Oncogenic and Wild-type Ras Play Divergent Roles in the Regulation of Mitogen-Activated Protein Kinase Signaling

Amy Young, David Lou, and Frank McCormick
ABSTRACT

H-Ras, K-Ras, and N-Ras regulate cellular growth and survival and are often activated by somatic mutation in human tumors. Although oncogenic lesions occur in a single Ras isoform within individual tumors, it is unclear whether the remaining wild-type isoforms play supporting roles in tumor growth. Here, we show that oncogenic and wild-type Ras isoforms play independent and nonredundant roles within the cell. Oncogenic Ras regulates basal effector pathway signaling, whereas wild-type Ras mediates signaling downstream of activated receptor tyrosine kinases (RTK). We show that both are necessary for exponential growth of Ras-mutant cell lines. Furthermore, we show that oncogenic Ras desensitizes signaling from EGF receptor (EGFR). Depletion of oncogenic Ras with siRNA oligonucleotides relieves this negative feedback, leading to the hyperactivation of EGFR and wild-type Ras signaling. Consistent with this model, combining oncogenic Ras depletion with EGFR inhibition potently increases cell death.

SIGNIFICANCE: The results of this study highlight a novel role for wild-type Ras signaling in cancer cells harboring oncogenic RAS mutations. Furthermore, these findings reveal that therapeutically targeting oncogenic Ras signaling alone may be ineffective owing to feedback activation of RTKs, and suggest that blocking upstream RTKs in combination with downstream effector pathways may be beneficial in the treatment of Ras-mutant tumors. Cancer Discov; 3(1); 112–23. ©2012 AACR.

See related commentary by Hayes and Der, p. 24.

INTRODUCTION

H-Ras, K-Ras, and N-Ras are the founding members of the Ras family of small GTPases, and regulate cell growth, differentiation, and survival (1, 2). Ras GTPases function as binary molecular switches, cycling between inactive GDP-bound and active GTP-bound states. In the basal state, Ras is predominately GDP bound. Activated receptor tyrosine kinases (RTK) recruit guanine nucleotide exchange factors (GEF) to promote the exchange of bound GDP for GTP on nearby Ras molecules. In its GTP-bound state, Ras interacts with downstream effectors to activate signaling pathways important for cell growth and survival, including the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways (3, 4). GTPase-activating proteins (GAP) facilitate the hydrolysis of bound GTP to GDP, returning Ras to its basal, inactive conformation and terminating downstream signaling. Ras, therefore, acts as a sensor of extracellular growth cues to ensure that signaling output through downstream effector pathways are of the appropriate intensity and duration.

The aberrant hyperactivation of Ras plays a causal role in human cancer, and an estimated 30% of human tumors harbor oncogenic somatic mutations in HRAS, KRAS, or NRAS (5, 6). Oncogenic RAS alleles differ from their wild-type counterpart by a single missense point mutation that results in an amino acid substitution typically at position 12, 13, or 61 (6, 7). These substitutions impair the rate of GAP-mediated GTP hydrolysis and consequently deregulate Ras signaling (5–7). Oncogenic mutation in any one of the 3 RAS genes is thought to be sufficient to constitutively activate downstream signaling and confer independence from upstream growth cues (8). Although the remaining 2 wild-type Ras isoforms remain subject to regulation by GAPs and GEFs, the signaling contribution of the wild-type Ras isoforms in this context has been largely unexamined.

Currently, no curative treatments for Ras-mutant cancers are available (9). Efforts to develop drugs that specifically block the activity of oncogenic Ras have been unsuccessful, although this remains an active area of investigation (10, 11). Clinical studies show that oncogenic KRAS mutations predict resistance to EGF receptor (EGFR) inhibitors; hence, the use of RTK inhibitors in this subset of cancers is usually contraindicated (12). Developing drugs that inhibit signaling pathways downstream of Ras has thus been a key focus of clinical development. Small-molecule compounds targeting the MAPK and PI3K effector pathways are currently under clinical investigation, as these pathways have been well characterized and shown to be critical in mediating Ras-driven tumorigenesis (9, 13, 14). However, the response to MAP–ERK kinase (MEK) inhibitors varies across Ras-mutant tumors, and clinical efficacy is thought to be limited by feedback activation of the PI3K pathway (14–17). Furthermore, Raf kinase inhibitors paradoxically activate MAPK signaling in cells harboring oncogenic RAS mutations, thereby precluding the use of these inhibitors in the treatment of RAS-mutant cancers (18–20). These findings highlight the complexity of effector pathway regulation and feedback mechanisms downstream of oncogenic Ras. Continued investigation of these signaling processes will be required to design effective targeted therapeutics.
In this study, we examine the regulation of MAPK and PI3K signaling in cancer cells with oncogenic RAS mutations. We use 3 cancer cell models, each of which harbors a homozygous RAS mutation: the T24 bladder cancer cell line (HRAS G12V), the MIA PaCa-2 pancreatic cancer cell line (KRAS G12C), and the RD rhabdomyosarcoma cell line (NRAS Q61H; Catalog of Somatic Mutations in Cancer, Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom). By depleting either the single oncogenic Ras isoform or the remaining 2 wild-type Ras isoforms, we delineate independent roles for oncogenic and wild-type Ras. Our studies uncover an unexpected role for wild-type Ras isoforms in mediating growth factor signaling and sustaining the proliferation of cancer cells harboring oncogenic RAS mutations. Furthermore, we show that oncogenic Ras regulates basal MAPK signaling and negatively regulates RTK signaling. Thus, although depletion of oncogenic Ras impairs basal signaling, the concomitant relief of negative feedback allows for compensatory activation of RTKs and wild-type Ras-mediated downstream signaling as a secondary driver of cell survival and proliferation.

RESULTS

Growth Factor Signaling Can Enhance Effector Pathway Signaling in Cancer Cell LinesHarboring Oncogenic RAS Mutations

Oncogenic mutations in RAS are thought to confer growth factor independence and constitutive activation of downstream signaling pathways. However, acute stimulation with EGF can still further enhance MAPK and PI3K signaling in cancer cells harboring oncogenic mutations in HRAS, KRAS, or NRAS, as evidenced by increased extracellular signal-regulated kinase (ERK) and Akt phosphorylation, respectively (Fig. 1A). Although the oncogenic Ras isoform is constitutively GTP bound, EGF stimulation induces GTP loading on the wild-type Ras isoforms. This finding suggests that cancer cells with oncogenic RAS mutations have not saturated the capacity to activate downstream signaling, but that heightened activity can be achieved through activation of wild-type Ras isoforms by RTKs.

Wild-type Ras Regulates Growth Factor Signaling

To directly test the requirement for wild-type Ras in growth factor signaling, cells were transfected with siRNA to deplete the expression of the 2 wild-type RAS isoforms. The cells were then serum starved overnight, and signaling was measured before and after acute stimulation with EGF (Fig. 1B). Indeed, siRNA-mediated depletion of the wild-type Ras isoforms attenuates the EGF-induced phosphorylation of ERK (Fig. 1B and C). These findings have been confirmed in additional cell lines and with several siRNA oligonucleotides (Supplementary Figs. SIA–S1C and S2). These results show that the wild-type Ras isoforms are required for the growth factor–induced activation of MAPK signaling in cancer cells with oncogenic RAS mutations. siRNA-mediated depletion of the wild-type Ras isoforms also attenuates the EGF-induced phosphorylation of Akt in MIA PaCa-2 and RD cells, indicating that growth factor–induced PI3K signaling is partially mediated by wild-type Ras in these cell lines (Fig. 1B). Interestingly, depletion of the oncogenic Ras isoform results in a more robust induction of EGFR, ERK, and Akt phosphorylation in response to EGF stimulation (Fig. 1B and C). The increase in signaling correlates with enhanced GTP loading on the wild-type Ras isoforms (Supplementary Fig. S1A and S1B), again highlighting a critical role for wild-type Ras in mediating growth factor signaling and suggesting that oncogenic Ras negatively regulates EGFR signaling, as we will discuss below.

We next examined whether effector pathway signaling could be suppressed by blocking RTK-mediated activation of wild-type Ras. The EphA2 RTK negatively regulates Ras/MAK signaling upon stimulation with its ligand ephrin-A1 (21, 22). We thus used ephrin-A1 to block wild-type Ras activation. Ephrin-A1 attenuates EGF-induced activation of MAPK signaling and coincides with reduced GTP loading on wild-type Ras isoforms (Supplementary Fig. S3A). Importantly, wild-type Ras isoforms are required to mediate this effect, given that ephrin-A1 has no effect on ERK signaling in RD cells depleted of wild-type Ras compared with a robust suppression of ERK signaling when the oncogenic N-Ras isoform is depleted (Supplementary Fig. S3B and S3C). Taken together, these data show that effector pathway signaling can still be modulated through wild-type Ras even in the presence of an oncogenic Ras isoform.

Oncogenic Ras Regulates Basal MAPK Signaling

Depletion of oncogenic Ras results in a subtle yet reproducible reduction in the basal levels of ERK phosphorylation in serum-starved cells (Fig. 1B and D) and in cells asynchronously growing in media supplemented with 10% serum (Fig. 2A and B), indicating that oncogenic Ras is critical in regulating basal MAPK signaling. In contrast, depletion of oncogenic Ras increases basal Akt signaling in T24 and RD cells (Fig. 2A, Supplementary Fig. S2), highlighting a divergence in the regulation of basal MAPK and PI3K signaling downstream of oncogenic Ras.

Intriguingly, depletion of the wild-type Ras isoforms modestly increases basal ERK phosphorylation in MIA PaCa-2 and RD cells under conditions of serum starvation or asynchronous growth in media supplemented with 10% serum (Figs. 1B and 1D; 2A and 2B). Several studies show that wild-type Ras can antagonize the transforming potential of its oncogenic counterpart in vitro and in vivo (23–25). Thus, the relief of antagonism on oncogenic Ras may serve as one potential mechanism by which basal ERK signaling increases in cells depleted of wild-type Ras. Depleting oncogenic Ras in combination with the 2 wild-type Ras isoforms prevents this increase in basal ERK phosphorylation, suggesting that the increased ERK phosphorylation observed under conditions of wild-type Ras depletion is indeed driven by oncogenic Ras (data not shown). These data are consistent with a model in which the wild-type Ras isoforms antagonize basal oncogenic Ras signaling to the MAPK pathway.

Wild-type and Oncogenic Ras Differentially Regulate Cell Proliferation

The results discussed above highlight unique roles for wild-type and oncogenic Ras in the regulation of effector pathway signaling: The oncogenic Ras isoform regulates basal MAPK signaling, whereas the wild-type Ras isoforms regulate growth factor signaling. Because Ras regulates cellular growth and survival, we next tested the requirement for the oncogenic and...
Figure 1. Wild-type Ras mediates growth factor–induced activation of MAPK signaling in cancer cells harboring oncogenic RAS mutations. A, cancer cell lines with oncogenic mutations in HRAS, KRAS, and NRAS were serum starved overnight before acute stimulation with EGF for 5 minutes. Levels of active GTP-bound Ras were determined by a Ras–GTP pulldown assay, and lysates were subjected to immunoblot analysis. B, cells were transfected with the indicated siRNAs for 48 hours and placed in serum-free medium overnight. Signaling was measured before and after 5 minutes of EGF stimulation. Lysates were subjected to immunoblot analysis. K-Ras protein levels are below the detection limit of the assay for the RD cell line. C, quantification of the fold-change in EGF-induced ERK phosphorylation (p-ERK) from the immunoblot analyses in B are depicted in histograms. Values are normalized to samples transfected with non-silencing siRNA. D, quantification of basal ERK phosphorylation levels from the immunoblot analyses in B are depicted in histograms. Values are normalized to samples transfected with non-silencing siRNA. p-EGFR, phosphorylated EGFR; p-ERK, phosphorylated ERK.
Figure 2. Oncogenic Ras regulates basal MAPK signaling. A, cells were transfected with the indicated siRNAs for 72 hours. Levels of active GTP-bound Ras were determined by a Ras–GTP pulldown assay, and lysates were subjected to immunoblot analysis. B, quantification of ERK phosphorylation levels from the immunoblot analyses in A are depicted in histograms. Values are normalized to samples transfected with non-silencing siRNA.

wild-type Ras isoforms in maintaining cellular proliferation. siRNA-mediated depletion of either the oncogenic or wild-type Ras isoforms reduces the proliferative capacity of cells asynchronously growing in media supplemented with 10% serum (Fig. 3). In all 3 cell lines tested, depletion of the oncogenic Ras isoform more potently inhibits cell growth than does depletion of the wild-type Ras isoforms. Despite sustaining steady-state ERK signaling, depletion of the 2 wild-type Ras isoforms also impedes the growth of each of the 3 cell lines, although not as drastically as depletion of the oncogenic isoform. This observation suggests that wild-type Ras might regulate cellular growth through additional pathways, whose roles are independent from maintaining steady-state ERK activation. Several studies show that transient activation of ERK signaling promotes cell proliferation, whereas sustained ERK proliferation promotes differentiation (26). Thus, we postulate that the growth defect observed in cells depleted of wild-type Ras may result from a defect in the growth factor–mediated transient activation of ERK and additional Ras effector pathways. Taken together, these data show that the differential regulation of basal MAPK signaling by wild-type and oncogenic Ras translates into independent and nonredundant roles in the regulation of cell growth, and that both are required to maintain optimal growth rates.

Oncogenic Ras Negatively Regulates EGFR Sensitivity

As mentioned above, silencing oncogenic Ras expression sensitizes cells to EGF stimulation (Fig. 1B and C), showing that oncogenic Ras negatively regulates EGFR sensitivity. It has been shown that pharmacologic MEK inhibition similarly sensitizes cells to EGF stimulation (17, 27–29). One mechanism by which MEK inhibition sensitizes EGFR signaling is by relieving an inhibitory ERK-mediated phosphorylation of EGFR at residue T669 (30–32). To examine whether silencing oncogenic Ras achieves the same effect, a time-course study of EGF stimulation was conducted in cells depleted of oncogenic Ras or treated with the MEK inhibitor U0126. Consistent with prior studies, pharmacologic MEK inhibition reduces...
basal phosphorylation of ERK and EGFR T669, and potentiates the EGF-induced phosphorylation of EGFR at the activating Y1068 residue, resulting in enhanced and sustained Akt signaling (Supplementary Fig. S4). Oncogenic Ras depletion produces a comparatively modest reduction in EGF T669 and basal ERK phosphorylation, but more robustly amplifies EGFR signaling, suggesting additional mechanisms by which oncogenic Ras attenuates EGFR signaling (Fig. 4A). It should be noted that sustained siRNA-mediated oncogenic Ras depletion (72 hours) may allow time for rewiring of the EGFR-MAPK axis, compared with short-term MEK inhibition (1 hour), and may explain the differences observed in the differential sensitivity to EGF stimulation. Nevertheless, these results show that depletion of oncogenic Ras sensitizes cells to the acute activation of EGFR signaling.

To directly test whether ectopic expression of oncogenic Ras is sufficient to attenuate EGFR signaling, HBL100 cells stably expressing GFP or oncogenic H-Ras, K-Ras, or N-Ras were serum starved overnight before acute stimulation with EGF. As expected, expression of oncogenic Ras increased basal levels of ERK phosphorylation. In addition, we observed a striking suppression of EGF-induced phosphorylation of EGFR, Akt, and ERK in cells expressing the ectopic oncogenic Ras isoforms when compared with the GFP-expressing control (Fig. 4B). The results support our observations that oncogenic Ras negatively regulates EGFR sensitivity.

To test whether oncogenic Ras negatively regulates the sensitivity of additional RTKs, cells depleted of oncogenic Ras were treated with a panel of growth factors. Depletion of oncogenic Ras heightens the sensitivity to additional growth factors in a cell line–dependent fashion (Supplementary Fig. S5), suggesting that oncogenic Ras may engage a program to globally desensitize signaling from upstream RTKs.

**Combining Oncogenic Ras Depletion with EGFR Inhibition Enhances Cell Death**

Although several small-molecule compounds targeting MEK are currently under clinical investigation, efficacy may be limited by feedback activation of EGFR-PI3K signaling (14–17). Similarly, recent studies show that colorectal cancer cells harboring BRAF V600E mutations are unresponsive to the small-molecule RAF inhibitor PLX4032 (vemurafenib) owing to feedback activation of EGFR, and that combining RAF and EGFR inhibition improves efficacy (33, 34). Our results indicate that feedback activation of EGFR may also be a mechanism of resistance to therapies targeting either oncogenic Ras or oncogenic Ras-driven MAPK signaling. We hypothesized that combining oncogenic Ras depletion with EGFR inhibition would block feedback EGFR activation. To test this idea, siRNA-transfected cells were serum starved overnight and pre-treated with the EGFR inhibitor erlotinib or vehicle control [dimethyl sulfoxide (DMSO)] before acute stimulation with EGF. Consistent with earlier results, depletion of oncogenic Ras markedly sensitizes cells to acute stimulation with EGF and results in increased downstream signaling. Treatment with erlotinib predictably blocks EGFR phosphorylation but notably attenuates EGFR-stimulated GTP loading on the wild-type Ras isoforms, and abrogates the phosphorylation of both ERK and Akt (Fig. 5). Thus, blocking the activation of EGFR prevents the hypersensitivity to EGF stimulation induced by oncogenic Ras.
**Figure 4.** Oncogenic Ras negatively regulates EGFR sensitivity. 

**A,** cells were transfected with the indicated siRNAs for 48 hours and placed in serum-free medium overnight. Signaling was measured before and after EGF stimulation. Lysates were subjected to immunoblot analysis. Lysates derived from T24, MIA PaCa-2, and RD cells were probed with H-Ras, K-Ras, or N-Ras antibodies, respectively. 

**B,** HBL100 cells stably expressing GFP or oncogenic H-Ras, K-Ras, or N-Ras were serum starved overnight, and signaling was measured before and after 5 minutes of EGF stimulation. Levels of active GTP-bound Ras were determined by a Ras–GTP pulldown assay, and lysates were subjected to immunoblot analysis. Endogenous levels of K-Ras are below the detection limit of the assay.
Regulation of Signaling by Wild-type and Oncogenic Ras

### DISCUSSION

The work presented here defines distinct roles for oncogenic and wild-type RAS in regulating effector pathway signaling in cancer cells harboring oncogenic RAS mutations. We show that oncogenic Ras regulates basal signaling and uncover an unexpected role for wild-type Ras in regulating growth factor signaling. Although oncogenic Ras constitutively activates MAPK signaling, acute activation of growth factor receptors can enhance this signaling by stimulating GTP loading on wild-type Ras (Fig. 7). Given that we detect this phenomenon in cell lines harboring oncogenic mutations in HRAS, KRAS, or NRAS, we conclude that these are not isoform-specific effects, but rather can be generalized to oncogenic and wild-type isoforms as a class. Collectively, these findings uncover an underappreciated role for wild-type Ras signaling in the context of oncogenic RAS mutations.

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**Figure 5.** Erlotinib blocks the feedback activation of EGFR and wild-type Ras signaling. Cells were transfected with the indicated siRNAs for 48 hours, placed in serum-free medium overnight, and treated with either DMSO or 10 μM erlotinib for 1 hour. Signaling was measured before and after 5 minutes of EGF stimulation. Levels of active GTP-bound Ras were determined by a Ras–GTP pulldown assay, and lysates were subjected to immunoblot analysis.

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Figure 6. Combining oncogenic Ras depletion with EGFR inhibition induces cell death. A, T24 cells were transfected with the indicated siRNAs for 48 hours and treated with a serial dilution of erlotinib for 72 hours. Relative cell proliferation values were determined by the CyQUANT Direct Cell Proliferation Assay. For each siRNA condition, data are expressed as the fraction of maximal cell growth at 72 hours after drug treatment. B, T24 cells were transfected with the indicated siRNAs for 48 hours and treated with 10 μmol/L erlotinib for 72 hours. Cells were fixed, stained with PI, and analyzed by flow cytometry to determine cell-cycle distribution. C, T24 cells were transfected with the indicated siRNAs for 48 hours and treated with 10 μmol/L erlotinib for 72 hours. Cells were stained with Annexin V-FITC and PI and analyzed by flow cytometry to determine the percentage of cells undergoing apoptosis. The fold difference in Annexin V–positive cells is shown relative to the condition in which cells were transfected with non-silencing siRNA and treated with 10 μmol/L erlotinib. D, T24 cells were transfected with the indicated siRNAs for 48 hours and treated with the indicated concentrations of erlotinib for 24 hours. Lysates were subjected to immunoblot analysis.

Intriguingly, we found that depletion of wild-type Ras increases basal ERK phosphorylation, suggesting that wild-type Ras antagonizes oncogenic Ras signaling. The mechanism by which this antagonism occurs is not fully understood. In the basal state, wild-type Ras is predominantly GDP bound. It is plausible that GDP-bound wild-type Ras inhibits oncogenic Ras signaling by sustaining unique and independent growth-inhibitory signaling pathways. Alternatively, different Ras isoforms might compete for common regulators, effectors, or proper localization. Prior studies indicate that wild-type Ras can antagonize the transforming potential of its oncogenic counterpart in a dose-dependent manner in vitro and in vivo (23–25), and show a strong selective pressure to lose expression of the corresponding wild-type allele of the oncogenic Ras isoform (35–37). Whereas these data support a tumor-suppressive role for the wild-type counterpart of the same oncogenic Ras isoform, our studies uncover a novel role for the remaining 2 wild-type Ras isoforms in antagonizing oncogenic Ras signaling.

It is unclear whether the specific inhibition of wild-type Ras activity, rather than the outright depletion of wild-type Ras expression, would similarly antagonize oncogenic Ras signaling. Early studies on Ras signaling show that membrane-targeted...
Several recent studies show that targeted inhibition of a single oncogenic pathway results in the feedback activation of compensatory pathways, thereby limiting the efficacy of monotherapy (33, 34, 41–45). Likewise, our study cautions against targeting oncogenic Ras signaling alone, and indicates that combined treatment with the appropriate RTK inhibitor is likely required, owing to the compensatory activation of upstream signaling. Determining the appropriate RTK to target will likely be context dependent and will be contingent upon factors such as tumor type, RTK expression levels, the availability and accessibility of relevant ligands, and whether autocrine RTK signaling is sustained.

The finding that oncogenic Ras mediates feedback suppression of RTKs may help explain the observation that KRAS-mutant tumors are often resistant to EGFR-based therapies and have variable responses to single-agent MEK inhibitor treatment (12, 14, 46). We hypothesize that oncogenic mutations in KRAS desensitize cells to EGFR-based therapies by at least 2 mechanisms. The first and most intuitive explanation is that oncogenic Ras increases basal effector signaling downstream of EGFR. Second, our data are consistent with an additional model in which oncogenic Ras rewires EGFR signaling dynamics. Indeed, several recent studies show that oncogenic K-Ras desensitizes cells to EGFR activation and inhibition by altering EGFR trafficking, turnover, and localization (29, 30, 32, 47–49). Suppression of basal MAPK signaling, whether by siRNA-mediated depletion of oncogenic Ras or by pharmacologic inhibition of MEK, resensitizes EGFR signaling and primes the receptor for activation. Accordingly, recent studies indicate that treatment with a MEK inhibitor alongside an EGFR inhibitor synergistically inhibits the growth of cancer cells harboring oncogenic KRAS mutations (29, 49). Taken together, these studies are consistent with our findings and suggest that targeting the MAPK pathway in combination with the appropriate RTK is potentially beneficial as well as necessary to effectively treat cancers harboring oncogenic Ras mutations. Importantly, this approach would concurrently block the RTK-mediated activation of wild-type Ras signaling while suppressing the basal levels of MAPK signaling sustained by oncogenic Ras.

**METHODS**

**Cell Culture**

The T24 cell line was kindly provided by Dr. Osamu Tetsu (University of California San Francisco, San Francisco, CA); all other cell lines were obtained from the American Type Culture Collection. No additional cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and incubated at 37°C in a 5% CO₂ incubator. For stable cell line generation, genes were cloned into pENTR or pDONR 221 vectors (Invitrogen), were transferred into a Gateway-compatible derivative of the pBNeo retroviral vector using recombination-mediated Gateway technology (Invitrogen), and were transduced into HBL100 cells using retroviruses. All Ras constructs are of human origin and have been described previously (2).

**Cell Stimulation and Drug Treatments**

For stimulation with recombinant EGF (Invitrogen), cells were serum starved overnight before acute stimulation with 30 ng/mL EGF for 5 minutes, unless otherwise noted. For analysis of cellular...
signaling after U0126 or erlotinib treatment, cells were serum starved overnight and pretreated with either 10 μM of drug or vehicle control (DMSO) for 1 hour before acute stimulation with EGF.

RNA Interference

Cells were transfected with 80 nmol/L siRNA (Qiagen) using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer’s instructions. siRNA oligonucleotide sequences are provided in the Supplementary Methods.

Immunoblot Analysis

Cells were washed twice in ice-cold PBS and lysed in 1% Triton lysis buffer [25 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 20 mmol/L NaF, 1 mmol/L Na3VO4, and 1 mmol/L dihydrothreitol (DTT)] supplemented with a protease inhibitor cocktail (Roche) and cleared by centrifugation. Protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad). Equal amounts of protein extracts were resolved using SDS-PAGE (NuPAGE; Invitrogen), transferred to a nitrocellulose membrane, and immunoblotted with primary antibodies. Primary antibodies were detected with secondary antibodies labeled with either IRDye800 (Rockland) or Alexa Fluor 680 (Molecular Probes) and were visualized using a LI-COR Odyssey scanner. A complete list of primary antibodies is provided in the Supplementary Methods.

Ras–GTP Assay

Cells were washed twice in ice-cold PBS and lysed in 1% TX100-TNM lysis buffer [20 mmol/L Tris pH 7.5, 5 mmol/L MgCl2, 150 mmol/L NaCl, 1% Triton X-100] supplemented with 1 mmol/L DTT, and protease and phosphatase inhibitors (Sigma-Aldrich), and were processed as described above. Equal amounts of protein from each sample were added to 10 μL of packed GST-Raf-RBD beads in 300 to 500 μL of 1% TX100-TNM lysis buffer and rotated at 4°C for 1 to 2 hours. Beads were washed 3 times with 1 mL of cold lysis buffer and boiled in lithium dodecyl sulfate (LDS) buffer (Invitrogen).

Cell Proliferation Analysis

Forty-eight hours after siRNA transfection, cells were trypsinized and seeded at equal densities in 12-well plates in triplicate for cell proliferation analysis. Samples were collected immediately at the time of seeding and were harvested at 24-hour intervals thereafter, as indicated. Accurate cell counts were obtained using a Coulter particle analyser.

Erlotinib Growth Inhibition Assay

Twenty-four hours after siRNA transfection, cells were trypsinized and seeded in 96-well plates at 3,500 cells per well. The following day, cells were treated with a serial dilution of erlotinib (6 replicates per drug concentration) and allowed to proliferate for an additional 72 hours. Cell proliferation was measured using CyQUANT Direct Cell Proliferation Assay (Life Technologies). For each siRNA condition, data are expressed as the fraction of maximal cell growth at 72 hours after erlotinib treatment, and dose–response curves were generated using GraphPad Prism software. All assays were conducted at least 3 independent times and representative curves are shown.

Analysis of Cell Cycle and Apoptosis

Forty-eight hours after siRNA transfection, cells were treated with erlotinib or DMSO and harvested 72 hours after drug treatment. For cell-cycle analysis, adherent and floating cells were collected, washed once with PBS, permeabilized with ice-cold 70% ethanol, resuspended in PBS, and incubated with RNase A and propidium iodide (PI). For analysis of apoptosis, adherent and floating cells were collected, washed with ice-cold PBS, and stained with Annexin V-FITC (BD Pharmingen) and PI. Cells were sorted using FACS Calibur (Becton, Dickinson and Co.). Ten thousand live cell events were collected per treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: A. Young, D. Lou, F. McCormick
Development of methodology: A. Young, D. Lou
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Young, D. Lou
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Young, D. Lou, F. McCormick
Writing, review, and/or revision of the manuscript: A. Young, D. Lou, F. McCormick
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Young, D. Lou
Study supervision: F. McCormick

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