Molecular mechanisms of the cooperation between Rac1/1b GTPases and the canonical Wnt signaling pathway in colorectal cancer

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Laboratory Medicine and Pathobiology

University of Toronto

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Abstract

Aberrant activation of the canonical Wnt signaling pathway accounts for the vast majority of colorectal cancers. The Rac1 GTPase is overexpressed in colon cancer, and its splice variant, Rac1b, is preferentially expressed in colon tumours. Rac1 and Rac1b have both been previously shown to crosstalk with the canonical Wnt signaling pathway in colon cancer; however, the specific means by which this crosstalk occurs were unclear. This study examines the molecular mechanisms of Rac1/1b in the cooperation with canonical Wnt signaling in colon cancer. In a colon cancer cell line with dysregulated Wnt signaling, the constitutively active Rac1 mutant, V12Rac1, was observed to transcriptionally upregulate the expression of a gene set associated with cellular migration. Further, V12Rac1-mediated promotion of cell migration was dependent on its nuclear localization. Previous work in our lab has shown a Rac1-specific activator, Tiam1, is present in the nucleus at the promoter of Wnt target genes upon Wnt3a stimulation; and that exogenous introduction of Tiam1 increased the expression of a Wnt-responsive reporter (TopFlash).
Given the importance of nuclear localization of Rac1 in the promotion of tumourigenic processes, we demonstrated that knockdown of endogenous Tiam1 reduced TopFlash expression, proving reverse specificity and strengthening the evidence of a nuclear role for Rac1. Since some functional differences exist between Rac1 and Rac1b, we also examined Rac1b for transcriptional targets following induction, and identified the RhoA effector, ROCK2, which has been previously associated with cell migration. ROCK2 demonstrated a positive correlation with Rac1b transcript expression in primary colon tumours as compared to matched normal tissue specimens. Interestingly, the observed induction in ROCK2 transcript did not translate into a detectable change in protein expression or kinase activity. Like Rac1, Rac1b also promotes cellular motility, which is dependent on nuclear localization. Cell migration can be negatively regulated by E-cadherin. Following Rac1b knockdown in HT29 cells, we show that Rac1b might contribute to motility through upregulation of the E-cadherin-repressor, Slug. Taken together, we provide greater insight into the mechanistic roles of Rac1 and Rac1b in transcriptionally regulating target genes to promote cellular processes, such as cell migration, in colon cancer with dysregulated canonical Wnt signaling.
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CHAPTER 1

General Introduction

1.1 Colorectal Cancer

Every year in Canada, an estimated 22,000 cases of colorectal cancer are diagnosed, and 9,100 patients die of the disease, making it the second-leading cause of death from cancer among adults.\[1\] The declining colorectal cancer mortality rates in both sexes (by 1.3% and 1.7% per year in males and females, respectively) are likely the results of improvements in early detection screening methods as well as treatment.\[1\] These encouraging statistics underscore the need for a greater understanding, especially in the etiology of molecular mechanisms that contribute to this and other cancers, in order to improve upon current therapies.

Colorectal cancer begins as a benign adenomatous polyp, which develops into an advanced adenoma with high grade dysplasia and then progresses to an invasive cancer.\[2\] Invasive cancers that are confined within the wall of the colon are curable, but if untreated, they spread to regional lymph nodes and then metastasize to distant sites.\[3\] Early stage tumours are curable by surgical excision, and up to 73% of cases of later stage disease are curable by surgery combined with adjuvant chemotherapy.\[4\] Recent
advances in chemotherapy have improved survival; however, typically advanced late-stage disease is incurable.[2]

The clinical presentation of a colorectal cancer results from interactions at many levels. The sequential accumulation of genetic mutations and epigenetic alterations are widely considered as the primary method of initiation and progression of benign adenomas to malignant adenocarcinomas. Colorectal cancers acquire many genetic changes, but certain signaling pathways are clearly singled out as key factors in tumour formation.[5-24] One of these changes, the activation of the Wnt signaling pathway, is regarded as the initiating event in colorectal cancer.[7]

1.2 The Wnt Signaling Pathway

The Wnt signaling pathway plays critical roles in the regulation of body axis formation, cell proliferation, and organogenesis in many organisms and is important for homeostatic self-renewal in several adult tissues.[25, 26] Inappropriate activation of Wnt signaling transduction causes human degenerative diseases as well as cancer.[26, 27] There are approximately 20 secreted Wnt proteins that have been identified, across 12 conserved Wnt subfamilies, and in several different animals.[27] Comparative genomic analysis across species underscored the pivotal role that Wnt genes play in organismal patterning throughout the animal kingdom.[27, 28] Frizzled (Fz) receptors are seven transmembrane molecules with a long amino-terminal extension called CRD (cysteine-rich domain). Wnt proteins bind directly to the CRD of Fz or Fz-like receptors, or a complex of Fz and a long single pass transmembrane molecule of the LRP (LDL receptor
related protein) class, identified as the gene *arrow* in Drosophila and as *LRP5* or *LRP6* in vertebrates.[29-34] It has been proposed, but not always confirmed, that Wnt molecules can also bind to LRP and form a trimeric complex with a Frizzled. The cytoplasmic tail of LRP may interact directly with Axin, one of the downstream components in Wnt signaling.[35]

In the cytoplasm, Wnt signaling branches into three distinct pathways via a cytoplasmic phosphoprotein Dishevelled (Dvl), namely the canonical Wnt/β-catenin signaling pathway, the noncanonical planar cell polarity (PCP) pathway and the Wnt/Ca^2+ pathway.[27, 29, 36-40] Regulation into one or more of the Wnt pathways are regulated, in addition to Dvl, by the presence of heterotrimeric G-proteins, Gαo and Gαq.[41] The canonical Wnt pathway requires both G proteins for proper signaling transduction, whereas Wnt/JNK signaling requires only the Gαo protein.[42-44]

### 1.2.1 Noncanonical Wnt Signaling Pathways

The noncanonical Wnt signaling pathways, also known as β-catenin-independent Wnt signaling, are pathways that upon stimulation by specific Wnt ligands, do not require the transcriptional activity of β-catenin. Vertebrate noncanonical Wnt signaling regulates processes as diverse as cochlear hair cell morphology, heart induction, dorsoventral patterning tissue separation, neuronal migration, and cancer. Relative to the widely studied canonical Wnt signaling pathway, the specific signaling events of the β-catenin-independent pathway are less defined; primarily because there are at least three signaling mechanisms that overlap with other signaling pathways. First, specific Wnt and frizzled
homologs can activate calcium/calmodulin-dependent kinase II (CamKII) and protein kinase C (PKC) in a Wnt/calcium pathway. Second, some frizzled receptors, like other seven transmembrane receptors, act through heterotrimeric GTP-binding proteins to activate phospholipase C (PLC) and phosphodiesterase (PDE). Lastly, in Drosophila, the planar cell polarity (PCP) pathway is mediated by Frizzled, which activates small GTP-binding proteins, and the Jun-N-terminal kinase (JNK).

**Wnt/Planar Cell Polarity Signaling**

The most studied β-catenin-independent signaling pathway is one that controls aspects of gastrulation movements through a mechanism similar to the *Drosophila* planar cell polarity (PCP).[45, 46] The term “planar cell polarity” is derived from the study of tissue polarity necessary to generate polarization within the plane of the epithelium, along an axis perpendicular to the apical-basal axis of the cell.[47] *Drosophila* is the hallmark model system for PCP signaling because of the many phenotypic changes in structure and orientation, including the perfectly aligned hairs on wing cells, the precisely arranged ommatidia in the facet eye, and the bristles on the thorax. In vertebrates, PCP was shown in several cellular processes, including convergent extension movements of the mesenchymal cells during gastrulation, ordered arrangement of hairs of mammalian skin and cilia of respiratory tract, orientation of stereocilia in the Organ of Corti, and orientation of axon extension.[48]

The PCP signaling pathway is evolutionarily conserved and constitutes three sequential steps, involving Daam1, Rho, Rac, Rho kinase (ROCK), JNK, and Profilin as
the primary members of this pathway. These key components are engaged in the regulation of cytoskeleton to execute cell polarity and cell movements.[47] In vertebrates, initiation of this pathway occurs with the binding of noncanonical Wnts (Wnt5a and Wnt11) to a Fz receptor, independent of the canonical Wnt co-receptor LRP5/6.[49, 50] As in the canonical Wnt signaling pathway, this interaction recruits the cytoplasmic scaffolding protein Dvl to the plasma membrane, where it is activated. At this point the pathway diverges into three different aspects of cytoskeleton reorganization in cell polarity and movement. In one signaling cascade, activated Dvl recruits and binds the carboxy-terminus of Daam1 (Dishevelled associated activator of morphogenesis 1), a Formin-homology protein, while the amino-terminus of Daam1 binds RhoA.[51] This interaction results in a Dvl-Daam1 complex that leads to activation of Daam1 and subsequently the activation of Rho via a Rho guanine nucleotide exchange factor, such as WGEF (Weakly similar to Rho GEF 5).[51] Rho activation consequently activates ROCK and myosin, and leads to cytoskeletal rearrangement and modification of the actin cytoskeleton.[52, 53] In the second branch of this signal transduction pathway, Daam1 mediates actin polymerization by its interaction with the actin binding protein Profilin. The third branch occurs independently of Daam1 and involves the activation of Rac GTPase by Dvl. This results in the stimulation of Jun-N-terminal kinase (JNK) activity leading to the phosphorylation of c-jun and assembly of the activator protein 1 (AP-1) transcription factor.[54, 55] While Rac and Rho GTPases are known to possess opposing activities, it appears as
Figure 1.1 Planar Cell Polarity transduction cascade.
Wnt signaling is transduced through Fz independent of LRP5/6 leading to the activation of Dvl. Dvl through Daam1 mediates activation of Rho which in turn activates Rho kinase (ROCK). Daam1 also mediates actin polymerization through the actin binding protein Profilin. Dvl also mediates activation of Rac, which in turn activates JNK. The signaling from Rock, JNK and Profilin are integrated for cytoskeletal changes for cell polarization and motility during gastrulation.
though their functions are coordinated for cell polarization and directional migration, however, the details of their orchestrated response remains poorly understood.

**Wnt/Calcium Signaling**

Calcium was implicated as a second messenger, independent of β–catenin, in studies using 1-cell zebrafish embryos that found the ER (endoplasmic reticulum) would release double the levels of intracellular Ca$^{2+}$ following injection with Wnt5a or Wnt11 mRNA.[56-58] Ectopic expression of rat Fz2 (RFz-2) was also capable of releasing intracellular Ca$^{2+}$, without affecting β–catenin stabilization; however, overexpression of Wnt8, which activates β–catenin, had no effect on calcium release.[59] Exposure to pertussis toxin and the α subunit of transducin, which inhibit G protein signaling, also inhibited calcium release following overexpression of Wnt5a.[43] These results supported a role for calcium in the Wnt signaling pathway in which Wnt5a, or another similar Wnt, stimulates the release of calcium via a frizzled receptor through trimeric G proteins.

The Wnt5a-mediated increase in intracellular calcium modulates several Ca$^{2+}$ sensitive proteins, including protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CamKII). In Xenopus embryos, injection of Wnt5a or Rfz-2 mRNA led to a threefold increase in CamKII activity relative to controls.[60] Similar results were observed with expression of the closely related Wnt11, whereas Wnt8 expression, which stabilizes β–catenin, had no effect on CamKII activity. *In vitro* studies also
Figure 1.2  **Wnt/Ca\(^{2+}\) signal transduction cascade.**

Wnt signaling via Fz mediates activation of Dvl via activation of G-proteins. Dishevelled activates the phosphodiesterase PDE which inhibits PKG and in turn inhibits Ca\(^{2+}\) release. Dvl through PLC activates IP\(_3\), which leads to release of intracellular Ca\(^{2+}\), which in turn activates CamK11 and calcineurin. Calcineurin activate NF-AT to regulate ventral cell fates. CamK11 activates TAK and NLK, which inhibit β-catenin/TCF function to negatively regulate dorsal axis formation. DAG through PKC activates CDC42 to mediate tissue separation and cell movements during gastrulation.
demonstrated that ectopic expression of Wnt5a and Rfz-2 resulted in translocation of PKC to the plasma membrane and stimulated PKC kinase activity.[61]

Similar to the Wnt/PCP pathway, further examination of the Wnt/Fz interaction revealed that this calcium signaling pathway is functioning through activation of Dvl, which can regulate calcium release through two cascade events (Figure 1.2). First, Dvl activates the phosphodiesterase (PDE) which inhibits protein kinase G (PKG) and subsequently inhibits calcium release.[58] The second cascade involves the release of calcium where Dvl, through Phospholipase C (PLC), activates IP3. Intracellular release of calcium activates CamKII and calcineurin. CamKII activates TGFβ activated kinase (TAK1) and Nemo-like kinase (NLK) which antoganizes the canonical Wnt signaling pathway, negatively regulating dorsal axis function.[62] Calcineurin can activate the transcription factor NF-AT to promote ventral cell fates. In addition to IP3, PLC can also activate the small GTPase CDC42, which regulates the process of tissue separation during gastrulation.[63]

1.2.2 Canonical Wnt Signaling Pathway

Signaling by the Wnt family of secreted glycolipoproteins is one of the fundamental mechanisms that direct cell proliferation, cell polarity, and cell fate determination during embryonic development and tissue homeostasis.[38] As a result, mutations in the Wnt pathway are often linked to human birth defects, cancer, and other diseases.[27] A critical and heavily studied Wnt pathway is the canonical Wnt pathway, which functions
Figure 1.3  **Canonical Wnt Signaling.**
When Wnt receptor complexes are not bound by ligand (left), the serine/threonine kinases, CK1 and GSK3, phosphorylate β-catenin. Phosphorylated β-catenin is recognized by a component of a dedicated E3 ubiquitin ligase complex. Following ubiquitination, β-catenin is targeted for rapid destruction via the proteasome. In the nucleus, the binding of Groucho to TCF (T cell factor) inhibits the transcription of Wnt target genes. Once bound by Wnt (right), the Frizzled (Fz)/LRP coreceptor complex activates the canonical signaling pathway. Fz interacts with Dvl and results in its phosphorylation. Wnts are thought to induce the phosphorylation of LRP by GSK3 and casein kinase I-γ (CK1γ), thus regulating the docking of Axin. The recruitment of Axin away from the destruction complex leads to the stabilization of β-catenin. In the nucleus, β-catenin displaces Groucho from Tcf/Lef to promote the transcription of Wnt target genes.
by regulating the amount of the transcriptional coactivator β-catenin, which controls key developmental gene expression programs.

In the absence of Wnt ligands, cytoplasmic β-catenin protein is constantly degraded by the action of the Destruction Complex, which is composed of the scaffolding protein Axin, the tumour suppressor adenomatous polyposis coli gene product (APC), and the two kinase families [casein kinase 1 (CK1) α, −δ, −ε, and glycogen synthase kinase 3 (GSK3) α and −β]. CK1 and GSK3 sequentially phosphorylate a series of highly conserved Ser/Thr residues near the amino terminal region of β-catenin. Phosphorylated β-catenin is recognized by the F box/WD repeat protein, β-TrCP, an E3 ubiquitin ligase subunit, and results in β-catenin ubiquitination and subsequently targeted for degradation via the proteosome.[27, 64, 65] This continual purging of β-catenin prevents β-catenin from entering the nucleus, and Wnt target genes are therefore repressed by the interaction of the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) family of proteins with Groucho/Grg/TLE proteins.[27, 66, 67]

The Wnt/β-catenin pathway is activated when a Wnt ligand binds to the Frizzled (Fz) receptor and its coreceptor, LRP6, or its close relative LRP5. Wnts are conserved in all metazoan animals. In mammals, complexity and specificity in Wnt signaling are in part achieved through 19 Wnt ligands, which are cysteine-rich proteins of approximately 350–400 amino acids that contain an N-terminal signal peptide for secretion. Murine WNT3A was the first purified and biochemically characterized Wnt protein.[68] The Fz family of proteins are seven-pass transmembrane receptors with an extracellular N-terminal cysteine-rich domain (CRD), while the LRP family of proteins are single-pass
transmembrane molecules.[29, 38, 65, 69] The formation of a Wnt-Fz-LRP5/6 complex leads to the phosphorylation of the scaffolding protein Dishevelled (Dvl), and also the phosphorylation of LRP5/6 at five conserved intracellular PPP(S/T)PX(S/T) motifs (colloquially known as PPPSPXS), which provide an optimal binding site for Axin. These PPPSPXS motifs are phosphorylated sequentially by GSK3 and CK1. It is recently postulated that upon phosphorylation of the five PPPSPXS sites by GSK3 and CK1, these five motifs act together in the recruitment of Axin-GSK3 to one of the sites, permitting the remaining phospho-PPPSPXS sites to inhibit GSK3 phosphorylation of β-catenin at serine 33, serine 37 and threonine 41.[70-72] In order for GSK3 to phosphorylate the three aforementioned β-catenin residues, β-catenin is stabilized and therefore escapes degradation with an initial priming event of phosphorylation at serine 45 by CK1. Given that each LRP6 molecule contains five phosphorylated PPPSPXS and that LRP6 upon Wnt activation may multimerize, a high local concentration of potential docking sites for a single Axin molecule exists, ensuring a tight association between LRP6 and Axin.

Dvl is the molecular branchpoint between the canonical and noncanonical Wnt signaling pathways. Upon Wnt activation in the canonical pathway, phosphorylation of Dvl, which is one of the first proteins activated on the cytoplasmic side of the plasma membrane, leads to the inactivation of the β-catenin destruction complex.[73, 74] Previous studies have suggested that phosphorylated Dvl interacts with the Axin component of the destruction complex, thereby leading to its inactivation. However, the specific mechanisms by which Dvl deactivates, whether through Axin or some other molecule, remains poorly defined. Recent studies have identified a nuclear role for Dvl
in mediating its effects on both canonical and noncanonical Wnt signaling pathways.

Gan et al. (2008) discovered that at the promoter of Wnt target genes, c-Jun functions as a scaffold in the β-catenin/TCF transcription complex bridging Dvl to TCF.[25] Another novel role for nuclear Dvl involving Rac1b is discussed in Chapter 4.

Stabilized (nonphosphorylated) β-catenin accumulates in the cytoplasm and translocates into the nucleus where it can displace the transcriptional repressor complex, Groucho/Grg/TLE, from the promoter of Wnt target genes, and binds with the amino-terminus of LEF/TCF (lymphoid enhancer/T cell factor) transcription factors.[42, 44, 75] Although β-catenin can localize to the nucleus on its own, it has been proposed that Tcf and Pygopus anchor β-catenin within the nucleus. Along with Bcl9, which bridges Pygopus to the N-terminal of β-catenin, this trimeric complex has been implicated in nuclear import/retention of β-catenin, and may also transactivate transcription. An array of potential co-activators, present at the promoters of Wnt target genes is increasing, however, their specific roles are not well understood.

1.2.3 Wnt Signaling and Colon Cancer

The hereditary nature of adenomatous polyposis, a form of human colon cancer in which numerous polyps form in the epithelium of the large intestine, has been recognized as early as 1900.[76, 77] However, it was not until 1987 that the rare inherited disease, familial adenomatous polyposis (FAP), was found to be associated with deletions of the specific chromosome region 5q21-22.[78, 79] FAP patients develop hundreds to thousands of adenomatous polyps in the colon, and without a total surgical resection of
the colon, some of these polyps will, with near certainty, develop to malignant carcinomas. Prior to the work of Kinzler, Vogelstein, and Polakis in 1993, there was no link between research on Wnt signaling and human cancer.[80, 81] These studies reported that an important biochemical interaction exists between APC, a tumour suppressor, and β-catenin through two types of repeat regions: three 15-amino acid and seven 20-amino acid repeats. APC mutations that disrupt these and other interactions are responsible for FAP. The vast majority of mutations, either in patients with FAP or in sporadic colorectal cancers (which make up 85% of all colorectal cancer cases), are truncating mutations.[76, 82, 83] Most APC mutations are frameshift, nonsense or splice-site mutations, which result in a truncation of about 50% of the protein.[84, 85] We have recently identified a rare founder deletion in the major promoter region of APC in a Canadian Mennonite family.[86] Since APC is a tumour suppressor gene, a single mutation is insufficient for the induction of adenomatous polyposis; a mutation must also occur on the second APC allele. Many APC mutations occur before the region responsible for mediating the interaction between APC and scaffolding proteins of the β-catenin destruction complex, AXIN1 and AXIN2.[87, 88] While loss-of-function mutations in AXIN1 and AXIN2 have been detected, they occur in rare colorectal cancer cases.[89, 90]

Gain-of-function mutations in the gene encoding β-catenin, CTNNB1, have been discovered in a fraction of sporadic colorectal human cancers.[5, 6, 91] The effect of these mutations is that β-catenin is able to evade phosphorylation and ubiquitylation, and subsequent proteasomal degradation. It was once believed, through limited sampling, that approximately 10% of sporadic colorectal cancers contain activating β-catenin
mutations. However, in more recent studies, this frequency has been amended to approximately 1%.[92]

Overall, mutations in the *APC, AXIN1, AXIN2* and *CTNNB1* genes generally cause tumour formation because of their ability to stabilize β-catenin, and bypass regulation by extracellular Wnts. The accumulated β-catenin then translocates to the nucleus where it interacts with the TCF/LEF transcription factors, and inappropriately activates transcription of Wnt target genes associated with the regulation of cell proliferation.[42, 44, 93-96] Recently, aberrant activation of canonical Wnt signaling in cancer has been associated with mutations in other components of the pathway. These include loss-of-function mutations in the recently discovered component of the β-catenin destruction complex (WTX, X-chromosome-linked Wilms tumour), silencing of inhibitory Wnt ligands (SFRPs and DKKs) by hypermethylation, and overexpression of Wnt proteins (eg. WNT2B), Frizzleds (eg. FZD10), or Dvl.[97-104]

While our knowledge of the complete etiology of human colon cancer is under constant revision as new evidence modifies older theories, it is still widely accepted that multiple mutations are necessary for the development of human malignancy. Mutations that cause aberrant activation of the Wnt signaling pathway are the only known genetic alterations present in early premalignant lesions in the intestine, such as aberrant crypt foci and small polyps.[105, 106] Mutations in the *APC* gene represent an early event in tumour progression, and are therefore considered as the ‘gatekeeper’ in colon carcinogenesis.[8, 17, 76, 107-109] However, mutations in genes from other pathways (eg. *KRAS, SMAD2, SMAD4*, and *TP53*) generally follow. Therefore, it is more fitting to claim that aberrant activation of developmental signaling pathways are responsible for
and promote tumour progression in the colon. Currently, there is no evidence to support a link between the noncanonical Wnt pathways and colon cancer.

### 1.3 Ras Superfamily of Small GTPases

There are more than 150 members of the Ras superfamily of small guanosine triphosphatases (GTPases) with evolutionary conserved orthologs found in *Drosophila, C. elegans, S. cerevisiae, S. pombe, Dictyostelium* and plants.[110, 111] This superfamily is divided between five sub-families: Rab, Ras, Arf, Rho, and Ran.[112] Additionally, there are a few proteins that are members of the Ras superfamily based upon their functional domain, but are not members of the aforementioned sub-families. All of these GTPases are similar to heterotrimeric G protein subunits in their biochemistry and function; however, Ras superfamily proteins function as monomeric G proteins. The differences in structure and post-translational modifications dictate the subcellular localization and the proteins which activate them, as well as which effectors will be regulated by them.

The Ras superfamily GTPases function as a molecular switch by cycling between a GTP-bound (active) and GDP-bound (inactive) state (Figure 1.4).[112-115] They have a high affinity binding for GDP and GTP, and a low intrinsic GTP hydrolysis and GDP/GTP exchange activities. Two regulatory proteins are responsible for the cycling between the two states. The guanine-nucleotide-exchange factors (GEFs) promote the formation of the active form, whereas GTPase-activating proteins (GAPs) accelerate the intrinsic GTPase activity to promote the inactive form.[111, 116, 117] The two
Figure 1.4 Regulation of GTPase cycle.

GDP-bound inactive GTPases are mainly cytoplasmic, maintained there by GDIs masking the C-terminal tail required for plasma membrane localization. Upon dissociation of the GDI, posttranslational modification can take place and GTPases translocate to the plasma membrane, where they can be activated by GEFs upon external stimuli from surface ligand-receptor systems such as adhesion receptors, G-protein coupled receptors (GPCR) and receptor tyrosine kinases (RTK). Upon activation by GEFs, Rho GTPases can bind different effector proteins, selection of which can be mediated by GEFs and induce downstream signaling pathways. GAPs inactivate the Rho GTPases and switch off the downstream signaling.
conformational states are similar in structure with the exception of two differences, the switch I and switch II regions.[118, 119] Regulatory and effector proteins sense the active or inactive state of the Ras GTPases because of the conformational change in these two regions.

Another critical biochemical feature of Ras superfamily proteins is the post-translational modification by lipids. At the carboxy-terminus of Ras and Rho family proteins exists a CAAX (C=cysteine, A=aliphatic, X=any amino acid) tetrapeptide sequence that, when coupled with residues immediately upstream (a polybasic region), comprise the membrane-targeting sequences responsible for proper subcellular localization.[120] For Ras and Rho family proteins, farnesyltransferase and geranylgeranyltransferase I recognize the CAAX motif and catalyze the covalent addition of farnesyl or geranylgeranyl isoprenoids, respectively, to the cysteine residue. The Rab family proteins are regulated in the same manner by the addition of geranylgeranyl isoprenoid to a distinct set of cysteine-containing c-terminal motifs (CC, CXC, CCX, CCXX, or CCXXX) by geranylgeranyltransferase II. Membrane localization is regulated at the N-termini by a myristate fatty acid in the Arf family proteins. Rho and Rab GTPases may be sequestered to the cytosol by guanine nucleotide dissociation inhibitors (GDIs), which mask the prenyl modification.[121] Some Ras superfamily members are not modified by lipids and still retain the ability to associate with membranes (eg. Rit, RhoBTB, Miro and Sar1), while other members are neither lipid modified nor membrane-bound (eg. Ran and Rerg).

There are two distinct sequence elements that serve as additional signals to the CAAX motif in the promotion of proper membrane-association and biological function.
These elements are positioned immediately upstream of the CAAX motif. One element is composed of clusters of polybasic amino acid residues, also termed the polybasic region (PBR), that provide a positive charge which facilitates association with acidic membrane-associated lipids.[122-127] Also present upstream of CAAX in some Rho GTPases is a second element of one or two cysteine residues that undergo post-translational modification by the fatty acid palmitate. Ras proteins that undergo the CAAX-signaled modifications but harbour PBR mutation or lack palmitoylated cysteines are mislocalized and are significantly compromised in their biological activities. A third sequence element that flanks the previously described elements may also contribute to dictating the precise subcellular localization of Ras and Rho GTPases, however, these sequences are largely uncharacterized.[128-130] The proper subcellular placement of Ras superfamily member proteins is critical for co-localization with activators and effectors that will ultimately result in appropriate downstream signal transduction.

1.3.1 Overview of the Rho GTPase sub-family

The Rho GTPase sub-family of Ras has been implicated in a wide variety of cellular processes, including cytoskeletal organization, cell adhesion to the substratum, cell polarity, and transcriptional activation.[131] Functional information on Rho-family studies has largely come from studies of RhoA, Rac1 and Cdc42. All three share common growth-promoting and anti-apoptotic functions, as well as regulation of gene expression, through activation of signaling molecules such as serum response factor, NF-κB, the stress-activated protein kinases and cyclin D1.[115, 127, 132] Additionally, they all
promote actin cytoskeleton reorganization, however, they differ in their ability to affect cellular morphology and motility.[131, 133]

RhoA shares a significant amino acid sequence identity with RhoB and RhoC (~85%); and all three Rho-related proteins promote actin-myosin contractility and, consequently, the formation of stress fibers and focal adhesions, regulating cell shape, attachment and motility.[134-138] Rac1 promotes actin polymerization and the formation of lamellipodia, which are curtain-like extensions that consist of thin protrusive actin sheets at the leading edge of migrating cells.[139] Cdc42 causes formation of filopodia, which are thin, finger-like cytoplasmic extensions that contain tight actin bundles and might be involved in the recognition of the extracellular environment.[140-142] The cytoskeletal rearrangements caused by activation of Rho GTPases play a key role in the process of cell motility. It is the adhesion, subsequent loss of attachment, and re-adhesion of lamellipodia and filopodia at a cell’s leading edge to the substratum that result in the coordinated and polarized movement of a cell.[143-145]

1.3.2 Rac1 Sub-class of Rho GTPases

Rac1 GTPase is ubiquitously expressed and interacts with specific effectors through domains that subsequently activate a multitude of signaling cascades. These downstream pathways ultimately influence diverse physiological outcomes, including cell–cell adhesion in epithelial cells in addition to its effects on the actin cytoskeleton and motility.[113, 131] A family of p21 activating kinases (PAK) was among the first described Rac1 effector proteins.[146, 147] PAK kinase activity is stimulated after
binding to GTP-bound Rac1, and leads to cytoskeletal dynamics, adhesion, and transcription.[146, 147] Rac1 signals through PAK to activate c-Jun N-terminal kinase (JNK), placing Rac1 between Ha-Ras and MEKK in a signaling cascade from growth factor receptors and v-Src to JNK activation. In addition, Rac1, through PAK, can influence transmembrane guanylyl cyclase activity and the second messenger cGMP production and mediates canonical JNK regulated Wnt-signaling to the TCF transcription factor.[148-150] Rac1 has also been shown to influence nuclear signaling through its effectors MLK2/3, which have been shown to activate the JNK pathway.[151, 152] It was recently shown that, following Rac1 activation, PAK1 participates in the destabilization of E-cadherin, and subsequently disruption of cell-cell contacts.[153] Rac1 signaling via the PAK effector proteins is described in greater detail in the next section.

Rac1 signaling can be important for cellular transformation via modulation of anti-apoptotic and cell cycle machineries. Rac1 positively regulates transcription at NFκB transcription factor-dependent promoters and facilitates phosphatidylinositol-3 kinase (PI3K)-dependent activation of AKT ser/thr kinase, thereby permitting the survival of transformed cells. Rac1 can also influence transformation through regulation of cyclin D1, a cell cycle protein that is frequently overexpressed in cancer.[149, 154-159] Rac1 is a critical component of a complex signaling network, given its ability to directly or indirectly impact a vast array of cellular signals.

Expression of a constitutively active form of Rac in Madine–Darby canine kidney (MDCK) cells or keratinocytes resulted in an increase in E-cadherin complex members and filamentous actin (F-actin) at cell–cell contacts, while a dominant negative mutant
was found to disrupt cell–cell adhesions.\cite{160-162} The cellular effects of Rac are dependent on numerous factors, including the cell-type, the type of stimulus, the subcellular localization, and concentration. Given the importance of Rac, disruption of the natural balance of Rac (and all small Rho GTPases) in a cell will ultimately lead to phenotypes of invasion and metastasis.\cite{131}

The process of cellular invasion is a complex state that involves alterations in cell–cell and cell–substrate adhesion, remodeling of the extracellular matrix, reorganization of the actin cytoskeleton, and an increase in cell motility. Identification of the Rac-specific GEF Tiam1 (T-lymphoma invasion and metastasis) in a retroviral insertional mutagenesis screen provided the first evidence of a role for Rac in invasion. Virus-infected T-lymphoma cells were selected repeatedly for \textit{in vitro} invasion through a layer of fibroblasts and the proviral insertions in invasive clones were used to identify the Tiam1 gene.\cite{163} Subsequently, Rac, and later Cdc42, were shown to provide the same invasive potential to these T-lymphoma cells.\cite{164, 165} Tiam1 activation is initiated upon localization to the plasma membrane through binding of its PH domain to the PI3-K lipid products.\cite{166, 167} Recent studies have shown that Tiam1 activation increases cellular migration and \textit{in vitro} invasion of a murine breast cancer epithelial cell line, SP-1, by mediating the effects of hyaluronic acid (HA) downstream of the HA receptor, CD44, and the cytoskeletal protein ankyrin.\cite{168, 169} Conversely, Tiam1 has also been shown to inhibit the migration of NIH3T3 fibroblasts through fibronectin-coated filters in an ECM-dependent manner, and to abrogate HGF/SF-induced scattering in Ras-transformed MDCK cells.\cite{170, 171} On fibronectin and laminin, Tiam1/Rac signaling inhibits invasion, while on collagen it favors motility.\cite{167} Our lab has recently shown
that, upon Wnt3a stimulation, Tiam1 is recruited to the promoter of canonical Wnt target genes and can form a complex with Rac1 and β-catenin.[172] These studies implicate Tiam1 as a Wnt-dependent contributor to tumourigenesis via Rac1 activation.

Several other studies provide further evidence for the role of Rac in cellular processes pertaining to motility and invasion. Expression of the laminin-receptor α6β4 integrin in the breast carcinoma cell line MDA-MB-435 promotes cellular invasion in a Rac and PI3-kinase-dependent manner.[173] Additionally, in T47D breast carcinoma epithelial cells, activated mutant forms of Rac and Cdc42 induce invasion through a collagen matrix. This invasion can be blocked by PI3-K inhibitors which, therefore, places PI3-kinase downstream of Rac and Cdc42 in this system.[156] Fibroblasts that are deficient in the tumour suppressor gene PTEN are more motile and contain higher levels of active Rac and active Cdc42 than wild-type cells. The motile behavior of these cells can be suppressed by Rac and Cdc42 dominant negative mutants.[174] Since PTEN is a lipid phosphatase that hydrolyzes PI(3,4,5)P3 generated by PI3-K, these experiments implicate PI3-kinase in cell migration in addition to Rac and Cdc42.[175, 176]

Numerous studies have established a role for Rho GTPases in integrin-mediated motility. Upon binding to ECM proteins, integrins assemble a focal adhesion complex, containing cytoskeletal proteins such as talin, α-actinin, vinculin, and paxillin, as well as focal adhesion kinase (FAK). Activated FAK then recruits Src family kinases, such as Src and Fyn, to the focal adhesion (FA), resulting in the phosphorylation of paxillin.[177] Expression of dominant negative forms of the Rho GTPases has been shown to interfere with these processes.[178] Furthermore, a complex of adaptor proteins has been shown to mediate integrin-dependent signaling upstream of Rac. FAK and Src kinases are
involved *in vivo* in the phosphorylation of the adaptor protein CAS (cellular apoptosis susceptibility), and phosphotyrosine residues of CAS are then recognized by the SH2 domains of the Crk proto-oncogene. When expressed in COS cells, CAS and Crk cooperate in stimulating migration toward vitronectin, which is abrogated by dominant negative RacN17.[179] In addition, Crk can bind to the docking protein DOCK180 (which was shown to activate Rac), and DOCK180 can enhance the migration-promoting potential of CAS and Crk.[180, 181]

1.3.3 *Effectors of Rac1 GTPase*

Several Rac1 effectors that mediate numerous cellular processes have been identified. Some effectors are specific for Rac1, while others can be activated by other small Rho GTPases, such as Cdc42 and RhoA.[131] Rac binds to WAVE (WASP-like Verprolin-homologous protein), localizing to membrane ruffles, and promotes actin polymerization in lamellipodia through activation of the Arp2/3 complex.[182] As described previously, cellular motility is achieved, in part, by cytoskeletal changes that are regulated by members of the PAK family of serine/threonine kinases.[131] PAKs are downstream effectors of Rac and Cdc42. Cell polarization and cell-cell adhesion are promoted by scaffolding proteins IQGAP and Par-6, also Rac1 effectors. Dysregulation of these effectors and subsequently cell-cell contacts suggests that Rac1-mediated motility is promoted through the disruption of the normal organization of cells.

PAK exerts its physiological effects on actin dynamics and adhesion through at least three mechanisms.[183-185] First, PAK inactivates MLCK (myosin light chain
kinase) by phosphorylation, and results in a decrease in MLC phosphorylation. Therefore, PAK-mediated inhibition of MLCK leads to a potential for motility by the disassembly of stress fibers and focal adhesions. In kidney cells that express activated PAK, decreased MLC phosphorylation resulted in a decrease in cell spreading on fibronectin. Expression of a kinase-defective PAK mutant in a breast cancer cell line, MDA-MB-435, restricted cellular invasion into a gel matrix substrate.[186] Active PAK has also been shown to increase MLC phosphorylation in human microvascular endothelial cells (HMEC-1), and, while PAK is not associated with lamellipodia formation, it still contributes to cell motility by its role in cell adhesion and contraction. Additionally, MLC phosphorylation was found increased in PAK-expressing MIH3T3 fibroblasts, resulting in increased directionality of haptotactic movement through a collagen gradient. Secondly, control of the actin cytoskeleton by PAK is also achieved through the phosphorylation of LIM-kinase, and subsequent phosphorylation of the actin-depolymerizing protein cofilin causing extreme membrane ruffling. Membrane ruffling is another phenotype associated with motility.[187] Thirdly, PAK can mediate its effect on actin dynamics and motility through a positive feedback loop with Rac1. A Rac GEF, PIX (also known as Cool) has been shown to interact with PAK and target it to focal contacts (FCs), which are integrin-dependent sites linked to the actin cytoskeleton.[188-192] PIX also interacts with the G-protein-coupled receptor kinase-interacting protein (GIT1), which when overexpressed, leads to loss of paxillin and the dissolution of FCs, and promotes cell migration.[193] Taken together, PAK plays a critical role though a variety of different regulatory mechanisms and signaling cascades in the promotion of Rac-dependent motility.
The scaffolding effectors of Rac, IQGAP1 and IQGAP2, regulate cell–cell adhesion through actin polymerization and sequestration of the adhesion complex molecule β-catenin.[194-197] Specifically, the IQGAP protein competes with α-catenin for binding to β-catenin, which prevents the attachment of the E-cadherin/α-catenin/β-catenin complex to the actin cytoskeleton and therefore disrupts cell–cell contacts.

Another scaffolding protein, Par-6, is an effector that is shared between Rac1 and Cdc42 as identified by yeast two-hybrid screens.[198, 199] Par-6 binds to atypical protein kinase C (aPKC) and forms a complex and, upon overexpression by Rac1, can disrupt the formation of tight junctions in MDCK cells.[200] This loss of cell-cell contacts results in the loss of contact inhibition and excessive cell growth.[131]

1.3.4  Rac1b GTPase splice variant of Rac1

An alternatively spliced variant of the Rac1 gene was found to be preferentially expressed in breast and colon tumours.[201, 202] At both the RNA and protein levels, the Rac1b variant was found to be overexpressed in malignant colon carcinomas when compared to levels in benign tissue.[203] The Rac1b transcript contains an additional 57 nucleotides corresponding to the inclusion of exon 3b into the Rac1 mRNA, which encodes a protein with an in-frame insertion of 19 amino acids between Rac1 residues 75 and 76.[204] This insertion is positioned immediately carboxyl-terminal to the switch II domain (Rac1 residues 60–76), which, in addition to the switch I domain (Rac1 residues 30–38), constitute the regions that change in conformation during GDP/GTP cycling. These regions are also important for Rac1 interaction with regulators and effectors.[112]
Therefore, the Rac1b insert alters the intrinsic biochemical properties of the protein and affects its interaction with regulators and effectors. As a result, \textit{in vitro} and \textit{in vivo} studies have demonstrated that Rac1b possesses an impaired intrinsic GTPase activity, and yet is still responsive to GAP activity. Thus, unlike the constitutively active mutants (G12V or Q61L) of Rac1, Rac1b can still be inactivated by GAP activity. However, Rac1b does not appear to be sequestered to the cytosol in an inactive state, as RhoGDI cannot bind Rac1b, and behaves as if it were constitutively active.[204, 205]

With the inclusion of exon 3b within a key functional region, there exists several differences between Rac1 and Rac1b. Classic Rac signaling pathways that are not activated by Rac1b include lamellipodia formation, the activation of PAK1, or c-Jun-NH2-kinase activities.[206] However, Rac1b retains the ability to stimulate the NFkB pathway and was shown to induce InBa phosphorylation, nuclear translocation of RelA, and transcriptional stimulation of a consensus NFkB promoter in a luciferase reporter construct.[207, 208]

In addition to functional differences between Rac1 and Rac1b, the mechanisms by which these two proteins are regulated are dissimilar. It appears likely that a large contributor to the effects that Rac1b has on tumourigenesis may stem from the stability of Rac1b over Rac1.[209] Visvikis \textit{et al}. (2008) demonstrated that Rac1, and not Rac1b, is ubiquitinated on Lys-147 through a JNK-dependent process.[209] Interestingly, like Rac1, Rac1b is found to be largely associated with the plasma membrane, a requirement for Rac ubiquitination; and the Lys-147 is accessible, and in similar conformation between both proteins. However, Rac1b ubiquitination could be stimulated through co-expression with the constitutively active Rac1 mutant (Rac1L61) suggesting a Rac1
feedback loop via downstream signaling. Since Rac1 ubiquitination could be impaired in experiments using a JNK inhibitor (SP600125) in a dose-dependent and time-dependent manner, the JNK pathway appears to render Rac1 sensitive to proteasomal degradation.[209] Therefore, Rac1b escapes degradation by way of its inability to activate JNK signaling.

Rac1b has been implicated in mediating the effects of the tumour microenvironment. Radisky et al. (2005) investigated the matrix metalloproteinase-3 (MMP-3) stromal enzyme in a component of the breast tumour microenvironment.[205] This study found that MMP-3 induced the expression of Rac1b which, together, stimulated the mitochondrial production of reactive oxygen species (ROS). The MMP-3-induced ROS caused DNA damage, genomic instability, and the transcription factor Snail which led to epithelial-mesenchymal transition (EMT).

Aside from this thesis, the only other protein with a statistical association to Rac1b in colon cancer is with a mutant form of the BRAF, which harbours an oncogenic V600E mutation.[206] The BRAF serine/threonine kinase is a member of the mammalian Raf gene family, and an integral part of the Raf/MEK/ERK pathway.[16, 19, 210] The BRAF^{V600E} stimulation of the MEK/ERK pathway does occur independent of Ras, however, at such low levels, mutant BRAF requires an additional mechanism for progression to Rac1 or NFκB activation. Matos et al. (2008) demonstrated that, in addition to their association, Rac1b and BRAF^{V600E} cooperate to sustain cell viability in colorectal cancer.[206]
The molecular regulation of Rac1b expression in tumours, while absent in normal tissue, is one complexity with few defining studies in literature. Goncalves et al. (2009) recently examined three cell lines (SW480, DLD-1, and HT29) for the presence of mutations in the Rac1 genomic fragment 286bp upstream to 224bp downstream of exon 3b, however, only wild-type sequences were found. They next investigated if the alternative splicing events were modulated by changes in the protein expression of individual splicing factors, and identified ASF/SF2 and SRp20 to exhibit antagonistic effects on alternative spliced exon 3b. Comparison of transcript and protein levels of these splicing factors in the Rac1b-expressing (HT29) and Rac1b non-expressing (SW480) colon cancer cell lines, and corroboration with experiments using a Rac1 mini-gene, demonstrated that ASF2/SF2 acts as an enhancer of for exon 3b inclusion, whereas SRp20 is a silencer promoting exon 3b skipping. Additionally, this study demonstrated that Rac1b expression could be regulated by the PI3-kinase and canonical Wnt signaling pathways. They found that the SFSR3 gene, which encodes for SRp20, is a target of β−catenin/Wnt signaling; therefore, inhibition of Wnt signaling, decreased SRp20, and increased Rac1b expression. Conversely, experiments utilizing a PI3-kinase inhibitor (LY294002) or overexpression of PTEN led to increased expression of endogenous ASF2/SF2, and subsequent increase in Rac1b expression. The experiments used to connect SRp20 and Wnt were performed in HT29 cells. Interestingly, HT29 cells harbour an APC mutation, and therefore, constitutive activation of β−catenin/TCF signaling, which should result in a constitutive suppression of Rac1b expression.
of the greatest endogenous expression of Rac1b, these results appear inconsistent. The authors concede that mechanisms underlying Rac1b overexpression remain unclear. However, they offer the fact that in addition to increased ASF/SF2 expression, HT29 carries the oncogenic B-RafV600E mutation, which is known to correlate with Rac1b overexpression, as plausible justifications.[206] Nevertheless, currently, this study provides the only explanation for the preferential expression of Rac1b in colon tumours, and likely other tumours as well.

1.4 Rho GTPases and cell migration

Intrinsic and directed cell motility can be influenced by polarity signaling machinery that arises from the regulated formation of lamellipodia. Polarization of a cell results in the establishment of the leading and trailing edges. The partitioning defective (Par) complex, which consists of PAR3, PAR6 and atypical protein kinase C (aPKC) is activated by the Rho GTPase, Cdc42, at the leading edge to stabilize microtubules.[213-215] Cdc42 interaction with the Par complex aids in the recruitment of the GEF T-lymphoma invasion and metastasis-inducing protein-1 (TIAM1) to the leading edge where it activates Rac1 GTPase.[216] A depletion of either TIAM1 or Par3 results in a decrease in front-rear polarization, an increase in random cell migration and reduces sensitivity to chemotactic cues.[217, 218] Activation of the RhoA effector, Rho-associated protein kinase 1 (ROCK1) can antagonize Rac1 activation by phosphorylating Par3 and disrupting the complex, preventing TIAM1 activation of Rac1.[171, 217-219]
As the key regulator of cell polarization, Cdc42 can also activate Rac1 by regulating the activity of p21-activated kinase 1 (PAK1), which subsequently recruits the Rac1-GEF, βPIX (also known as ARHGEF7) to the leading edge.[220] The regulation of Rac1 by Cdc42 in the migrating cell directs adhesion and cytoskeletal remodeling that is necessary for lamellipodium formation.[171, 221]

The trailing edge contributes to cellular motility and its directional orientation by generating contracting forces that pull the rear forward while restricting the formation of protrusions in the rear. Through its effector, ROCK1, RhoA activity results in the phosphorylation of myosin phosphatase and the regulatory light chain on myosin II, which increases actin-myosin contractility, disassembles focal adhesions, and triggers tail retraction.[222, 223]

Each step of the cell migration cycle act to regulate Rho GTPase signaling to achieve a well-balanced and directionally-persistent motility pattern. Novel insights into the balance between Rac1 and the canonical Wnt signaling pathway are addressed in this thesis.
1.5 Rac1/Wnt crosstalk in colon cancer

Our lab has previously demonstrated a novel crosstalk between the canonical Wnt signaling pathway and Rac1/Rac1b GTPases.[212, 224] We observed that a constitutively active Rac1 mutant (V12Rac1) and stabilized β-catenin cause a synergistic induction of TCF-dependent transcription in colon cancer cells. Expression of a dominant-negative mutant of TCF-4 was able to block this effect, indicating involvement of TCF-4 as a downstream target of Rac1. Conversely, in HCT116 (ATCC) colon cancer cells with constitutive activation of the Wnt pathway, a dominant-negative Rac1 mutant (N17Rac1) was able to downregulate endogenous Wnt signaling. We further demonstrated that Rac1 and Rac1b-mediated transcriptional activation depended on nuclear localization, since mutation in the nuclear localization signal (NLS) sequence in the C-terminal polybasic region (PBR) of Rac1 and Rac1b was unable to facilitate Wnt activation. We have shown that Rac1 and its GEF, Tiam1, associates with β-catenin and TCF4 at Wnt-responsive promoters (c-myc and Cyclin D1).[172] Additionally, V12Rac1 overexpression promoted redistribution of β-catenin from the cell membrane to the nucleus and reduced endogenous E-cadherin expression. Co-immunoprecipitation studies demonstrate that β-catenin and TCF-4 associate specifically with Rac1 only in its active, GTP-bound state.

We recently provided evidence that suggests Rac1b overexpression facilitates tumour progression by enhancing Dvl-3-mediated Wnt pathway signaling and induction of Wnt target genes specifically involved in decreasing the adhesive properties.[212] We observed that Rac1b downregulated endogenous E-cadherin expression and decreased
cell-cell adhesion of HCT116 colorectal cancer cells and this effect was further augmented by combined action of Rac1b and Dvl-3. We demonstrated that mutation of the nuclear localization signal sequence in the C-terminal polybasic region of Rac1b resulted in a complete loss of Rac1b stimulatory effects on TCF-mediated gene transcription and the suppressive effects seen on cell adhesion, indicating the importance of nuclear and membrane localization of Rac1b. Cumulatively, these studies have established crosstalk mechanisms between Rac1/Rac1b and canonical Wnt signaling.

1.5 Hypothesis and Objectives

Rac1 and Rac1b GTPases cooperate with the canonical Wnt signaling pathway to promote tumourigenesis in colon cancer. We hypothesize that Rac1 GTPase and its splice variant, Rac1b, cooperate with the canonical Wnt signaling pathway to promote tumourigenesis in colon cancer through the recruitment of specific subsets of target genes.

To characterize the contribution of Rac1 and Rac1b GTPases in colorectal carcinogenesis, the following aims were formulated:

I. Investigation of molecular mechanisms involved in Rac1 GTPase-mediated tumourigenesis in colorectal cancer cells with aberrantly active canonical Wnt signaling

II. Investigation of molecular mechanisms involved in Rac1b GTPase-mediated tumourigenesis in colorectal cancer cells with aberrantly active canonical Wnt signaling
CHAPTER 2

Rac1 GTPase cooperates with the canonical Wnt signaling pathway to promote tumourigenesis in colon cancer cells

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Chapter 2

Rac1 GTPase cooperates with the canonical Wnt signaling pathway to promote tumourigenesis in colon cancer cells

2.1 SUMMARY

The canonical Wnt signaling pathway is aberrantly active in the vast majority of colorectal tumours. Rac1 GTPase expression is upregulated in colon tumours. Our lab has demonstrated a novel crosstalk between Rac1 GTPase and the canonical Wnt signaling pathway in colon cancer; however, the specific mechanism underlying this cooperation was not completely elucidated. In this study, we investigated novel transcriptional targets of this crosstalk following induction of the constitutively active mutant, V12Rac1, in a stable-inducible HCT116 colon cancer cell line. Although, by microarray analysis, a significant change in the steady state transcript levels of a single gene was not detected, we identified a gene set comprising 13 genes that were differentially expressed in concert to promote cellular migration. Rho GTPases are classically known to be activated and initiate their downstream signaling at the plasma membrane; however, increasing evidence suggests that nuclear localization is critical for many of their functions. We used a V12Rac1 construct with the polybasic region (PBR) mutated to six Glutamines (PBRQ) which permits GTP loading, but restricts nuclear entry. In scratch wound assays, while V12Rac1 accelerated migration in the HCT116 colon cancer cell line, the V12Rac1 PBRQ mutant had no effect on motility as compared to the empty vector control. Therefore, it appears that the PBR domain, and ultimately
nuclear localization is critical for Rac1 to regulate cell migration in colon cancer cells. Given the importance of nuclear localization of Rac1 to promote tumourigenic processes, such as cell migration, our lab previously investigated the role of nuclear Rac1 at the promoter of Wnt-responsive genes. We determined that the Rac1 GTPase-specific GEF, Tiam1, can upregulate a TCF-responsive promoter, TopFlash, in HCT116 cells. In this study, we sought to confirm the reverse-specificity of this finding through RNAi experiments. We determined that Tiam1 knockdown resulted in a modest reduction in the TopFlash luciferase reporter assay. This finding complemented work completed in our lab in which Tiam1 transfection assays, in addition to ChIP experiments, demonstrated that Tiam1 potentiates Rac1-mediated stimulation of β-catenin/TCF-dependent transcription. Taken together, this study demonstrates a requirement for nuclear entry of Rac1 in its modulation of cell migration, likely through the subtle regulation of several downstream targets.

2.2 INTRODUCTION

Rac1 GTPase (Rac1) is a member of the Rho family of small GTP binding proteins.[131] In response to a variety of external stimuli, Rac1 cycles between inactive (GDP-bound) and active (GTP-bound) forms. This cycle is regulated by guanine exchange factors (GEFs) that activate Rac1, GTPase-activating proteins (GAPs) that convert Rac1 to its inactive GDP-bound form and guanine nucleotide dissociation inhibitors (Rho-GDIs) that inhibit Rac1 activation by sequestering it in the GDP state in the cytoplasm. Upon GTP-loading, the Rac1-GTPase acquires a conformational change that allows it to interact with
a variety of effector proteins. Rac1 is involved in signal transduction pathways that induce formation of lamellipodia, stimulate cell proliferation and cell motility, and activate NF-κB and JNK/MAPK protein kinases. Overexpression of Rac1, as well as altered expression of Rac1-specific regulators (GEFs, GAPs, and GDIs) or downstream effectors have been found in several cancers. Studies using activated point mutants of Rac1 (p.G12V or p.Q61L) indicate that aberrant activation of Rac1 can alter many cellular processes important for cancer progression.

The canonical β-catenin-mediated Wnt signaling pathway is aberrantly activated in the vast majority of colorectal cancers.[27] Wnt binding to frizzled receptors induces phosphorylation of Dishevelled (Dvl) protein, subsequently leading to stabilization and nuclear accumulation of β-catenin. Nuclear association of β-catenin with the TCF/LEF family of transcription factors leads to activation of Wnt target genes. Wnt signaling can also lead to the non-canonical (β-catenin independent) activation of the Wnt pathway involved in planar cell polarity, which is primarily implicated in developmental processes. Dvl is a key component that participates in both the non-canonical and canonical Wnt pathways, raising intriguing possibilities for its functional contribution at signal crossroads. Discovery of new protein partners, as well as the elucidation of novel functional interactions for known Wnt components, has implicated additional signaling pathways as potential regulators of Wnt signaling. These include the TGF-β, RAR, estrogen receptor and MAPK signaling pathways.

Our lab has demonstrated a novel crosstalk between the canonical Wnt signaling pathway and Rac1 GTPases.[172, 224, 225] We observed that a constitutively active Rac1 mutant (V12Rac1) and stabilized β-catenin cause a synergistic induction of TCF-
dependent transcription in colon cancer cells. Expression of a dominant-negative mutant of TCF-4 was able to block this effect, indicating involvement of TCF-4 as a downstream target of Rac1. Conversely, in HCT116 colon cancer cells with constitutive activation of the Wnt pathway, a dominant-negative Rac1 mutant (N17Rac1) was able to downregulate endogenous Wnt signaling. We further demonstrated that Rac1-mediated transcriptional activation depended on nuclear localization, since mutation in the nuclear localization signal (NLS) sequence in the C-terminal polybasic region (PBR) of Rac1 was unable to facilitate Wnt activation. We have shown that Rac1 and its GEF, Tiam1, associate with β-catenin and TCF4 at Wnt-responsive promoters (c-myc and Cyclin D1). Additionally, V12Rac1 overexpression promoted redistribution of β-catenin from the cell membrane to the nucleus and reduced endogenous E-cadherin expression. Co-immunoprecipitation studies demonstrate that β-catenin and TCF-4 associate specifically with Rac1 only in its active, GTP-bound state. While Rac1 GTPase and canonical Wnt signaling can independently facilitate tumourigenic properties, it is clear that their crosstalk results in a synergistic response, however, the specific mechanisms are unclear. The focus of the work presented in this chapter was to identify novel transcriptional targets of this crosstalk, and to determine how these two pathways might converge to promote carcinogenesis.
2.3 MATERIALS AND METHODS

2.3.1 Materials

Tissue culture reagents and fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies (Burlington, Ontario). Wild-type (WT) and activated mutant FLAG-tagged V12Rac1 constructs, and rabbit polyclonal caveolin1 antibody, were gifts from Dr. Jeff Wrana (SLRI, Mount Sinai Hospital, Toronto). Dominant-negative N17Rac1 was purchased from Upstate Biotechnology (Lake Placid, NY). pTopFlash and pFopFlash luciferase constructs, and Δ89β-catenin, WT TCF4, and dominant-negative TCF4 expression vectors were gifts from Dr. Benjamin Alman (Hospital for Sick Children, Toronto). Wild-type β-catenin construct was a gift from Dr. Liliana Attisano (University of Toronto, Canada). –1745CD1-LUC reporter plasmid was a gift from Dr. Tetsu Akiyama (University of Tokyo).

2.3.2 Cell Culture

HCT116 and HT29 cells were obtained from the ATCC, Rockville, MD. Both cell lines were cultured in McCoy’s 5A media and was supplemented with 10% FBS. Both cell lines were cultured at 37°C in a humidified atmosphere of 5%.

2.3.3 Rac1 Activation Assay

Rac1-GTP levels were determined using a non-radioactive Rac activity Assay kit (Upstate Biotechnology). Briefly, cell lysates were immunoprecipitated with a Glutathione S-transferase (GST) fusion-protein corresponding to the p21-binding domain
(residues 67-150) of human PAK1 bound to glutathione-agarose, run on 12% SDS-PAGE, and western blotted using monoclonal anti-Rac1 antibody. In vitro GTPγS and GDP protein loading were included for positive and negative controls, respectively.

2.3.4 Luciferase Reporter Gene Assays

Transfection experiments were carried out using Lipofectamine 2000 reagent (Invitrogen Life Technologies) according to manufacturer’s instructions. Cells were seeded in 24-well plates at a density of $2 \times 10^5$ cells per well 24 hours prior to transfection. Luciferase constructs (0.1 µg) containing either four tandem wild-type (pTopFlash) or mutant (pFopFlash) TCF4 binding sites, or cyclin D1 (-1745) promoter were transfected into cells to determine luciferase reporter activity. In addition, cells were co-transfected with an internal control (30 ng of pCMVβ-gal), along with pcDNA3.1 or dominant-negative TCF4 (DNTCF). The total amount of DNA per transfection was held constant at 1 µg total plasmid DNA by co-transfection of appropriate amounts of empty vector. Luciferase and β-galactosidase activity was measured 24 hr after transfection using commercially available kits from Promega (Madison, WI) and Stratagene, respectively. Luminescence was quantitated using a Berthold 96-well microplate luminometer. All transfections were carried out in triplicate on at least three independent occasions, and error bars represent SEM (standard error of the mean).

2.3.5 Western Blot Analysis

Cell extracts were prepared using Radio-Immunoprecipitation Assay (RIPA) buffer (0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1x phosphate buffered
saline (PBS), “Complete” mini EDTA-free protease inhibitor tablet (Roche, Mannheim, Germany). Immunoblotting was conducted using 10 µg of cell lysate, and resolved on a 10% SDS-PAGE gel. Resolved proteins were transferred to PVDF nylon membranes (Amersham Pharmacia Biotech, Quebec, Canada), and non-specific reactivity was blocked with Tris-buffered saline containing 0.1% Tween-20 and 5% non-fat dried milk. Antibodies specific for Myc tag (Santa Cruz Biotechnology) and β-actin were used according to the manufacturer’s specifications. Bound proteins were detected with appropriate horseradish peroxidase-conjugated secondary antibodies in the enhanced chemiluminescence system (ECL) (Amersham Pharmacia Biotech).

2.3.6 Generation of stable-inducible cell line

T-REX system (Invitrogen Life Technologies) was employed to generate HCT116 cell lines with stable inducible expression of V12Rac1 according to manufacturer's instructions. HCT116 cells were transfected in 10-cm dishes with 13 µg pcDNA6/TR per plate using LipofectAMINE 2000 (Invitrogen) transfection reagent. Forty-eight hours after transfection, cells were re-seeded into 15-cm plates at low density and transfectants were selected for in the presence of 5 µg/ml blasticidin. Resistant colonies were screened by Western blotting. Positive clones were subsequently transfected with 2 × myc-V12Rac1/pcDNA4/TO in 10-cm dishes as described above and selected for with 300 µg/ml zeocin. To screen resistant colonies, cells were treated with either 1 µg/ml doxycycline (dox) or vehicle (water), and protein lysates were harvested after 24 hours and analyzed by Western blotting. Positive clones were maintained in blasticidin and zeocin.
2.3.7 Quantitative Reverse Transcriptase (RT)-PCR

Total RNA was isolated using an RNeasy Mini kit (Qiagen, Valencia, CA) and analyzed by real-time RT-PCR. For RNAi experiments, cells were transfected with either: SMARTpool Rac1-specific siRNA, ONTARGETplus SMARTpool Tiam1-specific siRNA, siCONTROL non-silencing siRNA as a control, GAPDH-specific siRNA as a control (Dharmacon, Lafayette, CO.) or mock transfected using LipofectAMINE 2000 according to manufacturer's protocol. After 48 hours (Rac1 siRNA experiments) or 64 hours (Tiam1 siRNA experiments), total RNA was isolated and analyzed by real-time RT-PCR. The following primer pairs were used to amplify cDNA: c-Myc forward, 5'-GCCAAGCTCGTCTCAGAGAAG-3', and reverse, 5'-CAGAAGGTGATCCAGACTCTG-3'; Rac1 forward, 5'-ATGCAGGCCATCAAGTGTGTG-3', and reverse, 5'-ATGCAGGCCATCAAGTGTGTG-3'; Tiam1 forward, 5'-AGACGTACTCAGGCCATGTC-3', and reverse, 5'-ACCCAAATGTCGCAGTCAGG-3'; β-actin forward, 5'-ATCATGTTTGTGACAGTCTCAGAG-3', and reverse, 5'-CATCTCTTGCTCGAAGTCCA-3', and; GAPDH forward, 5'-ACCACAGTCCATGCACCATC-3', and reverse, 5'-TCCACCACCCCTGGTTGCTGTA-3'.

For statistical analyses, unpaired Student's t-test was performed.

2.3.8 Microarray Analysis

Genomic RNA expression analysis was performed using the University Health Network (UHN) Human 27k array, which is a set of two arrays; one array contains approximately
8,000 expressed sequence tags (ESTs) and annotated genes spotted in duplicate, and one array containing approximately 22,000 ESTs and genes spotted once. In total, there are 22,126 unique ESTs or genes that make up the Human 27k array. RNA (200 ng) isolates from the HCT116-TR-V12Rac1 colon cancer cells under various conditions were analyzed 24h post-treatment per the instructions of UHN Microarray Centre, Toronto, ON, Canada. Each microarray experiment was performed in triplicate.

Raw probe intensity values, provided by the UHN Microarray Centre, were analyzed using Microsoft Excel (Microsoft Corporation, CA under the supervision of Dr. Laurent Briollais. The raw data were analyzed using a modified t test formula, and genes that met the cut-off value (≥2) were ranked by fold change. Candidate genes were validated by QRT-PCR using the same microarray sample set, and using an independent sample set following V12Rac1 induction, and in another independent sample set using parental HCT116 cells transiently transfected with V12Rac1 (to rule out drug effects).

2.3.9 Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) is a computational method that determines whether an a priori defined set of genes shows statistically significant, concordant differences between two biological states (e.g. phenotypes). Subramanian et al. (2005) describe the algorithm used in the Gene Set Enrichment Analysis.[226] The GSEA software (Broad Institute/Massachusetts Institute of Technology) is freely available for download. Gene sets were generated using Gene Ontology identifiers. This analysis was performed with the assistance of Dr. Laurent Briollais, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada.
2.3.10 Wound Scratch Assay

Cells were cultured to confluence in 6-well dishes. Using a marker, a line was drawn on the underside of the plate to ensure consistency when capturing images. Using a sterile 200 ml pipet tip, three separate wounds were scratched through the cells, moving perpendicular to the line drawn. Images of the cells at 10X using phase contrast were captured using the QCapture software above and below the line for each well. Measurements were taken in Adobe Photoshop CS3 (Adobe Systems Incorporated, San Jose, CA, USA), and statistical analysis (t test) was performed in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).
2.4 RESULTS

2.4.1 Generation and characterization of a HCT116 cell line with stable inducible expression of V12Rac1

The main objective of this chapter was to identify novel transcriptional targets of V12Rac1 induction in a colon cancer cell line with dysregulated Wnt signaling. I first generated a stable-inducible cell line system to control for genetic variability inherent in cancer cells. A Rac1 mutant defective in intrinsic GTPase activity and existing constitutively in the GTP-bound caused by a p.Gly12Val missense substitution, was stably co-transfected into the HCT116 colon cancer cells with the Tet-Repressor (HCT116-TR-V12Rac1). In these cells, transcriptional repression of the V12Rac1 promoter is removed and expression is induced following treatment with doxycycline. Clones were examined by Western blotting and selected based on the greatest level of V12Rac1 expression following doxycycline treatment provided there was an observed total absence of expression when treated with a drug-vehicle control (distilled water). Clones #1, 2, 3, 5, and 6 were maintained for this study, while all others were discarded (Figure 2.1). At both the transcript and protein levels, V12Rac1 induction was observed with as little as 0.5 μg/ml doxycycline (Figure 2.2).

The HCT116-TR-V12Rac1 cell line was examined to confirm the ability of V12Rac1 to activate TopFlash, and Cyclin D1 promoters under various concentrations of doxycycline (0.00005 μg/ml, 0.05 μg/ml, and 0.1 μg/ml), as compared to treatment with the drug vehicle (double-distilled water) (Figure 2.3a). This activation was inhibited when cells were transiently transfected with a dominant-negative TCF4 construct,
Figure 2.1  HCT116 colon cancer cell line clones with stable-inducible expression of V12Rac1.
Western blot analysis of cell lysates at 24h post-induction with 0.01µg/ml doxycycline (D) as compared to treatment with the drug vehicle control, water (U). Clones without V12Rac1 expression in the uninduced lysates, were maintained and used in this study.
Figure 2.2. V12Rac1 expression inducible at mRNA and protein levels.

(A) Western blot analysis indicates constitutively active Rac1 (V12Rac1) induction with as little as 0.0005 µg/ml doxycycline treatment. Lysates were harvested at 24h post-induction. Rac1 and β-actin are 21 and 44 kDa, respectively. (B) RT-PCR analysis indicates V12Rac1 induction following doxycycline treatment as compared to endogenous Rac1 (uninduced). A slight dose-dependent expression gradient was observed. Lysates harvested 24h post-induction. NTC, no template control.
Figure 2.3  V12Rac1 activates transcription of Wnt-responsive and Cyclin D1 promoters.

(A) TopFlash, FopFlash or (B) Cyclin D1 luciferase reporter constructs were transiently co-transfected with dominant-negative TCF4 following V12Rac1 induction at various doxycycline concentrations (Uninduced [-] = 10 µl H2O, DOX [+]= 0.00005 µg/ml, DOX [++] = 0.05 µg/ml, DOX [+++]= 0.1 µg/ml) for 24h. Luciferase activity is expressed as total relative light units (RLU). Columns, average of experiments carried out in triplicate; bars, SE.
confirming that the observed effects are a result of V12Rac1 cooperating with the canonical Wnt signaling pathway (Figure 2.3b). The 0.05 µg/ml doxycycline concentration was used for microarray analysis experiments.

2.4.2 V12Rac1 induction stimulates the cell migration gene set in a colon cancer cell line with dysregulated canonical Wnt signaling

I sought to identify novel differentially expressed targets following Rac1 induction in the HCT116-TR-V12Rac1 cell line by microarray analysis. In preparation for microarray analysis, I isolated total RNA from HCT-TR-V12Rac1 cells under each of four conditions after 24h (Table 2.1). Each condition was performed in triplicate for a total of 12 test conditions. These twelve conditions were Cy5-labeled and hybridized along with the Cy3-labeled reference control RNA to the Human 27k (Human 19kss + Human 8k) UHN arrays, containing 22 126 unique ESTs or genes. Quality control measures were employed to ensure technical consistency, given the highly sensitive nature of microarray analysis. Examination of the quality (average 260/280 = 2.00) and integrity (Figure 2.4) of the RNA samples by nanodrop provided confidence that the sample preparation was optimal for microarray analysis. Following microarray analysis, one array (Chip 4C) had approximately twice the average number of gene intensities that fell below background, as compared to other replicates. On close visual inspection, it appeared that one grid had diminished fluor intensities, possibly due to poor hybridization, and the array was repeated for the RNA #4C sample.
Table 2.1  Transfection conditions for the HCT116-TR-V12Rac1 colon cancer cell line

<table>
<thead>
<tr>
<th>Sample (Replicates)</th>
<th>Transfection</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA #1 (A,B,C)</td>
<td>pcDNA3.1</td>
<td>Drug vehicle control (ddH₂O)</td>
</tr>
<tr>
<td>RNA #2 (A,C,D)</td>
<td>pcDNA3.1</td>
<td>Doxycycline (0.1 µg/ml)</td>
</tr>
<tr>
<td>RNA #3 (A,B,C)</td>
<td>dnTCF4</td>
<td>Drug vehicle control (ddH₂O)</td>
</tr>
<tr>
<td>RNA #4 (A,B,C)</td>
<td>dnTCF4</td>
<td>Doxycycline (0.1 µg/ml)</td>
</tr>
</tbody>
</table>
To identify candidate genes with differential expression due to the cooperative effects of Rac1 GTPase and the canonical Wnt signaling pathway, we performed a modified t test for three comparisons: Comparison #1 (RNA#2/RNA#1) identifies genes whose expression changes following Rac1 overexpression; Comparison #2 (RNA#3/RNA#1) identifies genes whose expression changes following inhibition of the canonical Wnt signaling through transfection of a dominant-negative TCF4 construct; and Comparison #3 (RNA#4/RNA#2) identifies genes whose expression is dependent on Rac1 and Wnt. The modified t test formula used in this statistical analysis is:

\[
\frac{\hat{\delta}}{\sqrt{B \hat{\sigma}^2 + (1 - B) \hat{\sigma}^2}}
\]

\(\hat{\delta}\) indicates Log2 Fold Change; \(\hat{\sigma}^2\) indicates variance based on data from a single gene; \(\hat{\sigma}^2\) indicates variance based on data from all genes; and \(B\) is a value between 0 and 1 called the “shrinkage factor”. As \(B\) approaches a value of 1, information will predominantly come from the variance for the single gene; when \(B = 0\), the returned value is the standard t-test (or Ordinary t) without shrinkage. The B value is a tool to help avoid eliminating genes based on its own variance due to one replicate chip with global low intensity as compared to its replicates. If one gene’s intensity is low due to technical reasons, then all genes on that specific chip should be affected equally.

Examination of candidate genes that pass statistical thresholds for all three comparisons should identify novel gene targets of the Rac1/Wnt crosstalk while controlling for off-target effects due to in vitro conditions, such as doxycycline treatment.

Candidate genes were selected for validation based on the following criteria:
• a modified $t$ test score greater than 2 for Comparisons #1 and #3
• the probe was annotated to a gene and the full gene sequence was available
• previous characterization in any pathway

The candidate gene list that passed the first criterion included 41 genes (Table 2.2). This list was ordered based on the Comparison #1 fold change, and six genes were selected for further validation by QRT-PCR (Figure 2.5). All six genes met the second and third criteria. However, following QRT-PCR analysis on an independent sample set of total RNA, none of the gene candidates passed the validation experiments (Figure 2.6). We postulated that perhaps the criteria were too strict given the number of conditions and replicates. Therefore, we examined only genes in the Comparison #1 pool that had a modified $t$ test score of two or more, greater than four-fold change in expression, as well as previous characterization in known pathways. This alternate strategy yielded 35 candidates (Appendix 1). We then examined the promoter regions of the 35 genes for putative TCF-binding elements (TBEs), and identified nine candidates with two or more TBEs. Validation experiments by QRT-PCR on these nine candidates in an independent sample set also did not confirm any viable target genes. Of note, comparison #1 results showed V12Rac1 had a fold-change of 2.24 and modified $t$ test score of 1.61 and was validated by QRT-PCR.

Possible reasons for not validating novel targets of Rac1/Wnt include: a) V12Rac1 does not transcriptionally regulate any genes in our cell system at the 24h timepoint; or b) the effect of V12Rac1 on targets is modest relative to the noise inherent in microarray technology. To address this, we postulated that changes in transcriptional expression of individual genes on the array may have been too subtle to be captured by the sensitive
Figure 2.4  Representative Nanodrop electropherograms of total mRNA for microarray analysis. These graphs illustrate the integrity of RNA.
Table 2.2  List of candidate genes that passed microarray analysis criteria. Candidates selected for validation are highlighted. FC, fold change. n=41

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<th>Gene Symbol</th>
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<th>Comparison #2</th>
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</table>
Figure 2.5  Candidate gene differential expression on microarray analysis data. Microarray analysis generates a single value that is representative of the three replicates, accounting for the lack of error bars.
Figure 2.6  Candidate gene expression validation from an independent sample. QRT-PCR analysis based on the six candidates.
QRT-PCR analysis; however, genes belonging to a group (gene set) that share common biological function, chromosomal location, or regulation may act in concert to promote tumourigenic properties. We next employed a computational method, Gene Set Enrichment Analysis (GSEA), which determines whether an *a priori* defined set of genes shows statistically significant, concordant differences between two biological states (Figure 2.7). For GSEA, genes from the Comparison #1 were ranked from 1 to 22 126 following a Bayesian t test. Genes at the top of the list are considered overexpressed following V12Rac1 induction, while genes at the bottom of the list are considered to be statistically underexpressed. The GSEA program begins at the top and continues down the list, calculating a score termed Enrichment Score (ES) that increases whenever it encounters a gene from the list of genes (gene set) that share a common characteristic, and decreases whenever it encounters a gene that is not in the list. The ES can be plotted against the ranked list; and, the proportion of genes that cluster with the increasing ES are considered to be the “Leading Edge” that drives the gene set under the given condition and contribute to a statistical p-value. Gene sets that are overrepresented at the extreme ends of the ranked gene list (overexpressed or underexpressed) are statistically correlated with the treatment conditions (eg. V12Rac1 induction).
Figure 2.7  Gene set enrichment analysis (GSEA).
GSEA is a microarray data analysis method that uses predefined gene sets and ranks of genes to identify significant biological changes in microarray data sets. GSEA is especially useful when gene expression changes in a given microarray data set are minimal or moderate. Following a Bayesian t test, genes are ranked with the overexpressed genes at the top and underexpressed genes at the bottom. The GSEA algorithm will predict whether the gene set is statistically contributing to the treatment. Genes that drive an Enrichment score are part of the Leading Edge (red).
Based on Gene Ontology identification numbers, we examined four gene sets: Apoptosis (448 genes spotted on these arrays), Wnt Pathway (95 genes), Cell Migration (47 genes), and Epithelial-Mesenchymal Transition (21 genes) (Figure 2.8). Using the raw data from the microarray analysis, GSEA identified the Cell Migration gene set to be statistically affected (p<0.05). The leading edge of the Cell Migration gene set is comprised of 19 probesets annotating to 13 genes (Appendix 2). Validation experiments were unable to detect statistical changes in these individual genes (Appendix 3). However, GSEA has demonstrated that their subtle changes in steady-state transcript levels act in concert to promote cell migration following V12Rac1 induction in the HCT116 colon cancer cell line.
Figure 2.8  Enrichment Plots for four gene sets following Gene Set Enrichment Analysis. Genes associated with cell migration (n=47), the Wnt pathway (n=95), apoptosis (n=448), and epithelial-mesenchymal transition (n=21) were included based on Gene Ontology identification (GO ID) numbers. Nominal p-values (p=0.0314, p=0.5024, p=0.6174, and p=0.6949, respectively) were calculated.
2.4.3 **V12Rac1 nuclear localization is required for the promotion of cellular migration in colon cancer**

Since Rac1 expression upregulates the cell migration pathway in colon cancer cells, we postulated that Rac1 must enter the nucleus to contribute to this pathway. Some Rho GTPases are targeted to the nucleus by the evolutionary conserved canonical nuclear localization signal sequence (K(K/R)X(K/R)) located in the C-terminal polybasic region (PBR). The PBR of Rac1 contains the NLS sequence KKRKRK. To determine the specific requirement of nuclear localization of the constitutively active Rac1 mutant, V12Rac1, in promoting cell migration, I used a V12Rac1 mutant with the NLS substituted for six Glutamines (V12Rac1 PBRQ), as compared to V12Rac1 and pcDNA3.1 (empty vector) in the colon cancer HCT116 cell line (Figure 2.9). While V12Rac1 transfection resulted in a marked increase in cell migration over the empty vector control as confirmed by wound scratch assay, the V12Rac1 PBRQ mimicked the empty vector control, demonstrating the importance of a functional polybasic region and nuclear localization in eliciting effects on cell migration.

2.4.4 **Knockdown of the Rac1 GTPase GEF, Tiam1, reduces Wnt-responsive promoter activation**

Given the importance of nuclear localization of Rac1 to promote tumourigenic processes, such as cell migration, our lab previously investigated the role of nuclear Rac1 at the promoter of Wnt-responsive genes. We previously showed, by chromatin immunoprecipitation (ChIP), that Rac1 lies resident at the promoters of Cyclin D1 and c-myc in HEK293T cells, and that upon stimulation with Wnt3A-conditioned media, the
Figure 2.9  V12Rac1 localization is critical for wound closure by wound scratch assay. A) Images of the wound width of plated cells following transfection with either V12Rac1 or V12Rac1 PBRQ, as compared to the empty vector control. B) Bar graph depicting the average wound width at 0h, 12h, 18h and 24h post-transfection. *p<0.05, n=6-8.
Rac1 GTPase-specific GEF, Tiam1, is recruited along with β-catenin to the promoters of these same Cyclin D1 and c-myc genes. We also previously demonstrated that Tiam1 transfection can upregulate the TopFlash reporter in HCT116 cells. However, we elected to establish if the reverse-specificity could be observed by RNAi experiments. Therefore, to determine whether Tiam1 can regulate the expression of canonical wnt target genes, we examined the expression of the wnt-responsive promoter (TopFlash) following Tiam1 knockdown in HCT116 cells. Tiam1 siRNA transfection resulted in a modest reduction in the TopFlash luciferase reporter assay (Figure 2.10). This finding complimented work completed in our lab in which Tiam1 transfection assays, in addition to ChIP experiments, demonstrated that Tiam1 potentiates Rac1-mediated stimulation of β-catenin/TCF-dependent transcription.[172]
Figure 2.10  Tiam1 knockdown decreases Wnt-responsive reporter transcription. (A) Tiam1 siRNA was transfected and examined by QRT-PCR for knockdown of endogenous Tiam1 in HCT116 cells. (B) TopFlash or FopFlash constructs were co-transfected with either Tiam1 siRNA or a nonsilencing siRNA control, and examined for luciferase light units relative to β-galactosidase. *p<0.05
2.5 DISCUSSION

The canonical Wnt signaling pathway is well recognized as a key contributor in tumourigenesis in colon cancers. More recently, it has become clearer that this classical pathway mediates its effects through cooperation with other pathways, including Rac1 GTPase. However, the specific effects of this crosstalk and the molecular mechanism have remained unclear.

Our lab has previously demonstrated that a constitutively active Rac1 mutant (V12Rac1) and stabilized β-catenin cause a synergistic induction of TCF-dependent transcription in colon cancer cells. Expression of a dominant-negative mutant of TCF-4 was able to block this effect, indicating involvement of TCF-4 as a downstream target of Rac1. Conversely, in HCT116 colon cancer cells with constitutive activation of the Wnt pathway, a dominant-negative Rac1 mutant (N17Rac1) was able to downregulate endogenous Wnt signaling. We further demonstrated that Rac1 and Rac1b-mediated transcriptional activation depended on nuclear localization, since mutation in the nuclear localization signal (NLS) sequence in the C-terminal polybasic region (PBR) of Rac1 and Rac1b was unable to facilitate Wnt activation. Additionally, V12Rac1 overexpression promoted redistribution of β-catenin from the cell membrane to the nucleus and reduced endogenous E-cadherin expression. Co-immunoprecipitation studies demonstrate that β-catenin and TCF-4 associate specifically with Rac1 only in its active, GTP-bound state. Taken together, it is clear that activation of Rac1 amplifies the signaling activity of stabilized/mutated β-catenin by promoting its accumulation in the nucleus, and synergizing with β-catenin to augment TCF/LEF-dependent gene transcription, however,
the specific mechanism by which this crosstalk promotes tumourigenesis was the primary focus of this chapter.

To determine the specific effects of active Rac1 GTPase on colon cancer cells with aberrantly active Wnt signaling, I generated a stable-inducible cell system that utilizes a removable repressor of the V12Rac1 promoter. The HCT116-TR-V12Rac1 stable-inducible cell line was used in this study for its following attributes: a) stably transfecting these cells and maintaining them under selection media (containing Zeocin and Blasticidin) ensures that every cell expresses the V12Rac1 and TR genes, and at a consistent and optimal dose for full repression, in comparison to transient transfection methods; b) the Tet-repressor allows for comparisons between V12Rac1 expression (doxycycline-treated), and no expression of V12Rac1 (mock-treated) under one cell system. Consistent V12Rac1 expression was achieved through doxycycline treatment after 24h. These conditions were used to examine transcriptional changes in candidate genes by microarray analysis following V12Rac1 induction. However, no genes could be validated in an independent system. After ruling out many technical possibilities for the lack of candidates, one potential reason remained; that is, the possibility that changes in individual gene expression could be so subtle as to elude detection by the highly sensitive microarray statistical analysis. Therefore, we sought to investigate whether sets of genes belonging to a single cellular process changed in transcriptional expression. By GSEA, we identified the set of genes with known cell migration properties, according to Gene Ontology, as differentially expressed following V12Rac1 induction. Of the 22126 genes across the two arrays, 47 genes belong to the Cell Migration set; and 13 of those genes formed the leading edge in the Enrichment Plot.
Since the constitutively active Rac1 mutant can modestly upregulate transcription in genes involved in cell migration after 24h, we next examined whether this subtle change in gene regulation would translate in a subtle change in cell migration as observed in a wound scratching assay. And since V12Rac1 has a nuclear localization signal sequence, we used the same wound scratch assay to determine whether Rac1 must translocate to the nucleus to affect cell migration. Our data demonstrated that V12Rac1 increases cell migration in the HCT116 cell line with significance after 24h post-transfection; and this effect is abolished with transfection of a V12Rac1 mutant harbouring a defective NLS. Taken together, our data suggests that in a cell system with stabilized β-catenin levels, active Rac1 can upregulate cell migration through the regulation of a subset of target genes, and this role of Rac1 is dependent on its subcellular localization.

Given that the NLS is adjacent to the CAAX motif, a vital domain for post-translational modification and membrane targeting, it is likely that mutating the PBR region may not only restrict Rac1 from entering the nucleus, but may also affect its ability for activation at the plasma membrane. The classical mechanism of activation of small Rho GTPases occurs at the plasma membrane. We have shown that V12Rac1 is present in the nucleus at the promoter of Wnt target genes. Therefore, while our data proves that proper localization of Rac1 is a requirement for Rac1 to act on cellular processes, such as cell migration, further characterization of the precise subcellular sites of Rac1 activity will further our knowledge and understanding of the complex role of Rac1 in colon tumourigenesis.
CHAPTER 3

Rac1b GTPase regulates ROCK2 transcription and promotes tumourigenesis in colon cancer

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Chapter 3

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3.1 SUMMARY

The Rac1 splice variant, Rac1b, contains a 57 nucleotide insertion that reduces GTP hydrolysis and escapes sequestration to the cytoplasm, therefore, behaving like it is constitutively active. The canonical Wnt signaling pathway is aberrantly active in colon cancer, with Rac1b preferentially expressed in tumour tissue such as in colon and breast. Like Rac1, Rac1b has been previously shown to cooperate with the canonical Wnt signaling pathway to synergistically upregulate downstream targets. To identify transcriptional targets of Rac1b in the context of a dysregulated canonical Wnt signaling colon cancer cells, we generated a stable-inducible Rac1b colon cancer cell line, HCT116, with aberrantly active canonical Wnt-signaling. A comparative microarray analysis of these cells with Rac1b induction relative to uninduced cells yielded two novel target genes following Rac1b induction, a RhoA effector (ROCK2) and a noncoding RNA (MALAT-1). Unlike MALAT-1, ROCK2 steady state transcript levels positively correlated with Rac1b expression a panel of 17 primary colon tumours and their matched normal colon tissue. However, if Rac1b translate the observed transcriptional effect on ROCK2 into changes in protein expression or activity, it is below the detectable limits of Western blotting and IP kinase assays. ROCK2 has been previously associated with cell
migration. Since Rac1b regulates ROCK2 steady state transcript levels, I examined whether Rac1b must enter the nucleus to mediate its effects on cell migration. I used a Rac1b construct with the PBR domain substituted for six Glutamines (Q6) in a scratch wound assay, and compared it against Rac1b and an empty vector control in HCT116 cells. I demonstrated that, while Rac1b-induced cells exhibited an accelerated migration response, the Rac1b Q6 mutant had no effect, mimicking the empty vector control. This suggests that nuclear entry is critical for Rac1b to mediate its affects on cell migration. In an effort to identify other tumourigenic processes under regulation by Rac1b, our lab previously demonstrated that Rac1b expression could decrease cell adhesion. This finding was likely caused by the ability of Rac1b to reduce E-cadherin expression. This study aimed to further this discovery by determining the specific mechanism for E-cadherin regulation by Rac1b. We designed a siRNA pool that targets the unique insertion region of Rac1b, and investigated the transcriptional expression levels of known E-cadherin regulators. Following knockdown of endogenous Rac1b in the HT29 colon cancer cell line, we observed an increase in E-cadherin expression by approximately 50%, and a concomitant decrease in Slug expression by approximately 25%. No change in the transcript levels of Snail was observed under similar conditions. This likely indicates that the suppressive role of Rac1b on E-cadherin is mediated through regulation of Slug in the HT29 colon cancer cell line. Taken all together, this study implicates Rac1b as a putative regulator of both cellular migration and adhesion in colorectal cancer cells.
3.2 INTRODUCTION

Rac1 is a member of the Rho family of small GTPases that regulate pathways involved in many cellular processes, such as lamellipodia formation and planar cell polarity.[127] These GTPases cycle between an active GTP-bound state and an inactive GDP-bound state. The exchange of GDP for GTP is promoted by guanine nucleotide exchange factors (GEFs). The GTP-bound state is recognized by effectors, which can elicit a downstream signaling response, and by GTPase-activating proteins (GAPs), which accelerates the slow intrinsic GTP hydrolysis reaction. Guanine nucleotide dissociation inhibitors (GDIs) interact with GTPases in the GDP-bound state, and sequester them from the membrane to the cytoplasm. Two structural regions (switch I and II) undergo conformational changes to reveal binding sites for their interacting partners.[131] Rac1b is a splice variant of Rac1 that contains a 19-amino acid insertion (exon 3b) at the end of the switch II region.[227] This insertion causes a structural change that inhibits the interaction of GDIs and escapes sequestration to the cytoplasm, thereby behaving in a constitutively active manner. Rac1b is found preferentially expressed in cancers, such as colorectal and breast.

We have previously identified a relationship between Rac1b GTPases and the canonical Wnt signaling pathway that cooperate to promote carcinogenic processes.[212, 225] Our lab has provided evidence that suggests Rac1b overexpression facilitates tumour progression by enhancing Dvl-3-mediated Wnt pathway signaling and induction of Wnt target genes specifically involved in decreasing the adhesive properties. We observed that Rac1b downregulated endogenous E-cadherin expression and decreased
cell-cell adhesion of HCT116 colorectal cancer cells and this effect was further augmented by combined action of Rac1b and Dvl-3. We demonstrated that mutation of the nuclear localization signal sequence in the C-terminal polybasic region of Rac1b resulted in a complete loss of Rac1b stimulatory effects on TCF-mediated gene transcription and the suppressive effects seen on cell adhesion, indicating the importance of nuclear and membrane localization of Rac1b. Cumulatively, these studies have established crosstalk mechanisms between Rac1b and canonical Wnt signaling. The precise mechanism of how this crosstalk contributes to tumourigenesis was the focus of this chapter.

3.3 MATERIALS AND METHODS

3.3.1 Materials

Tissue culture reagents and fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies (Burlington, Ontario). The FLAG-tagged Rac1b construct was a gift from Dr. Jeff Wrana (SLRI, Mount Sinai Hospital, Toronto). pTopFlash and pFopFlash luciferase constructs, and dominant-negative TCF4 expression vectors were gifts from Dr. Benjamin Alman (Hospital for Sick Children, Toronto). –1745CD1-LUC reporter plasmid was a gift from Dr. Tetsu Akiyama (University of Tokyo).
3.3.2 Cell Culture

HCT116 and HT29 cells were cultured in McCoy’s 5A medium supplemented with 10% FBS. Both cell lines were cultured at 37°C in a humidified atmosphere of 5% CO2.

3.3.3 Luciferase Reporter Gene Assays

Transfection experiments were carried out using Lipofectamine 2000 reagent (Invitrogen Life Technologies) according to manufacturer’s instructions. Cells were seeded in 24-well plates at a density of 2×105 cells per well 24 hours prior to transfection. Luciferase constructs (0.1 µg) containing either four tandem wild-type (pTopFlash) or mutant (pFopFlash) TCF4 binding sites or cyclin D1 (-1745) promoter were transfected into cells to determine luciferase reporter activity. In addition, cells were co-transfected with an internal control (0.03 µg of pCMVβ-gal), along with combinations of pcDNA3.1 (empty vector) or dominant-negative TCF4 (DNTCF). The total amount of DNA per transfection was held constant at 1 µg total plasmid DNA by co-transfection of appropriate amounts of empty vector. Luciferase and β-galactosidase activity was measured 24 hr after transfection using commercially available kits from Promega (Madison, WI) and Stratagene, respectively. Luminescence was quantitated using a Berthold 96-well microplate luminometer. All transfections were carried out in triplicate on at least three independent occasions, and error bars represent SEM (standard error of the mean).
3.3.4 Immunoprecipitation, Cell Fractionation, and Western Blotting

Cell extracts were prepared using Radio-Immunoprecipitation Assay (RIPA) buffer (0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1x phosphate buffered saline (PBS), “Complete” mini EDTA-free protease inhibitor tablet (Roche, Mannheim, Germany)). Immunoprecipitation was conducted using 300 µg of cell lysate, 2 µg of TCF4 antibody (Upstate Biotechnology) or β-catenin antibody (Transduction Labs, Lexington KY), and 50 µl of Protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA). Immunocomplexes were washed three times using RIPA buffer, resuspended in Laemmli buffer, and resolved on a 10% SDS-PAGE gel. Resolved proteins were transferred to PVDF nylon membranes (Amersham Pharmacia Biotech, Quebec, Canada), and non-specific reactivity was blocked with Tris-buffered saline containing 0.1% Tween-20 and 5% non-fat dried milk. Antibodies specific for FLAG (Sigma, St. Louis, MO), Myc tag (Santa Cruz Biotechnology) and β-catenin were used according to the manufacturer’s specifications. Bound proteins were detected by using appropriate horseradish peroxidase-conjugated secondary antibodies in the enhanced chemiluminescence system (ECL) (Amersham Pharmacia Biotech). For cell fractionation analysis, cells were separated into either cytoplasmic and nuclear fractions, or membrane fractions, using the NE-PER and MEM-PER kits, respectively (Pierce, Rockford, IL). Briefly, cells were grown in 60 mm dishes until approximately 80% confluence prior to transfection. Twenty-four hours post-transfection, cells were processed according to manufacturer’s instructions. Cytoplasmic and nuclear fractions (30 µg and 10 µg, respectively) were separated on 10% SDS-PAGE and immunoblotted with indicated antibodies.
3.3.5 Evaluation of the activity of ROCK2

Activation of ROCK2 was evaluated by immunoprecipitating ROCK2 followed by an in vitro kinase activity assay. Cells were lysed using RIPA lysis buffer. Approximately 150-400 µg of total cell lysates were incubated overnight at 4°C with 2 µg of rabbit anti-ROCK2 antibody (Santa Cruz Biotechnology). The mixture was incubated with 30-40 µl protein G PLUS agarose beads for 1 hour at 4°C. The beads were pelleted, and washed three times with PBS, and washed once with cold kinase buffer containing 100 µM ATP and 10mM MgCl$_2$. The mixture was then incubated for 30 minutes at 30°C with the kinase reaction buffer containing 100 µM ATP-$^{32}$P, 10mM MgCl$_2$, and 2.5 µg MYPT-1 (714-1004), a ROCK-specific substrate (Upstate Biotechnology). The reaction was terminated by boiling the samples in SDS sample buffer. The samples were run on a 10% polyacrylamide gel, and exposed to a PhosphoScreen overnight at room temperature. The amount of MYPT-1 phosphorylation was determined using the Storm 860 Molecular Imager (Molecular Dynamics, Sunnyvale, CA, USA). Quantitation analysis was performed using ImageQuant software (Molecular Dynamics).

Experiments were performed with the assistance of Dr. Jim Woodgett and Ms. Elizabeth Rubie, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada.
3.3.6 RNA interference assays

For endogenous gene knockdown experiments, the following small interfering RNAs (siRNA) were used: Rac1 siRNA was a SMARTpool reagent (Dharmacon, Lafayette, CO), whereas Rac1b knockdown was achieved via an equal mixture of two siRNAs against the target sequences 5’-GAAACGUACGGUAAGGAUA-3’ and 5’-GGCAAAAGACAAGCCGAUUG-3’. Gene expression data were normalized against transfections with a siCONTROL nontargeting siRNA (Dharmacon).

3.3.7 Quantitative Reverse Transcriptase (RT)-PCR

Total RNA was isolated using an RNeasy Mini kit (Qiagen, Valencia, CA) and analyzed by real-time RT-PCR as described [49]. For RNAi experiments, cells were transfected with either: SMARTpool Rac1-specific siRNA, ONTARGETplus SMARTpool Tiam1-specific siRNA, siCONTROL non-silencing siRNA as a control, GAPDH-specific siRNA as a control (Dharmacon, Lafayette, CO.) or mock transfected using LipofectAMINE 2000 according to manufacturer's protocol. After 48 hours (Rac1 siRNA experiments) or 64 hours (Tiam1 siRNA experiments), total RNA was isolated and analyzed by real-time RT-PCR. The following primer pairs were used to amplify cDNA: c-Myc forward, 5’-GCCAAGCTCGTCTCAGAGAAG-3’, and reverse, 5’-CAGAAGGTGATCCAGACTCTCTCAGAGAAG-3’; Rac1 forward, 5’-ATGCAGGCCATCAAGTGTGTG-3’, and reverse, 5’-ATGCAGGCCATCAAGTGTGTG-3’; Rac1 forward, 5’-TTACAACAGCAGGCATTTTTCTCT-3’, and reverse, 5’-
AGACGTACTCAGCCATGTC-3', and reverse, 5'ACCCAAATGTCGAGTCAGG-3'; β-actin forward, 5'-ATCATGTGGAGACCTTCAA-3', and reverse, 5'-CATCTCTTGCTGAGTCACC-3'; and; GAPDH forward, 5'-ACCACATGGCCATCAC-3', and reverse, 5'-TCCACCACCTGTTGCTGTA-3'.

For statistical analyses, unpaired Student's t-test was performed.

### 3.3.8 Microarray Analysis

Genomic RNA expression analysis was performed using the Affymetrix Gene 1.0 ST arrays, where each of the 28,869 genes is represented on the array by approximately 26 probes spread across the full length of the gene. RNA (200 ng) isolates from the HCT116-TR-Rac1b colon cancer cells under two treatment conditions (0.01 µg/ml doxycycline or 10 µl double-distilled water in 6 cm plates) at 7h and 15h post-treatment were analyzed per the instructions of The Centre for Applied Genomics (TCAG), The Hospital for Sick Children, Toronto, ON, Canada. Each microarray experiment was performed a minimum of three times (n=3-6).

Raw probe intensity values, provided by the TCAG, were analyzed using the Partek Genomics Suite software (Partek Incorporated, St. Louis, MO). Raw data contained in .CEL files from were imported into Partek, log2 transformed, background corrected by RMA, and quantile normalized before summarization methods. Summarization into probeset values were performed using either Tukey’s biweight, median polish, or average of the logs. Candidate genes for each summarization method were narrowed using a false discovery rate (FDR) of 0.1%. The final list of differentially
expressed genes were generated from probesets that were common by all three summarization methods and met the FDR cutoff. Candidate genes were validated by QRT-PCR using the same microarray sample set, an independent sample set, and an additional set of samples from HT29 colon cancer cells following Rac1b knockdown.

### 3.3.9 Scratch Wound Assay

Cells were cultured to confluence in 6-well dishes. Using a marker, a line was drawn on the underside of the plate to ensure consistency when capturing images. Using a sterile 200 µl pipet tip, three separate wounds were scratched through the cells, moving perpendicular to the line drawn. Images of the cells at 10X using phase contrast were captured using the QCapture software above and below the line for each well. Measurements were taken in Adobe Photoshop CS3 (Adobe Systems Incorporated, San Jose, CA, USA), and statistical analysis (t test) was performed in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).
3.4 RESULTS

3.4.1 Characterization of the HCT116 cell line with stable inducible expression of Rac1b GTPase

The main objective in this series of experiments was to identify novel transcriptional targets of Rac1b induction in a colon cancer cell line with dysregulated Wnt signaling. Our lab has previously generated a HCT116 cell line with stable inducible expression of a Flag-tagged Rac1b GTPase under control of Tet-operator in its promoter (HCT116-TR-Rac1b). However, this cell line was not characterized by microarray analysis.

Our strategy in identifying novel downstream targets of Rac1b overexpression in colon cancer included the attempt at identifying the earliest genes with transcriptional expression mediated by Rac1b. Therefore, prior to investigation of differentially expressed genes following Rac1b induction, we examined how quickly Rac1b protein expression could be visualized in the nucleus. High levels of Rac1b protein were observed in the nuclear fraction of HCT116-TR-Rac1b cells in as little as 7h post-treatment by doxycycline (0.01 µg/ml) as compared to treatment with the drug vehicle control; and this was corroborated at the transcript level by QRT-PCR analysis (Figure 3.1). Therefore, Rac1b has the potential to illicit a nuclear role in regulating transcription of novel gene targets in as little as 7h post-induction.
Figure 3.1  
**Rac1b stable-inducible colon cancer cell line.**
A) Rac1b expression at 7h, 11h, and 15h post-induction by doxycycline as compared to drug vehicle control was observed by Western blotting of whole cell lysates of stably transfected HCT116 cells. B) Rac1b expression was determined at 7h and 11h post-induction by doxycycline or the drug vehicle control and observed by Western blotting of fractionated cytosolic (CE) and nuclear (NE) lysates. C, drug-vehicle control; D, doxycycline.
3.4.2 Identification of differentially expressed gene candidates following Rac1b induction.

Microarray analysis was performed using the Affymetrix Gene 1.0ST arrays to ensure greatest chance at true-positive results. In an effort to obtain the greatest direct effect of Rac1b on differentially expressed genes that are critical for tumourigenic processes, total RNA from HCT-TR-Rac1b cells was harvested at 7h and 15h post-induction by doxycycline. For the 7h time point, total RNA from 12 replicates were isolated following Rac1b induction, and total RNA from 8 replicates were isolated following treatment with the drug vehicle control. Of those, the 6 replicates from each group that displayed the closest amount of Rac1b transcript (in the induced set) or Cyclin D1 (in the uninduced set) were forwarded to The Centre for Applied Genomics (TCAG), Hospital for Sick Children (Toronto) for microarray analysis. Statistical analysis was performed on the raw intensity using the Partek Genomics Suite and Ingenuity Pathway Analysis software programs. This GeneChip contains 764 885 probes that annotate to 28 869 genes for an average of 26 probes per gene. Given the relatively small Rac1b gene size, there are 6 probes that annotate to Rac1b, however, only 2 are unique in that they are located within the Exon 3b insertion. Therefore, we omitted data from the other four probes, and used the Rac1b gene intensity from the two probes as an internal control that overexpression was consistently achieved. Statistical analysis of the 7h sample set (induced and uninduced controls) yielded no candidate genes with only Rac1b passing statistical cutoffs. A Principal Component Analysis is a mathematical procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components. Examination of the PCA plot for the 7h timepoint revealed
the induced arrays overlapped with the control arrays, indicating a lack of consistent and distinct variability between the two sets (Figure 3.2A).

Microarray analysis was then performed on 3 replicates for each condition (induced and uninduced) for the 15h timepoint. The PCA plot demonstrated that the 15h samples clearly grouped together and in isolation from the control and 7h sample sets, indicating that the variance in the 15h samples are similar among themselves and different from the variance in the control samples (Figure 3.2A).

Since every annotated gene on the Affymetrix genechip array contains data from multiple probes that span the coding regions and surrounding sequences, there are many methods to summarizing these probes into one probeset value for the gene. To enrich for true positive candidate genes, we selected three popular summarization methods (Tukey’s biweight, median polish, and average of the logs) and generated a list of candidates for each method (FDR = 0.1%). Our final candidate list was generated on genes common to all methods, yielding 634 candidate genes (Figure 3.2B).

We selected three genes (MALAT1, ABCA5, and ROCK2) for further validation based on fold change, statistical probability ($p$ value), and pathway analysis as determined by the Ingenuity software. By microarray analysis, ROCK2 had the least expression change (2.5-fold difference, $p = 4.36 \times 10^{-7}$) of the three candidates, therefore, we used published primer pairs that target two different regions of the gene and looked for an element of consistency.[228, 229] Quantitative RT-PCR analysis of the same samples used for microarray analysis confirmed microarray results for all three candidates (Figure 3.3A). In an independent sample set, we observed MALAT1 and
Figure 3.2  Microarray analysis following Rac1b induction in colon cancer cells. 
A) Principal component analysis (PCA) plot displaying a segregation of cells 15 hours post-Rac1b induction (red) as compared to cells induced with a drug-vehicle control (green). Cells analyzed 7 hours post-induction of Rac1b are also indicated (blue). B) Venn diagram illustrating the number of genes differentially expressed following three summarization analyses. Each summarization analysis generated a list of differentially expressed genes with a false discovery rate of 0.1%. A total of 634 genes were common by all three summarization methods.
Figure 3.3  Rac1b regulates ROCK2 expression in colon cancer.  
A) QRT-PCR analysis on the same samples (15h) used in the microarray analysis, on three microarray candidates: MALAT1, ABCA5, and ROCK2 using two primer sets that target different regions of the ROCK2 transcript.  
B) QRT-PCR analysis of the three microarray candidates using three independent samples of HCT116 cells at 24h following Rac1b induction.  
C) QRT-PCR analysis of microarray candidate genes at 72h following siRNA knockdown of Rac1b in HT29 cells. *p<0.05, **p<0.01, ***p<0.001.
ROCK2 expression trends to mimic the initial sample set, however, expression of the ABCA5 transporter gene did not change in the Rac1b-induced samples as compared to controls (Figure 3.3B).

We examined the reverse specificity in another colon cancer cell line that harbors activated Wnt signaling, and endogenously express Rac1b. We used a cocktail of two siRNAs to knockdown the expression of endogenous Rac1b in the HT29 colon cancer cell line, as previously described. Rac1b was knocked down by 85% at 72h post-siRNA transfection, and caused a reduction in MALAT1 and ROCK2, with ROCK2 meeting statistical significance (p<0.01) as observed by QRT-PCR (Figure 3.3C). No effect was observed in the ABCA5 gene. Despite the genetic variability between the HT29 and HCT116 colon cancer cell lines, they both have dysregulated Wnt signaling, and they both display changes in ROCK2 expression following Rac1b level changes.

3.4.3 Rac1b correlates with ROCK2 transcriptional overexpression but not with MALAT1 in colon tumours as compared to matched normal tissue

Having established a novel role for Rac1b in regulating the expression of ROCK2 and MALAT1 transcripts in two colon cancer cell lines, I sought to investigate these associations in vivo. I identified a positive correlation (p <0.005) between transcript levels of Rac1b and ROCK2 on examination of mRNA from 17 colon tumours as compared to matched normal lysates (Figure 3.4A). In contrast, no significant correlation
Figure 3.4  Rac1b expression correlates with ROCK2 expression in colon tumours. Pearson r correlation analysis exhibited a statistically modest positive correlation (Pearson r = 0.6978, p<0.01) between Rac1b and ROCK2 transcripts (Panel A), but not with MALAT1 transcripts (Panel B) in matched tumour and normal colon tissues (n = 17).
Figure 3.5  Rac1b expression does not increase ROCK2 protein levels and does not lead to its activation in the HCT116 colorectal cancer cell line.

(A) ROCK2 expression was examined by western blotting at 15h, 20h, 24h, and 48h post-induction with Rac1b. U, treated with the drug vehicle (water); D, treated with 0.01 µg/ml doxycycline. (B, top) IP kinase activity assay for ROCK2 with $^{32}$P-MYPT1 as the substrate. Coomassie blue staining to demonstrate equal loading (B, bottom). (C) ROCK2 expression was examined in HEK293T cells at various timepoints post treatment with Wnt3a-conditioned media (W) as compared to the L-cell-conditioned media control (L).
was observed between Rac1b expression and MALAT-1 expression in the same set of cases (Figure 3.4B). Clinical information for these patients was not available.

### 3.4.4 Rac1b does not regulate ROCK2 protein expression or activity in colon cancer cells with dysregulated canonical Wnt signaling

Given that I demonstrated a novel role for Rac1b in mediating the steady state transcript levels of the RhoA effector, ROCK2, I next determined whether this observed effect translated into changes in ROCK2 protein expression and kinase activity. By western blotting, I could not detect an appreciable change in ROCK2 protein expression following Rac1b induction on examination at 15h, 20h, 24h, and 48h (Figure 3.5A). I next performed an IP kinase activity assay using an anti-ROCK2 antibody for immunoprecipitation and a ROK substrate protein corresponding to amino acids 714-1004 of chicken MYPT1 with an N-terminal MBP (Maltose Binding Protein) tag and did not detect an appreciable change in activity following Rac1b induction (Figure 3.5B).

While Rac1b alone could only contribute to slight changes in ROCK2 transcript levels in a system with an intrinsic activation of the canonical Wnt signaling pathway, I aimed to determine if a change in ROCK2 activity could be observed following Wnt signaling activation. HEK293T cells possess an intact Wnt signaling pathway that is not active, and can therefore be stimulated at multiple levels of the pathway. I treated HEK293T cells with Wnt3a-conditioned media, and compared both ROCK2 expression and activity to cells given a control treatment (L-cells conditioned media). I detected increases in both expression and activity as early as 30 min post-treatment, but these level
off by 6h post-treatment (Figure 3.5C). This indicates a very transient effect of Wnt signaling in mediating ROCK2 before basal levels are restored.

### 3.4.5 Rac1b nuclear localization is required for the promotion of cellular migration in colon cancer

Given our observations in Chapter 2.4.3 that delineates a role of Rac1 in mediating cell migration, and in establishing a clear requirement for proper subcellular Rac1 localization, I elected to investigate if these same findings are evident in the splice variant, Rac1b. By site-directed mutagenesis, our lab generated a Rac1b mutant similar to the V12Rac1 PBRQ (discussed in Chapter 2) that harbours six Glutamines in place of the NLS (Rac1b Q6). In contrast to V12Rac1, by wound scratch assay, the HCT116 cells transfected with Rac1b demonstrated a greater ability to close the wound after 24h relative to the empty vector control (Figure 3.6A,B). And similar to the V12Rac1 PBRQ, the Rac1b Q6 transfected cells mimicked empty vector demonstrating a clear requirement for proper subcellular localization for Rac1b. A western blot was performed on the lysates harvested at different timepoints to confirm that observed effects were not due to a reduction in expression (Figure 3.6C).
Figure 3.6  Rac1b localization is critical for wound closure by wound scratch assay.
A) Images of the wound width of plated cells following transfection with either Rac1b WT, Rac1b Q6 as compared to the empty vector control.  B) Bar graph depicting the average wound width at 0h, 12h, and 24h post-transfection.  *p<0.05,  **p<0.01, n=6-8.  C) Western blotting of representative lysates at 0h and 24h post-transfection demonstrates the presence of Rac1b Q6 expression after 24h.  n=6-8
3.4.6 Rac1b regulates Slug expression and reduces E-cadherin in colorectal cancer cells

Our lab has demonstrated a role for Rac1b in decreasing cell adhesion in colon cancer cells using an Innocyte Adhesion Assay (Calbiochem).[212] Additionally, Rac1b also decreased E-cadherin expression as observed by Western blotting; and Rac1b caused a marked reduction in the plasma membrane pool of E-cadherin as observed by fluorescence microscopy. Interestingly, both findings returned to basal (untransfected) levels following introduction of the Rac1b mutant, Rac1b Q6. This demonstrates that the PBR region is required for Rac1b-mediated Regulation of E-cadherin and cell adhesion. Specific mechanisms for the role of Rac1b on E-cadherin expression were not clear. E-cadherin expression can be regulated through the binding of its promoter region by repressors, Snail and Slug. Therefore, we speculated that Rac1b might elicit its effects on E-cadherin through one or more of its known repressors. We further postulated that RNAi knockdown of Rac1b in the HCT116 colon cancer cell line should result in decrease in one of the known E-cadherin repressors, and a subsequent increase in E-cadherin transcription.

We designed two siRNAs that target the Rac1b exon 3b insertion region and transfected them in an equal mixture to achieve the greatest amount of Rac1b knockdown. RNAi experiments were performed in comparison to an siRNA control that contains a scrambled sequence which does not target any known human genes. To ensure that our Rac1b siRNA cocktail does not result in off-target effects, we examined both endogenous transcript levels of Rac1 and Rac1b in HT29 cells, and compared the
Rac1b siRNA against the commercially available Rac1 siRNA (Figure 3.7A). As expected, the nonspecific Rac1 siRNA had a slight effect on Rac1b expression, and the Rac1b siRNA achieved approximately 70% Rac1b knockdown after 24h post-transfection and had no effect on Rac1 expression by QRT-PCR. We also examined the effects of Rac1b knockdown on the regulation of an endogenous Wnt target gene, cyclin D1 and observed approximately 40% reduction (Figure 3.7B). These results validated the effectiveness of our Rac1b siRNA and demonstrated that Rac1b expression contributes to inappropriate transcription of Wnt target genes in colorectal cancer cells. We then applied this Rac1b siRNA to determine a mechanism of E-cadherin repression. In HT29 cells, Rac1b knockdown resulted in an increase in E-cadherin expression by approximately 50%, and a concomitant decrease in Slug expression by approximately 25% (Figure 3.7C,D). No change in the transcript levels of Snail was observed under similar conditions. Taken together, this likely indicates that the suppressive role of Rac1b on E-cadherin is mediated through regulation of Slug in the HT29 colon cancer cell line.
Figure 3.7  E-cadherin and Slug expression following Rac1b knockdown in HT29 colon cancer cells.
(A) QRT-PCR of Rac1 and Rac1b expression following knockdown with Rac1b siRNA to establish specificity. Rac1b was designed in the lab to target Rac1b exon 3b, while the Rac1 siRNA is commercially available. (B-D) QRT-PCR of Cyclin D1, E-cadherin, and Slug transcript expression at 24h following Rac1b knockdown. All experiments were compared with a commercially available nonsilencing scrambled sequence control. This figure was adapted from Esufali S, Charames GS, Pethe VV, Buongiorno P, Bapat B. Cancer Res (2007) 67:2469-2479.
3.5 DISCUSSION

The Rac1b GTPase, which is preferentially expressed in tumours, is an attractive target for cancer therapy strategies, however the paucity of conclusions for its mechanistic action in the promotion of carcinogenesis is a testament to its complex nature. We identified ROCK2 and MALAT-1 as transcriptional targets of Rac1b overexpression in colon cancer. Additionally, we observed a positive correlation of mRNA expression between Rac1b and ROCK2 in colon tumours as compared to mRNA from matched normal colon tissue. However, the effects of Rac1b on ROCK2 protein expression and activity were below detectable limits.

Rho GTPases are implicated in a variety of physiological functions associated with changes in the actin cytoskeleton, such as cell migration, motility, migration, and contraction.[131] Rho GTPases signal through numerous downstream targets or effectors, including citron kinase, protein kinase N, and p21-activated protein kinase (PAK). The Rho-associated kinases (ROCKs) are one of the first identified effectors of RhoA. Although the two ROCK isoforms, ROCK1 and ROCK2, are both RhoA effectors, they are processed and regulated differently and are not functionally redundant. ROCK1 and ROCK2 share a 65% amino acid sequence homology [230], however, probes annotating to ROCK1 showed no significant change following Rac1b induction, demonstrating specificity for ROCK2 as a transcriptional target of Rac1b.

Recently, ROCK2 was found overexpressed in human hepatocellular carcinomas (HCCs) and closely associated with intrahepatic metastasis via tumour microsatellite formation.[231] Additionally, ROCK2 promoted HCC migration and also promoted
HCC invasion by regulating cell directional movement. ROCK2 knockdown significantly inhibited filopodia and lamellipodia formation in HCC cells. Rac1b can induce the formation of lamellipodia in fibroblasts [227]. Whether the tumourigenicity observed in these cancers is promoted by Rac1b regulation of ROCK2 expression, is unclear.

By subtractive hybridization between primary nonsmall cell lung tumours that did or did not metastasize, Ji P et al. (2003) identified a large 8kb noncoding transcript, MALAT-1 (Metastasis Associated in Lung Adenocarcinoma Transcript).[232] In a panel of pooled cDNA from various healthy human organs, colon tissue had intermediate levels of MALAT-1 expression (ranking fifth out of 23 different organs). We identified MALAT-1 to be differentially expressed in Rac1b overexpression and Rac1b knockdown analyses, yet we did not see a significant correlation between mRNA expression of Rac1b and MALAT-1 in colon tumours as compared to matched normal tissues. Although the chromosomal region that harbors MALAT-1 (11q13) has been frequently associated with tumourigenesis and metastasis, the functional role of MALAT-1 beyond a prognostic marker for survival is still to be determined.

In summary, Rac1b GTPase and the RhoA effector, ROCK2, have been previously shown to be independent contributors to many tumourigenic processes. Our results show a novel role for Rac1b in upregulating the expression of ROCK2, as well as a noncoding RNA (MALAT-1) in colon cancer. Additionally, we observed a positive correlation between Rac1b and ROCK2 transcripts in colon tumours as compared to matched normal colon tissue. Future studies aimed at identifying the precise functional implication of Rac1b in regulating the expression of ROCK2 and MALAT-1 in colon
cancer will increase our understanding of Rac1b in tumourigenesis, and may also be of prognostic value or provide novel therapeutic targets.
CHAPTER 4

Discussion and Future Directions

The experiments presented in this chapter were performed jointly with Dr. Vaijayanti Pethe.

4.1 Molecular mechanisms of tumourigenesis by Rac1/1b GTPases in colon cancer with dysregulated canonical Wnt signaling

The work of this thesis implicates a subtle but critical role of Rac1 and Rac1b in regulating the expression of genes that work together to promote cell migration in colon cancer. I demonstrate that Rac1 GTPase upregulates at least 19 genes associated with cellular migration. Since previous work in our lab has shown that Rac1 can sit at the promoter and contribute to the transcription of canonical Wnt target genes, it is possible that Rac1 acts at the promoters of these 19 genes. Lending further support for this notion are our results showing that nuclear localization of Rac1 or Rac1b modulates wound closure in a scratch wound assay.

One downstream transcriptional target of Rac1b induction in a colon cancer cell line with dysregulated canonical Wnt signaling is ROCK2, classically known as a RhoA effector. Changes in ROCK2 mRNA expression were observed in two different colon cancer cell lines in both Rac1b induction and Rac1b knockdown experiments. Additionally, Rac1b and ROCK2 demonstrated a modest correlation in primary tumours
with matched normal colon tissue. Yet, these transcriptional effects could not be corroborated at the protein level, or through an activity assay. Candidate selection following microarray analysis was based on three criteria: the modified t test score; fold change; and pathway analysis using the Ingenuity software. ROCK2 was not ranked as high as the other candidates selected in the modified t test score and fold change. ROCK2 was primarily selected based on a strong association with some of the top networks identified in the pathway analysis program. However, to maintain confidence in ROCK2 using the highly sensitive QRT-PCR analysis, two primer pairs were selected from the literature that target different regions of the gene. In all validation experiments, these two primer pairs were quite closely correlated. Therefore, given that Rac1b modestly upregulates the transcript levels of Rac1b, it is likely that translation into protein and subsequent activity falls below detection limits in comparative analysis. It is possible that the endogenous expression of ROCK2 in the two cell lines used (HCT116 and HT29) was near a saturation point, and that further stimulation by Rac1b may be insufficient for observation. It is already known that Rac1 and RhoA act together at opposite ends of a motile cell.[233] The possibility of Rac1b at the leading edge feeding an effector to RhoA at the trailing edge of the migrating colon cancer cell is interesting and should be further investigated.

Metastasis associated in lung adenocarcinoma transcript 1 (MALAT-1) was first described as a noncoding RNA that is associated with significantly worse survival of patients with stage I non-small cell lung cancer (NSCLC).[232] MALAT-1, in our experiments, exhibited consistent upregulation in response to Rac1b induction and could have potentially served as a biomarker, except that its transcriptional expression was not
linked to Rac1b expression levels in a primary colon tumours with matched normal tissue control.

APC interacts with a known effector of Rac and Cdc42, IQGAP1 (IQ motif-containing GTPase activating protein 1), which leads to the accumulation of APC/IQGAP complexes at the leading edge of the lamellipodia.[234-236] The interaction between IQGAP1, active Rac, and active Cdc42, promotes the binding to, and crosslinking with, actin filaments. Loss of APC or IQGAP1 inhibits the accumulation of Rac1 and Cdc42 at the leading edge of migrating cells, disrupts localized actin meshwork formation and inhibits overall cell migration in a wounding assay.[236] This suggests that these four proteins interact as a complex in migrating cells.[234] Since IQGAP1 can bind to truncated mutant APC in colon cancer cells, an APC interaction with the cytoskeleton could potentially be involved in the dysregulation of cell polarity and motility changes.[236]

The HCT116 cell line used in our studies has constitutively active Wnt signaling through an activating β-catenin mutation, rather than loss of expression of APC. Since we observed an increase in the migratory potential of these cells it is possible that the type of Wnt signaling mutation coupled with Rac overexpression may determine the effectiveness of cellular migration through the generation of lamellipodia. Therefore, it would be interesting to examine the migratory potential of colon cancer cells that either express Rac1 or not, and either harbour full-length APC, a truncated APC mutant, or loss of APC due to methylation as the second mutation event, in addition to cells with stabilized β-catenin through an activating mutation. Applying this same concept to Rac1b
studies, will lend further support for the cooperation between Rac1/1b GTPases and the canonical Wnt signaling pathway in the promotion of colon cancer.

4.2 The role of Rac1b in the nucleus

Mounting evidence suggests that although the classical mechanism of Rac1b function occurs through activation at the plasma membrane, it is becoming clearer that the specific oncogenic properties of Rac1b occur as a result of entering the nucleus. The ability of V12Rac1 to enter the nucleus and form a transcriptional complex at the promoters of Wnt target genes, lends support to a similar role for Rac1b.

A recent study showed that, in HEK293T cells, Wnt3a stimulation not only recruits Dvl-3 and β-catenin to the promoter of the Wnt-target gene, c-myc, but also recruits c-Jun.[25] This study also demonstrated that the binding of Dvl3 to TCF-4 and subsequently the c-myc promoter was dependent on c-Jun. We propose that Rac1b may also play a role in the recruitment of Dvl3 to the c-myc promoter.

In preliminary co-immunoprecipitation experiments, after inducing the expression of Rac1b using the HCT116/Rac1b stable-inducible cell line that we generated, we found that immunoprecipitation of Rac1b co-precipitated Dvl3, β-catenin and TCF4; but does not appear to affect the expression levels or the subcellular redistribution of β-catenin or Dvl3 (Figure 4.1). This leads us to speculate that the interplay between Rac1b and the canonical Wnt signaling may occur in the nucleus, involving Dvl3 and β-catenin. Using the tcf-binding elements (TBEs) on the promoters of c-myc and cyclin D1, we investigated if Rac1b, Dvl3 and β-catenin co-exist at these same promoters by chromatin
Figure 4.1  
Rac1b binds Dvl3, β-catenin, and TCF4 in colon cancer cells, but does not alter the expression or subcellular redistribution of β-catenin.

(A) By co-immunoprecipitation, Rac1b GTPase was pulled down with Dvl3, β-catenin, and TCF4 at 24h post-induction as compared to uninduced controls. (B) β-catenin and Dvl3 protein expression from cytonuclear fractionation of lysates at 15h and 24h post-induction with doxycycline. This experiment was conducted with the assistance of Dr. Vaijayanti Pethe. WCL, whole cell lysate; CE, cytoplasmic extract; NE, nuclear extract; U, uninduced; I, doxycycline-induced.
Figure 4.2  Rac1b expression leads to the recruitment of Dvl3 and β-catenin to the promoter of Wnt target genes.
By ChIP, (A) chromatin from HCT116 cells was subjected to immunoprecipitation using Dvl3, β-catenin, Flag, and TCF4 antibodies. (B) chromatin from HEK293T cells was subjected to immunoprecipitation using Dvl3, β-catenin, Flag or IgG antibodies. Enriched chromatin was analyzed by PCR using primers that flank the TBEs in Wnt target genes, c-myc and cyclin D1. GAPDH primers, which flank a region of the GAPDH promoter that is devoid of TBEs, were used as a negative control. I, induced with 0.01µg/ml doxycycline; U, uninduced; L, L-conditioned-media; W, Wnt3a-conditioned media; EV, pcDNA3.1 transfected; 1b, Rac1b-Flag transfected. This experiment was conducted with the assistance of Dr. Vaijayanti Pethe.
immunoprecipitation (ChIP). As anticipated, Rac1b is constitutively resident at these Wnt target promoters and Dvl3 and β-catenin are recruited upon stimulation by Wnt3a (Figure 4.2). Interestingly, upon examining cells without Wnt3a stimulation (treated with L-cell-conditioned media), we observed that Rac1b leads to the recruitment of Dvl3 and β-catenin to canonical Wnt target promoters independently of Wnt3a stimulation. However, it appears that Rac1b expression leads to a physical saturation of the space occupying the TBEs when comparing the Wnt3a stimulated versus unstimulated for the Rac1b transfectants. In spite of this, we have previously shown that Wnt3a induction in addition to Rac1b expression leads to synergistic downstream canonical Wnt signaling.[212] Future experiments aimed at determining whether Rac1b truly exists in a complex at these promoters (through 2-step co-immunoprecipitation plus ChIP), and determining whether Rac1b can activate Dvl3 (using phosphatase assays) in the nucleus, will help further elucidate the role of Rac1b in the nucleus. Taken together, we propose a model where the cooperation between Rac1b and Wnt stimulation occurs in the nucleus to drive transcription of canonical Wnt target genes.

Since one of the known differences between Rac1 and Rac1b is the inability of Rac1b to activate the JNK pathway, coupled with the lack of Rac1b expression in HEK293T cells, it is unlikely that Rac1b is recruiting c-Jun to the c-myc promoter. These observations likely implicate Rac1b in a new role that is parallel to c-Jun in the recruitment of Dvl-3 to the promoter of Wnt-target genes. Given the two studies of both c-jun and Rac1b in Dvl-3 recruitment, the relationship between these transcriptional players may be larger than the ostensible physical proximity.
Proposed model for the nuclear role of Rac1b in colon cancer.
Upon Wnt3a stimulation in a normal cell (left panel), β-catenin and Dvl proteins are recruited to the promoter of canonical Wnt target genes. In cancer, nuclear expression of Rac1b (centre panel) leads to the recruitment of β-catenin and Dvl to these same promoters, independent of Wnt stimulation. Rac1b expression in the presence of Wnt3a stimulation (right panel) results in a synergistic increase in transcription of canonical Wnt target genes.
In summary, the present study focused on elucidating the specific molecular mechanisms by which Rac1 and Rac1b cooperate with the canonical Wnt signaling pathway to promote tumourigenesis in colorectal cancer cells. The interplay between Rac1/1b and canonical Wnt signaling led to novel findings at multiple levels, both at a single gene level and at a pathway level. Notably, Rac1 appears to drive the transcriptional expression of a subset of genes implicated in cell migration in a colon cancer cell line with dysregulated canonical Wnt signaling; knockdown of the Rac1 activator, Tiam1, reduced Wnt-responsive transcription; both Rac1 and Rac1b significantly increased cell migration, however, the subcellular localization of both genes were critical for their role in motility; Rac1b can drive the transcriptional expression of a noncoding RNA, MALAT1, and the RhoA effector, ROCK2, but not its protein expression or activity; and Rac1b expression in primary colon tumours correlates with ROCK2 expression, but not MALAT1. Taken together, it appears that the larger roles of Rac1 and Rac1b GTPases in carcinogenesis may be to tap into the canonical Wnt signaling with subtle individual changes that generate a collectively greater impact in colon cancer. Based on these observations, it can be postulated that the quick actions of Rac1 and Rac1b in the upregulation of the Wnt target genes might be a mechanism by which Rac GTPases are not required to remain at the plasma membrane to be activated, but that a nuclear pool of Rac might create a favourable environment for energy-efficient transcription. In agreement, overexpression of Rac1b resulted in the translocation of Rac1b directly to the promoters of Wnt target genes, independent of Wnt activation, and more interestingly, in the recruitment of Dvl3 and β-catenin to the same promoters, also without Wnt activation. These results underscore the importance of future investigations
on how Rac1b might act on various levels (cytoplasmic and nuclear components) of the canonical Wnt signaling pathway in the absence of Wnt activation. Additionally, given the small size of Rac1 and Rac1b and their limited number of domains, further analysis of the mechanism by which Rac contributes to transcription and protein recruitment will also further our understanding of this complex group of proteins.
References


47. Wang Y. Wnt/Planar cell polarity signaling: a new paradigm for cancer therapy. 


82. Ashton-Rickardt PG, Dunlop MG, Nakamura Y, Morris RG, Purdie CA, Steel CM, Evans HJ, Bird CC, and Wyllie AH. High frequency of APC loss in sporadic


125. Roberts PJ, Mitin N, Keller PJ, Chenette EJ, Madigan JP, Currin RO, Cox AD, Wilson O, Kirschmeier P, and Der CJ. Rho Family GTPase modification and


Appendix 1.

List of genes differentially expressed following Rac1b induction in HCT116 colon cancer cells. Fold change and number of putative TBE sites up to 1000kb upstream of the translation initiation sequence are indicated. Genes that comprise the primary pool for validation are highlighted. Multiple listings of the same gene names are due to different probesets for the same gene locus. n=35

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Appendix 2.

List of genes associated with cell migration in the leading edge following Gene Set Enrichment Analysis (GSEA). Multiple listings of the same gene names are due to different probesets for the same gene locus. n=13

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\(n = 47\)  
\(p = 0.031\)
Appendix 3.

Validation of candidate genes identified by GSEA. VEGF ($p=0.0754$) and NRD1 ($p=0.2764$) were validated by QRT-PCR.