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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

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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.

MATERIALS AND METHODS

Cell lines and plasmids. The LS174T/W4, LS174T/dnTCF4, LS174T/siβCAT, 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).

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-*Apc*^{Min}/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 rhotekin 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

(Supplementary Figure 1E and Supplementary Table 1; Pearson's $r = 0.53$ and $P = 0.025$).

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. Hypermethylation of promoter CpG islands in the promoter region of tumour suppressor genes is an important mechanism silencing the expression of genes with tumour suppressor activity (Corté *et al*, 2012). Because the proximal promoter region of *RHOA* contains a CpG island spanning >1400 bp flanking its transcription start site (Supplementary Figure 4), we investigated whether methylation of this region was associated with loss of RHOA expression in colorectal tumours by determining the levels of *RHOA* promoter methylation in 30 colon cancer cell lines and using publicly available data from 370 primary colorectal tumours (Cancer Genome Atlas Network, 2012) (Supplementary Tables 1 and 2). Quantification of the levels of methylation of 16 CpG dinucleotides in the promoter of *RHOA* using HumanMethylation450 BeadChip arrays (Illumina) revealed low levels of methylation in colorectal tumours (Supplementary Figure 5A), and this finding was independently confirmed in colorectal cancer cell lines using either bisulphite sequencing of a region of 254 bp containing 15 CpGs flanking the RHOA transcription start site (Figure 1C and Supplementary Figure 6A) or methylation-specific PCR of the same region (Supplementary Figure 6B). Furthermore, no associations were observed between the levels of methylation and expression of RHOA in colorectal cancer cell lines and primary tumours (Figure 1D and E and Supplementary Figure 5B). These results indicate that *RHOA* promoter methylation does not regulate *RHOA* expression in colorectal tumours.

Post-transcriptional regulation of RHOA by miRNAs in colorectal tumours. Previous studies in other tumour types have demonstrated regulation of *RHOA* expression by microRNAs

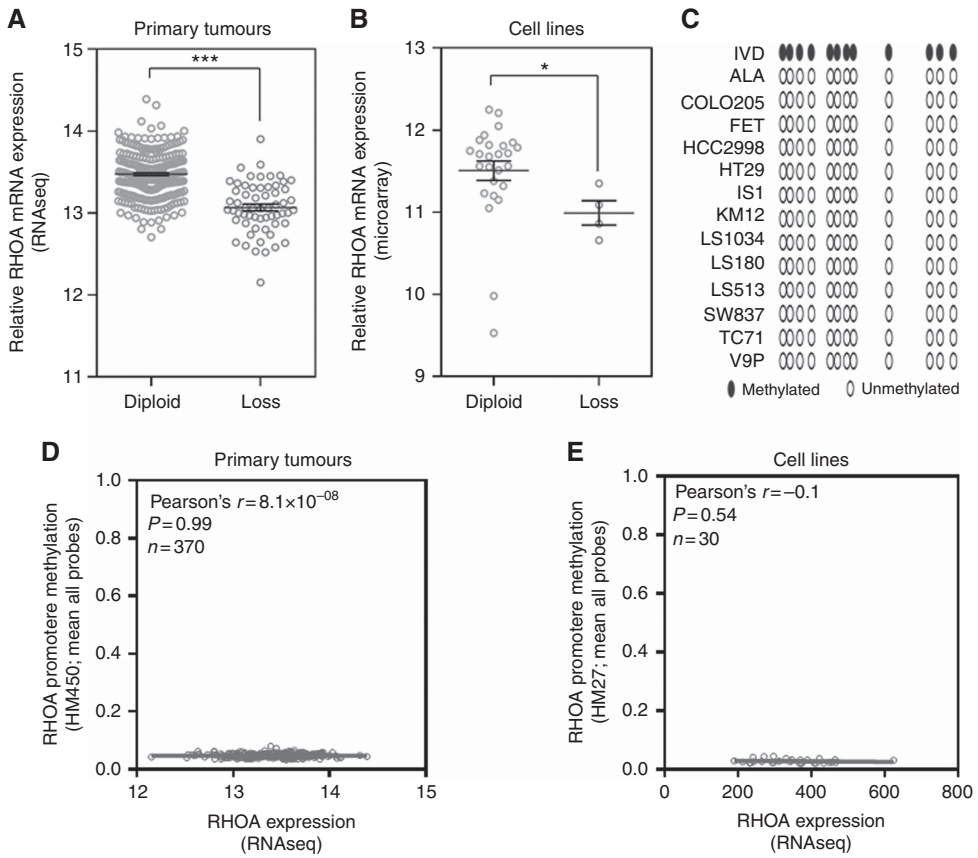

Figure 1. Genetic and epigenetic alterations of *RHOA* in colorectal tumours. (**A** and **B**) Scatter dot plot (and mean ± s.e.m.) showing *RHOA* mRNA levels as a function of *RHOA* copy number in 376 primary colorectal tumours (**A**) (TCGA) and 30 cell lines (**B**). (**C**) Results of bisulphite 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.

(Wong *et al*, 2015). To investigate this in colorectal cancer cells, we used a cohort of 195 primary colorectal tumours where the levels of mRNA and miRNA expression were determined by RNAseq (Cancer Genome Atlas Network, 2012) (Supplementary Table 2). This analysis identified 47 miRNAs whose expression was also significantly negatively correlated with *RHOA* expression (Supplementary Table 3). *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 RHOA (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 over-expression of the *miR-200a/b/429* cluster in LIM2405 cells significantly reduced the endogenous mRNA 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 *in 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 $r = 0.47$; $P = 7.97 \times 10^{-15}$). Moreover, quantification of the levels of RHOA (Arango *et al*, 2005) and SMAD4 (Alazzouzi *et al*, 2005; Alhopuro *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 $r = 0.48$; $P = 7.8 \times 10^{-11}$; 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 (Apc^{min} 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 Apc^{min} mice compared with the normal intestinal epithelium of either wild-type or Apc^{min} 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

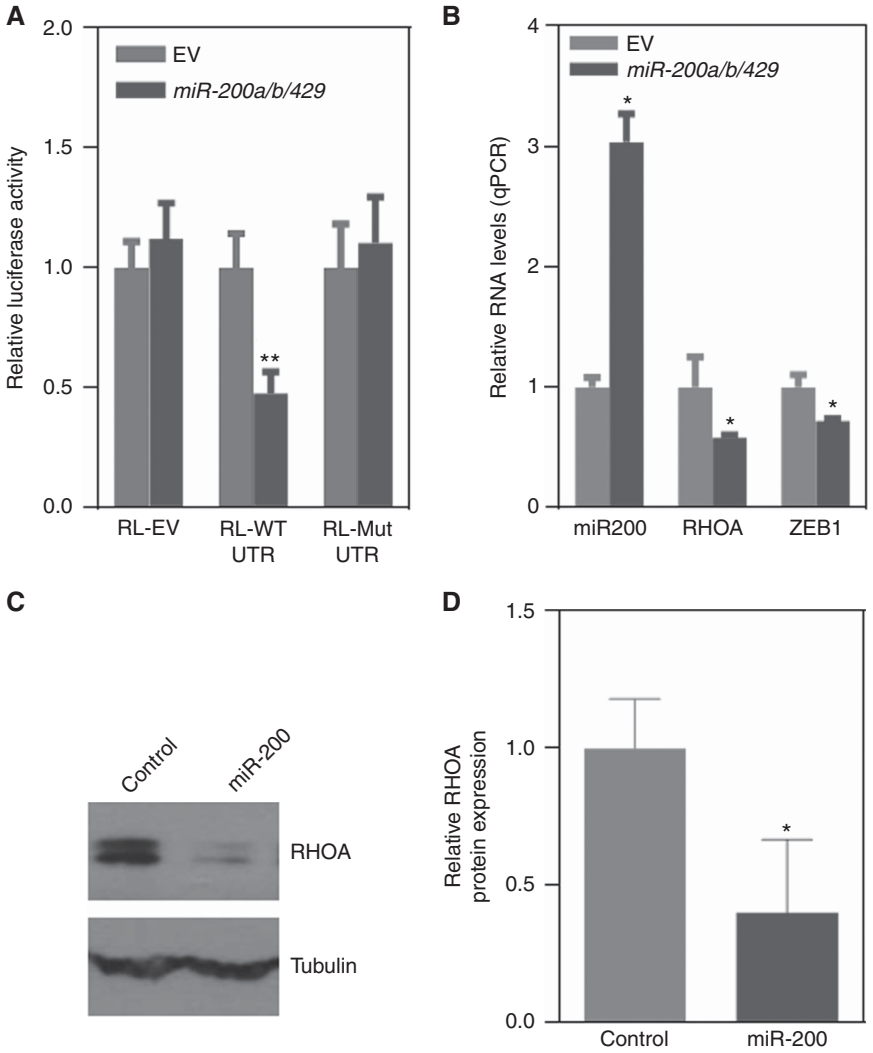

Figure 2. The *RHOA* expression is regulated by *miR-200a/b/429* in colorectal cancer cells. (**A**) The regulation of *RHOA* levels by *miR-200a/b/429* was assessed using a transient transfection assay with a luciferase reporter gene fused to the 3′-UTR of *RHOA* containing the wild-type (wt) or a mutant (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.

RHOA promoter are likely to be indirect. 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 $p21^{CIP1/WAF1}$ 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 $p21^{CIP1/WAF1}$ 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 $p21^{CIP1/WAF1}$ 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.

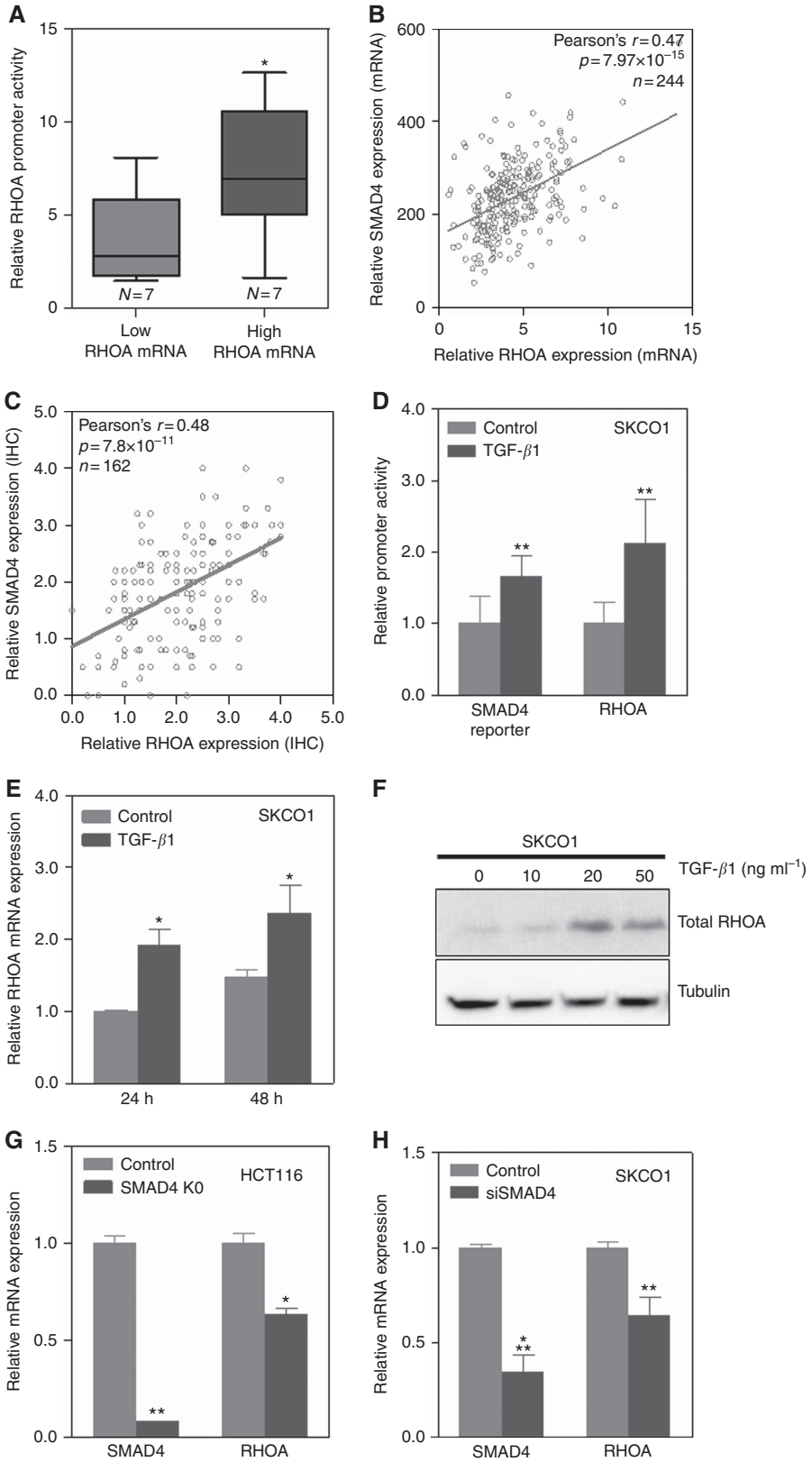

Figure 3. The *RHOA* regulation by TGF-β/SMAD pathway. (**A**) Activity of the promoter of *RHOA* in 14 colorectal cancer cell lines with low and high *RHOA* expression. (**B**) Correlation between the expression of *SMAD4* and *RHOA* mRNA levels (RNAseq) in 244 primary colorectal tumours (TCGA). (**C**) 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.

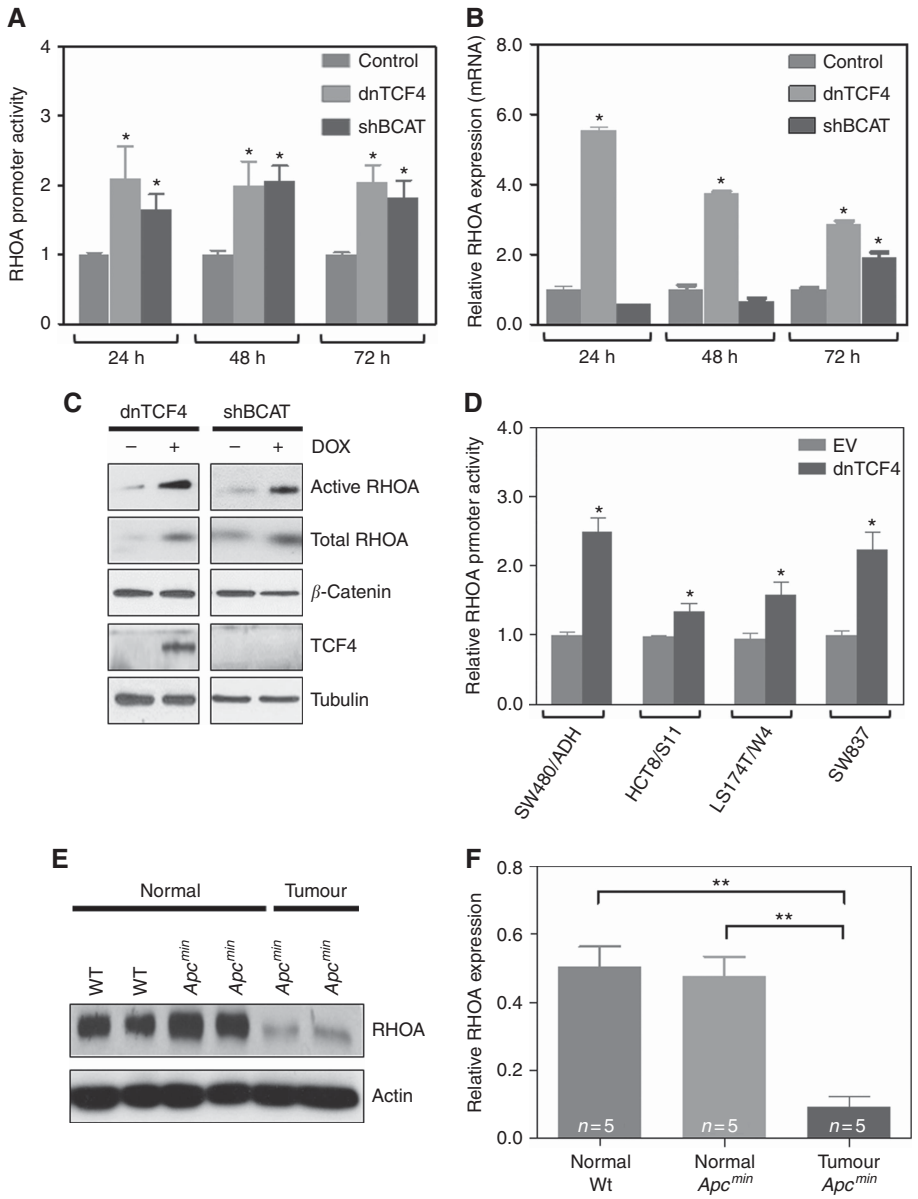

Figure 4. The *RHOA* regulation by Wnt signalling. (**A**) Changes in *RHOA* promoter activity after TCF4/β-catenin inactivation following doxycycline exposure in LS174T/dnTCF4 and LS174T/shBCAT cells. (**B**) Expression of *RHOA* (qPCR) in LS174T/dnTCF4 and LS174T/shBCAT cells after TCF4/β-catenin inactivation. (**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 Apc^{min} mice and in the intestinal tumours of Apc^{min} 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.

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 $p21^{CIP1/WAF1}$, through the sequestration of the SP1/SP3 complex (Gartel *et al*, 2001). Similar to the $p21^{CIP1/WAF1}$ 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 $r=0.37$; $P=5.8\times10^{-9}$). 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 $p21^{CIP1/WAF1}$ (Figure 6B and C), further indicating that SP1 transactivation is important to sustain *RHOA* expression in colon cancer cells.

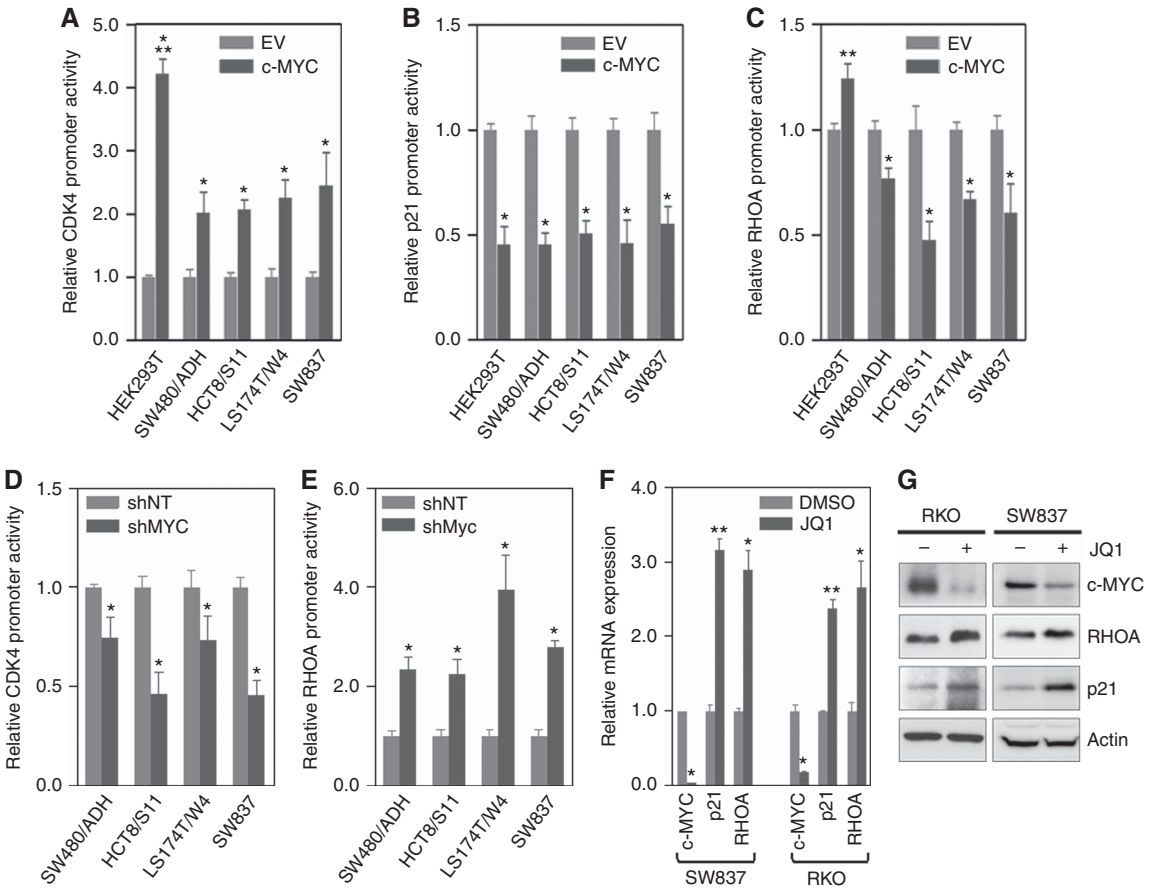

Figure 5. The *RHOA* regulation by c-MYC. (**A**) *CDK4*, (**B**) $p21^{CIP1/WAF1}$ and (**C**) *RHOA* promoter activity in SW480/ADH, HCT/S11, LS174T/W4 and SW837 colon cancer cells and kidney embryonic HEK293T cells with transient overexpression of c-MYC or the empty vector (EV) control construct. (**D**) *CDK4* and (**E**) *RHOA* promoter activity in response to c-MYC downregulation by a specific shRNA or a non-targeting (NT) control shRNA. (**F**) Relative mRNA expression of *c-MYC*, $p21^{CIP1/WAF1}$ and *RHOA* in SW837 and RKO colon cancer cells after JQ1 treatment for 24 h. (**G**) Western blot analysis showing the relative levels of c-MYC, $p21^{CIP1/WAF1}$ and RHOA in RKO and SW837 cells after JQ1 treatment. Actin was used as a loading control. The average (± s.e.m.) of three independent experiments each run in triplicate is shown for (**A–F**). 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.

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 $p21^{CIP1/WAF1}$ promoter after c-MYC downregulation with JQ1 treatment using a ChIP assay (compare blue (DMSO SP1) and green (JQ1 SP1) bars without stripes for the p21 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 known to be regulated by GEF and GAP proteins (Sahai and Marshall, 2002), in colon cancer cells, the promoter activity as well as the levels of RHOA mRNA and protein are significantly correlated with the levels of GTP-bound active RHOA, as determined by a pull-down assay based on the Rho binding domain (RBD) of the Rho effector Rhotekin. These data indicate that RHOA signalling is 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

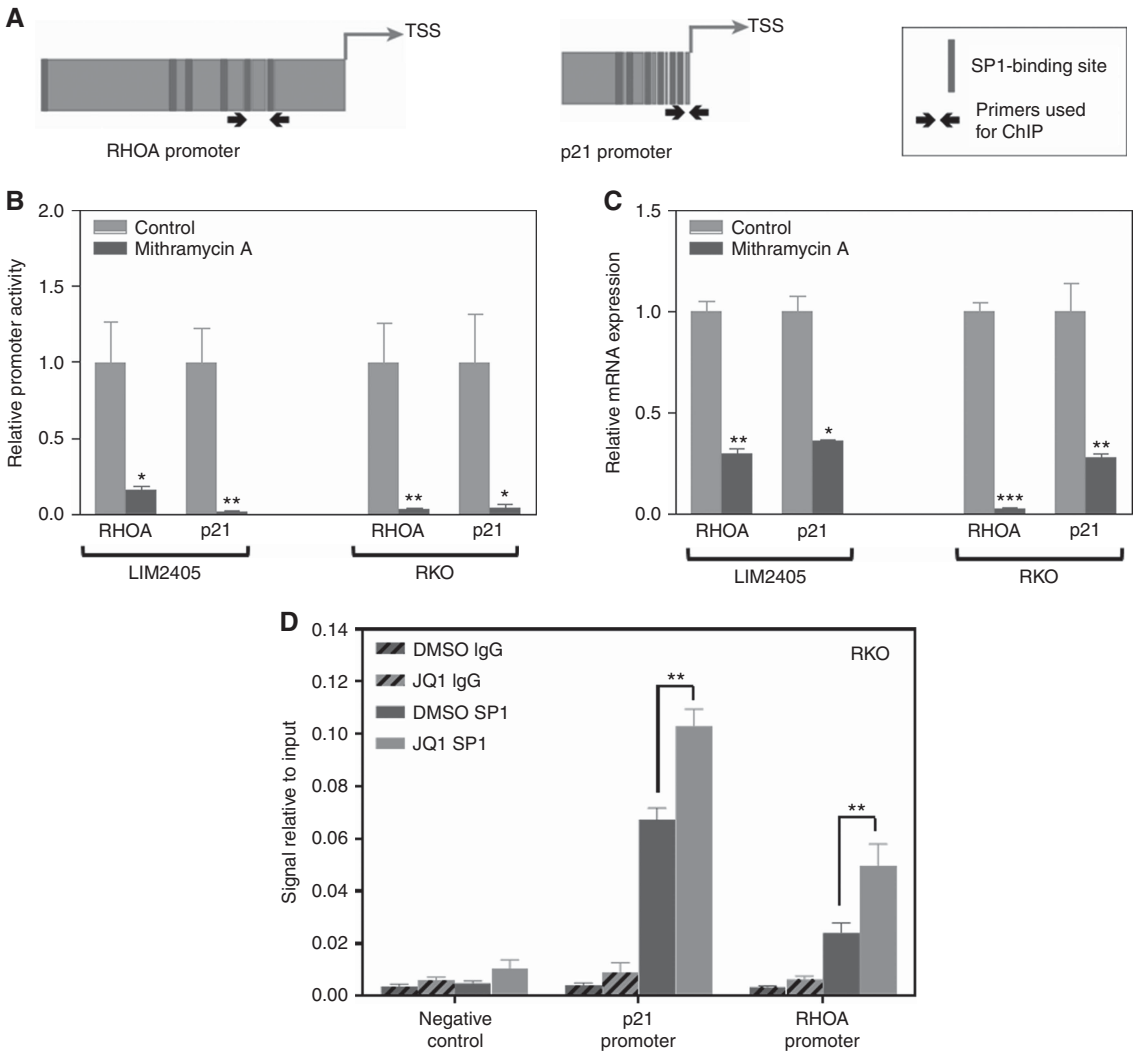

Figure 6. The c-MYC regulates *RHOA* levels through SP1. (**A**) Schematic representation of the *RHOA* and $p21^{CIP1/WAF1}$ promoters showing the localisation of SP1-binding sites and the primers used for qPCR after chromatin immunoprecipitation with an anti-SP1 antibody. (**B** and **C**) Effects of Mithramycin A treatment on the promoter activity (**B**) and the mRNA expression (**C**) of *RHOA* and $p21^{CIP1/WAF1}$. (**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). $p21^{CIP1/WAF1}$ 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.

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

cells. The *miR-200* family is located in two genomic clusters, *miR-200a/b/429* on chromosome 1 and *miR200c/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 evolutionary 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 colonic 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 $p21^{CIP1/WAF1}$ (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/MYC 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.

REFERENCES

Alazzouzi H, Alhopuro P, Salovaara R, Sammalkorpi H, Järvinen H, Mecklin J-P, Hemminki A, Schwartz S, Aaltonen LA, Arango D (2005) SMAD4 as a prognostic marker in colorectal cancer. *Clin Cancer Res* **11**: 2606–2611.

Alhopuro P, Alazzouzi H, Sammalkorpi H, Dávalos V, Salovaara R, Hemminki A, Järvinen H, Mecklin J-P, Schwartz S, Aaltonen LA, Arango D (2005) SMAD4 levels and response to 5-fluorouracil in colorectal cancer. *Clin Cancer Res* **11**: 6311–6316.

Arango D, Laiho P, Kokko A, Alhopuro P, Sammalkorpi H, Salovaara R, Nicorici D, Hautaniemi S, Alazzouzi H, Mecklin J-P, Järvinen H, Hemminki A, Astola J, Schwartz S, Aaltonen LA (2005) Gene-expression profiling predicts recurrence in Dukes' C colorectal cancer. *Gastroenterology* **129**: 874–884.

Bazzocco S, Dopeso H, Carton-Garcia F, Macaya I, Andretta E, Chionh F, Rodrigues P, Garrido M, Alazzouzi H, Nieto R, Sanchez A, Schwartz S, Bilic J, Mariadason JM, Arango D (2015) highly expressed genes in rapidly proliferating tumor cells as new targets for colorectal cancer treatment. *Clin Cancer Res* **21**: 3695–3704.

Betel D, Koppal A, Agius P, Sander C, Leslie C (2010) Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. *Genome Biol* **11**: R90.

Bi DP, Yin CH, Zhang XY, Yang NN, Xu JY (2016) MIR-183 functions as an oncogene by targeting ABCA1 in colon cancer. *Oncol Rep* **35**: 2873–2879.

Bienz M, Clevers H (2000) Linking colorectal cancer to Wnt signaling. *Cell* **103**: 311–320.

Bijkerk R, de Bruin RG, van Solingen C, Duijs JMGJ, Kobayashi K, van der Veer EP, ten Dijke P, Rabelink TJ, Goumans MJ, van Zonneveld AJ (2012) MicroRNA-155 functions as a negative regulator of RhoA signaling in TGF-beta-induced endothelial to mesenchymal transition. *MicroRNA* **1**: 2–10.

Braga EA, Loginov VI, Pronina I V, Khodyrev DS, Rykov S V, Burdennyy AM, Friedman M V, Kazubskaya TP, Kubatiev AA, Kushlinskii NE (2015) Upregulation of RHOA and NKIRAS1 genes in lung tumors is associated with loss of their methylation as well as with methylation of regulatory miRNA genes. *Biochem* **80**: 483–494.

Brannon A, Vakiani E, Sylvester BE, Scott SN, McDermott G, Shah RH, Kania K, Viale A, Oschwald DM, Vacic V, Emde A-K, Cercek A, Yaeger R, Kemeny NE, Saltz LB, Shia J, D Angelica MI, Weiser MR, Solit DB, Berger MF, D'Angelica MI (2014) Comparative sequencing analysis reveals high genomic concordance between matched primary and metastatic colorectal cancer lesions. *Genome Biol* **15**: 454.

Brunen D, Willems SM, Kellner U, Midgley R, Simon I, Bernards R (2013) TGF-β: an emerging player in drug resistance. *Cell Cycle* **12**: 2960–2968.

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

Chan C-H, Lee S-W, Li C-F, Wang J, Yang W-L, Wu C-Y, Wu J, Nakayama KI, Kang H-Y, Huang H-Y, Hung M-C, Pandolfi PP, Lin H-K (2010) Deciphering the transcriptional complex critical for RhoA gene expression and cancer metastasis. *Nat Cell Biol* **12**: 457–467.

Cheng D, Zhao S, Tang H, Zhang D, Sun H, Yu F, Jiang W, Yue B, Wang J, Zhang M, Yu Y, Liu X, Sun X, Zhou Z, Qin X, Zhang X, Yan D, Wen Y, Peng Z (2016) MicroRNA-20a-5p promotes colorectal cancer invasion and metastasis by downregulating Smad4. *Oncotarget* **7**(29): 45199–45213.

Cheng Z, Gong Y, Ma Y, Lu K, Lu X, Pierce LA, Thompson RC, Muller S, Knapp S, Wang J (2013) Inhibition of BET bromodomain targets genetically diverse glioblastoma. *Clin Cancer Res* **19**: 1748–1759.

Corté H, Manceau G, Blons H, Laurent-Puig P (2012) MicroRNA and colorectal cancer. *Dig Liver Dis* **44**: 195–200.

Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM, Kastritis E, Gilpatrick T, Paranal RM, Qi J, Chesi M, Schinzel AC, McKeown MR, Heffernan TP, Vakoc CR, Bergsagel PL, Ghobrial IM, Richardson PG, Young RA, Hahn WC, Anderson KC, Kung AL, Bradner JE, Mitsiades CS (2011) BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* **146**: 904–917.

Eppert K, Scherer SW, Ozcelik H, Pirone R, Hoodless P, Kim H, Tsui LC, Bapat B, Gallinger S, Andrulis IL, Thomsen GH, Wrana JL, Attisano L (1996) MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* **86**: 543–552.

Fernandes MS, Carneiro F, Oliveira C, Seruca R (2013) Colorectal cancer and RASSF family-a special emphasis on RASSF1A. *Int J Cancer* **132**: 251–258.

Gartel AL, Ye X, Goufman E, Shianov P, Hay N, Najmabadi F, Tyner AL (2001) Myc represses the p21(WAF1/CIP1) promoter and interacts with Sp1/Sp3. *Proc Natl Acad Sci USA* **98**: 4510–4515.

Giannakis M, Mu XJ, Shukla SA, Qian ZR, Cohen O, Nishihara R, Bahl S, Cao Y, Amin-Mansour A, Yamauchi M, Sukawa Y, Stewart C, Rosenberg M, Mima K, Inamura K, Nosho K, Nowak JA, Lawrence MS, Giovannucci EL, Chan AT, Ng K, Meyerhardt JA, Van Allen EM, Getz G, Gabriel SB, Lander ES, Wu CJ, Fuchs CS, Ogino S, Garraway LA (2016) Genomic correlates of immune-cell infiltrates in colorectal carcinoma. *Cell Rep* **15**: 857–865.

Howe JR, Roth S, Ringold JC, Summers RW, Järvinen HJ, Sistonen P, Tomlinson IP, Houlston RS, Bevan S, Mitros FA, Stone EM, Aaltonen LA (1998) Mutations in the SMAD4/DPC4 gene in juvenile polyposis. *Science* **280**: 1086–1088.

Hur K, Toiyama Y, Takahashi M, Balaguer F, Nagasaka T, Koike J, Hemmi H, Koi M, Boland CR, Goel A (2013) MicroRNA-200c modulates epithelial-to-mesenchymal transition (EMT) in human colorectal cancer metastasis. *Gut* **62**: 1315–1326.

Itoh K, Yoshioka K, Akedo H, Uehata M, Ishizaki T, Narumiya S (1999) An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. *Nat Med* **5**: 221–225.

Jian Q, An Q, Zhu D, Hui K, Liu Y, Chi S, Li C (2014) MicroRNA 340 is involved in UVB-induced dendrite formation through the regulation of RhoA expression in melanocytes. *Mol Cell Biol* **34**: 3407–3420.

Li LH, Nerlov C, Prendergast G, MacGregor D, Ziff EB (1994) c-Myc represses transcription in vivo by a novel mechanism dependent on the initiator element and Myc box II. *EMBO J* **13**: 4070–4079.

Liu M, Lang N, Chen X, Tang Q, Liu S, Huang J, Zheng Y, Bi F (2011) MiR-185 targets RhoA and Cdc42 expression and inhibits the proliferation potential of human colorectal cells. *Cancer Lett* **301**: 151–160.

Luongo C, Moser AR, Gledhill S, Dove WF (1994) Loss of Apc + in intestinal adenomas from min mice. *Cancer Res* **54**: 5947–5952.

MacDonald BT, Tamai K, He X (2009) Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* **17**: 9–26.

Mehus JG, Deloukas P, Lambeth DO (1999) NME6: a new member of the nm23/nucleoside diphosphate kinase gene family located on human chromosome 3p21.3. *Hum Genet* **104**: 454–459.

Mishra L, Shetty K, Tang Y, Stuart A, Byers SW (2005) The role of TGF-beta and Wnt signaling in gastrointestinal stem cells and cancer. *Oncogene* **24**: 5775–5789.

Moser AR, Pitot HC, Dove WF (1990) A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* **247**: 322–324.

Mouradov D, Sloggett C, Jorissen RN, Love CG, Li S, Burgess AW, Arango D, Strausberg RL, Buchanan D, Wormald S, O'Connor L, Wilding JL, Bicknell D, Tomlinson IPM, Bodmer WF, Mariadason JM, Sieber OM (2014) Colorectal cancer cell lines are representative models of the main molecular subtypes of primary cancer. *Cancer Res* **74**: 3238–3247.

Moustakas A, Souchelnytskyi S, Heldin C-H (2001) Smad regulation in TGF-β signal transduction. *J Cell Sci* **114**: 4359–4369.

Palomero T, Couronne L, Khiabanian H, Kim MY, Ambesi-Impiombato A, Perez-Garcia A, Carpenter Z, Abate F, Allegretta M, Haydu JE, Jiang X, Lossos IS, Nicolas C, Balbin M, Bastard C, Bhagat G, Piris MA, Campo E, Bernard OA, Rabadan R, Ferrando AA (2014) Recurrent mutations in epigenetic regulators, RHOA and FYN kinase in peripheral T cell lymphomas. *Nat Genet* **46**: 166–170.

Parsons R, Myeroff LL, Liu B, Willson JK, Markowitz SD, Kinzler KW, Vogelstein B (1995) Microsatellite instability and mutations of the transforming growth factor beta type II receptor gene in colorectal cancer. *Cancer Res* **55**: 5548–5550.

Rodrigues P, Macaya I, Bazzocco S, Mazzolini R, Andretta E, Dopeso H, Mateo-Lozano S, Bilić J, Cartón-García F, Nieto R, Suárez-López L, Afonso E, Landolfi S, Hernandez-Losa J, Kobayashi K, Ramón y, Cajal S, Tabernero J, Tebbutt NC, Mariadason JM, Schwartz S, Arango D (2014) RHOA inactivation enhances Wnt signalling and promotes colorectal cancer. *Nat Commun* **5**: 5458.

Sahai E, Marshall CJ (2002) RHO-GTPases and cancer. *Nat Rev Cancer* **2**: 133–142.

Sansom OJ, Meniel VS, Muncan V, Phesse TJ, Wilkins JA, Reed KR, Vass JK, Athineos D, Clevers H, Clarke AR (2007) Myc deletion rescues Apc deficiency in the small intestine. *Nature* **446**: 676–679.

Sansom OJ, Reed KR, Hayes AJ, Ireland H, Brinkmann H, Newton IP, Batlle E, Simon-Assmann P, Clevers H, Nathke IS, Clarke AR, Winton DJ (2004) Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. *Genes Dev* **18**: 1385–1390.

Seoane J, Le H-V, Massagué J (2002) Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature* **419**: 729–734.

Seshagiri S, Stawiski EW, Durinck S, Modrusan Z, Storm EE, Conboy CB, Chaudhuri S, Guan Y, Janakiraman V, Jaiswal BS, Guillory J, Ha C, Dijkgraaf GJP, Stinson J, Gnad F, Huntley M a, Degenhardt JD, Haverty PM, Bourgon R, Wang W, Koeppen H, Gentleman R, Starr TK, Zhang Z, Largaespada DA, Wu TD, de Sauvage FJ (2012) Recurrent R-spondin fusions in colon cancer. *Nature* **488**: 660–664.

Si J, Yu X, Zhang Y, DeWille JW (2010) Myc interacts with Max and Miz1 to repress C/EBPdelta promoter activity and gene expression. *Mol Cancer* **9**: 92.

Talamillo A, Grande L, Ruiz-Ontañon P, Velasquez C, Mollinedo P, Torices S, Sanchez-Gomez P, Aznar A, Esparis-Ogando A, Lopez-Lopez C, Lafita C, Berciano MT, Montero JA, Vazquez-Barquero A, Segura V, Villagra NT, Pandiella A, Lafarga M, Leon J, Martinez-Climent JA, Sanz-Moreno V, Fernandez-Luna JL (2017) ODZ1 allows glioblastoma to sustain invasiveness through a Myc-dependent transcriptional upregulation of RhoA. *Oncogene* **36**: 1733–1744.

Tögel L, Nightingale R, Chueh AC, Jayachandran A, Tran H, Phesse T, Wu R, Sieber OM, Arango D, Dhillon AS, Dawson MA, Diez-Dacal B, Gahman TC, Filippakopoulos P, Shiau AK, Mariadason JM (2016) Dual targeting of bromodomain and extraterminal domain proteins, and WNT or MAPK signaling, inhibits c-MYC expression and proliferation of colorectal cancer cells. *Mol Cancer Ther* **15**: 1217–1226.

Tsankov AM, Gu H, Akopian V, Ziller MJ, Donaghey J, Amit I, Gnirke A, Meissner A (2015) Transcription factor binding dynamics during human ES cell differentiation. *Nature* **518**: 344–349.

Van de Wetering M, Sancho E, Verweij C, De Lau W, Oving I, Hurlstone A, Van der Horn K, Batlle E, Coudreuse D, Haramis AP, Tjon-Pon-Fong M, Moerer P, Van den Born M, Soete G, Pals S, Eilers M, Medema R, Clevers H (2002) The B-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **111**: 241–250.

Wong C-M, Wei L, Au SL-K, Fan DN-Y, Zhou Y, Tsang FH-C, Law C-T, Lee JM-F, He X, Shi J, Wong CC-L, Ng IO-L (2015) MiR-200b/200c/429 subfamily negatively regulates Rho/ROCK signaling pathway to suppress hepatocellular carcinoma metastasis. *Oncotarget* **6**: 13658–13670.

Yogurtcu B, Hatemi I, Aydin I, Buyru N (2012) NPRL2 gene expression in the progression of colon tumors. *Genet Mol Res* **11**: 4810–4816.

Zhou J, Hayakawa Y, Wang TC, Bass AJ (2014) RhoA mutations identified in diffuse gastric cancer. *Cancer Cell* **26**: 9–11.

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