

The Roles of Rap1 in Cancer Metastasis and Pancreatic Islet Beta Cell Function

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Dissertation submitted in partial
fulfillment of the requirements for
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ABSTRACT

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Abstract

Signaling from the G protein, Rap1 is involved in several fundamental biological processes. Yet the mechanism or even consequence of Rap1 signaling in several biologies and diseases is still unclear. Rap1 has been implicated in cancer tumorigenesis, but its role in cancer invasion and metastasis is less well understood. Rap1 signals to pathways involved in cell adhesion, migration, and survival, suggesting that Rap1 may promote several processes associated with metastasis. Recent studies in another biological system have demonstrated that the Rap activator proteins, Epac, are important regulators of pancreatic β -cell insulin secretion. However, the role of Rap1 in β -cell biology has not yet been defined. Here we established roles for Rap1 in distinct signaling events and begin to answer some of the key questions about Rap1 function in two diverse biologies: cancer metastasis and pancreatic islet β -cell function.

Elucidating the mechanisms of prostate and breast cancer survival and metastasis are critical to the discovery of novel therapeutic targets. Examination of prostate cancer cell lines revealed cells with a high metastatic ability exhibited increased Rap1 activity and reduced expression of the negative regulator, Rap1GAP. Activation of Rap1 increased prostate and breast cancer cell migration and invasion, and inhibition of Rap1A activity via RNAi-mediated knockdown or ectopic expression of Rap1GAP markedly impaired cancer cell migration and invasion. Additional studies implicated integrins α 4, β 3, and α v β 3 in the mechanism of Rap1-mediated prostate and breast cancer migration. Furthermore, these same integrins and matrix metalloproteinases were shown to be involved in Rap1-induced prostate cancer invasion. Introduction of activated Rap1 into prostate cancer cells dramatically enhanced the rate and incidence of CaP metastasis in a mouse metastasis model. In another mouse xenograft model, blockade of Rap1 signaling by expression of Rap1GAP abrogated breast cancer metastasis. These studies support a role for aberrant Rap1 activation in prostate and breast cancer

metastatic progression, and suggest that targeting Rap1 signaling could provide a means to control metastasis of these cancers.

In a separate biological system, the effects of Rap1 signaling on pancreatic β -cells was directly examined. Activation of Rap1 was demonstrated to promote ribosomal protein S6 phosphorylation through the mTOR and p70 S6 kinase (S6K1) pathway, a known growth-regulatory pathway. This newly defined β -cell axis acts downstream of cAMP, in parallel with the stimulation of both Epac and PKA. Like previous studies on Epac, activation of Rap1 indeed increased glucose stimulated insulin secretion (GSIS) from rat islet β -cells; however, Rap1-mediated GSIS did not appear to signal through this new S6 pathway. Interestingly, Rap1 was shown to significantly increase islet cell proliferation and this indeed occurred through signaling to mTOR and S6. In summary, these findings represent a new link between cAMP signaling and the pathways controlling β -cell proliferation, and suggest that directly targeting this pathway may have beneficial therapeutic effects for patients with Type 2 diabetes. Furthermore, an additional benefit to targeting Rap1 signaling is the potentiation of insulin secretion, which could possibly prevent or reverse β -cell dysfunction (i.e., defects in both β -cell mass and insulin secretory capacity) in diabetes.

Dedication

To my parents, William and Jane Bailey

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List of Abbreviations

BrCa	Breast Cancer
CaP.....	Prostate Cancer
DMEM.....	Dulbecco's Modified Insect Medium
DTT.....	dithiothreitol
FBS.....	fetal bovine serum
G proteins.....	guanine nucleotide binding regulatory protein
GFP.....	green fluorescent protein
GFR.....	growth factor-reduced
GPCR.....	G protein-coupled receptor
GSIS.....	Glucose Stimulated Insulin Secretion
GST.....	glutathione S-transferase
RapGEF.....	Rap-specific guanine nucleotide exchange factor
RapGAP.....	Rap-specific GTPase activating protien
RBD.....	Ras-binding domain
RTK.....	Receptor Tyrosine Kinase
S6.....	S6 ribosomal protein
SDF-1.....	Stromal Derived Factor-1

Acknowledgements

First, I would like to start by thanking my advisor, Pat Casey. You always encouraged me to think freely and to be resourceful as a scientist, while at the same time providing your great wisdom and experience at exactly the right moments. Despite the physical distance, your guidance and patience has been invaluable in my journey. I thank the members of my committee, Gerry Blobe, Leslie Parise, and Ann Marie Pendergast for all their wonderful insight and support in my development as a scientist. Additionally I would like to thank Tim Fields for guiding me and helping me along my path.

I would also like to thank all my collaborators and friends at Duke. I would especially like to thank Pat Fueger and Chris Newgard for the expertise and assistance. It was a wonderful experience to be part of such a helpful and open scientific community; I have never hesitated to go door-to-door with my ice-bucket. To all my fellow grad student friends, it has been a great relief and enjoyment to share this experience with you.

To all the members of the Casey Lab, both past and present, I thank you from the very bottom of my heart. I have had so many mini mentors in this group that have helped me immensely. It has been a true pleasure and honor to work with everyone in this group. I would especially like to thank Michelle Kimple and Pat Kelly, for their tireless efforts and encouragement, and for their interest in my scientific and personal development. There also are not enough words to thank Ian Cushman, Juhi Juneja, Andy Nixon, Laura Stemmler, and Alan Embry for all their amazing advice and friendship.

Finally I would like to thank all my family and loved ones, who have made this possible. I would especially like to thank my parents, Bill and Jane, thank you for all your love and encouragement and for teaching me to do my best. To my sister and brother-in-law, thank you for always being there and for making Dylan- the most wonderful diversion from lab work. To John, there are no words to describe how much love, support, and motivation you give me in every aspect of life.

1. Introduction and Background

1.1 Introduction to G protein signaling

In concert with their regulators and effectors, guanine nucleotide-binding regulatory proteins (G proteins) are central components that control signal transduction pathways within a cell to impact almost every aspect of cell function and biology. Most G proteins fall under a large Ras superfamily (named after the Ras oncoprotein), including the monomeric G proteins classified into subfamilies termed Ras, Rho, Rab, and Arf, and the heterotrimeric G protein $G\alpha$ subfamily (1). Although very diverse, all members of this superfamily share the basic biochemical property of binding and hydrolyzing GTP, making them GTPases.

G proteins undergo conformational changes that are regulated by guanine nucleotide binding. Upon receiving a stimulus, a G protein adopts a conformation that allows it to release GDP and bind GTP. Accompanying GTP binding, further conformational changes then display a surface on the G protein that confers its ability to bind with high affinity to downstream effector proteins. The inactivation of a G protein occurs through GTP hydrolysis and release of the phosphate produced, which leaves the G protein bound to GDP. This GDP-bound conformation results in a release of the effector protein and attenuation of downstream signaling (2). Furthermore, GTP hydrolysis by the G protein can be enhanced by interaction with GTPase activating proteins (GAPs), resulting in a more rapid return to the inactive state (3). Conversely, guanine nucleotide exchange factors (GEFs), generally responding to the afore-mentioned stimulus, can activate a G protein by catalyzing the release of GDP and allowing the G protein to bind GTP, which is ten-fold more abundant than GDP in cells (1). An additional class of

regulatory proteins, guanine nucleotide dissociation inhibitors (GDIs) can bind to a GDP-bound G protein and inhibiting GDP release, forcing the protein to remain in its inactive state (4).

The GEF for a heterotrimeric G protein is generally a G-protein coupled receptor (GPCR), and the G protein then transmits signals upon receptor activation. In this model, following the dissociation GDP from the $G\alpha$ subunit and exchange for GTP, the GTP-bound $G\alpha$ dissociates from the $G\beta\gamma$ dimer and the receptor, and $G\alpha$ -GTP and $G\beta\gamma$ are then able to engage downstream effectors (5). Heterotrimeric G proteins are classified by the similarity of their α subunits, and are grouped into four families termed Gq, G12, Gs, and Gi. The Gq proteins are involved in calcium-mediated signaling via their ability to activate phospholipase C, and G12 proteins promote signaling through the monomeric G protein Rho and also via other pathways that are still being deciphered (6). The Gs family members activate adenylyl cyclase, thereby elevating intracellular cAMP levels that can enhance several biological processes, such as cancer metastasis and insulin secretion (7). The members of the Gi family are named for their ability to inhibit adenylyl cyclase, and thus are considered negative regulators of cAMP signaling processes.

1.2 Rap monomeric G protein signaling

Rap1 is a member of the Ras-proximal branch of the Ras protein subfamily, and Rap and Ras proteins have high sequence similarity, particularly in their effector regions. This would suggest that Ras and Rap1 share or compete for the same effectors (8). Based on its original description, Rap1 was termed Krev-1, as it was found to suppress K-Ras-mediated transformation in fibroblasts (9). Subsequently, Rap1 was reported to promote mitogenic

properties through activation of B-Raf, and thus is now seen as a growth enhancer in most B-Raf-containing cells (1).

There are five isoforms of Rap: Rap1A and 1B, which are 95% identical; and Rap2A, 2B, and 2C, which are 90% identical (hereafter, I will refer to Rap1A and 1B together as Rap1, and Rap2A, 2B, and 2C together as Rap2; all isoforms together will be termed Rap). Rap1 and Rap2 are approximately 60% identical to each other, differing mostly at the C-terminus (10). Due to their sequence similarity, they often appear to perform similar functions; thus, determining the distinct biological roles of each isoform still remains an area of intense investigation. One report in HEK293T cells suggests the half-life of GTP-bound Rap2 is significantly longer than that of GTP-bound Rap1, suggesting that Rap2 has a lower sensitivity to GAPs, resulting in a high GTP-bound to GDP-bound ratio (11). Although Rap1 and Rap2 are regulated by a similar set of GEFs and GAPs, it has been postulated that Rap2 functions as a slowly responding molecular switch in the Rap signaling cascade (11). Additionally, the Rap isoforms appear to have different sensitivities to RapGEFs, as preferential activation of one Rap isoform over another has been reported in some cell types (10).

1.3 The Regulators of Rap Activity

1.3.1 Rap guanine nucleotide exchange factors (GEFs)

Currently, more than 10 GEFs for Rap1 have been identified, yet, it remains unclear why there are so many RapGEFs. It has been hypothesized that their distinct subcellular locations and cell-type expression might influence Rap function (12). One obvious mechanism for a specific influence on Rap1 function of the various GEFs is the discrete activation of these GEFs by specific stimuli.

C3G was one of the earliest identified RapGEFs, and is responsible for Rap activation in response to several different ligands, cytokines, and growth factors. C3G exists in complex with the adapter protein, Crk, which activates C3G by phosphorylating Tyr504 in the protein (13). Crk also brings the Crk-C3G complex to the membrane through Crk SH2 (Src homology domain 2) domain interaction with the phosphotyrosine residues of docking proteins, non-receptor tyrosine kinases, or growth factor receptor tyrosine kinases (RTKs) (14). Specifically, Cas, Cbl, and IRS can serve as the scaffolds for Crk-C3G mediated activation of Rap1. These Crk-C3G scaffold molecules can also be phosphorylated at Crk binding sites by other cytoplasmic tyrosine kinases (like c-src), RTKs, and BCR/Abl (15). In addition to Rap1, C3G can also activate R-Ras to mediate different biological effects (16).

The CalDAG-GEF subfamily of RapGEFs is mainly stimulated through calcium and diacylglycerol (DAG) signaling. In particular, CalDAG-GEFI is regulated by calcium, and thought to be the main RapGEF downstream of phospholipase C (PLC) that is known to induce calcium signaling. It preferentially activates Rap1, but can also activate, Rap2 and N-, H-, and R-Ras (15). Upon sensing of DAG, CalDAG-GEFIII (RasGRP3) translocates to the membrane and can activate both Ras and Rap (17). The other members of this subfamily, CalDAG-GEFII (RasGRP1) and RasGRP4, do not activate Rap, but instead act on Ras.

The Epac subfamily of Rap GEFs is mainly stimulated by the second messenger, cAMP; hence, the name Epac: exchange protein directly activated by cAMP. There are two isoforms of Epac, Epac 1 and Epac2; both isoforms can trigger Rap1 activation and they also have Rap1-independent effectors such as Rim2 (18). Epac1 is widely expressed and contains a DEP (Dishevelled, Egl-10, and Pleckstrin) domain responsible for its localization to the membrane.

On the other hand, Epac 2 is mostly expressed in the brain and in pancreatic islet β cells and contains an additional, lower affinity cAMP-binding site at its N-terminus (12). The Epac proteins can signal independently of cAMP-dependent protein kinase (PKA) to activate Rap1. Finally, cAMP-mediated stimulation of Rap1 can also occur exclusively through PKA signaling or through a coordinated effort by both Epac and PKA pathways, this will be explained later in greater detail.

The PDZ-GEF family of Rap GEFs has been less studied with regard to Rap1 activation than the above-described families. Like Epac, it contains an autoinhibitory cAMP-binding domain, yet it has a low affinity for cAMP (12). These GEFs contain a Ras binding domain (RBD) that can bind to both Rap and Ras. As indicated by their name, PDZ-GEFs contain a PDZ (PSD-95, Dlg, and ZO-1/2) domain and are generally characterized as scaffolding proteins that recruit various members of a signaling cascade to the proper intracellular location. PDZ-GEFI is primarily found in neuronal cells and it can also activate other PDZ domain-containing proteins, like S-SCAM (19). There are two other family members, PDZ-GEFII and MR-GEF, which appear to act solely on Ras (12).

The last and most atypical RapGEF is DOCK4; its mechanism of activation is unknown. DOCK4 is a member of the CDM (Ced-5, DOCK180, myoblast city) gene family. The *DOCK4* gene is recurrently mutated in several human cancer cells (20). It is involved in Rap1-mediated regulation of adherens junction formation in osteoblasts and osteosarcoma cells (20).

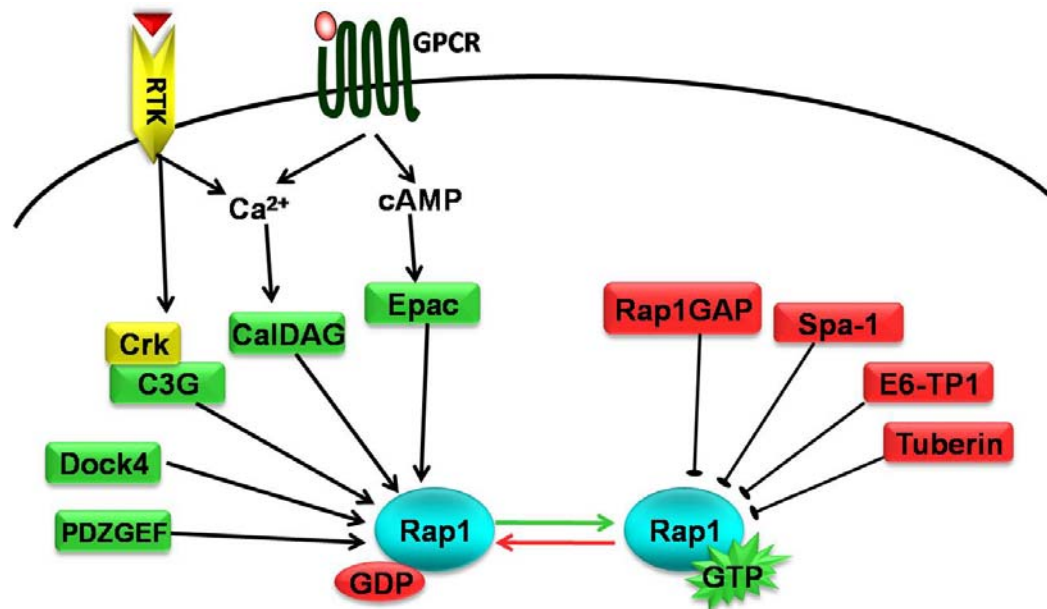


Figure 1: Summary of Rap1 Regulators

1.3.2 Rap GTPase activating proteins (GAPs)

The opposing regulatory group to the RapGEFs is RapGAPs, which negatively impact Rap signaling by increasing the intrinsic GTPase activity of Rap. All the RapGAPs are characterized as having a related catalytic GAP domain. RapGAPs are very important to the regulation of Rap1 in particular, since Rap1 has a relatively slow intrinsic GTPase activity, allowing them to mediate prolonged signal transduction events (21). This is because, unlike other G proteins, Rap1 does not have the conserved glutamine at residue 61 that is involved in the GTP hydrolysis step. Compared to the RapGEFs, there is less diversity in the identified RapGAPs, although their biological consequence is very significant as several disease states have been attributed to a loss of one of these proteins.

The first identified GAP specific for Rap1 was Rap1GAP, and its gene is located in a region where mutations or deletions have been found in several cancers, at chromosomal locus

1p36.5-35 (22). Rap1GAP (also referred to as rapGAP-I, Rap1GAP1a) acts on both Rap1 and Rap2. The Rap1GAP splice variant is termed Rap1GAPII (rapGAP-II, Rap1GAP1b) and has an N-terminus that is able to interact with heterotrimeric G α subunits, which will be discussed later in more detail (23). Another RapGAP, Rap1GAP2, is the product of a different gene with three splice variants encoding alternative N-termini: Rap1GAP2a, Rap1GAP2b, and Rap1GAP2c. Rap1GAP2a lacks exons 1 and 6; Rap1GAP2b includes exon 6; and Rap1GAP2c lacks exon 2 (24). The specific tissue expression of Rap1GAP2 is in the brain, platelets, and testis; it is also co-expressed with Rap1GAP1 in the pancreas and the stomach (24). The dual expression of both Rap1GAP1 and Rap1GAP2 in certain tissues suggests these GAPs may have different Rap selectivity or may serve to block different Rap effectors.

Another class of RapGAPs falls into the SPA-1 family. Generally restricted to expression in hematopoietic tissues, SPA-1 (signal-induced proliferation associated gene-1, also termed Sipa-1) is involved in leukemia, as displayed by the phenotypes of SPA-1 knockout mice (25). At the cellular level, SPA-1 reduces Rap1 activity and may be involved in actin dynamics by anchoring to the cell cytoskeleton by AF-6 or α -actin (15). Another member, co-named E6TP1 (E6 targeted protein 1) or SIPA1L1 (signal-induced proliferation associated protein 1 like 1), is a protein targeted for degradation by human papillomavirus E6 oncoprotein or by Wnt8-stimulated casein kinase 1 epsilon (CK1 ϵ) phosphorylation (26). The biological functions reported for E6TP1/SIPA1L1 include neuronal regulation and morphogenesis during vertebrate gastrulation (26, 27). The remaining two members SPAR (spine associated RapGAP) and SPA-Ls (SPA-1 like protein) are less studied in disease and are found in neuronal vesicles (28, 29).

The last RapGAP, Tuberin is the protein product of the *TSC2* gene, a gene lost in people suffering from tuberous sclerosis complex (TSC), resulting in numerous benign tumors (30). Tuberin contains a region of limited homology to the Rap1GAP catalytic domain and can stimulate GTP hydrolysis of Rap1A. Tuberin can also inhibit Rheb, Rab5, and possibly Rho (31, 32). The mechanism of action of Tuberin is homodimerization with the Hamartin protein, which is encoded by the *TSC1* gene(33). Loss of function of either protein results in aberrant growth and tumorigenesis (34).

1.4 The signaling targets of activated Rap1

There have been many potential Rap1 effectors identified, several of which can be activated through multiple Rap1GEFs and some with unknown activators upstream of Rap1. Depending on the cellular context, Rap1 activation of these effectors can have varying biological impacts.

1.4.1 Integrin-, Cell Adhesion-, and Actin-Related Molecules

Currently, Rap1 has been shown to activate a number of integrins ($\alpha 2$, $\alpha 4$, αL , $\beta 1-3$) and specific integrin heterodimers: $\alpha L\beta 2$ (LFA-1), $\alpha IIb\beta 3$, $\alpha 4\beta 1$ (VLA-4), $\alpha 5\beta 1$ (VLA-5), $\alpha v\beta 3$, and $\alpha M\beta 2$ (CR3) (8). Only two major Rap1 effectors for integrin signaling have been identified, RapL and RIAM, and not every Rap1-activated integrin has been shown to be associated with these specific effectors, suggesting there may be more that remain unidentified. Another common protein at Rap1-integrin complexes is talin, which also seems to be present with one of the following effectors. RapL (Nore 1b) is perhaps the most widely-studied Rap1 effector for integrin signaling. RapL contains a Ras association (RA) domain that recruits Rap1 to the cytoplasmic tail of integrins; in particular this has been demonstrated with the αL tail of LFA-1 ($\alpha L\beta 2$) (35).

binding to LFA-1 causes translocation of the Rap1-RapL complex to the membrane, whereby integrin affinity/avidity is increased and integrin adhesion to its ECM ligand is enhanced (36). The importance of Rap1 and RapL in integrin related processes has also been demonstrated by RapL knockout mice (37). These mice have T- and B-lymphocyte adhesion and transmigration defects, resulting in impaired homing of lymphocytes and dendritic cells to their target organs (37). The expression of RapL is limited; thus, it is likely other Rap1 effectors are involved in activation of integrin signaling (38).

Another Rap1 effector molecule involved in integrin signaling and actin dynamics is the Rap1-GTP-interacting adaptor molecule (RIAM). RIAM is highly expressed in hematopoietic cells and its paralogue, lamellipodin (Lpd), is found in fibroblasts and other somatic cells. Rap1 binds to the RA and pleckstrin homology (PH) domains of RIAM (38). Most recently, Rap1 was shown to complex with RIAM and talin, and this formation induce activation of integrin α IIb β 3 (39). In addition to regulating β 1, β 2 and β 3 integrins, RIAM can also bind the actin-related proteins profilin and Ena/VASP (40). The dual roles of RIAM in Rap1-mediated integrin and actin functions were demonstrated in T cells, where knockdown of RIAM inhibited Rap1-mediated integrin adhesion, inhibited Rap1 recruitment to actin at sites of adhesion, and reduced F-actin expression (38). In addition to impacting integrin-associated molecules, Rap1 effectors such as cadherin, Afadin, and Arap3, have also been identified as being involved in cell junction and actin-related functions. Rap1 has been linked to several cadherins. Via an impact on VE-cadherin, Rap1 can promote the assembly and maintenance of adherens junctions that control endothelial barrier function (41, 42). Studies show that Epac activation of Rap1 promotes its ability to interact with VE-cadherin and accelerate endothelial cell-cell junction formation (43).

The first evidence that Rap1 signaling impacted E-cadherin came from *Drosophila* cell-cell adhesion studies showing that ablation of Rap1 disrupted E-cadherin distribution of the epithelial cells on the fly wing (44). Furthermore, activation of Rap1 can occur at adherens junctions through its GEF, C3G, which can bind to the cytoplasmic tail of E-cadherin at immature junctions (45). At mature E-cadherin-based adherens junctions, C3G is displaced by β -catenin, which then interacts with PDZ-GEF to further activate Rap1 (46).

Another Rap1 effector that can also regulate E-cadherin function is Afadin (AF-6), (46). AF-6 is an adapter protein involved in various cell junctions, binding ZO-1 in tight junctions, nectins at adherens junctions, and profilin on the actin cytoskeleton (38). Rap1 binds to one of the RA domains at the N-terminus of AF-6, and this interaction appears to promote endocytosis of E-cadherin when it is not engaged in homophilic interactions, which is an important part of junction regulation (46). A slightly different model suggests that Rap1 may be involved in inducing interactions of E-cadherin and inhibiting its endocytosis. This model involves Rap1 activation of AF-6, which subsequently interacts with p120 catenin to elicit E-cadherin functions (46, 47). AF-6 has been shown to recruit the RapGAP, SPA-1, to inhibit Rap1 at sites of cell adhesion; this is perhaps a negative feedback loop for better temporal control of this process (48). Also, the association of Rap1 and AF-6 promotes dorsal closure in *Drosophila* (49).

Arap3 is another Rap1 effector protein that regulates the actin cytoskeleton, particularly through its regulation of RhoA. It has several domains, one of which is a Rap1-binding RA domain. It also has two GAP domains, one specific for Arf6 and the other for RhoA, and a PH domain that interacts with PIP3. Interestingly PIP3 binding enhances the ability of Arap3 to inhibit Arf6, and the binding of Rap1 enhances its ability to GAP and inhibit RhoA (50). Mutation

of either the RA or the RhoGAP domain on Arap3 produces a similar reduction of RhoA activity and lamellopodia formation (51).

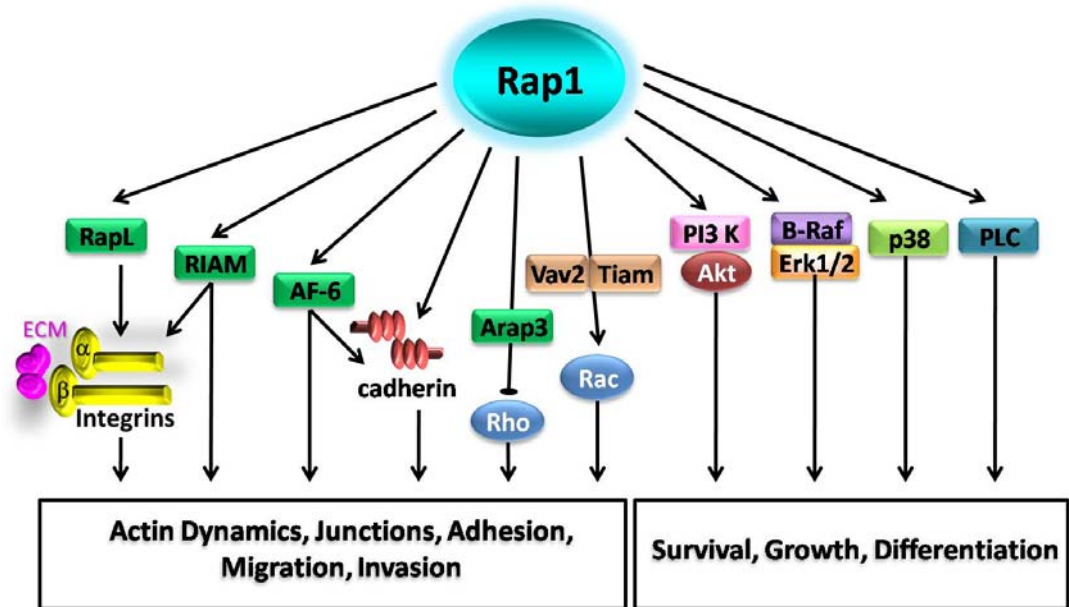


Figure 2: Rap1 Effectors

1.4.2 Monomeric G-proteins

As just alluded to, Rap1 signaling can also impact several G proteins from the Rho family. It is classically thought that Rac, CDC42, and Rho are three main Rho GTPases necessary for control of actin dynamics, coordinated cell movement, and migration. These Rho GTPases can activate each other, and furthermore, there is evidence that Rap1 can also participate in this signaling loop (43). As just mentioned, Rap1 may negatively affect Rho by binding the RhoGAP, Arap3, and enhancing its ability to reduce Rho activity (51). In contrast, Rap1 positively interacts with two RacGEFs, Vav2 and Tiam1, by binding to their PH domain and DH-PH catalytic region

(52). Rap1 recruits these GEFs to cell protrusions and extensions for the control of cell spreading (38). Additionally, it has been observed that Rac is required for Rap1-mediated cell spreading and that Rap1 activation can result in activation of Rac (53). In addition to impacting Rac, CDC42 can also be impacted by Rap1. In yeast, recruitment of the actin cytoskeleton to the site of bud formation results from the direct interaction of the Rap1 ortholog Bud1 with the CDC42GEF, CDC24 (54). In actin dynamics and adherens junction formation, activation of CDC42 by its GEF, FRG, also requires Rap1 (55). Additionally, CDC42 and the Par polarity complex are required for Rap1B-mediated fate determination of neurites to become axons (56).

Like Rap1, the Rheb branch is also in the Ras protein subfamily and is similar to the Rap branch in several ways. Rheb also has a slow intrinsic GTPase ability, so it is often negatively regulated by GAPs (1). It was originally thought to suppress Ras-mediated transformation and block MAPK signaling, like the early studies on Rap1 (57, 58). The main downstream effector of Rheb is mTOR (mammalian target of rapamycin), which regulates p70 S6 kinase (S6K1) and eukaryotic initiation factor 4E-binding protein (4E-BP1) to affect protein translation, cell growth and proliferation (1). Interestingly, the RapGAP, Tuberin, can also act as a GAP for Rheb (31). While no direct link to Rap1 has been reported, it is possible they may regulate each other by sequestering Tuberin.

As mentioned earlier, the first identified function of Rap1 was to impact Ras proteins. Rap1 can antagonize Ras signaling through interaction with Raf1, thereby blocking Erk activation from Ras-Raf1. Yet, it now appears that Rap1 preferentially interacts with B-Raf, not Raf1, to enhance Erk activation (12). It has also been postulated that like R-Ras, Rap1 may increase integrin signaling by reversing Ras-induced inhibition (14). Rap1 has also been indirectly linked

to another Ras family member, Ral. *In vitro*, Rap1 can be activated by binding to the Ras binding domains (RBD) of the RalGEFs: RalGDS, Rlf, and Rgl (59).

1.4.3 Protein Kinases

Rap1 signaling can also directly impact large signaling molecules, like protein kinases, which are known to be stimulated from various signaling sources. The role of Rap1 in Erk activation is mixed, and generally is decided by the relative expression of Raf-1 and B-Raf; the differential effect of Rap1 on Erk activation has been reviewed extensively (12, 14). To summarize, Rap1 can antagonize Ras stimulation of Erk by binding to Raf-1, in an inactive complex, and also stimulate Erk through interaction with B-Raf. It is thought that Rap1 interaction with a cysteine-rich region of both of the Raf isoforms is responsible for these differential effects (60). An excellent model for this opposing function of Rap1 signaling can be observed with constitutive Rap1 activation in the Spa-1 knockout (Spa-1^{-/-}) mouse. T-lymphocytes only express the Raf-1 isoform, and the T cells of Spa-1^{-/-} mice exhibit growth inhibition, are refractive to TCR stimulation, and become anergic due to constitutive Rap1 activation and Erk inhibition (61). Conversely, the myelocytes of these same Spa-1^{-/-} mice are hyperproliferative and serve as a model for chronic myelogenous leukemia (CML). Thus, constitutive Rap1 signaling enhances Erk activation in myelocytes, cells that express both B-Raf and Raf-1 (62). Even in cells with Raf-1 as the dominant isoform, Rap1 does not necessarily inhibit Erk signaling (63). The question remains, then, as to the need for different Erk signaling mechanisms when all four proteins, Ras, Raf-1, Rap1, B-Raf, are expressed in a given cell (14). One explanation can be found during the differentiation of megakaryocyte and neuronal cells. In these cells, it was demonstrated that Rap1/B-Raf is required for sustained Erk signaling, but not

transient Erk activation mediated by Ras/Raf-1 (64, 65). It has also been postulated that Rap1 activation of integrins can result in a positive feedback loop that activates Erk, termed outside-in signaling (15). Integrins can activate Erk through focal adhesion kinase (FAK) or cross-talk with RTKs (66, 67).

Although less defined than its interaction with Erk, Rap1 can also directly interact with the phosphatidylinositol 3-kinase (PI3-K) catalytic subunit p110 (68). This was first shown in thyroid cells, where Rap1 is involved in cAMP-stimulated PI3-K activation of Akt (69). This Rap1 activation appeared to be independent of the cAMP signaling protein, PKA; however, treatment with a PKA inhibitor could inhibit PI3-K/Akt activation (69). It is postulated that PKA phosphorylation of Rap1 or Rap1GAP may have some inhibitory effect, and recently this has been demonstrated for Rap1GAP and is a mechanism for PKA-mediated Rap1 activation (70). Thus, it would appear the cAMP-to-Rap1 axis can stimulate alternative pathways; this will be discussed in more detail in Chapter 5.

Other important signaling proteins with which Rap1 can interact are p38 MAP kinase and phospholipase c (PLC). Specifically, Rap1 was shown to antagonize IL-1-stimulated Ras activation of p38 MAPK in thymocytes and neuronal cells (71-73). The mechanical force of cell stretching can also activate Rap1 and mediate p38 activation (72). In neuroblastoma cells, Epac-activated Rap2B was shown to bind and activate PLC ϵ (74). Additionally, PLC- γ 1 can mediate TCR activation of Rap1 and increase T cell adhesion; this occurs through CalDAG-GEFI, which is activated by the PLC- γ 1 products calcium and DAG (75). Rap1 interaction with these proteins seems to be prevalent in neuronal cells and lymphocytes.

1.5 Rap1 signaling in cancer progression and metastasis

While Rap1 activity is linked to many cellular functions that impact numerous biological processes, a major focus of this thesis is on the implications of Rap1 signaling in cancer. As of late, the majority of reports correlate enhanced activation of Rap1 with cancer progression; however, it is clear there is much more to learn about the impact of Rap on tumorigenesis and tumor progression.

1.5.1 Rap1 signaling in cancer cell proliferation and tumorigenesis

1.5.1.1 Leukemia

Rap1 involvement in leukemia is primarily the result of aberrant Erk activation and growth deregulation. In leukemia, activation of Rap1 signaling has been implicated via three mechanisms: (1) the ability of BCR/ABL kinase to activate Rap1/B-Raf/Erk; (2) a proviral insertion in leukemia-prone BXH-2 mice that results in activation of CalDAG-GEFI to activate Rap1; and (3) alteration of the chromosomal region, 11q13.3, which houses the *SPA-1* gene, resulting in loss of that RapGAP and hence aberrant Rap1 activation (76-78). Most human leukemias arise from the presence of the Philadelphia chromosome, formed from the translocation of the long arms of chromosomes 9 and 22, from which the BCR-ABL fusion gene product is derived (79). BCR-ABL has been shown to have enhanced tyrosine kinase activity that can lead to Rap1 activation. The generation of *SPA-1*^{-/-} mice resulted in the development of a range of myeloid disorders, several of which resembled chronic myelogenous leukemia (CML) in the chronic phase and in blast crisis (25). In the bone marrow of *SPA-1*^{-/-} mice that were preleukemic, a large expansion of hematopoietic progenitors with high levels of active Rap1 and Erk activation was observed (25). Similarly, expression of mutationally activated Rap1 (Rap1-63E) in normal hematopoietic

progenitors enhanced hematopoiesis, and it was suggested that Rap1 enhanced progenitor interaction with stromal cells (25).

1.5.1.2 Solid tumors

Ectopic expression of Rap1B has been reported to induce proliferation and morphological transformation in Swiss 3T3 cells (80). In another study, vasoactive intestinal peptide (VIP), a neuropeptide, was shown to promote androgen-independent proliferation of prostate cancer cells through PKA-dependent Rap1 activation of Erk (81). Conversely, in osteosarcoma cells harboring a *DOCK4* gene deletion, restoration of the Rap1 activator suppressed anchorage-independent growth (20). In addition to these individual instances in cancer cells, several comprehensive studies have demonstrated major roles for Rap1 in cancer progression.

High levels of Rap1 expression were discovered in human oral cancer (82), and active Rap1 was strongly expressed in oropharyngeal squamous cell carcinoma (SCC) as compared to normal or non-malignant keratinocytes (83). In SCC cells, Rap1GAP expression inhibited MAPK activation and blocked cell-cycle progression, resulting in inhibition of *in vitro* proliferation and *in vivo* tumor growth of these cells (83). Several pancreatic tumors have reduced Rap1GAP expression, and restoring Rap1GAP expression abrogated pancreatic cancer cell proliferation, growth in soft agar, and tumor growth in mice (22). Similarly, a role for Rap1 in melanoma tumorigenesis was shown by identification of Rap1 activation in several human cutaneous metastatic melanomas, which was then correlated with increased Erk activity. Interestingly, of the three melanoma tissues with increases in Rap1-GTP, the only sample with significant Erk activation also expressed an activating *B-Raf* mutation, suggesting Rap1 activity might not be

responsible for the Erk activation. Although they had lower levels of Erk activation, Rap1 activation was seen in two other melanoma samples with wild type B-Raf. The *in vitro* melanoma cell line studies showed Rap1 could indeed impact growth and Erk activation, via Epac or hepatocyte growth factor (HGF) stimulation (84). The same group also discovered the *Rap1GAP* gene is downregulated in melanoma due to hypermethylation of its promoter, a mechanism for gene silencing, and demonstrated enforced expression of Rap1GAP reduced Erk activation (85). A high percentage of thyroid cancers express the RET/papillary thyroid carcinoma (PTC) oncoproteins, which result from the in-frame fusion of the RET receptor tyrosine kinase with other proteins. It was shown that RET/PTC activated Rap1 through C3G to stimulate Erk, resulting in increased proliferation and stress fiber formation (86). Recently, two other converging stories in thyroid cancer have identified the loss of Rap1GAP expression in papillary thyroid tumors (87) and that Rap1GAP downregulation contributes to Ras transformation and Erk activation of thyroid tumors (88).

1.5.2 Rap1 signaling in cancer cell adhesion

Maintaining adhesions that reduce mobility is vital to preventing tumor escape from the primary site; however, the ability of cells to adhere (and detach) is also essential for cell movement. Adhesion of cancer cells to platelets and leukocytes is vital to their survival and navigation of the circulatory system, as well as adhesion to endothelium, ECM, and cells of the metastatic site. The consequence of Rap1 signaling may have opposing roles in cancer progression, due to the effects of Rap1 on cell adhesion it has been reported to promote or inhibit cancer cell motility.

Inhibition of Rap1 in an ovarian cancer cell line disrupted cell junctions from a loss of E-cadherin on the cell surface (89). One of the first papers implicating Rap1 in cancer adhesion showed that depletion of the atypical RapGEF, DOCK4, disrupted Rap1 activation and adherens junctions (20). Furthermore, the authors detected a homozygous deletion of DOCK4 in osteosarcoma cells and several DOCK4 missense mutations in glioma, ovarian, prostate, and colon cancer cell lines. Yajnik *et al.* found that the expression of DOCK4 or Rap1 restored adherens junction formation in these cells (20). A slightly different story was reported in prostate cancer cells, PC3LN3, in which PLC γ 1 regulated Rap1GEF1 (C3G) and Rap1 activity (90). Knockdown of PLC γ 1 resulted in a reduction of Rap1 activity and reduced cell adhesion and cell spreading (and migration) of these cells (90). Interestingly, unlike the DOCK4 study showing a reduction in adhesion and an increase in invasion, in this study reduction of C3G-Rap1 activity decreased adhesion and migration. It would be interesting to know if these differences were the result of different cell types, different cancer stages, or different Rap1 GEF regulation of Rap1 effectors. Although no downstream mechanism or Rap1 effector was examined in the PLC γ 1-C3G-Rap1 study, it is possible that integrin regulation was involved, rather than cadherin regulation as in the DOCK4-Rap1 study.

1.5.3 Rap1 signaling in cancer cell migration and invasion

There are a few studies implicating the loss of Rap1 in enhancement of cancer cell invasion and migration. As mentioned above, in osteosarcoma cells with a *DOCK4* gene deletion, restoration of the Rap1 activator suppressed tumor invasion *in vivo*. It was speculated that this may be due to lost regulation of cell junctions (20). Interestingly, a new report from the same group that showed overexpression of Rap1GAP reduced Erk activation and delayed cell cycle

progression in SCC and that Rap1GAP expression increased SCC cell invasion (91). Mitra *et al.* suggested the mechanism of this invasion enhancement was that Rap1GAP expression augmented matrix metalloproteinase (MMP) 2 and MMP9 transcription and secretion in SCC cells (91). They also suggested that Rap1GAP might have a role as a biomarker for early N-stage aggressive SCC, as its expression correlated with MMP9 expression and high MMP9 expression at this tumor stage was indicative of poor survival (91).

The first evidence for the role of Rap1 in promoting invasion was recently demonstrated in pancreatic cancer, where a loss of Rap1GAP expression was seen in 60% of invasive pancreatic cancers (22). Zhang *et al.* also showed that loss of Rap1GAP promoted pancreatic cancer cell motility and invasion and suggested a mechanism involving modulation of integrin activity. Although no specific integrin was identified, an examination of general integrin function showed that FAK phosphorylation and F-actin formation were reduced in pancreatic cells expressing Rap1GAP (22). Additionally, injection of MiaPaCa-2 pancreatic cancer cells into the pancreas of SCID mice resulted in local invasion and liver metastasis, whereas no invasion or metastasis was observed in mice receiving cells expressing Rap1GAP (22). Similar results were seen in melanoma cells, where Epac-stimulated Rap1 was shown to induce melanoma cell migration *in vitro* and integrin $\alpha\beta 3$ activation. This was demonstrated by cell surface staining with WOW-1 (an antibody specific for activated $\alpha\beta 3$ integrin) and immunoblotting for phosphorylated Src (84). Additionally, the same group showed again in melanoma cells that Rap1GAP expression was reduced by hypermethylation of its promoter, and that re-expression of Rap1GAP resulted in a reduction of melanoma cell migration (85). We have recently identified that activation of Rap1 enhances prostate and breast cancer invasion and migration through a mechanism

involving integrins. Specifically, in prostate cancer, we identified the involvement of $\alpha v\beta 3$ and implicated a new integrin ($\alpha 4$) in Rap1-mediated cancer signaling. These findings are covered in detail in Chapters 3 and 4.

1.5.4 Rap1 signaling in other cancer-related processes

While less is known about other potential roles for Rap1 in cancer, several individual studies suggest there is much more to elucidate in regards to the role of Rap1 in cancer progression and metastasis. The potential importance of Rap1 in the early steps of tumorigenesis, are highlighted by studies indicating that Rap1 may contribute to organization of tissue architecture and in regulation of hormone receptors, which are important processes that are disrupted or aberrantly activated during cancer initiation. Specifically, Rap1 has also been described as an important regulator of breast architecture and cell polarity during breast acinar morphogenesis, which is effected by cell proliferation and death as well as cell-cell and cell-ECM signaling (92). Rap1 signaling is increased in malignant mammary cells and a reduction of Rap1 in these cells resulted in formation of correct breast acinar polarity and a reduction in tumor incidence (92). Additionally two studies describe Rap1 involvement in regulation of the androgen receptor. The neuropeptide VIP was shown to promote androgen-independent transactivation of the androgen receptor in prostate cancer cells through PKA-dependent Rap1 activation of Erk (81). Rap2 is involved in androgen-mediated transcriptional activity and androgen-stimulated growth of prostate cancer cells (93). Rap1 may also be involved in another important aspect of cancer progression, angiogenesis. Primary and metastatic tumor maintenance requires the initiation and formation of blood vessels. In prostate cancer cells, Rap1 is involved in stimulation of VEGF transcription induced by cell-cell contact; and this was

not observed in primary prostate epithelial cells (94). Rap1 also has recently been described as being a positive regulator of angiogenesis. Rap1A^{-/-} mice have reduced neovascularization after ischemia (95). Both Rap1A^{-/-} and Rap1B^{-/-} mice have reduced microvessel sprouting in response to VEGF and bFGF (96, 97). Furthermore, the endothelial cells of Rap1A^{-/-} and Rap1B^{-/-} mice have decreased FGF and VEGF stimulated Erk and p38 MPK activation (96, 97).

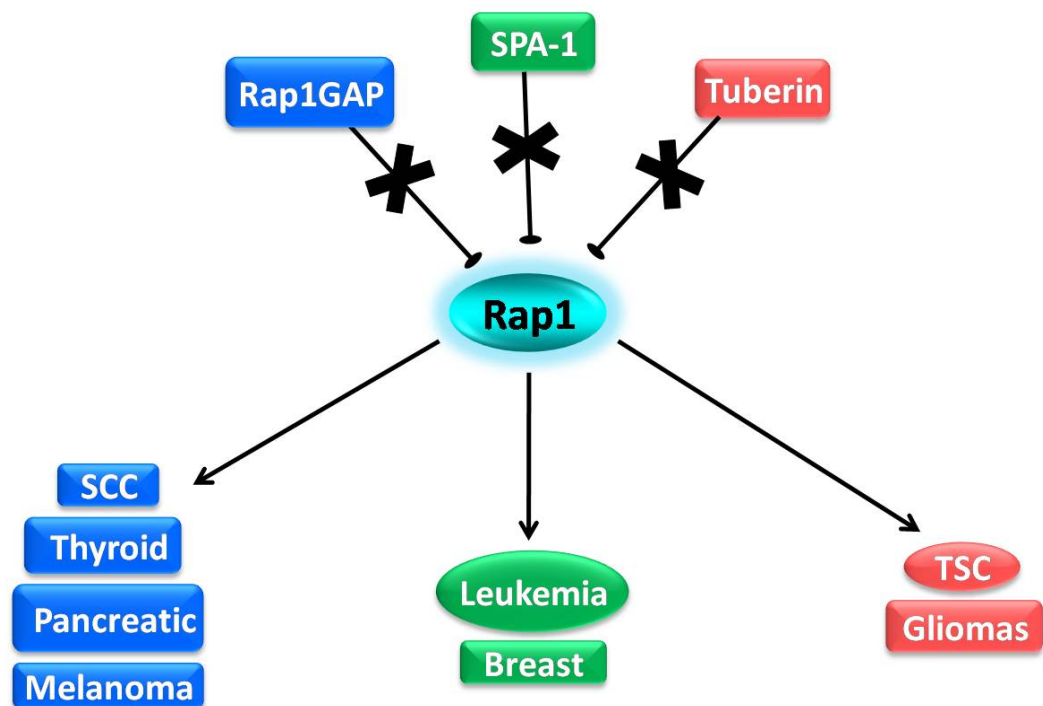


Figure 3: Rap1 signaling in Cancer

1.6 Rap1 signaling in pancreatic islet beta cell function

Most of the current implications of Rap1 in pancreatic islet β -cell function come from studies done on its RapGEF, Epac, and studies from our lab on Gz, a potential negative regulator of Rap1 activity. We have recently shown that Gz also negatively regulates glucose clearance

and insulin secretion in β -cells; these papers will be highlighted in the appendix of this thesis (98, 99). Several of the effects mediated by Epac in β -cells have either not examined Rap1 involvement or have been determined to be Rap1-independent (100).

1.6.1 Rap1 signaling in beta cell insulin secretion and exocytosis

As a compensation for increased blood glucose levels and peripheral insulin resistance often observed during overnutrition, the pancreatic islets of Langerhans, containing mostly insulin-secreting β -cells, begin to increase in size and insulin secreting capacity. Eventually, in a subset of insulin-resistant individuals, β -cell fatigue occurs, causing the loss of β -cell mass and insulin secretory capacity, a state referred to as β -cell dysfunction. The insufficient level of plasma insulin to regulate blood glucose levels results in type 2 diabetes, formerly known as adult-onset diabetes, but which is increasingly being seen in children and adolescents due to increasing population obesity.

As a potential treatment for patients with type 2 diabetes, glucagon like peptide-1 (GLP-1) analogs have been shown to stimulate insulin secretion, thereby lowering fasting blood glucose levels and reducing the elevation of blood glucose after eating (101). GLP-1 is a receptor-mediated activator of cAMP and an important regulator of β -cell signaling. Recently, it has been shown that GLP-1 can act through PKA and Epac to elicit β -cell signaling. In particular, Epac has been shown to be essential for GLP-1-mediated insulin secretion (100). Another important regulator of insulin secretion is glucose-dependent insulinotropic polypeptide (GIP), a gastrointestinal hormone. Like GLP-1, GIP stimulates insulin secretion from the pancreatic β -cell through GPCR activation of adenylyl cyclase and cAMP. GIP has been shown to regulate Erk through Rap1 in β -cells (102). Most recently, it has been directly demonstrated that Rap1 can

be activated by both of these incretins, GLP-1 and GIP, as well as cAMP in β -cells (103). Additionally, Shibasaki *et al.* went on to show that cAMP activation of Rap1 was mediated through Epac2 and was independent of PKA. In insulinoma cells and in islets, inhibition of Rap1 reduced cAMP (and glucose)-stimulated insulin secretion (103).

The mechanism by which Rap1 impacts insulin secretion may be through an impact on the exocytosis of insulin granules. It was demonstrated in Epac2^{-/-} mice that Epac2 regulated primarily the first phase of cAMP-potentiated insulin granule exocytosis, by increasing the number of insulin granules that were docked and/or primed to the β cell plasma membrane, ready for immediate fusion upon stimulation of secretion (103). Interestingly, cAMP activation of Rap1 can occur at low glucose concentrations, yet Rap1-mediated insulin granule fusion events do not occur at low glucose concentrations. Thus, it appears that Epac2/Rap1 does not trigger the exocytosis of insulin granules, but Shibasaki *et al.* hypothesize Epac2/Rap1 may facilitate insulin granule recruitment to the membrane or increase the pool of non-membrane docked granules (103). Additionally, it has been demonstrated that Epac is essential for GLP-1-mediated calcium (Ca^{2+}) signaling; as mentioned earlier, GLP-1 can activate Rap1 through Epac. In a cAMP-dependent and PKA-independent manner, Epac is responsible for fast Ca^{2+} -dependent exocytosis and movement of calcium from intracellular Ca^{2+} -stores, termed calcium induced calcium release (CICR). In addition to cAMP- or Ca^{2+} -stimulated secretory granule exocytosis in β -cells, Epac-Rap1 mediated CICR may be important in stimulating ATP production of the mitochondria (100). Rap1 may also be involved in the Epac-stimulated exocytosis in other secretory cells, like neurons, discussed later (104).

1.6.2 Rap1 interaction with the heterotrimeric G protein G α z

An interesting feature of several splice variants of both Rap1GAP1 and Rap1GAP2 is an N-terminal extension that is able to bind the G α subunit of heterotrimeric G-proteins. Specifically, Rap1GAPs have been shown to bind G α o, G α i, and/or G α z to promote Rap1GAP recruitment to the membrane and attenuation of Rap1 activation (105-107). However, there are differential effects from G α binding, as binding of G α o appears to inhibit GAP activity by recruiting Rap1GAP away from Rap1 (105). On the other hand, Rap1GAP binding to G α z forms a membrane-localized G α z-Rap1GAP-Rap1 tertiary complex that attenuates NGF stimulation of Rap1-Braf-Erk to limit PC12 cell differentiation (106). There are other important biologic functions of G α z that may also be Rap1-dependent.

A unique member of the Gi family of inhibitory heterotrimeric G proteins, Gz, is the only member of the Gi family that is insensitive to the inactivation by pertussis toxin (PTX) catalyzed ADP-ribosylation, due to the lack of an otherwise conserved C-terminal cysteine. Furthermore, unlike the ubiquitous expression seen for most Gi family members, the expression of Gz is limited mostly to neurons, some endocrine tissues, and platelets. This limited distribution highlights the potential therapeutic utility of Gz as a drug target. Common to other Gi family members, Gz inhibits adenylyl cyclase and consequently reduces cAMP levels. We have previously shown that Gz can specifically recruit Rap1GAP, the major GTPase activating protein for Rap1, to the membrane, where it inactivates Rap1, preventing its downstream signaling (106, 107). Additionally, we have found a role for Gz in pancreatic islet β -cell biology as a regulator of insulin secretion and glucose clearance; this will be discussed in the appendix in more detail (98, 99).

1.7 Other biological functions of Rap1 signaling

Several studies have examined the role of Rap1 in neurons and regulation of synaptic function (14). Rap1 has been shown, through calcium- and cAMP-mediated PKA activation, to signal to Erk for the release of intracellular calcium in neuronal cells and striatal neurons (108, 109). Additionally, NGF stimulation of Rap1 activates Erk, leading to neuronal differentiation and neurite outgrowth (65). Rap1 activation by the cannabinoid receptor (CB1) can also stimulate neurite outgrowth (110). Several RapGAPs have also been identified or studied in the nervous system. For example, SPA-Ls was identified in neuronal synaptic vesicles (29). The RapGAP, E6TP1 (SPAR), along with Rap2, can regulate the formation of dendritic spines in neurons (28).

Many important studies on the function of Rap1 originated from work in hematopoietic cells like platelets, leukocytes, and lymphocytes. In platelets, the major Rap isoform is Rap1B, and it has been shown to be essential for platelet adhesion, aggregation, and spreading (15). The mechanism of Rap1 function in platelets appears to be mainly through CalDAG-GEF stimulation of Rap1 to regulate integrins (α IIb β 3), rather than Erk regulation. In addition to the already mentioned implication of Rap1 in growth/Erk activation of leukocytes in leukemias, Rap1 is also involved in leukocyte adhesion. Studies from leukocytes have identified several cytokines (IL-3, thrombopoietin, erythropoietin, and SDF-1) that activate Rap1 and promote its enhancement of integrin (β 1) adhesion (15, 35). Interestingly, in T lymphocytes, Rap1 appears to inhibit Erk, likely because peripheral T cells are thought not to express B-Raf (62). As demonstrated in Spa1^{-/-} mice, Rap1 inhibition results in growth inhibition of T cells. However, Rap1 does enhance adhesion of T-cells through several integrins: α L β 2, α 4 β 1, and α 5 β 1 (15). Rap1-mediated integrin activation promotes T cell migration and antigen-dependent T cell

activation. Interestingly, in response to antigen, Rap1 can also increase T cell proliferation and IL-2 production (111). There is also a role for Rap1b in B cell development and homeostasis, as Rap1b^{-/-} mice display reduced trafficking of mature B cells and reduced T-dependent humoral immune response (112). Here I have introduced a mere sampling of the many diverse functions of the G protein Rap1 in the forest of signaling.

In this thesis, I will focus on the functions of the monomeric G protein Rap1 in the regulation of two particular cellular biologies. First, in Chapters 3 and 4, I analyze the role of activation of Rap1 in prostate cancer and breast cancer metastasis. In Chapter 5, I examine the role of Rap1 activation in pancreatic islet β -cell function. Finally, in the Appendix, I explore my contributions to the elucidation of the role of the heterotrimeric G protein, G α z, in the regulation of pancreatic islet β -cell function and glucose homeostasis. Together, the data presented in this thesis describe the important and diverse functions of both monomeric and heterotrimeric G proteins in cancer metastasis and diabetes.

2. Experimental Methods

Cell Lines. Prostate Cancer Cell Lines (Chapter 3)—The PC3-M, PC3, DU145, LNCaP, and LNCaP-c81 cell lines were obtained from the Duke University Medical Center Cell Culture Facility or American Type Culture Collection. The VCaP cells were from Donald McDonnell (Duke Univ.). The PC3 line was maintained in F-12K Kaignh's modified medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) from Hyclone (Thermo Fisher Scientific Inc., Waltham, MA). The PC3-M and DU145 cell lines were maintained in RPMI (Mediatech, Herndon, VA) supplemented with 10% FBS. The LNCaP and LNCaP-c81 cell lines were maintained in RPMI (Mediatech) supplemented with 10% FBS, 10mM HEPES, 1mM sodium pyruvate, and 4.5 g/L glucose. The VCaP line was maintained in DMEM with 10% FBS, non-essential amino acids, and 1mM sodium pyruvate. All cells were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO₂. Cells were passaged 1:4 every 3 days or at 80-90% confluency. Prior to the indicated assays, cells were starved with Starvation medium: DMEM supplemented with 0.1% fatty acid-free BSA, and 10 mM HEPES. **Breast Cancer Cell Lines (Chapter 4)**—MDA-MB-231 cells were obtained from the American Type Culture Collection and grown under the recommended conditions. ***β*-cell Lines (Chapter 5)**—The Ins-1-and Ins-1-derived glucose-responsive 832/13 insulinoma line (gifts of Christopher Newgard, Duke University Medical Center) were maintained in complete RPMI-1640 medium (11.1 mM glucose) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol at 37 °C in a humidified atmosphere containing 95% air and 5% CO₂. Cells were passaged 1:4 every 3 days.

Reagents (Chapters 3, 4, and 5). For immunoblot analysis the antibodies employed were: to Rap1 (Santa Cruz Biotechnology, Santa Cruz, CA), to Rap1GAP (EMD/Merck KGaA, San Diego, CA), and to Actin (Sigma-Aldrich, St. Louis, MO). The monoclonal anti-HA and anti-myc antibodies were obtained from Zymed Laboratories (San Francisco, CA). Phospho-ERK (T202/Y205), phospho-Akt (S473), phospho-S6 ribosomal protein (S235/236) and S6 ribosomal protein (S6) antibodies were from Cell Signaling (Beverly, MA), and total ERK and Akt antibodies were from BD Pharmingen. LI-COR Odyssey[®]-compatible secondary antibodies were from Rockland (Gilbertsville, PA) and Molecular Probes (Eugene, OR). The function-blocking monoclonal antibodies for integrins $\alpha 4$, $\beta 3$, $\alpha \nu \beta 3$ were from Cell Signaling (Beverly, MA), and blocking antibody to integrin $\alpha 2 \beta 1$ was from Abcam Inc. (Cambridge MA). MMP inhibitor GM6001 was from Sigma. Growth Factor-Reduced Matrigel was from BD Biosciences (Beverly, MA), SDF-1 α was from R&D Systems, Inc. (Minneapolis, MN), and D-Luciferin was from Xenogen Corp. (Caliper Life Sciences, Hopkinton, MA). 8-CPT-cAMP, 8-CPT-6Me-cAMP, and GLP-1 were from Biomol (Plymouth Meeting, PA). H89, the membrane-permeable peptide PKA inhibitor, EGF, LY29609 and rapamycin were from Calbiochem (La Jolla, CA).

Immunoblot analysis (Chapters 3, 4, and 5). Cells, grown to ~80-90% confluency, were washed once with cold PBS, and lysed with cold Lysis Buffer (described in the Rap1 Activity Assay). Lysates were cleared by centrifugation at 14,000 x *g* for 10 min at 4 °C. Supernatants were assayed for protein concentration (Bio-Rad, Hercules, CA), and then equal amounts of total protein from lysates were separated by SDS-PAGE and subjected to immunoblot analysis according to standard methods. Western blotting was performed using the Odyssey System (LICOR, Lincoln, Nebraska) according to the manufacturer's instructions.

Preparation of GST fusion proteins (Chapters 3, 4, and 5). The GST-RalGDS protein was produced in the BL21DE3 strain of *E. coli* (Novagen/Merck KGaA, San Diego, CA). Briefly, a transformed bacterial colony containing the GST-RalGDS construct was cultured to an optical density of 0.5-0.6, whereupon the cells were induced with 0.5 mM isopropyl-D-thiogalactopyranoside (Teknova) and cultures were grown for an additional 2.5 h at 37 °C. Cells were harvested by centrifugation for 15 min at 6,000 × g at 4 °C, and the resulting pellet resuspended in 2.5 ml buffer A [2.3 M sucrose, 50 mM Tris-HCl 7.7, 1 mM EDTA, and protease inhibitors mix followed by dilution with 10 ml buffer B (50 mM Tris-HCl 7.7, 10 mM KCl, 1 mM EDTA, 1 mM DTT, and 1:500 PI mix). Cells were disrupted and the lysates were cleared by centrifugation at 30,000 × g for 30 min, following which, the resulting supernatant was incubated for 2 h at 4°C with continuous rocking with Glutathione Sepharose 4B beads (Amersham Biosciences/GE Healthcare, Pittsburgh, PA) equilibrated in buffer B. Beads were washed three times in Buffer B by brief centrifugation and resuspension. Protein concentration was determined by Bradford assay. Fusion proteins were stored as bound to the beads at -80°C.

Rap1 Activity assays The levels of activated Rap1 were determined using pull-down assays with a GST fusion of the Ras-binding domain of RalGDS as previously described (113). Briefly cells, as indicated in the appropriate Figure legend, were allowed to grow until ~80% confluent and then starved for 18 h in starvation medium, described above. **(Chapters 3 and 4).** Cells stimulation was initiated by the addition of media containing 10% FBS or SDF-1 agonist for the time period noted in the appropriate Figure legend. Cells were washed once with ice-cold PBS, and lysed in a Lysis Buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM EDTA, 10 mM MgCl₂, 5% glycerol, 10 µg/ml leupeptin, and 10 µg/ml aprotinin).

Lysates were clarified by centrifugation, and the supernatants, adjusted to contain equal amounts protein and volume, were incubated with 50 μ g of GST-RalGDS-RBD and 40 μ l of a 50% slurry of Glutathione Sepharose 4B beads. This was mixed for 45 min at 4 $^{\circ}$ C, whereupon the beads were pelleted by centrifugation at 700 \times g and washed three times with cold lysis buffer. Pelleted material was resuspended in SDS-PAGE sample buffer, proteins separated by gel electrophoresis, and subjected to immunoblot analysis to detect Rap1. **(Chapter 5).** 4×10^6 Ins-1 cells were seeded in 10 cm dishes and allowed to grow for \sim 48 h. The cells were then infected with the indicated adenoviruses, and then starved for 18 h in starvation medium. Remaining assay performed as described above.

Silencing RNA interference studies (Chapter 3). The siRNA oligos were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) and based on a published Ambion sequence (84), Rap1A sense 5'-gcaggacagagcauuuucatt and antisense 5'-uaaaugcucuguccugcagtt (84). A random sequence that did not match any known sequence after National Center for Biotechnology Information Blast search was used as a control Scramble siRNA, sense 5'-uuccuuuuccguauucgcuutt and antisense 5'-cgcgaaucggaaaaggaugtt (84). siRNA against Rap1A was introduced into PC3 cells via transient transfection with Oligofectamine reagent (Invitrogen, Carlsbad, CA) for 72 hrs. Immunoblot analysis of total cell lysates was used to confirm the efficacy of knockdown of Rap1 expression in siRNA treated cells.

Retrovirus production (Chapters 3 and 4) Rap1-63E and Rap1GAP constructs were gifts of Lawrence Quilliam (University of Indiana Medical Center). Recombinant retroviruses were constructed by subcloning the Rap1-63E and Rap1GAP plasmids into the pMSCV-IRES -GFP vector (Clontech) and then cloning Luciferase into the pLPCX vector (Clontech). The DNA

constructs were co-transfected into the GP2-293 packaging line (Clontech) using FuGene (Roche Applied Science). Viral supernatants were collected 48 h later, clarified by filtration, and concentrated by ultracentrifugation. The concentrated virus was used to infect 1×10^6 cells in a 60-mm dish with 8 $\mu\text{g}/\text{ml}$ polybrene (Sigma). Stable cells were selected for GFP by FACS sorting and selected with 2 $\mu\text{g}/\text{ml}$ puromycin (Sigma).

Cell Migration assays (Chapters 3 and 4). For the migration assays, cells were added directly to transwell chamber filters (8- μm pore size, polycarbonate filter, 6.5-mm diameter; Costar) that had been washed with PBS. Cells were starved for 12-18 h in Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin, detached with Cellstripper™ (Mediatech), and 3×10^4 cells in 100 μl were placed into the upper chamber of the transwell. For experiments using SDF-1, the ligand (200ng/ml) was added to the cells after harvesting and for the duration of the experiment. The upper well of the transwell was then transferred into a well containing 600 μl of conditioned medium from MG-63 osteosarcoma cells (114). Cells were permitted to migrate for 4 h at 37 °C in a humidified incubator. Cells in the top well were removed with cotton swabs. The membranes were then stained using the Hema3 Staining Kit (Fisher Scientific), and the cells on the underside of the membrane were counted using a Nikon TS100 microscope. Four representative high-powered fields were selected and counted for each membrane.

Cell Invasion assay (Chapters 3 and 4). Invasion assays were performed similarly to the migration assays above, with a few modifications. For the invasion assays, cells were added to transwells previously coated with 50 μg of Growth Factor-Reduced Matrigel™ (BD Biosciences) for 4h. Following addition of the cells, the transwell was transferred into a well containing 600 μl

of growth medium containing 10% FBS. Cells were incubated for 24 h at 37 °C in a humidified incubator and cells in the top well were removed with cotton swabs. The transwell membranes were then stained and analyzed as described above in the Migration assay.

Real-Time PCR. The RT² Profiler™ PCR Arrays were obtained from SA Biosciences (PAHS-013). These 96-well array plates contained primers for 84 cell adhesion molecules and extracellular matrix genes, as well as the appropriate control genes. RNA from PC3 cells expressing activated Rap1-63E or Vector was isolated using a RT² qPCR-Grade™ RNA Isolation Kit (PA-001) according to manufacturer instructions. The RNA was converted into first strand cDNA with the RT² First Stand Kit. The cDNA of the individual cells were then analyzed on their respective RT²-PCR plates. RT-PCR experiments were performed on a BioRad iQ Real-Time PCR Cycler and analyzed according to manufacturer instructions. The gene expression results from the two cell lines were compared and are represented in the table as fold expression.

In Vivo Metastasis. All animal handling and procedures were conducted in accordance with an approved protocol by the Duke University Medical Center Institutional Animal Care and Use Committee and the NIH Guide for the Care and Use of Laboratory Animals. **(Chapter 3)** PC3 cells expressing firefly luciferase (PC3-Luc) were generated by retroviral transduction. Under an anesthesia mixture of (100mg/kg) ketamine and (5mg/kg) xylazine, 4-6week-old male athymic NCr nu/nu mice (NCI-Frederick) were injected with 1×10^5 PC3-Luc cells into their left cardiac ventricle. The intracardiac injections were performed as described by Guise *et al.* (115).

(Chapter 4) MB231 cells expressing firefly luciferase (MB231-Luc) were generated by retroviral transduction. 4-6week-old female athymic NCr nu/nu mice (NCI-Frederick) were injected with 1×10^5 231-Luc cells into their tail vein.

Bioluminescence Imaging (Chapters 3 and 4). Bioluminescence imaging was performed on all of the mice at 4-7-day intervals. This technique was performed as described previously by Kelly *et al.* (6). Briefly, mice were anesthetized by inhaling 1-3% isoflurane/air and were given 100 μ l of 15 mg/ml d-luciferin in PBS by i.p. injection. Ten minutes after injection, bioluminescence was imaged with a charge-coupled device camera (IVIS, Xenogen Corp/Caliper Life Sciences, Hopkinton, MA). Bioluminescence images were obtained with a 15-cm field of view, binning (resolution) factor of 8, 1/f stop, open filter, and with an imaging time of 30-60 sec. Bioluminescence from relative optical intensity was defined manually, and data were expressed as photon flux (photons/s per cm²/steradian). Background photon flux was defined from a relative optical intensity drawn over a mouse that was not given an injection of luciferin. Data was acquired and analyzed on the IVIS system coupled PC running IVIS Living Image software (Xenogen Corp/Caliper Life Sciences, Hopkinton, MA).

Proliferation assays (Chapter 4). MB231 cells stably expressing activated Rap1 (Rap1-63E), Rap1GAP, or Vector were plated in 96-well plates. Every 24hrs MTS reagent (CellTiter 96™ AQueous Non-Radioactive Cell Proliferation Assay, Promega) was added to triplicate wells according to manufacturer instructions, and the conversion of tetrazolium salt to a colored formazan product was measured colorimetrically at OD₄₉₀ as an indicator of living proliferating cells.

Plasmids and Constructs (Chapter 5). Rap1 (63E) and Rap1GAP constructs were gifts of Lawrence Quilliam (University of Indiana Medical Center). pCDNA3.1 plasmids containing Rap1 (wt), H-Ras (T19N), Rac1 (G12V), Rac1 (T19N), and myc-CDC42 (G12V) were obtained from the

UMR cDNA Resource (Rolla, Mo). The Adtrack-CMV vector was a gift of Bert Vogelstein (Johns Hopkins University Medical Center).

Adenoviral Infections (Chapter 5). Recombinant adenoviruses were constructed by subcloning human constructs into the Adtrack-CMV vector, and then recombining these with pAdEasy-1 in BJ5183 *E. coli* (Stratagene). The resulting DNA was transfected into HEK 293 cells with LipofectAMINE (Invitrogen). Next, the viruses were serially amplified and purified using Adeno-X™ Virus Purification Kits (BD Biosciences). Ins-1 cells were infected at multiplicity of infection (MOI) of 10 for 2 h at 37 °C.

Phospho-protein immunoblot analysis (Chapter 5). 1×10^6 Ins-1 cells were plated in 6-well plates and allowed to grow for two days. The cells were then infected with the indicated adenovirus as described above, and then starved for 18 h in starvation medium (RPMI-1640 medium (0.5 mM glucose), supplemented with 0.1% fatty acid-free BSA, 10 mM HEPES, 10 mM sodium pyruvate and 50 μ M β -mercaptoethanol). Cells were then stimulated with the indicated agonists washed once with ice-cold PBS, and lysed with ice-cold RIPA buffer (50 mM Tris, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 50 mM NaF, 150 mM NaCl, 2 mM DTT, 0.2 mM activated sodium vanadate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 3 μ g/ml leupeptin, 4 μ g/ml aprotinin, 30 μ M TPCK, 29 μ M TLCK, and 133 mM phenylmethylsulfonyl fluoride). Lysates were cleared by centrifugation at 16,000 $\times g$ for 10 min at 4 °C. Supernatants were assayed for protein concentration, and then equal amounts of total protein from lysates were separated by SDS-PAGE and subjected to immunoblot analysis according to standard methods.

Rat islet isolation (Chapter 5). As described previously (116), pancreatic islets were harvested from male Sprague-Dawley rats weighing approximately 250 g, under a protocol approved by the Duke University Institutional Animal Care and Use Committee. Approximately 200 rat islets per condition were cultured in 2 ml of RPMI medium (10% fetal calf serum, 8 mM glucose) and treated with adenoviruses at a concentration of 5×10^9 particles/ml medium for 18 h. Virus-containing medium was replaced with fresh culture medium, and islets were cultured for various times after treatment, as indicated in the figure legends, with fresh medium daily.

Microscopy (Chapter 5). Pools of ~ 200 rat islets were treated with various recombinant adenoviruses, treated with and without rapamycin, and groups of 5-10 islets per condition were picked. The islets were fixed with 2% paraformaldehyde and placed in warm agarose on tissue culture dishes. Fixed islet cell nuclei were Hoechst stained. The entire islet was analyzed and z-stack images were captured using a SP5 inverted confocal microscope (Leica) at the Duke University light microscopy core facility (supported by Sam Johnson). Three random slices (z-stack) per islet were chosen for analysis. The number of nuclei in a measured area was counted using Metamorph software, data represents the average nuclei counted from 4 areas/ image slice with 3 image slices/islet for 4-6 islets/group.

Insulin Secretion Assay (Chapter 5). The day before the assay pools of ~ 200 human or rat islets were treated with various recombinant adenoviruses, treated with and without rapamycin, and groups of 30 islets per condition were picked in triplicate. On the day of the assay, islets were preincubated with 2.8 mM glucose-containing Krebs Ringer Bicarbonate Buffer (KRBB: 4.38 mM KCl, 1.2 mM $MgSO_4$, 1.5 mM KH_2PO_4 , 129 mM NaCl, 5 mM $NaHCO_3$, 10 mM HEPES, 3.11 mM $CaCl_2$, 0.25% BSA, pH 7.4) for 2 h. Next, 1 ml of KRBB containing the desired

concentration of glucose was added for an additional 2 h. Following stimulation, the secretion media was removed and assayed for insulin levels (Coat-A-Count® [¹²⁵I]-Insulin radioimmunoassay, Diagnostic Products Corp., Los Angeles, CA). The remaining islets were washed once in PBS and incubated with a 150:47:3 ethanol:water:1.5% HCl insulin extraction buffer overnight at -20 °C, and the amount of secreted insulin was normalized to the total protein of the extract as measured by Bradford.

Proliferation Assays (Chapter 5) DNA synthesis rates were measured as described previously (117), briefly, [Methyl-³H]thymidine was added at a final concentration of 1 μCi/ml to pools of ~200 islets during the last 18 h of cell culture. Groups of 25 islets were picked in triplicate, washed, and centrifuged twice at 300 x g for 3 min at 4 C. DNA was precipitated with 500 μl cold 10% trichloroacetic acid (TCA) for 30 min and then solubilized and resuspended by addition of 80-100 μl 0.3 N NaOH. The amount of [³H]thymidine incorporated into DNA was measured by liquid scintillation counting and normalized to total cellular protein.

Statistical Analysis (Chapters 3, 4, and 5). Data were analyzed using GraphPad Prism v5 (GraphPad Software Inc., San Diego, CA). Data are given as mean ± SEM and compared by one- or two-way ANOVA as appropriate, followed by the relevant post-test to determine p values. A probability of p < 0.05 was considered significant.

3. Activation of Rap1 promotes prostate cancer metastasis

3.1 Introduction

In the United States, the second leading cause of cancer deaths in men is carcinoma of the prostate (CaP). Most of these deaths are caused by metastatic spread of the CaP to the bone. Much remains to be learned, however, about inhibiting the process of CaP metastasis. CaP metastasis is complex series of events, which include aberrant proliferation of CaP cells, remodeling of their environment, migration from their primary tumor, survival in circulation, attachment and migration to the metastatic site, and proliferation as micrometastases (118). During several steps in metastasis, cancer cell affinity and avidity for extracellular matrix (ECM) components is altered. Most of these changes are mediated by enhancing the expression or activation of integrins, a family of heterodimeric receptors for cell adhesion molecules that are crucial for cell behavior and motility (119). In prostate cancer, there are dramatic differences between the distribution and surface expression of integrins in pre-neoplastic and malignant tumor cells (120). Therefore, an attractive approach to CaP therapy is to target cells by antagonizing integrin function (121).

Integrin affinity and avidity is regulated by two different signaling methods (i) outside-in signaling from integrin interaction with the extracellular environment, and (ii) inside-out signaling from intracellular pathways involving monomeric G proteins such as Rap1(119). Rap1 is a member of the Ras family of monomeric G proteins that has received recognition for its role in tumorigenesis and cancer progression (122). Studies originating in leukocytes first demonstrated that Rap1 can enhance cell adhesion and migration and activate survival pathways (35, 38). GTPases like Rap1 are molecular switches regulated positively by guanine nucleotide exchange

factors (GEFs) and negatively by GTPase activating proteins (GAPs). Recent *in vivo* and *in vitro* data from several mouse models and several cancers provide evidence that aberrant Rap1 activation contributes to several malignancies (62). In cancer phenotypes attributed to increased Rap1 activity, one common thread seems to be a decrease in expression of a RapGAP by deletion or mutation at the location of the *Rap1GAP* or *Sipa1* gene (22, 25, 84, 123). In pancreatic cancer, melanoma, and chronic myelogenous leukemia there is a consensus that aberrant Rap1 activation leads to increased cancer cell proliferation and tumorigenesis (22, 25, 84). The consequence of Rap1 activation on invasion, however, seems to have a very cell and/or environment-specific context. In osteosarcoma cells, inactivation of DOCK4, a Rap1 activator, causes a reduction in Rap1 activation and adherens junction formation, which allows the cells to become more invasive (20). Additionally, Rap1GAP expression has been correlated with increased *in vitro* invasion of squamous cell carcinoma SCC (91). On the other hand, Rap1GAP overexpression led to a reduction in pancreatic cancer incidence and local invasion from the primary tumor site (22). Thus, it remains uncertain when and in which cancers Rap1 signaling positively or negatively regulates invasion and metastasis.

The differential roles of Rap1 require that its impact be addressed in different malignancies, and thus far the importance of Rap1 in CaP metastasis had not been examined. In this study, we found CaP cell lines with high metastatic potential had increased Rap1 activity levels and decreased Rap1GAP expression. Activation of Rap1 in CaP cells enhanced their migration and invasion via a process that involves $\alpha v\beta 3$, $\beta 3$ and $\alpha 4$ integrins. Additionally, activated Rap1 dramatically enhanced the rate and incidence of CaP metastasis *in vivo*. These

findings demonstrate a role for Rap1 in CaP metastasis and suggest a novel, targeted therapeutic strategy for prostate cancer.

3.2 Results

3.2.1 Rap1 activity is increased in progressively metastatic human prostate cancer cell lines

Anomalous Rap1 signaling has been reported in some types of cancers (122). This prompted our assessment of Rap1 activation status in prostate cancer. Since analyzing Rap1 activation status requires the use of fresh samples, it was necessary to do the study with prostate cancer cell lines in culture. For these studies, we chose a spectrum of established human prostate adenocarcinoma cell lines of varying invasiveness and metastatic potential (124). Two major criteria were used; first, the expression status of the androgen receptor, since expression of this receptor is lost in most human metastatic CaP, and second the ability or rate of tumor formation in mouse xenografts, if such data existed. The well-characterized RalGDS pull-down assay was used to directly examine levels of activated (GTP-liganded) Rap1. Additionally, immunoblot analysis was performed to assess levels of Rap1GAP, the negative regulator of Rap1, in the cell lines. The results of these analyses, shown in Fig. 4, demonstrated the general trend that the more invasive, androgen-independent cell lines DU-145, PC3, and PC3-M, had higher levels of Rap1-GTP and lower levels of Rap1GAP when compared to the less metastatic, androgen-sensitive, cell lines VCaP, LNCaP and LNCaP-c81 cells. Furthermore, this trend of decreased Rap1GAP expression and increased Rap1-GTP levels was observed in direct comparison of the LNCaP and PC3 cell lines with their more invasive derivative lines, LNCaP-c81 and PC3-M, respectively (Fig 4, Lanes 2-3 and 5-6). The PC3 cell line was chosen for the subsequent studies, as these cells have been widely used in similar *in vitro* and *in vivo* assays.

Furthermore, it was hoped that their moderate metastatic potential would allow assessment of both increasing and decreasing Rap1 activity on cellular functions related to metastasis.

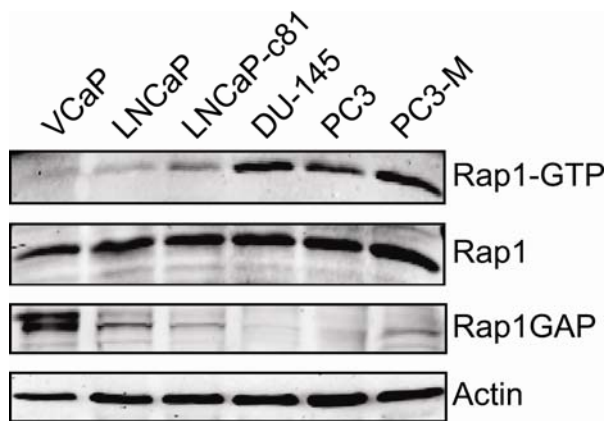


Figure 4: Rap1 activity is increased in progressively metastatic human prostate cancer cell lines. Top panel: Cell lysates (roughly in order of increasing metastatic potential, from left to right) were analyzed in a pull-down assay using a GST fusion of the activated Rap1-binding domain of RalGDS. The levels of precipitated Rap1 were determined by immunoblot analysis using an anti-Rap1 antibody. Levels of total Rap1, Rap1GAP, and actin (loading control) were also determined as shown. Data shown is from a single experiment that is representative of three separate experiments. All blots were cropped to display the relevant bands

3.2.2 Modulation of Rap1 activity impacts CaP cell migration and invasion

The finding that increased Rap1 activity correlated with more metastatic CaP cells prompted an evaluation of the impact of Rap1 activation on CaP cell metastatic behavior. Given that the classic metastatic site of prostate cancer is bone, we used conditioned media from bone-derived osteosarcoma cells (MG63) as the source of chemoattractant in the migration assays to mimic this bone microenvironment (114). We have confirmed this conditioned media contains ECM proteins, specifically vitronectin (integrin $\alpha v \beta 3$, and $\beta 3$ ligand) and fibronectin (integrin $\alpha 4 \beta 1$ ligand) (data not shown). Additionally, invasion assays through Matrigel™ were performed to more accurately model an environment with collagen, extracellular matrix proteins, and growth factors that are usually present in the metastatic microenvironment.

To assess the requirement for Rap1 function in PC3 cell migration a gain-of-function approach was utilized overexpressing Rap1-63E, a mutationally activated form of Rap1 which renders Rap1 in a GTP-bound active state in the cells. Retrovirus was generated to stably express Rap1-63E in PC3 cells and overexpression of Rap1-63E was verified by immunoblot analysis of protein levels. Comparison with control cells demonstrated that the PC3-Rap1-63E cells indeed had high levels of activated Rap1 (Fig 5A). Analysis of the migration and invasion properties of the PC3 cells revealed that overexpression of Rap1-63E increased these properties by 2.3 ± 0.52 -fold and 2.2 ± 0.08 -fold, respectively (Fig 5B).

Having determined that gain of function of Rap1 enhanced CaP cell migration and invasiveness, we asked whether loss of function could inhibit these properties of the PC3 cells. Again, a retrovirus approach was taken, this time to stably express Rap1GAP. Rap1GAP inhibits Rap1 signaling by stimulating the ability of Rap1 to hydrolyze GTP into GDP and thus maintains Rap1 in its inactive GDP-bound state. Comparison with vector control cells demonstrated that PC3-Rap1GAP-expressing cells indeed had reduced levels of Rap1-GTP (Fig 5A) and the migration and invasion assays performed in the cells demonstrated that Rap1GAP reduced PC3 cell migration and invasion by $47 \pm 0.08\%$ and $71 \pm 0.07\%$, respectively, as compared to vector control cells (Fig. 25). A second loss-of-function study utilizing RNAi-mediated knockdown of Rap1 expression was also employed. Transfection of PC3 cells with siRNA directed against Rap1A resulted in a marked reduction of Rap1 protein expression as assessed by immunoblot analysis (Fig 5C). Similar to Rap1GAP-expressing cells, knockdown of Rap1A expression significantly reduced both migration and invasion of PC3 cells compared to control cells treated with scrambled RNA oligos ($53 \pm 4.2\%$ and $67 \pm 2.7\%$, respectively, $p < 0.001$; Fig 5D). In an attempt to

further elucidate which Rap isoform was most important for PC3 cell migration and invasion. We also separately targeted Rap1B and Rap2 isoforms using specific RNAi constructs. These results were variable, but in all cases targeting Rap1A expression was most effective in impacting these cellular processes (data not shown). Together with the finding that expression of the activated form of Rap1A (Rap1A-63E) was sufficient to significantly enhance CaP migration and invasion (Fig 5B), these data suggest that Rap1A is the primary subtype responsible for these effects. Taken together, the results of the loss- and gain-of-function studies provide compelling evidence that Rap1A function plays an important role in CaP cell migration and invasion, and that activation of Rap1 enhances CaP cell migration and invasion.

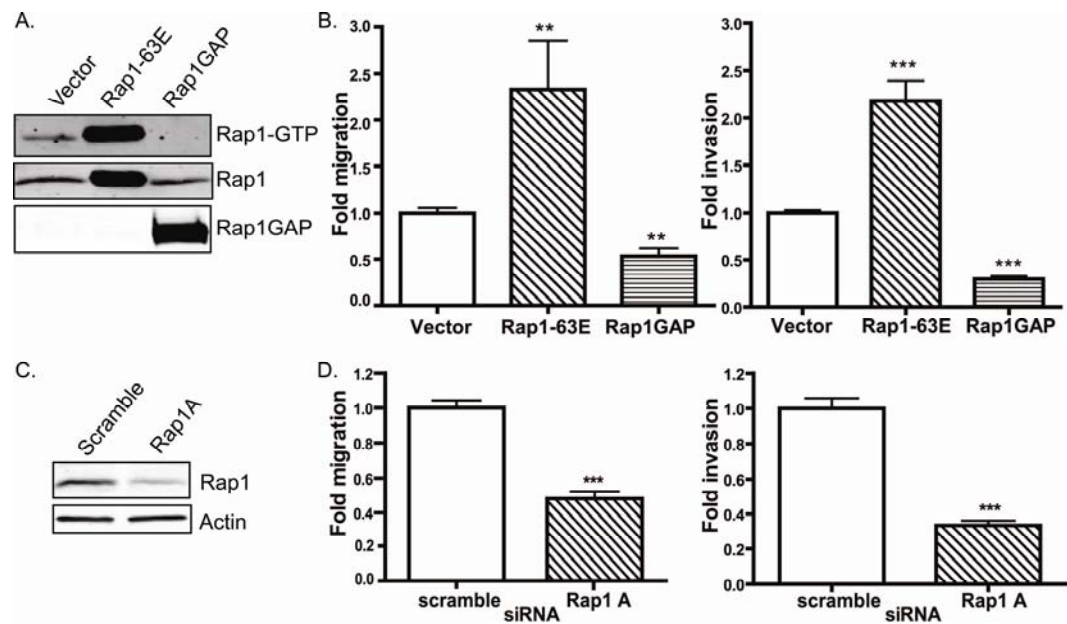


Figure 5: Modulation of Rap1 activity impacts CaP cell migration and invasion. Panels A and B. PC3 cells were transduced with retrovirus to establish stable expression of the indicated constructs--vector, activated Rap1 (Rap1-63E), or Rap1GAP. A, PC3 cells stably expressing the indicated constructs were lysed and levels of Rap1-GTP, total Rap1 and Rap1GAP were determined by immunoblot analysis (in the case of Rap1-GTP, analysis was performed following RalGDS pulldown; see *Materials and Methods* for details). B, Migration (left panel) and invasion (right panel) of PC3 cells stably expressing the indicated constructs described in panel A. For the migration assays, cells migrated toward a lower chamber containing conditioned media from

osteosarcoma cells and were assessed after 4 h. For the invasion assays, cells invaded through a Matrigel layer toward a lower chamber containing 10% serum and were assessed after 24 h. In both assays, the transwell filter membranes were fixed and stained to quantify the number of cells on the lower surface of the transwell filter. Panels C and D. siRNA oligonucleotides targeting Rap1A and a sequence-scrambled control were introduced into PC3 cells by transfection. Cells were assayed at ~80% confluence following 72 h of siRNA treatment, as described in *Materials and Methods*. C, Levels of Rap1 and actin (loading control) were determined by immunoblot analysis following introduction of the indicated siRNA. D, Migration (left panel) and invasion (right panel) assays of siRNA treated PC3 cells. The data represents that pooled from 3 (B) or 2 (D) independent experiments with at least 2 experimental replicates each, and is plotted as the ratio of the number of migrated or invaded cells vs. that of control cells (B, vector; D, scrambled siRNA) that were determined in parallel. ***, $p < 0.001$; **, $p < 0.005$

3.2.3 SDF-1 stimulation of CaP cell migration and invasion requires Rap1 function

Having determined that Rap1 was an important player in PC3 cell migration and invasion, we were keen to explore its potential role in an endogenous signaling process known to be involved in cancer metastasis. To this end, we stimulated Rap1 signaling by treatment of PC3 cells with SDF-1. Often present in the bone microenvironment, SDF-1 is a chemokine that has been shown to be upregulated in and associated with CaP progression (125). Indeed, when PC3 cells were treated with SDF-1, the level of activated Rap1 was markedly increased (Fig 6A). Furthermore, the ability of SDF-1 to elicit Rap1 activation was completely blocked in the PC3 cells overexpressing Rap1GAP (Fig 6A). Treatment of PC3 cells with SDF-1 stimulated cell migration (Fig 6B) and invasion (Fig 6C) by 1.4 ± 0.19 -fold and 1.7 ± 0.22 -fold, respectively, and this stimulation was eliminated in PC3-Rap1GAP cells. Similar to the untreated Rap1GAP cells, and cells with reduced Rap1A expression, SDF-1 treatment of these cells gave no stimulatory effect and they still maintained a reduced capacity to migrate and invade. Specifically, in both treated and untreated Rap1GAP cells, a ~50% reduction in migration (Fig 6B) and a ~60% reduction in

invasion (Fig 6C) were observed compared to untreated vector control cells. Additionally siRNA-mediated knockdown of Rap1A in both SDF-1 treated and untreated PC3 cells resulted in a ~50% reduction in invasion compared to control cells (Fig 6D). These results clearly demonstrate that SDF-1 stimulates Rap1 activation in CaP cells and that Rap1 function is required for SDF-1 stimulated CaP cell migration and invasion.

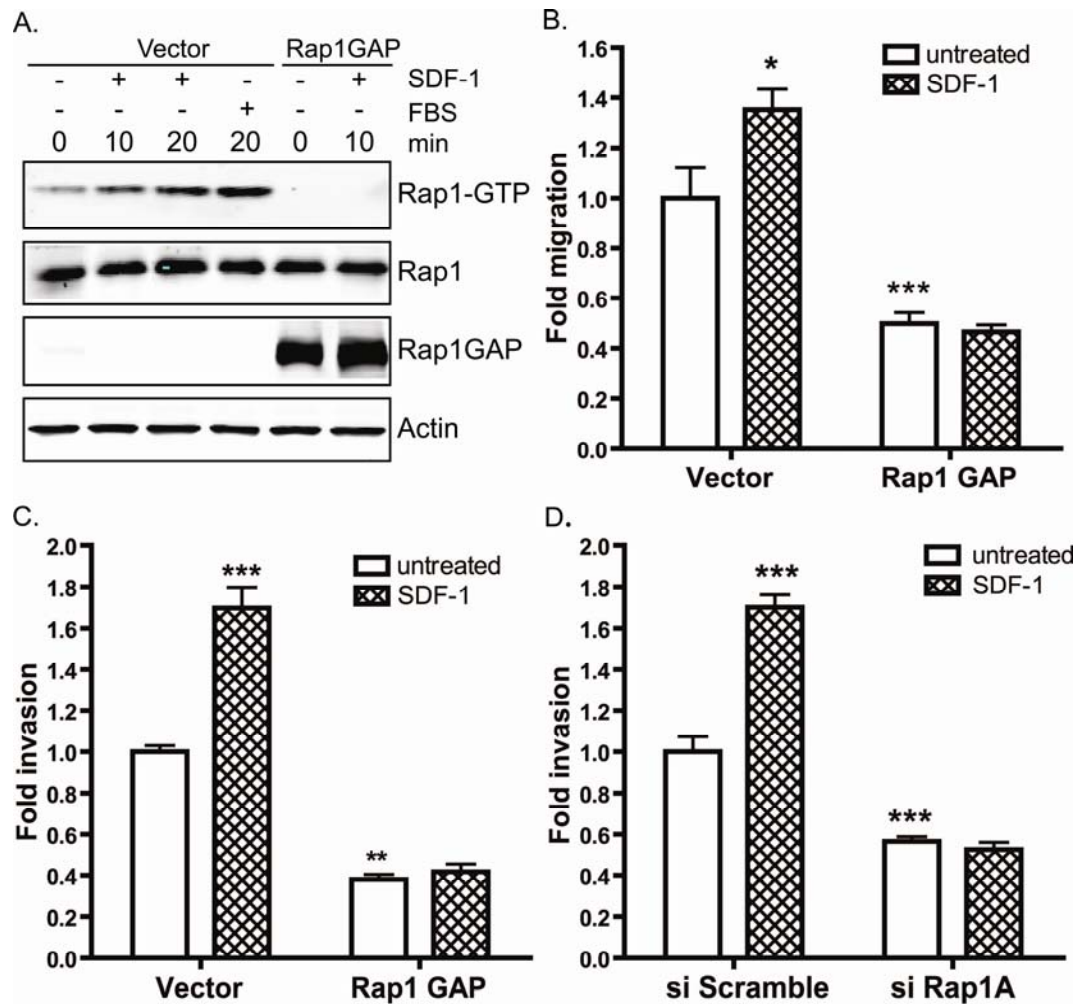


Figure 6: SDF-1 stimulation of CaP cell migration and invasion requires Rap1 function. A, PC3 cells stably expressing vector control or Rap1GAP were treated with SDF-1 ligand (200 ng/ml) or 10% FBS (control) for the time indicated. Cell lysates were analyzed for Rap1 activation in a RalGDS pull-down assay (as described in the legend to Fig. 1). The levels of (activated) Rap1-GTP, total Rap1, and Rap1GAP were determined by immunoblot analysis. B,

Migration of PC3 cells stably expressing the indicated constructs and treated with 200 ng/mL SDF-1 for 4 h (assays performed as in Fig 2B). *C*, Invasion of PC3 cells stably expressing the indicated constructs and treated with 200 ng/mL SDF-1 for 24 h (assays performed as in Fig. 2B). *D*, Invasion of PC3 cells treated with siRNA to Rap1A for 72 h and treated with 200 ng/mL SDF-1 for 24 h (assays performed as in Fig. 2D). Data represents that pooled from two independent experiments with at least 2 experimental replicates each, and is plotted as the ratio of the number of migrated or invaded cells vs. that of vehicle-treated control cells (*B,C* vector; *D*, scrambled siRNA) determined in parallel. ***, $p < 0.001$; **, $p < 0.005$; *, $p < 0.05$.

3.2.4 Rap1 promotes CaP migration and invasion through integrins

In lymphocytes and platelets, the downstream signaling event with which Rap1 has been most associated is integrin activation (8). Likewise, activation of several integrins, particularly $\alpha v \beta 3$, is thought to be an important component of CaP metastatic progression (126, 127). Thus, we sought to determine if the impact of Rap1 activation on PC3 cell migration and invasion involved integrins. As an initial screen to examine whether Rap1 signaling influenced the repertoire of integrins that a PC3 cell expresses, we performed real-time PCR analysis to compare the expression levels of a broad array of integrins in PC3 cells expressing the dominant-active Rap1-63E with vector control cells. Through this analysis, we found that PC3 cells expressing Rap1-63E exhibited 2.1- and 2.4-fold upregulated expression of integrins $\alpha 4$ and $\beta 3$, when compared to vector control. Hence, we examined the consequence of blocking these upregulated integrins in PC3 cell migration and invasion assays. Function-blocking monoclonal antibodies were employed to inhibit integrins $\alpha 4$, $\beta 3$, as well as and $\alpha v \beta 3$ -directed antibody since this integrin pair has been extensively linked to cancer progression.

In control cells expressing vector alone neither migration (Fig 7A) nor invasion (Fig 7C) was significantly decreased by treatment with a low concentration of integrin blocking antibodies (20 $\mu\text{g/ml}$). Importantly, Rap1-63E-mediated migration (Fig 7B) and invasion (Fig. 7D)

of PC3 cells was markedly (40-60%) abrogated by treatment with the same concentration of blocking antibodies targeting integrins $\alpha 4$, $\beta 3$, and $\alpha v\beta 3$. No effect was observed by addition of isotype-matched control IgG (not shown) or blocking antibody to integrin $\alpha 2\beta 1$ (Fig. 7), an integrin that is not activated by Rap1. These data indicate that the migratory and invasive properties of these three integrins are stimulated by activated Rap1A. These integrins are likely also involved in the migration and invasion capacity of the parental cells as well, as both processes were affected at higher concentrations of blocking antibody (data not shown).

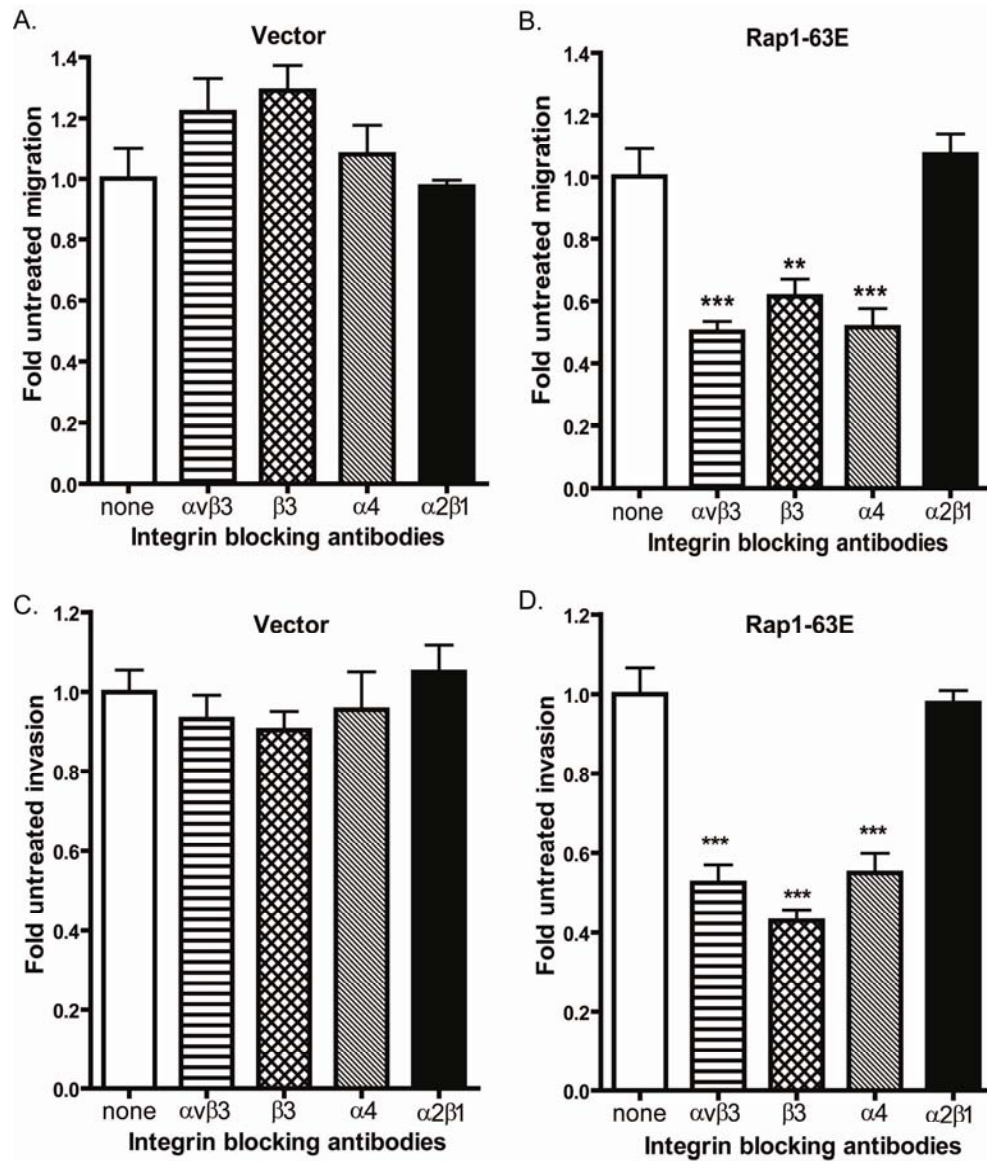


Figure 7: Rap1 promotes CaP migration and invasion through integrins. In all panels, PC3 cells stably expressing the indicated constructs were treated with specific integrin-blocking antibodies (20 $\mu\text{g/ml}$) for integrins $\alpha v\beta 3$, $\beta 3$, and $\alpha 4$, and migration and invasion of the cells was evaluated as described in the legend to Fig 2. Panels A and B. Migration of vector control cells (A) or Rap1-63E expressing cells (B) treated with integrin-blocking antibodies for 4 h. Panels C and D. Invasion of vector control cells (C) or Rap1-63E expressing cells (D) treated with integrin-blocking antibodies for 24 h. For all panels, data represents that pooled from two independent experiments with at least two replicates each and is plotted as ratio of the number of the migrated or invaded cells vs. the number of migrated or invaded cells in the vehicle-treated

control cells (labeled none) determined in parallel. ***, $p < 0.001$; **, $p < 0.005$; *, $p < 0.05$; compared to vehicle-treated control cells.

Additionally, since invasion through Matrigel requires breakdown of the matrix, was also examined a potential role of matrix metalloproteinases (MMPs) in Rap1-mediated invasion of these CaP cells. Treatment with a broad-spectrum MMP inhibitor (the GM6001 compound) revealed that Rap1-mediated invasion was much more sensitive to blockade of MMP activity than vector PC3 cells (Figure 8E). Together, these data indicate the ability of Rap1 to promote PC3 cell migration and invasion is dependent on integrin and MMP function, and particularly implicates integrins $\alpha 4$, $\beta 3$, and $\alpha v\beta 3$ in this process.

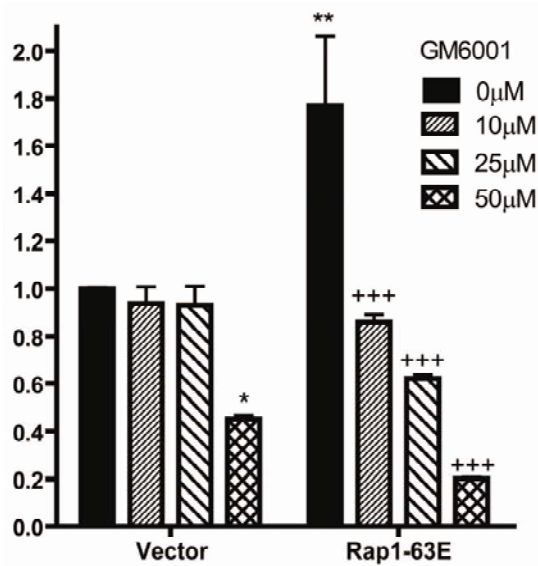


Figure 8: Rap1 promotes CaP invasion through MMPs. PC3 cells stably expressing vector control or Rap1-63E were treated with broad-spectrum MMP inhibitor GM6001 for 24 h. Invasion of the cells was evaluated as described in the legend to Fig 5. Data represents that pooled from two independent experiments with at least two replicates each and is plotted as the ratio of the number of invaded cells vs. the number of invaded cells in the vehicle-treated control cells determined in parallel. ***, $p < 0.001$; **, $p < 0.005$; *, $p < 0.05$; compared to vehicle-treated control cells. +++, $p < 0.001$; compared to vehicle-treated Rap1-63E cells.

3.2.5 Activation of Rap1 promotes CaP cell metastasis *in vivo*

The *in vitro* data supported a role for Rap1 in metastasis and these cell assays were valuable tools to examine the consequence of activated Rap1 in CaP cells and the mechanism of Rap1-mediated migration and invasion. However, it was important to demonstrate whether activation of Rap1 could impact CaP metastasis *in vivo*. To this end, we chose mice to model the later steps in CaP metastasis and to most accurately examine bone metastasis (128). Further, we elected to perform intracardiac injections based on the ability of this technique to permit metastasis to the bone, rather than injections into the tail vein, which usually result in lung metastasis (115, 128). In this approach, cells are injected into the left cardiac ventricle of mice to bypass the lungs and immediately introduce the cells into the systemic circulation.

To initiate the *in vivo* experiments, we generated PC3 cells stably expressing luciferase (PC3-Luc) to allow analysis of cancer progression in live animals using bioluminescent imaging technology. The PC3-Luc cells were engineered to stably express either vector control or the constitutively-active Rap1-63E and were then injected into athymic mice and their metastatic spread was followed. The mice were imaged immediately after the cells were injected to verify the cells had been correctly injected into the left cardiac ventricle, instead of in the right ventricle, which pumps blood directly into the lungs (data not shown). Bioluminescent signal detected in the entire mouse indicated an accurate injection, whereas signal detected only the lungs indicated an incorrect injection; such mice were not included in further analysis.

Following intracardiac injection of the engineered PC3 cells, mice were imaged every 4-7 days for 30 days or until humane endpoints had been reached following an IACUC-approved protocol. Images of the PC3-Luc cells in sedated mice were obtained between 5-15 minutes

following intraperitoneal injection of the luciferase substrate, D-luciferin. Bioluminescent imaging demonstrated a significantly increased formation of metastatic lesions, mostly in the bone, in the mice that received Rap1-63E cells compared to the mice injected with vector control PC3-Luc cells (Fig 9A). As later confirmed by necroscopy, these metastatic lesions were mostly observed in the mandible, tibia, femur, and thoracic cavity (not shown). A summary of the data obtained from this study is shown in Fig. 9, panel B-D. Metastasis-free survival is shown in panel 9B; this data indicates the first day a metastatic event was observed. As is readily apparent, metastasis-free survival was dramatically reduced in the mice injected with Rap1-63E-expressing PC3-Luc cells. All of these mice had detectable metastases within 15 days of injection, whereas only 2/5 of the vector control mice had detectable metastasis even after 30 days. In panel 5C, the average number of metastatic lesions per mouse is shown as a function of time. Again, a dramatic difference was observed, with all 6 mice bearing the Rap1-63E-expressing cancer cells developed metastatic lesions in multiple (3-10) regions. In contrast, the 2/5 mice bearing vector control cells exhibited visible metastases in only 1 to 3 regions (Fig 9C). Lastly, assessment of the individual number of metastatic lesions in each mouse at the termination of the study (Fig. 9D) again showed a marked difference in lesion formation between the two groups. Taken together, this *in vivo* data revealed a dramatic increase in the rate and incidence of metastatic lesion formation of PC3 cells expressing activated Rap1. Combined with the *in vitro* and *in vivo* data, this study provides a compelling demonstration of the potent effect of Rap1 activation on CaP migration, invasion, and metastasis.

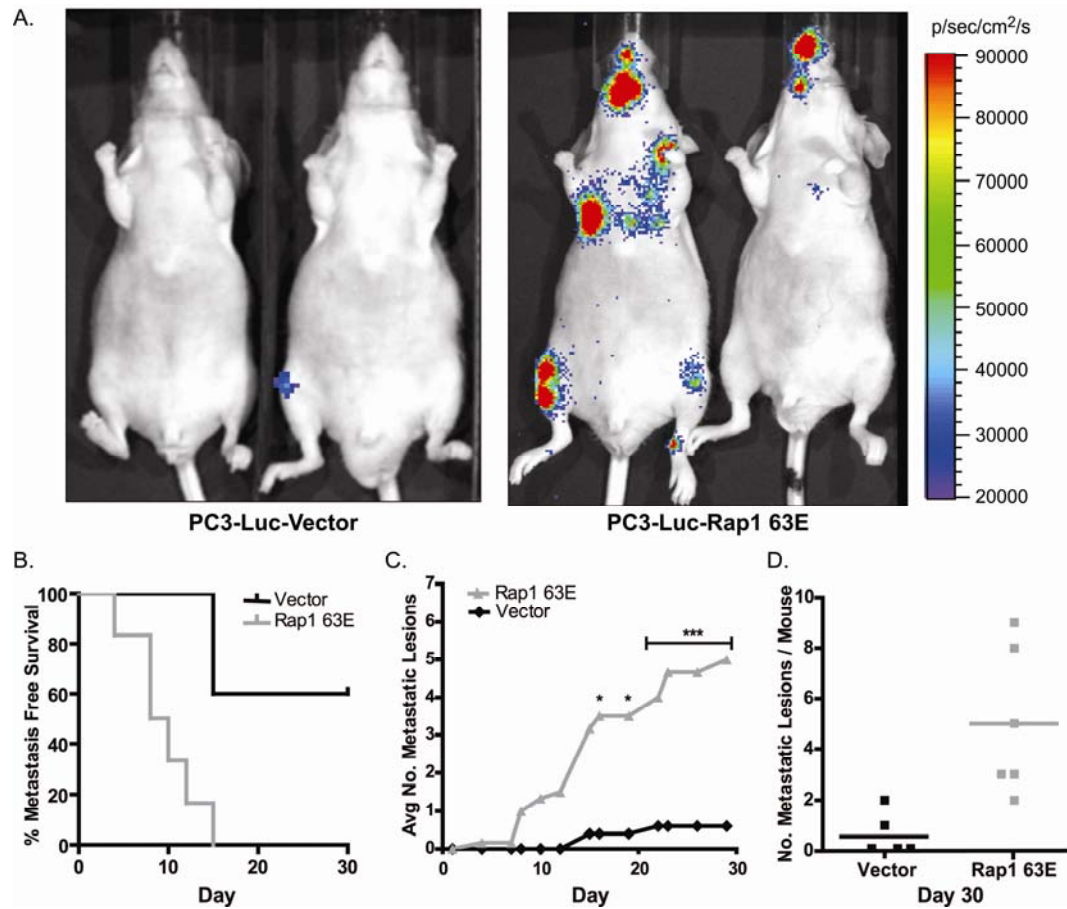


Figure 9: Activation of Rap1 promotes CaP cell metastasis *in vivo*. The metastasis assay was performed by intracardiac injection of 1×10^5 PC3-Luc cells expressing either vector control or Rap1-63E into the left cardiac ventricle of anesthetized 4 to 6-week-old athymic male mice. At approximately weekly intervals, the mice were anesthetized, IP injected with luciferin, and imaged with the IVIS® Imaging System (Xenogen) to visualize the metastatic dissemination of the PC3-Luc cells, as described in *Materials and Methods*. *A*, Images of mice day 22 post-intracardiac injection of PC3Luc-vector (left panel) or PC3Luc-Rap1-63E cells (right panel). Bone metastasis of the cells was detectable by bioluminescence in the tibia, mandible, and thoracic cavity of the mice (confirmed by necropsy). These images are pseudo-color representations of the mean bioluminescence (BL) detected; background BL was automatically subtracted out of the image based on the Igor Pro/Wavemetrics software program. *B*, Graphic representation of the percentages of mice that were metastasis free. *C*, Graphic representation of the average number of metastatic events observed per day. *D*, Graphic representation of the number of metastatic lesions per mouse at the conclusion of the study (day 30 post-intracardiac injection). Data from this figure represents vector control mice (N=5) and Rap1-63E mice (N=6). ***, $p < 0.001$; *, $p < 0.05$.

3.3 Discussion

Elucidating the precise contributions of Rap1 signaling to any particular cancer is complicated by cancer cell- and/or environment-specific differences that may alter the consequences of Rap1 activation. In the current study, we show activation of Rap1 promotes CaP metastasis. Our findings reveal that CaP cells with moderate to high metastatic potential have increased Rap1 activity and reduced expression of the negative Rap1 regulator, Rap1GAP (Fig. 4). These findings in CaP are similar to those reported in several cancers where a reduction or loss of Rap1GAP expression was observed and correlated with enhanced tumorigenesis or cancer progression (22, 83, 88).

In dissecting the role for Rap1 in CaP metastasis *in vitro*, results demonstrated activation of Rap1 enhanced CaP cell migration and invasion, and furthermore these processes were abrogated by Rap1 inhibition (Figs 5, 6). These findings corroborate with others that showed inhibition of Rap1, by Rap1GAP overexpression led to a reduction in local invasion of pancreatic cancer from the primary tumor site (22), and inhibited thyroid cancer cell migration and invasion (88). On the other hand, a reduction in Rap1 activation resulted in osteosarcoma cells becoming more invasive, a process ascribed to less adherens junction formation (20). Also differing from our results, inhibition of Rap1 through expression of Rap1GAP has been correlated with increased *in vitro* invasion of squamous cell carcinoma (91). These opposing findings highlight the need to assess the impact of Rap1 activation in individual cancer types, as clearly some aspect of the cells or their environment is important in dictating the response of the cell to Rap1 activation of migration and invasion.

Recently, a 'tumor microenvironment invasion model' has been proposed in which sequential stable genetic changes during metastasis may give rise to a tumor microenvironment that induces transient gene expression patterns that contribute to transient cell migration and invasion (129). Depending on changes in pH, blood supply, or oxygen tension, these microenvironment alterations could generate the transient expression of different integrins and adhesion molecules (some Rap1-regulated) to affect the invasive and metastatic ability of the tumor cells. The impact of Rap1 activation likely depends on the particular stage in metastatic progression of that cancer, since different integrins and ECM ligands are involved and expressed (3, 30). For example, the ability of CaP cells to escape from the laminin-rich primary tumor site has been attributed to alterations in CaP cell expression of laminin integrin receptors ($\alpha6\beta4$, $\alpha6\beta1$) (130). Our results implicate Rap1 in the later stages of CaP metastasis, particularly by the demonstration that Rap1-mediated CaP invasion and migration involves $\alpha v\beta3$ integrin, an integrin shown to be important for CaP cell navigation of the circulatory system and metastasis to the bone (130). This conclusion is supported by recent studies in other cell types showing that Rap1 signaling impacts activation and/or aggregation of integrins (8, 84, 131). Our results showing functional blockade of integrins $\alpha4$ and the $\alpha v\beta3$ heterodimer reduced Rap1-mediated migration and invasion provide the first direct evidence that activation of Rap1 impacts integrins involved in CaP migration, invasion, and metastasis to the bone.

In CaP metastasis to bone, a critical regulator of bone tropism, growth of cancer and osteoclastogenesis is SDF-1(125). SDF-1 has gained considerable attention for its role in tumor progression and metastasis as shown by numerous studies in solid and hematopoietic malignancies (132, 133). The current study demonstrates for the first time that SDF-1 stimulates

Rap1 in CaP cells and enhances CaP migration and invasion via a process that depends on Rap1 function (Fig. 6). While we do not suggest that all SDF-1/CXCR4-mediated events go through Rap1 or that Rap1-independent SDF-1 stimulated processes do not contribute to CaP metastasis; e.c. findings demonstrate that an SDF-1/Rap1 signaling axis exists in CaP migration and invasion. Combining the SDF-1 and the migration studies in media containing bone-trophic factors secreted from osteosarcoma cells, our results support a role for Rap1 in CaP metastasis to bone.

In addition to *in vitro* findings, the current study also demonstrated that Rap1 activation dramatically enhances CaP metastasis *in vivo*. Specially, in our *in vivo* model of PC3 cell metastasis, activation of Rap1 had a potent ability to promote the rate and incidence of CaP metastatic lesion formation (Fig 9). Through the use of this model, which involved intracardiac injection of PC3 luciferase expressing cells, we directly monitored the impact of activated Rap1 in CaP cells during the late steps of metastasis, after escape from the primary site (115). This model takes into account several steps involved in the metastatic process: survival in circulation, adhesion to and transmigration through the endothelial cell lining, migration to and invasion of the metastatic site, colonization and growth (134). Inferring from the literature and our study, we would propose that Rap1-mediated integrin activation may explain the dramatic rate and occurrence of metastatic lesion formation *in vivo*. Collectively, these findings indicate Rap1 may play roles at several points during CaP metastasis.

Previous studies have examined Rap proteins in other aspects of prostate cancer cell biology. In LNCaP cells, vasoactive intestinal peptide promoted androgen-independent transactivation of the androgen receptor through a PKA/Rap1/ERK pathway to enhance growth (81). Rap2A has been shown to regulate androgen sensitivity and androgen stimulated growth

of LNCaP cells (93). In CaP and other epithelial cancers, Rap1 function has been linked to cancer cell growth and proliferation through MAP kinase signaling (14, 122). Two recent studies also highlight other potential roles for Rap1 signaling in metastasis, these being the regulation of secretion of metalloproteinases and an involvement in angiogenesis (91, 97). Hence, it is important to note that Rap1 activation may be promoting several aspects involved in CaP progression.

In conclusion, the current study provides compelling evidence for a role of Rap1 in the metastatic progression of CaP. The increased Rap1 activity detected in the more metastatic human CaP cells was shown to contribute to processes fundamental to CaP metastasis, i.e., cell migration and invasion mediated through specific integrins. Extending these findings to an *in vivo* model, activation of Rap1 was shown to dramatically enhance the rate and incidence of CaP metastatic lesion formation. Taken together, the results from this study indicate regulation of the Rap1 signaling pathway may constitute an important therapeutic target in CaP metastasis.

4. Activation of Rap1 enhances the metastatic progression of Breast Cancer

4.1 Introduction

In the United States, the second leading cause of cancer deaths in women is breast carcinoma (BrCa). Most of these deaths are caused by metastatic spread of the BrCa, thus more effective ways to diagnose and treat this disease are needed. A great effort is being made to identifying prognostic markers for BrCa metastasis and to discover new methods of inhibiting the process of BrCa metastasis. BrCa metastasis is a complex series of events, and one of the rate-limiting steps in cancer metastasis is believed to be the acquisition of locally invasive phenotype (135). This invasive phenotype is accomplished in part by transient changes in tumor cell expression or activation of integrins, thus altering cancer cell affinity for extracellular matrix (ECM) components as well as its motility (119). Therefore, an attractive approach to BrCa therapy is to target cells by antagonizing integrin function (136).

As mentioned previously and demonstrated in prostate cancer, Rap1 is an important regulator of integrin affinity and avidity and has become recognized for its role in tumorigenesis and cancer progression (119, 122). The consequence of Rap1 activation on invasion and migration, however, depends on the specific context of the cancer-type and its environment. Inhibition of Rap1 in an ovarian cancer cell line disrupted cell junctions from a loss of E-cadherin on the cell surface, suggesting loss of Rap1 contributes to ovarian cancer cell motility (89). On the other hand, Rap1 has also been described as important regulator of breast architecture and cell polarity during breast acinar morphogenesis, which is effected by cell proliferation and death as well as cell-cell and cell-ECM signaling (92). In these studies, it was found that

activation of Rap1 signaling is increased in malignant mammary cells and reduction of Rap1 in these cells resulted in formation of correct breast acinar polarity and a reduction in tumor incidence. Thus, it remains uncertain in which cancers cells and at what cancer stages does Rap1 signaling positively or negatively regulates invasion and metastasis.

The differential roles of Rap1 require that it be addressed in different cancers and at different stages, and thus we sought to examine the biological impact of Rap1 activation in BrCa metastasis. In this study, we showed modulation of Rap1 regulated the proliferation of metastatic BrCa cells *in vitro*. We found that activation of Rap1 enhanced BrCa cell invasion and migration through a process that involves integrins. We also demonstrated that inhibition of Rap1 by expression of Rap1GAP1 resulted in reduced formation of BrCa metastatic lesions *in vivo*. These findings demonstrate a role of Rap1 activation in BrCa metastasis, suggesting a targeted therapeutic strategy for metastatic breast cancer.

4.2 Results

4.2.1 Modulation of Rap1 activity impacts metastatic breast cancer cell proliferation

The first study to assess the requirement for Rap1 function in BrCa cell proliferation was a gain- and loss-of-function approach utilizing overexpression of Rap1-63E and Rap1GAP. Rap1-63E is a mutationally activated form of Rap1 which renders Rap1 in a GTP-bound active state in the cells; while Rap1GAP inhibits Rap1 signaling by stimulating the ability of Rap1 to hydrolyze GTP into GDP and thus maintains Rap1 in its inactive GDP-bound state. Retrovirus was generated to stably express Rap1-63E and Rap1GAP in MB231 cells and this overexpression was verified by immunoblot analysis of protein levels. Comparison with control cells demonstrated

that the MB231-Rap1-63E cells indeed had high levels of activated Rap1-GTP and the MB231-Rap1GAP- cells had reduced levels of Rap1-GTP (data not shown). The proliferation of these cells was analyzed by an MTS assay, using a tetrazolium compound, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] , in combination with an electron coupling reagent, PES (phenazine ethosulfate). In this MTS system the metabolic activity of living proliferating cells produces a colorimetric change that can be measured by the conversion of a tetrazolium salt into a colored formazan product. In comparison with vector control MB231 cells, it was observed that stable expression of activated Rap1 resulted in a 1.2-fold increase in MB231 cell proliferation after seven days (Fig 1). In contrast the Rap1GAP expressing MB231 cells had 56.8% reduction in proliferation compared to control cells (Fig 1). These results indicated modulation of Rap1 activity alters metastatic BrCa cell proliferation, and thus activation of Rap1 may contribute to BrCa progression and metastasis.

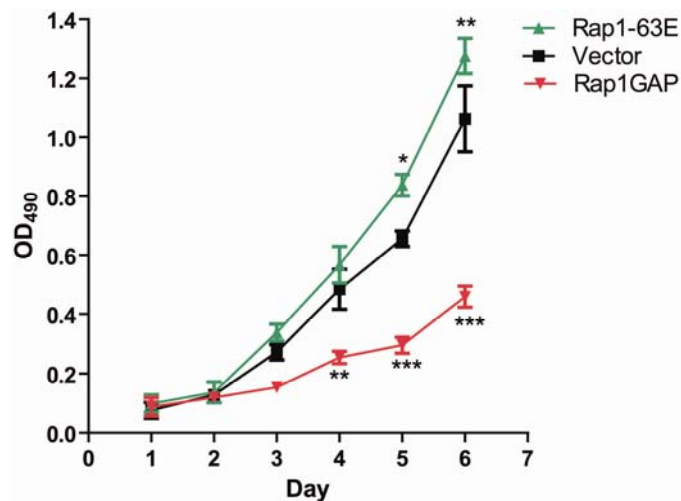


Figure 10: Modulation of Rap1 activity regulates BrCa cell proliferation. MB231 cells stably expressing activated Rap1 (Rap1-63E), Rap1GAP, or Vector were plated at Day 0. Every 24hrs MTS reagent was added to triplicate wells and the conversion of tetrazolium salt to a colored formazan product was measured calorimetrically at OD₄₉₀ as an indicator of living proliferating cells. ***, p < 0.001; **, p < 0.005; *, p < 0.05; compared to vector control cells.

4.2.2 Activation of Rap1 promotes metastatic BrCa cell migration and invasion

Our evaluation of the impact of Rap1 activation on BrCa cell metastatic behavior was prompted by a study finding that increased Rap1 activity correlated with disruption of the breast tissue architecture in malignant T4-2 BrCa cells (92). Thus, we hypothesized that Rap1 may contribute to metastatic BrCa cell motility. Given that the classic metastatic site of breast cancer is bone, we used conditioned media from bone-derived osteosarcoma cells (MG63) as the source of chemoattractant in the migration assays, to mimic this bone microenvironment (137). We have confirmed this conditioned media contains ECM proteins, specifically vitronectin and fibronectin. Additionally, invasion assays through Matrigel™ were performed to more accurately model an environment with collagen and extracellular matrix proteins that are usually present in the metastatic microenvironment.

The first study was to assess the requirement for Rap1 function in MB231 cell migration towards bone trophic factors. Again we utilized the MB231 cells expressing activated Rap1 (Rap1-63) and expressing Rap1GAP. Analysis of the migratory properties of the MB231 cells revealed that overexpression of Rap1-63E increased these properties by 1.8 ± 0.15 -fold (Fig 11A) while the Rap1GAP MB231 cell migration was reduced by $56 \pm 0.02\%$ (Fig 11B), compared to vector control. The second study assessed the ability of Rap1 to impact MB231 cell invasion through a Matrigel membrane, a crucial component of cancer cell escape from the primary site and infiltration in the metastatic site. Similarly, in the invasion assays the MB231-Rap1-63E cell invasion was dramatically enhanced by 4.9 ± 0.55 -fold, compared to vector control cells (Fig 11C). Repeating this assay with the MB231-Rap1GAP-expressing cells demonstrated that Rap1GAP

reduced MB231 cell invasion by and $42 \pm 0.05\%$, compared to vector control cells (Fig 11D). Taken together, the results of the loss- and gain-of-function studies provide compelling evidence that Rap1 function plays an important role in BrCa cell migration and invasion, and that activation of Rap1 enhances these properties in metastatic BrCa cells.

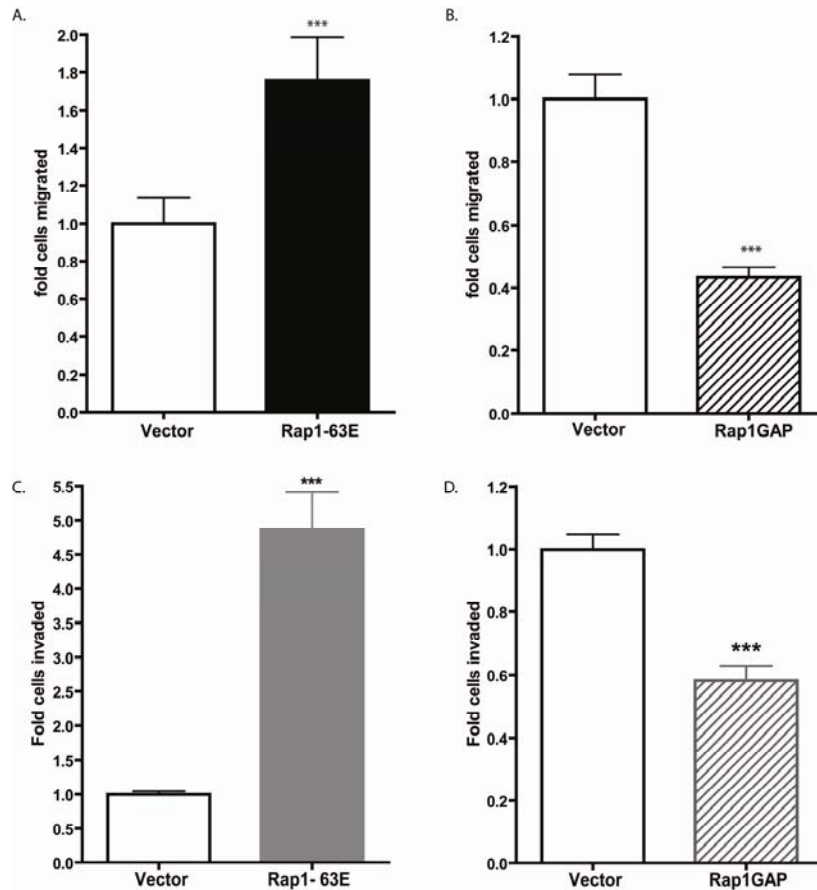


Figure 11: Modulation of Rap1 activity impacts BrCa cell migration and invasion. Panels A and B. MB231 cells were transduced with retrovirus to establish stable expression of the indicated constructs--vector, activated Rap1 (Rap1-63E), or Rap1GAP. *A and B*, Migration of MB231 cells stably expressing the indicated constructs. For the migration assays, cells migrated toward a lower chamber containing conditioned media from osteosarcoma cells and were assessed after 4 h. *C and D*, Invasion of MB231 cells stably expressing the indicated constructs. For the invasion assays, cells invaded through a Matrigel layer toward a lower chamber containing 10% serum and were assessed after 24 h. In both assays, the transwell filter membranes were fixed and stained to quantify the number of cells on the lower surface of the transwell filter. The data represents that pooled from 3 independent experiments with at least 2

experimental replicates each, and is plotted as the ratio of the number of migrated or invaded cells vs. that of control cells that were determined in parallel. ***, $p < 0.001$

4.2.3 Rap1 enhancement of BrCa migration involves integrins

In other cell types, the downstream signaling event with which Rap1 has been most associated is integrin activation, and thus impacting various changes in cell signaling, polarity, and migration, etc. (8). Likewise, activation of several integrins, particularly $\alpha v \beta 3$, is thought to be an important component BrCa metastatic progression (136, 137). Thus, we sought to determine if the impact of Rap1 activation in BrCa cell migration involved integrins. Again, the migration assays were utilized, however with a modification. Rather than using conditioned media as the chemoattractant source, these studies instead examined the ability of Rap1 to promote integrin-mediated migration of the cells towards ECM proteins, specifically, fibronectin, vitronectin, and collagen IV. Rap1 activation in MB231 cells dramatically enhanced integrin-mediated migration toward all three ECM; fibronectin by 7.66-fold (Fig 12A), vitronectin by 7.0-fold (Fig 12B), and collagen IV by 1.6-fold (Fig 12C). To examine the consequence of blocking integrin engagement with the ECM, function-blocking RGD peptides were used. These peptides mimic the RGD sequence found in the cell attachment site of several ECM and cell surface proteins, and this sequence is recognized by almost half of the known integrins (138). In solution, RGD peptides can block the RGD-directed integrin adhesion (138). Blockade of integrin function with RGD peptide treatment significantly abrogated Rap1-mediated migration toward all ECM, bringing it to the level of control MB231 cells (Fig 12). Interestingly, RGD peptides have also been shown to induce apoptosis through activation of caspase-3, and 24hr treatment with RGD peptide resulted in a 60% reduction of fibroblast cell survival (139). Despite, this impact on apoptosis, we feel our migration results from only 4hr treatment with RGD peptide are not likely

from an apoptotic effect, since the time of treatment is rather short and the reduction in migration is quite dramatic. The results from Fig 12 suggest the potential involvement of several RGD-directed integrins ($\alpha v\beta 3$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha 2\beta 1$) specific to these ECM in Rap1-mediated migration. These results indicate the ability of Rap1 to promote BrCa cell migration is dependent on integrin function, and implicates Rap1 regulation of several integrins in this process.

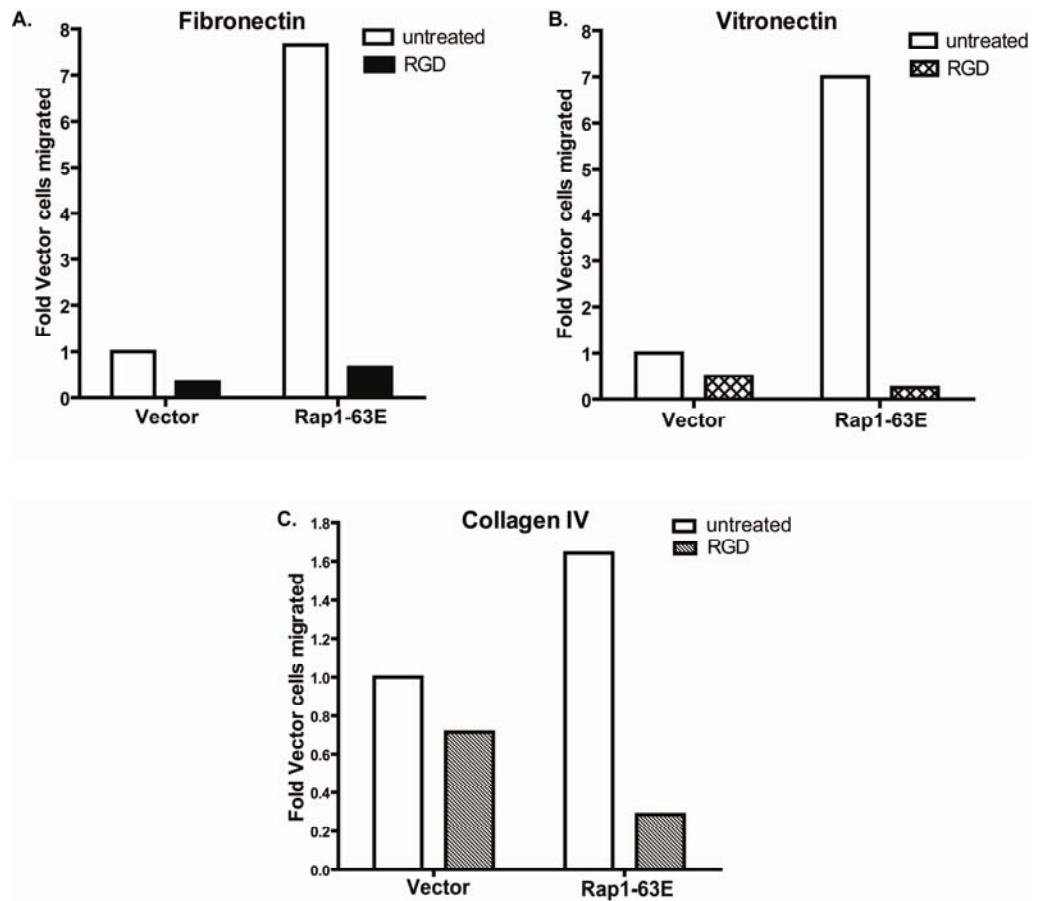


Figure 12: Rap1 promotes BrCa migration through integrins. In all panels, MB231 cells stably expressing the indicated constructs were treated with integrin-blocking RGD peptide (700 $\mu\text{g}/\text{ml}$), and migration of the cells was evaluated as described in the legend to Fig 7, except cells migrated toward various ECM, instead of conditioned media. Panels A and B. Migration of vector control cells or Rap1-63E expressing cells (A) toward fibronectin (B) toward vitronectin (C) toward collagen. For all panels, data represents two independent experiments with at least two replicates each and is plotted as ratio of the number of the migrated cells vs. the number of migrated vehicle-treated control cells determined in parallel.

4.2.4 Inhibition of Rap1 abrogates BrCa metastasis *in vivo*

The *in vitro* data supported a role for Rap1 in BrCa metastasis, and these cell –based assays were valuable tools to examine the consequence of activated Rap1 in BrCa cells and the mechanism of Rap1-mediated migration. However, it was important to demonstrate whether activation of Rap1 could impact BrCa metastasis *in vivo*. To this end, we chose mice to model the later steps in metastasis (128). Further, we elected to perform tail vein injections based on the ability of this technique to permit metastasis in mice (115, 128). In this approach, cells are injected into the lateral tail vein of mice to introduce the cells into circulation. To initiate the *in vivo* experiments, we generated MB231 cells stably expressing luciferase (MB231-Luc) to allow analysis of cancer progression in live animals using bioluminescent imaging technology. The MB231-Luc cells were engineered to stably express either vector control or Rap1GAP and were then injected into athymic mice and their metastatic spread was followed.

Following tail vein injection of the engineered BrCa cells, mice were imaged every 4-7 days for several weeks or until humane endpoints had been reached following an IACUC-approved protocol. Images of the MB231-Luc cells in sedated mice were obtained following intraperitoneal injection of the luciferase substrate, D-luciferin. Bioluminescent imaging demonstrated a significant formation of metastatic lesions in the mice that received vector control cells (Fig 13A). Mice that received MB231-Luc-Rap1GAP cells, however, never developed any detectable metastasis after 22 days (Fig 13B). As is readily apparent, a dramatic difference is observed, with none of the mice bearing the Rap1GAP-expressing cancer cells developed lesions, whereas the 3/5 vector control cell-bearing mice exhibited metastatic lesions (Fig 13).

Taken together, this *in vivo* data shows the dramatic decrease in the incidence of metastatic lesion formation of MB231 cells expressing Rap1GAP. This also suggests Rap1 activation may be important in BrCa cell survival in circulation or growth at the metastatic site. Combined with the *in vitro* and *in vivo* data, this study provides a compelling demonstration of the potent effect of Rap1 activation on BrCa migration, invasion, and metastasis

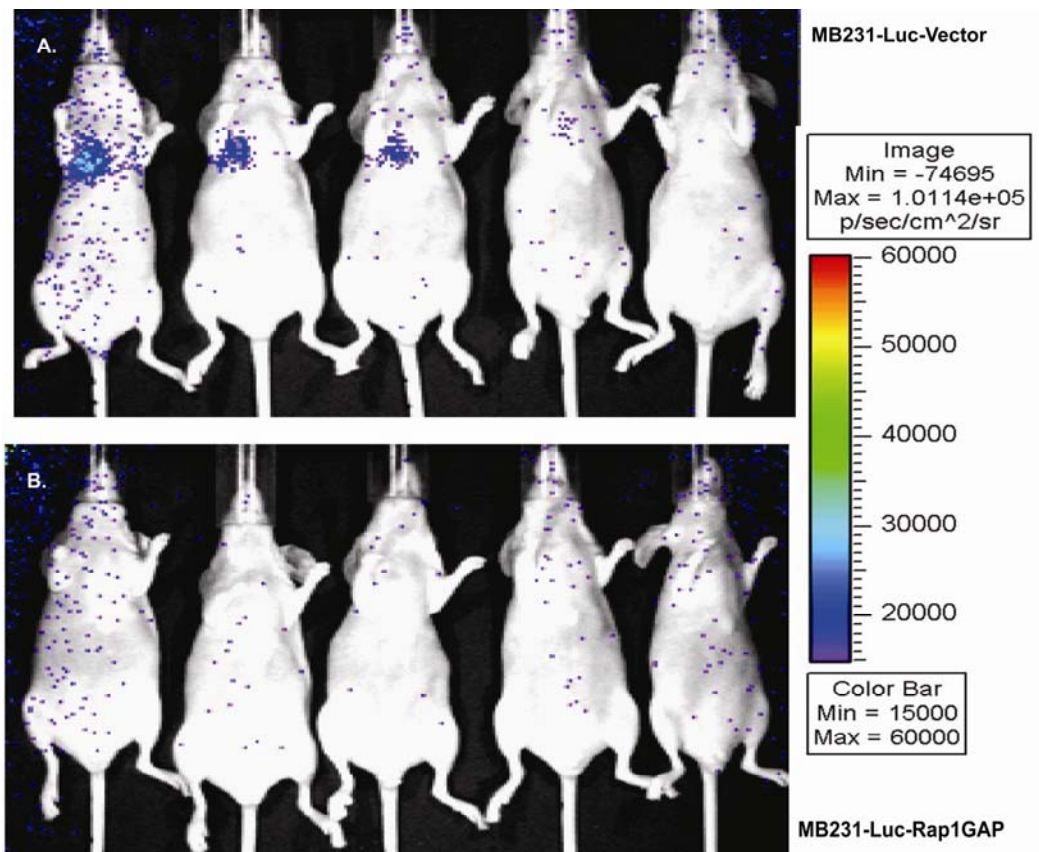


Figure 13: Inhibition of Rap1 abrogates BrCa cell metastasis *in vivo*. The metastasis assay was performed by tail vein injection of 1×10^5 MB231-Luc cells expressing either vector control or Rap1GAP into anesthetized 4 to 6-week-old athymic female mice. At approximately weekly intervals, the mice were anesthetized, IP injected with luciferin, and imaged with the IVIS® Imaging System (Xenogen) to visualize the metastatic dissemination of the MB231-Luc cells, as described in *Materials and Methods*. Images of mice day 22 post-injection of MB231Luc-vector (top panel) or MB231Luc-Rap1GAP cells (bottom panel). Lung metastasis of the cells was detectable by bioluminescence. These images are pseudo-color representations of the mean bioluminescence (BL) detected; background BL was automatically subtracted out of the image based on the Igor Pro/Wavemetrics software program.

4.3 Discussion

Recent studies have suggested a role for Rap1 in tumorigenesis and cancer progression (122). However, elucidating the precise contributions of Rap1 signaling to any particular cancer is complicated, as the consequences of Rap1 activation seems to be quite cell- and environment-specific. In the current study, we show activation of Rap1 promotes BrCa metastasis. These findings in BrCa are similar to our observations in CaP and other reports in SCC, pancreatic, and thyroid cancers where increased Rap1 activity was observed and correlated with enhanced tumorigenesis or cancer progression (22, 83, 88, 92). Differing from some of our findings in prostate cancer, here we reveal that modulation of Rap1 can impact metastatic BrCa cell proliferation as well (Fig. 11). This highlights the different roles and likely different signaling pathways impacted by Rap1 enhancement of cancer metastasis.

To our knowledge this study demonstrates for the first time that Rap1-mediated BrCa migration involves integrin function (Fig 13). Interestingly, Itoh *et al.* showed that EGFR inhibitor treatment of T4-2 BrCa cells reduced expression of $\beta 1$ integrin, but $\beta 1$ integrin expression was not affected in T4-2 cells expressing dominant active Rap1 (92). They suggested that high levels of Rap1 activation somehow prevented BrCa sensing of the multidimensional ECM environment, which then caused the uncoupling of the EGFR and $\beta 1$ integrin signaling pathway (92). While their study suggests Rap1 disrupts polarity to thereby impact integrins, our study suggests rather that Rap1 directly impacts integrins to change BrCa behavior. Our model is also supported by reports in other cancers suggesting a more direct impact of Rap1 on integrin function and activation (22, 84).

For breast cancer in particular, the databases created from the vast repository of BrCa patient samples have facilitated tissue data analysis in the study of breast cancer. The mechanistic data presented here correlated with several genetic studies implicating the loss of negative regulators (GAPs) of Rap1 in different subsets of BrCa patients. In particular, a mutation of the *Rap1GAP1* gene was found in a subset of breast cancer samples and thus was termed a 'candidate cancer gene' (140). This correlates very nicely with our results demonstrating expression of Rap1GAP reduces human BrCa proliferation, migration, invasion, and metastasis (Fig 11, 12, and 14).

Other studies have also identified mutations in BrCa that lead to an enhancement of Rap1 activation. Genetic studies uncovered a metastasis efficiency modifier locus (Mtes1) by quantitative trait loci (QTL) mapping, and further identified the RapGAP, *Sipa1* (or *SPA-1*) gene as the candidate gene at this locus (141). Additional analysis demonstrated that, unlike mouse strains with a low metastatic phenotype, mice with a high metastatic potential had a mutation in *Sipa1* that altered its ability to GAP and inhibit Rap1 (141). Further, Park *et al.* showed modulation of *Sipa1* expression in mouse mammary cells significantly altered pulmonary tumor formation in mice after subcutaneous injection of these cells (141). Another related study with genetic analysis of human BrCa samples found a strong association between three single nucleotide polymorphisms (SNPs) in the *SIPA1* gene that correlated with aggressive disease behavior in BrCa patients (123). Specifically, Crawford *et al.* showed *Sipa-1* variation was associated with (negative) tumor hormone receptor status and lymph node involvement at the time of diagnosis, both of which are factors that contribute to the metastatic potential of BrCa (123). Since they did not observe an association *Sipa-1* variation and tumor size, it would

interesting if perhaps *Rap1GAP1* was altered in correlation with tumor size, as our results showed that *Rap1GAP1* reduced proliferation of BrCa cells(Fig 11). Our work, combined with these studies, provide genetic and mechanistic evidence that Rap1 is an important regulator of BrCa progression that can be activated through several different mechanisms in breast cancer.

In conclusion, the current study provides evidence of a role for Rap1 in the metastatic progression of BrCa. Rap1 contributes to processes fundamental to BrCa growth and metastasis, i.e. cell invasion and migration mediated through specific integrins. Extending these findings to an *in vivo* model, activation of Rap1 was shown to dramatically enhance the rate and incidence of CaP metastatic lesion formation. Thus, targeting Rap1 may serve as an important diagnostic and/or treatment tool for metastatic breast cancer.

5. Rap1 signals through mTOR and ribosomal protein S6 to promote pancreatic islet β -cell function

5.1 Introduction

Type 2 diabetes is characterized by a decreased sensitivity of the body to the effects of insulin. Early in the pathogenesis of the disease, the β -cells of the pancreas are able to compensate for this reduced sensitivity by increasing insulin production. This increase in production is the result of increased insulin secretion from the individual β -cells and by an overall increase in β -cell mass, specifically from increased proliferation and cell size. It is these compensatory measures that slow the development of Type 2 diabetes, and it is their failure that marks the progression to the insulin dependent stage of the disease (142). As such, the pathways regulating these compensatory mechanisms have been widely studied and have become targets for the treatment of Type 2 diabetes (143).

At the center of many of these pathways is cAMP (144). It was long thought that cAMP acted exclusively through the cAMP dependent protein kinase (PKA); however, it is now clear that there exist other important intracellular targets for cAMP. Principal among these is a family of cAMP regulated guanine nucleotide exchange factors (GEFs) known as Epac (145, 146). The primary function of this family of proteins is believed to be the cAMP-induced activation of the Ras-like monomeric G protein Rap. However, recent studies have suggested that Epac can engage other downstream targets directly and thus stimulate effects independently of Rap.

The role of Epac in β -cell biology has been studied extensively. Early experiments demonstrated that Epac is required for cAMP-stimulated intracellular Ca^{2+} release (147, 148) and insulin secretion (18, 149). More recently, studies have demonstrated a role for Epac in

promoting resistance to fatty acid-induced apoptosis (150). Interestingly, the role of Rap in these Epac-mediated processes was not examined. Moreover, in some of these reports, the effects of Epac were attributed to Rap-independent pathways (18, 149). As result, the role of Rap signaling in the pancreatic β -cell is largely undefined. Thus, this study was initiated to identify a role for Rap signaling in the pancreatic β -cells by examining the pathway directly. Interestingly, we found that the activation of Rap1 indeed affects insulin secretion, but also promotes β -cell proliferation through the stimulation of mTOR, leading to an increase in the levels of phosphorylated, active ribosomal protein S6 (S6). This newly described pathway may be a potential target for treatments aimed at preventing or reversing β -cell dysfunction in Type 2 diabetes.

5.2 Results

5.2.1 Rap1a stimulates the phosphorylation of S6 ribosomal protein in an activation-dependent manner through mTOR and S6K1.

To gain insight into the function of Rap1 in the pancreatic β -cell, we used recombinant adenovirus to express Rap1a-63E in the rat insulinoma cell line, Ins-1 (151). After adenoviral infection and starvation, the cells were lysed and subjected to multiplex phospho-protein immunoblot analysis using antibodies towards proteins that represented a diverse set of cell signaling pathways. Of the ten phospho-proteins examined, Erk (T202/Y205), p38 MAPK (T180/Y182), SAPK/JNK (T183/Y185), Akt (S473), Stat 3 (Y705), p90 RSK/ribosomal S6 kinase (S380), S6 ribosomal protein (S235/236), and GSK-3 beta (S21/9), the most striking change was seen in the phosphorylation status of S6 (data not shown). Expression of Rap1a-63E reproducibly induced a 15-20 fold increase in S6 phosphorylation (Fig. 14). This change in phosphorylation appeared to be dependent on Rap1 activation, as expression of the wild-type

form of Rap1a resulted in only a modest increase in S6 phosphorylation (Fig. 14). Moreover, this effect appeared to be specific to Rap1. To elaborate, both the related Rac1 and CDC42 proteins have been shown to promote phosphorylation of S6 (152); but the activated forms of these proteins (Rac1-G12V and CDC42-G12V, respectively), induced little to no increase in the phosphorylation of S6 in the Ins-1 cell line (Fig. 14).

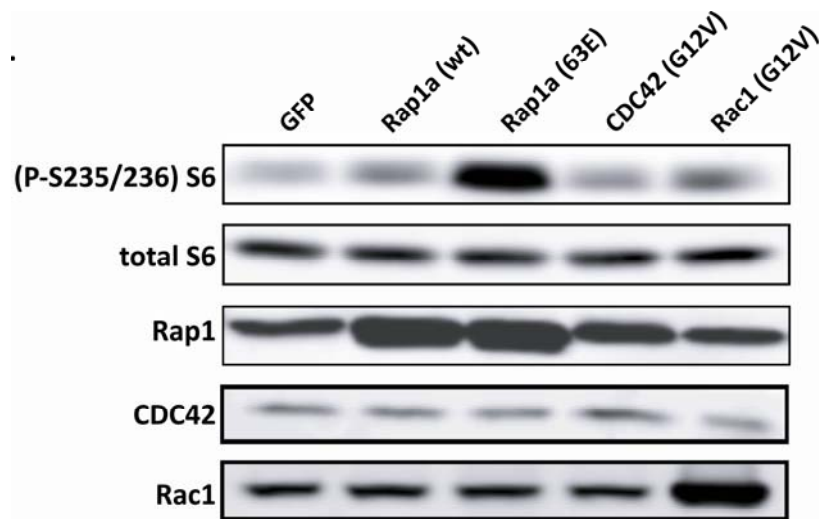


Figure 14: Rap1a stimulates the phosphorylation of S6 ribosomal protein in an activation-dependent manner. Ins-1 cells were transduced with adenovirus expressing the indicated proteins and then serum-starved into quiescence. Cells were then lysed, and equal amounts of protein were analyzed by immunoblotting for phosphorylated S6 using a specific antibody. Total S6, Rap1, Rac and CDC42 were also blotted as controls. The figure is representative of four independent experiments.

Since expression of activated Rap1a induced an increase in S6 phosphorylation in a β -cell line, we sought to determine if Rap1a was promoting S6 phosphorylation in pancreatic islets as well. Named after its inhibitor rapamycin, mammalian target of rapamycin (mTOR) is a known regulator of cell growth and proliferation, and one of its downstream targets is p70 S6 kinase

(S6K1), which phosphorylates and activates ribosomal protein S6 (153). Expression of Rap1a-63E in pancreatic islets also induced phosphorylation of S6, and additionally this activation was blocked by rapamycin treatment (Fig. 15). Thus, it appears in islets and b-cell lines that activated Rap1a promotes the phosphorylation of S6 by specifically promoting mTOR activation of S6K1.

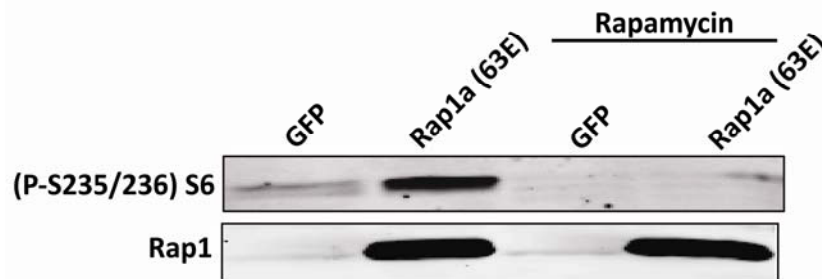


Figure 15: Rap1a stimulates the phosphorylation of S6 ribosomal protein in an activation-dependent manner through mTOR and S6K1. Rat pancreatic islets were transduced with adenovirus expressing the indicated proteins and then treated with or without rapamycin. Islets were then lysed, and equal amounts of protein were analyzed by immunoblotting for phosphorylated S6 using a specific antibody. Total Rap1 was also blotted as controls. The figure is representative of two independent experiments.

5.2.2 cAMP can act through Rap1 to stimulate S6 phosphorylation

In isolated rat islets maintained at low glucose concentrations, cAMP is able to stimulate S6K1 activity (154). As Rap1 is a known downstream effector of cAMP, we investigated the role of Rap1 signaling in this cAMP pathway. First, we confirmed that cAMP activates Rap1 in the Ins-1 cell line. Ins-1 cells were starved for 18 h in low glucose medium and then treated cells for various time periods with the cell-permeable cAMP analog, 8CPT-cAMP. Treatment of Ins-1 cells with this compound resulted in a significant increase in the levels of active, GTP-bound Rap1 (Fig. 16A). Next, we confirmed that cAMP was able to stimulate S6 activity in the Ins-1 cells. Treatment of the Ins-1 cells with 8CPT-cAMP resulted in a significant and sustained increase in

the phosphorylation of S6 (Fig. 16B), suggesting that both the cAMP-to-Rap1 and the cAMP-to-S6 pathways are present and active in the Ins-1 cells.

To determine if the activation of Rap1 is required for cAMP stimulation of S6 in the Ins-1 cells, we used a recombinant adenovirus to overexpress Rap1GAP, thus abolishing Rap1 signaling (42). Interestingly, expression of this construct in the Ins-1 cells resulted in a reduction of 8CPT-cAMP-stimulated S6 phosphorylation (Fig. 16C). The phosphorylation of S6 was not completely eliminated by Rap1GAP expression; however, thus we wanted to verify that another related pathway was not involved in cAMP stimulation of S6 as well. Since the closely related Ras pathway has been shown to mediate EGF-stimulated S6 phosphorylation, we wanted to determine if it was also involved in cAMP-mediated S6 phosphorylation, (155). We expressed a dominant-negative form of H-Ras (myc-HRas-17N) in Ins-1 cells, thereby blocking signaling through the Ras pathway. Expression of this construct completely abrogated EGF-stimulated S6 phosphorylation, as expected, yet it had no effect on 8CPT-cAMP-stimulated S6 phosphorylation (Fig 16D). This verified Ras was not additionally involved in cAMP –mediated S6 activation. Taken together, these results suggest that cAMP signals specifically through Rap1 to promote phosphorylation of S6 in the Ins-1 cell line.

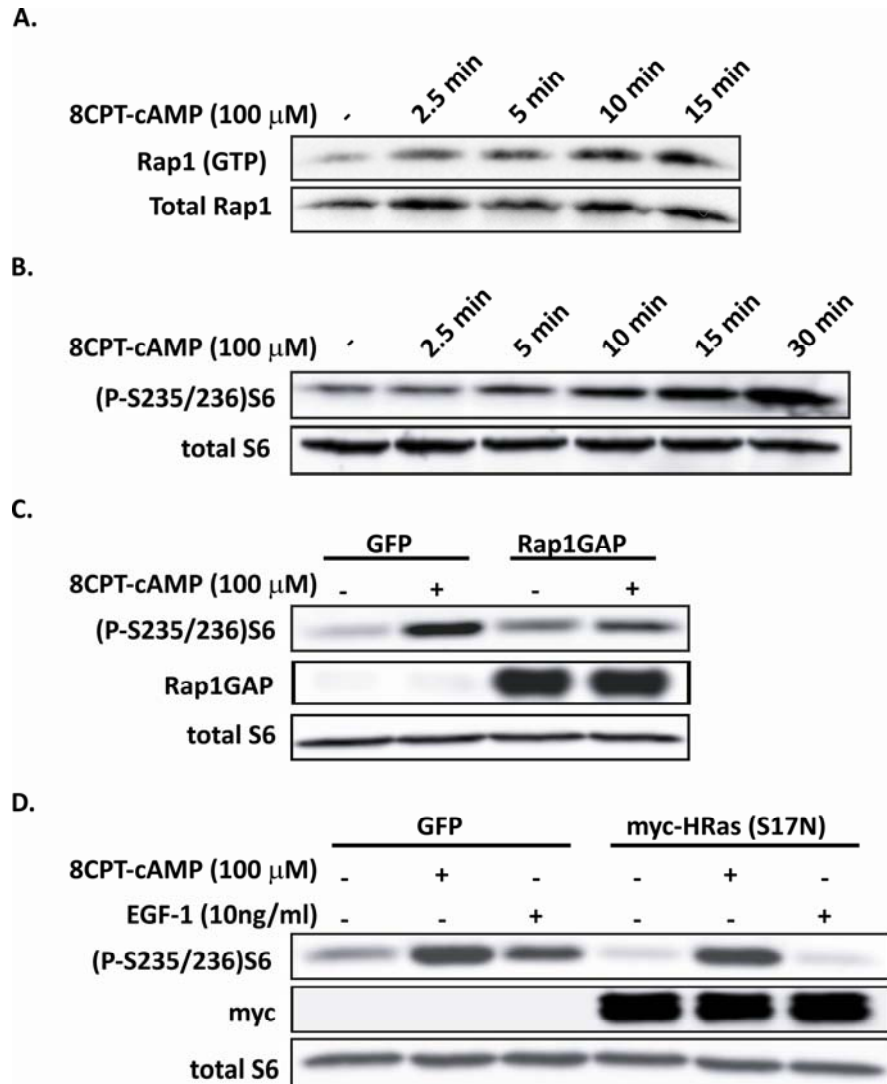


Figure 16: cAMP can act through Rap1 to stimulate S6 phosphorylation. *A*, cAMP induces Rap1 activation in the Ins-1 cell line. Ins-1 cells were serum- and glucose-starved into quiescence and then incubated with 100 μ M 8CPT-cAMP. Cell lysates were used in a pull-down assay using a GST fusion of the activated Rap1-binding domain of RalGDS. The levels of precipitated Rap1 were determined by immunoblot analysis using an anti-Rap1 antibody. Levels of total Rap1 were also determined as a control. *B*, cAMP induces S6 phosphorylation in the Ins-1 cell line. Ins-1 cells were treated as in (*A*) and then stimulated for various times with 100 μ M 8CPT-cAMP. Cells were then lysed, and equal amounts of protein were analyzed for phosphorylation of the S6 protein by immunoblot using an anti-phospho-S6-antibody. Levels of total S6 were also determined as a control. *C*, Expression of Rap1GAP partially inhibits cAMP-stimulated S6 phosphorylation. Ins-1 cell were transduced with adenoviruses expressing either GFP or Rap1GAP and then starved as in (*B*). The cells were the stimulated for 15 min with 100 μ M 8CPT-cAMP, lysed, and analyzed for S6 phosphorylation as in (*B*). *D*, Expression of dominant

negative Ras has no effect on cAMP induced S6 phosphorylation. Ins-1 cells were transduced with adenoviruses encoding either GFP or DN-H-Ras and then starved as in (B). The cell were then stimulated for 15 min with 100 μ M 8CPT-cAMP or 10 ng/ml EGF, lysed, and analyzed for S6 phosphorylation as in (B). All panels are representative of two or more separate experiments.

5.2.3 PKA cooperates with Rap1 in promoting cAMP-stimulated S6 activation

Work in isolated rat islets has suggested that some of the effects of cAMP on S6K1 are mediated by cAMP-dependent protein kinase (PKA) (154). Therefore, we tested whether PKA was involved in cAMP-stimulated S6K1 activity in the Ins-1 cell line. Supporting this, preincubation of the Ins-1 cells with a selective PKA inhibitor, myristoylated PKI (myr-PKI), resulted in a partial inhibition of 8CPT-cAMP-induced S6 phosphorylation (Fig 17). Moreover, when Ins-1 cells overexpressing Rap1GAP were treated with myr-PKI, 8CPT-cAMP-stimulated phosphorylation of S6 was completely abrogated (Fig. 17). Thus, it appears that PKA and Rap1 cooperate to stimulate S6K1 kinase activity downstream of cAMP in the Ins-1 cell line.

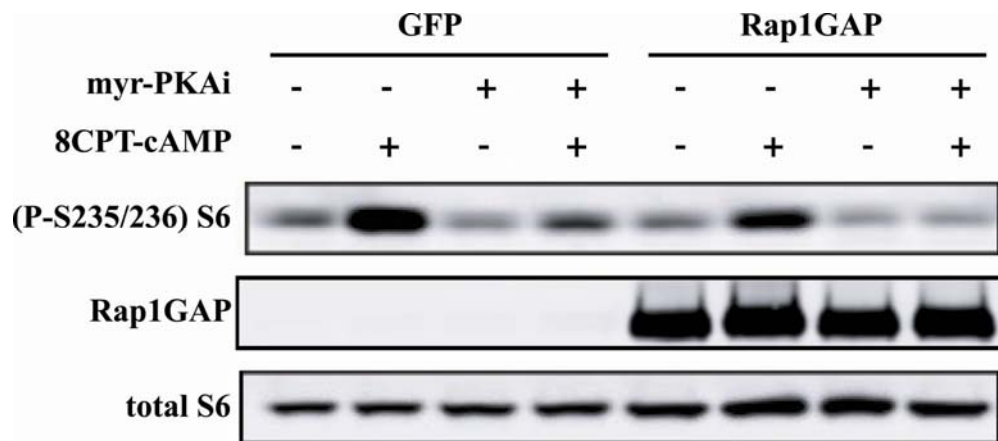


Figure 17: PKA cooperates with Rap1 in promoting cAMP-stimulated S6 activation. Ins-1 cell were transduced with adenovirus encoding either GFP or Rap1GAP and then glucose- and serum-starved. The cells were treated with or without 50 μ M myr-PKI for 30 min and then stimulated for the indicated for 15 min with 100 μ M 8CPT-cAMP. Cells were then lysed, and equal amounts of protein were analyzed for phosphorylation of the S6 protein by immunoblot

using an anti-phospho-S6-antibody. Levels of total S6 were also determined as a control. The figure is representative of three separate experiments.

5.2.4 cAMP stimulation of S6 activity is independent of PI3K/Akt

In many cell types, hormone-stimulated activation of S6K1 activity is mediated by a PI3 kinase (PI3K) dependent pathway (153, 156). As Rap1 signaling is able to activate PI3K in some cell types (69), we explored the role of this pathway in Rap1-stimulated S6K1 activity in the Ins-1 cells. Activation of PI3K is usually accompanied by phosphorylation of Akt. Interestingly, stimulation of the Ins-1 cells with either 8CPT-cAMP or IGF-1, which is thought to signal exclusively through the PI3K/Akt pathway (157), resulted in increased S6 phosphorylation. However, only stimulation with IGF-1 resulted in enhanced phosphorylation of Akt (Fig. 18, lanes 1-3). Moreover, inhibition of PI3K with LY294002 completely blocked IGF-1-stimulated S6 phosphorylation, but it had no effect on 8CPT-cAMP-induced S6 phosphorylation (Fig. 18). Taken together, these data suggest that cAMP stimulation of S6K1 activity is independent of the PI3K/Akt signaling pathway. Thus, Rap1 does not likely signal through PI3K/Akt downstream of cAMP, but cooperates in some other way with PKA to activate S6K1 phosphorylation of S6, affecting β -cell function.

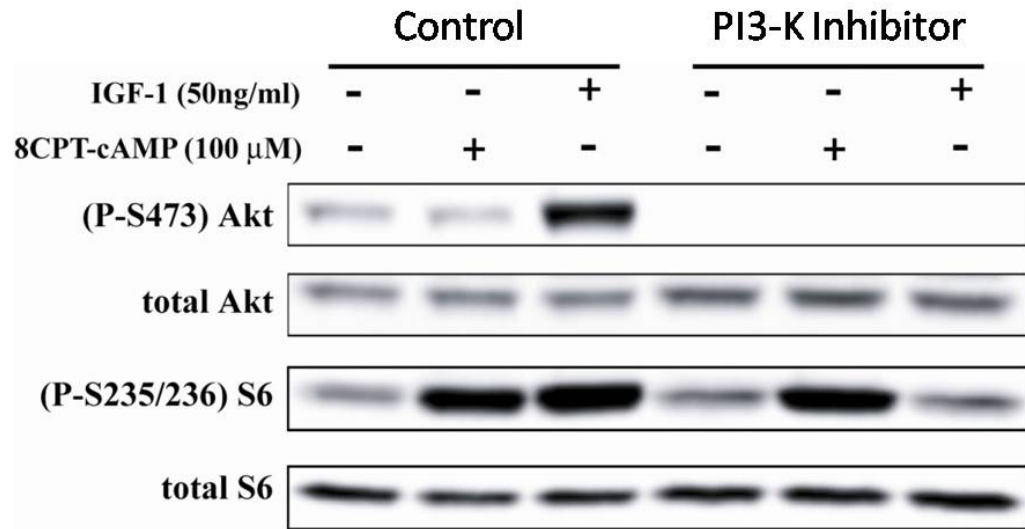


Figure 18: cAMP stimulation of S6 activity is independent of PI3K/Akt. Ins-1 cells were glucose- and serum-starved, treated with 30 μ M PI3K inhibitor, LY294002, for 30 min, and then stimulated for 15 min with 100 μ M 8CPT-cAMP or 10 ng/ml IGF-1. Cells were then lysed, and equal amounts of protein were analyzed for phosphorylation of S6 by immunoblotting with an anti-phospho-S6-antibody. Levels of total S6 were also determined as a control. The figure is representative of three separate experiments.

5.2.5 Activation of Rap1 promotes GSIS and β -cell proliferation, but only acts through mTOR and S6K1 to promote proliferation

After establishing the cAMP-Rap1-mTOR-S6K1-S6 signaling pathway in pancreatic β -cell lines and islets, we next sought to analyze this pathway in pancreatic islet biology. Physiologic response to the systemic insulin resistance of Type 2 diabetes is characterized by an increase in the both β -cell number and size (158). Also, activation of S6 has been linked to this increase in β -cell size (158). These findings suggest that Rap1 may play a role in β -cell size and function. To explore this hypothesis, we examined the effects of modulating Rap1a signaling in isolated rat pancreatic islets. Initially, we determine whether Rap1 signaling was able to increase islet cell size (hypertrophy) through activation of S6. The constitutively-activate form of Rap1a (Rap1a-

63E) was expressed in isolated pancreatic islets and the size of islet cells were determined with confocal microscopy by counting the number of nuclei per a defined area. Surprisingly, no significant change in islet cell size was observed from the expression of activated Rap1, or from rapamycin treatment (Fig. 19A).

In addition to increased β -cell size, another major hallmark of the physiologic response to the systemic insulin resistance in Type 2 diabetes is increased insulin secretion. Previous studies have demonstrated signaling through the RapGEF, Epac, potentiates glucose-stimulated insulin secretion (GSIS) and insulin granule dynamics (103, 145, 146). Expression of Rap1a-63E in pancreatic islets indeed enhanced GSIS (Fig. 19B). This result, coupled with previous reports showing Epac-potential of GSIS (103), suggests that this function of Epac is indeed mediated by Rap1. Additionally, to examine if our newly identified Rap1-S6 pathway was involved, rapamycin treatment was used to block the downstream signaling of mTOR-S6K1-S6. Interestingly, we observed that blockade of mTOR /S6 activation had no significant impact on the ability of activated Rap1 to increase GSIS (Fig 19B).

Stimulation of Erk through GPCR activation of cAMP has also been shown to involve Rap1 in β -cells (102), thus the potential impact of Rap1 on β -cell proliferation was also examined. Indeed, we found activation of Rap1 signaling, again by expression of Rap1a-63E, enhanced islet proliferation approximately two-fold as determined by [³H]-thymidine uptake, a measure of DNA synthesis (Fig. 5C). Also, we observed that rapamycin blockade of mTOR /S6 activation abrogated Rap1 enhancement of islet proliferation (Fig 19C). Thus, the Rap1/mTOR/S6 signaling pathway appears to specifically enhance Rap1-mediated β -cell proliferation, and not Rap1-

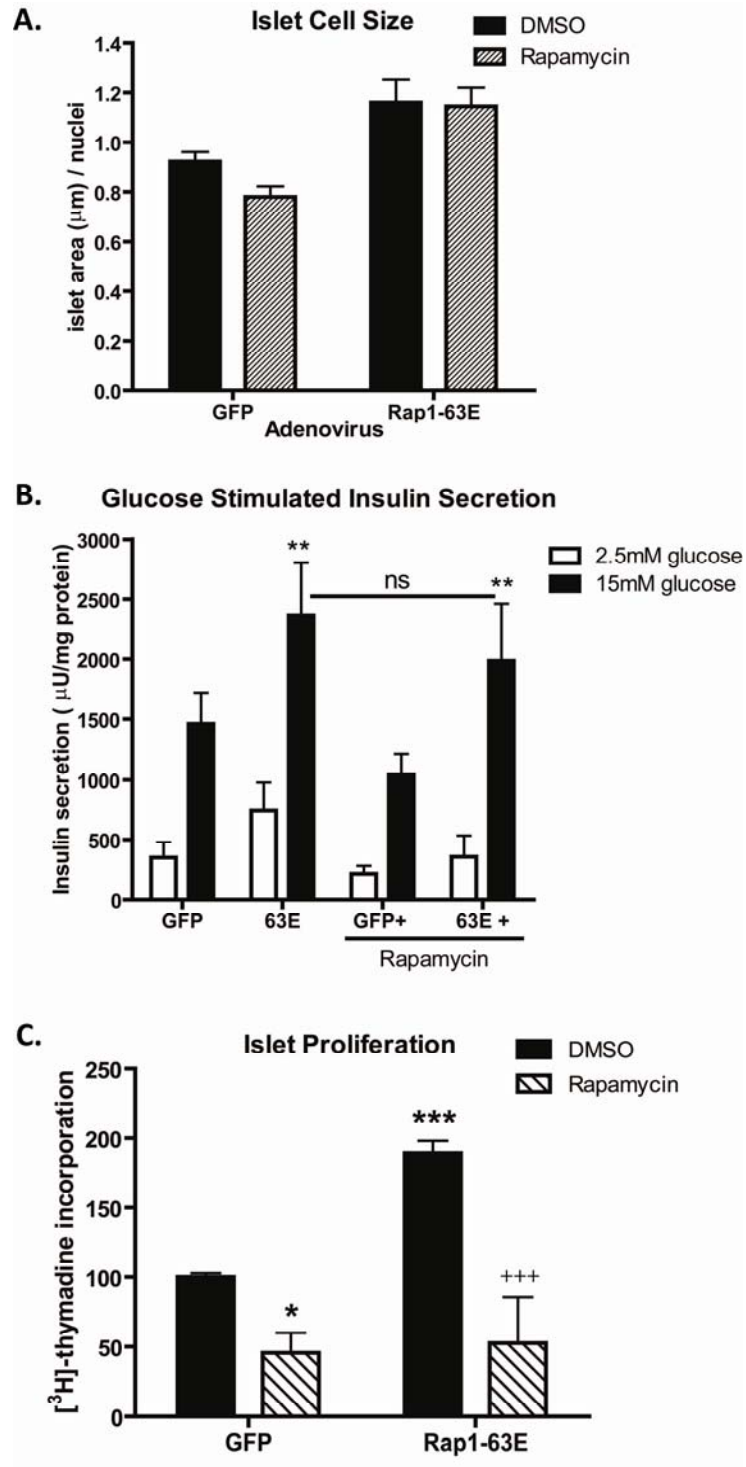


Figure 19: Activation of Rap1 promotes GSIS and β -cell proliferation, but only acts through mTOR and S6K1 to promote proliferation. Rat pancreatic islets were isolated and

infected with adenoviruses expressing vector or Rap1-63E, before the day of the assay, the islets were treated with or without rapamycin. *A*, fixed islet cell nuclei were Hoechst stained and analyzed on an a Leica SP5 confocal microscope. The number of nuclei in a measured area was counted using Metamorph software, data represents the average nuclei counted from 4 areas/ image slice with 3 image slices/islet for 4-6 islets/group. *B*, GSIS from islets expressing the indicated proteins was measured after 2 h incubation in basal (2.5 mM) or stimulatory (15 mM) glucose buffer. **, $p > 0.01$ compared to glucose-stimulated GFP control (for respective DMSO or rapamycin treated) islets. *C*, Islets were serum- and glucose-starved into quiescence, and then incubated with [³H]-thymidine. The amount of [³H]- thymidine uptake was then determined as measure of cell proliferation. All data is pooled from 3-4 separate experiments. ***, $p < 0.001$; *, $p > 0.05$; compared to DMSO treated GFP islets. +++, $p < 0.001$; compared to DMSO treated Rap1-63E islets.

mediated enhancement of GSIS. Together, these results indicate Rap1 is involved in at least two distinct signaling pathways impacting on different β -cell functions.

5.3 Discussion

In this study, we define Rap1 as regulator of β -cell proliferation and insulin secretion. Additionally, we show that the impact of Rap1 on β -cell proliferation is mediated through an activation of mTOR and resultant phosphorylation and activation of S6 ribosomal protein by S6K1. Previous studies involving deletion of the S6K1 gene have demonstrated the significance of S6K1 signaling in the biology of the mouse pancreatic β -cell. The phenotype of the S6K1-null mouse is predominantly characterized by a marked reduction in β -cell mass, resulting in a syndrome that mimics Type 2 diabetes (159). Interestingly, this defect appeared to be specific to the pancreatic β -cells, as the other tissues examined were mostly unaffected. In addition, a recent study found that phosphorylation-deficient S6 knock-in-mice have a growth defect that is selective for embryonic fibroblasts and β -cells and these mice have compromised glucose

homeostasis. (158). Both of these studies correlate well with our findings that Rap1 signaling through mTOR and phosphorylation of S6 play an important role in regulating β -cell function.

A recent study by Xie *et al.* demonstrates the impact of cAMP signaling on β -cell functions with a β -cell-specific $G\alpha_s$ deficient mouse (160). As expected from the loss of this stimulator of cAMP production, cAMP levels in the islets of these mice were significantly reduced. The major defect was not in insulin secretion, though, but rather a decrease in β -cell mass that was explained by a decrease in β -cell size and proliferation. Although we did not see an impact on cell size by introduction of activated Rap1, this report correlates well with our data showing the importance of Rap1 in cAMP- signaling in β -cell proliferation.

We were intrigued by the specificity of cAMP stimulating only Rap1/mTOR/S6K1 activity in the islets and β -cell line. Many studies have demonstrated roles for Rap in the activation of other kinases involved with proliferation, such as Erk (14) and PI3K/Akt (161, 162). Furthermore, other important β -cell ligands or incretins GLP-1 and GIP are able to stimulate both of these pathways in the Ins-1 cell line, and have recently been shown to activate Rap1 (103, 163, 164). However, cAMP-stimulated Rap1 had no effect on either ERK or PI3K/Akt phosphorylation. Thus it appears that incretin activation of Rap1 may signal through the Erk and PI3K/Akt cascades, while cAMP activation of Rap1 does not. Previous studies have reported that cAMP is able to activate S6K1 in several cell types, including isolated rat islets, pancreatic acinar cells (165), sertoli cells (166), Swiss 3T3 cells (167) and thyroid cells (168). Consistent with our findings in the Ins-1 β -cell line, cAMP appears to stimulate S6K1 activity through a PI3K-independent pathway in both sertoli (166) and thyroid cells (161) as well. However, in both cell types, cAMP stimulation of S6K1 appears to be completely PKA-dependent (69, 161, 166).

Furthermore, to our knowledge, only three studies have directly examined the effects of Rap1 on S6K1 activity (31, 69, 169); in none of these studies did activation of Rap1 stimulate S6K1. Thus, the involvement of Rap1 in cAMP-stimulated mTOR and S6K1 activity in the islets and β -cells appears to be a novel, cell type-specific phenomenon.

Several studies have demonstrated a role for the cAMP-stimulated RapGEF, Epac, in secretion in pancreatic cells. In particular, recent reports have indicated a role for Rap1 in β -cells and acinar cells (18, 103, 149, 170). Thus, it was not unexpected that expression of the activated form of Rap1a potentiated GSIS. Recently, in insulinoma cells and islets, Shibasaki *et al.* demonstrated inhibition of all Rap isoforms by Rap1GAP overexpression reduced cAMP-stimulated GSIS, but they did not show an impact on insulin secretion from glucose stimulation alone (103). Our results, however, differ slightly from theirs, as we demonstrated specific activation of the Rap1a isoform enhanced insulin secretion stimulated by glucose alone. Additionally, Shibasaki *et al.* showed that cAMP activation of Rap1 was mediated through Epac2 and was independent of PKA (103). In contrast to these results, our data indicate that Rap1 signaling in β -cells involves both PKA and Epac. This corroborates with a study showing that Rap1 can be activation from multiple stimuli and GEFs to promote amylase secretion from pancreatic acinar cells (170). Thus, Epac may function in concert with Rap to mediate GSIS (and possibly other secretory events). However, the fact that Rap1 stimulation of GSIS was independent of mTOR-S6 was rather interesting. Taken together, the combined results from our study and Shibasaki *et al.* suggests that Epac activated Rap1 impacts on GSIS, and Rap1 activation by both Epac and PKA contributes to mTOR/S6 activation and subsequent impact on β -cell proliferation.

The results of our study show that this previously unappreciated signaling axis, cAMP-Rap1-mTOR-S6, may be responsible for some of the physiologic effects of cAMP signaling on β -cell function. In summary, our findings represent a new link between cAMP signaling and the pathways controlling β -cell proliferation, and suggest that directly targeting this pathway may have beneficial therapeutic effects for patients with Type 2 diabetes. Furthermore, an additional benefit to targeting Rap1 signaling is the potentiation of insulin secretion, which could possibly prevent or reverse β -cell dysfunction (i.e., defects in both β -cell mass and insulin secretory capacity) in diabetes.

6. Discussion and Concluding remarks

The study of Rap1, and the signaling pathways it influences, has grown immensely in the past 5 years, particularly in the fields of cancer biology, endocrinology, and neurobiology. Despite (or perhaps because of) this intense investigation, the breadth of the Rap1 field appears to be widening rather than becoming more focused. Multiple, and often seemingly opposing, Rap1 signaling events can occur even in the same cell type, examples include such processes as activation of integrins and regulation of E-cadherin in thyroid cells (171) and activation of insulin secretion and cell proliferation in pancreatic β -cells. Furthermore, much remains unclear, such as the ability of Rap1 to enhance integrin adhesion, and whether this results in cell stability and anchoring or in cell motility and migration. I have presented data in this thesis that clarifies some of these discrepancies concerning Rap1 signaling in cancer cells and pancreatic islet β -cells.

Our group became interested in Rap1 signaling with our finding that the heterotrimeric G protein *G α z* could bind Rap1GAP1 and thereby influence Rap1 signaling. Subsequently the identification by a former student in the lab, Pat Kelly, that *G α z* was expressed in pancreatic islet β -cells as well as in the epithelium of the normal human prostate and in the tumor cells of adenocarcinoma of the prostate led us to probe the functional consequences of the *G α z*-Rap1GAP axis in these tissues and its potential contribution to disease progression. I chose to examine the potential impact of Rap1GAP-mediated negative regulation of Rap1 signaling, as this was a known signaling axis our group had previously characterized in PC12 cells (106). While others in our group more closely examined the functions of *G α z*, I chose to pursue a more

rigorous and comprehensive study of Rap1 signaling. I became intrigued, in particular, by the profound biological impact of modulating Rap1 function in prostate and breast cancer invasion.

In Chapters 3 and 4 of this thesis, I detail the results of my examination of the impact of Rap1 in prostate and breast cancer metastasis. My first set of analyses revealed that prostate cancer cells of increasing metastatic potential displayed increased Rap1 activation levels and decreased expression of a negative Rap1 regulator, Rap1GAP1. These findings bear similarity to reports on several other cancers (e.g., pancreatic, thyroid, melanoma, and CML) that showed a loss of Rap1GAP1 expression in advanced cancer compared to normal or non-invasive tissue (22, 25, 84, 87). Our prostate cancer correlation was not entirely consistent; however, as the extremely metastatic PC3-M cell line expressed more Rap1GAP than its parental cell line, although it still maintained significantly higher levels of Rap1 activity. This indicates that alternative mechanisms of Rap1 activation may play a role in PC3-M cells. One alternative mode of Rap1 regulation may be inhibition of Rap1GAP activity. For example, it has been demonstrated that PKA phosphorylation of Rap1GAP abolishes its GAP activity (70). Furthermore, other RapGAPs are altered in several cancers (e.g., leukemia, cervical cancer, breast cancer); therefore, it is plausible that these other RapGAPs may be disrupted in certain subsets of prostate cancer. Specifically, in leukemia and breast cancer, loss or mutation of the gene encoding SPA-1 (also called Sipa-1) has been well-documented (62, 123).

In addition to RapGAPs themselves, multiple RapGAP regulators aberrantly function in breast cancer; thus, it is possible that Rap1 activity is also altered in prostate cancer by modifications of its upstream regulators. For example, it was recently demonstrated that the bromodomain 4 protein (Brd4) can enhance SPA-1 activity, thus promoting inhibition of Rap1

(172). Expression of Brd4 abrogated both tumor growth and metastasis in mice (172). Most interestingly, Brd4 was shown to be a diagnostic marker for breast cancer, as its activation in human breast carcinomas induced a gene expression signature that predicted patient outcome in multiple breast cancer datasets (172). Similarly, genomic analysis revealed gene expression of the afore-mentioned potential negative regulator of Rap1, $G\alpha_z$, correlated with poor prognosis of breast cancer patients (173). Thus, it appears there are multiple mechanisms of Rap1 activation in breast cancer that may also be applicable in and may even be prognostic indicators for prostate cancer.

Our studies suggest Rap1 promotes similar functions in prostate and breast cancer metastasis, although we did observe differences in the ability of Rap1 to promote cancer cell proliferation. We showed modulation of Rap1 regulated the proliferation of metastatic breast cancer cells *in vitro*; however, we observed no significant impact on prostate cancer cell proliferation when we modulated Rap1 activity. This suggests in breast cancer, Rap1 may signal to additional or alternative signaling pathways than in prostate cancer. One of the major similarities we did observe was the importance of integrin function in Rap1-mediated migration and invasion of both prostate and breast cancer cells. In prostate cancer, we identified three specific integrins that Rap1 interacted with: α_4 , β_3 , and $\alpha_v\beta_3$. Specifically, we revealed the requirement for these integrins in Rap1-mediated prostate cancer invasion and migration. Although a Rap1 link to integrin α_4 had been shown before in lymphocytes (174), it had yet to be demonstrated in cancer cells. Rap1, however, did not impact the function of all integrins, as blockade of integrin $\alpha_2\beta_1$ had no effect on Rap1-mediated prostate cancer migration and invasion. In breast cancer, we obtained evidence that Rap1 may influence these same integrins

($\alpha 4$, $\beta 3$, and $\alpha v\beta 3$), by demonstrating the ability of Rap1 to induce MB231 cell migration towards fibronectin, vitronectin, and collagen IV, which are the ECM ligands for these integrins, among others.

Our analysis of Rap1 migration and invasion in breast and prostate cancer is consistent with the processes following the 'elongated invasion' model, which involves cell polarization, pseudopod extension, focal contact formation, ECM proteolysis, actinomyosin contraction, and detachment from the trailing edge (175). The impact of Rap1 activation on integrin function may also involve spatial organization of cell polarity, which is an important part of tissue integrity and architecture, as well as motility. Interestingly, Itoh *et al.* showed that treatment of T4-2 breast cancer cells with an EGFR inhibitor reduced expression of integrin $\beta 1$, but $\beta 1$ integrin expression was not affected in T4-2 cells expressing dominant-active Rap1. They suggested that high levels of Rap1 activation somehow prevented breast cancer cell sensing of the multidimensional ECM environment and thus uncoupled the EGFR and $\beta 1$ integrin signaling pathway (92). Their study suggests Rap1 activation disrupts polarity and thereby impacts integrins in a 3-D environment. Alternatively, our studies in both prostate and breast cancer indicates that stimulation of Rap1 directly impacts integrin activation to promote migration and invasion. The role of Rap1 in integrin signaling has generally been thought to occur through an 'inside-out' signaling mechanism, meaning the direct activation of the integrin emanates from an internal source rather than through an external event such as integrin engagement with the ECM (131). These slightly different results may be explained by the previous studies and ours being performed in different breast cancer cells, in different environments, and at different

stages in metastasis, or simply reflect the capacity of different Rap1 effectors (ours likely being RapL or RIAM) to produce similar outcomes in prostate and breast cancer cells.

My *in vivo* studies demonstrated that inhibition of Rap1 by Rap1GAP1 expression resulted in reduced formation of metastatic breast cancer lesions, while activated Rap1 dramatically enhanced the rate and incidence of prostate cancer metastasis. In particular, results from my intracardiac prostate cancer metastasis model are thus far the best demonstration of the potent ability of Rap1 activation to promote cancer metastasis. Other Rap1 metastasis models in pancreatic and breast cancers have revealed that inhibition of Rap1 reduces local invasion, but only within a relatively discrete anatomical region (22, 141). Another exciting observation from our prostate cancer metastasis model was the propensity of bone lesion formation. This may either be a function of using the bone metastasis-derived PC3 cells or may suggest a more specific function of Rap1 in prostate cancer metastasis to bone. This *in vivo* bone metastasis data also corroborates with our *in vitro* demonstration that Rap1 is stimulated by the bone trophic factor, SDF-1 and promotes migration toward bone-cell derived conditioned media, and that this process involves integrins important in metastasis to bone ($\alpha v \beta 3$ integrin). One interesting future study would be to see if activation of Rap1 produces a gene expression signature that correlates with signatures obtained from cancer that has metastasized to bone.

A relatively unexplored function in the field of Rap1 signaling is its ability to regulate gene expression. We performed real-time PCR analysis and found that expression of activated Rap1 modulated the expression of several adhesion and extracellular matrix genes. In particular, we found Rap1 activation modulated several integrins and MMPs that were crucial to Rap1-mediated invasion and migration. Clues to the mechanism of Rap1 regulation of gene

transcription are currently unknown, although links between Rap1 activation and transcriptional regulation have been reported in a few studies. For example, in squamous cell carcinoma (SCC), Rap1GAP expression decreased expression of cell cycle proteins, cyclin D1, cdk4, and cdk6 (83). Moreover, low levels of Rap2 can promote androgen-dependent transactivation of the androgen receptor and androgen-mediated transcription, whereas higher Rap2 concentrations inhibit these transcriptional events (93). These studies indicate that, in addition to their many other functions, Rap proteins may also play a role in transcriptional regulation. In future studies, it would be interesting to examine this aspect of Rap1 function in various cell contexts.

An emerging feature of Rap1 signaling is its ability to impact secretion. Our analysis in cancer metastasis implies that activation of Rap1 may facilitate MMP secretion, as invasion of prostate cancer cells with activated Rap1 are strikingly more sensitive to MMP inhibition. The only other example of Rap1 involvement with MMPs in cancer suggests the converse, as Rap1GAP expression in SCC cells enhanced invasion and promoted MMP9 secretion (91). Hence, while these results indicate Rap1 may regulate MMP secretion, it remains to be clarified whether, and in what contexts, Rap1 is a positive or negative regulator of MMP secretion.

Studies from endocrine cells perhaps best address the role of Rap1 in secretion; these studies unambiguously brand Rap1 as a positive regulator of secretion. In Chapter 5 of this thesis, we demonstrate that Rap1 promotes secretion of insulin from pancreatic islet β -cells. Prior studies in pancreatic acinar and β -cells have shown that activation of Rap1 facilitates amylase and insulin granule release, respectively (103, 170). Specifically, one group suggested that Epac2 activation of Rap1 does not trigger the exocytosis of insulin granules themselves, but may facilitate insulin granule recruitment to the membrane and/or increase the pool of

membrane-docked granules (103). They came to this hypothesis from the observation that cAMP activation of Rap1 occurred at low glucose concentrations, yet insulin granule fusion events occurred at high glucose concentrations (103). Whether this is a function of Epac2-specific activation of Rap1 or if other RapGEFs can activate Rap1 to promote different steps in the secretory pathway remains to be determined.

We have additionally elucidated another exciting function of Rap1 in pancreatic β -cells that appears to occur through a pathway distinct from GSIS. In chapter 5 of this thesis, I detail a novel Rap1 signaling pathway in pancreatic islet β -cells that leads to ribosomal protein S6 activation and impacts cell proliferation. This pathway involves Epac- and PKA-mediated cAMP stimulation of Rap1, leading to mTOR and S6 activation. Interestingly, although Rap1 has been shown to be able to signal through PI3K in other cell types, PI3K is not required for Rap1-mediated stimulation of S6 activation. Interestingly, in $S6K1^{-/-}$ mice, the pronounced effect on β -cell mass appeared to be due to a decrease in cell size, resulting in a syndrome characteristic of Type 2 diabetes (159). Hence, we were surprised to find that the most significant effects of the Rap1-mediated activation of mTOR and S6 was the enhancement of islet cell proliferation, and not cell size.

There are several possible mechanisms to explain how Rap1 promotes S6 kinase activity in the β -cells. Initially, we postulated that Rac 1 and CDC42 may be involved, as studies have demonstrated that Rap1 is able to stimulate Rac1 (52, 55) and CDC42 (55, 56) activity by recruiting the GEFs for these G proteins to the plasma membrane (52). Furthermore, it is known that Rac1 and CDC42 are able to promote S6K1 activity through a PI3K-independent mechanism (152). However, we feel that this mechanism is unlikely, as expression of activated Rac1 or

activated CDC42 failed to induce S6 phosphorylation to the same extent as activated Rap1. Alternatively, Rap1 may stimulate S6 phosphorylation through the activation of PLC- ϵ (176). Activation of PLC activity produces diacylglycerol (DAG), and this ubiquitous second messenger is able to stimulate a number of effectors (e.g., protein kinase C, protein kinase D, and diacylglycerol kinase) that could induce S6 activity (177) through PI3K-independent pathways. Alternatively, Rap1 may induce S6 activation through a more indirect pathway, possibly involving regulation of integrins. Interestingly, integrin binding has been reported to stimulate S6K1 activity (178), and in some systems this occurs through a PI3K-independent pathway (169). Thus, Rap1 stimulation of S6K1 in the β -cells could proceed through a mechanism dependent on Rap1 activation of integrins. In relation to our cancer metastasis studies, it would be interesting to determine if mTOR S6 activation was involved in the Rap1-mediated breast cancer proliferation we observed, as well as the integrin-mediated migration and invasion induced by Rap1 in both prostate and breast cancer.

Another intriguing model for Rap1 activation of mTOR and S6 signaling may involve the related G protein, Rheb. The main downstream effector of Rheb is mTOR, which regulates both S6K1 and eukaryotic initiation factor 4E-binding protein (4E-BP1) to affect protein translation, cell growth and proliferation (1). Interestingly, the TSC1 protein, tuberin, has been reported to act as a GAP for both Rap1 and Rheb (31). Hence, it is possible that activated Rap1 could sequester tuberin, thereby impacting its ability to act on Rheb and promoting increased levels of Rheb-GTP in cells. Thus, an important future studies would include assessment of the potential the involvement of Rheb in Rap1 activation of this novel mTOR-S6 pathway.

Another interesting future direction would be to analyze the ability of Rap1 to activate the mTOR-S6 pathway in other cell types and biologies. A recent report has implicated PI3K independence in thyroid stimulating hormone (TSH)-mediated stimulation of the mTOR-S6 pathway and thyroid proliferation(179). TSH can induce thyroid hyperplasia and has been linked to Rap1 via its ability to increase cAMP levels in cells; furthermore, Rap1 has been linked to thyroid growth and cancer (86, 180). Our finding that Rap1 can engage a mTOR-S6K1-S6 pathway in pancreatic β -cells should stimulate interest in addressing the role of this pathway in other contexts, such as cancer proliferation and growth.

G protein signaling, and specifically Rap1 signaling, is involved in several fundamental biological processes and diseases. As knowledge of the biological functions of Rap1 increases, so does our ability to connect previously-unlinked signal transduction cascades and to better understand the crosstalk between pathways. In this thesis, I have demonstrated the diverse roles of Rap1 in promoting several cellular processes: migration, invasion, integrin function, proliferation, and secretion. While the precise mechanisms are still not fully understood, my work has established a role for Rap1 in distinct signaling events and has begun to answer some of the key questions about Rap1 function in two diverse biologies: cancer metastasis and pancreatic islet β -cell function.

Appendices

A1. Real –Time PCR Array Gene Expression Analysis (For Chapter 3)

Table 1: RT-PCR Array Gene Expression analysis of ECM and Adhesion molecules

Unigene	GeneBank	Symbol	Description	Fold expression
Hs.218040	NM_000212	ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, CD61)	2.61474
Hs.1695	NM_002426	MMP12	Matrix metalloproteinase 12 (macrophage elastase)	2.45661
Hs.111779	NM_003118	SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	2.38943
Hs.409034	NM_001855	COL15A1	Collagen, type XV, alpha 1	2.18354
Hs.694732	NM_000885	ITGA4	Integrin, alpha 4 (CD49D, α 4 subunit of VLA-4)	2.12383
Hs.171311	NM_003638	ITGA8	Integrin, alpha 8	1.9816
Hs.57697	NM_001523	HAS1	Hyaluronan synthase 1	1.77358
Hs.314543	NM_001332	CTNND2	Catenin (cadherin-associated protein)	1.73708
Hs.166011	NM_001331	CTNND1	Catenin (cadherin-associated protein), δ 1	1.63203
Hs.476092	NM_003278	CLEC3B	C-type lectin domain family 3, member B	1.60956
Hs.200841	NM_000426	LAMA2	Laminin, alpha 2	1.51222
Hs.2936	NM_002427	MMP13	Matrix metalloproteinase 13 (collagenase 3)	1.47087
Hs.210283	NM_000093	COL5A1	Collagen, type V, alpha 1	1.43065
Hs.161839	NM_002424	MMP8	Matrix metalloproteinase 8 (neutrophil collagenase)	1.33484
Hs.2257	NM_000638	VTN	Vitronectin	1.16205
Hs.2256	NM_002423	MMP7	Matrix metalloproteinase 7 (matrilysin, uterine)	1.13813
Hs.81071	NM_004425	ECM1	Extracellular matrix protein 1	1.13813
Hs.133397	NM_000210	ITGA6	Integrin, alpha 6	1.11471
Hs.445981	NM_001903	CTNNA1	Catenin (cadherin-associated protein), α 1	1.10701

Hs.544577	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	1.05458
Hs.265829	NM_002204	ITGA3	Integrin, alpha 3 (CD49C, α 3 subunit of VLA-3)	1.03288
Hs.169875	NM_007112	THBS3	Thrombospondin 3	1.00463
Hs.101302	NM_004370	COL12A1	Collagen, type XII, alpha 1	1.00463
Hs.131433	NM_139025	ADAMT S13	ADAM metallopeptidase with thrombospondin type 1 motif, 13	1.00463
Hs.520640	NM_001101	ACTB	Actin, beta	-1.0163
Hs.536663	NM_002213	ITGB5	Integrin, beta 5	-1.0163
Hs.546267	NM_005941	MMP16	Matrix metallopeptidase 16 (membrane-inserted)	-1.0163
Hs.375129	NM_002422	MMP3	Matrix metallopeptidase 3 (stromelysin1, progelatinase)	-1.0234
Hs.368921	NM_001856	COL16A1	Collagen, type XVI, alpha 1	-1.0234
Hs.505654	NM_002205	ITGA5	Integrin, alpha 5 (fibronectin receptor, α)	-1.0377
Hs.523185	NM_012423	RPL13A	Ribosomal protein L13a	-1.0377
Hs.270364	NM_005559	LAMA1	Laminin, alpha 1	-1.0521
Hs.633514	NM_003255	TIMP2	TIMP metallopeptidase inhibitor 2	-1.0817
Hs.650585	NM_002291	LAMB1	Laminin, beta 1	-1.0892
Hs.502328	NM_000610	CD44	CD44 molecule	-1.1355
Hs.476218	NM_000094	COL7A1	Collagen, type VII, alpha 1	-1.1434
Hs.476018	NM_001904	CTNNB1	Catenin (cadherin-associated protein), β 1	-1.1434
Hs.654548	NM_001850	COL8A1	Collagen, type VIII, alpha 1	-1.1674
Hs.609663	NM_002293	LAMC1	Laminin, gamma 1 (formerly LAMB2)	-1.1837
Hs.508716	NM_001846	COL4A2	Collagen, type IV, alpha 2	-1.1837
Hs.524484	NM_002206	ITGA7	Integrin, alpha 7	-1.192
Hs.371147	NM_003247	THBS2	Thrombospondin 2	-1.192
Hs.474053	NM_001848	COL6A1	Collagen, type VI, alpha 1	-1.2002
Hs.420269	NM_001849	COL6A2	Collagen, type VI, alpha 2	-1.2002
Hs.271605	NM_007037	ADAMT S8	ADAM metallopeptidase with thrombospondin type 1 motif,	-1.2086

8				
Hs.371199	NM_003919	SGCE	Sarcoglycan, epsilon	-1.217
Hs.643357	NM_006988	ADAMT S1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	-1.2255
Hs.497636	NM_000228	LAMB3	Laminin, beta 3	-1.234
Hs.436873	NM_002210	ITGAV	Integrin, alpha V (vitronectin receptor, α polypeptide, CD51)	-1.2426
Hs.701968	NM_000362	TIMP3	TIMP metallopeptidase inhibitor 3	-1.2512
Hs.164226	NM_003246	THBS1	Thrombospondin 1	-1.2687
Hs.143250	NM_002160	TNC	Tenascin C (hexabrachion)	-1.2775
Hs.172631	NM_000632	ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	-1.3134
Hs.707987	NM_002211	ITGB1	Integrin, beta 1 (fibronectin receptor, β polypeptide, CD29)	-1.3226
Hs.375957	NM_000211	ITGB2	Integrin, beta 2	-1.3692
Hs.461086	NM_004360	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	-1.3692
Hs.412707	NM_000194	HPRT1	Hypoxanthine phosphor- ribosyltransferase 1	-1.3787
Hs.514412	NM_000442	PECAM1	Platelet/endothelial cell adhesion molecule (CD31 antigen)	-1.398
Hs.707983	NM_000201	ICAM1	Intercellular adhesion molecule 1 (CD54), ICAM	-1.398
Hs.591346	NM_001901	CTGF	Connective tissue growth factor	-1.4373
Hs.143751	NM_005940	MMP11	Matrix metallopeptidase 11 (stromelysin 3)	-1.4777
Hs.696076	NM_181501	ITGA1	Integrin, alpha 1	-1.4983
Hs.185597	NM_003119	SPG7	Spastic paraplegia 7	-1.5511
Hs.503878	NM_000615	NCAM1	Neural cell adhesion molecule 1	-1.5728
Hs.632226	NM_000213	ITGB4	Integrin, beta 4	-1.5948
Hs.522632	NM_003254	TIMP1	TIMP metallopeptidase inhibitor 1	-1.6058
Hs.83169	NM_002421	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	-1.651

Hs.80343	NM_002428	MMP15	Matrix metallopeptidase 15 (membrane-inserted)	-1.651
Hs.369397	NM_000358	TGFBI	Transforming growth factor, β - induced, 68kDa	-1.651
Hs.73800	NM_003005	SELP	Selectin P (granule membrane protein 140kDa, CD62)	-1.6974
Hs.521869	NM_000216	KAL1	Kallmann syndrome 1 sequence	-1.7451
Hs.82848	NM_000655	SELL	Selectin L (lymphocyte adhesion molecule 1)	-1.7573
Hs.143434	NM_001843	CNTN1	Contactin 1	-1.8704
Hs.203717	NM_002026	FN1	Fibronectin 1	-1.8704
Hs.695930	NM_004385	VCAN	Versican	-1.9097
Hs.2399	NM_004995	MMP14	Matrix metallopeptidase 14 (membrane-inserted)	-2.0046
Hs.534255	NM_004048	B2M	Beta-2-microglobulin	-2.0753
Hs.482077	NM_002203	ITGA2	Integrin, alpha 2 (CD49B, α 2 subunit of VLA-2 receptor)	-2.1043
Hs.436367	NM_000227	LAMA3	Laminin, alpha 3	-2.1785
Hs.2258	NM_002425	MMP10	Matrix metallopeptidase 10 (stromelysin 2)	-2.2553
Hs.297413	NM_004994	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa type IV collagenase)	-2.4509
Hs.409662	NM_021110	COL14A1	Collagen, type XIV, alpha 1	-2.7195
Hs.89546	NM_000450	SELE	Selectin E (endothelial adhesion molecule 1)	-3.793
Hs.513617	NM_004530	MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa type IV collagenase)	-4.1795
Hs.109225	NM_001078	VCAM1	Vascular cell adhesion molecule 1	-5.4014
Hs.174103	NM_002209	ITGAL	Integrin, alpha L (LFA-1; α)	-5.6308
Hs.313	NM_000582	SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I)	-7.3785

A2. G α_z Negatively Regulates Insulin Secretion and Glucose Clearance¹

A2.1 Introduction

Insulin secretion from the β -cells of the pancreas follows a biphasic pattern, in which an initial peak minutes after stimulation by glucose is followed by a lower-magnitude sustained phase over the duration glucose stimulation. This phenomenon is thought to be due to the interaction of two signaling pathways: the triggering pathway and the amplifying pathway (181, 182). In the initial (triggering) phase of insulin secretion, metabolism of glucose in the mitochondria leads to an increase in cytosolic ATP/ADP ratio, which causes the closure of the ATP-sensitive K⁺ channel (K_{ATP} channel)¹ and membrane depolarization. The resulting membrane depolarization opens voltage-dependent calcium channels (VDCCs), allowing an influx of extracellular Ca²⁺, and stimulation of calcium-induced calcium release from the endoplasmic reticulum. These intracellular changes lead to exocytosis of a readily-releasable pool of insulin granules, a process that involves ATP-dependent insulin granule priming, granule fusion, and insulin release. The amplifying pathway of insulin secretion is also dependent on glucose, but does not elevate the intracellular Ca²⁺ concentrations, and is therefore referred to as the K_{ATP} channel-independent pathway (183); the mechanisms underlying this pathway are not as well-defined as those of the triggering pathway, but may involve an increase in the efficiency of the exocytosis process and/or the replenishment of the readily-releasable granule pool.

Heterotrimeric G proteins are involved in both the positive and negative regulation of insulin secretion (184, 185). There are four subfamilies of the GTP-binding G protein α subunits,

¹ This is a publication in which I was a third author because of my contribution to many of the mouse studies (99).
Kimble ME, Joseph JW, Bailey CL, et al. Galphaz negatively regulates insulin secretion and glucose clearance.
J Biol Chem 2008;283:4560-7.

classified based on sequence similarities and signaling pathways engaged: $G\alpha_q$, $G\alpha_s$, $G\alpha_i$, and $G\alpha_{12}$. In terms of pancreatic β -cell physiology, acetylcholine can activate M3 muscarinic receptors that are coupled to $G\alpha_q$, leading to Ca^{2+} release from intracellular stores and activation of protein kinase C, both of which augment insulin secretory granule release (186). PACAP-, GLP-, and GIP-specific receptors all couple primarily to adenylyl cyclase-stimulating $G\alpha_s$ (187), leading to an increase in cAMP and activation of its downstream signaling cascades. cAMP action requires glucose and is primarily thought of as impacting the amplifying phase of insulin secretion (188), but has also been shown to have effects on proximal events such as expression of key genes involved in metabolism and augmentation of the closure of the K_{ATP} channel (187). Conversely, $G\alpha_i$ proteins have been shown to block insulin secretion both by inhibition of adenylyl cyclase and by direct regulation of the K_{ATP} channel, VDCCs, and/or the exocytosis process itself (184, 185). Examples of agonists that work through $G\alpha_i$ -coupled receptors are epinephrine, galanin, somatostatin, and E-prostaglandins (185), among several others.

Because of the lack of a required cysteine residue, $G\alpha_z$ is the only member of the $G\alpha_i$ subfamily of G protein α subunits, the largest of the four $G\alpha$ subfamilies (189), that is immune to inactivation by pertussis toxin (PTX)-catalyzed ADP-ribosylation (190). Other unique $G\alpha$ characteristics are a very low GDP-to-GTP exchange rate that is reduced 20-fold further in the presence of physiologic concentrations of Mg^{2+} and an extremely low intrinsic GTP hydrolysis rate that is 200-fold lower than that described for other $G\alpha$ subunits (191). $G\alpha_z$ also displays a limited expression profile, and protein expression has been demonstrated conclusively only in the brain, adrenal medulla, retina, platelets, pituitary, and pancreatic islets (98, 190, 192).

Initial work studying the role of $G\alpha_z$ in regulating insulin secretion took advantage of INS-1-derived 832/13 cells (a clonal pancreatic β -cell line) (151). As expected for a member of the $G\alpha_i$ subfamily, constitutively-activated $G\alpha_z$ inhibited GSIS from 832/13 cells (98). More importantly, inhibition of insulin secretion by prostaglandin E1 was PTX-insensitive, and could be blocked either by overexpression of the “Regulator of G protein Signaling” (RGS) domain of RGSZ1, a $G\alpha_z$ -specific deactivator (193), or by siRNA-mediated knockdown of $G\alpha_z$ expression (98). Together, these data indicated that endogenous $G\alpha_z$ in the 832/13 cells coupled to endogenous G protein-coupled receptors (GPCRs) to inhibit insulin secretion. However, the question of whether $G\alpha_z$ was important for regulation of GSIS *in vivo* remained unanswered.

The generation of a $G\alpha_z$ -knockout mouse has provided an opportunity to study the involvement of this $G\alpha$ protein in physiology. Thus far, the phenotypes associated with $G\alpha_z$ deletion are moderate consequences on platelet function (194, 195) and altered behavioral and CNS responses to serotonin, morphine, apomorphine, amphetamine, and dopamine agonists (195-198); the effect of $G\alpha_z$ knockout on insulin secretion and glucose homeostasis in these mice has not been determined. We show here that $G\alpha_z$ -null mice have increased insulin secretion after glucose challenge and a corresponding decrease in blood glucose levels. We also show a potentiation of insulin secretion in islets from $G\alpha_z$ -null mice. The findings from these studies may have implications for development of novel therapeutic agents for impaired β -cell function and Type 2 diabetes.

A2.2 Materials and Methods

Generation of the $G\alpha_z$ knockout mouse and animal care. $G\alpha_z$ knockout mice were originally derived by targeting the gene in the C57BL/6 mouse strain and backcrossing more

than 10 times into the Balb/c strain (194). Mice were housed 5 or less per cage, with a 12 h light/dark cycle and *ad libitum* access to chow and water. Animals were handled in accordance with the principles and guidelines established by the Duke University Animal Care and Use Committee. $G\alpha_z$ -null and wild-type control mice were generated by heterozygote matings to produce littermate controls.

Glucose tolerance tests. Nine- to eleven-week-old mice were fasted 12 h and an intraperitoneal (ip) glucose tolerance test (IPGTT) was performed as previously described (199). Because the glucose values measured during the assay in male mice of both genotypes were influenced more dramatically likely by the stress of the assay (data not shown), all experiments were conducted with female mice only. Plasma insulin was measured at 0 and 5 min during the IPGTT, and blood glucose values determined at 5, 10, 15, 30, 60, and 120 min post-injection. Glucose readings were taken from tail blood using a BD Logic™ Meter (BD, Franklin Lakes, NJ). The glucose readings were averaged within genotypes at each time point, giving the means \pm SE. Oral glucose tolerance tests (OGTTs) were conducted essentially as described for the IPGTTs, except that the glucose was administered by oral gavage after a 20 h fast, and the post-glucose blood sample for insulin determination was taken at 10 min instead of 5 min.

Plasma insulin ELISAs. During the IPGTTs, blood samples were collected into heparinized tubes and centrifuged at 3,000 x g for 15 min. The clarified plasma layer was transferred to a new tube, snap-frozen, and stored at -80 °C. On the day of the assay, the plasma samples were thawed, and 5 μ L was used for each replicate of an insulin ELISA according to the manufacturer's protocol (Rat Insulin ELISA Kit, Crystal Chem Inc., Downers Grove, IL). One to four replicates of each plasma sample were performed depending on total sample volume; the insulin values for

all of the replicates of a single sample were averaged, and the averages for all three experiments were used to calculate the means \pm SE.

Insulin Tolerance Tests. Insulin tolerance tests (ITTs) were performed essentially as previously described (199). Eleven-week-old female mice were fasted for 4 h and then injected ip with 2 U/kg B.W. insulin lispro, using a solution of 0.08 U/ml Humalog® and sterile insulin diluent (Lilly, Indianapolis, IN). Glucose readings were taken at 0, 30, 50, 80, and 120 min post-injection. The glucose readings were averaged within genotypes at each time point, giving the means \pm SE.

Pancreatic islet isolation and insulin secretion assays. Islet isolation and insulin secretion assays were performed essentially as previously described (200). After overnight incubation to recover secretion capacity, the islets were picked into a dish containing Krebs Ringer Bicarbonate Solution (KRB: 4.38 mM KCl, 1.2 mM MgSO₄, 1.5 mM KH₂PO₄, 129 mM NaCl, 5 mM NaHCO₃, 10 mM HEPES, 3.11 mM CaCl₂, 0.25% BSA, pH 7.4) with 2.8 mM D-glucose, and incubated at 37 °C/5% CO₂ for 1 h. Twelve islets were picked into a tube containing 250 μ l KRB with the indicated concentrations of glucose for each replicate, and incubated at 37 °C/5% CO₂ for 2 h. After the incubation, samples were taken for insulin assay (Coat-A-Count® Insulin, Diagnostic Products Corporation, Los Angeles, CA). The insulin secretion values for each glucose-concentration replicate (normalized to its DNA content) were averaged, and the averages from all three experiments were used to calculate the means \pm SE.

cAMP production assays. Islets were isolated and preincubated in KRB as described above for insulin secretion assays. For each replicate, twenty islets were picked into a tube containing 250 μ l KRB with 0.1 M of the phosphodiesterase (PDE) inhibitor 3-isobutyl-1-

methylxanthine (IBMX; Sigma, St. Louis, MO) and the indicated concentrations of glucose. When specified, 20 nM exendin-4 (Sigma) was added in addition to stimulatory glucose. After 15 min, the islets were pelleted by a centrifugal pulse and the KRB removed. Islets were immediately lysed for cAMP ELISA according to the manufacturer's protocol (cAMP Biotrak™ Enzymeimmunoassay System; GE Healthcare, Piscataway, NJ). Each sample replicate (5 replicates per glucose concentration) was measured in triplicate, and the averaged replicates for each concentration were averaged from all three experiments to calculate the means \pm SE.

Immunoblot analyses. Islets (100-150 of each genotype) were washed in PBS, pelleted at 500 xg for 5 min, re-suspended in 1X Laemmli sample buffer, and subjected to 15% SDS/PAGE. Proteins were transferred to nitrocellulose using a tank-transfer apparatus. The membrane was incubated with 1:1000 rabbit anti-G α_z (Santa Cruz Biotechnology, Santa Cruz, CA) followed by 1:3000 goat anti-rabbit-horseradish peroxidase (HRP) conjugate (GE Healthcare) according to standard protocols. HRP was detected using Western Lightning® chemiluminescence reagent (PerkinElmer, Boston, MA) and the membrane was exposed to film. The membrane was stripped with Restore™ Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, IL), washed, re-blocked, and incubated with 1:1000 mouse anti- α -tubulin (Santa Cruz Biotechnology) followed by 1:3000 goat anti-mouse-HRP conjugate (GE Healthcare). HRP was detected as above.

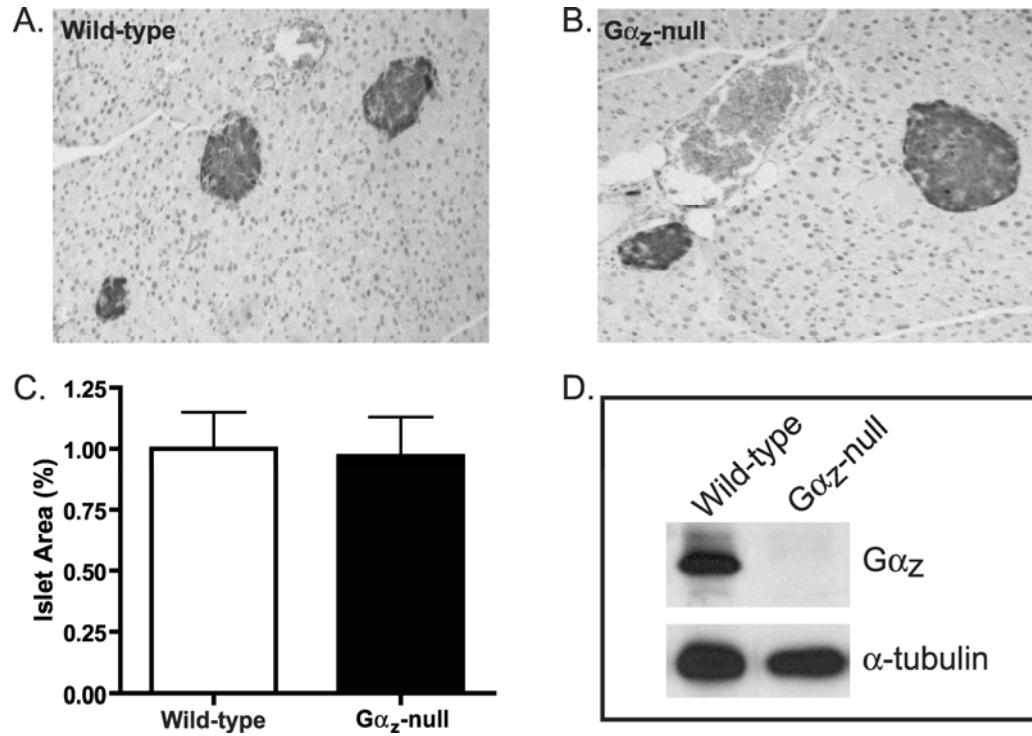
Immunohistochemical assays. Tissues were dissected, fixed, and sectioned as previously described (98). For insulin immunostaining, the HistoMouse-MAX kit (Invitrogen) was used with prediluted guinea pig anti-insulin primary antibody (Invitrogen) incubated for 1 h at room temperature. The substrate used for colorimetric detection of the HRP-coupled secondary antibody was 3,3'-diaminobenzidine. Sections were lightly counterstained with hematoxylin.

Islet area was determined using ImageJ software (NIH, Bethesda, MD) on two independent sections from three mice of each genotype.

Statistical analyses. Data were analyzed using GraphPad Prism v. 4 (GraphPad Software Inc., San Diego, CA). An unpaired t-test or two-way ANOVA was used to determine the P value as appropriate. A $P < 0.05$ was considered significant.

A2.3 Results

Islets from $G\alpha_z$ -null and wild-type mice are morphologically identical. Before beginning the task of analyzing the *in vivo* and *in vitro* insulin secretory capacity of $G\alpha_z$ -null islets, it was crucial to assess whether any potential differences in secretion that were observed might be due to an overall change in the islet morphology between the two genotypes. To assess potential morphological differences between the pancreatic islets of $G\alpha_z$ -null and wild-type control mice, pancreases from three mice of each genotype were dissected, fixed, paraffin-embedded, and stained for insulin (Figs. 20A and 20B). As insulin-containing β -cells comprise the majority of the mouse islet, measuring the β -cell area as a function of total pancreatic area analyzed is a reliable measure of the islet area. In fact, the measurements of islet area were nearly identical between the two genotypes (Fig. 20C), suggesting that there was no net increase in either β -cell number or size that could account for potential differences in insulin secretion from $G\alpha_z$ -null islets. Finally, extracts were prepared from 100-150 islets isolated from $G\alpha_z$ -null or wild-type control mice, subjected to SDS/PAGE, and transferred to nitrocellulose for immunoblotting. The membrane was probed for $G\alpha_z$ and α -tubulin content. Islets from $G\alpha_z$ -null mice were completely deficient in $G\alpha_z$, confirming the loss of $G\alpha_z$ expression as expected (Fig. 20D).



Appendix Figure 20: Morphogenic analysis of pancreatic islets of 10-12-week-old wild-type and $G\alpha_z$ -null mice. A and B, Representative insulin immunostaining of a pancreas section from a wild-type and $G\alpha_z$ -null mouse, respectively. Pancreases from 3 mice of each genotype were formalin-fixed, paraffin-embedded, and non-serial sections from each pancreas were stained with an anti-insulin antibody. C, Quantification of islet area using insulin-stained sections from wild-type and $G\alpha_z$ -null mice. Two independent pancreas sections from each of three mice of each genotype were used for determination of islet area as a function of pancreas area. D, Analysis of $G\alpha_z$ expression in islets isolated from $G\alpha_z$ -null and wild-type mice. Extracts were prepared from 100-150 islets of each genotype, and $G\alpha_z$ expression assessed by immunoblot analysis. α -tubulin levels were probed as control for protein levels. Results are representative of three independent experiments.

$G\alpha_z$ -null mice display increased glucose clearance correlating to increased plasma insulin levels. To determine the effect of knockout of $G\alpha_z$ on glucose homeostasis, IPGTTs were performed after a 12 h fast on $G\alpha_z$ -null mice and wild-type controls. During the IPGTTs, $G\alpha_z$ -null

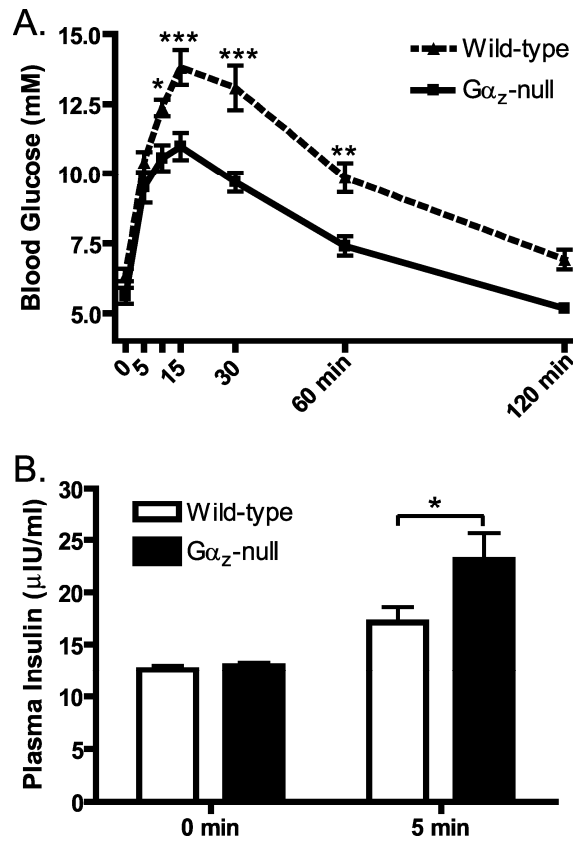
mice had blood glucose levels that were significantly different at 10, 15, 30, and 60 min after injection of glucose (Fig. 21A). Area under the curve (AUC) analyses on both the $G\alpha_z$ -null and wild-type IPGTT profiles indicated that, while the $G\alpha_z$ -null mice did display slightly lower fasting glucose levels (corresponding to t=0), the glucose AUC, which is inversely proportional to the glucose clearance, was significantly lower in the $G\alpha_z$ -null mice (449.0 ± 40.8 mM/120 min, wild-type; vs. 264.2 ± 30.3 mM/120 min, $G\alpha_z$ -null; Table 2). If the differences in blood glucose had remained the same at each time point, identical glucose AUC's would have been observed. Furthermore, the time to peak glucose level was shorter on average in the $G\alpha_z$ -null mice (mean: 17.3 ± 1.8 in wild-type vs. 12.3 ± 1.0 in $G\alpha_z$ -null mice; range: 10 to 30 min vs. 5 to 15 min, respectively), and the peak glucose levels were dramatically lower (about 3 mM less) (Table 2).

Appendix Table 2: Glucose clearance in $G\alpha_z$ -null vs. wild-type mice. The baseline was set as the glucose reading at t=0 min, and area under the curve (AUC) analyses performed on IPGTT data from $G\alpha_z$ -null and wild-type mice. Data are in mean \pm SE. *, $P < 0.05$, **, $P < 0.01$.

Parameter	Wild-type	$G\alpha_z$-null
Baseline (mM)	6.37 ± 0.22	$5.63 \pm 0.29^*$
AUC (mM/120 min)	449.0 ± 40.8	$264.2 \pm 30.3^{**}$
Peak time [min (range)]	17.3 ± 1.8 (10-30)	$12.3 \pm 1.0^*$ (5-15)
Peak glucose (mM)	14.30 ± 0.71	$11.18 \pm 0.49^{**}$

To determine whether increased insulin secretion after glucose load could account for the increased glucose clearance of the $G\alpha_z$ -null mice, plasma insulin concentrations were measured from blood samples collected before and during the IPGTTs. $G\alpha_z$ -null mice did not display significantly different fasting plasma insulin levels (corresponding to t=0 of the IPGTT); however, they did have significantly increased plasma insulin levels in the plasma sample taken 5 min after the glucose load (Fig. 21B). This increased insulin concentration is consistent with

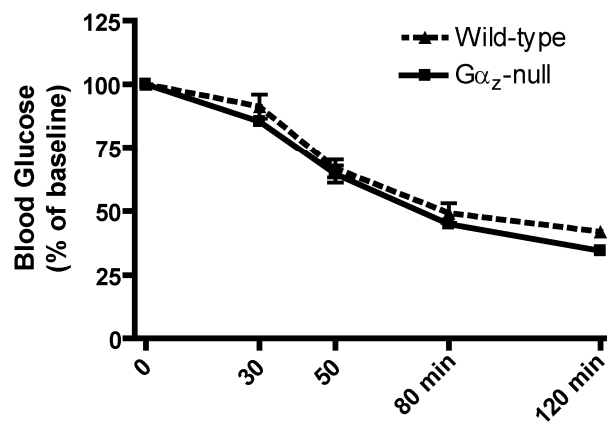
the faster time to peak blood glucose levels and the lower peak blood glucose levels observed in the $G\alpha_z$ -null mice (Fig. 21A and Table 2).



Appendix Figure 21: Glucose clearance and insulin secretion in $G\alpha_z$ -null mice and wild-type controls. A, Intraperitoneal glucose tolerance tests (IPGTTs) of 9-11-week-old wild-type control and $G\alpha_z$ -null mice, performed after a 12 h fast. Blood glucose levels were measured at progressive time points after injection of 2 g/kg D-glucose. N=14 (wild-type), 11 ($G\alpha_z$ -null). B, Plasma insulin levels in wild-type control and $G\alpha_z$ -null mice before glucose injection and 5 min after glucose injection during the IPGTT. Approximately 50-100 μ L of blood was massaged from the tail at t=0 and 5 min after glucose load to generate plasma samples for analysis. N=8 (wild-type), 6 ($G\alpha_z$ -null). *, P<0.05; **, P<0.01; ***, P<0.001.

To determine whether decreased glucose levels might be also be due to increased insulin sensitivity, ITTs were performed on 9-11-week-old female $G\alpha_z$ -null mice and wild-type

controls. After a 4 h fast, the $G\alpha_z$ -null mice had about 1 mM lower blood glucose levels than wild-type mice (data not shown), similar to the difference observed after a 12 h fast (Fig. 21A, t=0). Unlike during the IPGTTs, though, this difference remained nearly the same at each time point after injection of 2 U/kg insulin lispro; thus, no differences were observed in the change in blood glucose concentrations over time when plotted as percent of baseline between the wild-type and $G\alpha_z$ -null mice (Fig. 22). The results of this experiment suggest that the decreased blood glucose levels in the $G\alpha_z$ -null mice are not due to increased insulin sensitivity.

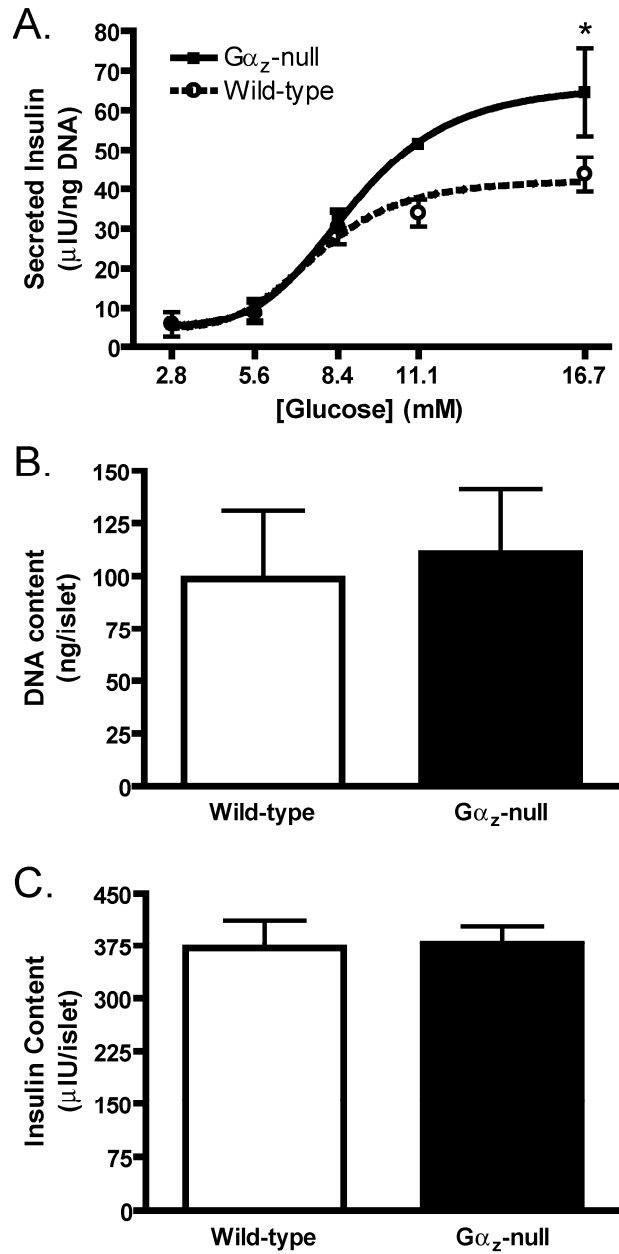


Appendix Figure 22: Insulin sensitivity of $G\alpha_z$ -null and wild-type mice. Insulin tolerance tests (ITTs) were performed on 9-11-week-old $G\alpha_z$ -null and wild-type control mice to assess whether the $G\alpha_z$ -null mice had increased insulin sensitivity. Blood glucose readings were taken after a 4 h fast, and were measured at progressive time points after injection of 2U/kg insulin lispro. The values were normalized to percent of baseline. N=6 (wild-type), 6 ($G\alpha_z$ -null).

Islets isolated from $G\alpha_z$ -null mice display an increased GSIS response. To determine whether increased plasma insulin levels after glucose load in the $G\alpha_z$ -null mice were due to increased pancreatic islet insulin secretion, islets were isolated from $G\alpha_z$ -null mice and wild-type littermate controls. After an overnight recovery in islet medium, insulin secretion assays were

performed at various concentrations of glucose. While the insulin secretion from $G\alpha_z$ -null and wild-type islets was nearly indistinguishable at 2.8 and 5.6 mM glucose, the $G\alpha_z$ -null islets began to secrete more insulin at stimulatory concentrations of glucose (8.4-16.7 mM; Fig. 23A). Curve-fit analyses of insulin secretion data from $G\alpha_z$ -null and wild-type islets reflected these observations; the baseline insulin secretion and EC_{50} of glucose for insulin secretion of the two genotypes were almost indistinguishable, while the maximal response was significantly higher in $G\alpha_z$ -null islets (Table 2). The results from these isolated islet secretion studies are consistent with both the improved glucose clearance and the increased plasma insulin levels observed in $G\alpha_z$ -null mice during the IPGTTs (Figs. 21A and 21B; Table 2).

To confirm that the differences observed were not due to increased islet number, size, or insulin content, DNA and insulin were extracted from each experimental replicate (12 islets per replicate) and their levels measured. Consistent with the absence of an effect of loss of $G\alpha_z$ expression on islet morphology (Fig. 20), there were no significant differences in the DNA or insulin content of the islets isolated from $G\alpha_z$ -null and wild-type mice (Figs. 23B and 23C). Together, these results provide compelling evidence that the differences observed between the GSIS response of $G\alpha_z$ -null and wild-type islets is due to an effect on GSIS itself, and not an artifact of a change in gross islet morphology or physiology.

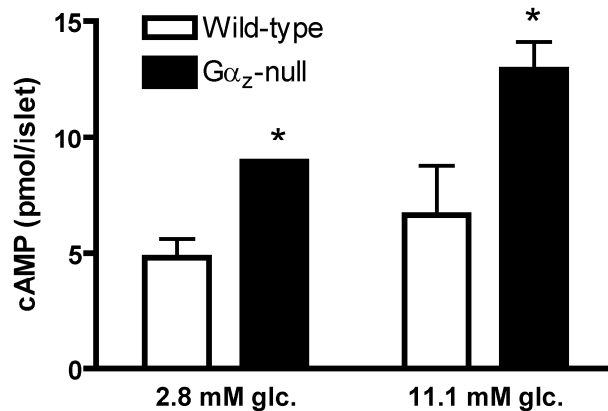


Appendix Figure 23: $G\alpha_2$ -null mice display an increased GSIS response. A, Insulin secretion of islets isolated from 10-12-week-old $G\alpha_2$ -null and wild-type control mice was elicited using increasing concentrations of glucose, and measured using an insulin RIA. $G\alpha_2$ -null mice demonstrated significantly higher insulin secretion at stimulatory glucose concentrations. B, DNA content per islet for $G\alpha_2$ -null and wild-type control mice. C, Insulin content per islet for $G\alpha_2$ -null and wild-type control mice. Insulin assays were performed and described under “Experimental Procedures”, and values were normalized to DNA content (see panel B). Each

experiment was performed on islets pooled from 2 mice of each genotype (6 mice each total), with 3-6 replicates for each glucose concentration. N=3. *, P<0.05.

A tonic inhibition of adenylyl cyclase is likely the mechanism for $G\alpha_z$'s inhibition of GSIS.

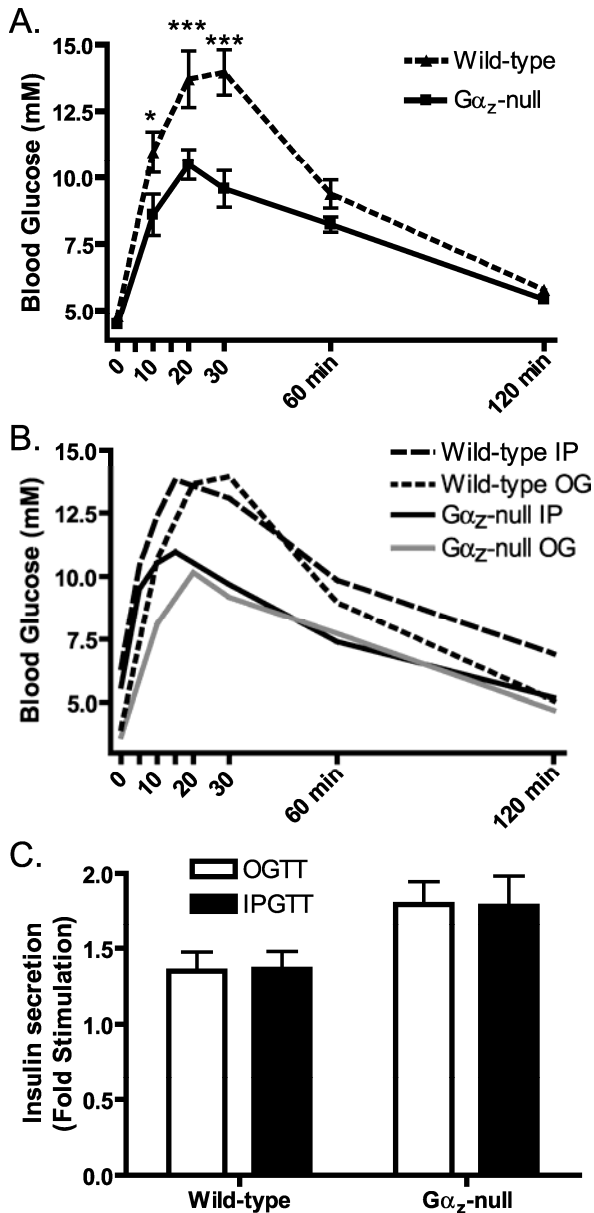
$G\alpha_z$ is a member of the $G\alpha_i$ subfamily of $G\alpha$ subunits, whose classical signaling mechanism is a decrease in cAMP production resulting from the inhibition of adenylyl cyclase. Furthermore, cAMP is a known modulator of multiple steps in the GSIS pathway. As such, an effect on cAMP levels seemed a logical mechanism for $G\alpha_z$'s action, and loss of this effect could underly the GSIS phenotype observed in $G\alpha_z$ -null mice. In order to determine whether an impact on modulation of cAMP levels could be the mechanism through which deletion of $G\alpha_z$ impacts on insulin secretion, cAMP levels were determined in islets isolated from $G\alpha_z$ -null and wild-type mice. An observed increase in cAMP production in $G\alpha_z$ -null islets would explain the correspondingly higher magnitude of insulin secretion in these islets (Figs. 2B and 4A). In fact, $G\alpha_z$ -null islets displayed significantly higher cAMP production in both low and stimulatory glucose concentrations (Fig. 5). These data suggest that genetic deletion of $G\alpha_z$ relieves a tonic inhibition of adenylyl cyclase, independent of stimulation with glucose or other agonists. As cAMP does not itself stimulate insulin secretion, but rather only potentiates GSIS, this finding also explains why increased insulin secretion is only observed in $G\alpha_z$ -null mice and islets under conditions of stimulatory glucose concentrations (Figs. 2B and 4A).



Appendix Figure 24: cAMP levels in Gα₂-null vs. wild-type control mice. cAMP levels in islets isolated from 16-week-old Gα₂-null and wild-type control mice were determined as described under “Experimental Procedures”. Islets were incubated in buffer containing 0.1 M IBMX and 2.8 mM or 11.1 mM glucose for 15 min, after which the medium was removed and islets were lysed for measurement of cAMP. Each experiment was performed on islets pooled from 2-3 mice of each genotype (8 wild-type and 7 Gα₂-null mice, total), with 3-5 replicates for each glucose concentration. N=3. *, P<0.05.

We also performed an OGTT analysis in order to examine the role of Gα₂ in maintaining glucose homeostasis in a more typical physiologic situation. Incretins such as GLP-1 and GIP, which activate G_s-coupled receptors in the β-cell and might oppose the actions of Gα₂, are released from the gastric mucosa upon GI absorption of glucose. Similar to that observed with IPGTT, during the OGTT Gα₂-null mice had blood glucose levels that were significantly different at 10, 20, and 30 min after injection of glucose (Fig. 6A). These glucose levels during the OGTT were almost indistinguishable from those observed during the IPGTT, as can be seen by overlay of the two sets of curves (Fig. 6B). The peak glucose levels occurred approximately 10 min. later and 5 min. later in the OGTT as compared to the IPGTT for wild-type and Gα₂-null mice, respectively, which is consistent with glucose having to be absorbed through the GI tract. Besides the lower starting glucose levels due to a longer fast in the OGTT, there were no

significant differences between the data obtained during IPGTT vs. OGTT within a genotype, except at 10 min. for the $G\alpha_z$ -null mice ($P < 0.05$), a reflection of the faster time to peak glucose. These results suggest that if $G\alpha_z$ plays a role in regulating incretin-potentiated insulin secretion, it is likely to be modest. This is supported by a similar increase in plasma insulin levels within each genotype during the OGTT as compared to the IPGTT (Fig. 6C). Furthermore, the stable GLP-1 analog exendin-4 (Ex-4; exenatide) elicited a similar augmentation of cAMP levels in islets isolated from wild-type and $G\alpha_z$ -null mice (data not shown).



Appendix Figure 25: The effect of incretins on glucose clearance and insulin secretion in $G\alpha_2$ -null mice and wild-type controls. A. Oral glucose tolerance tests (OGTTs) of $G\alpha_2$ -null mice and wild-type controls. Blood glucose levels were measured at progressive time points after administration of 2 g/kg D-glucose by OG to 20-week-old wild-type control and $G\alpha_2$ -null mice after a 20 h fast. N=8 (wild-type), 8 ($G\alpha_2$ -null). B. Blood glucose levels observed during the OGTT as compared to the IPGTT, as represented by overlay of the mean blood glucose levels from Fig 2A (IPGTT) and 6A (OGTT). C. The increase in insulin observed in the plasma of $G\alpha_2$ -null mice and wild-type controls with oral administration of glucose (OGTT) as compared to ip injection (IPGTT). *, $P < 0.05$; ***, $P < 0.001$.

A2.4 Discussion

It is currently appreciated that both β -cell dysfunction and insulin resistance are early and necessary events in the development of Type 2 diabetes (142). The present study focused on the role of $G\alpha_z$ in regulating insulin secretion in a mouse model system. $G\alpha_z$ has been previously shown to modulate an endogenous GSIS inhibitory pathway in an insulinoma cell line (98); therefore, we hypothesized that $G\alpha_z$ -null mice would display increased GSIS and correspondingly decreased blood glucose levels as compared to wild-type control mice. These phenotypes were indeed observed, and point to $G\alpha_z$ as a critical component of the cellular mechanisms responsible for β -cell homeostasis.

$G\alpha_z$ is the least understood member of the $G\alpha_i$ subfamily of heterotrimeric G proteins. Previous rodent models have been used to study the potential involvement of other $G\alpha_i$ subfamily members in insulin secretion. Injection of rats with pertussis toxin (PTX; originally named "islet activating protein") or incubation of isolated rat islets with PTX augmented the effects of various secretagogues, and blocked the ability of $G\alpha_i$ coupled receptor agonists, such as somatostatin and epinephrine, to inhibit GSIS (201). Interestingly, however, no insulin secretion phenotype has been reported in mouse lines deficient in the PTX-sensitive $G\alpha_i$ subfamily members, which include $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$. A likely explanation is that there exists some level of redundancy of these $G\alpha$ isoforms in islet cell biology, as has been shown in other systems (189). $G\alpha_o$ knockout mice do display changes in the nervous system structure and aberrant Ca^{2+} channel signaling in the heart, but are normoglycemic (202). Interestingly, loss of $G\alpha_{i2}$ expression does not impact insulin secretion, but instead leads to insulin resistance (203),

while expression of constitutively-active $G\alpha_{i2}$ in mouse liver and adipose tissue leads to enhanced glucose tolerance (204). This occurs because $G\alpha_{i2}$ signals to GLUT4 and regulates glucose uptake into peripheral tissues (205).

In contrast to $G\alpha_{i2}$ -null mice (205), there is no effect of loss of $G\alpha_z$ on insulin sensitivity (Fig. 3). This is not unexpected, as $G\alpha_z$ is not expressed in the liver, adipose tissue, or skeletal muscle (191, 206, 207). Conversely, we did find that $G\alpha_z$ -null mice do have lower blood glucose levels and increased glucose clearance, as demonstrated by GTTs (Fig. 2A). These effects appear to be due to a direct impact on insulin secretion, as $G\alpha_z$ -null mice have higher plasma insulin levels after glucose load than wild-type mice (Fig 2B), and islets isolated from $G\alpha_z$ -null mice secrete more insulin at stimulatory glucose concentrations (Fig. 4). Hence, although under conditions of ectopic expression, constitutively-active forms all $G\alpha_i$ subfamily members can inhibit GSIS *in vitro*, only loss of $G\alpha_z$ impacts GSIS *in vivo*, possibly because of the aforementioned abilities of the others to compensate for each other. This is of particular relevance in regards to the inhibition of adenylyl cyclase; $G\alpha_z$ displays specificity for adenylyl cyclases I and V, while all three $G\alpha_i$ isoforms block adenylyl cyclases V and VI (208). The possibility remains, though, that one or more of the PTX-sensitive $G\alpha_i$ proteins is required for inhibition of GSIS through a specific GPCR agonist (e.g. somatostatin, epinephrine, etc.).

A significant increase in GSIS was observed in isolated $G\alpha_z$ -null islets, even though no GPCR had been specifically engaged in the tissue treatments. This is not entirely unexpected, as the slow GTP hydrolysis rate of $G\alpha_z$ leads to a significant amount of active, GTP-liganded protein even under basal conditions (191), in contrast to the rapid hydrolysis rates of the other $G\alpha_i$ subfamily members. In support of this hypothesis, cAMP production was significantly increased

in $G\alpha_2$ -null islets in the absence of exogenous stimuli (Fig. 5), suggesting that $G\alpha_2$ tonically inhibits adenylyl cyclase in wild-type islets, and that in $G\alpha_2$ -null islets, this inhibitory constraint has been removed. cAMP augments insulin secretion dependent on the glucose concentration being above a threshold of approximately 5.6-7 mM (182, 209, 210). This is consistent with our observations that $G\alpha_2$ -null islets do not secrete more insulin at 2.8 mM or 5.6 mM glucose, and only begin to show increased insulin secretion at higher levels of glucose. Furthermore, increases in cAMP levels at suprathreshold glucose concentrations have been shown to correlate directly with augmentation of insulin secretion (209). At 11.1 mM glucose, the cAMP production in $G\alpha_2$ -null islets is approximately 1.9-fold that of wild-type islets (Fig. 5), which correlates well with a 1.5-fold increase in insulin secretion at this glucose concentration (Fig. 4A).

cAMP has been shown to augment insulin secretion by numerous mechanisms, including increasing glucokinase expression and/or activity, augmenting K_{ATP} channel closure, augmenting VDCC opening, enhancing calcium-induced calcium release, and directly enhancing the exocytosis process itself (184, 211). In addition, $G\alpha_i$ proteins have been implicated in regulation of the K_{ATP} channel and VDCCs, leading to hyperpolarization of the membrane and inhibition the influx of extracellular Ca^{2+} (184, 185). Furthermore, $G\alpha_i$ proteins have been localized to various degrees on secretory granules from pancreatic α - and β -cell s (212, 213), and to the trans-golgi network in the exocrine pancreas (214). $G\alpha_i$ subfamily members have also been implicated in directly inhibiting exocytosis from β -cell s (184), which, together with their subcellular localization, indicates a role for $G\alpha_i$ proteins in the regulation of a distal step in the stimulated secretion pathway. Taken together, the regulation of cAMP levels seems a likely

explanation for the effects of $G\alpha_i$ proteins on insulin secretion; in particular, those of the subfamily member that is the subject of the current study, $G\alpha_z$.

Agents which impact on cAMP levels are being tested and/or currently being used for the treatment of β -cell dysfunction in Type 2 diabetes. The incretin GLP-1, which activates G_s -coupled receptors and thereby increases cAMP levels, is rapidly degraded; but the stable GLP-1 analogs exenatide and liraglutide have been shown to be clinically effective Type 2 diabetes treatments (211, 215). In addition, inhibition of the enzyme which degrades GLP-1 and GIP, dipeptidyl peptidase (DPP)-IV, also leads to improved β -cell function and insulin sensitivity in Type 2 diabetes patients (211, 215). Inhibition of PDE3B, the enzyme responsible for degradation of cAMP in the β -cell, leads to increased insulin secretion, although its expression in the liver and adipose tissues prevents inhibitors from decreasing systemic blood glucose levels (211).

$G\alpha_z$ -null mice do not appear to have further augmented insulin secretion during the OGTT, suggesting that $G\alpha_z$ does not play a major role in the regulation of incretin-potentiated insulin secretion (Fig 6A-C). This is not entirely unexpected, as GLP-1-stimulated G_s appears to couple to ACVIII (216), which is not inhibited by $G\alpha_z$. Since $G\alpha_z$ and GLP-1 likely signal through different upstream pathways, it may be possible that $G\alpha_z$ inhibition might be used in combination with a GLP-1 analogue *in vivo*. Overall, the significant effect of knockout of $G\alpha_z$ on improving GSIS and glucose clearance, without significant negative systemic effects, makes $G\alpha_z$ worthy of further study, possibly as a potential target for impaired β -cell function therapeutics toward Type 2 diabetes.

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Biography

Background

Date of Birth

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Place of Birth

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Education

Ph.D. Molecular Cancer Biology, Duke University, Durham, NC, expected Spring 2009

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B.S. Chemistry, Biology (Minor), University of North Carolina at Chapel Hill, Chapel Hill, NC
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Publications

Bailey CL, Kelly P, and Casey PJ (reviewed and revised for acceptance Feb 2009) Activation of Rap1 promotes prostate cancer metastasis. Cancer Research

Kimple ME, Joseph JW, **Bailey CL**, Fueger PT, Hendry IA, Newgard CB, and Casey PJ. (2008) Galphaz negatively regulates insulin secretion and glucose clearance. J Biol Chem., 283 (8): 4560-7.

Kimple ME, Nixon AB, Kelly P, **Bailey CL**, Young KH, Fields TA, and Casey PJ. (2005) A role for Gz in pancreatic islet beta-cell biology. J Biol Chem., 280 (36): 31708-13.

Lee D, Cross SH, Strunk KE, Morgan JE, **Bailey CL**, Jackson IJ, and Threadgill DW. (2004) Wa5 is a novel ENU-induced antimorphic allele of the epidermal growth factor receptor. Mamm Genome, 15: 523-536.

Works in Progress

Bailey CL*, Kelly P*, Fueger PT, Newgard CB, Casey PJ, and Kimple ME. Rap1 activates mTOR and ribosomal protein S6 to promote pancreatic beta-cell function. (To be submitted March 2009)

Bailey CL and Casey PJ. The roles of Rap1 in cancer progression and metastasis. (To be submitted April 2009)

Honors and Fellowships

Department of Defense Prostate Cancer Research Program Predoctoral Fellowship 2005-2008

ASBMB Travel Award to Experimental Biology, San Diego, CA, 2008

Certificate in Cell and Molecular Biology, Duke University 2005