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Role of Rap Signaling in the Regulation of ERK Activation and Cell-Cell Adhesion

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Role of Rap Signaling in the Regulation of ERK Activation and Cell-Cell Adhesion

Abstract
Rap is a member of the Ras family of small GTPases. Mammalian Rap proteins regulate many biological processes including cell and matrix adhesion, migration, proliferation, cytoskeletal dynamics, cell polarity and secretion. Rap1 is activated by TSH and cAMP in thyroid cells independently of PKA. TSH regulates differentiated gene expression and proliferation. Many of these effects are mediated through PKA. There is disagreement as to whether cAMP signaling through Rap contributes to any of the cellular effects of TSH. Given that thyroid cells express high levels of B-Raf and that Rap1 activates ERK through B-Raf in many cells, we investigated whether ERK activation by TSH is mediated through Epac/Rap. We found that ERK activation by TSH was Rap-independent. Instead, TSH stimulates ERK activity through PKA, Ras, B-Raf and MEK in Wistar rat thyroid cells. Intriguingly, silencing B-Raf impaired TSH-stimulated DNA synthesis. This is the first report to identify B-Raf as a target of TSH and a contributor to TSH-mediated proliferation. The contribution of Epac/Rap to the effects of TSH in thyroid cells remains uncertain.

Rap1GAP, an important cellular regulator of Rap activity, is downregulated in many human tumors including melanomas, thyroid, prostate, pancreatic, breast and colon carcinomas. In some instances, Rap1GAP expression was further decreased with tumor progression, suggesting that depletion of Rap1GAP contributes to tumor progression. For this reason the consequences of restoring Rap1GAP expression in human tumor cells were investigated. Given the well-defined role of Rap in the regulation of cell adhesion, we examined the functional consequences of restoring Rap1GAP expression on cell-matrix and cell-cell adhesion in human colon cancer cell lines. Modest overexpression of Rap1GAP sufficient to abolish Rap activity impaired cell-matrix interaction, including cell spreading and the formation of focal adhesions. Moreover, restoring Rap1GAP inhibited the ability of cells to migrate to serum. Under the same conditions, Rap1GAP had no effect on the formation or maintenance of cell-cell contacts. This suggests that Rap1GAP is a more efficient inhibitor of Rap signaling to the extracellular matrix than to cell-cell adhesion. The mechanism underlying the specificity of the inhibitory effects of Rap1GAP on Rap signaling remain to be elucidated.

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ROLE OF RAP SIGNALING IN THE REGULATION OF ERK ACTIVATION AND
CELL-CELL ADHESION

LISA A. VUCHAK

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DEDICATION

I dedicate this thesis to my mother, father and grandparents. My mother, Audrey, has always viewed education as a top priority and made every sacrifice to give her children the best education possible. I thank her for her undying love and support for me throughout my life and especially throughout my graduate career. I thank my father, Stephen, for his love and understanding and for supporting me in whichever profession I chose. I thank my grandmother, Geraldine, for inspiring me to be the salutatorian of my high school class and to pursue a career in science. I thank my grandfather, Stephen, for the fond memories and his loving affection.
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ABSTRACT

ROLE OF RAP SIGNALING IN THE REGULATION OF ERK ACTIVATION AND
CELL-CELL ADHESION

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JUDY L. MEINKOTH, Ph.D.

Rap is a member of the Ras family of small GTPases. Mammalian Rap proteins regulate many biological processes including cell and matrix adhesion, migration, proliferation, cytoskeletal dynamics, cell polarity and secretion. Rap1 is activated by TSH and cAMP in thyroid cells independently of PKA. TSH regulates differentiated gene expression and proliferation. Many of these effects are mediated through PKA. There is disagreement as to whether cAMP signaling through Rap contributes to any of the cellular effects of TSH. Given that thyroid cells express high levels of B-Raf and that Rap1 activates ERK through B-Raf in many cells, we investigated whether ERK activation by TSH is mediated through Epac/Rap. We found that ERK activation by TSH was Rap-independent. Instead, TSH stimulates ERK activity through PKA, Ras, B-Raf and MEK in Wistar rat thyroid cells. Intriguingly, silencing B-Raf impaired TSH-stimulated DNA synthesis. This is the first report to identify B-Raf as a target of TSH and a contributor to TSH-mediated proliferation. The contribution of Epac/Rap to the effects of TSH in thyroid cells remains uncertain.
Rap1GAP, an important cellular regulator of Rap activity, is downregulated in many human tumors including melanomas, thyroid, prostate, pancreatic, breast and colon carcinomas. In some instances, Rap1GAP expression was further decreased with tumor progression, suggesting that depletion of Rap1GAP contributes to tumor progression. For this reason the consequences of restoring Rap1GAP expression in human tumor cells were investigated. Given the well-defined role of Rap in the regulation of cell adhesion, we examined the functional consequences of restoring Rap1GAP expression on cell-matrix and cell-cell adhesion in human colon cancer cell lines. Modest overexpression of Rap1GAP sufficient to abolish Rap activity impaired cell-matrix interaction, including cell spreading and the formation of focal adhesions. Moreover, restoring Rap1GAP inhibited the ability of cells to migrate to serum. Under the same conditions, Rap1GAP had no effect on the formation or maintenance of cell-cell contacts. This suggests that Rap1GAP is a more efficient inhibitor of Rap signaling to the extracellular matrix than to cell-cell adhesion. The mechanism underlying the specificity of the inhibitory effects of Rap1GAP on Rap signaling remain to be elucidated.
# TABLE OF CONTENTS

DEDICATION.......................................................................................................................... ii  
ACKNOWLEDGEMENTS ........................................................................................................ iii  
ABSTRACT............................................................................................................................... v  
TABLE OF CONTENTS ........................................................................................................ vii  
LIST OF FIGURES .................................................................................................................. ix  

1. CHAPTER 1: INTRODUCTION..............................................................................................1  
   1.1 cAMP signaling pathways ...............................................................................................2  
   1.2 RapGTPases ...................................................................................................................5  
   1.3 Thyroid cells: a paradigm for cAMP signaling ..............................................................18  
   1.4 Contribution of Rap to TSH signaling ..........................................................................22  
   1.5 Thyroid cancer ............................................................................................................ 23  
   1.6 Rap activity and tumor progression .............................................................................27  
   1.7 Colon Cancer ..............................................................................................................32  
   1.8 Goals of the thesis .......................................................................................................39  

2. CHAPTER 2: PROTEIN KINASE A AND B-RAF MEDIATE EXTRACELLULAR SIGNAL-REGULATED KINASE ACTIVATION BY THYROTROPIN .............................................41  
   2.1 Introduction ..................................................................................................................43  
   2.2 Results .........................................................................................................................44  
   2.3 Discussion ...................................................................................................................60
2.4 Materials and Methods........................................................................................................63

3. CHAPTER 3: RAPIGAP IMPAIRS CELL-MATRIX ADHESION IN THE
ABSENCE OF EFFECTS ON CELL-CELL ADHESION.................................................67
   3.1 Introduction......................................................................................................................68
   3.2 Results ............................................................................................................................70
   3.3 Discussion........................................................................................................................86
   3.4 Materials and Methods....................................................................................................89

4. CHAPTER 4: DISCUSSION ...............................................................................................93

5. REFERENCES.......................................................................................................................114
LIST OF FIGURES

Chapter 1

Figure 1.1 cAMP signaling pathway .................................................................3
Figure 1.2 Receptor-mediated activation of PKA ...............................................4
Figure 1.3 Rap signaling pathways .................................................................9
Figure 1.4 A schematic of cell-cell and cell-ECM contacts .............................14
Figure 1.5 A model of Rap activation during cell-cell contact disruption ...........17
Figure 1.6 Thyroid hormone biosynthesis .....................................................21
Figure 1.7 Tumor cell dissemination ..............................................................31
Figure 1.8 The anatomy of the colon ..............................................................33
Figure 1.9 Mutation of the Wnt pathway in colon carcinomas .........................35
Figure 1.10 Niche succession and clonal conversion .......................................38

Chapter 2

Figure 2.1 TSH stimulates cAMP-dependent MEK and ERK activation ............45
Figure 2.2 cAMP stimulates Rap-independent ERK activation .......................47
Figure 2.3 Rap1 is not coupled to ERK activation .........................................49
Figure 2.4 ERK activation in FRTL5 and PCCL3 cells does not require Rap activity .51
Figure 2.5 ERK activation is PKA-dependent .................................................53
Figure 2.6 Ras activity is required for ERK activation ....................................55
Figure 2.7 B-Raf is required for cAMP-stimulated ERK activation ....................57
Figure 2.8 B-Raf is required for TSH-dependent DNA synthesis .....................59

Chapter 3

Figure 3.1 Blockade of Rap activity induces morphological changes in colon cancer cells .................................................................71
Figure 3.2 Rap1GAP impairs cell-matrix adhesion .......................................73
Figure 3.3 Rap1GAP impairs cell migration ..................................................75
Figure 3.4 Rap1GAP does not impair cell-cell adhesion ..................................78
Figure 3.5 Rap1GAP does not impair the formation of cell contacts ..................81
Figure 3.6 Rap1GAP does not impair cell-cell adhesion in non-transformed epithelial cells .................................................................83
Figure 3.7 Cell-cell junction formation requires Rap1 ......................................85

Chapter 4

Figure 4.1 A model for ERK activation by TSH in WRT cells .........................97
Figure 4.2 A model for Rap1GAP regulation of cell-cell and cell-matrix adhesion ....105
Figure 4.3 Rap regulates cellular processes involved in metastasis ...................113
CHAPTER 1: INTRODUCTION
1.1 cAMP signaling pathways

3′-5′-cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger that is generated downstream from serpentine receptors coupled to G\textsubscript{\alpha}s (see Figure 1.1). Receptor activation stimulates adenylyl cyclase, resulting in increases in intracellular cAMP. cAMP is degraded by phosphodiesterases (PDEs). PDEs are enzymes that cleave phosphodiester bonds. PDEs cleave cAMP into 5′ AMP, providing a mechanism to regulate the duration and amplitude of the cAMP signal. cAMP induces differentiation in many cells, such as fibroblasts (Pastan and Johnson, 1974) and smooth muscle cells (Dubey et al., 1996), and proliferation in others, including neuroendocrine cells (Frodin et al., 1994), thyrocytes (Dremier et al., 2002), Schwann cells (Raff et al., 1978), salivary gland cells (Selye et al., 1961), Swiss 3T3 fibroblasts (Rozengurt et al., 1981) and anterior pituitary somatotrophs (Burton et al., 1991). The mechanism through which cAMP induces such pleiotropic effects remains to be elucidated. The downstream effectors of cAMP include PKA, cAMP-gated ion channels, and more recently, Exchange protein activated by cAMP, or Epac. PKA is a holoenzyme comprised of a regulatory (R) subunit dimer and two catalytic (C) subunits. cAMP binds to the R subunits of the PKA holoenzyme, allowing the catalytic subunits to dissociate and act as serine/threonine kinases both in the cytoplasm and nucleus to phosphorylate downstream effectors including transcription factors such as CREB and CREM (Figure 1.2).
Figure 1.1 cAMP signaling pathway.
The Go_s subunit of the GPCR stimulates adenylyl cyclase (AC) which generates cAMP, resulting in the activation of PKA and Epac, a RapGEF. Figure adopted from (Metrich et al., 2010).
Figure 1.2 Receptor-mediated activation of PKA.
cAMP binds the regulatory subunits of PKA, freeing the catalytic subunits to enter the
nucleus and phosphorylate downstream effectors such as CREB. Figure from
PKA activity can be localized to specific cellular compartments by virtue of the association of R and C subunits with A-kinase anchoring proteins (AKAPs) (reviewed in (Colledge and Scott, 1999)). Epac is a Rap-specific guanine nucleotide exchange factor (RapGEF) that activates Rap by inducing GDP dissociation, allowing Rap to bind to GTP (See section 1.2 for a discussion of Rap proteins). Epac was first identified as a cAMP-binding protein that activates Rap independently of PKA (de Rooij et al., 1998; Kawasaki et al., 1998). cAMP binds to the cyclic nucleotide domain on Epac, reorienting Epac into an open conformation and relieving autoinhibition of the regulatory domain. This exposes the GEF domain for Rap binding (Bos, 2006). cAMP activates Rap in a PKA-independent manner in many cell types including differentiated thyroid cells, Chinese hamster ovary (CHO) cells, Ovcar3 cells and endothelial cells (Cullere et al., 2005; de Rooij et al., 1998; Rangarajan et al., 2003; Tsygankova et al., 2001). Thus, the discovery of Epac led to new avenues in cAMP signaling.

1.2 RapGTPases

Rap is a member of the Ras superfamily of small GTPases and shares 50% sequence homology to Ras. Rap1A, or K-Rev-1, was initially discovered as a protein capable of reverting the phenotype of K-Ras-transformed cells (Kitayama et al., 1989). Ras and Rap contain identical effector domains and bind many of the same downstream effectors. It was proposed that the reversion of Ras transformation by Rap was due to the sequestration of downstream effectors. Although overexpression of Rap can impair Ras signaling, when expressed at physiological levels, the roles of Rap are distinct from those
of Ras (reviewed in (Stork, 2003)). As an example, activation of the PDGF receptor led to the activation of Ras and Rap1. Nevertheless, Ras-mediated ERK activation was not impaired (Zwartkruis and Bos, 1999). The mammalian Rap family is comprised of Rap1a/b and Rap2a/b/c, the products of distinct genes. Rap1a/b share 90% homology and the Rap2 isoforms 95% homology. Rap1 and Rap2 share 60% homology (Caron, 2003).

Like other GTPases, Rap is active when bound to GTP and inactive when bound to GDP (Figure 1.1). Cellular Rap activity is controlled by Rap-specific GEFs and RapGTPase activating proteins (RapGAPs). RapGEFs include C3G, PDZ-GEF1 and -2, Epac1, -2 and Repac, CalDAGGEFs and Dock4 (reviewed in (Quilliam et al., 2002)). RapGEFs are activated downstream from receptor tyrosine kinases (RTKs), G-protein-coupled receptors (GPCRs) and cytokine receptors (Bos et al., 2003). Activation of the Gs-coupled TSH receptor results in Epac-mediated Rap activation (Mei et al., 2002; Tsygankova et al., 2001). Epac1 contains a single cAMP-binding domain while Epac2 contains an additional cAMP-binding domain. C3G is activated by RTKs including the NGF receptor (Kao et al., 2001). PDZ-GEFs contain a PDZ and a Ras-association (RA) domain and provide a mechanism for integrating Rap- and Ras-mediated signaling. Repac also contains an RA domain. CalDAG-GEFs contain calcium and diacylglycerol (DAG) binding domains and exhibit differing small G-protein specificities. CalDAG-GEFI activates Rap1 and R-Ras, CalDAG-GEFII activates Ras and R-Ras and CalDAG-GEFIII activates Ras, Rap1, Rap2 and R-Ras (Bos et al., 2001; Pannekoek et al., 2009).
Rap activity is terminated by a family of RapGAPs that include Rap1GAPs (Rap1GAP, Rap1GAPII) and the SPA-1 family (SPA-1, SPAR, SPAL, E6TP1) (Bos et al., 2007; Donovan et al., 2002; Spilker and Kreutz, 2010). In Dictyostelium, Rap1GAP was translocated to the plasma membrane in response to chemoattractants (Jeon et al., 2007). Rap1GAPII interacts with several G-protein α-subunits (Mochizuki et al., 1999) and has shown to be recruited to the plasma membrane by Giai and Giaz (Meng and Casey, 2002; Mochizuki et al., 1999). Most RapGAPs contain domains in addition to the GAP domain (reviewed in (Spilker and Kreutz, 2010)). This suggests that these proteins associate with other proteins in signaling domains.

In its GTP-bound form, Rap interacts with many of the same downstream effectors that are activated by Ras, including Raf-1, B-Raf, PI3K, RalGEFs and Krit, although Krit binds Rap with a much higher affinity than Ras. Unlike Ras, interaction of Rap with Raf-1 does not result in its activation. Constitutively active Rap1 has been shown to modestly activate p110, the catalytic subunit of PI3K in 293T cells (Rodriguez-Viciana et al., 2004). In thyroid cells, Rap1 contributes to Akt activation by TSH and PI3K (Tsygankova et al., 2001). In B-cells, Rap2 inhibits PI3K activation of Akt (Christian et al., 2003). Rap1 binds to and activates B-Raf (reviewed in (Stork, 2003)). Rap1 signaling to B-Raf has been reported to activate ERK in PC12 and CHO cells and to inhibit ERK activation in C6 glioma and NB2A neuroblastoma cells (Qiu et al., 2000). The pleiotropic effects of Rap on ERK activation are shared by cAMP, which elicits opposing effects on ERK activation in different cell types. In Chapter 2, we explore the role of Rap1 in B-Raf-mediated activation of ERK in thyroid cells. Afadin (AF-6), an
adaptor protein that binds to ZO-1 in tight junctions, nectins in adherens junctions and profilin, an actin binding protein, is bound by both GTP-bound Ras and Rap1 (Boettner et al., 2000; Bos, 2005).

There are a number of Rap-specific effector proteins. Rap1 binds to RapL, which facilitates the interaction and activation of LFA-1 integrins by Rap1. Dominant negative RapL mutants impaired the clustering of integrins and adhesion to ICAM in T-cells (Katagiri et al., 2003). RIAM, Rap-GTP-interacting adaptor molecule, is another Rap-specific effector. RIAM links Rap1 activation to the actin cytoskeleton via binding to profilin. Inhibition of RIAM by gene silencing impaired integrin-mediated cell adhesion in Jurkat T cells (Lafuente et al., 2004). Rap1GTP also interacts with Arap3, a RhoAGAP and an Arf6GAP. The binding of activated Rap to Arap3 enhanced RhoGAP activity (Krugmann et al., 2004). The RacGEFs Vav2 and Tiam1 also interact with Rap1GTP. A role for Rap in the localization of RacGEFs at cell protrusions has been reported (Arthur et al., 2004).
Figure 1.3 Rap signaling pathways.
Rap is activated by receptor tyrosine kinases, G-protein-coupled receptors and cytokine receptors through the activation of RapGEFs. In its active form, Rap signals to a variety of effectors that function in integrin activation, proliferation, adhesion, actin cytoskeletal dynamics, secretion and cell polarity. Rap activity is terminated by RapGAPs. Figure adapted from (Bos, 2005).
The regulation of Rap1 by cAMP is complex. cAMP activates Epac, resulting in the activation of Rap. PKA can also activate Rap1 by phosphorylation of Src on serine 17, activating Rap through the Cbl/Crk/C3G pathway (Schmitt and Stork, 2002). Adding further complexity to the situation, PKA phosphorylates Rap1. As described above, there are two Rap1 proteins expressed in mammalian cells, Rap1a and Rap1b. PKA phosphorylates Rap1a at serine 180 (Lerosey et al., 1991) and Rap1b at serine 179 (Altschuler and Lapetina, 1993). Rap1a has a lysine residue at amino acid 179, while Rap1b has serine residues at both 179 and 180. PKA phosphorylates Rap1b primarily at serine 179, although if serine 179 is mutated, serine 180 can be phosphorylated by PKA (1993; Bokoch and Quilliam, 1990; Hoshijima et al., 1988; Kawata et al., 1989). The phosphorylation of Rap1 by PKA is widespread. PKA-mediated phosphorylation of Rap1 has been reported in rat thyroid, Rat-1, HL-60, PC12 cells and neutrophils (Bokoch et al., 1991; Lerosey et al., 1991; Quilliam et al., 1991; Quinn et al., 1989; Tsygankova et al., 2001; Vossler et al., 1997). In one report, phosphorylation of Rap1 by PKA did not alter Rap activity or cellular localization (Bokoch, 1993). In rat thyroid cells, blockade of PKA activity prolonged the duration of Rap1 activity (Tsygankova et al., 2001). A role for phosphorylation in the interaction of activated Rap1 with downstream effectors has been reported. Phosphorylation decreased the binding of Rap1 to cytochrome b in neutrophils (Bokoch et al., 1991). Rap1 phosphorylation mutants can alter Ras-mediated Raf-1 activation in COS-7 cells (Hu et al., 1999). More recently, phosphorylation of Rap was shown to induce a conformational change in a distal effector domain, further suggesting that phosphorylation of Rap by PKA regulates its binding partners (Edreira et
al., 2009). Some of the functions of Rap require both activation and phosphorylation. In differentiated thyroid cells, both activation of Rap1 by Epac and phosphorylation by PKA are required for TSH-stimulated DNA synthesis (Hochbaum et al., 2008; Ribeiro-Neto et al., 2002). Activation of Akt by TSH required PKA-mediated phosphorylation of Rap1 (Tsygankova et al., 2001).

Insights into the functions of Rap were initially provided from studies in lower organisms (reviewed in (Bos et al., 2001)). In yeast, the Rap1 homologue Bud1 regulates polarization and bud site selection by recruiting Cdc42, Cdc24 (a GEF for cdc42) and Bem1, a scaffolding protein with two SH3 domains (Gulli et al., 2000; Park et al., 1997; Park et al., 1999). In Dictyostelium, Rap1 regulates the actin cytoskeleton and membrane ruffling. In this organism, chemoattractants activate Rap1 and localize it to the leading edge of migrating cells. Here, Rap is required for the phosphorylation and disassembly of myosin II, resulting in the elaboration of membrane protrusions at the leading edge of chemotaxing cells (Jeon et al., 2007). Gain of function mutations in Rap resulted in aberrant cell fate specification in the eye in Drosophila (Hariharan et al., 1991). Loss of function Rap mutants induced defects in dorsal closure, ventral furrow closure and head involution in Drosophila embryos, suggesting that Rap1 is required for cell migration during development (Asha et al., 1999). In the wing, cells with mutant Rap1 dispersed into adjacent tissue. Rap was also required for normal eye, imaginal disk and ovary development (Knox and Brown, 2002). Together, these observations provided the first evidence that Rap functions in the regulation of cell-cell adhesion.
In mammalian cells, Rap1 regulates integrin activation, cell-matrix adhesion, cell-cell adhesion, cell polarity, vesicle trafficking, proliferation, migration, secretion/exocytosis and cytoskeletal dynamics (Boettner and Van Aelst, 2009; Bos et al., 2003; Caron, 2003). The localization of Rap1b drove axonal specification of neurites in rat hippocampal neurons and activated Rap1 induced a polarized morphology in lymphocytes (Schwamborn and Puschel, 2004; Shimonaka et al., 2003). Activated Rap1 enhanced the proliferation of hematopoietic progenitor cells (Ishida et al., 2003). Similarly, microinjection of activated Rap1 into Swiss 3T3 cells induced DNA synthesis (Yoshida et al., 1992). Constitutively active Rap1b stimulated cAMP-stimulated DNA synthesis in PCCL3 thyroid cells, which also required the phosphorylation of Rap1b (Ribeiro-Neto et al., 2002). Rap1 induced amylase secretion in rat parotid acini after isoproterenol stimulation (D'Silva et al., 1998). Epac-mediated activation of Rap mobilized intracellular calcium stores in pancreatic beta cells, a process linked to exocytosis (Kang et al., 2003).

Of the myriad of functions regulated by Rap1, the regulation of integrin-dependent cell-matrix adhesion has been most thoroughly investigated (reviewed in (Bos et al., 2003; Bos et al., 2001; Caron, 2003)). Integrins are transmembrane glycoproteins composed of α and β subunits which serve to link the actin cytoskeleton to the matrix via focal adhesions (Hynes, 2002). Rap1 activates β1, β2 and β3 integrins and, in this way, regulates integrin affinity and avidity or “inside-out signaling” (Boettner and Van Aelst, 2009; Caron et al., 2000; Katagiri et al., 2000; Reedquist et al., 2000; Tsukamoto et al., 1999). In platelets, activation of Rap1 by PMA recruits talin and RIAM to α2β3
integrins. It is believed that Rap induces a conformational change in talin that results in its binding to and activation of α2β3 (Boettner and Van Aelst, 2009; Han et al., 2006). Silencing RIAM impaired Rap1-dependent integrin activation and adhesion to fibronectin in Jurkat cells (Boettner and Van Aelst, 2009). Rap1-dependent integrin activation in platelets and T-cells has been demonstrated in mouse models (Chrzanowska-Wodnicka et al., 2005; Sebzda et al., 2002). Rap1-mediated integrin activation functions in a variety of biological processes including T-cell and antigen-presenting cell interactions (Katagiri et al., 2002), macrophage phagocytosis (Caron et al., 2000) and chemokine-induced migration in B-cells (McLeod et al., 2002).

Rap plays complex roles in the regulation of E-cadherin-based cell-cell contacts. E-cadherin is a major component of adherens junctions. E-cadherin is a type I transmembrane protein. Its extracellular domain is comprised of five cadherin repeats that encode calcium binding sequences. Upon calcium binding, the extracellular domains undergo homophilic interactions with E-cadherin molecules on adjacent cells. The intracellular domain is highly conserved among cadherins. The cytoplasmic tail of an E-cadherin homodimer binds to β-catenin. β-catenin, in turn, binds α-catenin to link E-cadherin to the actin cytoskeleton (see Figure 1.4). p120 catenin binds to the juxtamembrane domain of E-cadherin and regulates the stability of E-cadherin at the plasma membrane (Peifer and Yap, 2003). p120 catenin may also play a role in cadherin clustering or adhesive activity (Wheelock and Johnson, 2003).
Figure 1.4 A schematic of cell-cell and cell-ECM contacts.
E-cadherin is a major component of adherens junctions, a calcium-dependent type of cell-cell contact. E-cadherin binds β-catenin and α-catenin to link E-cadherin to the actin cytoskeleton. Adapted from (Niessen, 2007).
In *Drosophila*, cells expressing mutant Rap displayed aberrant distribution of E-cadherin and dispersed into surrounding wild-type cells. In this organism, Rap1 was found to localize to the junction between two daughter cells during cytokinesis (Knox and Brown, 2002). These landmark studies provided the first evidence that Rap1 regulates cell-cell adhesion. In epithelial cells, the role of Rap in the regulation of cell-cell contacts is incompletely understood. In osteosarcoma cells, inactivating mutations in the RapGEF DOCK4 induced aberrant adherens junctions suggesting that Rap1 is required for cell-cell adhesion (Yajnik et al., 2003). Expression of activated Rap1 in Ras-transformed MDCK cells restored an epithelial morphology (Price et al., 2004). Similarly, stable expression of activated Rap1 in these cells conferred resistance to cell scattering by scatter factor or HGF (Asuri et al., 2008). MDCK cells overexpressing Epac were also resistant to scattering by HGF when stimulated with 8CPT-cAMP (Price et al., 2004). 8CPT-cAMP (also referred to as EcAMP or 0007) is a cAMP analog that selectively activates Epac (See figure 1.1). In MCF-7 cells, overexpression of Rap1GAP impaired nascent cell-cell contact formation and the recruitment of E-cadherin to the membrane, but had no effect on the maintenance of cell-cell contacts in confluent cells. Interestingly, in the same study, expression of RapV12 impaired the recruitment of E-cadherin to cell-cell contacts (Hogan et al., 2004). These results agree with an earlier report that sustained high level expression of activated Rap1 led to aberrations in adherens junctions (Boettner et al., 2000). The regulation of cell-cell contacts by Rap1 in endothelial cells seems to be more straightforward. Activation of Epac in HUVEC cells decreased cell permeability (Fukuhara et al., 2005; Wittchen et al., 2005). Furthermore,
cells treated with EGTA to disrupt cell contacts reformed contacts faster in calcium-supplemented medium containing EcAMP (Wittchen et al., 2005). Overexpression of Rap1GAP increased cell permeability (Fukuhara et al., 2005; Wittchen et al., 2005) and inhibited the recruitment of β-catenin to the membrane in HUVECs (Wittchen et al., 2005).

The above findings support a role for Rap1 in promoting E-cadherin (or VE-cadherin)-mediated cell-cell adhesion. However, in many epithelial cell lines, the disruption of E-cadherin-mediated cell-cell contacts activates Rap1. Rap1 is activated upon the disruption of E-cadherin-based cell-cell contacts in MCF-7, FRT, COS7, NIH3T3, GD25, sEnd1 cells and MDCK cells, and inactivated as cell-cell contacts reform (Asuri et al., 2008; Balzac et al., 2005). Balzac and colleagues found that the activation of Rap upon cell-cell disruption was E-cadherin-dependent and independent of integrin signaling. E-cadherin ligation was also required to inactivate Rap when contacts reform with calcium addition (see Figure 1.5) (Asuri et al., 2008; Balzac et al., 2005).
Figure 1.5 A model of Rap activation during cell-cell contact disruption.
Disruption of cell-cell contacts through calcium chelation by treatment with EGTA, thrombin or by incubation in low calcium medium activates Rap1. Cell contact reformation was correlated with a decrease in Rap1 activity which required the ligation of E-cadherin transdimers. DECMA, an antibody that blocks E-cadherin transligation, prevented Rap1 inactivation by calcium.
It has been suggested that Rap1 is activated upon cell-cell contact disruption as a repair mechanism to reinitiate contact re-formation (Asuri et al., 2008). Alternatively, Balzac et al. proposed that Rap1 integrates signals that induce the disruption of cell-cell contacts with those that induce the formation of focal adhesions. In the report by Balzac et al., disruption of cell-cell contacts resulted in the endocytosis of E-cadherin and Rap1, which transiently co-localized in endosomes. Microinjection of Rap1GAP impaired focal adhesion formation, suggesting that Rap1 activation by the disruption of cell-cell contacts is required for the subsequent formation of focal adhesions (Balzac et al., 2005). Collectively, these data highlight the complexity of the role of Rap in the regulation of cell-cell adhesion. To date, overexpression of activated Rap1 has been associated with conflicting effects on the formation and maintenance of cell-cell contacts in epithelial cells. In Chapter 3, the requirement of endogenous Rap in the formation and maintenance of cell-cell contacts is further explored.

1.3 Thyroid cells: a paradigm for cAMP signaling

Thyroid epithelial cells provide an excellent model for the study of cAMP signaling. The physiological regulator of the thyroid follicular cell is thyroid-stimulating hormone, TSH (or thyrotropin). TSH is a glycoprotein hormone that is secreted from the anterior pituitary gland upon stimulation by TRH (thyroid-releasing hormone) released from the hypothalamus. Although the TSH receptor is coupled to a large number of G-proteins, most of the cellular effects of TSH are mediated through Gαs-mediated activation of adenylyl cyclase and the generation of cAMP (reviewed in (Kimura et al.,
TSH regulates the differentiated function of thyroid follicular cells and is required for the proliferation of these cells (Dumont et al., 1992; Zakarija and McKenzie, 1989).

Virtually all aspects of thyroid hormone synthesis are regulated by TSH. TSH induces the expression of the sodium/iodide symporter (NIS), thyroperoxidase (TPO) and thyroglobulin (Tg), proteins that function in thyroid hormone biosynthesis. Dietary iodide is actively transported from the circulation into thyroid follicular cells via NIS (see Figure 1.6). Iodide is transported to the apical cell surface where it is oxidized to its active form by TPO. TPO carries out organification or the coupling of iodinated tyrosyl residues to Tg, a precursor to thyroid hormone. The coupling of two di-iodotyrosyl residues results in the formation of $3,5,3',5'$-tetraiodothyronine or thyroxine, T4. The coupling of one mono-iodotyrosyl and one di-iodotyrosyl residues results in the formation of triiodothyronine or T3. Iodinated Tg is stored in the lumen as colloid. Upon an appropriate signal, Tg is taken up into the follicular cell by endocytosis. Endosomes then fuse with lysosomes, resulting in the proteolytic cleavage of Tg, generating free amino acids and T4 and T3.

All of the steps in TSH-induced thyroid hormone synthesis are believed to be PKA-dependent. In dog thyrocytes, microinjection of a PKA inhibitor blocked TSH-induced accumulation of Tg and TPO (Dremier et al., 1997). Two distinct PKA-selective cAMP analogs were sufficient to induce NIS, TPO and Tg expression in dog thyroid cells and iodide uptake and T3/4 secretion in dog thyroid tissue slices (Dremier et al., 2007). However, one study reported PKA-dependent and -independent regulation of the
upstream enhancer element in the NIS promoter (NUE) by TSH, suggesting that the situation may be more complex (Ohno et al., 1999; Taki et al., 2002).
Figure 1.6 Thyroid hormone biosynthesis.
TSH stimulates thyroid hormone production. Iodide is actively transported into the follicular cell by NIS. Here, iodine is oxidized and one or two iodinated tyrosines are coupled to Tg, a precursor to thyroid hormone. Iodinated Tg is stored in the follicular lumen colloid. When required, Tg is endocytosed into the follicular cells and cleaved by lysosomal enzymes, releasing free amino acids and T4/T3 which diffuse across the membrane and enter the circulation.
1.4 Contribution of Rap to TSH Signaling

In addition to its effects on thyroid function, TSH is strictly required for the proliferation of thyroid follicular cells. Although TSH is not a mitogen on its own (Lewis et al., 2004), it collaborates with serum growth factors, primarily insulin-like growth factor I (IGF-I), to stimulate thyroid cell proliferation (Kimura et al., 2001; Medina and Santisteban, 2000; Zakarija and McKenzie, 1989). The contribution of TSH to cell proliferation can be reproduced by cholera toxin (acting at the level of G\textsubscript{\alpha}s), forskolin (acting at the level of adenylyl cyclase) and cAMP analogs. However, while cAMP elevating agents and analogs mimic the effects of TSH, activation of PKA did not (Dremier et al., 1997; Tsygankova et al., 2000). In WRT cells, microinjection of purified PKA catalytic subunit failed to reproduce the effects of cAMP analogs on DNA synthesis (Tsygankova et al., 2000). This paradox was explained by the identification of additional pathways activated by cAMP, including cAMP-mediated activation of Ras and Epac/Rap.

More recent studies conducted in rat thyroid epithelial cell lines revealed that the activation of Rap1 by Epac as well as the phosphorylation of Rap1 by PKA are required for TSH-induced DNA synthesis (Hochbaum et al., 2008; Ribeiro-Neto et al., 2002). Thyroid epithelial cells expressing activated Rap1 enhanced TSH-stimulated DNA synthesis and cells expressing dominant negative Rap1 impaired TSH-stimulated DNA synthesis (Ribeiro-Neto et al., 2002). Additionally, the expression of mutant Rap1 with a point mutation at the PKA phosphorylation site impaired TSH-induced DNA synthesis (Ribeiro-Neto et al., 2002). In this study, dominant negative Epac hindered DNA synthesis by cAMP. Specifically, the membrane localization of Epac via its DEP domain
was essential for cAMP-mediated mitogenesis (Hochbaum et al., 2008). Overexpression of Rap1GAP in WRT cells impaired TSH-stimulated DNA synthesis and proliferation (Tsygankova et al., 2004). Together, these data indicate that both Rap and PKA are required for cAMP-stimulated proliferation in thyroid cells. The mechanism through which Rap1 contributes to cell proliferation is not understood. It has been suggested that Rap1 mediates cAMP-dependent ERK activation in cells that express B-Raf. B-Raf is highly expressed in thyroid cells and B-Raf activating mutations are common in thyroid tumors. In Chapter two, we investigate the contribution of Rap1 and B-Raf to the regulation of ERK activity by TSH.

1.5 Thyroid Cancer

Thyroid cancer is the most common endocrine cancer. The incidence of thyroid cancer has been steadily rising in the past two decades, largely due to advances in diagnosis (Giusti et al., 2010). In 2009, there were 37,200 new cases and 1,630 deaths due to thyroid cancer in the United States (NCI). Thyroid carcinomas are derived from follicular epithelial cells (thyroid hormone-producing cells) or parafollicular C cells (calcitonin-producing cells). The vast majority of thyroid carcinomas are derived from follicular cells. These are classified as papillary thyroid carcinoma (PTC) or follicular thyroid carcinoma (FTC) based on histology. PTC and FTC are well-differentiated tumors that can be treated by surgery, followed by hormone replacement therapy. The prognosis for patients with differentiated thyroid tumors is favorable. Today, the overall survival rate is 93% for patients with PTC and 85% for patients with FTC (Hundahl et al.,
The most significant factor in prognosis was age. The cancer mortality rates were lowest in patients under 40 years of age (Mazzaferri and Jhiang, 1994). Risk factors for PTC and FTC include radiation exposure, reduced iodine intake, lymphocytic thyroiditis, hormonal factors, and family history (Kondo et al., 2006). Unfortunately, many thyroid tumors progress to poorly-differentiated carcinomas, the most extreme of which is anaplastic thyroid carcinoma (ATC). ATC is an extremely aggressive cancer that is invariably fatal, most commonly within 6 months following diagnosis. Loss of NIS expression in undifferentiated thyroid tumors renders radioiodide therapy ineffective. Chemotherapy is relatively ineffective in these tumors, and up to several years ago, supportive care was the only treatment for ATC. With the discovery of activating mutations in B-Raf in PTC in 2003 (Kimura et al., 2003), targeted therapies have been exploited in the treatment of thyroid cancer (reviewed in (Woyach and Shah, 2009)).

There are several common genetic alterations present in thyroid carcinomas. In PTC, the most common mutations include activating mutations in B-Raf (45%) and RET/PTC chromosomal rearrangements (20%), both of which result in sustained ERK activation. RET is a RTK for the glial cell-derived neurotrophic factor (GDNF) family that is involved in the growth, survival, differentiation and migration of cells derived from the neural crest (Fagin, 2002). The rearrangement occurs when the RET intracellular kinase domain is juxtaposed to a variety of partners, driving dimerization and activation of the RET kinase domain (Fagin, 2002). There are up to 15 different RET fusion proteins, including RET/PTC1, RET/PTC2 and RET/PTC3. The genes fused to the RET gene include H4/D10S170, R1α (regulatory subunit of PKA) and the RFG/ELE1
gene, respectively (Fagin, 2002). B-Raf and RET/PTC alterations are rarely found in the same tumor, suggesting that the major advantage conferred by these alterations is hyperactivation of the ERK pathway (Nikiforov, 2008).

Ras mutations are most common in FTCs (40-50%), but also occur in PTCs (10-20%). Activating mutations in Ras, PAX8-PPARY rearrangements and PI3K activation are the most common alterations found in FTC (Gilfillan, 2010; Nikiforov, 2008). Both gene amplification and activating mutations in the PI3K catalytic subunit are found in FTCs (Saji and Ringel, 2010). Anaplastic thyroid carcinomas are believed to arise de novo or from PTCs as a result of the accumulation of additional mutations. ATCs are characterized by a wide spectrum of mutational events including loss of p16 and mutations in p53, β-catenin, PIK3CA, Axin, APC and PTEN (Ball et al., 2007; Smallridge and Copland, 2010).

Hyperactivating mutations of the TSHR and activating mutations in Gaα are common in thyroid adenomas and not seen in carcinomas, suggesting that chronic cAMP signaling is not sufficient for tumor progression. Mutations in Epac or Rap have not been reported in thyroid adenomas or carcinomas (Matsuse et al., 2009; Vanvooren et al., 2001). Interestingly, our lab discovered that Rap1GAP is downregulated in thyroid adenomas and its expression further decreased in carcinomas, suggesting that Rap activity may be increased in thyroid tumors (Nellore et al., 2009).

Ribeiro-Neto and colleagues studied the effect of sustained Rap1 activity in the thyroid gland in mice. A transgenic mouse model was constructed wherein activated Rap1 was targeted to the thyroid gland. This transgene also encoded conditional
dominant negative Rap1 (RapN17), which upon tamoxifen treatment, resulted in the loss of activated Rap and the expression of dominant negative Rap (Ribeiro-Neto et al., 2004). This model provided a tool to investigate the role of Rap1 in tumor initiation and maintenance. Mice expressing activated Rap1 developed enlarged multilobular glands, suggestive of hyperplasia. In the presence of goitrogens to increase circulating TSH, a small proportion of mice developed invasive lesions, but only over prolonged times. When young animals were treated with tamoxifen to induce the expression of dominant negative Rap, the oncogenic phenotype was lost, as assessed by decreased Ki67 labeling and reduced tumor volume. The requirement for circulating TSH suggests that Rap behaves as a conditional oncogene, perhaps requiring TSH-mediated phosphorylation for its activity. In addition, these findings suggest that Rap activity is required for tumor maintenance (Ribeiro-Neto et al., 2004).

More recently, the effects of Rap1GAP downregulation was explored in WRT cells. WRT cells express abundant levels of Rap1GAP. Acute silencing of Rap1GAP expression increased cell number, suggesting that Rap1GAP functions as a tumor suppressor in thyroid cells (Tsygankova et al., 2007). Interestingly, stable or transient expression of the Ras oncogene in WRT cells extinguished Rap1GAP expression suggesting that Rap1GAP downregulation may contribute to Ras transformation (Tsygankova et al., 2007). The expression of Rap1GAP is lost from thyroid carcinoma cell lines. Overexpression of Rap1GAP in follicular thyroid carcinoma cells impaired Rap activity, cell spreading, migration and invasion. Overexpression of Rap1GAP also inhibited DNA synthesis as measured by BrdU incorporation, and the growth of colonies.
in soft agar. These findings were recently confirmed by others, who showed that overexpression of Rap1GAP impaired cell migration, invasion and growth in agar in papillary and anaplastic thyroid carcinoma cell lines (Zuo et al., 2010).

1.6 Rap activity and tumor progression

It is now clear that downregulation of Rap1GAP is widespread in human tumors. The expression of Rap1GAP expression is decreased in thyroid, colon and pancreatic carcinomas and melanomas (Nellore et al., 2009; Tsygankova et al., 2010; Tsygankova et al., 2007; Zhang et al., 2006a; Zheng et al., 2009; Zuo et al., 2010). Where it has been examined, the expression of Rap1GAP was further decreased in invasive versus benign lesions. These results suggest a role for Rap1GAP depletion in tumor progression. Many studies have analyzed the phenotypic effects of overexpressing Rap1GAP in tumor cells in vitro. Stable overexpression of Rap1GAP impaired DNA synthesis, focal adhesion formation and cell migration in human melanoma cell lines (Zheng et al., 2009). Stable overexpression of Rap1GAP in pancreatic cancer cell lines impaired proliferation and growth in agar, random cell motility and invasion (Zhang et al., 2006a). Oropharyngeal squamous cell carcinoma (SCC) cells that stably overexpressed Rap1GAP exhibited decreased proliferation and MAPK signaling. The inhibitory effects on MAPK were not seen in pancreatic carcinoma cell lines that expressed Rap1GAP (Zhang et al., 2006a). In prostate cancer cell lines, Rap1GAP impaired migration and invasion (Bailey et al., 2009). Primary tumors formed when SCC cells stably overexpressing Rap1GAP were injected subcutaneously into mice were smaller compared to controls (Zhang et al.,
In vivo, injection of breast carcinoma cells expressing activated Rap1 resulted in a higher percentage of mice with large tumors compared to vector controls (Itoh et al., 2007). In contrast to these studies, Rap1GAP-overexpressing SCC cells exhibited enhanced invasion secondary to the upregulation of matrix metalloproteinases (Mitra et al., 2008).

A small number of studies have focused on the contribution of Rap1GAP to metastasis. Intracardiac injection of prostate carcinoma cells expressing activated Rap1 resulted in enhanced metastasis in mice (Bailey et al., 2009). When Rap1GAP-overexpressing pancreatic cancer cells were injected into the pancreas of mice, the primary tumors formed were smaller, and decreased metastasis to the liver and lymph nodes was observed (Zhang et al., 2006a). When injected into the tail vein of mice, A20 B-cell lymphoma cells expressing Rap1GAP exhibited a decrease in the formation of liver metastases. Interestingly, when these lesions were explanted and analyzed, the expression of Rap1GAP was dramatically reduced (Lin et al., 2010). By contrast, primary tumors that arose following subcutaneous injections retained the expression of Rap1GAP. These data suggest that downregulation of Rap1GAP may be required for metastasis (Lin et al., 2010). It is likely that Rap activity plays multiple roles in tumor cell dissemination. In addition to alterations in cell-cell and cell-matrix adhesion, increased Rap activity promotes cell migration as well as adhesion to endothelial cells that could enhance extravasation from the vasculature (see Figure 1.7). Extravasation occurs where the tumor cells leave the vasculature and enter the new organ through endothelial cell transmigration (Chambers et al., 2002). Interestingly, B16F1 melanoma
cells expressing constitutively active Rap or Rap1GAP impaired extravasation from the vasculature and, consequently, the formation of lung metastases. These results suggest that the cycling of Rap activity is required for movement across the endothelial cell wall (Freeman, 2010 #256).

Our laboratory took a different approach to assess the contribution of Rap1GAP depletion to tumorigenesis. Rather than overexpress Rap1GAP, we decided to abolish its expression and investigate whether loss of Rap1GAP conferred aggressive features to tumor cells. More than ten thyroid carcinoma cell lines were screened for Rap1GAP expression (Tsygankova, unpublished). None of these cell lines retained the expression of Rap1GAP. We then screened a panel of human breast and colon carcinoma cell lines. This analysis identified several tumor cell lines that retained Rap1GAP expression, including HT29 colon carcinoma cells. We used this cell line as a model to investigate whether depleting Rap1GAP was associated with functional effects in tumor cells. Stable silencing of Rap1GAP in HT29 cells was sufficient to induce durable increases in both Rap1 and Rap2 activity (Tsygankova et al., 2010). Strikingly, Rap1GAP-depleted cells exhibited profound changes in cell morphology. Unlike parental HT29 cells which exhibited a high degree of cell-cell contact, Rap1GAP-depleted cells were more dispersed. Loss of Rap1GAP enhanced adhesion and spreading on collagen IV, consistent with the established role of Rap in promoting integrin activation and interaction with the extracellular matrix. Unexpectedly, Rap1GAP-depleted cells exhibited defects in cell-cell adhesion, including weakened adhesion and that aberrant localization of the adherens junction proteins, E-cadherin, β-catenin and p120-catenin
(Tsygankova et al., 2010). These effects were a consequence of unrestrained Rap activity as silencing the expression of Rap1 and Rap2 restored the localization of E-cadherin, β-catenin and p120-catenin to cell-cell contacts. (Tsygankova et al., 2010). Given the well-documented role of Rap in promoting cell-cell adhesion, which seemed to be at odds with our findings, we decided to further investigate the role of Rap1GAP in the regulation of cell-ECM and cell-cell adhesion in colon cancer cells. In Chapter 3, we demonstrate that modest overexpression of Rap1GAP in HCT116 colon cancer cells impairs cell-matrix adhesion in the absence of effects on cell-cell adhesion. This suggests that the role of Rap1GAP in the regulation of cellular adhesion is more complex than is currently appreciated.
Figure 1.7 Tumor cell dissemination.
Tumor cells degrade the basement membrane and invade the extracellular matrix. Following intravasation, tumor cells circulate through the blood stream to distal sites. Tumor cells are then physically trapped in capillary beds of a new organ, extravasate and give rise to metastatic lesions. This figure is adopted from (Guo and Giancotti, 2004).
1.7 Colon Cancer

In the United States and worldwide, colon cancer is the fourth most common cancer in men and women after skin, prostate/breast and lung cancer (NCI) (Parkin et al., 2005). It is estimated that there will be 102,900 new cases of colon cancer and 39,670 new cases of rectal carcinomas this year. A total of 51,370 deaths from this disease are anticipated this year (NCI). Risk factors for developing colorectal carcinoma include age (50 years and older), the presence of colorectal polyps, a family history of colon cancer, hereditary syndromes such as familial adenomatous polyposis (FAP), which is caused by a mutation in the APC gene, cigarette smoking, diet and persons with inflammatory bowel diseases such as inflammatory bowel syndrome and Crohn’s disease (reviewed in (Markowitz and Bertagnolli, 2009)).

The colon absorbs water from solid waste before its elimination. In humans, the colon is 4-5 feet long and is comprised of the ascending colon, the transverse colon, the descending colon and the sigmoid colon (see Figure 1.8). Layers of circular and longitudinal smooth muscle line the colon to drive peristalsis. The epithelial lining of the colon is composed of intestinal crypts, housing goblet cells that secrete mucus and stem cells that support the continuous renewal of the epithelium. (anatomy of the colon reviewed in Grey, H. Anatomy of the human body. http://www.bartleby.com/107/249.html).
Figure 1.8 The anatomy of the colon.
This figure illustrates the ascending, traverse, descending and sigmoid colon along with a cross-section depicting each layer of the colon wall. The crypts greatly increase the total surface area of the colonic epithelium. Stem cells reside at the base of the crypts and differentiation occurs as the cells migrate upward. Cells at the apex undergo apoptosis and are shed. The epithelial lining in the intestine is renewed every 4-5 days in humans (van der Flier and Clevers, 2009).
Most polyps are benign, but some progress to invasive carcinoma. The progression of CRC is measured in stages I-IV, depending on the degree of invasion of the tumor into surrounding tissues and the presence of distant metastases. According to the TNM (tumor, nodes, metastasis) staging system, stage I cancers are confined to the submucosa or invade the major muscular layer. Stage II cancers extend through the muscular layer. Stage III cancers are metastatic to mesenteric lymph nodes and stage IV cancers are metastatic to distant organs (Markowitz et al., 2002). Stage I and II invasive carcinomas can be cured with surgery. Stage III or IV colorectal cancer require surgery as well as chemotherapy, however most stage IV carcinomas are incurable (reviewed in (Markowitz and Bertagnolli, 2009)).

The most prevalent mutational event in colorectal carcinoma is inactivating mutations in APC, which occurs in 80% of sporadic colorectal carcinomas. APC mutations result in a failure to degrade β-catenin, which subsequently accumulates in the cytoplasm and enters the nucleus. In the nucleus, β-catenin binds to T-cell factor-lymphocyte enhancer factor family members to form a transcription factor complex that drives the transcription of Wnt target genes (see Figure 1.9) (reviewed in (Goss and Groden, 2000)). Of the few carcinomas that do not harbor an APC mutation, 50% have mutations in the GSK3β binding site of β-catenin, rendering the protein resistant to degradation by GSK3β. The progression from adenoma to carcinoma involves a mutation in the Wnt signaling pathway, followed by mutations in K-Ras, SMAD4 and p53(Cho and Vogelstein, 1992).
Figure 1.9 Mutation of the Wnt pathway in colon carcinomas.
The most common mutations in colon carcinoma are in the Wnt signaling pathway. Most commonly, inactivating APC mutations occur, resulting in constitutive signaling by β-catenin. Activating mutations in β-catenin are common in CRCs that do not harbor APC mutations.
TP53 gene encodes p53, a protein that regulates cell cycle arrest. SMAD4 is a component of the TGF-β signaling pathway. Activating mutations in K-Ras and B-Raf drive constitutive MAPK signaling. Mutations in PI3KCA and co-amplification of AKT and PAK4 have also been reported (Markowitz and Bertagnolli, 2009). The upregulation of COX-2, an enzyme that mediates the production of Prostaglandin E2, or the loss of 15-PDGH, an enzyme that regulates the degradation of Prostaglandin E2, are also believed to contribute to the development of colorectal carcinoma (Markowitz and Bertagnolli, 2009).

Colon cancer is believed to arise from stem cells that reside at the base of the crypts (reviewed in (Humphries and Wright, 2008)). These progenitor cells undergo differentiation as they migrate upwards, and the cells at the apex of the crypts are shed every few days, making it impossible for them to acquire the mutations required for transformation. According to one model, clonal expansion arises from a single stem cell. A mutation in a stem cell can confer a growth advantage and give rise to a monocryptal clone by niche succession, which occurs through genetic drift, selective advantage or hitchhiking. Genetic drift occurs when one stem cell line is amplified by a symmetric division into two daughter stem cells, and all of the cells in crypt are descendants of this stem cell (See Figure 1.10). Symmetric divisions are a natural occurrence in crypts and clonal succession of these stem cells occurs naturally every 8.2 years. Neutral mutations can “hitchhike” along with these stem cells to dominate the niche in subsequent succession cycles. Selective advantage occurs when a mutation in a stem cell confers a growth advantage over the other stem cells (Leedham and Wright, 2008). Monoclonal
conversion occurs when the mutated stem cells occupy the entire niche (Humphries and Wright, 2008). The mutated stem cells expand to adjacent tissue through crypt fission where two daughter crypts are formed from the original crypt. The resulting crypts increase in size and undergo crypt fission again, a process referred to as the crypt cycle.

The mutator phenotype hypothesis suggests that early mutations in stem cells occur in genes that maintain genetic stability, paving the way for an increased mutation rate in stem cells that drives tumorigenesis. Subsequently acquired mutations are believed to confer a selective growth advantage, resulting in clonal expansion (Leedham and Wright, 2008).

There is also evidence that some adenomas are polyclonal in nature. This could occur through random collision of independent neoplastic clones that expand in close proximity (Humphries and Wright, 2008).
Figure 1.10 Niche succession and clonal conversion.
A. Stem cells reside at the crypt base.  B. Stem cells can undergo symmetric division, resulting in two daughter stem cells (b). If a stem cell acquires a mutation that confers a selective mutation (b, blue cell), it will expand clonally to dominate the niche (e). D. An illustration of a crypt undergoing niche succession and monoclonal conversion. Figure adopted from (Humphries and Wright, 2008).
1.8 Goals of the thesis

The overarching goal of this thesis was to gain insight into the biological roles of Rap in nontransformed cells and in tumor cells. Cyclic AMP signals through diverse effectors that include PKA and Epac/Rap. Cyclic AMP is an important regulator of thyroid cell proliferation and function, which is regulated by the glycoprotein hormone TSH. Although the role of PKA is well understood in these cells, the role of Rap has been much more mysterious. We set out to assess whether Rap functioned in thyroid cells as an intermediate in signaling from TSH/cAMP to ERK. The results indicated that Rap1 activity was not sufficient to activate ERK, nor was Rap1 required for the effects of cAMP on ERK activation. Our data identified a pathway through which cAMP activates PKA, Ras and B-Raf upstream from MEK/ERK activation. This differs from reports that suggest that Rap1 mediates cAMP-stimulated ERK activation in cells that express B-Raf (Stork and Schmitt, 2002). Our data is the first to suggest that PKA activates B-Raf in thyroid cells, and that B-Raf is required for TSH-stimulated proliferation.

The second part of my thesis explores the role of Rap1GAP in the regulation of cellular adhesion. Rap1GAP is downregulated in a wide array of human tumors. Earlier studies from our laboratory revealed that silencing Rap1GAP disrupted cell-cell adhesion, a result that was most unexpected based on the role of Rap in promoting cell-cell adhesion. To further assess the function of Rap1GAP in human tumor cells, we assessed the functional consequences associated with modest overexpression of Rap1GAP in colon cancer cells. This analysis revealed that Rap1GAP impairs Rap activity and cell adhesion, spreading and migration on a collagen matrix. Surprisingly,
Rap1GAP did not impair cell-cell adhesion under the same experimental conditions. This suggests Rap1GAP is not a universal inhibitor of cell-cell adhesion and that its role in the regulation of cell-cell adhesion is more complex than what is currently perceived. A greater understanding of the roles of Rap and Rap1GAP in normal and transformed cells may provide new insight into hormone signaling and tumor cell biology.
CHAPTER 2: PROTEIN KINASE A AND B-RAF MEDIATE
EXTRACELLULAR SIGNAL-REGULATED KINASE ACTIVATION BY
THYROTROPIN

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ABSTRACT

TSH regulates thyroid cell proliferation and function through cAMP-mediated signaling pathways that activate PKA and Epac/Rap1. The respective roles of PKA versus Epac/Rap1 in TSH signaling remain unclear. We set out to determine whether PKA and/or Rap1 mediate ERK activation by TSH. Neither blocking Rap1 activity nor silencing the expression of Rap1 impaired TSH or forskolin-induced ERK activation in Wistar rat thyroid cells. Direct activation of Epac1 failed to stimulate ERK activity in starved cells, suggesting that Epac-induced Rap1 activity is not coupled to ERK activation in rat thyroid cells. By contrast, PKA activity was required for cAMP-stimulated ERK phosphorylation, and sufficient to increase ERK phosphorylation in starved cells. Expression of dominant negative Ras inhibited ERK activation by TSH, forskolin and 6MB-cAMP, a selective activator of PKA. Silencing the expression of B-Raf also inhibited ERK activation by TSH, forskolin and 6MB-cAMP, but interestingly, not that stimulated by insulin or serum. Depletion of B-Raf impaired TSH-induced DNA synthesis, indicating a functional role for B-Raf in TSH-regulated proliferation. Collectively, these results position PKA, Ras and B-Raf as upstream regulators of ERK activation, and identify B-Raf as a selective target of cAMP elevating agents in thyroid cells. These data provide the first evidence for a functional role for B-Raf in TSH signaling.
2.1 INTRODUCTION

Although the TSH receptor couples to multiple G proteins (Laugwitz et al., 1996), most of the effects of TSH are mediated through Gsα and cAMP (reviewed in (Kimura et al., 2001; Medina and Santisteban, 2000)). Cyclic AMP activates multiple downstream targets including PKA, guanine nucleotide exchange factors (GEFs) for Rap (de Rooij et al., 1998; Kawasaki et al., 1998) and Ras (Pak et al., 2002; Pham et al., 2000) and certain ion channels. The respective contributions of PKA, Rap1 and Ras to the effects of TSH are unclear. There is general agreement that TSH activates endogenous Rap1 via a PKA-independent mechanism (Dremier et al., 2000; Iacovelli et al., 2001; Tsygankova et al., 2001). Rap1 activation is presumably mediated by Epac1, which is highly expressed in thyroid cells (Dremier et al., 2007; Hochbaum et al., 2008; Iacovelli et al., 2001; Mei et al., 2002). However, the functional significance of Rap1 activation by TSH is controversial. Activation and phosphorylation of Rap1 are required for TSH-stimulated DNA synthesis in rat thyroid PCCL3 cells (Ribeiro-Neto et al., 2002). Roles for both Epac and PKA in TSH-induced DNA synthesis in these cells were reported (Hochbaum et al., 2008). In contrast, studies in canine thyroid cells showed that PKA, and not Epac/Rap1, mediates the effects of TSH, including those on cell proliferation (Dremier et al., 2007).

In cells where cAMP stimulates ERK activity, Rap1 has been shown to induce B-Raf-dependent activation of ERK (reviewed in (Stork and Schmitt, 2002)). As B-Raf is highly expressed in thyroid cells (Mitsutake et al., 2005), we explored whether Rap1 mediates the effects of TSH on ERK activity in Wistar rat thyroid (WRT) cells.
Unexpectedly, based on a report that TSH stimulates Rap1-dependent ERK activity in FRTL-5 rat thyroid cells (Iacovelli et al., 2001), our studies identified PKA as the primary mediator of cAMP-stimulated ERK activity. They further revealed that Ras and B-Raf function downstream from PKA in the regulation of ERK activity and cell proliferation. These findings identify Ras and B-Raf as important components of TSH-mediated signaling pathways that converge on ERK activation and cell proliferation.

2.2 RESULTS

**TSH stimulates MEK1 and ERK activity through cAMP.** TSH stimulates rapid and transient activating phosphorylation of ERK in rat thyroid cells (Iacovelli et al., 2001). In WRT cells, ERK phosphorylation was stimulated maximally at 2-3 min and decreased to near basal levels by 15 min after TSH addition (Figure 2.1A). Forskolin elicited similar effects, demonstrating that increased cAMP is sufficient to activate ERK (Figure 2.1B). The effects of TSH/cAMP on MEK1 activity have not been reported. TSH and forskolin induced activating phosphorylation of MEK1 with a time course similar to that of ERK phosphorylation. Pretreatment with the MEK inhibitor UO126 blocked ERK activation by cAMP elevating agents (Figure 2.1C). These data indicate that the transient activation of ERK by TSH and cAMP is a result of stimulatory effects on MEK1, and focused our analysis on upstream regulators of MEK1 activity.
Figure 2.1 TSH stimulates cAMP-dependent MEK and ERK activation.
Starved WRT cells were stimulated with (A) TSH or (B) forskolin (fsk) for the times indicated (min). (C) Cells were pretreated with UO126 (10µM) for 30 min prior to stimulation. Total cell lysates were analyzed for ERK and MEK activation by western blotting for phospho-ERK (ERK-P) and phospho-MEK1/2 (MEK-P). Equal protein loading was confirmed by western blotting for Rac1 or ERK2.
**Rap1 is not required for cAMP-stimulated ERK activity.** TSH and forskolin activate endogenous Rap1 in canine and rat thyroid cells (Dremier et al., 2000; Iacovelli et al., 2001; Tsygankova et al., 2001). To assess whether Rap mediates cAMP-stimulated ERK activity, Rap activity was inhibited by overexpressing Rap1GAP, a negative regulator of Rap activity. As the transfection efficiency of rat thyroid cells in transient assays is very low, an adenovirus was used to drive efficient expression of Rap1GAP. Preliminary studies were conducted to determine the lowest dose of virus sufficient to block TSH- and forskolin-stimulated Rap1 activity. When infected at 500 particles/cell, Rap1GAP was expressed in virtually all cells (Figure 2.2A) and completely blocked Rap1 activity stimulated by TSH and forskolin (Figure 2.2B). Under these conditions, Rap1GAP failed to impair ERK activation by TSH or forskolin (Figure 2.2C, D). Controls for Rap1GAP adenoviral infections including mock- (see methods), LacZ-, and Rap1GAPN290A-infected cells all failed to impair TSH- or forskolin-stimulated ERK activation (Figure 2.2C and data not shown). Rap1GAPN290A contains a point mutation in the GAP domain, rendering the GAP domain catalytically inactive. This result was unexpected based on an earlier report that cAMP stimulates Rap1-dependent ERK activation in FRTL-5 rat thyroid cells (Iacovelli et al., 2001).
Figure 2.2 Cyclic AMP stimulates Rap-independent ERK activation. (A) WRT cells infected with Rap1GAP adenovirus (AdRap1GAP, 500 particles/cell) were fixed and stained for HA-Rap1GAP at days 1 and 2 post-infection. Nuclei were stained with DAPI. (B) Cells infected with AdRap1GAP were starved for 24 h, stimulated with forskolin (F) or TSH (T) for 2 min (on day 2 post-infection), subjected to RalGDS-RBD pull down assay and subsequently blotted for activated Rap1 (Rap1GTP). Total cell lysates were subjected to western blotting for Rap1 and Rap1GAP. (C) Cells infected with AdRap1GAP were starved and stimulated with TSH (upper panels) or forskolin (lower panels) and total cell lysates analyzed for ERK phosphorylation and Rap1GAP expression. Equal protein loading was confirmed by western blotting for ERK2. (D) The results from three experiments are summarized.
Hence, to further explore the contribution of Rap1 to cAMP-stimulated ERK activation, the effects of silencing Rap1 expression were investigated. WRT cells were transfected with two sets of Rap1-directed siRNAs that target different regions of the Rap1 message or scrambled siRNAs as a control. Although the expression of Rap1 was markedly reduced in cells transfected with Rap1-directed siRNAs, ERK activation was not affected (Figures 2.3A, B). To assess whether direct activation of Rap by Epac was sufficient to stimulate ERK activity, starved cells were stimulated with the Epac agonist, EcAMP (Enserink, 2002). Similar to the effects of TSH, EcAMP stimulated Rap1 activity (Figure 2.3C). Nonetheless, EcAMP failed to stimulate ERK phosphorylation (Figure 2.3D). Collectively, these data exclude a major role for Rap1 in the regulation of ERK activity by cAMP in WRT cells.
Figure 2.3 Rap1 is not coupled to ERK activation.
(A) WRT cells transfected with Rap1 siRNAs (Rap#1, #2) were starved, stimulated with forskolin for 2 min and ERK phosphorylation, Rap1 and Akt expression (as a loading control) analyzed. Rap1 expression was reduced by more than 80% following transfection with Rap1-directed siRNAs. (B) The results from three experiments are summarized. (C) Starved cells were stimulated with EcAMP or TSH for the indicated times (min). Lysates were subjected to RalGDS-RBD pull down assay and blotted for Rap1GTP. Total cell lysates were analyzed for Rap1 expression. (D) Starved cells were stimulated with EcAMP for the indicated times (min) and analyzed for ERK phosphorylation. Western blotting for actin was used to ensure equal protein loading.
Because our studies were conducted in WRT cells and those reported previously in FRTL-5 cells (Iacovelli et al., 2001), we extended our analysis to FRTL-5 and PCCL3 cells, both continuous lines of rat thyroid follicular cells (see (Kimura et al., 2001; Medina and Santisteban, 2000)). Overexpression of Rap1GAP failed to inhibit cAMP-stimulated ERK activity in both rat thyroid cell lines (Figure 2.4). These data differ from the single published report that analyzed the mechanism through which TSH stimulates ERK and concluded that cAMP stimulates Rap1-dependent ERK activity (Iacovelli et al., 2001).
Figure 2.4 ERK activation in FRTL5 and PCCL3 cells does not require Rap activity.
FRTL-5 (A) and PCCL3 cells (B) infected with Rap1GAP adenovirus (500 particles/cell) were starved for 24 hr, stimulated with forskolin for the indicated times (min) and subjected to western blotting for ERK phosphorylation, Rap1GAP and Akt expression, the latter to confirm equal protein loading. Similar results were obtained in two experiments in each cell line.
**PKA mediates cAMP-stimulated ERK activation.** Having excluded a role for Rap in cAMP-regulated ERK activity, we next investigated the role of PKA. PKI, a highly specific inhibitor of PKA, was employed in these experiments. Overexpression of PKI using an adenovirus markedly reduced PKA activity as determined by western blotting for substrates phosphorylated by PKA using a PKA-phospho-substrate antibody (Figure 2.5A, middle panel). Under these conditions, PKI significantly impaired cAMP-stimulated ERK activity (Figures 2.5A, B). To determine whether PKA activity was sufficient to stimulate ERK activity, starved cells were treated with the selective PKA agonist, 6MB-cAMP. 6MB-cAMP stimulated activating phosphorylation of both ERK and MEK1 with a similar time course (Figure 2.5C). Thus, PKA activity is required for cAMP-stimulated ERK activity and sufficient to increase MEK1 and ERK activity.
Figure 2.5 ERK activation is PKA-dependent.
(A) WRT cells infected with LacZ or PKI adenoviruses were starved overnight and stimulated with forskolin for the indicated times (min). ERK and PKA substrate phosphorylation were analyzed by western blotting. ERK2 expression documented equal protein loading. (B) PKI significantly reduced forskolin-stimulated ERK activation (*p<0.05 at 2 and 5 min). (C) Starved cells were stimulated with 6MB-cAMP for the indicated times (min), and analyzed by western blotting for phospho-ERK, phospho-MEK and Rac1 as a loading control. MEK1 activation by 6-MB-cAMP was analyzed in a single experiment.
Ras is required for cAMP-stimulated ERK activity. Studies were next conducted to elucidate the mechanism through which PKA stimulates ERK activity. Having excluded a role for Rap in the regulation of ERK activity, the contribution of Ras was investigated. Expression vectors encoding FLAG-tagged dominant negative RasN17 and HA-tagged ERK1 were co-transfected into WRT cells and ERK activation assessed in HA immunoprecipitates. This approach circumvented difficulties associated with low transfection efficiency by monitoring ERK activity selectively in transfected cells. Forskolin stimulated activating phosphorylation of HA-ERK1, and co-transfection with FLAG-RasN17 but not empty vector impaired ERK activation (Figure 2.6A). To determine whether the requirement for Ras was upstream or downstream of PKA, the effects of RasN17 on 6MB-cAMP-stimulated ERK activation were examined. Expression of RasN17 inhibited ERK phosphorylation by 6MB-cAMP (Figure 2.6B), supporting a role for Ras downstream from PKA in the regulation of ERK activity.
Figure 2.6 Ras activity is required for ERK activation.
(A) WRT cells transfected with HA-ERK and FLAG-RasN17 or empty vector were starved, stimulated with forskolin for 2 min and HA-ERK immunoprecipitated (IP) using an HA antibody and subjected to western blotting for phospho-ERK and HA-ERK expression. Whole cell extracts (CE) were analyzed for FLAG-RasN17 expression. (B) Cells transfected as described in (A) were stimulated with 6MB-cAMP for 2 min. Two experiments using forskolin and 6-MB-cAMP, and a single experiment using TSH were performed with similar results.
**ERK activation by cAMP requires B-Raf.** WRT cells express Raf-1 (data not shown) and B-Raf (Figure 2.7). Although B-Raf plays a major role in the regulation of ERK activity in human thyroid tumor cells (Melillo et al., 2005), the role of B-Raf in the regulation of ERK by TSH is unknown. To assess whether B-Raf mediates the effects of TSH on ERK activity, the expression of B-Raf was silenced. Decreasing the expression of B-Raf using two different sets of siRNAs significantly impaired TSH- and forskolin-stimulated ERK phosphorylation (Figures 2.7A, B, D). Silencing B-Raf induced a similar reduction in MEK1 phosphorylation by forskolin and TSH (data not shown). To exclude the possibility of ERK activation by Raf-1, silencing experiments were performed. Silencing Raf-1 had no effect on TSH- or forskolin-stimulated ERK activation (data not shown). Interestingly, in contrast to the effects observed using cAMP elevating agents, silencing B-Raf did not impair insulin-stimulated ERK activation (Figure 2.7A). Similar results were observed for serum-stimulated ERK activity (data not shown). Silencing Raf-1 impaired insulin- and serum-stimulated ERK activation (data not shown).

To assess whether activation of ERK by PKA required B-Raf, the consequences of silencing B-Raf on ERK activation by 6MB-cAMP were examined. As for the cAMP elevating agents, ERK activation by 6MB-cAMP was reduced in cells depleted of B-Raf (Figures 2.7C, D). These findings indicate that PKA lies upstream from B-Raf in the regulation of ERK activity, and that the requirement for B-Raf in ERK activation appears to be selective to cAMP elevating agents in these cells.
Figure 2.7 B-Raf is required for cAMP-stimulated ERK activation.
(A) WRT cells transfected with scrambled- (Scr) versus B-Raf-directed siRNAs were starved, stimulated with forskolin (2 min), insulin (5 min) or (B) TSH (2 min) and subjected to western blotting for phospho-ERK and B-Raf. Equal protein loading was confirmed by blotting for ERK2 or Akt. Depletion of B-Raf using an independent set of siRNAs (B-Raf#2) inhibited ERK activation by forskolin and TSH. (C) siRNA-transfected cells were stimulated with 6MB-cAMP for the times indicated (min) and analyzed for phospho-ERK, B-Raf and Akt as a loading control. (D) The decrease in ERK activation in B-Raf-depleted cells was statistically significant (**p<0.01).
B-Raf is required for TSH-stimulated DNA synthesis. To assess the contribution of B-Raf to the biological effects of TSH, the consequences of silencing B-Raf expression on DNA synthesis were examined. As TSH fails to stimulate proliferation in the absence of cooperating growth factors (reviewed in (Kimura et al., 2001; Medina and Santisteban, 2000)), DNA synthesis was examined in cells starved in the presence of 0.2% calf serum (Figure 2.8A). TSH-stimulated DNA synthesis was significantly reduced in the presence of the MEK1 inhibitor (UO126). Depletion of B-Raf also inhibited TSH-induced DNA synthesis. In contrast, silencing B-Raf did not impair insulin-stimulated DNA synthesis. Thyroid cell proliferation is maximal in growth medium containing TSH, insulin and serum (3H). Importantly, although modest in magnitude, silencing the expression of B-Raf, or treatment with the MEK1 inhibitor significantly decreased 3H-stimulated DNA synthesis (Figure 2.8B). We did not anticipate a more marked decrease in 3H-stimulated DNA synthesis, as other pathways, most notably those mediated through PI3K and mTOR, make substantial contributions to thyroid cell proliferation in vitro (Cass and Meinkoth, 1998; Cass et al., 1999);(Kimura et al., 2001; Medina and Santisteban, 2000) and in vivo (Miller et al., 2009; Yeager et al., 2008). These data confirm that B-Raf contributes to the proliferation of non-transformed thyroid cells, possibly through the ability of cAMP elevating agents to stimulate B-Raf dependent ERK activation.
Figure 2.8 B-Raf is required for TSH-dependent DNA synthesis.

Cells pretreated with UO126 (10µM) for 1 h or depleted of B-Raf were stimulated with (A) TSH, insulin or (B) 3H growth medium and DNA synthesis analyzed. The % BrdU-positive nuclei (13.2% for TSH, 19.1% for insulin, 52.6% for 3H versus 2.0% for starved cells) in mitogen-stimulated cells was set to 1. The decrease in DNA synthesis in UO126-treated (**p<0.01 for TSH, **p<0.01 for 3H) and B-Raf-depleted (***p<0.001 for TSH, *p<0.05 for 3H) cells was statistically significant.
2.3 DISCUSSION

Given the important role played by Rap1 in the activation of B-Raf (reviewed in (Stork and Schmitt, 2002)), together with the frequent mutational activation of B-Raf in thyroid tumors (reviewed in (Kondo et al., 2006; Xing, 2005), we investigated whether Rap1 contributes to ERK activation by TSH/cAMP. Our findings indicate that PKA, and not Rap1, mediates B-Raf-dependent ERK activation by TSH. A role for endogenous Rap1 in the regulation of ERK by TSH/cAMP was excluded based on two major lines of evidence. Overexpression of Rap1GAP failed to impair TSH/cAMP-stimulated ERK activity, and silencing the expression of Rap1 had no effect on ERK activation. Additionally, direct activation of Epac with the specific agonist EcAMP (Enserink, 2002) failed to stimulate ERK activity in starved cells. Collectively, these data show that Rap1 activation is neither required for nor sufficient to induce ERK activation in WRT cells. These results differ from the single report published to date that concluded that Rap1 was required for TSH/cAMP-induced ERK activity in FRTL-5 cells (Iacovelli et al., 2001). There are several explanations for this discrepancy. These authors demonstrated the requirement of Rap through the overexpression of dominant negative Rap, RapN17. The expression of RapN17 resulted in a modest decrease of ERK activity by TSH, although it was impossible to compare this reduction to a vector control, as the control data was offered on a separate western blot and there was no summation or quantification of multiple replicates of this experiment provided. Additionally, dominant negative Rap sequesters additional small G-proteins including Ras. Therefore, it is feasible RapN17 is impairing Ras-dependent ERK activity. In our hands, overexpression of Rap1GAP had
no effect on cAMP-stimulated ERK activity in either FRTL-5 or rat thyroid PCCL3 cells, another widely used model of rat thyroid cells (reviewed in (Kimura et al., 2001; Medina and Santisteban, 2000)). Clearly, further work is required before an accepted model for the regulation of ERK by TSH can be derived.

Our findings indicate that PKA mediates cAMP-stimulated ERK activation. Inhibition of PKA activity using the highly selective inhibitor PKI impaired TSH/cAMP-stimulated ERK activity. Moreover, treatment with the selective PKA analog 6-MB-cAMP was sufficient to stimulate activating phosphorylation of MEK1 and ERK in starved cells. The molecular mechanism through which PKA regulates ERK activity was explored. Silencing the expression of B-Raf impaired ERK activation by TSH, forskolin and 6MB-cAMP, documenting a role for B-Raf in the regulation of ERK by cAMP and PKA. Having excluded a role for Rap1 in the regulation of ERK activity, our analysis focused on Ras. TSH activates Ras in rat thyroid cells (Iacovelli et al., 2001; Tsygankova et al., 2000). Expression of dominant negative Ras inhibited TSH-, forskolin- and 6MB-cAMP-stimulated ERK activity. Although there are precedents for PKA-dependent Ras activation (Ambrosini et al., 2000; Obara et al., 2007; Yang et al., 2003), we reported that TSH activates Ras through a PKA-independent mechanism, experiments that were conducted in thyroid cells overexpressing human H-Ras (Tsygankova et al., 2000). Unfortunately, we have been unable to detect the activation of endogenous Ras in WRT cells, even in response to serum mitogens (Vuchak, unpublished). Iacovelli et al reported that TSH activates endogenous Ras in FRTL-5 cells and that Ras is required for ERK activation. Unfortunately, the mechanism through which Ras was activated was not
explored (Iacovelli et al., 2001). It is conceivable that PKA-dependent and -independent mechanisms of Ras activation co-exist in thyroid cells, similar to what has been reported for Rap1 where PKA-dependent and -independent modes of activation have been described. Alternatively, the requirement for Ras in PKA-dependent ERK activation could reflect effects of PKA on Ras signaling. TSH influences downstream events in Ras signaling. TSH transiently impaired ERK activation by RasV12S35, an effector domain mutant that signals preferentially through Raf, whereas it enhanced ERK activation by RasV12G37, a mutant that signals via RalGDS and Ral proteins (Miller et al., 1998). In the absence of TSH, Raf-1 was required for DNA synthesis stimulated by microinjected cellular Ras protein, whereas in the presence of TSH, Ras-stimulated DNA synthesis did not require Raf1 (al-Alawi et al., 1995). Ciullo et al. reported that TSH stimulated the association of Ras with the p85 regulatory subunit of PI3K through PKA-mediated phosphorylation of p85. Moreover, these authors showed that cAMP disrupted Ras/Raf-1 complexes at least partly through PKA (Ciullo et al., 2001; De Gregorio et al., 2007). Collectively, these studies indicate that there are multiple sites of crosstalk between PKA- and Ras-mediated signaling.

Our data is the first to ascribe a functional role to B-Raf in TSH signaling. Silencing the expression of B-Raf impaired DNA synthesis stimulated by TSH. Depleting B-Raf also induced a modest decrease in DNA synthesis stimulated by 3H growth medium, which contains TSH, insulin and serum. In that depletion of B-Raf failed to inhibit insulin- or serum-induced ERK activation, these data suggest that the requirement for B-Raf in cell proliferation resides downstream of TSH. This is consistent
with previous studies showing that expression of dominant negative Ras (Ciullo et al., 2001; Kupperman et al., 1993; Medina and Santisteban, 2000) or treatment with MEK1 inhibitors (Iacovelli et al., 2001; this report) impairs TSH-stimulated DNA synthesis. Even in canine thyroid cells where PKA mediates all or many of the effects of TSH (Dremier et al., 2007), treatment with a MEK1 inhibitor impaired TSH-stimulated DNA synthesis (Vandeput et al., 2003). Other ligands that activate Gs-coupled receptors have been shown to signal through B-Raf. PTH stimulated PKA- and B-Raf-dependent ERK activation in CHO cells expressing the PTH1 receptor (Wang et al., 2008). Silencing the expression of B-Raf impaired MSH-induced ERK activation in melanocytes (Dumaz et al., 2006).

In conclusion, our findings highlight important roles for PKA, Ras and B-Raf in TSH signaling to ERK. The requirement for Ras in TSH-induced ERK activation provides further evidence that Ras functions in TSH signaling (al-Alawi et al., 1995; Ciullo et al., 2001; Iacovelli et al., 2001; Kupperman et al., 1993; Medina and Santisteban, 2000; Tsygankova et al., 2000). Mutations in Ras and B-Raf are prevalent in thyroid tumors. Patients with Carney’s syndrome, caused by inactivating mutations in the gene for the PKA regulatory subunit R1α exhibit an increased frequency of thyroid tumors (reviewed in (Boikos and Stratakis, 2007)). Loss of heterozygosity for PRKAR1A and increased PKA activity have been observed in thyroid tumors (Sandrini et al., 2002). Elucidating sites of crosstalk between these important signaling molecules in non-transformed thyroid cells may reveal novel insight into the molecular basis of thyroid cancer.
2.4 MATERIALS AND METHODS

Reagents. Phospho-ERK (Thr202/Tyr204), phospho-MEK1/2 (Ser221), phospho-(Ser/Thr) PKA substrate, DYKDDDDK FLAG epitope, Akt and MEK1 antibodies were from Cell Signaling Technology (Beverly, MA). ERK2, B-Raf, Rap1 and Rap1GAP antibodies were from Santa Cruz (Santa Cruz, CA). Rac1 antibody was from Upstate (Lake Placid, NY). HA antibody was kindly provided by Dr. Jeffrey Field (Department of Pharmacology, University of Pennsylvania). Sheep anti-BrdU antibody was from BioDesign (Carmel, NY). AlexaFluor 488 donkey anti-sheep IgG was from Invitrogen (Eugene, OR). Crude bovine TSH and forskolin were from Sigma (St. Louis, MO). Glutathione sepharose beads were from GE Healthcare (Piscataway, NJ). 8-(4-chlorophenylthio)-2′-O-methyladenosine 3′,5′-cyclic monophosphate (EcAMP) and N6-monobutrylcytidine-3′, 5′- cyclic monophosphate (6MB-cAMP) were from Axxora LLC (San Diego, CA). UO126 was purchased from Promega (Madison, WI).

Cell culture. WRT, PCCL3 and FRTL5 rat thyroid cells were cultured in Coon’s modified Ham’s F12 medium supplemented with calf serum (5%), insulin (10 µg/mL), TSH (1 mU/mL), and transferrin (5 µg/mL), referred to as 3H growth medium. Cells were starved in basal medium (Coon’s modified Ham’s F12 medium devoid of growth factors and serum) for 48 h prior to stimulation with TSH (1 mU/mL), forskolin (10 µM), insulin (10 µg/mL), EcAMP (100 µM) or 6MB-cAMP (1 mM). Starved cells were pretreated with UO126 (10 µM) for 1 h prior to stimulation.
**Viral infection.** Rap1GAP adenovirus was constructed as previously described (Tsygankova et al., 2007). The PKI adenovirus was a kind gift from Dr. Mark A. Giembycz (Department of Pharmacology & Therapeutics, University of Calgary). LacZ and Rap1GAPN290A viruses acted as negative controls for Rap1GAP or PKI infections. Cells were infected overnight in basal medium, transferred to growth medium for 6 h and starved in basal medium overnight. On day 2 post-infection, cells were stimulated and total cell lysates prepared for western blotting. Mock-infected cells received the same treatment as adenoviral-infected cells, but received no adenovirus during the infection procedure.

**Rap activation assay.** Rap1 activation was assessed as previously described (Tsygankova et al., 2007) except that lysates were collected in Triton-based lysis buffer (20mM TrisHCl, 100mM NaCl, 10mM MgCl₂, 1.0% Triton-X-100, 100 μM pefabloc, 1 μg/mL pepstatin, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 200 μg/mL Na₃VO₄).

**Transient transfection of siRNA.** siRNA duplexes were introduced into cells using the Amaxa Nucleofector (Germany) as described previously (Tsygankova et al., 2007). Cells (1.5x10⁶) were transfected with siRNA duplexes (200-1000 nM) in suspension, plated overnight in growth medium, transferred to basal medium for 24 h and subsequently stimulated. B-Raf siRNAs were from Invitrogen (Carlsbad, CA). Scrambled siRNAs, Rap1 siRNAs and a second set of B-Raf siRNAs were from Qiagen (Valencia, CA).

**Immunoprecipitation.** The Flag-RasN17 plasmid was generously provided by Dr. Phillip Stork (Vollum Institute, Oregon Health and Sciences University). The HA-ERK1 plasmid was a kind gift from Dr. Margaret Chou (Department of Pathology and Lab
Plasmids (5 µg) were co-transfected using the Amaxa Nucleofector. Cells were starved for 24 h prior to treatment. At 48 h post-transfection, cells were stimulated and then lysed in 20 mM Tris pH 7.8, 100 mM NaCl, 0.5% Triton, 80 mM β-glycerophosphate, 20 mM NaF, 2 mM EDTA, 100 µM pefabloc, 1 µg/mL pepstatin, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 200 µg/mL Na₃VO₄. Lysates were clarified by centrifugation and the supernatant pre-cleared with protein G agarose beads (Invitrogen, Carlsbad, CA). Proteins (200 µg) were precipitated with HA antibody for 2 h at 4˚C, protein G agarose (100 µL) added for 1 h, and beads collected by centrifugation. After washing 3 times in PBS, 2X Laemmli buffer was added, the samples boiled for 5 min, and subjected to western blotting.

**Western blotting.** Western blotting was performed as previously described (Tsygankova et al., 2007). Proteins were visualized via chemiluminescence using the FUJI-LAS 3000 system and Multi Gauge V3.0 software (Fuji, Japan). Western blot quantification was measured using densitometry.

**DNA synthesis.** Following transfection with siRNAs and plating overnight, cells were starved for 24 hr in basal medium supplemented with calf serum (0.2%), stimulated with TSH (1mU/mL) or insulin (10µg/mL) for 28 hr and BrdU added for the final 4 hr (24-28 hr). In parallel, starved cells were stimulated with 3H growth medium for 20 hr and labeled with BrdU for 4 hr (16-20 hr). Cells were fixed in 3.7% formaldehyde/PBS, stained with sheep anti-BrdU, AlexaFluor488 anti-sheep IgG and 4’,6-diamidino-2-phenylindole (DAPI) (to stain nuclei). At least six fields (>200 cells) were scored in a
blinded fashion. Parallel dishes of cells were harvested and analyzed by western blotting to confirm silencing in each experiment.

Statistics. At least three biological replicates were performed for all experiments unless otherwise indicated. Statistical significance was determined using student’s t-test. Data sets were considered statistically significant when p<0.05 (* p<0.05, ** p<0.01, *** p<0.001).
CHAPTER 3: RAP1GAP IMPAIRS CELL-MATRIX ADHESION IN THE ABSENCE OF EFFECTS ON CELL-CELL CONTACTS

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ABSTRACT

The significance of the frequent downregulation of Rap1GAP in human tumors is unknown. In previous studies we demonstrated that silencing Rap1GAP expression in human colon cancer cells resulted in sustained increases in Rap activity, enhanced spreading on collagen and the weakening of cell-cell contacts. The latter finding was unexpected based on the role of Rap in strengthening cell-cell adhesion and reports that Rap1GAP impairs cell-cell adhesion. We now show that Rap1GAP is a more effective inhibitor of cell-matrix compared to cell-cell adhesion. Overexpression of Rap1GAP in human colon cancer cells impaired Rap2 activity and the ability of cells to spread and migrate on collagen IV. Under the same conditions, Rap1GAP had no effect on cell-cell adhesion. Overexpression of Rap1GAP did not enhance the dissociation of cell aggregates nor did it impair the formation of cell-cell contacts. Additionally, the accumulation of β-catenin and E-cadherin at cell-cell contacts was not altered by Rap1GAP overexpression. To further explore the role of Rap1GAP in the regulation of cell-cell adhesion, Rap1GAP was overexpressed in non-transformed thyroid epithelial cells. Although the formation of cell-cell contacts required Rap1, overexpression of Rap1GAP did not impair cell-cell adhesion. These data indicate that the expression of Rap1GAP is compatible with cell-cell adhesion and that the role of Rap1GAP in the regulation of cell-cell adhesion may be more complex than is currently appreciated.

3.1 INTRODUCTION
Rap1GAP (RapGTPase activating protein) is one member of a family of negative regulators of Rap proteins (Rap1a/b, Rap2a/b/c in mammalian cells). The expression of Rap1GAP is decreased in tumors of the pancreas, thyroid, skin and colon (Nellore et al., 2009; Tsygankova et al., 2010; Tsygankova et al., 2007; Zhang et al., 2006a; Zheng et al., 2009; Zuo et al., 2010). Rap1GAP expression is further decreased in invasive compared to benign lesions, suggesting that depletion of Rap1GAP enhances tumor progression (Nellore et al., 2009; Zhang et al., 2006a; Zheng et al., 2009; Zuo et al., 2010). The advantages conferred to tumor cells by the downregulation of Rap1GAP are unknown. Overexpression of Rap1GAP in vitro impaired tumor cell proliferation, migration and invasion (Bailey et al., 2009; Mitra et al., 2008; Tsygankova et al., 2007; Zhang et al., 2006a; Zhang et al., 2006b; Zheng et al., 2009). Overexpression of Rap1GAP elicited variable effects on tumor formation (Freeman et al., 2010; Lin et al.; Zhang et al., 2006a; Zhang et al., 2006b), but consistently impaired metastasis (Bailey et al., 2009; Freeman et al., 2010; Lin et al.; Zhang et al., 2006a). We previously reported that silencing Rap1GAP expression in human colon cancer cells impaired cell-cell adhesion and enhanced spreading on collagen (Tsygankova et al., 2010). The weakening of cell-cell contacts together with alterations in matrix adhesion are hallmarks of tumor progression. These data suggest that loss of Rap1GAP endows cells with the ability to disseminate and provide a potential rationale for the progressive downregulation of Rap1GAP in human tumors.

The notion that loss of Rap1GAP enhances matrix adhesion is not surprising. Silencing Rap1GAP induced sustained increases in Rap activity (Tsygankova et al.,...
Activated Rap enhances cell-matrix adhesion by regulating integrin affinity and avidity (Bos et al., 2003; Caron et al., 2000; Reedquist et al., 2000). However, Rap activity promotes cell-cell adhesion, a finding that is difficult to reconcile with the observation that silencing Rap1GAP weakened cell-cell adhesion. Given the importance of alterations in cellular adhesion in tumor progression together with the widespread downregulation of Rap1GAP in human tumors, we further explored the role of Rap1GAP in the regulation of cell adhesion. Surprisingly, overexpression of Rap1GAP in human colon cancer cells and in thyroid epithelial cells impaired matrix adhesion in the absence of effects on cell-cell adhesion. These data indicate that the role of Rap1GAP in the regulation of Rap signaling is more complex than is currently appreciated.

3.2 RESULTS

Rap1GAP impairs cell-matrix adhesion

To analyze the role of Rap1GAP in the regulation of cell adhesion, an adenovirus was used to transiently overexpress Rap1GAP in HCT116 cells. Dose response experiments revealed that infection with the Rap1GAP adenovirus at 5 IU/cell was sufficient to block endogenous Rap2 activity (Figure 3.1A, lane 2). Rap1 activity was undetectable, most likely due to the low level of Rap1 expression in these cells (data not shown). Cells overexpressing Rap1GAP exhibited dramatic alterations in cell morphology. Rap1GAP-expressing cells were more compact and less spread than LacZ-infected cells (Figure 3.1B). There was no difference in the morphology of LacZ and mock-infected cells (data not shown).
Figure 3.1 Blockade of Rap activity induces morphological changes in colon cancer cells. (A) HCT116 cells were infected with Rap1GAP adenovirus at 0, 5, 10, or 21 infectious units (IU)/cell and Rap activity monitored (PD, pull-down). Whole cell lysates (WCL) were subjected to western blotting for Rap1 and Rap1GAP. (B) HCT116 cells were imaged at 48 hours post-infection with Rap1GAP or LacZ adenoviruses.
To determine if Rap1GAP impaired cell spreading, Rap1GAP-expressing cells were plated on collagen IV for various times and cell area measured. Rap1GAP-infected cells were less spread than mock- or LacZ-infected cells (Figure 3.2A). Measurements of cell area confirmed that Rap1GAP significantly impaired cell spreading (Figure 3.2B). When plated on poly-L-lysine, Rap1GAP-expressing cells were similar in size to mock- and LacZ-infected cells, suggesting that Rap1GAP selectively impairs integrin-mediated spreading (data not shown). To explore consequences on integrin signaling, focal adhesions were analyzed by immunostaining for autophosphorylated FAK (phospho-FAKY397). Overexpression of Rap1GAP dramatically reduced the size and number of focal adhesions (Figure 3.2C). These results demonstrate that Rap1GAP impairs cell-matrix adhesion in human colon cancer cells.
Figure 3.2 Rap1GAP impairs cell-matrix adhesion. (A) Mock-, LacZ- and Rap1GAP-infected cells were plated on collagen IV for various times (240 min shown here), fixed and stained for F-actin with rhodamine phalloidin. (B) Cell area was measured and quantified using morphometry. At least 3 random fields (approximately 100 cells) were measured for each time point. The graph illustrates results from a representative experiment. (C) Mock-, LacZ- and Rap1GAP-infected cells were plated on collagen IV for 90 min, fixed and stained for phospho-FAK (Y397). Arrows indicate examples of focal adhesions.
Rap1GAP inhibits cell migration

Stable overexpression of Rap1GAP inhibited cell migration in pancreatic, thyroid and melanoma cell lines (Tsygankova et al., 2007; Zhang et al., 2006a; Zheng et al., 2009). To assess whether Rap1GAP directly inhibits migration, Rap1GAP was transiently overexpressed in HCT116 cells. Confluent monolayers of Rap1GAP- and LacZ-infected cells were wounded with a pipet tip and wound closure monitored. Increased expression of Rap1GAP significantly impaired wound closure (Figure 3.3). These data demonstrate that transient overexpression of Rap1GAP is sufficient to inhibit Rap activity and Rap-mediated signals that control matrix adhesion and cell migration.
Figure 3.3 Rap1GAP impairs cell migration. Confluent mock-, LacZ- and Rap1GAP-infected cells were wounded and images acquired immediately and 24 hours later. The distance migrated by mock-infected cells was set to 1 (see methods).
Cell-cell adhesion is not disrupted by Rap1GAP

Overexpression of Rap1GAP disrupted VE-cadherin-mediated cell-cell adhesion in endothelial cells (Fukuhara et al., 2005; Wittchen et al., 2005). To assess the functional effects of Rap1GAP on cell-cell adhesion in colon cancer cells, cell dissociation assays were conducted. Cells were released from tissue culture dishes and subject to dissociation by pipetting in the presence of calcium, which maintains E-cadherin-mediated cell-cell contacts or in EGTA to disrupt calcium-dependent contacts. Although expressed at levels sufficient to inhibit Rap2 activity (Figure 3.1A), spreading on collagen IV (Figure 3.2A) and cell migration (Figure 3.3), Rap1GAP did not render cells more sensitive to dissociation (Figure 3.4A).

In MDCK cells, overexpression of Rap1GAP induced the loss of β-catenin from cell-cell contacts (Asuri et al., 2008). To assess if the same was true in colon cancer cells, Rap1GAP-infected cells were fixed and stained for HA to identify cells expressing HA-Rap1GAP and with antibodies to β-catenin or E-cadherin. The proportion of cells with β-catenin (Figure 3.4B) or E-cadherin at cell-cell contacts was similar in Rap1GAP-expressing and control cells (β-catenin: mock-85.3%, LacZ-84.6%, Rap1GAP-87.7%; E-cadherin: mock-77.8%, LacZ-71.8%, Rap1GAP-76.1%). At least 50 pairs of cells were analyzed for each condition.

Cell surface expression of E-cadherin was further analyzed in trypsin sensitivity assays. Cells were disrupted directly on tissue culture dishes (total in Figure 3.4C) or trypsinized in the presence of EGTA or calcium prior to disruption. As expected, treatment with EGTA removed E-cadherin from the cell surface, while calcium protected
cell surface E-cadherin (Figure 3.4C). Consistent with the results obtained in immunostaining, overexpression of Rap1GAP did not reduce cell surface expression of E-cadherin. Collectively, these data show that overexpression of Rap1GAP is not sufficient to weaken cell-cell adhesion or to disrupt E-cadherin-mediated cell-cell contacts in human colon cancer cells.
Figure 3.4 Rap1GAP does not impair cell-cell adhesion. (A) LacZ- and Rap1GAP-infected cells were trypsinized in the presence of calcium or EGTA for 10 minutes, pipetted 10X, plated and images acquired immediately. (B) At 24 hours post-infection, mock-, LacZ- and Rap1GAP-infected cells were plated overnight, fixed and stained for β-catenin and HA-Rap1GAP. (C) Mock-, LacZ- and Rap1GAP-infected cells were disrupted directly on tissue culture dishes (total cell lysates) or trypsinized in the presence of calcium or EGTA prior to lysis. Lysates were subjected to western blotting for E-cadherin.
Effects of Rap1GAP on the formation of cell-cell contacts

Overexpression of Rap1GAP delayed, but did not inhibit the formation of cell-cell contacts in MCF-7 breast cancer cells (Hogan et al., 2004). Based on this, we explored whether Rap1GAP impairs the formation of cell-cell contacts. HCT116 cells were dissociated into single cells, transferred to EGTA- or calcium-containing medium and the cells allowed to aggregate for 1 hour. Rap1GAP-overexpressing cells formed aggregates in a manner that was indistinguishable from that seen in control cells (Figure 3.5A). Measures of the dissociation index confirmed that Rap1GAP had no effect on the formation of aggregates (Figure 3.5B).
Figure 3.5 Rap1GAP does not impair the formation of cell contacts. (A) LacZ- and Rap1GAP-infected cells were released, dissociated into single cells and allowed to aggregate for 1 hour in the presence of calcium. (B) Cell aggregation was quantified by calculating the dissociation index (see methods).
These experiments were conducted in HCT116 cells, which express Rap2. The role of Rap2 in E-cadherin-mediated cell-cell adhesion is unclear. Therefore, we extended our analysis to Wistar rat thyroid (WRT) epithelial cells, which express abundant levels of Rap1. Rap1GAP was transiently overexpressed in these cells at the lowest dose sufficient to inhibit Rap1 activity (Tsygankova et al., 2007; Vuchak et al., 2009). At 48 hours post-infection, cell association assays were conducted. Rap1GAP-expressing cells formed aggregates in a manner indistinguishable from mock- and LacZ-infected cells (Figure 3.6A). In addition, β-catenin accumulated at cell-cell contacts in Rap1GAP-expressing and control cells (Figure 3.6B). The proportion of Rap1GAP-expressing cells with β-catenin at cell-cell contacts was similar to that in mock- and LacZ-infected cells (experiment 1: mock-92.0%, LacZ-94.9%, Rap1GAP-93.8%; experiment 2: mock-83.6%, LacZ-83.3%, Rap1GAP-78.6%). At least 90 pairs of cells were analyzed per condition.
Figure 3.6 Rap1GAP does not impair cell/cell adhesion in non-transformed epithelial cells. (A) Cell association assays were conducted in mock-, LacZ- and Rap1GAP-infected WRT cells. After analysis, aggregates were collected and subjected to western blotting for Rap1GAP. Actin was analyzed to document equal protein loading. (B) Mock-, LacZ- and Rap1GAP-infected cells were plated on laminin-coated coverslips overnight. Cells were fixed and stained for β-catenin and HA-Rap1GAP.
To exclude the possibility that cell-cell contact formation was Rap1-independent in these cells, the expression of Rap1 was silenced using RNA interference. Depletion of Rap1 disrupted the integrity of cell-cell contacts in growing cells (Figure 3.7A, 3H). The junctions between cells were more irregular and disorganized in the absence of Rap1. β-catenin was lost from cell/cell contacts when cells were deprived of calcium (Figure 3.7A, -calcium). Interestingly, under these conditions, Rap1-depleted cells were consistently more dissociated from one another compared to control cells. Within 1 hour of transfer to calcium-supplemented medium, control cells spread and formed cell-cell contacts that labeled for β-catenin (Figure 3.7A, + calcium). The reformation of cell-cell contacts was delayed in Rap1-depleted cells at 1 hour (Figure 3.7A, +calcium). Silencing Rap1 did not abolish the reformation of cell-cell contacts as Rap1-depleted cells and control cells stained more similarly for β-catenin at cell-cell contacts after 4 hours (data not shown).

We analyzed Rap1 activity during the disruption and reformation of cell-cell contacts. EGTA activated Rap1 and calcium decreased Rap1 activity (Figure 3.7B), results that show that the dynamic regulation of cell-cell adhesion is accompanied by changes in Rap1 activity similar to reports in other epithelial cells (Asuri et al., 2008; Balzac et al., 2005). Nonetheless, overexpression of Rap1GAP had no effect on Rap1-mediated cell-cell adhesion.
Figure 3.7 Cell-cell junction formation requires Rap1. (A) Cells transfected with scrambled or Rap1-directed siRNAs were maintained in 3H growth medium (left panels), transferred to low calcium medium for 1 hour (middle panels) or deprived of calcium for 1 hour and then transferred to calcium-containing medium for 1 hour (right panels). Cells were fixed and stained for β-catenin. Total cell lysates were subjected to western blotting to confirm Rap1 depletion. (B) WRT cells were treated with EGTA for 30 min or EGTA followed by addition of calcium-containing medium for 5, 30, or 60 min. Rap1 activation was monitored. Total cell lysates were subject to western blotting for Rap1.
3.3 DISCUSSION

Although widely used as a tool to inhibit Rap activity, little is known about the cellular functions regulated by Rap1GAP. Given the roles of Rap in promoting cell-matrix and cell-cell adhesion, it has been assumed that Rap1GAP inhibits both processes similarly. We previously reported that downregulation of Rap1GAP impaired cell-cell adhesion in human colon cancer cells, suggesting a role for Rap1GAP in the regulation of cell-cell adhesion (Tsygankova et al., 2010). This finding was unexpected based on reports that overexpression of Rap1GAP impaired VE-cadherin- (Fukuhara et al., 2005; Sakurai et al., 2006; Wittchen et al., 2005) and E-cadherin-mediated cell-cell adhesion (Asuri et al., 2008). This prompted us to further explore the effects of Rap1GAP on cell-cell adhesion.

Transient overexpression of Rap1GAP in HCT116 colon cancer cells impaired spreading on collagen, decreased focal adhesions and inhibited migration. However, under these conditions Rap1GAP did not render cells more sensitive to dissociation, nor did it impair the formation of calcium-dependent cell aggregates. The accumulation of β-catenin and E-cadherin at cell-cell junctions was not perturbed in Rap1GAP-overexpressing cells. It has been suggested that Rap is required for the early stages of cell-cell adhesion, but not for the maintenance of cell-cell contacts (Hogan et al., 2004). Although we cannot exclude a transient delay in the formation of cell-cell contacts, this was not apparent in the experiments that were conducted. In addition, overexpression of Rap1GAP in HT29 colon cancer cells in which endogenous Rap1GAP expression was chronically suppressed restored the accumulation of E-cadherin and p120-catenin at cell-
cell contacts (Tsygankova et al., 2010). Integrins are activated and subsequently inactivated during cell spreading. Nevertheless, overexpression of Rap1GAP exhibited demonstrable effects on cell spreading even when analyzed at 48 hours after infection. It is surprising that no effects on cell-cell adhesion were observed. Clearly, we cannot exclude the possibility that small pools of Rap remain active in Rap1GAP-overexpressing cells.

Mammalian cells express Rap1 and Rap2, each of which undergoes alternative splicing (Rap1a/b, Rap2a/b/c). Rap2 is the predominant isoform expressed in HCT116 cells. Like Rap1, Rap2 promotes integrin activation (Jenei et al., 2006; McLeod et al., 2004). Our findings provide the first demonstration that Rap2 promotes the spreading of human colon cancer cells. Activation of Rap2 promotes the interaction of B cells with T cells by activating the LFA-1 surface antigen (McLeod et al., 2004). A role for Rap2 in the stabilization of β-catenin in Xenopus embryos has been demonstrated (Choi et al., 2008). However, it is not clear that Rap2 regulates E-cadherin-mediated cell adhesion. The absence of inhibitory effects on cell-cell adhesion in Rap1GAP-expressing HCT116 cells could simply indicate that cell-cell adhesion is independent of Rap2. Moreover, HCT116 cells harbor an activating mutation in β-catenin that could alter the regulation of cell-cell adhesion (Sparks et al., 1998). For these reasons, we extended our analysis to non-transformed thyroid epithelial cells that express Rap1 (Tsygankova et al., 2004; Tsygankova et al., 2001). Overexpression of Rap1GAP blocked Rap1 activity (Tsygankova et al., 2007; Vuchak et al., 2009), but not cell-cell adhesion or the accumulation of β-catenin at cell-cell junctions. Silencing Rap1 delayed the formation of
cell-cell contacts and Rap1 was activated when cell-cell contacts were disrupted and inactivated as cell-cell contacts formed. Thus, cell-cell adhesion in these cells is Rap1-mediated.

RapGAPs are multi-domain proteins that contain a variety of protein/protein interaction and other motifs in addition to a conserved GAP domain (Bos et al., 2007; Spilker and Kreutz, 2010). An N-terminally extended isoform of Rap1GAP (Rap1GAP-II) contains a GoLoco motif that mediates high affinity interactions with heterotrimeric G-protein α subunits (Jordan et al., 1999; Meng et al., 1999; Mochizuki et al., 1999). Spa-1 and Rap1GAP bind to AF6, first identified as the fusion partner of ALL-1 protein in human acute myeloid leukemia (Prasad et al., 1993). AF6 is a scaffolding protein that co-localizes Spa-1 or Rap1GAP and activated Rap at sites of cell-matrix attachment (Su et al., 2003). There appear to be different consequences of AF6 on Rap1 activity. AF6 has been shown to sequester activated Rap1, limiting its interaction with effectors and protecting it from RapGAP-mediated GTP hydrolysis (Mitsutake et al., 2005). On the other hand, AF6 enhanced the ability of RapGAP to inactivate Rap1 (Su et al., 2003). The consequences of Rap1GAP on Rap activity at different cellular sites may be influenced by the binding partners expressed in particular cells. Clearly, the regulation of Rap activity is complex. In addition to limiting the duration of Rap activity, RapGAPs may play a role in determining the cellular sites of Rap signaling (Ohba et al., 2003).

Based on previous findings (Tsygankova et al., 2010), we speculate that loss of Rap1GAP in human tumors renders tumor cells more sensitive to dissociation, thereby facilitating tumor cell dissemination. In support of that notion, we show that Rap1GAP
overexpression is compatible with cell-cell adhesion in tumor cells and in non-transformed epithelial cells. Further studies are required to determine how Rap1GAP contributes to the regulation of cell-cell adhesion, and whether loss of Rap1GAP in other tumor cells facilitates their dissemination.

3.4 MATERIALS AND METHODS

Reagents. Rap1GAP (sc-28189), β-catenin (sc-7199), HA polyclonal (sc-805) and HRP secondary antibodies were from Santa Cruz Biotechnology. Monoclonal HA antibody was kindly provided by Dr. Jeffrey Field (Department of Pharmacology, University of Pennsylvania). Glutathione sepharose beads (17-0756-01) were from GE Healthcare. E-cadherin antibody was from EMD Biosciences (205601). Phospho-Fak (Y397) antibody (44624G), rhodamine-conjugated phalloidin (R415) and Alexa-fluor conjugated secondary antibodies (A21202, A21203 and A21207) were from Invitrogen. For Amaxa transfections, Cell Line Nucleofector Kit V was used (VCA-1003). Scrambled (1027280), Rap1 (SI01968722, SI03090010) and Rap1GAP (SI01737050) siRNAs were from Qiagen. Rap2 antibody (610215), Matrigel (356230) and collagen IV (354233) were from BD Biosciences. Laminin was from Collaborative Biomedical Products (CB40232EA)

Cell lines and reagents. HCT116 human colon carcinoma cells were a generous gift from Dr. John Lynch (Department of Medicine, University of Pennsylvania). Cells were propagated in McCoy’s 5A medium supplemented with 10% fetal bovine serum (FBS). Wistar rat thyroid (WRT) cells were propagated in Coon’s modified Ham’s F-12 medium
supplemented with calf serum (5%), insulin (10µg/ml), TSH (1 mU/ml) and transferrin (5 µg/ml)(referred to as 3H growth medium). WRT cells were starved in basal medium (Coon’s modified Ham’s F-12 medium devoid of growth factors and serum). Low-calcium medium was from Invitrogen (SMEM, 11380).

**Adenovirus infection.** Rap1GAP and LacZ adenoviruses were constructed and purified as described previously (Tsygankova et al., 2007). Control (LacZ) and Rap1GAP viruses were infected at an equal MOI (multiplicity of infection) of 5 infectious units (IU)/cell in HCT116 cells and 10 IU/cell in WRT cells. HCT116 cells were infected overnight in growth medium. WRT cells were infected overnight in basal medium and transferred to 3H growth medium the following day.

**Western blotting.** Western blotting was performed as described previously (Vuchak et al., 2009). Proteins were detected using the FUJI LAS-3000 system and Multi Gauge 3.0 software (Fuji).

**Rap activation.** Rap activation was assessed as described previously using the RalGDS Rap-binding domain fused to GST to selectively retrieve GTP-bound Rap1 or Rap2 (Tsygankova et al., 2001). For Rap activation experiments in WRT cells, log phase cells were treated with EGTA (4mM) alone or EGTA followed by transfer to calcium-containing media for various times and Rap1 activation was measured.

**Cell spreading.** HCT116 cells were plated in serum-containing growth medium on collagen IV for various times, fixed and stained with rhodamine phalloidin. Cell area was measured using a Zeiss Axiophot fluorescence microscope and Zeiss Axiovision software.
**Wound assays.** Cells were infected with LacZ or Rap1GAP virus overnight. Cells were plated in 35mm dishes with a line made down the center. At 24-48 hrs post-infection, confluent cell monolayers were wounded perpendicularly to the line (5-6 wounds/dish). Images were captured immediately and after 24 hrs using a Nikon Eclipse TE2000 microscope and analyzed using Image J software. The area measured after 24 hrs was subtracted from the area measured at 0 hours. The area closed by mock-infected control cells was set to 1. All experiments were terminated prior to complete wound closure.

**Cell dissociation assays.** Cell dissociation assays were performed as described previously (Nagafuchi et al., 1994). Cells were treated with 0.01% trypsin in the presence of EGTA (2mM) or calcium (2mM) for 10 min. Cells were triturated 15 times, plated and images acquired immediately using a Nikon Eclipse TE2000 microscope.

**Cell association assays.** Cell association assays were performed as described previously (Balzac et al., 2005; Urushihara et al., 1979). HCT116 cells were dissociated into single cells in 0.01% trypsin/EGTA (2mM). Two x 10^6 cells were collected by centrifugation, washed and suspended in McCoy’s medium containing EGTA (2mM) or calcium (2mM). Cells were plated on agarose-coated dishes to prevent matrix attachment and rocked for 1hr for HCT116 cells and 16 hr for WRT cells. Images were acquired using a Nikon Eclipse TE2000 microscope. The number of particles as a function of the total number of cells (Np/Nt or dissociation index) was determined.

**Trypsin sensitivity assays.** Experiments were performed as described previously (Miyashita and Ozawa, 2007). Cells were lysed directly on tissue culture dishes (total cell lysates) or trypsinized (0.01%) in the presence of calcium (2mM) or EGTA (2mM).
prior to lysis in RIPA buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.5% deoxycholate; 1% NP-40; 0.1% sodium dodecyl sulfate (SDS); 10 mM NaF; 2 mM Na₃VO₄; aprotinin, leupeptin, and Pefabloc (each at 10 µg/mL). Lysates were subjected to western blotting for E-cadherin.

**Immunostaining.** For cell spreading and immunostaining for focal adhesions, HCT116 cells were plated on collagen IV for 15, 90, 120 and 240 minutes. For analysis of adherens junction proteins, HCT cells were plated on matrigel and WRT cells on laminin. Cells were fixed in MeOH:acetone (1:1) for 15 min at room temperature, stained with primary antibodies diluted in PBS, 5mg/ml bovine serum albumin, 0.2% Triton-X-100 for 1 hr at 37°C and then with Alexa-fluor-conjugated secondary antibodies for 1 hr at 37°C. Images were captured using a Zeiss Axiohhot fluorescence microscope and Zeiss Axiovision software. All images for a given antibody within an experiment were captured for the same times.

**Silencing experiments.** WRT cells were transfected with Rap1 (400nM) or scrambled siRNAs (400nM) using Amaxa electroporation and plated onto glass slips. At 72 hrs post-transfection, cells were transferred to low calcium medium (SMEM) for 1 hr and then refed with 3H growth medium for various times. Cells were fixed and stained for β-catenin.

**Statistics.** All experiments were performed a minimum of two biological replicates. Statistical significance was determined using the Student’s t test. Data sets were considered statistically significant when p<0.05 (* p<0.05, ** p<0.01, *** p<0.001).
CHAPTER 4: DISCUSSION
The biological effects of TSH are mediated through cAMP. Cyclic AMP activates PKA, cyclic-nucleotide gated ion channels and members of the Epac family. Epac is highly expressed in thyroid cells (de Rooij et al., 1998; Kawasaki et al., 1998). Together with the important role of cAMP in the regulation of differentiated function and cell proliferation, these data suggest that Epac/Rap mediates some of the biological effects of TSH. What these are has remained rather mysterious. Studies conducted in canine thyroid cells revealed that the effects of TSH on differentiated gene expression and function are mediated primarily through PKA (Dremier et al., 2007; Van Sande et al., 1989). Expression of the thermostable protein kinase inhibitor, a highly selective inhibitor of PKA activity (Walsh et al., 1971) impaired TSH-stimulated TPO and Tg expression, iodide transport and DNA synthesis (Dremier et al., 1997). Later studies from the same group revealed that 6MB-cAMP, a PKA-selective agonist, induced iodide transport, thyroid hormone secretion and cytoskeletal arrangements in a manner that was indistinguishable from TSH. Conversely, treatment with an Epac-selective agonist had no effect (Dremier et al., 2007; Van Sande et al., 1989).

Somewhat different results were reported in rat thyroid PC-CL3 cells. In these cells, Rap1 activation by Epac and Rap1 phosphorylation by PKA were required for the regulation of Akt activity (Lou et al., 2002) and DNA synthesis by TSH (Hochbaum et al., 2008). Expression of dominant negative Epac impaired TSH-stimulated DNA synthesis, while expression of activated Rap1 enhanced TSH-stimulated DNA synthesis (Hochbaum et al., 2008). Expression of a mutant Rap1 lacking the PKA phosphorylation site (Rap1bS179A) impaired TSH-stimulated DNA synthesis (Ribeiro-Neto et al., 2002).
The reason for the discrepant results regarding the role of Epac in TSH-induced proliferation in the different thyroid cell models remains to be elucidated.

There is a prevailing model that Rap1 activates ERK in cells that express B-Raf (Stork and Schmitt, 2002). B-Raf is highly expressed in the thyroid gland of mice (Mitsutake et al., 2005), in differentiated rat thyroid cell lines including WRT, PC-CL3 and FRTL-5 and in human thyrocytes (reviewed in (Kimura et al., 2001)). The important role of B-Raf in thyroid cells is underscored by the frequent mutational activation of B-Raf in PTC (Kimura et al., 2003). Hence, we reasoned that a likely role for Epac/Rap1 in thyroid cells would be the activation of ERK through B-Raf. In Chapter 2, we investigated the role of Rap1 in TSH signaling pathways that impinge upon ERK activation in WRT cells. We demonstrated that ERK is rapidly and transiently activated by TSH in a cAMP-dependent fashion. Surprisingly, ERK activation was independent of Rap1 activity or expression. Overexpression of Rap1GAP or silencing the expression of Rap1 had no effect on the ability of TSH or forskolin to activate ERK. Additionally, treatment with E-cAMP, an Epac-selective agonist, activated Rap1 but failed to stimulate ERK activation. In contrast, treatment with the PKA selective agonist, 6MB-cAMP, activated ERK with a time course similar to that of TSH or forskolin. Blockade of PKA activity by expressing PKI inhibited cAMP-stimulated ERK activation. These data indicate that cAMP activates ERK via PKA. We further examined whether PKA-mediated activation of ERK required B-Raf. Silencing B-Raf expression impaired TSH, forskolin and 6MB-stimulated ERK activation. We previously reported that Ras functions downstream from cAMP in TSH signaling (Kupperman et al., 1993). To assess
whether Ras was involved in ERK activation, dominant negative Ras was expressed. Dominant negative Ras impaired ERK activation by TSH and 6MB-cAMP. Taken together, our data suggests that PKA activates a Ras- and B-Raf-dependent pathway that culminates in ERK activation (Figure 4.1). This is the first study to identify B-Raf as a target of TSH. Interestingly, silencing B-Raf or treatment with the MEK inhibitor UO126 impaired DNA synthesis stimulated by TSH and serum. These data establish a role for B-Raf in TSH-dependent proliferation in thyroid cells.
Figure 4.1 A model for ERK activation by TSH in WRT cells.
TSH activates ERK in Wistar rat thyroid cells through a PKA-, Ras-, B-Raf-, and MEK-dependent pathway. Rap1 activation or knockdown had no effect on ERK activation by TSH.
Our findings confirm a prominent role for PKA in TSH signaling in rat thyroid cells, similar to the many studies conducted in primary canine thyrocytes. Those studies did not examine the role of PKA in ERK activation, as ERK activation by TSH was not detected in these experiments (Lamy et al., 1993). This may be a function of the protocol employed, where the earliest time examined was 5 min (Lamy et al., 1993). There are a number of experiments that are required to confirm the working model shown in Figure 4.1. Our working model proposes that PKA activates Ras, leading to the downstream activation of B-Raf, MEK and ERK. Future studies should determine whether PKA activates Ras. This could be assessed using the PKA agonist 6-MB-cAMP or overexpression of the PKA catalytic subunit in pull down assays for Ras activation. Earlier studies from our laboratory demonstrated PKA-independent activation of Ras by cAMP (Tsygankova et al., 2000). This does not exclude the possibility that cAMP may also elicit PKA-dependent effects on Ras. There are precedents for PKA-mediated Ras activation. In cortical neurons, forskolin activated Ras via a PKA-dependent mechanism (Ambrosini et al., 2000). Activation of β2-adrenergic receptors stably expressed in CHO resulted in cAMP-mediated activation of ERK that was both PKA- and Ras-dependent (Enserink, 2002). The β2-adrenergic receptor is stimulated by isoproterenol and acts through Gαs to activate cAMP. In cerebellar granule cells, cAMP activated ERK in a PKA- and Ras-dependent manner (Obara et al., 2007). In addition to measuring direct effects on Ras activity, it would be useful to confirm that silencing Ras expression impairs cAMP-mediated activation of ERK.
Further studies might include determining whether PKA associates with RasGEFs using co-immunoprecipitation experiments or *in vitro* binding assays. Interestingly, the RasGEF RasGRF-1 is phosphorylated on serine 916 (mouse) and serine 927 (human) by PKA (Norum et al., 2005). This phosphorylation was not required for activation of Ras by cAMP (Norum et al., 2005) and phosphorylation of this site by forskolin did not enhance Ras activity by RasGRF-1 (Mattingly, 1999). Further studies from the same group later showed that phosphorylation at this site was required for maximal neurite outgrowth in PC12 cells (Yang et al., 2003). A screen of the RasGEFs expressed in thyroid cells would identify potential candidates to analyze.

It is feasible that PKA does not activate Ras, but alters the effectors that are activated by Ras. In this scenario, activation of Rap1 would be cAMP-mediated, as has been reported (Tsygankova et al., 2000), and PKA would dictate the effectors activated by Ras, presumably channeling Ras-mediated signals to B-Raf. It was reported that PKA inhibits complexes between Ras and Raf-1 and promotes the interaction of Ras with PI3K in thyroid cells (Ciullo et al., 2001). There are reports that PKA phosphorylates Raf-1 at serines 43 and 233, creating a binding site for 14-3-3 proteins, leading to the inhibition of Raf-1 activity (reviewed in (Gerits et al., 2008)). Another potential mechanism through which PKA inhibits Raf-1 would be through the inhibition of p21-activated kinase (PAK) activity by PKA (Edin and Juliano, 2005; Gerits et al., 2008). PKA-mediated phosphorylation and inactivation of PAK would prevent PAK activation of Raf-1 through phosphorylation of Raf-1 on serine 338.
There is also precedence for direct PKA effects on B-Raf. PKA phosphorylated B-Raf *in vitro* (MacNicol and MacNicol, 1999; Peraldi et al., 1995). When the catalytic domain of PKA was expressed with the catalytic domain of B-Raf, PKA inhibited B-Raf activity. However, PKA phosphorylated and activated full-length B-Raf when both proteins were co-expressed in PC12 cells (MacNicol and MacNicol, 1999). Another report revealed that the level of 14-3-3 bound to B-Raf determined whether B-Raf was a substrate for PKA (Qiu et al., 2000). In this study, cAMP activated Rap1 in four cell lines, but activated MAPK in only two of the four cell lines. All of the cell lines expressed similar levels of B-Raf and 14-3-3. The cell lines where cAMP did not activate MAPK had five-fold less 14-3-3 associated with B-Raf than the lines that activated MAPK (Qiu et al., 2000). This suggests that 14-3-3 protects B-Raf from inactivation by PKA and may explain the tissue-specific effects of cAMP on B-Raf activity (Qiu et al., 2000). The sites on B-Raf that are phosphorylated by PKA remain to be determined. B-Raf lacks the equivalent of serine 43 on Raf-1. It has been suggested that PKA phosphorylates serine 728, equivalent to serine 621 site in Raf-1 as this region is highly homologous to Raf-1, but this remains to be determined (Dumaz and Marais, 2005). Besides regulating B-Raf activity or association with Ras, it is conceivable that PKA regulates the localization of B-Raf, perhaps inducing its association with scaffolds that also bind MEK and/or ERK.

Silencing B-Raf impaired the effects of TSH on DNA synthesis. Whether this is due to abolishing ERK activation remains to be determined. It would be interesting to determine whether expression of activated B-Raf enhances proliferation in WRT cells, as
might be expected based on the proliferative effects of B-Raf in thyroid carcinoma cell lines.

Although we were unable to ascribe a role to Rap1 in the regulation of ERK activity in differentiated thyroid cells, we were intrigued by the possible role of Rap in thyroid cancer. In 1994, our laboratory speculated that Rap1GAP functioned as a tumor suppressor based on its ability to inhibit thyroid cell proliferation when overexpressed (Tsygankova et al., 2004). Additionally, silencing Rap1GAP enhanced the proliferation of thyroid cells (Tsygankova et al., 2007). We went on to show that expression of Rap1GAP was downregulated in thyroid carcinoma cell lines, and that expression of activated Ras silenced the expression of Rap1GAP (Tsygankova et al., 2007). Importantly, we found the expression of Rap1GAP to be high in the normal thyroid gland and downregulated in thyroid tumors (Tsygankova et al., 2007). Further studies revealed that the expression of Rap1GAP is decreased with tumor progression (Nellore et al., 2009). These studies were recently confirmed by other investigators in the field (Zuo et al., 2010). It is now clear that the expression of Rap1GAP is downregulated in a variety of human tumors, suggesting that unrestrained Rap activity contributes to tumor progression.

The significance of downregulation of Rap1GAP in human tumors has been explored largely in overexpression studies (Nellore et al., 2009; Zhang et al., 2006a; Zhang et al., 2006b; Zheng et al., 2009; Zuo et al., 2010). Collectively, these analyses revealed that overexpression of Rap1GAP in tumor cells had wide reaching effects, including the inhibition of cell migration, invasion, proliferation and survival. The
relevance of these conclusions to the situation in human tumors is unclear given the high degree of overexpression achieved in many of these studies. Our laboratory took a different approach to discern the contribution of Rap1GAP downregulation to tumor cells. We sought to explore the functional consequences associated with silencing Rap1GAP expression in human tumor cells. We screened a large panel of human thyroid cell lines and found that Rap1GAP was downregulated in all of the lines analyzed (Tsygankova, unpublished). This prompted us to explore the expression of Rap1GAP in other human tumor lines. We found that Rap1GAP was expressed at variable levels in breast (Vuchak, unpublished) and colon carcinoma cell lines (Tsygankova et al., 2010). We demonstrated that Rap1GAP is downregulated in primary colon carcinomas (Tsygankova et al., 2010). Our screen identified HT29 colon carcinoma cells as a tumor cell line that retained Rap1GAP. Hence, we set out to explore the functional significance of silencing Rap1GAP in these cells. We demonstrated that silencing Rap1GAP was sufficient to induce sustained increases in both Rap1 and Rap2 activity (Tsygankova et al., 2010). Intriguingly, Rap1GAP-depleted cells exhibited profound alterations in cell adhesion. Unlike HT29 cells, which exhibit a high degree of cell-cell contact, Rap1GAP-depleted cells were dispersed. Additionally, the accumulation of E-cadherin, β-catenin and p120-catenin at cell-cell contacts was disrupted by Rap1GAP depletion (Tsygankova et al., 2010). The ability of Rap1GAP-depleted cells to adhere and spread on a collagen matrix was markedly enhanced. Furthermore, loss of Rap1GAP enhanced cell migration and invasion (Tsygankova, submitted). These results are consistent with the hypothesis that downregulation of Rap1GAP enhances tumor cell dissemination.
In Chapter 3, we broadened our investigation of Rap signaling in colon cancer cells. Specifically, we wished to assess the contribution of Rap1GAP to the regulation of cell-cell and cell-matrix adhesion in tumor cells. There is abundant evidence that Rap1 regulates integrin signaling (Bos et al., 2003; Caron, 2003) and cell-cell adhesion (Kooistra et al., 2007). In particular, Rap1 has been shown to promote cell-cell adhesion. Therefore, we were surprised to find that increased Rap activity in cells depleted of Rap1GAP was associated with a decrease in cell-cell adhesion (Tsygankova et al., 2010). Based on this, we set out to more thoroughly investigate the role of Rap1GAP in the regulation of cell adhesion. We decided to use HCT116 colon carcinoma cells as a model system. The expression of Rap1GAP in HCT116 cells is considerably lower than that in HT29 cells, suggesting that these cells provided a model in which to examine the effects of both Rap1GAP overexpression and depletion. HCT116 cells exhibit significant Rap2 activity in exponentially growing cells. Rap1 expression and activity was not detected in these cells. We devised conditions to overexpress Rap1GAP at the lowest dose that was sufficient to block Rap2 activity. Using these conditions, the contribution of Rap1GAP to the regulation of cell-matrix and cell-cell adhesion was explored. Overexpression of Rap1GAP induced a more compact morphology. To assess if these morphological changes reflected impaired cell-matrix adhesion, the ability of Rap1GAP-expressing cells to plate and spread on collagen IV was examined. In these acute experiments, Rap1GAP impaired cell adhesion, spreading and the formation of focal adhesions. These effects were functionally significant, as overexpression of Rap1GAP impaired the migration of these cells to serum. These initial observations confirmed that blocking Rap2 activity by
overexpressing Rap1GAP elicited functional consequences. Surprisingly, overexpression of Rap1GAP did not render cells more sensitive to dissociation under conditions of calcium chelation, nor did it impair the formation of cell-cell contacts. Moreover, the accumulation of E-cadherin and β-catenin at cell contacts was not altered by blocking Rap2 activity. These data were surprising given that Rap1 activation is associated with enhanced cell-cell contact formation (reviewed in (Kooistra et al., 2007)). Given that HCT116 cells harbor a mutation in the gene for β-catenin and that the role of Rap2 in the regulation of cell-cell adhesion is largely unknown, similar studies were conducted in an alternate cell line. Wistar rat thyroid cells, or WRT cells, are a non-transformed thyroid epithelial cell line that are wild-type for β-catenin and express high levels of Rap1. Similar to the results obtained in HCT116 cells, overexpression of Rap1GAP did not weaken cell-cell adhesion or alter the localization of β-catenin at cell-cell contacts. Silencing the expression of Rap1 in these cells disrupted cell-cell contacts. This documents that Rap1 is required for cell-cell adhesion in these cells. Based on this, our findings suggest that cell-cell adhesion requires the cycling of Rap1 activity and/or that Rap1GAP selectively impairs the effects of Rap on cell-matrix adhesion (see Figure 4.2).
Figure 4.2 A model for Rap1GAP regulation of cell-cell and cell-matrix adhesion. We suggest that Rap1GAP preferentially impairs Rap signaling to the ECM. Overexpression of Rap1GAP in cell lines that express different forms of Rap (Rap1 versus Rap2) impaired matrix adhesion in the absence of inhibition of cell-cell adhesion.
Interestingly, silencing the expression of Rap1 and Rap2 restored cell-cell contacts in Rap1GAP-depleted HT29 cells (Tsygankova et al., 2010). These results support the notion that unrestrained Rap activity disrupts cell-cell adhesion in some cells.

Quite little is known regarding the effects of Rap1GAP on cell-cell adhesion. Microinjection of Rap1GAP into MCF-7 breast cancer cells did not disrupt cell-cell adhesion, although it delayed the formation of cell-cell contacts (Hogan et al., 2004). These authors concluded that Rap1 activity plays a transient role in the formation of cell-cell contacts. Interestingly, these authors noted that microinjection of RapV12 also delayed the formation of cell-cell contacts. Although they did not discuss the significance of these findings, these results support the notion that the cycling of Rap1 activity may be important for the maintenance of cell-cell adhesion. In another report, the authors also noted that overexpression of high levels of RapV12 induced aberrant cell-cell contacts in MCF-7 cells (Boettner et al., 2000). These reports differ from others that showed that stable expression of RapV12 restored cell-cell adhesion in Ras-transformed MDCK cells (Price et al., 2004). Similarly, stable expression of RapV12 in wild-type MDCK cells strengthened cell-cell adhesion and impaired cell scattering by HGF (Asuri et al., 2008). The basis for the discrepant effects is unknown, but could be related to cell line differences or the level of overexpression.

Somewhat differently, two studies have shown that the disruption of cell-cell contacts activates Rap1 in epithelial cells (Asuri et al., 2008; Balzac et al., 2005). One group concluded that Rap1 is activated as a mechanism to restore cell-cell contacts (Asuri et al., 2008) while the other concluded that Rap1 activation upon the disruption of cell-
cell contacts is required for the formation of integrin-dependent focal contacts (Balzac et al., 2005). It is noteworthy that in both reports, the formation of E-cadherin-mediated cell-cell contacts was required for Rap1 inactivation. These data suggest that Rap1GAP plays an important role in the inactivation of Rap1 that is apparently required in some cell types to allow the formation of cell-cell contacts (see model in Figure 4.2).

Based on our own data and that from the literature, our working hypothesis is that Rap1GAP is required for the maintenance of cell-cell adhesion. This would explain our findings in HT29 cells that silencing Rap1GAP disrupted cell-cell adhesion and the findings from others that high level expression of activated Rap1 resulted in aberrant cell-cell junctions. In Chapter 3, we show that modest overexpression of Rap1GAP is sufficient to impair cell-matrix adhesion, but does not alter the formation or maintenance of cell-cell adhesion. We hypothesize that the differences between our findings and those of Hogan in MCF-7 cells reflect differences in the level of expressed Rap1GAP. RapGAP microinjection into MCF-7 cells resulted in vast overexpression of the protein, but E-cadherin recruitment still occurred 2 hrs after the calcium switch. Perhaps expressing endogenous levels of RapGAP would not have resulted in a delay in cell contact re-formation. The differences in our findings and the findings in endothelial cells may point toward differences in the regulation of cell-cell adhesion in epithelial versus endothelial cells. Wittchen and colleagues used adenoviral infection of Rap1GAP, however whether the lowest concentration to inhibit Rap activity was used is unclear. Fukuhara showed that modest levels of Rap1GAPII (adenoviral infection) blocked Rap1 activity and increased the permeability of HUVEC cells, but it is unclear if this level of
Rap1GAP expression was sufficient to block Rap2 activity, which is also expressed in HUVECs (Fukuhara et al., 2005).

Our data also suggest that Rap1GAP preferentially impairs cell-matrix adhesion. There are several potential mechanisms through which Rap1GAP could elicit such specific effects. RapGAPs interact with cellular proteins in addition to Rap. This is especially true for members of the Spa-1 family, which contain a variety of protein-protein interaction domains (Bernards and Settleman, 2004; Spilker and Kreutz, 2010). Spa-1 interacts with RNA processing -1 homolog (Rrp-1b) and mammalian bromodomain protein-4 (Brd4), and these interactions elicit effects on Spa-1 activity. Rap1GAP exhibits a much simpler domain structure, however it has been shown to interact with the scaffolding protein AF6 or afadin (Su et al., 2003; Zhang et al., 2005). This is intriguing from several respects. First, activated Rap1 binds to AF6, suggesting that this protein may co-localize Rap1 and Rap1GAP (Boettner et al., 2000; Su et al., 2003). In support of that idea, ternary complexes of Rap1, Spa-1 and AF6 have been reported (Su et al., 2003). In addition, AF6 has been localized to cell-cell contacts (Boettner et al., 2000) and to sites of cell-matrix adhesion (Su et al., 2003). In principle, AF6 could localize Rap1GAP to distinct cellular sites. Inducible expression of AF6 in HeLa cells caused the redistribution of Spa-1 from the cytosol to sites of cell-ECM adhesion, where Rap1GTP also co-localized (Su et al., 2003). Expression of AF6 enhanced the detachment of cells from the matrix, suggesting that AF6 enhances Spa-1 activity at sites of cell-ECM attachment. Whether AF6 associates with Rap1GAP and/or Rap in HCT-116 or WRT cells is unknown.
Another potential explanation for our findings is that Rap1GAPII, rather than Rap1GAP specifically inhibits Rap activity at cell-cell contacts. Rap1GAPII contains an N-terminal extension with a GoLoco domain that has been shown to mediate its interaction with heterotrimeric G protein α subunits. Rap1GAPII binds to the Gi α-subunit, recruiting Rap1GAPII to the plasma membrane and impairing Rap activity at the membrane (Mochizuki et al., 1999). A Rap1GAP has also been shown to associate with inactive Goαo at the membrane, sequestering Rap1GAP and enhancing Rap activation (Jordan et al., 1999). The same year another laboratory reported a Rap1GAP isoform that binds Goαz at the membrane (Meng and Casey, 2002; Meng et al., 1999). There are several different Rap1GAP isoforms and it is unclear if these reports all utilized what is now referred to as Rap1GAPII, but the RapGAPs used in these reports all contain a GoLoco domain for membrane binding. The experiments presented in Chapter 3 were performed using Rap1GAP, a Rap1GAP that does not contain a GoLoco domain. Therefore, the effects of Rap1GAPII on cell-cell adhesion could be tested.

Collectively, these data highlight the need for further studies to clarify the mechanism by which Rap1GAP and Rap regulate cell-cell contact formation. To assess whether Rap1GAP is required for cell-cell contact formation in HCT116 cells, as it is in HT29 cells, Rap1GAP expression could be silenced in HCT116 cells. If Rap1GAP is required for cell-cell adhesion, silencing Rap1GAP should disrupt cell-cell contacts in these cells (Tsygankova et al., 2010). Calcium switch assays could be conducted in Rap1GAP-depleted cells to assess whether the formation of cell-cell contacts is delayed or disrupted. To assess whether the cycling of Rap activity is required for proper contact
formation, activated forms of Rap1 and Rap2 could be overexpressed. This would also
determine if both Rap proteins regulated cell-cell adhesion. There are reports that Rap1
and Rap2 have distinct roles. In the nervous system, Rap1 is important for long-term
depression of synaptic transmissions and inhibits glutamatergic synaptic transmission
(Imamura et al., 2003; Zhu et al., 2002) while Rap2 is required to remove AMPA
receptors during synaptic depotentiation. Inhibition of Rap2A promotes dendrite growth
in neurons (Kawabe et al., 2010; Zhu et al., 2005). There are also examples where these
proteins function similarly. Activation of both Rap1 and Rap2 promoted integrin
activation and cell-cell adhesion (McLeod et al., 2004). Although this report is widely
cited as evidence that Rap2 regulates cell-cell adhesion, this was based on the inhibition
of cell-cell adhesion mediated through LFA-1 and ICAM-1 by overexpression of
Rap1GAPII (McLeod et al., 2004). Both Rap1 and Rap2 were expressed in these cells,
hence it is not clear that cell-cell adhesion was mediated by Rap2. Future studies could
examine the role of Rap2 in the regulation of cell-cell adhesion in HCT116 and other
cells that express high levels of Rap2. It would be interesting to determine whether
silencing the expression of Rap2 deregulates cell-cell adhesion either in growing cells or
upon the acute disruption and re-formation of cell-cell contacts. In addition,
overexpression of dominant negative Rap would assess the requirement for Rap1 or Rap2
activity in the formation and maintenance of cell-cell adhesion. We would anticipate that
locking Rap on or off would elicit similar effects on cell-cell adhesion. There is
precedent for this notion where activated and dominant negative forms of other small
GTPases, including Rac1 elicited similar effects (Braga et al., 1999; Braga et al., 1997).
Very little is known regarding the cellular localization of Rap1GAP. Most studies have described Rap1GAP as a cytoplasmic protein. It remains plausible that there are discrete pools of Rap1GAP that reside at cell-cell contacts. Localization studies would allow us to determine if Rap1GAP resides at cell-cell contacts in growing cells, or is recruited there under conditions of cell-cell contact formation. Preliminary studies assessing the localization of endogenous Rap1GAP were uninformative, in part due to the high level of RapGAP expression which may have masked pools of Rap1GAP at cell-cell junctions. The generation of GFP-Rap1GAP fusion proteins would afford an opportunity to monitor Rap1GAP localization in living cells. It would be most interesting to compare the localization of Rap1GAP and Rap1GAPII in the same cell types, and to compare their effects on cell-cell adhesion.

AF6 is expressed in WRT cells. Preliminary studies examining the localization of AF6 in these cells were compromised by the poor quality of commercial AF6 antibodies. A number of investigators have generated antibodies suitable for immunostaining (Boettner et al., 2000). It would be interesting to examine the localization of AF6 in WRT and HCT116 cells, and to assess whether AF6 and Rap1GAP co-localize. This could be approached by generating GFP and YFP tagged proteins to analyze protein distribution in living cells. As an alternative strategy, co-precipitation experiments could be conducted analyzed the association of endogenous proteins or following their overexpression. Silencing AF6 could be performed to determine whether this results in the inhibition of cell-cell adhesion by Rap1GAP.
Overall, our data challenges the notion that Rap1GAP inhibits cell-cell adhesion and suggests that Rap1GAP may be required for cell-cell adhesion. This would provide a potential explanation for the widespread downregulation of Rap1GAP expression that occurs with progression in human tumors. Expression of activated Rap1A in prostate cancer cell lines enhanced the incidence of metastasis in a mouse model (Bailey et al., 2009). Stable expression of activated Rap1 in human breast cancer cells resulted in enhanced invasion into the vasculature (Itoh et al., 2007). An implication of these findings is that downregulation of Rap1GAP alters the balance in cell-cell and cell-matrix adhesion, an event that could enhance tumor cell dissemination. In support of this notion, a recent report suggests that the downregulation of Rap1GAP is required for metastasis. Overexpression of Rap1GAP in B16F1 melanoma cells had no effect on tumor formation, but inhibited metastasis (Freeman et al., 2010). When primary tumors and the small number of metastases that formed were explanted and analyzed for Rap1GAP, Rap1GAP expression was retained in primary tumors and extinguished in metastatic lesions (Freeman et al., 2010). In addition, melanoma cells expressing either Rap1GAP or Rap1V12 failed to extravasate from the microvasculature (Freeman et al., 2010). This provides evidence that the cycling of Rap may be particularly important for extravasation and the formation of metastases. Collectively, these data along with many others highlight role for Rap in many of the cellular processes that are required for tumor cell dissemination (Figure 4.3). Future studies to explore the functional significance of depleting Rap1GAP on tumor cell dissemination and the formation of metastatic lesions may provide novel targets that could be used to attenuate tumor spread.
Figure 4.3 Rap regulates cellular processes involved in metastasis.
Dowregulation of Rap1GAP reproduces early steps in invasive growth including the weakening of cell-cell adhesion and enhanced interaction with the matrix (Tsygankova et al., 2010). Rap activation has been shown to promote cell motility in vitro (McLeod et al., 2002; Zheng et al., 2009) and invasion and metastasis in vivo (Bailey et al., 2009; Lin et al.). The cycling of Rap activity appears to be important for extravasation and the formation of metastatic lesions (Freeman et al., 2010). Figure from (Huber et al., 2005).
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