Mechanisms of inactivation of the tumour suppressor gene RHOA in colorectal cancer

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Background: Reduced RHOA signalling has been shown to increase the growth/metastatic potential of colorectal tumours. However, the mechanisms of inactivation of RHOA signalling in colon cancer have not been characterised.

Methods: A panel of colorectal cancer cell lines and large cohorts of primary tumours were used to investigate the expression and activity of RHOA, as well as the presence of RHOA mutations/deletions and promoter methylation affecting RHOA. Changes in RHOA expression were assessed by western blotting and qPCR after modulation of microRNAs, SMAD4 and c-MYC.

Results: We show here that RHOA point mutations and promoter hypermethylation do not significantly contribute to the large variability of RHOA expression observed among colorectal tumours. However, RHOA copy number loss was observed in 16% of colorectal tumours and this was associated with reduced RHOA expression. Moreover, we show that miR-200a/b/429 downregulates RHOA in colorectal cancer cells. In addition, we found that TGF-β/SMAD4 upregulates the RHOA promoter. Conversely, RHOA expression is transcriptionally downregulated by canonical Wnt signalling through the Wnt target gene c-MYC that interferes with the binding of SP1 to the RHOA promoter in colon cancer cells.

Conclusions: We demonstrate a complex pattern of inactivation of the tumour suppressor gene RHOA in colon cancer cells through genetic, transcriptional and post-transcriptional mechanisms.

The small GTPases of the RHO family, including RHOA, RAC and CDC42, are molecular switches that cycle between a GTP-bound active form and a GDP inactive form, and participate in key aspects of the oncogenic process such as proliferation, polarisation, apoptosis, adhesion, migration, invasion and metastasis (Itoh et al, 1999; Sahai and Marshall, 2002). Activation of RHO proteins is most commonly reported to be oncogenic and has been shown to promote transformation of different cell types (Sahai and Marshall, 2002). However, we have recently shown that RHOA (Ras homologue family member A) has tumour suppressor activity in colorectal cancer and the loss of this GTPase significantly contributes to tumour progression and metastasis, largely through the activation of canonical Wnt signalling (Rodrigues et al, 2014) and low RHOA expression is associated with poor patient
Regulation of RHOA levels in CRC

prognosis (Arango et al, 2005). Tumour suppressor genes are inactivated during the tumourigenic process by a variety of mechanisms, including genetic and epigenetic alterations, overexpression of regulatory microRNAs or direct transcriptional silencing of the promoter, and the molecular mechanisms regulating the loss of RHOA expression and activity in colorectal cancer cells are not well understood.

Large exome/genome sequencing studies of colorectal tumours have demonstrated low mutation frequency of RHOA in colorectal tumours (Cancer Genome Atlas Network, 2012; Seshagiri et al, 2012). Consistent with the oncogenic activity of RHOA in most tumour types investigated, reduced promoter methylation levels associated with RHOA overexpression have been reported in different tumour types (Braga et al, 2015). In addition, RHOA levels have been shown to be regulated by miR-155, miR-340 and miR-200a/b/429 in endothelial cells (Bijkerk et al, 2012), melanocytes (Jian et al, 2014) and hepatocellular carcinoma (Wong et al, 2015), respectively. However, the regulation of RHOA by miRNAs in colorectal cancer cells has not been investigated in detail.

Activation of canonical Wnt signalling and inactivation of TGF-β signalling play key roles in the initiation and progression of colorectal tumours (Mishra et al, 2005). Both pathways convey extracellular signals from the membrane to the nucleus, regulating the transcription of multiple genes that participate in tumour growth and metastasis. Canonical Wnt signalling is a master regulator of proliferation and differentiation in normal intestinal epithelial cells and colon cancer cells (Mishra et al, 2005). Almost 80–90% of colorectal tumours have hyperactive Wnt signalling, most frequently because of inactivating mutations in APC that leads to the nuclear accumulation of β-catenin and the transcriptional activation of multiple targets, such as c-MYC and Cyclin D1 (Bienz and Clevers, 2000). On the other hand, TGF/SMAD signalling, an important regulator of cell growth and differentiation, is initiated by TGF-β superfamily ligands binding to type II TGF-β receptors, leading to the activation of SMAD intracellular mediators that translocate then into the nucleus, where they regulate transcription (Moustakas et al, 2001). TGF-β signalling is frequently silenced in colorectal tumours through different mechanisms, such as mutations in TGFBR2 or SMAD4 (Parsons et al, 1995; Eppert et al, 1996; Alazzouzi et al, 2005).

In this study, we found that multiple mechanisms contribute to the inactivation of RHOA signalling in colorectal cancer cells. The loss of the chromosomal region containing RHOA (3p21) was associated with reduced expression of this GTPase. Moreover, miR-200a/b/429 was found to inhibit the expression of RHOA in colorectal cancer cells. Importantly, we also demonstrate that transcriptional downregulation of RHOA through the TGF-β and Wnt signalling pathways is an important mechanism contributing to the inactivation of RHOA signalling.

Western blot and RHOA activity determination. The GTP-bound RHOA levels and western blotting were carried out as previously described (Rodrigues et al, 2014). The antibodies used are described in the Supplementary Methods.

CpG island methylation assays and microarray analysis. The DNA methylation status in the 1145 bp CpG island located in the promoter of RHOA was assessed on colon cancer cell lines by methylation-specific PCR (MSP-PCR), bisulphite sequencing and HumanMethylation27 Beadchips (Illumina, San Diego, CA, USA). Relative mRNA levels were determined by microarray analysis (HG-U133 Plus 2.0 chips; Affymetrix, Santa Clara, CA, USA) as previously reported (Bazzocco et al, 2015).

Animal experiments. C57BL/6J-ApcMin/J mice were obtained from The Jackson Laboratory (Stock No: 002020, Bar Harbor, ME, USA). All animal experiments were carried out under protocols approved by the Vall d’Hebron Ethical Committee for Animal Experimentation.

Immunohistochemistry and quantitative RT–PCR. The expression of RHOA and SMAD4 in Dukes’ C colorectal tumours have been reported before (Alazzouzi et al, 2005; Arango et al, 2005). Total RNA was reverse transcribed and relative levels of the indicated genes were assessed by real-time PCR using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). All primers used are detailed in the Supplementary Methods.

ChIP assay. The RKO cells were treated for 24 h with 1 μM JQ1 (or vehicle DMSO) and fixed with formaldehyde (Thermo Scientific, Waltham, MA, USA). Cell pellets were resuspended in nuclei lysis buffer and sonicated with a Bioruptor instrument (Diagenode, Liege, Belgium). Then, 200 μg of the cleared supernatant was immunoprecipitated with anti-SP1 antibody (Millipore 07-645, Burlington, MA, USA) or unspecific rabbit IgG. After washing, samples were treated with proteinase K and DNA recovered (Qiagen PCR product purification kit, Hilden, Germany). Relative levels of RHOA, p21 and a negative control (gene desert in chromosome 12) were quantified by real-time PCR as detailed in the Supplementary Methods.

RESULTS

RHOA expression in colorectal tumours. The expression of the small GTPase RHOA is frequently downregulated (Supplementary Figure 1A) and contributes significantly to the progression of colorectal cancer (Rodrigues et al, 2014). To investigate the mechanisms regulating the expression of RHOA in colorectal cancer cells, we first assessed the RHOA mRNA levels in a panel of 59 colorectal cancer cell lines using RNA sequencing (Mouradov et al, 2014). Significant variability in RHOA expression was observed (Supplementary Figure 2A and Supplementary Table 1) and the mRNA expression levels in a subset of 34 of these cell lines was confirmed by microarray analysis (Bazzocco et al, 2015) (Supplementary Figure 2B; Pearson’s r = 0.52 and P = 0.0018). The levels of RHOA protein were then assessed in a subset of 18 colon cancer cell lines using western blot analysis, confirming a wide range of RHOA expression in colorectal cancer cell lines also at the protein level (Supplementary Figure 2C and Supplementary Table 1). The range of expression in primary colorectal tumours and cell line models was comparable (Supplementary Figure 1B–G). A significant correlation between the levels of RHOA mRNA and protein was observed in colorectal cancer cells (Supplementary Figure 2D; Pearson’s r = 0.6 and P = 0.018). We then used a rhodin pull-down assay to assess the levels of active RHOA in 18 colorectal cancer cell lines (Supplementary Figure 2C) and observed a significant correlation between total RHOA expression and GTP-bound active RHOA.

MATERIALS AND METHODS

Cell lines and plasmids. The LS174T/W4, LS174T/dnTCF4, LS174T/siJCAT, SW480/ADH, HCT8/S11 and HCT116-SMAD4−/− were kindly provided by other researchers, as detailed in the Supplementary Methods. Other cell lines used in this study (LIM2405, HCT116, RKO, SK-CO-1, SW837 and HEK293T) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All plasmids used are described in the Supplementary Methods.

Luciferase reporter assays. Renilla luciferase (pRL-TK; Promega, Madison, WI, USA) was used as a control for transfection efficiency in luciferase reporter assays using the Dual-Luciferase Assay Kit (Promega).

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Regulation of RHOA levels in CRC

Genetic inactivation of RHOA in colorectal tumours. Previous studies have shown that RHOA is not frequently mutated in colorectal tumours (Arango et al, 2005). Meta-analysis of RHOA mutation frequencies in 983 colorectal primary tumours (Cancer Genome Atlas Network, 2012; Seshagiri et al, 2012; Brannon et al, 2014; Giannakis et al, 2016) showed that only 1.0% (10 out of 983) of colorectal tumours have RHOA mutations and that these are randomly distributed throughout its coding sequence (Supplementary Figure 3). Therefore, the low incidence of mutations observed in RHOA cannot explain the large variability of RHOA levels observed in colorectal tumours. However, RHOA copy number losses in chromosome 3p21 affecting RHOA were observed in 16% (60 of 376) of colorectal tumours from The Cancer Genome Atlas (TCGA) initiative (Cancer Genome Atlas Network, 2012) and this was associated with reduced RHOA expression (Figure 1A and Supplementary Table 2). In good agreement, we observed RHOA losses in 13.8% (4 out of 30) of colorectal cancer cell lines, and this was associated with significantly lower RHOA mRNA expression (Figure 1B and Supplementary Table 1). Therefore, deletions of chromosome 3p21 affecting RHOA could account for the reduced levels of RHOA observed in a subset of colorectal tumours.

Epigenetic inactivation of RHOA in colorectal tumours. Hyper-methylation of promoter CpG islands in the promoter region of RHOA was demonstrated regulation of RHOA expression in colorectal tumours. Previous studies in other tumour types have demonstrated regulation of RHOA expression by microRNAs.

![Image](https://example.com/image.png)

Figure 1. Genetic and epigenetic alterations of RHOA in colorectal tumours. (A and B) Scatter dot plot (and mean ± s.e.m.) showing RHOA mRNA levels as a function of RHOA copy number in 376 primary colorectal tumours (TCGA) and 30 cell lines (B). (C) Results of bisulphite sequencing in 12 colon cancer cell lines. IVD: in vitro methylated DNA used as control. (D) Correlation between mRNA RHOA expression (RNAseq) and the average methylation of all probes in the RHOA promoter in the HumanMethylation450 arrays in a cohort of 370 primary colorectal tumours (TCGA). (E) Correlation between mRNA expression (microarray) and average RHOA promoter methylation (HumanMethylation27) in 30 colorectal cancer cell lines. Student's t-test *P<0.05; ***P<0.0001. A full colour version of this figure is available at the British Journal of Cancer journal online.
Regulation of RHOA levels in CRC

Transcriptional regulation of RHOA in colorectal tumours. Detailed in *silico* analysis predicted evolutionarily conserved binding sites for 14 of these miRNAs (Supplementary Table 3). Importantly, the expression of 12 of these 14 miRNAs was significantly elevated in colorectal tumours compared with the normal colonic mucosa (Supplementary Table 3), suggesting that different miRNAs may contribute to the downregulation of RHOA observed in colorectal tumours.

Of these 14 miRNAs, *miR-200b* and *miR-429*, which are located in the *miR-200a/b/429* cluster on chromosome 1 and are transcribed together, showed the highest homology to HOMA (TargetScan), and are highly conserved from Xenopus to humans (Betel *et al.*, 2010). To investigate the possible regulation of RHOA by *miR-200a/b/429*, we transiently co-transfected the *miR-200a/b/429* genomic cluster along with a reporter construct expressing luciferase fused to the RHOA 3'-UTR that was either wild type or mutant for the putative *miR-200a/b/429* binding site. LIM2405 colon cancer cells were used for these experiments as they express low endogenous levels of *miR-200a/b/429* (Supplementary Figure 7A). Overexpression of *miR-200a/b/429* significantly reduced luciferase reporter activity in the cells transfected with the wild-type RHOA 3'-UTR construct compared with the mutant construct (Figure 2A), providing evidence of direct regulation of RHOA expression by these microRNAs. Moreover, stable overexpression of the *miR-200a/b/429* clusters in LIM2405 cells significantly reduced the endogenous miRNA levels of RHOA, as well as ZEB1, a known target of *miR-200a/b/429* (Hur *et al.*, 2013) (Figure 2B–D and Supplementary Figure 7B). Collectively, these results demonstrate that the *miR-200a/b/429* cluster significantly contributes to the downregulation of RHOA observed in colorectal cancer cells. Moreover, we identified additional microRNAs that could further modulate the levels of RHOA in colorectal cancer cells (Supplementary Table 3).

Transcriptional regulation of RHOA in colorectal tumours. Regulation of the activity of the promoter is a key mechanism controlling gene expression. To assess the extent to which RHOA expression is regulated at the transcriptional level we utilised a reporter construct containing the 2 kb promoter region immediately upstream of the RHOA transcription start site driving the expression of luciferase (Chan *et al.*, 2010). Transient transfection of this reporter construct in 14 colon cancer cell lines revealed significantly higher RHOA promoter activity in cell lines with high endogenous RHOA mRNA expression (Figure 3A), suggesting that regulation of the transcriptional activity of the RHOA promoter is an important mechanism determining the levels of RHOA expression in colorectal cancer cells.

Detailed *silico* analysis of the proximal RHOA promoter identified the presence of potential binding sites for at least 57 transcription factors (Supplementary Table 4). To identify physiological regulators of RHOA transcription in colon cancer cells, we correlated the levels of RHOA mRNA with the expression of these transcription factors assessed by RNAseq in a cohort of 244 primary colorectal tumours (TCGA; Supplementary Table 2) (Cancer Genome Atlas Network, 2012). Of the 57 transcription factors with putative binding motifs in the RHOA promoter, the expression of 44 (77.2%) of them was significantly correlated with the expression of RHOA (Supplementary Table 4), suggesting that they may directly regulate RHOA expression in colorectal tumours. Notably, these included binding sites for SMAD4 and c-MYC that are key effectors of the TGF-β and Wnt signalling, two of the most commonly deregulated pathways in colorectal cancer.

Transcriptional regulation of RHOA by TGF-β signalling in colorectal cancer cells. SMAD4 is a transcription factor regulated by the TGF-β pathway and has important tumour suppressor activity in colorectal tumours (Howe *et al.*, 1998; Alazzouzi *et al.*, 2005). SMAD4 mRNA levels showed a significant positive correlation with RHOA mRNA levels in 244 primary colorectal tumours (Supplementary Tables 2 and 4, Figure 3B; Pearson’s r = 0.47; P = 7.97 × 10⁻¹¹). Moreover, quantification of the levels of RHOA (Arango *et al.*, 2005) and SMAD4 (Alazzouzi *et al.*, 2005; Alhoupou *et al.*, 2005) protein by immunohistochemistry in a tissue microarray containing triplicate samples of 162 primary colorectal tumours confirmed a significant association between RHOA and SMAD4 expression (Pearson’s r = 0.48; P = 7.8 × 10⁻¹¹; Figure 3C), further suggesting an important role for SMAD4 in the regulation of RHOA expression in these tumours.

To investigate whether TGF-β signalling functionally regulates the expression of RHOA, SKCO1 colon cancer cells, which have a functional TGF-β signalling pathway (Brunen *et al.*, 2013), were stimulated with TGF-β1. As expected, TGF-β1 resulted in increased SMAD4 transactivation activity (Figure 3D). In addition, TGF-β1 enhanced RHOA promoter activity (Figure 3D) and elevated RHOA mRNA and protein expression (Figure 3E and F). SMAD4 has previously been shown to bind to the RHOA promoter in human embryonic stem cells (Tsankov *et al.*, 2015). Importantly, targeted inactivation of SMAD4 in HCT116 and SKCO1 colon cancer cells resulted in reduced RHOA mRNA levels (Figure 3G and H). These results collectively indicate that RHOA is transcriptional target of SMAD4 in colon cancer cells.

Transcriptional regulation of RHOA by Wnt signalling in colorectal cancer cells. Canonical Wnt signalling is a master regulator of proliferation and differentiation of both normal intestinal epithelial cells and colon cancer cells (MacDonald *et al.*, 2009). We therefore investigated whether Wnt/β-catenin also regulates RHOA expression using LS174T cells where TCF4/β-catenin signalling can be inactivated by doxycycline-dependent overexpression of a dominant negative form of TCF4 (LS174T-dnTCF4) or an shRNA targeting β-catenin (LS174T-shBCAT) (Van de Wetering *et al.*, 2002). As expected, doxycycline treatment of both LS174T-dnTCF4 and LS174T-shBCAT cells significantly reduced the transcriptional activity of TCF4/β-catenin as assessed using a luciferase reporter construct (Supplementary Figure 8A and B). Importantly, this decrease in TCF4/β-catenin activity was associated with increased promoter activity (Figure 4A), mRNA expression (Figure 4B) and protein levels and activity (Figure 4C) of RHOA. The transcriptional regulation of RHOA by TCF4/β-catenin was further confirmed by transient transfection of dominant negative TCF4 in four additional colon cancer cell lines (SW837, HCT8/S11, SW480/ADH and LS174T/W4; Figure 4D and Supplementary Figure 8C).

In addition, we used a mouse model of intestinal tumourigenesis carrying heterozygous germline Apc mutations (*Apcmin* model) (Moser *et al.*, 1990) to assess the effects of Wnt pathway activation on RHOA levels in vivo. Heterozygous Apc mutations in normal intestinal epithelial cells did not affect the levels of RHOA protein (Figure 4E and F and Supplementary Figure 8D–F). However, the sporadic loss of the wild-type allele in these cells results in hyperactivation of Wnt/β-catenin and the formation of intestinal tumours (Luongo *et al.*, 1994; Sansom *et al.*, 2004) and reduced RHOA expression was observed in the intestinal tumours of *Apcmin* mice compared with the normal intestinal epithelium of either wild-type or *Apcmin* mice (Figure 4E and F and Supplementary Figure 8D–F). Collectively, these results indicate that RHOA expression is repressed by aberrant Wnt signalling in colorectal cancer cells.

Transcriptional regulation of RHOA by c-MYC in colorectal cancer cells. Because the promoter region of RHOA used for these experiments does not contain binding sites for the TCF4/β-catenin transcriptional complex, the effects of Wnt signalling on the
The c-MYC is an important transcriptional target of TCF4/β-catenin and has been reported to regulate the expression of RHOA in prostate and breast cancer cells (Chan et al., 2010). Moreover, c-MYC and RHOA mRNA expression were negatively correlated in a cohort of 244 primary colorectal tumours (Supplementary Table 4).

We have previously shown that RHOA negatively regulates Wnt/c-MYC signalling in the colon cancer cells lines SW480/ADH, HCT8/S11, LS174T/W4 and SW837. To investigate the possible reciprocal regulation of the RHOA promoter by c-MYC in colon cancer cells, we assessed the effects of ectopic expression of c-MYC in the same cell lines as well as in HEK293T human embryonic kidney cells. c-MYC can enhance the promoter activity of multiple targets, such as CDK4, through its binding to E-boxes or inhibit the promoter activity of other target genes, such as p21CIP1/WAF1 through alternative mechanisms (Seoane et al., 2002). As expected, overexpression of c-MYC led to a significant activation of the CDK4 promoter and reduced the activity of the p21CIP1/WAF1 promoter (Figure 5A and B). In addition, and consistent with previous reports (Chan et al., 2010), c-MYC overexpression in HEK293T cells led to a significant increase in the transcription of RHOA (Figure 5C). However, ectopic expression of c-MYC in four different colon cancer cell lines (SW837, HCT8/S11, SW480/ADH and LS174T/W4) resulted in decreased RHOA promoter activity (Figure 5C).

To further confirm the regulation of RHOA expression by c-MYC, we downregulated c-MYC levels either using c-MYC-specific shRNA (Figure 5D and E) or treatment with the BET bromodomain inhibitor JQ1 (Figure 5F and G) that has previously been shown to reduce the expression of c-MYC (Delmore et al., 2011; Tögel et al., 2016). Transient shMYC transfection resulted in increased activity of the RHOA promoter in all four colon cancer cell lines (Figure 5E). In addition, JQ1 treatment led to increased mRNA and protein levels of RHOA as well as p21CIP1/WAF1 that was used as a positive control (Cheng et al., 2013) (Figure 5F and G). Collectively, these results demonstrate that c-MYC negatively regulates the activity of the promoter of RHOA and leads to reduced RHOA mRNA expression in colorectal cancer cells.

Figure 2. The RHOA expression is regulated by miR-200a/b/429 in colorectal cancer cells. (A) The regulation of RHOA levels by miR-200a/b/429 was assessed using a transient transfection assay with a luciferase reporter gene fused to the 3′-UTR of RHOA containing the wild-type (wt) or a mutant (Mut) miR-200a/b/429 binding site, co-transfected with a vector expressing miR-200a/b/429 or the corresponding empty vector (EV) in LIM2405 colon cancer cells. (B) Stable overexpression of miR-200a/b/429 in LIM2405 colon cancer cells resulted in significant downregulation of the endogenous RHOA mRNA, as well as the known miR-200a/b/429 target gene ZEB1. (C and D) Representative western blot (C) and the mean of three independent experiments (D) showing reduced RHOA protein expression in LIM2405 cells with stable overexpression of miR-200a/b/429. All histograms show the average (± s.e.m.) of three independent experiments. Student’s t-test *P<0.05; **P<0.01. A full colour version of this figure is available at the British Journal of Cancer journal online.
Figure 3. The RHOA regulation by TGF-β/SMAD pathway. (A) Activity of the promoter of RHOA in 14 colorectal cancer cell lines with low and high RHOA expression. (B) Correlation between the expression of SMAD4 and RHOA mRNA levels (RNAseq) in 244 primary colorectal tumours (TCGA). (C) Correlation between the expression of SMAD4 and RHOA protein expression (immunohistochemistry) in a cohort of 162 primary Dukes’ C colorectal tumours. (D–F) Changes in RHOA promoter activity (D) (luciferase reporter assay), RHOA mRNA expression (E) (qPCR) and RHOA protein expression (F) (western blotting) after treatment of SKCO1 colon cancer cells with TGF-β1 for 24 h with the indicated concentrations. (G and H) The SMAD4 and RHOA mRNA expression (qPCR) in HCT116 knockout cells compared with control HCT116 cells (G) and after SMAD4 downregulation in SKCO1 cells using siRNA (H). The average (± s.e.m.) of three independent experiments each run in triplicate is shown in (A, D, E, G and H). Student’s t-test *P<0.05; **P<0.01; ***P<0.001. A full colour version of this figure is available at the British Journal of Cancer journal online.
c-MYC represses the RHOA promoter through its interactions with SP1. c-MYC has been shown to repress the promoter activity of target genes such as USF, C/EBPα or albumin through its binding to initiator elements (Li et al., 1994). However, detailed in silico analysis of the RHOA promoter did not identify the presence of any consensus initiator elements. Alternatively, c-MYC can repress the expression of some genes, such as p21CIP1/WAF1, through the sequestration of the SP1/SP3 complex (Gartel et al., 2001). Similar to the p21CIP1/WAF1 promoter, the proximal promoter of RHOA contains several SP1 binding sites (Figure 6A), and the levels of SP1 were significantly correlated with the expression of RHOA in a series of 244 primary colorectal tumours (Supplementary Tables 2 and 4; Pearson’s $r = 0.37; \, P = 5.8 \times 10^{-9}$). A chromatin immunoprecipitation (ChIP) assay demonstrated a significant enrichment in SP1-bound DNA fragments spanning the RHOA promoter region in untreated colon cancer cells compared with negative controls (compare blue bars with (DMSO IgG) and without (DMSO SP1) stripes for the RHOA promoter in Figure 6D), demonstrating SP1 binding to the RHOA promoter and further suggesting that SP1 is important for RHOA transcription. Treatment with the SP1 inhibitor mithramycin resulted in a significant reduction of the promoter activity and the expression of RHOA, as well as the positive control p21CIP1/WAF1 (Figure 6B and C), further indicating that SP1 transactivation is important to sustain RHOA expression in colon cancer cells.

Figure 4. The RHOA regulation by Wnt signalling. (A) Changes in RHOA promoter activity after TCF4/β-catenin inactivation following doxycycline exposure in LS174T/dnTCF4 and LS174T/shBCAT cells. (B) Expression of RHOA (qPCR) in LS174T/dnTCF4 and LS174T/shBCAT cells after TCF4/β-catenin inactivation. (C) Total and active RHOA in LS174T/dnTCF4 and LS174T/shBCAT cells after TCF4/β-catenin inactivation. The levels of TCF4, β-catenin and tubulin are also shown. (D) Changes in RHOA promoter activity in SW480/ADH, HCT8/S11, LS174T/W4 and SW837 colon cancer cells with transient overexpression of dnTCF4. (E) Western blot showing the relative expression of RhoA in the normal intestinal epithelial cells of control wild-type mice (WT), in the normal intestinal epithelial cells of Apcmin mice and in the intestinal tumours of Apcmin animals. Actin was used as a loading control. (F) Histogram showing the average (± s.e.m.) RhoA protein expression quantified from five different animals. The average (± s.e.m.) of three independent experiments each run in triplicate is shown in (A, B and D). Student’s t-test *$P<0.05$; **$P<0.01$. A full colour version of this figure is available at the British Journal of Cancer journal online.
SW837 colon cancer cells and kidney embryonic HEK293T cells with transient overexpression of c-MYC or the empty vector (EV) control construct.

**p21CIP1/WAF1**

SP1 binding to the

Here, we have carried out a comprehensive analysis of the possible mechanisms downregulating RHOA activity in colorectal tumours, and found that RHOA signalling is regulated by multiple mechanisms that result in a significant reduction of RHOA expression and activity in a high proportion of these tumours.

To investigate whether c-MYC downregulates the expression of RHOA by interfering with the capacity of SP1 to activate RHOA transcription, we assessed possible changes in the levels of SP1 bound to the RHOA promoter following c-MYC downregulation with JQ1. As a control (Gartel et al, 2001), we showed increased SP1 binding to the p21CIP1/WAF1 promoter after c-MYC down-regulation with JQ1 treatment using a ChIP assay (compare blue (DMSO SP1) and green (JQ1 SP1) bars without stripes for the RHOA promoter in Figure 6D), Importantly, reduced c-MYC levels after JQ1 treatment resulted in a significant increase in SP1 bound to the RHOA promoter (compare blue (DMSO SP1) and green (JQ1 SP1) bars without stripes for the RHOA promoter in Figure 6D), consistent with the notion that c-MYC downregulates RHOA levels in colon cancer cells by reducing SP1 binding to the RHOA proximal promoter.

**DISCUSSION**

RHOA is a small GTPase that in normal epithelial cells functions as an important regulator of cytoskeleton structure, differentiation, polarisation, cell adhesion and motility, and is activated in most tumour types where it has been studied, mostly through its overexpression (Itoh et al, 1999; Sahai and Marshall, 2002). In contrast, we have previously shown that RHOA expression is frequently lost or reduced in colorectal tumours and that low levels of expression are associated with poor patient prognosis (Arango et al, 2005), probably because of context-dependent differences in RHOA signalling. Moreover, we have recently reported that the loss of RHOA is an important driver of colorectal cancer progression and metastasis (Rodrigues et al, 2014). However, the underlying molecular mechanisms responsible for RHOA inactivation in colorectal tumours have not been previously investigated. Here, we have carried out a comprehensive analysis of the possible mechanisms downregulating RHOA activity in colorectal tumours, and found that RHOA signalling is regulated by multiple mechanisms that result in a significant reduction of RHOA expression and activity in a high proportion of these tumours.

The RHO GTPases function as molecular switches that cycle between a GTP-bound active state and a GDP-bound inactive state. Here we found that although the levels of active RHOA-GTP are determined to a large extent by the transcriptional activity of the RHOA promoter, although other layers of regulation further fine-tune RHOA activity on colorectal tumours.

Whereas frequent recurrent RHOA mutations have been reported in subtypes of T-cell leukaemia/lymphoma (Palomero et al, 2001), we showed increased RHOA expression and activity in a high proportion of colorectal tumours, and found that RHOA signalling is regulated by multiple mechanisms that result in a significant reduction of RHOA expression and activity in a high proportion of these tumours.
et al., 2014) and gastric tumours (Zhou et al., 2014), RHOA mutations are not frequent (1.0%; 10 out of 983) in colorectal tumours, indicating that this is not a common mechanism regulating RHOA signalling in this tumour type. However, RHOA is located on the short arm of chromosome 3 (3p21.3), a region frequently deleted in colorectal tumours (Mehus et al., 1999) and that is known to contain several candidate tumour suppressor genes, including NPRL2 (Yogurtcu et al., 2012) and RASSF1 (Fernandes et al., 2013). Here we show that copy number losses in 3p21.3 are associated with a significant reduction in RHOA expression, suggesting that these deletions could target RHOA among other genes in this region.

We show here that despite the presence of a dense CpG island in the promoter of RHOA, uniformly low levels of methylation were observed in this region in primary colorectal tumours and cell lines, and no associations were observed with RHOA mRNA expression, indicating that aberrant promoter hypermethylation does not significantly contribute to RHOA silencing in colorectal cancer. Interestingly, the transcription start site (TSS) of RHOA is located only three base pairs away from the TSS of TCTA (T-cell leukaemia translocation altered), a gene that is transcribed in the opposite direction. The TSSs of both RHOA and TCTA are contained within the CpG island investigated here and likely contribute to the regulation of the expression of both genes. As previous studies have shown that TCTA may have an oncogenic function, this could explain the absence of methylation in this CpG island spanning the TSS of RHOA and TCTA, even in tumours with CpG island methylator phenotype (CIMP).

Using genome-wide transcriptomic analysis of both mRNA and miRNA, we identified 12 miRNAs whose expression shows a significant negative correlation with RHOA mRNA levels, have putative binding sites in the 3’-UTR of RHOA and are significantly overexpressed in colorectal tumours compared with the normal colonic mucosa. As expected, some of these miRNAs, including miR-183 (Bi et al., 2016), miR-20a (Cheng et al., 2016) and miR-185 (Liu et al., 2011), have previously been shown to directly regulate RHOA levels and/or to be involved in colorectal carcinogenesis. Here, we further investigated the possible role of miR-200a/b/429 on the regulation of RHOA expression in colorectal cancer.

Figure 6. The c-MYC regulates RHOA levels through SP1. (A) Schematic representation of the RHOA and p21CIP1/WAF1 promoters showing the localisation of SP1-binding sites and the primers used for qPCR after chromatin immunoprecipitation with an anti-SP1 antibody. (B and C) Effects of Mithramycin A treatment on the promoter activity (B) and the mRNA expression (C) of RHOA and p21CIP1/WAF1. (D) Results of a ChIP assay showing relative levels of SP1 bound to the RHOA promoter in RKO cells after treatment with JQ1 or control vehicle (DMSO). p21CIP1/WAF1 was used as a SP1-binding positive control and gene desert in chromosome 12 (chr12:61 667 747–61 667 824) as negative control. Nonspecific polyclonal IgG was used as a negative control for the ChIP assay. The average (± s.e.m.) of three independent experiments run in triplicate is shown for (B–D). Student’s t-test *P<0.05; **P<0.01; ***P<0.001. A full colour version of this figure is available at the British Journal of Cancer journal online.
cells. The miR-200 family is located in two genomic clusters, miR-200a/b/429 on chromosome 1 and miR-200c/141 on chromosome 12. We found that miR-200a/b/429 overexpression significantly reduced the levels of RHOA expression in LIM2405 colon cancer cells. These results are in good agreement with a recent report where miR-200b was found to reduce RHOA expression in hepatocellular cells (Wong et al., 2015).

Although the function of a protein can be post-transcriptionally modulated, transcriptional control at the promoter level represents a major mechanism of gene expression regulation. Here, we identified a set of 44 transcription factors with evolutionarily conserved binding sites in the promoter of RHOA, whose expression is significantly correlated with RHOA expression in a cohort of 244 colorectal tumours, suggesting that they may be important for the transcriptional regulation of RHOA. Two of these transcription factors, SMAD4 and c-MYC, are key regulators of the two most frequently deregulated signalling pathways in colorectal tumours, TGF-β and Wnt respectively, and we demonstrate here that they are important for the regulation of RHOA levels in colon cancer cells.

Inactivation of the TGF-β signalling pathway is important for colorectal cancer progression, as illustrated by the frequent alterations observed in TGF-β receptors (Parsons et al., 1995) or the SMAD intracellular mediators such as SMAD4 inactivation (Alazzouzi et al., 2005). Although TGF-β signalling has been shown to activate RHOA in a SMAD-independent manner (Moustakas et al., 2001), we show here that SMAD4 inactivation leads to a 30–40% reduction of the levels of RHOA in colon cancer cells, suggesting that RHOA activation may significantly contribute to TGF-β-mediated tumour suppression in colorectal epithelial cells, although the specific RHOA-dependent mechanisms remain to be elucidated. Wnt signalling is a master regulator of proliferation and differentiation of the normal intestinal epithelium and colon cancer cells (MacDonald et al., 2009). Aberrant activation of Wnt signalling in colorectal tumours leads to the accumulation of nuclear β-catenin that binds to the transcription factor TCF4, driving the expression of multiple β-catenin/TCF4 target genes, and c-MYC has been shown to be an important determinant of the oncogenic effects of Wnt hyperactivation in colorectal tumours (Sansom et al., 2007). c-MYC positively regulates the expression of RHOA in glioblastoma, prostate and breast cancer cells as well as kidney embryonic cells through the direct binding to E-boxes in the RHOA promoter (Seoane et al., 2002; Si et al., 2010; Talamillo et al., 2017). Surprisingly, however, we found that in colon cancer cells SP1 is required for active transcription of RHOA, and that c-MYC interferes with the binding of SP1 to the RHOA promoter, thus downregulating RHOA expression, as previously shown for the CDK inhibitor p21CIP1/WAF1 (Gartel et al., 2001). Therefore, as we have recently shown that the loss of RHOA enhances Wnt signalling and significantly contributes to colon cancer progression and metastasis (Rodrigues et al., 2014), we describe here the existence of a positive cross-regulatory feedback loop, where reduced RHOA leads to enhanced Wnt/β-catenin signalling that in turn further reduces RHOA levels. Importantly, these data illustrate how the same transcription factor can regulate in opposite directions the transcription of the same gene in two different cancer cell types, highlighting the complexity of gene expression regulation in the human genome.

In conclusion, we demonstrate a complex pattern of inactivation of RHOA in colon cancer cells involving overlapping genetic, transcriptional and post-transcriptional mechanisms in colon cancer cells. Deletions affecting RHOA are associated with reduced RHOA expression and miR-200a/b/429 regulates the expression of this GTPase in colorectal cancer cells. In addition, we demonstrate that RHOA is regulated by the transcription factors SMAD4 and c-MYC, key mediators of the most commonly deregulated signalling pathways on colorectal cancer, TGF-β and Wnt respectively.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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