

The activity of Gli transcription factors is essential for Kras-induced pancreatic tumorigenesis

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Pancreatic ductal adenocarcinoma (PDAC), one of the most aggressive human malignancies, is thought to be initiated by *KRAS* activation. Here we find that transcriptional activation mediated by the Gli family of transcription factors, although dispensable for pancreatic development, is required for Kras-induced proliferation and survival in primary pancreatic epithelial cells in culture and for Kras-driven pancreatic intraepithelial neoplasia and PDAC formation in vivo. Further, ectopic Gli1 activation in the mouse pancreas accelerates Kras-driven tumor formation, underscoring the importance of Gli transcription factors in pancreatic tumorigenesis. Interestingly, we demonstrate Gli-regulated I-kappa-B kinase epsilon (IKBKE) and NF-kappa-B activity in pancreatic cancer cells and show that this activity is a critical downstream mediator for Gli-dependent PDAC cell transformation and survival. Together, these studies demonstrate the requirement for Gli in Kras-dependent pancreatic epithelial transformation, suggest a mechanism of Gli-NF-kappa-B oncogenic activation, and provide genetic evidence supporting the therapeutic targeting of Gli activity in pancreatic cancer.

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer mortality in the United States, with a 5-y survival rate of less than 5% (1, 2). PDAC is thought to arise from precursor lesions termed “pancreatic intraepithelial neoplasias” (PanINs), which are characterized by mutations in *KRAS* and are believed to be initiating events in this cancer (3, 4). The importance of activating *KRAS* mutations in PDAC development is underscored further by studies of mouse models of the disease (5–7). Thus, understanding of the molecular and genetic mechanisms in Kras-dependent pancreatic tumorigenesis is essential for the development of early diagnostic and treatment tools.

The Gli transcription factors Gli1, Gli2, and Gli3 are effectors of the Hedgehog (Hh) signaling pathway. In mammalian cells, the Hh ligands bind to the 12-pass transmembrane receptor, Patched1 (Ptch1), leading to activation of the seven-pass membrane protein, Smoothened (Smo). Smo-mediated intracellular signal transduction controls the activity of the Gli proteins, resulting in transcriptional responses in target tissues (8–10). Dysregulation of Hh–Gli signaling likely is involved in multiple aspects of PDAC formation (11–18), but its exact roles remain poorly characterized. Unlike Hh-related tumors associated with Gorlin syndrome, mutations in cell-surface molecules such as Ptch and Smo have not been identified in human pancreatic cancers (11, 19–21). However, Hh ligands are highly expressed in human and mouse PDAC (12, 13, 15, 18), and emerging evidence suggests that the ligands regulate pancreatic tumor development through a paracrine mechanism. In this model, Hh ligands produced by epithelial tumor cells signal to the adjacent stroma, thereby altering the tumor microenvironment and regulating tumor growth (16, 17, 22, 23). This model is supported further by recent studies demonstrating that neither genetic removal nor activation of *Smo* in the pancreatic epithelium affects Kras-induced pancreatic tumor formation (18, 23). Consistent

with this finding, treatment of a PDAC mouse model with a Smo antagonist led to alterations in the tumor stroma and enhanced tumor response in combination with gemcitabine (17).

Surprisingly, Gli expression within the pancreatic tumor epithelium is maintained despite *Smo* deletion and does not correlate with Hh ligand levels (18), suggesting a more complex regulation of the pathway. Several recent studies also have shown that Gli1 gene expression in PDAC cells is regulated by oncogenic pathways such as Kras and TGF- β , independently of Hh ligand input and the canonical intracellular pathway through Ptch and Smo (18, 24). Gli1 also is required for PDAC cell survival and transformation in culture (18), and ectopic activation of Gli2 in the mouse pancreas induces the formation of undifferentiated tumors (14). Interestingly, sequencing of human PDAC specimens identified mutations in genes encoding Gli transcription factors, including *GLI1* and *GLI3* (3). Together, these data suggest a cell-autonomous regulation of Gli activity in pancreatic tumor epithelial cells, independent of Hh ligands. However, the requirement for Gli activation within the tumor epithelium has not been established in vivo, and the underlying molecular consequences of Gli-mediated transcription during pancreatic tumorigenesis remain unexplored.

In this study, we describe the generation of a mouse model of epithelium-specific inhibition of Gli transcriptional activity in Kras-induced pancreatic cancer. We find that although blocking Gli-induced transcription does not affect pancreatic development, it potentially inhibits Kras-driven PanIN and PDAC formation. Conversely, ectopic Gli1 expression accelerates Kras-mediated pancreatic tumorigenesis. Using gene-expression profiling and functional assays, we identify downstream regulation of the I-kappa-B kinase epsilon (IKBKE) and NF-kappa-B pathway as a mechanistic link between Gli transcriptional activation and PDAC cell transformation.

Results

Gli Activity Is Not Required for Pancreatic Development. To investigate the role of Gli transcriptional activity in pancreatic de-

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velopment, we used a conditional *Rosa26* knock-in allele of Gli3T (*R26-Gli3T*), which allows ectopic expression of Gli3T protein from the ubiquitously expressed *Rosa26* locus following Cre-mediated recombination (25). Gli3T is a C-terminally truncated form of Gli3 that acts as a dominant repressor of Gli transcription. Overexpression of Gli3T specifically inhibits Gli1- and Gli2-dependent gene transcription but not lymphoid enhancer-binding factor-1 or serum-response factor-mediated gene transcription in cultured NIH 3T3 cells (Fig. 1 *A–C*). These results show the specificity and effectiveness of the Gli3T allele in blocking Hh/Gli transcriptional activation.

We crossed *R26-Gli3T* mice to *Ptf1a-Cre* transgenic mice that direct Cre recombinase expression to the epithelial lineages of the mouse pancreas (26). *Ptf1a-Cre;R26-Gli3T* mice were born at the expected frequency, and their pancreata showed normal parenchymal architecture and cytodifferentiation up to the age of 12 mo, the longest time examined ($n = 10$) (Fig. 1 *E* and *J*). In the *R26-Gli3T* allele, a 3×Flag tag and an internal ribosomal entry site (IRES)-Venus unit also are inserted at the C terminus of Gli3T to facilitate identification of transgene expression (25). Therefore, we used detection of the Flag tag with immunoblotting (Fig. 1*D*) and Venus fluorescence on cryosections (Fig. 1*N*) to confirm Cre-mediated expression of Gli3T in pancreatic tissue from *Ptf1a-Cre;R26-Gli3T* mice. We analyzed the expression of the endocrine markers insulin and glucagon and the acinar cell marker amylase and found no significant differences between Gli3T-expressing and control pancreata (Fig. 1 *F–H* and *K–M*). These data show that Gli3T inhibition does not affect differentiation of the pancreatic epithelial lineages and suggest that cell-autonomous Gli activity is largely dispensable for the proper development of mouse pancreas.

Gli Activation Is Required for Formation of Kras-Induced PanIN Lesions.

We next investigated the specific role for Gli transcription in regulating Kras-initiated tumor development in vivo. We generated a mouse model in which simultaneous activation of Kras and inhibition of Gli transcription was achieved by breeding mice harboring a conditionally activated Kras allele (*LSL-Kras^{G12D}*) (27) with *Ptf1a-Cre;R26-Gli3T* mice. As reported previously, Cre-mediated activation of the *LSL-Kras^{G12D}* allele in the mouse pancreas results in the development of slowly progressive PanINs (5). At 6 mo of age, *Ptf1a-Cre;LSL-Kras^{G12D}* mice developed early PanIN lesions, most of which were classified histologically as PanIN1A and PanIN1B (Fig. 2*A*). These lesions showed a high proliferation index, demonstrated by Ki67 immunohistochemistry (IHC), and showed evidence of epithelial transformation with associated mucin accumulation as detected by Alcian blue staining (Fig. 2 *C* and *E*). By 12 mo of age, the pancreata of the *Ptf1a-Cre;LSL-Kras^{G12D}* mice displayed evidence of more advanced lesions, including PanIN2 and PanIN3 (Fig. 2*G*).

In contrast, inhibition of Gli activity resulted in a dramatic reduction in Kras-driven tumorigenesis. *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli3T* mice ($n = 15$) examined at 6 mo and 12 mo of age showed a largely normal parenchymal architecture in the pancreas with little evidence of epithelial transformation (Fig. 2 *B* and *H*). Most of the cells in the pancreas were nonproliferating, as determined by Ki67 staining, and there was no reactive stroma (Fig. 2 *D* and *F*), suggesting a critical requirement for Gli transcriptional activation in Kras-induced PanIN lesion formation in vivo.

Pancreatic ductal epithelial transformation is a critical step in the development of Kras-initiated PanIN lesions. Thus, we examined the effect of Gli3T expression on Kras-induced phenotypes in primary pancreatic duct epithelial cells (PDECs) in culture. Consistent with our previous work (15), we found that

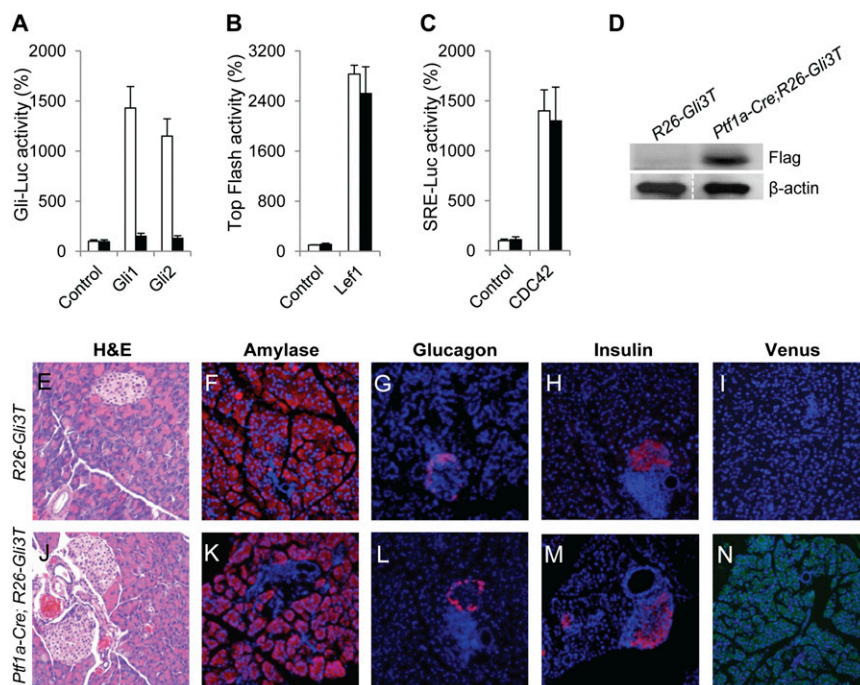


Fig. 1. Gli transcriptional activation is dispensable for pancreatic development. (*A–C*) Gli3T blocks Gli-dependent transcriptional activity. Gli3T inhibited a Gli-mediated luciferase reporter activity induced by Gli1 and Gli2 in transfected NIH3T3 cells (*A*). Gli3T does not inhibit TCF4-induced TopFlash reporter activity (*B*) or serum-response factor-dependent SRE-luc reporter activity induced by the CDC42 GTPase (*C*). Open bars represent control cells; black bars represent Gli3T-expressing cells. Data shown are expressed as mean \pm SD. (*D*) Western blot analysis of Gli3T^{Flag} expression in pancreatic lysates from 6-month-old *R26-Gli3T* and *Ptf1a-Cre;R26-Gli3T* mice. β -actin image was assembled from cropped lanes of the same western blot analysis. (*E–N*) H&E staining (*E* and *J*), immunofluorescence staining of amylase (*F* and *K*), glucagon (*G* and *L*), and insulin (*H* and *M*), and Venus fluorescence (*I* and *N*) in pancreata derived from *R26-Gli3T* (*E–I*) and *Ptf1a-Cre;R26-Gli3T* (*J–N*) mice.

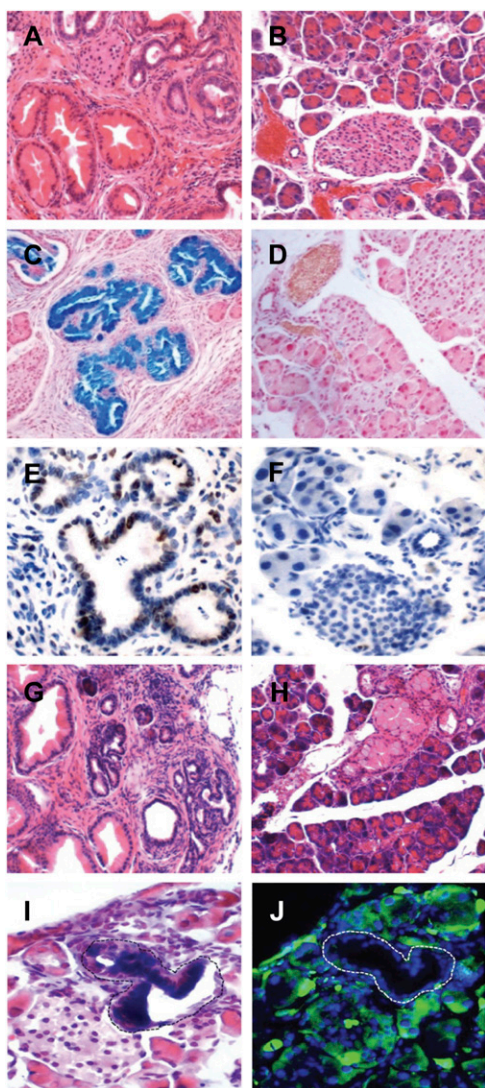


Fig. 2. Gli-mediated transcription is required for Kras-initiated PanIN formation. (A–F) Representative histological sections from *Ptf1a-Cre;LSL-Kras^{G12D}* (A, C, and E) and *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli3T* (B, D, and F) mice stained with H&E (A and B), Alcian blue (C and D), or an antibody against Ki67 (E and F). (G–J) PanIN lesions detected in *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli3T* mice. H&E staining shows widespread advanced PanIN formation in *Ptf1a-Cre;LSL-Kras^{G12D}* pancreata (G), but only rare PanIN1 lesions were detected in a 12-mo-old *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli3T* mouse (H and I). Note robust Venus fluorescence in adjacent acinar and endocrine cells indicating expression of Gli3T. The boundary of the lesions from the adjacent slides is delineated by dashed black (I) and white (J) lines.

Kras activation induced the proliferation of PDECs and enhanced their survival in response to challenge by apoptotic stimuli (Fig. S1). However, Gli3T expression abrogated Kras-induced PDEC survival after exposure to cycloheximide (Fig. S1A) and also impaired Kras-induced proliferation in PDECs (Fig. S1B). Together, our in vivo and in vitro data suggest that Gli activation is critical for Kras-initiated pancreatic tumorigenesis, possibly by mediating Kras-induced epithelial cell proliferation and survival.

Interestingly, we did detect a few rare PanIN1 lesions in three *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli3T* mice (Fig. 2 H and I). This observation suggests that the Gli requirement eventually might be overcome or that the lesions that developed failed to express Gli3T. To differentiate between these possibilities, we assayed for transgene expression in these lesions. Because of the high

background in detecting the Gli3T C-terminal Flag tag with immunofluorescence staining or IHC, we took advantage of the IRES-Venus unit inserted in the *R26-Gli3T* allele that allows detection of transgene expression by Venus fluorescence on cryosections. We found that the lesions from *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli3T* mice were Venus negative compared with the adjacent normal-appearing islet and acinar tissues (Fig. 2 I and J), indicating that the Gli3T transgene was not expressed in these lesions. These results suggest that the development of rare lesions in animals bearing the *R26-Gli3T* allele probably is the result of inefficient Cre recombination and the failure to express Gli3T.

Gli Activation Is Required for Formation of Kras-Dependent PDAC.

Our data suggest that Gli transcriptional activity is required for Kras-initiated formation of precursor PanIN lesions. To test whether Gli activity is required for progression to adenocarcinoma, we generated compound mice bearing a single floxed *Trp53* allele (28) in addition to the *LSL-Kras^{G12D}*, *R26-Gli3T*, and *Ptf1a-Cre* alleles. Mice negative for the *R26-Gli3T* allele developed pancreatic carcinomas rapidly, with a median latency of 111 d (range 85–164 d) (Fig. 3A). These tumors were predominantly moderately to poorly differentiated ductal adenocarcinomas that frequently were invasive and metastatic, with dissemination to lymph nodes, the adjacent intestine, liver, peritoneal cavity, and lungs (Fig. 3B, i–v). Non-tumor-bearing pancreatic tissue displayed acinar atrophy and contained numerous PanINs (PanIN1 to PanIN3) (Fig. 3B, i). By contrast, mice bearing the *R26-Gli3T* allele developed carcinomas with a significantly longer latency, with a median age of 193 d (range 115–270 d; $P < 0.001$ by log-rank test) (Fig. 3A). Histologically, the tumors that developed in the *R26-Gli3T*-positive animals were indistinguishable from those in *R26-Gli3T*-negative mice and commonly were metastatic (Fig. 3B, vi–ix). Characterization of tumors by immunostaining for Ki67 or the pancreas progenitor marker PDX1 and immunoblotting for AKT and ERK phosphorylation showed no differences between tumors induced in *R26-Gli3T*-positive and -negative animals (Fig. S2).

Despite the delayed kinetics, the eventual formation of pancreatic tumors in animals bearing the *R26-Gli3T* allele again raises the question whether the tumors that developed failed to express Gli3T or whether *Trp53* deletion obviates the need for Gli activity, as suggested by a previous study (29). Thus, we assayed for the presence of Gli3T protein in lysates from tumors and from cell lines derived from these tumors by immunoblotting with an anti-Flag antibody. Although Gli3T protein could be detected readily in 293T cells transfected with a Gli3T expression construct (and in pancreas tissue from *Ptf1a-Cre;R26-Gli3T* mice; Fig. 1), Gli3T could not be detected in any of the tumor (Fig. 3C, Upper) or cell line (Fig. 3C, Lower) lysates. To determine whether these tumors were derived from cells that failed to undergo recombination, we analyzed DNA extracted from tumors and cell lines with PCR primers that differentiate between unrecombined and recombined *R26-Gli3T* alleles. The unrecombined allele was detected readily in all samples isolated from mice carrying the *R26-Gli3T* allele, whereas only one tumor sample tested positive for the presence of the recombined allele (Fig. 3D). Interestingly, a cell line derived from the tumor that tested positive for recombination was negative for the presence of the recombined *R26-Gli3T* allele, suggesting that the cells in the tumor carrying the recombined allele represented a minority of the sample or were normal epithelial cells entrapped within the mass of the tumor. Collectively, our data demonstrate that Gli transcriptional activity is required for pancreatic carcinoma development in vivo.

Gli1 Accelerates Kras-Initiated Pancreatic Tumorigenesis. Prior work suggested that Kras regulates Gli1 expression in PDAC cells and that Gli1 activity is critical for PDAC cell survival and trans-

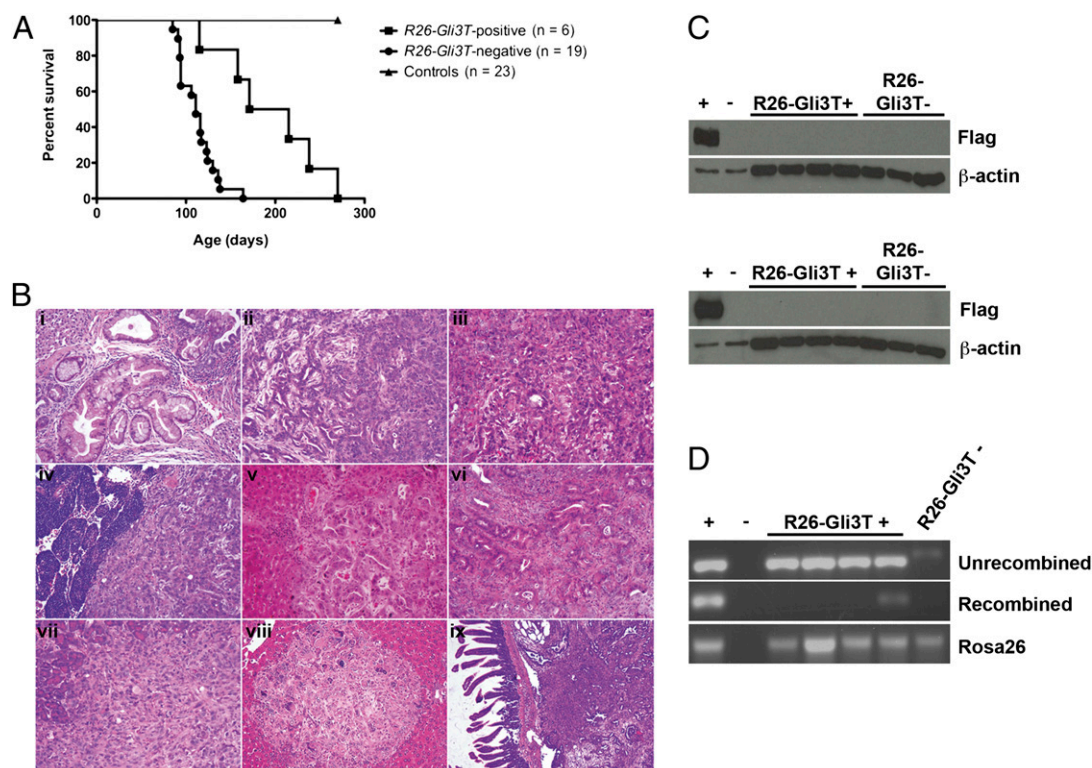


Fig. 3. Gli transcriptional activation is required for PDAC formation. (A) Kaplan–Meier survival curve for *Ptf1a-Cre;LSL-Kras^{G12D};Trp53^{flox/wt}* mice, *Ptf1a-Cre;LSL-Kras^{G12D};Trp53^{flox/wt};R26-Gli3T* mice, and *Ptf1a-Cre* and *LSL-Kras^{G12D}*-negative littermate controls. $P < 0.001$ for comparison between *R26-Gli3T*-positive and -negative animals. (B) Representative H&E-stained histological sections from lesions arising in *Ptf1a-Cre;LSL-Kras^{G12D};Trp53^{flox/wt}* (i–v) and *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli3T* (vi–ix) mice. PanIN lesions (i), glandular PDAC (ii), undifferentiated carcinoma (iii), lymph node metastasis (iv), and liver metastasis (v) were identified in *Ptf1a-Cre;LSL-Kras^{G12D};Trp53^{flox/wt}* mice. Glandular PDAC (vi), undifferentiated carcinoma (vii), liver metastasis (viii), and intestinal invasion (ix) were observed in *Ptf1a-Cre;LSL-Kras^{G12D};Trp53^{flox/wt};R26-Gli3T* mice. (C) Immunoblotting for Gli3T protein (using an anti-Flag antibody) in lysates from *R26-Gli3T*-positive (+) and -negative (–) tumors (Upper) and cell lines derived from these tumors (Lower). (D) PCR of genomic DNA to detect the recombination status of the *R26-Gli3T* allele in *R26-Gli3T*-positive tumor samples. Amplification of the native *Rosa26* locus was used as a control.

formation in vitro (18). However, the tumorigenic activity of Gli1 has not been examined in the pancreas in vivo. Therefore, we used a recently established conditional *Rosa26* knockin allele of Gli1, *R26-Gli1* (30, 31), to express Gli1 ectopically in the mouse pancreas.

Ptf1a-Cre;R26-Gli1 mice were generated and monitored for more than 12 mo. Gli1 activation alone did not initiate pancreatic tumors, and the pancreatic development and cytodifferentiation appeared normal in *Ptf1a-Cre;R26-Gli1* mice compared with control *R26-Gli1* mice (Fig. S3). However, activation of both *Kras* and Gli1 within the pancreas dramatically accelerated tumorigenesis. Although the pancreas appeared normal at birth, the health of the triple-transgenic *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1* mice deteriorated rapidly, and the majority of the animals were killed between 6 and 12 wk of age, although a few survived to the age of 10 mo (Fig. 4A). At 2 mo of age, the *Ptf1a-Cre;LSL-Kras^{G12D}* mice demonstrated only a few early PanIN1 lesions, as reported previously (5), whereas the vast majority of the pancreas contained well-organized acinar, islet, and ductal tissue (Fig. 4C). In contrast, the *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1* mice had widespread formation of PanIN lesions resulting in almost complete disruption of normal pancreatic architecture. These lesions showed a variety of nuclear atypia in the ductal epithelium, some corresponding to human PanIN3 lesions (Fig. 4D). Cytokeratin 8 staining confirmed the epithelial phenotype (Fig. 4E). A prominent desmoplastic stromal response, confirmed by smooth muscle actin staining, was present also (Fig. 4E).

Molecular analysis of the *Kras*/Gli1 advanced lesions revealed that cell proliferation as measured by Ki67 staining was increased

significantly compared with the lesions with *Kras* activation alone (Fig. 4F and G and Fig. S4A). Consistent with a previous report (32), we also detected senescence-associated β -galactosidase expression in early PanIN lesions from 2-mo-old *Ptf1a-Cre;LSL-Kras^{G12D}* mice (Fig. 4H), whereas high-grade PanIN lesions from age-matched *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1* mice did not exhibit detectable senescence-associated β -galactosidase staining (Fig. 4I and Fig. S4B), suggesting escape from *Kras^{G12D}*-induced growth arrest/senescence. However, we did not detect invasive PDAC in the *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1* mice, and metastasis was not observed in any of the mice we examined. Interestingly, we detected large multilocular cystic lesions resembling mucinous cystic neoplasms (Fig. 4J) in two of the *Ptf1a-Cre;LSL-KRAS^{G12D};R26-Gli1* mice, one at 6 wk of age and the other at 8 mo of age, in addition to the PanIN lesions we described above. The cysts from the *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1* mice were as large as 2 cm in diameter and were lined by columnar epithelial cells, where abundant mucin production was demonstrated by reaction with Alcian blue (Fig. 4K). IHC for estrogen receptor (ER) and progesterone receptor (PR) in these cystic lesions showed the presence of ER- or PR-positive cells in the stroma (Fig. S4C and D), a key feature of human mucinous cystic neoplasms. Taken together, these results suggest that Gli1 activation is not sufficient to initiate PDAC but synergizes with *Kras* to promote pancreatic tumor formation in vivo.

Gli-Dependent Transcriptional Program in PDAC Cells. The results from our loss-of-function and gain-of-function genetic analyses underscore the functional importance of Gli activation in pan-

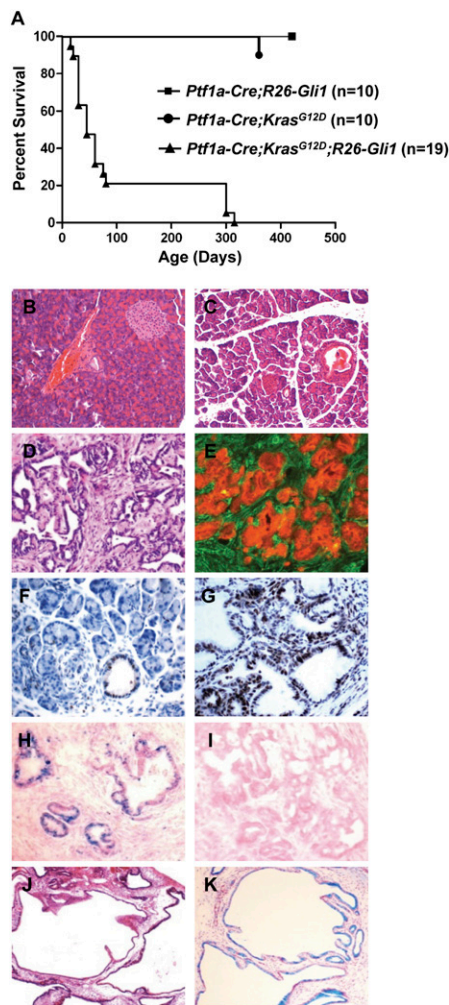


Fig. 4. Gli1 promotes Kras-initiated pancreatic tumorigenesis. (A) Survival of *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1* mice is significantly less than that of *Ptf1a-Cre;LSL-Kras^{G12D}* and *Ptf1a-Cre;R26-Gli1* mice. (B–D) H&E staining of pancreata derived from 2-mo-old *Ptf1a-Cre;R26-Gli1* (B), *Ptf1a-Cre;LSL-Kras^{G12D}* (C), and *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1* (D) mice. (E) Immunostaining for cytokeratin 8 (red) and α -smooth muscle actin (green) in a *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1*-derived pancreatic lesion. (F and G) IHC for Ki67 in *Ptf1a-Cre;LSL-Kras^{G12D}* (F) and *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1* (G) pancreata. (H and I) Senescence-associated β -galactosidase staining of pancreatic lesions from *Ptf1a-Cre;LSL-Kras^{G12D}* (H) and *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1* (I) mice. (J) H&E staining of pancreatic cystic lesions from a 1-mo-old *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1* mouse. (K) Alcian blue staining revealing abundant mucin production in epithelial cells in the cysts.

creatic ductal epithelial transformation. However, the Gli-mediated transcriptional program in pancreatic cancer remains largely unexplored.

Panc1 and MiaPaCa2 are human PDAC cell lines that contain activating mutations in *KRAS* and require Gli1 activity for survival and maintenance of their oncogenic properties (18). To examine further the Gli-mediated transcriptional regulation in these cells, Panc1 or MiaPaCa2 cells were transfected with a Gli3T expression vector, infected with shRNA constructs against Gli1, or treated with a small-molecule Gli inhibitor, GANT61. We found that Gli3T effectively blocked Gli-dependent transcriptional activation in the Panc1 or MiaPaCa2 cells (Fig. 5A), consistent with the down-regulated expression of the Gli target genes, *Ptch1* and *Gli1* in mouse mutant *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli3T* pancreas (Fig. S5A). In agreement with previous studies (18), we found that inhibition of Gli-mediated

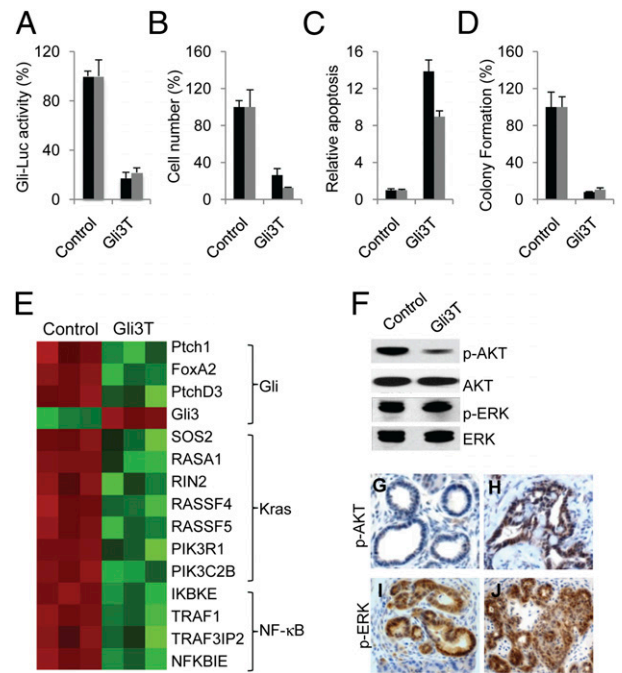


Fig. 5. Gli-mediated transcriptional program in pancreatic cancer cells. (A) Changes in Gli-luciferase reporter activity in Gli3T-expressing and control Panc1 (black bars) and MiaPaCa2 (gray bars) cells. (B–D) Cell proliferation, measured by MTT assay (B), apoptosis (C), and anchorage-independent soft-agar colony formation (D) by FACS-isolated Gli3T-expressing and control Panc1 (black bars) and MiaPaCa2 (gray bars) cells. Data shown are expressed as mean \pm SD. (E) Heat map illustrating mRNA expression of selected genes in Gli3T-expressing and control Panc1 cells. Red, high expression; green, low expression. (F) Immunoblot analysis of the levels of AKT phosphorylation (p-AKT), total AKT, ERK phosphorylation (p-ERK), and total ERK, in isolated Gli3T-expressing and control Panc1 cells. (G–J) IHC for p-AKT (G and H) and p-ERK (I and J) in pancreatic lesions from 2-mo-old *Ptf1a-Cre;LSL-Kras^{G12D}* (G and I) and *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1* (H and J) mice.

transcription by expression of Gli3T, shRNAs against Gli1, or treatment of GANT61 inhibited cell proliferation, increased apoptosis, and impaired anchorage independent growth in these cell lines (Fig. 5B–D and Fig. S5F–K). Together, these data demonstrated the importance of Gli transcriptional activation in human PDAC cells.

To identify Gli-dependent downstream target genes, we performed gene-expression profiling on Gli3T-expressing Panc1 cells and vector controls. We transfected Panc1 cells with a Gli3T-IRES-nuclear GFP expression construct, and 24 h posttransfection we isolated GFP-positive cells by FACS and performed expression profiling using Affymetrix chips. As expected, we detected up-regulation of *Gli3* that likely reflects the expression of the ectopic Gli3T transgene. We identified 265 genes that were significantly down-regulated by Gli3T (Table S1); among them, *PTCH1* and *FOX42* are known transcriptional targets of the Hh–Gli pathway (Fig. 5E). Interestingly, we also identified several genes involved in regulating Ras intracellular signal transduction, including *SOS2* (RasGEF), *RASA1* (RasGAP), *RIN2*, and *RASSF4/5* (Fig. 5E), suggesting possible feedback regulation of Kras signaling in cancer cells influenced by Gli activity.

The PI3K–AKT and MEK–ERK pathways are Kras-stimulated signaling pathways that have been implicated in tumorigenesis (33). In our transcriptional profiling, we found that two subunits of PI3K, *PIK3R1* and *PIK3C2B*, were among the genes whose expression was down-regulated significantly by Gli3T (Fig. 5E), indicating a possible interaction between Gli and PI3K–AKT signaling. Thus, we further examined the status of

these two critical Kras downstream pathways in PDAC cells. We found that Akt phosphorylation was down-regulated markedly in Gli3T-expressing Panc1 cells (Fig. 5F) but was elevated in mouse pancreatic tumors with both Kras and Gli1 activation (Fig. 5G and H). In contrast, ERK phosphorylation was not changed significantly in mouse PanIN lesions and human cancer cells upon Gli regulation (Fig. 5F, I, and J). These results are in agreement with a previous report that showed activation of AKT, but not ERK, when an active form of Gli2 was expressed in mouse pancreas (14).

Hh-Gli regulates Wnt signaling in several developmental, tissue regeneration and tumorigenic contexts (16, 34–37). Interestingly, a recent study suggested that Wnt signaling also may be regulated by Hh signaling in pancreatic tumors (38). Thus, we examined the canonical Wnt activity in Panc1 cells where Gli transcription activity was inhibited by Gli3T. Quantitative RT-PCR (qPCR) analysis showed that the expression of a well-established Wnt target gene, *AXIN2*, was not inhibited by Gli3T expression in Panc1 and MiaPaCa2 cells (Fig. S6). This result is consistent with the absence of Wnt pathway target genes from the list of differentially expressed genes identified by our microarray experiments (Table S1) and with the absence of β -catenin nuclear accumulation in the PanIN lesions with both Kras and Gli1 activation (Fig. S6). These data also are consistent with our genetic result that Gli3T expression did not affect embryonic pancreatic development (Fig. 1), where canonical Wnt signaling plays a prominent role (39, 40). Together, these data suggest that regu-

lation of canonical Wnt signaling may not play a major role in our Gli-dependent pancreatic tumor models.

Gli-Mediated Regulation of IKBKE and NF- κ B Activation in Pancreatic Tumors. Our gene-expression profile analysis detected an enrichment of genes associated with the NF- κ B pathway, including *IKBKE* (*IKK ϵ*), *TRAF1*, *TRAF3IP2*, and *NFKBIE* (Fig. 5E). IKBKE (*IKK ϵ*) is a I κ B kinase (IKK)-related kinase involved in the activation of the NF- κ B pathway (41, 42) and recently was identified as a breast cancer oncogene (43). Importantly, elevated IKBKE expression was shown recently to be a common feature of PDAC (44). Thus, we examined whether IKBKE/NF- κ B activation constituted a downstream mechanism of Gli transcription in pancreatic cancer.

We first examined whether NF- κ B transcriptional activity is regulated by Gli-mediated transcription. Panc1 cells expressing Gli3T or shRNAs against Gli1 or treated with GANT61 exhibited a significantly lower level of NF- κ B activity than cells expressing a control plasmid as assayed by measuring the activity of a synthetic NF- κ B reporter gene (Fig. 6A). Inhibition of Gli transcriptional activation in Panc1 cells also resulted in marked down-regulation of IKBKE gene expression as measured by qPCR or immunoblot analysis (Fig. 6B and C). To determine the functional importance of IKBKE in PDAC cells, we used two shRNA constructs against human *IKBKE* to silence IKBKE expression in Panc1 or MiaPaCa2 cells and confirmed knockdown efficiency by qPCR (Fig. 6D and Fig. S7A). We found that inhibition of IKBKE expression reduced cell numbers relative to

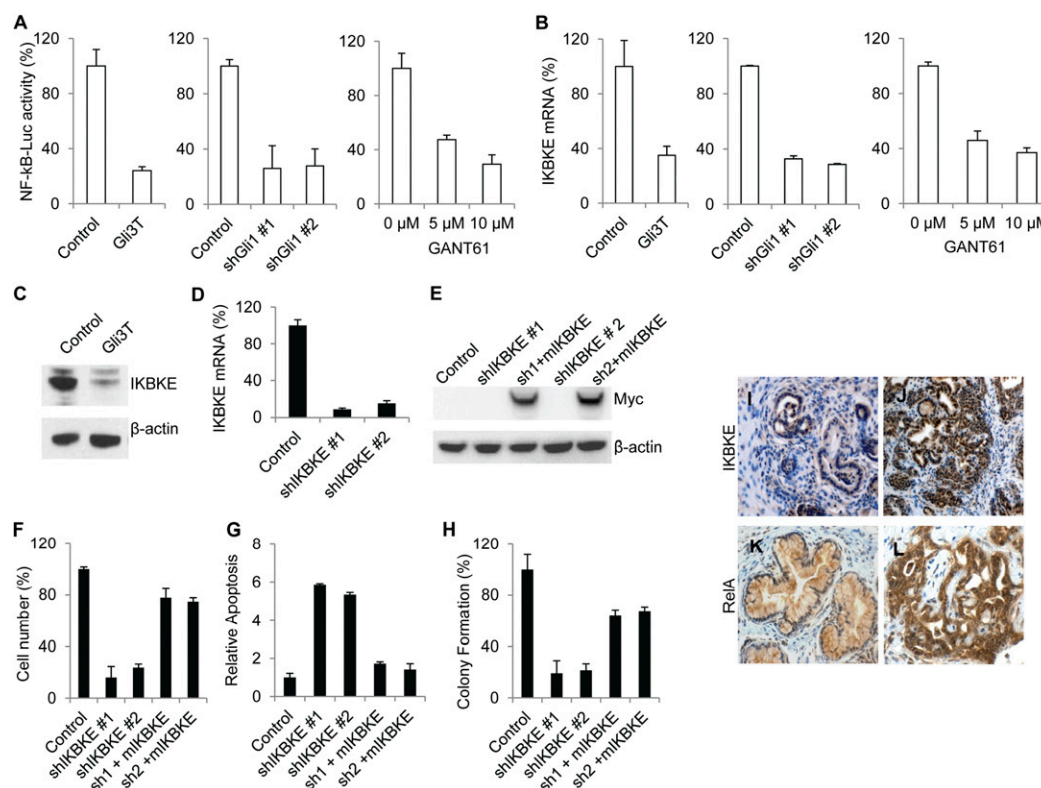


Fig. 6. Gli regulates IKBKE and NF- κ B activity in pancreatic cancer cells. (A) Inhibition of NF- κ B reporter activity by Gli3T, shRNAs against Gli1, and GANT61 in Panc1 cells 48 h after transfection. (B) qPCR analysis of *IKBKE* mRNA expression in response to Gli3T expression, Gli1 knockdown, and GANT61 treatment. (C) Immunoblot analysis of IKBKE expression in control and Gli3T-expressing Panc1 cells. (D) qPCR analysis of *IKBKE* mRNA level in Panc1 cells expressing shRNA constructs against GFP (control) or IKBKE (shIKBKE #1 and shIKBKE #2). (E) Immunoblot analysis for expression of myc-tagged mouse IKBKE (mIKBKE) in Panc1 cells expressing shRNA constructs against GFP or IKBKE with or without mouse IKBKE expression. Data shown are expressed as mean \pm SD. (I–L) IHC staining of IKBKE (I and J) and RelA (K and L) in pancreatic lesions from *Ptf1a-Cre;LSL-Kras^{G12D}* (I and K) and *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1* (J and L) mice. Note the predominantly cytoplasmic staining of RelA in J and increased nuclear staining in K.

a nonsilencing shRNA control, as measured by a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Fig. 6F and Fig. S7C). Further, IKBKE knockdown increased apoptosis, as illustrated by caspase 3 cleavage (Fig. 6G and Fig. S7D), and impaired the ability of PDAC cells to form colonies in soft agar (Fig. 6H and Fig. S7E). Importantly, these phenotypes were rescued by coexpression of a mouse IKBKE expression vector that is resistant to the two shRNAs targeting human IKBKE (Fig. 6 E–H and Fig. S7 B–E). Together, these data indicate that IKBKE is required for the survival and transformation phenotypes of human PDACs.

Next, we determined whether IKBKE and NF- κ B activity are regulated by Gli-mediated transcription in vivo. RelA is a member of the NF- κ B family, and nuclear accumulation of RelA indicates activation of the canonical NF- κ B pathway. We therefore performed IHC for IKBKE and RelA in tissue samples from *Ptf1a-Cre;LSL-Kras^{G12D}* and *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1* mice at the age of 2 mo. We found that, compared with those in *Ptf1a-Cre;LSL-Kras^{G12D}* mice, epithelial cells in *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1* mice exhibited significantly increased IKBKE expression and nuclear RelA staining (Fig. 6 I–L). In summary, these observations demonstrate activation of IKBKE/NF- κ B signaling by Gli proteins in PDAC cells and highlight a potential mechanism for the observed requirement for Gli transcription for PDAC development in vivo.

Discussion

Noncanonical Gli Function in Pancreatic Tumor Cells. Noncanonical Gli regulation has been reported and implicated in several oncogenic settings (29, 45–48). A growing body of evidence also suggests a cell-autonomous noncanonical Gli regulation in pancreatic cancer that is distinct from the Hh ligand-dependent paracrine effect on the tumor stroma (3, 11, 18, 24, 49). Our results here, together with a previous report (14), show that, unlike Smo activation (23), Gli1 or Gli2 activation is able to cooperate with Kras to promote pancreatic tumorigenesis. Moreover, *GLI1* and *GLI3* recently were reported to be mutated in human PDAC-derived cells (3), and the expression of Gli1 and Gli3 can be regulated in Smo-null mouse pancreatic tumor cells (18). Together, these studies support the noncanonical model and indicate a broad involvement of Gli misregulation in pancreatic cancer.

Using a dominant repressor Gli3T allele that inhibits all Gli-mediated transcriptional activation, we demonstrate that Gli transcriptional activity is specifically required for pancreatic tumor formation in vivo, although it is dispensable for normal pancreatic development. Importantly, our data show that Gli activity is required not only for pancreatic tumor initiation but also for the maintenance of established PDAC cells. Given the demonstrated importance of Hh ligands on the desmoplastic stroma, our results suggest that Gli proteins are attractive therapeutic targets (45, 50) in PDAC, because their inhibition would affect both the tumor epithelium and the reactive stroma.

Currently it is not well understood why the pancreatic epithelium is refractory to Ptch/Smo-mediated canonical signaling or how Kras potentially regulates Gli expression levels (18, 49, 51); however, recent work points to an interesting potential connection with the primary cilium (52). Significant Gli signal up-regulation was observed within the pancreatic epithelium after disruption of primary cilium (52), a cellular organelle that is associated intimately with Hh–Gli signal transduction (53). Interestingly, another recent study showed that Kras-mediated transformation of the pancreatic duct epithelium correlates the loss of this organelle in PanIN and PDAC cells in vivo (54). Thus, Kras activation may lead to loss of the primary cilium, and this loss might facilitate the Hh ligand-independent activation of Gli activity in tumor epithelium.

Gli1 Activation in Pancreatic Cancer. Our results on the cooperation of Gli1 with Kras provided evidence for the in vivo tumorigenic potential of Gli1 in the pancreas. However, it is interesting to note the phenotypic differences between Gli1 and Gli2 activation in pancreatic tumor initiation. Gli1 is unable to initiate pancreatic tumorigenesis on its own, whereas activation of Gli2 by the *CLEG2* allele drives pancreatic neoplasia, albeit in the development of undifferentiated tumors that do not progress via PanINs (14). Several possibilities may account for the difference. First, there are differences in the design of the transgenes. The *R26-Gli1* allele (30) allows Cre-dependent expression of the full-length Gli1 protein from the ubiquitously expressed *Rosa26* locus, whereas in the *CLEG2* allele (14) a dominant active version of Gli2, Gli2 Δ N, is expressed via control of the *CAGGS* promoter, a highly active hybrid *CMV/ β -actin* promoter. Furthermore, Gli2 Δ N consists of an N-terminally truncated form of the protein that lacks an N-terminal repressor domain, perhaps resulting in resistance to posttranslational regulation (55). Second, different Cre drivers were used in these two studies. Compared with *Ptf1a-Cre* (26), the *Pdx1-Cre* transgene (56) used in the Gli2 Δ N study directs Cre expression in earlier embryonic pancreatic progenitor cells. However, it is possible that the phenotypic difference indeed may reflect distinct properties of these two Gli proteins; differential transcriptional outputs mediated by Gli1 and Gli2 have been reported (57).

However, in the context of Kras-initiated pancreatic tumorigenesis, both Gli1 and Gli2 showed remarkable capacity to accelerate tumor development. Ectopic expression of either Gli1 or Gli2 Δ N, together with Kras activation, resulted in the formation of advanced PanIN lesions by 2 mo of age. Only minimal early PanIN1A lesions are detected at this age in mice with Kras activation alone. Extensive fibrosis also was evident in both Gli1/Kras and Gli2 Δ N/Kras tumors, a feature similar to desmoplasia observed in human PDAC. Despite the frequent formation of advanced lesions at an early age, we did not observe invasive or metastatic PDAC in the *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1* mice that survived to 10 mo of age, suggesting the requirement for additional oncogenic alterations, possibly loss of p53 or p16 function (6, 7). Another intriguing possibility is that paracrine signaling of Hh ligands to the reactive stroma is involved in the stimulation of metastasis. It would be interesting to test whether Gli activation, together with Hh ligand up-regulation in postnatal mouse pancreas, may lead to the development of metastatic PDAC.

Unique Gli Transcriptional Program in PDAC. Despite the importance of Kras in PDAC, the transcriptional output regulated by this signaling in pancreatic cancer cells remains poorly characterized. Our data here, in agreement with prior studies, place Gli transcription factors downstream of Kras in pancreatic cancer, and our gene-expression profiling studies potentially describe a noncanonical transcriptional regulation controlled by Gli proteins in PDAC cells. Interestingly, we did not detect down-regulation of typical Hh-Gli mitogenic targets, such as *cyclin D1* and *MYC*, that are commonly found in Hh-related tumors (58–60). Although we cannot rule out the possibility that Gli3T inhibition may not recapitulate fully the Gli1/2-null phenotypes in PDAC cells, our data support the idea that Gli proteins may exert a unique transcriptional program in pancreatic cancer cells, a notion that is consistent with recent studies showing that Gli-mediated transcriptional output is highly context dependent (25, 31, 61). Our data also suggest that canonical Wnt signaling is unlikely to play a major role in Gli-dependent pancreatic epithelial transformation. Instead, we identified a cluster of Gli-dependent genes that possibly are involved in selective feedback regulation of Kras-stimulated signal transduction in PDAC cells. Further, our findings that Gli3T expression in Panc1 cells suppresses Akt phosphorylation and that Gli activity is required for the elevated expression of the PI3K subunits *PIK3R1* and

PIK3C2B in these cells support the intriguing possibility that Gli proteins may contribute, directly or indirectly, to the selective activation of the PI3K-AKT pathway.

IKBKE and NF- κ B Activation in Pancreatic Cancer. NF- κ B activity has long been linked to inflammation-related tumorigenesis (62–64). There also is increasing recognition of intrinsic dysregulation of the pathway in solid tumors. Alteration of NF- κ B pathway components was a common finding in a recent comprehensive genomic screen of somatic copy-number alterations across human cancers (65). Further, activation of the NF- κ B pathway, possibly via noncanonical IKK kinases such as IKBKE and TBK1, has been shown recently to be critical for several epithelial tumors, including breast and lung carcinomas that harbor *KRAS* mutations (42, 43, 66, 67). Our results in human PDAC cells and mouse tumors indicate enhanced NF- κ B activity in the tumor epithelium, a finding that is consistent with previous studies on NF- κ B activation in human PDAC cells (68, 69). Our data further suggest that the IKK-related kinase IKBKE may play an important functional role in PDAC downstream of Gli proteins. IKBKE levels are elevated in pancreatic tumors with concomitant expression of activated *Kras* and *Gli1*, and IKBKE knockdown impairs the survival and transformation of PDAC cells. *IKBKE* recently was identified as a breast cancer oncogene (43), and recent work demonstrated elevated levels in PDAC samples (44). Therefore it will be interesting to test whether *IKBKE* functions as an oncogene in PDAC as well. Our finding that multiple components of the NF- κ B pathway are regulated downstream of *Gli3T* also suggests a potential link between *Kras* activation and NF- κ B mediated, in part, by Gli transcription factors. Interestingly, although our data show cell-autonomous regulation of IKBKE and NF- κ B activity by Gli, a recent report suggested that production of the Shh ligand may be regulated by NF- κ B activity in PDAC cells (70). Clearly, further in vivo studies are warranted to elucidate the mechanism of and the requirement for the interplay among these critical pathways in the pathogenesis of pancreatic cancer.

Materials and Methods

Mouse Strains. *Ptf1a-Cre*, *LSL-Kras^{G12D}*, *Trp53^{fllox}*, *R26-Gli3T*, and *R26-Gli1* mice have been described previously (25–28, 31). *Ptf1a-Cre;LSL-Kras^{G12D}*, *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli3T*, and *Ptf1a-Cre;LSL-Kras^{G12D};R26-Gli1* mice were obtained by crossing *LSL-Kras^{G12D}* to *Ptf1a-Cre;R26-Gli3T* or *Ptf1a-Cre;R26-Gli1* mice, respectively. Offspring from the cross of *LSL-Kras^{G12D};R26-Gli3T* to *Ptf1a-Cre;Trp53^{fllox/fllox}* mice were followed longitudinally for tumor development for 270 d. All mouse experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School.

Tissue Collection and Histology. After animals were killed, primary pancreatic tissues and metastatic lesions were separated in pieces and fixed in 4% (wt/vol) paraformaldehyde. For paraffin sections, tissues were dehydrated, embedded in paraffin blocks, and cut at a thickness of 6 μ m. For frozen sections, tissues were dehydrated in 30% (wt/vol) sucrose and embedded in Optimal Cutting Temperature compound (OCT; Sakura Finetek), and sections were cut at a thickness of 12 μ m. For RNA and protein analysis, tissues were flash frozen in liquid nitrogen. Tissue sections (6 μ m) were stained with H&E using standard reagents and protocols.

Generation of Cell Lines from Tumor Tissue. Pancreatic tumors were dissected and minced in cold DMEM containing 10% (vol/vol) FBS. The tumor tissue was then plated in a 10-cm tissue culture dish and allowed to adhere for 2 d. Media was then changed and tumor pieces removed. The epithelial character of the isolated cells was confirmed by staining for pancreas epithelial markers such as cytokeratins 8 and 19.

IHC, Immunofluorescence, and Immunoblotting. For IHC, high-temperature antigen retrieval was conducted in sodium citrate solution (pH 6.0) on paraffin sections for 30 min. Sections were blocked in a buffer containing 5% (wt/vol) BSA and 0.1% (vol/vol) Triton X-100 in PBS and then were incubated overnight at 4 °C in primary antibodies diluted in blocking buffer. Primary

antibodies used were Ki67 (1:500; Abcam); phospho-AKT (1:50; Cell Signaling); phospho-ERK (1:500; Cell Signaling); IKBKE (1:50; Santa Cruz); RelA (1:50; Santa Cruz); β -catenin (1:400; BD Transduction); estrogen receptor (1:1,000; Santa Cruz); progesterone receptor (1:1,000; Santa Cruz); and PDX1 (1:5,000; gift of Chris Wright, Vanderbilt University, Nashville, TN). Signal detection was accomplished with biotinylated secondary antibodies in the Vectastain ABC kit (Vector Labs).

For immunofluorescence, OCT sections were washed with PBS and incubated in blocking buffer containing 5% (vol/vol) sheep serum, 1% (vol/vol) FBS, and 0.1% (vol/vol) Triton X-100 in PBS. Sections then were incubated overnight at 4 °C in primary antibodies diluted in blocking buffer. Primary antibodies used were glucagon (1:3,000; gift of Andrew Leiter, University of Massachusetts Medical School, Worcester, MA); amylase (1:800; Sigma); insulin (1:100; Abcam); smooth muscle actin (1:500; Sigma); and cytokeratin-8 (1:100; Developmental Studies Hybridoma Bank). Alexa Fluor fluorescent conjugated secondary antibodies (Invitrogen) were used for detection at a concentration of 1:500 diluted in blocking buffer. Slides then were mounted in mounting medium containing DAPI.

For immunoblotting, the primary antibodies used were FlagM2-HRP (1:1,000; Sigma); β -actin (1:1,000; Sigma); phospho-AKT (1:1,000; Cell Signaling); phospho-ERK (1:1,000; Cell Signaling); AKT (1:1,000; Cell Signaling); ERK (1:1,000; Cell Signaling); IKBKE (1:1,000; Sigma); and Myc (1:1,000; Developmental Studies Hybridoma Bank). HRP-conjugated secondary antibodies used for detection were obtained from Jackson Laboratories.

Alcian Blue Staining and Senescence-Associated β -Galactosidase Staining. For Alcian blue staining, paraffin sections were hydrated and stained for 30 min at room temperature using Alcian blue reagent (IHC World). Sections were counterstained with Nuclear Fast Red. For senescence-associated β -galactosidase staining, frozen sections were washed in PBS and stained overnight using Senescence β -galactosidase staining solution (Cell Signaling Technology). Sections were counterstained using eosin.

Cell Proliferation, Apoptosis, and Soft-Agar Assays. *Gli3T*-expressing GFP-positive Panc1 or MiaPaCa2 cells were isolated 24 h after transfection using flow cytometry. For shRNA knockdown, Panc1 or MiaPaCa2 cells were infected with pLKO-based lentiviruses expressing shRNAs and were selected for 4 d using puromycin. For GANT61 inhibition, Panc1 or MiaPaCa2 cells were treated with 5 μ M or 10 μ M GANT61 dissolved in DMSO.

For the MTT-based cell-proliferation assay, the cells were seeded at a density of 3,000 cells per well in a 96-well plate, treated with 5 mg/mL MTT 5 d after seeding, and lysed in DMSO 4 h later. Absorbance was measured at 595 nm. The assay was performed in triplicate.

For the apoptosis assay, cells were plated in chamber slides after cell sorting, drug selection, or GANT61 treatment. Cells were fixed in 4% paraformaldehyde. Immunostaining with a polyclonal antibody against cleaved caspase 3 (1:400; Cell Signaling) was used as a marker for apoptosis. Apoptotic cells were counted in three wells, and relative apoptosis was measured by comparing the number of apoptotic cells with the control sample. SD was used to calculate error bars.

For the anchorage-independent growth assay using soft-agar, cells were seeded at a density of 6,000 cells per well in a six-well plate of 0.3% agarose in DMEM containing 10% (vol/vol) FBS. Colonies from 12 fields of view were counted 14 d later. Assays were performed in triplicate.

Luciferase Reporter Analysis. NIH 3T3 or Panc1 cells were cotransfected with luciferase reporter constructs, GliB5-Luc (gift of H. Kondoh, Osaka University, Osaka, Japan), TOPflash (Addgene), serum-response element luciferase [SRE-Luc; gift of D. Wu, Yale University, New Haven, CT], NF- κ B-Luc (gift of F. Chan, University of Massachusetts Medical School, Worcester, MA), and expression vectors for Renilla luciferase, *Gli3T*, *Gli1*, *Gli2*, *Lef1*, and *CDC42*. For *Gli1* knockdown, the cells were cotransfected with shRNAs targeting *Gli1* along with NF- κ B luciferase and Renilla expression plasmids. For GANT61 treatment, the Panc1 cells were cotransfected with NF- κ B reporter and Renilla Luciferase and then were treated with 5 μ M or 10 μ M GANT61 6 h after transfection. Luciferase assays were conducted 48 h after transfection using the dual-luciferase reporter kit (Promega). Assays were conducted in triplicate.

Detection of Recombination at the *R26-Gli3T* Locus. PCR was performed on isolated genomic DNA using the primer pair (forward: 5'-GTAGTCCAG-GTTTCCTTGATG-3'; reverse: 5'-TGCTACTTCCATTGTGCACGTC-3') for detection of the unrecombined *R26-Gli3T* allele and (forward: 5'-GTAGTC-CAGGGTTTCCTTGATG-3'; reverse: 5'-GGACTTTCATCTCATTTGAAG-3') for detection of the recombined allele. Primers to the native *R26* locus were

used as a control (forward: 5'-GGAGCGGGAGAAATGGATATG-3'; reverse: 5'-AAAGTCGCTCTGAGTTGTTAT-3').

qRT-PCR. cDNA synthesis was conducted using Invitrogen SuperScript II kit. Primers used for qRT-PCR were human IKBKE (forward: 5'-TGGTGCAGAGTATCAAGC-3'; reverse: 5'-TACAGGCAGCCACAGAACAG-3'); mouse IKBKE (forward: 5'-GCGGAGGCTGAATCACCAG-3'; reverse: 5'-GAAAGCCCGAACGTGTTCTCA-3'); Axin2 (forward: 5'-AGTGTGAGGTCCACGGAAAC-3'; reverse: 5'-CTTCACACTGCGATGATTT-3'); human GAPDH (forward: 5'-ATGGGGAAGTGAAGGTCG-3'; reverse: 5'-GGGGTCATTGATGGCAACAATA-3'); mouse GAPDH (forward: 5'-AGGCCGGTGTGATGATGTC-3'; reverse: 5'-TGCCTGCTTACCACCTTCT-3'); β -actin (forward: 5'-TGACAGGATGAGGAGAG-3'; reverse: 5'-CTGGAAAGGTGGACAGTGAGG-3'); human Gli1 (forward: 5'-CCAGCGCCAGACAGAG-3'; reverse: 5'-GGCTCGCCATAGCTACTGAT-3'); mouse Gli1 (forward: 5'-GTCGGAAGTCTATTACAGC-3'; reverse: 5'-CAGTCTGCTCTTCCCTGC-3'); human Patched-1 (forward: 5'-CCACAGAAGCGCTCTACA-3'; reverse: 5'-CTGTATTTTCGCCCTTC-3'); and mouse Patched-1 (forward: 5'-AACAAATTCACCAACCTC-3'; reverse: 5'-TGTCTTCATTCAGTTGATGTG-3'). All qPCR assays were conducted in triplicate.

Gli1 and IKBKE Knockdown. Panc1 or MiaPaCa2 cells were infected with pLKO-based lentiviruses encoding shRNAs targeting human *Gli1* (shGli1#1: CATC-CATCAGATCGCATT; shGli1#2: GCTCAGCTGTGTGAATTAT) and human *IKBKE* (shIKBKE#1: TGGGCAGGAGCTAATGTTTCG; shIKBKE#2: GAGCATTG-GAGTACCTTGTGA). Infected cells were selected in 5 μ M puromycin for 4 d. An MTT assay, soft-agar assay, and caspase staining for apoptosis were conducted as described previously.

PDEC Proliferation and Survival. PDECs from *Keratin19-tv-a;R26-Gli3T* double-transgenic mice were isolated and cultured as described previously (15). The

retroviral constructs *RCAS-GFP* and *RCAS-KrasG12D-IRES-GFP* have been described previously (15). *RCAS-Cre* was a gift from Eric Holland (Memorial Sloan-Kettering Cancer Center, New York, NY). Isolated PDECs were infected with either *RCAS-KrasG12D-IRES-GFP* or *RCASGFP* and subsequently were infected with *RCAS-Cre* to induce Gli3T expression or with *RCAS-GFP* as a control. Cell proliferation and survival assays were performed as previously described (15).

Affymetrix Gene Chip Analysis. Panc1 cells were transfected with a GFP-expressing vector carrying Gli3T (pCIG-Gli3T) or a GFP-expressing empty vector (pCIG). GFP-positive cells were isolated 24 h after transfection using flow cytometry. RNA was isolated, labeled, and hybridized to mouse GeneST1.0 chips (Affymetrix) according to Affymetrix protocols. Three independent biological samples were used for chip analysis. Statistical analyses were performed using R, a system for statistical computation and graphics (<http://www.r-project.org>). Genes with adjusted *P* value <0.05 and absolute fold change ≥ 1.5 were considered potential targets for further investigation.

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