Oncogene. Author manuscript; available in PMC 2011 February 12.

Published in final edited form as:

Oncogene. 2010 August 12; 29(32): 4567–4575. doi:10.1038/onc.2010.202.

The activating mutation R201C in *GNAS* promotes intestinal tumourigenesis in *Apc*$^{Min/+}$ mice via activation of Wnt and ERK1/2 MAPK pathways

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Abstract

Somatically acquired, activating mutations of *GNAS,* the gene encoding the stimulatory G-protein Gsα subunit, have been identified in kidney, thyroid, pituitary, leydig cell, adrenocortical and more recently, in colorectal tumours, suggesting that mutations such as R201C may be oncogenic in these tissues. To study the role of *GNAS* in intestinal tumourigenesis, we placed *GNAS* R201C under the control of the A33-antigen promoter (*Gpa33*), which is almost exclusively expressed in the intestines. The *GNAS* R201C mutation has been shown to result in the constitutive activation of Gsα and adenylate cyclase and to lead to the autonomous synthesis of cAMP. *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice showed significantly elevated cAMP levels and a compensatory upregulation of cAMP-specific phosphodiesterases in the intestinal epithelium. *GNAS* R201C alone was not sufficient to induce tumourigenesis by 12 months but there was a significant increase in adenoma formation when *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice were bred onto an *Apc*$^{Min/+}$ background. *GNAS* R201C expression was associated with elevated expression of Wnt and extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase (ERK1/2 MAPK) pathway target genes, increased phosphorylation of ERK1/2 MAPK, and increased immunostaining for the proliferation marker Ki67. Furthermore, the effects of *GNAS* R201C on the Wnt pathway were additive to inactivation of *Apc*. Our data strongly suggest that activating mutations of *GNAS* cooperate with inactivation of *APC* and are likely to contribute to colorectal tumourigenesis.

Keywords

cAMP; colorectal cancer

Introduction

Genome-wide exon re-sequencing studies of human cancers have identified a number of frequently mutated genes that are associated with cancer formation and progression, however their functional significance is unknown (Sjoblom et al 2006, Wood et al 2007). One potentially interesting candidate is *GNAS*, the gene encoding the stimulatory G-protein

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Conflicts of interest

The authors declare there are no conflicts of interest.

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alpha subunit (Gsα), situated on human chromosome 20q13.3. Heterotrimeric G-proteins that are composed of α, β and γ-subunits mediate signal transduction from a large number of hormone and growth factor-activated seven-transmembrane receptors to diverse intracellular signalling pathways (Weinstein et al 2004). Ligand-bound G-protein coupled receptors activate the Gs-protein through promoting the exchange of GDP for GTP on Gsα, which results in dissociation from the receptor and the βγ-complex. The free Gsα subunit interacts with adenylate cyclase to stimulate the synthesis of cAMP until hydrolysis of GTP returns it to the inactive GDP-bound form, which reassociates with the βγ-complex to enter a new cycle (Weinstein et al 2004).

Somatically acquired, activating mutations of *GNAS* have been identified in adrenal hyperplasia and ovarian cysts, as well as thyroid carcinomas (5%), adrenocortical, pituitary (22-40%), kidney (17%), and leydig cell tumours (67%) (Fragoso et al 1998, Hayward et al 2001, Kalfa et al 2006, Landis et al 1989, Palos-Paz et al 2008, Taboada et al 2009). Furthermore, several reports have documented the presence of thyroid, pituitary and adrenocortical tumours in patients with McCune-Albright Syndrome, a mosaic disease caused by sporadic, post-zygotic, activating mutations of *GNAS* (Chen et al 2004, Collins et al 2003, Fragoso et al 2003, Happle 1986, Kirk et al 1999, Weinstein et al 2004, Yang et al 1999). The common mutations of *GNAS* that have been identified in tumours, including R201C, R201H and Q227R, are thought to inhibit GTP hydrolysis and result in the constitutive activation of Gsα and its effector adenylate cyclase, leading to autonomous synthesis of cAMP (Landis et al 1989). Collectively, these data suggest that activating mutations of *GNAS* can modify cell growth and may be oncogenic, however, how *GNAS* functions as an oncogene remains unclear.

Interestingly, a number of studies have reported an association between McCune-Albright Syndrome and multiple gastrointestinal polyps (MacMahon 1971, Weinstein et al 1991). More recently *GNAS* R201C mutations were identified in 9% of colorectal tumours (3/35; (Sjoblom et al 2006, Wood et al 2007)). The most frequent early event in >80% of sporadic colorectal carcinomas is loss of function mutations of the adenomatous polyposis coli (*APC*) gene, which is also mutated in the germline of patients with familial adenomatous polyposis (Nishisho et al 1991). APC forms a complex with Axin and glycogen synthase kinase 3β (GSK-3β), which results in the phosphorylation of β-catenin and consequently, its degradation via the ubiquitin proteasome system (Castellone et al 2005). $Apc^{Min/+}$ mice represent a valuable model of intestinal tumourigenesis, since sporadic loss of heterozygosity of the wild-type allele of *Apc* recapitulates the initiation of adenomagenesis observed in humans (Levy et al 1994). A number of groups have shown that loss of cyclooxygenase-1 or -2 (COX) dramatically reduces tumour formation in $Apc^{Min/+}$ mice (reviewed in ref. (Taketo 2006)). Accordingly, the expression of *PTGS1* and *2* (COX-1 and 2, respectively) has been shown to be upregulated in approximately 80% of colorectal adenomas and carcinomas and COX inhibition represents a valuable therapeutic target (Eberhart et al 1994). COX-1 and -2 synthesize the proinflammatory metabolite prostaglandin E2 which activates prostaglandin receptors 2 and 4 (EP2 and EP4), resulting in activation of Gsα, adenylate cyclase and cAMP synthesis. The effects of COX-2 on intestinal tumour formation have been shown to be mediated by EP2 receptor activation (Castellone et al 2005), suggesting that both the spacial and temporal production of cAMP is important in intestinal tumourigenesis. The promotion of intestinal tumourigenesis by COX-2 is thought to be due to the direct binding of activated Gsα to the regulator of G-protein signalling domain of Axin, which promotes the release of GSK-3β from the complex and its inactivation. Furthermore, upon loss of *APC*, the threshold of Wnt pathway activation by constitutively active Gsα is thought to be lowered (Castellone et al 2005). Therefore, loss of APC, or activation of Gsα is predicted to lead to reduced degradation and increased nuclear translocation of β-catenin. Constitutively active β-catenin/T-cell factor/

lymphocyte enhancer factor (TCF-LEF)-mediated transcription leads to the expression of growth-promoting genes, and the transformation of normal crypts into the earliest colorectal cancer precursor lesions, called dysplastic aberrant crypt foci or monocryptal/oligocryptal adenomas (reviewed in ref. (Radtke and Clevers 2005)).

Activating mutations of either *KRAS* or *BRAF* are found in 40-50% of colorectal cancers (Bos 1989, Brink et al 2003) and lead to activation of the extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase (ERK1/2 MAPK) pathway, which enhances proliferation, neoplastic transformation, differentiation and survival of many cell types (Barbacid 1987). cAMP has been shown to have opposing effects on cell growth: cAMP either inhibits or stimulates ERK1/2 MAPK-mediated cell proliferation and/or differentiation in a cell-type specific manner (reviewed in ref. (Stork and Schmitt 2002)). Whether elevated levels of cAMP result in the activation of the ERK1/2 MAPK pathway and growth of intestinal cells is not known.

Here, we have generated mice that specifically express *GNAS* R201C in the intestinal stem cells and all epithelial cell lineages from E14.5 into adulthood (Abud et al 2000, Johnstone et al 2000) to assess whether the mutation participates in the formation and/or progression of colorectal cancer *in vivo*.

Results

Generation of an intestine-specific conditional *Gpa33*$^{tm1(GnasR201C)Wtsi}$ allele

Full-length human *GNAS* cDNA was synthesized to introduce the putative oncogenic mutation R201C. The mutation R201C was chosen over R201H and Q227R because is it found more frequently in cancers, however, all mutations have both been shown to have a similar affect on adenylyl cyclase stimulation (Landis et al 1989). To express the mutant *GNAS* R201C cDNA specifically in the intestinal epithelium we obtained and modified a *Lox-Stop-Lox* targeting vector to place it under the control of the endogenous *Gpa33*-antigen gene promoter (Fig 1A; ref 30). This approach was used to avoid complications that may arise from imprinting at the endogenous *Gnas* locus and extra-intestinal phenotypes such as those observed in McCune-Albright Syndrome patients (Plagge et al 2008). Upon Cre-mediated recombination of the loxP sites, *GNAS* R201C cDNA is predicted to be expressed bicistronically from the *Gpa33* gene locus (Fig. 1A). In adult mice *Gpa33* is exclusively expressed throughout the epithelium of intestinal tract, thus directing mutant *GNAS* R201C expression exclusively to proliferating and differentiating intestinal epithelial cells and crypt stem cells (Abud et al 2000). The linearised conditional *Gpa33*$^{tm1(GnasR201C)Wtsi}$ vector was electroporated into Bruce4 C57BL/6J mouse ES cells and correctly targeted clones were identified by Southern blot analysis (Fig. 1B) and PCR. The F1 conditional *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ males generated were crossed with *CMV-Cre*$^{-/-}$ females. A total of 69 progeny were obtained from 10 litters, 34 were *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$, 35 were *Gpa33*$^{+/+}$ and all were heterozygous for *CMV-Cre*. This is the expected 1:1 Mendelian ratio, therefore expression of mutant *GNAS* R201C from the *Gpa33* locus does not cause embryonic lethality.

Expression of *GNAS* R201C from the *Gpa33* locus

RT-PCR expression analysis was performed on RNA extracted from a range of tissues from *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ and wildtype (*Gpa33*$^{+/+}$) littermates using primers specific for the *Gpa33* locus (exon 5) and the mutant *GNAS* cDNA. As expected, a 1.1kb product was only generated from intestinal tissue from *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice (Fig. 1C). The transcript was not present in other non-intestinal tissue from *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice or in intestinal tissue from wildtype littermate controls. Cloning and sequencing of the

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product generated from the intestines of *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice revealed the expected spliced transcript contained *Gpa33* exons 5, 6 and 7 followed by LoxP-IRES-*GNAS* R201C cDNA sequences.

Enhanced cAMP levels and upregulation of cAMP-specific phosphodiesterases in the intestinal epithelium of *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice

The *GNAS* R201C mutation is thought to constitutively activate Gsα and adenylate cyclase and to lead to autonomous production of cAMP (Landis et al 1989). We used immunofluorescence and flow cytometry to show that expression of *GNAS* R201C leads to a 1.5-fold increase in cAMP levels in the intestinal epithelium of *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice relative to wildtype littrmate controls (P<0.05; Fig. 1D). In the long-term, high levels of cAMP are subject to negative feedback control by upregulation of phosphodiesterases (Conti and Beavo 2007). We therefore determined the expression of the cAMP-specific phosphodiesterases 4a, 4b, 7a, 8a and 8b by qRT-PCR. Expression of *Pde4a* and *Pde8b* were upregulated 2.5–fold in the intestinal epithelium of *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice relative to control mice (P=0.05), whereas expression of *Pde4b* and *Pde8a* were unchanged (Fig. 1E). Expression of *Pde7a* was negligible in this tissue.

Gpa33$^{tm1(GnasR201C)Wtsi/+}$ mice do not develop intestinal polyps by 12 months of age

To establish the phenotypic effect of expression of mutant *GNAS* R201C in the intestine we aged a cohort of 21 *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ and 20 wildtype littermate controls (*Gpa33*$^{+/+}$) mice to 12 months. Macroscopic and histological analysis did not reveal any polyp formation in the intestines suggesting that by itself, the activating *GNAS* R201C mutation is not sufficient to generate intestinal neoplasms (Fig. 2A).

The *GNAS* R201C mutation promotes intestinal adenoma formation in *Apc*$^{Min/+}$ mice

To further explore the role of *GNAS* R201C mutations in intestinal adenoma formation we crossed *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice with *Apc*$^{Min/+}$ mice. Mice were sacrificed at 16 weeks to determine the number, size and location of intestinal adenomas in the small and large intestines. The mean number of adenomas in *Apc*$^{Min/+}$ mice (C57BL6) was 30 ± 2.9 (mean ± SE, n = 12), which is comparable to data obtained in other studies (Fig. 2A; ref (Su et al 1992). The activating R201C mutation of *GNAS* resulted in a mean adenoma number of 62 ± 5.2 (n = 11), a 2-fold increase (P=0.0001; Fig. 2A&B). The majority of intestinal adenomas were in the small intestine and showed a distribution very similar to that of *Apc*^{Min} mice (Supplemental Fig. 1). There was no difference in the dysplastic grade of adenoma between the two cohorts of mice - all were low-grade, non-invasive, tubular or tubulovillous adenomas typical of *Apc*^{Min} mice (Fig. 2B&C). Collectively these data show that activation of *GNAS* cooperates with inactivation of *Apc* in the formation of intestinal adenomas.

Activation of Wnt and ERK1/2 MAPK pathways and enhanced proliferation in the intestines of *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice

To identify a mechanism for the enhanced formation of adenomas in *Apc*$^{Min/+}$ mice carrying the mutant *GNAS* R201C allele, we examined two of the most important signaling pathways in intestinal tumourigenesis, the ERK1/2 MAPK and Wnt pathways. cAMP signalling has been shown to activate ERK1/2 MAPKs in a cell-type specific manner (McCubrey et al 2007, Stork and Schmitt 2002). Immunohistochemical analysis of the small intestines revealed a significant increase in the number of nuclei that stained positively for phosphorylated-ERK1/2 MAPK within the crypt region (P= 0.0453; Fig.3A) of *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ relative to wildtype littermate control mice. In agreement with this, Western blot analysis showed a 2-fold increase in the amount of phosphorylated-

ERK1/2 MAPK relative to total ERK1/2 MAPK (P=0.05; Fig. 3B). Phosphorylated-ERK1/2 MAPK activates transcription factors such as c-Myc and consequently leads to enhanced expression of the transcription factor c-Fos (McCubrey et al 2007). Expression of *Fos* was increased 2-fold in *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice relative to wildtype littermate control mice (P=0.02; Fig. 3C).

Gsα has been implicated in the aberrant activation of Wnt signalling by COX2 and prostaglandin E2 (Castellone et al 2005), therefore we examined the expression of two Wnt target genes, *Myc* and *Birc5* (survivin) by qRT-PCR (Giles et al 2003). Both *Myc* and *Birc5* were upregulated 2-fold suggesting significant activation of the Wnt pathway in *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ intestines when compared to control mice (P=0.05; Fig. 3C). To test whether the effects of loss of *Apc* and activation of *Gnas* on the Wnt pathway were additive, we determined the expression of *Myc* and *Birc5* in intestinal polyps. *Myc* was upregulated 1.5-fold and *Birc5* was upregulated 2-fold in intestinal polyps from *Apc*$^{Min/+}$ *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice compared with polyps from *Apc*$^{Min/+}$ mice (P<0.05 for both; Supplemental Fig. 2). *Pgts2* (COX-2) expression has been reported to be upregulated by both the Wnt and ERK1/2 MAPK pathways (Araki et al 2003), however, we found no evidence to suggest that *Pgts2* is upregulated in *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ intestines (Fig. 3C), indicating that the effects of *GNAS* R201C occur independently and downstream of COX-2.

c-Myc and c-Fos transcription factors coordinate the expression of genes that drive cellular proliferation and/or differentiation. There was a significant increase in the number of nuclei that stained positively for Ki67 within the crypt region of *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice relative to *Gpa33*$^{+/+}$ (P= 0.005; Fig. 3A), suggesting that activation of *GNAS* R201C within the intestine augments proliferation. Immunohistochemical and histological analysis of the differentiated cells of the intestinal tissue revealed no difference in the number of enteroendocrine (P=0.1282), Paneth (P=0.8102), goblet (P=0.1735), mitotic cells (P=0.1735) and apoptotic fragments (P=1.0; Supplemental Fig. 3) suggesting that the activating R201C mutation of *GNAS* has no effect on differentiation or apoptosis of intestinal cells.

Discussion

Somatically acquired, activating mutations of *GNAS* have been identified in kidney, thyroid, leydig cell, pituitary and adrenocortical tumours (Fragoso et al 1998, Hayward et al 2001, Kalfa et al 2006, Landis et al 1989, Palos-Paz et al 2008, Taboada et al 2009). However, a recent mutational analysis of 35 colorectal cancers by Sjoblom *et al.*, (1-2) was the first study to suggest that the R201 mutations of *GNAS* occurs frequently (9%) in colorectal cancers. Furthermore, a number of studies have reported an association between McCune-Albright Syndrome, a mosaic disease caused by sporadic, post-zygotic, activating mutations of *GNAS,* and multiple gastrointestinal polyps (MacMahon 1971, Weinstein et al 1991). However, how *GNAS* functions in this context remains unclear.

To examine the role of *GNAS* R201C in intestinal tumourigenesis we generated mice expressing *GNAS* R201C under the control of the *Gpa33*-antigen promoter, which is almost exclusively expressed by intestinal stem cells and all epithelial cell lineages from E14.5 into adulthood (Abud et al 2000, Johnstone et al 2000). *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice were shown to express mutant *GNAS* R201C in the intestine which led to a significant increase in cAMP levels in the intestinal mucosa, in agreement with a previous report indicating that the R201C mutation leads to constitutive activation of Gsα protein (Landis et al 1989). We show that *GNAS* R201C alone was not sufficient to induce tumourigenesis by 12 months, but there was a 2-fold increase in adenoma formation when *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice

were crossed with *Apc*$^{Min/+}$ mice. Furthermore, we have shown that constitutive activation of Gsα in intestinal cells results in increased activation of two of the most important signaling pathways in the development of colorectal cancers - the Wnt and ERK1/2 MAPK pathways.

Several reports have suggested that both the spatial and temporal production of cAMP are important in the promotion of intestinal tumourigenesis by COX-2 (reviewed by (Taketo 2006)). cAMP differentially regulates cell growth through the compartmental organization of cAMP signalling which is predominantly the result of cell-type specific isoform expression of phosphodiesterases, adenylate cyclases, protein kinase A (PKA) and cAMP effectors such as Rap-1 and B-raf (Conti and Beavo 2007, Houslay and Milligan 1997, Stork and Schmitt 2002). We found that the 1.5-fold increase in cAMP was accompanied by a 2.5-fold upregulation of the cAMP-specific phosphodiesterases *Pde4a* and *Pde8b* but there was no change in the expression of *Pde4b* and *Pde8a*. Since high levels of cAMP are subject to negative feedback control by upregulation of phosphodiesterases (Conti and Beavo 2007), our data suggest that Gsα R201C is localized to the same cellular compartment(s) as Pde4a and Pde8b. In many cells cAMP serves to inhibit cell growth via inhibition of ERK1/2 MAPK, however, activation of ERK1/2 MAPK by cAMP has been shown to occur in a cell-type specific manner via a number of mechanisms (Stork and Schmitt 2002). Briefly, cAMP has been shown to activate ERKs in a PKA-dependent manner; by stimulation of the PKA/Src/Rap-1/B-raf/MEK cascade, by stimulation of a PKA/Ras/B-raf or Raf-1/MEK cascade or through PKA-mediated inhibition of specific protein tyrosine phosphatases that inhibit ERKs. For Rap-1 to activate ERKs, the Raf isoform B-Raf must be expressed, as is the case in intestinal cells. cAMP has also been shown to activate ERKs independently of PKA, for example in the crypt-like intestinal cell-line T84 (Nishihara et al 2004, Rudolph et al 2004), and this is thought to occur through activation of cAMP GTPase exchange factors which then directly activate the Rap-1/B-raf/MEK cascade. Furthermore, cAMP-mediated activation of ERK1/2 MAPK can stimulate proliferation and/or differentiation depending on the stimulus and cell type. In *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$mice we saw both enhanced levels of activated phospho-ERK1/2 MAPK and increased levels of proliferation, as shown by an increased number of nuclei that stained positively for activated phospho-ERK1/2 MAPK and the proliferative marker Ki67 in the crypt region of the intestines. We found no changes in the markers for enteroendocrine, Paneth and goblet cells or apoptotic bodies, suggesting that cAMP may stimulate the proliferation of intestinal cell types in an ERK1/2 MAPK-dependent fashion, but that it does not affect the differentiation or apoptosis of these cells. Although we have not established the exact mechanism of cAMP-mediated activation of ERK1/2 MAPK following constitutive activation of Gsα in intestinal cells, activation of ERK1/2 MAPK is a significant contributory factor in the genesis of colorectal cancer. ERK1/2 MAPK activation is the result of activating mutations in either *KRAS* or *BRAF* in 40-50% of colorectal cancers (Bos 1989, Brink et al 2003).

Expression of activated *K-Ras* from its endogenous locus or a transgene, and therefore activation of ERK1/2, by itself has been shown to be insufficient to induce neoplasia within the murine intestine (Haigis et al 2008, Luo et al 2007, Luo et al 2009, Sansom et al 2006), however, in combination with inactivation of *Apc,* tumourigenesis is promoted (Haigis et al 2008, Luo et al 2007, Luo et al 2009, Sansom et al 2006). In support, our data suggest that the intestinal expression of mutationally activated *GNAS* and subsequent increase in activated ERK1/2 within the intestine was insufficient (by 12 months) to induce tumourigenesis alone, but in combination with inactivation of *Apc,* tumour number doubled.

Aberrant activation of the Wnt/β-catenin pathway is an initiating event in the vast majority of colorectal adenomas and cancers. The upregulation of c-Myc following activation of β-catenin is thought to be a critical mediator of the phenotypes of *APC* inactivation in the

intestine (reviewed by (Wilkins and Sansom 2008)), which include cytoskeleton organization, apoptosis, cell cycle control and cell adhesion (Sieber et al 2000). Other models predict increased stem cell survival after loss of function of *APC* which can be explained by enhanced expression of the Wnt target genes (Zhang et al 2001). Upregulation of the Wnt target genes *Myc* and *Birc5* (survivin) in *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$mice is likely to be due to the binding of constitutively activated Gsα to the regulator of G-protein signalling domain of Axin, thereby promoting the release of GSK-3β from the APC/Axin complex and leading to reduced degradation of β-catenin (Castellone et al 2005). Our data suggest that a 2-fold upregulation of *Myc* and *Birc5* is not sufficient to induce intestinal tumourigenesis in *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$mice, by 12 months at least. However, clinical evidence from McCune-Albright Syndrome patients suggests that constitutive activation of Gsα is sufficient to induce intestinal tumour formation, although the *APC* status of these lesions is unknown (MacMahon 1971, Weinstein et al 1991). APC and Gsα are both reported to bind to the regulator of G-protein signalling domain of Axin and it is thought that APC hinders the activation of the Wnt pathway by Gsα. Upon loss of *APC*, the threshold of Wnt pathway activation by constitutively active Gsα is thought to be lowered (Castellone et al 2005). In agreement, we found that the effects of loss of *Apc* and activation of *Gnas* were additive since expression of the Wnt targets *Myc* and *Birc5* were increased further in polyps from double mutant mice when compared those from *Apc*$^{Min/+}$ mice (Supplemental Fig. 2). These findings may explain the increase in tumour formation, accompanied by no increase in tumour severity in *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$*Apc*$^{Min/+}$ mice and why *GNAS* R201C and *APC* mutations have been found to co-occur in intestinal tumours (Sjoblom et al 2006, Wood et al 2007).

Interestingly, cortisol-producing adrenocortical tumours have been associated with a number of syndromes other than McCune-Albright that feature abnormalities of cAMP–signaling. Examples are Cushing's syndrome due to mutations in *GNAS*, primary pigmented nodular adrenocortical disease (Carney Complex) due to mutations of the PKA regulatory subunit type 1A (*PRKAR1A*), macronodular adrenocortical disease associated with aberrant expression of G-protein–coupled receptors in the tumour tissue and more recently, micronodular adrenocortical hyperplasia with inactivating mutations of phosphodiesterases *PDE11A* and *PDE8B* (Horvath et al 2006, Horvath et al 2008, Kirschner et al 2000, Stratakis 2003). Moreover, somatic activating mutations of the β-catenin gene (*CTNNB1*), which cause activation of the Wnt pathway, are the most frequent genetic defects in adrenocortical tumours and activating mutations of either *BRAF, KRAS, NRAS* or *EGFR*, which all result in activation of ERK1/2 MAPK, are also frequently found in this type of tumour (Kotoula et al 2009). Given the involvement of the phosphodiesterase *Pde8b*, Wnt and ERK1/2 MAPK pathways in intestinal tumourigenesis that is driven by activating mutations of Gsα in mice, it may be hypothesized that the above signalling molecules are localized to the same cAMP-signalling compartment in these two cell types and are involved in the development of both colorectal and adrenocortical cancers.

With the advancement of next generation sequencing technologies genome-wide profiling of somatic mutations in human cancers is becoming a reality, therefore, a number of potentially interesting cancer genes are being identified. Data suggests further, that only a few genes are commonly mutated "mountains" and a much larger number of gene "hills" are mutated at low frequency. Our data suggests that genes such as *GNAS,* which are mutated at a modest frequency in colorectal cancer, may also represent 'drivers' of tumourigenesis, making functional validation critical for establishing their diagnostic and therapeutic potential. Our data show that the R201C activating mutation of *GNAS* causes augmentation of the both the Wnt and ERK1/2 MAPK pathway in the intestinal epithelium of mice, and that the mutation co-operates with inactivation of *APC* in intestinal tumour formation *in vivo*. Presumably, the activating R201H mutation of *GNAS* that has been identified in other human cancers

contributes to tumour formation via the same mechanism. Taken together with previous human colorectal cancer mutational analysis (Sjoblom et al 2006, Wood et al 2007) our data strongly suggests that activating mutations of *GNAS* are likely, together with inactivation of APC, to contribute to colorectal tumourigenesis.

Materials and Methods

Generation of conditional *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice

The human full length *GNAS* cDNA was synthesized to contain the codon change R201C. This cDNA was inserted into the *pGpa33*^{LSL} targeting vector (Fig. 1; ref (Orner et al 2002) and targeted to the *Gpa33* locus in C57BL/6J Bruce4 ES cells. Correctly targeted clones were identified by Southern blotting (probe primers: F- TGAGTTAGGGCTGCTTGCTT and R- ATGGGTTCTGAGGATGATGC) on *Pst*I digested genomic DNA.

Mice

Targeted ES cells were injected into albino C57BL/6J bastocysts and chimeras bred to C57BL/6J mice for germline transmission. All procedures were carried out in accordance with Home Office guidelines. Conditional *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ and *CMV-Cre*$^{-/-}$ mice (Su et al 2002) were crossed to produce *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ and *Gpa33*$^{+/+}$ littermates (heterozygous for CMV-*Cre*) for phenotyping. *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ *Cre*$^{+/-}$ mice were crossed with *Apc*$^{Min/+}$ mice to produce *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$*Apc*$^{Min/+}$ mice and littermate controls. Mice were genotyped by PCR for the *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ allele using the primers: 1-CGAGGGAGGGCTAACTTTCT 2-AAGAAGTGCTCCACCAATGC and 3-CGTCCTGACCTCTGGAATCT, which when multiplexed detect the targeted and wildtype alleles of *Gpa33*. Expression of *GNAS* R201C was confirmed by RT-PCR using primers F-ACATCACCGTGGCACCCAGACCTCCCTC and R-ATCTTTTTGTTGGCCTCACG. Mice were genotyped by PCR for *Apc*^{Min} using standard methods (Dietrich et al 1993). For assessment of adenoma formation the small and large (colon and rectum) intestines were collected. The small intestine was subdivided into three equal segments - denominated duodenum, jejunum, and ileum. All were examined macroscopically for number, size and location of adenomas, and embedded in paraffin wax.

Flow cytometric analysis of cAMP levels

The intestinal epithelial cells were scraped away from the muscular wall of the ileum (n= 7 *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$and n= 5 *Gpa33*$^{+/+}$) using a scalpel and sieved (70 μm) with ice-cold 4% formaldehyde in Ca^{2+}- and Mg^{2+}-free PBS. The cells were fixed for 10 min, washed in PBS, resuspended in PBS containing 0.2% Tween-20 (Sigma, Gillingham, UK) for 10 min, washed again and resuspended in 1 ml 3% BSA (Sigma: A3803). Two μg/ml anti-cAMP antibody (Abcam, Cambridge, MA, USA: ab24851) was added to test samples and incubated for 3 h. The cells were washed and incubated in 3% BSA containing 40 μg/ml FITC-IgG (Abcam: ab6785) for 1 h. The median fluorescence was determined by flow cytometry (FC-500, Beckman Coulter, High Wycombe, UK) and data were analysed using FlowJo (v7). Each test sample was normalised to the corresponding no primary antibody control and data were statistically analysed using two-tailed Student's *t*-test.

Immunohistochemistry and immunoblotting

Immunostaining was performed using the DAKO Autostainer Plus with the rabbit VECTASTAIN ELITE ABC horseradish peroxidase kit (Vector Laboratories, Burlingame, CA, USA). Primary antibodies: anti-Ki67 (DCS - Innovative Diagnostik-Systeme, Hamburg, Germany), anti-chromogranin A (Abcam: ab15160), and anti-phospho-Thr202/Tyr204 p44/42 ERK1/2 MAPK (Cell Signalling Technology, Danvers, MA, USA: 20G11). Goblet

cells were counted from Alcian blue stained sections. Paneth cells, mitotic cells and apoptotic fragments were counted from H&E stained sections. In all cases, a total of 30 crypts/villus were counted from the small intestine of 6 *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$and 6 *Gpa33*$^{+/+}$ mice and compared using the Mann–Whitney U test. Western blotting was performed using standard methods. Primary antibodies: anti-p44/42 ERK1/2 MAPK (Cell Signalling Technology: 137F5) and anti-phospho-Thr202/Tyr204 p44/42 ERK1/2 MAPK. Band density was quantified using Image J software (NIH), normalized against β-Actin and analysed using two-tailed Student's *t*-test.

Quantitative RT-PCR (qRT-PCR)

Epithelial cells of the normal intestinal mucosa were scraped away from the muscular wall of the small intestine (n=11 of each genotype) using a scalpel, snap frozen in liquid nitrogen and total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA). Polyps of 3 mm in diameter were dissected away from the muscular wall of the small intestine (n=6 of each genotype), snap frozen in liquid nitrogen and total RNA was isolated using the RNeasy Mini Kit (Qiagen, UK). RNA was DNase treated using Turbo DNase (Ambion, Austin, TX, USA) and 1-2 μg total RNA was reverse transcribed using the BD Sprint kit with random hexamers (ABI, Foster City, CA, USA) according to the manufacturers' protocols. Quantitative PCRs were performed with SYBR Green (ABI) on the ABI 7900HT sequence detection system in accordance with the manufacturer's instructions, using cDNA obtained from 20 ng total RNA (see Supplemental Table 1 for primer sequences). The final quantitation was determined relative to the average C_T of the house-keeping genes *Gapdh* and *Actb* (Livak and Schmittgen 2001). Data were statistically analysed using two-tailed Student's *t*-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: Work in the DJ Adams Laboratory and the MJ Arends Laboratory is funded by Cancer Research UK (CR-UK) and the Wellcome Trust. We thank Dr. Mattias Ernst for providing the *Gpa33* targeting vector.

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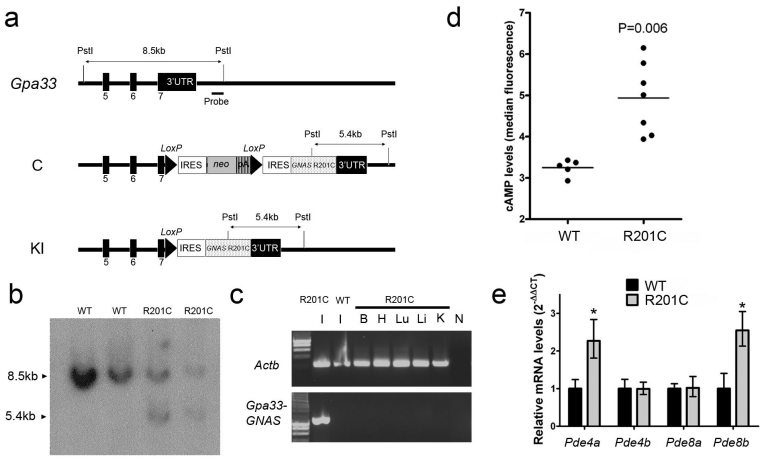

Figure 1.

Generation and validation of the intestine specific *GNAS* R201C allele. *A*, Schematic illustration of the *Gpa33* wildtype locus (*Gpa33*, upper panel), *Gpa33*$^{tm1(GnasR201C)Wtsi}$ conditional targeted locus (C, middle panel) and the *Gpa33*$^{tm1(GnasR201C)Wtsi}$ knock-in locus (KI, lower panel). Exons (numbered black rectangles), the positions of relevant *PstI* sites and the predicted sizes of fragments (thin lines with arrows) and probes (small black rectangle) are shown. *B*, Southern blot analysis of *PstI* digested DNA from targeted *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ (R201C) and control (WT) ES cells. *C*, RT-PCR expression analysis of *GNAS* R201C transcripts (*Gpa33-GNAS*) and β-Actin (*Actb*) expression in tissues taken from *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ (R201C) mice and wildtype littermate controls (WT). I-small intestine, B-brain, H-heart, Lu-lung, Li-liver, K-kidney and N-blank water control. *D*, Flow cytometric analysis of cAMP levels in intestinal epithelial cells. *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ (R201C) and wildtype (WT) tissues were analyzed. *E*, Quantitative RT-PCR analysis of cAMP-specific phosphodiesterases *4a, 4b, 7a, 8a* and *8b* in the intestine of *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ (R201C) and wildtype littermate control mice (WT). *P<0.05.

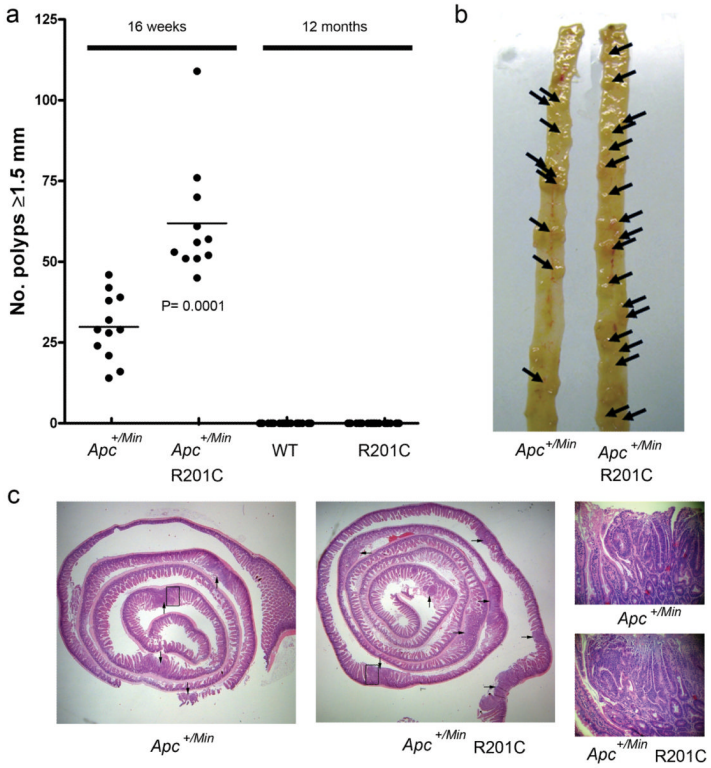

Figure 2.

Gpa33$^{tm1(GnasR201C)Wtsi}$ (R201C) promotes intestinal adenoma formation in *Apc*$^{Min/+}$ mice when compared to *Apc*$^{Min/+}$ controls but alone does not promote tumor formation. *A*, Macroscopic counts of intestinal tumours (≥1.5 mm in size) at 16 weeks in *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ *Apc*$^{Min/+}$ mice and *Apc*$^{Min/+}$ littermates. No tumours were found in *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ or wildtype littermate controls (WT) aged to 12 months. *B*, Representative photograph of small intestines from *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$*Apc*$^{Min/+}$ and *Apc*$^{Min/+}$ littermate (arrows indicate adenomas). *C*, Representative H&E stained section of a Swiss roll from an *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$*Apc*^{Min} and *Apc*$^{Min/+}$ littermate (arrows indicate adenomas, boxes enlarged to right). Histological analysis revealed no difference in the dysplastic grade of adenoma between *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$*Apc*^{Min} and *Apc*$^{Min/+}$ littermates.

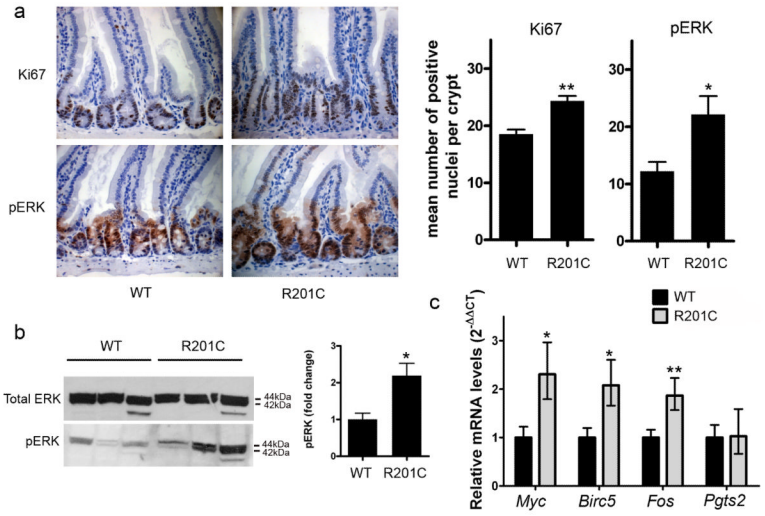

Figure 3.

Activation of Wnt and ERK1/2 MAPK pathways in the intestines of *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice. *A*, Immunohistochemical analysis of Ki67 and phosphorylated ERK1/2 MAPK showed an increase in the number of nuclei that stained positively within the crypt region of *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ mice (R201C) when compared to control mice littermate controls (WT). *B*, Western blot analysis of intestinal tissue from *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ (R201C) and littermate controls mice (WT) showed an increase in phosphorylated ERK1/2 MAPK . *C*, Quantitative RT-PCR analysis of *Myc, Birc5, Fos* and *Pgts2* in the intestine of *Gpa33*$^{tm1(GnasR201C)Wtsi/+}$ (R201C) And littermate control mice (WT).

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