NEOPLASIA
www.neoplasia.com

Volume 12 Number 6 June 2010 pp. 443–452 443

Oncogenic *KRAS* Desensitizes Colorectal Tumor Cells to Epidermal Growth Factor Receptor Inhibition and Activation[1,2]

Winan J. van Houdt*, Frederik J.H. Hoogwater*, Menno T. de Bruijn*, Benjamin L. Emmink*, Maarten W. Nijkamp*, Danielle A.E. Raats *, Petra van der Groep†, Paul van Diest†, Inne H.M. Borel Rinkes* and Onno Kranenburg*

*Department of Surgery, University Medical Center Utrecht, Utrecht, The Netherlands; †Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands

Abstract

Epidermal growth factor receptor (EGFR)–targeting therapeutics have shown efficacy in the treatment of colorectal cancer patients. Clinical studies have revealed that activating mutations in the *KRAS* protooncogene predict resistance to EGFR-targeted therapy. However, the causality between mutant KRAS and resistance to EGFR inhibition has so far not been demonstrated. Here, we show that deletion of the oncogenic *KRAS* allele from colorectal tumor cells resensitizes those cells to EGFR inhibitors. Resensitization was accompanied by an acquired dependency on the EGFR for maintaining basal extracellular signal–regulated kinase (ERK) activity. Deletion of oncogenic KRAS not only resensitized tumor cells to EGFR inhibition but also promoted EGF-induced NRAS activation, ERK and AKT phosphorylation, and c-FOS transcription. The poor responsiveness of mutant KRAS tumor cells to EGFR inhibition and activation was accompanied by a reduced capacity of these cells to bind and internalize EGF and by a failure to retain EGFR at the plasma membrane. Of 16 human colorectal tumors with activating mutations in *KRAS*, 15 displayed loss of basolateral EGFR localization. Plasma membrane localization of the EGFR could be restored *in vitro* by suppressing receptor endocytosis through Rho kinase inhibition. This caused an EGFR-dependent increase in basal and EGF-stimulated ERK phosphorylation but failed to restore tumor cell sensitivity to EGFR inhibition. Our results demonstrate a causal role for oncogenic KRAS in desensitizing tumor cells not only to EGFR inhibitors but also to EGF itself.

Neoplasia (2010) 12, 443–452

Introduction

The epidermal growth factor receptor (EGFR) is widely expressed in the gastrointestinal tract and stimulates proliferation of a range of cell types, including epithelial cells [1]. Most colorectal tumors are initiated by inactivating mutations in the tumor suppressor gene *APC* [2]. Loss of functional *APC* is sufficient to initiate the formation of intestinal polyps in mice, and this is accompanied by increased EGFR expression and activity [3]. Partial loss of EGFR function, or pharmacological inhibition of the EGFR, greatly reduces polyp development in this model [4]. The EGFR is also frequently overexpressed in human colorectal tumors when compared with normal intestinal tissue, and this is associated with increased metastatic potential and poor prognosis [5–7]. EGFR-targeting therapeutics have shown promising clinical activity in a minority of colorectal cancer patients [8–12]. The presence of activating mutations in the *KRAS* gene in these tumors is a reliable predictor of tumor resistance to anti-EGFR therapy [13,14]. Conversely,

Abbreviations: EGFR, epidermal growth factor receptor; ERK, extracellular signal–regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PI3K, phosphatidylinositol-3-kinase

Address all correspondence to: Onno Kranenburg, PhD, Department of Surgery, University Medical Center Utrecht, Heidelberglaan 100, 3584CX Utrecht, the Netherlands.
E-mail: o.kranenburg@umcutrecht.nl

[1]This work was supported by grants from the Dutch organization for Medical Scientific Research (ZON-Mw no. 920-03-382 and no. 920-03-534 to W.J.v.H. and M.W.N.), the PON Foundation (to F.J.H.H.), the Association for International Cancer Research (no. 07-552 to M.T.d.B.), and the Dutch Cancer Society (no. 2009-4367 and 2003-3711 to B.L.E. and M.W.N.).

[2]This article refers to supplementary materials, which are designated by Figures W1 to W3 and are available online at www.neoplasia.com.

Received 24 December 2009; Revised 17 March 2010; Accepted 18 March 2010

DOI 10.1593/neo.92088

high expression of EGFR ligands predicts response to anti-EGFR therapy but only in the subset of wild-type *KRAS* tumors [15,16]. Although these clinical studies have firmly associated activating mutations in *KRAS* with resistance to EGFR-targeted therapy, so far, it has not been demonstrated that signaling by the KRAS oncoprotein is the underlying cause of resistance to EGFR inhibition. For instance, it is possible that colorectal tumors with *KRAS* mutations preferentially develop in an (epi)genetic background of EGFR independence. Such EGFR independence has previously been shown in a minority of tumors that are driven by *APC* loss only [4]. Constitutive activation of KRAS and its downstream signaling pathways may reduce the dependency on upstream activators such as the EGFR. However, the EGFR activates multiple distinct mitogenic signaling pathways of which the GRB2/SOS/RAS pathway is only one [17]. In addition, activation of the extracellular signal–regulated kinase (ERK) pathway by EGFR ligands is very different in time and amplitude than activation of this pathway by a constitutively active endogenous KRAS mutant protein. For these reasons, we set out to assess the causal relationship between the presence of endogenous oncogenic KRAS and EGFR independence.

Materials and Methods

Cell Culture

The colorectal cancer cell lines HCT116, CT26, and DLD1 were purchased from ATCC (Manassas, VA). The HCT116 cells lacking $KRAS^{D13}$ (HKH2) with their own HCT116 control and the DLD1 cells lacking $KRAS^{D13}$ (DKO4) with their own DLD1 control were obtained from Dr Shirasawa and were previously described [18]. We previously established CT26 cell lines in which the endogenous $Kras^{D12}$ allele is stably suppressed by mutant-specific RNA interference, using a lentiviral vector (CT26-KrasKD) [19]. Control CT26 cells were transduced with a lentiviral short hairpin RNA (shRNA) construct targeting luciferase (see below). All these cell lines were cultured in Dulbecco's modified Eagle medium (DMEM; Dulbecco, ICN Pharmaceuticals, Zoetermeer, The Netherlands) supplemented with 5% (vol./vol.) fetal calf serum, 2 mM glutamine, 0.1 mg/ml streptomycin, and 100 U/ml penicillin. L145 cells were derived directly from a tumor biopsy of a patient operated on for colorectal liver metastases in our hospital. The tissue fragment was washed with PBS and was mechanically dissociated. Enzymatic digestion (thermolysin [Sigma, St Louis, MO] 0.05% for 2 hours at 37°C) was performed in DMEM/F12. Single-cell suspensions were obtained by filtering through a 40-μm-pore size nylon cell strainer (BD Falcon, Breda, The Netherlands). Spheroids formed spontaneously by culturing in DMEM/F12 (Gibco, Breda, The Netherlands) supplemented with 0.6% glucose (BDH Laboratory Supplies, Soulbury, UK), 2 mM L-glutamine (Biowhittaker, Walkersville, MD), 9.6 μg/ml putrescin (Sigma), 6.3 ng/ml progesterone (Sigma), 5.2 ng/ml sodium selenite (Sigma), 25 μg/ml insulin (Sigma), 100 μg/ml apotransferrin (Sigma), 5 mM HEPES (Gibco), 0.005 μg/ml trace element A (Cellgro, Manassas, VA), 0.01 μg/ml trace element B (Cellgro), 0.01 μg/ml trace element C (Cellgro), 100 μM β-mercaptoethanol (Merck, Schiphol, The Netherlands), 10 ml of antibiotic-antimycotic (Gibco), 4 μg/ml gentamicin (Invitrogen, Molecular Probes, Leiden, The Netherlands), 0.002% lipid mixture (Sigma), 5 μg/ml glutathione (Roche, Woerden, The Netherlands), and 4 μg/ml heparin (Sigma). The human intestinal epithelial cells (HIECs) were a kind gift from Dr Beaulieu, and these have been described before [20]. All cells were kept at 37°C in a humidified atmosphere containing 5% CO_2.

Antibodies and Inhibitors

The following antibodies were from Cell Signaling Technology, Inc, Leiden, The Netherlands: rabbit anti–phospho–mitogen-activated protein kinase (MAPK) p44/42 (Thr 202/Tyr 204), rabbit anti–phopsho-Akt (Ser 473), rabbit anti-EGFR (no. 2232; used for HCT116 and HKH2 cells), and rabbit anti–phospho-EGFR antibodies (Tyr 845, 992, 1068, 1045; nos. 2231, 2234, 2235, and 2237). EGFR phosphorylation status was determined by probing the Western blots with a mixture of antibodies 2231, 2234, 2235, and 2237. For EGFR detection in mouse CT26 and CT26-KrasKD cells, we used rat anti-mouse EGFR (clone 176436; R&D Systems, Minneapolis, MN). Goat anti-Akt1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary peroxidase-conjugated antibodies were from Dako (Glostrup, Denmark).

Gefitinib (Iressa/ZD1839) was kindly provided by AstraZeneca (Macclesfield, United Kingdom). Cetuximab was kindly provided by Merck (Darmstadt, Germany). Erlotinib (Tarceva/OSI1774) was purchased from LC Laboratories (Woburn, MA). The MAPK/ERK kinase (MEK) inhibitor U0126 was from Promega (Madison, WI), the phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002 and the Rho kinase (ROK) inhibitor Y27632 were from Sigma, and the Rac inhibitor (Rac1-Inh) was from Calbiochem (Darmstadt, Germany).

Viability Assay

Cells were plated at a density of 5000 cells/well in DMEM containing 5% fetal calf serum in 96-well plates. Cell viability of treated or mock-treated cells was then analyzed for three to six consecutive days by standard 3-(4,5 dimethylthiazolyl-2)-2,5-diphenyltetrazoleumbromide (MTT) assays (Roche Diagnostics, Almere, The Netherlands) according to the manufacturer's instructions.

Analysis of EGF Signaling

Cells were plated at day 0 at a density of 200,000 cells/well on six-well plates. After an overnight incubation in serum-free medium, the cells were stimulated with the indicated concentrations of EGF and were harvested after the indicated periods of stimulation. Western blot analysis was performed according to standard procedures. Quantity One (Bio-Rad, Hercules, CA) software was used to quantify the signal intensities and ratios between samples loaded on the same gel.

Ras Assay

The RAS-binding domain of RAF1 fused to glutathione-*S*-transferase and coupled to glutathione-sepharose was used as an affinity matrix for activated RAS. The assay and subsequent Western blot analysis with isoform-specific antibodies were performed exactly as described [21].

Reverse Transcription–Polymerase Chain Reaction

Cells were plated at day 0 at a density of 2.10^6 cells on 10 cm disks. After overnight serum starvation, cells were stimulated with EGF and reverse transcription–polymerase chain reaction was performed. RNA was isolated using the TRIzol method, and complementary DNA was synthesized by Superscript 2 (Invitrogen). The primers used were as follows:

c-FOS forward: 5′-GTCTTCACCACCATGGAG-3′ and
c-FOS reverse: 5′-CCACCTTCTTGATGTCATC-3′,
GAPDH forward: 5′-CCTACCCAGCTCTGCTTCAC-3′, and
GAPDH reverse: 5′-GTGGGAATGAAGTTGGCACT-3′.

Live Cell Imaging

Cells were seeded in a Lab-Tek Chambered no. 1.0 Borosilicate Coverglass System (Nalge Nunc International, Rochester, NY) and were mounted on a Zeiss Axiovert 200M microscope (Carl Zeiss Microimaging, Inc, Sliedrecht, The Netherlands) for live cell imaging under 5% CO_2 at 37°C overnight. LysoTracker and Alexa 488–labeled EGF (Invitrogen) were added to the wells, and images were captured every 10 seconds using a Photometrics CoolSNAP HQ charge-coupled device camera (Scientific, Tucson, AZ). Images were processed using MetaMorph software (Universal Imaging, Downingtown, PA).

Immunofluorescence

Cells were grown on glass coverslips and either were stimulated with EGF or were left unstimulated. Cells were fixed by the addition of 3.7% formaldehyde to the culture medium for 10 minutes. Cells were then permeabilized with 0.05% Triton X-100 in 1% PBS/BSA. Coverslips were blocked in PBS with 3% BSA for 1 hour. Anti-EGFR primary antibodies were incubated at room temperature overnight. Secondary antibodies were incubated for 1 hour at room temperature. Coverslips were mounted using Vectashield with 4′,6-diamidino-2-phenylindole (H-1200; Vector Labs, Burlingame, CA). Confocal images were acquired using a Zeiss Meta Axiovert 200M confocal microscope (Carl Zeiss, Microimaging, Inc), with a 40× 1.3 NA objective.

Tissue Microarray

A tissue microarray (TMA) was used to analyze the expression of EGFR in colorectal tumors. The TMA was constructed as previously described in detail [22]. In brief, samples from surgical resections of 55 patients with colorectal cancer with known KRAS mutation status were selected for the TMA. For each sample, three different cylindrical tissue cores were included in the TMA. Immunostaining was performed using standard procedures. After incubating with 3% hydrogen peroxide, antigen retrieval was achieved by boiling in 10 mM citrate buffer pH 6.0. Sections were blocked with 5% goat serum in TBS and incubated with a rabbit α-EGFR (no. 2232; Cell Signaling Technology, Inc) at 4°C overnight. The HRP-conjugated secondary antibody (DPVM-55HRP; Immunologic, Duiven, the Netherlands) was detected with 3,3′-diaminobenzidine substrate (D4418; Sigma). Slides were counterstained with hematoxylin, were rinsed with water, dehydrated in ethanol, cleared in xylene, and mounted on coverslips. The intensity and localization of EGFR staining and the percentage of positive cells were determined in each separate tissue core by two independent experienced observers blinded to the cores' identities. Staining intensity was scored as follows: 0 = negative, 1 = weak, 2 = moderate, 3 = strong, 4 = very strong. The percentages of positive cells were categorized as follows: less than 1% as 0, 1% to 25% as 1, 25% to 50% as 2, and greater than 50% as 3. The staining coefficient was determined as the product of the staining intensity and the percentage category, so with a maximum score of 12. The EGFR antibody was validated by Western blot analysis using lysates of colorectal cancer cells and by immunohistochemistry on formalin-fixed paraffin-embedded sections of normal human colon and skin tissue. The staining and scoring procedures were independently repeated by a third independent observer.

Statistical Analysis

Differences between the distinct treatment groups were evaluated using the Student's *t* test. Asterisks indicate statistical significance on the basis of two-tailed analyses of the data sets. Differences with $P < .05$ were considered statistically significant.

Results

Loss of Oncogenic KRAS Sensitizes Tumor Cells to EGFR Inhibition

To assess the causal relationship between oncogenic *KRAS* and *EGFR* independency, we made use of colorectal cancer cell lines with an activating mutation in *KRAS* (HCT116, DLD1, and CT26) and their isogenic derivatives lacking oncogenic *KRAS* (HKH2, DKO4, and CT26-KrasKD) [18,19]. Treatment of HCT116 and HKH2 cells with the anti-EGFR antibody cetuximab or with the small-molecule inhibitors gefitinib or erlotinib had no effect on parental (KRAS-mutant) HCT116 cells but strongly reduced cell proliferation of KRAS–wild-type HKH2 cells (Figure 1*A*). In addition, cetuximab only marginally affected DLD1 cell proliferation but strongly inhibited DKO4 proliferation (Figure 1*B*). Likewise, gefitinib had no effect on control CT26 cells, but it suppressed cell proliferation on knockdown of the mutant *KRAS* allele (Figure 1*C*). Cetuximab could not be used in this cell system because it does not recognize mouse EGFR. Gefitinib and cetuximab also strongly reduced the proliferation of primary HIECs [20] expressing wild-type *KRAS*. In contrast, neither gefitinib nor cetuximab had any effect on the proliferation of freshly isolated colorectal cancer stem cells with an activating mutation in *KRAS* (L145; $KRAS^{D12}$; Figure 1*D*). In all these cases, the cells expressing mutant *KRAS* were significantly more resistant to cell growth inhibition by EGFR-targeting therapeutics than their isogenic KRAS-deleted/suppressed counterparts (Figure W1). Cell death was not observed in any of the previously mentioned experiments. These results suggest that oncogenic *KRAS* is causally involved in reducing tumor cell dependency on EGFR activity.

Oncogenic KRAS Reduces EGFR Control of ERK Phosphorylation

The classic RAS/RAF/MEK/ERK pathway is one of the mitogenic signaling pathways that is activated in response to EGFR stimulation. We first assessed the contribution of EGFR activity to basal levels of ERK phosphorylation. Treatment of cells lacking oncogenic *KRAS* (HKH2, CT26-KrasKD, and HIEC) with either cetuximab or gefitinib caused a strong decrease in the levels of basal ERK phosphorylation, which corresponds with the observed reduction in cell proliferation (Figures 2, *A–C*, and W1). In contrast, basal ERK phosphorylation was unaffected in tumor cells expressing mutant *KRAS* (Figure 2, *A–C*). Thus, EGFR is a major determinant of basal ERK phosphorylation in the absence but not in the presence of oncogenic *KRAS*.

EGF stimulation of HCT116 and HKH2 cells showed that the immediate early response gene *c-FOS* was readily induced in HKH2 cells but not in HCT116 cells (Figure 3*A*). Oncogenic *KRAS* strongly suppressed EGF-stimulated activation of ERK phosphorylation (Figure 3*B*). Because NRAS is an efficient activator of the ERK pathway, we examined how oncogenic *KRAS* affected EGF stimulation of (wild-type) NRAS activity. EGF strongly stimulated NRAS activity in cells expressing wild type or no KRAS but not in the parental cells expressing oncogenic *KRAS* (Figure 3*C*). This suggests that the desensitizing effect of oncogenic *KRAS* on EGF-stimulated ERK pathway activation lies upstream of RAS activation. Indeed, receptor-independent ERK pathway activation at the level of RAF by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate was unaffected, or even more pronounced, in the presence of oncogenic *KRAS* (Figure 3*D*). We conclude that oncogenic *KRAS* strongly reduces the impact of both EGFR inhibition and activation on ERK phosphorylation.

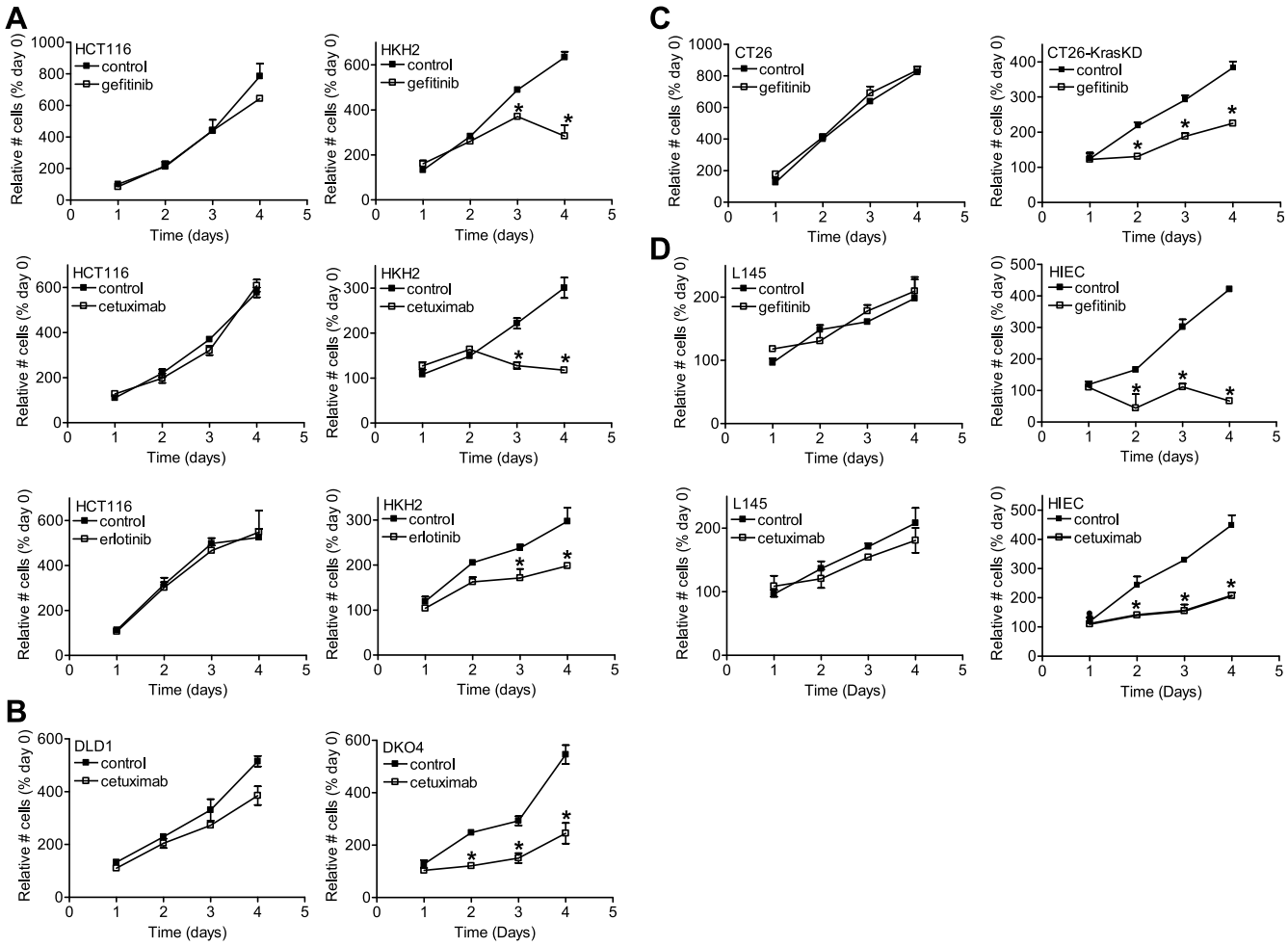

Figure 1. Deletion of oncogenic *KRAS* sensitizes colorectal tumor cells to EGFR inhibition. (A) HCT116 and HKH2 cells were seeded in 96-well plates and were treated with cetuximab (20 μg/ml), erlotinib (5 μM), or gefitinib (2 μM) for four consecutive days in triplicate. Mitochondrial activity was determined by MTT assays. (B) The experiment was performed as in panel A, using cetuximab (20 μg/ml) to treat DLD1 and DKO4 cells. (C) The experiment was performed as in panel A, using gefitinib (2 μM) to treat CT26 cells expressing luciferase-targeting shRNA (CT26) and CT26 cells in which endogenous $Kras^{D12}$ is stably suppressed by RNAi [19]. (D) The experiment was performed as in panel A, using cetuximab (20 μg/ml) or gefitinib (2 μM) on L145 cells. L145 cells were freshly established from a liver metastasis harboring a $KRAS^{D12}$ mutation. Primary human epithelial cells (HIEC [20]) express only wild-type *KRAS*. *Statically significant differences, $P < .05$.

Next, we tested whether oncogenic *KRAS* selectively suppressed EGF-stimulated ERK pathway activation or whether other pathways were suppressed as well. Tyrosine-phosphorylated (activated) EGFR binds the p85 subunit of PI3K, which results in the activation of AKT. EGF stimulation of *KRAS* wild-type and mutant cells shows that this pathway is also strongly suppressed by oncogenic *KRAS* (Figure 3*E*).

KRAS Alters EGFR Localization and Reduces EGF Binding and Internalization

The previously mentioned results suggest that oncogenic *KRAS* desensitizes cells to EGF at the level of the EGFR. Indeed, basal and EGF-stimulated EGFR phosphorylation was strongly impaired in the presence of oncogenic *KRAS* (Figure 4*A*). Live cell imaging using Alexa 488–labeled EGF showed that HKH2 cells readily bound and internalized EGF. A large proportion of EGF ended up in the lysosomes as expected (Figure 4*B*). In contrast, whereas parental HCT116 cells express EGFR (Figure 4*A*), they bound and internalized far less fluorescent EGF, although some internalization could still be observed (Figure 4*B*). Therefore, we examined the localization of the EGFR by immunofluorescence analysis. Strikingly, in parental HCT116 cells, most of the EGFR were found in intracellular vesicles (Figure 4*C*), and their distribution did not change after receptor stimulation (Figure 4*D*). In HKH2 cells, however, the EGFR predominantly localized to the plasma membrane (Figure 4*C*) and was internalized after stimulation with EGF (Figure 4, *B* and *D*), or after re-expression of $KRAS^{G13D}$ (Figure W2). In line with these results, prolonged stimulation with EGF, or with cetuximab, caused down-regulation of the EGFR in cells expressing wild-type *KRAS* but not in cell expressing mutant *KRAS* (Figure 4*E*). Also, in the CT26 cell system, we found that suppression of the oncogenic $Kras^{D12}$ allele restored plasma membrane localization of the EGFR (Figure 5*A*). Stimulation of CT26 cells did not affect intracellular localization of the EGFR. However, stimulation of CT26-KrasKD cells caused EGFR clustering and internalization, similar to what was observed in HKH2 cells (Figure 5*A*). In line with these results, CT26-KrasKD cells, but not CT26 cells, efficiently internalized fluorescent EGF (Figure 5*B*).

Next, we tested whether the relationship between oncogenic *KRAS* and altered EGFR localization was also observed in human colorectal tumors. To this end, we analyzed EGFR localization in a panel of 55

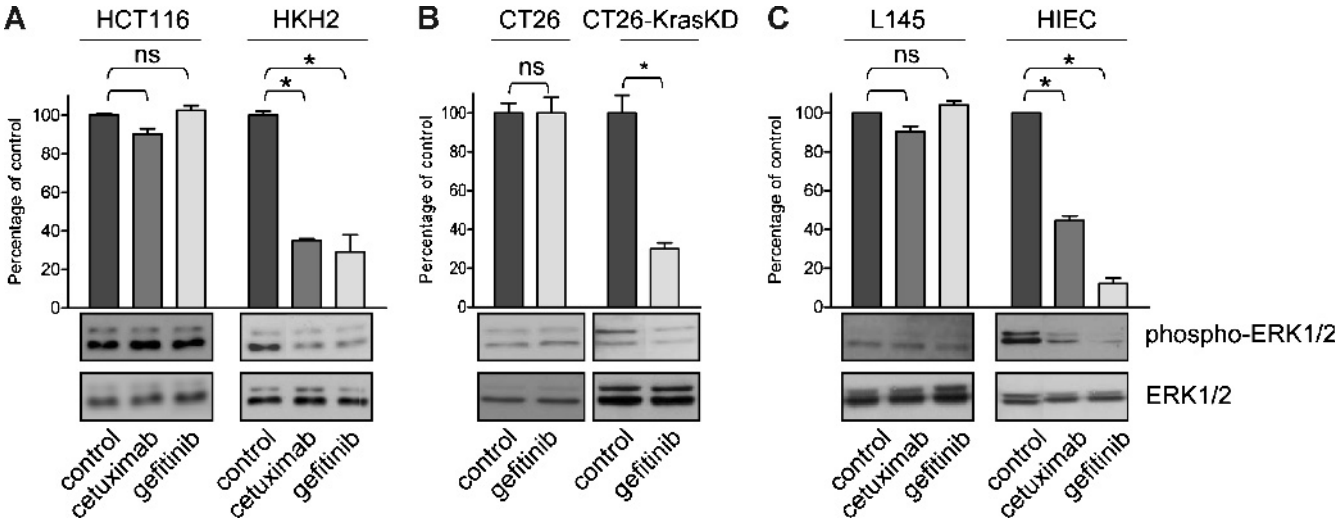

Figure 2. EGFR activity is required for maintenance of ERK phosphorylation in wild-type *KRAS* cells but not in mutant *KRAS* cells. (A) HCT116 cells and HKH2 cells were treated overnight with the indicated EGFR inhibitors, and phosphorylated and total ERK levels were assessed by Western blot analysis. Bar diagrams represent means and SEM of three independent experiments. (B) As in panel A, using CT26 cells expressing luciferase-targeting shRNA (CT26) and CT26-KrasKD. (C) As in panel A, using L145 cells and HIEC. *Statically significant differences, $P < .05$.

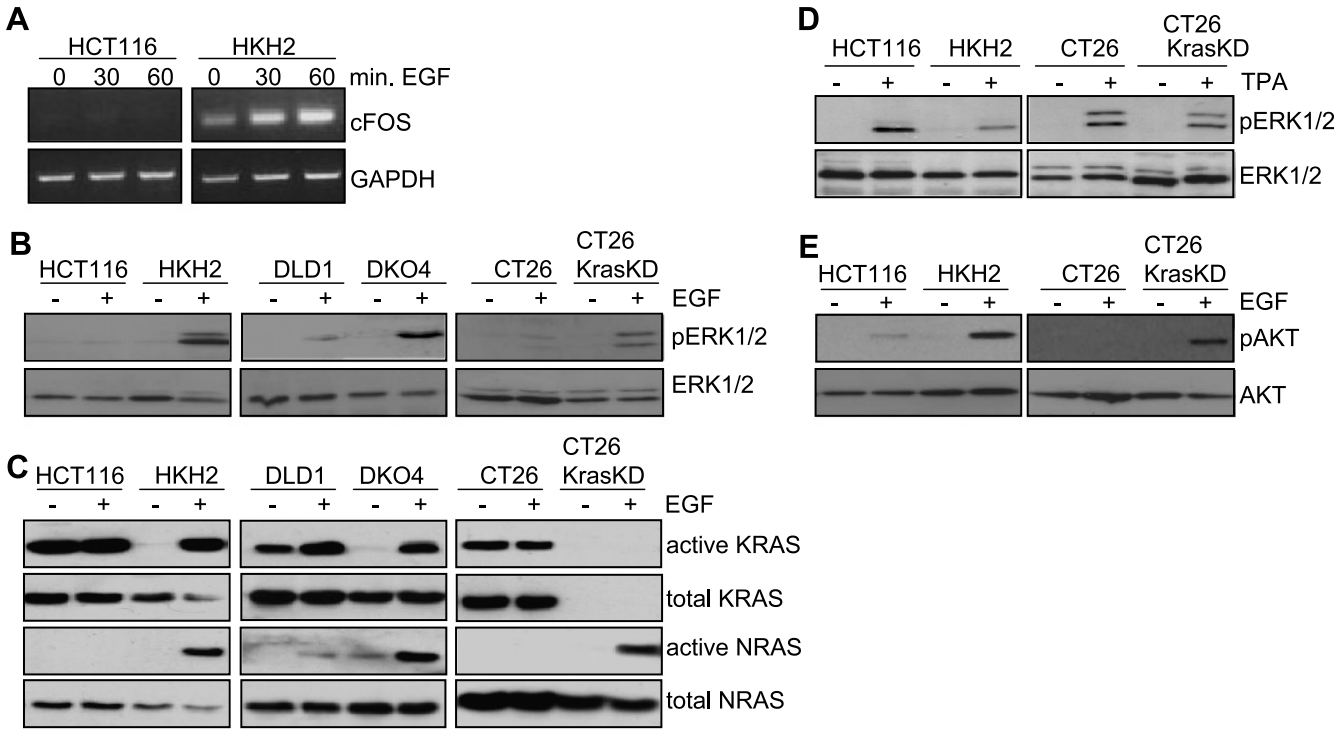

Figure 3. Oncogenic *KRAS* suppresses EGFR signaling. (A) Serum-starved HCT116 and HKH2 cells were stimulated with 20 ng/ml EGF for 0, 30, and 60 minutes. c-*FOS* messenger RNA levels were determined using reverse transcription–polymerase chain reaction. (B) Serum-starved HCT116, DLD1, and CT26 cells and their isogenic derivatives lacking oncogenic *KRAS* (HKH2, DKO4, and CT26-KrasKD) were stimulated with 20 ng/ml EGF for 5 minutes. The levels of phosphorylated and total ERK were determined by Western blot analysis. (C) Cells were cultured and stimulated as in panel B. Ras activity assays were performed using the Ras-binding domain (RBD) of Raf1 fused to glutathione-*S*-transferase immobilized on glutathione-sepharose. Lysates and RBD-Raf1–bound proteins were analyzed for the presence of NRAS and KRAS by Western blot analysis. (D) Cells were cultured as previously mentioned and stimulated with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (5 nM) for 5 minutes. The levels of phosphorylated and total ERK were determined by Western blot analysis. (E) Cells were cultured as previously mentioned and stimulated with 20 ng/ml EGF for 5 minutes. The levels of phosphorylated and total AKT were determined by Western blot analysis.

colorectal carcinomas with known *KRAS* mutation status using antibody no. 2232 from Cell Signaling Technology (Figures 6 and W3). Of these tumors, 12 showed normal basolateral EGFR staining, 32 showed diffuse staining throughout the tumor cells, and 11 were negative (Figure 6, *A* and *B*). In this panel, 16 tumors had an activating mutation in *KRAS*. Strikingly, only 1 of these tumors displayed normal basolateral staining, 10 displayed diffuse staining, and 5 had lost EGFR staining altogether (Figure 6*B*). A χ^2 test revealed that the unequal distribution of *KRAS* mutations in tumors with basolateral EGFR staining (1/12) *versus* tumors with either negative or diffuse staining (15/43) showed a trend toward statistical significance ($P = .073$). This result suggests that *KRAS* mutations may be associated with loss of normal basolateral EGFR localization in human colorectal tumors, which could influence the responsiveness of these tumors to EGFR ligands.

ROK Inhibition Restores EGFR Plasma Membrane Localization and EGF Signaling but Not EGFR Dependency

The previously mentioned results suggest that desensitization of HCT116 and CT26 cells to EGF could be due to the loss of EGFR localization from the plasma membrane. We reasoned that inhibition of *KRAS* signaling could restore EGFR plasma membrane localization. Therefore, we treated HCT116 cells with inhibitors targeting MEK, Rac, PI3K, and ROK and evaluated the response of these cells to EGF.

PI3K or Rac inhibition completely abolished AKT (but not ERK) phosphorylation, but it did not sensitize the tumor cells to EGF. Likewise, MEK inhibition abolished ERK (but not AKT) phosphorylation, but it did not sensitize the tumor cells to EGF. However, inhibition of ROK sensitized cells to EGF-stimulated phosphorylation of ERK1/2 and AKT, albeit not as efficiently as after deletion of mutant *KRAS* (Figure 7, *A* and *B*). ROK inhibition also restored EGF-induced tyrosine phosphorylation of the EGFR (Figure 7*C*). Furthermore, immunofluorescence analysis showed that the sensitization of HCT116 cells to EGF by ROK inhibition was accompanied by restoration of EGFR localization to the plasma membrane (Figure 7*D*). Next, we tested whether restoration of EGF signaling in ROK-inhibited cells would be accompanied by a newly acquired dependency on EGFR signaling. To this end, HCT116 cells were treated with cetuximab or gefitinib and the ROK inhibitor Y27632, either alone or in combination. Figure 7*E* shows that the proliferation of HCT116 cells was not significantly affected by treatment with the drugs, either alone or in combination,

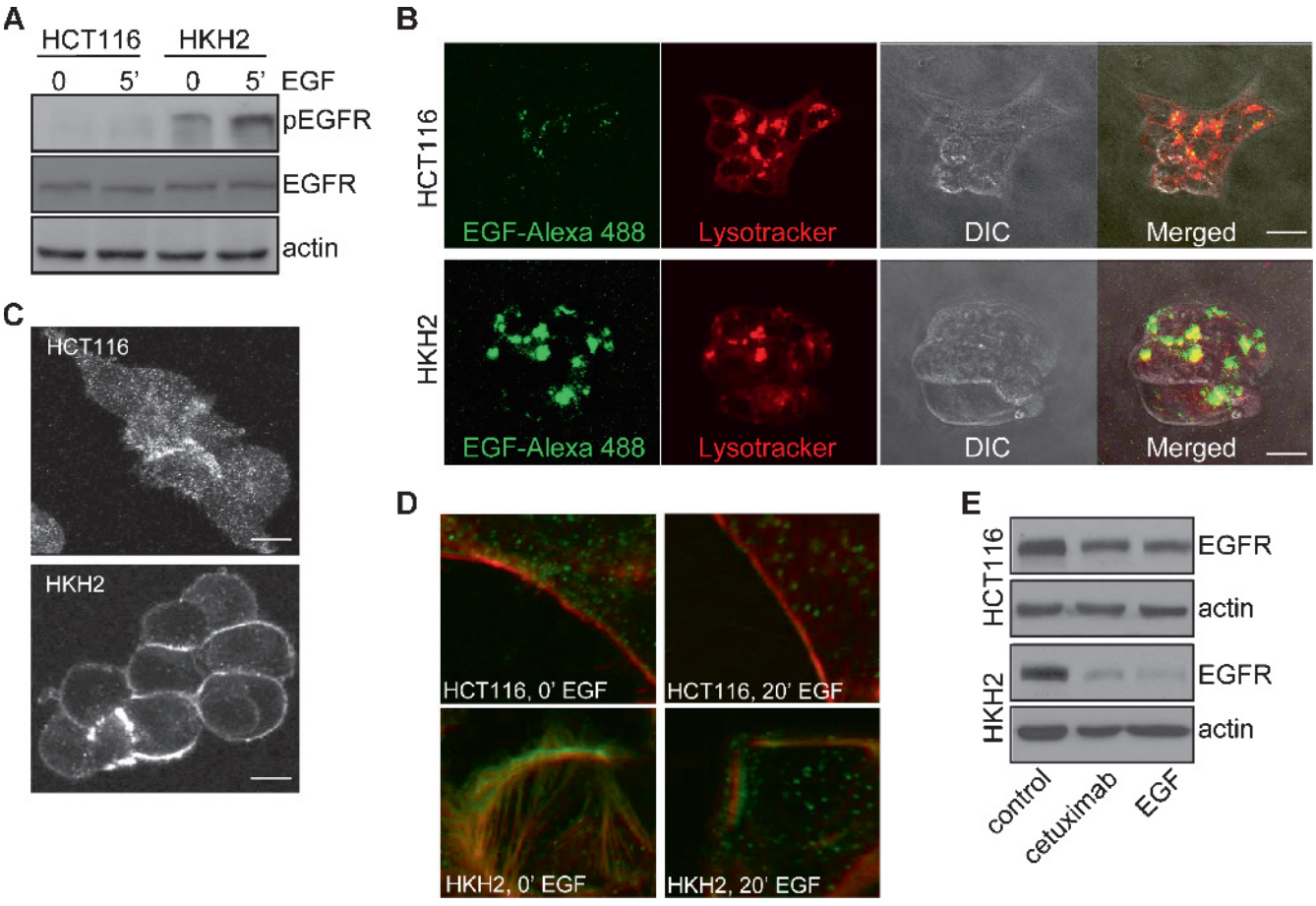

Figure 4. Aberrant EGFR localization and reduced EGF internalization in HCT116 cells. (A) Serum-starved HCT116 and HKH2 cells were stimulated with 20 ng/ml EGF for 0 or 5 minutes. Total and phosphorylated EGFR levels were determined by Western blot analysis. (B) Serum-starved HCT116 and HKH2 cells were stimulated with Alexa 488–conjugated EGF (30 ng/ml; 20 minutes) in the presence of LysoTracker. The uptake of fluorescent EGF and its trafficking to lysosomes were analyzed by live cell imaging. Final images are shown. (C) HCT116 cells were grown on glass coverslips, and EGFR localization was studied by immunofluorescence analysis. (D) Serum-starved HCT116 cells were stimulated with 20 ng/ml EGF (0 or 20 minutes). EGFR (green) and F-actin (red) distributions were then analyzed by immunofluorescence. (E) HCT116 cells were incubated overnight with EGF (20 ng/ml) or cetuximab (20 μg/ml) under serum-free conditions. EGFR and actin levels were determined by Western blot analysis.

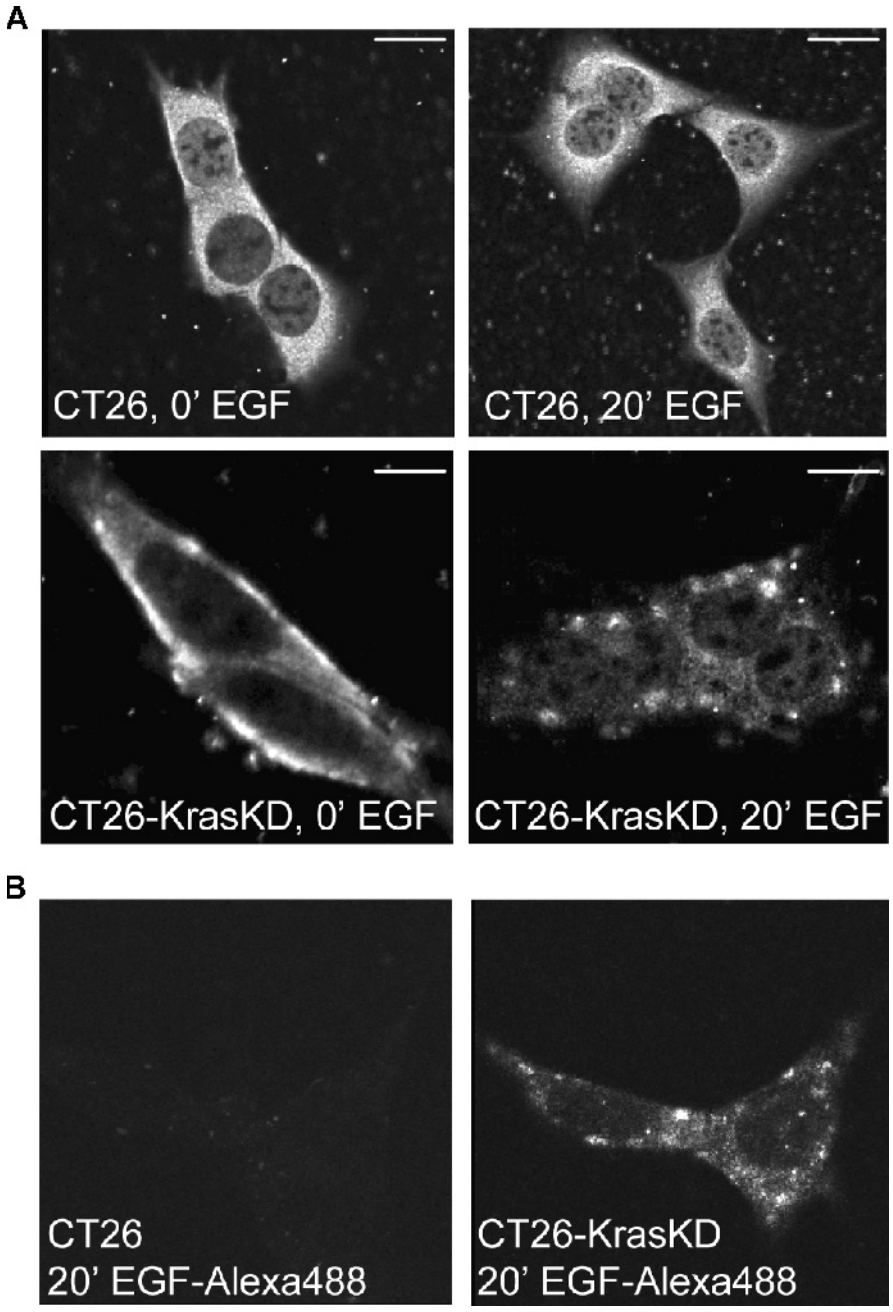

Figure 5. Aberrant EGFR localization and reduced EGF internalization in CT26 cells. (A) CT26 control cells and CT26-KrasKD cells were grown on glass coverslips. Cells were serum-starved overnight and were subsequently stimulated with 20 ng/ml EGF for 20 minutes. Coverslips were then stained for EGFR and were analyzed by immunofluorescence. (B) CT26 and CT26-KrasKD cells were stimulated with Alexa 488–conjugated EGF (30 ng/ml; 20 minutes). Cells were then fixed and analyzed for the uptake of fluorescent EGF by confocal microscopy.

although both the ROK and EGFR inhibitors were effective in inducing and suppressing EGFR-dependent ERK phosphorylation, respectively. Next, we tested the effect of ROK inhibition on EGF signaling in CT26 cells. In line with the results previously mentioned, ROK inhibition by Y27632 promoted ERK phosphorylation by EGF (Figure 7*F*) and restored EGFR plasma membrane localization in CT26 cells (Figure 7*G*). Also, in this cell system, Y27632 did not restore tumor cell sensitivity to EGFR inhibition by gefitinib (Figure 7*H*). Taken together, the results suggest that ROK inhibition restores EGFR plasma membrane localization and EGF signaling, but it does not restore tumor cell dependency on EGFR activity.

Discussion

The association of KRAS mutations with resistance to EGFR inhibitors has been demonstrated in a large number of clinical studies, both in patients with colorectal cancer and lung cancer [14]. Our study shows that oncogenic *KRAS* is causally involved in mediating resistance to EGFR-targeted therapeutics. Constitutive signaling by oncogenic *KRAS* may reduce the requirement for EGFR activity as an upstream RAS activator. Although we did not observe reduced basal phosphorylation of the ERK or AKT protein kinases after deletion of oncogenic *KRAS*, maintenance of the activity of these pathways became dependent on EGFR signaling. This lends support to the hypothesis that *KRAS*

renders cells less dependent on the EGFR for maintaining the activity of these critical signaling pathways, albeit at relatively low basal levels.

Oncogenic *KRAS* desensitized cells not only to EGFR inhibition but also to EGFR activation by altering its intracellular localization. EGFR localization, internalization, and trafficking are controlled by a complex network of signaling molecules [23,24]. After EGF stimulation, the EGFR is downregulated through ubiquitination and lysosomal degradation. Alternatively, internalized EGFR can recycle back to the plasma membrane or can be retained inside the cell. The reduction in cell surface EGFR in *KRAS* mutant cells limits its availability to EGF and to EGFR-targeting antibodies. We have so far not been able to identify the KRAS effector pathway(s) that cause(s) altered EGFR localization. Although suppression of ROK signaling partially restored EGFR localization to the plasma membrane and EGF responsiveness, the activity of RhoA or ROK was reduced rather than elevated in mutant *KRAS* cells when compared with wild-type *KRAS* cells (W.J.v.H. and M.d.B., unpublished observations). This suggests that basal ROK activity is required for EGFR internalization but that mutant *KRAS* does not stimulate this pathway to accelerate EGFR internalization.

RNA interference (ERK1, ERK2, ERK1 + ERK2, ARAF, BRAF, CRAF, RalA, and RalB) and inhibitor studies (sorafenib, U0126, LY294002, and Rac1) failed to implicate these classic KRAS effector pathways in desensitizing HCT116 and/or CT26 cells to EGF (W.J.v.H., unpublished observations). Possibly, either a combination of effectors or subtle alterations in effector protein activity or localization are required for altering EGFR localization rather than robust changes in expression levels or activity.

From clinical studies, it has become clear that wild-type *BRAF* is required for response to EGFR therapy in metastatic colorectal cancer [25]. In addition, the Raf/vascular endothelial growth factor receptor inhibitor sorafenib has been used in combination with EGFR inhibitors in the treatment of several solid malignancies [26]. The rationale for this was to simultaneously target the tumor cells (EGFR inhibition) and the vasculature (vascular endothelial growth factor receptor inhibition). However, sorafenib is also a potent RAF kinase inhibitor, and RAF kinases are critical KRAS-activated signal transducers in lung cancer cells [27]. Therefore, it is possible that sorafenib could sensitize colorectal tumor cells to EGFR inhibition by suppressing KRAS/RAF signaling. Our *in vitro* results do not support such a simple mechanism because neither sorafenib treatment nor RNAi-mediated suppression of RAF kinases could restore tumor cell sensitivity to EGF or EGFR inhibition (W.J.v.H., unpublished observations). This suggests that other RAS effector pathways and/or a combination of effector pathways mediate KRAS-dependent resistance to EGFR inhibition.

Taken together, mutant *KRAS* causes intracellular retention of the EGFR, which dampens the tumor cell response to EGF. Interestingly, high levels of EGFR ligands predict tumor responsiveness to cetuximab but only in tumors with wild-type *KRAS* [15,16]. Our results suggest that in mutant *KRAS* tumors, the EGFR is likely to be a relatively inefficient signal transducer because of its absence from the basolateral membrane. We propose that oncogenic *KRAS* fixes the activation state of its effector pathways at levels that are relatively low when compared with those achieved after EGF stimulation of *KRAS* wild-type cells but are high enough to sustain cell proliferation and viability.

EGFR protein levels, as determined by immunohistochemistry, are not associated with the response of colorectal tumors to cetuximab [28,29]. Our results show that a minor population of human colorectal cancer tumors with wild-type *KRAS* shows proper polarized basal/basolateral localization of the EGFR, similar to what is observed in normal colon tissue. Interestingly, the effect of EGFR activation or inhibition on colorectal cancer cell proliferation was previously shown to be dependent on cell polarity: Only when stimulated or inhibited at the basolateral side does modulation of EGFR activity affect tumor cell proliferation [30]. Possibly, polarized EGFR staining, rather than total protein levels, may identify a subset of wild-type *KRAS* tumors that respond to EGFR-targeted therapy. This hypothesis should

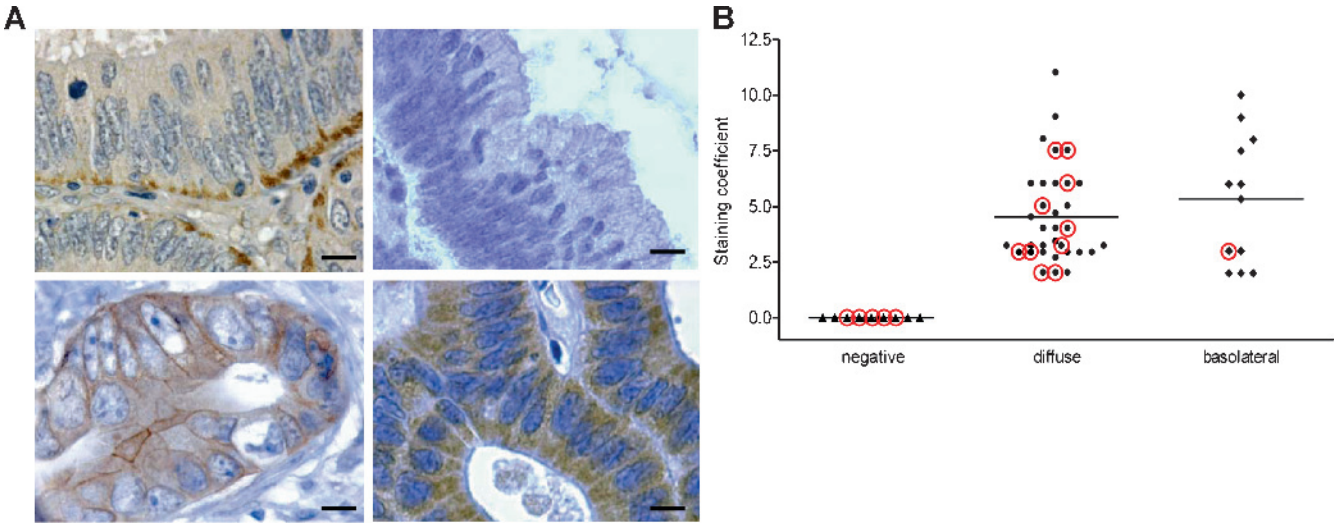

Figure 6. Aberrant localization of the EGFR in colorectal tumors expressing oncogenic *KRAS*. A TMA containing a panel of colorectal tumors with known *KRAS* mutation status was used to study EGFR localization. (A) We distinguished three types of staining. 1) Basolateral and membranous; examples are shown in the left upper and left lower images. 2) Negative; an example is shown in the right upper image. 3) Diffuse throughout the tumor cells with negative membrane staining; an example is shown in the right lower image. (B) The staining coefficient was determined as the product of the staining intensity on a 0 to 4 scale (with 0 = negative, 1 = weak, 2 = moderate, 3 = strong, 4 = very strong) and the percentage positive cells on a 0 to 3 scale (with <1% = 0, 1%-25% = 1, 25%-50% = 2, >50% = 3). The staining scores for all tumors (with a maximum score of 12) were then plotted. The tumors with activating mutations in *KRAS* are circled in red.

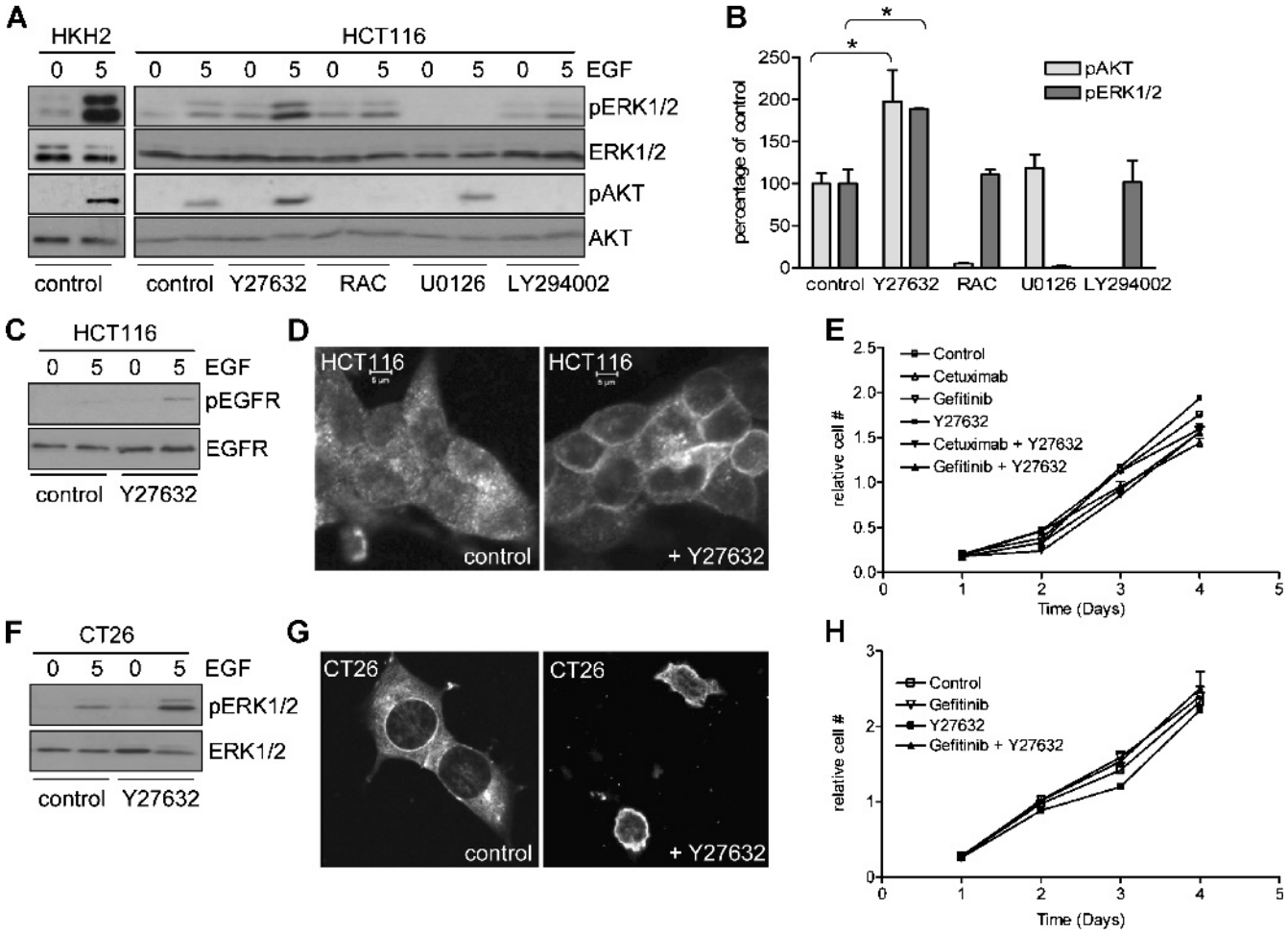

Figure 7. ROK inhibition restores EGFR localization and signaling but not EGFR dependency. (A) HCT116 cells were serum-starved overnight in the presence of 20 μM Y27632, 10 mM Rac1-Inh, 10 μM U0126, or 10 μM LY294002 or in the absence of inhibitors (control). HKH2 cells served as a positive control. The cells were then stimulated with 20 ng/ml EGF for 5 minutes (5) or were left unstimulated (0). ERK1/2 and AKT phosphorylation were then determined by Western blot analysis. (B) The intensities of the pERK1/2 and pAKT signals before and after EGF stimulation were measured using Quantity One software. The percentage of signal intensities (n = 3) in treated *versus* untreated cells was then plotted. (C) HCT116 cells were serum-starved overnight in the presence or absence of 20 μM Y27632. The cells were then stimulated with 20 ng/ml EGF for 5 minutes (5) or were left unstimulated (0). EGFR expression and phosphorylation were determined by Western blot analysis. (D) HCT116 cells were grown on glass coverslips in the presence or absence of 20 μM Y27632. EGFR localization was then determined by immunofluorescence. (E) HCT116 cells were treated for 4 days with 20 μg/ml cetuximab or 2 μM gefitinib either alone or in combination with 20 μM Y27632. Mitochondrial activity was then assessed by MTT assays. All data points represent means of triplicates ± SEM. (F) CT26 cells were serum-starved overnight in the presence or absence of 20 μM Y27632. The cells were then stimulated with 20 ng/ml EGF for 5 minutes (5) or were left unstimulated (0). ERK expression and phosphorylation were then determined by Western blot analysis. (G) CT26 cells were grown on glass coverslips in the presence or absence 20 μM Y27632. EGFR localization was then determined by immunofluorescence. (H) CT26 cells were treated for 4 days with 2 μM gefitinib either alone or in combination with 20 μM Y27632. Mitochondrial activity was then assessed by MTT assays. All data points represent means of triplicates ± SEM.

be tested in the tumors of cetuximab-treated cohorts of colorectal cancer patients.

Restoration of proper EGFR localization and signaling in colorectal cancer cells with oncogenic KRAS is possible (by ROK inhibition), but this does not restore tumor cell dependency on EGFR signaling. EGFR unresponsiveness is therefore uncoupled from EGFR independency. The results suggest that the combination of ROK inhibitors with EGFR inhibitors does not seem to be a logical combination strategy to pursue in the clinic at the moment. Identification of (the combination of) KRAS-activated effector pathways that mediate EGFR independency remains a major challenge for future studies.

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Reference to Figure W2

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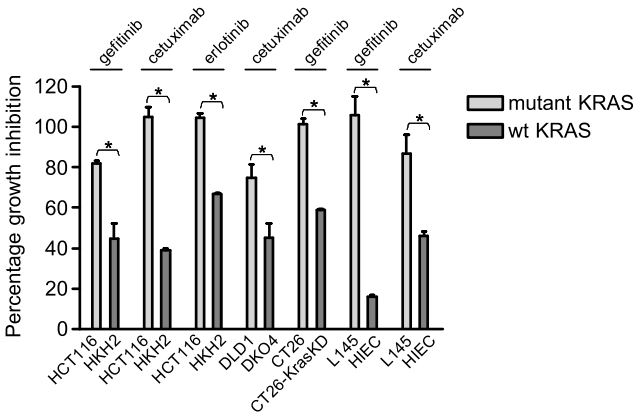

Figure W1. Deletion of oncogenic *KRAS* allows growth inhibition by EGFR-targeting therapeutics. Cells were treated for 4 days with the indicated compounds (Figure 1). MTT values of inhibitor-treated cells were then plotted as percentage of untreated controls. The effect of EGFR inhibition in *KRAS* mutant cells was minimal (20% at most), whereas *KRAS* deletion/suppression allowed growth inhibition in all cases. All differences between isogenic wild-type and mutant *KRAS* cell lines were statistically significant (*$P < .05$).

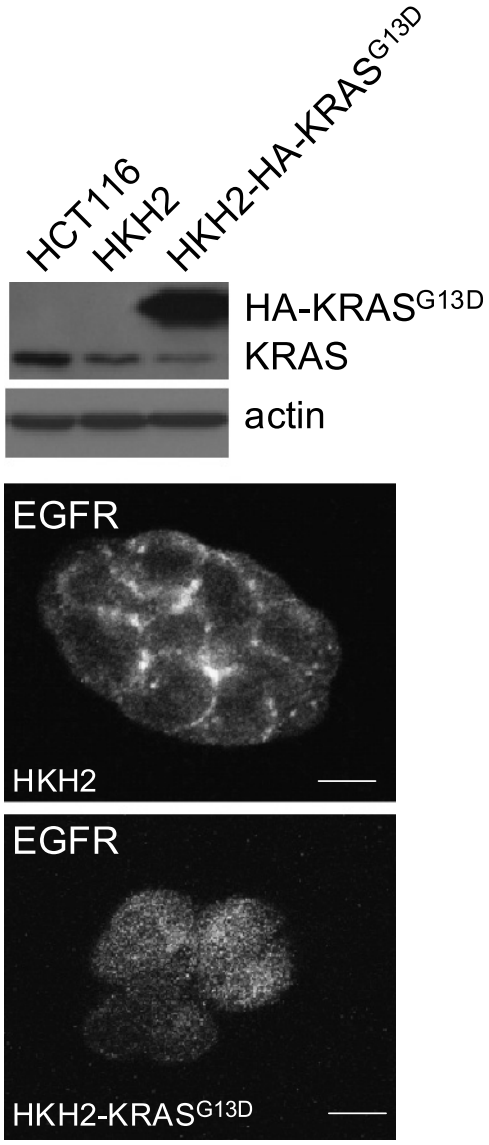

Figure W2. $KRAS^{G13D}$ expression causes loss of EGFR membrane localization in HKH2 cells. HKH2-$KRAS^{G13D}$ cells and control-transfected HKH2 cells were analyzed by Western blot analysis for the expression of *KRAS* (upper panel) [1], and for EGFR localization by immunofluorescence analysis using rabbit antihuman EGFR (2232; Cell Signaling Technology; lower panel).

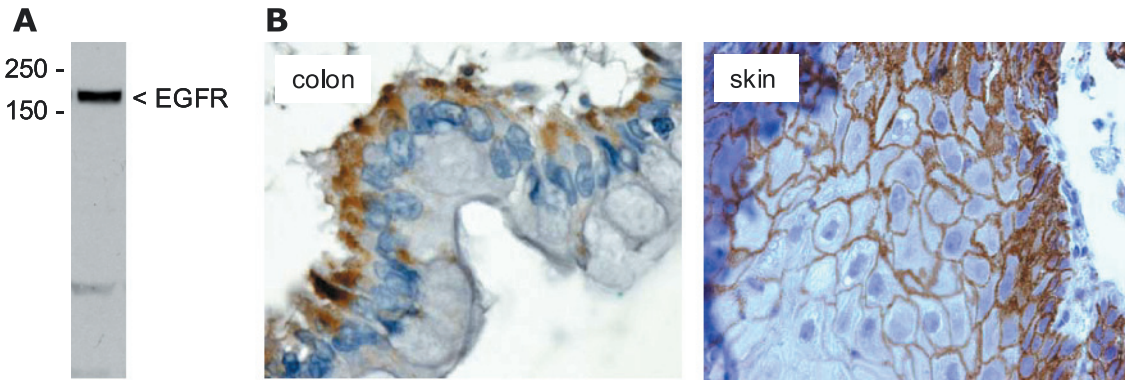

Figure W3. Validation of anti-EGFR antibody no. 2232. (A) Western blot analysis of 50 μg of a HCT116 cell lysate. (B) Immunohistochemistry of skin and normal colon showing membrane (skin) and polarized basal (colon) staining.