The Role of Ral GTPases in Human Oncogenic Transformation

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

The genes encoding the Ras family of small GTPases are mutated to yield constitutively active GTP-bound oncoproteins in one-third of all human cancers. In many other cancers lacking Ras mutations, Ras is activated by other means. One common example of such activation is found in breast cancer, in which epidermal growth factor receptor (EGFR) family receptor tyrosine kinases, including EGFR and HER2 (ErbB-2/Neu), are frequently amplified and overexpressed, which in turn activates Ras. In human cells, activation of the Ral guanine nucleotide exchange factor, or RalGEF, effector pathway is necessary for Ras-mediated tumorigenesis and metastasis. RalGEFs activate the two highly similar Ral GTPases, RalA and RalB. While RalA has been shown to be required for Ras-mediated tumorigenesis, RalB is important for tumor metastasis. Activated Ral GTPases bind to and activate a limited number of effector proteins, including RalBP1, Sec5, and Exo84, to affect numerous diverse activities of the cell. This dissertation research sought to determine which of these well-characterized Ral effector proteins were required for oncogenic mutant Ras-induceded tumorigenesis and metastasis of human cells, as well as to examine the role of RalA in breast cancer cells that can activate Ras through EGFR and HER2 overexpression.

RNA interference-mediated loss-of-function analysis demonstrated that Sec5 and Exo84 are required for oncogenic Ras-mediated tumorigenesis, and, at least in part, metastasis. Additionally, both gain-of-function and inhibition studies showed that RalA

activation is induced by EGFR and HER2 in breast cancer cell lines stimulated with EGF. Furthermore, stable suppression of RalA expression inhibited tumorigenic growth of breast cancer cells, and RalA activation was shown to be higher in a majority of mammary adenocarcinomas versus matched patient normal mammary tissue. These studies provide new insights into the importance of RalA activation in breast cancer, as well as the molecules downstream of RalA and RalB that may be responsible for mediating their effects on tumorigenesis and metastasis.

Dedication

All of my accomplishments in research are dedicated in loving memory of my grandparents, Albert and Victoria Salim. Along with my parents, my grandparents were my biggest supporters, and I know that they would be very proud.

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EGF	epidermal growth factor
EH	eps homology
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotife exchange factor
GGTI	geranylgeranyl transferase inhibitor
GTP	guanosine triphosphate
HEK	human embryonic kidney
HEK-HT	HEK cells expressing hTERT and the early region of SV40
MDCK	Madin-Darby canine kidney
MEF	mouse embryo fibroblast
PH	pleckstrin homology
RBD	Ras or Ral binding domain
SCID	severe combined immunodeficiency
SH	Src homology

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1. Introduction

1.1 Ras GTPases

Ras Function and Regulation in Normal Cells

The Ras proteins are members of the large superfamily of low molecular weight guanosine triphosphatases, or GTPases, that are evolutionarily conserved from yeast to humans (1). There are three Ras genes in mammalian cells, H-Ras, K-Ras, and N-Ras, that encode four closely related 21 kilodalton proteins, H-Ras, K-Ras4A, K-Ras4B, and N-Ras (2,3). The Ras proteins share 85% amino acid sequence identity and are expressed in almost all cell types (4). Knockout studies of the K-Ras gene in mice have resulted in embryonic lethality, demonstrating an essential role in normal development, whereas deletion of H-Ras or N-Ras, either alone or in combination, did not result in any detectable developmental defects (5-9).

The Ras proteins, like all small GTPases, cycle between an inactive guanosine diphosphate (GDP)-bound state, and an active GTP-bound state (Figure 1). When Ras proteins bind GTP they undergo a conformational change allowing them to interact with, and activate, numerous downstream target proteins, referred to as effectors (1,4). As such, the membrane-bound Ras proteins have been positioned as critical intermediaries, and function as molecular transmitters that relay extracellular signals to diverse intracellular signaling networks affecting numerous activities of the cell (3,4). *In vitro*, purified Ras proteins possess a low level of intrinsic GTP hydrolysis activity, and a slow intrinsic rate of GDP/GTP nucleotide exchange. Within the cell, however, guanine nucleotide cycling is catalyzed by two main classes of regulatory proteins. Guanine nucleotide exchange factors, or GEFS, promote exchange of GDP for GTP, activating Ras proteins and promoting their interactions with effectors. On the other hand, GTPase activating proteins, or GAPs, promote the hydrolysis of GTP to GDP, thus returning Ras to an inactive state and attenuating its signals (1,4,10).

Several GEFs that facilitate the activation of Ras proteins have been identified. SOS is a ubiquitously expressed RasGEFs, which mediates the activation of Ras proteins by a large variety of stimuli, including tyrosine kinase-coupled receptors, heterotrimeric G-protein-coupled receptors, and receptor tyrosine kinases such as the epidermal growth factor receptors (EGFRs) (3,4,10,11). Following ligand-stimulated activation of receptor tyrosine kinases, the receptor undergoes autophosphorylation of critical tyrosine residues in the cytoplasmic domain, leading to interaction with the Src homology 2 (SH2) domain of the adaptor protein growth factor receptor-bound protein 2 (Grb2). SH domains function to promote protein-protein interactions, and SH2 domains recognize specific phosphorylated tyrosine residues. The Grb2 SH2 domain is flanked by tandem SH3 domains, and it is through these domains that Grb2 is bound to SOS. Activation of receptor tyrosine kinases thus results in the recruiting of SOS to the plasma membrane where Ras is also localized. The increased local concentration and proximity of SOS to Ras results in activation of Ras through the increased guanine nucleotide exchange of GDP for GTP. In the GTP-bound form, Ras is able to bind to and stimulate the activation of its downstream effector signaling cascades (3,4,12).

Aberrant Ras Signaling and Cancer

The Ras genes were first discovered as retroviral oncogenes that had been hijacked from the host genome by the Kirsten and Harvey rat sarcoma viruses, and were termed v-Ki-Ras and v-Ha-Ras (13,14). Ras oncogenes were also identified in the genome of human tumors, and it was later revealed that the genes identified encoded mutated and constitutively activated Ras proteins (2,4). It is now evident that mutated and constitutively activated forms of Ras are found in nearly one-third of all human cancers (15). The mutated Ras genes acquire a single point mutation, found to occur at amino acid 12 or 13 resulting in a glycine to valine, cysteine, or aspartic acid substitution, or at amino acid 61, resulting in a glutamine to lysine substitution (15). These mutations all inhibit the intrinsic GTPase activity of Ras, as well as the ability of GAPs to hydrolyze GTP on Ras, thus causing Ras to remain in an activated state and trigger chronic activation of downstream effectors, which ultimately leads to cellular transformation (3,4).

Ras mutations occur most frequently in K-Ras, accounting for about 85% of all Ras mutations, followed by N-Ras, which accounts for about 15% of the total, then H-Ras, which is responsible for less than 1% of total Ras mutations. The different Ras isoforms are often mutated in distinct tissues. For example, K-Ras is mutated in 90% of all pancreatic adenocarcinomas, 35% of non small-cell lung adenocarcinomas, and 45% of colorectal cancers. N-Ras is mutated in 30% of all acute myelogenous leukemias and liver cancers, and 15% of melanomas, and H-Ras is mutated in 10% of all bladder and kidney cancers (4).

In addition to activation of Ras signaling by point mutations to the Ras genes themselves, several other mechanisms of aberrant, constitutive activation of Ras signaling exist. These include RasGAP deletion or silencing resulting in the accumulation of GTP-bound Ras, mutation, amplification, or overexpression of upstream receptor tyrosine kinases leading to aberrant receptor activation and Ras signaling, and mutation or amplification of Ras effector pathway members, leading to their over-stimulation (4).

Deletion or silencing of RasGAPs, which facilitate hydrolysis of GTP to GDP and thus serve as negative regulators of Ras signaling, can lead to accumulated GTP-bound, active Ras. One significant known example of RasGAP deletion is the loss of neurofibromin, which is encoded by the *NF1* gene, leading to the neurofibromatosis type I disorder. This inherited syndrome leads to the development of tumors in tissues of neural crest origin. The loss of both copies of *NF1* leads to activation of Ras and malignant tumor formation (3,16-19). An example of RasGAP silencing is promoter hypermethylation of *RASSF1A*, which has been observed in various primary tumor types, including lung, liver, kidney, breast, and pancreas, among others (20).

Ras proteins bind to and activate several downstream effector proteins, initiating diverse signaling cascades. These signaling cascades include Raf/MEK/ERK, PI3-kinase/Akt, and RalGEF/Ral (21), and will be described in more detail in the next section (Figure 2). Activating mutations and gene amplification have been observed in the Raf and PI3-kinase pathways in human cancers. Mutations in the B-Raf kinase have been identified in approximately 70% of melanomas and 15% of colorectal cancers. B-Raf mutations occur in the kinase domain, resulting in constitutive activation of the protein (4). The PI3-kinase pathway is aberrantly activated by amplification of PI3-kinase in ovarian cancer, and amplification of Akt2 in ovarian and breast cancers (22,23). Additionally, the PI3-kinase pathway is frequently activated in tumors by loss of the tumor suppressor gene encoding PTEN (24). PTEN serves as a negative regulator of PI3-kinase/Akt signaling, and deletion of PTEN occurs in 30 to 40% of human cancers (25). In comparison to the Raf and PI3-kinase pathways, mutations leading to activation of the RalGEF/Ral pathway have been less frequently identified, and their role in tumorigenesis is not as well-characterized. The mutations in this pathway have been identified by large-scale tumor genomic screens, and are found to occur in several of the RalGEFs. These include mutations in RalGDS, Rgl1, Rgl2, and Rgr in colon, breast, and skin cancers, and lymphoma, respectively (26). The functional relevance of these mutations to cancer formation and progression remains to be elucidated.

While Ras mutations occur in approximately one-third of all human cancers, tumors that lack activating mutations in Ras or its downstream signaling pathways can stimulate Ras signaling through aberrant activation of upstream receptor tyrosine kinases (3,4). The most common example of amplified and overexpressed receptor tyrosine kinases involves the epidermal growth factor receptor family members EGFR and HER2 (ErbB2/Neu), which are overexpressed in many types of cancer, including ovarian and breast (3,4,27). HER2 is overexpressed in approximately 30% of all breast cancers, and is associated with aggressiveness of disease and poor prognosis (28-30). Overexpression of these receptor tyrosine kinases leads to their chronic activation, recruiting SOS and thereby activating Ras. In addition to overexpression, a mutation in EGFR leading to the expression of a truncated and activated receptor occurs in glioblastomas and other cancer types (31). EGFR family members are also activated by autocrine production of growth factors by tumor cells, leading to inappropriate activation of downstream signaling molecules (4).

Ras Effector Signaling Pathways

The wide range of mechanisms leading to deregulated Ras signaling in cancer, whether through mutations in the Ras genes themselves, or alterations to proteins upstream or downstream of Ras, underscores the importance of understanding Ras signaling pathways. Given the frequency of Ras activation in human cancers, much work has been done to decipher the signaling pathways downstream of Ras. When Ras is in the GTP-bound activated form it can bind to and activate various targets, or effectors, to affect numerous activities of the cell, including proliferation, cell survival, cytoskeletal reorganization, migration, and other aspects that can contribute to cellular transformation (4,21). Although other Ras effectors do exist, the three most well-characterized Ras effectors are the Raf family of serine/threonine kinases, the phosphatidylinositol 3-kinases (PI3Ks), and a family of RalGEFs, which serve as guanine nucleotide exchange factors for the Ras-like small GTPases RalA and RalB (Figure 2) (4,21).

The Raf family of serine/threonine kinases is composed of the structurally similar members Raf-1, B-Raf, and A-Raf. This was the first discovered Ras effector pathway and has been the most-studied and perhaps the best-characterized (4,21). The activation of Ras promotes the translocation of the normally cytosolic Raf to the plasma membrane where it is activated through interaction with Ras, which relieves an N-terminal autoinhibitory effect on the Raf kinase domain (32-34). Activated Raf kinases phosphorylate and activate the dual specificity kinases MEK1 and MEK2, also known as mitogen-activated protein kinase kinase (MAPKK) 1 and 2, or MAP/Erk kinases. Activated MEK kinases phosphorylate and activate the extracellular signal-regulated kinases ERK1 and ERK2, which are also known as mitogen activated protein kinase (MAPK) 1 and 2 (4,21,35). Activated MAP kinases translocate to the nucleus where they phosphorylate and regulate several transcription factors, including Elk1, c-FOS, and c-

JUN (36). One effect of these regulatory interactions is the expression of D-type cyclins leading to cell cycle progression (4,37).

The second-best characterized Ras effectors are the phosphatidylinositol 3-kinases (PI3Ks). Activated Ras promotes the translocation of PI3Ks, which consist of a p85 regulatory subunit and a p110 catalytic subunit, to the plasma membrane, where Ras interacts with the regulatory subunit and causes a conformational change that activates the catalytic subunit (21,38,39). Activated PI3K phosphorylates phosphatidylinositol 4,5- bisphosphate (PIP_2) to produce the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which can bind to numerous proteins through pleckstrin homology (PH) domains (4,21). These include the Akt serine/threonine kinase, also known as protein kinase B (PKB), and the 3-phosphoinositide-dependent protein kinase-1 (PDK1) (40,41). PDK1 activates various protein kinases, including Akt. Akt phosphorylates a large number of target proteins leading to diverse signaling events, including the promotion of cell survival (4,42). PI3K can also activate the small GTPase Rac, which is involved in regulation of actin cytoskeleton dynamics, as well as activating the transcription factor nuclear factor-κB (NF-κB) (4,43-45). PIP3-mediated signaling is attenuated through its dephosphorylation by the lipid phosphatase and tumor suppressor phosphatase and tensin homolog, or PTEN (46,47).

The third group of well-characterized Ras effectors with a role in Ras-mediated transformation is a family of Ral guanine nucleotide exchange factors, or RalGEFs, including Ral guanine nucleotide dissociation stimulator (RalGDS), RalGDS-like gene (Rgl), Rgl2/Rlf, and Rgl3, which possess a C-terminal Ras binding domain (RBD) (26,48). RalGDS was first identified as a homolog of yeast RalGEFs, and was later identified as a Ras interactor in yeast two-hybrid screens (49-51). Activated Ras binds RalGEFs through their RBD, recruiting RalGEFs to serve as activators of the Ras-like small GTPases RalA and RalB. Additionally, PDK1 binding to RalGDS, independent of PDK1 kinase activity, has been shown to relieve an N-terminal auto-inhibitory effect on the catalytic domain of RalGDS, enhancing its nucleotide exchange activity. Activation of the RalGEF signaling pathway affects numerous cellular processes, including growth factor receptor endocytosis, polarized vesicle trafficking, cell morphology changes, and the regulation of several transcription factors (26,48).

The activation of Raf kinases, PI3-kinases, and RalGEFs by activated Ras allows for the stimulation of a diverse array of signaling pathways affecting critical cellular processes. The aberrant activation of these effector signaling pathways contributes to the transformed phenotype, supporting many aspects of tumorigenic growth (4,21).

1.2 Tools for Studying Oncogenic Ras Signaling

The development of several key tools used to study the relative contribution of the various Ras effector signaling pathways was critical for advancing the understanding of oncogenic Ras signaling. These reagents include Ras effector domain mutants, constitutively active Ras effectors, and a genetically defined human cell model system, which, when used in combination, proved to be very informative.

Ras Effector Domain Mutants

A significant set of molecular tools that have been used to dissect the importance of the various Ras signaling pathways are the Ras effector domain mutants (10,50,52-56). These mutants bind preferentially to one of the three major Ras effectors (Figure 2). These mutants are created by introducing a single point mutation in the core effector binding domain (amino acids 32-40). Specifically, the effector domain mutations are threonine 35 to serine (T35S) or aspartic acid 38 to glutamic acid (D38E), which preferentially bind to Raf-1 over PI3K and RalGDS, tyrosine 40 to cysteine (Y40C), which preferentially binds PI3K instead of Raf-1 or RalGDS, and glutamic acid 37 to glycine (E37G), which binds preferentially to RalGDS versus Raf-1 or PI3K. Combination of these point mutations with an activating mutation to Ras, such as glycine 12 to valine (G12V), has been used to specifically activate the Raf, PI3K, or RalGEF signaling pathways. While activated Ras harboring each of the three effector domain mutations has been shown to induce the activation of its downstream signaling molecules (39,55,57-59), the potential non-specific activation of Ras effectors, both known and potentially unknown, is possible when these mutants are overexpressed. However, Ras effector domain mutants have been widely-used important tools for the study of Ras signaling.

Constitutively Active Ras Effectors

Another set of molecular tools that have been used to dissect Ras signaling are constitutively activated forms of the three main Ras effectors. Since the Ras effectors are recruited to the plasma membrane and activated by GTP-bound Ras, the constitutively activated effectors are targeted to the plasma membrane. This is accomplished by fusing the membrane-targeting C-terminal region of Ras (CAAX) in frame to the C-terminus of the effector proteins. Specifically, Raf-1-CAAX, p110-CAAX, and Rlf-CAAX induce activation of the Raf, PI3K, and RalGEF pathways, respectively (39,54,56,59-61). Raf can also be activated by specific N-terminal deletion (62-65). Constitutively active Ras effectors provide more specific activation of distinct branches of Ras signaling than Ras effector domain mutants. However, there are multiple members of each Ras effector family (three Raf proteins, etc.), and they may activate distinct downstream molecules to different degrees in different cell types. Effector domain mutants and constitutively active effectors have been complementary to each other, and have helped to evaluate the effects of the various Ras signaling pathways (10).

The Genetically Defined Human Cell Model System

Much of what is known about cellular transformation has been derived from studies using rodent cells such as NIH 3T3 mouse fibroblasts. While these studies have provided a wealth of information, there are several important fundamental differences between rodent and human cells with regard to the process of transformation (21). For example, prolonged culture of mouse embryonic fibroblasts leads to their spontaneous immortalization, whereas similar treatment of human cultured fibroblasts leads to cellular senescence (66). Additionally, primary rodent cells have been shown to be transformed by certain combinations of oncogenes that fail to transform primary human cells (67,68). It has also been shown that mutations in p53 or p16/Arf are sufficient to complement mutant Ras and transform mouse embryonic fibroblasts, but not primary human fibroblasts (21,69). These findings suggest that human cells require more steps to become transformed than other cell types. Given these differences, among many others, the development of a human cell culture based model system for studying Ras signaling was necessary.

One obstacle to studying Ras oncogenesis in human cells was the observation that overexpression of Ras in primary human cells caused cellular senescence (69). This limitation was overcome, however, by the enforced expression of a distinct combination of genes known to be involved in human cancers. Specifically, a variety of normal human primary cell types were converted to a tumorigenic state by the retroviral, stable expression of hTERT, the early region of the SV40 tumor virus, and an oncogenic mutant H-Ras harboring the G12V mutation (H-Ras^{G12V}) (70). hTERT is the catalytic subunit of telomerase, which is responsible for maintaining telomere length and is essential for the immortalization of human cells (71-74). It is activated in 85% of human cancers to promote unlimited proliferation (75). The early region of SV40 encodes two oncoproteins, the large and small t antigens (T-Ag and t-Ag, respectively). T-Ag contributes to the transformation of human cells by disrupting the functions of the p53 and Rb tumor suppressors (76,77), and t-Ag inhibits protein phosphatase 2A (PP2A), which in turn stabilizes c-Myc (78-81). The changes initiated by T-Ag and t-Ag have been observed frequently in human cancers. Notably, expression of oncogenic Ras was required for the primary cells to become tumorigenic (70), establishing a genetically defined model system that could be used to dissect the contributions of the various Ras effector signaling pathways to Ras-mediated tumorigenesis of human cells.

The introduction of this genetically defined human cell model system provided the opportunity to directly compare and contrast Ras-mediated transformation in human and murine cells. It also provided a setting in which to analyze previous observations that had been made in rodent cell model systems. Based on work done in murine cells, the Raf branch of Ras signaling was thought to be the most important for Ras-mediated tumorigenesis because of the ability of constitutively active mutants of Raf and MEK to transform NIH 3T3 mouse fibroblasts (32,61,64,82). While dominant-negative mutants of PI3K have been shown to inhibit Ras transformation of NIH 3T3 cells, active PI3K alone was not able to transform NIH 3T3 cells. Activated PI3K was able to cooperate with active Raf, however, to cause enhanced transforming activity (39).

Constitutively active RalGEFs also lacked transforming ability in NIH 3T3 cells, but were able to enhance transformation in cells expressing active Raf (54,56). Also, like the other two Ras effectors, expression of dominant negative Ral was found to inhibit Ras-mediated transformation of NIH 3T3 cells (56). Additionally, the Ras effector domain mutant that preferentially activates the Raf pathway (Ras12V35S) was shown to be the most potent of the effector domain mutants for transforming NIH 3T3 cells (52,55). These data suggested that Ras-induced transformation of murine cells was primarily due to the Raf kinases.

To compare Ras-mediated transformation between identically transformed mouse and human cells, normal murine and human fibroblasts were infected with retroviruses encoding large and small t antigens, and the human fibroblasts were also infected with hTERT (83). hTERT was not expressed in the murine fibroblasts because murine fibroblasts already express endogenous telomerase activity (70). The resulting cells were then infected with retroviruses encoding oncogenic H-Ras^{G12V}, or three different H-Ras^{G12V} effector domain mutants that preferentially activate Raf, PI3K, or RalGEFs. As expected, in mouse fibroblasts the T35S Ras mutant, which preferentially activates the Raf kinases, was the most transforming, as assessed by anchorage-independent growth (83). However, in human fibroblasts only the E37G effector domain mutant, which activates RalGEFs, was able to support transformed growth. This effect was also seen in human epithelial cells, and was confirmed by replacing the effector domain mutants with constitutively active Ras effectors. Additionally, it was shown that expression of the E37G effector domain mutant was necessary to support tumorigenic growth of various human cell types (66,83). These findings demonstrated distinct requirements for Ras-mediated transformation of human, versus mouse cells, suggesting that the RalGEF signaling pathway was critical for the transformation of human cells. Consequently, understanding the contributions of proteins downstream of RalGEFs to their effects on transformed growth would be essential to further elucidate Ras oncogenesis.

1.3 Ral GTPases

The Ras-like GTPases RalA and RalB constitute a family of proteins within the Ras branch of small GTPases. They were isolated over twenty years ago from a hybridization screen of a B-lymphocyte cDNA library using degenerate probes that corresponded to a region of Ras and Ras-related proteins that was highly conserved from yeast to mammals (84). Ral GTPases share 58% identity to the Ras GTPases H-Ras, K-Ras, and N-Ras, and are grouped together with these proteins as well as Rap1, Rap2, TC21, M-Ras, and R-Ras in the Ras branch of small GTPases (48). Most of the sequence similarity between Ral and Ras is found in regions known to interact with guanine nucleotides (85). RalA and RalB share 85% sequence identity to each other at the amino acid level.

Like all GTPases, RalA and RalB act as molecular signal transmitters to the cell by cycling between an inactive GDP-bound state and an active GTP-bound state (Figure 1) (86). The process of GDP to GTP exchange on Ral proteins is mediated by a family of RalGEFs. In the active GTP-bound state, Ral proteins bind to a unique set of downstream targets that mediate their effects on cells (26,48). Attenuation of Ral signaling requires hydrolysis of GTP through complementation of weak intrinsic catalytic activity by GAPs. While RalGAP activity has been biochemically detected in cell extracts, the molecular identity of RalGAP(s) has remained unknown (26,87).

Regulation of Ral Activity by RalGEFs

The Ral GTPases began to attract significant attention following the discovery that, in addition to Raf kinases and PI3-kinases, a set of guanine nucleotide exchange factors with specificity for Ral proteins were also direct effectors of oncogenic Ras (48). The first RalGEFs were identified as Ras-binding proteins from a yeast two-hybrid screen, and were then shown *in vitro* to bind preferentially to GTP-bound Ras (10,49,50,88,89). The family of Ras-responsive RalGEFs includes the first identified member, Ral guanine nucleotide dissociation stimulator (RalGDS), RalGDS-like gene (Rgl), Rgl2/Rlf, and Rgl3, which possess a C-terminal Ras binding domain (RBD) (4,85,90). Ral proteins can also be activated by Ras-independent mechanisms involving RalGDS and GEFs that lack Ras-binding domains (RalGPS1 and RalGPS2), and instead appear to be mobilized by phosphatidylinositide second messengers through a C-terminal pleckstrin homology (PH) domain. These mechanisms of Ral activation are not well understood (26,48).

Since Ras proteins can be activated in response to a broad variety of stimuli, RalA and RalB have the potential to mediate processes initiated by diverse extracellular signals. Upon activation, GTP-bound Ras has been shown to bind to and recruit RalGEFs to Ral proteins at the plasma membrane leading to their activation (60,91). The intrinsic exchange activity of RalGEFs has also been shown to be enhanced by a complementary mechanism involving Ras activation of PI3-kinase. Specifically, epidermal growth factor (EGF)-mediated activation of PI3K promoted the binding of the PI3K effector 3-phosphoinositide-dependent protein kinase-1 (PDK1) to the amino-terminus of RalGDS (92). The interaction of PDK1 with RalGDS relieved an auto-inhibitory effect of the amino-terminus on the catalytic domain of RalGDS, enhancing its nucleotide exchange activity. Interestingly, the enhancement of RalGDS activity by PDK1 binding was independent of PDK1 protein kinase activity. Thus, RalGDS activation is caused by Ras-induced relocalization to the plasma membrane and enhancement of its catalytic activity through the Ras-PI3K-PDK1 pathway (48).

Once activated, Ral GTPases bind to and alter the activity of a relatively limited number of effector proteins (Figure 3). Despite their high sequence similarity to the Ras proteins H-Ras, K-Ras, and N-Ras, Ral proteins mediate their functions through a unique set of target proteins, likely due, in part, to divergence from Ras in the effector binding region (26). These Ral effectors include Ral binding protein 1, or RalBP1 (also known as RLIP76), which has *in vitro* Rac/Cdc42 GAP activity (93-95), Sec5 and Exo84, which are two members of the multi-subunit exocyst complex (96,97), the actin binding protein filamin (98), and the Y-box transcription factor ZO-1-associated nucleic acid binding protein (ZONAB) (99). Additionally, both RalA and RalB constitutively bind to phospholipase D1 (PLD1) (100). These effectors link Rals to diverse cellular processes, including receptor-mediated endocytosis, polarized vesicle trafficking, and remodeling of the actin cytoskeleton.

Normal Functions of Ral GTPases

Ral GTPases and Vesicle Trafficking

A growing body of evidence has implicated RalA and RalB in the regulation of vesicle trafficking events (48). This role for Ral proteins was suggested by early studies that showed that, in addition to being colocalized with Ras at the plasma membrane, a large fraction of Ral proteins were also present on intracellular vesicles representing components of both the endocytic and exocytic compartments (101-103). Further evidence has supported a role for Ral GTPases in exocytic events such as the polarized delivery of basolateral membrane proteins in epithelial cells, and the regulation of secretion in epithelial and neuronal cells. Additionally, Ral proteins have been linked to receptor-mediated endocytosis (48).

Ral GTPases and Endocytosis

Ral proteins were first linked to the process of receptor-mediated endocytosis through GTP-dependent interaction with Ral binding protein 1 (RalBP1) (93-95). RalBP1 was the first identified Ral effector, and was originally distinguished by its Rac/Cdc42 GAP domain, although the relevance of its role in regulating Rac/Cdc42 activity *in vivo* has been elusive. RalBP1 has been linked to receptor-mediated endocytosis through interaction with proteins that bind to other sites on RalBP1, including the epidermal growth factor receptor substrates partner of RalBP1 (POB1) (104), and Reps1 (105). POB1 and Reps1 both contain Eps homology (EH) domains, which are found in many proteins associated with endocytosis (106). RalBP1 can also bind to the μ2 subunit of the AP2 adaptor complex, which is involved in promoting the interaction of AP2 with integral membrane proteins and recruiting them into clathrin-coated pits (107). POB1 also binds to proteins involved in vesicle sorting such as epsin and Eps15 through its EH domain (108). Epsin can also interact with the AP2 complex and participate in endocytosis as an adaptor linking cargo and vesicles (109). Reps1 binds to Rab11-FIP2 and influences EGF receptor internalization, and is thought to act as an adaptor targeting certain endocytic vesicles to the recycling endosome (48,110).

In addition to the implications of the involvement of Ral GTPases in endocytosis through RalBP1-interacting proteins, studies have also provided more direct evidence for the involvement of Ral and RalBP1 in this process. For example, overexpression of a constitutively activated mutant RalB inhibited the internalization of both EGF and insulin receptors, and was dependent on Ral binding to RalBP1 (107,111). Additionally, it was further demonstrated that RalBP1 binding to POB1 was involved in Ral-dependent regulation of endocytosis, and that POB1 interaction with epsin was also important for this process (112).

Ral proteins have also been linked to endocytosis through their unregulated interaction with PLD1 (48). In particular, EGF receptor internalization was shown to be blocked when PLD activation was suppressed, and accelerated when PLD was overexpressed (113). Additionally, Ral activity has been found to be required for EGF-mediated activation of PLD, and in these studies has also been shown to be required for EGF receptor internalization following EGF stimulation (114).

Ral GTPases and Polarized Delivery of Basolateral Membrane Proteins

Ral GTPases were first linked to polarized delivery of membrane components through studies that identified Sec5 and Exo84 as effectors of active Ral proteins (96,97,115). Sec5 and Exo84 are two members of the multiprotein exocyst complex. The hetero-octameric exocyst complex was first identified in yeast, where it functions to direct the targeting of secretory vesicles to specific sites on the plasma membrane (116). The analogous mammalian exocyst complex (117) is responsible for the proper targeting and tethering of certain secretory vesicles to specific dynamic plasma membrane domains, including the basolateral but not apical surface of epithelial cells, sites of synapse formation, the abscission plane of mitotic cells, and the leading edge of migrating epithelial cells (26,96,118-120).

Direct evidence for the involvement of Ral proteins in the delivery of basolateral membrane proteins came from gain-of-function and loss-of-function analyses examining the importance of the Ral-exocyst interaction (96,120). Loss-of-function studies either inhibited the function of both Ral proteins through the expression of the Ral binding domain of RalBP1 or Sec5, which binds to activated Ral and serves a dominant-negative function, or suppressed RalA expression by short interfering RNAs (siRNAs). In either case, membrane proteins that would normally be delivered to the basolateral surface of the plasma membrane, such as EGFR, were found to be localized incorrectly to the apical as well as the basolateral surface, suggesting a defect in polarized sorting. Additionally, RalA depletion was shown to disrupt exocyst complex assembly, suggesting that activated RalA may regulate exocyst complex assembly and promote polarized delivery of membrane proteins (96).

Subsequent studies using inducible expression of RalA and RalB at near-endogenous levels further defined the role of Ral proteins in polarized delivery of membrane proteins in epithelial cells, and examined key differences between RalA and RalB (120). These studies showed that inducible expression of a constitutively activated mutant RalA, but not RalB, enhanced polarized delivery of basolateral membrane proteins such as E-cadherin. Additionally, constitutively active RalA was shown to bind more efficiently to members of the exocyst complex than constitutively active RalB. Furthermore, this study examined differences in the amino acid sequences of RalA and RalB. While RalA and RalB share 85% amino acid sequence identity, their region of greatest sequence divergence is in their C-terminal "hypervariable domain," consisting of the last 30 C-terminal amino acids (amino acids 176 – 206).

To determine the importance of the hypervariable domain, chimeric Ral proteins were created, in which the hypervariable domains were swapped (120). This created a RalA protein that had its hypervariable domain replaced by that of RalB (RalA/B), and a RalB protein that had its hypervariable domain replaced by that of RalA (RalB/A). By swapping these C-terminal regions, constitutively activated RalB/A was able to bind members of the exocyst and promote basolateral delivery of E-cadherin more efficiently than constitutively active RalB. This suggested that the C-terminal differences in RalA and RalB may affect their abilities to interact with the exocyst and perform their functions in polarized membrane delivery.

The C-terminus of small GTPases is known to effect their cellular localization, and this study went on to show that RalA and RalB localize differently in cells, utilizing the RalA/B and RalB/A chimeras to show that the difference is due to their C-terminal hypervariable domains (120). Specifically, endogenous RalA was found to be localized at the plasma membrane and diffusely punctate throughout the cytoplasm, while endogenous RalB was found to be localized mainly throughout the cytoplasm.

Expression of the constitutively activated mutants revealed that activated RalA localized strongly at the plasma membrane and demonstrated perinuclear staining, colocalizing with recycling endosomes. Activated RalB localized primarily to the plasma membrane with no observed perinuclear staining.

The observations that activated RalA enhanced polarized secretion of basolateral proteins and promotes exocyst assembly through direct interactions with Sec5 and Exo84, together with the finding that siRNA-mediated RalA depletion inhibited polarized secretion, strongly positioned RalA as a key regulator of maintaining polarity in epithelial cells. The lack of RalB involvement in enhancing polarized secretion, combined with its possible role in regulating receptor-mediated endocytosis, makes it appealing to speculate that RalA and RalB may serve distinct roles in mediating vesicle trafficking, with RalA regulating exocytic processes and RalB regulating endocytic processes (48). This hypothesis remains untested, however, and future studies will be necessary to examine its validity.

Ral GTPases and Neurosecretion

In addition to their role in polarized delivery of membrane components, Ral GTPases have also been linked to neurosecretion. In neurons, the exocyst has been shown to be involved in delivering synaptic vesicles and calcium channels to new synapses, and its abundance in synaptic endings of adult animals suggests that the exocyst may contribute to the maintenance of synaptic function as well (120). Ral inhibition by expression of a Ral binding domain in PC12 cells was also found to block secretion (96). Additionally, expression of a dominant-negative mutant form of RalA in transgenic mice suppressed neurosecretion associated with the readily releasable pool of synaptic vesicles docked at the plasma membrane (121).

Ral GTPases and Insulin Secretion

Similar findings were made recently in pancreatic islet beta cells involving the regulated exocytosis of insulin (122). RalA was shown to interact with the exocyst complex and localize to the membrane of a mouse insulin-secreting beta cell line, where it was presumed to facilitate the tethering or docking of insulin granules at the plasma membrane. Additionally, shRNA-mediated knockdown of RalA decreased insulin secretion from isolated mouse islets in response to both low and high glucose. Furthermore, knockdown of RalA in the rat INS-1 832/13 insulinoma cell line impaired depolarization-induced exocytosis by affecting release of the readily releasable pool of insulin granules and mobilization of granules from reserve pools. This was similar to the role of RalA in neurosecretion involving the readily releasable pool of synaptic vesicles (121), suggesting that RalA may facilitate replenishment of readily releasable pools of synaptic vesicles and insulin granules mobilized from the cytoplasm and targeted to the plasma membrane, positioning RalA as a key regulator of exocytosis, mediated by interaction with the exocyst complex.

Ral GTPases, Cellular Morphology, and Migration

The Ral GTPases have been implicated in the processes of cellular morphology changes and migration in a number of studies, although the exact mechanisms of RalA and RalB involvement in these processes remain somewhat unclear, and may involve numerous effectors.

Ral GTPases have been linked to the process of filopodia formation through interactions with distinct effector proteins. A RalA-exocyst interaction was shown to be important for mediating filopodia formation in response to cytokines and Cdc42 activation. Specifically, a dominant negative mutant form of RalA as well as an anti-Sec5 antibody, were both shown to inhibit filopodia formation in fibroblasts. Additionally, filopodia formation induced by RalA was not blocked by disrupting membrane transport, suggesting that Ral and the exocyst may regulate actin reorganization (123). RalA has also been linked to filopodia formation through interaction with filamin. Expression of a constitutively activated mutant RalA led to filopodia formation, and the effect was not blocked by expression of a dominant negative Cdc42. However, filopodia formation induced by activated RalA was blocked by expression of the Ral binding domain of filamin, suggesting that filamin was necessary for the formation of filopodia induced by RalA. Additionally, this study suggested that Cdc42 acts through RalA and filamin to induce filopodia (98). The Ral effector RalBP1 has proposed Rac/Cdc42 GAP activity, adding a level of complexity to the mechanism by which Ral proteins may regulate filopodia formation (48).

Ral GTPases have also been implicated in cell migration by a number of studies. For example, activation of Ral proteins by a constitutively activated mutant RalGEF or expression of constitutively activated RalA stimulated increased motility of skeletal myoblasts (124), while the expression of a dominant-negative mutant Ral inhibited migration of *Drosophila* ovary epithelial cells (125). Ral activation has also been implicated in cytoskeletal changes associated with chemoattractant peptide-mediated cell stimulation (126). Additionally, RalA activation has been shown to be high at the lamellipodia of migrating Madin-Darby canine kidney cells, and inhibition of Ral proteins by the Ral binding domain of RalBP1 perturbed migration of these cells (127).

Ral proteins have been further implicated in cell motility of bladder cancer cells, in which dominant negative mutant RalA substantially inhibited T24 bladder cancer cell motility (128). Further studies demonstrated that RalA and RalB may serve different functions in serum-stimulated motility of bladder and prostate cancer cells, showing that siRNA-mediated knockdown of RalB, but not RalA, inhibited cell migration. Additionally, expression of constitutively activated mutant RalA inhibited migration, whereas expression of constitutively activated mutant RalB stimulated migration (129).

The importance of RalB for cell migration was separately demonstrated by the finding that siRNA-mediated knockdown of RalB, but not RalA, inhibited the migration of normal rat kidney cells associated with wound healing. Knockdown of Sec5 also inhibited migration, indicating that the RalB-exocyst interaction may be important for this effect. In further support of this idea, migration led to increased RalB activation and interaction with the exocyst, promoting exocyst assembly and localization to the leading edge of migrating cells (130).

The suggested distinct roles of RalA and RalB in cell migration are similar to the situation involving Ral proteins and polarized delivery of membrane components, where RalA was shown to promote basolateral delivery but RalB was not (120). This further suggests that while RalA and RalB are 85% identical, they may mediate distinct cellular functions.

Ral GTPases and Gene Expression

Ral GTPases have been implicated in the regulation of a number of transcription factors, although it remains unclear how they do so in nearly all cases reported. Much of the evidence suggesting that Ral proteins affect gene transcription has come from expression of activated RalGEFs. For example, expression of a constitutively activated RalGEF, such as Rlf-CAAX, has been shown to activate the ternary complex factor (TCF), which stimulates the serum response element c-Fos, and may promote cell proliferation (59). Expression of constitutively activated Rlf has also been shown to promote the phosphorylation and activation of c-Jun through activation of the Jun N-terminal kinase (131). Additionally, Rlf activation promoted the phosphorylation and activation of AFX, a member of the Forkhead transcription factor family (132). The activation of these transcription factors was also blocked by the expression of dominant negative Ral. However, some of the effects seen, such as the activation of AFX, may involve more complex Ral-independent mechanisms, because they are not always induced by expression of constitutively activated Ral proteins (48,132).

Experiments using constitutively activated mutant forms of Ral proteins have also shown that Ral GTPases may regulate transcription factor activity. Expression of a constitutively activated mutant form of RalA led to phosphorylation and activation of the transcription factor Stat3, through activation of c-Src. Ral activation was also shown to be required for c-Src and Stat3 activation in response to EGF stimulation, and expression of a dominant negative RalA inhibited EGF-stimulated c-Src activation (133). Expression of a constitutively activated mutant form of RalB induced expression of NF-κB, leading to increased expression of cyclin D1. The increase in cyclin D1 expression was shown to be dependent on NF-κB activation and binding to a site in the cyclin D1 promoter, and was also suggested to occur through a mechanism independent of Ral binding to PLD1 or RalBP1 (134). Like all of the previous studies implicating Ral GTPases in the regulation of gene expression, the specific effector pathway utilized by Ral proteins to mediate effects on transcription is unclear.

Unlike the situations mentioned above in which Ral regulation of gene expression occurs through undefined interactions, RalA has been directly associated with the Y-box transcription factor ZO-1 associated nucleic acid binding protein, or ZONAB (99). ZONAB was identified as a RalA-GTP-dependent binding protein in a yeast two-hybrid screen. The interaction between RalA and ZONAB was shown to be cell density-dependent. Specifically, the endogenous RalA-ZONAB interaction increased under conditions of increased cell density. The increased interaction was associated with altered localization of RalA, which was shown to be punctate at low cell density, but localized mainly at the plasma membrane with ZONAB at high cell density. ZONAB has previously been shown to act as a transcriptional repressor regulating the ErbB-2 promoter through binding to an inverted CCAAT box (135). This regulation has been shown to be cell density-dependent, with a pool of ZONAB localizing to the nucleus and repressing transcription at low density, and relocalizing to relieve transcriptional repression at higher cell densities. A reporter gene assay in which luciferase activity was driven by a version of the ErbB-2 promoter containing the ZONAB binding site showed that expression of a constitutively activated mutant form of RalA in low density cells led to a relief of ZONAB transcriptional repression (99). Additionally, expression of the Ral binding domain of RalBP1, which served a dominant-negative function, led to decreased transcription under conditions of high cell density, when ZONAB does not usually repress transcription. These findings suggest that under conditions of low cell density, ZONAB localizes to the nucleus and represses transcription of ErbB-2, and at higher cell density, ZONAB interacts with Ral at tight junctions, relieving transcriptional repression. It remains to be determined whether RalA plays a role in other ZONAB functions, such as modulating the expression of cell cycle regulators including cyclin D1 and PCNA (136).

Ral GTPases and Cancer

Following the discovery of Ral GTPases, several experiments were done to evaluate their effect on cellular transformation. This was done because RalGEFs were identified as effectors of Ras, one of the most frequently identified oncogenes in human cancers, and because oncogenic Ras was shown to stimulate the activation of Ral GTPases. Early studies in rodent model systems provided mixed results regarding the importance of Ral proteins for transformed growth, and it was not until the development of the genetically defined human cell model system that the importance of Ral proteins in mediating RalGEF and Ras-induced transformation was realized.

Early Studies on Ral GTPases and Transformation

When Ral proteins were first identified as Ras-related proteins, their transforming ability was evaluated in rodent cell model systems in the same way that Ras was evaluated (48). While early studies showed that activated RalGEFs could enhance the proliferation rate of cells grown in low serum and promote anchorage-independent growth of some lines of NIH 3T3 cells, constitutively activated variants of Ral had no activity in NIH 3T3 transforming assays (10,48,56,59). However, support for a role of Ral proteins in Ras-induced transformation was provided by an observation that dominant-negative inhibition of Ral proteins partially inhibited Ras transformation (54). In addition, transfection of activated RalA with limiting amounts of Ras was shown to augment focus formation in NIH 3T3 cells (54), and transformation of RalGDS with an activated mutant Raf also increased transformation in the same assay (56). The expression of constitutively activated mutant RalA in 3Y1 rat fibroblasts overexpressing EGF receptor was shown to enhance transformation, as assessed by anchorage-independent growth (114). Taken together, these studies suggested that RalGEFs were either mediating transformation through undiscovered effectors other than Ral proteins, or that Ral proteins were necessary but not sufficient for transformation. Further studies began to refute the former hypothesis and lend credence to the latter, instead.

Support for the Involvement of Ral GTPases in Transformation

Further evidence supporting the role of Ral proteins in transformation was provided by studies in human tumor cell lines. For example, the expression of an activated mutant RalA was shown to promote anchorage-independent growth of HT1080 human fibrosarcoma cells, whereas expression of a dominant-negative mutant RalA inhibited the effect (137,138). Activating mutations in TC21, which is a very close relative of Ras, have been shown to lead to increased levels of activated GTP-bound RalA in breast cancer cells, causing increased proliferation, which was inhibited by suppression of Ral activity (139). Ral activation was also shown to be required for estrogen-independent growth of breast cancer cells overexpressing the EGF receptor, and expression of a dominant negative mutant RalA inhibited the proliferation of these cells (140).

While several studies had implicated Ral GTPases in the process of cellular transformation, the mechanistic contributions of RalA and RalB to this process remained unclear. One of the first studies to examine whether RalA and RalB served distinct or overlapping functions in transformation was carried out using RNA interference. Specifically, both non-transformed and tumor-derived human cell lines were treated with RalA and RalB siRNA, followed by assessment of proliferation and cell survival of adherent and suspension cultures of the treated cells (138). RalA siRNA treatment had no effect on cell proliferation under adherent conditions, but reduced the proliferation of transformed cells in suspension culture. Notably, expression of an oncogenic mutant Ras in mammary epithelial cells sensitized them to the effect of RalA knockdown. RalB siRNA treatment was shown to induce apoptosis of cells grown in suspension, and similar to the effect of RalA siRNA, was more dramatic in transformed versus non-tumorigenic cell lines, as well as Ras-transformed versus non-Ras-transformed mammary epithelial cells. These results suggested that RalA and RalB may serve distinct, yet cooperative, functions in mediating transformed phenotypes, similar to the distinction between RalA and RalB function in regulating polarized delivery of basolateral membrane proteins (120). Moreover, these findings suggested that cancer cells, versus non-tumorigenic cells, may develop an increased dependency on Ral signaling.

The idea that RalA and RalB may collaborate to allow cancer cells to evade restrictions on both cell proliferation and survival was further examined and supported by a study that utilized inhibition of geranylgeranyltransferase (GGT) I (141). GGT inhibitors (GGTIs) are currently undergoing advanced preclinical studies for use as anti-cancer agents targeting both cancer cell proliferation and survival. Both RalA and RalB undergo C-terminal geranylgeranylation which targets them to the plasma membrane, and GGTIs prevent this modification, inhibiting Ral function. This study made use of GGTI-resistant variants of RalA and RalB that were modified by replacement of C-terminal geranylgeranylated sequences with farnesylation-specific ones, allowing the proteins to be alternatively prenylated and still localize to the membrane. Expression of a GGTI-resistant farnesylated RalA rescued anchorage-independent cell proliferation but did not prevent GGTI-induced apoptosis of adherent cells, whereas expression of GGTI-resistant farnesylated RalB conferred resistance to GGTI-induced apoptosis but not inhibition of anchorage-independent proliferation of pancreatic cancer cells. These results suggested that GGTIs target RalA and RalB-mediated support of cancer cell proliferation and survival to account, at least in part, for their biological activity.

While RalA and RalB share 85% amino acid sequence identity, one possible level of regulation that may contribute to their seemingly distinct functions in transformation, as well as other cellular processes, is post-translational modification by phosphorylation. In a screen for substrates of Aurora-A kinase, serine 194 of RalA was identified as being phosphorylated by Aurora-A kinase (142). This serine residue is located in the C-terminal hypervariable domain of RalA and is not found in RalB. Additionally, phosphorylation of serine 194 by Aurora-A was shown to be important for promoting migration and anchorage-independent growth of MDCK cells expressing Aurora-A, since an activated mutant of RalA promoted these effects, while an activated RalA containing a serine 194 to alanine mutation did not.

Further evidence for the possible significance of RalA phosphorylation came from the identification of RalA as a target of the protein phosphatase 2A (PP2A) Aβ tumor suppressor (143). Mutations and gene deletions of PP2A Aβ have been identified in human lung, breast, and colon cancers (144-148). In addition to serine 194, serine 183 of RalA was also identified as being phosphorylated, though the kinase responsible is not known (143). Both phosphorylated serines were identified as PP2A Aβ substrates, and were also associated with increased RalA-GTP levels. Additionally, only complementation of RNAi-mediated knockdown of RalA with wild-type RalA, but not RalA mutants with serine to alanine substitutions at position 183 or 194, was able to rescue anchorage-independent growth and tumor formation of HEK cells transformed in part by PP2A Aβ suppression. This suggested that dephosphorylation of RalA could contribute to PP2A Aβ tumor suppressive function.

Analogous phosphorylation of serine 181 of K-Ras has been shown to disrupt the association of a stretch of lysine residues within the C terminus with the negatively charged inner surface of the plasma membrane, resulting in relocalization and altered signaling (149). In RalA, serines 183 and 194 flank a region of positively charged residues, suggesting that phosphorylation of RalA may similarly alter its subcellular localization, causing it to shuttle between internal membranes and the plasma membrane (26). Further studies will be necessary to evaluate the relevance of RalA phosphorylation to its effects on transformation.

Genetic evidence for the importance of Ral GTPases in Ras-mediated transformation was provided by knockout of the RalGEF RalGDS, generating mice with a homozygous deletion of RalGDS (150). These mice were developmentally normal and fertile, with no major defects observed in any of the organs analyzed, suggesting that RalGDS is dispensable for normal mouse development. This may be due to compensation by other RalGEFs during development. In order to assess the role of RalGDS in Ras-mediated tumorigenesis, a well-established chemical carcinogenesis protocol was used, in which tumors are initiated in keratinocytes by topical application of 7,12-dimethylbenz[*a*]anthracene (DMBA) and subsequently promoted by 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment. This treatment results in the development of benign papillomas with a high incidence of H-Ras mutations, and some of the tumors progress to squamous cell carcinomas (151). RalGDS-null mice displayed a significant delay in the onset of papilloma formation, as well as decreased number and size of papillomas, when compared to wild-type littermates (150). Additionally, MEFs isolated from wild-type and RalGDS-null embryos were immortalized and infected with oncogenic H-Ras to assess the effect of RalGDS deletion on Ras-induced transformation in cell culture. RalGDS-null MEFs formed 50% less foci than their wild-type counterparts, and the reduction in transformation was rescued by re-introduction of RalGDS expression by retroviral infection. Taken together, these data support a critical role for the RalGEF-Ral pathway in mediating Ras-induced tumorigenesis.

Ral GTPases and Metastasis

In addition to transformed and tumorigenic growth, Ral proteins have also been implicated in the process of tumor cell metastasis. The ability of constitutively activated Ras and its three effector domain mutants to promote metastatic growth was first studied in hamster embryo fibroblasts (152). In this setting, the Ras effector domain mutant that selectively activates RalGEF-Ral signaling was the most effective at promoting metastatic growth. Expression of a constitutively activated RalGEF, Rlf-CAAX, or an activated mutant RalA were also capable of enhancing metastasis. Likewise, suppression of Ral proteins by expression of a dominant-negative mutant RalA inhibited the metastatic growth of a highly aggressive hamster embryo fibroblast cell line.

A similar study in human prostate cancer cell lines showed that expression of the three Ras effector domain mutants in a prostate cancer cell line with low metastatic activity promoted tissue-specific metastasis (153). While the Raf and PI3-kinase-activating Ras effector domain mutants enhanced brain metastasis, the RalGEF-specific effector domain mutant enhanced bone metastasis, which is the most common site of prostate cancer metastasis. Expression of Rlf-CAAX also promoted bone metastasis, and inhibition of Ral function by either dominant-negative RalB or shRNA targeting either RalA or RalB resulted in a significant decrease in bone metastasis. These studies suggested that Ral GTPases may be important for mediating aspects of metastatic growth, such as intravasation, survival in the bloodstream, extravasation, or organ colonization.

Ral GTPases, Tumorigenesis, and Metastasis: Insights from the Genetically Defined Human Cell Model System

While the Ral GTPases had been implicated in tumorigenesis and metastasis, several questions still surrounded the nature of the involvement of RalA and RalB in these processes in human cells. Several earlier studies had suggested that the two proteins, while 85% identical at the amino acid level, may serve distinct functions in the cell. To elucidate the exact contributions of RalA and RalB in Ras-mediated transformed and tumorigenic growth, as well as metastatic growth, our lab utilized the genetically defined human cell model system (70). This system was used to dissect which signaling pathways downstream of oncogenic Ras were essential for the transformation of human cells, demonstrating that, unlike the situation in rodent cell models where the Raf signaling cascade is most important for transformation, the RalGEF pathway was critical for the transformation of human cells (66,83).

To examine the relative contributions of RalA and RalB to Ras-mediated oncogenesis, HEK cells expressing hTERT and the early region of SV40 (HEK-HT cells), which are dependent on activated Ras signaling to become tumorigenic, were stably infected with activated mutants of RalA and RalB, and only RalA-expressing cells were able to support anchorage-independent growth in soft agar (154). This was also the case in human fibroblasts. Effector binding mutants of RalA were also expressed in HEK-HT cells to address which Ral effectors might be important for mediating transformed growth, and a mutant that lacked PLD1 binding was still able to promote anchorage-independent growth similar to activated RalA, whereas mutants that lacked either RalBP1 or exocyst binding were less transforming.

As mentioned earlier, RalA and RalB show the greatest sequence divergence in their C-terminal hypervariable domains, and chimeric Ral proteins in which the hypervariable domains of RalA and RalB were swapped demonstrated that these domains were responsible for the distinct localization of RalA and RalB (120). These Ral chimeras were used to assess whether the localization differences of RalA and RalB may contribute to their differential effects on transformation (154). Specifically, the constitutively activated RalA/B and RalB/A chimeras were expressed in HEK-HT cells along with activated RalA and RalB and their transforming abilities were compared. While activated RalA was the most transforming and activated RalB the least, replacing the C-terminal hypervariable domain of RalB with that of RalA made the RalB/A chimera more transforming than RalB. Swapping the C-terminus of RalA with that of RalB made the RalA/B chimera less transforming than RalA, suggesting that the C-terminal hypervariable domain, and thus Ral localization, is important for transformation.

By loss-of-function analyses, stable knockdown of RalA, but not RalB, was shown to reduce RalA, Rlf-CAAX, H-Ras$^{G12V\ E37G}$, and H-Ras^{G12V}-induced transformation (154). Additionally, stable knockdown of RalA, but not RalB, significantly impaired the tumorigenic growth of H-Ras^{G12V}–transformed HEK-HT cells as well as HT1080 fibrosarcoma cells, J82 bladder carcinoma cells, and SW620 colon adenocarcinoma cells. Furthermore, RalA was shown to be in the GTP-bound activated state in a panel of K-Ras-mutant pancreatic cancer cell lines, and knockdown of RalA in several of these cell lines reduced their anchorage-independent growth. Taken together, these results demonstrated the critical role of RalA in mediating oncogenic Ras-induced tumorigenesis of human cells.

The role of RalA and RalB in mediating transformed and tumorigenic growth was further investigated in a panel of pancreatic cancer cell lines, a cancer characterized by oncogenic K-Ras mutation (155). Once again, RalA was the Ral GTPase necessary for anchorage-independent and tumorigenic growth of pancreatic cancer cells, as determined by stable shRNA-mediated knockdown. Additionally, Ral proteins were more highly activated in pancreatic adenocarcinoma patient samples versus normal pancreatic tissue. In addition to tumorigenesis, the relative contributions of RalA and RalB to metastasis were also evaluated (155). H-Ras^{G12V}-transformed HEK-HT cells stably expressing RalA or RalB shRNA displayed reduced lung metastases, although RalB knockdown resulted in a more dramatic decrease. The same result was observed in two metastatic pancreatic cancer cell lines as well, and metastasis to the adrenal gland caused by one of the two pancreatic lines was only abolished by RalB, but not RalA, knockdown. These data suggested that RalA and RalB may serve distinct functions in tumorigenesis and metastasis, with RalA activation necessary early in the process of tumorigenesis, and RalB function necessary for some aspect of metastatic growth, such as survival in the bloodstream or extravasation and organ colonization.

1.4 Research Goals

While work from our lab and others had demonstrated a critical role for the RalGEF-Ral signaling cascade in mediating oncogenic Ras-induced tumorigenesis and metastasis of human cells, several key issues still needed to be addressed. In particular, the Ral effectors responsible for propagating the transforming signal from Ras remained unclear. Therefore, one of the major goals of my dissertation research was to evaluate which of the known Ral effectors were responsible for supporting Ras-mediated oncogenesis. Since most of the cellular functions that Ral proteins were implicated in regulating, including maintenance of cellular polarity, receptor-mediated endocytosis, cell migration, and morphology changes, involved either RalBP1 or the exocyst complex subunits Sec5 and Exo84, I examined whether these Ral effectors were necessary for Ras-induced transformed and tumorigenic growth of human cells. I also examined whether these effectors were important for oncogenic Ras-induced metastasis.

While Ras itself is mutated in approximately one-third of all human cancers, it is also activated by other means in many cancers lacking Ras mutations. One common example is found in breast cancer, in which EGFR family receptor tyrosine kinases, including EGFR and HER2 (ErbB-2/Neu), are frequently amplified and overexpressed, leading to aberrant Ras activation (3,4,28,156). While the RalGEF-Ral signaling branch has been shown to be necessary for Ras-mediated tumorigenic growth, its function in breast cancer is unclear. Given the importance of the RalGEF-Ral effector branch of Ras signaling in human cells, and the work demonstrating that RalA is necessary for tumorigenesis of human cells mediated by this signaling pathway, I hypothesized that RalA may be activated in breast cancers in which EGFR and HER2 were overexpressed, and that such activation may be associated with, and possibly even necessary for, tumorigenic growth. Therefore, another major goal of my dissertation research was to test this hypothesis.

Both of these studies represented novel work, since an evaluation of the role of Ral effectors in Ras-induced tumorigenesis and metastasis had not been carried out, and the role of RalA activation in breast cancer was unclear. Therefore, the work described here provides new insights into the importance of RalA activation in breast cancer, as well as the molecules downstream of RalA that may be responsible for mediating its transforming activity. Taken together, these data offer a more complete understanding of the specific pathway involved in the RalA-mediated support of Ras-induced oncogenic transformation of human cells.

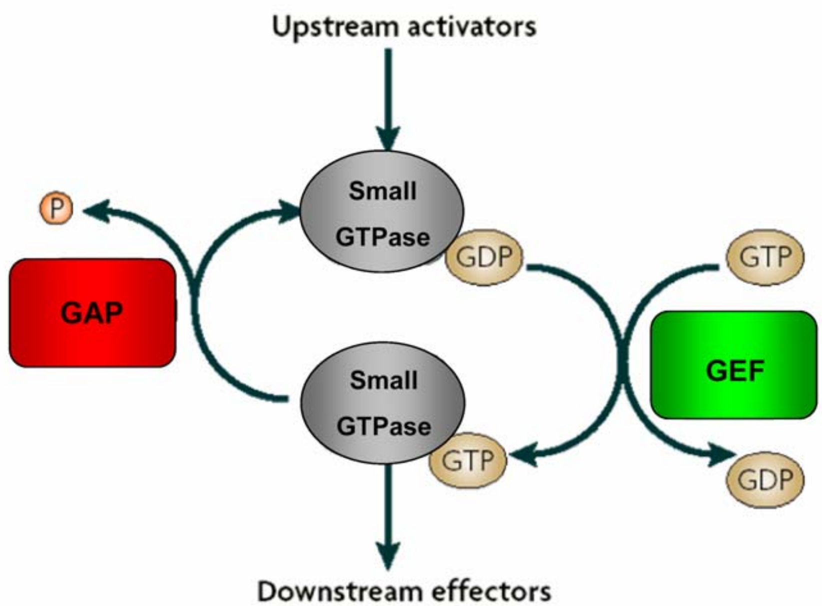

Figure 1. The G-protein Cycle.

The activity of small GTPases such as Ras and Ral is activated by guanine nucleotide exchange factors (GEFs), and inactivated by GTPase activating proteins. Adapted from Bodermann and White, Nature Reviews Cancer, 2008 (26).

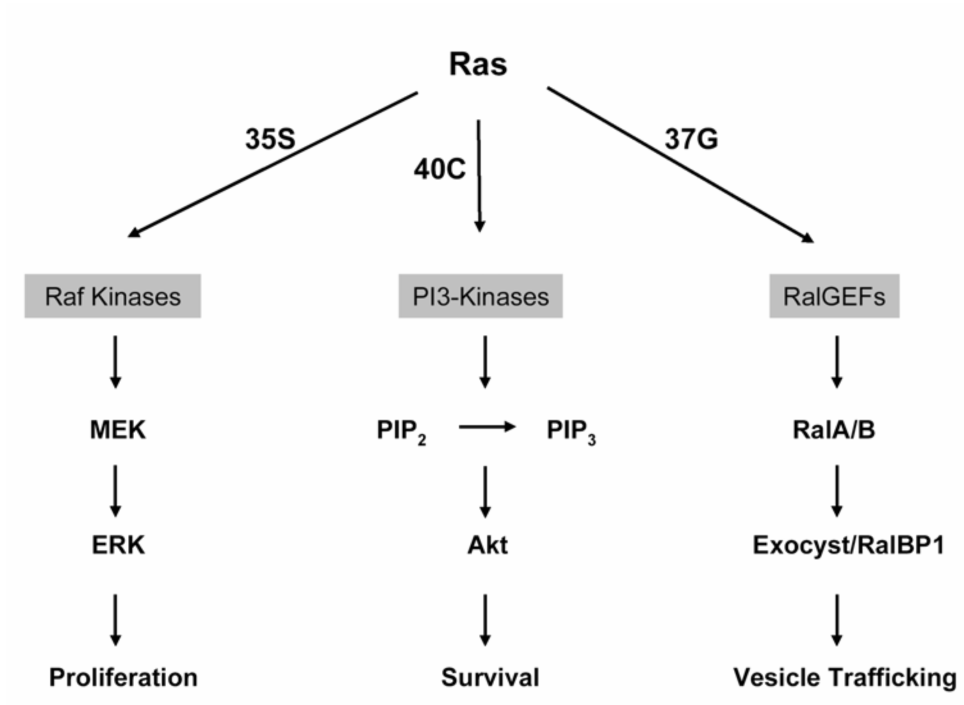

Figure 2. Ras Signaling.

Ras activation of the three best-characterized downstream effector signaling pathways. Effector domain mutants that preferentially activate one of the three pathways are indicated.

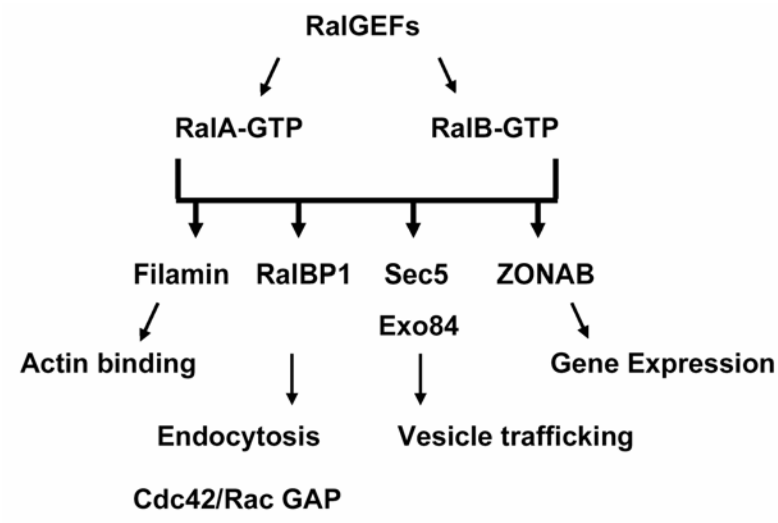

Figure 3. Ral Signaling.

Ral activation-dependent interaction with effector proteins and the cellular effects mediated by the various effectors.

2. Materials and Methods

2.1 HER2 and EGFR Dependant Activation of RalA in Breast Cancer

Cell lines- Human breast cancer cell lines SKBR3, BT474, MDA-MB-231, MDA-MB-468 were obtained from ATCC and cultured in RPMI medium (Gibco) supplemented with 10% fetal bovine serum (Gibco). MDA-MB-231-RalA shRNA or –scramble control cells were generated by stably infecting MDA-MB-231 cells with a retrovirus derived from pSUPER-retro-puro-RalA shRNA or scramble (154) and selecting for stable polyclonal populations by puromycin resistance as previously described (157). 293T cells were transiently transfected with pcDNA3 encoding NeuNT, or empty vector as a control, using FUGENE 6 transfection reagent (Roche) according to the manufacturer's protocol. After 36 hours, 293T cells were serum-starved overnight and then collected, and Ral activation assay was performed.

Human mammary tissue samples- Matched frozen surgical samples of normal human mammary tissue and ductal adenocarcinoma were obtained from the Duke University Medical Center Excess Tissue Bank, devoid of all patient identity information. Samples were pathologically verified as normal or tumor tissue by a board-certified pathologist.

Immunoblotting- For immunoblot analyses independent of Ral activation assays, nearly confluent cells were collected from 10 cm plates and lysed in a general lysis buffer composed of 25 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton-X, 1 mg/ml pepstatin A, 1 mg/ml leupeptin, 1.5 mg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, and 1 mM NaF. Cell suspensions were then passed through a 21-gauge needle several times and rocked at 4^oC for 15 minutes before being spun down to produce cleared lysates. All lysates (for both standard immunoblotting and Ral activation assays) were resolved on a 10% SDS-PAGE gel, transferred to PVDF membrane (Millipore), and blotted with the following antibodies according to the manufacturers' recommendations: anti-RalA (BD Transduction Laboratories), anti-actin (Sigma), anti-HER2 (Oncogene, Ab3), anti-phospho-p44/42 MAPK (Cell Signaling Technology, #9106). Proteins were detected using ECL detection reagents (GE Healthcare/Amersham) in accordance with the manufacturer's protocol.

For Ral activation assays, cells were either collected from nearly confluent 10 cm plates or prepared by grinding frozen tissue with mortar and pestle, and lysed in Ral activation lysis buffer composed of 50 mM Tris pH 7.5, 200 mM NaCl, 10 mM $MgCl_2$, 1% NP40, 1% aprotinin, 1 mM PMSF, and 0.5 mM DTT. In the case of ground frozen tissues, ground tissue was incubated on ice with Ral activation lysis buffer for 10 – 15 minutes before proceeding with lysis. Cell suspensions were then passed through a 21-gauge needle several times and rocked at 4^oC for 15 minutes before being spun down to produce cleared lysates. Activated Ral-GTP was immunoprecipitated as previously described (155,158), using glutathione-sepharose beads (Glutathione Sepharose 4B, Amersham Biosciences) bound to a recombinant glutathione S-transferase fusion protein containing the GTP-dependent Ral binding domain of RalBP1. This was followed by

SDS-PAGE and immunoblotting for RalA with anti-RalA antibody to detect RalA-GTP. Total RalA and actin were also detected by immunoblotting the same non-immunoprecipitated lysates prepared for Ral activation assays.

Trastuzumab, cetuximab, and lapatinib treatment- The indicated human breast cancer cell lines were cultured in serum free media for 48, 24 or 12 hours in the presence or absence of 20 μg/ml cetuximab (ImClone Systems), 30 μg/ml trastuzumab (Genentech), or 1 μM lapatinib (LC Laboratories), respectively. Media was then supplemented with 100 ng/ml EGF (Sigma) for 5 minutes at 37^{o}C, and cells were collected for measurements of phosporylated ERK1/2, RalA-GTP, total RalA, and actin levels.

Tumorigenesis assay- 2 X 10^{6} MDA-MB-231 cells stably expressing either RalA shRNA or a scramble sequence were mixed with Matrigel (BD) and injected into the mammary fat pad of five SCID/beige female mice per cell line. Tumors were measured at regular intervals using Vernier calipers, and tumor volumes were calculated as previously described (157). When scramble control tumors reached maximum allowable tumor volume, mice were euthanized and tumors removed from both experimental groups and analyzed for total and GTP-bound RalA levels. All procedures involving mice were done according to a protocol approved by the Duke University Institutional Animal Care and Use Committee.

2.2 Sec5 and Exo84 are Required for Oncogenic Ras-mediated Tumorigenesis

Cell Lines- Human embryonic kidney cells stably expressing the early region of SV40, the catalytic subunit of telomerase hTERT (HEK-HT), and either H-Ras^{G12V} or Rlf-CAAX were previously described (70,83,154,157). Cell lines were stably infected with retroviruses generated from the described vectors encoding the indicated shRNAs. Specifically, Rlf-CAAX-transformed HEK-HT cells were stably infected with a retrovirus derived from pSUPER-retro-puro encoding RalBP1 shRNA-1, Sec5 shRNA-1, Exo84 shRNA-1, or a vector control. Ras^{G12V}-transformed HEK-HT cells were stably infected with a retrovirus derived from pSUPER-retro-puro encoding RalBP1 shRNA-1, Sec5 shRNA-1, Sec5 shRNA-2, Exo84 shRNA-1, Exo84 shRNA-2, or a scramble control. Ras^{G12V}-transfected HEK-HT cells expressing Sec5 shRNA-1 were further infected with pSUPER-retro-GFP/neo Exo84 shRNA-1 to generate cells expressing Sec5 shRNA-1 and Exo84 shRNA-1. Stable polyclonal populations were enriched by either culture in media supplemented with puromycin, as previously described (157), or sorting for GFP-positive cells by FACS analysis.

Plasmids- pSUPER-retro-puro was engineered to encode RalBP1 shRNA-1, Sec5 shRNA-1 and 2, and Exo84 shRNA-1. pSUPER-retro-GFP/neo was engineered to encode Exo84 shRNA-1. pSM2c encoding Exo84 shRNA-2 was obtained from the Duke University RNA Interference Facility. shRNAs targeted human sequences and were as follows: RalBP1 shRNA-1 (5′-GTA GAG AGG ACC ATG ATG T) (159), Sec5 shRNA-1

(5′-GG TCG GAA AGA CAA GGC AGA T) (160), Sec5 shRNA-2 (5′-CGG CAG AAT GGA TGT CTG C) (160), Exo84 shRNA-1 (5′-GGT GCC ACT TTA CTC TAT A) (97), Exo84 shRNA-2 (5′- ACA ATA TAA TTT GAA TGG CTA A).

Real-time RT-PCR- Total RNA was isolated from cells (Qiagen RNeasy mini kit, Qiagen Inc., Valencia, CA), and reverse-transcribed (iScript cDNA synthesis kit, Bio-Rad, Hercules, CA). Real-time PCR analysis of described mRNA levels was performed on triplicate samples using iTaq SYBR Green Supermix with ROX (Bio-Rad). The primers used were: RalBP1 5′-ACT GTG CAG ATC AGC AAT CG (forward) and 5′-CCT GAT CTC CTC CTT GAT GC (reverse), Exo84 5′-CTG CTT GAG AAG GTG GAA GG (forward) and 5′-GGT AGC CAC CAA CAA GCA AT (reverse), and Sec5 5′-GAT CCT TCA GCT CAT GCA CA (forward) and 5′- GAC TGA GAT GGC CCA ACA CT (reverse). Pre-validated primers and probes against 18S RNA were included as controls (Applied Biosystems, Foster City, CA). Measurements were performed on an ABI Prism 7000 sequence detection system.

Soft agar- 50,000 cells per 3 cm plate were suspended in soft agar as previously described (154,157) and colonies greater than 30 cells were scored after 4 weeks. Assays were performed in triplicate and at least twice independently.

Tumor growth- 7×10^6 cells mixed with Matrigel (BD, Franklin Lakes, NJ) were injected subcutaneously into the flank of 4 SCID/beige mice per cell line, after which tumor volumes were determined at regular intervals as described previously (154,157). All procedures with mice were done according to a protocol approved by the Duke University Institutional Animal Care and Use Committee,

Immunohistochemistry- Tumors arising in mice from injection of Ras^{G12V}-transformed HEK-HT cells expressing the indicated shRNAs were excised, fixed in formalin, paraffin embedded, sectioned, and H&E stained, or used for immunohistochemical detection of Ki67. Ki67-positive cells were counted around the proliferative tumor periphery. Statistical significance was determined by Student's *t*-test. A p value of < 0.05 was considered significant.

2.3 Ral GTPases, Tumorigenesis, and Metastasis: A "Tail" of Two Proteins

Exo84 is Required for Oncogenic Ras-mediated Metastatis of Human Cells

Cell Lines- Human embryonic kidney cells stably expressing the early region of SV40, the catalytic subunit of telomerase hTERT (HEK-HT), and H-Ras^{G12V} were previously described (70,83,154,157). Ras^{G12V}-transformed HEK-HT cells stably expressing RalBP1 shRNA-1, Sec5 shRNA-1, Exo84 shRNA-1, or both Sec5 shRNA-1 and Exo84 shRNA-1 were described in Section 2.2.

Plasmids- pSUPER-retro-puro or pSUPER-retro-GFP/neo were engineered to encode RalBP1, Sec5, and Exo84 shRNAs , as described in Section 2.2.

Metastasis Assay by Tail-Vein Injection- One million cells of the indicated lines were resuspended in 200 μl of sterile phosphate buffered saline and injected into the tail vein of four SCID/beige mice per cell line, as previously described (155). Mice were monitored at regular intervals for signs of weight loss, hair loss, respiratory distress, and general malaise. Upon detection of any of these parameters in a single mouse of a treatment group, all mice were euthanized. Internal organs were examined for metastasis, and lungs were removed from the euthanized mice to assess metastatic growth. All procedures with mice were done according to a protocol approved by the Duke University Institutional Animal Care and Use Committee.

The C-terminal Hypervariable Domain of Ral GTPases Contributes to Their Ability to Support Tumorigenic Growth

Generation of Cell Lines- Ras^{G12V}-transformed HEK-HT cells stably expressing RalA shRNA were previously described (154). These cells were stably infected with a retrovirus derived from pBabe-GFP encoding RalA/B, RalB/A, or no transgene as a vector control, as previously described (154,157). Stable polyclonal populations were enriched by sorting for GFP-positive cells by FACS analysis.

Tumor growth- 7×10^6 cells mixed with Matrigel (BD Biosciences) were injected subcutaneously into the flank of 4 SCID/beige mice per cell line, after which tumor volumes were determined at regular intervals as described previously (154,157). All procedures with mice were done according to a protocol approved by the Duke University Institutional Animal Care and Use Committee.

3. HER2 and EGFR Dependant Activation of RalA in Breast Cancer

Members of the EGF receptor tyrosine kinase family play a role in regulating the proliferation and/or survival of a variety of epithelial tumors, including breast cancer. Activated HER2 and EGFR stimulate the Ras small GTPases, which in turn bind to a number of effector proteins, primarily Rafs, PI3Ks and RalGEFs. While it is well-established that activation of Rafs and PI3Ks downstream of HER2 and EGFR promote mammary tumorigenesis, little is known regarding the role of RalGEFs, the third major effector arm of Ras signaling. In other cancers, oncogenic mutant Ras binds to and activates RalGEFs, which converts the small GTPases RalA and RalB to an active GTP-bound state. Of these two Ral proteins, RalA plays a more dominant role in transformation and tumorigenesis, and hence we investigated the role of RalA in HER2-overexpressing and EGFR-positive breast cancer. We now report that pharmacologic inhibition of HER2 and/or EGFR by treatment with trastuzumabb, cetuximab, and lapatinib reduced the level of activated GTP-bound RalA in a panel of human breast cancer cell lines, whereas expression of activated HER2 increased RalA-GTP levels. Furthermore, we show that shRNA-mediated knockdown of RalA reduced the tumorigenic growth of MDA-MB-231 breast cancer cells in mice, and lastly that RalA was detected in the activated GTP-bound state in tumor versus normal mammary tissue.

Taken together, these results support the notion that HER2 and EGFR activation of RalA contributes to breast cancer.

3.1 Introduction

Genes encoding receptor tyrosine kinases of the epidermal growth factor receptor (EGFR) family, including HER2 (ErbB2/Neu), are commonly dysregulated in breast cancer. For example, gene amplification of HER2 occurs in approximately twenty-five percent of primary human breast tumors (28-30,156). The expression of EGFR family members is implicated in many breast cancer phenotypes, including enhanced cell proliferation, survival, motility, and adhesion, and is associated with poor prognosis (28,29,161,162). As such HER2 overexpression is perhaps the most consistent oncogene marker for breast cancer (3,29,30).

EGFR family members transmit their signals to the cell, in part, by stimulating the conversion of the Ras small GTPases from an inactive GDP-bound state to an active GTP-bound state (3,4,163). Once activated, Ras proteins undergo a conformational change that fosters binding of proteins that mediate Ras signaling. In many cancers, Ras is mutated to remain in the active oncogenic state, and it is well established that oncogenic Ras promotes many cancer cell phenotypes in cultured cells, and tumorigenesis in mice (4,21). While Ras mutations are rare in breast cancer, there is considerable experimental evidence suggesting that deregulated Ras activation and signaling may promote breast cancer development (3,163). For example, expression of an activated Ras protein in MCF-7 breast cancer cells or non-tumorigenic MCF-10A breast epithelial cells enhanced tumorigenicity and led to tumorigenic transformation, respectively (164-166). Furthermore, transgenic mice expressing oncogenic Ras under the control of a mammary tissue-specific promoter develop mammary adenocarcinomas (163). Additionally, overexpression of Ras has been detected in human breast tumors and has been associated with poor prognosis (163,167-170).

Activated Ras binds to and stimulates numerous downstream effector proteins involved in diverse signaling networks affecting numerous activities of the cell, such as proliferation, apoptosis, and differentiation, which have all been implicated in cancer (4). The best-characterized Ras effectors are the Raf kinases, phosphatidylinositol 3-kinases (PI3Ks), and more recently, Ral guanine nucleotide exchange factors (RalGEFs) (4,21). The Raf family of serine/threonine kinases activate the dual-specificity MEK kinases, which phosphorylate and activate the ERK mitogen-activated protein kinases (MAPKs), leading to increased cell proliferation. HER2 overexpressing breast cancer cell lines have been shown to exhibit increased ERK activation in comparison to cells with low HER2 expression levels (171). ERK is also commonly overexpressed and activated in primary breast tumors (163,172-174). EGFR or HER2 activation of ERK in MCF10A cells has been shown to block anoikis, while MEK inhibitor treatment sensitized MDA-MB-231 breast cancer cells to anoikis (175,176). The second-best characterized Ras effectors, the PI3K lipid kinases, regulate phosphoinositide lipid metabolism, leading to the activation of the Akt serine/threonine kinase, and promoting a number of cancer cell phenotypes, including cell survival. Activation of the PI3K/Akt pathway is thought to be involved in both the initiation and progression of human breast cancer (177,178). Activating mutations of PI3K have been identified in breast cancer cell lines and primary breast tumors (177). Additionally, the overexpression of activated Akt1 accelerated Neu-induced mammary tumor development in transgenic mice (179). The third class of Ras effectors with a role in transformation are the family of RalGEFs, which serve as activators of the Ras-related small GTPases RalA and RalB.

Unlike the Raf and PI3K pathways, less is known regarding the RalGEF-Ral pathway in human cancers, and less further on its role in breast cancer. Expressing activated RalGEFs can weakly transform, a variety of cell types, while *RalGDS*$^{-/-}$ mice are resistant to chemically induced skin tumorigenesis (83,150). Activated RalA, but not RalB, is also weakly transforming (154). RNAi-mediated knockdown of RalA in oncogenic Ras-transformed cells or tumor cell lines reduced anchorage-independent growth and tumor initiation (138,154,155), whereas knockdown of RalB reduced cell migration (129,130), survival of transformed cells in suspension (138), and consistent with these phenotypes, metastasis (155). In the cancer most commonly characterized by oncogenic Ras mutations, pancreatic cancer (15), Rals are commonly activated (154,155). In regard to breast cancer, expression of an activated RalGEF promoted anchorage-independent growth of SV40-transformed human mammary epithelial cells (66), and infection of rat mammary ducts *in vivo* with a retrovirus encoding a mutant version of oncogenic Ras (G12V, E37G) that preferentially activates RalGEFs over the other effector pathways, at least in some settings, induced mammary tumors after a long latency (180). Lastly, RalA has been shown to be more highly activated in transformed versus non-transformed mammary epithelial cell lines (181), and in MCF-7 breast cancer cells overexpressing EGFR versus having low EGFR levels following EGF stimulation (140).

EGFR family members have been directly implicated in the pathogenesis of a variety of epithelial malignancies including breast cancer. Given that these receptors activate Ras, and that the RalGEF substrate RalA, in particular, is required for oncogenic Ras-driven tumorigenesis, we explored the activation status and requirement of RalA, representing the last major Ras effector pathway, in breast cancer.

3.2 Results

RalA is Activated Upon HER2/Neu Expression

HER2 is amplified and/or overexpressed in approximately twenty-five percent of all human primary breast cancers, and is associated with a poor prognosis (28-30). HER2 signals extensively through activation of the small GTPase Ras (3,163), and of the three major effectors of Ras, only the RalGEF-Ral pathway remains to be studied for its role in breast cancer. Given that in the RalGEF-Ral pathway, the protein RalA in particular is required for oncogenic Ras-driven tumorigenesis, we focused our analysis on RalA, testing whether HER2 activation stimulated RalA. Human 293T cells were therefore transiently transfected with a construct expressing constitutively activated HER2/Neu (NeuNT) (182) or no transgene as a control, and appropriate expression was confirmed by immunoblot analysis (Figure 4). Active GTP-bound RalA levels were assessed by incubating lysates from the transiently transfected serum-starved 293T cells with a recombinant, glutathione-sepharose-bound GST-tagged polypeptide encoding the Ral binding domain of the Ral effector protein RalBP1 (GST-RBD), which specifically interacts with GTP-bound Ral. RalA-GTP was detected by immunoblot analysis of the GST-RBD/ Ral-GTP complex with an α-RalA antibody (155,158). Cells expressing the activated Neu protein reproducibly exhibited approximately 2-fold more active RalA-GTP compared to vector control cells. NeuNT expression did not affect total RalA levels, as measured by immunoblot. Thus, activated HER2/Neu increases the level of GTP-bound, active RalA.

Trastuzumab Inhibition of HER2 Suppresses RalA Activation

To address whether the observation that ectopic expression of HER2 activates RalA could be recapitulated in a more clinically relevant and physiological setting, we tested whether inhibition of endogenously expressed HER2 with a pharmacologic inhibitor used in the clinic, trastuzumab (162,183), reduced activation of endogenous active RalA in breast cancer cell lines. Specifically, the breast cancer cell lines SKBR3 and BT474, which are known to overexpress HER2 (184,185), were cultured in serum-free media supplemented with either the humanized monoclonal anti-HER2 antibody trastuzumab, or sterile water as a vehicle control, after which cells were stimulated with EGF. In both cell lines, trastuzumab treatment was confirmed to be effective, as evident by the reduction in ERK1/2 phosphorylation in the trastuzumab-treated versus control-treated cells (Figure 5A). Importantly, the level of RalA-GTP was also reduced by approximately 15 - 50% by trastuzumab treatment in both cell lines (Figure 5A). The modest decrease in ERK1/2 phosphorylation in trastuzumab-treated cells following EGF stimulation may have been due to activation by HER2-independent EGFR family members. Thus, the drug used to clinically treat HER2-positive breast cancers reduces RalA activation.

Cetuximab Inhibition of EGFR Suppresses RalA Activation

While HER2 overexpression is observed in twenty-five percent of human breast cancers, HER2 belongs to the EGFR family of receptor tyrosine kinases. Moreover, a significant amount of breast cancers are also characterized by expression of EGFR (28). EGFR-positive cancers, such as colorectal and head and neck squamous-cell carcinoma, are treated clinically with anti-EGFR antibodies such as cetuximab (162,186). We therefore tested whether, like HER2, pharmacologic inhibition of endogenously expressed EGFR with the clinical drug cetuximab reduced activation of endogenous RalA in MDA-MB-231 and MDA-MB-468 breast cancer cell lines, which are known to express EGFR (184,185). Specifically, treatment of the serum-starved cells with cetuximab reduced not only EGF-induced ERK1/2 phosphorylation, thereby validating that cetuximab suppressed activated EGFR, but also RalA-GTP by 25 – 50% compared to cells treated with PBS as a vehicle control (Figure 5B). Thus, a drug clinically used to treat EGFR-positive cancers reduces RalA activation.

Lapatinib Inhibition of HER2 and EGFR Suppresses RalA Activation

To independently validate that EGFR and HER2 activation leads to RalA activation we utilized a different type of drug used clinically for the treatment of breast cancer, namely the small molecule dual EGFR/HER2 tyrosine kinase inhibitor lapatinib (162,187). We treated serum-starved SKBR3, BT474, and MDA-MB-231 cells with either lapatinib, or DMSO as a vehicle control, and as above, measured ERK1/2 phosphorylation and RalA-GTP levels following stimulation with EGF. In all three cell lines, lapatinib-treated cells exhibited reduced ERK1/2 phosphorylation, again confirming that the drug inhibited HER2 and EGFR, and reduced RalA-GTP levels by up to 50% compared to vehicle control-treated cells (Figure 5C). Thus, inhibition of endogenous HER2 or EGFR in multiple human breast cancer cells, with two different classes of drugs used in the clinic to treat breast cancers, reduced endogenous RalA activation, suggesting that RalA is a *bona fide* downstream effector of these receptor tyrosine kinases.

Knockdown of RalA Expression Inhibits Tumorigenic Growth of Breast Cancer Cells

Given that activated HER2 increased, and pharmacologic inhibition of HER2 and EGFR decreased RalA-GTP levels, respectively, we next assessed the biological significance of RalA activation in breast cancer cells. Specifically, tumorigenic MDA-MB-231 cells, which have elevated levels of endogenous EGFR (184,185), were stably infected with retroviruses encoding either shRNA targeting RalA or a scramble sequence as a control (154,155). Appropriate knockdown of RalA was confirmed by immunoblot (Figure 6A). The resultant two stable cell lines were orthotopically injected into the mammary fat pad of five immunocompromised mice each, and tumor volume was recorded at regular intervals. Tumors arising from MDA-MB-231 cells in which RalA expression was knocked down had an approximately one week longer latency, and reduced growth kinetics, growing approximately two-fold slower, compared to the scramble control counterpart (Figure 6B). Additionally, when scramble control tumors reached maximum allowable tumor volume, tumors arising from RalA knockdown cells were approximately 55% smaller by average volume. The difference in volume was not statistically significant, however, but this was due to delayed growth of one of the five scramble control tumors, likely due to an error in injection of that mouse. Moreover, when tumors did finally grow, the level of RalA, as assessed by immunoblot was elevated in every one of the five tumors compared to the same cells before injection, suggestive of a strong selective pressure for cells with restored RalA expression (Figure

6A). Collectively, these data suggest that loss of RalA is a potent barrier to tumorigenic growth of breast cancer cells.

Elevated RalA-GTP Levels in Human Breast Cancer

To test if activation of RalA occurs in human breast cancer, we measured the level of RalA-GTP in five matched pairs of ductal adenocarcinomas and normal mammary tissue isolated from patients diagnosed with breast cancer. All five tumor specimens selected were immunohistochemically shown to be HER2-positive (data not shown). Analysis of total and GTP-bound RalA revealed that while the level of total RalA did not vary dramatically between matched normal and tumor tissue, in four out of five cases RalA-GTP levels were 1.5 – 3 fold higher in the tumor versus matched normal tissue (Figure 7). While this analysis was only performed once due to limited material, these data suggest that RalA activation may play an important role in human HER2-positive mammry tumorigenesis.

3.3 Discussion

We now demonstrate that ectopic expression of activated HER2 increased, while pharmacologic inhibition of endogenous HER2 and/or EGFR in multiple breast cancer cell lines by different classes of drugs (antibody and small molecule inhibitor) decreased, the levels of active GTP-bound endogenous RalA. Consistent with these results, RalA was also activated in four of five human ductal adenomcarcinoma samples compared to matched patient normal mammary tissues. These results strongly suggest that the inappropriate constitutive activation of these receptor tyrosine kinases in breast cancer leads to the sustained activation of RalA. Presumably, the activation of RalA in this manner is due to stimulation of Ras downstream of HER2 or EGFR, given that stimulation of these receptors leads to Ras activation (3,163), Ras is in the activated state in breast cancer cells (163,188), and constiutively active (oncogenic) Ras is known to activate RalA (154,155). However, sequence differences have been detected in the RalGEF Rgl1 in breast cancer tissues (189), raising the possibility of other modes of activating RalA in breast cancer. While RalA has been shown to be activated in oncogenic Ras-driven cancer cells and tumor specimens (154,155), our studies now demonstrate other cancer settings that lead to activated Ras also increase RalA-GTP levels; specifically, activation of receptor tyrosine kinases. Thus, RalA activation may occur more broadly in human cancers.

Prompted by the finding that RalA-GTP levels in breast cancer cell lines were reduced by pharmacologic inhibition of HER2 and EGFR, we tested and demonstrated that knockdown of RalA in a breast cancer cell line reduced its tumorigenic potential. Thus, consistent with the observation that knockdown of RalA reduces tumorigenesis in settings in which the protein is activated by oncogenic Ras (154,155), knockdown of RalA in a setting in which the protein was activated by EGFR also reduced tumorigenesis.

Although therapies targeting HER2 and EGFR are currently clinically approved for the treatment of breast and other solid tumors insight into their mechanism of anti-tumor activity remains controversial, and has limited their clinical impact. For example, we and others have shown that lapatinib inhibits the phosphorylated, activated form of HER2 with downstream inhibitory effects on activation of PI3K-Akt and MAPK-ERK in HER2-positive breast cancer cell lines, tumor xenografts, and HER2-positive primary breast cancers (190,191). Although inhibition of HER2 and/or EGFR phosphorylation by lapatinib might be necessary, it is not sufficient to induce tumor cell apoptosis and clinical response. We now provide important insight into the effect of HER2 and EGFR targeted therapies on RalA, and how this might play a key role in mediating the anti-tumor activity of these targeted agents. RalA is not only informative as to mechanisms of tumorigenesis in HER2-positive and EGFR-expressing breast cancers, and how HER2 and EGFR targeted therapies exert their anti-tumor effects, but may also provide valuable insight into mechanisms of therapeutic resistance. For example, persistent activation of RalA through redundant mechanisms of regulation, independent of HER2 and EGFR signaling, might lead to therapeutic resistance to targeted agents such as lapatinib, trastuzumab, or cetuximab. Moreover, gain-of-function mutations in the PI3K catalytic domain, or activating Ras mutations render solid tumors resistant to HER2 and EGFR targeted therapies (192-194). Whether similar mutations in RalA exist in tumors, and if so, whether they too play a role in therapeutic resistance, warrants further exploration.

With the development and widespread clinical use of targeted therapies, treatment decisions in cancer are increasingly being made on the molecular profile of tumors rather than histology alone. Identifying the mechanism(s) of anti-tumor activity of targeted therapies such as lapatinib, trastuzumab, or cetuximab, is essential in order to inform as to which tumor profiles will predict for response, or conversely resistance. The evidence presented here indicates that the state of RalA activation at baseline and in response to HER2 or EGFR targeted therapy represents a potential predictor of, and pharmacodynamic indicator associated with response to trastuzumab, lapatinib, or cetuximab. Furthermore, insight into the regulation of RalA and its downstream effectors provides additional potential tractable targets for therapeutic intervention to enhance the clinical efficacy of HER2 and EGFR targeted therapies in breast and other solid tumors.

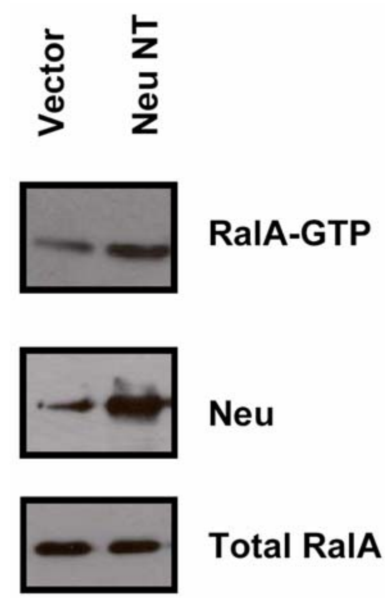

Figure 4. The effect of activated HER2/Neu expression on RalA-GTP levels.

Detection of activated GTP-bound RalA via association with effector protein domain specific for the activated version of the protein, followed by immunoblot analysis with RalA antibody in 293T cells transfected with empty vector control (Vector) or NeuNT. Detection of transfected NeuNT in the same cell lines by immunoblot analysis with HER2/Neu antibody. Total RalA serves as a loading control. Representative immunoblot of 3 independent experiments.

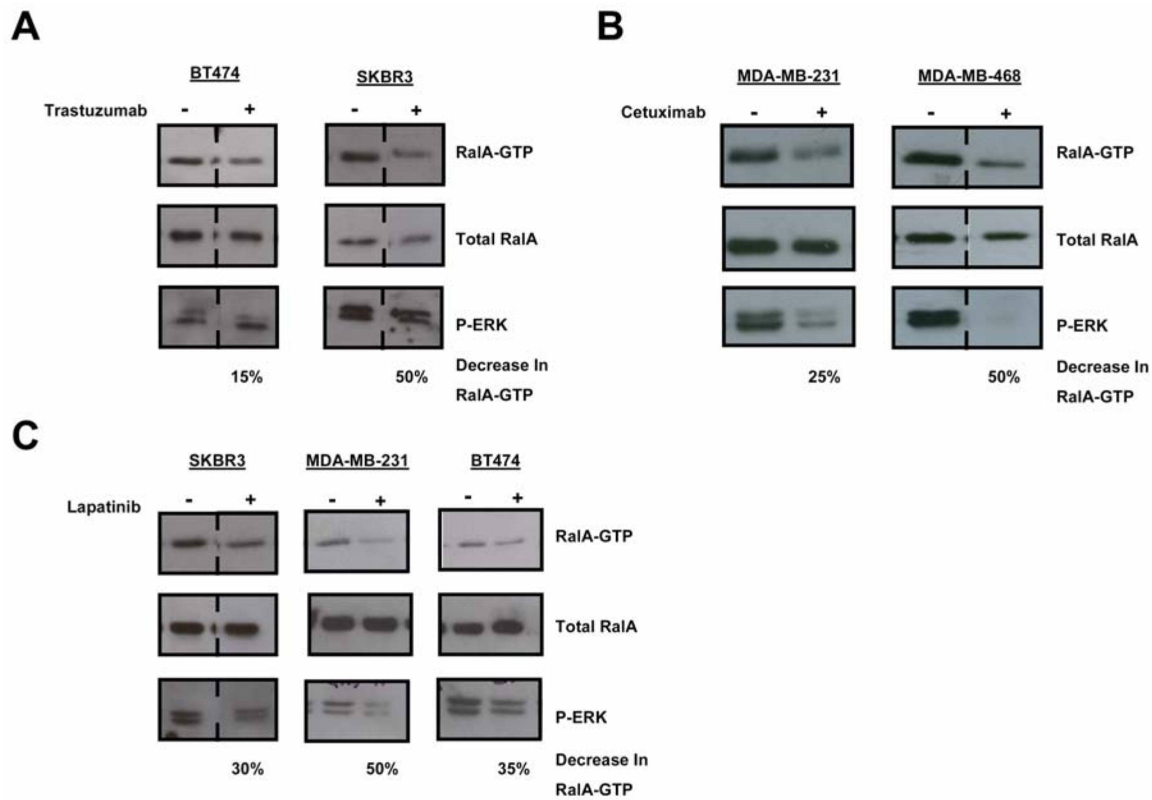

Figure 5. The effect of EGFR and HER2 inhibition on RalA-GTP levels.

A - C. Detection of activated GTP-bound RalA via association with effector protein domain specific for the activated version of the protein, followed by immunoblot analysis with RalA antibody in the indicated breast cancer cell lines treated with (+) or without (-) the indicated EGFR and HER2 inhibitors. Detection of MAPK activation in the same cell lines by immunoblot analysis with phosphor-specific antibody for phosphorylated ERK1/2 (P-ERK). Total RalA serves as a loading control. Representative immunoblots of 2 independent experiments. Decrease in RalA-GTP of inhibitor-treated cells versus vehicle control treated cells was determined using ImageJ image quantitation software, and was normalized to total RalA.

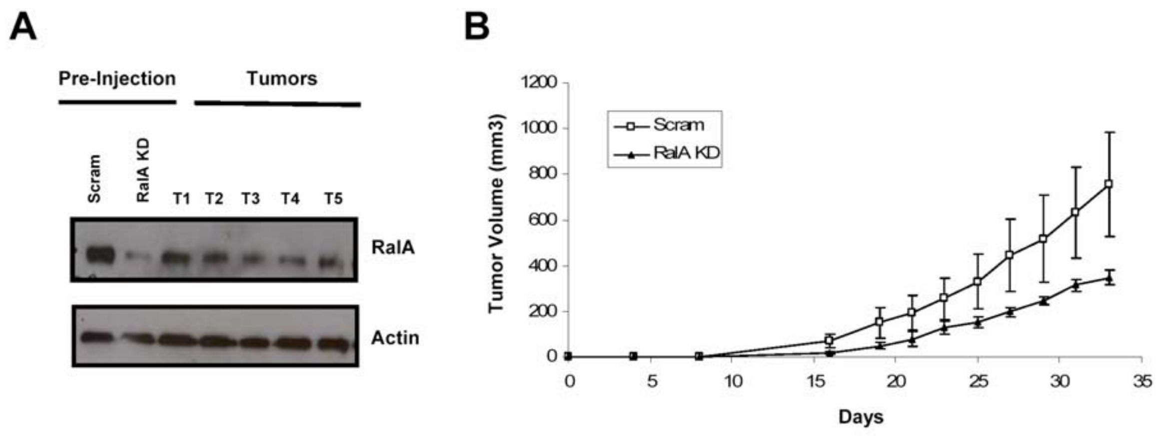

Figure 6. The effect of RalA knockdown on tumorigenic growth of breast cancer cells.

A .Detection of RalA by immunoblot analysis in MDA-MB-231 human breast cancer cells stably expressing a RalA scramble sequence (Scram) or RalA shRNA (RalA KD) before injection (Pre-Injection) into immunocompromised mice, or in resultant RalA KD tumors (T1-T5). Actin serves as a loading control. *B*. Average tumor volume ± standard error versus time, of five mice injected with MDA-MB-231 cells stably expressing a RalA scramble sequence (□), or RalA shRNA (▲). Thomas Ribar assisted with mouse injections.

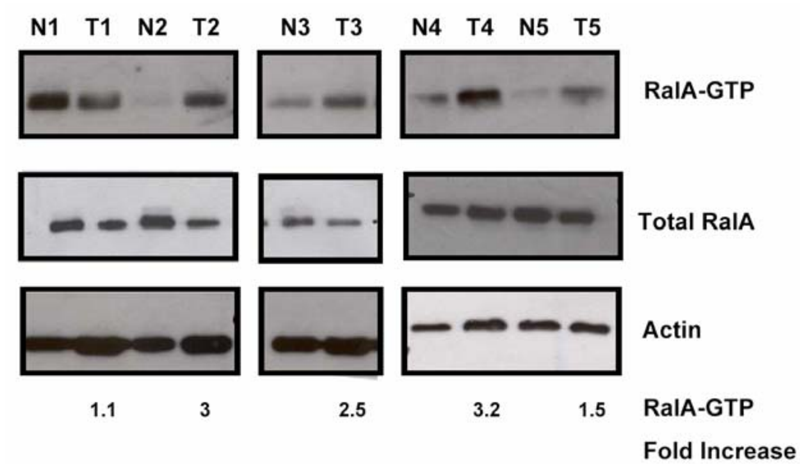

Figure 7. RalA-GTP levels in matched normal and breast tumor samples.

Detection of activated GTP-bound RalA via association with effector protein domain specific for the activated version of the protein, followed by immunoblot analysis with RalA antibody in a panel of 10 matched mammary tissue samples obtained from patients. Total RalA and actin serve as loading controls. (N represents normal tissue, T represents ductal adenocarcinoma tissue). Fold increase in RalA-GTP in tumor versus normal tissue was determined using ImageJ image quantitation software, and was normalized to total RalA.

4. Sec5 and Exo84 are Required for Oncogenic Ras-mediated Tumorigenesis

The genes encoding the Ras family of small GTPases are mutated to yield constitutively active GTP-bound oncogenic proteins in one-third of all human cancers. Oncogenic Ras binds to and activates a number of proteins that promote tumorigenic phenotypes, including the family of Ral guanine nucleotide exchange factors, or RalGEFs. Activated RalGEFs convert the Ral family of small GTPases, comprised of RalA and RalB, from an inactive GDP-bound state to an active GTP-bound state. As both RalA and RalB have been implicated in a variety of tumorigenic phenotypes, we sought to determine which proteins downstream of Rals promote transformation and tumorigenesis. Here we report that shRNA-mediated knockdown of the Ral effector proteins Sec5 and Exo84, but less so in the case of RalBP1, reduced oncogenic RalGEF-mediated transformation and oncogenic Ras-driven tumorigenic growth of human cells. These results suggest that Rals promote oncogenic Ras-mediated tumorigenesis through, at least in part, Sec5 and Exo84.

4.1 Introduction

Mutated and constitutively activated forms of the small GTPase Ras are found in nearly one-third of all human cancers (15). Oncogenic Ras binds to a number of effector proteins to promote transformation and tumorigenesis, including the family of Ral guanine nucleotide exchange factors (RalGEFs) (21,83,85,150). Recruiting RalGEFs to their substrates, the Ras-like GTPases RalA and RalB, converts Rals from an inactive GDP-bound state to an active GTP-bound state (48,84,86). Ral activation is necessary for oncogenic Ras-mediated transformation of a broad variety of human cell types (66,83,154,155). Specifically, RalA is required for anchorage-independent growth and tumorigenesis, whereas RalB is important for cell survival, especially of cells in suspension, as well as for cell migration, and metastasis (129,130,138,154,155). Activated Rals bind to a limited number of effector proteins, the best-documented being RalBP1 (RLIP76), Sec5, and Exo84 (26,48,93,96,97). RalBP1 contains a Rac/Cdc42 GAP domain (94) and is involved in the regulation of a subset of endocytic pathways (107,111,159). Sec5 and Exo84 constitute two members of the octameric exocyst complex, which participates in the targeting and tethering of secretory vesicles to specific plasma membrane domains, including the basolateral surface of epithelial cells, the mitotic abscission plane, and the leading edge of migrating epithelial cells (26,120). Interactions with RalBP1, Sec5, and Exo84 link Ral proteins to receptor-mediated endocytosis, the maintenance of epithelial cell polarity, cytokinesis, and cell motility (26,48,96,107,111,120,130,195,196). The contribution of these effector proteins to Ral-dependent support of transformed and tumorigenic growth of human cells is unclear. Given the frequency of Ras mutations in human cancers, and the critical role of Rals in many different oncogenic Ras-mediated tumor phenotypes, we examined whether the

Ral effectors RalBP1, Sec5 or Exo84 were required for transformation or tumorigenesis of human cells.

4.2 Results

Sec5 and Exo84 are Required for RalGEF-mediated Transformation of Human Cells

Oncogenic Ras promotes transformed cell growth through, in part, activation of RalGEFs (66,83). Upon activation by RalGEFs, Ral proteins bind to a limited number of effectors, including RalBP1, Sec5, and Exo84, which have been implicated in receptor-mediated endocytosis, the maintenance of epithelial cell polarity, cytokinesis, and cell motility (26,48,96,107,111,120,130,195,196). To identify how oncogenic Ras promotes transformation through Rals, we first tested whether knockdown of RalBP1, Sec5, or Exo84 inhibited transformation. Transformed growth was assessed using human embryonic kidney cells stably expressing the early region of SV40 and the catalytic subunit of telomerase, hTERT (HEK-HT). These cells are genetically defined and grow in soft agar, a transformed phenotype characteristic of many cancer cell lines, upon activation of endogenous Ral proteins by expression of Rlf-CAAX (154), a constitutively active version of the RalGEF family member Rlf (59,88). Rlf-CAAX-expressing HEK-HT cells were stably infected with retroviruses encoding shRNA (shRNA-1) against RalBP1 (159), Sec5 (160), Exo84 (97) or no shRNA to serve as a vector control. Appropriate knockdown of the targeted mRNA was confirmed by quantitative real-time RT-PCR

(Figure 8A). Specifically, RalBP1, Sec5, and Exo84 mRNA levels were reduced by 86%, 77%, and 90%, respectively. The resultant cell lines were then seeded in soft agar, and four weeks later the number of colonies were counted as a measure of transformation. shRNA-mediated knockdown of RalBP1 resulted in a 50% decrease in colonies growing in soft agar, while knockdown of either exocyst complex component Sec5 or Exo84 nearly abolished transformation, resulting in an 83% or 86% decrease in the number of colonies compared to control cells, respectively (Figure 8, B and C).

Sec5 and Exo84 are Critical for Oncogenic Ras-mediated Tumorigenesis of Human Cells

To address whether the decrease of *in vitro* soft agar growth of Rlf-CAAX-transformed HEK-HT cells reflected a decrease in tumorigenic growth *in vivo*, expression of the same three Ral effector proteins was knocked down by shRNA in the tumorigenic version of HEK-HT cells in which Rlf-CAAX was replaced with oncogenic Ras^{G12V} (70). Specifically, Ras^{G12V}-expressing HEK-HT cells were stably infected with the aforementioned retroviruses encoding shRNA (shRNA-1) targeting RalBP1, Sec5, Exo84, or a scramble sequence as a control. Reduced expression of RalBP1, Sec5, and Exo84 was assessed by real-time RT-PCR (Figure 9*A*). RalBP1, Sec5, and Exo84 mRNA levels were decreased by 64%, 78%, and 80%, respectively. The resultant stable cell lines were then injected subcutaneously into the flank of four immunocompromised mice each, and tumor volume was recorded at regular intervals. Scramble control cells generated tumors with a latency of 13 days, and reached maximum tumor volume by 3 weeks, as previously reported (154). Knockdown of RalBP1 had little effect on the tumorigenic growth of HEK-HT Ras^{G12V} cells, as tumors formed at the same time and grew with nearly identical kinetics as those derived from scramble control cells (Figure 9, B and C). However, consistent with the soft agar results (Figure 8C), knockdown of either Sec5 or Exo84 prolonged the latency period of tumorigenesis and impeded subsequent tumor growth. Specifically, tumors arising from cells in which Sec5 or Exo84 were knocked down had a latency of 26 days, twice as long as scramble control cells, and reached maximum volume by around 5 weeks, 2 weeks longer than it took scramble control cells to reach the same size (Figure 9, B and C). Knockdown of Sec5 or Exo84 thus reduced the tumorigenic potential of oncogenic Ras-transformed human cells.

To independently validate that knockdown of Sec5 or Exo84 reduced tumor growth we generated retroviruses encoding a second shRNA (shRNA-2) against a different region of the Sec5 (160) and Exo84 mRNA (Figure 9A). Ras^{G12V}-transformed HEK-HT cells were infected with these retroviruses and stable cell lines were assayed for Sec5 and Exo84 expression. These second shRNAs reduced Sec5 expression by 62% and Exo84 expression by 51%, as assessed by real-time RT-PCR (Figure 9A). Thus, these shRNAs knocked down the expression of the desired target mRNA, although not as efficiently as the first shRNAs. The resultant stable cell lines were injected into immunocompromised mice as before, and tumor volume was recorded at regular intervals. Consistent with the previous findings, cells expressing these independently generated Sec5 and Exo84 shRNAs exhibited a clear reduction in tumorigenic growth (Figure 9B). Moreover, there was a correlation between tumor size and the degree of Sec5 or Exo84 expression (Figure 9, A and B, compare knockdown level and subsequent tumor size of Sec5 and Exo84 shRNA-1 to shRNA-2). Sec5 and Exo84 thus act as critical mediators of oncogenic Ras-induced tumorigenesis.

Since suppression of Sec5 and Exo84 expression had the greatest effects on transformation and tumorigenesis, and both proteins are members of the exocyst complex, we tested whether simultaneously reducing the expression of both of these exocyst components would further reduce the level of tumor growth. Specifically, Ras^{G12V}-transformed HEK-HT cells were sequentially infected with the aforementioned retroviruses encoding Sec5 shRNA-1 and Exo84 shRNA-1. Appropriate knockdown of the targeted mRNA was confirmed by quantitative real-time RT-PCR (Figure 9A). Sec5 and Exo84 expression levels were reduced by 59% and 58%, respectively. Tumors arising in mice injected with this cell line grew the slowest, with a latency of 29 days, and displayed impaired growth kinetics (Figure 9, B and C). Thus the concerted loss of Sec5 and Exo84 function further reduced tumor growth.

Knockdown of Sec5 and/or Exo84 is Associated with a Minor Proliferation Defect *in vivo*

To ascertain the nature of the defect in tumor growth upon knockdown of either Sec5 or Exo84, tumors arising from Ras^{G12V}-transformed HEK-HT cells stably expressing RalBP1, Sec5, Exo84, or both Sec5 and Exo84 shRNA were removed and immunohistologically compared to tumors arising from scramble control cells. Specifically, tumors arising from the aforementioned cells were excised, formalin fixed, paraffin embedded, and sectioned. H&E staining of the tumors did not reveal any gross histological differences compared to scramble control tumors (Figure 10A, top). Since Ral proteins have been previously linked to proliferation (48), changes in cell proliferation were assessed by Ki67 immunohistochemical staining. Tumors derived from RalBP1 knockdown cells had no statistically significant difference in the number of Ki67-positive cells ($P>0.05$) compared to scramble control tumors. (Figure 10A, bottom and 3B) However, tumors eventually arising from Sec5, Exo84, or Sec5 and Exo84 double knockdown cells exhibited a small, but statistically significant 10% decrease in the number of Ki67-positive cells ($P<0.05$) compared to scramble control tumors (Figure 10A bottom and 10B). The relatively minor effect may be a consequence of tumors overcoming the loss of Sec5 and Exo84 by the time they can be excised and examined. Nevertheless, the detected decrease in Ki67 staining does suggest that knockdown of Sec5 and/or Exo84 reduces proliferation of tumorigenic cells *in vivo*.

4.3 Discussion

Inappropriate activation of the small GTPase Ras plays a critical role in oncogenesis. Mutated and constitutively activated forms of Ras are found in nearly one-third of all human cancers (15). Consequently, there has been considerable effort to define the effector pathway downstream of Ras signaling responsible for mediating its oncogenic effects. To this end, a critical role for the RalGEF/Ral effector pathway in Ras-mediated transformed and tumorigenic growth of a variety of human cell types has been demonstrated (66,83,154,155). RalGTPases signal through a small set of effector proteins, the most documented being RalBP1 and the exocyst complex components Sec5 and Exo84. In the current study we have examined which Ral effectors mediate Ras-driven oncogenesis.

We now demonstrate that the Ral effectors Sec5 and Exo84 are critical mediators of oncogenic Ras signaling. Stable knockdown of either Sec5 or Exo84 in RalGEF-transformed cells led to decreased anchorage-independent colony formation, similar to that previously observed upon knockdown of RalA in the same cells (154), arguing that these effectors mediate much of the transforming signal downstream of Rals. In support of this hypothesis, it was previously shown that a mutation that reduced the binding of RalA to Sec5 and Exo84 resulted in a greater reduction of transformation by activated RalA than a mutation that reduced binding to RalBP1 (154). Stable suppression of Sec5 and Exo84 had no gross effect on the ability of the cells to proliferate in monolayer culture as compared to the control cells (data not shown), consistent with previous observations that knockdown of RalA or RalB did not alter the growth of cultured adherent cells (129,130,138,154,155), suggesting a defect in cell transformation.

With regard to Ras-mediated tumorigenesis, we show that stable knockdown of Sec5 or Exo84, and even more significantly knockdown of both Sec5 and Exo84 together, in highly tumorigenic Ras^{G12V}-transformed cells significantly prolonged the latency period of tumorigenesis and impeded subsequent tumor growth. Immunohistochemical analysis of Ki67 staining revealed that the outgrowing tumors formed from Sec5 and/or Exo84 knockdown cells displayed less proliferation than those derived from scramble control or RalBP1 knockdown cells.

While Rals have been implicated in the regulation of a number of diverse cellular processes, our current findings suggest that interactions with the exocyst complex components Sec5 and Exo84 may contribute to their oncogenic activity. Interactions with these proteins link Rals to basolateral membrane protein targeting and maintenance of epithelial cell polarity, the maintenance of cytokinesis, and cell migration (96,130,195,196). While deregulation of these processes would likely have significant repercussions, the exact mechanism by which the effects of Ral proteins on exocyst function may be linked to Ras-mediated transformation of human cells remains to be elucidated.

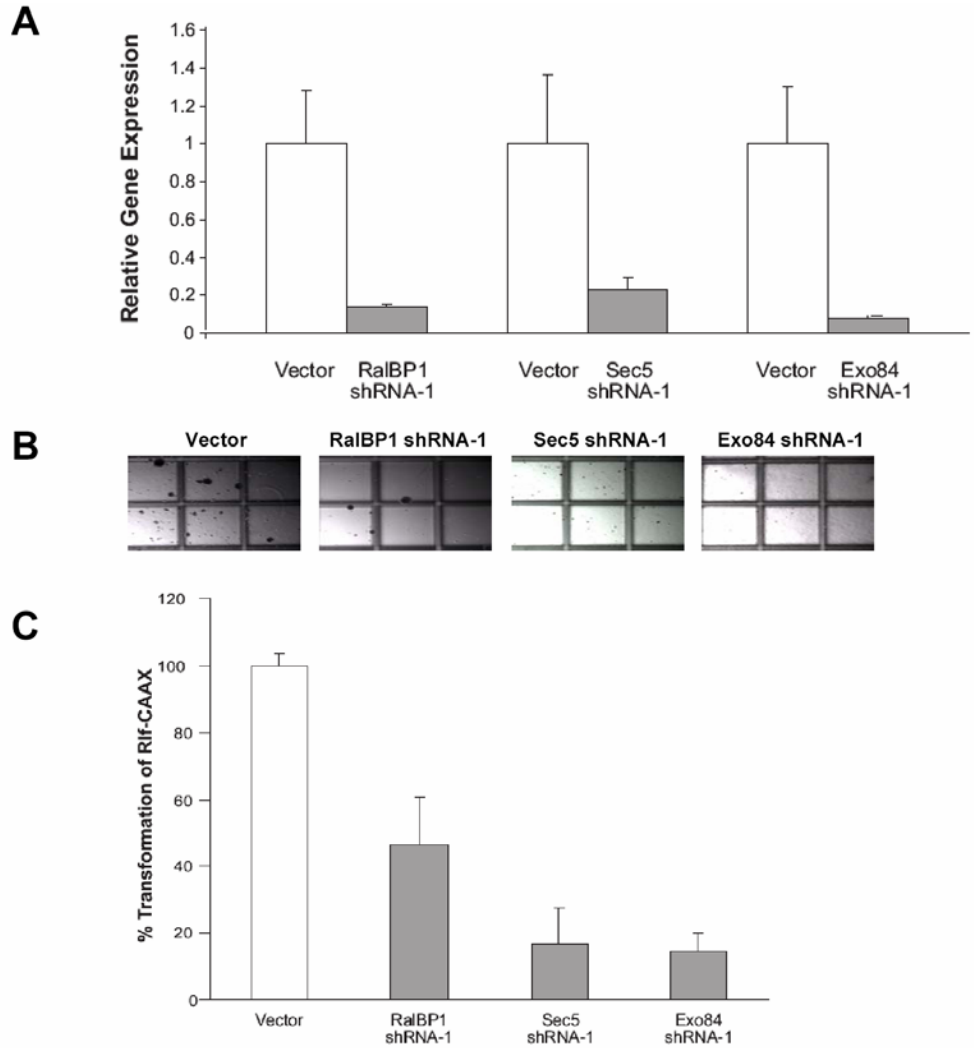

Figure 8. The effect of RalBP1, Sec5, and Exo84 knockdown on RalGEF-mediated transformation of human cells.

A, Relative mRNA level ± standard deviation of the indicated transcripts in Rlf-CAAX-transformed HEK-HT cells stably infected with the indicated shRNAs, as determined by real-time RT-PCR. *B*, Photographs demonstrating representative anchorage-independent growth of Rlf-CAAX-transformed HEK-HT cells stably expressing the indicated shRNAs and *C*, graphical representation expressed as the average percentage of colonies (>30 cells) ± standard deviation normalized to vector control cells. Kian-Huat Lim assisted with soft agar assays.

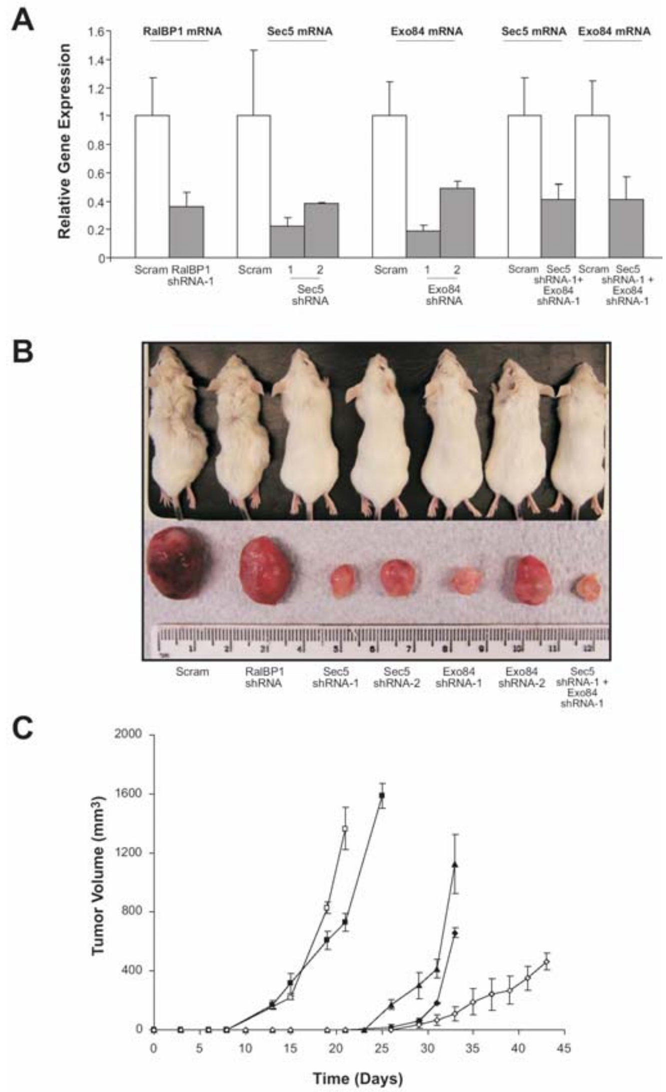

Figure 9. The effect of RalBP1, Sec5, and Exo84 knockdown on oncogenic Ras-mediated tumorigenesis of human cells.

A, Relative mRNA level ± standard deviation of the indicated transcripts in Ras^{G12V}-transformed HEK-HT cells stably infected with the indicated shRNAs, as determined by real-time RT-PCR. *B*, Photograph of a representative mouse flank and excised tumor resulting from injection of Ras^{G12V}-transformed HEK-HT cells expressing the indicated shRNAs, taken when scramble control cells reached maximum tumor volume. *C*, Average tumor volume ± standard error versus time, of at least three mice from a representative experiment, injected with Ras^{G12V}-transformed HEK-HT cells stably expressing a scramble control shRNA (□), RalBP1 shRNA-1 (■), Sec5 shRNA-1 (▲), Exo84 shRNA-1 (♦), or Sec5 shRNA-1 and Exo84 shRNA-1 (◊). Kian-Huat Lim and Stacey Adam assisted with mouse injections.

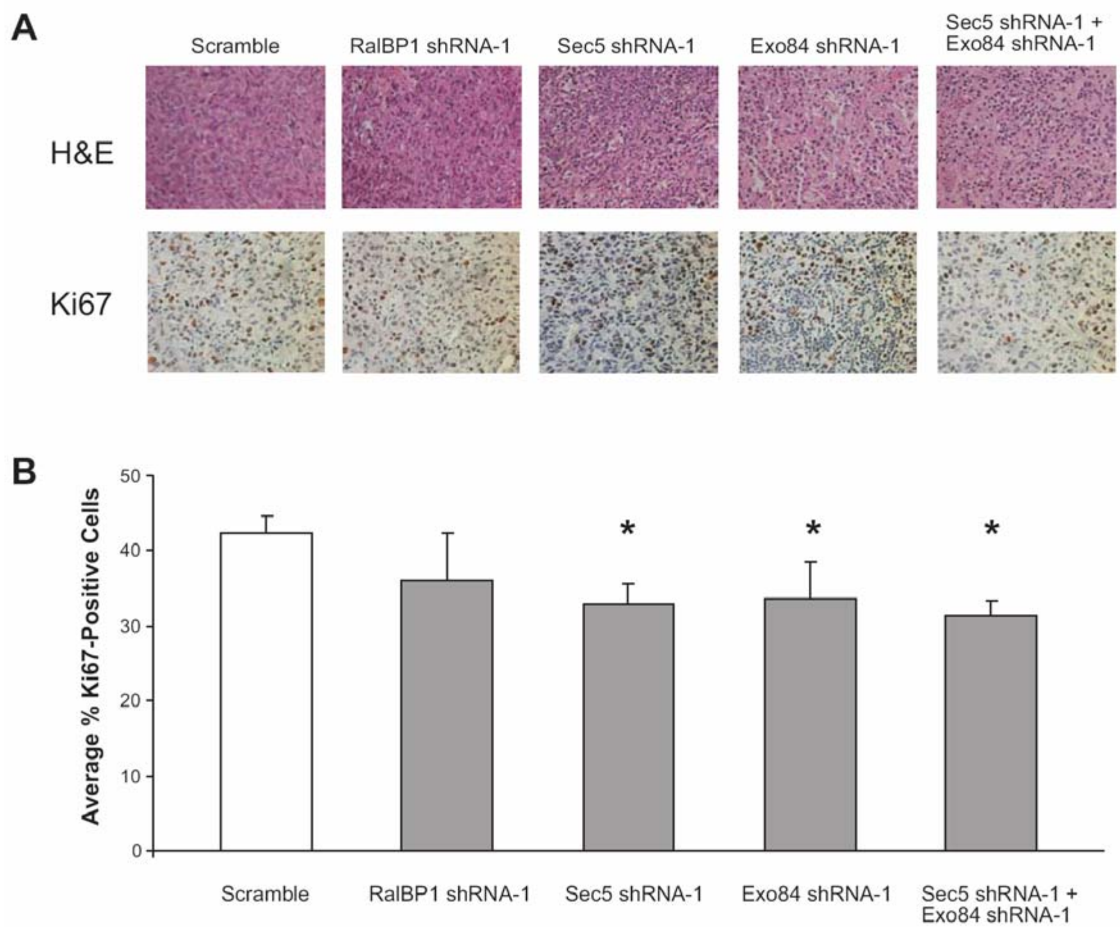

Figure 10. Ki67 staining of tumors derived from cells expressing RalBP1, Sec5, and Exo84 shRNA.

A, Representative H&E (*top*) and Ki67 (*bottom*) staining of tumors arising in mice from injection of Ras^{G12V}-transformed HEK-HT cells expressing the indicated shRNAs (Magnification, X400). *B,* Average percentage of Ki67-positive cells ± standard deviation from at least three independent fields consisting of a total of at least one thousand cells from two different tumors. * $P < 0.05$, as assessed by Student's *t*-test.

5. Ral GTPases, Tumorigenesis, and Metastasis: A "Tail" of Two Proteins

5.1 Introduction

While RalA has been shown to be necessary for oncogenic Ras-mediated tumorigenesis of human cells, RalB has been shown to be important for metastasis (154,155). Specifically, stable shRNA-mediated knockdown of RalB in oncogenic Ras-transformed HEK-HT cells or two K-Ras mutant metastatic pancreatic cancer cell lines has been shown to reduce metastatic growth more effectively than RalA kncockdown (155). Additionally, metastasis to the adrenal gland caused by one of the two pancreatic lines was abolished by RalB, but not RalA, knockdown. These data suggested that RalA and RalB, while 85% identical at the amino acid level, may serve distinct functions in tumorigenesis and metastasis, with RalA activation necessary early in the process of tumorigenesis, and RalB function necessary for some aspect of metastatic growth, such as survival in the bloodstream or extravasation and organ colonization. RalA and RalB have also previously been shown to play distinct roles in processes such as polarized delivery of membrane proteins to the basolateral surface of epithelial cells, and migration of bladder and prostate cancer cell lines (120,129). While RalB was shown to be required for Ras-mediated metastasis, the Ral effectors supporting metastatic growth were not examined.

As mentioned, RalA and RalB are highly similar proteins (Figure 11). The greatest region of sequence divergence between the two proteins is found in the C-terminal hypervariable domain. This region has been shown to be important for subcellular localization and certain functions of Ral GTPases (120,154). Specifically, the importance of the hypervariable domain was evaluated by the generation of chimeric Ral proteins, in which the hypervariable domains (consisting of the last 30 C-terminal amino acids) of RalA and RalB were swapped (120). This created a RalA protein that had its hypervariable domain replaced by that of RalB (RalA/B), and a RalB protein that had its hypervariable domain replaced by that of RalA (RalB/A). When the two chimeras were expressed and assayed for localization and functional activity, they behaved similarly to the Ral protein containing the same hypervariable domain (120,154). For example, the RalB/A chimera was more transforming than RalB, suggesting that the RalA C-terminus contributes to transforming ability, as assessed by anchorage-independent growth (154). However, the importance of the hypervariable domain in mediating RalA-dependent support of Ras-induced tumorigenesis was not examined.

Given the distinct roles of RalA and RalB in tumorigenesis and metastasis, I examined whether the Ral effectors RalBP1, Sec5, or Exo84 were required for metastatic growth of oncogenic Ras-transformed human cells. I also tested whether the Ral C-terminal hypervariable domains contributed to the ability of the proteins to affect tumorigenesis.

5.2 Results and Discussion

Exo84 is Required for Oncogenic Ras-mediated Metastasis of Human Cells

Oncogenic Ras promotes metastatic growth, in part, through, activation of RalGEFs (152,153). Of the two known RalGEF substrates, RalA and RalB, RalA has been shown to be necessary for Ras mediated tumorigenesis, while RalB has been shown to be more important for metastasis (154,155). Upon activation by RalGEFs, Ral proteins bind to a limited number of effectors, including RalBP1, Sec5, and Exo84 (48). Of these three Ral effectors, Sec5 and Exo84 have been shown to be required for oncogenic Ras-mediated transformed and tumorigenic growth of human cells (Chapter 4). To investigate the role of each of these proteins in metastasis, I determined whether stable knockdown of RalBP1, Sec5, or Exo84 altered the metastatic potential of oncogenic Ras-transformed HEK-HT cells.

Specifically, Ras^{G12V}-expressing HEK-HT cells were stably infected with retroviruses encoding RalBP1 shRNA-1, Sec5 shRNA-1, Exo84 shRNA-1, both Sec5 and Exo84 shRNA-1, or a scramble sequence as a control. These cell lines were previously described in Chapter 4. Appropriate knockdown of the targeted mRNA was confirmed by quantitative real-time RT-PCR (Figure 9A). These cell lines were injected into the tail vein of four immunocompromised mice each. The mice were monitored regularly until any of the three groups of mice manifested signs of respiratory distress or significant weight loss, at which point all mice were euthanized and their internal organs were examined for evidence of metastasis.

Mice injected with scramble control-treated cells exhibited signs of respiratory distress and cachexia by approximately four weeks. Upon dissection, the mice were found to have severe damage and colonization of both lungs by metastatic tumors (Figure 12). At the time when control mice exhibited physiological signs of metastasis, mice injected with Exo84 knockdown cells showed the most normal appearance of the remaining groups. Upon gross analysis of the lungs of all the remaining groups, visible metastatic nodules were only found to be significantly fewer in the Exo84 knockdown mice, compared to control animals (Figure 12).

The observation that Exo84 knockdown reduced metastatic potential was consistent with the role of this protein in the establishment of tumors, as described in Chapter 4. Perhaps the most surprising observation was that Sec5 knockdown did not appear to reduce the amount of visible metastatic nodules, even though it has been shown to be required for tumorigenic growth. Knockdown of RalBP1 did not appear to affect metastatic growth compared to controls, consistent with its inability to affect tumorigenic growth. One possible explanation for the lack of an effect on reducing visible metastases by Sec5 knockdown, either alone or in combination with Exo84, may be that an effect elicited by Sec5 was evident at an earlier time point that was missed, and subsequently cells had time to select for a population with lower shRNA expression.

This hypothesis is supported by the previous finding that RalB knockdown resulted in a lack of visible metastatic nodules in the same HEK-HT cell line when compared to a scramble control (155). It would therefore be informative to repeat the experiment and observe the status of lung metastases at intermediate time points. It would also be pertinent to determine the expression levels of the various Ral effectors in the metastatic nodules to determine whether the cells had undergone an *in vivo* selection process to gain a growth advantage.

These finding suggested that Ral-exocyst interactions may be important for supporting Ras-mediated metastasis, and warrant further investigation. A role for RalB in cell migration has previously been suggested (129,130). RalB-mediated effects on migration have even been linked to increased interaction with the exocyst complex (130). Additionally, RalB has been shown to be important for survival of transformed cells in suspension culture (138). Both of these effects were shown to be specific to RalB, and not RalA. Because cell migration and survival in suspension are both relevant components of tumor cell metastasis (197), the RalB-exocyst interaction may support metastatic growth of tumor cells through promoting these processes.

The C-terminal Hypervariable Domain of Ral GTPases Contributes to Their Ability to Support Tumorigenic Growth

While RalA and RalB represent two highly similar small GTPases, they show the most significant sequence divergence in their C-terminal hypervariable domains (Figure 11). This region has previously been shown to affect subcellular localization with and protein function (120,154). The evaluation of this C-terminal domain was facilitated by the generation of chimeric Ral proteins, in which the hypervariable domains of RalA and RalB were swapped (Figure 13A). To determine whether this domain affects the differential functions of RalA and RalB in tumorigenesis, I utilized the Ral chimeras and Ras-transformed HEK-HT cells in which RalA was stably knocked down. These cells were used to examine which Ral chimera, RAlA/B or RalB/A, could rescue the tumorigenic ability of these cells when injected subcutaneously in immunocompromised mice.

Specifically, Ras^{G12V}-transformed HEK-HT cells stably expressing RalA shRNA (154) were infected with retroviruses encoding RalA/B, RalB/A, or no transgene, as a control. These cell lines were injected subcutaneously into the flank of four immunocompromised mice per cell line. Subsequent tumor growth was monitored at regular intervals. The first cell line to exhibit palpable tumors was the one in which the RalB/A chimera had been expressed. Subsequent tumor growth demonstrated that these cells grew at the greatest rate, as compared to the other two lines (Figure 13B). Cells in which the RalA/B chimera was expressed grew at a rate that was in between that of the RalB/A chimera and empty vector-treated cells (Figure 13B). These data suggest that the RalA C-terminus contributes to the ability of RalA to support tumorigenic growth, since it was able to affect the ability of a protein that was otherwise RalB (RalB/A) to rescue tumorigenic growth. This is notable because a wildtype RalB protein would not be expected to rescue a reduction in tumorigenic growth caused by RalA knockdown, since RalB is not required for tumorigenic growth (154,155). Further studies are necessary to directly compare the ability of RalB/A and RalA itself to rescue the decreased tumor growth mediated by RalA knockdown.

These data suggest that the distinct functions of RalA and RalB in tumorigenesis may be mediated through their differing C-termini, which are known to affect subcellular localization of the two proteins. The hypervariable domain of RalA also contains two serine residues that have been identified as phosphorylation sites, which have been shown to be important for its transforming activity (142,143). Interestingly, these serine residues are not conserved in RalB. Analogous phosphorylation of K-Ras has been shown to affect its localization, and thus phosphorylation of RalA may similarly alter its subcellular localization, causing it to shuttle between internal membranes and the plasma membrane (26,149). Therefore, differential subcellular localization of RalA and RalB, as well as further regulation of RalA localization by phosphorylation, may allow cells to regulate RalA activity and diversify the functions of the two Ral proteins.

5.3 Conclusions

Preliminary data examining the role of Ral effectors in supporting oncogenic Ras-mediated metastasis of human cells suggests that the exocyst complex, or at least the Exo84 subunit, may be important for this process. Additionally, the C-terminal hypervariable domain of RalA has been shown to be important for affecting tumorigenic activity of Ras-transformed cells. In addition to the experiments proposed above, it would also be informative to examine whether RalA phosphorylation was essential for the RalB/A chimera to rescue reduced tumorigenesis mediated by RalA knockdown. This could be accomplished by mutating the phosphorylated serine residues to alanine residues, either alone or in combination in the RalB/A chimera, and then testing the ability of the mutant chimeras to rescue tumorigenic growth compared to wildtype RalA and Ral chimeras. The subcellular localization of the serine to alanine mutant chimeras could also be analyzed to determine whether phosphorylation altered subcellular localization. Additionally, it would be interesting to test the ability of the Ral chimeras to rescue reduced metastasis of Ras-transformed cells mediated by RalB-knockdown. Taken together, these studies may help to elucidate the distinct functions of RalA and RalB in tumorigenesis and metastasis.

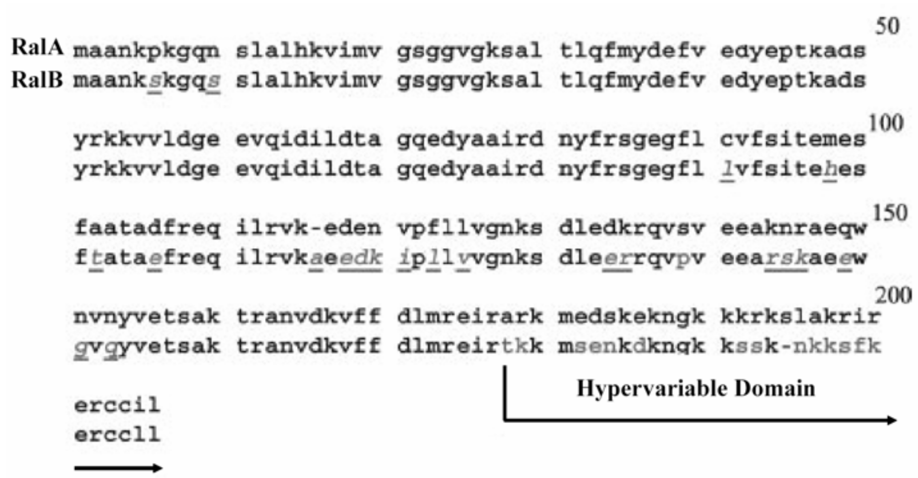

Figure 11. RalA and RalB amino acid sequence alignment.

Amino acid sequence alignment of RalA and RalB indicating sequence differences and the C-terminal hypervariable domain, which was swapped in the Ral chimeras. Adapted from Shipitsin and Feig, Molecular and Cellular Biology, 2004 (120).

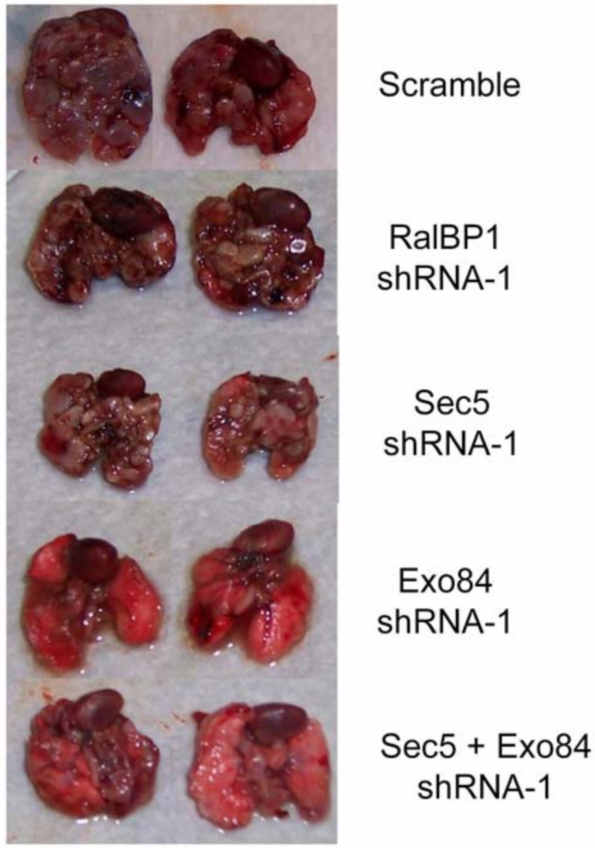

Figure 12. The effect of RalBP1, Sec5, and Exo84 knockdown on oncogenic Ras-mediated metastasis of human cells.

Representative pictures of lung metastases resulting from intravenous injection of Ras^{G12V}-transformed HEK-HT cells stably expressing the indicated siRNAs, after removal of the lungs from the mice. Stacey Adam assisted with tail-vein injections.

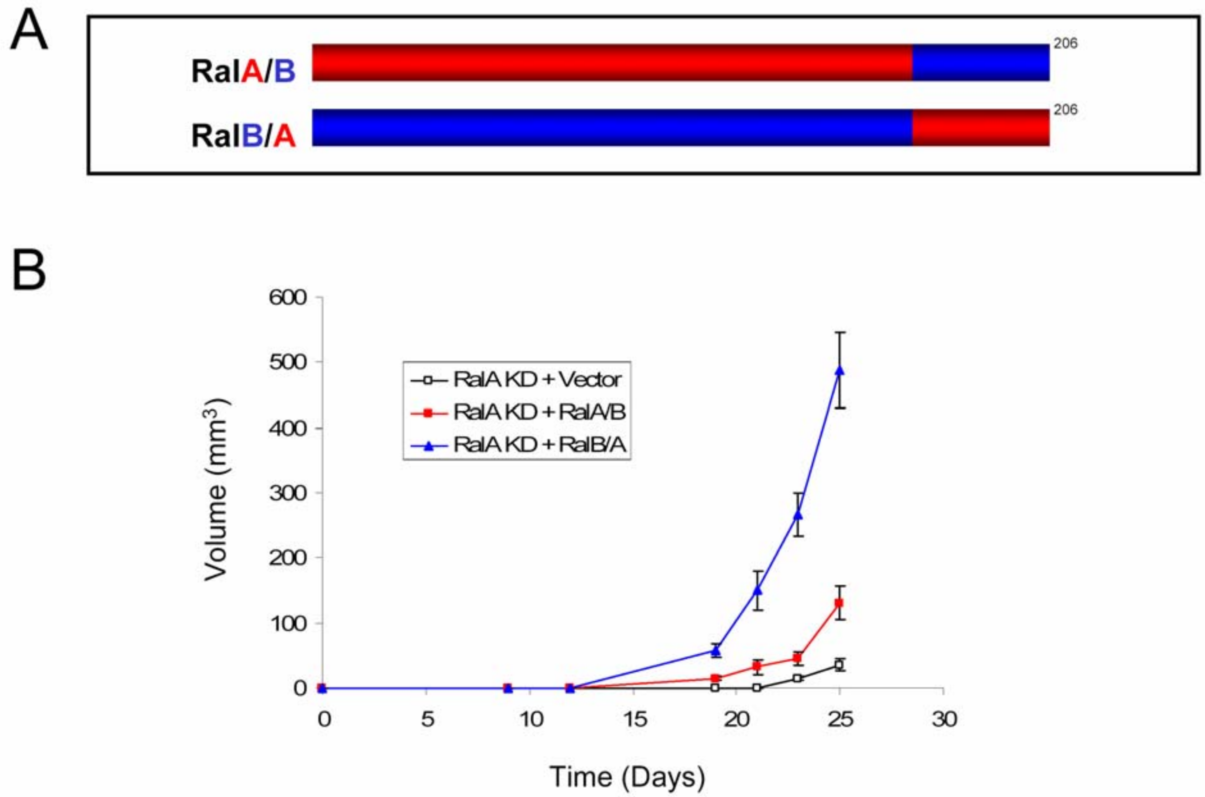

Figure 13. The role of the Ral hypervariable domain in oncogenic Ras-mediated tumorigenesis.

A. Representation of Ral chimera proteins in which the C-terminus of RalA or RalB is replaced with the C-terminus of the opposite Ral. *B.* Average tumor volume ± standard error versus time, of Ras^{G12V}-transformed HEK-HT cells stably expressing RalA shRNA and the indicated shRNA-resistant wildtype Ral chimeras or an empty vector control, injected into the flank of four immunocompromised mice per cell line.

6. Conclusions and Future Directions

6.1 Summary

Members of the Ras family of small GTPases are aberrantly activated to promote tumorigenic growth in a significant number of human cancers (4,15,21). Ras-mediated tumorigenic growth of human cells requires activation of the RalGEF-Ral effector pathway (66,83). Of the two highly similar Ral GTPases, RalA and RalB, RalA has previously been shown to be necessary for Ras-mediated tumorigenic growth, whereas RalB has been shown to be more important for metastatic growth (154,155). While our lab and others have demonstrated the involvement of RalA and RalB in tumorigenesis and metastasis of oncogenic mutant Ras-transformed human cells, the Ral effectors mediating these processes were unknown. Additionally, the role of RalA in cancers that activate Ras through mechanisms other than activating mutations, such as breast cancer, in which Ras is activated by amplification and overexpression of upstream growth factor receptor tyrosine kinases, was unclear (3,163). Therefore, my dissertation research focused on addressing these subjects to further elucidate the role of Ral GTPases in human oncogenic transformation.

HER2 and EGFR Dependant Activation of RalA in Breast Cancer

In order to address whether RalA activation was important for tumorigenesis of mammary epithelial cells, I first examined whether RalA was activated in response to HER2/Neu overexpression, since HER2 is commonly overexpressed in mammary tumors, is known to activate Ras, and is associated with poor prognosis (28-30). I found that expression of activated Neu in 293T cells induced RalA activation. Conversely, human breast cancer cell lines overexpressing HER2 and EGFR treated either with monoclonal antibodies against HER2 or EGFR, or a small molecule dual EGFR/HER2 kinase inhibitor, displayed reduced EGF-induced RalA activation in all cell lines tested. Taken together, these results suggested that RalA is activated downstream of EGFR and HER2 overexpression, and that clinical inhibitors of EGFR and HER2 signaling may exert their efficacy, in part, through reducing RalA activation.

To determine whether the activation of RalA in breast cancer cells was biologically relevant to their tumorigenesis, I knocked down RalA in the tumorigenic EGFR-overexpressing MDA-MB-231 human breast cancer cell line (184,185). Stable knockdown of RalA in these cells reduced the rate at which they formed tumors after orthotopic injection into the mammary fat pad of immunocompromised mice, compared to scramble control treated cells, suggesting that RalA activation may be an important part of their tumorigenic growth. To assess the clinical relevance of RalA activation in HER2-overexpressing breast cancers, a panel of ductal adenocarcinoma and matched patient normal mammary tissue samples was obtained. Protein was isolated from these samples, and RalA activation was directly compared between matched tissue pairs. This analysis demonstrated that RalA was preferentially activated in tumor versus normal tissue, suggesting that RalA activation may indeed be clinically relevant to the development and/or progression of human breast cancer.

Sec5 and Exo84 are Required for Oncogenic Ras-mediated Tumorigenesis

To further elucidate the Ral-dependent pathway supporting oncogenic Ras-mediated tumorigenesis of human cells, the known Ral effectors RalBP1, Sec5, and Exo84 were stably knocked down in HEK-HT cells transformed by Rlf-CAAX, an activated RalGEF. While depletion of RalBP1 resulted in a slight reduction of transformed growth, knockdown of either Sec5 or Exo84 resulted in the most significant inhibition of anchorage-independent growth, and was similar to the effect seen in the same cells treated with RalA shRNA (154). Given the effect of these Ral effectors on reducing transformed growth, I next examined whether they were similarly required for Ras-mediated tumorigenic growth by knocking down the same Ral effectors in tumorigenic Ras^{G12V}-transformed HEK-HT cells. While stable suppression of RalBP1 did not affect tumorigenic growth as assessed by xenograft in immunocompromised mice, knockdown of either Sec5 or Exo84 significantly increased the latency period and decreased the tumorigenic growth of these cells. Furthermore, simultaneous knockdown of Sec5 and Exo84 in the same cell line had the most dramatic effects on inhibiting tumor growth. These results suggest that the Ral effectors Sec5 and Exo84 are necessary for Ras-mediated tumorigenic growth, and that they may function together to mediate their effect on tumorigenesis.

Ral GTPases, Tumorigenesis, and Metastasis: A "Tail" of Two Proteins

In addition to their involvement in transformed and tumorigenic growth, the role of Ral effectors in supporting oncogenic Ras-mediated metastasis was also examined, since the RalGEF-Ral effector pathway has been implicated in this process (152,153,155). To evaluate whether RalBP1, Sec5, or Exo84 were important for Ras-induced metastasis, the Ras^{G12V}-transformed HEK-HT cells described above, in which expression of the Ral effectors was suppressed, were utilized. Stable shRNA-mediated knockdown of Exo84 decreased the amount of visible metastatic nodules, compared to scramble control treatment, in the lungs of immunocompromised mice following tail-vein injection. These preliminary findings suggest that the exocyst complex, or at least Exo84, may be important for mediating oncogenic Ras-induced metastasis, in addition to its role in tumorigenesis.

In addition to the work mentioned above, the role of the Ral C-terminal hypervariable domain in mediating differential tumorigenic activities of RalA and RalB was examined. The hypervariable domain of RalA and RalB represents the region of greatest sequence divergence between the two otherwise highly similar proteins, and has been implicated in regulating subcellular localization, post-translational modification, and proper function of Ral GTPases in polarized delivery of membrane proteins and anchorage-independent growth (120,142,143,154). To determine the importance of the C-terminal hypervariable domain for Ral-dependent support of Ras-mediated tumorigenesis, chimeric Ral proteins were utilized, in which the C-terminal domains of RalA and RalB had been swapped (120,154). The Ral chimeras were stably expressed in Ras^{G12V}-transformed HEK-HT cells stably expressing RalA shRNA, and their ability to rescue tumorigenic growth of these cells when injected subcutaneously into immunocompromised mice was evaluated. The C-terminal hypervariable domain of RalA was shown to be important for tumorigenesis, since a RalB/A chimera (RalB with the RalA hypervariable domain) was better able to rescue tumorigenic growth of RalA knockdown cells than a RalA/B chimera. These results suggest that the RalA hypervariable domain is responsible, at least in part, for mediating tumorigenic growth of Ras-transformed cells, suggesting that differences between RalA and RalB function in tumorigenesis, and potentially metastasis, may be due to differential subcellular localization or phosphorylation, which have both previously been shown to be mediated by this region.

6.2 New Insights and Future Directions

The work described here provides new insights, not only into the importance of RalA activation in breast cancer, which was previously unknown, but also into the molecules downstream of Ral GTPases that may be responsible for mediating effects on tumorigenesis and metastasis. Taken together, these data offer a more complete understanding of the specific pathway involved in the Ral-mediated support of Ras-induced oncogenic transformation of human cells, presenting several new lines of experimentation to follow in order to further elucidate Ral function.

The Role of RalA, Sec5, and Exo84 in Tumorigenesis

I have shown that RalA activation is induced by overexpression of EGFR family members in human breast cancer cell lines and patient tumor samples, and that RalA function may be necessary for tumorigenic growth of breast cancer cells. Additionally, I have demonstrated an essential role for the Ral effectors Sec5 and Exo84 in the Ras-mediated tumorigenic growth of genetically defined human cells. The mechanism by which Sec5 and Exo84 contribute to the RalA-dependent support of Ras-mediated tumorigenesis, as well as the role of RalA in mammary tumorigenesis may be gleaned from the normal cellular functions of Ral GTPases.

Ral GTPases have been implicated in the regulation of a number of cellular processes, both directly and through interactions with distinct effector proteins (26,48). Ral involvement in one or more of these processes may be able to account for their role in supporting tumorigenesis and metastasis. While sharing 85% amino acid sequence identity, RalA and RalB have been shown to play distinct roles in mediating Ras-induced transformed and tumorigenic growth of human cells. For example, RalA, but not RalB, is required for anchorage-independent growth and tumorigenesis of a variety of human cancer cell types (154,155). Additionally, RalB appears to be the more important Ral protein for mediating Ras-induced metastasis (155). These differing roles of RalA and RalB are therefore likely to be supported by distinct functions of the two proteins.

One such example of a cellular process that is differentially affected by RalA and RalB is vesicle trafficking. Activated RalA, but not RalB, has been shown to promote the polarized delivery of basolateral membrane proteins, including EGFR, to the surface of the plasma membrane, and knockdown of RalA has been associated with mislocalization of such proteins (96,120). Regulation of this process is associated with RalA-dependent assembly of the exocyst complex to promote polarized delivery, and is dependent upon RalA localization mediated by the C-terminal hypervariable domain (96,120). In contrast to the role of RalA in basolateral delivery, RalB has been implicated in the process of receptor-mediated endocytosis of growth factor receptors, including EGFR (107,111). RalB function in endocytosis is dependent upon interaction with RalBP1, which interacts with the EGFR substrate POB1, which contains an Eps homology domain (104). Eps homology domains are found in many proteins associated with endocytosis and vesicle sorting, including epsin and Eps15, two proteins that bind to POB1 (106,108). RalBP1 also binds to the μ2 subunit of the AP2 adaptor complex, which is involved in promoting the interaction of AP2 with integral membrane proteins and recruiting them into clathrin-coated pits (107). Given these observations, it has been speculated that RalA and RalB may serve distinct roles in mediating vesicle trafficking, with RalA regulating exocytic processes and RalB involved in endocytosis (48).

The role of Ral-GTPases in various aspects of vesicle trafficking has not been studied in the context of transformation, although the findings presented in this dissertation suggest that the exocyst complex components Sec5 and Exo84 are required for RalGEF-mediated anchorage-independent growth and oncogenic Ras-mediated tumorigenesis. It is therefore possible that in normal cells, RalA and RalB may act in a coordinated manner to regulate certain aspects of vesicle trafficking, with RalA-exocyst interactions mediating exocytic events and RalB-RalBP1 interactions involved in endocytosis. The balance of these two processes may in turn affect signaling to downstream effector proteins, and disruption of this balance in cancer cells may contribute to inappropriate stimulation of downstream targets.

While Ral GTPases and certain Ral effectors have been implicated in growth factor receptor trafficking, it is also possible that these proteins may contribute to transformation through affecting the trafficking and/or secretion of other molecules. In *Drosophila*, exocyst components, including Sec5, associate with recycling endosomes and promote E-cadherin delivery to the plasma membrane and facilitate recycling of E-cadherin to adherens junctions (198). Expression of an activated mutant RalA in MDCK cells has also been shown to enhance delivery of E-cadherin and other unknown proteins to the basolateral membrane (120), however the consequences of Ral or exocyst-dependent E-cadherin delivery/recycling in cancer cells remains to be determined. Nevertheless, these findings suggest that Ral-mediated exocyst-dependent trafficking of molecules to the plasma membrane occurs, and in cancer cells the trafficking and/or recycling of specific cargo may contribute to transformed growth. It is also possible that Ral and the exocyst may support autocrine or paracrine stimulation of cell signaling pathways influencing transformation by promoting the secretion of specific ligands. In support of this theory, oncogenic Ras has been shown to induce secretion of IL6, and inhibition of IL6 in Ras-transformed cells inhibited tumorigenic growth (199). It would therefore be interesting to similarly examine whether Ral activation induces the secretion of molecules involved in the process of tumorigenic growth, and if so, the role of Sec5 and Exo84 in the secretion of such molecules could also be examined.

In breast cancer, the amplification and overexpression of EGFR and HER2 leads to their ligand-independent activation through spontaneous dimerization, resulting in chronic stimulation of downstream signaling pathways (3,156). Internalization and degradation of EGFRs, as well as recruitment of these receptors to the plasma membrane are thus important mechanisms of regulating signal potentiation (156,200). One mechanism by which RalA may contribute to the tumorigenesis of such mammary epithelial cells may involve the process of Sec5 and Exo84-mediated trafficking of growth factor receptors to the plasma membrane, promoting their inappropriate activation and chronic signaling. Therefore it would be informative to examine whether knockdown of RalA, RalB, RalBP1, Sec5, or Exo84 influences EGFR and HER2 localization. Knockdown of Sec5 and Exo84 would be expected to decrease the amount of properly localized growth factor receptors at the plasma membrane, based on the role of the exocyst complex in regulating basolateral delivery of EGFR (96). I have shown that RalA is activated downstream of EGFR and HER2 in breast cancer cells, and is important for their tumorigenic growth. It would be interesting to examine whether Sec5 and Exo84 are similarly important for tumorigenic growth of these cells. Even if RalA promotes trafficking of EGFRs in cancers that depend upon activation of these receptors, the fact that oncogenic Ras depends on RalA for tumor growth argues that other mechanisms also drive tumorigenesis, either through more general effects on protein secretion, as described above, or through alternative mechanisms, discussed in detail below.

A complimentary mechanism by which RalA may promote tumorigenesis of breast cancer cells could be through the activation of transcription factors such as Stat3 and ZONAB. Expression of constitutively activated RalA has previously been shown to activate Stat3, and RalA activation has also been shown to be required for EGF-induced activation of Stat3 (133). ZONAB was recently identified as a RalA binding protein, and acts as a transcriptional repressor of ErbB-2 (99). Interaction of RalA with ZONAB has been shown to alleviate transcriptional repression of ErbB-2. Additionally, expression of activated Ras has also been shown to overcome transcriptional repression by ZONAB, suggesting that Ras signals through RalA to perform this function. Thus, in breast cancer, RalA activation may lead to Stat3 activation and/or alleviate transcriptional repression of ErbB-2. It would therefore be interesting to examine Stat3 activation, as well as the effect of stable suppression of Stat3 in breast cancer cell lines in which RalA is activated and required for tumorigenic growth. In parallel, it would be informative to perform a similar evaluation of ZONAB function in breast cancer cells by examining the effects of depletion of ZONAB on ErbB-2 transcription and tumorigenic growth.

It is also possible that both vesicle trafficking and regulation of gene expression act in concert to promote tumorigenesis. For example, activated RalA may interact with ZONAB to alleviate transcriptional repression of ErbB-2, while interactions with Sec5 and Exo84 may promote targeting of ErbB-2 to the plasma membrane where its overexpression can lead to improper activation and chronic downstream signaling. This type of mechanism could also set up a feedback loop, leading to sustained expression and localization of ErbB-2, amplifying its signals, and ultimately contributing to tumorigenesis.

In addition to affecting vesicle trafficking, the exocyst complex is required for the appropriate completion of cytokinesis (195,201-204). Cytokinesis involves actino-myosin ring formation and cleavage furrow ingression, generating a cytoplasmic bridge, requiring mobilization of secretory vesicles and exocytosis for abscission (202,205-211). The exocyst is involved in late cytokinesis and is recruited to the midbody for the resolution of the intracellular bridge (202). Recently, a role for Ral GTPases in exocyst-dependent completion of cytokinesis has been demonstrated, in which RalA and RalB make distinct contributions to cytokinesis (195). Specifically, siRNA-mediated depletion of RalA resulted in cytokinesis regression characterized by improper cell spreading and furrow regression, resulting in binucleate cells. Knockdown of RalB did not affect intracellular bridge formation, but did result in abscission failure. The increase in binucleate cells caused by RalA knockdown was reversed by complementation with wildtype RalA but not RalA effector domain mutants that failed to bind Sec5 or Exo84, suggesting that RalA contributes to cytokinesis through these effectors. Abscission defects caused by RalB knockdown were rescued by wildtype RalB or an effector domain mutant unable to bind to Sec5, but not by a RalB effector domain mutant unable to bind to Exo84, suggesting that RalB contributes to cytokinesis through interaction with Exo84. These results indicated that RalA and RalB make distinct contributions to cytokinesis through both shared and distinct exocyst components. Additionally, knockdown of Sec5 led to an increase in the percentage of binucleate cells and could be rescued by expression of wildtype Sec5, but not Sec5 that was unable to bind to RalA, further implicating Ral regulation of the exocyst in the completion of cytokinesis. The extent of the effects of RalA and RalB depletion on inducing binucleate cells and failed abscission, respectively, are unknown in the context of transformed and tumorigenic cells. The promotion of proper cell division by RalGEF-induced Ral-mediated interactions with the exocyst complex components Sec5 and Exo84 may, however, contribute to tumorigenic growth by supporting proliferation of tumor cells. Along these lines, oncogenic Ras has been found to promote anchorage-independent cytokinesis in human fibroblasts (212). It would therefore be interesting to examine the effects of RalA, RalB, Sec5, and Exo84 knockdown on cytokinesis in tumorigenic Ras-transformed cells.

In addition to exocyst-dependent functions, it is possible that Sec5 and Exo84 may affect tumorigenic growth through exocyst-independent signaling. This mechanism has recently been supported by the discovery of a novel Sec5-interacting protein, TBK1 (181). Sec5 depletion was found to induce apoptosis in certain transformed cell lines, and this phenotype was duplicated by TBK1 knockdown. RalB was found to activate TBK1 by promoting interaction of Sec5 and TBK1, suggesting that Ral-dependent anti-apoptotic signaling through Sec5 and TBK1 activation may promote tumor cell survival. While this study demonstrated apoptosis associated with both RalB and Sec5 RNAi-mediated knockdown, RalB knockdown does not induce apoptosis in all cell lines (129,130,154,155). This could be due to a difference in cell type, the degree of RalB knockdown, or the method by which RNA-interference was performed (stable retroviral infection versus transient transfection). Regardless of the discrepancy, the effects of Sec5-mediated TBK1 signaling on tumorigenic growth were not evaluated, and the importance of this pathway for Ras-induced tumorigenic growth is unknown. It would therefore be interesting to examine the effects of stable knockdown of TBK1 on transformed and tumorigenic growth of RalGEF and oncogenic Ras-transformed cells, respectively, in order to assess the contribution of this signaling pathway to Ral-dependent support of tumorigenesis. These findings also raise the possibility that Exo84, like Sec5, may interact with unidentified effectors contributing to exocyst-independent effects on tumorigenesis. Proteomic screens for Ex084 and Sec5 interacting proteins may shed some light on such effector proteins.

Ral GTPases, the Exocyst, and Metastasis

Oncogenic Ras promotes metastatic growth, in part, through activation of RalGEFs (152,153). While RalA has been shown to be necessary for oncogenic Ras-mediated tumorigenesis of human cells, RalB has been shown to be more important for metastasis (154,155). Specifically, stable shRNA-mediated knockdown of RalB in oncogenic Ras-transformed HEK-HT cells or two K-Ras mutant metastatic pancreatic cancer cell lines has been shown to reduce metastatic growth more effectively than RalA kncockdown (155). Additionally, metastasis to the adrenal gland caused by one of the two pancreatic lines was abolished by RalB, but not RalA, knockdown.

The data presented here suggests that the exocyst, or at least Exo84, may be the Ral effector supporting metastatic growth, since knockdown of Exo84 reduced the amount of visible metastatic nodules in the lungs of mice following tail-vein injection, when compared to scramble controls. The differing levels of contribution of RalA and RalB to metastasis (155), and the possible involvement of the exocyst in this process, may be explained by a mechanism involving differential regulation of cellular functions important for some aspect of metastatic growth. To this end, distinct roles for RalB and the exocyst have been observed in cell migration (129,130) and proliferation of cells in suspension culture (138).

A role for RalB in cell migration has previously been suggested (129,130). RalB-mediated effects on migration have also been linked to increased interaction with the exocyst complex (130). Additionally, RalB has been shown to be important for transformed cell survival in suspension culture (138). Both of these effects were demonstrated by knockdown of RalB expression, and were shown to be specific to RalB, and not RalA. Because cell migration and survival in suspension are both relevant components of tumor cell metastasis (197), the RalB-exocyst interaction may support metastatic growth of tumor cells through promoting these processes. Further evaluation of the role of Sec5 and Exo84 in Ras-induced metastasis will be necessary to confirm their importance. It would be interesting to examine whether stable knockdown of either Sec5 or Exo84 could reduce metastasis of CFPac-1 pancreatic cancer cells to the adrenal gland following tail vein injection, which was previously observed upon RalB, but not RalA, knockdown (155). Additionally, it would be interesting to evaluate the contribution of Sec5 and Exo84 to migration of these cells and other cell lines in which RalB mediates metastasis. The effect of Sec5 and Exo84 knockdown on cell survival in suspension could also be studied in order to help define which functions of RalB may be mediated by the exocyst to affect metastatic potential. If RalB-mediated effects on metastasis are due to interactions with the exocyst promoting migration and survival in suspension, then knockdown of the exocyst components Sec5 and Exo84 would be expected to inhibit these processes. It is also possible that either Exo84 or Sec5 may be more important for metastatic growth, since data presented here suggests that Exo84 knockdown had a more pronounced effect on metastatic growth of Ras-transformed cells. This could be due to the existence of distinct subcomplexes containing Sec5 and Exo84 (213), which may be differentially regulated by RalA and RalB during specific cellular events, such as migration. Indeed, RalA and RalB share Exo84 as an effector for cytokinesis, while Sec5 is specifically utilized by RalA, but not RalB, in cytokinesis (195), further establishing the possibility that distinct exocyst complex components may be utilized by RalA and RalB to mediate specific functions.

The Ral Hypervariable Domain and Differential Regulation of Ral GTPase Functions

RalA and RalB are 85% identical at the amino acid level, sharing identical effector-binding domains. The two proteins exhibit the greatest amount of sequence divergence in the C-terminal hypervariable domain. Previous findings have demonstrated that this domain is responsible for subcellular localization, post-translational modification, and proper functions in promoting basolateral membrane protein delivery and anchorage-independent growth (120,154). Therefore, while RalA and RalB may be expected to bind similarly to effectors, key differences in their hypervariable domains may enable the two proteins to serve distinct cellular functions.

To examine whether differences in the C-terminal hypervariable domains of RalA and RalB were important for differences in their abilities to mediate tumorigenic growth, I utilized Ral chimeras, in which the C-terminal regions of RalA and RalB were swapped (120,154). These chimeras were stably expressed in oncogenic Ras-transformed HEK-HT cells stably expressing RalA shRNA, and were assayed for tumorigenesis following subcutaneous injection in mice. The RalB/A chimera was shown to be more efficient at rescuing tumorigenic growth than the RalA/B chimera, suggesting that the C-terminal hypervariable domain of RalA is important for its function in promoting tumorigenic growth.

As mentioned above, the hypervariable domain of Ral GTPases is important for subcellular localization of the two proteins (120,154). RalA and RalB localize differently in cells, and studies utilizing activated Ral chimeras have demonstrated that targeting RalB to RalA-specific regions causes RalB to function more like RalA, increasing its ability to enhance polarized delivery of membrane proteins (120) and anchorage-independent growth (154). While RalA and RalB share identical effector binding domains, distinct subcellular localization of the two proteins may regulate their interactions with specific effectors. In the case of polarized delivery of membrane proteins, the RalB/A chimera displayed greater binding to exocyst subunits than RalB, suggesting that altered subcellular localization caused increased interaction with the exocyst complex, stimulating enhanced delivery of membrane proteins (120).

Additionally, RNAi-mediated knockdown of RalA and RalB altered the subcellular distribution of exocyst components in distinct ways, suggesting that the two proteins may differentially regulate subcellular localization of effectors (214). Taken together, these data suggest that RalA and RalB may perform different cellular functions, at least in part, due to distinct patterns of subcellular localization affecting interactions with, and localization of effector proteins.

Like Ral GTPases, Ras and Rho GTPases also exhibit a high level of amino acid sequence identity between family members, with the greatest amount of sequence divergence found in C-terminal sequences. Moreover, Ras and Rho family members perform distinct cellular functions, and these differences have been ascribed to the differences in C-terminal hypervariable domains between the different Ras and Rho family members (215-217). Like Ral proteins, the hypervariable domains of Ras and Rho family members affect subcellular localization. In the case of Ras proteins, oncogenic K-Ras has been shown to be a more potent activator of Rac than oncogenic H-Ras, inducing greater membrane ruffling and cell motility. This effect was due to C-terminal differences in the two proteins, because an H-Ras-K-Ras-C-terminus chimera behaved exactly like K-Ras (218). Furthermore, altering Ras localization through C-terminal mutation has been associated with altered effector utilization (215). Similarly, an alteration in the use of effectors due to distinct subcellular localization mediated by differences in the Ral hypervariable domains could therefore contribute to the ability of the RalB/A chimera to rescue tumorigenic growth of Ras-transformed, RalA knockdown cells.

Additionally, RalA is phosphorylated on two serine residues in its C-terminal hypervariable domain, which are not found in RalB, and this phosphorylation has been shown to be important for the transforming activity of RalA (142,143). Analogous phosphorylation of K-Ras has been associated with altered protein localization, and thus phosphorylation of RalA may similarly alter its subcellular localization, causing it to shuttle between internal membranes and the plasma membrane (26,149). RalA has also been identified as a substrate for the tumor suppressor PP2A Aβ. Mutations and gene deletions of PP2A Aβ have been identified in human cancers, including lung, colon, and breast (144-148). Taken together, these data suggest that the phosphorylation status of RalA may influence its subcellular localization and activation, allowing it to shuttle between different effector proteins, mediating their distinct functions.

To further evaluate the role of RalA phosphorylation on tumorigenesis, it would be informative to examine whether RalA phosphorylation is essential for the RalB/A chimera to rescue reduced tumorigenesis caused by RalA knockdown in Ras-transformed cells. This could be accomplished by mutating the phosphorylated serine residues to alanines, either alone or in combination, in the RalB/A chimera, and then testing the ability of the mutant chimeras to rescue tumorigenic growth compared to wildtype RalA and Ral chimeras. If phosphorylation of either of the two serine residues is important for RalA-mediated rescue of tumorigenic growth, then mutation of these residues should interfere with the ability of the RalB/A chimera to rescue tumorigenesis. The subcellular localization of the mutant chimeras could also be analyzed to determine whether any effects of loss of phosphorylation on tumorigenesis were due to altered localization. In parallel, it would be informative to determine whether phosphorylation of RalA at either serine residue altered interactions with Ral effectors.

One of the two serine residues of RalA described above is known to be phosphorylated by Aurora-A kinase, while the kinase responsible for phosphorylating the other serine residue is unknown (142,143). It would be very informative to determine where RalA phosphorylation was taking place in the cell. Immunofluorescence of RalA and Aurora-A kinase could be performed to determine where in the cell the two proteins interact. This could help to explain whether RalA phosphorylation was occurring at the plasma membrane and causing the protein to relocalize, or if RalA was being phosphorylated in a different part of the cell, trapping it at a specific site where its function may be necessary. Alternatively, cancer cells may utilize phosphorylation of RalA to enhance its transforming activity. Taken together, these studies may help to elucidate the mechanisms by which the two highly similar Ral proteins are able to promote distinct cellular functions.

Insights to be Gained from Ral Knockout Mice

The generation of RalA and RalB conditional knockout mice has recently been undertaken by our lab. These mice will serve as valuable tools to address the normal functions of Ral GTPases in mouse development, and will also be important for the evaluation of genetically induced tumorigenesis. Given the results of the work presented here, one particularly interesting experiment would be to induce RalA knockout in mammary tissue through expression of a mammary-specific promoter-driven Cre recombinase, and to cross these mice to transgenics overexpressing activated Neu driven by a mammary-specific promoter. Transgenic Neu mice are well-characterized and commonly used to evaluate mammary tumorigenesis, since they develop mammary tumors in a relatively short amount of time and with fairly high efficiency, depending on the strain used (219). Based on the findings suggesting that RalA activation may be important in breast cancer cells, I would expect the progeny of such a cross to exhibit a delay in the onset of mammary tumor formation. Such a study would provide valuable insight into the role of RalA activation in mammary tumorigenesis in a biologically relevant setting. Additionally, similar studies could be performed in which tissue-specific RalA knockout mice could be generated and crossed to other tissue-specific transgenic mice modeling cancers such as lung and pancreatic.

Conclusion

The work described here provides new insights, not only into the importance of RalA activation in breast cancer, which was previously unknown, but also into the molecules downstream of RalA and RalB that may be responsible for mediating their effects on tumorigenesis and metastasis. Taken together, these data offer a more complete understanding of the specific pathway involved in the Ral-mediated support of Ras-induced oncogenic transformation of human cells, presenting several new lines of experimentation to follow in order to further elucidate Ral function. Given the importance of Ral GTPases in Ras-mediated tumorigensis and metastasis, a better understanding of Ral function in this commonly activated oncogenic pathway may ultimately lead to new therapeutic strategies for the treatment of human cancers in which Ral activation is involved.

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Biography

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Born
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Education

University of Maryland Baltimore County, Baltimore, MD	B.S.	Biochemistry and Molecular Biology	1999-2003
Duke University, Durham, NC	Ph.D.	Molecular Cancer Biology	2003-2009

Research Experience
Summer 2000 – Visiting Scientist, Analytical Chemistry Laboratory, National Cancer Institute-Frederick, Frederick, MD. Project: The Separation of a Natural Product Extract with Potential Anti-cancer Activity.

Summer 2001, 2002 – Production Technician, Laboratory of Molecular Technology, National Cancer Institute-Frederick, Frederick, MD. Project: Analysis of Methylation Patterns of Genes Implicated in Cancer.

2002-2003 – Undergraduate Research, PI: Dr. Dan Fabris, University of Maryland Baltimore County, Baltimore, MD. Project: Applying Electrospray Ionization Mass Spectrometry to the Identification of Cancer Biomarkers.

Fall 2003 – Rotation Student, PI: Dr. Patrick Casey, Duke University, Durham, NC. Project: Analysis of the Kinetic Mechanism of Human Isoprenylcysteine carboxylmethyltransferase (Icmt) Inhibitors.

Winter 2003 – Rotation Student, PI: Dr. Christopher Counter, Duke University, Durham, NC. Project: Examining the Role of Nbs1 at the Telomere.

Spring 2004 – Rotation Student, PI: Dr. Ann Marie Pendergast, Duke University, Durham, NC. Project: The Role of Abl Interactor Proteins in Cytoskeletal Rearrangement During Infection by Shigella Flexneri.

Summer 2004-2009 – PI: Dr. Christopher Counter, Duke University, Durham, NC. Project: Examining the Role of Ral-GTPases In Human Oncogenic Transformation.

Honors

President's Scholarship, University of Maryland Baltimore County

Dean's List, University of Maryland Baltimore County

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Publications

Xu, H., Issaq, S.H., McCloud, T.G., Issaq, H.J. (2001) The Separation of a Penicillium Fungal Extract by Thin Layer Chromatography and HPLC: A Comparative Study. *Journal of Liquid Chromatography and Related Technologies*, **24**:625-633.

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