p65 (Fig. 2*C*). RNA interference of GSK-3α or combined GSK-3α/β reduced p65 phosphorylation in Panc-1 cells (Fig. 2*C*). Conversely, knockdown of GSK-3β had a greater effect on basal p65 phosphorylation in MiaPaCa-2 cells (Fig. 2*C*). Taken together, these data suggest that both GSK-3 isoforms play individual roles in maintaining constitutive NF-κB DNA binding and transcriptional activity in Panc-1 and MiaPaCa-2 cells.

GSK-3α promotes pancreatic cancer cell growth and survival. Constitutive NF-κB activity has been reported to play mitogenic and antiapoptotic roles in pancreatic cancer cell lines (8, 9). Pharmacologic GSK-3 inhibition and GSK-3 RNA interference were used to determine a role for GSK-3 in controlling proliferation and survival of Panc-1 and MiaPaCa-2 cells. Consistent with a previous report (21), GSK-3 inhibition (AR-A014418) decreased growth of Panc-1 cells in a dose-dependent manner (5, 15, and 30 μmol/L) over a 96-hour time course (Fig. 3*A*). Similar results were observed in MiaPaCa-2 cells (data not shown). Next, we used RNA interference to determine the individual requirements for GSK-3α and GSK-3β on cell viability. We observed a significant reduction in Panc-1 cell growth following GSK-3α RNA interference at 96 hours after siRNA transfection when compared with nontargeting control and GSK-3β siRNA (Fig. 3*B*). Similar results were observed in MiaPaCa-2 cells (data not shown). Moreover, the knockdown of GSK-3α in Panc-1 cells resulted in a significant reduction in the total number of cells at 72 and 96 hours after siRNA transfection (Supplementary Fig. S2*A*). Western blot analysis confirmed knockdown of GSK-3 isoforms at each time point after siRNA transfection (Supplementary Fig. S2*B*). Importantly, we did not observe complete GSK-3 knockdown until 72 and 96 hours after siRNA transfection (Supplementary Fig. S2*B*). These data could explain why the effects of GSK-3α knockdown were only seen at later time points after siRNA transfection.

The induction of caspase-3 cleavage was also measured to determine whether the loss of cell viability following the disruption of GSK-3 was due to apoptosis. An increase in caspase-3 cleavage following 30 μmol/L pharmacologic GSK-3 inhibition was observed over 24 and 48 hours in Panc-1 (Fig. 3*C*). The efficiency of GSK-3 inhibition was indicated by the reduced basal phosphorylation of a GSK-3 substrate, glycogen synthase at Ser^{461} (Fig. 3*C*). Moreover, GSK-3α knockdown induced caspase-3 cleavage 48 hours post-transfection of siRNA (Fig. 3*D*). Similar results were observed in MiaPaCa-2 cells (data not shown). A luminescent caspase-3/7 assay was used to confirm a significant increase in caspase activity following the knockdown of GSK-3α or combined GSK-3α/β in Panc-1 cells (Supplementary Fig. S3). Notably, pharmacologic GSK-3 inhibition was more effective than GSK-3 knockdown in suppressing cell growth and inducing caspase-3 cleavage. These observations could indicate unknown off-target effects of pharmacologic GSK-3 inhibition (Fig. 3; see Discussion). Taken together, we show that the loss of GSK-3α is more efficient than GSK-3β in suppressing cell growth and survival in Panc-1 and MiaPaCa-2 cells. Importantly, these observations correlate with the ability of GSK-3α to regulate constitutive NF-κB activity.

Constitutive NF-κB activity in pancreatic cancer cells is IKK dependent. In addition to the studies shown above relative to GSK-3, studies have also shown IKK to be essential in regulating constitutive NF-κB signaling in pancreatic cancer cells (8–10). To confirm these findings, NF-κB DNA binding activity was analyzed in the presence of an established small-molecule IKKβ inhibitor (compound A; ref. 25). Compound A treatment for 24 hours showed dose-dependent suppression of constitutive NF-κB DNA binding

activity in Panc-1 and MiaPaCa-2 cells (Fig. 4*A*). We note that IKKβ inhibition is more effective in blocking constitutive NF-κB DNA binding activity in MiaPaCa-2 cells. Next, RNA interference to IKKα and IKKβ was used to determine the individual requirements of IKK subunits for constitutive NF-κB DNA binding activity and transcriptional activation. The knockdown of IKK subunits was specific and significantly reduced protein levels relative to nontargeting siRNA control (Fig. 4*B*). We observed a decrease in p65/p50 DNA binding activity following the knockdown of either IKK subunit in MiaPaCa-2 cells (Fig. 4*C*). Interestingly, knockdown of IKKα alone or combined IKKα/β knockdown caused significant loss of NF-κB DNA binding activity in Panc-1 cells (Fig. 4*C*). The individual role that each IKK subunit plays in regulating basal NF-κB transcriptional activity was measured by an NF-κB reporter assay. RNA interference against either IKK subunit caused substantial suppression of reporter activity in both Panc-1 and MiaPaCa-2 cells (Fig. 4*D*). However, the suppressive effects of IKKα and combined IKKα/β knockdown were stronger than IKKβ knockdown alone (Fig. 4*D*). Thus, our data suggest that both IKK subunits play a role in regulating constitutive NF-κB activity in pancreatic cancer cells, but IKKα may play a more significant role in Panc-1 cells.

IKK regulates pancreatic cancer cell growth and survival. To determine the functional significance of NF-κB regulation by IKK in pancreatic cancer cells, growth and survival were measured following IKKβ inhibition (compound A) or IKK RNA interference. We showed that a higher concentration of compound A (5 μmol/L) was required to significantly reduce Panc-1 cell growth (Fig. 5*A*). However, MiaPaCa-2 cells were more sensitive to increasing concentration of compound A, as shown by significant loss of cell growth after 48-hour treatment at 1, 3, and 5 μmol/L (Fig. 5*A*). These results are consistent with our previous observations of compound A being more effective in suppressing constitutive NF-κB DNA binding in MiaPaCa-2 cells (Fig. 4*A*). Cell growth was also measured in cells at 48, 72, and 96 hours posttransfection of IKKα and IKKβ siRNA. Furthermore, we used p65/RelA RNA interference to test the dependency of NF-κB for cell growth. In Panc-1 cells, loss of either IKK subunit or p65/RelA significantly reduced cell growth 48 hours after siRNA transfection (Fig. 5*B*). However, knockdown of IKKα and p65/RelA was more effective in suppressing cell growth at the 72- and 96-hour time points (Fig. 5*B*). In MiaPaCa-2 cells, knockdown of IKKα, IKKβ, and p65/RelA each resulted in a significant reduction in cell growth 96 hours after siRNA transfection (Fig. 5*B*). We note that MiaPaCa-2 cell growth is more sensitive to the loss of both IKK subunits, whereas Panc-1 cell growth is more dependent on IKKα. Whereas siRNA effects are not complete in their ability to knock down individual proteins, these experiments show that the IKK subunits and p65/RelA are involved in the growth and survival of pancreatic cancer cells.

The induction of caspase-3 cleavage was also measured to determine whether the reduction of cell growth following the disruption of IKK was due to apoptosis. We observed a dose-dependent increase in caspase-3 cleavage on 24-hour treatment with compound A in Panc-1 cells (Fig. 5*C*). Similar results were obtained with MiaPaCa-2 cells (data not shown). Additionally,

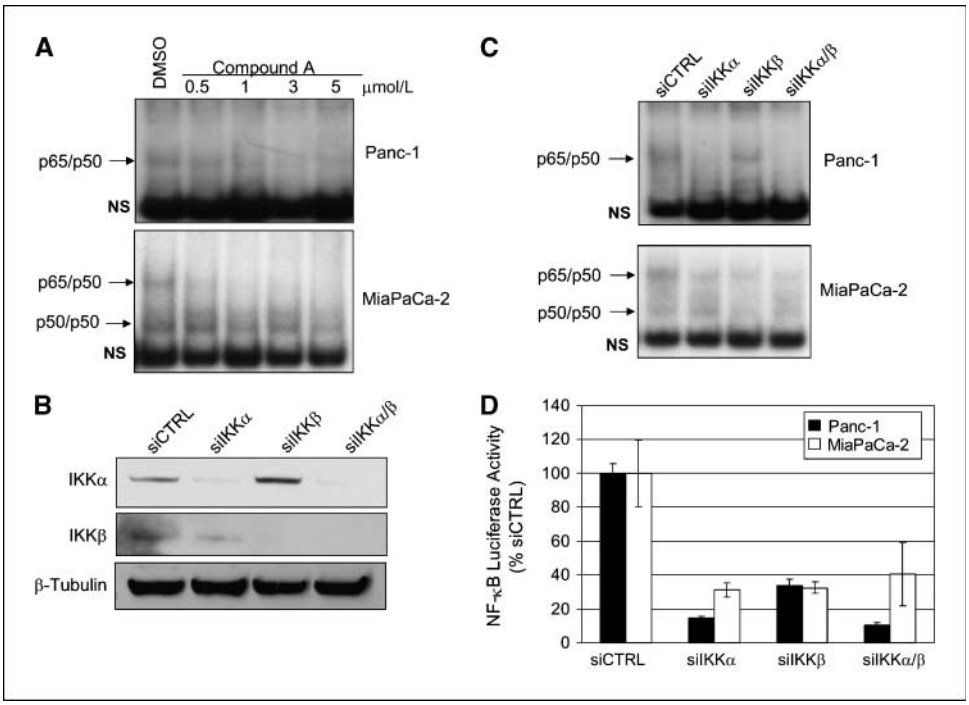

Figure 4. IKK is required for constitutive NF-κB activation. *A,* Panc-1 and MiaPaCa-2 cells were treated with DMSO or 0.5, 1, 3, and 5 μmol/L of IKKβ inhibitor (compound A) for 24 h. Nuclear extracts were harvested and EMSA was done using ^{32}P-labeled NF-κB–specific probe. *Arrows,* NF-κB complexes as determined by supershift analysis in Fig. 1*A*. *B,* Panc-1 cells were transiently transfected with 100 nmol/L siRNA targeted against IKKα, IKKβ, combined IKKα/β, and nontargeting control for 48 h. Cytoplasmic extracts were harvested from cells transfected with siRNA as described above. Extracts were separated by SDS-PAGE and immunoblots were done with the specified antibodies. *C,* EMSA was done on nuclear extracts harvested from cells transfected with siRNA as described above. *D,* Panc-1 and MiaPaCa-2 cells were transfected with siRNA as described above for 24 h. Cells were subsequently transfected with 3X-NF-κB and Renilla luciferase reporter constructs for 24 h. Luciferase activity was measured in triplicate and indicated as percent activity relative to nontargeting control. Reporter activities from cells transfected with IKK siRNA were all found to be significantly different relative to nontargeting control.

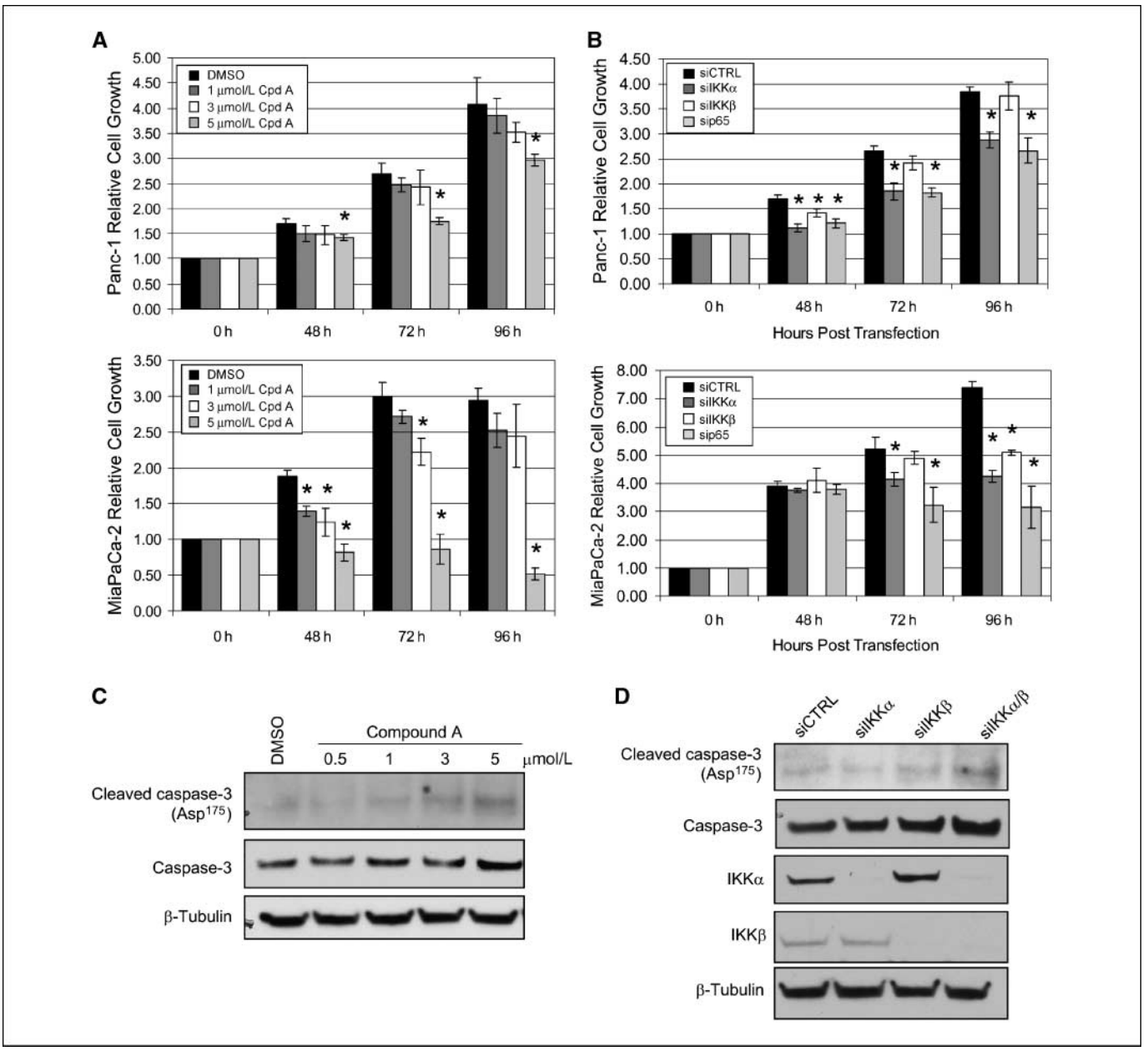

Figure 5. Blockade of IKK suppresses cell proliferation. *A,* Panc-1 and MiaPaCa-2 cells were treated with DMSO or 1, 3, and 5 μmol/L of IKKβ inhibitor (compound A) for 48, 72, and 96 h. Cell growth was measured in triplicate at each time point using a colorimetric MTS tetrazolium assay. *B,* Panc-1 and MiaPaCa-2 cells were transiently transfected with 100 nmol/L siRNA targeted against IKKα, IKKβ, p65, and nontargeting control. Cell growth was measured as described above at 48, 72, and 96 h posttransfection. Data were normalized to the initial cell density before compound A treatment or siRNA transfection. *, $P < 0.05$, relative to DMSO or nontargeting control. *C,* Panc-1 cells were treated with compound A as described above for 24 h. Whole cell extracts were harvested and separated by SDS-PAGE. *D,* Panc-1 cells were transiently transfected with 100 nmol/L siRNA targeted against IKKα, IKKβ, combined IKKα/β, and nontargeting control. Whole cell extracts were harvested, separated by SDS-PAGE, and immunoblotted with the specified antibodies.

maximal caspase-3 cleavage was induced in Panc-1 cells following knockdown of both IKKα and IKKβ subunits (Fig. 5*D*). Furthermore, we observed a significant increase in caspase-3/7 activity following RNA interference against either IKKα or IKKβ (Supplementary Fig. S4). Overall, our data suggest that differential NF-κB regulation by IKK subunits correlates with cell growth and survival in Panc-1 and MiaPaCa-2 cells.

GSK-3 is required for constitutive IKK activity. The mechanism by which GSK-3 regulates constitutive NF-κB signaling in pancreatic cancer is poorly understood. Our data thus far have shown that loss of GSK-3 isoforms and IKK subunits suppresses constitutive NF-κB activity, cell growth, and survival in Panc-1 and MiaPaCa-2 cells. To determine whether GSK-3 regulates constitutive IKK activity in pancreatic cancer cells, the effects of pharmacologic GSK-3 inhibition on IKK-dependent IκBα phosphorylation were measured via an *in vitro* IKK kinase assay. Panc-1 cells were treated for 24 hours with GSK-3 inhibitor (AR-A014418) at 25 and 50 μmol/L. Endogenous IKK complexes were immunoprecipitated with an IKKα-specific antibody. Immunoprecipitates were incubated with GST-IκBα, and an *in vitro* kinase reaction was

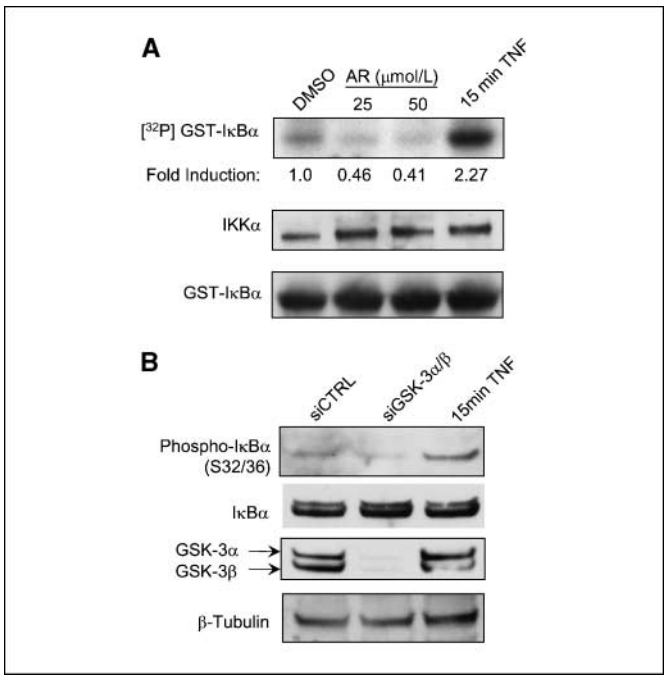

Figure 6. GSK-3 inhibition suppresses constitutive IKK kinase activity. *A,* Panc-1 cells were treated with DMSO or GSK-3 inhibitor (AR-A014418) at 25 and 50 μmol/L for 24 h. As control, cells were treated with 10 ng/mL TNF-α for 15 min. Whole cell extracts were harvested. IKKα antibody was used to immunoprecipitate IKK complex. Kinase activities within immunocomplexes were assayed following incubation with wild-type GST-IκBα substrate in the presence of [γ-^{32}P]ATP. Autoradiography was quantified and indicated as fold induction relative to DMSO. Immunoblots were done against IKKα and GST to confirm equal loading. *B,* Panc-1 cells were treated with 10 ng/mL TNF-α for 15 min or transiently transfected with 100 nmol/L siRNA targeted against GSK-3α/β and nontargeting control for 48 h. Whole cell extracts were harvested, separated by SDS-PAGE, and immunoblotted with the specified antibodies.

done. GSK-3 inhibition reduced phosphorylation of GST-IκBα in a dose-dependent manner (Fig. 6*A*). Moreover, the basal phosphorylation status of IκBα at Ser$^{32/36}$ was measured in Panc-1 cells following the knockdown of both GSK-3 isoforms. Consistent with our *in vitro* kinase assay data, the loss of GSK-3 also resulted in a reduction of endogenous IκBα phosphorylation (Fig. 6*B*) and is consistent with an inhibition of IKK activity. Notably, these data provide the first evidence that GSK-3 regulates cancer cell–associated NF-κB by maintaining IKK activity.

Discussion

GSK-3β has previously been reported to play an essential role in maintaining constitutive NF-κB reporter activity and expression of NF-κB target genes in pancreatic cancer cells (21). In this study, we show that both GSK-3 isoforms function to regulate constitutive NF-κB activity in Panc-1 and MiaPaCa-2 cells. Although both GSK-3 isoforms contribute to regulating NF-κB activity, GSK-3α was shown to be primarily required for cell growth (Fig. 3*B*; Supplementary Fig. S2*A*). Moreover, the suppression in cell growth following the loss GSK-3α may be due to increased apoptotic signaling, as shown by an induction of caspase-3 activity (Fig. 3*D*; Supplementary Fig. S3). We note that pharmacologic inhibition of GSK-3 was more effective than GSK-3α knockdown in suppressing Panc-1 cell growth (Fig. 3*A* and *B*). Although the GSK-3 inhibitor (AR-A014418) was not shown to affect a panel of closely related protein kinases (26), unknown off-target effects cannot be ruled out when interpreting growth inhibition in pancreatic cancer cells. Importantly, we show that complete knockdown of GSK-3 and significant growth suppression were not observed until later time points after siRNA transfection (Supplementary Fig. S2). These data may also explain why GSK-3α siRNA was less effective in suppressing cell growth relative to GSK-3 pharmacologic inhibition. Overall, our data suggest that GSK-3 isoforms are not functionally redundant in regulating NF-κB activity and cell growth in pancreatic cancer cells.

IκB kinases play a central role in regulating NF-κB signal transduction. Therefore, efforts have been made in using IKK as a therapeutic target to block constitutive NF-κB activity. In this report, we use an IKKβ-specific small-molecule inhibitor (compound A) that has been shown to be effective in blocking constitutive (27) and inducible (25) NF-κB activity in various cell lines. Our data show that compound A is also efficient in inhibiting constitutive NF-κB activity (Fig. 4) and cell proliferation (Fig. 5) in pancreatic cancer cells. In addition to IKKβ inhibition, we show that IKKα is the predominant regulator of constitutive NF-κB activity and cell growth in Panc-1 (Figs. 4 and 5). Notably, higher concentrations of the IKKβ inhibitor (compound A) were required to diminish Panc-1 cell growth. These data raise the possibility that compound A may target IKKα at higher doses. Indeed, compound A was shown to inhibit recombinant IKKα activity at higher concentrations (K_i for ATP, 135 nmol/L; ref. 25). Overall, we show that the individual requirements for IKKα and IKKβ for driving NF-κB activity vary between Panc-1 and MiaPaCa-2 cells. These data underscore the heterogeneity between pancreatic carcinoma cell lines and prompt the need to understand the variety of oncogenic mutations that potentiate NF-κB activity. Moreover, our results emphasize the need for IKKα-specific small-molecule inhibitors to target constitutive NF-κB activity in select pancreatic carcinoma cell types.

The mechanism by which GSK-3 regulates constitutive NF-κB activity in pancreatic cancer is not fully understood. Our data suggest that GSK-3 and IKK may function together to regulate constitutive NF-κB activity. Previous reports have speculated on whether IKK is required for GSK-3–dependent NF-κB activity. Analyses from our group and others provide evidence that GSK-3β functions independent of the IKK complex during TNF-α–induced NF-κB signaling (19, 20, 28). Moreover, GSK-3 was also suggested to function independent of IKKβ in pancreatic cancer cell lines (21). In this regard, our current data show GSK-3–dependent regulation of constitutive IKK activity in certain pancreatic cancer cell lines. We show that inhibition (AR-A014418) or knockdown of both GSK-3 isoforms suppressed constitutive IKK activity in Panc-1 cells (Fig. 6). Notably, this abrogation in constitutive IKK activity correlated with reduced NF-κB activity, cell growth, and survival. Thus, we provide the first evidence that links GSK-3 and IKK in constitutive NF-κB signaling in pancreatic cancer cells.

Pancreatic cancer is a highly aggressive, metastatic disease known for its dismal mortality rate. Despite current chemotherapeutic efforts to improve clinical prognosis, pancreatic cancer still remains to be among the most drug-resistant tumors. Consequently, there is growing interest on specifically targeting deregulated signaling pathways that drive the molecular pathogenesis of pancreatic cancer. We emphasize in this report the critical roles that GSK-3 and IKK play in maintaining constitutive NF-κB signaling. Collectively, our data provide new

insight into GSK-3–dependent NF-κB regulation and further establish GSK-3 and IKK as potential therapeutic targets for pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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