Cancer Cell
Article

Oncogenic KRas Suppresses Inflammation-Associated Senescence of Pancreatic Ductal Cells

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DOI 10.1016/j.ccr.2010.10.020

SUMMARY

Mutational activation of KRas is the first and most frequently detected genetic lesion in pancreatic ductal adenocarcinoma (PDAC). However, the precise role of oncogenic KRas in the pathogenesis of PDAC is not fully understood. Here, we report that the endogenous expression of oncogenic KRas suppresses premature senescence in primary pancreatic duct epithelial cells (PDEC). Oncogenic KRas-mediated senescence bypass is conferred by the upregulation of the basic helix-loop-helix transcription factor Twist that in turn abrogates $p16^{INK4A}$ induction. Moreover, the KRas-Twist-$p16^{INK4A}$ senescence bypass pathway is employed in vivo to prevent inflammation-associated senescence of pancreatic ductal epithelium. Our findings indicate that oncogenic KRas could contribute to PDAC initiation by protecting cells from entering a state of permanent growth arrest.

INTRODUCTION

Ras proteins comprise a family of signal-transducing GTPases that are frequently mutated in human cancers. Oncogenic Ras mutations lock the protein in its GTP-bound form thus permitting its constitutive interaction with and activation of multiple effectors (Downward, 2003). The pathogenic role of oncogenic Ras has been attributed primarily to its promoting effects on cell proliferation and cell survival. In contrast, in normal primary cells oncogenic Ras can cause a permanent proliferative arrest known as premature senescence (Serrano et al., 1997). The induction of senescence by oncogenic Ras is largely mediated by the upregulation of inhibitors of cell proliferation including $p16^{INK4A}$, $p19^{ARF}$, $p21^{CIP}$, and p53 and is thought to serve as a tumor suppressive process by preventing the expansion of cells bearing mutant Ras (Lowe et al., 2004). However, the capacity of oncogenic Ras to provoke senescence varies considerably depending on cellular context and biological setting. For example, the ectopic expression of oncogenic Ras in fibroblasts at supraphysiological levels can trigger senescence, whereas expression of oncogenic Ras at physiological levels fails to engage the senescence machinery (Serrano et al., 1997; Tuveson et al., 2004). In addition, although some studies using mouse models of oncogenic KRas-driven tumorigenesis have documented the presence of senescent preneoplastic lesions in lung, colon, and pancreatic tissues (Bennecke et al., 2010; Collado et al., 2005; Morton et al., 2010), others have reported that senescence could not be detected in oncogenic KRas-expressing tissues (Tuveson et al., 2004). Thus, it remains unclear to what extent the implementation of the senescence program is linked to the oncogenic potential of mutated Ras.

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in the United States and carries a median survival of <6 months (Jemal et al., 2009; Warshaw and Fernandez-del Castillo, 1992). A distinguishing molecular feature of PDAC is the presence of activating KRas mutations in >90% of tumors (Almoguera et al., 1988). Because of their unusually high prevalence and their detection at very early stages of disease, KRas mutations are considered a key genetic determinant in the initiation of PDAC. In support of this postulate, mice engineered to express mutated KRas

Significance

Cellular senescence, a state of stable proliferative arrest, is a potent tumor suppressor mechanism and senescence bypass represents an important step in tumor development. In this study, we demonstrate that inflammatory stress can provoke a $p16^{INK4A}$-dependent senescence of pancreatic ductal cells, and that this response is abrogated by oncogenic KRas through the upregulation of Twist and the consequent suppression of $p16^{INK4A}$ expression. These findings identify a mechanism by which oncogenic KRas may contribute to pancreatic tumorigenesis and warrant the evaluation of oncogenic KRas-mediated senescence escape as a potential target for therapeutic intervention.

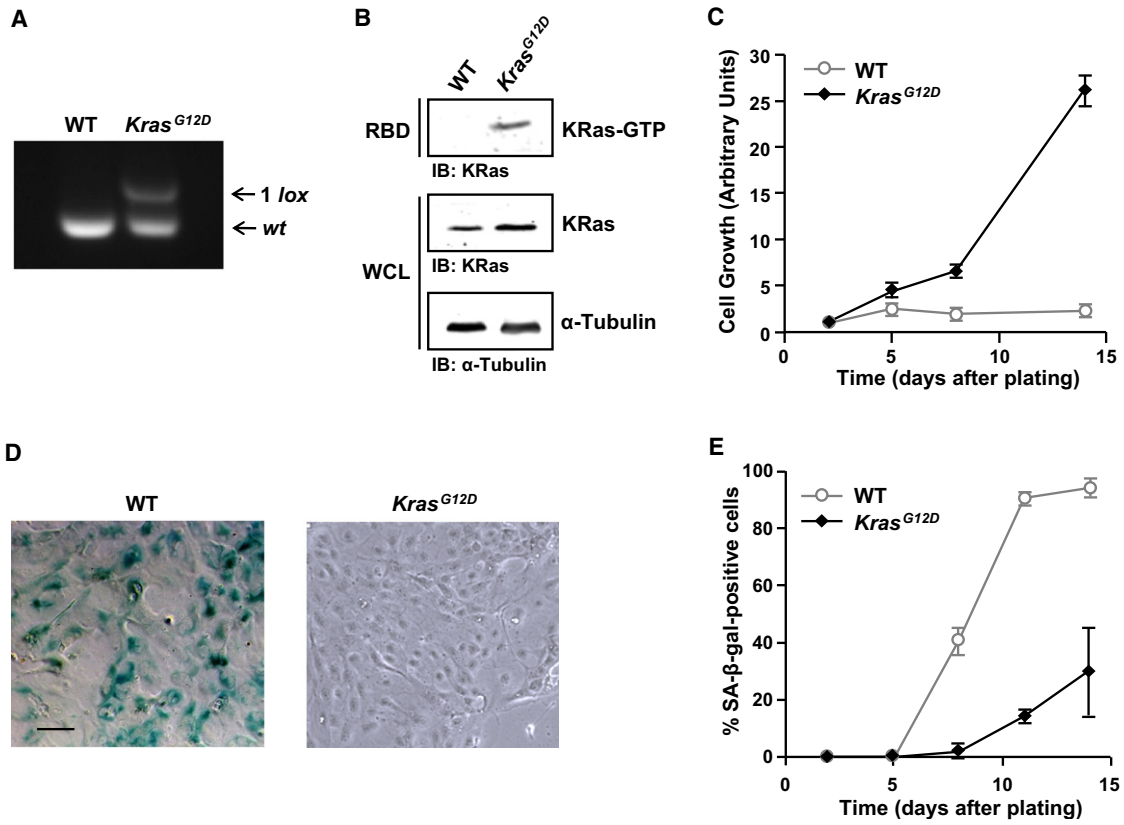

Figure 1. Oncogenic KRas Protects PDEC from Undergoing Premature Senescence

(A) PCR analysis of genomic DNA prepared from *LSL-Kras*^{G12D} PDEC infected with adenoviral-GFP (WT) or adenoviral-Cre (*Kras*^{G12D}). The excision-recombination event of the LSL cassette leaves behind a single LoxP (1 *lox*) site.

(B) Measurement of Ras activation in WT and *Kras*^{G12D} PDEC by GST-RBD pull-down assay. α-Tubulin serves as a loading control. IB: immunoblot; WCL: whole cell lysates.

(C) Growth analysis of WT and *Kras*^{G12D} PDEC. The number of DAPI-stained nuclei was counted in nine random fields of view (FOV) each containing at least 50 cells at day 2, 5, 8, and 14. The average number of nuclei present in the FOV at each time point was then normalized to the average number of nuclei per FOV at day 2. Error bars indicate standard deviation (SD). Data are representative of five independent experiments.

(D) SA-β-gal staining of WT and *Kras*^{G12D} PDEC cultures at day 8. Scale bar represents 100 μm.

(E) Quantification of SA-β-gal staining in WT and *Kras*^{G12D} PDEC cultures at day 2, 5, 8, 11, and 14. Cells were counterstained with Hoechst 33342 for β-gal quantification. Error bars indicate SD (n = 6 FOV). Data are representative of five independent experiments. See also Figure S1.

specifically in the pancreas sustain a spectrum of neoplastic lesions that mirror histologically those observed in humans (Hingorani et al., 2003). Thus, understanding the mechanisms by which KRas mutations contribute to PDAC development is critical for the identification of effective strategies to detect and treat PDAC. To clarify the relationship between the mutational activation of KRas, induction of senescence and pancreatic tumorigenesis, we have examined the consequences of endogenous oncogenic KRas expression in primary pancreatic duct epithelial cells (PDEC), a potential cell of origin for PDAC.

RESULTS

To assess the role of oncogenic KRas in pancreatic tumorigenesis, we have used a previously described cell culture system for primary mouse PDEC (Agbunag and Bar-Sagi, 2004; Agbunag et al., 2006). The endogenous expression of oncogenic KRas in these cells was achieved by their isolation from conditional oncogenic KRas (*LSL-Kras*^{G12D}) knock-in mice (Jackson et al., 2001) followed by infection with recombinant adenoviruses encoding Cre recombinase. As a control, PDEC derived from the same mice were infected with recombinant adenoviruses encoding green fluorescent protein (GFP). The excision of the LSL cassette was verified by PCR (Figure 1A) and the expression of the *Kras*^{G12D} allele was indicated by the pronounced increase in the levels of KRas-GTP (Figure 1B).

We have previously shown that primary PDEC undergo premature senescence in culture (Agbunag and Bar-Sagi, 2004). Consistent with these observations, control PDEC, hereafter referred to as wild-type (WT), ceased growing within 5 days after plating, adopted an enlarged flattened morphology, and displayed senescence-associated-β-galactosidase (SA-β-gal) activity starting at day 8 and peaking at days 12–15 (Figures 1C–1E; see Figure S1 available online). In contrast, the vast majority of KRas^{G12D}-expressing PDEC, hereafter referred to as *Kras*^{G12D}, continued proliferating and showed no SA-β-gal

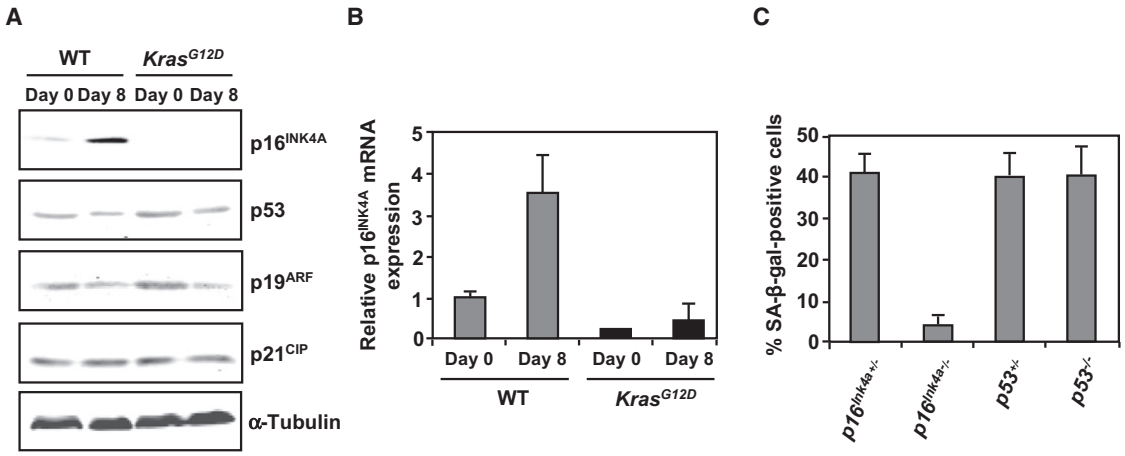

Figure 2. Oncogenic KRas Confers Bypass of Premature Senescence in PDEC through the Suppression of p16^{INK4A} Induction

(A) Western blot analysis for senescence effectors in WT and *Kras*^{G12D} PDEC cultures at day 0 and day 8. Equal loading was verified with anti-α-tubulin.

(B) Quantitative RT-PCR analysis of p16^{INK4A} in WT and *Kras*^{G12D} PDEC cultures at day 0 and day 8. Error bars indicate SD (n = 3).

(C) Quantification of SA-β-gal staining in *p16*$^{Ink4a+/-}$, *p16*$^{Ink4a-/-}$, *p53*$^{+/-}$, *p53*$^{-/-}$ PDEC cultures at day 8. Error bars indicate SD (n = 6 FOV). Data are representative of three independent experiments.

activity (Figures 1C–1E; Figure S1). These findings indicate that oncogenic KRas can protect PDEC from premature senescence.

To investigate the mechanisms by which oncogenic KRas represses PDEC senescence, we first assessed the expression levels of the major senescence effectors p16^{INK4A}, p19^{ARF}, p21^{CIP}, and p53 in WT and *Kras*^{G12D} PDEC by western blot analysis. As shown in Figure 2A, in WT PDEC the levels of p19^{ARF}, p21^{CIP}, and p53 remained essentially unchanged as the cells matured in culture. In contrast, p16^{INK4A} protein and message levels increased markedly (Figures 2A and 2B) suggesting that the induction of premature senescence in PDEC might depend preferentially on p16^{INK4A} upregulation. To test this idea directly, we examined the senescence phenotype of PDEC isolated from *p16*$^{Ink4a\ -/-}$ mice and *p53*$^{-/-}$ mice (Jacks et al., 1994; Serrano et al., 1996) by SA-β-gal staining. Loss of p53 had no effect on the extent of PDEC senescence, whereas p16^{INK4A} deficiency led to a significant reduction in PDEC senescence (Figure 2C). The senescence phenotype of the PDEC used as controls for these experiments (*p16*$^{Ink4a+/-}$ and *p53*$^{+/-}$) was indistinguishable from that observed in WT PDEC (data not shown). These observations suggest that premature senescence of PDEC requires the induction of p16^{INK4A} but not p53. Noticeably, the induction of p16^{INK4A} expression was abrogated in *Kras*^{G12D} PDEC (Figures 2A and 2B) indicating that oncogenic KRas might confer senescence bypass via the suppression of p16^{INK4A} induction.

Next we sought to determine the mechanism by which oncogenic KRas prevents the upregulation of p16^{INK4A}. We focused our attention on the basic helix-loop-helix transcription factor Twist (also known as Twist1) because of its documented ability to override premature senescence by abrogating p16^{INK4A} expression (Ansieau et al., 2008) and its recently reported genetic interactions with Ras (Hurlbut et al., 2009). To examine the effect of oncogenic KRas on Twist expression, the levels of Twist mRNA and protein in WT and *Kras*^{G12D} PDEC were compared. As illustrated in Figures 3A and 3B, Twist levels were markedly increased in *Kras*^{G12D} PDEC indicating that Twist transcription might be regulated by oncogenic KRas signaling. Of note, oncogenic KRas did not induce the expression of the Twist1 homolog, Twist2 (formerly known as Dermo-1) (Li et al., 1995) (data not shown). Initial analysis of Ras effector pathways that might target Twist transcription failed to implicate the Ras-ERK and Ras-PI3K signaling axes (data not shown). To establish whether Twist is essential for oncogenic KRas-mediated senescence bypass in PDEC, Twist expression was suppressed in *Kras*^{G12D} PDEC using RNA interference. A significant attenuation of Twist mRNA and protein expression was attained using two independent targeting sequences (Figures 3C and 3D). The reduction in Twist expression coincided with a specific increase in p16^{INK4A} expression (Figures 3D and 3E) and was accompanied by the induction of SA-β-gal activity (Figures 3F and 3G). In contrast, the knockdown of Twist in *p16*$^{Ink4a-/-}$ PDEC was without an effect on SA-β-gal activity (Figure S2A). Together, these results indicate that Twist is a critical mediator of oncogenic KRas-dependent suppression of p16^{INK4A} expression and senescence bypass in PDEC. Although Twist has been shown to cooperate with Ras in the induction of the epithelial-mesenchymal transition (EMT) (Ansieau et al., 2008), the expression of Twist in *Kras*^{G12D} PDEC was not accompanied by the activation of the EMT program, as reflected by the persistence of expression of the epithelial marker E-cadherin, and absence of induction of the mesenchymal markers vimentin and α-smooth muscle actin (α-SMA) (Figures S2B–S2D).

To investigate the physiological relevance of senescence abrogation by oncogenic KRas, we set out to identify an in vivo context in which premature senescence occurs in a p16^{INK4A}-dependent manner. Because inflammation is a well-established risk factor for pancreatic cancer (Lowenfels et al., 1993; Malka et al., 2002) and pro-inflammatory signals have been implicated in senescence induction (Acosta et al., 2008; Kuilman et al., 2008), we examined whether inflammatory conditions may trigger senescence in pancreatic ductal epithelium in vivo. To

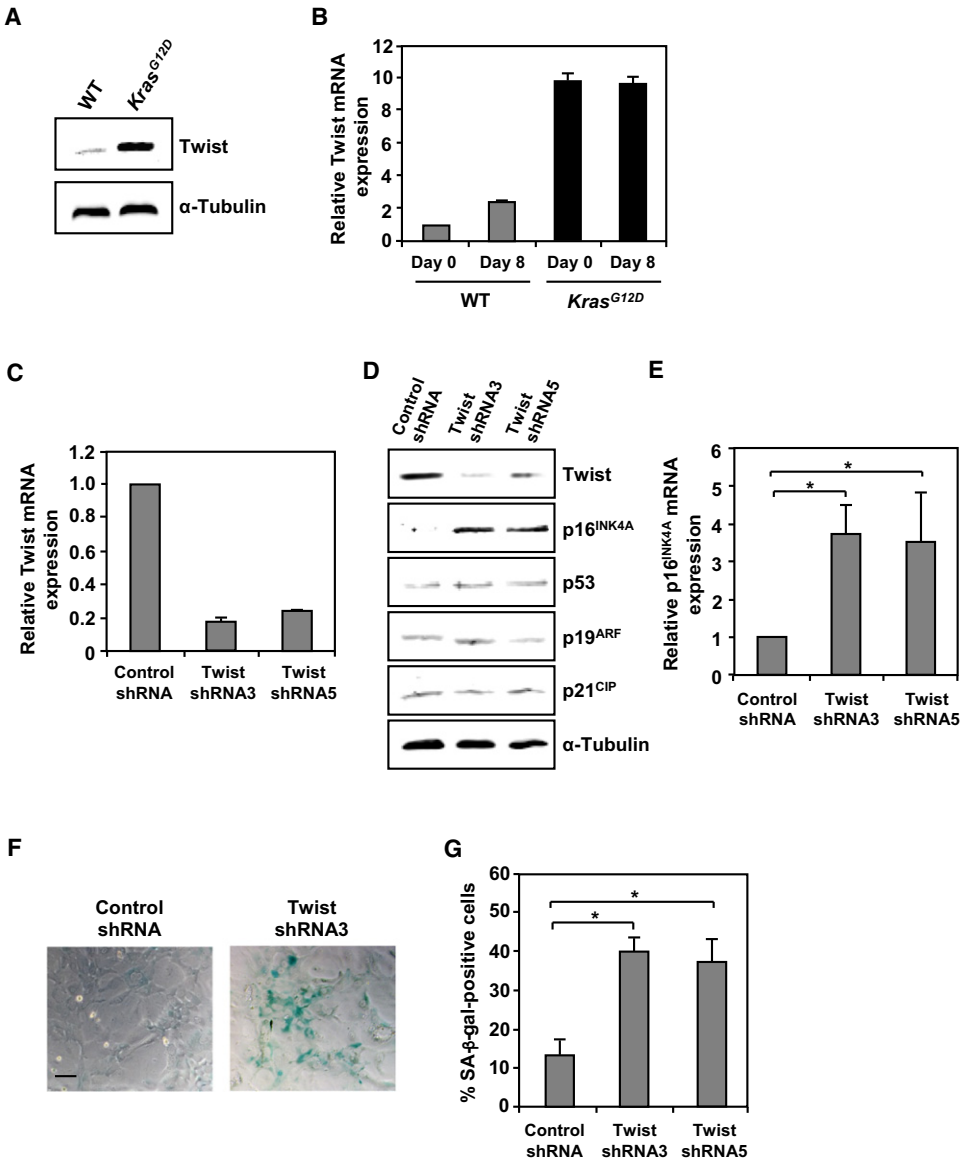

Figure 3. Oncogenic KRas-Mediated Bypass of Premature Senescence in PDEC Is Dependent on Twist

(A) Western blot analysis for Twist in WT and *Kras*^{G12D} PDEC cultures at day 8. α-Tubulin serves as a loading control.

(B) Quantitative RT-PCR analysis of Twist in WT and *Kras*^{G12D} PDEC cultures at day 0 and day 8. Error bars indicate SD (n = 3).

(C) Quantitative RT-PCR analysis of Twist in *Kras*^{G12D} PDEC cultures 8 days after infection with recombinant lentiviruses encoding shRNA targeted against *Gfp* (control shRNA) or *Twist* (Twist shRNA3 or Twist shRNA5). Error bars indicate SD (n = 3).

(D) Western blot analysis for Twist and senescence effectors in *Kras*^{G12D} PDEC cultures 8 days after infection with recombinant lentiviruses encoding control shRNA, Twist shRNA3, or Twist shRNA5. Equal loading was verified with anti-α-tubulin.

(E) Quantitative RT-PCR analysis of p16^{INK4A} in *Kras*^{G12D} PDEC cultures 8 days after infection with recombinant lentiviruses encoding control shRNA, Twist shRNA3, or Twist shRNA5. Error bars indicate SD (n = 3). *p value < 0.05.

(F) SA-β-gal staining of *Kras*^{G12D} PDEC cultures 8 days after infection with recombinant lentiviruses encoding control shRNA or Twist shRNA3. Scale bar, 100 μm.

(G) Quantification of SA-β-gal staining in *Kras*^{G12D} PDEC cultures 8 days after infection with recombinant lentiviruses encoding control shRNA, Twist shRNA3, or Twist shRNA5. Error bars indicate SD (n = 6 FOV). *p value < 0.05. Data are representative of three independent experiments. See also Figure S2.

elicit pancreatic inflammation, mice were subjected to a series of eight hourly intraperitoneal injections of supraphysiological levels of cerulein over 2 consecutive days. This protocol has been shown to induce exocrine pancreatic injury followed by a mild inflammatory response (Carriere et al., 2009; Jensen et al., 2005; Willemer et al., 1992). Three days after the last cerulein injection, pancreata were harvested and subjected to SA-β-gal assays. As illustrated in Figure 4A, mock-injected pancreata had no detectable SA-β-gal activity, whereas pancreatic ducts in cerulein-treated pancreata displayed pronounced SA-β-gal activity. Consistent with the growth arrest state attributed to senescent cells, the SA-β-gal-positive ductal cells were negative for

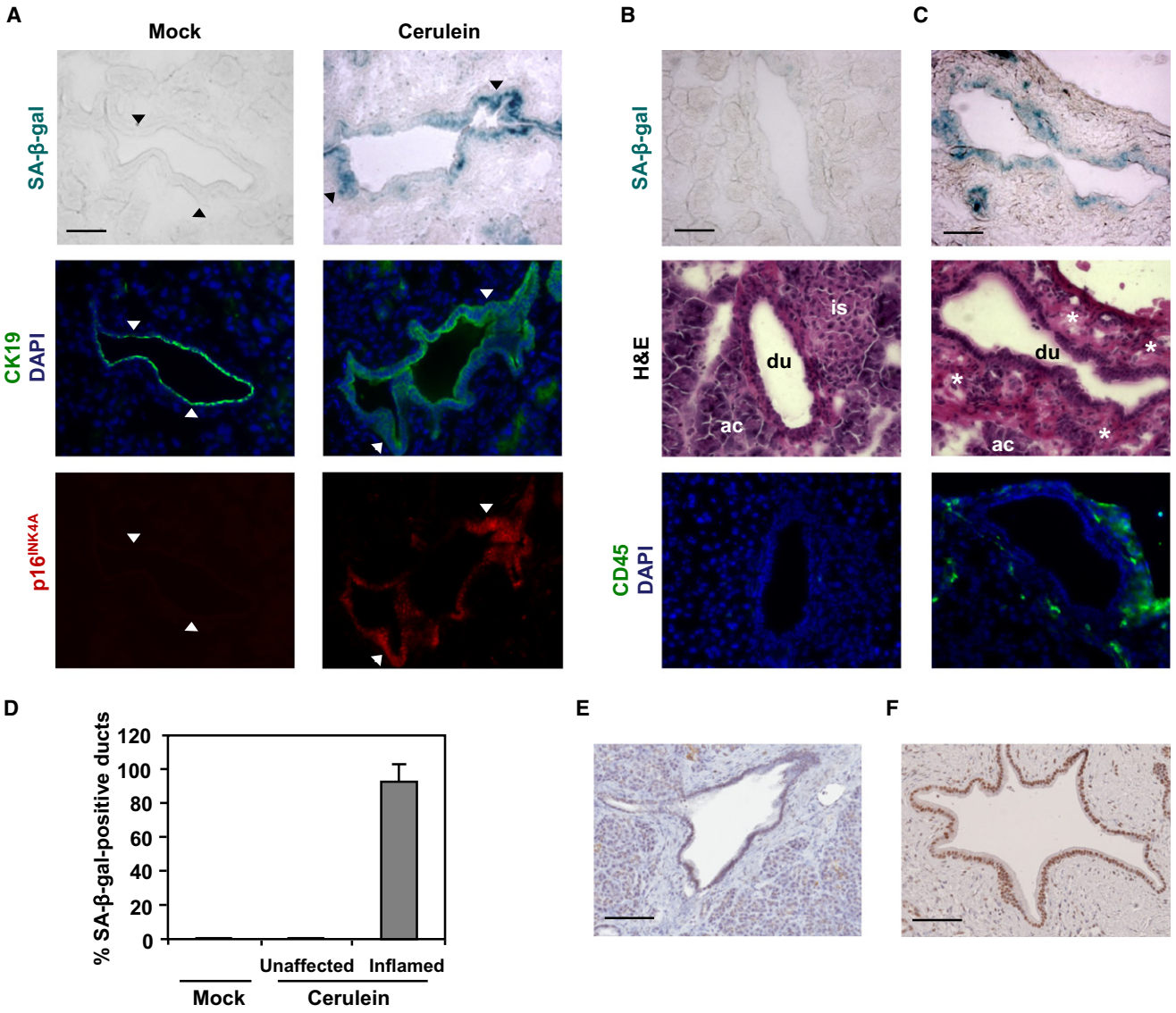

Figure 4. Inflammatory Insult Triggers Premature Senescence in Pancreatic Ductal Epithelium In Vivo

(A–D) Cerulein was administered to 1-month-old mice as 8 hourly intraperitoneal injections (50 ng/g of body weight/injection) for 2 days. Three days after the last cerulein injection, pancreata were harvested. At least three sections were analyzed per animal (n = 5 per genotype per treatment). (A) SA-β-gal staining and immunofluorescence staining for $p16^{INK4A}$ and CK19 on consecutive sections of pancreata from WT mice treated with cerulein or saline (mock). CK19 was used to identify ductal cells. Nuclei were counterstained with DAPI. Arrowheads mark corresponding areas. (B and C) SA-β-gal staining, hematoxylin and eosin (H&E) staining, and immunofluorescence staining for CD45 on consecutive sections of pancreata from WT mice treated with cerulein. Pancreatic ducts in unaffected (B) or inflamed (C) areas are from the same tissue section. CD45 was used to identify leukocytes. Nuclei were counterstained with DAPI. ac: acinus; du: duct; is: islet; asterisk: immune infiltrate. (D) Quantification of SA-β-gal staining in pancreata from WT mice treated with cerulein or saline (mock). Pancreatic ducts bearing ≥10% β-gal-positive cells in each duct were scored as positive. At least four pancreatic ducts were scored in each mouse. Error bars indicate SD (n = 5). (E and F) Immunohistochemical analysis of $p16^{INK4A}$ in human chronic pancreatitis tissue samples. Representative areas of normal pancreatic (E) and pancreatitis (F) tissues are shown. Scale bars represent 50 μm (A–C) and 100 μm (E and F). See also Figure S3.

the proliferation marker Ki67 (Figure S3). It should be noted that the inflammatory response after cerulein-induced injury is focal. Significantly, only pancreatic ducts that were adjacent to inflamed areas as evident by loss of acinar cells and presence of immune infiltrates stained positively for SA-β-gal activity (Figures 4B–4D). This spatial correlation indicates that the pancreatic ductal epithelium can be induced to undergo premature senescence in vivo in response to inflammatory insults.

To explore the relationship between inflammation-induced premature senescence in pancreatic ducts and $p16^{INK4A}$ expression, the levels of $p16^{INK4A}$ were assessed in pancreata from mock-injected and cerulein-injected mice by immunofluorescence staining. In agreement with published data documenting the lack of $p16^{INK4A}$ expression in the mouse pancreas (Krishnamurthy et al., 2004), pancreatic ducts from mock-injected mice were negative for $p16^{INK4A}$ staining (Figure 4A). In contrast,

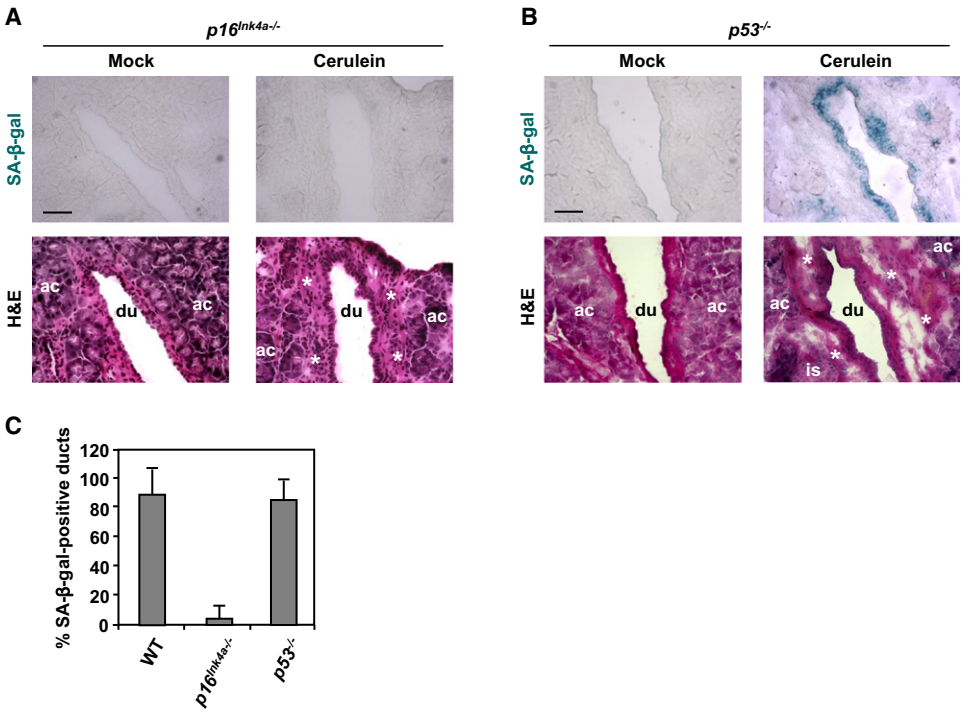

Figure 5. Inflammation-Induced Premature Senescence in Pancreatic Ductal Epithelium Depends on p16^{INK4A} Upregulation

Cerulein was administered as described in Figure 4. At least three sections were analyzed per animal (n = 5 per genotype per treatment).

(A and B) SA-β-gal staining and H&E staining on consecutive sections of pancreata from *p16*$^{Ink4a-/-}$ (A) and *p53*$^{-/-}$ mice (B) treated with cerulein or saline (mock). Ac: acinus; du: duct; is: islet; asterisk: immune infiltrate. Scale bars, 50 μm.

(C) Quantification of SA-β-gal staining in pancreata from WT, *p16*$^{Ink4a-/-}$, and *p53*$^{-/-}$ mice treated with cerulein. Only pancreatic ducts in inflamed areas were counted. At least four pancreatic ducts were scored in each mouse. Error bars indicate SD (n = 5). See also Figure S4.

p16^{INK4A} staining was robust in pancreatic ducts from cerulein-injected animals (Figure 4A). Significantly, human chronic pancreatitis was found to be also associated with the ductal upregulation of p16^{INK4A} (Figures 4E and 4F). These results along with the observation that strong p16^{INK4A} immunostaining coincided with high SA-β-gal activity (Figure 4A) raised the possibility that inflammation-induced premature senescence in pancreatic ducts is conferred by the upregulation of p16^{INK4A}. To test this idea, we treated *p16*$^{Ink4a-/-}$ mice with cerulein and analyzed the pancreata for SA-β-gal activity. As shown in Figures 5A and 5C, inflammation-induced premature senescence of the ductal epithelium was rescued by p16^{INK4A}-deficiency. By comparison, pancreatic ducts from cerulein-injected *p53*$^{-/-}$ mice retained the capacity to undergo premature senescence (Figures 5B and 5C). The inflammatory response per se did not appear to be altered by p16^{INK4A} deficiency as determined by gross inspection of tissue sections and the abundance of CD45-positive cells (Figure S4). These observations indicate that p16^{INK4A} is required for inflammation-associated premature senescence in pancreatic ducts and p53 is dispensable for this process.

Given our in vitro findings that oncogenic KRas inhibits premature senescence in PDEC via the suppression of p16^{INK4A}, we next asked whether oncogenic KRas can circumvent inflammation-induced premature senescence in pancreatic ductal epithelium in vivo. The *LSL-Kras*^{G12D} allele was conditionally activated in the pancreas by interbreeding *LSL-Kras*^{G12D} mice with *p48-Cre* mice (Kawaguchi et al., 2002). Lineage tracing studies previously demonstrated uniform expression of Cre recombinase throughout the pancreas in *p48-Cre* mice (Kawaguchi et al., 2002) and PCR analysis confirmed the presence of the recombined allele in pancreatic ducts of *p48-Cre;LSL-Kras*^{G12D} mice used for the experiments (Figure S5A). As illustrated in Figures 6A–6C, pancreatic ducts in *p48-Cre;LSL-Kras*^{G12D} mice treated with cerulein failed to senesce, and the abrogation of senescence was associated with suppression of p16^{INK4A} expression. These results indicate that endogenous expression of oncogenic KRas in vivo protects the pancreatic ductal epithelium from undergoing premature senescence by suppressing the induction of p16^{INK4A} in response to inflammation. Significantly, Twist levels were increased in pancreata from *p48-Cre;LSL-Kras*^{G12D} mice (Figure 6D) implicating Twist in the protective effects of oncogenic KRas on inflammation-associated senescence. Consistent with our in vitro findings, the expression of Twist in pancreata from *p48-Cre;LSL-Kras*^{G12D} mice was not associated with EMT induction as demonstrated by persistent expression of E-cadherin and absence of α-SMA induction in pancreatic ducts (Figures S5B and S5C).

DISCUSSION

In the present study, we describe a previously unrecognized functional facet of oncogenic KRas—the capacity to suppress premature senescence of pancreatic ductal epithelium.

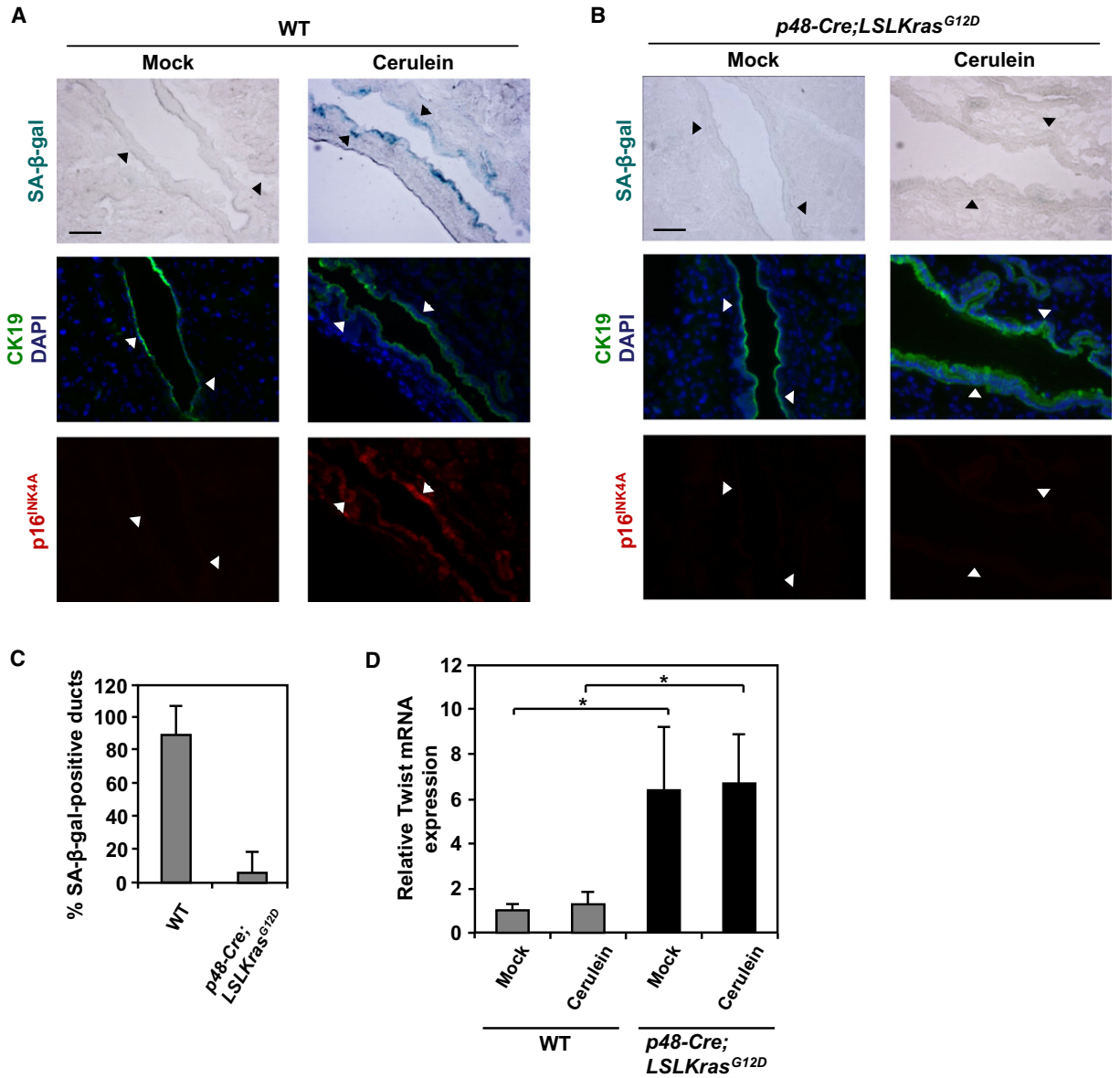

Figure 6. Inflammation-Associated Premature Senescence of Pancreatic Ductal Epithelium In Vivo Can be Blocked by Oncogenic KRas through the Suppression of p16^{INK4A}

Cerulein was administered as described in Figure 4. At least three sections were analyzed per animal (n = 5 per genotype per treatment).

(A and B) SA-β-gal staining and immunofluorescence staining for p16^{INK4A} and CK19 on consecutive sections of pancreata from WT (A) and *p48-Cre;LSL-Kras^{G12D}* (B) mice treated with cerulein or saline (mock). CK19 was used to identify ductal cells. Nuclei were counterstained with DAPI. Arrowheads mark corresponding areas. Scale bars, 50 μm.

(C) Quantification of SA-β-gal staining in pancreata from WT and *p48-Cre;LSL-Kras^{G12D}* mice treated with cerulein. Only pancreatic ducts in inflamed areas were counted. At least four pancreatic ducts were scored in each mouse. Error bars indicate SD (n = 5).

(D) Quantitative RT-PCR analysis of Twist in pancreata from WT and *p48-Cre;LSL-Kras^{G12D}* mice treated with cerulein or saline (mock). Error bars indicate SD (n = 5 per genotype). *p value < 0.05. See also Figure S5.

Because in the setting of evolving pancreatic neoplasms, activating KRas mutations are the first genetic alteration to be detected (Hruban et al., 2000), senescence bypass could serve as a mechanism to impart selective advantage to the cellular precursors of pancreatic cancer. Moreover, the enhanced fitness that oncogenic KRas confers on pancreatic ductal epithelium may explain why KRas mutations can be tolerated by these cells.

The propensity of cultured primary cells to undergo premature senescence has been described before and has been attributed to the induction of p16^{INK4A} expression in response to stress conditions imposed by an inappropriate growth environment (Ben-Porath and Weinberg, 2004; Sherr and DePinho, 2000). The senescence displayed by cultured PDEC likely reflects a similar mechanism as indicated by the increase in p16^{INK4A} levels observed when the cells are maintained in culture. In vivo

however, the link between $p16^{INK4A}$ upregulation and senescence induction has not been firmly established. Although $p16^{INK4A}$ levels are elevated in aging or stressed senescent tissues in vivo, so are p53 levels, and studies using genetically engineered mice have implicated both $p16^{INK4A}$ and p53 in affecting senescence under these conditions (Campisi and d'Adda di Fagagna, 2007; Collado et al., 2007). Thus, it has been postulated that the relative contribution of the $p16^{INK4A}$ and p53 pathways to senescence might depend on the cellular context and the type of stress signals (Campisi and d'Adda di Fagagna, 2007). Our present findings implicate selectively $p16^{INK4A}$ in a heretofore unreported senescence response invoked in vivo by inflammatory stress in the absence of oncogene activation. The precise nature of the signals elicited by the inflammatory milieu to promote the $p16^{INK4A}$-dependent induction of PDEC senescence remains to be established. However, experimental acute pancreatitis triggered by cerulein administration has been shown to induce an increase in the levels of proinflammatory cytokines such as TNF-α, IL-6, IL-8, and IL-10 (Fu et al., 1997; Norman, 1998; Van Laethem et al., 1998). IL-6 and IL-8 have both been implicated in the senescence response to oncogenic stress (Acosta et al., 2008; Kuilman et al., 2008), thus raising the possibility that they could play a similar role in the setting of pancreatic inflammation.

The in vivo and in vitro abrogation of senescence by oncogenic KRas reported here represents a departure from the more frequently described senescence-triggering effect of oncogenic Ras. The latter has been linked predominantly to hyperproliferative signals that result from a high Ras gene dosage (DeNicola and Tuveson, 2009; Ji et al., 2009; Sarkisian et al., 2007). Our findings demonstrate that PDEC harboring an endogenous allele of oncogenic KRas not only fail to activate the senescence program but are wired to suppress senescence through the upregulation of Twist. This observation is consistent with an earlier finding that Twist suppresses the senescence of immortalized human prostate cell lines (Kwok et al., 2007). Moreover, a connection between Twist expression and senescence bypass has been documented recently in the setting of oncogenic stress and has been attributed to the activity of Twist as a transcriptional repressor of the two senescence inducers $p16^{INK4A}$ and $p21^{CIP}$ (Ansieau et al., 2008). PDEC expressing oncogenic KRas rely on the same mechanism, namely Twist-dependent transcriptional repression of $p16^{INK4A}$, to override premature senescence caused by environmental stress. As such, the coincident expression of oncogenic KRas and Twist in PDEC may facilitate the initiation of the tumorigenic state through the inactivation of a proliferative barrier imposed by $p16^{INK4A}$ expression. The increased expression of Twist displayed by human pancreatic cancer cells lends further support to this idea (Satoh et al., 2008). It is noteworthy that in studies utilizing mouse models that are different from the one employed in this study, the endogenous expression of oncogenic KRas in the pancreas has been reported to trigger senescence in preneoplastic lesions (Collado et al., 2005; Morton et al., 2010). The factors that might contribute to this apparent discrepancy remain to be delineated but could in principle include both cell autonomous and noncell autonomous determinants related to strain-dependent variations in the expression levels of oncogenic KRas, the types of KRas activating mutation, and the Cre-driving promoters.

The loss of $p16^{INK4A}$ function occurs in ~90% of pancreatic cancers and is brought about by mutation, deletion and epigenetic silencing. However, the inactivation of $p16^{INK4A}$ is generally seen at a later stage of neoplastic progression subsequent to the acquisition of oncogenic KRas mutations (Moskaluk et al., 1997; Wilentz et al., 1998). Therefore, the expression of Twist may allow oncogenic KRas-expressing PDEC to escape senescence at an earlier stage when the $p16^{INK4A}$ locus has not been disrupted yet. The pressure to lose $p16^{INK4A}$ function as the disease advances might reflect alterations in Twist transcriptional repressive activity possibly resulting from changes in the abundance and/or post-translational modifications of Twist binding partners. The finding that the oncogenic KRas-Twist axis is exploited in the context of inflammation-associated senescence is of particular interest given the compelling evidence both from human studies and experimental models for a strong link between pancreatitis and an increased risk of pancreatic cancer (Carriere et al., 2009; Gidekel Friedlander et al., 2009; Guerra et al., 2007; Lowenfels et al., 1993; Malka et al., 2002; Morris et al., 2010). Accordingly, Twist expression could critically affect the impact of inflammatory conditions on the tumorigenic potential of pancreatic ductal cells harboring oncogenic KRas.

The induction of Twist by Ras is not unprecedented (Liu et al., 2009) and studies in *Drosophila* have reported genetic interactions between Twist and Ras (Hurlbut et al., 2009). However, the precise signaling events by which Ras stimulates Twist expression have not been defined. Indeed, it appears that multiple Ras effector pathways can contribute to an increase in Twist transcripts. These include the PI3K and ERK pathways as well as the NF-κB, JAK/STAT, and GSK-3β/β-catenin pathways (Dupont et al., 2001; Hong et al., 2009; Howe et al., 2003; Pham et al., 2007; Sosic et al., 2003; Zhu and Tan, 2005). The relative engagement of these pathways would vary depending on cell type and environmental conditions. Thus, the contribution of the Ras-Twist-$p16^{INK4A}$ axis to senescence bypass is likely to be context-dependent. Furthermore, although Twist has been implicated in the induction of EMT programs in epithelial cells (Yang et al., 2004), our findings indicate this aspect of Twist function is not essential for its senescence suppressing activity.

In addition to being locked in a state of permanent growth arrest, senescent cells have been noted to secrete an increased amount of pro-inflammatory mediators including cytokines, chemokines, growth factors, and extracellular proteases (Coppe et al., 2010; Kuilman and Peeper, 2009). Hence the induction of ductal cell senescence under conditions of pancreatic injury and inflammation may constitute a mechanism to maintain and amplify the inflammatory microenvironment. Given the protumorigenic impact of inflammatory conditions, senescent lesions within the pancreas may play a direct role in fueling the neoplastic process by promoting the unscheduled growth of precursor cells harboring KRas mutations.

EXPERIMENTAL PROCEDURES

Mice and Cerulein Treatment

The *LSL-Kras*^{G12D}, *p16*$^{Ink4a-/-}$, *p53*$^{-/-}$, and, *p48-Cre* strains have been previously described (Jacks et al., 1994; Jackson et al., 2001; Kawaguchi et al., 2002; Sharpless et al., 2001). One-month-old mice were subjected to eight hourly intraperitoneal injections of cerulein (50 ng/g of body weight/injection) on 2 consecutive days. Pancreata were harvested 3 days after the last

injection. All animal care and procedures followed National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at NYU School of Medicine.

Isolation, Culture, and Infection of PDEC

Isolation and culture of PDEC were carried out as previously described (Agbunag et al., 2006). PDEC were isolated from 2–3-month-old mice and propagated in Matrigel (Becton Dickinson). GFP pAdEasy-1 and Cre pAdEasy-1 adenoviral vectors were gifts from Gustavo Leone. Purified adenovirus particles were obtained by CsCl equilibrium centrifugation. Six hundred adenovirus particles/cell were added to the PDEC suspension. In a microfuge tube, cell/virus mixtures were rocked every 15 min for 1 hr at 37°C, and then embedded in Matrigel. Two days later, the infection was repeated. PDEC were propagated in Matrigel, and then transferred to 1% Matrigel-coated plated at the time of the experiment. Lentiviral vectors containing shRNAs directed against the *Twist* gene (shRNA Twist3 and shRNA Twist5) and control shRNA directed against the *Gfp* gene were kind gifts from the Robert A. Weinberg laboratory (Yang et al., 2004). $Kras^{G12D}$ PDEC or $p16^{Ink4a-/-}$ PDEC were infected with lentivirus (multiplicity of infection = 20) using 10 μg/ml polybrene (Chemicon), cultivated on Matrigel with medium containing 2% Matrigel for 3 days, and transferred to a 1% Matrigel-coated plate.

$Kras^{G12D}$ Allele Recombination Assay

For verification of Cre-mediated recombination, DNA was prepared from *LSL-*$Kras^{G12D}$ PDEC infected with adenoviral-GFP (WT) or adenoviral-Cre ($Kras^{G12D}$), or from pancreatic ducts or pancreata from 1-month-old WT or *p48-Cre;LSL-*$Kras^{G12D}$ mice by using DNeasy Blood and Tissue kit (QIAGEN). Genomic DNA was amplified by PCR to demonstrate 265 bp and 305 bp products that are specific for the WT and recombined alleles, respectively (Jackson et al., 2001).

Ras Activation Assay

The levels of Ras-GTP were determined by the GST–RBD pull-down assay, as described previously (Boykevisch et al., 2006). The following antibodies were used: mouse anti-KRas (Santa-Cruz) and mouse anti-α-Tubulin (Sigma).

Senescence-Associated-β-Galactosidase Assay

Adherent cells or frozen sections of pancreatic tissue were fixed with 2% formaldehyde/0.2% glutaraldehyde in phosphate buffer solution (PBS) for 3–5 min, washed with PBS, stained at 37°C for 12–16 hr in X-Gal solution (1 mg/ml X-Gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mM $MgCl_2$ in PBS at pH6.0), and counterstained with Hoechst 33342 (Invitrogen) for β-gal quantification. Slides were examined on a Zeiss Axiovert 200M microscope. For quantification of SA-β-gal staining in PDEC cultures, at least 150 cells/fields of view were counted. In the pancreatic tissue, pancreatic ducts bearing 10% or more β-gal-positive cells in each duct were scored as positive.

Immunoblot Analysis

Cells were lysed with 25 mM Tris at pH7.5, 120 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1% NP-40, 10% glycerol, 10 μg/ml aprotinin, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM Na_2VO_4, 0.25% sodium deoxycholate, 10 mM NaF, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 10 mM benzamidine. Cell lysates were separated by electrophoresis in sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with primary antibodies. Subsequently, membranes were incubated with IRDye 800-conjugated goat anti-rabbit (Rockland) or Alexa Fluor 680 goat anti-mouse (Molecular Probes) and visualized with the Odyssey Infrared Imaging System (Li-Cor). The following antibodies were used: rabbit anti-$p16^{INK4A}$ (Santa-Cruz), rabbit anti-$p19^{ARF}$ (gift from Charles J. Sherr), mouse anti-$p21^{CIP}$ (Becton Dickinson), mouse anti-p53 (Santa-Cruz), rabbit anti-Twist (Santa-Cruz), and mouse anti-α-Tubulin (Sigma).

Quantitative RT-PCR

Total RNA was extracted from PDEC or pancreata by using RNeasy mini kit (QIAGEN) and reverse transcribed with QuantiTect reverse transcription kit (QIAGEN). PCR reactions were performed using the SYBR Green PCR Master Mix (USB). Expression levels were normalized by cyclophilin A.

Human Pancreas Specimens

The use of human tissue for this study was reviewed and approved by the Institutional Review Board of NYU School of Medicine and samples were obtained after informed consent. Sections (5 μm) were cut from formalin-fixed paraffin-embedded samples for the purpose of immunohistochemistry.

Histology and Immunofluorescence

Pancreata were removed and snap frozen in OCT compound (Tissue-Tek). Sections of 8 μm were air-dried and hydrated in 70% ethanol. Hematoxylin and eosin (H&E) staining was performed by incubation in hematoxylin (Sigma) followed by eosin (Sigma). Sections were dehydrated (70%, 95%, 100% ethanol) and mounted with Permount (Fisher). For immunofluorescence of pancreata, frozen sections were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.25% Triton X-100 for 10 min, and blocked with 10% serum/0.1% Tween-20 for 1 hr. Slides were incubated with primary antibodies diluted in 1% BSA/0.5% Tween-20 overnight at 4°C. Slides were then incubated with Alexa Fluor-labeled secondary antibodies (Invitrogen) diluted in 1% BSA for 1 hr and mounted using Vectashield mounting medium with DAPI (Vector Laboratories). For immunofluorescence of PDEC, cells grown on 1% Matrigel-coated coverslips were fixed in 3% paraformaldehyde for 30 min, permeabilized with 0.2% Triton X-100 for 10 min, and blocked with 2% BSA for 1 hr. PDEC were incubated with primary antibodies diluted in 2% BSA for 1 hr followed by Alexa Fluor-labeled secondary antibodies (Invitrogen) diluted in 2% BSA for 1 hr. PDEC were then incubated with DAPI for 15 min and mounted with Immuno-mount (Shandon) containing 0.04% paranitrodiphenylene (Sigma). Slides were examined on a Zeiss Axiovert 200M microscope. The following antibodies were used: rat anti-CD45 (Becton Dickinson), rat anti-CK19 (TromaIII, developed by Rolf Kemler and obtained from Developmental Studies Hybridoma Bank), mouse anti-E-cadherin (Becton Dickinson), rabbit anti-Ki67 (Novocastra), rabbit anti-$p16^{INK4A}$ (Santa-Cruz), and mouse anti-α-SMA (Sigma).

Immunohistochemistry

Pancreata were fixed in 10% formalin overnight and embedded in paraffin. For immunohistochemistry, slides (5 μm) were deparaffinized, rehydrated, quenched in 0.6% hydrogen peroxide/methanol for 15 min, and antigens were boiled for 15 min in a microwave oven in 10 mM sodium citrate (pH 6.0) for antigen retrieval. Sections were blocked with 5% serum/1% BSA/0.5% Tween-20 for 1 hr. Slides were incubated with primary antibodies diluted in blocking buffer overnight at room temperature. Following the primary antibody, slides were incubated with biotinylated secondary antibodies (Vector Laboratories) followed by ABC solution (Vector Laboratories) and developed with 3,3′-diaminobenzidine tetrahydrochloride for 15 min. Slides were counterstained with hematoxylin, dehydrated, and mounted with Permount (Fisher). Slides were examined on a Zeiss Axiovert 200M microscope. The following antibodies were used: mouse anti-$p16^{INK4A}$ (Santa-Cruz) and rat anti-CD45 (Becton Dickinson).

Statistical Analyses

Data were analyzed by Student's t test (paired, two-tailed) and results were considered significant at p value < 0.05. Results are presented as mean and standard deviation.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at doi:10.1016/j.ccr.2010.10.020.

ACKNOWLEDGMENTS

We are grateful to T. Jacks, D.A. Tuveson, R.A. DePinho, C.V. Wright, R.A. Weinberg, G. Leone, H.C. Crawford, C.J. Sherr, R. Kemler for mice and reagents. We thank C.H. Hajdu for support and advice with human pancreatic tissue collection, J. Mallen-St. Clair for advice on the animal experiments, L.J. Taylor for help with data analysis and manuscript preparation, E. Hernando for

helpful suggestions, J.L. Zhou for technical assistance, and all members of the Bar-Sagi laboratory for comments and discussions. This work was supported by National Institutes of Health Grant CA055360 (D.B.-S.).

Received: April 1, 2010
Revised: August 4, 2010
Accepted: September 20, 2010
Published: November 15, 2010

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