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EGF Receptor Signaling Is Essential for K-*Ras* Oncogene-Driven Pancreatic Ductal Adenocarcinoma

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SUMMARY

Clinical evidence indicates that mutation/activation of EGF receptors (EGFRs) is mutually exclusive with the presence of K-*RAS* oncogenes in lung and colon tumors. We have validated these observations using genetically engineered mouse models. However, development of pancreatic ductal adenocarcinomas driven by K-*Ras* oncogenes are totally dependent on EGFR signaling. Similar results were obtained using human pancreatic tumor cell lines. EGFRs were also essential even in the context of pancreatic injury and absence of p16Ink4a/p19Arf. Only loss of p53 made pancreatic tumors independent of EGFR signaling. Additional inhibition of PI3K and STAT3 effectively prevented proliferation of explants derived from these p53-defective pancreatic tumors. These findings may provide the bases for more rational approaches to treat pancreatic tumors in the clinic.

INTRODUCTION

Patients with pancreatic ductal adenocarcinoma (PDAC) have an average survival of less than a year with fewer than 5% surviving more than 5 years (Vincent et al., 2011). Current standard of care for PDAC patients is Gemcitabine, a nucleoside analog that only prolongs survival for few weeks (Burris et al., 1997; Li et al., 2004). Hence, there is an urgent medical need to find more effective therapeutic approaches to treat this deadly disease (Hidalgo, 2010).

PDAC is likely to stem from a process known as acinar to ductal metaplasia that involves either transdifferentiation of adult acinar cells or misdifferentiation of their progenitors into ductal-like cells. These cells can subsequently progress into malignant adenocarcinoma through a series of histopathological lesions known as pancreatic intraepithelial neoplasias (PanINs) (Maitra and Hruban, 2008). Early pancreatic lesions including low-grade PanINs already carry mutations in K-*RAS* oncogenes, along with loss or inactivation of the *P16INK4a* tumor suppressor (Kanda et al., 2012). High-grade lesions develop upon accumulation of further mutational events, mainly involving inactivation of other tumor suppressors such as *TP53*, *SMAD4*, or *BRCA2* (Hong et al., 2011). Exome sequencing analysis of PDAC genomes has revealed an incredibly complex pattern of mutations affecting as many as 12 different signaling pathways (Jones et al., 2008). In a recent study describing the exomic sequence of different areas of a single PDAC tumor, Campbell et al. (2010) have illustrated the perverse molecular evolution of these tumors even before they spread to other organs.

In 2007, a clinical trial combining Gemcitabine with the EGFR inhibitor, Erlotinib, reported some responses in a limited number of PDAC patients (Moore et al., 2007). Yet, the overall results were not sufficiently significant for the FDA to recommend the

Significance

Previous clinical studies have suggested a therapeutic benefit of Erlotinib, an EGFR inhibitor, in pancreatic ductal adenocarcinoma patients. Here, we show that these observations may have a mechanistic base. EGFRs are expressed during pancreatic injury and in preneoplastic PanIN lesions. Loss of p53, but not of p16INK4a/p19ARF tumor suppressors, relieved the need of tumor cells to maintain EGFR signaling. Yet, loss of EGFRs increased tumor latency and survival. Tumor explants lacking p53 and EGFRs were sensitive to the combined inhibition of PI3K and STAT3. Thus, successful treatment of advanced human pancreatic tumors may require inhibition of at least four distinct signaling cascades including those driven by K-RAS, EGFRs, PI3K, and STAT3.

combination of these two drugs as standard of care. These observations are intriguing because the EGFR is known to signal upstream of K-RAS and hence, its inhibition should have little or no effect on downstream K-RAS-driven oncogenic signals (Yarden and Sliwkowski, 2001). Indeed, in nonsmall lung adenocarcinoma (NSCLC) mutations in *EGFR* and in K-*RAS* are mutually exclusive (Shigematsu et al., 2005). Likewise, a large clinical trial carried out in patients with advanced colorectal carcinomas (CRC) has determined that patients carrying tumors with K-*RAS* mutations do not benefit from treatment with Cetuximab, a monoclonal antibody that blocks EGFR signaling (Karapetis et al., 2008). In spite of these odds, we decided to interrogate by genetic means whether EGFRs might play a role in K-*Ras* oncogene-driven PDAC using a well-characterized genetically engineered mouse (GEM) model for this disease (Guerra et al., 2007, 2011).

RESULTS

Acinar to Ductal Metaplasia Requires EGFR Signaling Even in the Presence of K-*Ras* Oncogenes

Pancreatic acinar to ductal metaplasia is a precursor of the preneoplastic PanIN lesions that eventually lead to PDAC development (Parsa et al., 1985). In normal mice, generation of acinar to ductal metaplasia is largely dependent on activation of EGFRs (Means et al., 2005). Because EGFRs signal through the Ras pathway, we examined whether expression of a constitutive active K-Ras oncoprotein could bypass the requirement for EGFR activity during the generation of metaplasia. Pancreatic cell explants obtained from K-*Ras*$^{+/LSLG12Vgeo}$;*Elas*-tTA/*tetO*-Cre mice (from now on *ElasK-Ras*^{G12V}) in which the K-*Ras*^{G12V} oncogene is selectively expressed in acinar cells, efficiently transdifferentiated into metaplastic ductal-like cells leading to the generation of 5- to 10-fold more metaplastic structures than those not expressing the oncogene (Figure S1A available online). Yet, K-Ras^{G12V}-driven metaplasia was still largely dependent on activation of EGFRs because addition of their cognate ligands EGF or TGFα, effectively increased the number of metaplastic figures (Figure S1B). Ablation of *Egfr* alleles significantly reduced, but did not eliminate the ability of acinar cell explants to generate metaplastic structures (Figures S1B–S1D). These observations suggest that EGF and TGFα may contribute to acinar to ductal metaplasia by activating additional receptors, at least in vitro. Pancreatic acinar cells also expressed high levels of amphiregulin, but not of other members of the EGFR family of ligands (Figure S1E).

Human and Mouse Pancreatic Lesions Express Abundant EGFRs

Mouse acinar cells did not express detectable levels of EGFRs regardless of whether they expressed a K-*Ras* oncogene or not (Figure 1A). In contrast, PanINs, regardless of their grade, were decorated with high levels of the receptor (Figure 1A) (Ueda et al., 2004; Hingorani et al., 2005). Elevated expression of EGFRs was maintained during tumor progression including well-differentiated glandular structures within PDAC tumors (Figure 1A). However, expression levels decreased in poorly differentiated tumor cells (Figure 1B) (Ueda et al., 2004; Hingorani et al., 2005). Human normal pancreata also displayed undetectable levels of EGFRs (Figure 1C). However, morphologically normal acinar cells of pancreatitis patients expressed significant levels of EGFRs in a manner highly reminiscent of the result obtained in pancreata derived from mice exposed to caerulein (Figures 1B and 1C).

These observations are in agreement with an early study describing overexpression of EGFRs in patients with chronic pancreatitis (Korc et al., 1994). We also observed that metaplasias present in pancreatitis biopsies displayed elevated levels of EGFRs (Figure 1C). Low-grade and high-grade PanINs present in human PDAC tumors also expressed high levels of EGFRs (Figure 1C). Interestingly, their pattern of expression in tumored areas closely resembled that observed in mouse PDACs (Figure 1B). Whereas well-differentiated tumor glands were uniformly decorated with EGFR antibodies, less-differentiated glands expressed significantly lower levels of the receptors (Figure 1C). Finally, metastatic cells localized in a regional lymph node retained detectable, albeit somewhat attenuated levels of EGFRs (Figure 1C). These observations indicate that induction of EGFRs in acinar cells of injured pancreata as well as in PanIN and PDAC lesions is a common event in mouse and human pancreatic tissues.

EGFRs Are Essential for the Generation of K-*Ras* Oncogene-Driven PanIN Lesions

To determine whether development of PanIN lesions and PDAC tumors require EGFR signaling, we generated *ElasK-Ras*^{G12V}; *Egfr*$^{+/+}$ and *ElasK-Ras*^{G12V}; *Egfr* $^{lox/lox}$ strains and analyzed their pancreata at 1 year of age. These mice were not exposed to doxycycline to allow expression of the *Elastase*-driven Cre recombinase during late embryonic development (E16.5). Cre-mediated recombination allowed concomitant expression of the resident K-*Ras*^{G12V} oncogene and ablation of the floxed *Egfr* alleles in acinar cells (Figure S2A). As illustrated in Figure 2A, control *ElasK-Ras*^{G12V};*Egfr*$^{+/+}$ littermates (12 out of 13 animals, 92%) exhibited abundant low- and high-grade PanIN lesions (average of 16 and 5 lesions per pancreata, respectively). Moreover, three animals (23%) displayed sizable PDAC tumors. Animals heterozygous for the *Egfr* locus also harbored low- and high-grade PanIN lesions albeit at reduced numbers (average of 5 and 2.5 lesions per pancreata, respectively). Likewise, only one out of ten heterozygous mice carried a PDAC tumor (Figure 2A).

In contrast, careful analysis of serial sections of pancreata from 1-year-old *ElasK-Ras*^{G12V}; *Egfr* $^{lox/lox}$ animals (n = 24) only revealed the presence of a limited number of PanIN lesions (ten low-grade and two high-grade PanINs) in eight mice. More importantly, all of these lesions expressed EGFRs due to incomplete recombination of the floxed *Egfr* alleles (Figures S2B and S2C). Similar results were obtained in older mice sacrificed at 2 years of age (data not shown). These observations indicate that EGFRs are essential for the induction of PanINs and PDAC by K-*Ras* oncogenes.

Adult Mice Also Require EGFR Signaling for PDAC Development

To exclude the possibility that these observations were due to developmental defects in acinar cells lacking EGFRs during embryonic development, we exposed *ElasK-Ras*^{G12V};*Egfr*$^{+/+}$

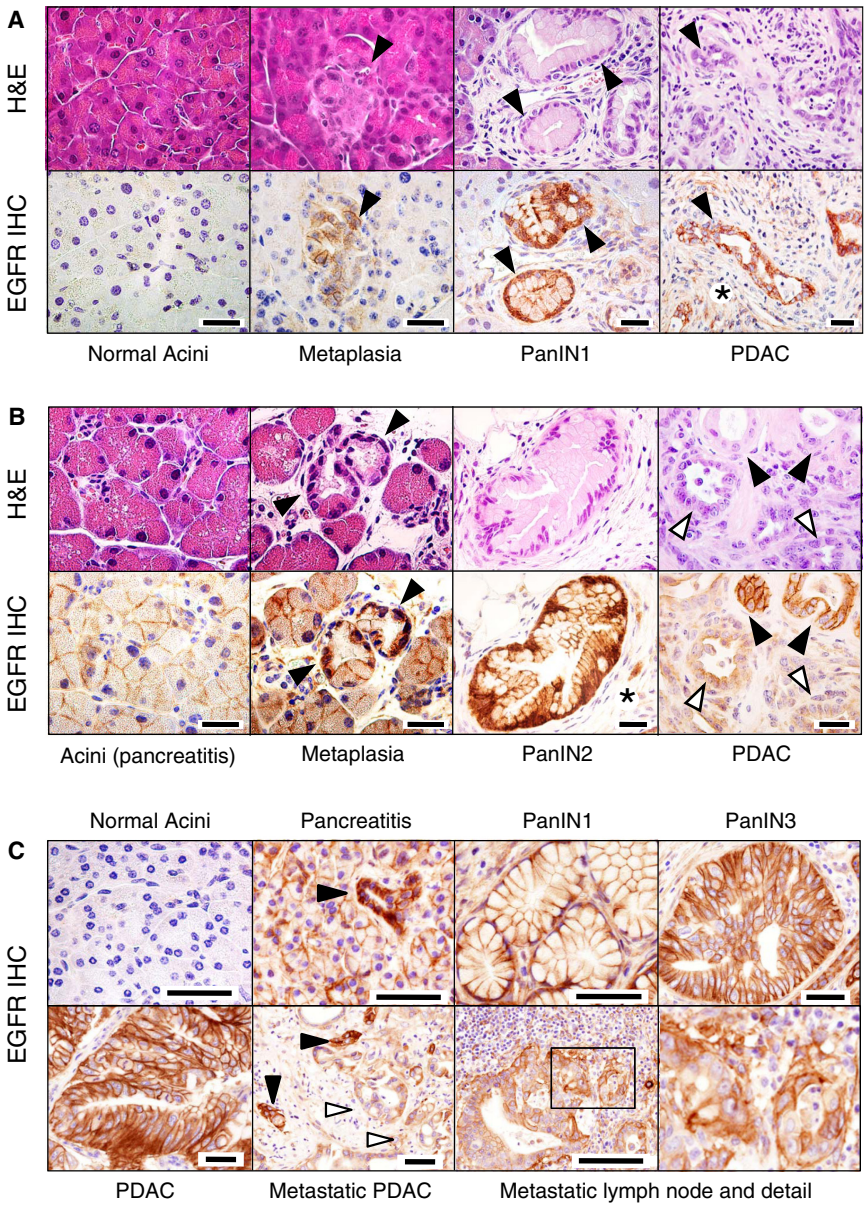

Figure 1. Expression of EGFR in Pancreas of *ElasK-Ras*^{G12V} Mice and of Patients with Pancreatitis and PDAC

(A) Serial paraffin sections obtained from *ElasK-Ras*^{G12V} mice not exposed to doxycycline depicting normal acini, acinar to ductal metaplasia, PanIN1, and PDAC were stained with hematoxylin and eosin (H&E) or with antibodies against the EGFR (EGFR IHC). Lesions are indicated by solid arrowheads. Asterisk indicates stroma cells positive for EGFR immunostaining. Scale bars represent 20 μm.

(B) Serial paraffin sections obtained from *ElasK-Ras*^{G12V} mice exposed to doxycycline from conception to P60 and to caerulein from P90 to P180 depicting acini, acinar to ductal metaplasia, PanIN2 and PDAC were stained with H&E or with antibodies against the EGFR (EGFR IHC). Lesions are indicated by solid arrowheads. Open arrowheads indicate less-differentiated glands within a PDAC. Asterisk indicates stroma cells positive for EGFR expression. Scale bars represent 20 μm.

(C) EGFR IHC of human pancreatic biopsies depicting normal pancreata, pancreata from patients with pancreatitis, PanIN lesions (PanIN1 and PanIN3), nonmetastatic PDAC and a metastatic lymph node with amplified detail. Lesions are indicated by solid arrowheads. Open arrowheads indicate less-differentiated glands within the metastatic PDAC. Scale bars represent 50 μm. See also Figure S1.

and *ElasK-Ras*^{G12V};*Egfr*$^{lox/lox}$ littermates to doxycycline from conception until adulthood (8 weeks of age) to prevent expression of the Cre recombinase. As previously reported, induction of PanIN lesions in these mice requires a pancreatic insult (Guerra et al., 2007). Analysis of 14-month-old *ElasK-Ras*^{G12V};*Egfr*$^{lox/lox}$ mice (n = 14) treated with caerulein for 3 months (P90–P180), that is, 1 year after turning on expression of the resident K-*Ras*^{G12V} oncogene, revealed complete absence of EGFR positive low- and high-grade PanIN lesions or PDAC tumors (Figure 2B). Only three mice carried PanIN lesions, all of which expressed EGFRs (data not shown). Mice examined at 2 years of age displayed a total of nine low-grade and three high-grade PanIN lesions in three out of the seven mice analyzed, all of which retained EGFR expression (data not shown).

As summarized in Figure 2B, control *ElasK-Ras*^{G12V};*Egfr*$^{+/+}$ littermates exhibited the expected number of lesions (Guerra et al., 2011). All mice (n = 13) developed low-grade PanINs (average of 18 lesions per pancreata) and more than 90% (12 out of 13) displayed high-grade PanINs (average of 16 lesions per pancreata). Only one mouse out of 13 (8%) had a full-blown PDAC tumor (Figure 2B). Ablation of a single *Egfr* allele yielded similar results regarding the number of mice affected (80% of the animals carried PanIN lesions and 10% a PDAC tumor). However, the average number of lesions per pancreata was significantly lower (Figure 2B). These observations strongly support the concept that initiation of PDAC tumors requires at least two independent signaling inputs mediated by the EGFR and the K-Ras oncoprotein.

EGFRs Cooperate with Resident K-*Ras* Oncogenes by Activating AKT and STAT3 Signaling Pathways

In an attempt to shed light on the mechanism by which the EGFR cooperate with the resident K-Ras^{G12V} oncoprotein to induce pancreatic lesions, we examined the status of AKT, a well-known downstream effector of the PI3K/AKT survival pathway and STAT3, a mediator of inflammatory cytokines that has been recently implicated in PDAC development (Corcoran et al., 2011; Fukuda et al., 2011; Lesina et al., 2011). As illustrated in Figure S2D, pancreata of untreated *ElasK-Ras*^{G12V} mice display acinar cells that do not express either EGFR or detectable levels

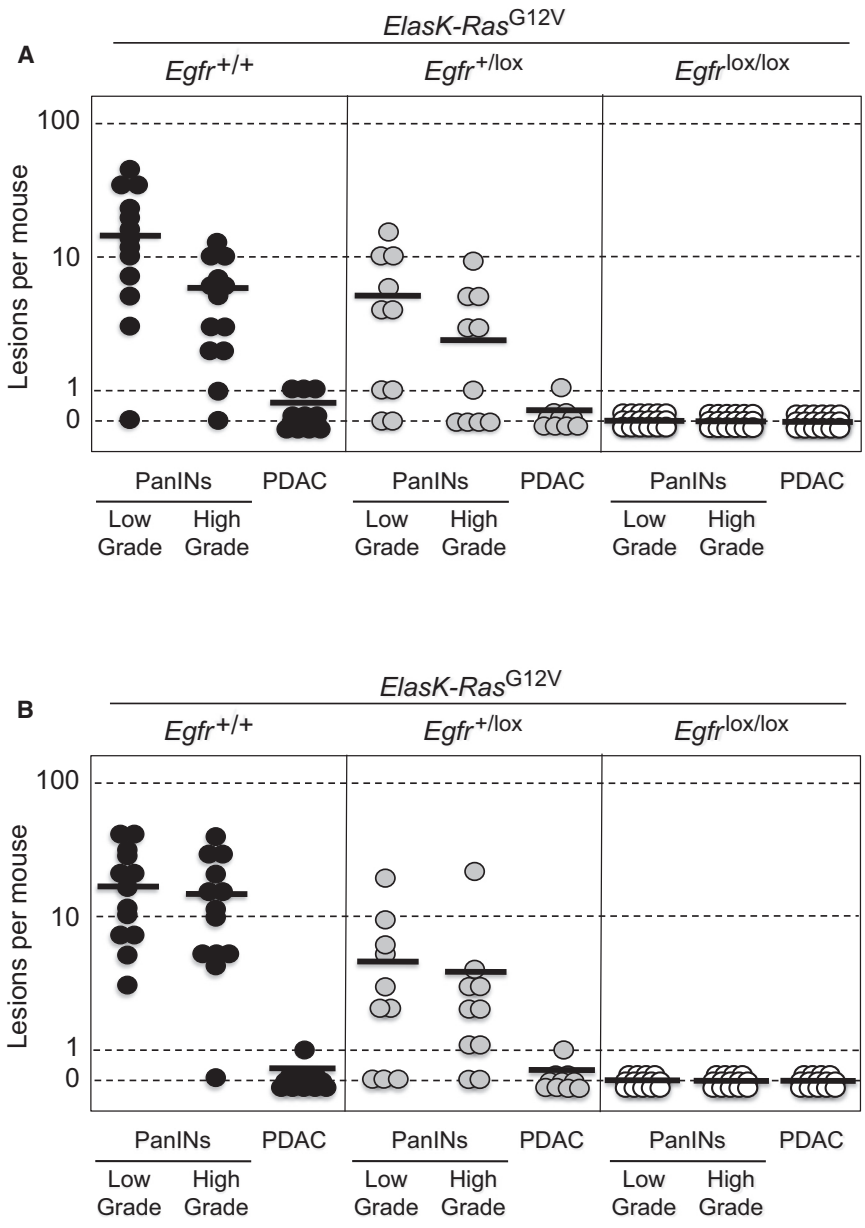

Figure 2. Induction of PanINs and PDAC Tumors by an Endogenous K-*Ras*^{G12V} Oncogene Requires Expression of the EGFR

(A) Number of low- and high-grade PanINs and PDACs per mouse in untreated, 1-year-old *ElasK-Ras*^{G12V} mice carrying the indicated *Egfr* alleles. *ElasK-Ras*^{G12V};*Egfr*$^{+/+}$ (solid circles), *ElasK-Ras*^{G12V};*Egfr*$^{+/lox}$ (gray circles), and *ElasK-Ras*^{G12V};*Egfr*$^{lox/lox}$ (open circles) mice. In these mice, Cre recombinase-mediated expression of the endogenous K-*Ras*^{G12V} oncogene and ablation of the conditional *Egfr*^{lox} alleles took place in a percentage (30%) of acinar cells during late embryonic development.

(B) Number of low- and high-grade PanINs and PDACs per mouse in 14-month-old *ElasK-Ras*^{G12V} mice carrying the indicated *Egfr* alleles. *ElasK-Ras*^{G12V};*Egfr*$^{+/+}$ (solid circles), *ElasK-Ras*^{G12V};*Egfr*$^{+/lox}$ (gray circles), and *ElasK-Ras*^{G12V};*Egfr*$^{lox/lox}$ (open circles) mice. These mice were exposed to doxycycline from conception to P60, a time at which Cre recombinase-mediated expression led to the concomitant activation of the resident K-*Ras*^{G12V} oncogene and ablation of the conditional *Egfr*^{lox} alleles in acinar cells. Mice were subsequently treated with caerulein from P90 to P180.

Horizontal bars indicate the average number of lesions per mouse for each genotype. See also Figure S2.

of phosphorylated AKT or STAT3. Thus indicating that expression of the resident K-Ras^{G12V} oncoprotein does not activate these pathways, at least in this cellular context. In contrast, pancreata of *ElasK-Ras*^{G12V} mice treated with caerulein for 3 months exhibit uniform expression of EGFRs along with nuclear phospho-AKT and phospho-STAT3 proteins through the entire pancreas. Since K-Ras^{G12V} expression in these mice only takes place in about 30% of their acinar cells, activation of the EGFR/AKT/STAT3 axis must be independent of K-*Ras* oncogene signaling (Guerra et al., 2007, 2011). As expected, pancreatic lesions including metaplasias and PanINs, also display activated phospho-AKT and phophop-STAT3 molecules in response to EGFR expression, suggesting that activation of the PI3K/AKT and STAT3 signaling pathways play a role in the induction of these lesions (Figure S2E). Finally, the presence of nuclear phospho-AKT and phospho-STAT3 in PDAC tumors also suggests that activation of these effector molecules might be required for tumor progression (data not shown).

Human Pancreatic Ductal Tumor Cell Lines Are Dependent on EGFR Signaling Regardless of the Presence of K-*RAS* Oncogenes

Next, we interrogated whether cell lines derived from human PDACs also depend on EGFR signaling for proliferation. We selected eight well-characterized tumor cell lines with different pattern of mutations. Six of them, AsPc1, CFPAC, IMIMPC-2, MIAPaCa, PANC1, and SKPC, harbor K-*RAS* oncogenes along with inactivation of *P16INK4a* and *TP53* tumors suppressor genes (Table 1). CFPAC and SKPC cells also have a deleted *SMAD4* locus. The remaining pancreatic tumor cell lines BxPc3 and T3M4, carry a wild-type K-*RAS* locus. Yet, they also have mutated or silenced *P16INK4a* and *TP53* loci and one of them, BxPc3, a mutated *SMAD4* locus (Table 1). Knockdown of EGFRs using two independent shRNAs efficiently inhibited proliferation (>70%) of AsPc1, BxPc3, MIAPaCa, and T3M4 cells. Two cell lines, PANC1 and IMIMPC-2, were only partially inhibited whereas the remaining cell lines, CFPAC and SKPC, were resistant (Table 1). Thus, the effect of EGFR signaling on proliferation appears to be independent of the presence of K-*RAS* oncogenes. Knockdown of EGFR expression only inhibited the PI3K pathway, as determined by

Table 1. Human Pancreatic Ductal Adenocarcinoma Cell Lines Are Sensitive to Inhibition of EGFR and MEK Signaling

	Mutation				*EGFR* Knockdown	Treatment			
						Erlotinib		PD325901	
Tumor	*K-RAS*	*P16INK4a*	*TP53*	*SMAD4*	% Inhibition	IC_{50}	IC_{90}	IC_{50}	IC_{90}
AsPc1	G12D	Frameshift	Frameshift	WT	72.3	>200.0 μM	>200 μM	190.0 μM	>200 μM
CFPAC	G12V	Methylated	Mutation	Deletion	5.2	20.0 μM	>200 μM	22.5 μM	>200 μM
IMIMPC-2	G12D	Deletion	Mutation	WT	32.5	>200.0 μM	>200 μM	0.8 μM	>200 μM
MIAPaCa	G12C	Deletion	Mutation	WT	97.0	66.8 μM	>200 μM	8.5 μM	>200 μM
PANC1	G12D	Deletion	Mutation	WT	59.9	>200.0 μM	>200 μM	>200.0 μM	>200 μM
SKPC	G12V	Methylated	Mutation	Deletion	08.7	70.0 μM	>200 μM	0.5 μM	>200 μM
BxPc3	WT	Mutation	Mutation	Mutation	81.0	23.5 μM	>200 μM	0.3 μM	>200 μM
T3M4	WT	Methylated	Mutation	WT	90.1	12.2 μM	>200 μM	0.1 μM	>200 μM

WT, wild-type. See also Figure S3.

phosphorylation of AKT, in those cells carrying a wild-type K-*RAS* locus (Figure S3).

Five of these tumor cell lines, including K-*RAS* oncogene-positive CFPAC, MiaPaCa and SKPC cells, and K-*RAS* oncogene-negative BxPc3 and T3M4 cells were partially sensitive to Erlotinib (Table 1). Erlotinib treatment did not result in complete inhibition of cell proliferation (IC_{90}) even at concentrations as high as 200 μM. The SKPC cell line, whereas partially sensitive to Erlotinib, was refractory to EGFR knockdown (Table 1). This discrepancy might be explained by either the inability of the shRNAs to effectively knockdown the high levels of EGFRs present in this cell line or to the off-target effect of Erlotinib on related tyrosine protein kinase receptors (Figure S3). We also examined the effect of directly inhibiting the RAS pathway by using the MEK inhibitor, PD325901. Four cell lines carrying K-*RAS* oncogenes, CFPAC, IMIMPC-2, MiaPaCa, and SKPC cells were sensitive to this inhibitor. Interestingly, the BxPc3 and T3M4 cell lines that have a wild-type K-*RAS* locus, were also highly sensitive to the MEK inhibitor, suggesting that these cells may have activated their RAS pathway by mechanisms other than mutating their K-*RAS* locus (Table 1). Finally, AsPc1 and PANC1 cells were resistant to MEK inhibition in spite of carrying K-*RAS* oncogenes, suggesting that in these cells K-*RAS* oncogenes may no longer play an essential role in maintaining their proliferative properties (Table 1).

K-RAS^{G12V}-Driven Lung and Intestinal Tumors Do Not Require EGFR Signaling

The above results, taken together, indicate that proliferation of pancreatic ductal tumor cells have a dual requirement for EGFR and K-RAS signaling. These observations are at odds with extensive clinical data in human NSCLCs, in which oncogenic mutations in the *EGFR* and K-*RAS* loci are mutually exclusive (Shigematsu et al., 2005). Likewise, CRC patients carrying K-*RAS* oncogenes do not benefit from treatments involving inhibition of EGFR signaling (Karapetis et al., 2008). To determine whether the results described above only occur in the context of mouse tumor models or are an intrinsic property of pancreatic tumors, we ablated the *Egfr* locus in two well-characterized GEM models of lung and intestinal tumors induced by the same endogenous K-*Ras*^{G12V} oncogene used to initiate pancreatic lesions. In these models, expression of the resident K-*Ras*^{G12V} oncogene is mediated by activation of an ubiquitously expressed Cre-ERT2 inducible recombinase knocked-in at the locus encoding the large subunit of RNA polymerase II (*RERT* strain; see Guerra et al., 2003). For the lung model, *RERT;K-Ras*^{G12V};*Egfr*$^{+/+}$ (n = 17), *RERT;K-Ras*^{G12V};*Egfr*$^{+/lox}$ (n = 13), and *RERT;K-Ras*^{G12V};*Egfr*$^{lox/lox}$ (n = 25) littermates were treated at weaning with a single dose of 4-hydroxy-tamoxifen (4OHT) (Guerra et al., 2003; Puyol et al., 2010). As illustrated in Figure 3A, all mice died of lung tumors between 63 and 72 weeks of age. Mice displayed similar number of adenomas (average of 15 per mouse) and adenocarcinomas (average of three per mouse) regardless of genotype. None of the tumors analyzed expressed EGFRs by IHC analysis (Figure 3B). Moreover, PCR analysis of tumor DNA only revealed recombined *Egfr null* alleles (data not shown), thus indicating that tumor development had occurred in the absence of EGFRs.

Similar results were obtained in a GEM model of intestinal tumors. *RERT;K-Ras*^{G12V};*Apc*$^{lox/lox}$;*Egfr*$^{lox/lox}$ mice (n = 16) along with control *RERT;K-Ras*^{G12V};*Apc*$^{lox/lox}$;*Egfr*$^{+/lox}$ (n = 17) and *RERT;K-Ras*^{G12V};*Apc*$^{lox/lox}$;*Egfr*$^{+/+}$ (n = 7) littermates were treated at weaning for 2 weeks (3 days per week) with 4OHT. These mice displayed similar tumor burden including adenomas and adenocarcinomas (data not shown) and did not survive beyond 20 weeks of age (Figure 3C). As expected, tumor cells, regardless of genotype, failed to express EGFRs (Figure 3D). These results, taken together, indicate that the requirement of EGFR signaling for the onset of neoplastic pancreatic lesions driven by K-*Ras* oncogenes is unique to this tumor type. Moreover, the similarity between the results obtained in clinical trials and in mouse models of lung and intestinal cancer reinforces the concept that GEM tumor models faithfully reproduce those events observed in cancer patients.

Loss of Senescence Does Not Override the Need for EGFR Signaling during PanIN and PDAC Development

The EGFR is known to promote survival signals that might be essential to overcome the oncogene-induced senescence characteristic of the early stages of pancreatic tumor development (Collado et al., 2005; Guerra et al., 2011). Indeed, most human PDACs carry a mutated or silenced *P16INK4a/P14ARF* locus, an event likely to override senescence (Hong et al., 2011). Thus, we reasoned that ablation of the p16INK4A/p19ARF tumor suppressors (from now on p16/p19), might bypass the requirement for EGFR signaling during tumor

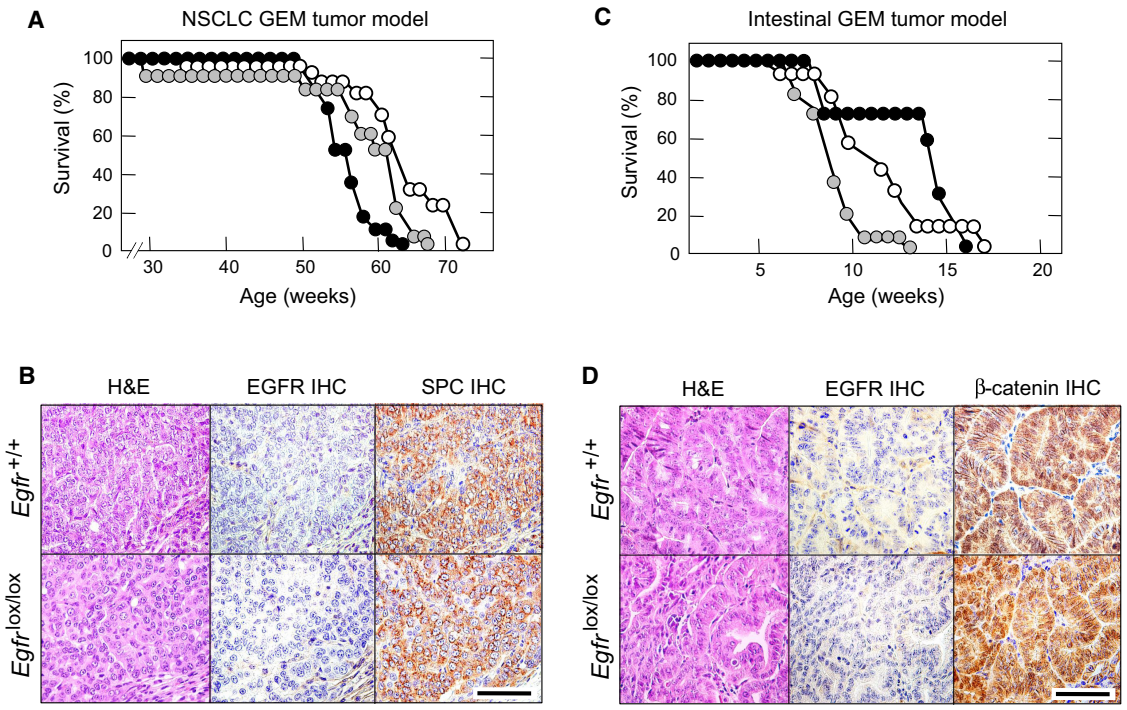

Figure 3. Ablation of EGFRs Has No Effect on K-*Ras*^{G12V}-Driven Lung and Intestinal Tumors

(A) Survival of *RERT;K-Ras*^{G12V};*Egfr*$^{+/+}$ (solid circles), *RERT;K-Ras*^{G12V};*Egfr*$^{+/lox}$ (gray circles), and *RERT;K-Ras*^{G12V};*Egfr*$^{lox/lox}$ (open circles) mice treated with a single injection of 4OHT at P21 to induce NSCLCs.

(B) H&E staining and EGFR and pro-surfactant protein C (SPC) immunostaining (IHC) of consecutive paraffin sections showing representative adenocarcinoma lesions from 9-month-old *RERTK-Ras*^{G12V} mice carrying either (top) wild-type *Egfr* or (bottom) conditional *Egfr* alleles. Scale bar represents 50 μm.

(C) Survival of *RERT;K-Ras*^{G12V};*Apc*$^{lox/lox}$;*Egfr*$^{+/+}$ (solid circles), *RERT;K-Ras*^{G12V};*Apc*$^{lox/lox}$;*Egfr*$^{+/lox}$ (gray circles), and *RERT;K-Ras*^{G12V};*Apc*$^{lox/lox}$;*Egfr*$^{lox/lox}$ (open circles) mice treated with 4OHT (3 days per week, for 2 weeks) at P21 to induce intestinal tumors.

(D) H&E staining and EGFR and β-catenin immunostaining (IHC) of consecutive paraffin sections showing representative intestinal tumor lesions from 2-month-old *RERT;K-Ras*^{G12V};*Apc*$^{lox/lox}$ mice carrying either (top) wild-type *Egfr* or (bottom) conditional *Egfr* alleles. Scale bar represents 50 μm.

initiation. Conditional floxed *p16/p19* alleles were introduced into *ElasK-Ras*^{G12V} mice carrying wild-type or floxed *Egfr* alleles and their pancreata examined at 16 weeks of age, before they displayed any obvious signs of overt tumor development. These mice were not exposed to doxycycline to allow expression of the resident K-*Ras*^{G12V} oncogene during late embryonic development (Guerra et al., 2007). As summarized in Figure 4A, six out of eight mice carrying wild-type EGFRs displayed abundant low- and high-grade lesions. Moreover, five animals had developed at least a PDAC tumor at this time. In contrast, none of the mice carrying *Egfr*$^{lox/lox}$ alleles (n = 6) displayed PanIN lesions or PDAC (Figure 4A).

Mice with the above genotypes were allowed to age. Littermates carrying wild-type *Egfr* alleles, either in homozygocity (n = 12) or heterozygocity (n = 30) succumbed to the disease before they reached 10 months of age (Figure 4B). Postmortem analysis revealed multiple lesions including invasive and metastatic PDAC as well as anaplastic carcinomas that metastasized to multiple organs (Aguirre et al., 2003; Guerra et al., 2011). As expected, none of the low-grade PanIN lesions contained senescent cells as determined by staining for β-galactosidase activity (data not shown). In contrast, *ElasK-Ras*^{G12V};*p16/p19*$^{lox/lox}$;*Egfr*$^{lox/lox}$ mice (n = 7) sacrificed at 1 year of age did not carry any PanIN lesion positive for EGFRs in spite of careful analysis of multiple serial sections (data not shown). Only four animals had a total of six low-grade and two high-grade PanIN "escaper" lesions positive for EGFRs (Figure 4C). Seventeen additional *ElasK-Ras*^{G12V};*p16/p19*$^{lox/lox}$;*Egfr*$^{lox/lox}$ mice were allowed to age beyond 1 year. All of them remained in good health condition at 80 weeks of age (Figure 4B). Histological examination of their pancreata at this time did not reveal any lesions (data not shown). These observations indicate that abrogation of senescence by inactivation of the *p16/p19* tumor suppressors does not relieve pancreatic tumor cells of their need for EGFR signaling.

Loss of p16/p19 tumor suppressors also accelerates tumor development in adult mice providing they have undergone chronic or temporary pancreatitis (Guerra et al., 2011). Analysis of pancreata of *ElasK-Ras*^{G12V};*p16/p19*$^{lox/lox}$;*Egfr*$^{lox/lox}$ mice (n = 7) 12 months after turning on K-Ras^{G12V} expression (8 months after completing caerulein treatment), also failed to reveal EGFR-negative PanIN lesions or PDAC tumors. Control *ElasK-Ras*^{G12V};*p16/p19*$^{lox/lox}$;*Egfr*$^{+/+}$ littermates (n = 10) died at the expected age (40 weeks of median survival) and displayed multiple PanIN lesions as well as PDACs, in some cases with perineural invasion, invasion of the intestinal wall and lymph node metastasis, as previously described (Guerra et al., 2011). These observations indicate that the requirement for EGFR

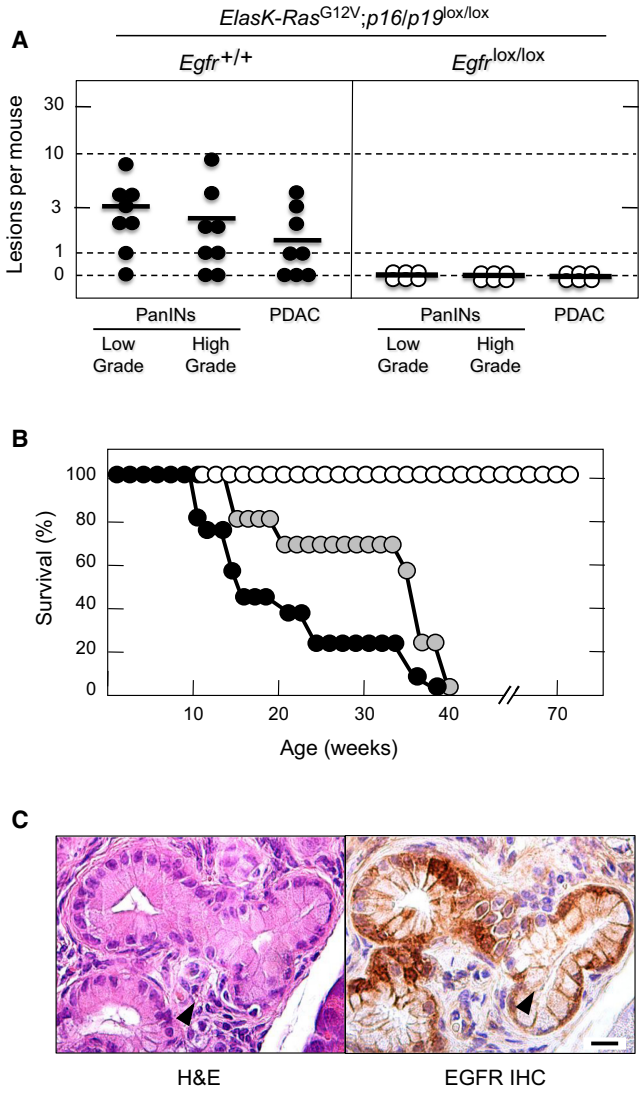

Figure 4. Loss of p16/p19 Tumor Suppressors Does Not Abrogate the Need for EGFR Expression during PanIN and PDAC Development

(A) Number of low- and high-grade PanINs and PDAC lesions per mouse in untreated, 16-week-old *ElasK-Ras*^{G12V};*p16/p19*$^{lox/lox}$ mice carrying either wild-type (solid circles) or conditional (open circles) *Egfr* alleles. In these mice, expression of a Cre recombinase in pancreatic acinar cells during late embryonic development results in the concomitant expression of the endogenous K-*Ras*^{G12V} oncogene and in the ablation of the conditional *p16/p19* and *Egfr* alleles. PanIN lesions positive for EGFR expression in *ElasK-Ras*^{G12V};*p16/p19*$^{lox/lox}$;*Egfr*$^{lox/lox}$ mice (see below) were not scored. Horizontal bars indicate the average number of lesions per mouse.

(B) Survival of untreated *ElasK-Ras*^{G12V};*p16/p19*$^{lox/lox}$; *Egfr* $^{+/+}$ (solid circles), *ElasK-Ras*^{G12V};*p16/p19*$^{lox/lox}$;*Egfr*$^{+/lox}$ (gray circles), and *ElasK-Ras*^{G12V};*p16/p19*$^{lox/lox}$;*Egfr*$^{lox/lox}$ (open circles) mice.

(C) H&E (left) and EGFR IHC (right) of consecutive paraffin sections showing an occasional PanIN lesion observed in *ElasK-Ras*^{G12V};*p16/p19*$^{lox/lox}$;*Egfr*$^{lox/lox}$ animals positive for EGFR expression (arrowhead). Scale bar represents 50 μm.

signaling during PanIN and PDAC development cannot be compensated by loss of the p16/p19 tumor suppressors even in the context of an inflammatory response induced by pancreatitis.

Loss of p53 Triggers Oncogenic Pathways Independent of EGFR Signaling

Human PDAC tumors harbor mutations in classical tumor suppressor genes such as *TP53*, *SMAD4*, or *BRCA2* (Hong et al., 2011). Likewise, mice expressing an endogenous K-*Ras* oncogene during embryonic development in the absence of a functional p53 protein develop aggressive PanINs and PDAC tumors that result in the death of the animals within their first 4 to 5 months of life (Hingorani et al., 2005; Guerra et al., 2007). Similar results have been obtained in the context of adult K-*Ras* oncogene expression followed by pancreatic damage (Guerra et al., 2011). To examine the effect of abrogating EGFR signaling in the absence of p53, we inserted conditional *p53*^{lox} alleles in the *ElasK-Ras*^{G12V} strain in the presence of wild-type or floxed *Egfr* alleles. Animals were sacrificed at 10 weeks of age before they showed signs of overt tumor development. As summarized in Figure 5A, control mice carrying wild-type *Egfr* alleles (n = 7) displayed abundant low- and high-grade lesions and PDAC, averaging ten high grade PanINs and four PDAC tumors per mouse. Interestingly, mice carrying conditional *Egfr* alleles (n = 5) also displayed neoplastic lesions but with reduced incidence (Figure 5A).

When we allowed these mice to age, all animals carrying wild-type *Egfr* alleles (n = 10) succumbed to pancreatic tumors around 20 weeks of age with a median survival of 12 weeks (Figure 5B). Similar results were obtained with heterozygous mice (n = 13) (Figure 5B). At the time of death or humane end point, these mice displayed multiple PanIN lesions and PDAC tumors including a lung metastasis in one of the animals. *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$;*Egfr*$^{lox/lox}$ mice (n = 13) also developed low- and high-grade PanINs as well as PDAC tumors (Figure 5B). Moreover, three of these mice also had macroscopic metastasis at different locations such as peritoneum, diaphragm, liver, and lung (data not shown). However, mice in which the conditional *Egfr* alleles have been ablated died by 40 weeks of age and displayed a median survival 83% longer than that of littermates expressing EGFRs (22 versus 12 weeks) (Figure 5B). Similar results were obtained with mice that expressed the resident K-*Ras*^{G12V} oncogene during adulthood and were treated for 3 months with caerulein. Whereas *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$;*Egfr*$^{lox/lox}$ animals (n = 26) died before reaching 60 weeks of age with a median survival of 38 weeks, control *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$;*Egfr*$^{+/lox}$ mice (n = 8) were dead at 35 weeks of age with a median survival of 27 weeks (data not shown). Thus, ablation of EGFR signaling resulted in an increased survival time of 40%.

Tumor development in mice carrying conditional *Egfr* alleles was not due to incomplete recombination because the large majority of the PanIN lesions and PDAC tumors did not express EGFRs when analyzed by IHC (Figure S4A). Moreover, PCR analysis of DNA isolated from tumor cells also failed to reveal unrecombined *Egfr*^{lox} alleles in most lesions (data not shown). Finally, histopathological analysis of PanIN and PDAC tumors lacking p53 and EGFRs did not reveal significant differences with those present in control animals (Figure S4A). These observations, taken together, indicate that loss of p53 activates oncogenic pathways that bypass the requirement of EGFR signaling for tumor development.

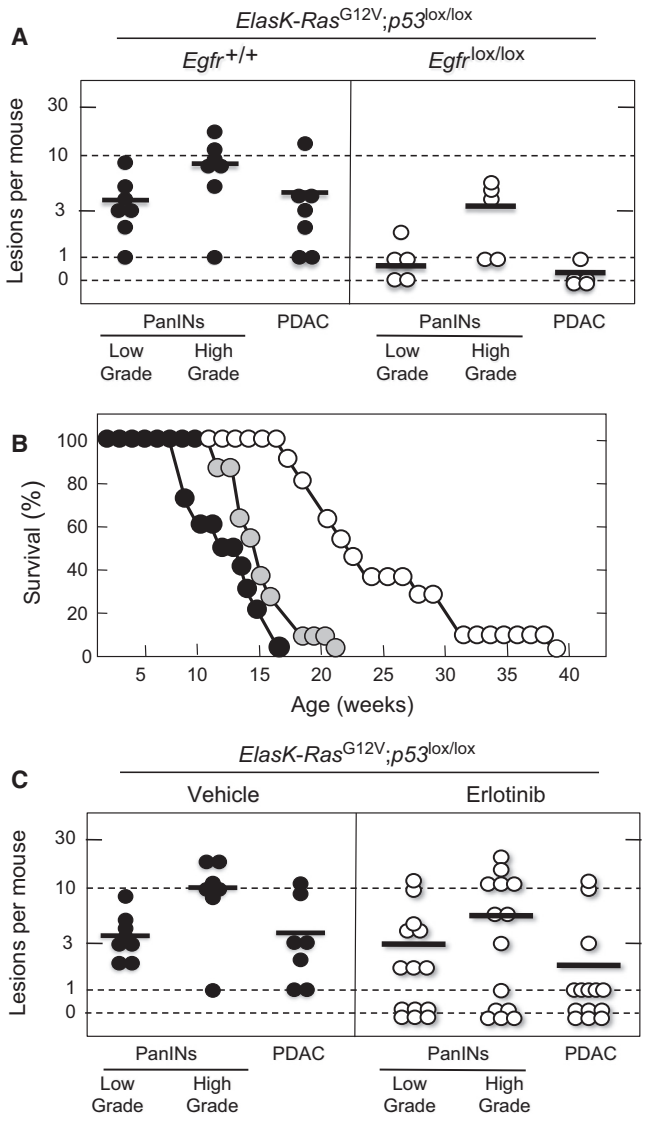

Figure 5. Loss of EGFRs Delays but Does Not Prevent PanIN and PDAC Development in the Absence of p53

(A) Number of low- and high-grade PanINs and PDACs per mouse in untreated, 10-week-old *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$ mice carrying either wild-type (solid circles) or conditional (open circles) *Egfr* alleles. In these mice, expression of a Cre recombinase in pancreatic acinar cells during late embryonic development results in the concomitant expression of the endogenous K-*Ras*^{G12V} oncogene and in the ablation of the conditional *p53* and *Egfr* alleles. Horizontal bars indicate the average number of lesions per mouse.

(B) Survival of untreated *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$;*Egfr*$^{+/+}$ (solid circles), *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$;*Egfr*$^{+/lox}$ (gray circles), and *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$;*Egfr*$^{lox/lox}$ (open circles) mice.

(C) Inhibition of EGFR signaling by Erlotinib treatment reduces the number of PanIN and PDAC lesions. Number of low- and high-grade PanINs and PDACs per mouse in 6-week-old *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$ mice treated for 4 weeks with vehicle (solid circles) or Erlotinib (open circles). Horizontal bars indicate the average number of lesions per mouse. The decrease in the number of PDAC tumors in the Erlotinib-treated cohort was statistically significant ($p < 0.05$).

See also Figure S4.

Inhibition of EGFR Signaling with Erlotinib Interferes with PDAC Development In Vivo

Previous studies have shown that Gefitinib can slow down progression of pancreatic precursor lesions to PDAC (Mohammed et al., 2010). To determine whether EGFRs are required for the progression of PanIN lesions in a more aggressive GEM model, 6-week-old *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$ mice were treated with either vehicle or Erlotinib (100 mg/Kg) for 4 weeks. A small group of mice (n = 3) analyzed at the start of the treatment revealed low- and high-grade PanINs in each of the animals. Moreover, two of the mice already carried small PDACs (Figure S4B). At the end of the 4-week treatment, all mice that received vehicle (n = 7) displayed a significant increase in the number of lesions (Figure 5C). In contrast, Erlotinib treatment led to disappearance of all lesions in three out of the 14 animals included in this cohort. Moreover, three additional Erlotinib-treated mice contained PanIN lesions but no PDAC tumors (Figure 5C). Overall, the Erlotinib-treated cohort had fewer lesions than the control group, indicating a limited but reproducible therapeutic effect of this EGFR inhibitor on PDAC development (Figure 5C).

IHC analysis of lesions in mice treated with Erlotinib for 4 weeks revealed robust inhibition of pAKT but not of pSTAT3 or pERK when compared with samples obtained from control mice treated with vehicle (Figure S4C). As an additional control, we examined the status of pAKT, pSTAT3 and pERK in *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$ mice carrying either wild-type or conditional *Egfr* alleles. As expected, ablation of EGFR expression resulted in inhibition of pAKT but not of pSTAT3 or pERK (Figure S4D). Interestingly, full blown PDACs retained pAKT IHC (Figure S4E), a result confirmed by western blot analysis (Figure S5 and data not shown). These observations suggest that loss of p53 might activate the PI3K pathway by a mechanism independent of EGFR signaling.

EGFR Signaling Is Required for Proliferation of PDAC Tumor Cell Explants

Knockdown of EGFR expression effectively slowed proliferation of cell explants derived from PDAC tumors isolated from *ElasK-Ras*^{G12V} mice (Figure 6A). Similar results were obtained using explants from tumors obtained from *ElasK-Ras*^{G12V};*p16/p19*$^{lox/lox}$ and *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$ animals (Figure 6A). These tumor explants, regardless of genotype, were also sensitive to Erlotinib (Figure 6B). As expected, Erlotinib had a cytostatic effect because inhibition required continuous exposure to the drug (data not shown). Knockdown of EGFRs in these tumor explants significantly inhibited phosphorylation of STAT3 (Figure S5). Phosphorylation of ERK proteins was also ameliorated by EGFR knockdown (Figure S5), possibly an indirect consequence of the limited proliferation of these cells in the absence of EGFRs (Figure 6A). Interestingly, phosphorylation of AKT, a marker for the activation of the PI3K/AKT survival pathway was downregulated in all explants except in those lacking p53 (Figure S5).

Cooperation between PI3K and STAT3 Signaling Pathways in the Absence of EGFRs

We reasoned that availability of mouse PDAC tumor explants lacking EGFRs and p53 may allow us to identify additional signaling pathways that contribute to tumor development.

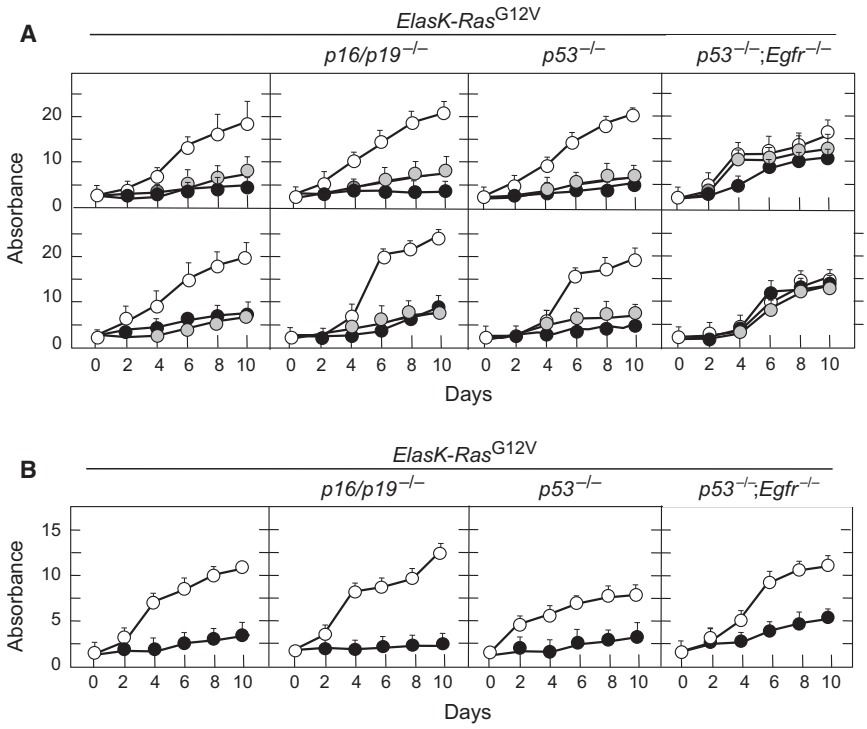

Figure 6. EGFR Expression Is Required for Proliferation of PDAC Tumor Explants In Vitro

(A) PDAC cell explants derived from tumors present in mice with the indicated genotypes were infected with lentiviral vectors expressing two independent shRNAs against the *Egfr* (solid and gray circles) or shRNA control (open circles). Results are the average of two experiments carried out with two independent cell explants done in triplicate. Errors bars mean SD.

(B) PDAC cell explants derived from tumors present in mice with the indicated genotypes were either untreated (open circles) or treated with Erlotinib (solid circles). Erlotinib was used at a final concentration of 50 μM, a concentration that corresponds with the average IC_{90} for these cell explants. Results are the average of two experiments carried out with two independent cell explants done in triplicate. Errors bars mean SD. See also Figure S5.

Unexpectedly, Erlotinib partially inhibited proliferation of PDAC explants from *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$;*Egfr*$^{lox/lox}$ mice. These results are most likely due to off-target effects because shRNAs against the *Egfr* locus did not have any inhibitory effect on the proliferation rate of these cells (Figures 6A and 6B). As illustrated in Figure 7, inhibition of PI3K with ETP-46992 (Martínez González et al., 2012) a selective inhibitor for the p110α and δ catalytic subunits only induced partial inhibition (Figure S6). However, combination of this inhibitor with Erlotinib resulted in robust inhibition of these tumor cell explants even in the absence of EGFRs and p53 (Figure 7).

As indicated above, ablation or inhibition of EGFRs in *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$ mice blocked AKT but not STAT3 phosphorylation (Figures S4C and S4D). Interestingly, knockdown of STAT3 expression did not inhibit proliferation of tumor cell explants, regardless of their genotype. However, addition of the PI3K inhibitor to the *Stat3* shRNA robustly inhibited proliferation of all tumor cell explants (Figure 7). These observations indicate that the STAT3 pathway also contributes to tumor development at least in the absence of p53. Moreover, in this context, PI3K may signal by pathways independent of AKT (Vogt and Hart, 2011). Thus, successful treatment of PDAC tumors in the clinic may require compound inhibition of at least four distinct signaling cascades including those driven by K-RAS, EGFR, PI3K, and STAT3.

DISCUSSION

Current dogma indicates that malignant progression of tumor cells selects against mutations in components of the same signaling pathway such as that driven by the EGFR and their downstream effectors, the RAS proteins. Indeed, clinical observations in NSCLCs, a tumor type that present frequent mutations in both *EGFR* and K-*RAS*, have indicated that they are mutually exclusive (Shigematsu et al., 2005). Similar results have been obtained in CRC patients. According to a large clinical trial, only patients containing nonmutated K-*RAS* genes benefit from treatment with EGFR inhibitors (Karapetis et al., 2008). This dogma, however, might not apply to PDAC tumors. Although *EGFR* mutations have been found in a very small percentage (<3%) of human pancreatic cancers, they can coexist with K-*RAS* mutations (Oliveira-Cunha et al., 2012). Moreover, as illustrated in this study, initiation of K-*Ras* oncogene-driven PanIN lesions and PDAC is absolutely dependent on EGFR signaling. This requirement is not abrogated even in the absence of the p16/p19 tumor suppressors, indicating that the absolute requirement for EGFR signaling in not involved in overcoming senescence. Only ablation of p53 overrules this requirement. Yet, neoplastic lesions lacking p53 take significantly longer to develop in the absence of EGFRs. Ongoing efforts to establish the mutational spectra of mouse PDAC exomic sequences might help us to identify those additional pathways activated by loss of p53.

Pancreatic injury results in the immediate induction of EGFR expression in acinar cells, leading to activation of the PI3K/AKT and STAT3 signaling pathways. EGFR expression, along with activation of AKT and STAT3, is maintained during PanIN progression to PDAC and only becomes attenuated in poorly differentiated glands of advanced PDAC tumors. The EGFR is also expressed in human biopsies obtained from patients suffering from pancreatitis and PDAC tumors, thus supporting the concept that GEM models faithfully reproduce PDAC development in an experimental setting. Previous studies have shown that overexpression of TGFα in the presence of a resident K-*Ras*^{G12D} oncogene accelerated progression of PanIN lesions to metastatic cancer and led to the development of cystic papillary lesions that resembled human intraductal papillary mucinous neoplasms (IPMN) (Siveke et al., 2007). These observations,

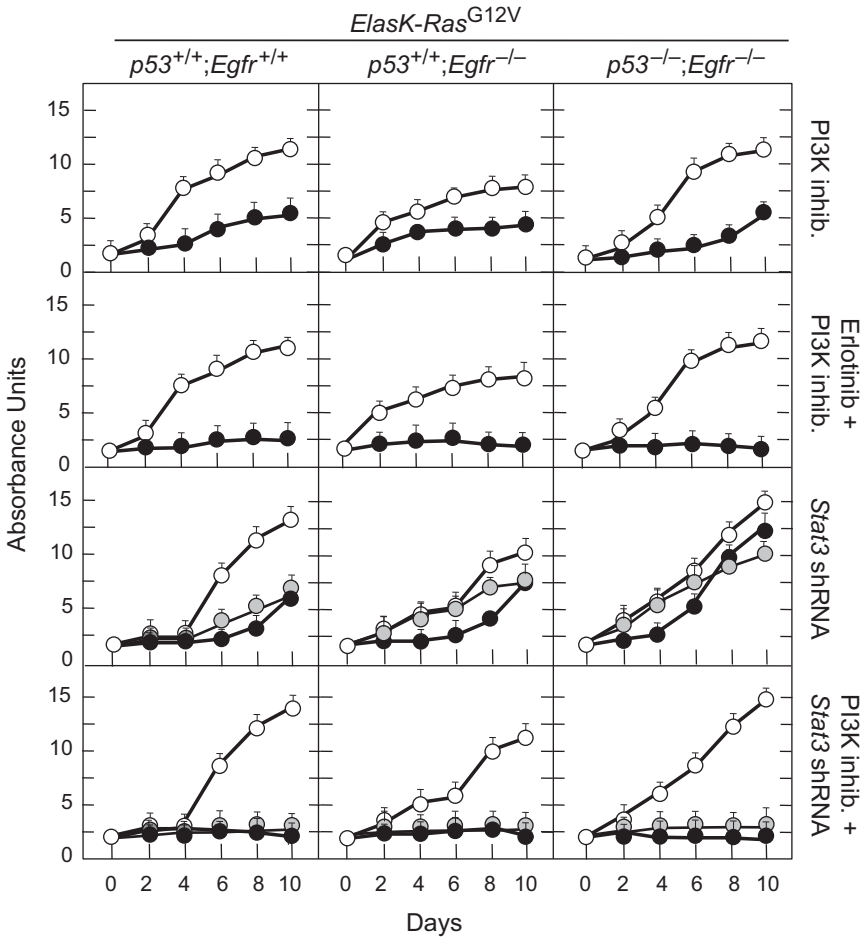

Figure 7. Loss of p53 Activates STAT3 and PI3K Pathways

PDAC cell explants derived from tumors present in mice with the indicated genotypes were treated with the indicated inhibitor(s) or infected with lentiviral vectors expressing two independent shRNA against *Stat3* (solid and gray circles). Control cells were either left untreated or infected with a shRNA control (open circles). Erlotinib was used at a final concentration of 50 μM, a concentration that corresponds with the average IC_{90} for these cell explants. ETP-46992, a selective PI3Kp110α and p110δ inhibitor, was used at a final concentration of 20 μM, a concentration that corresponds to the average IC_{90} for these tumor explants. Each graph represents the average of two experiments carried out with two independent cell explants. Each sample was carried out in triplicate. Errors bars mean SD. See also Figure S6.

taken together, suggest that upregulated expression of EGFRs leading to the activation of the PI3K/AKT and STAT3 pathways might be one of the early responses that predispose acinar cells to undergo neoplastic changes upon activation of K-*Ras* oncogenes. It is interesting to note that in acinar cells, activation of the PI3K/AKT pathway is mediated by induction of EGFRs and not by the resident K-*Ras* oncogenes. These observations may explain why, in spite of the presence of K-*Ras* oncogenes, EGFR signaling is essential to induce neoplastic lesions in pancreatic acinar cells.

Current GEM models of PDAC do not allow target ablation in existing tumors. Yet, our results demonstrating that expression of EGFRs are essential for the proliferation of tumor cell explants strongly suggest that EGFRs signaling is essential beyond the early stages of tumor development. Indeed, knockdown or pharmacological inhibition of EGFRs blocked proliferation of certain human pancreatic tumor cells lines. Likewise, EGFR inhibitors have been shown to limit progression of pancreatic lesions in mouse xenograft models (Ng et al., 2002; Durkin et al., 2006) as well as in K-*Ras* oncogene-driven GEM models (Mohammed et al., 2010; this study). These results suggest that clinical observations showing a limited beneficial effect of EGFR inhibitors in combination with Gemcitabine in patients with PDAC tumors need to be further explored (Moore et al., 2007).

Finally, our results using genetic as well as pharmacologic approaches, illustrate that blocking EGFR signaling only produces limited therapeutic benefit in the context of p53 inactivation, a mutation present in most human PDAC tumors. Availability of tumor cells lacking EGFRs and p53 has allowed us to demonstrate a synergistic activity between PI3K inhibitors and attenuation of STAT3 expression. These observations suggest that loss of TP53 might "reactivate" the PI3K/AKT and STAT3 signaling pathways by mechanisms independent of EGFR in human tumors. Further support for a key role of the EGFR/PI3K/AKT axis in PDAC development comes from preliminary studies in which ablation of the *Pten* tumor suppressor locus in *ElasK-Ras*^{G12V};*Egfr*$^{lox/lox}$ mice leads to efficient tumor development (C.N., I.H., C.G., and M.B., unpublished observations). Recent observations regarding a limited but significant tumor inhibitory effect by inhibitors of the Notch pathway (Plentz et al., 2009; Cook et al., 2012) may open the door to design therapeutic strategies in combination with PI3K and STAT3 inhibitors. Yet, to induce complete regression of aggressive PDAC tumors it will be necessary to unveil additional signaling pathways amenable to pharmacological inhibition. Recent progress in overcoming the stromal barrier characteristic of PDAC tumors (Olive et al., 2009; Von Hoff et al., 2011; Frese et al., 2012; Jacobetz et al., 2012; Provenzano et al., 2012) should facilitate testing these drug combinations in relevant GEM models of pancreatic cancer as a preliminary step prior to their use in a clinical setting.

EXPERIMENTAL PROCEDURES

Mice

The *ElasK-Ras*^{G12V} strain has been previously described (Guerra et al., 2007). In these mice, the *Elas*-tTA/*tetO*-Cre transgenes drive expression of the bacterial Cre recombinase from the *Elastase* promoter under the negative control of doxycycline (Tet-off system). Other strains of mice used in this study include *Egfr*^{lox}, *p16/p19*^{lox}, *p53*^{lox}, *Apc*^{lox}, and *RERT*. The original references

describing these strains appear in the Supplemental Experimental Procedures. All experiments were approved by the CNIO Ethical Committee and performed in accordance with the guidelines for Ethical Conduct in the Care and Use of Animals as stated in The International Guiding Principles for Biomedical Research involving Animals, developed by the Council for International Organizations of Medical Sciences (CIOMS).

Mouse Treatments

To prevent expression of the *Elastase*-driven Cre recombinase in *ElasK-Ras*^{G12V} mice, doxycycline (2 mg/ml; Sigma) was provided in the drinking water as a sucrose solution (5% w/v) to pregnant mothers from the time of conception and to their offspring until the time we activated expression of the resident K-*Ras*^{G12V} oncogene. Pancreatitis was induced by intraperitoneal injections of caerulein for 3 months (125 μg/Kg, 5 days per week; Sigma). For induction of NSCLC, *RERT;K-Ras*^{G12V} mice carrying wild-type or floxed *Egfr* alleles were treated at P21 with a single dose of 4OHT (0.5 mg/ml in oil). For intestinal tumors, *RERT;K-Ras*^{G12V};*Apc*$^{lox/lox}$ mice carrying wild-type or floxed *Egfr* alleles were treated 3 days per week for 2 weeks with 4OHT (0.5 mg/ml in oil) starting at P21. Erlotinib treatment was carried out in 6-week-old *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$ mice by oral gavage (100 mg/Kg in 0.5% methylcellulose with 0.1% Tween80) for 4 weeks. Control mice received the same treatment without Erlotinib.

Histopathology and Immunohistochemistry

Specimens were fixed in 10% buffered formalin and embedded in paraffin. For histopathological analysis, pancreata were serially sectioned (3 μm thick) and every ten sections stained with hematoxylin and eosin (H&E). Remaining sections were kept for immunohistochemical studies with β-catenin (1:750, goat polyclonal; Santa Cruz Biotechnology, Sc-1496), pAKT (pS473) (1:175, rabbit monoclonal EP2109Y; Epitomics 2118-1), EGFR (1:100, rabbit monoclonal; Epitomics 1902-1), SPC (1:175, rabbit polyclonal; Millipore, AB3786), and pSTAT3 (Tyr705) (1:100, rabbit monoclonal D3A7; Cell Signaling Technology, 9145) antibodies. Following incubation with the primary antibodies, positive cells were visualized using 3,3-diaminobenzidine tetrahydrochloride plus (DAB+) as a chromogen. For human samples (see below), immunostaining for EGFRs was preformed as described above.

PDAC Cell Explants

To generate mouse PDAC explants, freshly isolated tumors were minced with sterile razor blades, digested with collagenase P (1.5 μg/ml) in Hanks' balanced salt solution (HBSS) for 30 min at 37°C, and cultured in DMEM with 10% of fetal bovine serum (FBS). After 48 hr, media was supplemented with Geneticin (75 μg/ml) to select for K-Ras^{G12V} expressing cells. All studies were done on cells cultivated for less than ten passages. Their corresponding genotypes were verified by PCR analysis.

Cell Culture and Inhibitor Treatments

Mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryos and propagated according to standard 3T3 protocols. Human tumor cell lines PANC1, MIAPaCa-2, SKPC, T3M4, were purchased from ATCC. BxPc3 was kindly provided by M. Hidalgo (CNIO). AsPc1, CFPAC, and IMIMPC-2 by F.X. Real (CNIO). These cell lines as well as the PDAC explants were seeded in 96-well plates at a density of 1,000 cells/well or 300 cells/well, respectively, and incubated for 24 hr in DMEM media supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (GIBCO-Invitrogen) before adding the corresponding inhibitor. Inhibitors, including EGFR inhibitor Erlotinib (LC laboratories), MEK inhibitor PD0325901 (Pfizer), and PI3K inhibitor ETP-46992 (CNIO) (Martínez González et al., 2012), were dissolved in DMSO to yield the appropriate final concentrations. Three sets of control wells were included on each plate, containing either medium without drug or medium with the same concentration of DMSO. Cells were exposed to inhibitors for 10 to 14 days. Fresh drug was added every 2 days. For shRNA knockdown assays, human or mouse tumor cells were infected with MISSION shRNAs (Sigma) directed against human *EGFR* (TRC0000121206 and TRC0000121203), mouse *Egfr* (TRCN0000055218 and TRCN0000055221), and mouse *Stat3* (TRCN0000071454 and TRCN0000071456) sequences. Non-Target shRNA Control vector (SHC002, Sigma) was used as a negative control. Cells were selected with puromycin (2 μg/ml) for 5 days before seeding and maintained in DMEM media supplemented with 10% FBS and puromycin. Proliferation rates were determined by the (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche). The resulting absorbance was measured with a microplate reader at 544 nm (EnVision 2104 Multilabel Reader, Perkin Elmer, Waltham, MA). Results represent the average of three independent experiments in which all samples were assayed in triplicate.

Human Samples

Studies using human material were approved by the Ethics and Institutional Review Board of the Grupo Hospital de Madrid (CBBA/4 2008; REF: PI 275). All subjects gave informed consent.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2012.08.001.

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