GSK-3β Governs Inflammation-Induced NFATc2 Signaling Hubs to Promote Pancreatic Cancer Progression

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Abstract

We aimed to investigate the mechanistic, functional, and therapeutic role of glycogen synthase kinase 3β (GSK-3β) in the regulation and activation of the proinflammatory oncogenic transcription factor nuclear factor of activated T cells (NFATc2) in pancreatic cancer. IHC, qPCR, immunoblotting, immunofluorescence microscopy, and proliferation assays were used to analyze mouse and human tissues and cell lines. Protein–protein interactions and promoter regulation were analyzed by coimmunoprecipitation, DNA pulldown, reporter, and ChIP assays. Preclinical assays were performed using a variety of pancreatic cancer cell lines, xenografts, and a genetically engineered mouse model (GEMM). GSK-3β–dependent SP2 phosphorylation mediates NFATc2 protein stability in the nucleus of pancreatic cancer cells stimulating pancreatic cancer growth. In addition to protein stabilization, GSK-3β also maintains NFATc2 activation through a distinct mechanism involving stabilization of NFATc2–STAT3 complexes independent of SP2 phosphorylation. For NFATc2–STAT3 complex formation, GSK-3β–mediated phosphorylation of STAT3 at Y705 is required to stimulate euchromatin formation of NFAT target promoters, such as cyclin-dependent kinase-6, which promotes tumor growth. Finally, preclinical experiments suggest that targeting the NFATc2–STAT3–GSK-3β module inhibits proliferation and tumor growth and interferes with inflammation-induced pancreatic cancer progression in Kras(G12D) mice. In conclusion, we describe a novel mechanism by which GSK-3β fine-tunes NFATc2 and STAT3 transcriptional networks to integrate upstream signaling events that govern pancreatic cancer progression and growth. Furthermore, the therapeutic potential of GSK-3β is demonstrated for the first time in a relevant Kras and inflammation-induced GEMM for pancreatic cancer.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is among the most lethal of solid malignancies with more than 40,000 new cases diagnosed in the United States each year, thus posing a significant health problem at the beginning of the twenty-first century (1). Mouse models and genetic studies have highlighted that pancreatic carcinogenesis occurs through chronicologic accumulation of genetic alterations, of which activating mutations of the Kras oncogene represent a signature event that governs a multitude of mitogenic signaling events driving tumor initiation and progression (2). Importantly, preneoplastic cells and pancreatic cancer cells show a marked upregulation of inflammatory cytokines and transcription factors and are surrounded by a pronounced inflammatory microenvironment that enables Kras-mutant cells to progress toward frank malignancy (3–8). Moreover, chronic pancreatitis represents an important risk factor for the development of pancreatic cancer, underscoring the crucial role of inflammation in the process of malignant transformation in the pancreas (9–11). Blockade of these inflammatory factors, either directly or indirectly via interference with relevant signaling nodes, is likely to be important for effective Kras-directed therapy.

Interestingly, a recent study discovered Kras-dependent epigenetic induction of the glycogen synthase kinase 3β (GSK-3β) in a genetically engineered mouse model (GEMM) of PDAC. The authors showed that Kras signaling recruits the ETS/p300 transcriptional complex to the GSK-3β promoter, whose increased expression stimulated cancer cell growth (12). GSK-3β is a constitutive active and multifunctional Ser/Thr kinase, which, due to
its broad substrate specificity, is involved in numerous cellular processes (13, 14). GSK-3β was initially recognized as an important tumor suppressor in various cancers predominantly acting through destabilization of the oncogene β-catenin (15). However, GSK-3β also plays important promotormigenic roles in several human malignancies, most notably in pancreatic cancer, regulating proliferation and survival (16–20). Increased expression of active GSK-3β and nuclear accumulation of active GSK-3β significantly correlated with a dedifferentiated state in pancreatic cancer cells (18). Furthermore, GSK-3β is commonly involved in inflammatory responses such as enhanced cytokine production driven by NF-κB and STAT3 pathways (21–24), and inhibition was shown to inhibit tumor cell growth via downregulation of NF-κB–mediated expression of antiapoptotic and proinflammatory genes (18, 25). Thus, GSK-3β has recently received increasing attention as a therapeutic target in preclinical and clinical therapeutics. However, the downstream effects of GSK-3β are complex and by far not restricted to the NF-κB pathway. Therefore, further studies are needed to fully comprehend the multiple molecular and cellular mechanisms by which GSK-3β contributes to the pathogenesis of cancer.

NFAT (nuclear factor of activated T cells) proteins are oncogenic transcription factors, which are induced upon inactivation of its broad substrate specificity, is involved in numerous cellular processes (13, 14). GSK-3β was initially recognized as an important tumor suppressor in various cancers predominantly acting through destabilization of the oncogene β-catenin (15). However, GSK-3β also plays important promotormigenic roles in several human malignancies, most notably in pancreatic cancer, regulating proliferation and survival (16–20). Increased expression of active GSK-3β and nuclear accumulation of active GSK-3β significantly correlated with a dedifferentiated state in pancreatic cancer cells (18). Furthermore, GSK-3β is commonly involved in inflammatory responses such as enhanced cytokine production driven by NF-κB and STAT3 pathways (21–24), and inhibition was shown to inhibit tumor cell growth via downregulation of NF-κB–mediated expression of antiapoptotic and proinflammatory genes (18, 25). Thus, GSK-3β has recently received increasing attention as a therapeutic target in preclinical and clinical therapeutics. However, the downstream effects of GSK-3β are complex and by far not restricted to the NF-κB pathway. Therefore, further studies are needed to fully comprehend the multiple molecular and cellular mechanisms by which GSK-3β contributes to the pathogenesis of cancer.

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**Materials and Methods**

**Cell culture**

The human pancreatic adenocarcinoma cell line IMm-PC1 was provided by FX Real (Instituto Municipal de Investigacion Medica, Barcelona, Spain), Suit-028 and Suit-007 were kindly gifted from T. Iwamura (Miyazaki Medical College, Miyazaki, Japan). PaTu8988t were obtained from H.P. Elsaesser (Department of Cell Biology, Marburg, Germany). LinxA cells were purchased provided by FX Real (Institute Municipal de Investigacion Medica, Barcelona, Spain), Suit-028 and Suit-007 were kindly gifted from T. Iwamura (Miyazaki Medical College, Miyazaki, Japan). PaTu8988t were obtained from H.P. Elsaesser (Department of Cell Biology, Marburg, Germany). LinxA cells were purchased from Open Biosystems. All cell lines were obtained between 2004 to 2010. Mouse KrasG12D;p53−/− cells were established from mouse Pdx1-KrasG12D;p53−/− pancreatic tumors and generously gifted by J. Siveke (Technische Universität München, Munich, Germany) in 2012. Testing and authentication of human cell lines were not performed by the authors. All human pancreatic cancer cells were cultured in DMEM (Gibco, Invitrogen Corp.), supplemented with 10% FCS and 1% penicillin/streptomycin (Gibco). LinxA cells were maintained in DMEM (PAA Laboratories GmbH), supplemented with 10% FCS and 100 μg/mL hygromycin (Carl Roth GmbH). Mouse KrasG12D;p53−/− cells were grown in DMEM, 10% FCS, 1% nonessential amino acids (Gibco), and 1% penicillin/streptomycin (Gibco). GSK-3β inhibitor AR-A014418 was purchased from Calbiochem, caerulein, ionomycin, and IL6 were from Sigma-Aldrich. GSK-3β inhibitor 9-ING-41 has been previously described (33).

**Plasmids**

The full-length human NFATc2 expression vector as well as the bacterial expression vector pENTR11 NFATc2 were kindly provided by A. Rao (Department of Signaling and Gene Expression, La Jolla Institute for Allergy and Immunology, La Jolla, CA). GSK-3β S9A was from J. Woodgett (Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada). The wt STAT3 and STAT3 Y705F constructs were from J. Darnell (Laboratory of Molecular Cell Biology, The Rockefeller University, New York, NY), pBabe VS-GSK-3β S9A was from B. Manning (Department of Genetics and Complex Diseases, Harvard T.H. Chan School of Public Health, Boston, MA). The NFAT-luc reporter construct harboring three consensus sites for NFAT fused to a luciferase gene was purchased from Stratagene. The HA-tagged NFATc2 construct was generated by cloning of HA-wt NFATc2 into the pcDNA3.1 (+) vector supplied by Invitrogen into HindIII and Xbal restriction sites. The pSP2 mutation was generated as previously described (31).

**Generation of viral vectors and stable cell lines**

Retroviral vectors and stable cell lines were generated as described previously (28). Briefly, HA-tagged NFATc2 constructs were cloned into a pQMXP vector (BD Biosciences) and transfected into the packaging cell line LinxA. The supernatant containing retroviral particles was filtered through a 0.45-μm filter and supplemented with 2 μg/mL polybrene (Millipore) to infect the target cells PaTu8988t, Suit-028, and Suit-007. Spin infection was carried out at 2,500 × g and at 37°C. Transduced cells were selected with 1 μg/mL puromycin (Sigma-Aldrich) for at least two weeks. The successful overexpression of the NFATc2 constructs was tested by Western blotting.

**qPCR analysis**

RNA from cell lines was isolated using RNeasy Mini Kit (Qiagen), and first-strand cDNA was synthesized from 2 μg total RNA using random primers and Omniscript Reverse Transkriptase Kit (Qiagen). xs-13 was used as housekeeping gene. The following primer pairs were used: xs-13, 5′-ctgaggaagagaggaaggg-3′, 5′- gcttatcttctggttcaaa-3′, CDK-6, 5′-tccgctcattgcagtcgc-3′, 5′- caagcgctttactctctgtt-3′. All primers were supplied by Biomers. Real-time PCR experiments were performed in triplicates and are displayed ± SD.

**Transient transfection and siRNA**

The transient transfection of expression constructs was carried out 24 hours after seeding using TransFast (Promega) and Lipofectamine 2000 (Invitrogen) as transfection reagents according to the manufacturer’s protocol. The RNAi oligonucleotide sequences were as follows: NFATc2, 5′-ccauuuacacagagcaaatg-3′; STAT3, 5′-gacaaccaucaggaaucc-3′ (Applied Biosystems); GSK-3β, siGENOME ON-TARGETplus SMARTpool duplex (Thermo Scientific). The Silencer Negative Control from Ambion (Applied Biosystems) was used as a negative control.
Subcellular fractionation and coimmunoprecipitation

Subcellular fractionation was performed as described previously (2). Briefly, cells were washed with ice-cold PBS and centrifuged at 5,000 g at 4°C. Pellets were subsequently resuspended in buffer A (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, and protease inhibitors) for 30 minutes and consequently centrifuged for 20 minutes at 5,000 x g. Supernatants were transferred to new tubes and centrifuged for 20 minutes at 12,000 x g. Pellets were resuspended in buffer C (20 mmol/L HEPES, pH 7.9, 0.4 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, and protease inhibitors) and incubated on ice for additional 30 minutes. The final centrifugation step was carried out at 12,000 x g for 10 minutes.

For coimmunoprecipitation, cells were lysed with lysis buffer (50 mmol/L HEPES; pH 7.5–7.9, 150 mmol/L NaCl, 1 mmol/L EGTA, 10% glycerin, 1% Triton X-100, 100 mmol/L NaF, and 10 mmol/L Na2P2O7 × 10 H2O) containing protease inhibitors. Five hundred micrograms of the total lysates or 100 μg of nuclear lysates were immunoprecipitated with the indicated antibodies and protein G- or A-agarose (Roche Diagnostics).

For isolation of proteins from murine tissues, the tissues were homogenized using a mortar and subsequently mixed with lysis buffer. After sonication, cells were centrifuged at 12,000 x g for 10 minutes and subjected to immunoblotting.

Chromatin immunoprecipitation

Chromatin immunoprecipitation assays using IgG (Upstate Biotechnology) or antibodies specific to NFATc2, H3K4me3 (all Abcam), RNA Polymerase II (Millipore), or STAT3 (Cell Signaling Technology) were performed as described previously (27). Bound regions were detected by qPCR using the following primer: CDK-6 promoter: 5′-tccgctctcagctgc-3′, 5′-caagccgcttaatccttcctggtt-3′ (Biomers).

Reporter gene assays

Cells were seeded in 12-well plates and were transfected after 24 hours with the indicated constructs. Luciferase activity was measured using the Lumat LB 9501 Luminometer (Berthold Technologies) were performed as described previously (27). Bound regions were detected by qPCR using the following primer: CDK-6 promoter: 5′-tccgctctcagctgc-3′, 5′-caagccgcttaatccttcctggtt-3′ (Biomers).

DNA pulldown assays

For DNA pulldown assays, 100 μg to 150 μg of nuclear protein per sample was incubated for 3 hours with 1 μg of biotinylated double-stranded oligonucleotides containing the consensus NFAT-binding site (5′-ctcaaggagaaaaaggttcatg-3′ and 5′-catgagagaggggtttactg-3′). DNA–protein complexes were further incubated with streptavidin–agarose beads (Sigma-Aldrich) for 1 hour, washed twice with lysis buffer, and subjected to immunoblotting.

Proliferation assays

Cell proliferation was measured by 3H-thymidine and bromodeoxyuridine (BrdUrd) incorporation. Cells were seeded in 24-well plates and cultured in medium containing 10% FCS. Nineteen hours after the indicated treatment or transfection, 3H-thymidine (0.5 μCi/well) was added during the last 5 hours of incubation. The cells were washed with 5% trichloroacetic acid, and the acid-insoluble fraction was dissolved by incubation with 1 μmol/L NaOH for 30 minutes at 37°C. Radioactivity was evaluated with a scintillation counter (Pharmacia). For BrdUrd assays, (transfected) cells were seeded in 96-well plates and treated as indicated. Incorporation of BrdUrd was assayed with Cell Detection ELISA, BrdU Kit (Roche) according to the manufacturer’s instructions. The intensity of incorporation was measured with a DLRéady Luminometer (Berthold Technologies). All proliferation assays were performed in triplicate in at least three independent experiments.

Mouse strains and in vivo experiments

Chronic pancreatitis was induced in 8-week-old Pdx1-Cre, KrasG12D mice by single daily intraperitoneal injections of caerulein (0.2 mg/kg BW; Sigma-Aldrich) and the GSK-3β inhibitor 9-ING-41 (20 mg/kg BW) 3 days per week for a period of 4 weeks (33). Mice were sacrificed at 12 weeks of age.

For the nude mice studies, 6- to 8-week-old pathogen-free athymic female nu/nu mice were obtained from Harlan Winkelmann. A total of 1 × 105 IMIM-PC1 was mixed with Matrigel (BD Biosciences) and injected subcutaneously in both flanks of 12 nude mice. After the establishment of visible tumors, mice were randomized into two groups and were treated 3 times weekly with either 10% DMSO or 10 mg/kg of AR-A014418 (Calbiochem). Mice and tumor sizes were monitored twice weekly. Furthermore, we injected 1 × 106 parental PaTu8988t control cells or PaTu8988t cells stably overexpressing wt NFATc2 and NFATc2 pS2 subcutaneously into the flank of 6 nude mice each and weekly measured the tumor sizes. The tumor volumes (V) were estimated using the formula V = 4/3 × π × (L/2) × (W/2) × (H/2) where L = length, W = width, and H = height. After 4 weeks, mice were sacrificed and tumors were explanted, analyzed, and subjected to IHC and qPCR or Western blot analyses. Animal experiments were carried out using protocols approved by the Institutional Animal Care and Use Committee at the University of Marburg (Marburg, Germany).

Ki67 proliferation analysis

High-power images (20× objective) were taken from Ki67-stained slides from at least 3 animals for each group. Nuclei positive for Ki67 were counted as actively proliferating cells in 20 metaphasic lesions and were related to total numbers of nuclei per lesion. Data were expressed as percentage of positive nuclei per total nuclei.

Fluorescence microscopy, IHC, and Western blotting

Immunofluorescence and IHC were performed on formalin-fixed, paraffin-embedded tumor sections as described previously (28). The following antibodies were used for immunofluorescence: HA (1:1,000) and STAT3 (1:200; all Cell Signaling Technology) and for IHC: NFATc2 (1:200; Abcam), p-NFATc2 (S213/217/221; 1:50, Santa Cruz Biotechnology), STAT3 (1:100, Cell Signaling Technology), p-STAT3 (Y705; 1:50, Cell Signaling Technology), p-GSK-3β (S21/22; 1:500, Cell Signaling Technology), GSK-3β (1:200, Epitomics), and CD45 (BD Pharmingen, 1:100). Immunofluorescence was performed using standard methods on uncultured, macrodissected tumor protein lysates or lysates from cultured cell lines. Membranes were probed with NFATc2 (Abcam), p-NFATc2 (S213/217/221; Abcam), Flag (Sigma-Aldrich), HA, STAT3, GSK-3β, CDK-6, p-STAT3 (Y705), p-GSK-3β (S21/22), GSK-3β (1:200, Epitomics), and CD45 (BD Pharmingen, 1:100). Immunoblotting was performed using standard methods on uncultured, macrodissected tumor protein lysates or lysates from cultured cell lines. Membranes were probed with NFATc2 (Abcam), p-NFATc2 (S213/217/221; Abcam), Flag (Sigma-Aldrich), HA, STAT3, GSK-3β, CDK-6, p-STAT3 (Y705), p-GSK-3β (S21/22), GSK-3β (1:200, Epitomics), and CD45 (BD Pharmingen, 1:100). Immunofluorescence was performed using standard methods on uncultured, macrodissected tumor protein lysates or lysates from cultured cell lines. Membranes were probed with NFATc2 (Abcam), p-NFATc2 (S213/217/221; Abcam), Flag (Sigma-Aldrich), HA, STAT3, GSK-3β, CDK-6, p-STAT3 (Y705), p-GSK-3β (S21/22), GSK-3β (1:200, Epitomics), and CD45 (BD Pharmingen, 1:100). Immunofluorescence was performed using standard methods on uncultured, macrodissected tumor protein lysates or lysates from cultured cell lines. Membranes were probed with NFATc2 (Abcam), p-NFATc2 (S213/217/221; Abcam), Flag (Sigma-Aldrich), HA, STAT3, GSK-3β, CDK-6, p-STAT3 (Y705), p-GSK-3β (S21/22), GSK-3β (1:200, Epitomics), and CD45 (BD Pharmingen, 1:100).

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TMA staining and analysis

All staining carried out on human specimens was approved by the Mayo Clinic Institutional Review Board (Rochester, MN). Written confirmation was obtained from each patient. Tissue microarrays (TMA) containing samples from PDAC patients were stained as indicated and analyzed for NFATc2 and p-STAT3 expression in the Pathology Research Core. TMA slides were placed in the BOND III stainer (Leica Biosystems) for online processing. They were treated with Epitope Retrieval 2 Solution for 20 minutes and stained with NFATc2 (Abcam) or p-STAT3 (Cell Signaling Technology) for 15 minutes, and detection was achieved using the Polymer Refine Detection Kit per manufacturer’s instructions (Leica Biosystems). Counterstaining was performed for 5 minutes with hematoxylin. Slides were dehydrated through increasing concentrations of alcohol, cleared in xylene, and cover-slipped in xylene-based mounting media.

Data analysis

The TMAs were evaluated for NFATc2 and p-STAT3 expression by a trained pancreatic pathologist and were scored as positive (score 1, 2, or 3) or negative (score 0). Stain positivity across the core, which stained with the primary antibody detected wild-type NFATc2, but not a mutant NFATc2 lacking these phosphorylation sites (Supplementary Fig. S1C). Notably, whereas GSK-3β depletion resulted in diminished SP2 phosphorylation, expression of a constitutively active mutant (GSK-3β S9A) increased SP2 phosphorylation (Supplementary Fig. S1D and S1E). Immunoblot and immunohistologic analyses from GSK-3i–treated xenograft tumors revealed reduced levels of p-NFATc2, p-glycogen synthase (GS), and Ki67 ($P < 0.01$; Fig. 1G and Supplementary Fig. S1F and 1G). Moreover, nuclear NFATc2 protein levels were substantially reduced in pancreatic cancer cells treated with the GSK-3i, whereas overexpression of an NFATc2 mutant mimicking constitutive phosphorylation (NFATc2 pSP2) rescued the effect of GSK-3i inhibition in NFATc2 expression (Fig. 1H and Supplementary Fig. S1H).

It is noteworthy that in immune cells, GSK-3β–mediated phosphorylation of the SP2 region terminated DNA binding of NFAT proteins and induced shuttling of NFAT proteins to the cytoplasm, followed by inactivation of NFAT activity ($34–36$).

To address this issue in pancreatic cancer, we performed immunoblot analysis and DNA pulldown assays that detected high NFATc2 pSP2 levels in the cytoplasm as well as efficient binding to the NFAT consensus sequence GGAAA (Fig. 1I). Moreover, reporter gene assays demonstrated profound activation of an NFAT-responsive “cisNFAT” promoter by NFATc2 pSP2 compared with wild-type NFATc2 (Fig. 1J). Functionally, stable NFATc2 pSP2 expression significantly stimulated tumor growth of PaTu8988t xenograft tumors after 4 weeks (Fig. 1K). Immunohistochemical analysis of human PDA tissues showed nuclear NFATc2 expression (Fig. 1L). To extend and quantify this finding, we analyzed a human TMA consisting of 217 human pancreatic cancer tissues for NFATc2 expression and found a positive maximum nuclear tumor staining intensity in 96 of 217 (44.2%) of subjects, indicating the presence of active p-NFATc2, especially in neoplastic regions (Supplementary Table S1A). Collectively, our data suggest that contrary to the function in immune cells, SP2 phosphorylation by GSK-3β in pancreatic cancer cells maintains active NFATc2 protein in the nucleus, resulting in accelerated pancreatic cancer growth in vivo.

Results

GSK-3β confers pancreatic tumor growth stimulation by maintenance of NFAT expression levels through specific phosphorylation

To confirm and extend existing data on GSK-3β inhibition in pancreatic cancer, we applied a GSK-3β inhibitor (hereafter designated GSK-3i) to block kinase activity in different established pancreatic cancer cell lines. As expected, diminished GSK-3β activity was accompanied by a time- and dose-dependent decrease of cancer cell proliferation (Fig. 1A and B). We next treated nude mice bearing IMIM-PC1 xenograft tumors 3 × weekly with GSK-3i and observed a significant tumor growth inhibition (Fig. 1C and Supplementary Fig. S1A), supporting the notion of a growth-promoting role of GSK-3β in pancreatic cancer.

Interestingly, previous work by our group had pointed toward an oncogenic GSK-3β/NFATc2 stabilization pathway in pancreatic and breast cancer cells (31). To further investigate the GSK-3β/NFATc2 axis, we assessed NFATc2 expression levels in pancreatic cancer cell lines after pharmacologic inhibition or genetic depletion of GSK-3β. Treatment with the GSK-3i reduced nuclear NFATc2 protein expression in a dose-dependent fashion, as did transfection with a GSK-3β–specific siRNA (Fig. 1D and E and Supplementary Fig. S1B). Similarly, tissues derived from nude mice following GSK-3i treatment showed substantially less NFATc2 (Fig. 1F).

As GSK-3β was reported to specifically phosphorylate three conserved serines in the NFATc2 SP2 (S213, S217, and S221) region, we aimed to assess the functional relevance of this post-translational modification. Importantly, a phospho-specific SP2 antibody detected wild-type NFATc2, but not a mutant NFATc2 lacking these phosphorylation sites (Supplementary Fig. S1C). Moreover, whereas GSK-3β depletion resulted in diminished SP2 phosphorylation, expression of a constitutively active mutant (GSK-3β S9A) increased SP2 phosphorylation (Supplementary Fig. S1D and S1E). Immunoblot and immunohistologic analyses from GSK-3i–treated xenograft tumors revealed reduced levels of p-NFATc2, p-glycogen synthase (GS), and Ki67 ($P < 0.01$; Fig. 1G and Supplementary Fig. S1F and 1G). Moreover, nuclear NFATc2 protein levels were substantially reduced in pancreatic cancer cells treated with the GSK-3i, whereas overexpression of an NFATc2 mutant mimicking constitutive phosphorylation (NFATc2 pSP2) rescued the effect of GSK-3i inhibition in NFATc2 expression (Fig. 1H and Supplementary Fig. S1H).

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GSK-3β regulates NFATc2 activity through stabilization of complex formation with STAT3

Next, we assessed whether NFATc2 activity is also regulated by GSK-3β or whether the observed elevated NFATc2 pSP2 activation levels were due to increased protein levels following nuclear stabilization. Activation of the NFAT-responsive “cisNFAT” promoter correlated well with GSK-3β activity (Fig. 2A and B; Supplementary Fig. S2A and S2B). However, expression of the NFATc2 pSP2 construct, which is resistant to degradation upon GSK-3β inhibition, could not activate the cisNFAT promoter following GSK-3 depletion (Fig. 2A and Supplementary Fig. S2B). This was surprising, as we expected that recombinant NFATc2 pSP2 expression levels would overrule GSK-3β depletion. Moreover, expression of constitutively active GSK-3β (S9A) resulted in enhanced activity of the cisNFAT promoter when in the presence of wt or pSP2 NFATc1. Therefore, our data suggest that GSK-3β not only regulates NFATc2 protein stability through SP2 phosphorylation but also stimulates transcriptional activation by a distinct mechanism.
Figure 1.
GSK-3β stabilizes NFATc2 in vitro and in vivo. A and B, PaTu8988t (A) and IMIM-PC1 (B) cells were treated with indicated concentrations of GSK-3β inhibitor AR-A014418 (hereafter referred to as GSK-3i) and analyzed for BrdUrd incorporation after 24 hours and 48 hours. C, nude mice bearing IMIM-PC1 xenograft tumors were treated 3× weekly with 10 mg/kg BW GSK-3i (n = 5) or 10% DMSO (n = 5) intraperitoneally. Tumors were extracted after 4 weeks. The tumor growth was normalized to the respective starting volumes. Means ± SE, *P < 0.05; **P < 0.01. D, PaTu8988t cells were starved and nuclear translocation of NFATc2 was induced by ionomycin treatment (0.5 μmol/L). Cells were then treated for 1 hour with 2.5, 25, and 50 μmol/L of GSK-3i and analyzed for NFATc2 and p-GS expression. Reduction of the well-described GSK-3β target p-GS serves as treatment control. E, PaTu8988t cells were transfected with GSK-3β siRNA, and lysates were tested for NFATc2 and GSK-3β expression after 48 hours. F and G, tumor lysates from three representative mice treated with 10% DMSO or GSK-3i were assessed for NFATc2 and p-GS expression. Reduction of the well-described GSK-3β target p-GS serves as treatment control. E, PaTu8988t cells were transfected with GSK-3β siRNA, and lysates were tested for NFATc2 and p-GS expression after 48 hours. F and G, tumor lysates from three representative mice treated with 10% DMSO or GSK-3i were assessed for NFATc2 and p-GS expression. Reduction of the well-described GSK-3β target p-GS serves as treatment control. H, nuclear extracts from Suit-028 cells with stable expression of wt NFATc2 and NFATc2 pSP2 were treated for 3 hours with 25 μmol/L GSK-3i and then subjected to immunoblot analysis with the indicated antibodies. Lamin a/c serves as loading control. I, nuclear lysates from Suit-007 cells with stable expression of wt NFATc2 and NFATc2 pSP2 were subjected to DNA pulldown assays with oligonucleotides bearing the NFAT consensus sequence. DNA-bound NFATc2 was then analyzed by immunoblotting. J, PaTu8988t cells with stable wt NFATc2 and NFATc2 pSP2 expression or untransfected control cells were transfected with calcineurin and a reporter construct harboring three NFAT consensus binding sites (cisNFAT). Luciferase activity was measured after 24 hours. Displayed are means ± SD related to the control (first bar). K, nude mice were injected with PaTu8988t cells with stable wt NFATc2 or NFATc2 pSP2 expression or control cells, and tumor volumes were estimated after 4 weeks (n ≥ 5). Shown are means ± SE, *P < 0.05; **P < 0.01. L, representative IHC pictures of human PDA showing nuclear p-NFATc2 expression.
We recently established an oncogenic NFATc1–STAT3 cooperativity governing pancreatic cancer progression (27). Thus, we were interested whether other NFAT family members such as NFATc2 also interact and cooperate with STAT3 to promote cancer growth and whether this cooperation is regulated by GSK-3β. We detected comparable and robust physical interaction of endogenous STAT3 with all NFATc2 SP2 mutants (Supplementary Fig. S2C). Correspondingly, STAT3 overexpression remarkably elevated NFATc2 activation of the cisNFAT promoter, irrespective of NFATc2 phosphorylation on SP2 (Fig. 2C). We next assessed whether this NFATc2/STAT3 complex formation is regulated by GSK-3β in pancreatic cancer. Significantly, GSK-3β inhibition disrupted the physical interaction of STAT3 with both wt and pSP2 NFATc2 (Fig. 2D). In addition, depletion of STAT3 abrogated the ability of GSK-3β S9A to enhance the transactivation of the cisNFAT promoter, irrespective of NFATc2 phosphorylation on SP2 (Fig. 2C). We next assessed whether this NFATc2/STAT3 complex formation is regulated by GSK-3β in pancreatic cancer. Significantly, GSK-3β inhibition disrupted the physical interaction of STAT3 with both wt and pSP2 NFATc2 (Fig. 2D). We next sought to characterize the NFATc2–STAT3 complex and its functional implication in pancreatic cancer. Immunohistochemical costaining of 217 human pancreatic cancer tissues revealed that 81 (37.3%) of cases were positive for both nuclear NFATc2 and nuclear p-STAT3 (Fig. 3A; Supplementary Table S1B). Remarkably, those tumors that showed NFATc2 expression stained positive for p-STAT3 in 84% (Fig. 3A). These observations were confirmed by immunofluorescence studies detecting simultaneous nuclear NFATc2 and STAT3 expression levels in pancreatic cancer cells (Fig. 3B). Interestingly, the inflammatory cytokine IL6 could induce a robust physical STAT3–NFATc2 interaction that was further enhanced by the addition of serum (Fig. 3C) and that required STAT3 Y705 phosphorylation (Fig. 3D). In addition, DNA pulldown assays demonstrated an interaction of the transcription factors on the NFAT consensus sequence that was also dependent on STAT3 phosphorylation (Fig. 3E). The expression of mutant STAT3 Y705F most notably antagonized the activating effect of wt STAT3 on the NFAT-responsive "cisNFAT" promoter, indicating that STAT3 phosphorylation is not only required for complex formation but also for efficient NFATc2 transactivation (Fig. 3F). It is noteworthy that GSK-3β treatment reduced STAT3

**Figure 2.** SP2-independent regulation of NFATc2 activity by GSK-3β. Luciferase reporter gene assay demonstrating regulation of the cisNFAT promoter in PaTu8988t cells with stable wt NFATc2 and NFATc2 pSP2 expression or control cells following transfection with GSK-3β siRNA (A) or GSK-3β S9A (B). Means ± SD. C, PaTu8988t cells with stable expression of indicated constructs were transfected with wt STAT3 and cisNFAT and subjected to reporter gene assay. Shown are means ± SD normalized to controls. D, PaTu8988t cells with stable expression of indicated constructs were treated with 25 μmol/L GSK-3i, and after 3 hours nuclear lysates were subjected to coimmunoprecipitation and subsequent immunoblotting as indicated. E, reporter gene assay of Suit-007 cells with stable expression of indicated constructs after transfection with wt STAT3 and cisNFAT and subsequent administration of 25 μmol/L GSK-3i for 6 hours. Means ± SD. Successful knockdown of STAT3 is shown on right.
Figure 3.
STAT3 Y705 phosphorylation is required for efficient physical and functional cooperation with NFATc2. A, TMA analysis of 217 cases of PDACs shows nuclear NFATc2 expression in 44% of cases, of which 84% also stained positive for p-STAT3. B, Immunofluorescence staining displays nuclear colocalization of STAT3 (green) and HA-tagged NFATc2 (red) in PaTu8988t cells with stable expression of HA-NFATc2. Nuclei are visualized by DAPI staining (blue). C, Suit-007 cells with stable wt NFATc2 expression were transfected with Flag-tagged wt STAT3 and incubated with serum-free or serum medium for 24 hours with subsequent stimulation with IL6 (50 ng/mL). Lysates were subjected to coimmunoprecipitation and Western blotting as indicated. D, coimmunoprecipitation demonstrating reduced interaction of nuclear HA-tagged wt NFATc2 with Flag-tagged STAT3 Y705F compared with wt STAT3 in Suit-028 cells with stable HA-NFATc2 expression. E, PaTu8988t cells were transfected with STAT3 constructs as indicated and subjected to DNA pulldown assays with oligonucleotides bearing the NFAT consensus sequence. F, reporter gene assay demonstrating the inducibility of cisNFAT upon transfection of wt STAT3 and STAT3 Y705F in Suit-028 control cells and cells with stable expression of wt NFATc2 under serum-free conditions. Means ± SD. G, proliferation of Suit-007 cells with and without stable expression of wt NFATc2 following 72 hours of STAT3 siRNA transfection. Means ± SD. Efficient knockdown was tested by immunoblotting and is shown in Fig. 2E.
phosphorylation at Y705 in different pancreatic cancer cell lines (Supplementary Fig. S3A–S3C), suggesting that GSK-3β stabilizes NFATc2–STAT3 complexes by maintaining STAT3 phosphorylation. Finally, proliferation assays revealed that the loss of STAT3 expression reversed the NFATc2-dependent stimulation of cancer cell proliferation, highlighting its importance for NFATc2-induced growth stimulation (Fig. 3G). Accordingly, cell proliferation of PaTu8988t was reduced after STAT3 siRNA transfection, independent of SP2 phosphorylation (Supplementary Fig. S3D).

In summary, p-STAT3 (Y705) efficiently binds to and transactivates NFATc2 on responsive promoters under mitogenic and inflammatory conditions, and in turn, acts to sustain NFATc2-dependent cell growth in a GSK-3β-dependent manner. Collectively, GSK-3β maintains NFATc2 activation through stabilization of NFATc2–STAT3 complexes independent of SP2 phosphorylation (Supplementary Fig. S3D). In summary, p-STAT3 (Y705) efficiently binds to and transactivates NFATc2 on responsive promoters under mitogenic and inflammatory conditions, and in turn, acts to sustain NFATc2-dependent cell growth in a GSK-3β-dependent manner. Collectively, GSK-3β maintains NFATc2 activation through stabilization of NFATc2–STAT3 complexes independent of SP2 phosphorylation, presumably through phosphorylation of STAT3 at Y705.

We next analyzed the GSK-3β-dependent NFATc2/STAT3 interaction on the previously described NFATc2 target promoter, CDK-6 (Fig. 4A and B). Importantly, GSK-3i treatment resulted not only in the loss of NFATc2 binding to the CDK-6 promoter but also reduced the binding of STAT3 (Fig. 4C). Furthermore, binding of RNA-polymerase II and the activating histone modification H3K4-me3 was significantly decreased upon GSK-3i administration (Fig. 4C), followed by loss of CDK-6 gene and protein expression (Fig. 4D and E). Consistent with the role of GSK-3β in promoting the transactivation of NFATc2–STAT3 complexes, CDK-6 expression was decreased after upon GSK-3i treatment (Supplementary Fig. S3E). Taken together, our data strongly point to a multilevel regulatory impact of GSK-3β on NFATc2, which is, on one hand, mediated by SP2 phosphorylation-dependent stabilization of protein levels in the nucleus and on the other hand by recruitment of NFATc2–STAT3 complexes to target promoters and subsequent transcription of cell-cycle promoters independent of SP2 phosphorylation.

Targeting the NFATc2–STAT3–GSK-3β cooperativity interferes with inflammation-induced pancreatic cancer progression in KrasG12D mice

To assess the therapeutic potential of targeting the identified NFATc2–STAT3–GSK-3β cooperativity in vivo, we utilized a well-established model of pancreatitis-associated carcinogenesis to ensure profound induction of NFATc2 and STAT3, as these transcription factors were shown to be overexpressed or activated in the majority of Kras-mutated tumor specimen, especially upon inflammatory conditions (37, 38). In line with previously published data, we observed that cancer progression was massively accelerated when transgenic mice with pancreas-specific expression of oncogenic KrasG12D were challenged 3× weekly with the CCK analogue
caerulein to induce a persistent organ inflammation (9, 10). Mice developed the full spectrum of precursor lesions ranging from noninvasive PanIN1 to PanIN2/3 and carcinoma in situ lesions (Fig. 5A and B). This was accompanied by induced p-NFATc2 (SP2) and p-STAT3 (Y705) levels (Fig. 5C and D). When mice were additionally treated with a GSK-3i, however, cancer progression was significantly blocked in terms of proliferation, neoplastic transformation, and tumor incidence, as evidenced by restored normal ducts, decreased precursor lesions (Fig. 5B), and significantly reduced Ki67 expression levels (Fig. 5E). Moreover, we observed a reduced tendency to develop tumors upon GSK-3i treatment as indicated. Scale bars, 100 μmol/L. D, tissues from Pdx1;Kras<sup>G12D</sup> mice after treatment as indicated were assayed for p-STAT3, p-NFATc2, and NFATc2 expression. E, proliferation index was measured in Ki67-stained pancreatic sections (n ≥ 3) of Pdx1;Kras<sup>G12D</sup> mice after indicated treatment. Means ± SE. *P < 0.05; **P < 0.01; ***P < 0.001.

Discussion
Pancreatic cancer exhibits a high therapeutic resistance against cytotoxic and apoptosis-inducing agents (39), and although genetic approaches targeting Kras in mice have shown encouraging results (40, 41), the oncogene is still deemed ‘undruggable’ in mice and man. Persistent inflammation contributes to tumor progression and therapeutic resistance in pancreatic cancer, and the activation of proinflammatory cytokines and transcription factors is a molecular feature of this disease. Although GSK-3β is not induced by inflammation itself, it governs a variety of inflammatory responses such as enhanced cytokine production driven by NF-κB and STAT3 pathways (21–24). Interestingly, the anti-inflammatory potential of GSK-3 inhibition was evidenced in our study by a clear reduction of immune cell infiltrations in GEMM of pancreas cancer.

NFAT proteins represent another class of major proinflammatory transcription factors that were originally described as inducible factors binding to the IL2 promoter in activated T
cells (42). Strongly induced upon inflammatory stimuli, NFAT proteins have been shown to be overexpressed in the majority of invasively growing cancers, presumably in those with persistent inflammation, and to dramatically accelerate Kras-driven carcinogenesis in the pancreas (27, 28, 30). However, as therapeutic targeting of transcription factors is difficult, it is critical to identify upstream regulatory pathways that may be targetable for cancer therapeutics.

Here, we investigated the role of GSK-3β on the regulation and activation of NFATc2 in pancreatic cancer progression and growth. We propose the following model: in contrast to results in immune cells, where GSK-3β phosphorylates three serine residues in the N-terminal SP2 domain and thus terminates activation of p-NFATc2 as a potentially specific and diagnostic tool (left). In addition, GSK-3β stabilizes NFATc2/STAT3 complex formation independent of and dominant to SP2 phosphorylation resulting in target gene expression (right).

Figure 6. Proposed model of GSK-3β-mediated regulation of the NFATc2 metabolism in pancreatic cancer cells. Activation of the phosphatase calcineurin following an intracellular calcium influx dephosphorylates NFAT proteins and stimulates their nuclear import. There, GSK-3β-mediated SP2 phosphorylation protects NFATc2 from degradation and further stimulates DNA binding and transcriptional activation of target genes (43, 44). In line with these observations, we have previously shown that NFATc1 induces STAT3 expression with subsequent formation of NFATc1/STAT3 nucleoprotein complexes in pancreatic cancer cells. Likewise, STAT3 affected NFATc1 transcriptional activity by maintaining its DNA binding on distant enhancers and hence stimulated enhancer–promoter communication leading to the expression of oncogenes such as EGFR, cyclin D3, or Wnt proteins (27, 28, 30).

From a therapeutic point of view, targeting of central upstream regulators, and subsequently the complete proinflammatory oncogenic network, appears feasible and may help overcome drug resistance and apoptosis inhibition. Notably, caution has been raised in the therapeutic field owing to the putative tumor suppressive functions of GSK-3β as inhibition may result in the activation of the Wnt pathway and subsequent tumorigenesis. Reassuringly, however, Wnt signaling and β-catenin nuclear accumulation are not perturbed in GSK-3β−deficient mice (14). Also, lithium, a known inhibitor of GSK-3, causes a reduced cancer morbidity in psychiatric patients compared with the general population and inhibits proliferation in hepatocellular carcinoma cell lines (45, 46).

Collectively, our data suggest that GSK-3β fine-tunes NFATc2 and STAT3 transcriptional networks to integrate upstream signaling events to eventually govern pancreatic cancer progression and growth. Furthermore, the therapeutic potential of GSK-3β to delay inflammation-induced malignant transformation is demonstrated for the first time in a relevant GEMM for
pancreatic cancer. In a broader sense, our data add to the molecular understanding of GSK-3β-mediated tumorigenesis and may thus open new avenues for individually tailored therapies. For instance, one can imagine that patients with high expression of NFATc2 may benefit the most from GSK-3β inhibitor-based therapy. Our results are particularly germane given the fact that a large number of clinical GSK-3β inhibitors are available and currently being tested in several disease entities, including pancreatic cancer patients.

Disclosure of Potential Conflicts of Interest

A.P. Kozikowski is the owner and founder of, has ownership interest (including patents) in, and is a consultant/advisory board member for Actuate Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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GSK-3β Governs Inflammation-Induced NFATc2 Signaling Hubs to Promote Pancreatic Cancer Progression

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