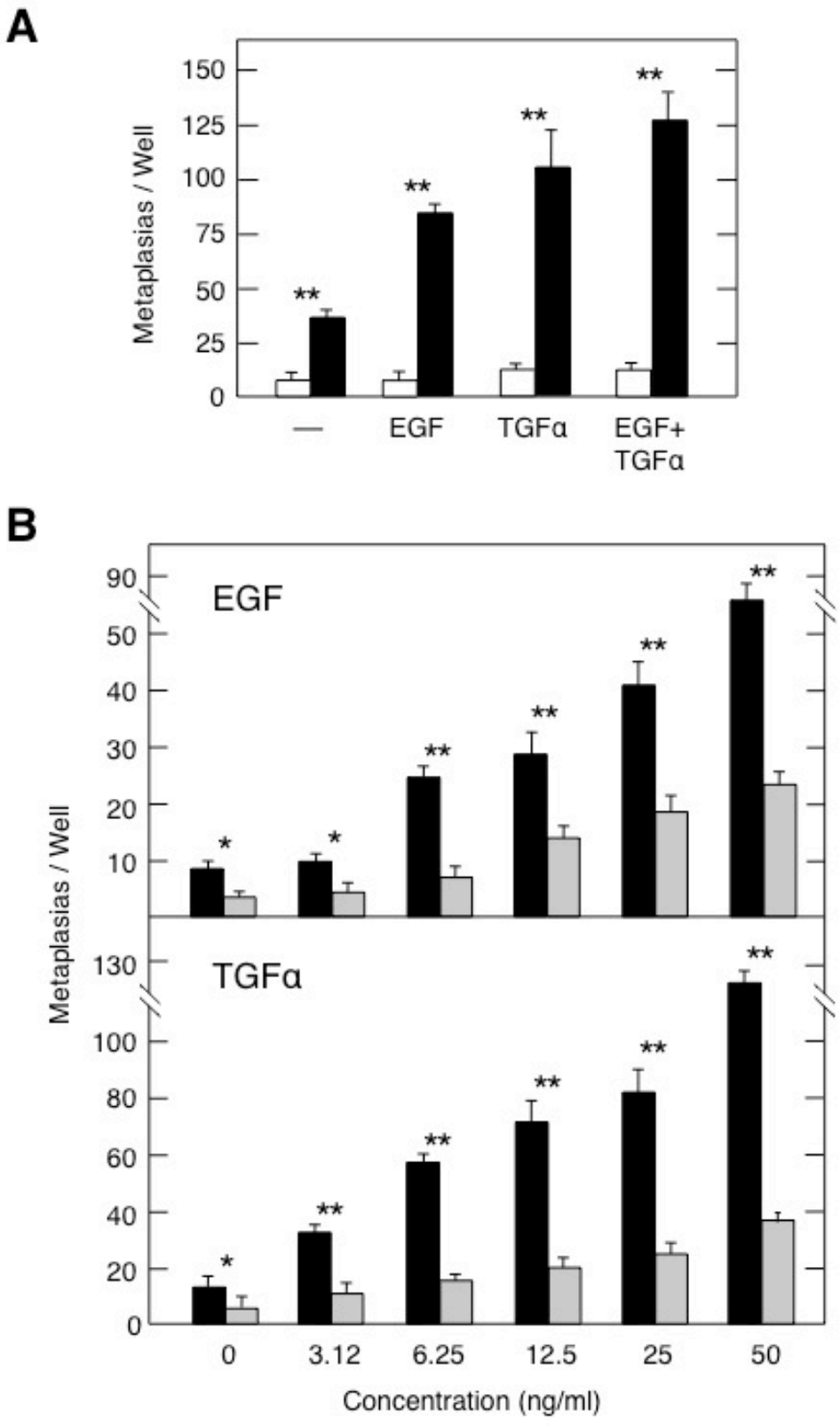
SUPPLEMENTAL DATA



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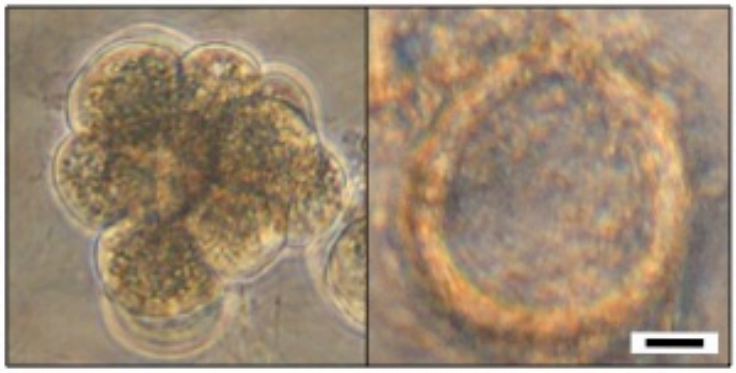
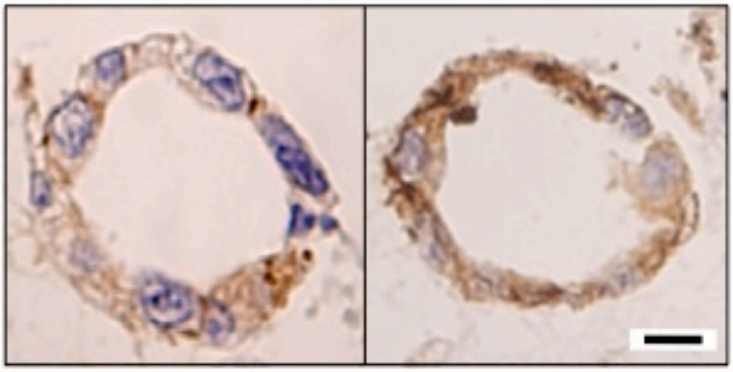


Day 0 Day 5

D



EGFR IHC CK19 IHC

E

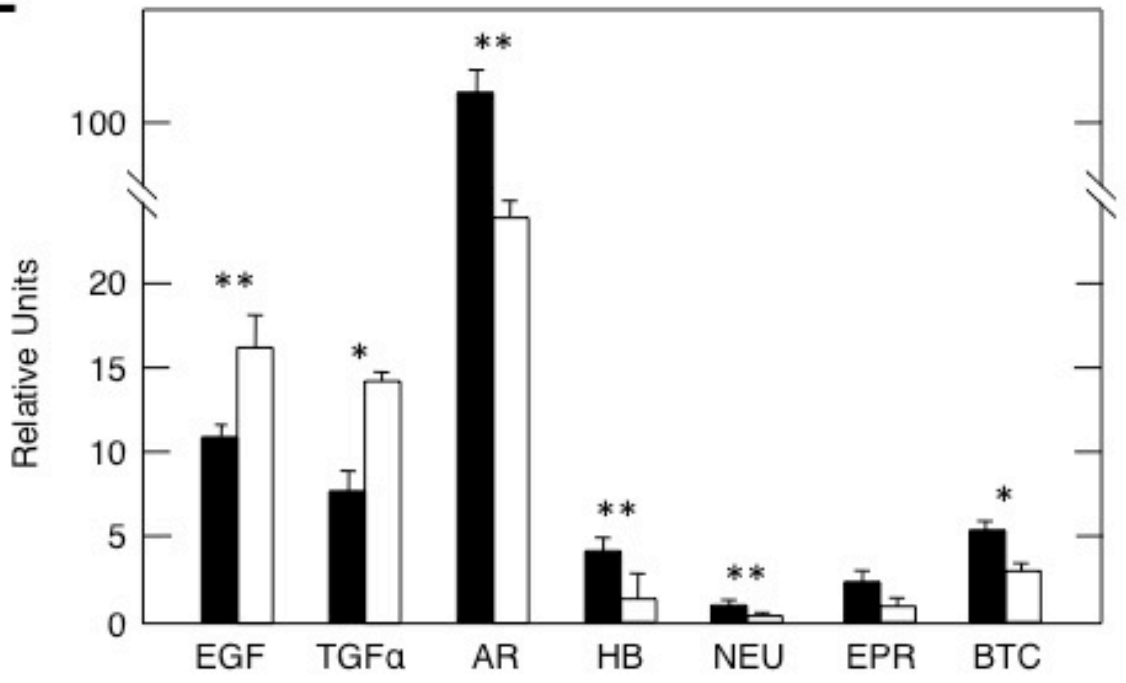


Figure S1, related to Figure 1. Acinar cells expressing an endogenous K-*Ras*^{G12V} oncogene require EGFR signaling to undergo metaplasia *in vitro*

(A) Acinar cell explants isolated from pancreata of untreated 6 to 8 week old *ElasK-Ras*$^{+/+}$ (open bars) or *ElasK-Ras*^{G12V} (solid bars) mice (n=3) were incubated with serum in the presence of the indicated growth factors at a final concentration of 50 ng/ml. Geneticin (75 µg /ml) was added to the *ElasK-Ras*^{G12V} cultures to select for K-Ras^{G12V} expressing acinar cells. The number of metaplasias was determined after five days in culture.

Data shown represent mean ± SD. ** $p < 0.01$.

(B) Acinar explants isolated from pancreata of untreated 6 to 8 weeks old *ElasK-Ras*^{G12V};*Egfr*$^{+/+}$ mice (solid bars) or *ElasK-Ras*^{G12V};*Egfr*$^{lox/lox}$ mice (grey bars) were incubated with serum in the presence of (**top**) EGF and (**bottom**) TGFα at the indicated concentrations. The number of metaplasias figures was determined after five days in culture in the presence of Geneticin (75 µg /ml) (n=3).

Data shown represent mean ± SD. * $p < 0.05$; ** $p < 0.01$.

(C) Representative images of acinar cells explants at the time of plating (day 0) and of the resulting mataplasias (day 5) after five days of incubation. There were no significant morphological differences due to genotype or growth factor treatment.

Scale bar represents 10 µm.

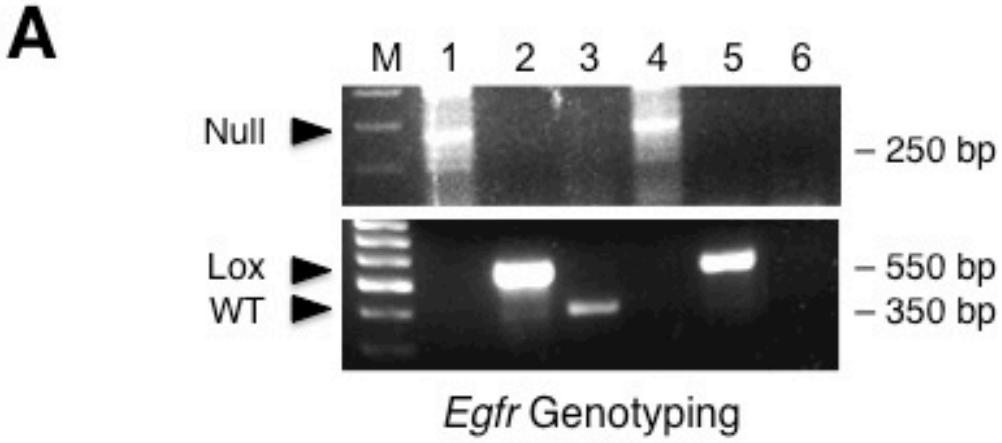
(D) EGFR (EGFR IHC) and Cytokeratin 19 (CK19 IHC) immunostaining of representative metaplasias.

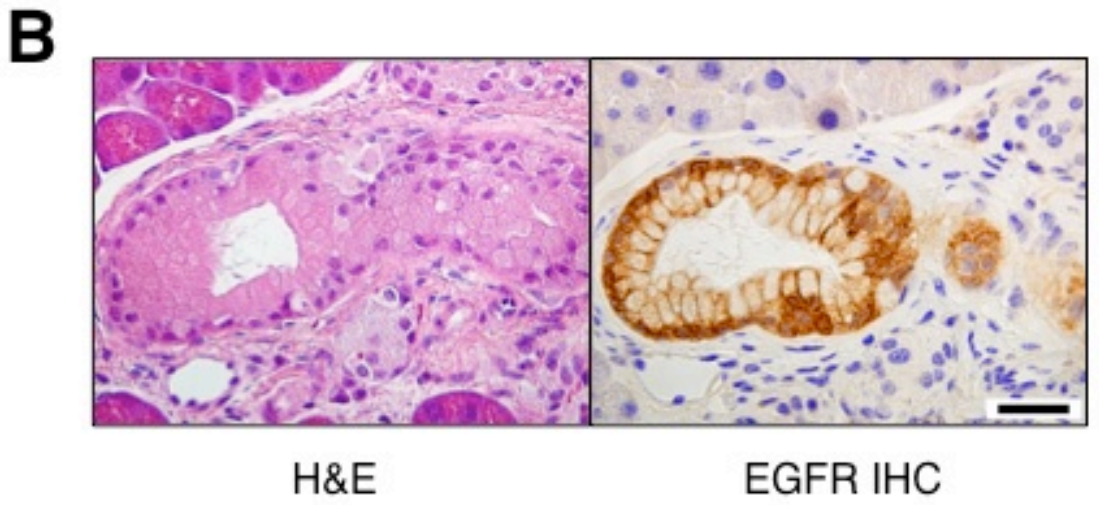
Scale bar represents 10 µm.

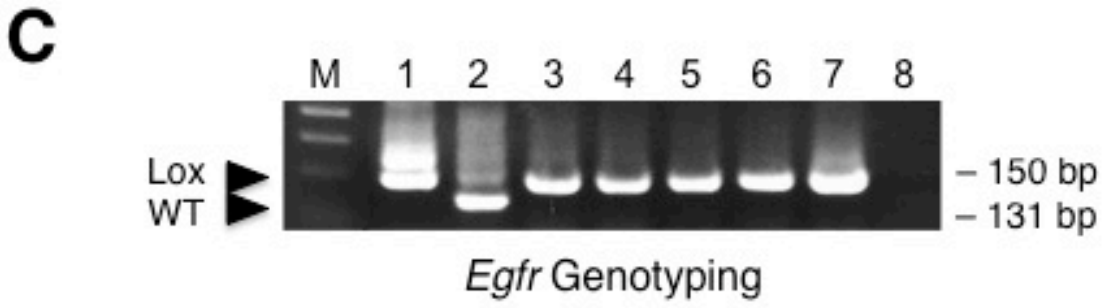
(E) Real-time quantitative PCR analysis of EGFR ligands including epidermal growth factor (EGF), transforming growth factor alpha (TGF-a), amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), neuregulin (NEU), epiregulin (EPR) and betacellulin (BTC) mRNA levels, represented as arbitrary units in isolated acinar cell explants obtained from 6 to 8

weeks old *ElasK-Ras*^{G12V};*Egfr*$^{+/+}$ mice (solid bars) or *ElasK-Ras*^{G12V};*Egfr*$^{lox/lox}$ mice (open bars).

Data shown represent mean ± SD. * $p < 0.05$; ** $p < 0.01$.


A
M 1 2 3 4 5 6
Null
– 250 bp
Lox
WT
– 550 bp
– 350 bp
Egfr Genotyping


B
H&E
EGFR IHC


C
M 1 2 3 4 5 6 7 8
Lox
WT
– 150 bp
– 131 bp
Egfr Genotyping

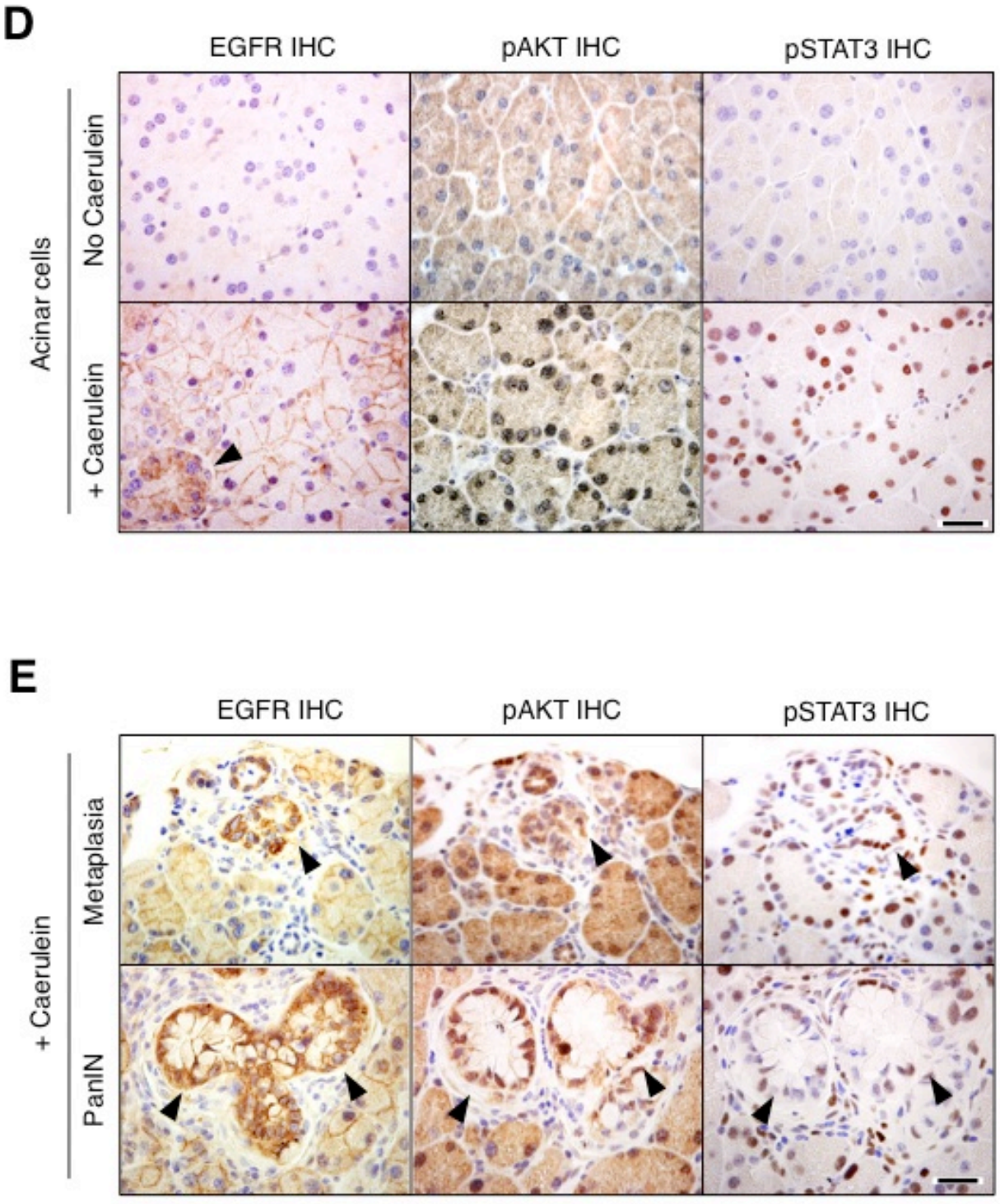


Figure S2, related to Figure 2. Expression of EGFR in acinar cells and PanIN lesions expressing K-*Ras*^{G12V} oncogenes

(A) Cre-recombinase mediated expression ablation of floxed *Egfr* alleles in acinar cells.

PCR analysis of *Egfr* alleles in DNA extracted from:

Lane 1: Control *RERTK-Ras*^{G12V};*Egfr*$^{-/-}$MEFs.

Lane 2: Control *RERTK-Ras*^{G12V};*Egfr*$^{lox/lox}$ MEFs.

Lane 3: Control *RERTK-Ras*^{G12V};*Egfr*$^{+/+}$ MEFs.

Lane 4: X-Gal positive acinar cells from pancreata of *ElasK-Ras*^{G12V};*Egfr*$^{lox/lox}$ mice.

Lane 5: X-Gal negative acinar cells from pancreata of *ElasK-Ras*^{G12V};*Egfr*$^{lox/lox}$ mice.

Lane 6: No DNA.

Lane M: Size markers.

Migration of the amplified DNA bands corresponding to the wild type (WT), floxed (lox) and *null* (null) *Egfr* alleles is indicated by arrowheads. The size of the DNA bands is also indicated.

(B) "Escaper" PanIN lesions present in *ElasK-Ras*^{G12V};*Egfr*$^{lox/lox}$ mice not exposed to doxycycline and positive for EGFR expression.

(Left) H&E staining.

(Right) EGFR IHC of consecutive paraffin sections illustrating a low-grade PanIN.

Scale bar represents 25 μm.

(C) PCR analysis of *Egfr* alleles in DNA extracted from PanIN lesions isolated by laser-capture microdissection.

Lane 1: Control *RERT;K-Ras*^{G12V};*Egfr*$^{lox/lox}$ MEFs.

Lane 2: Control *RERT;K-Ras*^{G12V};*Egfr*$^{+/+}$ MEFs.

Lanes 3-7: Cells from "escaper" PanIN lesions present in *ElasK-Ras*^{G12V};*Egfr*$^{lox/lox}$ mice not exposed to doxycycline displaying unrecombined floxed *Egfr* alleles.

Lane 8: No DNA.

Lane M: Size markers.

Migration of the amplified DNA bands corresponding to the wild type (WT) and floxed (lox) *Egfr* alleles is indicated by arrowheads. The size of the DNA bands is indicated.

(D) Expression of EGFRs (EGFR IHC), phosphorylated AKT (pAKT IHC) and phosphorylated STAT3 (pSTAT3 IHC) in serial paraffin sections of pancreata from *ElasK-Ras*^{G12V} mice not exposed to doxycycline.

(**Upper panel**) one year old *ElasK-Ras*^{G12V} mice

(**Lower panel**) six month old *ElasK-Ras*^{G12V} mice treated with caerulein from P90 to P180.

Scale bar represents 20 μm.

(**E**) Expression of EGFRs (EGFR IHC), phosphorylated AKT (pAKT IHC) and phosphorylated STAT3 (pSTAT3 IHC) in serial paraffin sections of pancreata from *ElasK-Ras*^{G12V} mice exposed to doxycycline from conception to P60 to prevent expression of the resident K-*Ras*^{G12V} oncogene until adulthood and subsequently treated with caerulein from P90 to P180. Mice were sacrificed just after caerulein treatment. Sections were immunostained with the corresponding antibodies.

(Upper panel) Representative metaplasias (solid arrowheads).

(Bottom panel) Representative low-grade PanINs (solid arrowheads).

Scale bar represents 20 μm.

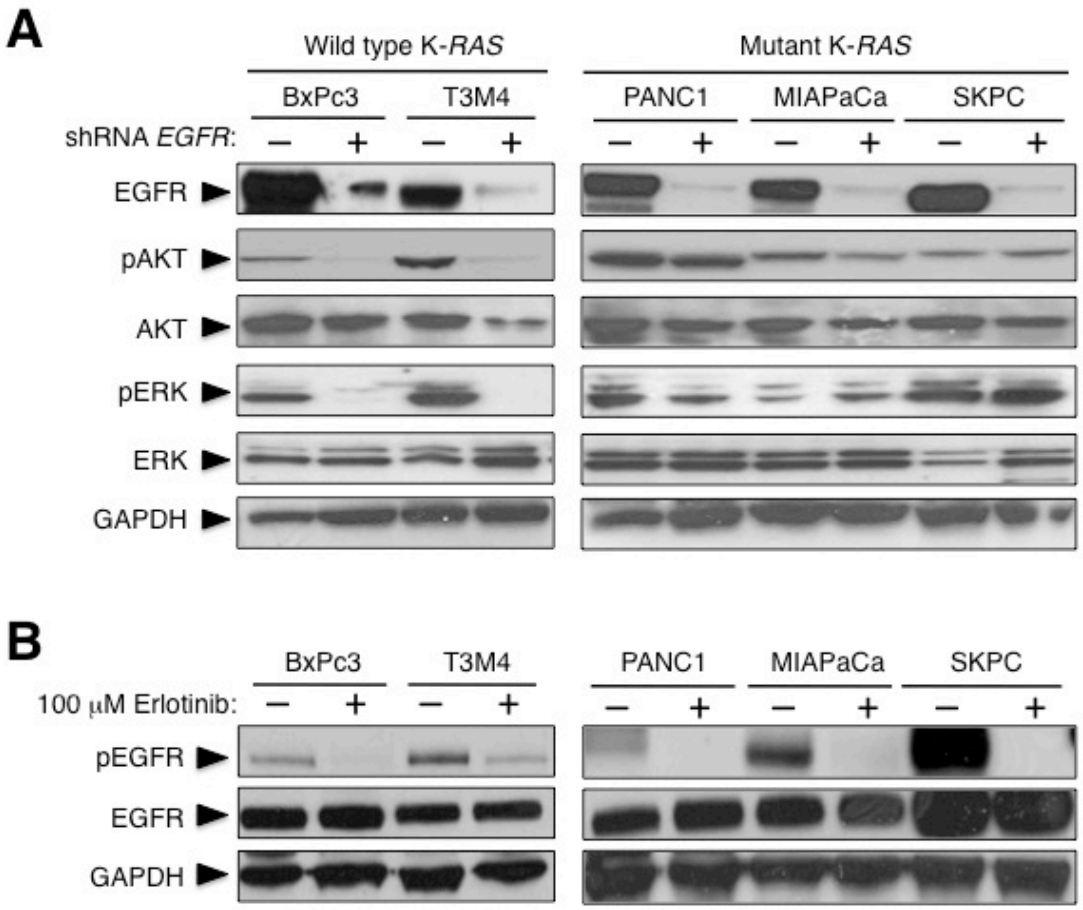


Figure S3, related to Table 1. Inhibition of EGFR signaling in human pancreatic tumor cell lines

(**A**) Western blot analysis of total EGFR (EGFR), phosphorylated AKT (Ser473) (pAKT), total AKT (AKT), phosphorylated ERK1/2 (Thr202/Tyr204) (pERK) and total ERK1/2 (ERK) expression in lysates prepared from human pancreatic tumor cell lines carrying a wild type (BxPc3, T3M4) or a mutated K-*RAS* locus (MIAPaCa, PANC1, SKPC). Cells were infected with lentiviral vectors carrying a control shRNA (–) or a shRNA specific against human *EGFR* sequences (+). Samples were collected 10 days after lentiviral infection.

GAPDH is shown as a loading control.

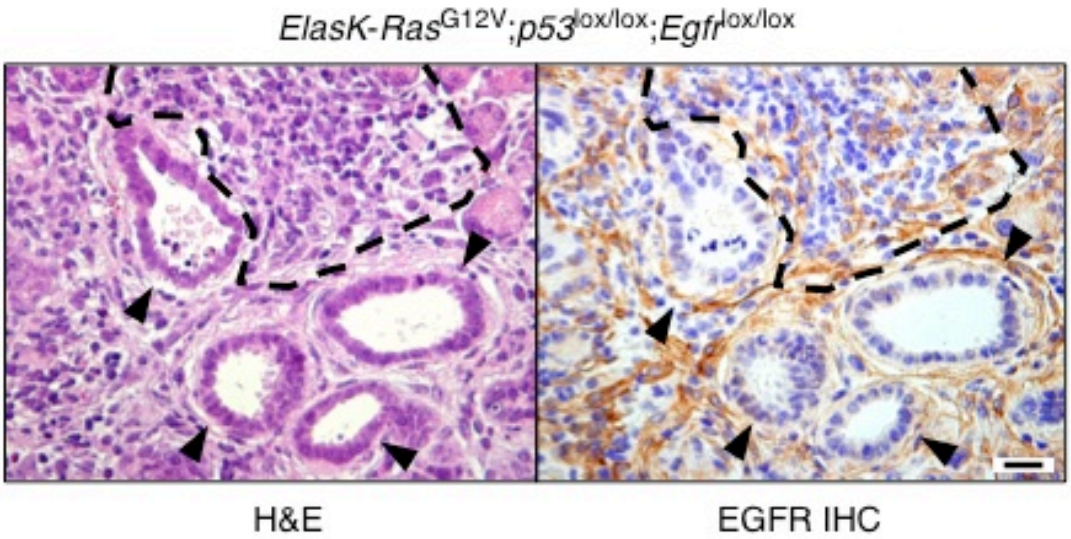
Migration of the corresponding proteins is indicated by arrowheads.

(**B**) Western blot analysis of phosphorylated (pEGFR) and total (EGFR) EGFRs in lysates prepared from the human pancreatic tumor cell lines described above not-treated (–) or treated (+) with 100 μM Erlotinib for 2 hr before harvesting.

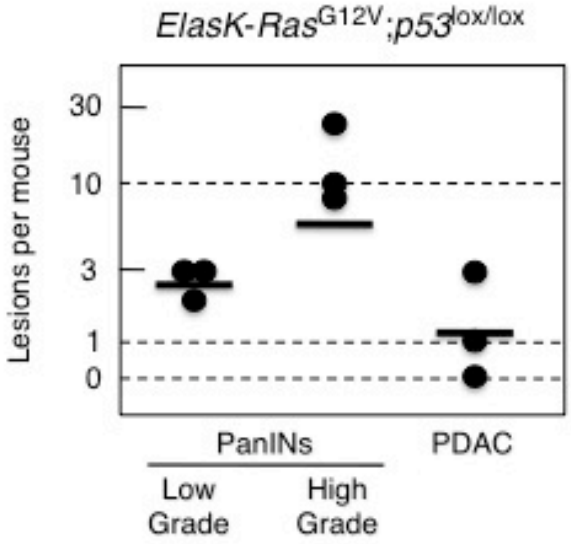
GAPDH was used as a loading control.

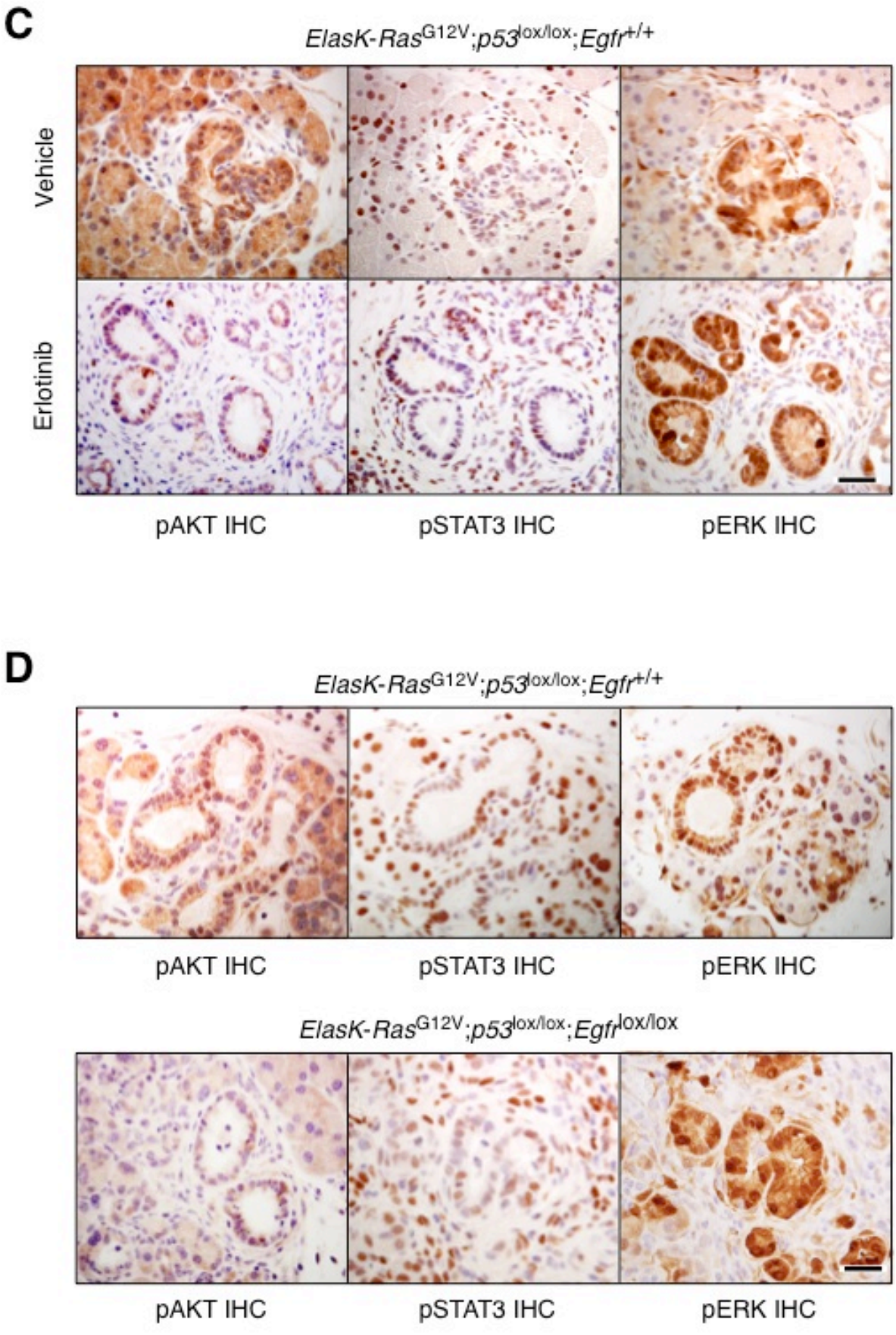
Migration of the corresponding proteins is indicated by arrowheads.

A


ElasK-Ras^{G12V};p53$^{lox/lox}$;Egfr$^{lox/lox}$
H&E
EGFR IHC

B


ElasK-Ras^{G12V};p53$^{lox/lox}$
Lesions per mouse
30
10
3
1
0
PanINs
PDAC
Low Grade
High Grade


C
ElasK-Ras^{G12V};p53$^{lox/lox}$;Egfr$^{+/+}$
Vehicle
Erlotinib
pAKT IHC
pSTAT3 IHC
pERK IHC
D
ElasK-Ras^{G12V};p53$^{lox/lox}$;Egfr$^{+/+}$
pAKT IHC
pSTAT3 IHC
pERK IHC
ElasK-Ras^{G12V};p53$^{lox/lox}$;Egfr$^{lox/lox}$
pAKT IHC
pSTAT3 IHC
pERK IHC

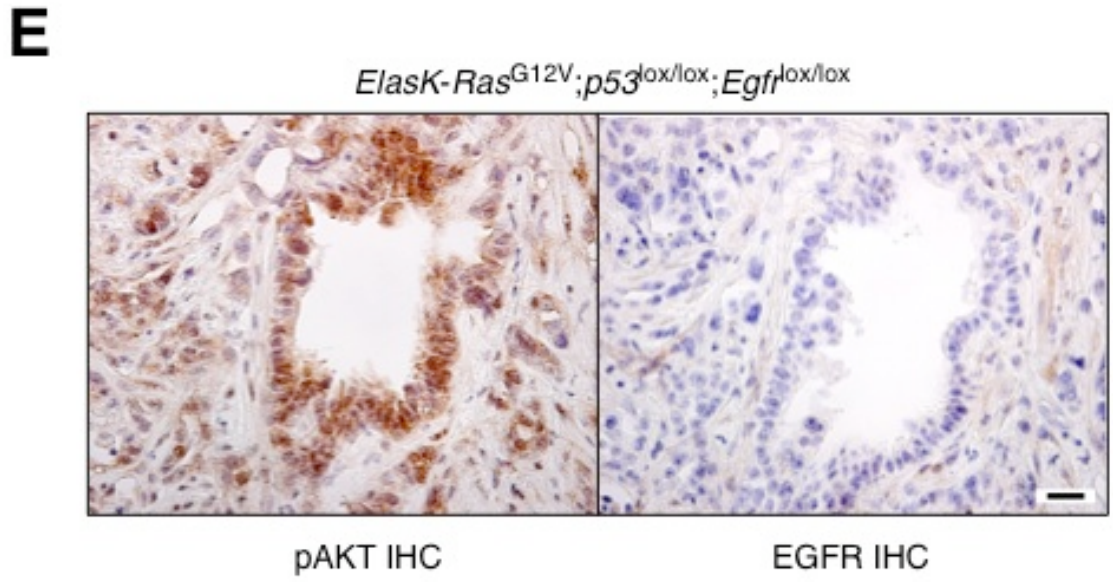


Figure S4, related to Figure 5. EGFR inhibition in PDAC tumors lacking the p53 tumor suppressor

(**A**) H&E and EGFR IHC of consecutive paraffin sections of pancreata from *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$;*Egfr*$^{lox/lox}$ showing representative high-grade PanIN (arrowheads) and PDAC (area within dotted line) lesions. EGFR immunostaining is present in stromal but not in tumor cells.

Scale bar represents 50 μm.

(B) Number of low- and high-grade PanINs and PDACs per mouse in six week old *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$ mice (n=3) sacrificed before Erlotinib treatment (see Figure 5C for results of Erlotinib treatment). Horizontal bars indicate the average number of lesions per mouse.

(**C**) Expression of phosphorylated AKT (pAKT IHC), phosphorylated STAT3 (pSTAT3 IHC) and phosphorylated ERK (pERK IHC) in serial paraffin sections of pancreata from *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$;*Egfr*$^{+/+}$ mice not exposed to doxycycline and treated with either vehicle (**upper panels**) or Erlotinib (**lower panels**) for 4 weeks.

Scale bar represents 20 μm.

(**D**) Expression of phosphorylated AKT (pAKT IHC), phosphorylated STAT3 (pSTAT3 IHC)

and phosphorylated ERK (pERK IHC) in serial paraffin sections of pancreata from either *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$;*Egfr*$^{+/+}$ (**upper panels**) or *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$;*Egfr*$^{lox/lox}$ (**lower panels**) mice not exposed to doxycycline.

Scale bar represents 20 μm.

(**E**) Expression of phosphorylated AKT (pAKT IHC) and EGFRs (EGFR IHC) in consecutive paraffin sections of a representative PDAC tumor obtained from *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$;*Egfr*$^{lox/lox}$ mouse not exposed to doxycycline.

Scale bar represents 50 μm.

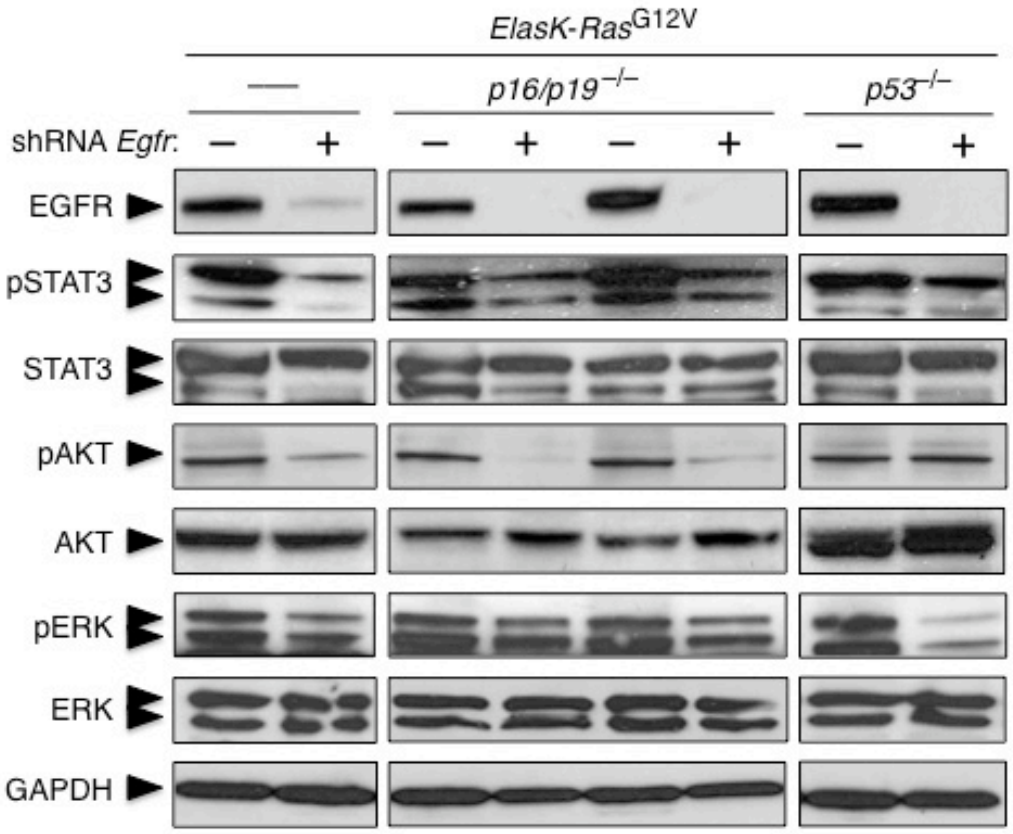


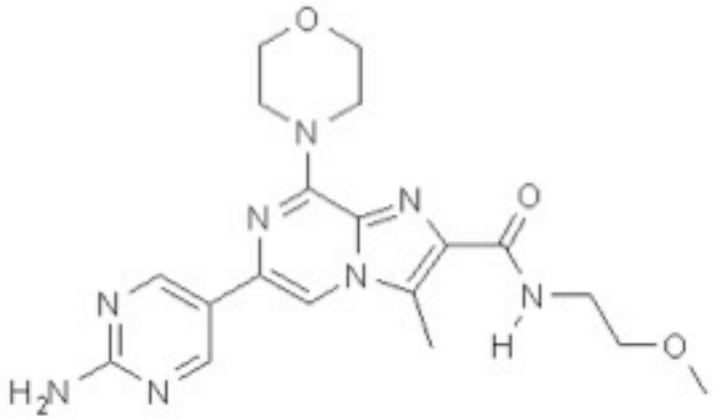
Figure S5, related to Figure 6. Knockdown of EGFR expression in cell explants derived from K-Ras^{G12V}-driven mouse PDAC tumors

Western blot analysis of total EGFR (EGFR), phosphorylated STAT3 (Tyr705) (pSTAT3), total STAT3 (STAT3), phosphorylated AKT (Ser473) (pAKT), total AKT (AKT), phosphorylated ERK1/2 (Thr202/Tyr204) (pERK) and total ERK1/2 (ERK) expression in lysates prepared from explants derived from individual tumors obtained from untreated *ElasK-Ras*^{G12V}, *ElasK-Ras*^{G12V};*p16/p19*$^{lox/lox}$ and *ElasK-Ras*^{G12V};*p53*$^{lox/lox}$ mice. Cells were infected with lentiviral vectors carrying a control shRNA (–) or a shRNA specific against mouse *Egfr* sequences (+). Samples were collected 10 days after lentiviral infection.

GAPDH is shown as a loading control.

Migration of the corresponding proteins is indicated by arrowheads.

A



ETP-46992

B

Target Kinase	IC50
PI3K p110α	1.0 nM
PI3K p110α^{H1047R}	2.3 nM
PI3K p110β	94.1 nM
PI3K p110γ	62.9 nM
PI3K p110δ	8.1 nM
mTOR	3,690.0 nM
DNA PK	484.3 nM

Figure S6, related to Figure 7. Structure and *in vitro* selectivity of the PI3K inhibitor ETP-46992

(**A**) Chemical structure of ETP-46992

(**B**) *In vitro* IC50 concentrations against the indicated human kinases. PI3K p110α^{H1047R} corresponds to the PI3K isoform more frequently mutated in human tumors.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice

Strains of mice used in this study include *ElasK-Ras*^{G12V} (Guerra et al., 2007), *Egfr*^{lox} (Natarajan et al., 2007), *p16/p19*^{lox} (Krimpenfort et al., 2001), *p53*^{lox} (Jonkers et al., 2001), *Apc*^{lox} (Shibata et al., 1997) and *RERT* (Guerra et al., 2003).

Laser-capture microdissection and PCR analysis

Cells were obtained by laser capture microdissection (LCM) using a PALM microbeam Zeiss Axio Observer (Carl Zeiss) from pancreas cryosections (10 μm thick) stained with X-Gal to identify cells expressing (blue staining) and non expressing (not stained) K-*Ras*^{G12V} oncogenes, as previously described (Guerra et al., 2003). Cells from PanIN lesions were also obtained by LCM from paraffin sections (3 μm thick). The cap containing captured cells was mixed with NID buffer (50 mM KCl, 10 mM TrisHCl pH 7.5, 2 mM $MgCl_2$, 0,45% NP40, 0,45% Tween 20) containing 400 μg/ml proteinase K and incubated overnight at 55ºC. Samples were boiled for 10 minutes to inactivate the proteinase K and submitted to PCR amplification in the presence of 1 mM primers, 2 mM Mg, and 0.5 UI Taq polimerase in a total volume of 25 μl. For samples derived from cryosections, *EGFR null* alleles were identified with forward EGFRΔFv (‘5-AGCAGCCTCCCTCCTCTTCTTCC-3’) and reverse EGFRΔRv (‘5-GTTGGGTGAGCCTGTTACTTGTGC-3’) primers at 94ºC for 5 minutes followed by 30 cycles of amplification (94ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 30 seconds), and a final extension at 72ºC for 10 minutes. These primers yielded a diagnostic 250 bp DNA fragment (Figure S2A). To detect the wild type and floxed *EGFR* alleles we used forward EgfrF_15C9 (‘5- CTCTTGACTGCTGCCAACTTAG-3’) and reverse EgfrR_7B9 (‘5-GAGATCTCCACACTTCCAGGTCA-3’) primers at 94ºC for 1 minute followed by 35 cycles of amplification (94ºC for 30 seconds, 60ºC for 30 seconds, 72ºC for 30 seconds) and a final

extension at 72ºC for 7 minutes. PCR amplification with these primers gives DNA products of 350 bp and 550 bp for the wild type and the floxed *EGFR* alleles, respectively. For samples derived from paraffin blocks (500 to 3,000 cells) we used a nested PCR strategy. For the first amplification step, we used the external forward EGFRAMPLFv ('5-TCCATTCAATCCAAAGTCTCTGAG-3') and reverse EGFRAMPLRv ('5-TGTGATGAGGGTCCCTGA-3') primers that yielded DNA products of 234 bp and 253 bp for wild type and floxed *EGFR* alleles, respectively under the PCR conditions described below. 2 µl of these reactions were used as templates for the second round of PCR amplification using the internal forward EGFRFV1 ('5-AGCAGCCCTGACTTTGCA-3') and reverse EGFRRV2 ('5-CTTGGTCCCCTAATTTAGGCC-3) primers to yield 131 bp and 150 bp DNA fragments diagnostic for wild type and floxed *EGFR* alleles, respectively. Cycling conditions for both PCR were 94ºC for 1 minute followed by 35 cycles of amplification (94ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 30 seconds) and extension at 72ºC for 10 minutes.

Acinar to ductal metaplasia assay

Cell explants of normal adult mouse pancreas were established by modification of previously published protocols (De Lisle and Logsdon, 1990; Githens et al., 1994; Wagner et al., 2002). Whole pancreas was harvested and digested in 0.2 mg/ml collagenase-P (Roche) at 37°C. Following multiple washes with Hank's balanced salt solution (HBSS) supplemented with 5% fetal bovine serum (FBS), collagenase P-digested pancreatic tissue was sequentially filtered through 100 µm polypropylene mesh (Spectrum Laboratories, Laguna, CA). The filtrate was passed through a 20% FBS cushion at 1000 rpm. The cellular pellet was re-suspended in RPMI 1640 media (Gibco BRL, Gaithersburg, MD) supplemented with penicillin G (1000 U/ml), streptomycin (100 µg/ml) with 10% FBS. An equal volume of neutralized rat-tail collagen type I (RTC) (Collaborative Biomedical Products, Bedford, MA) was added to the cellular

suspension. The cellular/RTC suspension was supplemented with 0.1 mg/ml soybean trypsin inhibitor (Sigma Chemicals, St Louis, MO) and 1 μg/ml dexamethasone (Sigma). Cellular/RTC suspension (500 μl) was pipetted into each well of a 24 well plate (Corning, Corning, NY) pre-coated with 200 μl of RTC. After solidification of the RTC, media supplemented with penicillin G/streptomycin (100 μg/ml) and FBS (at above mentioned concentrations) was added. Cultures were maintained at 37°C in a 5% CO_2 incubator for 5 days with medium replaced on days 1 and 3. Cell explants derived from *ElasK-Ras*^{G12V} mice were processed as above except that Geneticin was added to the media at a final concentration of 75 μg/ml to select for those cells expressing the resident K-*Ras*^{G12V} oncogene (about 30% of all acinar cells) (Guerra et al., 2007). These K-*Ras*^{G12V} expressing cells can be easily identified by X-Gal staining thanks to the b-galactosidase activity of the Geo protein co-expressed with the K-Ras^{G12V} oncoprotein (Guerra et al., 2003). Cell explants were maintained in the presence or absence of recombinant human TGFα (PePro Tech 100-16A) and/or EGF (Sigma E4127) at the indicated concentrations.

Western Blot Analysis

Thirty micrograms of protein extracts were separated on SDS/PAGE gels (Bio-Rad), transferred to a nitrocellulose membrane and blotted with antibodies raised against AKT (1:250, Cell Signaling Technology; 9272), pAKT (p-Ser473) (1:250, Cell Signaling Technology; 9271), EGFR (1:100, Epitomics; 1902-1), pEGFR (p-Tyr1068) (1:100 Epitomics, 1727-1), ERK (1:500, Santa Cruz Biotechnology; sc-93), pERK (p-Thr202/Tyr204) (1:250 Cell Signaling Technology; 9101), STAT3 (1:250, Cell Signaling Technology; 9132), pSTAT3 (p-Tyr705) (1:250, Cell Signaling Technology; 9131) and GAPDH (1:5000, Sigma; G8795). Primary antibodies were detected with peroxidase-labeled goat anti-rabbit or mouse IgG

(1:1000) for 45 min. All membranes were visualized using ECL and exposure to ECL Hyperfilm (GE Healthcare Amersham).

RNA analysis

Total RNA from mouse PDAC cell explants and from isolated acinar cells was extracted using TRIZOL (Invitrogen) and analyzed by real-time light cycler quantitative polymerase chain reaction (qRT-PCR). Reverse transcription was performed using random primers and Ready-To-GoTM You-Prime First-Strand Beads (GE Healthcare). qRT-PCR was performed using DNA Master Sybr Green I mix (Applied Biosystems) in an ABI PRISM 7700 thermocycler (Applied Biosystem). Primers used in quantitative RT-PCR include:

Locus	Primer	Oligonucleotide sequence	AT(ºC)	Product size
Amphiregulin	Forward Reverse	5′-CTGCTGGTCTTAGGCTCAGG-3′ 5′-TTTCGCTTATGGTGGAAACC-3′	55	145 bp
Betacellulin	Forward Reverse	5′-GCACAGGTACCACCCCTAGA-3′ 5′-GCCCCAAAGTAGCCTTTCTC-3′	60	148 bp
EGF	Forward Reverse	5′-GGGAAAATGTGTCTCCCTCA-3′ 5′-TGTCCCATCATCGTCTGGTA-3	50	120 bp
Epiregulin	Forward Reverse	5′-CGCTGCTTTGTCTAGGTTCC-3′ 5′-GGGATCGTCTTCCATCTGAA-3′	50	122 bp
HB-EGF	Forward Reverse	5′-GACCCATGCCTCAGGAAATA-3′ 5′-AGAGTCAGCCCATGACACCT-3′	50	128 bp
mGAPDH	Forward Reverse	5′-CCCACTAACATCAAATGGGG-3′ 5′-CCTTCCACAATGCCAAAGTT-3′	60	275bp
Neuregulin	Forward Reverse	5′-AGTGTGCGGAGAAGGAGAAA-3′ 5′-TGGCAACGATCACCAGTAAA-3′	60	124 bp
TGFα	Forward Reverse	5′-ATCACCTGTGTGCTGATCCA-3′ 5′-TGTCTCAGAGTGGCAGCAAG-3′	60	129 bp

m*GAPDH* was used as the housekeeping gene for input normalization of all qRT-PCR assays.

Statistical Analyses

All the experiments presented were performed at least 2 times. Student´s t test was used as indicated and a value of $p< 0.05$ or $p<0.01$ was considering as a threshold for significance.

One-way from ANOVA test was used in the case of Erlotinib treatment data and a value of $p<0.05$ was considering as statistically significant.

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response selectively in pancreatic duct cells. Gastroenterology. *122*, 1898-1912.