Oncogenic *K-RAS* Is Required to Maintain Changes in Cytoskeletal Organization, Adhesion, and Motility in Colon Cancer Cells

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Abstract

***RAS* oncogenes are thought to play a role at multiple stages of tumorigenesis. The role and mechanisms by which *RAS* oncogenes maintain the transformed state of human cancer cells are poorly understood. Here, we have studied the role of oncogenic *K-RAS* in maintaining cytoskeletal disruption, cell adhesion and motility in metastatic colon carcinoma cells. Targeted deletion of *K-RAS*^{G13D} from HCT116 colon carcinoma cells restored their ability to assemble stress fibers and focal adhesions/complexes, accompanied by increased cell-matrix adhesion and reduced motility. We further show that oncogenic K-Ras induces high Rho activity, but uncouples Rho from stress fiber formation. This uncoupling required the maintenance of high levels of the activator protein-1 family member, Fra-1, via a mitogen-activated protein/extracellular signal-regulated kinase–dependent pathway. We also show that PI3-kinase signaling is required for the motility of HCT116 cells downstream of oncogenic K-Ras. Our findings suggest that mutated *K-RAS* oncogenes are essential for maintenance of the transformed and invasive phenotype of human colon cancer cells.** (Cancer Res 2005; 65(4): 1244-50)

Introduction

Colorectal cancer develops when progressive accumulation of genetic and epigenetic changes leads to the conversion of normal colonic epithelium to dysplasia, followed by adenomas and adenocarcinomas (1, 2). Mutations in the *K-RAS* gene arise early during the colonic transformation process in ~40% of colon carcinomas (3). These mutations, which invariably occur at codons 12, 13, or 61, prevent efficient GTP hydrolysis and thus render the proteins in an activated state. As a result, multiple Ras effector pathways which control fundamental biological processes such as proliferation, apoptosis, and cell motility become constitutively activated and/or deregulated.

A major feature of Ras-transformed cells is a remodeled actin cytoskeleton which causes poor adhesion, increased motility, invasiveness, and contact-independent growth (4–6). Two of the best characterized Ras effectors involved in reorganization of the actin cytoskeleton are Raf kinases and PI3-kinases. The contribution these effectors to remodeling of the cytoskeleton seems to be cell type–specific. For example, whereas PI3-kinase signaling was required for active Ras to induce membrane ruffling and cytoskeletal rearrangement in REF-52 fibroblasts and endothelial cells, cytoskeletal changes in Swiss 3T3 cells and Madin-Darby canine kidney cells required mitogen-activated protein kinase activity (7–10). The Rho GTPases, Rho, Rac, and Cdc42, are key regulators of the cytoskeleton and are required for Ras-mediated transformation (11–15). At the leading edge of migrating cells, Rac regulates the formation of lamellipodial protrusions, whereas Cdc42 induces filopodial extensions by activating the actin polymerization apparatus at these sites. In contrast, Rho regulates the formation of stress fibers and focal adhesions. These processes require the Rho effectors, Rho-kinase (ROCK) and mDia (16). ROCK enhances actomyosin contractility and the assembly of stress fibers, whereas mDia aligns these filaments into parallel bundles (17). ROCK-mediated stress fiber formation results from its ability to increase phosphorylation of myosin light chains and by activating LIM kinase, which phosphorylates cofilin, thereby preventing it from severing actin filaments and sequestering actin monomers (18, 19). As a result, ROCK activation can either facilitate cell movement by promoting rear retraction or inhibit motility by increasing adhesion to the extracellular matrix (20, 21). In addition, Rho signaling via ROCK and mDia can also regulate the formation of intercellular adherens junctions (22). Recent studies have shown that by increasing Fra-1 levels, oncogenic Ras signaling via extracellular signal-regulated kinase (ERK) inhibits the activation of β1-integrin, leading to the inhibition of RhoA activity and stress fiber formation (23). The depression of RhoA activity in these colon carcinoma cells was also required for ERK to activate Rac via uPAR.

In addition to being involved in tumor initiation and progression, several studies have shown that oncogenic Ras plays a crucial role in tumor maintenance (24–26). The majority of studies on the function of oncogenic Ras have involved overexpression of mutant Ras proteins in nonhuman cells which do not contain many of the additional genetic mutations found in cancer cells. Thus, the mechanisms whereby endogenous levels of oncogenic Ras maintain different aspects of the transformed phenotype of human cancer cells remain poorly understood. In the present study, we investigated the role of oncogenic *K-RAS* in maintaining changes in the motility, adhesion, and actin cytoskeleton in metastatic human colon cancer cells. As a model system, we used HCT116 cells which have one wild-type and one mutant (G13D) *K-RAS* allele, and compared them with two isogenic daughter cell lines in which the mutant *K-RAS* was deleted by homologous recombination (27). These cells lines thus enable analysis of the role of endogenous *K-RAS*^{G13D} in mediating the transformed phenotype. Because these cells also harbor additional mutations in genes such as *CTNNB1* and *TGFβ-RII* (28, 29), they allow K-Ras signaling to be studied in the context of other commonly mutated genes in colon cancer. Our results show that the presence of *K-RAS*^{G13D} in HCT116 cells uncouples Rho activation from stress fiber formation via a mitogen-activated

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protein/ERK (MEK)-ERK-Fra-1 pathway. This contributes to the poor adhesion of these cells to the extracellular matrix and their enhanced motility. signaling from oncogenic K-Ras via PI3-kinase was also required to maintain the motile phenotype of HCT116 cells.

Materials and Methods

Cell Culture and Reagents. HCT116 cells were grown in DMEM supplemented with 10% FCS. Hkh-2 and Hke-3 were grown in the same medium containing 0.3 mg/mL G418. UO126 (Promega, Southampton, United Kingdom), LY294002 (Cell Signaling, Inc., Hertfordshire, United Kingdom) and Y27632 (Calbiochem, Lutterworth, United Kingdom) were dissolved in DMSO added to the culture medium at concentrations of 5, 8, and 10 μmol/L, respectively. TAT-C3 was a generous gift from Dr. Mike Olson (Beatson Institute for Cancer Research, United Kingdom) and used at 25 μg/mL. All treatments were carried out for 24 hours in growth medium unless otherwise indicated. Cells were transfected using LipofectAMINE 2000 according to the manufacturer's instructions. pGEX-PBD and pGEX-rhotekin were kindly provided by Dr. John Collard (Netherlands Cancer Institute, Netherlands). The pCMV-Fra-1 construct was a generous gift from Dr. Vladimir Berezin (University of Copenhagen, Denmark). Fra-1 short interfering RNA oligonucleotides were purchased from Ambion (Cambridgeshire, United Kingdom) and transfected into cells using Oligofectamine (Qiagen, Crawley, United Kingdom). The pEXVmyc-K-Ras^{G13D} construct was a kind gift from Dr. John Hancock (University of Queensland, Australia). Antibodies to p-ERK and ERK were purchased from Sigma (Dorset, United Kingdom), whereas antibodies to p-Akt, Akt, and phospho-myosin light chain were from Cell Signaling, Inc. The anti-RhoA and Fra-1 antibodies were from Santa Cruz (Calne, United Kingdom) and the anti-Rac1 antibody was from UBI (Buckingham, United Kingdom). All other antibodies were from BD Transduction Labs (Oxford, United Kingdom).

Immunofluorescence. Cells were seeded onto glass cover slips 24 hours prior to treatments. When only the cytoskeleton was stained, cells were fixed with 4% formaldehyde in citrate buffer solution [10 mmol PIPES (pH 6.8), 138 mmol KCl, 3 mmol $MgCl_2$, 2 mmol EGTA, 0.32 mol/L sucrose] for 20 minutes, permeabilized in TBS/0.5% Triton X-100 for 10 minutes, then blocked in TBS/2% bovine serum albumin/0.1% Triton X-100 for 10 minutes. For costaining experiments, cells were fixed with 4% formaldehyde in PBS for 15 minutes, then permeabilized in PBS/0.1% Triton X-100. The anti-Fra-1 antibody was used at 2 μg/mL and the anti-myc tag antibody (clone 4A6) was used at 10 μg/mL, whereas anti-paxillin and anti-E-cadherin antibodies were used at 2.5 μg/mL in blocking solution for 1 hour at room temperature. Cells were washed in TBS/0.1% Triton X-100, incubated with a 1:200 dilution of Alexa-488 or Alexa-594 secondary antibodies (Molecular Probes, Leiden, the Netherlands) for 1 hour and washed as above. Phalloidin preconjugated to FITC (100 μg/μL in blocking solution; Sigma) was added to cells for 20 minutes at room temperature. Cells were examined using a confocal microscope (Leica SP2, Milton Keynes, United Kingdom).

Biological Assays. Cells used in wounding assays were seeded into 12-well tissue culture dishes, precoated with 0.1 mg/mL collagen. When confluent, a wound was made down the middle of the layer of cells with a cell scraper. Fresh media containing 1% FCS was added to the cells, with or without pharmacologic inhibitors (5 or 8 μmol/L of UO126 or LY40029, respectively). Wound closure was examined using time-lapse photography over a period of 3 hours. Triplicate wells were set up for each condition and three to four fields of view were examined per well. The rate of motility was quantified by determining the percentage of wound closure over a 3-hour period. For adhesion assays, cells were starved overnight in DMEM containing 2% FCS, trypsinized, then resuspended to a concentration of 10^4 cells/mL in the starvation medium. The cell suspension (100 μL) was seeded in each well of a 96-well plate which had been precoated with 100 μL of a 0.1 mg/mL collagen. Plates were incubated for 25 minutes in a humidified incubator at 37°C. Wells were then washed four times in PBS and the adherent cells photographed using a digital camera under phase contrast at 5× magnification. Quantification was done by overlaying a grid onto each image and counting the number of cells per field of view.

Biochemical Methods. The levels of GTP-bound RhoA, Rac1, and Cdc42 were measured as described previously (30). Briefly, cells were grown in 10 cm diameter dishes until 50% to 70% confluent, then washed once in ice-cold PBS containing 0.5 mmol $CaCl_2$ and 0.5 mmol $MgCl_2$. Cells were lysed in 0.5 mL GTPase-lysis buffer [50 mmol Tris (pH 7.4), 100 mmol NaCl, 2 mmol $MgCl_2$, 10% glycerol, and 1% NP40] containing phenylmethylsulfonyl fluoride, leupeptin, benzamidine, aprotinin, and dithiothreitol. Lysates were equalized for protein content and incubated with 40 μg GST-PBD on beads (for 60 minutes) or an equivalent amount of GST-rhotekin on beads (for 30 minutes). The beads were washed thrice with lysis buffer without protease inhibitors. One dish of cells was used for each pulldown. To prepare lysates for immunoblotting, phosphatase inhibitors were also added to the lysis buffer. E-cadherin immunoprecipitates were made from cells grown in 10 cm tissue culture dishes to 95% confluency. After washing in ice-cold PBS, cells were lysed in 2 mL of lysis buffer [50 mmol Tris (pH 7.6), 200 mmol NaCl, 5 mmol $MgCl_2$, 0.5% NP40, 1 mmol phenylmethylsulfonyl fluoride, 2 mmol NaF, 1 mmol sodium orthovanadate, 5 μg/mL leupeptin, and 2.2 μg/mL aprotinin] and the lysates were clarified by centrifugation. Total protein (700 μg) was incubated overnight with 15 μL of anti-E-cadherin antibody (BD Transduction Labs) coupled to protein G-Sepharose beads. The immunoprecipitates were washed four times with lysis buffer and an aliquot used for SDS-PAGE and immunoblotting.

Results

Targeted Deletion of Oncogenic *K-RAS* Alters Cell Adhesion and Motility in Colon Cancer Cells. HCT116 cells are a metastatic human colorectal adenocarcinoma cell line which has one wild-type and one mutant (G13D) *K-RAS* allele. To investigate the role of endogenous levels of oncogenic K-Ras in maintaining the motility, adhesion, and actin cytoskeleton in these cells, we compared parental HCT116 cells with two isogenic derivative cell lines (Hke-3 and Hkh-2) in which the *K-RAS*^{G13D} allele was deleted by homologous recombination (27). The deletion of endogenous *K-RAS*^{G13D} from HCT116 cells resulted in cell lines exhibiting a more flattened morphology (Fig. 1*A*), a feature which correlated with their increased adhesiveness to collagen (Fig. 1*B*). In wound-healing assays, Hke-3 and Hkh-2 cells were less motile than their parental counterparts and closed wounds at a slower rate (Fig. 1*C*). Although *RAS* oncogenes have been implicated in the disruption of intercellular junctions, we found that cell-cell junctions remained intact in all three cell lines under normal growth conditions (data not shown). To test whether formation of these junctions differed, HCT116 and Hke-3 were first grown in calcium-free medium, then switched to normal growth medium to allow junctions to reform (Fig. 1*D*). In both cell lines, cadherin-mediated adherens junctions formed within 3 hours after the calcium switch, indicating that oncogenic K-Ras did not alter the formation of these junctions. In addition, E-cadherin immunoprecipitates prepared from HCT116 and Hke-3 cells contained similar amounts of major components of the adherens junction complex (Fig. 1*E*). These results show that although oncogenic K-Ras was required to maintain the poor cell-matrix adhesion and enhanced motility of HCT116 cells, it did not overtly alter their intercellular adhesion.

Oncogenic *K-RAS* Maintains a Disrupted Actin Cytoskeleton by Deregulating Rho Signaling. HCT116 cells were devoid of stress fibers and focal complexes/adhesions with actin localized predominantly to the periphery (Fig. 2*A* and *B*). The endogenous *K-RAS* oncogene was required to maintain both these characteristics of HCT116 cells because *K-RAS*^{G13D}-deleted cells were able to assemble stress fibers and focal complexes/adhesions. The reappearance of stress fibers and focal adhesions in the knockout cells correlated with an increase in phosphorylation of myosin II light chains and cofilin, two downstream targets of the Rho-ROCK

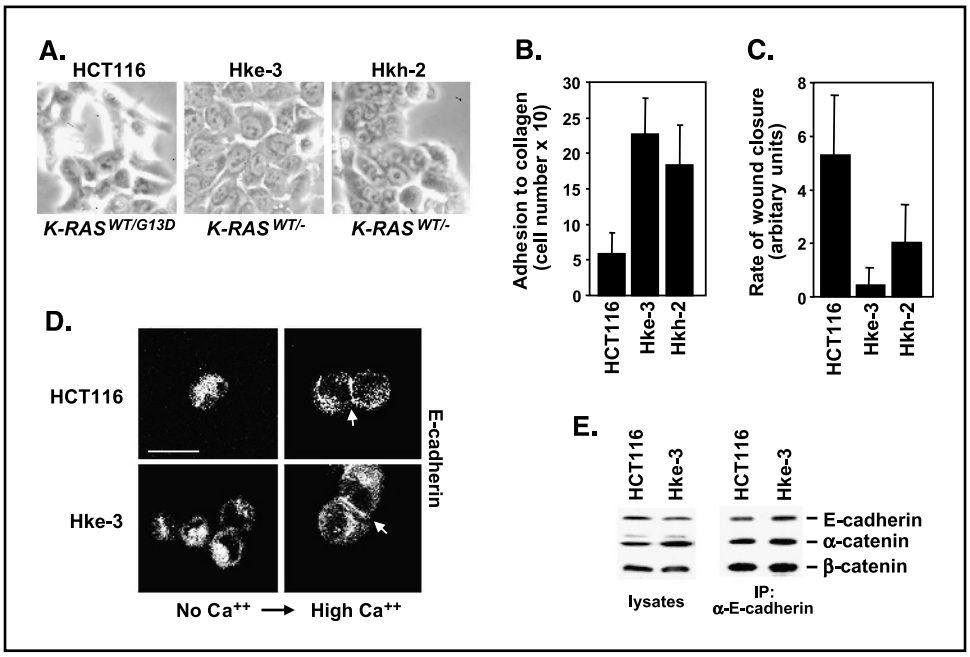

Figure 1. Effect of deletion of *K-RAS*^{G13D} cells on the adhesion and motility of HCT116 cells. *A,* phase-contrast images of HCT116, Hke-3, and Hkh-2 cells; *B,* adhesion of cell lines to collagen-coated tissue culture plates; *C,* relative rates of wound closure by cell lines; *D,* HCT116 and Hke-3 cells were incubated in calcium-free growth medium for 24 hours, then switched to medium containing high calcium for 3 hours. Cells were then fixed and stained for E-cadherin (*bar,* 20 μmol/L); *E,* immunoblotting of E-cadherin immunoprecipitates from HCT116 and Hke-3 cells with antibodies to α-catenin and β-catenin.

pathway (Fig. 2*C*). In addition, we found that transient reintroduction of K-Ras^{G13D} into the knockout cells resulted in the loss of stress fibers (Fig. 2*D*). This reorganization of the actin cytoskeleton by oncogenic K-Ras may explain why HCT116 cells adhere poorly to collagen and are motile.

As Rho signaling has been shown to regulate stress fiber assembly (16), we asked if changes in the activities of Rho GTPases could account for the K-Ras^{G13D}-mediated changes in the actin cytoskeleton. To this end, we did pulldown assays to capture Rho-GTP (using GST-rhotekin) or GTP-bound forms of Rac (using GST-PBD). Deletion of *K-RAS*^{G13D} reduced Rho-GTP levels in the two knockout cell lines by ~40% to 60% (Fig. 3*A*). Rac1-GTP levels were also reduced in these cells, but the extent of the reduction was smaller (~20-40%). Thus, despite having higher levels of Rho-GTP than the oncogenic *K-RAS*-deleted cells, HCT116 cells were devoid of stress fibers and focal adhesions, suggesting that Rho signaling was impaired in these cells. By contrast, stress fiber formation in Hkh-2 cells (and Hke-3 cells; results not shown) required Rho-ROCK signaling because treatment of these cells with a cell-permeable Rho inhibitor, TAT-C3 or the ROCK inhibitor, Y-27632, resulted in the loss of stress fibers (Fig. 3*B*). In addition to its effects on stress fiber formation, Rho signaling in Ras-transformed cells has been shown to suppress the expression of p21^{cip} (31, 32). Consistent with this, treatment of HCT116 cells with TAT-C3 increased p21^{cip} levels, indicating that this function of Rho remained intact (Fig. 3*C*). These results show that differences in the levels of Rho-GTP cannot account for the absence of stress fibers in HCT116 cells and suggest that oncogenic K-Ras maintained a disrupted actin cytoskeleton by selectively uncoupling Rho from stress fiber formation.

Oncogenic *K-RAS* Disrupts the Actin Cytoskeleton and Maintains Motility via MEK- and PI3-Kinase-Dependent Pathways. Two of the best characterized effectors of Ras are Raf and PI3-kinases. Raf kinases activate the ubiquitous MEK-ERK pathway,

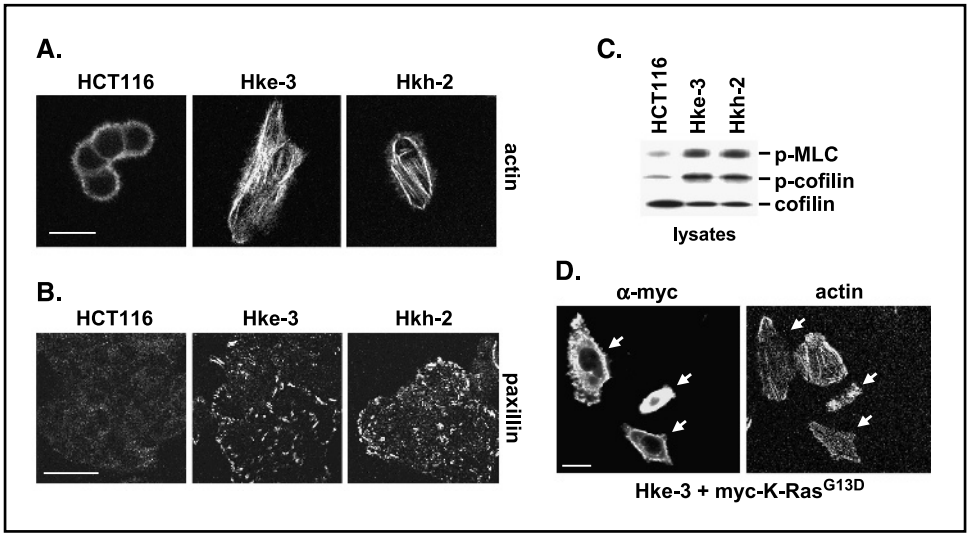

Figure 2. Effect of deletion of *K-RAS*^{G13D} on the actin cytoskeleton and focal adhesion/complex formation in HCT116 cells. HCT116, Hke-3, and Hkh-2 cells were fixed and stained with (*A*) phalloidin-FITC (*bar,* 20 μmol/L) or (*B*) anti-paxillin antibodies (*bar,* 40 μmol/L); *C,* immunoblotting of whole cell lysates of cell lines with antibodies to phospho-myosin II light chain (a mix of antibodies to serine 19 and serines 18/19), phospho-cofilin (serine 3) and cofilin; *D,* Hke-3 cells were transfected with myc-tagged K-Ras^{G13D}, then fixed and costained with an anti-myc tag antibody and phalloidin-FITC (*bar,* 20 μmol/L).

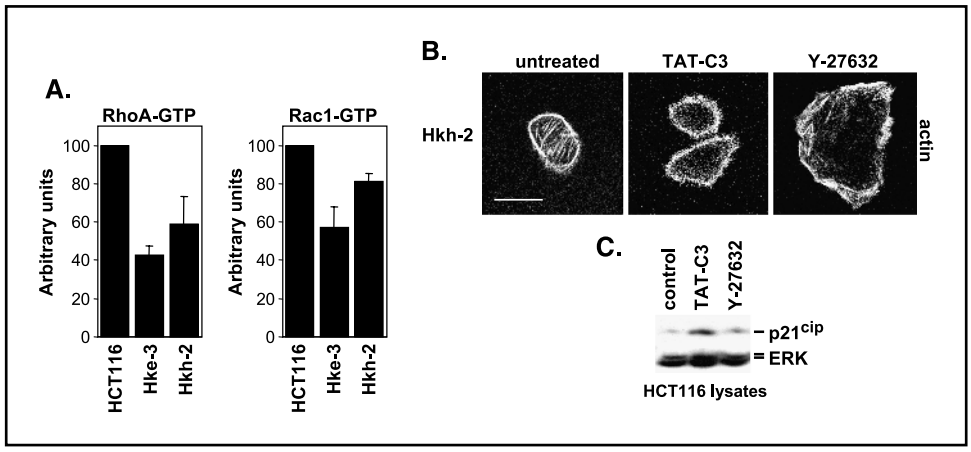

Figure 3. Impairment of Rho-mediated stress fiber formation by oncogenic K-Ras. *A,* pulldown assays measuring activities of RhoA (using GST-rhotekin beads) or Rac1 (using GST-PBD beads) in HCT116, Hke-3, and Hkh-2 cells. GTPase activities from three independent experiments were quantified by densitometry and their relative levels are shown as mean ± SE; *B,* staining of Hkh-2 cells with phalloidin-FITC before and after treatment with TAT-C3 or Y-27632 for 24 hours (*bar,* 20 μmol/L); *C,* immunoblotting of HCT116 cell lysates with a $p21^{cip}$ antibody before and after treatment with TAT-C3 or Y-27632 for 24 hours.

whereas PI3-kinases have numerous downstream targets, the best characterized being Akt. Interestingly, deletion of oncogenic *K-RAS* did not reduce phospho-ERK levels in subconfluent cultures growing cells, indicating that endogenous levels of $K\text{-}Ras^{G13D}$ do not cause hyperstimulation of ERK activity in HCT116 cells (Fig. 4*A*). Phospho-Akt levels were higher in Hke-3 cells than in HCT116 and Hkh-2 cells, suggesting that differences in Akt activity could not specifically be attributed to the presence of oncogenic K-Ras. We used pharmacologic inhibitors to test the role of these pathways in mediating the effects of oncogenic K-Ras on the actin cytoskeleton and motility. After initial experiments to determine the lowest concentration of UO126 and LY294002 that would effectively inhibit MEK and PI3-kinase signaling, respectively, we treated HCT116 cells with these inhibitors for 24 hours (Fig. 4*B* and results not shown). In wound-healing assays, UO126 and LY294002 reduced the rates of wound closure by HCT116 cells, indicating that both these pathways were required for the motility of these cells (Fig. 4*C*).

Next, we studied the effect of the inhibitors on the actin cytoskeleton. UO126, but not LY294002, restored stress fibers in HCT116 cells (Fig. 4*D*). However, both inhibitors increased the formation of focal adhesions (Fig. 4*E*). In cells treated with a combination of both inhibitors, stress fibers were more pronounced than in UO126-treated cells, although focal adhesions became larger, suggesting reduced turnover. Analysis of phospho-cofilin levels showed that inhibition PI3-kinase did not affect cofilin phosphorylation on its own, but enhanced the phosphorylation and inhibition of cofilin mediated by UO126, thus providing a mechanism for increased stress fiber formation (Fig. 4*F*). These results show that whereas MEK signaling alone is sufficient to disrupt stress fiber assembly in HCT116 cells, PI3-kinase signaling plays an accessory role in this process.

Inhibition of MEK Recouples Rho to Stress Fiber Formation. To test if inhibition of MEK signaling was sufficient was to recouple Rho to stress fiber formation, HCT116 cells treated with UO126 were subsequently incubated with TAT-C3 to inhibit Rho activity

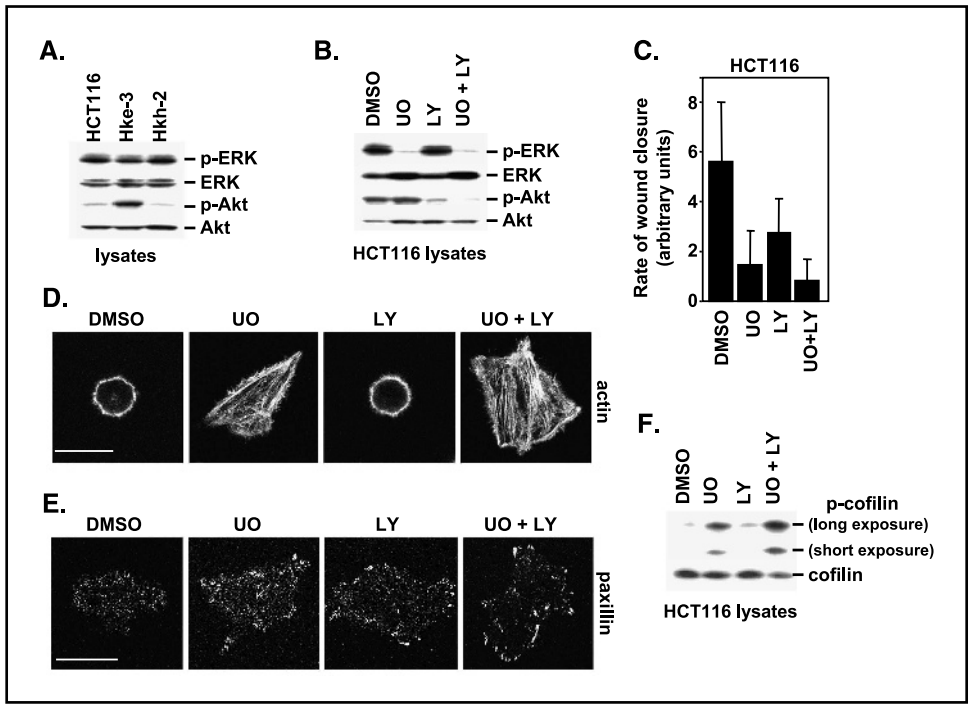

Figure 4. Effect of MEK and PI3-kinase inhibitors on the motility, actin cytoskeleton, and focal adhesions/complexes in HCT116 cells. *A,* phospho-ERK and phospho-Akt levels in whole cell lysates of HCT116, Hke-3, and Hkh-2 cells; *B,* phospho-ERK and phospho-Akt levels in whole cell lysates of HCT116 cells after treatment with 5 μmol/L UO126, 8 μmol/L LY294002, or a combination of both inhibitors for 24 hours; *C,* relative rates of wound closure by HCT116 cells after treatment with inhibitors as in (*B*); *D,* staining of HCT116 cells with phalloidin-FITC and anti-paxillin antibodies after treatment with inhibitors as in (*B*), *bar,* 20 μmol/L; *E,* phospho-cofilin levels in whole cell lysates of HCT116 cells after treatment with inhibitors as in (*B*).

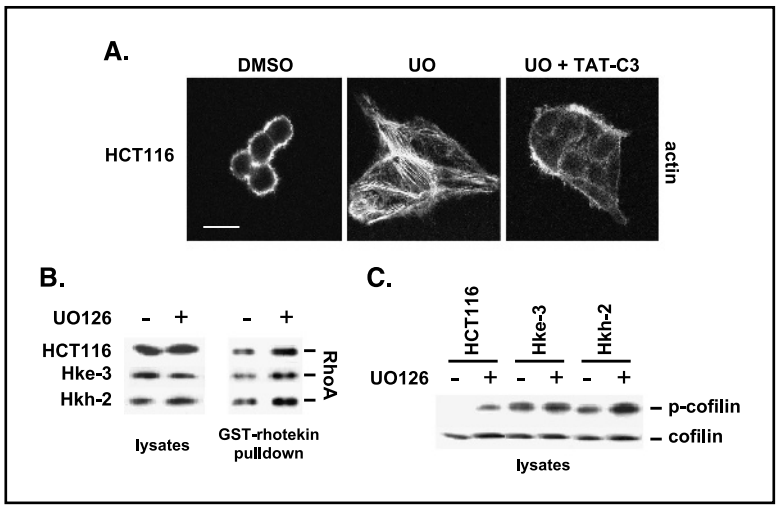

Figure 5. Effect of MEK inhibition on Rho signaling. *A,* cells treated with DMSO, 5 μmol/L UO126, or a combination of UO126 and TAT-C3 for 24 hours were fixed and stained with phalloidin-FITC (*bar,* 20 μmol/L); *B,* pulldown assays measuring RhoA-GTP levels in HCT116, Hke-3, and Hkh-2 cells after treatment with DMSO or 5 μmol/L UO126 for 24 hours. Levels of GTP-bound proteins and their levels in whole cell lysates were visualized by immunoblotting with anti-RhoA antibodies; *C,* phospho-cofilin levels in lysates of HCT116, Hke-3, and Hkh-2 cells after treatment with UO126.

and stained for actin (Fig. 5*A*). The stress fibers formed in UO126-treated cells were lost upon TAT-C3 treatment, indicating that MEK inhibition indeed recoupled Rho to stress fiber formation. Analysis of Rho-GTP levels showed that UO126 slightly increased Rho-GTP levels in all three cell lines, indicating that this inhibitory effect of MEK signaling on Rho activity did not require the presence of oncogenic Ras (Fig. 5*B*). In contrast to HCT116 cells where this increase in Rho activity correlated with the reappearance of stress fibers and an increase in cofilin phosphorylation, in the oncogenic *K-RAS*-deleted cells, it did not have a major effect on phospho-cofilin levels but caused a strengthening of stress fibers (Fig. 5*C* and results not shown). Taken together with the finding that HCT116 cells have higher Rho-GTP levels than the oncogenic *K-RAS*-deleted cells (Fig. 3*A*), our results show that oncogenic K-Ras uses a MEK signaling pathway to uncouple Rho from the assembly of stress fibers, thus maintaining a disrupted actin cytoskeleton.

Elevated Levels of Fra-1 Are Required to Maintain the MEK-Mediated Uncoupling of Rho from Stress Fiber Formation in HCT116 Cells. In a recent study, increased levels of the activator protein-1 family member, Fra-1, were reported to regulate Rho signaling and motility in colon cancer cells (23). Surprisingly, although phospho-ERK levels were similar in HCT116 and the oncogenic K-Ras-deleted cell lines (Fig. 4*A*), Fra-1 levels were markedly elevated in HCT116 cells (Fig. 6*A*). In all three cell lines, Fra-1 expression was dependent on MEK activity (Fig. 6*B*). We then tested if the higher Fra-1 levels in HCT116 cells were required to maintain the disruption of actin cytoskeleton. Reducing Fra-1 levels using Fra-1 short interfering RNA oligonucleotides resulted in the reappearance of stress fibers in these cells (Fig. 6*C*) confirming the results from a recent study (23). Conversely, expression of Fra-1 in Hke-3 cells caused the loss of stress fibers (Fig. 6*D*). These results thus provide genetic evidence that elevated Fra-1 levels resulting from $K\text{-}RAS^{G13D}$ signaling via MEK are required to maintain disruption of the actin cytoskeleton in HCT116 cells.

Discussion

The mechanisms whereby *RAS* oncogenes maintain the transformed characteristics of human cancer cells are poorly understood and may differ from those required for tumor initiation. In the present study, we show that oncogenic K-Ras uses a MEK-ERK-Fra-1 pathway to deregulate Rho signaling and thus maintain a disrupted actin cytoskeleton in HCT116 colon cancer cells. This contributes to the poor cell-matrix adhesion and enhanced motility of these cells. In addition, we show that PI3-kinase signaling is also required for the motility of these cells.

Because Ras proteins have multiple effectors, we have used a genetic approach to identify phenotypic characteristics of HCT116 colon carcinoma cells which required the presence of the mutant *K-RAS* allele (27). We then investigated the role of some of the major Ras effector pathways in regulating these processes. This approach allows the function of the endogenous oncogene in human cancer cells to be studied without the need for over-expression, a common caveat of many previous studies.

Despite mutations in *K-RAS* and *CTNNB1*, HCT116 cells retained the ability to form adherens junctions. Although our results show that the formation of these junctions were not affected by the presence of $K\text{-}RAS^{G13D}$, the oncogene may play a role in regulating the strength of these junctions after cells reach confluency and contribute to contact-independent proliferation of HCT116 cells.[4] The ability of *RAS* oncogenes to disrupt cell-cell junctions seems to be cell type–specific, indicating that additional factors may be required for this event to take place (10, 22, 33–35). In contrast to cell-cell junctions, $K\text{-}RAS^{G13D}$ was clearly required to maintain poor adhesion of HCT116 cells to the extracellular matrix component, collagen. In addition, oncogenic K-Ras signaling was required to maintain the motility of these cells. The profound disruptive effect of the K-Ras oncogene on the actin cytoskeleton and on the ability of cells to form focal adhesions/complexes may be a major reason for these characteristics of HCT116 cells.

When tracing the molecular mechanisms that maintain this disruption of the actin cytoskeleton, we found that oncogenic K-Ras was responsible for deregulated Rho signaling. In HCT116 cells, high levels of RhoA-GTP (in comparison to the $K\text{-}RAS^{G13D}$-deleted cells) were unable to induce stress fiber formation. However, the high Rho activity was required to maintain low levels of $p21^{cip}$, as described previously (31, 32). Thus, the presence of oncogenic K-Ras

[4] C.B. Pollock, W. Kolch, and A.S. Dhillon, unpublished data.

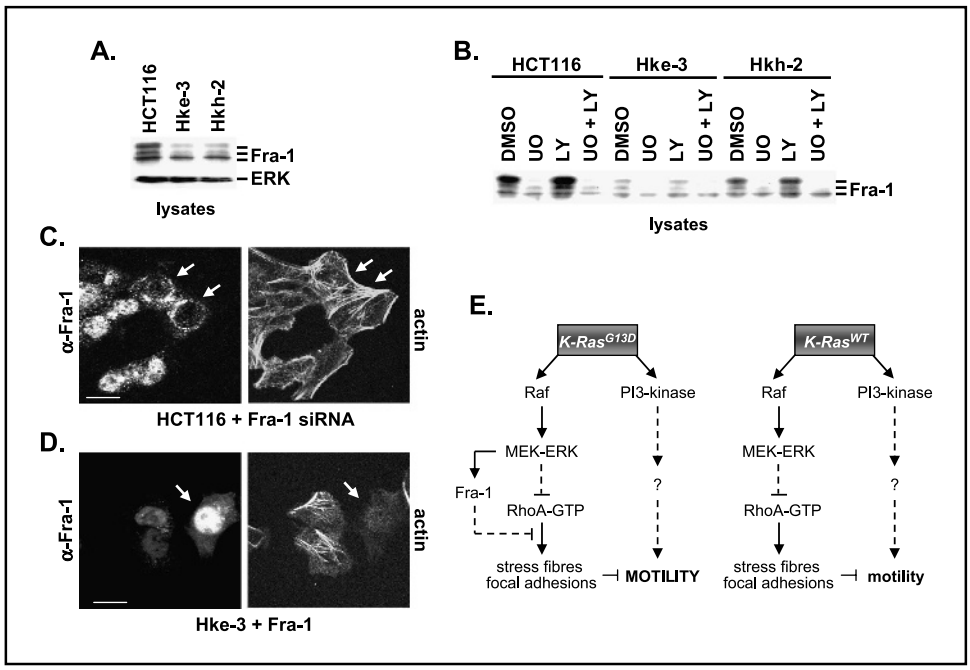

Figure 6. Role of Fra-1 in oncogenic K-Ras-mediated inhibition of stress fiber formation. *A,* Fra-1 levels in lysates of HCT116, Hke-3, and Hkh-2 cells; *B,* Fra-1 levels in cell lines treated with 5 μmol/L UO126, 8 μmol/L LY294002, or a combination of both inhibitors for 24 hours; *C,* HCT116 cells were transfected with Fra-1 short interfering RNA oligonucleotides, then fixed and costained with phalloidin-FITC and an anti-Fra-1 antibody (*bar,* 40 μmol/L); *D,* Hke-3 cells were transfected with pCMV-Fra-1, then fixed and costained with phalloidin-FITC and an anti-Fra-1 antibody (*bar,* 40 μmol/L); *E,* model of oncogenic K-Ras regulatedsignaling pathways involved in maintaining actin cytoskeletal disruption, poor cell-matrix adhesion and motility in HCT-116 cells. See Discussion for details.

specifically uncoupled Rho from its ability to organize the actin cytoskeleton but not other effector pathways. Such an uncoupling has been previously reported in Ras-transformed fibroblasts but its role in human cancer cells is less clear (32, 35, 36). In fibroblasts, several mechanisms seem to be involved in the uncoupling process, including changes in ROCK expression, ROCK localization and cytosolic pools of $p21^{cip}$, which can bind to ROCK inhibit its function. We did not observe differences in either ROCK expression or $p21^{cip}$ localization in the cell lines (results not shown). Our results instead suggest that elevated levels of the activator protein-1 family member, Fra-1, are required to maintain the uncoupling of Rho from actin reorganization in HCT116 cells. This conclusion is supported by the observation that reducing Fra-1 levels in HCT116 cells or increasing Fra-1 levels in Hke-3 cells reversed their phenotype with respect to their actin cytoskeleton. Surprisingly, whereas MEK-ERK signaling was required to maintain the elevated Fra-1 levels in HCT116 cells, an increase in ERK activity was not. This may be explained by recent reports that Fra-1 expression in Ras-transformed cells is dependent on both transcriptional autoregulation and MEK-dependent posttranscriptional stabilization (37, 38).

It was also recently reported the ERK-dependent elevation in Fra-1 levels in colon cancer cells reduced integrin-mediated activation of Rho, leading to a disruption of stress fiber formation (23). Our data, however, show that the increase in Rho activity upon inhibition if MEK-ERK signaling can occur even in cells having low Fra-1 levels, as is the case with the oncogenic *K-RAS* knockout cells. Our results support a model in which oncogenic K-Ras uses MEK-ERK-Fra-1 signaling to specifically uncouple Rho activation from stress fiber formation (Fig. 6*E*). It is not presently clear how high Fra-1 levels mediate this uncoupling process, but proteins which can disrupt signaling by Rho effectors or which regulate the spatiotemporal control of Rho signaling may be good candidates.

Our findings support those of several studies showing the critical role of activator protein-1 family members such as Fra-1 in regulating multiple aspects of transformation, including proliferation, cell motility, and morphology (23, 39–42). Taken together with the observation that cells stably transformed with *H-RAS* or *K-RAS* oncogenes have increased levels of Fra-1 (43, 44), our findings suggest that the MEK-ERK-Fra-1 pathway is a crucial pathway involved in both the initiation and maintenance of transformation by *RAS* oncogenes.

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