ABSTRACT

N-RAS is one member of a family of oncoproteins that are commonly mutated in cancer. Activating mutations in NRAS occur in a subset of colorectal cancers, but little is known about how the mutant protein contributes to the onset and progression of the disease. Using genetically engineered mice, we find that mutant N-RAS strongly promotes tumorigenesis in the context of inflammation. The protumorigenic nature of mutant N-RAS is related to its antiapoptotic function, which is mediated by activation of a noncanonical mitogen-activated protein kinase pathway that signals through STAT3. As a result, inhibition of MAP–ERK kinase selectively induces apoptosis in autochthonous colonic tumors expressing mutant N-RAS. The translational significance of this finding is highlighted by our observation that NRAS mutation correlates with a less favorable clinical outcome for patients with colorectal cancer. These data show for the first time the important role that N-RAS plays in colorectal cancer.

SIGNIFICANCE: Little is known about N-RAS function in normal biology or in cancer. Our study links the antiapoptotic function of mutant N-RAS to its ability to promote colorectal cancer in an inflammatory context. In addition, our study pinpoints a therapeutic strategy for this distinct colorectal cancer subtype. Cancer Discov; 3(3); 294–307. ©2013 AACR.
INTRODUCTION

The RAS protein family consists of 4 highly homologous enzymes (H-RAS, N-RAS, K-RAS4A, and K-RAS4B) that are identical over the first 85 amino acids, 85% identical over the next 80 amino acids, and largely divergent within the C-terminal 24 amino acids, a domain that is referred to as the hypervariable region (HVR). Proteins of the RAS family act as GDP/GTP-regulated switches to mediate cellular responses to extracellular signals (1). When bound to GTP, RAS proteins assume a conformation that allows them to engage and activate a multitude of downstream effectors. Each of the RAS isoforms can be locked into its GTP-bound activated state via missense mutation, typically at amino acid 12, 13, or 61 (2). Mutant RAS proteins accumulate in the GTP-bound conformation owing to defective intrinsic GTPase activity and/or resistance to inactivation by GTPase-activating proteins (3). Such activating mutations are common in human cancers. Consistent with the high degree of homology shared by members of the family, biochemical assays have failed to uncover significant differences between RAS isoforms. Nevertheless, genetic studies have revealed apparent isoform-specific phenotypes. In human cancers, for example, mutations in different RAS genes are preferentially associated with distinct tumor types. KRAS mutations are extremely common in cancers of the pancreas, colon, and lung, whereas NRAS mutations predominate in melanoma and hematopoetic cancers (4). Given the highly conserved enzymatic function of the RAS isoforms, it remains unclear what accounts for their differing mutational frequencies. Although variable expression levels and/or patterns in specific tissues might underlie some of the observed biologic differences (5), emerging evidence supports the idea that each RAS isoform is truly functionally unique. For example, we reported the generation of mice genetically engineered to express mutationally activated N-RAS (N-RAS\textsuperscript{G12D}) and K-RAS (K-RAS\textsuperscript{G12D}) specifically in the intestinal epithelium (6). Whereas activating mutations in KRAS occur in 40% of human colorectal cancers, NRAS mutations occur in only 3% (7–9). In the mouse colonic epithelium, K-RAS\textsuperscript{G12D} induces hyperproliferation that manifests as chronic intestinal hyperplasia and, in the context of mutant APC, strongly enhances the transition from a benign adenoma to a malignant adenocarcinoma. In contrast, N-RAS\textsuperscript{G12D} does not affect basal homeostasis or tumor progression, but instead inhibits the ability of intestinal epithelial cells to undergo apoptosis in response to stress (6). At present, it is unclear how or if the antiapoptotic phenotype associated with mutationally activated N-RAS contributes to the initiation and progression of colorectal cancer.

Because N-RAS is the least studied of the RAS family GTPases, its isoform-specific oncogenic properties are not well characterized. Unlike K-RAS and H-RAS, N-RAS is not activated by specific cytokines [e.g., interleukin (IL)-3] or growth factors (e.g., EGF; ref. 10). In myeloma cells, however, expression of mutationally activated N-RAS produces a transcriptional response similar to that after treatment with IL-6 (11, 12). Although loss of mutant N-RAS in some cell types is associated with a reduction in proliferation (13), N-RAS function has also been linked to the regulation of apoptosis. For example, knockdown of mutant NRAS in melanoma cells causes apoptosis, suggesting that mutant N-RAS provides a steady-state survival signal (14). In our study, we have examined the molecular mechanisms underlying the antiapoptotic function of N-RAS in colonic...
epithelial cells and connected these mechanisms to the ability of mutant N-RAS to promote colorectal cancer.

**RESULTS**

**Activated N-RAS Promotes Colorectal Cancer in the Context of Inflammation**

We have previously shown that expression of N-RAS$^{G12D}$ in the colonic epithelium has no effect on basal homeostasis, but instead protects the epithelium from apoptosis induced by acute exposure to dextran sodium sulfate (DSS; ref. 6). On the basis of this observation, we hypothesized that NRAS mutations might arise in colorectal cancer under circumstances of chronic apoptotic stimulus. Inflammation is a strong risk factor for colorectal cancer, and it can also promote apoptosis of epithelial cells (15). To determine whether activated N-RAS affects colonic epithelial homeostasis in the context of inflammation, we induced colitis in N-RAS wild-type (Villin-Cre, referred to as WT) and N-RAS–mutant (Villin-Cre; Nras$^{LSL-G12D/+}$) animals by treating them with cycles of DSS (5 days on, 10 days off).

Animals expressing N-RAS$^{G12D}$ were relatively resistant to the chronic effects of DSS, as measured by weight loss and activation of T cells in the mesenteric lymph nodes (Supplementary Fig. S1A–S1C). Whereas Villin-Cre; Nras$^{LSL-G12D/+}$ animals developed lower grade inflammation than WT mice, the colonic epithelium was hyperproliferative in N-RAS mutants compared with WT animals (Supplementary Fig. S1D). These observations indicate that expression of mutant N-RAS in the colonic epithelium plays a dual role in this mouse model: (i) it suppresses the initial DSS-induced apoptosis that is required for the initiation of colitis, and (ii) it promotes hyperproliferation in the context of colitis.

In WT mice, the extent of colitis typically increases as animals receive sequential cycles of DSS. After 9 cycles of DSS, WT animals developed severe colitis that was associated with epithelial damage and bleeding ulcers (Fig. 1A and B). The colitis that developed in animals expressing N-RAS$^{G12D}$ was markedly reduced, although focal regions of inflammation were present (Fig. 1A and B). Remarkably, half of the animals expressing N-RAS$^{G12D}$ (but none of the WT controls)
developed colonic adenocarcinomas exhibiting high levels of nuclear β-catenin (Fig. 1A and C), suggesting that activated N-RAS promotes colorectal cancer driven by inflammation.

An alternative way to induce colorectal cancer driven by inflammation is to pretreat animals with a single dose of the colon-specific carcinogen azoxymethane (AOM), followed by 3 cycles of DSS (16). Under this protocol, 5 of 7 WT animals developed colonic tumors, with most animals developing just a single macroscopically visible tumor (Fig. 1D). All of the animals expressing N-RAS\(^{G12D}\) (12 of 12) developed at least a single tumor, with most animals developing multiple (Fig. 1D).

Histologically, the tumors expressing mutant N-RAS were not clearly different from those expressing WT N-RAS (Fig. 1E and Supplementary Fig. S1E). One explanation for this observation is that AOM/DSS-induced tumors from WT mice acquire \(N\)-ras mutations. To explore this concept, we sequenced exons 2 (containing amino acids 12/13) and 3 (containing amino acid 61) of \(N\)-ras in tumors from WT mice treated with AOM/DSS. We found zero mutations in 21 tumors that were evaluated, indicating that \(N\)-ras is not commonly mutated in AOM/DSS-induced tumors from WT animals.

Because mutant N-RAS enhanced proliferation of the normal epithelium in the context of inflammation, we expected N-RAS-mutant tumors to be hyperproliferative relative to those that developed in a WT background. Unexpectedly, they were not (Supplementary Fig. 1F). Consistent with our previous observation that N-RAS\(^{G12D}\) suppresses stress-induced apoptosis, however, we did detect a difference in the basal levels of apoptosis in WT and N-RAS-mutant colonic tumors (Fig. 1F). Altogether, these observations suggest that mutationally activated N-RAS promotes colorectal cancer by suppressing the chronic apoptotic stimulus provided by inflammation.

 Mutant Forms of RAS Exhibit Unique Apoptotic Phenotypes and Signaling Properties

To uncover the molecular mechanisms underlying the anti-apoptotic function of N-RAS, we generated human colonic epithelial cell lines that differ in their RAS genotype. Although these cell lines expressed similar levels of RAS (Supplementary Fig. S2), they differed significantly in their proliferative and apoptotic phenotypes—only mutant K-RAS induced hyperproliferation (Supplementary Fig. S3A), whereas only mutant N-RAS suppressed apoptosis in response to butyrate, a carboxylic acid that induces cell death through both intrinsic and extrinsic apoptotic pathways (Fig. 2A and Supplementary Fig. S3B). These data are consistent with our previous observations that mutant N-RAS alone can function to suppress apoptosis in colonic epithelial cells (6, 17).

Because no known human colorectal cancer cell lines expressing mutationally activated N-RAS exist, our experiments relied on cell lines in which N-RAS\(^{G12D}\) was ectopically expressed from a retrovirus. A caveat to these studies is that the antiapoptotic phenotype associated with mutant N-RAS might be due to overexpression, rather than due to a true functional difference among family members. To address specifically whether the mutant N-RAS in our colorectal cancer cell line was overexpressed relative to endogenous mutant N-RAS found in other cancers, we conducted RAS activity assays on a panel of cell lines (Supplementary Fig. 4A). This analysis confirmed that the levels of N-RAS-GTP in our colorectal cancer cells are comparable with the levels found in cell lines expressing endogenous mutant N-RAS.

Mutant forms of RAS are thought to engage multiple downstream effectors to transmit their oncogenic signal. To determine whether mutant N-RAS exhibits unique signaling properties when compared with mutant K-RAS and H-RAS, we used quantitative Western blotting to measure the effects of RAS activation on downstream effector pathways in serum-starved cells (Supplementary Fig. S4B). N-RAS\(^{G12D}\) activated extracellular signal–regulated kinase (ERK) and AKT (relative to WT), but failed to activate RALA, c-Jun-NH\(\_2\)kinase (JNK), or p38 mitogen-activated protein kinase (MAPK) (Fig. 2B). The signaling properties associated with mutant K-RAS differed somewhat from mutant N-RAS, for example, in the activation of AKT, but H-RAS seemed to exhibit signaling properties identical to that of N-RAS (Fig. 2B).

Although this signaling analysis did not reveal whether a specific pathway is required for N-RAS function, it did show that the phenotypic differences between N-RAS and the other family members (e.g., H-RAS) cannot be explained by differential signaling through canonical effector pathways.

 N-RAS Signals Specifically through RAF-1 to Suppress Apoptosis

While N-RAS\(^{G12D}\) is clearly able to engage multiple downstream pathways, it remained unclear whether activation of a particular effector pathway is necessary or sufficient to mediate its antiapoptotic phenotype. To address this issue, we generated cell lines expressing N-RAS\(^{G12D}\) along with a secondary mutation that restricts signaling to a particular effector pathway (Supplementary Fig. S2)—the T35S mutant binds preferentially to RAF, the E37G mutant shows preference for RALGDS, and the Y40C mutant binds preferentially to phosphoinositide-3-kinase (PI3K, ref. 18). N-RAS\(^{G12D/T35S}\) fully phenocopied N-RAS\(^{G12D}\) with respect to its ability to confer resistance to butyrate-induced apoptosis (Fig. 2C). This observation indicates that binding to RAF is sufficient for mutant N-RAS to protect cells from apoptosis. To confirm this result, we pretreated cells with AZ-628, a pan-RAF inhibitor, before induction of apoptosis with sodium butyrate. Inhibition of RAF had no effect on the apoptotic phenotype of WT cells, but reversed the antiapoptotic phenotype of N-RAS\(^{G12D}\) (Fig. 2D), indicating that RAF signaling is required downstream of N-RAS.

RAF is a protein family that consists of 3 highly related serine/threonine kinases: A-RAF, B-RAF, and C-RAF (also called RAF-1). To determine whether one RAF family member mediates the N-RAS antiapoptotic phenotype, we used lentivirus-mediated short hairpin RNA (shRNA) to knock down RAF levels in cells expressing WT or mutant N-RAS (Supplementary Fig. S5A). Knockdown of RAF1, but not ARAF or BRAF, reverted the N-RAS phenotype toward that of WT (Fig. 2E, Supplementary Fig. S5B), suggesting that RAF-1, in particular, mediates the antiapoptotic function of N-RAS\(^{G12D}\).

To explore this concept further, we expressed different activated forms of RAF in colorectal cancer cells that were WT for N-RAS. Expression of mutationally activated RAF-1 protected cells from butyrate-induced apoptosis to the same extent as activated N-RAS (Fig. 2F). Expression of B-RAF or A-RAF did not phenocopy N-RAS, even though all 3 forms of RAF could activate ERK to the same extent.
Figure 2. Mutant N-RAS signals through RAF-1 to confer resistance to apoptosis induced by sodium butyrate. A, apoptotic phenotypes of colon cancer cell lines with mutant forms of RAS. Cells expressing WT RAS or H-RAS G12V were sensitive to induction of apoptosis by sodium butyrate (NaBu, 3 mmol/L for 24 hours). Retroviral expression of N-RAS G12D conferred resistance, whereas endogenous K-RAS G12D conferred hypersensitivity. It should be noted that codon 13 mutations in RAS have been shown to elicit attenuated transforming activity when compared with codon 12 mutations. B, canonical effector pathway signaling in cells expressing mutant RAS. Quantitative Western blotting revealed that mutant K-RAS, N-RAS, and H-RAS activated ERK, whereas only N-RAS and H-RAS activated AKT. For ERK, AKT, JNK, and p38, activation was measured as the ratio of phospho to total protein. For RALA, activation was measured as the ratio of GTP-bound protein to total protein. C, apoptotic phenotypes of colon cancer cell lines that express N-RAS G12D secondarily mutated within the effector-binding domain. Like N-RAS G12D, N-RAS G12D/T35S conferred resistance to butyrate-induced apoptosis. D, requirement of RAF for the antiapoptotic phenotype of N-RAS G12D. Treatment of WT cells with AZ-628 did not affect the response to butyrate, but inhibition of RAF in cells expressing N-RAS G12D reverted the antiapoptotic phenotype. E, effect of RAF knockdown on N-RAS G12D function. Cells expressing N-RAS G12D required RAF-1, but not A-RAF or B-RAF, to fully suppress butyrate-induced apoptosis. F, apoptotic phenotypes of colon cancer cell lines that express mutant forms of RAS. RAF-1 Y340/341D fully phenocopied N-RAS G12D. Mutationally activated A-RAF Y305/305D and WT B-RAF (which has high endogenous kinase activity due to aspartic acid substitutions at the analogous positions) did not suppress butyrate-induced apoptosis. G, ERK activation by mutant RAF. A-RAF, B-RAF, and RAF-1 all activated ERK to the same extent. ERK activation was measured by quantitative Western blotting. In all panels, error bars ± SEM.
N-RAS in Colorectal Cancer

Altogether, our data have shown that N-RAS alone suppressed butyrate-induced apoptosis, even though all of the RAS family members could activate ERK (Fig. 2B). Moreover, we found that RAF-1 was also unique within the RAS kinase family in its ability to suppress apoptosis, whereas all of the RAF family members activated ERK to roughly the same extent (Fig. 2G). On the basis of these observations, we considered the possibility that (i) a MAP-ERK kinase (MEK)/ERK-independent pathway functions cooperatively downstream of N-RAS/RAF-1 to suppress butyrate-induced apoptosis, or (ii) noncanonical MAPK pathways mediate the antiapoptotic function of mutant N-RAS.

N-RAS Activates STAT3

Mutationally activated N-RAS can confer cytokine-independent growth upon previously IL-6–dependent cell lines (11, 12). Studies of mouse models have also shown that IL-6 signaling plays an important role in inflammation-driven colorectal cancer (19, 20). By extension, we explored whether N-RAS actively functions within the IL-6 signaling pathway to control the response of colorectal cancer cells to apoptotic stimuli. To begin, we treated WT and mutant cells with exogenous IL-6. When exposed to IL-6, cells expressing WT N-RAS became partially resistant to butyrate-induced apoptosis (Fig. 3A). IL-6 treatment failed to further protect cells expressing mutant N-RAS, suggesting that the downstream pathway activated by IL-6 was already activated in cells expressing N-RASG12D (Fig. 3A).

The primary downstream effector of IL-6 receptor (IL-6R) function is the STAT3 transcription factor (21). Cells expressing N-RASG12D or RAF-1Y340/341D expressed 2-fold higher levels of STAT3 phosphorylated on Tyr705 than did cells expressing WT RAS or mutant forms of K-RAS and H-RAS (Fig. 3B). Surprisingly, although ERK was highly activated in cells expressing mutant N-RAS, we found that STAT3 was not changed in the phosphorylation state of Ser727, an ERK phosphorylation site (Supplementary Fig. S6A). STAT3 is a transcription factor that regulates the expression of genes involved in proliferation and survival (21). We found that colorectal cancer cells expressing mutant N-RAS expressed higher levels of the canonical STAT3 target genes CCND1 and SOCS3 relative to WT (Fig. 3C). Similarly, we found that genes negatively regulated by STAT3 (JUN and JUNB) were expressed at lower levels in cells expressing N-RASG12D (Fig. 3C).

We next analyzed the apoptotic phenotypes of WT and mutant cells that lack STAT3. Similar to loss of RAF-1, loss of STAT3 reverted the N-RAS phenotype, but had no significant effects on the response of WT cells to butyrate (Fig. 3D). We also found that a small-molecule inhibitor of STAT3 (Statatin) could revert the apoptotic phenotype of N-RAS mutant cells to WT (Fig. 3E). Taken together, our results indicate that mutant N-RAS signals through STAT3 to regulate the cellular response to butyrate.

Our data suggested that N-RASG12D suppresses apoptosis by activating RAF-1 and STAT3, but it was unclear whether these signals were dependent on, or independent of, one another. To explore this question, we examined the phosphorylation status of STAT3 in cells that were treated with CI-1040, a MEK inhibitor (22). MEK is the only bona fide effector of RAF signaling. Inhibition of MEK led to a decrease in the phosphorylation status of STAT3 in cells expressing N-RASG12D (Fig. 3F). Alternatively, shRNA-mediated knockdown of STAT3 had no effects on the response of WT cells to butyrate (Supplementary Fig. S6B). These data suggest that N-RASG12D activates STAT3 downstream of MEK.

N-RAS-GTP Forms a Complex with RAF-1 and STAT3

How does N-RAS activate STAT3 and why is it the only family member that can do so? One possibility is that mutant N-RAS induces an autocrine IL-6 feedback loop that activates STAT3 downstream of IL-6R. Contrary to this hypothesis, we did not detect an increase in secreted IL-6 in the culture media of cells expressing N-RASG12D (Supplementary Fig. S6C). Moreover, conditioned medium from cells expressing mutant N-RAS was not able to activate STAT3 in WT cells (Supplementary Fig. S6D). On the basis of these data, we believed that it was more likely that mutant N-RAS was directly activating STAT3 by forming a complex with it. Indeed, we found that N-RASG12D, but not K-RASG12D or H-RASG12V, communoprecipitated with STAT3 (Fig. 3G and Supplementary Fig. S6E and S6F). Consistent with our observation that mutant RAF-1 also activated STAT3, RAF-1 immunoprecipitated with STAT3 (Supplementary Fig. S6G). These results indicate that activated N-RAS forms an antiapoptotic signaling complex that includes STAT3 and RAF-1.

Mutant N-RAS Signals from Cholesterol-Rich Microdomains to Suppress Apoptosis

Mutant forms of N-RAS and H-RAS are equal in their ability to activate ERK, but only N-RAS can activate STAT3. We hypothesized that their differing abilities to activate STAT3 may result from a difference in subcellular localization. Consistent with this hypothesis, replacing the H-RAS HVR with that of N-RAS allowed H-RAS to suppress butyrate-induced apoptosis, suggesting that the antiapoptotic phenotype of mutant N-RAS is specified by its localization (Fig. 4A). On the plasma membrane, GTP-bound forms of N-RAS and H-RAS are thought to differ in their distributions among cholesterol-rich microdomains (23, 24). Following biochemical purification, we found that mutant N-RAS, but not mutant H-RAS, could be detected in caveolin-rich fractions (Fig. 4B). Consistent with our proposed mechanism of action of mutant N-RAS, we found that ERK and STAT3 were highly enriched in the same fractions as N-RAS (Fig. 4B). Caveolae can be dissociated by exposure to filipin, an antifungal agent isolated from Streptomyces filipensis, or to methyl-β-cyclodextrin (MβCD). The ability of N-RAS to communoprecipitate with STAT3 was affected by treatment of cells with filipin (Fig. 3G), and transient exposure to filipin or MβCD abrogated the antiapoptotic phenotype of mutant N-RAS (Fig. 4C), suggesting that, in colorectal cancer cells, N-RAS signals from caveolae to suppress apoptosis.

Mutant N-RAS Activates ERK and STAT3 in Primary Cancers

To determine whether mutant N-RAS signals through ERK and STAT3 in primary colorectal cancers, we examined their activation states in tumors from mice and humans by immunohistochemistry. In tumors from AOM/DSS-treated animals, there was no detectable difference in ERK activation between WT and N-RAS–mutant tumors, primarily because ERK was strongly
Figure 3. Mutant N-RAS signals through STAT3 to confer resistance to apoptosis induced by sodium butyrate. 

A, effect of exogenous IL-6 on butyrate-induced apoptosis. IL-6 significantly suppressed apoptosis in WT cells, but failed to do so in cells expressing N-RAS<sup>G12D</sup>. 

B, activation of STAT3 by mutant N-RAS and RAF-1. Expression of N-RAS<sup>G12D</sup> or RAF-1<sup>Y340/341D</sup> led to hyperphosphorylation of STAT3 at a positive regulatory site, Tyr705. 

C, expression of STAT3 target genes. Two genes known to be upregulated by activated STAT3, CCND1 and SOCS3, were also upregulated in cells expressing N-RAS<sup>G12D</sup>. Two genes known to be downregulated by activated STAT3, IFIT3 and IFI35, were also downregulated in cells expressing N-RAS<sup>G12D</sup>. 

D, effect of STAT3 knockdown on N-RAS<sup>G12D</sup> antiapoptotic function. Treatment of WT cells with Statist, a small-molecule STAT3 inhibitor, did not significantly affect the response to butyrate. 

E, effect of STAT3 inhibition on N-RAS<sup>G12D</sup> antiapoptotic function. Treatment of WT cells with Statist in cells expressing N-RAS<sup>G12D</sup> reverted the antiapoptotic phenotype. 

F, effect of MEK inhibition on STAT3 activation. Treatment with CI-1040 decreased STAT3 phosphorylation on Tyr705 ("P < 0.05, Wilcoxon rank-sum test"). The phosphorylation of ERK was measured as a control for the activity of the MEK inhibitor. 

G, N-RAS interacts with STAT3. α-STAT3 antibody was able to immunoprecipitate both STAT3 and N-RAS in cells expressing N-RAS<sup>G12D</sup>. This complex was dependent upon the presence of STAT3, N-RAS, and RAF-1. Transient exposure to filipin negatively affected complex formation. In this experiment, to control for the overall amount of N-RAS expressed in cells, "WT" denotes cells that ectopically overexpress WT N-RAS.

In A-F, error bars ± SEM.
N-RAS in Colorectal Cancer

What is the most effective way to target the MAPK pathway therapeutically? Over the past several years, a handful of highly specific MEK inhibitors have entered the laboratory and clinic (22, 25–27). Given that we had already shown that inhibition of MEK with CI-1040 could abrogate the activation of STAT3 downstream of N-RAS (22) (Fig. 3F), we next tested whether it could revert the antiapoptotic phenotype associated with mutant N-RAS. As with pharmacologic inhibition of RAF, inhibition of MEK suppressed the N-RAS apoptotic phenotype (Fig. 6A). Next, we treated mice bearing AOM/DSS-induced tumors with CI-1040. Acute inhibition of MEK induced apoptosis in tumors expressing N-RAS (22) but not in WT tumors (Fig. 6B). Together, these data suggest that MEK inhibitors could be highly efficacious for patients with N-RAS mutant colorectal cancer.

A Therapeutic Strategy for Colorectal Cancers Expressing Mutant N-RAS

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One major question remains: How significant is mutationally activated N-RAS to colorectal cancer in human patients? To address this question, we determined the N-RAS mutational status of 581 colorectal cancers from patients treated at Memorial Sloan-Kettering Cancer Center (MSKCC; New York, NY). Consistent with previous reports (8, 9), we found activating mutations in N-RAS in approximately 3% of cases (17 of 581; Supplementary Table S1). Importantly, when compared with patients whose cancers were WT for NRAS, KRAS, and BRAF, patients with NRAS-mutant cancers experienced significantly worse overall survival, similar to those with mutations in KRAS (Fig. 6C). This observation highlights the importance of identifying new therapies—for example, MEK inhibition—for colorectal cancers expressing mutant N-RAS.

The research article discusses the role of N-RAS in colorectal cancer and the potential therapeutic strategies targeting N-RAS. It highlights the importance of identifying N-RAS-mutant cancers, which have worse survival outcomes compared to WT colorectal cancers. The article also mentions the development of highly specific MEK inhibitors to target the MAPK pathway.
Figure 5. Activation of ERK and STAT3 in primary tumors expressing mutant N-RAS. A, immunohistochemistry for phospho-ERK in autochthonous mouse and human tumors. B, quantification of phospho-ERK staining in primary tumors. Staining was considerably less variable in mouse tumors (n = 10 for WT mouse tumors, n = 7 for N-RAS G12D mouse tumors, n = 17 for WT human cancers, and n = 19 for NRAS-mutant human cancers). C, enrichment plot for NRAS-mutant colorectal cancer. Genes related to ERK signaling were significantly enriched in cancers expressing mutant N-RAS. D, immunohistochemistry for phospho-STAT3 in autochthonous mouse and human tumors. E, evaluation of phospho-STAT3 staining in primary colonic tumors from animals treated with AOM/DSS (n = 10 for WT tumors, n = 8 for N-RAS G12D tumors, n = 17 for WT human cancers, n = 19 for NRAS-mutant human cancers). F, enrichment plot for NRAS-mutant colorectal cancer. Genes downregulated by STAT3 were negatively enriched in cancers expressing mutant N-RAS.
N-RAS in Colorectal Cancer

DISCUSSION

Colorectal cancer is a paradigm for the cooperative action of mutations in oncogenes and tumor suppressor genes (28). Understanding the disease at a level that will allow for a priori prediction of viable therapeutic targets will require a mechanistic elucidation of each of the mutational events that contribute to initiation and progression. Of particular importance is whether specific mutational events are cooperative or redundant, or whether they may contribute to the development of distinct subtypes of colorectal cancer. For example, on the basis of mutational analysis of colorectal cancer, mutations in K-RAS and NRAS seem to be redundant, as they are not typically found in the same tumor (Supplementary Table S1; refs. 8, 9). As a result, one might predict that a common therapeutic strategy would work for cancers that carry mutations in either of these 2 genes. Yet our functional analysis showed that mice expressing mutational activated forms of K-RAS or N-RAS in the colonic epithelium exhibited essentially non-overlapping phenotypes (6). We interpret these observations to mean that K-RAS and NRAS mutations are mutually exclusive, not because they are redundant, but because they are selected for under distinct tumorigenic contexts. The differential apoptotic function of N-RAS and K-RAS led us to speculate that NRAS mutations might arise specifically under circumstances of chronic apoptotic stimulus. Indeed, our studies of genetically engineered mice indicated that N-RASG12D enhanced colon cancer development in the context of inflammation (Fig. 1). Both chronic (as in inflammatory bowel disease) and acute inflammation contribute to the progression of colorectal cancer (29).

Using both in vitro and in vivo systems, we sought to uncover the molecular mechanisms that underlie the unique antiapoptotic function of N-RAS. In other cell types, mutant N-RAS has been correllatively linked to numerous survival and apoptotic pathways, including BCL2, AKT, JNK, and p38 (30–33). Our data failed to implicate these previously identified pathways in mediating N-RAS function in colonic epithelial cells, suggesting that the pathways used by mutant N-RAS to suppress apoptosis are context dependent. Our data indicated that N-RASG12D requires only RAF-1 to suppress apoptosis (Fig. 2C–E). Although each RAS family member is believed to bind all of the RAF family members, the specific engagement of RAF-1 by mutant N-RAS has also been observed in melanoma cells (34). Although N-RAS signals through RAF-1 to activate the canonical MAPK pathway, the RAF-dependent activation of ERK was not sufficient to explain the antiapoptotic phenotype associated with mutant N-RAS. On the basis of previous reports of N-RAS function in myeloma cells (11, 12), we examined whether STAT3 plays a role in the antiapoptotic function of N-RAS and RAF-1. Indeed, cells expressing mutant N-RAS exhibited hyperphosphorylation of STAT3 on Tyr705, and STAT3 was required for the antiapoptotic function of N-RASG12D (Fig. 3). Interestingly, STAT3 was also activated by endogenous mutant N-RAS when it was expressed in hematopoietic cells, suggesting that the noncanonical MAPK pathway may function in other contexts as well (35).

Because STAT3 functions as a transcription factor, we confirmed that it was functionally activated by measuring the expression levels of known STAT3 targets in cell lines and primary cancers expressing mutant N-RAS (Fig. 3C, SF). GSEA of mRNA
expression data from human colorectal cancers failed to identify enrichment for genes that are upregulated by STAT3 in cancers expressing mutant N-RAS. In contrast, GSEA identified a significant negative enrichment for genes that are downregulated by STAT3 in cancers expressing mutant N-RAS (Fig. 5F). Although it is somewhat difficult to interpret a lack of correlation in GSEA (i.e., the failure to find significant overexpression of STAT3 targets), this observation suggests that mutant N-RAS may specifically regulate the transcriptional repressive function of STAT3. Even though STAT3 is primarily known as a transcriptional activator, and many of its targets (e.g., c-Jun D1) are thought to promote cancer, it can also associate with the KAP1 corepressor to inhibit gene expression (36). The breadth and biologic significance of the genes negatively regulated by STAT3 are not clear, but these genes may play an important role in the antiapoptotic function of functionally activated N-RAS.

Our finding that N-RAS is unique among the RAS family members in its ability to bind STAT3 is not surprising, as other RAS family members also have unique binding partners. Galectins, for example, are a family of proteins characterized by their ability to bind β-galactoside. Mutant H-RAS binds galectin-1 more efficiently than does mutant K-RAS, and this interaction stimulates RAF activation at the expense of PI3K activation (37). Conversely, activated K-RAS binds more efficiently to galectin-3, resulting in prolonged activation of RAF and attenuation of PI3K and RAL signaling (38, 39). We speculate that isoform-specific downstream effectors account, at least in part, for the functional differences among RAS family members.

Why is N-RAS the only family member that can interact with and activate STAT3? Each RAS family member is subjected to its HVR (40). These posttranslational modifications affect its ability to bind STAT3 is not surprising, as other RAS family members in its ability to bind to farnesylation and palmitoylation within its HVR (40). These posttranslational modifications affect the trafficking of RAS family members through the cell, as well as their overall steady-state localization. Within membranes, RAS family members have been found to localize to distinct microdomains in a manner that is dependent upon their nucleotide binding state. N-RAS-GTP, for example, was found in cholesterol-rich lipid rafts, whereas H-RAS-GTP localized to disordered membrane (23, 24). In contrast, we failed to detect N-RAS in LYN kinase-positive lipid raft fractions in colorectal cancer cells, but we did find that N-RAS and H-RAS purified in distinct membrane fractions (Fig. 4B). Moreover, disruption of cholesterol-rich microdomains via treatment with filipin or MJ1CD abrogated resistance to apoptosis in cells expressing N-RAS (41, 42), indicating that microdomain localization is a major determinant underlying the unique antiapoptotic phenotype of mutant N-RAS.

As such, this subtle feature of RAS localization becomes of central importance to the oncogenic function of this protein. Our studies clearly establish that N-RAS, unlike K-RAS and H-RAS, can suppress stress-induced apoptosis because it activates a noncanonical MAPK pathway. A major question resulting from this observation is whether this mechanism can be taken advantage of as a therapeutic strategy. In human cell lines, pharmacologic inhibition of MEK abrogated the antiapoptotic function of mutant N-RAS (Fig. 6A). Two recent studies of large human cell line panels also connected N-RAS mutation to sensitivity to MEK inhibitors (41, 42). We also found that inhibition of MEK induced apoptosis in primary mouse tumors expressing mutant N-RAS (Fig. 6B), providing a critical in vivo validation of the in vitro results. These observations suggest that MEK inhibitors may be useful in the clinic to treat patients with N-RAS mutant colorectal cancer. This result is especially significant because, as we have shown previously, colorectal cancers expressing mutant K-RAS are not sensitive to MEK inhibition (6). Taken together, these experiments connect the antiapoptotic function of mutationally activated N-RAS to its oncogenic potential. Moreover, although we have shown that mutation of N-RAS affects the survival of patients with colorectal cancer, our biochemical data suggest a viable therapeutic strategy (inhibition of MAPK signaling) for these patients.

METHODS

Human Studies

Clinical data were collected on patients under protocols approved by the Institutional Review Boards of MSKCC and the Massachusetts General Hospital (MGH; Boston, MA). Tumor-associated mutations were identified as previously described (9). GSEA was conducted as described (43) on published RNA sequencing data from The Cancer Genome Atlas (44). Individual samples were separated on the basis of their KRAS, NRAS, and BRAF genotypes. The gene set corresponding to 39 targets down-regulated by STAT3 was manually collated from ref. (45).

Induction of Colitis and Cancer in Mice

All experiments involving animals were approved by the MGH Sub委员会 on Research Animal Care. Animals expressing K-RAS(G12D) and N-RAS(G12D) in the intestinal epithelium were previously described (6). Intestine-specific activation of RAS was achieved by crossing to mice expressing Villin-Cre, which directs expression of Cre recombinase to all crypts of the small intestinal and colonic epithelium (46). Eight-week-old mice were used in all experiments.

To stimulate colitis, mice were treated with 3 or 9 cycles of 2.5% DSS in the drinking water (5 days on, followed by 10 days off). Mice were weighed on a daily basis, and colonoscopy was conducted to monitor disease progression. At the end of the treatment, mice were sacrificed and tumor number was assessed by macroscopic examination of the dissected colon. The tissue was then fixed overnight in 10% formalin and processed for histologic analyses.

In the AOM/DSS model of inflammation-induced colon cancer, animals were treated with a single injection of AOM (10 mg/kg) on the first day and were then exposed to 3 cycles of 2.5% DSS (7 days on, 14 days off). Body weight was monitored daily. Five weeks after ending the treatment period, animals were sacrificed. Tumor number was scored by macroscopic examination of the dissected colon. The tissue was then fixed overnight in 10% formalin and processed for histologic analyses.

Mouse Colonoscopy

On the day before colonoscopy, animals were fasted for 18 to 24 hours, during which time they were given NuLYTELY, a bowel-cleansing solution of polyethylene glycol. Before the procedure, animals were anesthetized with Avertin (250 mg/kg). After sedation, the colon was flushed with PBS to remove any remaining fecal debris. Colonoscopy was conducted using a veterinary endoscope from Karl Storz Endoscopy.

CI-1040 Treatment of Mice

CI-1040 was obtained from Pfizer. N-RAS mutant and WT mice were submitted to AOM/DSS treatment to induce tumor formation. Tumor development was monitored by colonoscopy, and only those mice harboring tumors were selected for CI-1040 treatment. These mice were treated with 2 doses per day of 100 mg/kg of CI-1040 for
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...7 days. At the end of the treatment, tumors were excised and snap frozen to extract protein for Western blot analyses. The levels of apoptosis were examined by quantitative Western blotting using an antibody against cleaved caspase-3.

**Immunohistochemistry**

Immunohistochemistry was conducted on sections (5 μm) of paraffin-embedded mouse and human tissues. Antibodies for phospho-Histone H3 (pH3, Ser10), phospho-ERK (Thr202/Tyr204), and phospho-STAT3 (Tyr705) were from Cell Signaling Technology. The monoclonal antibody against CTNNB1 was from BD Biosciences. Immunohistochemistry was conducted following the manufacturer's instructions for each antibody. Proliferative indices were quantified via H3. The proliferative index of the epithelium was determined by counting the number of pH3-positive cells per crypt. To achieve statistical significance, at least 50 crypts were counted per sample. The proliferative index of tumors was assessed by counting the percentage of pH3-positive epithelial cells. Several magnification fields were analyzed to account for intratumoral variability.

For phospho-ERK, both human and mouse tumors were classified according to the intensity of the staining and the relative number of positive tumor glands. Phospho-STAT3 scoring was based on intensity of the staining, as its expression was relatively homogeneous throughout the tumors.

**Cell Culture, Infections, and Apoptotic Assays**

DLD-1, DKh-8, HCT-116, and HKe-3 human colorectal cancer cells have been described (47) and were provided to us by Dr. Robert Coffey (Vanderbilt University). Their identities were confirmed by RBD pulldown analysis (Supplementary Fig. S2). Melanoma cell lines were provided by Dr. Lawrence Kwong (Dana-Farber Cancer Institute, Boston, MA). They were not verified.

The pBabe retrovirus system was used to generate DKs-8 and HKe-3 cells expressing mutant forms of RAS. The DKh-8 and DBN isogenic pairs were used for all experiments, except for those described in Supplementary Fig. S3B. Gene knockdown was achieved with pSico Lentiviral shRNAs (48). To analyze apoptotic responses and cell signaling, cells were plated at 80% confluence with complete medium for 24 hours and then incubated in medium without serum for 12 hours following treatment with butyrate or sodium butyrate for the indicated timeframes. Apoptotic cells were quantified using the fluorescein isothiocyanate (FITC)-Annexin V Apoptosis Detection Kit I according to the manufacturer’s instructions (BD Biosciences). Each experiment was carried out at least twice, with each independent trial including biologic triplicates. All statistical analyses were conducted by the Wilcoxon Rank-Sum test using the MStat computer program.

**Real-Time PCR**

The mRNA expression levels of STAT3 target genes were measured by real-time PCR. RNA was extracted using the RNeasy Mini Kit (Qiagen). cDNA was synthesized with 2 μg of pure RNA using a SuperScript III Reverse Transcriptase kit (Invitrogen). One microliter of cDNA (diluted 1:5) was added to a 20 μL real-time PCR mixture containing 1X TaqMan Universal PCR Master Mix, No AmpErase UNG, and 1X TaqMan MGB specific probes. TaqMan expression assays were purchased from Applied Biosystems. Standard TaqMan thermocycling conditions were used. Real-time PCR assays were conducted on 3 biologic replicates.

**Expression Vectors**

The pBabe(puro)-H-RAS(G12D), pBabe(puro)-RAF1(T201/202)/G12/19, vectorhs have been described previously (49). pBabe(puro)-N-RAS(G12D), pBabe(puro)-N-RAS(G12D)-H-HVR, pBabe(puro)-H-RAS(G12D)-N-HVR, pBabe(puro)-RAF1(T201/202), pBabe(puro)-B-RAF, and pBabe(puro)-A-RAF(T304/308D) were generated for this study. The RAS effector loop domain mutants T3SS, E37G, and Y40C were generated in pBabe-N-RAS(G12D) by site-directed mutagenesis. The shRNAs for N-RAS, STAT3, ARAF, BRAF, and RAF-1 were designed with pSicoOligomaker and then cloned into pSicoR (48). Target sequences for specific shRNAs are listed in Supplementary Table S2.

**Membrane Fractionations**

Cellular fractionation was carried out using the simplified method for the preparation of detergent-free lipid rafts described by Macdonald and Pike (50). Briefly, cells were seeded in 150-mm dishes at similar densities. When the cells were approximately 90% confluent, protein was extracted following the protocol described by Macdonald and Pike (50). Fifty microliters of each fraction were used to analyze the distribution of N-RAS, H-RAS, ERK, STAT3, caveolin 1, LYN, and transferrin receptor (TFR) by quantitative Western blotting.

**Drug Treatments**

AZ-628 was obtained from AstraZeneca. Cells were pretreated with AZ-628 (1 μM) for 1 hour before treatment with butyrate or isoletin. Protein and MβCD were purchased from Sigma. In specified experiments, cells were transiently treated with filipin (1 μg/ mL) or MβCD (10 μM) for 2 hours, at which point the medium was replaced with serum-free medium ± sodium butyrate (3 μM/mL).

**Immunoblotting and GTPase Activity Assays**

For Western blotting, protein lysates were harvested with immunoprecipitation assay buffer. Quantitative Western blots were conducted using the LI-COR Odyssey. Antibodies to the following proteins were used: ERK1/2, phospho-ERK1/2 (Thr202/Tyr204, Thr185/Tyr187), AKT, phospho-STAT3 (Ser473), JNK, phospho-STAT3 (Thr183/Tyr185), p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), STAT3, and phospho-STAT3 (Tyr705 or Ser727) from Cell Signaling Technology. Additional antibodies included β-tubulin from Sigma and N-RAS, H-RAS, K-RAS, A-RAF, B-RAF, and RAF-1 from Santa Cruz Biotechnology. Secondary antibodies were from LI-COR. RAS and RAL activities were assessed with assay kits from Millipore. For RAS activity, N-RAS-specific antibody was used. For RAL, the RALA antibody included with the kit was used.

**Immunoprecipitations**

For immunoprecipitations, protein lysates were collected in 1X cell lysis buffer (Cell Signaling Technology). Immobilized STAT3 (79D7) rabbit antibody was used to purify STAT3 complexes from lysates. For immunoprecipitation of N-RAS, a mouse monoclonal antibody was used (Santa Cruz Biotechnology).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: Y. Wang, S. Velho, C.J. Der, M. Philips, D.B. Solit, K.M. Haigis
Development of methodology: Y. Wang
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