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Mutant Kras Promotes Hyperplasia and Alters Differentiation in the Colon Epithelium But Does Not Expand the Presumptive Stem Cell Pool

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Abstract

BACKGROUND & AIMS—Adenomatous polyps are precursors to colorectal cancer (CRC), whereas hyperplastic polyps (HPPs) have a small risk of progression to CRC. Mutations in *KRAS* are found in ~40% of CRCs and large adenomas and a subset of HPPs. We investigated the reasons that HPPs with *KRAS* mutations lack malignant potential; we compared the effects of *Kras/KRAS* activation to those of *Adenomatous polyposis coli (Apc)/APC* inactivation, which promotes adenoma formation.

METHODS—We activated a *KrasG12D* mutant allele or inactivated *Apc* alleles in mouse colon epithelium and analyzed phenotypes and expression of selected genes and proteins. The mouse data were validated using samples of human HPPs and adenomas. Signaling pathways and factors that contribute to Kras/KRAS-induced phenotypes were studied in intestinal epithelial cells.

RESULTS—Activation of *Kras* led to hyperplasia and serrated crypt architecture akin to that observed in human HPPs. We also observed loss of Paneth cells and increases in goblet cell numbers. Abnormalities in *Kras*-mediated differentiation and proliferation required mitogen-activated protein kinase (MAPK) signaling and were linked to activation of the Hes1 transcription factor. Human HPPs also had activation of *HES1*. In contrast to *Apc/APC* inactivation, *Kras/KRAS* activation did not increase expression of crypt stem cell markers in colon epithelium or colony formation in vitro. *Kras*/*KRAS* activation was not associated with substantial induction of $p16^{INK4a}$ protein expression in mouse colon epithelium or human HPPs.

CONCLUSIONS—Although *Kras/KRAS* mutation promotes serrated and hyperplastic morphological features in colon epithelium, it is not able to initiate adenoma development, perhaps in part because activated Kras/KRAS signaling does not increase the number of presumptive stem cells in affected crypts.

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Keywords

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Introduction

While it has long been recognized that many colorectal cancers (CRCs) arise from adenomatous polyps, it is now appreciated that about 15% of CRCs likely arise via an alternative pathway – the so-called serrated adenoma pathway. [1, 2] Hyperplastic polyps (HPPs) are common lesions in the colorectum, and HPPs also display a serrated (sawtooth) gland morphology. However, unlike serrated adenomas, the epithelium in HPPs does not show dysplasia, and most HPPs, particularly small lesions in the distal colon, are presumed to have nominal if any risk of progression to CRC.[2, 3] Regardless of the progression pathway, CRCs arise due to accumulated defects in genes regulating cell metabolism, proliferation, differentiation, and death.[4] The gene lesions lead to novel or increased oncogene function or loss of tumor suppressor gene (TSG) function.

Inactivation of the *APC* (*adenomatous polyposis coli*) TSG is viewed as an initiating event in most adenomas and CRCs.[5] The APC protein functions as a regulator of the β-catenin protein in the "canonical" (β-catenin-dependent) Wnt signaling pathway.[6] In cells where both *APC* alleles are inactivated, coordinated phosphorylation and destruction of β-catenin is disrupted, mimicking β-catenin stabilization by Wnt ligands. β-catenin can complex with TCF (T cell factor) DNA binding proteins to co-activate transcription. The TCF-dependent transcription program induced by β-catenin stabilization in CRC appears to resemble that normally present in presumptive stem cells at the intestinal/colonic crypt base.[7]

Other somatic defects, such as *KRAS* and *p53* mutations, collaborate with *APC* inactivation to promote progression of some adenomatous lesions to CRC.[4, 5] *KRAS* somatic mutations are found in about 40% of CRCs.[1] Mutant *KRAS* alleles have a key role in CRC cells, when present, as inactivation of mutant *KRAS* abrogated tumorigenic properties of CRC cells in *in vitro* and animal studies.[8] Of note, *KRAS* mutations are present in some HPPs, including about 50% of so-called goblet cell-rich HPPs arising usually in the left colon and rectum.[2] Prior studies of the phenotypic effect of *Kras* mutant alleles in mouse small intestine and/or colon have yielded highly variable results, ranging from no effect at all,[9, 10] to increased goblet cell numbers,[11] reduced Paneth cell numbers,[12] epithelial hyperplasia,[11, 12] epithelial architectural changes,[12] and even the appearance of grossly visible tumors.[13]

In light of the data indicating *KRAS* mutations are functionally significant in human CRCs but also present in colon tumors with negligible malignant potential (e.g., HPPs), we sought to explore the functional consequences of *Kras* mutations in mouse colon epithelium and to compare the results to human HPPs. Using a *CDX2P-G22Cre* transgene, which expresses Cre in crypts of the terminal ileum, cecum, and colon[14] to activate a $Kras^{LSL\text{-}G12D}$ mutant allele,[15] we found dramatic epithelial hyperplasia and crypt architecture changes in the colon, reminiscent of those seen in human HPPs. We also observed Paneth cell loss and increased goblet cells. We established *Kras* mediated the differentiation and proliferation abnormalities via the mitogen activated protein kinase (MAPK) signaling pathway. We also implicated the transcriptional regulator Hes1 in altered cell fate. Notably, our studies revealed that in contrast to *Apc/APC* inactivation, *Kras* activation does not enhance expression of markers of the presumptive crypt stem cell pool or enhance colony formation in culture, leading us to propose that *Kras/KRAS* mutation does not initiate colorectal adenoma-carcinoma progression perhaps in part because activated Kras/KRAS signaling by itself cannot enhance formation of crypt stem cells.

Results

Activation of Kras^{G12D} Mutant Allele in Mouse Colon Epithelium Promotes Hyperplastic Polyp-like Features

We used the *CDX2P-G22Cre* transgenic mouse line (referred to as *G22Cre*),[14] which expresses *Cre* in epithelial cells in terminal ileum, cecum, colon and rectum, to activate a latent oncogenic *Kras$^{LSL-G12D}$* allele.[15] Cre-mediated recombination removes transcriptional stop elements, resulting in expression of the mutant *Kras$^{LSL-G12D}$* allele under control of its endogenous regulatory elements. Using mice carrying a gene for enhanced yellow fluorescence protein (EYFP) at the *Gt(ROSA)26Sor* locus[16] to report on *Cre* expression, we found G22*Cre* activated EYFP in epithelial cells of 40–50% crypts in proximal colon and 10–20% crypts in terminal ileum (Supplementary Figure 1*B* and 1*E*), consistent with prior data on *G22Cre* activity.[14] In *G22Cre* mice carrying the EYFP reporter and the *Kras$^{LSL-G12D}$* gene, more than 90% of crypts in proximal colon and terminal ileum had Cre-activated EYFP expression in epithelial cells (Supplementary Figure 1*C* and *F*). These findings imply *Kras$^{LSL-G12D}$* activation may enhance proliferation and/or survival advantage of intestinal epithelial cells.

In *G22Cre Kras$^{LSL-G12D}$* mice of one to two months of age, we observed marked hyperplasia of more than 90% of crypts in the terminal ileum, cecum, and colon compared to that of control mice (i.e. wild type mice and *Kras$^{LSL-G12D}$* mice lacking the *G22Cre* transgene) (Figure 1*A* and *B*). No evidence of dysplasia, like that seen in adenomatous epithelium following Cre-mediated inactivation of both *Apc* alleles,[14] was observed in *G22Cre Kras$^{LSL-G12D}$* mice. Besides hyperplasia, the *G22Cre Kras$^{LSL-G12D}$* colon epithelium showed serrated glandular morphology and increased goblet cells, reminiscent of epithelium in goblet cell-rich type human HPPs with activating somatic mutations in the *KRAS* or *BRAF* genes (Figure 1*C* and Supplementary Table 1). [2, 3] Increases in proliferating cells and decreases in apoptotic cells per crypt were seen in *G22Cre Kras$^{LSL-G12D}$* epithelium (Figure 1, *D–F* and Supplementary Figure 2). Because the *G22Cre Kras$^{LSL-G12D}$* mice developed one or two progressive papillary squamous lesions at the anal verge (due to stochastic Cre activation in caudal-derived epithelial cells), we could not study *G22Cre Kras$^{LSL-G12D}$* mice beyond two months age. We used a tamoxifen-regulated Cre transgene (EGFP-IRES-*CreER^{T2}*) integrated into the locus encoding leucine-rich-repeat-containing G-protein coupled receptor 5 (Lgr5), a presumptive intestinal crypt stem cell marker,[17] as another approach to activate *Kras^{G12D}*. In four *Lgr5-EGFP-IRES-CreER^{T2} Kras $^{LSL-G12D}$* mice studied between 2 and 8 months after tamoxifen treatment, mutant *Kras* activation led to hyperplastic effects in intestine and colon epithelium (Supplementary Figure 3), without evidence of dysplasia or adenoma formation. For both drivers, the effects of Kras activation on Paneth cell loss, increased Goblet cells, increased proliferation, decreased cell death, and crypt hyperplasia all appeared restricted to the crypts where Kras was activated, without evidence of effects on adjacent non-targeted crypts (Supplementary Figure 4 and data not shown).

Kras Activation Alters Expression of Cell Cycle Regulatory Proteins

Phospho-Erk1/2 levels were increased in Kras-activated epithelium (Supplementary Figure 5*A*). We found increased expression of the DNA synthesis (S)-phase cyclin E2 protein and decreased expression of cyclin-dependent kinase (Cdk) inhibitory proteins p27^{Kip1} and p57^{Kip2} in Kras-activated epithelium (Supplementary 5*A*). Expression of the p19^{ARF} protein was decreased, and no change in p16^{INK4a} protein expression was seen (Supplementary 5*A* and *B*). Transcript and protein levels were concordantly changed only for cyclin E2. Other genes showed either no change (e.g., *p19^{ARF}*, *p27^{Kip1}*) or divergent effects on transcript versus protein levels (e.g., *p16^{Ink4a}*, *p57^{Kip2}*) (Supplementary Figure 5*C*). These data imply

that in colon epithelium protein levels of the Cdk inhibitory factors studied was largely post-transcriptional. Our results showing no change in $p16^{INK4a}$ protein levels in epithelium of two month old *G22Cre Kras*$^{LSL\text{-}G12D}$ mice are consistent with our analysis of 12 human HPPs, where we found $p16^{INK4a}$ expression in 1–5 cells per crypt of only 5–10% of crypts of 10 of 12 HPPs studied (Supplementary Figure 5*D* and Supplementary Table 1). The other 2 HPPs had no detectable $p16^{INK4a}$ staining. The data indicate $p16^{INK4a}$ protein is not substantially induced in human HPPs, though elevated $p16^{INK4a}$ protein expression has been reported in human serrated adenomas and carcinomas.[18] Our findings of no significant increase in $p16^{INK4a}$ protein expression in epithelium of *G22Cre Kras*$^{LSL\text{-}G12D}$ mice contrasts with the reported significant increase in $p16^{INK4a}$ protein levels when a *Villin-Cre* transgene was used to activate *Kras* in small intestine and colon.[18] Explanations for the divergent findings will be discussed below.

MEK (MAPK kinase) Inhibitor CI-1040 Treatment Blocks Hyperplastic Changes Induced by *Kras*^{G12D} Activation

Ras oncogenes have been shown to engage multiple downstream pathways and effectors including the Raf-MAPK, phosphatidylinositol-3-kinase (PI3K), and Ral protein pathways.[19, 20] Mutant KRAS/Kras proteins are poor activators of PI3K signaling,[21] and some human CRCs with *KRAS* mutations also carry activating mutations in the PI3K signaling pathway.[22] In CRCs, *BRAF* and *KRAS* mutations are mutually exclusive,[22] arguing KRAS oncogenic signaling depends on downstream RAF-MAPK signaling. In a prior study where an intestinal fatty acid binding protein liver (*Fabpl*) regulatory element-driven Cre transgene targeted *Kras*^{G12D} in small intestine and colon tissues, the mice showed colon epithelial hyperplasia that responded to MEK inhibitor CI-1040 treatment. [11] We found a four-day treatment with CI-1040 dramatically inhibited colon epithelial hyperplasia in *G22Cre Kras*$^{LSL\text{-}G12D}$ mice (Supplementary 6*A* and *B*) and restored $p27^{Kip1}$ levels to those seen in control mice (Supplementary 6*C*).

Kras^{G12D} Activation-Induced Effects on Cell Fate in Intestine and Colon Epithelium are Dependent on MAPK Signaling

Prior studies where a *Fapbl-Cre* transgene[11] or a *Villin-Cre* transgene[12] was used to activate the *Kras*$^{LSL\text{-}G12D}$ allele revealed increased goblet cells[11, 12] and, in the case of the *Villin-Cre* transgene, reduced Paneth cells.[12] We confirmed *G22Cre*-mediated *Kras*^{G12D} activation increased goblet cell numbers per crypt in small intestine and colon (Figure 2, *A–C*), and led to a complete loss of lysozyme-expressing Paneth cells in small intestine (Figure 2*A* and *B*), and did not alter enteroendocrine cell numbers (data not shown). We obtained very similar effects on Paneth cells using the *Lgr5-EGFP-IRES-CreER*^{T2} transgene to activate *Kras*$^{LSL\text{-}G12D}$ (Supplementary Figure 7*A*). A 4-day treatment of *G22Cre Kras*$^{LSL\text{-}G12D}$ mice with the MEK inhibitor CI-1040 restored Paneth cells and reduced goblet cells (Figure 2, *C–F*), indicating the key role of MAPK signaling in *Kras*-induced effects on cell fate specification and differentiation. In light of the persistence of *Kras*-mutant small intestinal crypts lacking Paneth cells for up to 8 months, our findings raise questions about the recently suggested role for Paneth cells to maintain a crypt niche for intestinal stem cells. [23]

MAPK and Notch Pathway Activation Collaborate in Mediating Effects on Goblet and Paneth Cells in Kras/KRAS-Activated Epithelium

Prior studies implicated Notch signaling in cell fate determination in intestinal epithelium.[24, 25] We thus sought to address effects of Kras/KRAS activation on the Notch pathway target genes *Hes1* and *Hey1*. Increased *Hes1* and *Hey1* transcripts were found in *Kras*-activated mouse ileal epithelium (Figure 3*A*). Increased Hes1 protein expression following Kras activation was confirmed by Western blot (Figure 3*A*) and by immunohistochemical (IHC) staining (Figure 3*B*). Human HPPs with *KRAS* or *BRAF*

mutations showed strong HES1 staining throughout most hyperplastic crypts (Figure 3*D* and Supplementary table 1), whereas HES1 staining in normal colon epithelium was limited to the lower third of the crypt (Figure 3*C*), in the region populated mostly by transit amplifying cells.

In *Kras*-activated mouse intestinal epithelium, phospho-Mek-expressing cells replaced the phospho-Mek-negative Paneth cells normally present at the crypt base (Figure 3*E*). While *Hes1* is a NOTCH pathway target gene, Hes1 has been reported to be activated via MAPK signaling in neuroblastoma cells.[26] Given prior reports that *Hes1*−/− mice show premature Paneth cell development,[27] we hypothesized MAPK-dependent Hes1 activation may underlie Paneth cell loss. Using rat IEC-6 intestinal cells stably expressing a G12V mutant *KRAS* cDNA, we found the MEK inhibitors PD98059 or U0126 blocked *KRAS*-induced activation of Hes1 and Notch1 (Figure 4*A* and *B*). Similarly, the MEK inhibitor CI-1040 blocked Hes1 induction in *Kras*-activated mouse epithelium in vivo (Figure 3*B*). We detected a strong increase in levels of the activated (cleaved) cytosolic domain of Notch1 in IEC-6/KRAS G12V cells (Figure 4*C*). To assess if *KRAS*-induced activation of Hes1 depends on Notch signaling, we blocked NOTCH signaling in IEC-6/KRAS G12V cells with the γ-secretase inhibitor, DAPT. DAPT potently blocked Notch activation (Figure 4*C*), but only partially inhibited the *KRAS* effect on Hes1 (Figure 4*D*). Our data indicate Kras/KRAS activation can induce *Hey1/Hes1* expression via MAPK signaling, and MAPK-dependent induction of Hes1 is only partially dependent on Notch. Hes1 activation may contribute to the Paneth cell loss observed, but a role for Hes1 activation in goblet cell loss is inconsistent with prior data showing that NOTCH loss-of-function leads to increased goblet cell numbers.[24, 25]

Kras/KRAS Activation Does Not Expand Expression of Crypt Stem Cell Markers in Colon Epithelium and Does Not Enhance Colony Formation In Vitro

Prior studies of van de Wetering et al suggested *APC* inactivation and the resultant β-catenin dysregulation in intestinal epithelium leads to a TCF-dependent transcription program resembling that in normal intestinal stem cells.[7] Presumptive markers for intestinal and colon stem cells include the Wnt pathway-regulated target gene *Lgr5*,[17] as well as the *Musashi-1* (*Msi1*) and *olfactomedin-4* (*Olfm4*) genes,[28–30] neither of which is known to be regulated by Wnt signaling. We found dramatically induced expression of all three presumptive crypt stem cell markers in mouse colon epithelium when both *Apc* alleles were inactivated (*G22Cre APC*$^{fl/fl}$, Figure 5*A*). In contrast, we observed a decrease in the relative abundance of *Lgr5* and *Msi1* gene expression in *Kras*-activated colon epithelium and no change in *Olfm4* expression (Figure 5*B*). The relative decrease in Lgr5 and Msi1 expression following Kras activation reflects the dilution of the cell population in a crypt that express Lgr5 and Msi1, as Kras crypts were hyperplastic with substantially greater numbers of epithelial cells per crypt than normal crypts. Only a few cells near the crypt base in normal mouse colon epithelium and in Kras-activated colon epithelium expressed Msi1 (Figure 5*C* and Supplementary Figure 7*B*). In contrast, Msi1 expression was broadly activated in Apc-mutant crypts. Using the *Lgr5-CreER*^{T2} targeted allele, where integrated enhanced green fluorescence protein (EGFP) sequences mark cells with endogenous *Lgr5*-expression,[17] we found *Kras*-activated epithelium showed only the few EGFP-positive cells at crypt base in colon and terminal ileum that were similarly visualized in normal epithelium (Figure 5*D* and Supplementary Figure 7*A*). Expression of *LGR5* and MSI1 in normal human colon epithelium and HPPs was detected in a minority of cells at the crypt base (Figure 5*E* and Supplementary Table 1). In contrast, *LGR5* and MSI1 were broadly expressed in glands of human colon adenomas (Figure 5*E*).

To address the functional significance of these stem cell marker expression differences, we studied gene expression and colony forming ability following Kras activation or β-catenin

dysregulation in IEC-6 cells. In IEC-6/KRAS G12V cells, reduced Msi1 expression was seen, though no significant inhibition of *Lgr5* expression was found (Supplementary Figure 8*A*). The colony forming ability of IEC-6/KRAS G12V cells was similar to that of control IEC-6 cells, whereas expression in IEC-6 cells of a cancer-derived S33Y missense mutant form of β-catenin to model consequences of APC inactivation led to a 2-fold increase in the number and size of colonies (Figure 5*F* and Supplementary Figure 8*B*). Our studies and data do not conclusively define whether β-catenin dysregulation enhanced colony formation because of a general over-expression of Wnt target genes or because of a Wnt-induced intestinal cell phenotype. Nonetheless, our data indicate that while *Apc/APC* inactivation is associated with enhanced crypt stem cell gene expression and phenotypes, *Kras/KRAS* activation is not linked to an expansion of the crypt progenitor pool in intestinal and colon epithelium.

Kras Activation Enhances CD133 Expression, Marking a Potential Transit Amplifying Population, Via MAPK-Dependent Signaling

The CD133 (prominin-1) protein has been reported as a possible cancer stem cell marker, including in CRC.[31] CD133 expression is not restricted to stem cells but is also prominently expressed in the transit amplifying pool in normal intestinal epithelium.[32] In colon and terminal ileum epithelium from *G22Cre Kras*$^{LSL\text{-}G12D}$ mice, we found the fraction of cells expressing CD133 and also the staining intensity of the positive cells substantially increased (Figure 6*A*, *C*, and *D*). In line with this data, CD133 transcript levels were increased 2.5-fold (Figure 6*E*). Interestingly, in proximal colon of *G22Cre Kras*$^{LSL\text{-}G12D}$ mice, the proliferating cell population marked by Ki67 staining (Figure 6*B*) was only a subset of the CD133-positive population, while in small intestine, cells expressing detectable CD133 levels roughly corresponded to the transit amplifying cells and stem cells (Figure 6*C* and *D*, and data not shown). Thus, CD133 expression may mark different cell populations in small intestine and colon. In IEC-6 cells, KRAS G12V expression dramatically increased *CD133* expression and the MEK inhibitors PD98059 and U0126 potently suppressed the induction (Figure 6 *E* and *F*).

No significant difference in CD133 transcript levels was seen between *Apc*-mutant and control colon epithelium (Figure 6*E*), in spite of the fact that *Apc*-mutant epithelium shows a major expansion of presumptive stem cells. We also found β-catenin activation in IEC-6 cells modestly inhibited CD133 expression (Supplementary Figure 8*C*). Our observations are therefore consistent with prior arguments that CD133 expression may not specifically mark colon crypt progenitor cells,[32] but might instead mark a transit amplifying cell population. Overall, we conclude Kras-induced hyperplasia is caused by a MAPK-dependent increase of the transit amplifying cells marked by CD133 and Ki67, without concomitant increase of crypt stem cells.

Discussion

Roughly 40% of human CRCs and larger adenomas carry *KRAS* activating mutations.[5, 33] Mutant *KRAS* alleles are functionally significant in advanced CRC cells.[8] In light of these data on *KRAS* mutations in CRC pathogenesis, it has not previously been clear why human HPPs with somatic *KRAS* (or *BRAF*) mutations have nominal risk of progressing to CRC.[2] One well-documented difference between colon adenomas, which are precursor lesions for CRC, and HPPs is that most adenomas have *APC* inactivation and resultant β-catenin dysregulation, whereas HPPs lack defects in *APC* or β-catenin.[2]

We hypothesized *Kras*/*KRAS* mutations might not increase stem cell numbers in the glands of hyperplastic colon epithelial lesions. Using the presumptive stem cell markers Lgr5, Msi1, and Olfm4, we demonstrated that, in contrast to *Apc*/*APC* inactivation, *Kras*/*KRAS*

activation does not increase the presumptive colon stem cell pool in hyperplastic epithelium of the mouse or in human HPPs. Hence, while HPP epithelium is more proliferative than normal epithelium, because stem cell numbers are not increased in HPP crypts, HPP epithelium may be at no greater risk than normal epithelium to acquire rate-limiting mutations for CRC development. This stands in contrast to adenomatous epithelium, which appears to show a substantial increase in stem cells in each crypt. In the work here we used multiple markers presumed to define intestinal stem cells. While Lgr5 is both a presumptive marker of intestinal stem cells and a direct target of β-catenin/TCF action,[17, 34] there are no data to suggest that Msi1 or Olfm4 are directly regulated by β-catenin/TCF. As such, increased Msi1 and Olfm4 expression in Apc/APC-mutant epithelium presumably reflects an increase in stem cells and not simply activation of β-catenin/TCF target genes. Furthermore, besides the effects on stem cell markers in mouse and human tissues, we found that in IEC-6 cells *Kras/KRAS* activation does not activate crypt stem cell marker expression or stimulate colony formation in vitro.

Our findings on hyperplasia and altered differentiation arising from expression of mutant $Kras^{G12D}$ allele are similar to data from Haigis and Trobridge,[11, 12] where *Villin-Cre* or *Fabpl-Cre* transgenes were used to activate $Kras^{G12D}$, respectively. However, our findings contrast with data from transgenic overexpression of mutant *Kras* in intestinal cells or activation of mutant Kras with the *AhCre* transgene, where essentially no proliferation or differentiation effects were reported.[9, 10] Differences in phenotypes observed in the various studies with Cre recombinase transgenes likely reflect differences in the particular cells expressing the mutant Kras protein, and transgenic over-expression studies may also be influenced by expression level-dependent effects. Finally, we note our studies using the *CDX2-G22Cre* and *Lgr5-Cre* transgenes and those of Trobridge et al. using a *Villin-Cre* transgene to activate $Kras^{G12D}$ differ from those in the recent paper from Bennecke et al. where it was reported that $Kras^{G12D}$ activation with a *Villin-Cre* transgene led to hyperplasia and a serrated morphology but no observed differences in cell proliferation or death.[18] The differences between the Trobridge et al. and Bennecke et al. papers, both using the *Villin-Cre* transgene, are difficult to reconcile, though subtle differences in mouse genetic backgrounds may have played a role. With regard to our results versus those of Bennecke et al., the *CDX2-G22Cre* and *Lgr5-Cre* transgenes we used are likely expressed in different subsets of epithelial cells than the *Villin-Cre* transgene, particularly progenitor cells at the crypt base. While the data of Bennecke et al. suggest a tumor suppressive role for $p16^{INK4a}$-dependent cellular senescence in their particular model, the lack of clearly elevated $p16^{INK4a}$ expression in a significant fraction of the epithelial cells of the 12 human HPPs analyzed in our study argues against a major role for $p16^{INK4a}$ induction in most human HPPs. This raises the possibility that the studies of Bennecke and colleagues might better model the situation for dysplastic serrated adenomas, which are known to have malignant potential. $p16^{INK4a}$-dependent cellular senescence could play a tumor suppressive role in progression of these particular lesions.[18]

In addition to hyperplastic changes, we also observed altered differentiation that was dependent on MAPK signaling, namely, increased goblet cells and Paneth cell loss. To our knowledge, our studies are the first to show increased HES1 expression in human HPPs. While Hes1 is a NOTCH pathway target gene, it is also activated via MAPK signaling.[26] Kras-induced activation of HES1 in our *in vitro* system was completely dependent on MAPK signaling and only partially dependent on NOTCH1 signaling. While we saw NOTCH1 activation (as measured by its cleaved intracellular form) upon expression of mutant *KRAS*, in our model, *HES1* expression is only partially driven by NOTCH signaling (possibly downstream of MAPK signaling). *Hes1*-deficient newborn (P0) mice show precocious differentiation of Paneth cells;[27] thus, increased Hes1 expression could have contributed to the absence of Paneth cells in our studies. In turn, it seems unclear if

increased NOTCH-independent expression of HES1 could increase goblet cell numbers, as goblet cell increases have previously been linked to NOTCH signaling loss-of-function.[24, 25]

The observation that *Kras* mutation can trigger malignant progression in some mouse tissues, such as lung,[15] but not in colon, highlights tissue-specific differences in effects for particular oncogene lesions in the mouse and more than likely in man as well. Our findings do not implicate a tumor suppressive role for $p16^{INK4a}$ in the pathogenesis of human HPPs,[18] but our data do offer an intriguing possible explanation for why *KRAS* mutations in human colon are not sufficient to generate a clonally related cell population with enhanced potential to progress to malignancy. Hence, future studies of the differing effects of *Kras/KRAS* in promoting tumor development in one tissue versus another should consider organ-specific mechanisms of stem cell expansion and not solely senescence-related effects of Kras/KRAS. Finally, insights into the presumed cooperative effects of APC, KRAS, and other mutations in human colorectal tumorigenesis will require further studies, as the effects of KRAS mutations in concert with other gene defects likely reflect complex tissue and genotype-dependent interactions.[35]

Material and Methods

Mice

Kras$^{LSL\text{-}G12D/+}$ mice have been described previously.[15] We crossed *Kras*$^{LSL\text{-}G12D/+}$ mice to *CDX2P9.5-G22Cre* (*G22Cre*) transgenic mice to activate the mutant *Kras* allele in the intestinal tract.[14] To monitor Cre expression, *G22Cre* mice or *G22Cre Kras*$^{LSL\text{-}G12D/+}$ mice were intercrossed with mice carrying the *Gt*(*ROSA*)*26Sor* $^{tm1(EYFP)Cos}$*/J* reporter allele (The Jackson Laboratory, Bar Harbor, ME). To target *Apc* alleles, *G22Cre* mice or *CDX2P 9.5-NLS Cre* mice were intercrossed with mice homozygous for *Apc* targeted alleles (*Apc*$^{loxP/loxP}$, 580S) as described.[14, 36] To study effects of *Kras* activation on Lgr5 expression, we crossed *Kras*$^{LSL\text{-}G12D/+}$ and *Lgr5-EGFP-IRES-creER*^{T2} (*B*6.129P2-*Lgr5*$^{tm1(cre/ERT2)Cle}$/J) mice.[17] The resultant mice were injected twice intraperitoneally with tamoxifen (200 mg/kg weight; Sigma-Aldrich, St. Louis, MO) dissolved in corn oil. For MEK inhibition, 25-day-old *G22Cre Kras*$^{LSL\text{-}G12D/+}$ mice were injected intraperitoneally with CI-1040 (50mg/kg weight) three times daily for 4 days.

Cell Lines and Plasmids

The rat intestinal epithelial line IEC-6 was obtained from American Type Culture Collection (ATCC). Polyclonal IEC-6 lines stably expressing mutant KRAS (a glycine to valine mutation at codon 12; G12V) or control vector were obtained following infection with pPGS-CMV-CITE-neo vector-based retroviruses.[37] For MEK inhibitor studies, IEC-6/KRAS G12V cells and control cells were exposed to 50μM PD98059 (Cell Signaling Technology, Danvers, MA), or 20μM U0126 (Cell Signaling Technology) for 24 hr. To block Notch function, cells were treated with γ-secretase inhibitor, DAPT (Sigma-Aldrich) at concentrations of 10μM, and 20μM for 24 hr.

Immunohistochemistry and Immunofluorescence

Sections of paraffin-embedded human or mouse tissues were subjected to IHC analysis as described.[37] Hes1 Immunostaining on human HPP samples was scored on a four level scale for intensity (−, absent; +/−, weak; +, moderate; ++, strong) in the cell nucleus. The Student t-test was used to determine statistical significance between groups with strong or moderate versus weak/no Hes1 staining. The de-identified human colon specimens were provided by Dr. Joel Greenson from the Department of Pathology at the University of Michigan Health System (Ann Arbor, MI). Standard immunofluorescence staining was performed on 6-μm frozen sections. See Supplementary Method for details.

In Situ Hybridization

A 969bp PCR product spanning Lgr5 nucleotides 24 to 992 was subcloned into the TA-cloning vector pGEM-T easy (Promega Corp., Madison, WI). Digoxigenin-labeled riboprobes were prepared using the DIG RNA labeling kit (Roche Applied Sciences). *In situ* hybridization assays on 4 human hyperplastic polyps, 3 human colon adenomas and 3 normal colon samples were performed as described.[37]

Western Blot Analysis

Western blot analyses on lysates from mouse colon mucosa or terminal ileum or IEC-6 cells were performed as described.[37] See Supplementary Materials for information on antibodies.

Quantitative Reverse Transcription (RT)-PCR (qRT-PCR)

cDNA was synthesized using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). qRT-PCR was performed with an ABI Prism 7300 Sequence Analyzer using a SYBR green fluorescence protocol (Applied Biosystems). Primer sequences will be provided upon request.

Statistical Analysis

All data were evaluated by Student's t test and asterisks denote significance with $P < .05$. Error bars denote S.D.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APC	adenomatous polyposis coli
Cdk	cyclin dependent kinase
EGFP	enhanced green fluorescent protein
ERK	extracellular signal-regulated kinase
EYFP	enhanced yellow fluorescent protein
Fabpl	fatty acid binding protein liver
H&E	hematoxylin and eosin
HPP	hyperplastic polyp
IHC	immunohistochemical
ISH	in situ hybridization
Lgr5	leucine-rich-repeat-containing G-protein coupled receptor 5
MAPK	mitogen activated protein kinase

MEK	MAPK kinase
Msi1	Musashi-1
Olfm4	Olfactomedin-4
PI3K	phosphatidylinositol-3-kinase
TCF	T-cell factor
TSG	tumor suppressor gene

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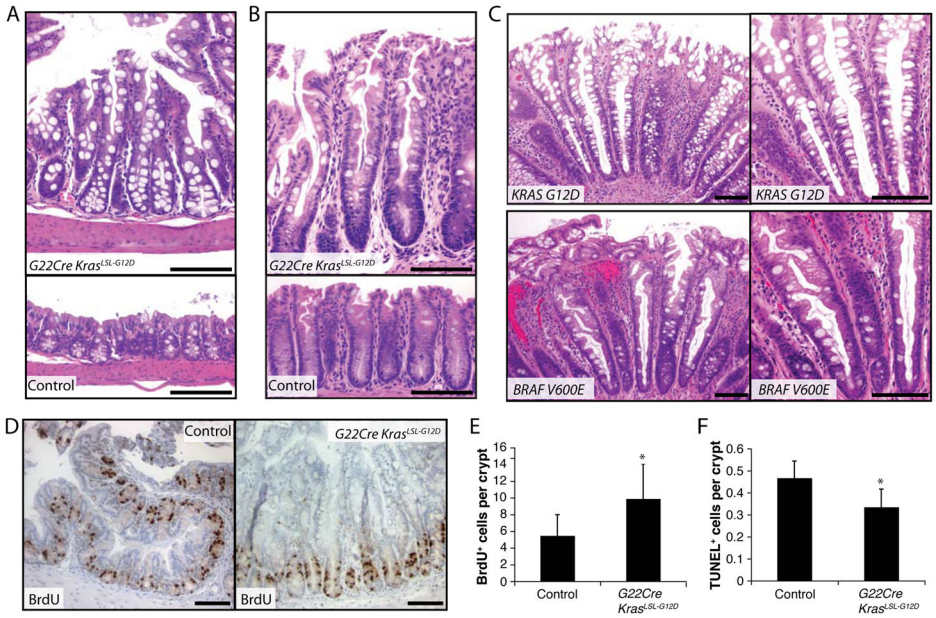

Figure 1.

Hyperplastic and hyperproliferation changes in mouse and human colon epithelium following *Kras/KRAS* pathway activation. (*A* and *B*) Hematoxylin and eosin (H&E) staining of proximal (*A*) and distal (B) colon tissues from a *G22Cre Kras*$^{LSL\text{-}G12D}$ mouse (A and B, upper) or a wild type mouse (*A* and *B*, lower). (*C*) H&E staining of a human HPP with *KRAS* G12D mutation (upper) and a HPP with *BRAF* V600E mutation (lower). Proliferating cells per crypt in mouse proximal colon were assessed by BrdU staining (*D*); BrdU incorporation was quantified (panel *E*; n = 4 mice and 160 crypts counted; **P* < .001). (*F*) Apoptotic cells in proximal colon were assessed by TUNEL assay (n=4 mice; **P* < .02). Scale bars, 100 μm.

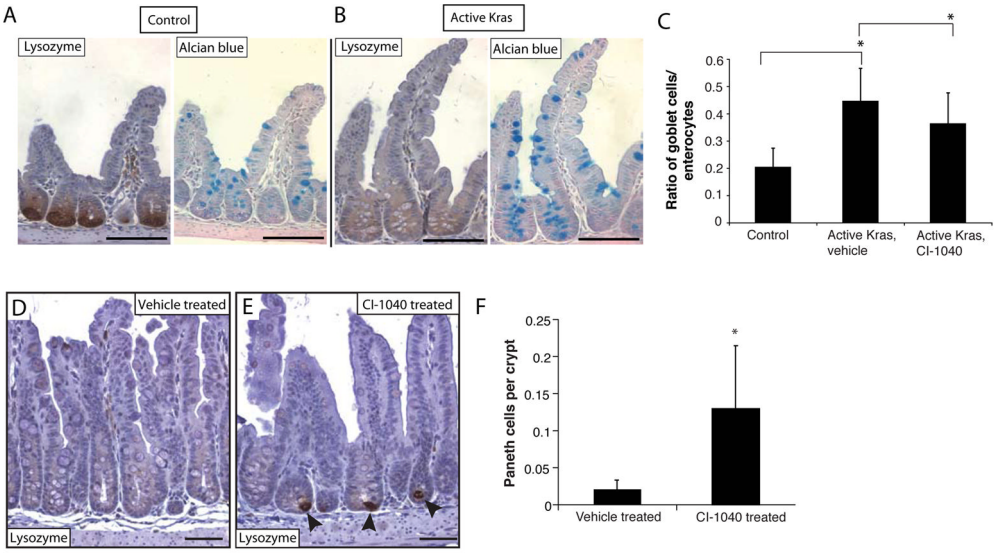

Figure 2.

Mutant *Kras* activation promotes increased goblet cells and Paneth cell loss, via RAS-MAPK-dependent signaling. Paneth cells, with lysozyme staining (left side, panels *A* and *B*) and goblet cells, with Alcian Blue staining (right side, panels *A* and *B*) in ileum. Scale bars, 100 μm. (*C*) Ratio of goblet cells vs. enterocytes in ileal crypts following CI-1040 treatment. (*D* and *E*) Recovery of Paneth cells in ileum of *G22Cre Kras*$^{LSL\text{-}G12D}$ mice following CI-1040 treatment. Scale bars, 50 μm. Arrowheads indicate lysozyme-positive Paneth cells. (*F*) Quantification of Paneth cells from *G22Cre Kras*$^{LSL\text{-}G12D}$ mice treated with vehicle or CI-1040. *$P < .001$.

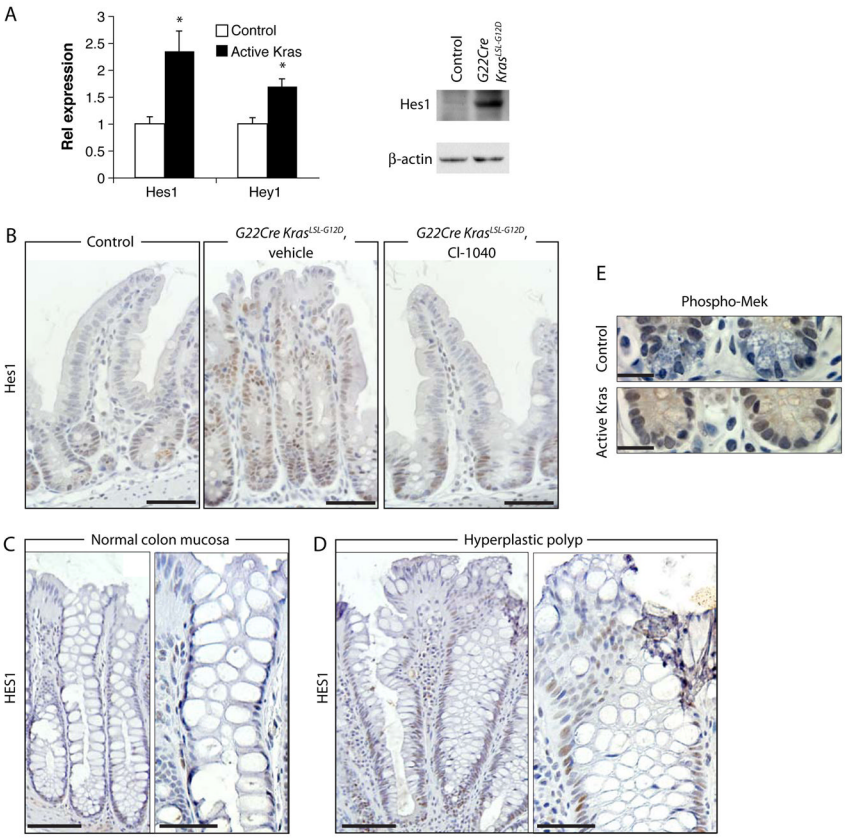

Figure 3.

MAPK and Notch pathway activation collaborate in mediating effects on goblet and Paneth cells in Kras-activated epithelium. RNA and proteins were isolated from distal ileum of four *G22Cre Kras*$^{LSL\text{-}G12D}$ mice and four control mice. (*A*) *Hes1* and *Hey1* expression was measured by qRT-PCR and normalized to U6 expression (left). **P* < .01. Hes 1 Western blot analysis shown at right. (*B*) IHC staining for Hes1 in ileum of a wild type mouse (left), vehicle-treated (middle), or CI-1040-treated (right) *G22Cre Kras*$^{LSL\text{-}G12D}$ mice. Scale bars, 50 μm. (*C* and *D*) Hes 1 IHC analysis in normal human colon mucosa (*C*) or HPPs (*D*). Representative images of low (*C* and *D*, left) or higher magnification (*C* and *D*, right) are shown. Scale bars, 100 μm for panels *C* and *D*, left, and 50 μm for panels *C* and *D*, right. (*E*) IHC detection of phospho-MEK at the ileum crypt base. Scale bar, 20 μm.

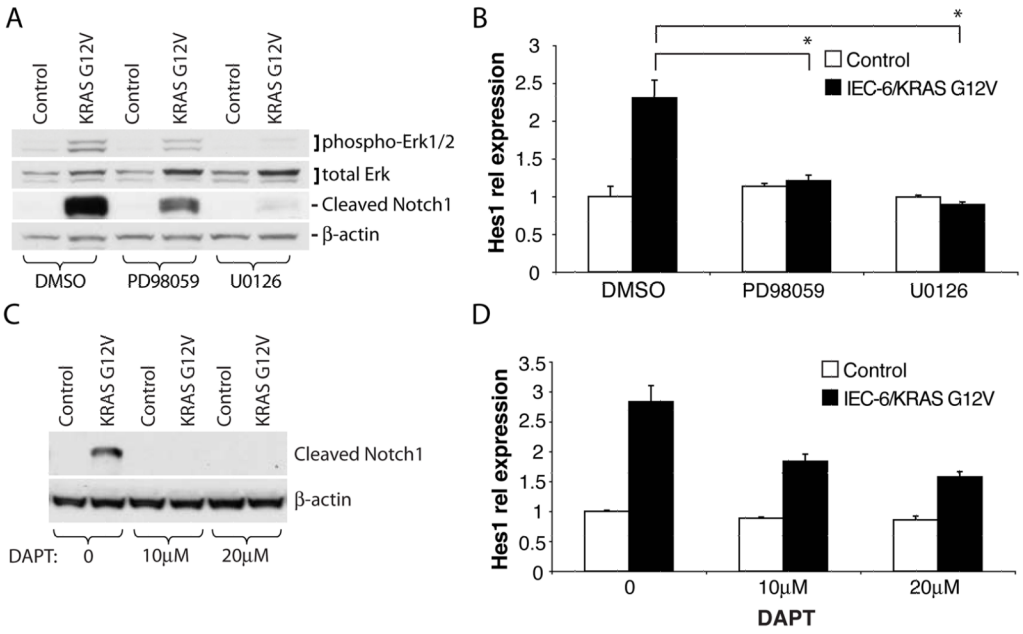

Figure 4.

IEC-6 cells stably expressing a human KRAS G12V mutant or control vector were treated for 24 hr with MEK inhibitors (50 μM PD98059, 20 μM U0126) or DMSO (*A* and *B*), or with DMSO or γ-secretase inhibitor DAPT at concentrations of 10μM, and 20μM (*C* and *D*). Western blot analysis of phospho-Erk, total Erk, cleaved Notch1 and β-actin in (*A*), or cleaved Notch1 and β-actin in (*C*). *Hes1* expression was measured by qRT-PCR and normalized to *β actin* expression (*B* and *D*). In (*A*), **P* < .01.

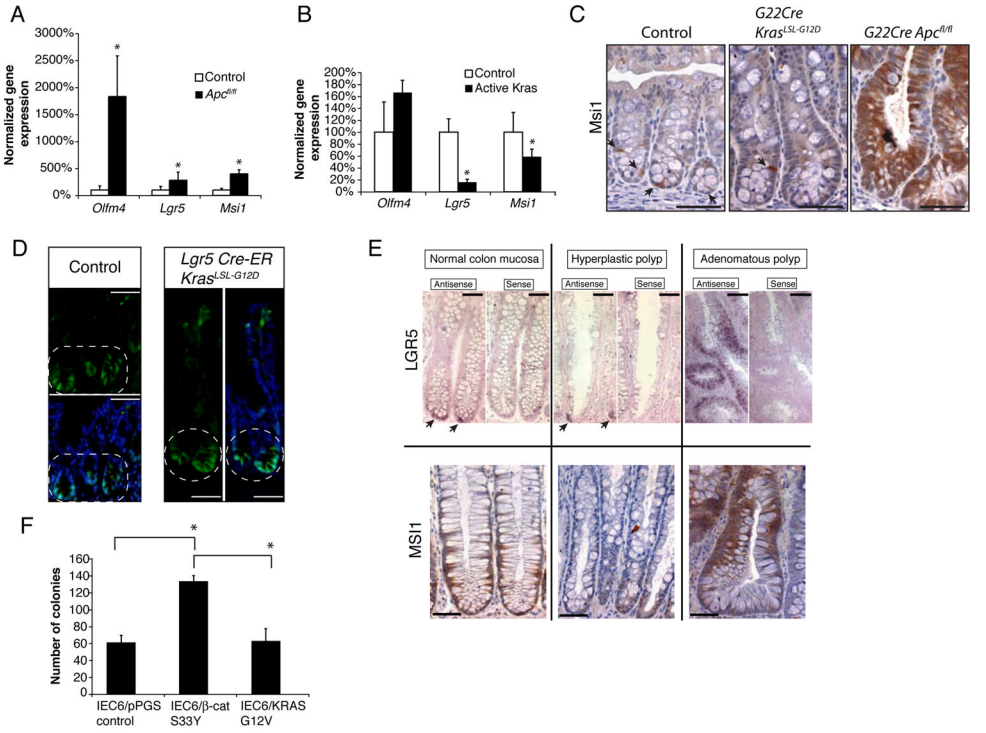

Figure 5.

Kras/KRAS activation does not increase presumptive colon stem cells. (*A* and *B*) RNA was extracted from proximal colon epithelium of four *G22Cre Apc*$^{fl/fl}$, seven *G22Cre Kras*$^{LSL\text{-}G12D}$, and seven control mice. Expression of *Olfm4, Lgr5 and Msi1*, relative to *U6*, was measured by qRT-PCR. (*C*) IHC staining for Msi1 in proximal colon of wild type, *G22Cre Kras*$^{LSL\text{-}G12D}$, and *G22Cre Apc*$^{fl/fl}$ mice. Arrows indicate Msi1-expressing cells near the crypt base. Scale bars, 50 μm. (*D*) GFP imaging in proximal colon tissues counter-stained with Hoechst 33342. *Lgr5-EGFP-IRES-CreER*^{T2} *Kras*$^{LSL\text{-}G12D}$ mice and *Lgr5-EGFP-IRES-CreER*^{T2} control mice were analyzed after tamoxifen treatment to activate *Cre*. The circled region denotes *Lgr5* expression restricted to the crypt base. Scale bars, 50 μm. (*E*) *LGR5* and *MSI1* expression in human HPPs, adenomas, and normal colon mucosa. (upper panels) *In situ* hybridization was performed with digoxigenin-labeled *LGR5* antisense or control *LGR5* sense riboprobes. Arrows indicate *LGR5*-expressing cells at the crypt base. Scale bars, 50 μm for normal mucosa and HPPs, and 100 μm for adenomas. (lower panels) IHC analysis of MSI1 expression. Scale bars, 50 μm. (*F*) Colony formation assay with IEC-6 cells expressing S33Y (serine 33 substitution with tyrosine) mutant β-catenin, KRAS G12V, or control vector. **P* < .01.

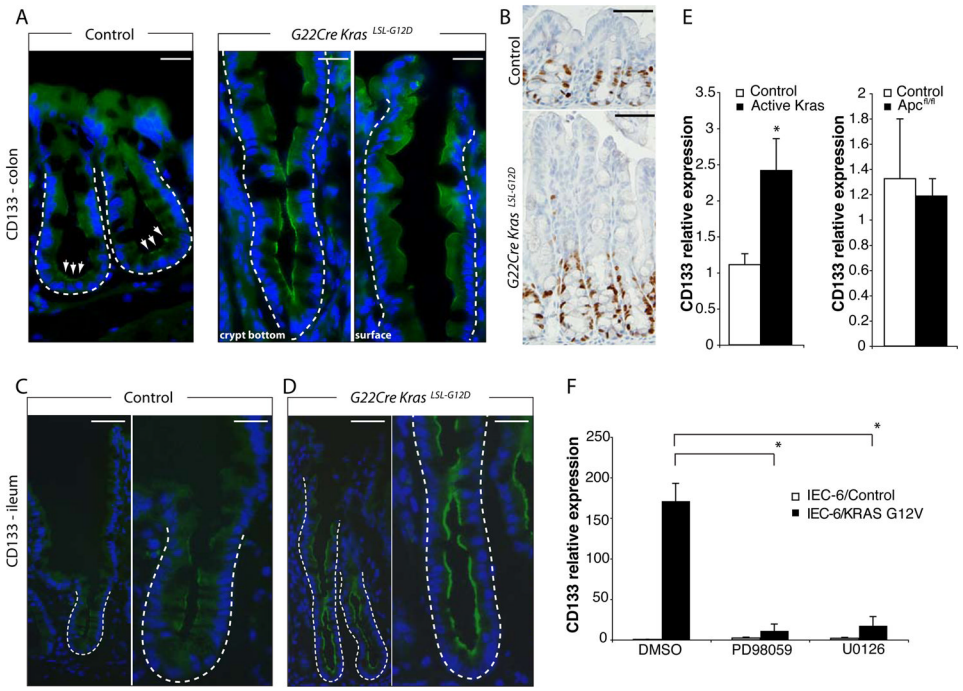

Figure 6.

Kras activation induces CD133 expression. (*A*) Proximal colon tissues from wild type (left) or *G22Cre Kras*$^{LSL\text{-}G12D}$ (right) mice were stained for CD133 (green), and counter-stained with Hoechst 33342 (Blue). In panel *A*, right side, the crypt base (left) and a region close to the luminal surface (right) from a single crypt are shown. Each crypt is outlined with dashed lines. White arrows indicate the CD133-positive cells at the crypt base region in normal epithelium. Scale bars, 20 μm. (*B*) IHC staining for Ki67 in proximal colon of same mice as (*A*). Scale bars, 50 μm. CD133 (green) immunofluorescence in mouse distal ileum of wild type control (*C*) or *G22Cre Kras*$^{LSL\text{-}G12D}$ mice (*D*). Representative low (left) or high magnification (right) images are shown. The outlined areas indicate CD133-positive cells, which may include progenitor and transit amplifying cells. Scale bars, 50 μm for left panels, and 20 μm for right panels. (*E*) qRT-PCR analysis for *CD133* expression in mouse proximal colon epithelium. (*F*) IEC-6/KRAS G12V or IEC-6/Control cells were treated with 50 μM PD98059, 20 μM U0126 or DMSO, and *CD133* gene expression was measured by qRT-PCR and normalized to *β-actin* expression. For *E* and *F*, **P* < .01.