Cell Stem Cell
Article

ROS Production and NF-κB Activation Triggered by RAC1 Facilitate WNT-Driven Intestinal Stem Cell Proliferation and Colorectal Cancer Initiation

Kevin B. Myant,[1] Patrizia Cammareri,[1] Ewan J. McGhee,[1] Rachel A. Ridgway,[1] David J. Huels,[1] Julia B. Cordero,[1] Sarah Schwitalla,[2] Gabriela Kalna,[1] Erinn-Lee Ogg,[1] Dimitris Athineos,[1] Paul Timpson,[1] Marcos Vidal,[1] Graeme I. Murray,[3] Florian R. Greten,[2] Kurt I. Anderson,[1] and Owen J. Sansom[1,*]

[1]Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Glasgow G61 1BD, UK
[2]Institute of Molecular Immunology, Klinikum rechts der Isar, Technische Universität München, 81675 Munich, Germany
[3]Department of Pathology, Division of Applied Medicine, School of Medicine and Dentistry, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK
*Correspondence: o.sansom@beatson.gla.ac.uk
http://dx.doi.org/10.1016/j.stem.2013.04.006

SUMMARY

The Adenomatous Polyposis Coli (*APC*) gene is mutated in the majority of colorectal cancers (CRCs). Loss of APC leads to constitutively active WNT signaling, hyperproliferation, and tumorigenesis. Identification of pathways that facilitate tumorigenesis after APC loss is important for therapeutic development. Here, we show that RAC1 is a critical mediator of tumorigenesis after APC loss. We find that RAC1 is required for expansion of the LGR5 intestinal stem cell (ISC) signature, progenitor hyperproliferation, and transformation. Mechanistically, RAC1-driven ROS and NF-κB signaling mediate these processes. Together, these data highlight that ROS production and NF-κB activation triggered by RAC1 are critical events in CRC initiation.

INTRODUCTION

Initiating mutations in colorectal cancer (CRC) most commonly target the *APC* tumor suppressor gene (Kinzler and Vogelstein, 1996). APC is a negative regulator of WNT signaling that is required to target β-catenin for proteosomal degradation. The loss of the *APC* gene results in the accumulation of β-catenin in the nucleus, subsequent activation of WNT transcriptional targets, and ultimately adenoma formation (Korinek et al., 1997; Morin et al., 1997; Sansom et al., 2004). Normal intestinal homeostasis is maintained by a number of intestinal stem cells (ISCs). Recent experiments have shown the presence of a cycling stem cell population marked by LGR5 along with a longer-lived population that can repopulate the crypt after injury (Barker et al., 2007; Buczacki et al., 2013). LGR5 expression marks crypt columnar stem cells that have a readily identifiable transcriptional profile termed the ISC signature. Genetic deletion of *Apc* within LGR5 ISCs leads to rapid adenoma formation, whereas deletion within more differentiated lineages leads to poorly proliferative lesions that fail to progress without additional oncogenic mutations (Schwitalla et al., 2013). Interestingly, although an established WNT target, LGR5 is only expressed in a subset of APC-deficient tumor cells. This may be due to LGR5 marking cells with the highest levels of WNT signaling or to co-operation of multiple pathways in conferring the LGR5 ISC phenotype.

Our previous studies have shown that the WNT target gene *Myc* is required for the phenotypes induced by *Apc* loss and that reduced levels of MYC slows intestinal tumorigenesis (Athineos and Sansom, 2010; Sansom et al., 2007). These studies set a precedent that targeting the downstream effectors of WNT signaling may be of therapeutic benefit in colorectal cancer. In the case of MYC, although there is great interest in it as a therapeutic target (Soucek et al., 2008), in vivo inhibitors are still in development. Therefore identification of other pathways downstream of *Apc* loss, in particular those highly active in LGR5 ISCs may provide candidates to target APC-deficient cells.

One candidate pathway is RAC signaling. RAC1 is a GTPase that acts as a key signaling node modulating a diverse set of cellular processes including proliferation, apoptosis, migration, and invasion. It influences a variety of signaling pathways including MTOR, NF-κB, JNK, and reactive oxygen species (ROS) production (Ellenbroek and Collard, 2007). RAC1 cycles between an inactive GDP and active GTP-bound state and is controlled by guanine nucleotide exchange factors (GEFs) (which activate RAC1) and GTPase-activating proteins (which inactivate RAC1). We previously identified MYC-dependent upregulation of a number of RACGEFs after *Apc* loss, and RACGEFs are commonly overexpressed during tumor progression (Lindsay et al., 2011; Sansom et al., 2007). RAC1-activating mutations have also been discovered in melanoma and a constitutively active RAC1 isoform termed RAC1B identified in colon and lung tumor samples can promote lung tumorigenesis (Hodis et al., 2012; Krauthammer et al., 2012; Zhou et al., 2013). RAC1 is also required for KRAS-mediated tumorigenesis in skin and the lung, but the mechanism of how RAC1 loss suppresses tumorigenesis is still unclear (Kissil et al., 2007; Samuel et al., 2011). Therefore, it is important to elucidate how RAC1 drives tumorigenesis.

Two studies suggest that RAC1 is required for nuclear localization of β-catenin and WNT signaling (Phelps et al., 2009; Wu et al., 2008). First, Wu and colleagues demonstrated that RAC1 is required for β-catenin nuclear localization in the developing

limb bud and the effects of its deletion phenocopy those of β-catenin. As nuclear localization of β-catenin is a key event during CRC initiation if this dependency remains once *Apc* is deleted, it would suggest that RAC1 would be an excellent therapeutic target. Additionally, Phelps and colleagues demonstrated that in zebrafish, *apc* loss alone was insufficient to cause nuclear localization of β-catenin (Phelps et al., 2009). Here the consequence of *apc* loss was perturbed differentiation, which was dependent on the transcriptional repressor Ctbp1. Additional activation of Kras was required for nuclear accumulation of β-catenin and hyperproliferation after *apc* loss. The authors proposed that RAC1 activation downstream of constitutive Kras activity was the mechanism that allowed the nuclear accumulation of β-catenin. These results are controversial as a number of studies have shown that (1) β-catenin can be nuclear localized in human adenomas without KRAS mutation (Obrador-Hevia et al., 2010), (2) WNT target genes can be upregulated in human adenomas without KRAS mutation (Sabates-Bellver et al., 2007), and (3) murine studies have shown nuclear β-catenin and WNT target gene upregulation in adenomas from the $Apc^{Min/+}$ mouse (Sansom et al., 2004; Segditsas et al., 2008).

Therefore, given this evidence for a role of RAC1 in WNT signaling, we investigated the importance of RAC1 activation after *Apc* deletion. We show that a number of *RacGEF*s and *Rac1b* are upregulated after *Apc* deletion, which leads to increased activity of RAC1. While *Rac1* deletion did not stop the nuclear accumulation of β-catenin and activation of the majority of the TCF/LEF targets, it attenuated hyperproliferation after *Apc* loss and subsequent tumorigenesis. Finally, we show that this is due to RAC1-mediated control of ROS production and NF-κB activation.

RESULTS

RAC1 Is Activated after *Apc* Loss

Previous microarray analysis revealed upregulation of several RACGEFs after *Apc* loss (Sansom et al., 2007). We found significant overexpression of *Vav3* and *Tiam1* transcripts after *Apc* loss in a MYC-dependent manner (as synchronous deletion of *Apc* and *Myc* returned expression levels to wild-type) (Figure 1A). Chromatin immunoprecipitation (ChIP) revealed that MYC was bound to the *Tiam1* promoter, demonstrating it is a direct MYC target (Figure S1A available online). Moreover, we observed increased expression of *Rac1b* but not *Rac1* (Figure 1A). To investigate whether this led to increased RAC1 activation, we performed immunohistochemistry (IHC) using an antibody raised against RAC-GTP (Samuel et al., 2011). We observed increased, specific positivity for RAC-GTP in APC-deficient tissue that was dependent on MYC (Figure 1B). Pull-downs for active RAC1 in wild-type and APC-deficient intestinal extracts confirmed these results (Figure S1B). Levels of RAC1 protein and mRNA remained unchanged (Figures 1A and S1C). To assess RAC activation in human CRC, we stained a TMA containing 50 normal and 650 primary CRC tumor cores for RAC-GTP (Figures 1C and S1D) (Duncan et al., 2008; O'Dwyer et al., 2011). We found a significant ($p \leq 0.0001$) increase in RAC-GTP staining intensity at all tumor stages compared to normal tissue, indicating that RAC activation is an early event in CRC. We also stained a commercial TMA for RAC-GTP, TIAM1, VAV3, and MYC and found a significant correlation between the expressions of these proteins (Figures 1D and S1E). These data are consistent with previous studies suggesting that RAC1 expression (and presumably activation) is elevated in CRC (Espina et al., 2008). Therefore, our data suggest that in mammalian cells, activation of RAC1 occurs downstream of WNT activation.

Rac1 Deletion Inhibits Hyperproliferation and LGR5 ISC Expansion after *Apc* Loss

To assess the functional significance of RAC1 activation after *Apc* loss, we generated *vil-Cre-ER*^{T2} $Apc^{fl/fl}$ $Rac1^{fl/fl}$ (APC Rac1) mice (el Marjou et al., 2004; Walmsley et al., 2003). Four days after cre induction, we observed loss of both *Rac1* and *Rac1b* mRNA and RAC1 protein (Figures 1A and S2A). In contrast to induced *vil-Cre-ER*^{T2} $Apc^{fl/fl}$ mice (APC), the intestines from APC Rac1 mice had smaller hyperproliferative crypts with significantly reduced incorporation of BrdU (Figures 2A and S2B). Acute deletion of Rac1 alone (*vil-Cre-ER*^{T2} $Rac1^{fl/fl}$) did not affect crypt size, proliferation, or ISC lineage-tracing capacity (Figures 2A and S2C), although increased apoptosis of villi enterocytes and intestinal barrier breakdown was observed at later time points (data not shown). This prevented later time point analysis and ruling out of potential subtle homeostatic effects over the longer term. The *vil-Cre-ER*^{T2} transgene also induces recombination in the colonic epithelium and the RAC1-dependent intestinal proliferation phenotype was recapitulated in this tissue (Figure S2D). Thus, RAC1 is a critical component in permitting crypt progenitor hyperproliferation after *Apc* loss in the intestinal and colonic epithelia. Given previous studies reporting that RAC1 is required for accumulation of nuclear β-catenin (Phelps et al., 2009; Wu et al., 2008), we predicted that this and downstream target gene activation should be impaired in APC Rac1 intestines. However, IHC analysis showed nuclear β-catenin in APC Rac1- (and APC-) deleted intestines (Figure 2B). Moreover, quantitative RT-PCR (qRT-PCR) of selected TCF/LEF targets that are deregulated after *Apc* loss were unchanged in APC Rac1 intestines (Figure S2E) (Sansom et al., 2007). Thus, RAC1 is not required for β-catenin nuclear localization and/or its functional activity in the absence of APC. To delineate the mechanism, we compared global gene expression changes between APC and APC Rac1 intestines by microarray. Gene set enrichment analysis (GSEA) identified a significant overlap between our data set and the ISC signatures associated with LGR5+ or EPHB2 high ISCs (chi-square test with Yates's correction, LGR5 $p < 0.0001$, EPHB2 $p = 0.001$) (Barker et al., 2007; Merlos-Suárez et al., 2011; van der Flier et al., 2009). We confirmed that the ISC signature was upregulated after *Apc* loss and reverted back to wild-type levels in APC Rac1 intestines using qRT-PCR (Figure 2C). To visualize changes in the LGR5-expressing population, we generated inducible *vil-Cre-ER*^{T2} WT, Rac1, APC, and APC Rac1 mice carrying the $Lgr5^{GFP\text{-}CREER}$ transgene (Barker et al., 2007). LGR5-GFP expression is restricted to the base of the crypt in wild-type and RAC1-deficient mice by IHC (Figure S2F). After *Apc* loss, there is a significant increase in the number of LGR5-GFP-positive cells that are suppressed in APC Rac1 intestines (Figure 2D). Moreover, scoring the location of LGR5-expressing cells revealed a striking expansion of the stem cell "zone" that codeletion of *Rac1* prevented (Figure 2E).

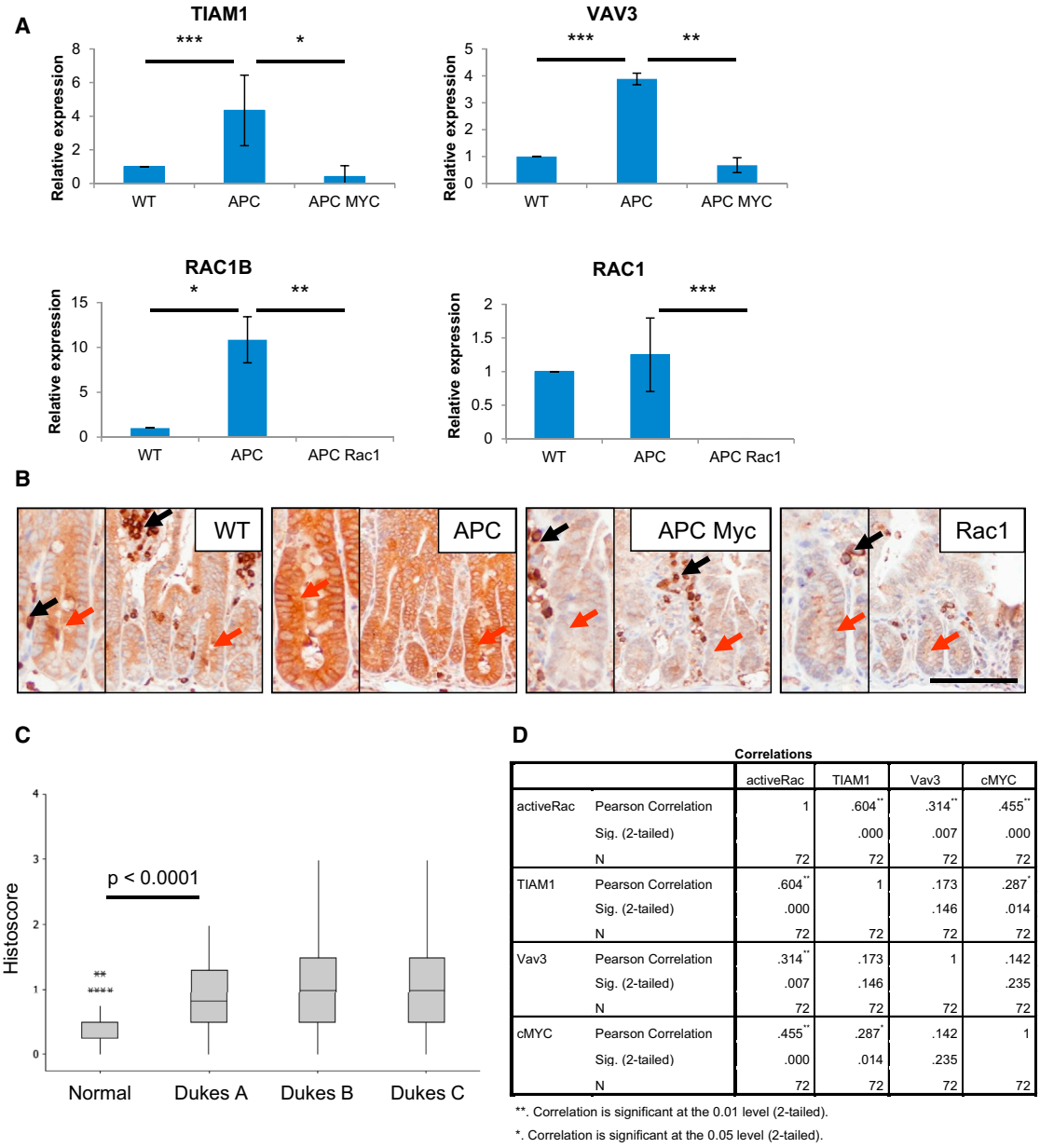

Figure 1. RAC1 Activity Is Increased after *Apc* Loss

(A) qRT-PCR of *Tiam1*, *Vav3*, *Rac1b*, and *Rac1* (error bars represent SD; *p < 0.05, **p < 0.01, ***p < 0.001; t test, n = 3).

(B) IHC for RAC-GTP in WT, APC, APC Myc, and Rac1 intestines. Red arrows indicate epithelial expression, and black arrows show immune cells in the villus compartment with very high positivity.

(C) Histoscores of human CRC TMA cores stained for RAC-GTP (Mann Whitney).

(D) Table comparing levels of active RAC, TIAM1, VAV3, and MYC in human CRC samples. All experiments are 4 days after induction. Scale bars represent 100 μm. See also Figure S1.

This was confirmed using multiphoton microscopy on intestines (Figures S2G and S2H). To investigate whether *Rac1* deletion suppressed the proliferative potential of all ISC populations, we purified crypts from APC and APC Rac1 intestines and cultured them ex vivo. Remarkably, while APC crypts formed colonies, APC Rac1 crypts did not, suggesting that RAC1 is required for the clonogenic capacity of all intestinal cell types (Figure S2I). Therefore, RAC1 is required for two critical constituents of *Apc* loss, progenitor hyperproliferation and LGR5 ISC expansion.

Generation of ROS by RAC1 Allows LGR5 ISC Signature Expansion upon *Apc* Loss

As RAC1 integrates many pathways, a plethora of downstream effectors could account for the phenotype that we observed. One critical cellular process requiring RAC1 is the generation of

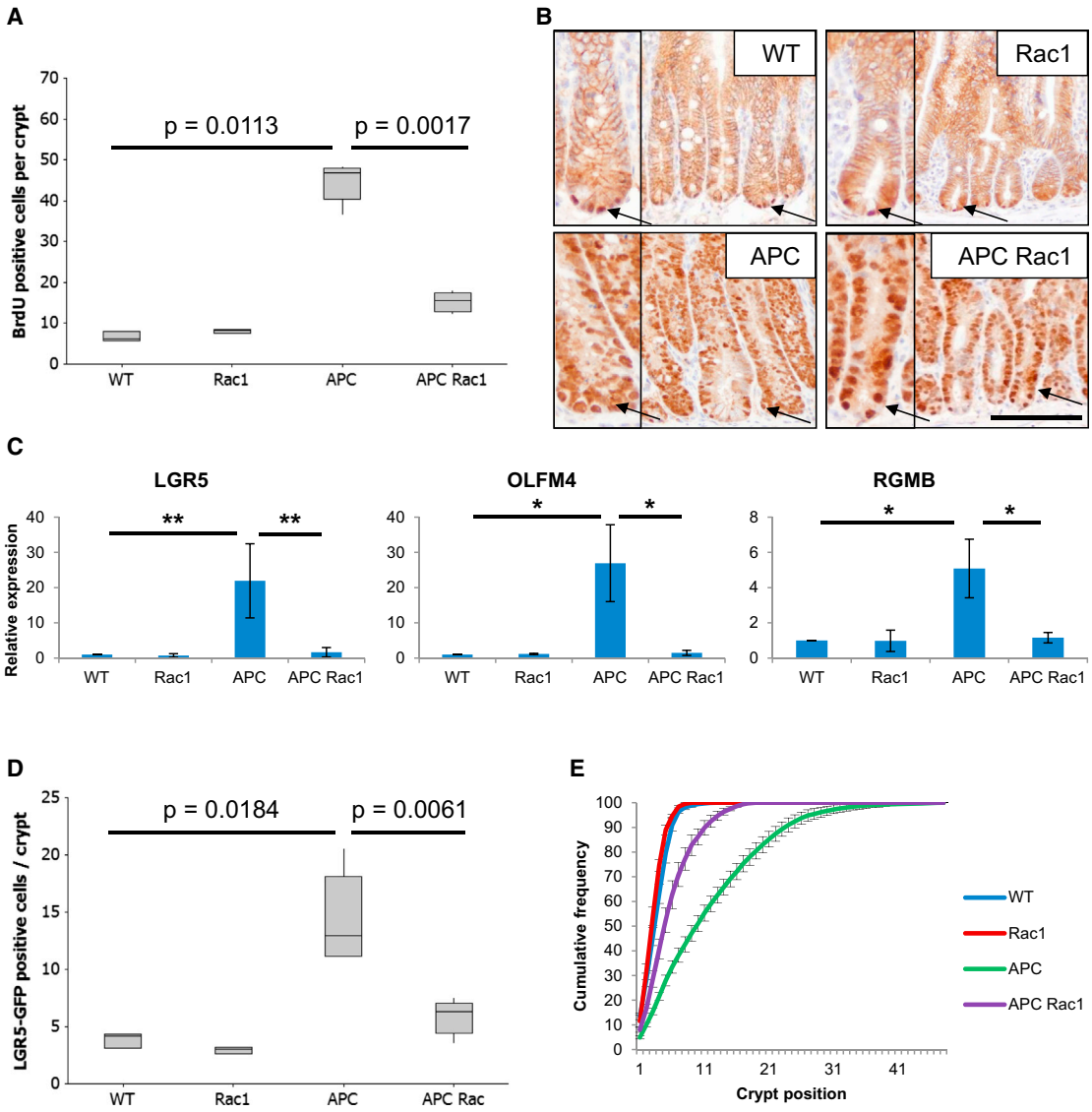

Figure 2. RAC1 Is Required for Hyperproliferation and LGR5 ISC Expansion after *Apc* Loss

(A) Quantification of BrdU IHC in WT, Rac1, APC, and APC Rac1 intestines (Mann Whitney, n = 4).

(B) β-catenin IHC on WT, Rac1, APC, and APC Rac1 intestines. Nuclear β-catenin is found throughout the crypt in APC and APC Rac1 tissue (black arrows). Histoscoring identified a significant increase in nuclear positivity after *Apc* loss ($p = 0.04$, Mann Whitney, n = 3) that was unchanged in APC Rac1.

(C) qRT-PCR of three ISC markers (error bars represent SD; t test, n = 3, $^*p < 0.05$, $^{**}p < 0.01$).

(D) Quantification of LGR5-GFP+ cell numbers.

(E) Cumulative frequency scoring of LGR5-GFP cell position (error bars represent SEM). All experiments 4 days after induction. Scale bars represent 100 μm. See also Figure S2 and Tables S1 and S2.

ROS. RAC1 is a member of the superoxide-generating NADPH oxidase complex, and RAC1B overexpression has been shown to lead to ROS production (Bromberg et al., 1994; Radisky et al., 2005). The generation of ROS by NADPH oxidase involves the conversion of NADPH to NADP+ so its activity can be evaluated by the ratio of these molecules. We observed a shift in the NADP+/NADPH ratio in APC intestinal extracts compared to WT, indicating increased complex activity that was RAC1 dependent (Figure S3A). To analyze ROS levels within the intestinal epithelium, we stained WT, Rac1, APC, and APC Rac1 intestines with the ROS-responsive dye dihydroethidium (DHE). We observed a significant increase in staining intensity in APC but not APC Rac1 intestines (Figures 3A and 3B). Thus, *Apc* loss leads to increased ROS generation in the intestinal epithelium via activation of the RAC1-containing NADPH oxidase complex. Moreover, DHE staining indicated that in WT tissue ROS generation was particularly high at the crypt base (Figure 3A). Scoring DHE fluorescence intensity based on crypt cell position showed that ROS generation was highest at cell positions 1–3 (where LGR5+ cells reside) and was absent in RAC1-deficient intestines (Figure S3B). To confirm that LGR5+ cells have high levels of ROS, we isolated intestinal epithelial cells from mice carrying

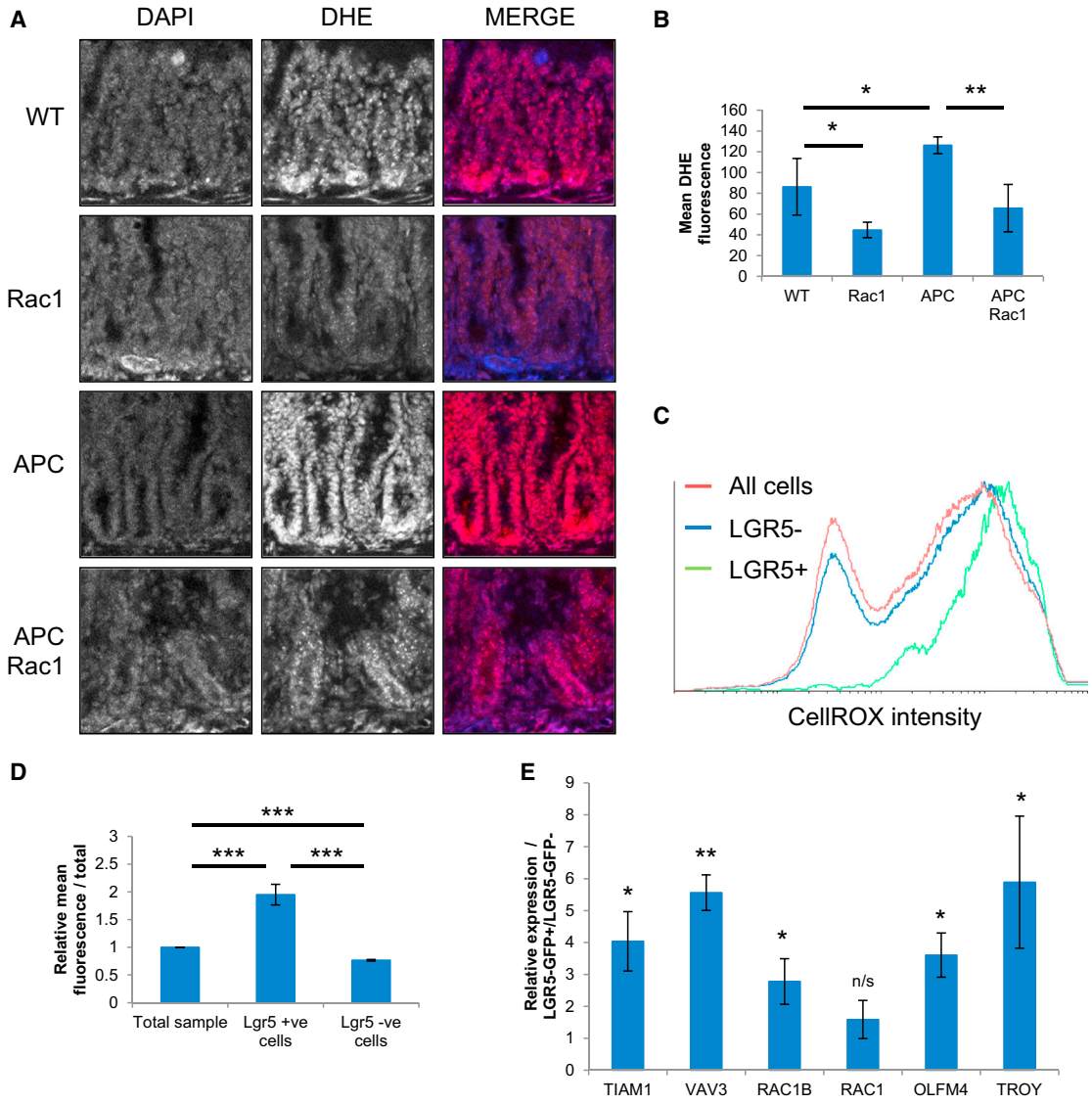

Figure 3. RAC1 Is Required for ROS Production in the Intestine

(A) Dihydroethidium (DHE) staining of frozen sections from WT, Rac1, APC, and APC Rac1 intestines; DAPI counterstain.

(B) Quanitification of DHE staining (error bars represent SD; t test, n = 3, *p < 0.05, **p < 0.01).

(C) Representative fluorescence-activated cell sorting (FACS) plot of CellROX Deep Red-stained epithelial cells from an *Lgr5*$^{GFP\text{-}CREER}$-expressing mouse.

(D) Quantification of CellROX Deep Red staining (error bars represent SEM; t test, n = 3, ***p < 0.001).

(E) Expression of Rac signaling components and control ISC markers in LGR5-GFP+ cells relative to LGR5-GFP− cells (error bars represent SEM; t test, n = 3, *p < 0.05, **p < 0.01). See also Figure S3.

the *Lgr5*$^{GFP\text{-}CREER}$ transgene (Barker et al., 2007), stained them with the ROS-responsive dye CellROX Deep Red, and analyzed them by flow cytometry. We found that LGR5-GFP-expressing cells contained around 2-fold more ROS than the cell population as a whole (Figures 3C, 3D, and S3C). As ROS generation at the crypt base was Rac1 dependent (Figures 3A and S3B), we investigated the expression of *Tiam1*, *Vav3*, and *Rac1b* in LGR5+ cells sorted by qRT-PCR and found that all were significantly enriched, though *Rac1* levels were unchanged (Figure 3E).

We hypothesized increased RAC1-driven ROS generation may be a critical process in conferring the ISC/progenitor phenotypes associated with *Apc* loss. We tested this hypothesis in two ways. First, we assessed whether reduction of intracellular ROS levels through NAC treatment could recapitulate the phenotypes associated with *Rac1* deletion. Remarkably, treatment of APC-deficient mice with NAC strongly suppressed overexpression of the ISC signature genes (*Lgr5*, *Olfm4*, and *Rgmb*) (Figure 4A). This suppression did not extend to non-ISC WNT target genes, indicating that WNT signaling was not perturbed by this treatment (Figure S4A). NAC treatment also significantly attenuated proliferation in APC intestines (Figures 4B and S4B). Importantly, NAC treatment had no effect on proliferation, number of LGR5-GFP cells, or gross villus histology in WT mice (4 day and 8 week time points) (Figure 4B and data not shown). Second,

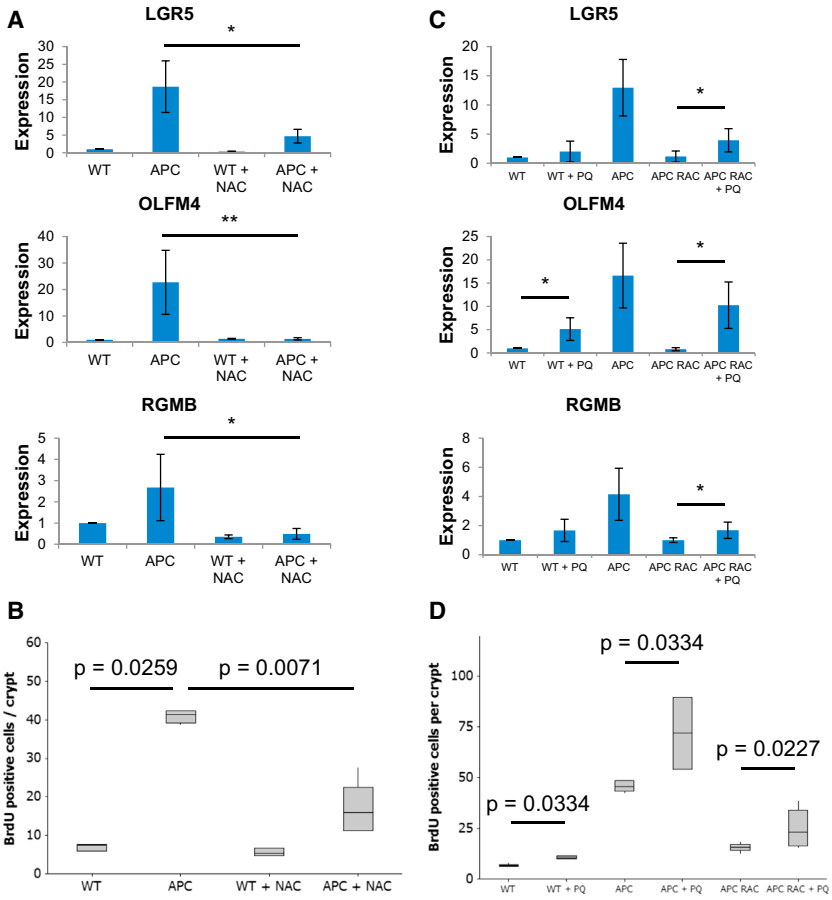

Figure 4. ROS Is Required for Expansion of the ISC Signature

(A) qRT-PCR of three ISC markers (error bars represent SD; t test, n = 3, *p < 0.05, **p < 0.01).

(B) Quantification of BrdU staining of intestinal crypts from WT, APC, WT + NAC, and APC + NAC mice (Mann Whitney, n = 4).

(C) qRT-PCR of three ISC markers, downregulated in APC Rac1-deficient intestines (AR) and upregulated upon treatment with paraquat (AR + PQ) (error bars represent SD; t test, n = 3, *p < 0.05, ***p < 0.001). APC + PQ has been removed for clarity and is included in Figure S4C.

(D) Quantification of BrdU positivity (Mann Whitney, n = 6). All experiments are 4 days after induction. Scale bars represent 100 μm. See also Figure S4.

we asked whether induction of ROS could rescue the reduced ISC signature in APC Rac1 intestines. Systemic treatment with the ROS-inducing compound paraquat led to increased expression of ISC marker genes in APC Rac1 intestines (Figures 4C and S4C). In situ microscopy of APC Rac1 mice carrying the *Lgr5*$^{GFP\text{-}CREER}$ transgene demonstrated a significant expansion of the ISC zone upon paraquat treatment (Figure S4D). In WT and APC-deficient mice treated with paraquat, the majority of ISC markers were unaffected. We also observed a significant increase in proliferation in the intestines of the three cohorts of paraquat-treated mice (Figures 4D and S4E). Thus, induction of ROS can induce proliferation and, in the absence of RAC1, can partially compensate for its loss.

RAC1-Driven NF-κB Signaling Mediates LGR5 ISC/Progenitor Hyperproliferation

We have recently demonstrated a requirement for the NF-κB transcription factor P65 in ISC expansion after β-catenin activation (Schwitalla et al., 2013). Given the remarkable overlap with this study, we addressed whether NF-κB signaling is an important mediator of LGR5 ISC/progenitor proliferation after *Apc* loss. We observed a significant reduction of NF-κB signaling components P65, IKKβ, and acetyl-P65 in APC Rac1 compared to APC-deficient intestines (Figures 5A, S5A, and S5B). Also, NF-κB binding activity was perturbed in APC Rac1 compared to APC intestinal extracts (Figure S5C). We also observed an increase in p65 binding to the promoters of *Lgr5*, *Olfm4*, and *Rgmb* after *Apc* loss that was lost upon codeletion of *Rac1* (Figures 5B and 5C). Thus, efficient activation of NF-κB signaling in the intestine requires RAC1. We next sought to functionally determine whether constitutive NF-κB signaling can compensate for *Rac1* deletion. We crossed APC Rac1 mice to mice carrying the *R26Stop*^{FL}*ikk2ca* allele (an inducible, constitutively active *Ikk2* allele from now on referred to as IKK) to generate APC Rac1 IKK mice (Sasaki et al., 2006) (Figure 5D). This model permits constitutive activation of NF-κB signaling in the absence of RAC1. This was sufficient to almost completely rescue the proliferation defect and partially rescue ISC marker expression in APC Rac1 mice (Figures 5E, 5F, and S5D). Thus, NF-κB signaling is an important downstream effector of RAC1 signaling in promoting the phenotypes of *Apc* loss. We noted that neither increased ROS nor NF-κB activation were able to completely rescue the APC Rac1 deletion phenotype. This is perhaps unsurprising given that we also observed effects on a number of other pathways downstream of RAC1 signaling, such as STAT3 and MTOR, which could impinge on the phenotypes of *Apc* loss (Figure S5E).

Rac1 Deletion Prevents Transformation after *Apc* Loss

Our previous studies have shown that *Apc* deletion within ISCs using *Lgr5*$^{GFP\text{-}CREER}$ leads to rapid tumorigenesis (Barker et al., 2009). Given that *Rac1* deletion prevents increased *Lgr5* expression within *Apc*-deficient crypts, we hypothesized that this would reduce the tumor-forming capacity of ISCs. To test this, we generated control *Lgr5*$^{GFP\text{-}CREER}$ *Apc*$^{fl/fl}$ (Lgr5 APC) and experimental *Lgr5*$^{GFP\text{-}CREER}$ *Apc*$^{fl/fl}$ *Rac1*$^{fl/fl}$ (Lgr5 APC Rac1) mice. To induce recombination, we gave mice a daily injection of tamoxifen for 4 days (a total of four injections). Most control Lgr5 APC mice developed a lethal intestinal adenoma burden by 20 days; however, none of the Lgr5 APC Rac1 did until much later time points (Figure 6A). When they did succumb to adenoma formation, all adenomas expressed *Rac1*, indicating that they most likely arose from cells in which *Rac1* deletion

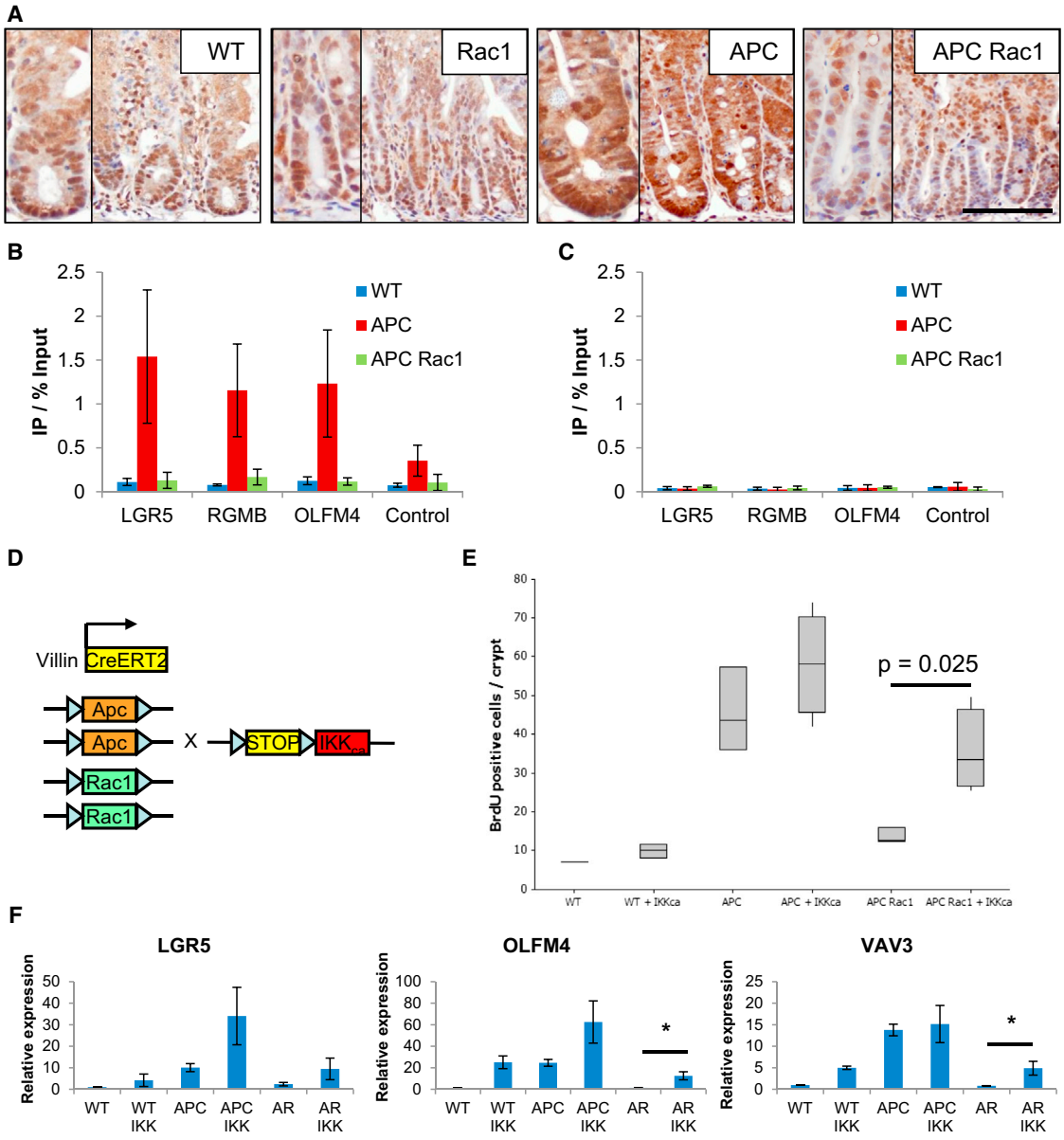

Figure 5. NF-κB Signaling after *Apc* Loss Requires RAC1

(A) P65 IHC shows decreased expression in APC Rac1 crypts.

(B) ChIP of P65 to the *Lgr5*, *Olfm4*, and *Rgmb* promoters and a control region (error bars represent SD; Mann Whitney, n = 3, p = 0.04).

(C) Control ChIP showing no binding to ISC promoters of a nonspecific IgG (error bars represent SD).

(D) Crossing strategy to generate Vil APC Rac1 IKK mice.

(E) Quantification of BrdU positivity (Mann Whitney, n = 4).

(F) qRT-PCR of three ISC markers showing increased expression in APC Rac1 mice after NF-κB activation (error bars represent SEM; t test, n = 3, *p < 0.05). All experiments were performed 4 days after induction. Scale bars represent 100 μm. See also Figure S5.

had not occurred (Figure S6A). Histological analysis revealed that in contrast to the adenomas found in controls, Lgr5 APC Rac1 mice developed numerous cystic structures and micro adenomas (Figures 6B and 6E). These lesions phenotypically resemble those that arise via *Apc* deletion outside the stem cell zone (Barker et al., 2009) and had a very low proliferative index (Figure 6E). Despite high levels of nuclear β-catenin, they had reduced levels of LGR5-GFP, suggesting that RAC1 activity is essential for tumor growth and the efficient expansion of LGR5 cells after *Apc* loss (Figures 6F, 6G, and S6B). LGR5 also marks colonic stem cells, and deletion of *Apc* with *Lgr5*$^{GFP\text{-}CREER}$ leads to formation of colonic adenomas (Barker et al., 2009). At the time of sacrifice, few colonic adenomas were macroscopically visible in either Lgr5 APC or Lgr5 APC Rac1 mice. However, microscopic analysis uncovered numerous aberrant crypt foci and micro adenomas in the colons of Lgr5 APC mice that stained

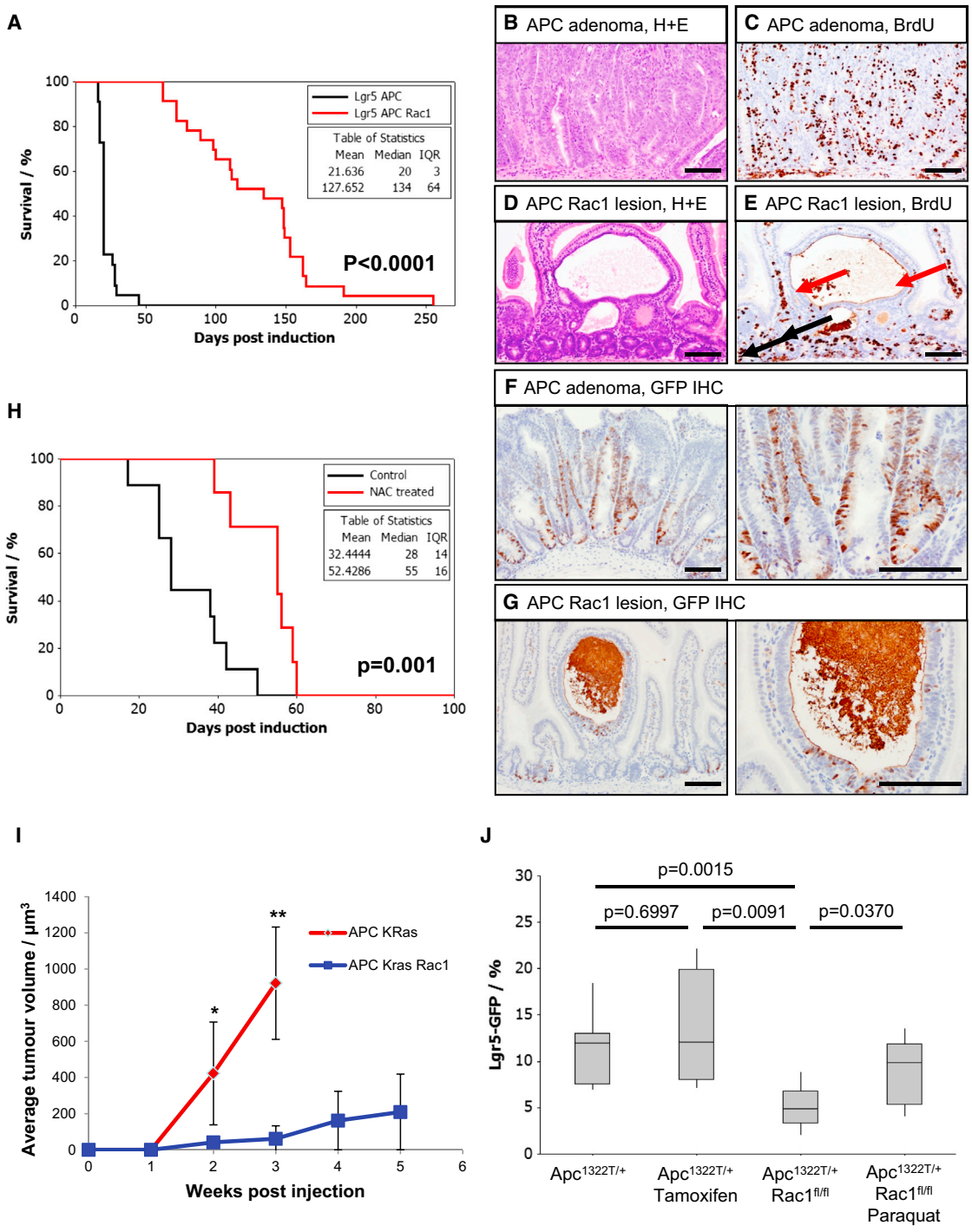

Figure 6. RAC1 Is Required for Tumorigenesis Downstream of *Apc* Loss

(A) Compared to Lgr5 APC, Lgr5 APC Rac1 mice were strongly protected against tumorigenesis (Kaplan Meier, $p < 0.0001$, n = 15).

(B and C) Lgr5 APC mice developed adenomas (B, H&E) that were highly proliferative (C, BrdU).

(D and E) Lgr5 APC Rac1 mice predominantly developed small intestinal lesions or cysts (D, H&E) that were poorly proliferative (E, BrdU IHC, red arrows). BrdU incorporation was higher in neighboring normal intestine (black arrows).

(F and G) GFP IHC showing high levels of Lgr5-GFP staining in Lgr5 APC adenomas (F) compared to Lgr5 APC Rac1 lesions (G).

(H) NAC treatment protected Lgr5 APC mice from intestinal tumorigenesis (Kaplan Meier, $p = 0.001$, n = 7).

(I) Tumor volume scores of allografts derived from 100 APC Kras and APC Kras Rac1-purified crypts (error bars represent SD; t test, n = 3, $^*p < 0.05$, $^{**}p < 0.01$).

(J) Quantification of FACS analysis demonstrating a significant reduction in LGR5-GFP positivity in tumors deficient in RAC1 (Mann-Whitney, $n \geq 5$). Scale bars represent 200 µm. See also Figure S6.

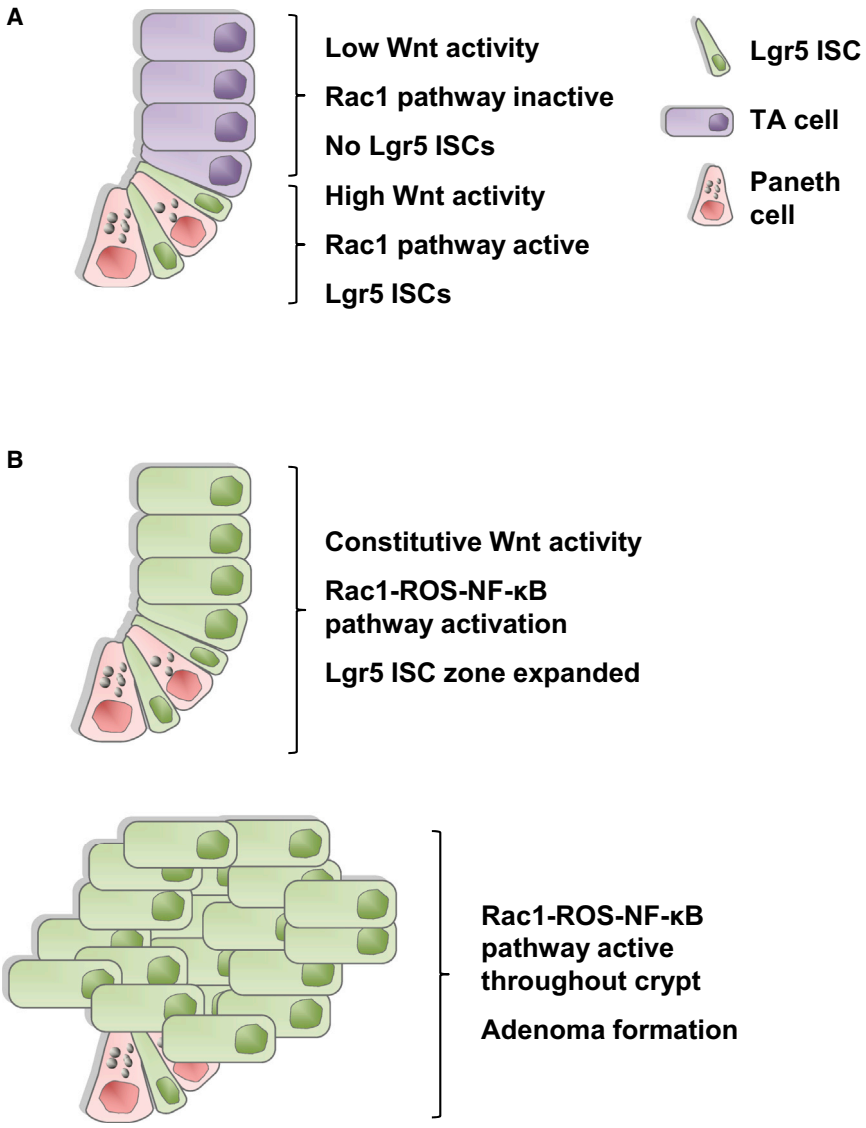

Figure 7. Model of RAC1-Mediated Control of Intestinal Tumor Initiation

(A) Normal small intestinal crypt with different cell populations outlined. LGR5 ISCs have high Wnt signaling leading to high RAC1-ROS-NF-κB pathway activation.

(B) Upon loss of *Apc*, RAC1-ROS-NF-κB pathway activation is increased throughout the crypt, permitting the expansion of the LGR5 ISC zone and progenitor cell hyperproliferation. The activation of the RAC1-ROS-NF-κB pathway throughout the crypt is critical for intestinal adenoma formation.

positive for nuclear β-catenin (Figure S6C and data not shown). We observed a significant reduction in the number of these lesions in the colons of Lgr5 APC Rac1 mice (Figure S6D), with the majority of these mice (6/8) being tumor free. Thus, RAC1 is required for intestinal and colonic adenoma formation after *Apc* loss. To test whether ROS production by RAC1 was also important, we treated Lgr5 APC mice with NAC. This led to a significant extension of tumor-free lifespan, further emphasizing the role ROS plays in intestinal transformation (Figure 6H).

As CRC progresses, tumors accumulate additional mutations, of which the activation of $KRAS^{G12D}$ (Kras) is one of the most common (Cancer Genome Atlas Network, 2012). Therefore, we generated *vil-Cre-ER*^{T2} *Apc*$^{fl/fl}$ *Kras*$^{G12D/+}$ (APC Kras) and *vil-Cre-ER*^{T2} *Apc*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Rac1*$^{fl/fl}$ (APC Kras Rac1) mice. Three days after induction, we purified crypts from these mice and assessed their tumor formation capacity by injecting them into nude mice. Strikingly, crypts from APC Kras intestines were able to efficiently form tumors, whereas those from APC Kras Rac1 animals did not (Figure 6I). In the two out of six cases in which delayed tumor formation occurred from Apc Kras Rac1 crypts, they were found to express *Rac1*, indicating that these grew from rare crypts that had not lost *Rac1* (data not shown). Together, these data strongly indicate an essential role for RAC1 in intestinal adenoma formation.

A recent study has demonstrated that LGR5 marks a population of cancer stem cells (CSCs) within mouse intestinal adenomas (Schepers et al., 2012). As RAC1 permits the proliferation of LGR5 ISCs after *Apc* loss, we asked whether RAC1 is important for maintaining this population in established tumors. Specifically, we wanted to assess the impact of deletion of *Rac1* from the LGR5+ population within adenomas. For this, we intercrossed mice carrying the *Apc*^{1322T} allele (Pollard et al., 2009) to *Lgr5*$^{GFP\text{-}CREER}$ mice. To test recombination within tumors, we aged *Lgr5*$^{GFP\text{-}CREER}$ *Apc*^{1322T} carrying the *Rosa26-RFP* reporter (Figure S6E) to 60 days, when mice have small tumors and induced recombination with tamoxifen. In agreement with previously published work, we observed lineage tracing within both wild-type epithelium and in adenomas, with marked RFP-positive clones 3 weeks after induction in both (Figures S6F and S6G) (Schepers et al., 2012). Given that we were able to induce recombination within adenomas, we aged *Lgr5*$^{GFP\text{-}CREER}$ *Apc*$^{1322T/+}$ *Rac1*$^{fl/fl}$ mice until they developed signs of intestinal tumor burden (weight loss and anemia) and induced recombination. *Rac1* deletion significantly reduced the number of LGR5-GFP-positive cells within the tumors (Figure 6J). Moreover, the reduction in the number of LGR5-GFP-positive cells was partially ablated by concurrent treatment of mice with paraquat (Figure S6J). Thus, RAC1 is required for LGR5 maintenance in intestinal adenomas.

DISCUSSION

Here, we demonstrate a critical requirement for RAC1 in intestinal transformation after *Apc* loss (Figure 7). In contrast to

previous studies suggesting that RAC1 is required for nuclear localization of β-catenin (Wu et al., 2008), we find that RAC1 acts downstream of constitutive WNT signaling to promote progenitor cell proliferation and expansion of the LGR5 ISC population. This may reflect a difference between the effects of WNT3A stimulation and *Apc* loss or may indicate a tissue-specific role for RAC1 in β-catenin activation. We find that the dependence on RAC1 for crypt hyperproliferation is translated to a tumor model driven by *Apc* loss in LGR5 ISCs. These findings are striking as other oncogenic mediators overexpressed after *Apc* loss, including CYCLIN D1/D2, CD44, and MBD2, show much less dramatic effects on crypt hyperproliferation or intestinal tumorigenesis (Cole et al., 2010; Phesse et al., 2008; Zeilstra et al., 2008). As RAC1 also suppresses proliferation of APC-deficient cells after KRAS activation, we propose that it is essential for intestinal transformation.

Mechanistically, these phenotypes depend on RAC1-driven production of ROS and NF-κB signaling. The role of ROS in stem cell maintenance, cellular transformation, and CSC survival appears to be context and tissue specific. In proliferative neural stem cells, high levels of ROS regulate self-renewal by driving PI3K/AKT signaling (Le Belle et al., 2011). Conversely, hematopoietic stem cells are sensitive to ROS levels and higher levels limit their lifespan (Ito et al., 2006). Additionally, although production of ROS via NOX1 upregulation is important for tumorigenesis (Mitsushita et al., 2004), it has been reported that low levels of ROS in CSCs help protect against radiotherapy-induced DNA damage (Diehn et al., 2009). Our data suggest that after *Apc* loss, LGR5 ISC/progenitor cell expansion is a critical process during tumor initiation and is dependent on RAC1-driven ROS production. Given that LGR5 ISCs maintain high levels of ROS but are not dependent on it, we would hypothesize that increased RAC1-ROS is critical for proliferation outside the normal niche (Figure 7). RAC1 is also required for efficient NF-κB signaling after *Apc* loss and, importantly, constitutive activation of NF-κB in the absence of RAC1 partially rescued the attenuated proliferation and ISC expansion phenotypes. We also found that loss of RAC1 prevented p65 recruitment to the promoters of ISC genes after *Apc* deletion. Our findings are similar to those observed with p65 deletion after β-catenin activation and suggest that after *Apc* loss, LGR5 ISC marker genes require both β-catenin and p65 for full transcriptional activation (Schwitalla et al., 2013). RAC1 deficiency prevents this by impairing NF-κB signaling, despite β-catenin nuclear accumulation. The finding that ROS and NF-κB both play important roles in intestinal tumor initiation indicates a role for inflammation in this process. Interestingly, a recent report outlines a role for inflammatory pathways in progenitor cell transformation in the foregut (Liu et al., 2013). Thus, it is tempting to speculate that inflammatory pathways are common mediators of stem/progenitor transformation in multiple tissues.

The LGR5 ISC Signature and CRC

There is evidence of two different ISC populations (Barker et al., 2007; Sangiorgi and Capecchi, 2008). One is highly proliferative, located at the crypt base, and marked by LGR5 and the other is relatively quiescent, label retaining, and expresses BMI1. The focus of our study was on the proliferative LGR5 population and we have not directly addressed whether RAC1 is required for the transformation of BMI1 ISCs. A level of hierarchy between ISC populations has been proposed. Under normal homeostatic conditions, Lgr5 ISCs populate the intestine, but this population can be depleted during times of intestinal stress. When this occurs, Bmi1+ cells can produce Lgr5 ISCs and subsequently repopulate the intestine (Tian et al., 2011). Interestingly, LGR5+ cells are also capable of generating the +4 population (Takeda et al., 2011). This would argue against a strict hierarchical relationship between these populations and instead indicate that, depending on various contexts, these ISC populations are interchangeable. The location, function, and plasticity of ISC populations are still subject to keen debate. Recent data has argued that LGR5+ cells express markers of alternative ISC populations and that immediate progenitor cells can regain stemness after crypt damage (Muñoz et al., 2012; van Es et al., 2012). Perhaps the simplest explanation is that LGR5+ ISCs are defined by their crypt location and progenitor cells can regain ISC properties if they enter the LGR5+ niche. Interestingly, we observed LGR5+ cells throughout the crypt after *Apc* loss, and this is dependent on RAC1. Perhaps this is why RAC1 deletion and ROS inhibition do not affect normal LGR5 ISC function, i.e., these factors are critical for unrestrained proliferation outside the conventional niche. It should be noted that despite being expanded after *Apc* loss, the LGR5+ zone does not encompass all APC-deficient cells and *Apc* deletion alone in LGR5− cells does not lead to tumorigenesis. Thus, other mutations are probably required to permit tumor formation from non-ISCs. We have recently shown that activation of KRAS in concert with *Apc* loss can induce dedifferentiation of villus enterocytes, leading to tumor formation (Schwitalla et al., 2013). It would be interesting to determine whether this process is RAC1 dependent. Given the plasticity of the normal intestine, this may also relate to how intestinal CSCs behave. If multiple CSC populations exist and are interconvertible, then so-called stem cell therapies, which specifically target a particular subpopulation of cells would be unlikely to prove beneficial. As specific CSC populations are depleted, they may be rapidly repopulated from alternative "reserve" CSCs or progenitor populations. In this case, it would be important to target both the CSCs and the proliferative capacity of their descendants. In this regard, our data demonstrate that RAC1 would be a particularly attractive candidate to target these populations. *Rac1* deletion suppressed both LGR5 ISC and progenitor hyperproliferation after deletion of *Apc* and prevented tumor formation from LGR5+ cells. Importantly, deleting *Rac1* also suppressed proliferation of cells with activated KRAS, thus expanding the potential range of tumors likely to respond to RAC1 inhibition.

It is currently unclear whether LGR5 marks APC-deficient ISCs. LGR5 marks a CSC population with tumor lineage-tracing properties in murine adenomas and LGR5+ APC-deficient cells have enhanced in vitro clonogenicity over LGR5− cells, but no in vivo clonogenicity data are available (Schepers et al., 2012). We have shown that LGR5 ISC expansion, in vitro clonogenicity of APC crypts, and adenoma initiation are RAC1 dependent. However, as sorted cells from adenomas do not transplant, we were not able to determine their clonogenicity in vivo. Thus, we cannot state categorically that RAC1 is required for APC-deficient ISC function. Two recent studies have suggested very different outcomes for CRC patients with high levels of LGR5

ISC markers. Merlos-Suarez and colleagues have shown that expression of EphB receptors is closely correlated with LGR5 and other ISC markers in CRC, and those cancers with high levels of these markers had a greater chance of relapse (Merlos-Suárez et al., 2011). In contrast, de Sousa E Melo and colleagues showed that while high levels of WNT signaling marks CSC populations, those cancers in which the promoters of LGR5 ISC markers were methylated were more likely to relapse (de Sousa E Melo et al., 2011). Thus, it is not clear how well LGR5 ISC marker expression defines CSCs in human tumors and how this is linked to disease progression.

RAC1 as a Therapeutic Target?

We previously demonstrated that deletion of *Myc* was sufficient to rescue all of the phenotypes of *Apc* loss. Although *Rac1* deletion did not completely recapitulate this, the comprehensive suppression of tumor formation suggests that RAC1 is required for transformation after *Apc* loss. As the majority of CRCs contain *APC* mutations, this has been the focus of our study. However, it would be interesting to determine whether *Rac1* deletion also suppresses tumorigenesis in *Apc*-independent murine CRC models (Heid et al., 2011; Li et al., 2012). Thus, RAC1 may be a worthwhile therapeutic target during the early stages of CRC. Interestingly, our data indicate that ROS and NF-κB are two parallel pathways involved in promoting ISC/progenitor proliferation downstream of RAC1. It should be noted that activation of ROS or NF-κB was not sufficient to completely rescue the phenotypes associated with *Rac1* loss. This is important in two respects. First, it demonstrates how robust the requirement for RAC1 is to intestinal tumor formation. As a key signaling node that integrates numerous downstream signaling pathways, the potential therapeutic benefits of targeting it should be high. Also, it would likely increase the range of tumors that would be sensitive to its inhibition and reduce the scope for drug resistance to develop. Second, it indicates that the RAC1 phenotype may also involve additional downstream pathways. Indeed, activation of two other known target pathways of RAC1, MTOR (Saci et al., 2011) and STAT3 (Simon et al., 2000), were attenuated in intestines lacking APC and RAC1. Both of these pathways have been shown to reduce though not prevent polyposis in the $Apc^{Min/+}$ mouse (Fujishita et al., 2008; Musteanu et al., 2010). This may also explain why our results do not perfectly phenocopy studies on any of the RAC1-modified factors that we have examined. For example, *Ikkβ* deletion in an AOM/DSS-driven model does not completely prevent tumorigenesis, whereas in our model, *Rac1* deletion does (Greten et al., 2004). Thus, the combined suppression of a number of downstream effectors appears to have a much more profound effect than deleting them individually. Thus, if efficient RAC1 inhibitors remain elusive, it may be worth combining inhibition of these downstream pathways. Another question that remains is what happens at more advanced stages of CRC. Would other mutations overcome the dependence of RAC1? It is interesting to note that in the case of mTOR inhibition, additional KRAS mutation strongly suppresses the efficacy of rapalogs in mouse models (Hung et al., 2010). We have demonstrated that RAC1 is essential for the transformation of KRAS-mutated crypts. Given previous reports that the transformation activity of KRAS is dependent on RAC1 (Qiu et al., 1995), it seems likely that RAC1 would remain an efficacious target even in the setting of a KRAS mutation.

In summary, we have elucidated an important axis downstream of WNT/MYC signaling after *Apc* loss that is crucial for LGR5 ISC/progenitor hyperproliferation and hence tumorigenesis.

EXPERIMENTAL PROCEDURES

Mouse Experiments

All experiments were performed under the UK Home Office guidelines. The background of mice were as follows: *AhCre* experiments were at least ten generations C57Bl6J, Apc^{1322T} experiments were at least five generations C57Bl6J, *VilCre-ER* experiments were performed on a mixed background (50% C57Bl6J, 50% S129). The alleles used for this study were as follows: $c\text{-}Myc^{fl}$ (Sansom et al., 2007), *AhCre* (Ireland et al., 2004), Apc^{580S} (Sansom et al., 2007), $Rac1^{fl}$ (Walmsley et al., 2003), *VilCre-ER* (el Marjou et al., 2004), *Lgr5-CreER* (Barker et al., 2007), *ROSA-tdRFP* (Luche et al., 2007), and $R26Stop^{FL}ikk2ca$ (Sasaki et al., 2006). Recombination by *VilCreER* was induced with one intraperitoneal (i.p.) injection of 80 mg/kg tamoxifen per day for 4 days. Analyses of VilCreER-induced mice were at day 4 after induction. GFP lineage tracing was performed by inducing recombination by *AhCre* using a single i.p. injection of 80 mg/kg β-napthoflavone and analyzing GFP expression 14 days later. Mice carrying the *Lgr5-CreER* transgene were given one i.p. injection of 120 mg/kg tamoxifen, followed by one daily i.p. injection of 80 mg/kg tamoxifen for 3 days. This protocol was also used for inducing recombination within preformed adenomas. For ROS inhibition, 0.5% N-acetyl cysteine was added to drinking water for 1 week prior to Cre induction by tamoxifen treatment. Treatment with paraquat was 10 mg/kg i.p. injection every other day starting 2 days prior to tamoxifen induction.

Immunohistochemistry

Standard immunohistochemistry techniques were used throughout this study. Primary antibodies and concentrations can be found in the Supplemental Experimental Procedures. For each antibody, staining was performed on at least three mice of each genotype and average staining intensity over the entire tissue area was scored. Representative images are shown for each staining.

Microarray Analysis

We reverse transcribed 1 μg of total RNA isolated from intestinal tissue was to cDNA and hybridized to Affymetrix Mouse Genome 430 2.0 microarrays. Cell files of six samples were normalized and analyzed in Partek Genomics Suite Software. RMA normalization and log2 transformation of the data was followed by the differential gene expression analysis using ANOVA and post hoc linear contrasts between all pairs of experimental conditions. Multiple test corrections were performed for all calculated p values. Finally, the fold change values were considered in ranking genes of interest. Enrichment analysis was performed with chi-square test with Yates's correction (Gold et al., 2007).

Additional methods are in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes six figures, Supplemental Experimental Procedures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.04.006.

ACKNOWLEDGMENTS

All authors are supported by Cancer Research UK. K.B.M. is funded by an AICR grant. The research leading to these results has received funding from the European Union Seventh Framework Programme FP7/2007-2013 under grant agreement number 278568. Thanks to histology, microscopy, and the BSU for enabling this work to be performed and Florian Greten for discussions. We thank Victor Tybulewicz for *Rac1* flox and Ian Tomlinson for $Apc^{1322T/+}$ mice. The *ROSA-tdRFP* mice were imported from the EMMA repository (strain 02112) and were a kind gift from Hans Jorg Fehling. We thank David Tuveson and Tim Humpton for help with ROS staining. We thank Bryan Miller for help

preparing the manuscript. O.J.S., K.B.M., and F.R.G. conceived and designed the project. K.B.M., P.C., E.J.M., R.A.R., D.J.H., S.S., G.K., E.-L.O., D.A., and P.T. performed the experiments and analyzed the data. K.B.M., O.J.S., P.C., D.J.H., E.J.M., and K.I.A. interpreted the data. O.J.S, K.B.M., P.C., and R.A.R. wrote the manuscript.

Received: January 10, 2012
Revised: November 27, 2012
Accepted: April 8, 2013
Published: May 9, 2013

REFERENCES

Athineos, D., and Sansom, O.J. (2010). Myc heterozygosity attenuates the phenotypes of APC deficiency in the small intestine. Oncogene *29*, 2585–2590.

Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P.J., and Clevers, H. (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature *449*, 1003–1007.

Barker, N., Ridgway, R.A., van Es, J.H., van de Wetering, M., Begthel, H., van den Born, M., Danenberg, E., Clarke, A.R., Sansom, O.J., and Clevers, H. (2009). Crypt stem cells as the cells-of-origin of intestinal cancer. Nature *457*, 608–611.

Bromberg, Y., Shani, E., Joseph, G., Gorzalczany, Y., Sperling, O., and Pick, E. (1994). The GDP-bound form of the small G protein Rac1 p21 is a potent activator of the superoxide-forming NADPH oxidase of macrophages. J. Biol. Chem. *269*, 7055–7058.

Buczacki, S.J., Zecchini, H.I., Nicholson, A.M., Russell, R., Vermeulen, L., Kemp, R., and Winton, D.J. (2013). Intestinal label-retaining cells are secretory precursors expressing Lgr5. Nature *495*, 65–69.

Cancer Genome Atlas Network. (2012). Comprehensive molecular characterization of human colon and rectal cancer. Nature *487*, 330–337.

Cole, A.M., Myant, K., Reed, K.R., Ridgway, R.A., Athineos, D., Van den Brink, G.R., Muncan, V., Clevers, H., Clarke, A.R., Sicinski, P., and Sansom, O.J. (2010). Cyclin D2-cyclin-dependent kinase 4/6 is required for efficient proliferation and tumorigenesis following Apc loss. Cancer Res. *70*, 8149–8158.

de Sousa E Melo, F., Colak, S., Buikhuisen, J., Koster, J., Cameron, K., de Jong, J.H., Tuynman, J.B., Prasetyanti, P.R., Fessler, E., van den Bergh, S.P., et al. (2011). Methylation of cancer-stem-cell-associated Wnt target genes predicts poor prognosis in colorectal cancer patients. Cell Stem Cell *9*, 476–485.

Diehn, M., Cho, R.W., Lobo, N.A., Kalisky, T., Dorie, M.J., Kulp, A.N., Qian, D., Lam, J.S., Ailles, L.E., Wong, M., et al. (2009). Association of reactive oxygen species levels and radioresistance in cancer stem cells. Nature *458*, 780–783.

Duncan, R., Carpenter, B., Main, L.C., Telfer, C., and Murray, G.I. (2008). Characterisation and protein expression profiling of annexins in colorectal cancer. Br. J. Cancer *98*, 426–433.

el Marjou, F., Janssen, K.P., Chang, B.H., Li, M., Hindie, V., Chan, L., Louvard, D., Chambon, P., Metzger, D., and Robine, S. (2004). Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis *39*, 186–193.

Ellenbroek, S.I., and Collard, J.G. (2007). Rho GTPases: functions and association with cancer. Clin. Exp. Metastasis *24*, 657–672.

Espina, C., Céspedes, M.V., García-Cabezas, M.A., Gómez del Pulgar, M.T., Boluda, A., Oroz, L.G., Benitah, S.A., Cejas, P., Nistal, M., Mangues, R., and Lacal, J.C. (2008). A critical role for Rac1 in tumor progression of human colorectal adenocarcinoma cells. Am. J. Pathol. *172*, 156–166.

Fujishita, T., Aoki, K., Lane, H.A., Aoki, M., and Taketo, M.M. (2008). Inhibition of the mTORC1 pathway suppresses intestinal polyp formation and reduces mortality in ApcDelta716 mice. Proc. Natl. Acad. Sci. USA *105*, 13544–13549.

Gold, D.L., Coombes, K.R., Wang, J., and Mallick, B. (2007). Enrichment analysis in high-throughput genomics - accounting for dependency in the NULL. Brief. Bioinform. *8*, 71–77.

Greten, F.R., Eckmann, L., Greten, T.F., Park, J.M., Li, Z.W., Egan, L.J., Kagnoff, M.F., and Karin, M. (2004). IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell *118*, 285–296.

Heid, I., Lubeseder-Martellato, C., Sipos, B., Mazur, P.K., Lesina, M., Schmid, R.M., and Siveke, J.T. (2011). Early requirement of Rac1 in a mouse model of pancreatic cancer. Gastroenterology *141*, 719–730, 730, e1–e7.

Hodis, E., Watson, I.R., Kryukov, G.V., Arold, S.T., Imielinski, M., Theurillat, J.P., Nickerson, E., Auclair, D., Li, L., Place, C., et al. (2012). A landscape of driver mutations in melanoma. Cell *150*, 251–263.

Hung, K.E., Maricevich, M.A., Richard, L.G., Chen, W.Y., Richardson, M.P., Kunin, A., Bronson, R.T., Mahmood, U., and Kucherlapati, R. (2010). Development of a mouse model for sporadic and metastatic colon tumors and its use in assessing drug treatment. Proc. Natl. Acad. Sci. USA *107*, 1565–1570.

Ireland, H., Kemp, R., Houghton, C., Howard, L., Clarke, A.R., Sansom, O.J., and Winton, D.J. (2004). Inducible Cre-mediated control of gene expression in the murine gastrointestinal tract: effect of loss of beta-catenin. Gastroenterology *126*, 1236–1246.

Ito, K., Hirao, A., Arai, F., Takubo, K., Matsuoka, S., Miyamoto, K., Ohmura, M., Naka, K., Hosokawa, K., Ikeda, Y., and Suda, T. (2006). Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. Nat. Med. *12*, 446–451.

Kinzler, K.W., and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. Cell *87*, 159–170.

Kissil, J.L., Walmsley, M.J., Hanlon, L., Haigis, K.M., Bender Kim, C.F., Sweet-Cordero, A., Eckman, M.S., Tuveson, D.A., Capobianco, A.J., Tybulewicz, V.L., and Jacks, T. (2007). Requirement for Rac1 in a K-ras induced lung cancer in the mouse. Cancer Res. *67*, 8089–8094.

Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Vogelstein, B., and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science *275*, 1784–1787.

Krauthammer, M., Kong, Y., Ha, B.H., Evans, P., Bacchiocchi, A., McCusker, J.P., Cheng, E., Davis, M.J., Goh, G., Choi, M., et al. (2012). Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. Nat. Genet. *44*, 1006–1014.

Le Belle, J.E., Orozco, N.M., Paucar, A.A., Saxe, J.P., Mottahedeh, J., Pyle, A.D., Wu, H., and Kornblum, H.I. (2011). Proliferative neural stem cells have high endogenous ROS levels that regulate self-renewal and neurogenesis in a PI3K/Akt-dependant manner. Cell Stem Cell *8*, 59–71.

Li, A., Ma, Y., Jin, M., Mason, S., Mort, R.L., Blyth, K., Larue, L., Sansom, O.J., and Machesky, L.M. (2012). Activated mutant NRas(Q61K) drives aberrant melanocyte signaling, survival, and invasiveness via a Rac1-dependent mechanism. J. Invest. Dermatol. *132*, 2610–2621.

Lindsay, C.R., Lawn, S., Campbell, A.D., Faller, W.J., Rambow, F., Mort, R.L., Timpson, P., Li, A., Cammareri, P., Ridgway, R.A., et al. (2011). P-Rex1 is required for efficient melanoblast migration and melanoma metastasis. Nat Commun *2*, 555.

Liu, K., Jiang, M., Lu, Y., Chen, H., Sun, J., Wu, S., Ku, W.Y., Nakagawa, H., Kita, Y., Natsugoe, S., et al. (2013). Sox2 cooperates with inflammation-mediated Stat3 activation in the malignant transformation of foregut basal progenitor cells. Cell Stem Cell *12*, 304–315.

Luche, H., Weber, O., Nageswara Rao, T., Blum, C., and Fehling, H.J. (2007). Faithful activation of an extra-bright red fluorescent protein in "knock-in" Cre-reporter mice ideally suited for lineage tracing studies. Eur. J. Immunol. *37*, 43–53.

Merlos-Suárez, A., Barriga, F.M., Jung, P., Iglesias, M., Céspedes, M.V., Rossell, D., Sevillano, M., Hernando-Momblona, X., da Silva-Diz, V., Muñoz, P., et al. (2011). The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse. Cell Stem Cell *8*, 511–524.

Mitsushita, J., Lambeth, J.D., and Kamata, T. (2004). The superoxide-generating oxidase Nox1 is functionally required for Ras oncogene transformation. Cancer Res. *64*, 3580–3585.

Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K.W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science *275*, 1787–1790.

Muñoz, J., Stange, D.E., Schepers, A.G., van de Wetering, M., Koo, B.K., Itzkovitz, S., Volckmann, R., Kung, K.S., Koster, J., Radulescu, S., et al. (2012). The Lgr5 intestinal stem cell signature: robust expression of proposed quiescent '+4' cell markers. EMBO J. *31*, 3079–3091.

Musteanu, M., Blaas, L., Mair, M., Schlederer, M., Bilban, M., Tauber, S., Esterbauer, H., Mueller, M., Casanova, E., Kenner, L., et al. (2010). Stat3 is a negative regulator of intestinal tumor progression in Apc(Min) mice. Gastroenterology *138*, 1003–1011, e1–e5.

O'Dwyer, D., Ralton, L.D., O'Shea, A., and Murray, G.I. (2011). The proteomics of colorectal cancer: identification of a protein signature associated with prognosis. PLoS ONE *6*, e27718.

Obrador-Hevia, A., Chin, S.F., González, S., Rees, J., Vilardell, F., Greenson, J.K., Cordero, D., Moreno, V., Caldas, C., and Capellá, G. (2010). Oncogenic KRAS is not necessary for Wnt signalling activation in APC-associated FAP adenomas. J. Pathol. *221*, 57–67.

Phelps, R.A., Chidester, S., Dehghanizadeh, S., Phelps, J., Sandoval, I.T., Rai, K., Broadbent, T., Sarkar, S., Burt, R.W., and Jones, D.A. (2009). A two-step model for colon adenoma initiation and progression caused by APC loss. Cell *137*, 623–634.

Phesse, T.J., Parry, L., Reed, K.R., Ewan, K.B., Dale, T.C., Sansom, O.J., and Clarke, A.R. (2008). Deficiency of Mbd2 attenuates Wnt signaling. Mol. Cell. Biol. *28*, 6094–6103.

Pollard, P., Deheragoda, M., Segditsas, S., Lewis, A., Rowan, A., Howarth, K., Willis, L., Nye, E., McCart, A., Mandir, N., et al. (2009). The Apc 1322T mouse develops severe polyposis associated with submaximal nuclear beta-catenin expression. Gastroenterology *136*, 2204–2213, e1–e13.

Qiu, R.G., Chen, J., Kirn, D., McCormick, F., and Symons, M. (1995). An essential role for Rac in Ras transformation. Nature *374*, 457–459.

Radisky, D.C., Levy, D.D., Littlepage, L.E., Liu, H., Nelson, C.M., Fata, J.E., Leake, D., Godden, E.L., Albertson, D.G., Nieto, M.A., et al. (2005). Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. Nature *436*, 123–127.

Sabates-Bellver, J., Van der Flier, L.G., de Palo, M., Cattaneo, E., Maake, C., Rehrauer, H., Laczko, E., Kurowski, M.A., Bujnicki, J.M., Menigatti, M., et al. (2007). Transcriptome profile of human colorectal adenomas. Mol. Cancer Res. *5*, 1263–1275.

Saci, A., Cantley, L.C., and Carpenter, C.L. (2011). Rac1 regulates the activity of mTORC1 and mTORC2 and controls cellular size. Mol. Cell *42*, 50–61.

Samuel, M.S., Lourenço, F.C., and Olson, M.F. (2011). K-Ras mediated murine epidermal tumorigenesis is dependent upon and associated with elevated Rac1 activity. PLoS ONE *6*, e17143.

Sangiorgi, E., and Capecchi, M.R. (2008). Bmi1 is expressed in vivo in intestinal stem cells. Nat. Genet. *40*, 915–920.

Sansom, O.J., Reed, K.R., Hayes, A.J., Ireland, H., Brinkmann, H., Newton, I.P., Batlle, E., Simon-Assmann, P., Clevers, H., Nathke, I.S., et al. (2004). Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. Genes Dev. *18*, 1385–1390.

Sansom, O.J., Meniel, V.S., Muncan, V., Phesse, T.J., Wilkins, J.A., Reed, K.R., Vass, J.K., Athineos, D., Clevers, H., and Clarke, A.R. (2007). Myc deletion rescues Apc deficiency in the small intestine. Nature *446*, 676–679.

Sasaki, Y., Derudder, E., Hobeika, E., Pelanda, R., Reth, M., Rajewsky, K., and Schmidt-Supprian, M. (2006). Canonical NF-kappaB activity, dispensable for B cell development, replaces BAFF-receptor signals and promotes B cell proliferation upon activation. Immunity *24*, 729–739.

Schepers, A.G., Snippert, H.J., Stange, D.E., van den Born, M., van Es, J.H., van de Wetering, M., and Clevers, H. (2012). Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. Science *337*, 730–735.

Schwitalla, S., Fingerle, A.A., Cammareri, P., Nebelsiek, T., Göktuna, S.I., Ziegler, P.K., Canli, O., Heijmans, J., Huels, D.J., Moreaux, G., et al. (2013). Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. Cell *152*, 25–38.

Segditsas, S., Sieber, O., Deheragoda, M., East, P., Rowan, A., Jeffery, R., Nye, E., Clark, S., Spencer-Dene, B., Stamp, G., et al. (2008). Putative direct and indirect Wnt targets identified through consistent gene expression changes in APC-mutant intestinal adenomas from humans and mice. Hum. Mol. Genet. *17*, 3864–3875.

Simon, A.R., Vikis, H.G., Stewart, S., Fanburg, B.L., Cochran, B.H., and Guan, K.L. (2000). Regulation of STAT3 by direct binding to the Rac1 GTPase. Science *290*, 144–147.

Soucek, L., Whitfield, J., Martins, C.P., Finch, A.J., Murphy, D.J., Sodir, N.M., Karnezis, A.N., Swigart, L.B., Nasi, S., and Evan, G.I. (2008). Modelling Myc inhibition as a cancer therapy. Nature *455*, 679–683.

Takeda, N., Jain, R., LeBoeuf, M.R., Wang, Q., Lu, M.M., and Epstein, J.A. (2011). Interconversion between intestinal stem cell populations in distinct niches. Science *334*, 1420–1424.

Tian, H., Biehs, B., Warming, S., Leong, K.G., Rangell, L., Klein, O.D., and de Sauvage, F.J. (2011). A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. Nature *478*, 255–259.

van der Flier, L.G., van Gijn, M.E., Hatzis, P., Kujala, P., Haegebarth, A., Stange, D.E., Begthel, H., van den Born, M., Guryev, V., Oving, I., et al. (2009). Transcription factor achaete scute-like 2 controls intestinal stem cell fate. Cell *136*, 903–912.

van Es, J.H., Sato, T., van de Wetering, M., Lyubimova, A., Nee, A.N., Gregorieff, A., Sasaki, N., Zeinstra, L., van den Born, M., Korving, J., et al. (2012). Dll1+ secretory progenitor cells revert to stem cells upon crypt damage. Nat. Cell Biol. *14*, 1099–1104.

Walmsley, M.J., Ooi, S.K., Reynolds, L.F., Smith, S.H., Ruf, S., Mathiot, A., Vanes, L., Williams, D.A., Cancro, M.P., and Tybulewicz, V.L. (2003). Critical roles for Rac1 and Rac2 GTPases in B cell development and signaling. Science *302*, 459–462.

Wu, X., Tu, X., Joeng, K.S., Hilton, M.J., Williams, D.A., and Long, F. (2008). Rac1 activation controls nuclear localization of beta-catenin during canonical Wnt signaling. Cell *133*, 340–353.

Zeilstra, J., Joosten, S.P., Dokter, M., Verwiel, E., Spaargaren, M., and Pals, S.T. (2008). Deletion of the WNT target and cancer stem cell marker CD44 in Apc(Min/+) mice attenuates intestinal tumorigenesis. Cancer Res. *68*, 3655–3661.

Zhou, C., Licciulli, S., Avila, J.L., Cho, M., Troutman, S., Jiang, P., Kossenkov, A.V., Showe, L.C., Liu, Q., Vachani, A., et al. (2013). The Rac1 splice form Rac1b promotes K-ras-induced lung tumorigenesis. Oncogene *32*, 903–909.