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The role of RalA and RalB in cancer

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The Role of RalA and RalB in Cancer

By

Samuel C. Falsetti

A dissertation submitted in partial fulfillment
Of the requirements for the degree of
Doctor of Philosophy
Department of Molecular Medicine
College of Medicine
University of South Florida

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Dedication

This thesis is dedicated to my greatest supporter, my wife. Without her loving advice and patience none of this would be possible.

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I would like to extend my sincere gratitude to my wife, Nicole. She is the most inspirational person in my life and I am honored to be with her; in truth, this degree ought to come with two names printed on it. Thanks to my parents and grandparents for inspiring a love of learning and nurturing creativity in me from an early age. I wish to extend my thanks to the rest of my family: my sister Adrianna; my in-laws Bob, Gail, Michael, and Robert; and of course my adopted sisters Courtney and Andrea. Also thanks to Audrey Shor for lots of afternoon coffee sessions and invaluable career advice, Adam Carie for being a great friend as well as an excellent source of advice, Jim Hawker, Michelle Blaskovich, Cindy Boo-shay, Ryan Floyd, Kazi Aslamuzzaman, De-an Wang, Maria Balasis, Laura Francis, Lisa White, and Barbara Roberto. Additionally, I would like to thank the many members of the Sebti Lab who have also contributed to my career development: Kara Forinash, Norbert Berndt, Kun Jiang, Kuichin Zhu, Kristine Sedey and Iain Duffy. A special thanks goes to my committee members whose patience and advice has been invaluable and without whom this work would not be possible: Larry Solomonson, Gloria Ferreira, Gary Reuther, Srikumar Chellapan, Doug Cress and my wonderful outside advisor, Channing Der. Last, but most certainly not least, I wish to extend my most sincere thanks to my mentor Dr. Saïd Sebti who has been the most invaluable source of scientific advice and inspiration over the last five years and without him none of the following work would be possible.

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The Role of RalA and RalB in Cancer

Samuel C. Falsetti

ABSTRACT

Ras genes are frequently mutated in human cancers and present compelling targets for therapeutic intervention. While previous attempts to directly inhibit oncogenic Ras function have largely been unsuccessful use of targeted agents to inhibit the three primary oncogenic pathways activated by mutated Ras: RalGEF-Ral, PI3K-Akt and Raf-MEK-Erk, is an area of intense investigation. Here, we describe the ability of a novel pharmacological inhibitor of geranylgeranyltransferase I, GGTI-2417, to inhibit Ral prenylation and localization. We further used a Ral rescue system to selectively preserve RalA and RalB function and localization during GGTI-2417 treatment and determine the precise roles for inhibition of Ral prenylation in the GGTI anti-cancer response. Specifically, we determined inhibition of RalA is required for GGTI-attenuation of anchorage independent growth whereas inhibition of RalB is required for inhibition of proliferation, induction of apoptosis, suppression of survivin and induction of p27^{Kip1}. We next determined the role of RalGEF-Ral signaling as well as PI3K-Akt and Raf-MEK-Erk signal transduction pathways in an *in vitro* model of human ovarian surface epithelial (T80 HOSE) cell Ras-dependent transformation. Using both small interfering RNA (siRNA) and pharmacological inhibitors of Ral, PI3K and MEK we determined that Ras signaling via Ral and PI3K but not MEK is required for ovarian oncogenesis.

Furthermore, stable expression of Ras mutants unable to activate Raf-MEK-Erk signaling were able to robustly transform T80 cells. Since we had confirmed the importance of Ral proteins to human epithelial malignancies we next sought to explore the molecular interactions governing Ral transformation using a proteomics approach to rapidly identify proposed Ral interacting partners. Using immunoprecipitation of transiently overexpressed FLAG-tagged RalA and RalB followed by 1D-gel separation and tandem MS/MS analysis we determined a database of proposed Ral interacting proteins. One of these, RACK1, is a validated RalA and RalB interacting protein which is at least partially required for Ras and Ral transformation. These results provide both a strong impetus and a solid basis for future studies into the mechanisms of RalA- and RalB- dependent transformation.

List of Abbreviations

AIG: Anchorage independent growth

EGFR: Epidermal growth factor receptor

FTase: Farnesyltransferase

FTIs: Farnesyltransferase inhibitors

GAP: GTPase activating protein

GBLP1: guanine nucleotide binding-like protein-1

GEF: Guanine nucleotide exchange factor

GGTase I: Geranylgeranyltransferase I

GGTI: Geranylgeranyltransferase I inhibitors

HDJ2: Human DNAJ-2

HEK: Human embryonic kidney cells

HMECS: Human mammary epithelial cells

HOSE: Human ovarian surface epithelial cells

hTERT: human telomerase catalytic subunit

IEC-6: Rat intestinal epithelial cells-6

PI3K: phosphotydil inositol 3,4,5-triphosphate kinase

PLC-E: Phospholipase C epsilon

PLD-1: Phospholipase D-1

PP2A: Protein phosphatase-2A

RACK1: Receptor for activated protein kinase C-1

RalA: Ras-like A

RalB: Ras-like B

RalBP1: Ral binding protein-1

RalGDS: Ral-guanine nucleotide dissociation stimulator

RalGEF: Ral-guanine nucleotide exchange factor

RasGAP: Ras GTPase activating protein

RIE: Rat intestinal epithelial cells

ROSE: Rat ovarian surface epithelial cells

RTK: Receptor tyrosine kinase

SCID: Severely compromised immuno-deficient mouse

siRNA: Small interfering RNA

shRNA: Small hairpin RNA

SOS: Son of sevenless

SV40T: simian virus-40 large T antigen

S40t : simian virus-40 small t antigen

ZONAB : ZO-1 binding protein

Chapter 1

Introduction

By

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The Ras Superfamily

In order to understand the role of Ral in cancer it is necessary to understand the role of Ras genes in oncogenesis. Members of the Ras and Rho branches of the Ras superfamily of small GTPases are critically involved in the regulation of many biological events critical to the regulation of cellular homeostasis such as cell cycle control, cell survival, death, differentiation, development and growth (11, 93). The aberrant activation or inactivation of Ras family proteins is believed to be important in the induction of oncogenesis. In addition to the three Ras proteins (H-, N- and K-Ras), other Ras family proteins with validated roles in oncogenesis include R-Ras, Ral, Rheb, Di-Ras and Noey2/ARHI small GTPases. Rho family GTPases (e.g., RhoB, RhoC, Rac1b, DBC2) are also implicated in oncogenesis (73). There are 156 known genes in the Ras superfamily and at least 166 known Ras superfamily isoforms (see Table 1 on following page).

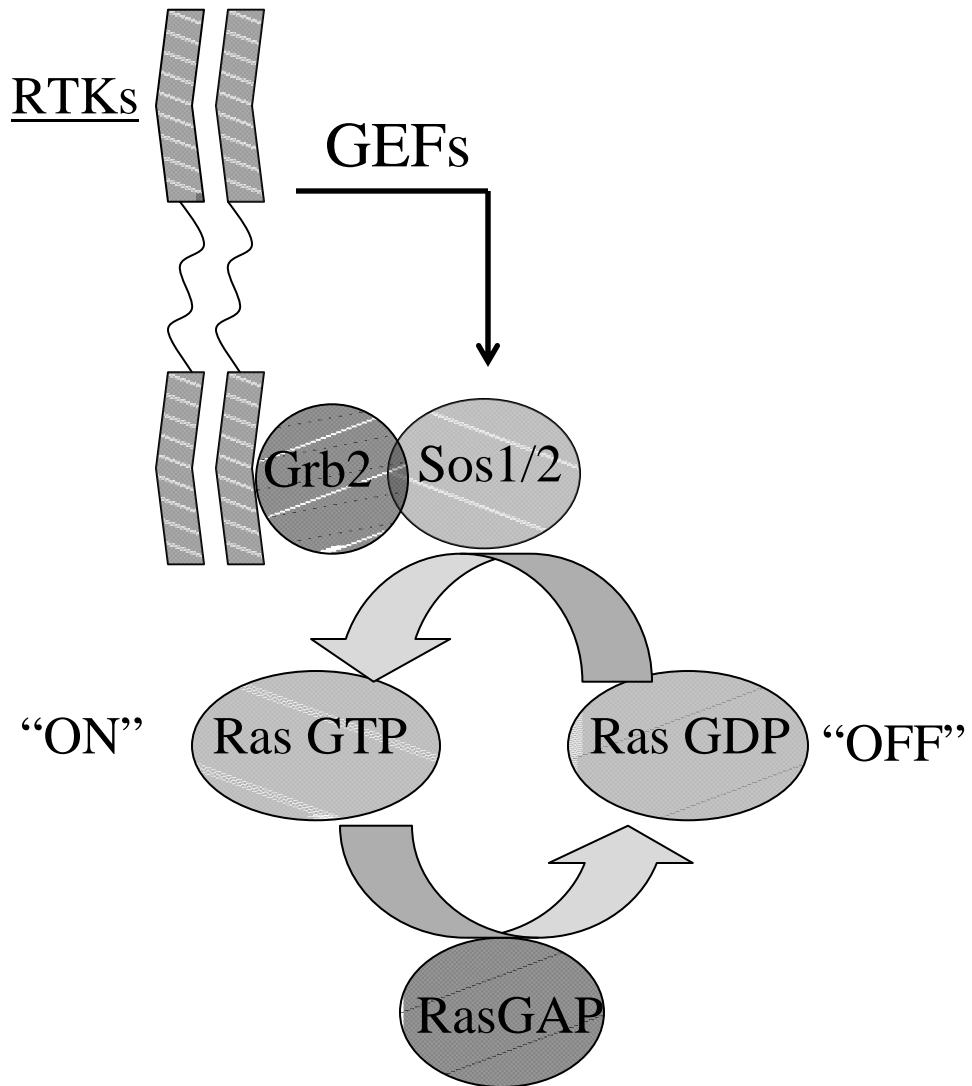
Table 1: The Ras Superfamily

Family	Human genes	Known isoforms
Ras	36	39
Ral	2	2
Rho	20	22
Rab	61	63
Sar1/ Arf	27	30
Ran	1	1
Other	9	10
Total	156	166

Adapted from Wennerberg K, Rossman KL, Der CJ. *Journal of Cellular Science*.2005; 118(5); 843-846.

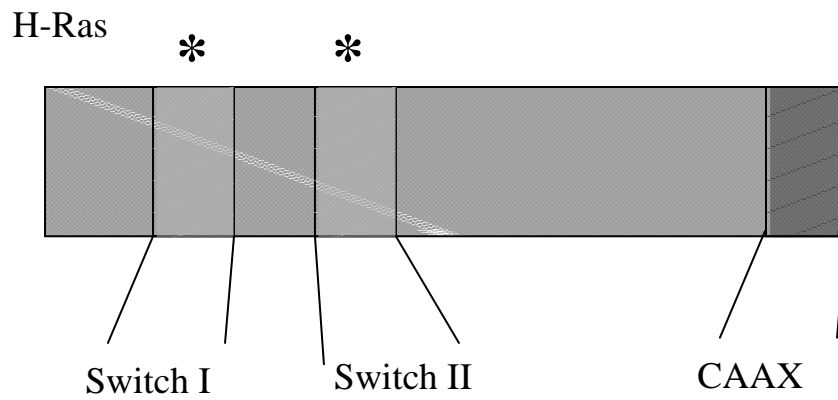
Members of the Ras superfamily are also referred to as molecular switches which cycle from the “on” (GTP-bound) to “off” position (GDP-bound) through an intrinsic GTPase activity (Figure 1, following page). Two classes of proteins regulate the catalytic rate of Ras superfamily GTPase activity: GTPase activating proteins (GAPs) and guanine-nucleotide exchange factors (GEFs). GAP proteins activate the intrinsic GTPase activity of Ras family members causing Ras to remain in a preferentially GDP-bound state whereas GEFs catalyze the exchange GDP for GTP causing Ras to be in a preferentially GTP-bound state (2, 16, 18, 46, 102). Mutational activation of Ras proteins typically involves point mutation of key residues, for example amino acids 12 and 61 of Ras (see Figure 2, following page), essential for GAP binding; this gradually reduces the GTPase activity of Ras and keeps it in a persistently activated, GTP-bound state (17, 63, 79, 87, 95, 96). The nucleotide bound state dictates the orientation of the effector loop regions of Ras family proteins; when GTP is bound the effector loop regions become accessible to the Ras-binding regions of cognate effector proteins activating multiple downstream effector pathways (60).

Figure 1: The Ras GTP-GDP Cycle



Abbreviations: RTKs (receptor tyrosine kinases), SOS (son of sevenless), Ras-GTP (GTP-bound Ras), Ras-GDP (GDP-bound Ras), GEFs (guanine nucleotide exchange factors), RasGAP (Ras GTPase activating protein)

Figure 2: Ras GTPase Domains and Point Mutations



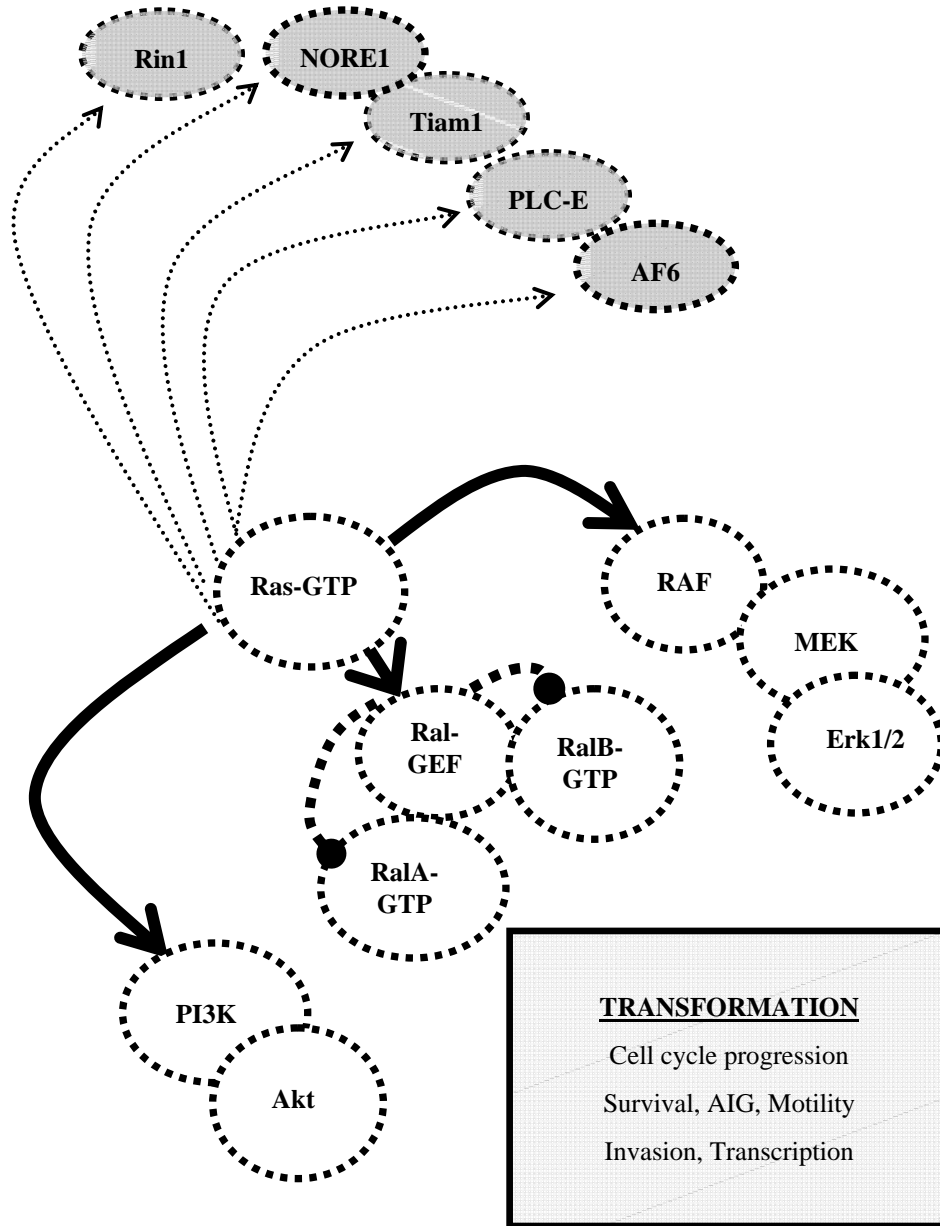
*** Amino acid 12**

*** Amino Acid 61**

Adapted from Pai EF, Krnngel U, Petsko GA, et al .EMBO Journal.
1990; 9(8); 2351-9.

Ras proteins engage over twenty known effectors, which in turn activate a variety of other proteins. Three of the Ras effector pathways, phosphatidylinositol tri-phosphate kinase (PI3K)/Akt, Raf/MEK/Erk and Ral guanine nucleotide dissociation stimulator (RalGDS)/Ral (7, 21, 35, 81) are at least partially required for constitutively activated Ras to initiate oncogenesis in certain cell types (26, 31, 41, 45, 64, 65, 71) [Figure 3, see following page]. Ras proteins, as the archetypical oncogenic small GTPases, are the most intensively studied members of the Ras superfamily. However, the vast majority of known small GTPases, despite being similar to Ras, are far less extensively studied and play undefined roles in oncogenesis. Furthermore, these gene products have, at least potentially, as many or more effectors as Ras. Some of these proteins, such as members of the Rac and Ral subfamilies, are known to interact directly with effectors which are distinct from those of Ras. Taken as a whole these observations suggest that the true range of cellular pathways governed by small GTPases remain to be determined.

Figure 3: Ras Signaling and Transformation



Abbreviations: Ras-GTP (GTP-bound Ras), Ral-GEF (Ral guanine nucleotide exchange factor), RalA/B-GTP (GTP-bound Ral), PLC-E (phospholipase C epsilon), AIG (anchorage independent growth)

The Role of Prenylation in Ras Superfamily Function

The oncogenic functions of the Ras and Rho proteins require posttranslational processing by prenyltransferase enzymes (37, 39, 101). The two enzymes responsible for prenylation of Ras family proteins are farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I) (6, 8, 9, 55, 74, 75, 100, 101), which covalently attach the 15-carbon farnesyl and 20-carbon geranylgeranyl lipids, respectively, to the cysteine of proteins with the carboxy terminal tetrapeptide consensus sequence CAAX, (C is cysteine, A is any aliphatic amino acid and X is any carboxyl-terminal amino acid), see figure 2. In general, FTase farnesylates proteins in which X is methionine or serine (70), whereas GGTase I geranylgeranylates proteins in which X is leucine or isoleucine (24). Ras proteins that are mutationally rendered unprenylatable lose their oncogenic activity and fail to properly localize within the cell (37, 39). Similarly, prenylation of other proteins in the Ras and Rho families is essential to their activities (1, 37, 49).

The fact that prenylation is required for the oncogenic activity of small GTPases prompted us and others to design FTase and GGTase I inhibitors (FTIs and GGTIs) as potential anticancer drugs (28, 50, 68, 105). While numerous studies have shown that FTIs suppress oncogenic and tumor survival pathways, the actual mechanism by which FTIs inhibit tumor growth is not known (67, 76). Thus, while designed originally as anti-Ras inhibitors, the Ras isoforms most commonly mutated in human cancers (N- and K-Ras) escape FTI inhibition by undergoing alternative prenylation by GGTase I (72, 85, 94). Therefore, the critical farnesylated proteins that FTIs target to induce these effects are not known (67, 76). Similarly, the GGTase I substrates important for the anti-tumor

activity of GGTIs are not clearly understood yet. While Rac1 and Rac3 Rho family proteins have been implicated as candidate targets for GGTIs (38), other important targets remain to be identified. Previous studies have demonstrated that GGTIs, at least partially require, the inhibition of Akt serine/threonine kinase and expression of survivin (15, 83), however it remains unknown how inhibition of geranylgeranylated proteins results in downregulation of these critical targets. Furthermore, GGTIs also induce p21^{waf}, inhibit CDK activity, phosphorylation of Rb and lead to G₀/G₁ cell cycle accumulation (61, 68, 83-85, 90). In animal models, GGTIs both inhibit tumor growth in nude mouse xenografts and induce tumor regression in transgenic mice (82, 83). The GGTase I substrates targeted by GGTIs to induce their anti-neoplastic effects are not known. Logical candidates include other Ras and Rho family proteins with roles in oncogenesis, such as the *Ras*-like RalA and RalB small GTPases.

The increasing evidence for Ral GTPases in oncogenesis prompted our interest in evaluating Ral GTPases as important targets for GGTI anti-tumor activity. Both RalA and RalB C-termini contain a CAAX sequence that predicts prenylation and RalA has been shown to be geranylgeranylated (43). Furthermore, RalA and RalB are involved in many oncogenic steps that are inhibited by GGTIs. Therefore, in chapter two of this thesis we investigated whether some of the anti-neoplastic effects of GGTIs are mediated by inhibition of the geranylgeranylation and function of RalA and/or RalB.

Tissue- and Species- Specific Mechanisms of Ras Transformation

Understanding the mechanisms of Ras transformation has become a critical component of developing a new generation of targeted therapeutics. Over 30% of human cancers have mutationally activated Ras (32). As we review in this section, over twenty years of research, using a variety of *in vitro* model systems, has defined a series of species and tissue-specific mechanisms of Ras-transformation. These *in vitro* cell systems fall into two general categories: spontaneously immortalized- and genetically defined- model systems; both of these are minimally transformed systems which provide a platform to assess the effects of stable expression of oncogenic Ras (30, 32). Furthermore, these systems provide an easily evaluable means of determining the mechanism by which Ras drives species- and tissue- specific transformation. In this section we first review the mechanisms of Ras-transformation in spontaneously immortalized cell systems followed by the more recently used genetically defined models of human cancer.

Use of Spontaneously Immortalized Cell Systems to Study Ras Transformation

Three of the Ras signaling pathways, RalGEF/Ral, PI3K/AKT and Raf/Mek/Erk have been primarily implicated in the ability of oncogenic Ras to initiate and maintain the transformed phenotype (41, 45, 51, 75, 78). The Raf/Mek/Erk pathway has been widely assumed to be the primary pathway of Ras transformation in all cell types. This inaccurate assessment is mostly derived from initial reports which, correctly, determined that the transformative capabilities of Ras oncogenes relied upon activity of the Raf/Mek/Erk pathway in spontaneously immortalized mouse fibroblast NIH-3T3 cells (41, 45). However, a variety of other spontaneously immortalized cell systems have been used to describe an array of species- and tissue- specific requirements for Ras transformation.

Spontaneously immortalized rat intestinal epithelial (RIE) cells have been used as a model system to assess the involvement of the Raf, Akt and Ral signaling pathways in Ras mediated transformation. Specifically, Sheng and colleagues (77) found that pharmacologic blockade of PI3K/Akt inhibited Ras mediated transformation however, myristoylated-Akt was not sufficient for transformation but was transforming when co-expressed with a constitutively activated C-Raf mutant (Delta-Raf-22W). Further work by Der and colleagues (64) compared transformation of NIH-3T3 side by side with RIE cells. While both Ras and a constitutively activated C-Raf (Raf-CAAX) could transform NIH-3T3 only Ras could independently transform RIE cells. Gangarosa and colleagues (27) compared the ability of K-Ras^{12V}, H-Ras^{12V} and Raf-CAAX to transform RIE (and the similar intestinal epithelial cell line-6, IEC-6 cells) by measuring the ability of the

oncogenes to elicit an external epidermal growth factor receptor (EGFR) autocrine loop. In these cells oncogenic Ras, but not Raf, could elicit this autocrine loop and transform RIE and IEC6 cells. Further work by this group in collaboration with Der and colleagues (65) demonstrated that while Ras transformation in RIE cells could be blocked by the MEK inhibitor PD98059, Ras mutants defective for Raf activation could still transform RIE cells, albeit at a reduced potency. These results argue that while both Raf and PI3K signaling are partially required for Ras transformation of rat intestinal epithelial cells neither is sufficient to independently initiate oncogenesis.

Immortalized human mammary epithelial cells (MCF-10A) derived from dysplastic mammary tissue were used as a model system to assess the involvement of various Ras downstream signaling molecules in Ras mediated transformation. Der and colleagues (64) compared transformation of NIH-3T3 side-by-side with MCF-10A cells. Both oncogenic Ras and Raf-CAAX could transform NIH-3T3 but only oncogenic Ras was sufficient to transform MCF-10A cells. The Salomon group (57) further determined that oncogenic Ras induced an EGFR-autocrine loop in MCF-10A cells and determined that both EGFR activation and MEK activity were required for full transformation. These results argue for a more complex mechanism of transformation in MCF-10A cells than in NIH-3T3 cells.

Spontaneously immortalized rat ovarian surface epithelial (ROSE) cells have been used to determine the requirements for Ras mediated transformation. In these cells the Der group (88) determined that stable expression of H-Ras^{12V} activated Raf/MEK/ERK signaling but did not activate PI3K signaling. Here, constitutively active Raf could partially reconstitute both Ras-transformation and associated morphology changes. The

Erickson group further defined this system (12) and determined that all three effector pathways (Raf, PI3K, and RalGDS) were required for full transformation and the production of cathepsin L.

Use of Genetically Defined Cell Systems to Study Ras Transformation

While spontaneously immortalized cell systems have proven to be useful models for the study of transformation requirements it is important to note that spontaneous immortalization proceeds through poorly defined processes. Conversely, genetically tractable models of human epithelial immortalization, while experimentally defined, offer an alternative means of investigating mechanisms of specific oncogene induced transformation in cells with a defined genetic background which closely mimics the genetic abnormalities present in the early and late stages of human carcinogenesis (51).

Human embryonic kidney cells sequentially immortalized by the catalytic subunit of human telomerase (hTERT) and the SV40-large T and –small t antigens (which inactivate the key tumor suppressors p53, Rb and protein phosphatase-2A), or HEK-HT cells, are derived from the embryonic mesoderm. HEKs are a mixed lineage cell line known to express both epithelial and mesenchymal markers that become fully transformed by stable expression of oncogenic Ras (30). The Counter group determined that H-Ras^{12V37G} was, by itself, sufficient for transformation in this cell line (31). This concept was further supported by the demonstration that constitutively activated Rlf or activated RalA, but not the related RalB, was capable of transforming these cells (51). In contrast to these results the Weinberg group further defined the requirements for Ras-mediated transformation of this system (69). Rangarajaram and colleagues found that only paired expression of H-Ras^{12V37G} (only activating RalGDS) and H-Ras^{12V40C} (only activating PI3K), but not H-Ras^{12V35S} (only activating Raf) and H-Ras^{12V40C} nor H-Ras^{12V37G} and H-Ras^{12V35S}, could fully transform HEK-HT cells. This concept was further

supported by evidence that stable expression of constitutively activated Akt and Rlf (a Ral-GEF) could induce transformation in soft agar.

In contrast, human mammary epithelial cells (HMECs) immortalized by hTERT and SV-40-large T antigen, HMECs, become fully transformed by stable expression of oncogenic Ras (19) but differ in the Ras-downstream signaling requirements from HEK-HTs. The Weinberg group has investigated the requirements for Ras-mediated transformation of this system (69) and determined that only co-activation of all three primary Ras effectors (Raf, RalGDS, and PI3K) can transform HMEC cells.

A variety of other human-derived genetically defined models of transformation have been used to address mechanisms of Ras transformation. Human fibroblasts immortalized by hTERT and SV40-large T antigen, BJ fibroblasts, become fully transformed by stable expression of oncogenic Ras (30). The Weinberg group further defined the requirements for Ras-mediated transformation of this system (69). Rangarajan and colleagues found that only paired expression of H-Ras^{12V35S} (only activating Raf-1) and H-Ras^{12V37G} (only activating RalGDS), but not H-Ras^{12V35S} and H-Ras^{12V40C} not H-Ras^{12V37G} and H-Ras^{12V40C}, could fully transform BJ fibroblasts. Human esophageal epithelial cells immortalized by hTERT and SV40, EPC2 cells, can be fully transformed by either H-Ras12V, c-Myc or activated AKT (42).

A variety of other tissue types following the same sequence of genetic events (hTERT activation, inactivation of p53 and Rb, and transduction of oncogenic Ras), but using non-viral approaches, have been used to create other genetically defined models of human cancer. Counter and colleagues (40) report the creation of an alternative human mammary epithelial cell system, the first human myoblast cell system, and an alternative

human embryonic kidney cell system. Linardic and colleagues report the creation of rhabdomyosarcoma model systems from human skeletal muscle cell precursors and committed human skeletal muscle myoblasts (53). Also, human bronchial epithelial cells immortalized by hTERT, CDK4 and siRNA to p53, HBECs, can be partially transformed by stable transduction of K-Ras^{12V} or mutant EGFR (E746-A750 del or LL859R). The Minna group determined that either Ras or EGFR could induce transformation and invasion in a 3-dimensional organotypic culture (103).

Additionally, while not genetically defined nor able to be fully transformed human thyroid epithelial primary cells can be induced to a hyper-proliferative state by expression of oncogenic Ras which closely resembles the phenotype of an early stage thyroid tumor, follicular adenoma. The Wynford-Thomas group (4) used these cells to investigate the contributions of the Ras-effectors Raf, PI3K and RalGDS to induction of thyrocyte hyperproliferation. Co-expression of the three Ras effector mutants which only bind RalGDS, Raf, and PI3K could fully recapitulate the hyperproliferative state induced by fully oncogenic Ras while combinations of any of these two mutants was not sufficient.

Importantly for our studies, human ovarian surface epithelial cells immortalized by stable expression of hTERT and SV40-large T antigen, T80 cells, have been recently established as models for human ovarian oncogenesis. These cells were created by the Bast group (54) and are fully transformed following stable expression of either H-Ras^{12V} or K-Ras^{12V}. A similar model system, created by Kuskari and colleagues (48), could also be transformed by stable expression of either H-Ras^{12V} or c-erbB-2.

Use of an *In Vitro* Model of Human Ovarian Cancer to Study the Mechanism of Ras Transformation: Implications for RalA and RalB

Ovarian cancer is the primary cause of death among gynecological malignancies and is the 5th leading cause of cancer deaths among women in the United States (29). Despite the high incidence of ovarian cancer among women and the relative lethality of the disease little is known about the precise mechanism of transformation of human ovarian surface epithelial (HOSE) cells. Indeed, multiple mechanisms of ovarian surface epithelial cell transformation have been proposed and are believed to be sub-type specific (3). The two most common sub-types of ovarian cancer, high-grade serous and mucinous ovarian carcinoma (29), are commonly diagnosed at late-stage and are frequently lethal due to a high-propensity for both metastasis and drug-resistant recurrence (3, 29). It is thus of critical importance to define the mechanism of ovarian epithelial cell transformation both for a more precise understanding of the underlying genetic risk factors as well as for the development of targeted therapies .

Epithelial neoplasias, such as ovarian cancers, exhibit multiple genetic aberrations in key tumor suppressor genes, so-called “cellular gatekeepers” such as p53 and Rb (32). Indeed, over 50% of ovarian cancers exhibit missense mutations in P53 (47, 56). Furthermore, over 80% of human ovarian cancers examined have been shown to have mutational defects in one or more of the genes involved in the Rb tumor suppressive pathway, such as CDK4, cyclin D1, and Rb (33). Activation of human telomerase activity is another well-recognized defect of human epithelial neoplasms (32) with ovarian cancer being no exception to this rule (13). In addition to p53, Rb and hTERT genetic

abnormalities activation of one or more proto-oncogenes is a pivotal fourth event in the development of malignant disease.

One of the most well studied mechanisms of oncogenesis in human cancer is mutational activation of members of the Ras gene family. Mutations in Ras gene family members (H-, K-, and N- Ras) are known to occur in approximately 30% of human cancers (78). Numerous studies have demonstrated that mutational activation of K-Ras, through missense mutations at codons 12 and 13, occurs with high frequency (27-69%) in certain subtypes of ovarian cancers (14, 25, 36, 62, 89). Similar mutations in the H-Ras proto-oncogene have been determined to occur in ovarian cancer subtypes though there is considerable disagreement over the frequency (20, 89, 104). For example, while one study has found that H-Ras activations occur with variable frequency depending on subtype (33% of borderline ovarian tumors, 13% of mucinous adenocarcinomas, and 7% of serous adenocarcinomas and in 6% of primary invasive ovarian carcinomas) (89) others have found H-Ras mutations occur infrequently (0-12.5%) (20, 104) .

A role for Ras mutation in ovarian cancer progression is especially well recognized in mucinous sub-type ovarian tumors where the incidence of K-Ras proto-oncogene mutation increases profoundly during the progression of disease (62). For example, Mok and colleagues found a high, progression-dependent incidence of K-Ras activation at codons 12 and 13 in 13% of mucinous adenomas, 33% of mucinous tumors at the borderline, and 46% of mucinous carcinomas (62). Similar results, in which K-Ras mutation incidence increases dramatically throughout disease progression, have been reported by many other groups (14, 20, 36, 62, 89). These studies have primarily examined mutations at codons 12 and 13 however, activating Ras mutations are also

known to occur in some cancers at codon 61; thus it is possible that the actual incidence of Ras mutations in mucinous ovarian cancer has been underestimated.

K-Ras mutation have also been detected, albeit to a lesser degree (0-12%), in high-grade serous sub-type ovarian cancer, the most common form of ovarian malignancy (62). It is interesting to note that a much higher incidence of Ras mutation exists in low-grade serous malignancy. Indeed, over 68% of low grade serous tumors have either a K-Ras or B-Raf mutation (59). Furthermore, these mutations occur in a mutually exclusive (59) manner suggesting that the primary mechanism of K-Ras mutation in low-grade serous is through B-Raf activation. Additionally, the presence of K-Ras proto-oncogene mutations in up to 48% of borderline ovarian epithelial tumors (62) indicates a role for K-Ras mutation in the early events of low-grade serous ovarian neoplasia. Given the lack of B-Raf mutations in high-grade serous carcinoma the precise mechanism of Ras mediated transformation in this tumor sub-type remains a mystery, as does the etiology of disease progression. While genetic analysis of patient tumors of multiple ovarian subtypes suggest Ras mutation is a frequent occurrence analysis of downstream Ras effectors, such as Raf, has not yielded a consensus view of the mechanism for Ras transformation. However, other methods are available for determining the necessary components of ovarian cell-type specific Ras transformation.

Recently, Der and colleagues described the mechanism of Ras transformation in spontaneously immortalized rat ovarian surface epithelial (ROSE) cells (88). They assessed the transformative ability of retrovirally transduced H-Ras and constitutively active mutants of the Ras downstream effectors Raf, Ral- GDS and the catalytic subunit of PI3K (p110) to determine, for the first time, which Ras effectors are capable of

independent ovarian cell transformation. In this system Raf, but not p110 or Ral-GDS, was capable of independently transforming in a manner similar to H-Ras^{12V}. Interestingly, H-Ras^{12V} was unable to activate PI3K catalytic activity or stimulate activation of Akt in this cell line. These results suggest that Ras effector utilization varies in a tissue specific manner. While this system has been useful in suggesting which signaling cascades are required for Ras transformation in ovarian epithelial cells, the ROSE system is neither genetically defined nor of human origin and thus, while suggestive of a role for Raf in ovarian transformation, has unknown prognostic significance for human ovarian oncogenesis.

Since that seminal discovery by Der and colleagues a genetically defined human model of ovarian malignancy has been developed which recapitulates the critical events in neoplasia (54). The T80 cell line is derived from primary human ovarian surface epithelial cells engineered to express SV40T and hTERT. Upon the addition of retrovirally transduced H-Ras^{12V} these cells, referred to as T80H, acquire anchorage independent growth capability in soft agar, a reliable estimate of transformation, and are capable of forming xenograft tumors in a SCID mouse model. This transformation requires continued expression of H-Ras (98) and, as such, is a bona fide model of Ras-dependent, ovarian cell-type-specific transformation. In chapter 3 of this thesis we have used these cells as well as our own stable K-Ras^{12V} expressing variant (T80K) to investigate the required downstream signaling components of Ras transformation in human ovarian surface epithelial cells.

Ral Proteins are Central Mediators of Oncogenesis

In further confirmation of a role for the Ral small GTPases in oncogenesis both RalA and RalB, which are over 85% similar, have been identified as critical divergent mediators of multiple tumorigenic processes, including metastasis, invasion, anchorage independent growth, survival and cell motility (10, 22, 66, 91, 92, 97). Specifically, depletion of RalA by siRNA has been shown to inhibit anchorage independent proliferation of multiple human cancer cell lines, such as the HeLa cervical and SW680 prostate cancer cell lines (10) as well as multiple human pancreatic cancer cell lines (52). Also, RalA expression is required for anchorage independent growth of sequentially Ras transformed human ovarian (see Chapter 3) and kidney cells (51). Similarly, stable depletion of RalA, but not RalB, by shRNA has been shown to inhibit tumor formation and metastasis of multiple human pancreatic cancer cell lines in athymic nude mice (52). Further confirming a role for RalA in metastasis, stable overexpression of constitutively activated RalA has been shown to promote both standard and experimental metastasis *in vivo* (86, 91) and in human prostate cancer cells, stable expression of activated RalA promotes bone, but not brain, metastasis (99). Even in non-epithelial cancers there is emerging evidence that RalA is involved in tumorigenicity; for example, in human HT1080 fibrosarcoma cells stable overexpression of activated RalA promotes anchorage independent growth (97). In agreement with these findings two cellular processes thought to promote metastasis and anchorage independent growth: cell motility and invasion, are inhibited by siRNA-mediated depletion of RalA in multiple human renal cancer cell lines (66).

While RalA has been well validated to play an essential role in anchorage independent processes in multiple tissue types the role of RalB has been determined to be far more tissue specific. For example, while depletion of either RalA or RalB inhibits anchorage independent growth in human ovarian epithelial cells transformed by either H- or K-Ras^{12V} (Chapter 3), only depletion of RalA, but not RalB, inhibits H-Ras^{12V} transformation of HEK-HT cells (51). We, as well as others have previously determined a role for RalB, but not RalA in the survival of multiple human cancer cell lines (10, 22). Specifically, White's group has found that depletion of RalB by siRNA induces apoptosis in both HeLa and SW680 human cancer cell lines (10). Similarly, our results in chapter two have established, using a chemical biology approach, that inhibition of a RalB, but not RalA, survival pathway underlies the apoptotic response to geranylgeranyltransferase I inhibitors (GGTIs) (22). Specifically, Ral proteins have been shown to require geranylgeranylation for localization and interaction with other proteins (34, 44, 58, 80). Importantly, stable expression of GGTI-resistant RalB in the human pancreatic carcinoma cell line MiaPaCa2 renders cells resistant to GGTI-induced apoptosis (22). Taken as a whole, these studies strongly suggest a role for RalB tumor survival in a wide array of tissue types as well as a tissue specific role in anchorage independent growth.

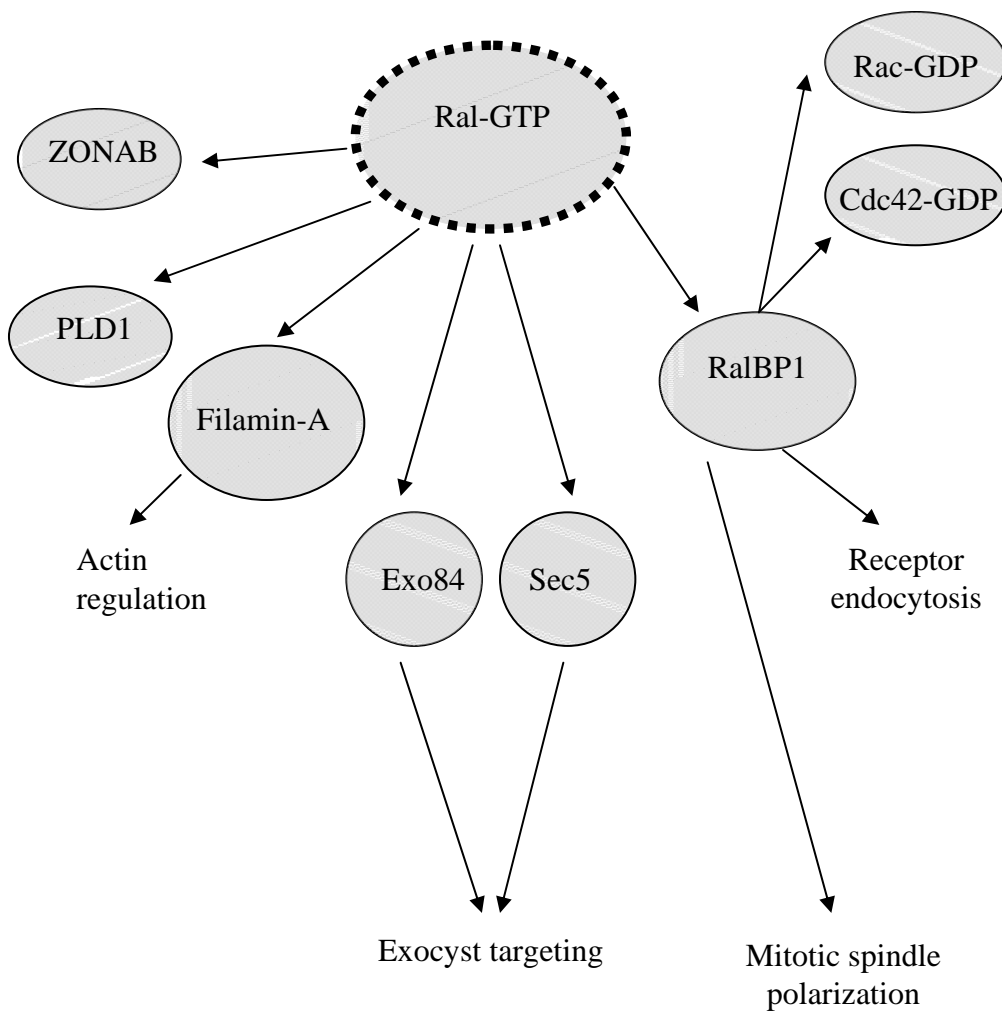
Ral Effectors and Control of Cellular Processes

Despite the wealth of information regarding the role of Ral small GTPases in transformation very little is known about the pathways through which Ral exerts these effects. For example, while RalA and RalB bear a high homology to Ras and over 20 effectors are known for the Ras isoforms (5, 78), only 6 Ral effectors have been described: phospholipase D1 (PLD1), the exocyst components Sec5/ Exo84, Filamin A, ZO-1 N-terminally associated binding protein (ZONAB) and Ral binding protein-1 (RalBP1/RLIP) (23). Precise roles for these proteins in the various transformation specific processes that are regulated by RalA and RalB remain poorly defined. Despite the fact that RalA and RalB are commonly thought of as Ras effector proteins, both RalA and RalB are found in the hyperactivated state independently of Ras in human pancreatic tumors (51, 52). Thus, novel means of Ral activation and inactivation may constitute an important and undescribed mechanism of transformation. However, no Ral GTPase activating proteins (RalGAPs), which would negatively regulate RalA and RalB, have been described to date. Also, RalA and RalB are known to regulate diverse physiological processes such as signaling via STAT3, NF-KB, JNK and AFX (23). However, the intermediate proteins in these signal transduction pathways through which RalA and RalB exert these functions remain unknown.

In an effort to more fully understand the protein interactions that govern the biological activities of RalA and RalB we have used proteomic analysis, in chapter 4 of this thesis, to describe a proposed database of Ral interacting proteins for the first time. We have uncovered a wealth of potential RalA and RalB interacting partners and used a

systems biology approach to analyze the broad themes that emerge from this proposed database. We have further demonstrated that one of these proteins, receptor for activated C-kinase (also known as GBLP1 or RACK1), is a potential RalA and RalB interacting protein that is required for both Ras and Ral mediated transformation of human ovarian surface epithelial (T80) cells. In addition to uncovering a critical role for RACK1 expression in both Ras- and Ral- mediated transformation our proteomics study is the first large-scale analysis of the Ral “interactome”. This database will provide the emerging field of Ral study with a wealth of potential RalA and RalB biological effectors and potential regulators of Ral cellular activities.

Figure 4: Ral Signaling



Abbreviations: Ral-GTP (GTP-bound Ral), PLD1 (phospholipase D1), RalBP1 (Ral Binding Protein 1), CDC-GDP (GDP-bound CDC42), Rac-GDP (GDP-bound Rac)

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Chapter 2:

Geranylgeranyltransferase I Inhibitors Target RalB to Inhibit Anchorage-dependent Growth and Induce Apoptosis, and RalA to Inhibit Anchorage-independent Growth

By

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All the work in this chapter was performed by Samuel C. Falsetti except for the cloning of the various constructs that was done in collaboration with De-an Wang and the chemical synthesis that was performed by Hairuo Peng and Dora Carrico

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Abstract

Geranylgeranyltransferase I inhibitors (GGTIs) are presently undergoing advanced preclinical studies and have been shown to disrupt oncogenic and tumor survival pathways, to inhibit anchorage-dependent and –independent growth and to induce apoptosis. However, the geranylgeranylated proteins that are targeted by GGTIs to induce these effects are not known. Here we provide evidence that the Ras-like small GTPases RalA and RalB are exclusively geranylgeranylated and that inhibition of their geranylgeranylation mediates, at least in part, the effects of GGTIs on anchorage-dependent and –independent growth and tumor apoptosis. To this end, we have created the corresponding carboxyl terminal mutants that are exclusively farnesylated, verified that they retain the subcellular localization and signaling activities of the wild type geranylgeranylated proteins and that Ral GTPases do not undergo alternative prenylation in response to GGTI treatment. By expressing farnesylated, GGTI-resistant RalA and RalB in Cos7 cells and the human pancreatic MiaPaCa2 cancer cells followed by GGTI-2417 treatment we demonstrated that farnesylated RalB, but not RalA, confers resistance to the pro-apoptotic and anti-anchorage-dependent growth effects of GGTI-2417. Conversely, farnesylated RalA but not RalB expression renders MiaPaCa2 cells less sensitive to inhibition of anchorage-independent growth. Furthermore, farnesylated RalB, but not RalA, inhibits the ability of GGTI-2417 to suppress survivin and induce p27Kip1 protein levels. We conclude that RalA and RalB are important, functionally distinct targets for GGTI-mediated tumor apoptosis and growth inhibition.

Introduction

Members of the Ras and Rho branches of the Ras superfamily of small GTPases are critically involved in the regulation of many biological events critical to the regulation of cellular homeostasis such as cell cycle control, cell survival, death, differentiation, development and growth (9, 53). The aberrant activation or inactivation of Ras family proteins is believed to be important in the induction of oncogenesis. In addition to the three Ras proteins (H-, N- and K-Ras), other Ras family proteins with validated roles in oncogenesis include R-Ras, Ral, Rheb, Di-Ras and Noey2/ARHI small GTPases. Rho family GTPases (e.g., RhoB, RhoC, Rac1b, DBC2) are also implicated in oncogenesis (41).

The oncogenic functions of the Ras and Rho proteins require posttranslational processing by prenyltransferase enzymes (20, 23, 56). The two enzymes responsible for prenylation of Ras family proteins are farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I) (3-5, 29, 42, 43, 55, 56), which covalently attach the 15-carbon farnesyl and 20-carbon geranylgeranyl lipids, respectively, to the cysteine of proteins with the carboxy terminal tetrapeptide consensus sequence CAAX, (C is cysteine, A is any aliphatic amino acid and X is any carboxyl-terminal amino acid). In general, FTase farnesylates proteins in which X is methionine or serine (37), whereas GGTase I geranylgeranylates proteins in which X is leucine or isoleucine (12). Ras proteins that are mutationally rendered unprenylatable lose their oncogenic activity and fail to properly localize within the cell (20, 23). Similarly, prenylation of other proteins in the Ras and Rho families is essential to their activities (1, 20, 25) .

The fact that prenylation is required for the oncogenic activity of small GTPases prompted us and others to design FTase and GGTase I inhibitors (FTIs and GGTIs) as potential anticancer drugs (14, 26, 35, 57). While numerous studies have shown that FTIs suppress oncogenic and tumor survival pathways, the actual mechanism by which FTIs inhibit tumor growth is not known (34, 44). Thus, while designed originally as anti-Ras inhibitors, the Ras isoforms most commonly mutated in human cancers (N- and K-Ras) escape FTI inhibition by undergoing alternative prenylation by GGTase I (40, 51, 54). Therefore, the critical farnesylated proteins that FTIs target to induce these effects are not known (34, 44). Similarly, the GGTase I substrates important for the anti-tumor activity of GGTIs are not clearly understood yet. While we have implicated Rac1 and Rac3 Rho family proteins as candidate targets for GGTIs (22), other important targets remain to be identified. Clues to what these targets may be are suggested by our previous studies demonstrating that GGTIs inhibit the activation of the Akt serine/threonine kinase and expression of survivin (10, 49). Furthermore, GGTIs also induce p21^{waf}, inhibit CDK activity, phosphorylation of Rb and lead to G0/G1 cell cycle accumulation (30, 35, 49-52). In animal models, GGTIs both inhibit tumor growth in nude mouse xenografts and induce tumor regression in transgenic mice (48, 49). The GGTase I substrates targeted by GGTIs to induce their anti-neoplastic effects are not known. Logical candidates include other Ras and Rho family proteins with roles in oncogenesis.

Recently, the *Ras*-like RalA and RalB small GTPases have been shown to play critical roles in Ras-mediated growth transformation of human cells (16, 27). Ral GTPases are activated by Ral guanine nucleotide exchange factors (RalGEFs; e.g.,

RalGDS), and RalGEFs function as key downstream effectors of activated Ras (38). Critical evidence for the important role of the RalGEF-Ral effector pathway in Ras-mediated oncogenesis is provided by studies in cell culture and mouse model systems. We showed previously that this effector pathway, and not the Raf-MEK-ERK mitogen-activated protein kinase effector pathway, is sufficient and necessary to promote Ras-mediated tumorigenic growth transformation (16). Similar observations were made by Weinberg and colleagues (36), who found cell type differences in the importance of RalGEF-Ral signaling in Ras transformation. Marshall and colleagues found that mice deficient in one RalGEF (RalGDS) are developmentally normal, but showed impaired skin tumor growth caused by carcinogen-induced Ras activation (15). An unexpected outcome of the study of the role of Ral GTPases in oncogenesis has been the distinct functions of the highly related RalA and RalB isoforms. Although RalA and RalB share strong sequence (85% identity) similarities, White and colleagues found that RalA is important for tumor cell anchorage-independent proliferation, whereas RalB promotes tumor cell survival (8). We recently determined that RalA, but not RalB is critical for anchorage-independent and tumorigenic growth of pancreatic carcinoma cells, whereas RalB and to a lesser degree RalA is critical for pancreatic carcinoma invasion and metastasis (28). The distinct functions of RalA and RalB may be due, in part, to distinct downstream effector utilization (7, 45).

The increasing evidence for Ral GTPases in oncogenesis prompted our interest in evaluating Ral GTPases as important targets for GGTI anti-tumor activity. Both RalA and RalB C-termini contain a CAAX sequence that predicts prenylation, and RalA has been shown to be geranylgeranylated (24). Furthermore, RalA and RalB are involved in

many oncogenic steps that are inhibited by GGTIs. Therefore, in this manuscript we investigated whether some of the anti-neoplastic effects of GGTIs are mediated by inhibition of the geranylgeranylation and function of RalA and/or RalB. To this end, we have demonstrated that RalA and RalB are exclusively geranylgeranylated, generated farnesylated variants of Ral and verified that they are GGTI-insensitive, and used these variants to rescue cancer cells from the anti-neoplastic effects of GGTIs. Our data suggest that inhibition of RalB mediates the effects of GGTIs on survivin, p27Kip1, apoptosis and anchorage-dependent growth, whereas inhibition of RalA mediates, at least in part, the effects of GGTIs on anchorage-independent growth.

Materials and Methods

Synthesis of CAAX Peptidomimetic—The GGTase I-specific peptidomimetics GGTI-2417 and GGTI-2418 were synthesized as described previously (33). The FTase-specific peptidomimetics FTI-2148 and FTI-2153 were synthesized as described previously (48).

Cloning of Ral A and Ral B mutants- We used our previously described RalA and RalB pBABE expression constructs (16) as template DNA for site directed mutagenesis PCR driven by Platinum Taq polymerase (Invitrogen, Carlsbad, CA) according to manufacturers instructions with the following primers (Qiagen, Valencia, CA): FLAG-RalA-CCIL was generated using (F-5'GCCGGATCCATG GATTACAAGGATGACGACGATAAGATCGTCGACTACCTAGCAAATAAGCCC-3', R-5'GCCGGATCCTTATAAAAATGCAGCATCTTTCTCTGATTC-3'), FLAG-RalA-CCIS was generated using (F-5'GCCGGATCCATGGATTACAA GGATGACGACGATAAGATCGTCGACTACCTAGCAAATAAGCCC-3', R-5'GCCGGATCCTTATGAAATGCAGCATCTTTCTCTGATTC-3'), FLAG-RalA-SCIL was generated using (F-5'GCCGGATCCATGGATTACAAGGATGACGACGA TAAGATCGTCGACTACCTAG CAAATAAGCCC-3', R-5'GCCGGATCCTTATA AAATGCAGGATCTTTCTCTGATTC-3'); FLAG-RalB-CCLL was generated using (F-5'GCCGGATCCATGGATTACAAGGATGACGACGATAAGATCGCT GCCAACAAGAGTAAGGGCCAG-3', R-5'GCCGGATCCTCATAGTAAGCAA CATCTTTCTTTAAACT-3'), FLAG-RalB-CCLS was generated using (F-5'GCCGGATCCATGGATTACAAGGATGACGACGATAAGATCGCTGCCAA CAAGAGTAAGGGCCAG-3', R-5'GCCGGATCCTCATGATAAGCAACATCT TTCTTTAAACT-3'), FLAG-RalB-SCLL was generated using (F-5'GCCGGAT

CCATGGATTACAAGGATGACGACGATAAGATCGCTGCCAACAAGAGTA
AGGGCCAG-3', R-5'GCCGGATCCTCATAGTAAGCAAGATCTTTCTTTAAAA
CT-3'). These PCR fragments were subcloned into pBABE molony-murine retroviral
plasmid by single enzyme digest with BamHI (New England Biolabs, Ipswich, MA)
using standard restriction enzyme conditions. Plasmid sequences were verified by
standard Sanger sequencing reaction.

In vitro transcription/translation/prenylation assay- Plasmid DNA was amplified in a
PCR reaction using AccuPrime Taq polymerase (Invitrogen, Carlsbad, CA) using 1µg
plasmid DNA by forward primer, T7-FLAG (RalA:

5'GCCGGATCCTAATACGACTCACTATAGGGTTCGACTACCTAGCAAATAAGCC
C—3', RalB: GCCGGATCCTAATACGACTCACTATAGGGGCTGCCAAC

AAGAGTAAGGGCCAG) and gene specific reverse primers, the same primers as
mentioned previously in the cloning protocol. Subsequent cDNA was isolated using
QIAquick PCR purification column (Qiagen, Valencia, CA) and 500ng was used for T7-
in vitro- transcription-translation (TnT coupled rabbit reticulocyte lysate system,
Promega, Madison, WI). Briefly, reaction components were assembled on ice according
to manufacturers protocol along with either 5 µCi [H³] farnesyl pyrophosphate, 5 µ Ci
[H³]Geranylgeranyl pyrophosphate or 10 µ Ci [S³⁵] methionine in the presence or
absence of GGTI-2418 or FTI-2148. The reaction was incubated at 30°C for 120 min and
the reaction was stopped by addition of an equal volume of 2x SDS-PAGE sample buffer.
The samples were then loaded onto 12 % acrylamide SDS-PAGE gel and separated at 50
V. Gels were then fixed in methanol/acetic acid (50% methanol, 10% glacial acetic acid,
40 % ddH₂O) for 30 min while gently shaking. Gels were then rinsed and incubated for

30 min with Amplify reagent (Amersham Biosciences, Piscataway, NJ) for 30 min while gently shaking. Gels were then transferred to Whatman paper at 80°C for 2 h on a BioRad Model 583 gel drier using a BioRad HydroTech Vacuum Pump (BioRad, Hercules, CA). Gels were visualized by autoradiography using Kodak BioMax FX film (Kodak, Rochester, NY) at -80°C.

Cells and Culture—Human tumor cell line MiaPaCa2 (pancreatic carcinoma), monkey embryonic kidney cell Cos7 and murine NIH-3T3 fibroblasts were purchased from ATCC (Manassas, VA) and grown in Dulbecco's modified minimal essential media (Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator at 5% CO₂.

Transfection procedure- Cells were grown to 50-70% confluence and transfected with Transit-LT1 (Mirus, Madison, WI) according to the manufacturer's instructions. Briefly, 3 µl of Transit LT1 reagent was suspended per 1 ml of OPTI-MEM media (Invitrogen, Carlsbad, CA) and allowed to equilibrate at 24-27°C for 15 min. Approximately 1 µg of plasmid per ml of media was suspended and allowed to complex with the liposomes for 15 min at 24-27°C. Cells were briefly washed with OPTI-MEM and 2 ml containing 2 µg media and 6 µl Transit LT1 was plated on top of the cells and incubated at 37°C for 6 h. Two ml of DMEM containing 10% FBS, without penicillin-streptomycin, was added and the cells were further incubated at 37°C overnight.

Immunofluorescence- Cos7 cells were seeded at a 50% cell density in 6-well plates containing sterilized glass coverslips and allowed to attach overnight. Cells were transfected overnight using TransitLT1 in Opti-MEM media following the manufacturer's instructions. Media was replaced with complete growth media containing either DMSO, 25 µM FTI-2153 or 25 µM GGTI-2417 and incubated at 37°C, 5% CO₂

for 48 h. Cells were then aspirated and washed twice with sterile PBS (pH 7.4) and fixed in 4% paraformaldehyde. Cells were then rinsed twice with sterile PBS (pH 7.4) and permeabilised, on ice, with 0.1% Triton X-100. Cells were then blocked for one hour with 1% BSA, rinsed twice and incubated overnight with 1:100 anti-FLAG M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO) or 1:100 anti-Hemagglutin (HA) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were rinsed three times in sterile PBS (pH 7.4) and then incubated for one hour with secondary antibody, FITC-conjugated rabbit anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were then washed twice in sterile PBS, mounted using Vectashield mounting reagent containing DAPI for nuclear visualization and analyzed using a Leica DMIL florescent microscope (Leitz, Wetzlar, Germany) at 525 nm (FITC) and 365/420 nm DAPI.

Luciferase assay – NIH-3T3 cells were plated (10^6 cells well) in 6-well plates and transfected using standard calcium phosphate-mediated transfection with 4 μ g of either pBABE, HA-H-Ras12V, FLAG-RalA72L-F, FLAG-RalA72L-GG, FLAG-RalA72L-S, FLAG-RalB72L-F, FLAG-RalB72L-GG, or FLAG-RalB72L-S; 1 μ g of NF-KB-pTAL firefly luciferase plasmid (BD Biosciences, Franklin Lakes, NJ); and 0.2 μ g Renilla luciferase plasmid for 5 h (both from BD Biosciences, Franklin Lakes, NJ). Cells were then incubated in full growth media overnight followed by serum starvation for 24 h in DMEM supplemented with 0.5% fetal calf serum. Cells were then lysed, and luciferase activity was determined using ProMega dual luciferase assay system according to the manufacturer's instruction.

Western blotting—Cells were treated with GGTI-2417 for 48 h, harvested, and lysed in HEPES lysis buffer as described previously (51). Proteins were then resolved by 12.5% SDS-PAGE and immunoblotted with antibodies against unprenylated Rap1A/Krev-1(121), p21WAF1 (C-190), Akt 1-2 (N19), and RhoB (119), RhoA (26C4), anti-Hemagglutinin (HA) monoclonal antibody and survivin (FL142) (all from Santa Cruz Biotechnology, Santa Cruz, CA), p27KIP1 (G173–524), (from Pharmingen, San Diego, CA), phospho-serine 473 Akt (Cell signaling, Danvers, MA) and β -actin (AC15) and anti-FLAG M2 monoclonal antibody (both from Sigma-Aldrich, St. Louis, MO). The ECL blotting system (NEN Life Science Products, Boston, MA) was used for detection of positive antibody reactions.

Trypan blue dye exclusion assay- Adherent cells were harvested using trypsinisation and pooled with suspension cells from media supernatant by pelleting at 300g for 5 min at 4°C. The cells were then aspirated and resuspended in an appropriate volume of media by pipetting gently up and down. Two 20 μ l aliquots were removed and combined with an equal volume of trypan blue and allowed to mix for 2 min. A 10 μ l volume was loaded onto a hemacytometer and cells were scored as live or dead based on trypan blue dye exclusion.

TUNEL analysis- Cells were seeded into 60-mm-diameter dishes and grown in DMEM supplemented with 5% FCS for 24 h and then treated with GGTI-2417 for 48 h, which we determined to be the optimal timepoint for induction of apoptosis. Apoptosis was determined by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) by using an *in situ* cell death detection kit (Roche, Indianapolis, IN). The cells were trypsinized, and cytospin preparations were obtained by centrifugation at 1,500xg

(using a Cytospin-3 centrifuge, Thermo-Shandon) Cells were fixed with freshly prepared paraformaldehyde (4% in phosphate-buffered saline [PBS], pH 7.4). Slides were rinsed with PBS, incubated in permeabilization solution, and cross-reacted with TUNEL reaction mixture for 60 min at 37°C in a humidified chamber. The slides were rinsed, mounted and analyzed under a light microscope.

Creation of retrovirus- Retrovirus was created by transient transfection of HEK-293T human embryonic kidney cells with pVPACK-Ampho, pVPACK-gag-pol and pBABE retroviral plasmids according to manufacturers protocol (Invitrogen Carlsbad, CA). Briefly 293T cells were seeded at 2.5×10^6 cells in a 60 mm² dish. 3 µg of each plasmid was combined and brought to a volume of 225 µl in Rnase/Dnase free water following which 25 µl 2.5 M CaCl₂ was added dropwise while gently vortexing. This solution was then added to 250 µl 2x HEPES-buffered saline dropwise and incubated at room temperature for 5 min. The total volume of 500 µl was then added to the 293T cells and incubated for 8 h at 37°C. The medium was then removed and cells were incubated for 48 h. The supernatant was then passed through a 0.45 µm nylon low protein-binding filter (Fisherbrand, Houston, TX) to remove cells and cell debris. Retroviral titers were determined by serial dilution and infection of NIH-3T3 cells followed by selection and colony formation in puromycin.

Cellular fractionation – Membrane and cytosolic fractions were isolated using the MEM-PER membrane extraction kit (Promega, Madison, WI). Briefly, 1×10^6 Cos7 cells were transfected with the appropriate plasmid and collected seventy-two hours later. Cells were lysed and membrane and cytosolic fractions were isolated according to manufacturer's protocol. Membrane and cytosolic fractions were diluted five fold and

diluted further in 2x Laemmli sample buffer (Biorad, Carlsbad, CA). Proteins were separated by SDS-PAGE and visualized by Western blotting.

Creation of stable cell lines- MiaPaCa2 cells were seeded into 6-well plates at a 40% confluency and incubated with 8 µg/ml polybrene (Millipore/Specialty Media, Billerica, MA) and 5×10^5 viral particles for 8 h. Medium was changed to complete growth media and cells were incubated at 37°C, 5% CO₂ for 24 h. Media was then replaced with complete growth media containing 4 µg/ml puromycin and incubated until colonies formed. All colonies were pooled and taken as a single polyclonal population and cultured in complete media containing 2 µg /ml puromycin.

MTT metabolism assay- Cells were seeded in a 96-well plate at a density of 1,500 cells per well and allowed to attach overnight. Cells were then incubated for 96 h with varying concentrations of GGTI-2417 or appropriate DMSO control. Media was aspirated after 96 h, the optimal time for study of proliferation using this assay, and replaced with complete medium containing 1 mg/ml MTT and incubated for 3 h at 37°C in 5% CO₂ humidified incubator. Medium was then aspirated and DMSO was added. Cells were incubated 5 min at room temperature while shaking following which absorbance was determined at 495 nm.

Soft agar clonogenicity assay- For soft agar growth assays, the cell lines were seeded at a cell density of 1,500/well in triplicate in 12-well culture dishes in 0.3% agar over a 0.6% bottom agar layer. Various concentrations of GGTI-2417 or vehicle (DMSO) were included in the 0.3% agar layer of cells. Cultures were fed and treated with drug or vehicle once weekly until colonies grew to a suitable size for observation (colony growth rates were 10-14 days for RalA stable MiaPaCa2 cells and 3 weeks for RalB stable

MiaPaCa2 cells). Colonies were photographed after overnight incubation with 1 mg/ml MTT in the respective cell growth media. The growth of colonies in the presence of inhibitor was compared with the control colonies treated with vehicle.

Statistical analysis- Statistical analysis was performed using standard student's T-Test via either Microsoft Excel (Microsoft, Redmond, WA) or GraphPad Software (San Diego, CA) statistical analysis tools.

Results

Generation of CAAX box mutants that are farnesylated or unprenylated versions of both RalA and RalB

It has been demonstrated previously that RalA is geranylgeranylated and that both RalA and RalB require the prenyl-accepting cysteine for proper localization (18, 24). However, direct evidence that RalA and RalB are exclusively geranylgeranylated, and thus validated targets of GGTI's, is lacking. Furthermore, whether the nature of the prenyl group influences the subcellular location and function of Ral proteins is not known. To this end, we used site-directed mutagenesis to generate two CAAX box mutant forms of wild type or GTPase-deficient /constitutively activated (Q72L) RalA and RalB proteins. The first missense mutation replaced the carboxyl-terminal leucine with a serine residue to switch the prenyltransferase specificity from GGase I (RalA-CCIL and RalB-CCLL) to a site preferred by the related enzyme FTase (RalA-CCIS and RalB-CCLS). The second mutation replaced the prenylated cysteine with a serine residue to prevent prenylation (RalA SCIL and RalB- SCLL). Both GTP-locked (RalA72L, RalB72L) and wild type RalA and RalB were used to generate the CAAX box mutants. Plasmid constructs containing these Ral cDNA sequences were then used in *in vitro* transcription-translation-prenylation assays in rabbit reticulocyte lysates with radiolabeled [³⁵S]-labeled-methionine, [³H]-labeled farnesyl pyrophosphate (FPP) and [³H]-labeled geranylgeranyl pyrophosphate (GGPP) to determine the relative strength of translation, farnesylation and geranylgeranylation, respectively. Figure 5A (see following pages) shows that RalA-CCIL and RalB-CCLL were geranylgeranylated while replacement of the carboxyl-terminal amino acid with a serine resulted in incorporation

of a farnesyl instead of a geranylgeranyl moiety. Replacement of the prenylation site cysteine with a serine residue (CCIL and CCLL to SCIL and SCLL) rendered both RalA and RalB unprenylated (Figure 5A, following pages).

Geranylgeranylated and farnesylated RalA and RalB are not alternatively prenylated in the presence of GGTIs and FTIs, respectively.

It has been well documented that certain Ras family members are capable of being alternatively prenylated when FTase is inhibited (40, 51, 54). We reasoned that it is possible that RalA and/or RalB (geranylgeranylated or farnesylated forms) could be alternatively prenylated when GGTase I or FTase is inhibited. In order to determine whether the wild type RalA-CCIL and RalB-CCLL were indeed targets only of GGTase I and could not be prenylated by FTase upon GGTase I inhibition, we added GGTI-2418, a potent and selective competitive inhibitor of GGTase I (33), to the reticulocyte lysate transcription-translation-prenylation mixture (Figure 5B, following pages). Wild type RalA-CCIL and RalB-CCLL were geranylgeranylated and were not alternatively farnesylated when GGTase I was inhibited by GGTI-2418, and their geranylgeranylation was little affected by inhibition of FTase by our previously characterized FTase-specific inhibitor, FTI-2148 (48). Furthermore, RalA-CCIS and RalB-CCLS remained farnesylated when GGTase I was inhibited and did not become geranylgeranylated when FTase I was inhibited (Figure 5B, following page). Minor variations in the apparent incorporation efficiency of the radiolabeled prenyl groups in the absence or presence of inhibitors, was seen regularly but without consistency. These results suggest that RalA-CCIL and RalB-CCLL are targets of GGTIs but not FTIs, and conversely, that the RalA-CCIS and RalB-CCLS CAAX box mutants are targets of FTIs, but not GGTIs. Furthermore, neither the geranylgeranylated or farnesylated Ral proteins are alternatively prenylated under the pressure of GGTI or FTI treatment, respectively. Therefore, for the rest of the manuscript we will refer to RalA-CCIL and RalB-CCLL as RalA-GG and

RalB-GG, respectively. Similarly, we will refer to RalA-CCIS and RalB-CCLS as RalA-F and RalB-F, respectively.

Figure 5. RalA-CCIL and RalB-CCLL are geranylgeranylated whereas the mutants RalA-CCIS and RalB-CCLS are farnesylated

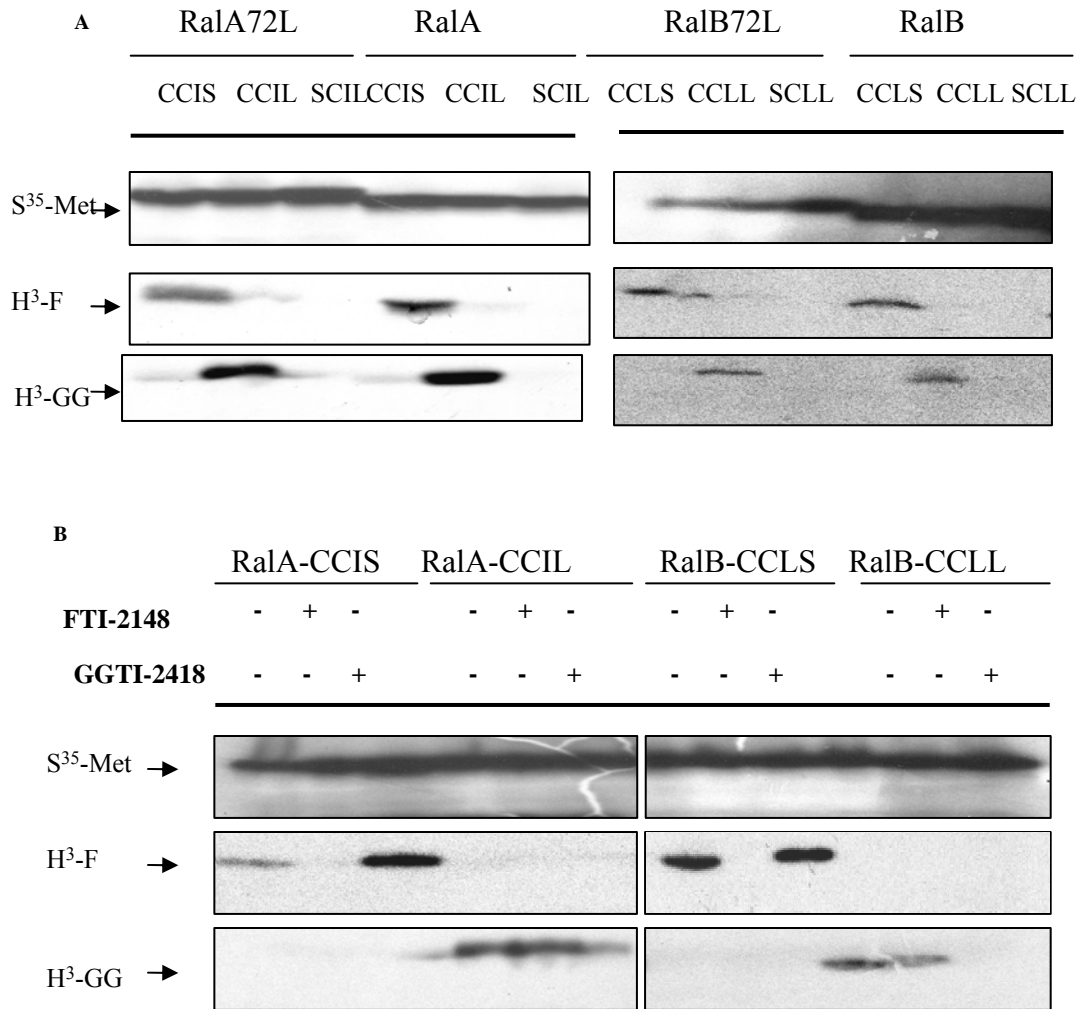


Figure 5. RalA-CCIL and RalB-CCLL are geranylgeranylated whereas the mutants RalA-CCIS and RalB-CCLS are farnesylated. A) Ral DNAs were used in transcription-translation-prenylation assays using either radiolabeled [³⁵S]-methionine, [³H]-FPP or [³H]-GGPP then run on SDS-PAGE and visualized by autoradiography as described in Materials and Methods. Results are representative of two independent experiments. B) RalA-CCIS, RalA-CCIL, RalB-CCLS or RalB-CCLL DNAs were transcribed, translated and prenylated using either [³⁵S]-Methionine, [³H]-FPP or [³H]-GGPP in the presence of either vehicle (DMSO), 250 nM FTI-2148 or 250 nM GGTI-2418 then run on SDS-PAGE and visualized by autoradiography. Results are representative of two independent experiments.

Prenylation is required for proper subcellular localization of RalA and RalB and both RalA and RalB are mislocalized in response to prenyltransferase inhibition.

The results of Figure 1 demonstrated, in a reconstituted cell reticulocyte system, that the wild type RalA-GG, RalB-GG, and CAAX box mutant RalA-F and RalB-F variants are not alternatively prenylated *in vitro*. We next determined whether prenylation is required for proper localization in intact cells and whether GGTI-2417 treatment is sufficient to disrupt localization of wild type RalA-GG and RalB-GG. We also wanted to determine if the RalA-F and RalB-F mutants display a localization similar to RalA-GG and RalB-GG. To this end, we ectopically expressed FLAG epitope-tagged RalA-GG, RalA-F, RalA-S, RalB-GG, RalB-F or RalB-S in Cos-7 cells followed by treatment with the indicated prenyltransferase inhibitors. We found that both the geranylgeranylated and farnesylated forms of RalA and RalB demonstrated a similar localization to the plasma membrane (Figure 6, following pages). This was confirmed by membrane/cytosol cellular fractionations where RalA-GG and RalB-GG as well as RalA-F and RalB-F localized to the membrane fractions (Figure 7A, following pages). The fact that RalA and RalB localize similarly is interesting but different than results by Feig et al (45). The reason for this difference at present is not known. The unprenylated versions of RalA (RalA-S) and RalB (RalB-S) were diffused in the cytoplasmic and the perinuclear regions indicating that prenylation is required for proper plasma membrane localization of RalA and RalB (Figure 6, following pages). Furthermore, in response to GGTase I inhibition RalA-GG and RalB-GG were unable to localize to the plasma membrane while no change in membrane localization was observed when FTase activity was inhibited. As expected, H-Ras was predominantly localized to the plasma membrane,

and this was only affected by FTI but not GGTI treatment. Similarly, in response to FTase inhibition, RalA-F and RalB-F were unable to localize to the plasma membrane while no change in membrane association was observed when treated with GGTase I inhibition (Figure 6, following pages). These data demonstrate that RalA-GG and RalB-GG are targets of GGTase I inhibitors and that the farnesylated Ral variants will be useful reagents to determine if inhibition of Ral GTPases contribute to the biological effects of GGTI-2417 treatment.

Figure 6. Geranylgeranylated (GG) and farnesylated (F) RalA and RalB localize similarly and require prenylation for correct localization

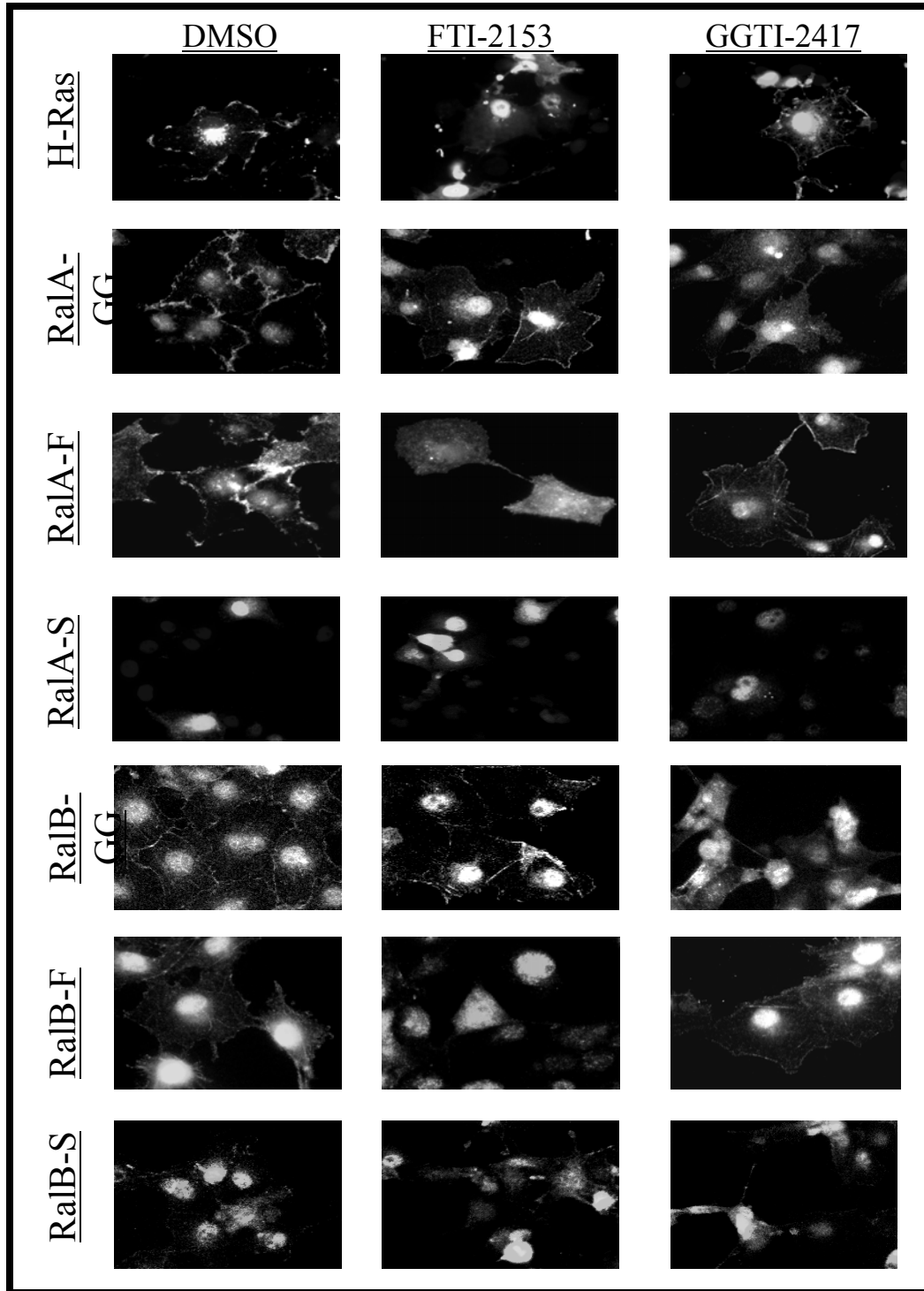


Figure 6. Geranylgeranylated (GG) and farnesylated (F) RalA and RalB localize similarly and require prenylation for correct localization. Cos7 cells grown on coverslips were transiently-transfected with plasmids expressing the indicated proteins and treated with the indicated inhibitors (25 μ M), and were then analyzed for FLAG-Ral or HA-H-Ras distribution using immunofluorescence as described under Materials and Methods. Nuclei were visualized using DAPI stain. Results are representative of three independent experiments.

Farnesylated and geranylgeranylated RalA and RalB are equivalent in ability to activate NF- κ B responsive promoter elements

We next wanted to verify that Ral function was retained when modified by farnesylation. It has been reported previously that the constitutively activated RalB72L mutant protein is capable of activating NF- κ B-dependent transcription (17). We therefore determined if farnesylation and geranylgeranylation of Ral are equivalent in their ability to mediate Ral activation of an NF- κ B -dependent promoter. In order to assess the contribution of the prenyl moiety to NF- κ B-driven transcription we transiently transfected NIH-3T3 cells with either the pBabe-puro empty vector, or encoding constitutively activated H-Ras (H-Ras12V), RalB72L-GG, RalB72L-F, RalB72L-S, RalA72L-GG, RalA72L-F or RalA72L-S. As expected, H-Ras12V stimulated an approximately four-fold increase in NF- κ B luciferase activity (Figure 7, following page). As previously reported, RalB72L-GG activated NF- κ B-dependent promoter activity. Importantly, RalB72L-F activated NF- κ B luciferase activity to a similar extent as the geranylgeranylated version (approximately two-fold). Similar to the results with RalB, ectopic expression of RalA72L-GG and RalA72L-F both stimulated four-fold activation of NF- κ B. Importantly, neither nonprenylated RalB-S nor RalA-S stimulated activation of NF- κ B (Figure 7, following page). These data demonstrate that prenylation is required for RalA and RalB activation of NF- κ B and that farnesyl and geranylgeranyl moieties are equivalent in supporting Ral activation of NF- κ B.

Figure 7. Farnesylated and geranylgeranylated RalA and RalB are equivalent in mediating activation of NF- κ B promoter activity.

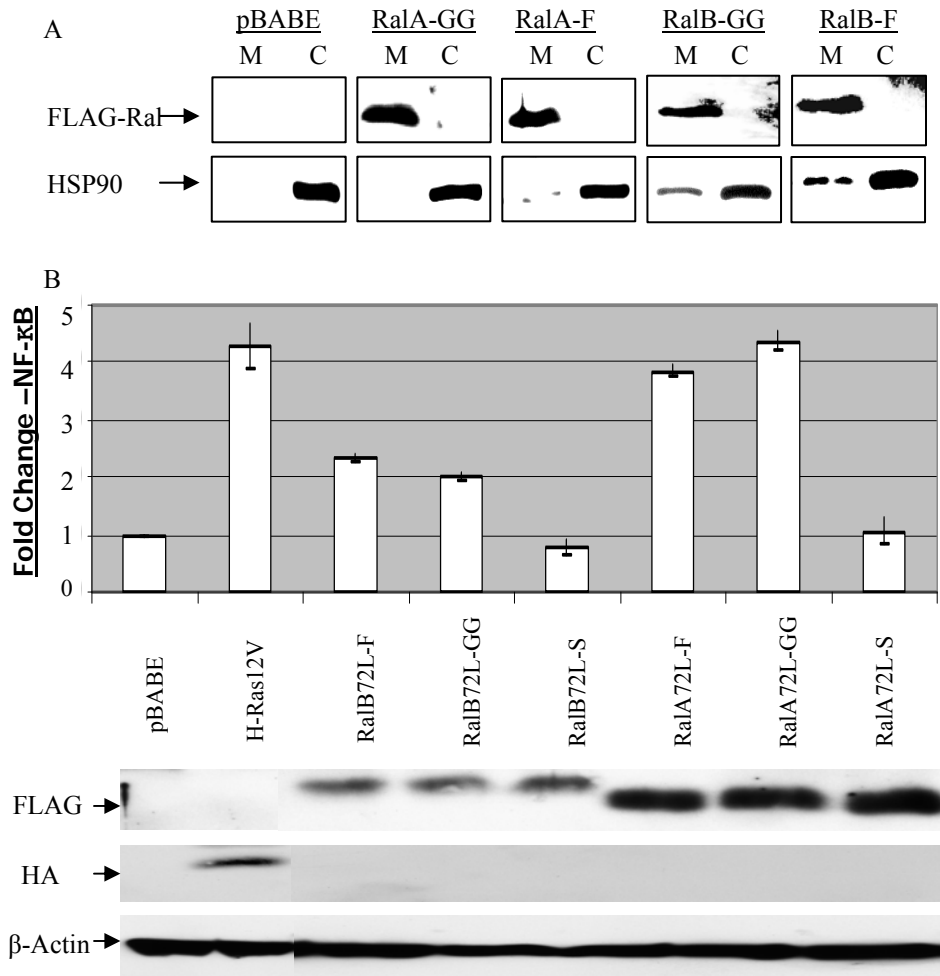


Figure 7. Farnesylated and geranylgeranylated RalA and RalB are equivalent in mediating activation of NF- κ B promoter activity. A) Cos7 cells were transiently transfected with the indicated plasmids and membrane (M) and cytosolic (C) fractions were isolated and probed by Western blotting; HSP90 was used as a cytosolic fraction marker. Results are representative of 2 independent experiments. B) NIH-3T3 cells were serum-starved and transiently co-transfected with plasmid expressing the indicated proteins and a NF- κ B reporter plasmid. NF- κ B promoter activity was detected following transfection as described under Materials and Methods. Expression was analyzed by western blot analysis. Results are representative of three independent experiments.

Ectopic expression of RalB-F renders Cos7 cells resistant to inhibition of proliferation, induction of cell death and apoptosis by GGTI-2417

Since the farnesylated and geranylgeranylated forms of Ral proteins displayed similar subcellular localization and signaling properties, we reasoned that, if Ral GTPases are important functional targets of GGTI, ectopic expression of farnesylated, but not geranylgeranylated, RalA and/or RalB would rescue cells from the effects of GGTI-2417 inhibition. Figure 8A (see following pages) shows that ectopic expression of farnesylated RalB, but not RalA, rendered cells less sensitive to the induction of cell death by GGTI-2417. There were no statistically significant differences between all transfected groups (pBabe empty vector control, RalA-F, RalA-GG, RalB-GG) in cell death except in the RalB-F expressing Cos7 cells ($P < 0.05$ at 50, 100 and 150 μM). For example, GGTI-2417 induced 39% cell death at 50 μM in RalB-GG transfected Cos7 cells but when RalB-F was expressed GGTI-2417 induced only 7% cell death (Figure 8A, following pages). Similarly, RalB-F expressing Cos7 cells were 4.51-fold more resistant to inhibition of proliferation by GGTI-2417 than RalB-GG expressing cells (IC_{50} of 131 μM and 29 μM , respectively) (Figure 8B, following pages).

We next determined whether RalA-F and/or RalB-F could rescue cells from GGTI-2417 treatment-induced programmed cell death (apoptosis) by TUNEL assay. Figure 8D (see following pages) shows that only ectopic expression of farnesylated RalB, but not farnesylated RalA, demonstrated a significantly protective effect from GGTI-2417-induced apoptosis. GGTI-2417 increased the fraction of apoptotic cells from about 18 to 32% in RalB-GG but only from 17 to 20% in RalB-F (Figure 8D, following pages). In contrast, GGTI-2417 potently induced apoptosis in Cos7 cells expressing either RalA-

GG or RalA-F, with GGTI-2417 treatment increasing the fraction of apoptotic cells from approximately 15% to 30% in both cell populations (Figure 8D, following pages). These results suggest that inhibition of RalB and not RalA geranylgeranylation is an important target for GGTI-mediated apoptosis. All cell lines displayed similar accumulation of non-geranylgeranylated Rap1A following GGTI-2417 treatment (Figure 8C, following pages). No changes were observed in gel mobility of the human homologue of DNAJ-2 (HDJ2), an exclusively farnesylated protein, indicating that FTase activity was not blocked by this treatment (Figure 8C, following pages).

Figure 8. Ectopic expression of farnesylated RalB, but not RalA, renders cells less sensitive to GGTI-2417 inhibition of survival and proliferation and induction of apoptosis in Cos7 cells

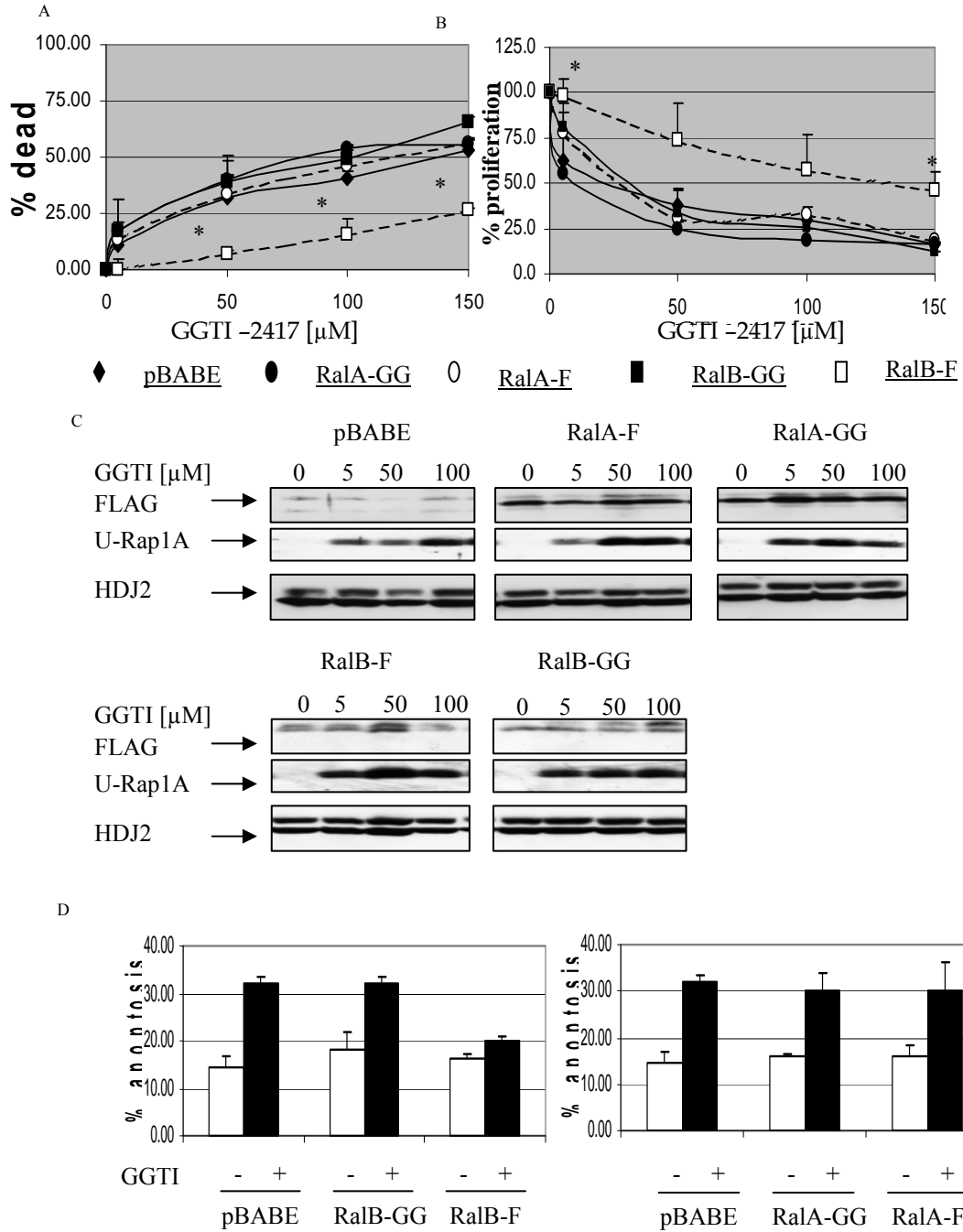


Figure 8. Ectopic expression of farnesylated RalB, but not RalA, renders cells less sensitive to GGTI-2417 inhibition of survival and proliferation and induction of apoptosis in Cos7 cells. A-B) Inhibition of RalB prenylation is required for GGTI induction of cell death and inhibition of proliferation. Cos7 cells were transfected with the indicated plasmids then treated with GGTI-2417 or vehicle (DMSO) control. Cell viability was determined by the trypan blue dye exclusion assay. Data shown are the average of three independent experiments. C) Cell lysates from A-B were analyzed for expression by western blot analysis as described in Materials and Methods. D) Inhibition of RalB prenylation is required for GGTI induction of apoptosis. Cos7 cells were transiently-transfected with plasmids expressing the indicated proteins and then treated for 72 h with 50 μ M GGTI-2417 or DMSO control, collected and analyzed for apoptosis via TUNEL assay as described in Materials and Methods. Data shown are the average of three independent experiments. (* $p < 0.05$)

MiaPaCa2 human pancreatic cancer cells stably expressing farnesylated RalB are resistant to the anti-proliferative and pro-apoptotic effects of GGTI-2417 compared to geranylgeranylated RalB-expressing cells

Since Cos7 are immortalized non-transformed cells of non-human primate origin we endeavored to create a more relevant cell system to characterize the growth inhibitory and pro-apoptotic effects of GGTI-2417. We first determined by western blotting that both RalA and RalB are efficiently targeted by GGTI-2417 in MiaPaCa2 cells. Figure 9A (see following pages) shows that GGTI-2417 concentrations as low as 1 μ M inhibited RalA and RalB geranylgeranylation. We next established populations of MiaPaCa2 cells stably expressing RalA and RalB prenyl isoforms through retroviral transduction and selection (Figure 9B, following pages). We then used these cells to examine the effects of ectopic WT or farnesylated RalA and RalB expression on GGTI-2417-mediated inhibition of proliferation of MiaPaCa2 cells by both MTT metabolism viability (Figure 9C) and trypan blue dye exclusion assays (Figure 9D, following pages). Similar to our observations in Cos7 cells, MiaPaCa2 cells stably expressing farnesylated as opposed to geranylgeranylated RalB were less sensitive to the anchorage-dependent anti-proliferative effects of GGTI-2417. Specifically, using the MTT assay RalB-GG expressing cells were inhibited by 50% at 1.6 \pm 0.3 μ M GGTI-2417 whereas RalB-F expressing cells (IC₅₀ of 8.3 \pm 1.4 μ M, p value = 0.0015) were more than 5-fold resistant. There were no statistically significant differences between RalB-GG and empty vector cells. Furthermore, no statistically significant differences in proliferation inhibition sensitivity were observed for empty vector, RalA-F or RalA-GG expressing cells (Figure 9C, following pages). Similar results were obtained using total cell counting by trypan blue

dye exclusion assay demonstrating no difference in sensitivity between RalA-F and RalA-GG expressing cells and a greater than 6-fold statistically significant difference ($p < 0.0001$) in IC50 values between RalB-GG and RalB-F expressing cells (IC50 values of $4.8 \pm 1.6 \mu\text{M}$ and greater than $30 \mu\text{M}$, respectively) (Figure 9D, following pages). There were no statistically significant differences between empty vector ($5.6 \pm 1.7 \mu\text{M}$) and RalB-GG ($4.8 \pm 1.6 \mu\text{M}$) cells. These data both confirmed and extended our observations in Cos7 cells and further indicated that inhibition of RalB, not RalA, prenylation is an important target for GGTI inhibition of anchorage-dependent cell proliferation.

To further examine the role of Ral proteins in GGTI-2417 induction of apoptosis, we determined if ectopic expression of RalA-GG, RalA-F, RalB-GG or RalB-F altered MiaPaca2 sensitivity. Specifically, we observed that GGTI-2417 treatment induced a greater than three-fold increase in apoptosis in all cell lines except those expressing RalB-F (Figure 9F, following pages). Of primary importance, GGTI-2417 induced a 3.6-fold induction of apoptosis (1.6 to 6.07%) in RalB-GG expressing cells as compared to no statistically significant induction of cell death in RalB-F expressing cells (1.42 to 1.56%). This indicates that, consistent with the Cos7 results, RalB and not RalA is a critical target for the apoptotic cell death caused by GGTI-2417 treatment of MiaPaCa2 cells.

Figure 9. Stable expression of farnesylated RalB, but not RalA, promotes resistance to the anti-proliferative and pro-apoptotic effects of GGTI-2417 in MiaPaCa2 cells.

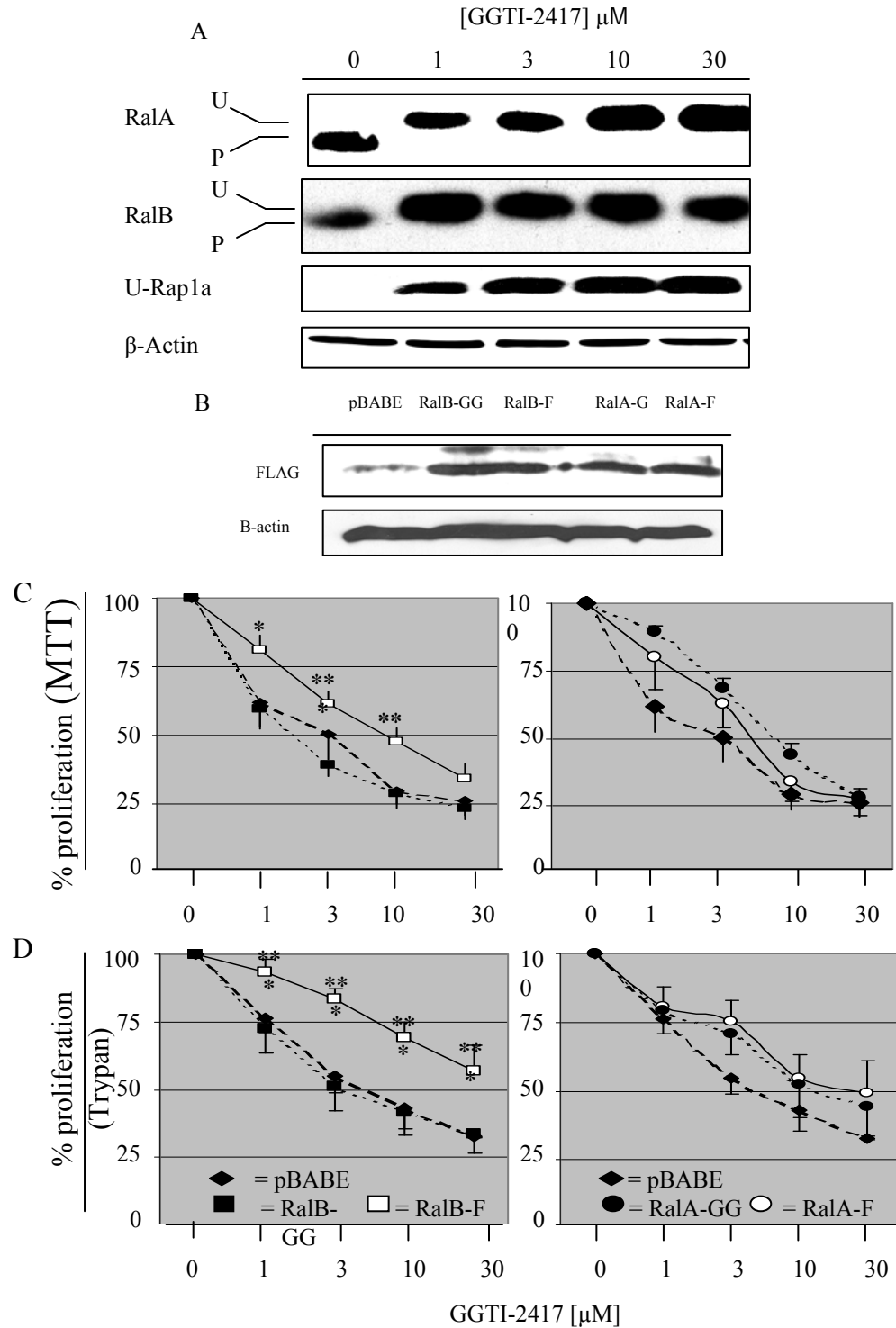


Figure 9. Stable expression of farnesylated RalB, but not RalA, promotes resistance to the anti-proliferative and pro-apoptotic effects of GGTI-2417 in MiaPaCa2 cells.

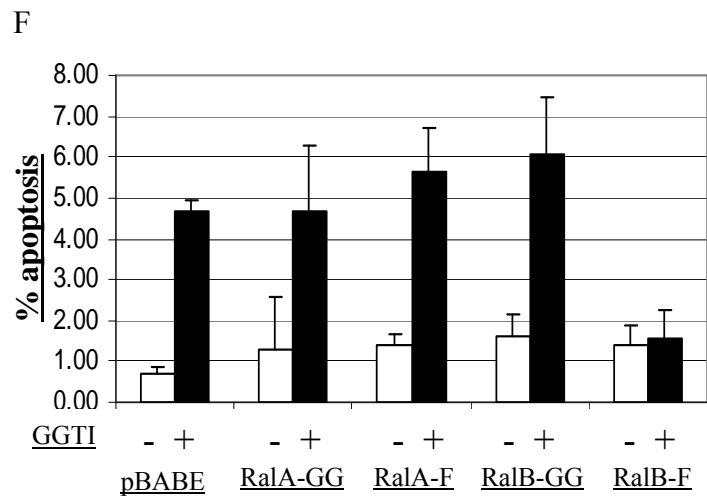
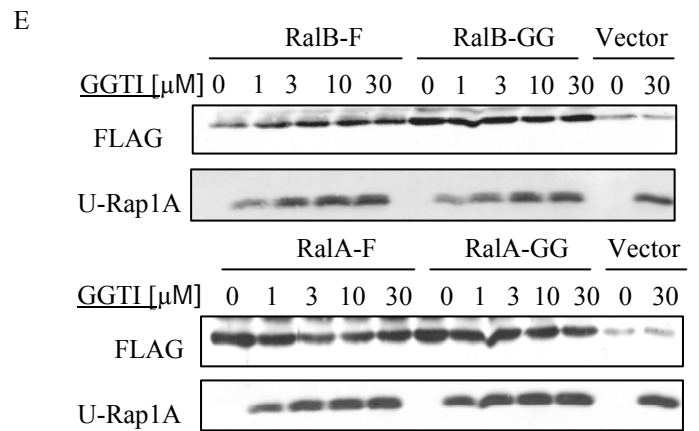


Figure 9. Stable expression of farnesylated RalB, but not RalA, promotes resistance to the anti-proliferative and pro-apoptotic effects of GGTI-2417 in MiaPaCa2 cells. A) Dose response to determine the efficacy of GGTI-2417 in inhibiting RalA and RalB prenylation in MiaPaCa2 cells. MiaPaCa2 cells were treated with various concentrations of GGTI-2417 for 48h and processed for Western blotting as described under Materials and Methods. B) Stable MiaPaCa2 cell lines were created using retroviral infection of puromycin resistance marker along with the indicated transgenes as described under Materials and Methods. Cells were lysed and expression was assessed by western blot analysis. C) Stably expressing MiaPaCa2 cells were treated with the indicated concentrations of GGTI-2417 for 96 h. Proliferation was measured by the MTT viability assay as described in Materials and Methods. Data shown are the average of at least three independent experiments. D) MiaPaCa2 cells stably expressing the indicated Ral proteins were treated with the indicated concentrations of GGTI-2417 for 72 h. Proliferation was assessed by trypan blue dye exclusion assay as described in Materials and Methods. Data shown are the average of at least three independent experiments. E) MiaPaCa2 cells stably expressing the indicated Ral proteins were treated with GGTI-2417 (30 μ M), lysed and expression was assessed by western blot analysis. Data shown are representative of three independent experiments. F) MiaPaCa2 cells stably expressing the indicated Ral proteins were treated with GGTI-2417 for 48 h and apoptosis was assessed by TUNEL assay as described in Materials and Methods. Data shown are the average of three independent experiments. (*, p<0.05; **, p<0.01, ***,p<0.001)

Stable expression of farnesylated RalA, but not RalB, induces partial resistance to inhibition of anchorage-independent growth by GGTI-2417 in MiaPaCa2 cells

We next determined whether GGTI-2417 inhibition of growth in soft agar might be due, in part, to inhibition of RalA and/or RalB function. To this end, we used a soft agar clonogenicity assay to measure the effects of GGTI-2417 on MiaPaCa2 cells stably expressing geranylgeranylated or farnesylated RalA or RalB. We found that MiaPaCa2 cells stably expressing either RalB-GG or RalB-F did not differ in sensitivity to GGTI-2417 treatment. However, MiaPaCa2 cells stably expressing RalA-F were less sensitive to inhibition of soft agar growth by GGTI-2417 than those cells stably expressing ectopic RalA-GG (Figure 10, following page). For example, at 30 μ M GGTI-2417, RalA-GG and RalA-F inhibited colony formation by 41.3 ± 5.9 and 17.8 ± 8.0 , respectively ($p < 0.001$). Taken as a whole these data implicate RalA, but not RalB, inhibition of prenylation as a potential mechanism for GGTI reversion of the transformed growth phenotype of MiaPaCa2 cells as measured by anchorage-independent soft agar growth.

Figure 10. Stable expression of farnesylated RalA, but not RalB, induces resistance to inhibition of anchorage independent growth by GGTI-2417 in MiaPaCa2 cells.

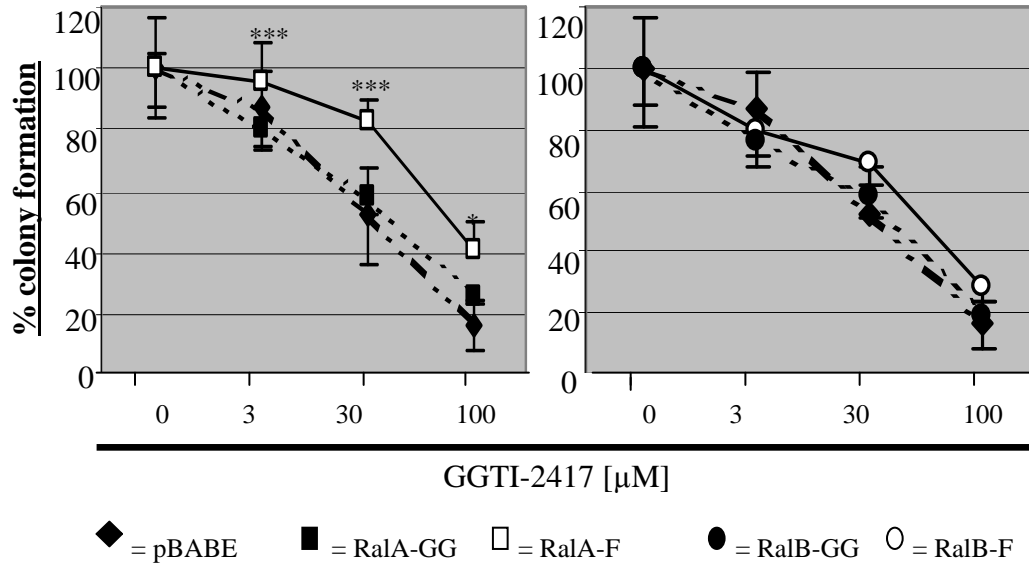


Figure 10. Stable expression of farnesylated RalA, but not RalB, induces resistance to inhibition of anchorage independent growth by GGTI-2417 in MiaPaCa2 cells. MiaPaCa2 cells stably-expressing the indicated Ral proteins were seeded into 12-well plates in 0.3% soft agar and treated with the indicated concentrations of GGTI-2417 for 10 days as described in Material and Methods. Data shown are the average of two independent experiments repeated in triplicate. (*, $p < 0.05$; ***, $p < 0.001$)

Stable expression of farnesylated RalB but not RalA in MiaPaCa2 cells inhibits the ability of GGTI-2417 to increase p27^{Kip1} and decrease survivin protein levels.

Recent data from our lab showed that GGTIs treatment affects several signal transduction pathways critical to cell cycle division and tumor cell survival (30, 35, 49-52)¹. In order to examine the contribution of inhibition of the geranylgeranylation of RalA and/or RalB to the effects of GGTI-2417 on signaling pathways, we treated MiaPaCa2 cells stably expressing RalA-GG, RalA-F, RalB-GG and RalB-F with GGTI-2417. In control, empty vector-transfected MiaPaCa2 cells, GGTI-2417 treatment resulted in inhibition of RalA and Rap1 geranylgeranylation, increased the protein levels of p27^{Kip1}, RhoA and RhoB and decreased the levels of two anti-apoptotic proteins, activated phosphorylated Akt (P-Akt) and survivin (Figure 11, following page). Expression of RalA-F, RalA-GG, or RalB-GG did not affect the ability of GGTI-2417 to induce its effects on these signaling molecules. However, expression of RalB-F inhibited the ability of GGTI-2417 to increase p27^{Kip1} and to decrease survivin protein levels. For example, while p27^{Kip1} protein levels were increased in cells expressing RalB-GG (3.12 ± 0.88 fold) cells expressing RalB-F showed a 50% (1.63 ± 0.64 fold) attenuated increase of p27^{Kip1} protein levels (p=0.0073). Furthermore, GGTI-2417 treatment reduced survivin levels by 46 ± 11% in empty vector and RalB-GG expressing cells. However, MiaPaCa2 cells expressing RalB-F were less sensitive to depletion of survivin levels, with only a 12 ± 3% reduction of survivin levels following GGTI-2417 treatment (p=0.0006). By contrast expression of either RalA-F or RalA-GG had no effect on GGTI

¹ and Kazi A, A Carie, MA Blaskovich, C Bucher, V Thai, S Moulder, H Peng, D Carrico, E Pusateri, AD Hamilton and SM Sebt, Inhibition of Protein Geranylgeranylation Requires p27^{Kip1} to Induce Human Breast Cancer Cell Death: Implications for breast cancer therapy, submitted for publication.

reduction of survivin or induction of p27Kip1. To determine if knockdown of RalA or RalB affects the levels of survivin or p27Kip1, we used siRNA to specifically inhibit RalA and RalB expression in parental MiaPaCa2 cells. We found that knockdown of RalB, but not RalA, inhibited the survivin protein levels by 70.2%, whereas neither RalA nor RalB knockdown affected the levels of p27Kip1 (data not shown).

Figure 11. RalB-F, not RalA-F, inhibit the ability of GGTI-2417 to increase p27Kip1 and decrease survivin protein levels.

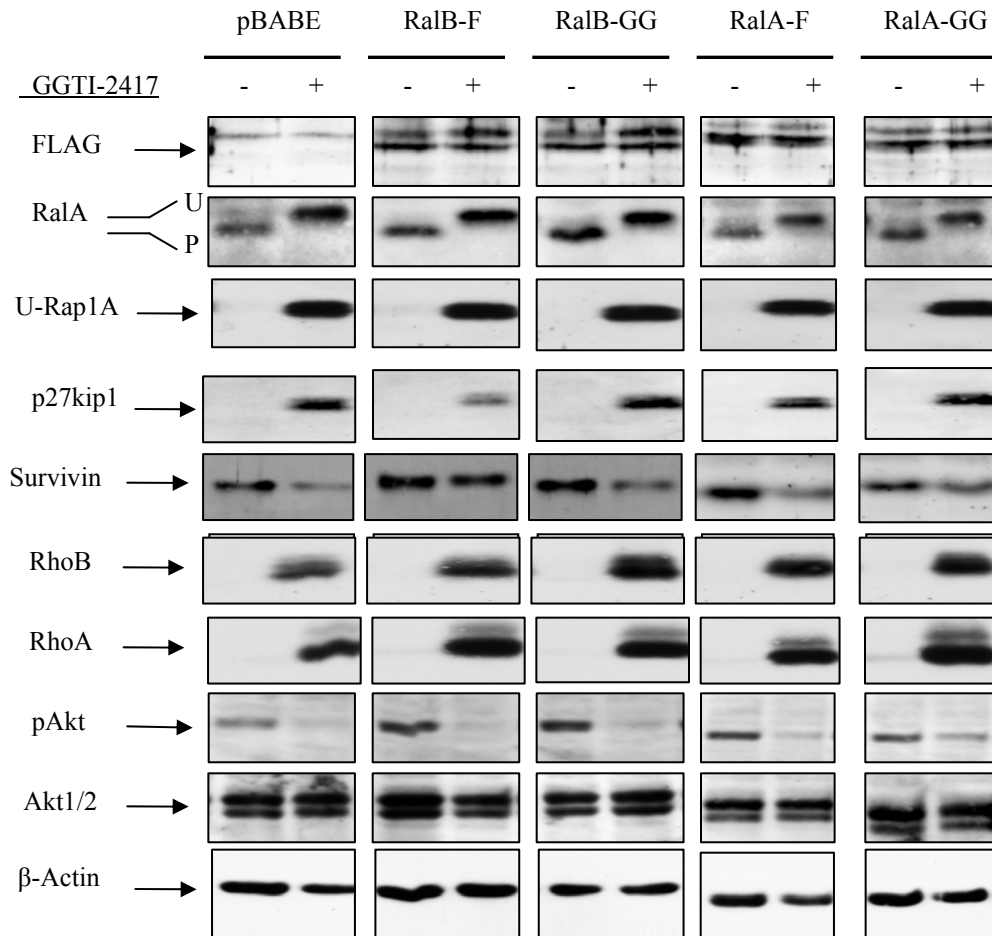


Figure 11. RalB-F, not RalA-F, inhibit the ability of GGTI-2417 to increase p27Kip1 and decrease survivin protein levels. Stably expressing MiaPaCa2 cells were seeded into 6-well plates and treated with GGTI-2417 (30 μ M) for 48 h. Cells were lysed and expression was assessed by western blot analysis. Data shown are representative of three independent experiments.

Discussion

Inhibition of protein geranylgeranylation strongly attenuates multiple tumorigenic pathways such as anchorage-dependent and –independent tumor growth and protection from apoptosis (30, 35, 49-52). However, many proteins are substrates for GGTase I-mediated protein geranylgeranylation, and it is not known which of these are critical targets for the anti-neoplastic effects of GGTIs (56). Since recent studies demonstrated the important roles of the RalA and RalB small GTPases in human oncogenesis (7, 8, 15, 16, 28, 36, 38, 45), we evaluated the possibility that these GGTase I substrates are important targets for GGTIs. In this study we have demonstrated that the GTPases RalA and RalB are geranylgeranylated by GGTase I, require prenylation for proper localization and are downstream targets of pharmacological inhibitors of GGTase I. We designed farnesylated, GGTI-insensitive, variants of Ral GTPases and found that farnesylated RalB, but not RalA, confers resistance to the pro-apoptotic and anti-anchorage-dependent growth effects of GGTI-2417 on Cos7 and MiaPaCa2 pancreatic carcinoma cells. Conversely, farnesylated RalA, but not RalB expression renders MiaPaCa2 cells less sensitive to inhibition of anchorage-independent growth. Finally, we determined that farnesylated RalB, but not RalA inhibited the ability of GGTI-2417 to suppress survivin and induce p27Kip1. We conclude that RalA and RalB are important, functionally distinct targets for GGTI-mediated anti-neoplastic effects. Additionally, our studies extend recent observations showing that the highly related RalA and RalB proteins serve distinct functions in oncogenesis.

The determination of the GGTase I substrates that are critical for the anti-tumor activity of GGTIs is complicated by the fact that over 60 CAAX-terminating proteins

may be substrates for this prenyltransferase (56). We previously established and validated a substitutive chemical biology approach to define the contribution of inhibition of specific GGTase I substrates to GGTI inhibition of cell survival, proliferation, transformation and cell signaling (22). In this study, we applied this approach and we generated farnesylated mutants of both RalA and RalB and showed that they localized similarly to authentic geranylgeranylated wild-type versions and similarly activated NF- κ B and were resistant to inhibition by GGTI treatment. Thus, this substitutive chemical biology approach is a valid means of uncoupling RalA and RalB from GGTase I dependency while preserving their wild type subcellular localization and signaling activity.

Our results demonstrate a partial requirement for inhibition of RalA prenylation in the inhibition of anchorage-independent growth by GGTI-2417. Since expression of farnesylated RalB did not attenuate the ability of GGTI-2417 to inhibit anchorage-independent growth we reason that the ability of RalA to regulate clonogenicity is a divergent function from that of the 85% identical protein RalB. The ability of farnesylated RalA but not RalB to regulate anchorage-independent growth is similar to results reported by Chien et al (15) where depletion of RalA but not RalB by siRNA inhibited anchorage independent growth in HeLa and SW480 cancer cell lines. However, other processes commonly associated with anchorage-independent proliferation such as cell migration and chemotaxis bear a requirement for RalB as opposed to RalA; such results were first reported by Oxford et al (32) where depletion of RalB but not RalA by siRNA inhibited cell migration in a panel of renal carcinoma cell lines. In particular, we

found that RNAi-mediated suppression of RalA, but not RalB greatly impaired the soft agar growth of MiaPaCa2 and other pancreatic carcinoma cells (27, 28).

Interestingly, we found further divergent functions for the Ral family of small GTPases. Specifically, inhibition of RalB, but not RalA, prenylation was required for GGTI-mediated inhibition of proliferation and induction of apoptosis. In both the Cos7 and the MiaPaCa2 cell lines expression of RalB-F but not RalA-F rendered cells less sensitive to GGTI-mediated inhibition of proliferation and induction of apoptosis. In MiaPaCa2 cells this rescue was concurrent to abrogation of GGTI effects on increasing p27Kip1 and decreasing survivin protein levels, but not on inhibiting Akt activation levels and inducing RhoA and RhoB levels, both of which we have shown previously to be induced by GGIs (11). These results suggest that, at least in the MiaPaCa2 pancreatic carcinoma cells, the ability of RalB-F to abrogate the GGTI anti-proliferative and pro-apoptotic mechanism of action is associated with abrogation of p27Kip1 induction and suppression of survivin levels. In further support of this we used siRNA to specifically inhibit RalA and RalB expression and found that knockdown of RalB, but not RalA, strongly attenuated survivin expression but did not affect p27Kip1 levels. These results concur with previous data that some tumor cells have at least a partial requirement for RalB in suppressing apoptosis (8) and suggest that RalB maintenance of survivin expression could be an important mechanism by which RalB promotes tumor cell survival. These results agree with our previous finding that ectopic expression of survivin partially abrogates GGTI induction of programmed cell death (10). Taken as a whole the results of this study raise interesting questions about the mechanism of GGTI-mediated anti-neoplastic activity. Both RalA and RalB are important regulators of many cellular

processes that have not previously been implicated in the GGTI mechanism of action. One of the most critical of these in oncogenesis is cellular trafficking both at the receptor level through endocytosis and through formation and delivery of the exocyst complex (2, 6, 13, 21, 47), a process reported to be required for the transforming ability of RalA and RalB (27). While both RalA and RalB utilize the exocyst complex RalA seems to primarily utilize the exocyst for transformation whereas RalB preferentially utilizes components of the exocyst for exocyst-independent functions in regulating cell mobility (7, 39). In fact, RalA and RalB are the only known binding partners for two competitive regulatory elements of the exocyst; Exo84 and Sec5. Therefore, the inhibition of RalA and RalB by GGTI-2417 represents a novel mechanism to inhibit exocyst function in transformed cells.

Beyond the exocytic and endocytic trafficking pathways RalA and RalB may regulate important interactions with the actin cytoskeleton through their association with RalBP1, Rho GTPase activating protein, and filamin, an actin-binding partner (31). Indeed, while many small GTPases, such as Cdc42, Rac, and Rho, bind filamin, only RalA and RalB bind filamin in a GTP-dependent manner. Therefore, the effects of GGTIs on cytoskeletal organization may be mediated by inhibition of RalA and/or RalB geranylgeranylation. In the course of our study we observed that RalB-F, but not RalA-F, inhibited GGTI-induced cell rounding in MiaPaCa2 cells concurrent with inhibition of actin fiber formation (data not shown). This is indicative of a disruption in the cell-cell junctions and the underlying actin driven membrane formations. This raises the provocative hypothesis that at least part of GGTI effects could be due to inhibition of normal cell-cell patterning. This is consistent with the fact that RalA is required for

basolateral sorting and delivery of E-cadherin (45), an important component of cell junctions and sheet patterning. Furthermore, it has been previously reported that cadherins mediate growth suppression by potently inducing p27Kip1 levels in a variety of cell lines (46). It is thus an intriguing possibility that aberrant activation of Ral proteins could mediate further aberrations in vesicle sorting of cadherins and contribute to suppression of p27Kip1 levels. Disruption of Ral-mediated vesicle sorting of cadherins by GGTIs could constitute a mechanism for the anti-neoplastic activity of GGTase I inhibition. Further, cadherin clustering and expression levels can regulate survivin levels (19). This suggests that sorting of basolateral and apical membrane proteins, such as cadherins, could be a Ral-dependent pathway that is required for tumor cell proliferation and survival and is sensitive to perturbation by GGTIs.

The anti-neoplastic activity of GGTIs is likely to be a consequence of inhibiting the function of multiple, functionally distinct GGTase I substrates. However, our chemical biology approach has clearly delineated non-overlapping roles for RalA and RalB in anchorage-independent and -dependent growth and demonstrates that inhibition of RalA and RalB geranylgeranylation is an important step in the mechanism of action of GGTIs. Specifically, we have identified three novel pathways that are associated with the GGTIs response: first, a RalB-dependent induction of the p27Kip1 pathway; second, a RalB maintenance of the survivin pathway and third, a RalA anchorage-independent proliferation pathway. The first two pathways are associated with the ability of GGTI to inhibit anchorage-dependent proliferation and survival. Further characterizing these pathways should prove to be an interesting and important contribution to the study of Ral biology. We feel these results should prompt a thorough examination of GGTI

mechanism of action with particular attention focused on the Ral family in both future clinical trials and in preclinical models.

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Chapter 3

Ras Transformation in a Genetically Defined Human Ovarian Cancer Model Requires Akt and Ral but not Raf

By

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Abstract

In addition to the high prevalence of inactivating mutations of p53 and Rb tumor suppressors, oncogenic mutations of the Ras genes are also common in human ovarian cancer; however, the downstream effectors required for Ras transformation in this disease are not known. Using a genetically defined human ovarian epithelial cell model in which stable expression of H- or K-Ras^{12V} drives transformation of ovarian surface epithelial cells (T80) immortalized by SV40 Large T antigen (SV40T) and activated human telomerase reverse transcriptase catalytic subunit (hTERT), we have delineated a requirement for Ral A/B and Akt1/2 but not Raf-1 or Mek1/2 expression and activation in Ras transformation.. Knockdown of expression of the RalGDS effectors RalA and RalB, and the PI3K effectors Akt1/2, but not Raf-1 and Mek1/2 inhibited H- and K-Ras transformation. Furthermore, stable expression of K-Ras^{12V} effector loop mutants revealed that activation of either RalGDS with Ras^{12V37G} or PI3K with Ras^{12V40C}, but not Raf-1 with Ras^{12V35S}, was sufficient to transform T80 cells. In further support of Ral and Akt as the primary transforming components of the H- and K-Ras signaling cascade perturbation of Ral or Akt but not Raf-1 signaling pathways by pharmacologic inhibitors attenuated Ras transformation. These results sharply contrast with previous studies which have reduced the requirements for Ras transformation in spontaneously immortalized rat ovarian surface epithelial cells to Ras/Raf signaling.

Introduction

Ovarian cancer is the primary cause of death among gynecological malignancies and is the 5th leading cause of cancer deaths among women in the United States (12). Despite the high incidence of ovarian cancer among women and the relative lethality of the disease little is known about the precise mechanism of transformation of human ovarian surface epithelial (HOSE) cells. Indeed, multiple mechanisms of ovarian surface epithelial cell transformation have been proposed and are believed to be sub-type specific (3). The two most common sub-types of ovarian cancer, high-grade serous and mucinous ovarian carcinoma (12), are commonly diagnosed at late-stage and are frequently lethal due to a high-propensity for both metastasis and drug-resistant recurrence (3, 12). It is thus of critical importance to define the mechanism of ovarian epithelial cell transformation both for a more precise understanding of the underlying genetic risk factors as well as for the development of targeted therapies for treatment.

Epithelial neoplasias, such as ovarian cancers, exhibit multiple genetic aberrations in key tumor suppressor genes, so-called “cellular gatekeepers” such as p53 and Rb(16). Indeed, over 50% of ovarian cancers exhibit missense mutations resulting inactivation of p53 (25, 30). Furthermore, over 80% of human ovarian cancers examined have been shown to have mutational defects in one or more of the genes involved in the Rb tumor suppressive pathway, such as CDK4, cyclin D1, and Rb (17). Activation of human telomerase (hTERT) activity is another well-recognized defect of human epithelial neoplasms including ovarian cancer [3,7]. In addition to p53, Rb and hTERT genetic abnormalities, activation of one or more proto-oncogenes is a pivotal fourth event in the development of human epithelial malignant disease.

Mutations in Ras proto-oncogene family members (H-, K-, and N- Ras) are known to occur in approximately 30% of human cancers (40). Numerous studies have demonstrated that mutational activation of K-Ras, through missense mutations at codons 12 and 13, occurs with high frequency (27-69%) in certain subtypes of ovarian cancers (7, 11, 20, 33, 44). Similar mutations in the H-Ras proto-oncogene have been determined to occur in ovarian cancer subtypes though there is considerable disagreement over the frequency (9, 44, 53). A role for Ras mutation in ovarian cancer progression is especially well recognized in mucinous sub-type ovarian tumors where the incidence of K-Ras proto-oncogene mutation increases profoundly during the progression of disease (33). For example, Mok and colleagues found a high, progression-dependent incidence of K-Ras activation at codons 12 and 13 in 13% of mucinous adenomas, 33% of mucinous tumors at the borderline, and 46% of mucinous carcinomas (33). Similar results have been reported by many other groups (7, 9, 20, 33, 44). K-Ras mutation have also been detected, albeit to a lesser degree (up to 12% only), in high-grade serous sub-type ovarian cancer, the most common form of ovarian malignancy (33). It is interesting to note that a much higher incidence of Ras mutation exists in low-grade serous malignancy. Indeed, over 68% of low grade serous tumors have either a Ras or Raf mutation (31), and Ras mutations have been found in up to 48% of borderline ovarian epithelial tumors (33) indicating a role for Ras mutations in the early events of low-grade serous ovarian neoplasia.

Recently, genetically defined models of human epithelial transformation have been developed which have enabled, for the first time, the description of oncogene stimulated transformation in cells of human epithelial origin (13-15, 21, 27, 29, 37).

Sequential inactivation of the tumor suppressors p53 and retinoblastoma (Rb) protein by ectopic expression of SV40-T-antigen (TAg) or polyoma middle T antigen (PoMT), coupled with stable expression of human telomerase catalytic subunit (hTERT) in human epithelial and fibroblastic cell lines recapitulates the critical events in the development of human neoplastic disease and creates a minimally transformed model for the study of oncogenic signaling pathways (13, 14). Creation of these models has prompted a reexamination of the essential downstream signaling components of the Ras oncogene. Previous reports in the spontaneously immortalized NIH-3T3 murine fibroblast model identified the Raf-MEK-Erk pathway, but not phosphatidylinositol 3' kinase (PI3K)-Akt or Ral guanine nucleotide dissociation stimulator (RLGDS)-Ral pathways, as being necessary and sufficient to promote Ras-mediated growth and morphologic transformation (38). However, recent work has demonstrated tissue- and species-specific requirements for Ras downstream effectors, other than Raf, in malignant transformation (15, 27, 37). While in one study [19], the only Ras effector pathway to show independent transforming activity in the human embryonic kidney cell system (HEK-HT) was RalGEF-RalA small GTPase pathway, whereas in another study [23] both Ras/RalGEF and Ras/PI3K pathways were required for Ras transformation in the same HEK-HT model. In contrast, in human mammary epithelial cells (HMECs), which are minimally transformed by hTERT and TAg, Ras transformation requires concurrent activation of Raf, RalGEF, and PI3K. Yet in another study [26], spontaneously immortalized rat ovarian surface epithelial (ROSE) cells, Raf but not PI3K or RalGEF, was independently transforming. Therefore, the requirements for Ras transformation appear to be highly dependent on the tissue and species of origin.

For ovarian cancer, while the ROSE cell model has been useful in suggesting which signaling cascades are required for Ras transformation in ovarian epithelial cells, it is neither genetically defined nor of human origin. Thus, while suggestive of a role for Raf in ovarian transformation, the ROSE model has unknown prognostic significance for human ovarian oncogenesis. Recently, a genetically defined human model of ovarian malignancy has been developed which recapitulates the critical events in neoplasia (29). The T80 cell line is derived from primary human ovarian surface epithelial cells engineered to express TAg and hTERT. Upon the addition of retrovirally transduced H-Ras^{12V}, these cells, referred to as T80H, acquire anchorage independent growth capability in soft agar and are capable of forming xenograft tumors in a SCID mouse model. This transformation requires continued expression of H-Ras (49) and, as such, is a bona fide model of Ras-dependent, ovarian cell-type-specific transformation. Using this system, as well as our own stable K-Ras^{12V} expressing variant (T80K), we selectively attenuated Ras signaling to the three primary Ras signal transduction pathways (RalGEF-Ral, PI3K-Akt, and Raf-MEK-Erk) using a combination of genetic and pharmacological approaches, as well as ectopic expression of Ras effector loop mutants to determine which of the downstream signaling components are required for human ovarian surface epithelial cell transformation. In contrast to the ROSE cell model we have determined that RalGEF-Ral and PI3K-Akt but not Raf-MEK-Erk are the primary pathways of Ras transformation of human ovarian epithelial cells.

Materials and Methods

Cells and culture—Human ovarian surface epithelial cells T80 and T80H were cultured as described previously (29). The T80K, T80K^{12V35S}, T80K^{12V37G}, T80K^{12V40C}, stably expressing cell lines were maintained in similar culture conditions as the T80 and T80H cells [22]. Human HEK-293T was a kind gift from Dr. Gary Reuther and were maintained in Dulbecco's modified minimum essential medium (DMEM) 10% fetal bovine serum and 1% penicillin/ streptomycin at 10% CO₂ and 37°C.

Plasmids- Retroviral plasmids pBABE-puro containing the K-Ras^{12V}, KRas^{12V35S}, KRas^{12V37G} and KRas^{12V40C} transgenes were a kind gift from Dr. Gary Reuther (15).

Creation of retrovirus- Retrovirus was created by transient transfection of HEK-293T human embryonic kidney cells with pVPACK-Ampho, pVPACK-gag-pol and pBABE retroviral plasmids as previously described (10). Briefly, 293T cells were seeded at 2.5×10^6 cells in a 60mm² dish. 3 µg of each plasmid was combined and brought to a volume of 225 µl in RNase/DNase free water, following which 25 µl 2.5M CaCl₂ was added dropwise while gently vortexing. This solution was then added to 250 µl 2x HEPES Buffered Saline dropwise and incubated at room temperature for 5 min. The total volume of 500 µl was then added to the 293T cells and incubated for 8 hs at 37°C. The media was then removed and cells were incubated for 48 h. The supernatant was then passed through a 0.45 µm nylon low protein-binding filter to remove cells and cell debris.

Creation of stable cell lines- T80 cells were seeded into 6 well plates at a 40% confluency and incubated with 10 µg/mL polybrene (Millipore/ Specialty Media, Billerika, MA) and a 1:3 dilution of retrovirus containing conditioned media for 72 h. Media was then replaced with complete growth medium containing 1.0 µg/mL

puromycin and incubated until colonies formed. All colonies were pooled and taken as a single polyclonal population and cultured in complete medium.

Small interfering RNA (siRNA) sequences- siRNA sequences targeting RalA and RalB were purchased from Dharmacon using previously described sequences (6). Specifically, RalA: 5'-GACAGGUUUCUGUAGAAGAdTdT-3', RalB: 5'-GGUGAUCAUGGUUGGCAGCdTdT-3'. Pre-designed chemically synthesized siRNA targeting Akt1/2 (Cat #6211, Cell Signaling Technology Inc., Danvers, MA), Raf1 (Cat #M-003601-00, Dharmacon, Lafayette, CO) MEK1 (Cat #6420, Cell Signaling Technology Inc., Danvers, MA) and MEK2 (Cat #6431, Cell Signaling Technology Inc., Danvers, MA) were used according to manufacturer's recommendation.

Small interfering RNA (siRNA) transfection procedure- Cells were grown to 50% confluence and transfected with Oligofectamine transfection reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Briefly, 5 μ l of oligofectamine reagent was suspended per 1 mL of OPTI-MEM media (Invitrogen, Carlsbad, CA) and allowed to equilibrate at 24-27°C. A 100 nM final concentration of siRNA was suspended and allowed to complex with the liposomes. Cells were briefly washed with OPTI-MEM and the transfection mix was plated on top of the cells and incubated at 37°C. Following 8 h incubation 2 mL of complete growth media, without penicillin-streptomycin, was added and the cells were further incubated at 37°C overnight. The media was replaced after 24 h with complete growth medium for 24 h. Experiments were performed in triplicate.

Pharmacological inhibitors – Cells were treated for 48 h with either DMSO vehicle (Sigma-Aldrich, St. Louis, MO), 20 μ M LY29004 (Calbiochem, San Diego, CA), 10 μ M

U0126 (Sigma-Aldrich, St. Louis, MO), or 10 μ M GGTI-2417 (36). Experiments were performed in triplicate.

Western blotting—Cells were harvested, and lysed in HEPES lysis buffer as described previously (10). Proteins were then resolved by 11.5% SDS-PAGE gel and immunoblotted with antibodies against phosphorylated-Ser473 Akt1/2 (9217, Cell Signaling Technologies Inc., Danvers, MA), phosphorylated-Thr202/Tyr 204 Erk1/2 (9101, Cell Signaling Technologies Inc., Danvers, MA), Akt1/2 (N-19, Santa Cruz Biotechnology, Santa Cruz, CA), Erk1/2 (p44/p42 MAP Kinase, 9102, Cell Signaling Technologies Inc., Danvers, MA), RalA (61022, BD Biosciences Pharmingen, Franklin Lakes, NJ), H-Ras(C-20, Santa Cruz Biotechnology, Santa Cruz, CA), K-Ras (F234, Santa Cruz Biotechnology, Santa Cruz, CA), RalB (04037, Millipore, Billerika, MA), β -actin (AC15, Sigma-Aldrich, St. Louis, MO) and anti-FLAG M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO). The ECL blotting system (NEN Life Science Products, Boston, MA) was used for detection of positive antibody reactions. Experiments were performed in triplicate.

Soft agar clonogenicity assay- For soft agar growth assays, the cell lines were seeded at a cell density of 5000/well in triplicate in 12-well culture dishes in 0.3% agar over a 0.6% bottom agar layer as previously described (10). Cultures were fed once weekly until colonies grew to a suitable size for observation (approximately 14 days). Colonies were photographed after overnight incubation with 1 mg/ml MTT in cell growth media.

Colony number was visually determined and quantified. Experiments were performed in triplicate and 3 independent experiments were performed.

Results

Differential transforming activity and signaling activation by H- and K- Ras12V isoforms in the T80 human ovarian surface epithelial (HOSE) cell line.

The incidence of K-Ras mutation in ovarian malignancy is far greater than that of H-Ras (3, 7, 9, 20), suggesting a preferential role for K-Ras in the development of ovarian neoplasia. Additionally, while Ras isoforms are commonly assumed to activate the same three primary transformative pathways; Raf/MEK/Erk, PI3K/Akt and RalGDS/Ral, the degree to which H- and K- Ras isoforms activate these pathways vary according to both physical association constants (8, 48) as well as cell-type specific differences (15, 26, 37, 43). Therefore, we first wanted to examine differences in both the transformative capability and the signaling pathway utilization of oncogenic, mutated H- and K-Ras^{12V} in the HOSE T80 cell model where hTERT is overexpressed and p53 and Rb are inactivated by Tag as described in Liu et al (29). To this end, we created a T80 cell line stably expressing oncogenic K-Ras^{12V} (T80-K) as described under Materials and Methods. The T80 cell line stably expressing oncogenic H-Ras^{12V} (T80H) was obtained from Dr. Bast (29). We first compared the downstream effector utilization of the two Ras isoforms by SDS-PAGE and western blot analysis as well as Ral-GTP binding assay (figures 12A and 12B, see following pages). Both H- and K-Ras^{12V} potently stimulated GTP loading of RalA and RalB to a similar extent. However, while both H-Ras^{12V} and K-Ras^{12V} induced phosphorylation of Akt1/2 and Erk1/2, K-Ras^{12V} was relatively less potent; indicating a differential specificity of the Ras isoforms for activation of Akt and Erk in the human ovarian cancer cell model. Finally, we used anchorage-independent growth on soft agar to show that ectopic expression of both mutant H-Ras^{12V} and K-

Ras^{12V} promotes soft agar clonogenicity in the T-80 model. Figure 12C shows that whereas T80 grew only 42 ± 5 colonies, T80-H and T80-K grew 183 ± 28 ($p < 0.05$) and 114 ± 13 ($p < 0.01$) colonies, respectively.

Figure 12. Differential transforming activity and signaling activation by H- and K- Ras^{12V} in human ovarian surface epithelial (HOSE) cells

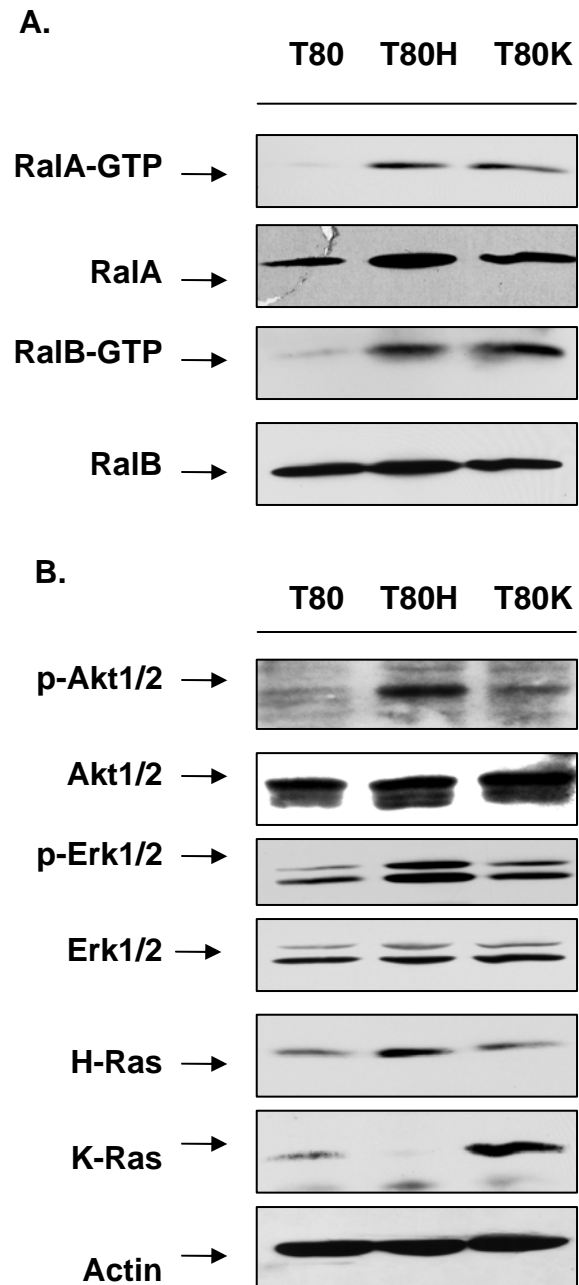
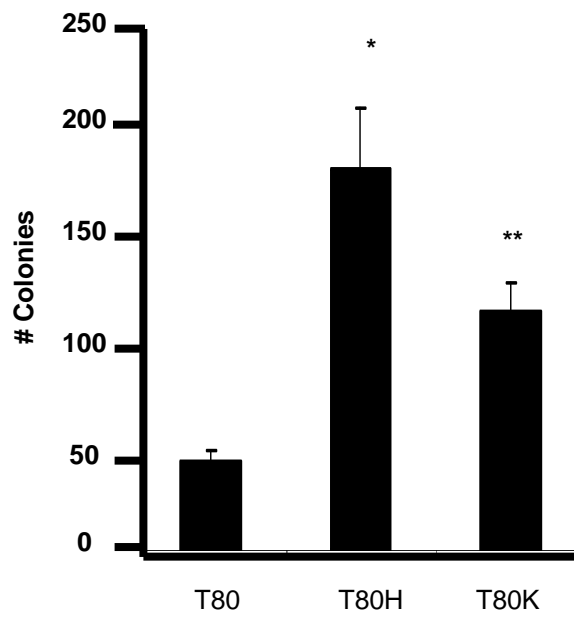


Figure 12. Differential transforming activity and signaling activation by H- and K- Ras^{12V} in human ovarian surface epithelial (HOSE) cells

C.



* = P<0.05, ** = P<0.01 compared to T80

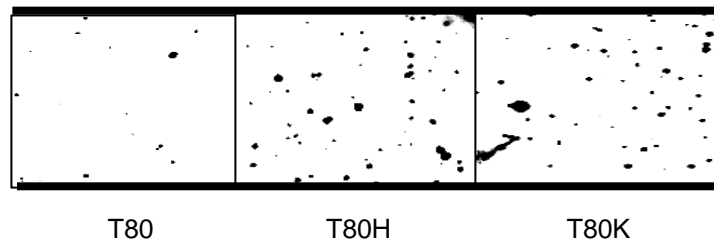


Figure 12. Differential transforming activity and signaling activation by H- and K- Ras^{12V} isoforms in the T80 human ovarian surface epithelial (HOSE) cell line. T80 cells stably transduced with H- Ras^{12V} (T80H) or K- Ras^{12V} (T80K) were collected and lysed. Proteins were separated by SDS-PAGE and used for Ral-GTP quantitation using GST-RalBP pull down assay (A) or western blot analysis (B); results are representative of three experiments. T80, T80H and T80K cells were seeded into 0.3% soft agar for two weeks, colonies were manually scored (C); T80 cells formed 43+/-5 colonies per well, T80H formed 183+/-28 colonies per well and T80K formed 114+/-13 colonies per well. Results are representative of three experiments each performed in triplicate.

Ras transformation of human ovarian surface epithelial cells requires expression of RalA/B and Akt1/2 but not Raf1 or Mek1/2.

We next determined the requirements for expression of the Ras effectors RalA, RalB, Akt1/2, and Raf1 and Mek1/2 in both H- and K- Ras^{12V} mediated transformation of T80 cells. For these analyses, we used siRNA to RalA, RalB, Akt1/2 and Raf1 and Mek1/2 to specifically deplete each gene product and systematically evaluate the effects of this knockdown on Ras transforming capability as described under Materials and Methods. Figure 13A (see following pages) shows that in both T80H and T80K RalA siRNA knocked down RalA, but not RalB, expression whereas RalB siRNA knocked down RalB, but not RalA, expression; these results demonstrated, by western blot analysis, that each siRNA was specific. Figure 13B (see following pages) shows that knockdown of expression of RalA potently inhibited H-Ras^{12V} and K-Ras^{12V} transformation of T80 cells by 56%±7% and 54%±5%, respectively (all p values were less than 0.01). Similarly, knockdown of expression of RalB potently inhibited H-Ras^{12V} and K-Ras^{12V} transformation of T80 cells by 54%±11% and 66%±3%, respectively (all p values were less than 0.01). Knockdown of Akt1/2 expression in both T80H and T80K cells (Figure 14A, see following pages) inhibited transformation by 46%±3% and 42%±5%, respectively (Figure 14C, all p values were less than 0.05). In contrast knockdown of Raf1 expression (Figure 3A, see following pages) did not have any statistically significant effect on the clonogenicity of T80K and T80H cells (Figure 14C, see following pages). Since inhibition of Raf1 expression did not result in reduction of Ras transformation we aimed to further evaluate the role of Raf/MEK/Erk signaling by knocking down the expression of Mek1/2. While inhibition of Mek1/2 expression

resulted in the attenuation of Erk1/2 phosphorylation (figure 14B, see following pages), it did not inhibit either H-Ras^{12V} or K-Ras^{12V} transformation (figure 14C, see following pages). These results suggest that RalA, RalB and Akt1/2, but not Raf1 or Mek1/2, are essential components of Ras mediated human ovarian epithelial cell transformation.

Figure 13. Ras transformation of HOSE cells requires expression of RalA/B.

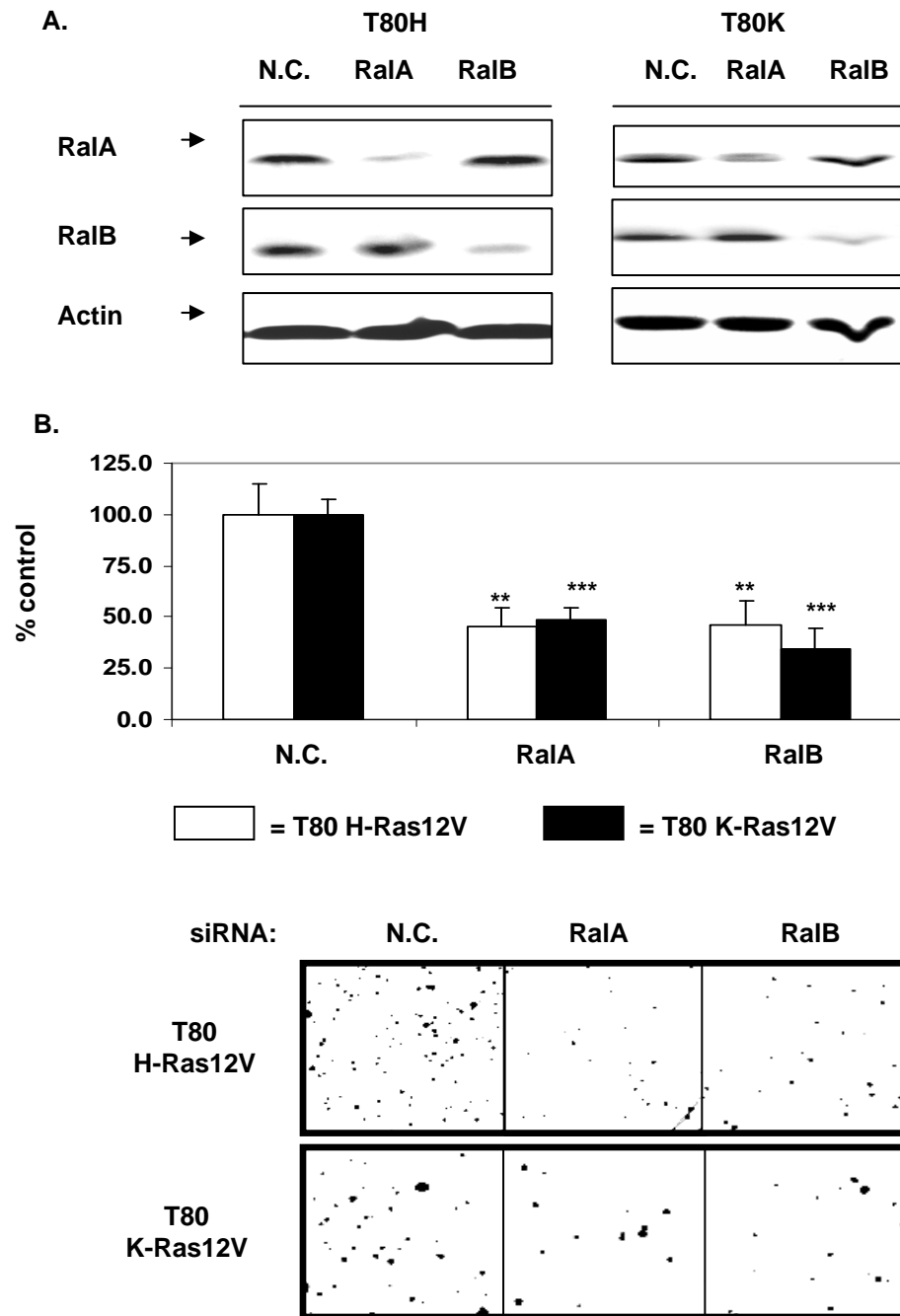


Figure 13. Ras transformation of HOSE cells requires expression of RalA and RalB. Small interfering RNA (siRNA) specific to RalA and RalB or a negative control siRNA were transfected for 48 hours into T80, T80H and T80K cells. Cells were collected, counted using trypan blue dye exclusion assay and collected for western blot analysis (A) and an equal number of cells were plated for soft agar assay (B) and scored manually at the end of two weeks; for T80-H N.C. cells 153 \pm 11 cells = 100%, for T80-K N.C. cells 115 \pm 17 cells = 100%; results are representative of three experiments each performed in triplicate.

Figure 14. Ras transformation of HOSE cells requires expression of Akt1/2 but not Raf1 or MEK1/2.

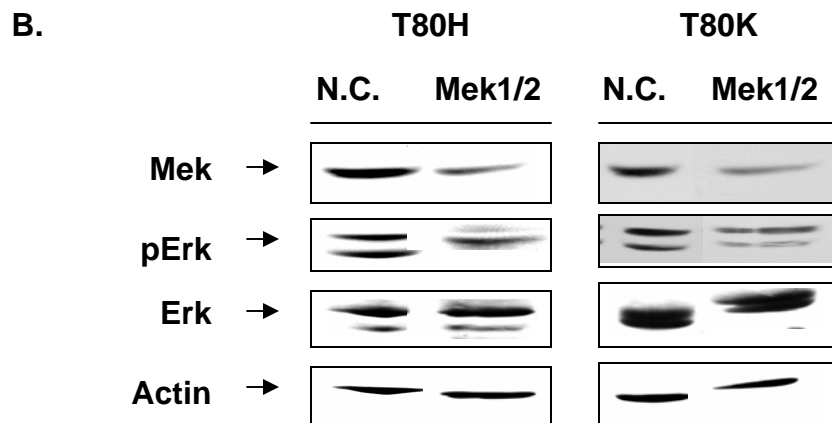
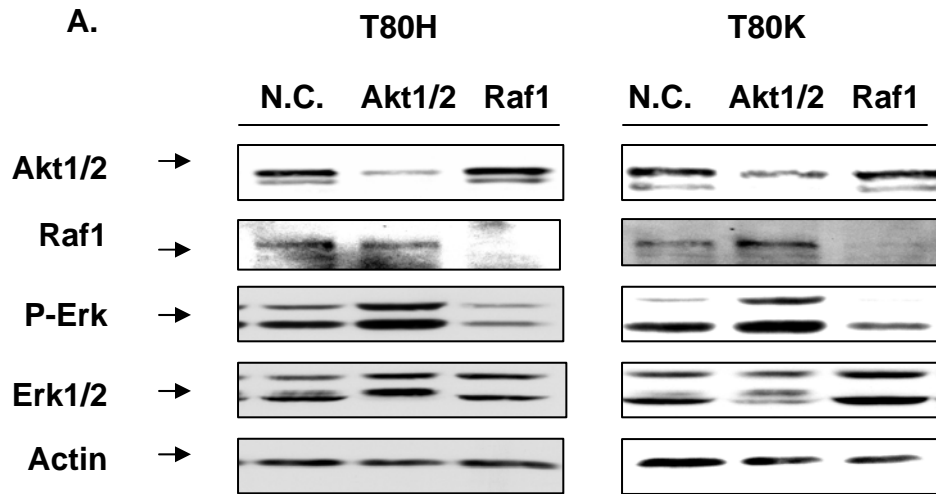


Figure 14. Ras transformation of HOSE cells requires expression of Akt1/2 but not Raf1 or MEK1/2.

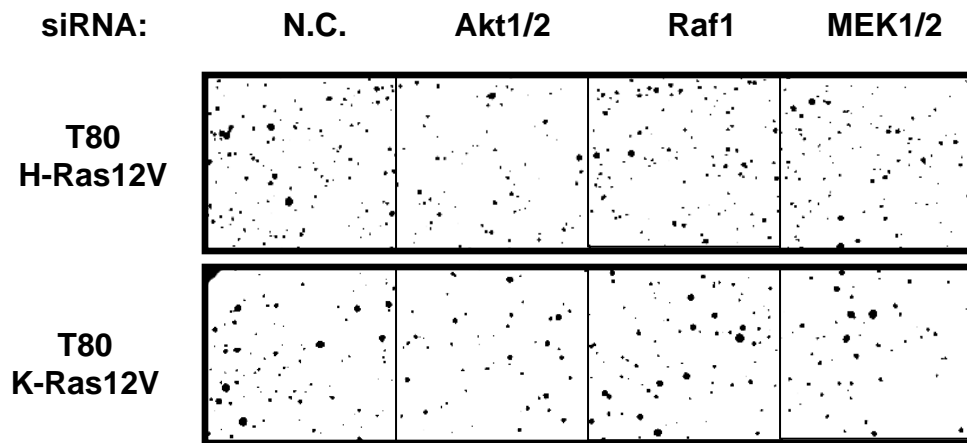
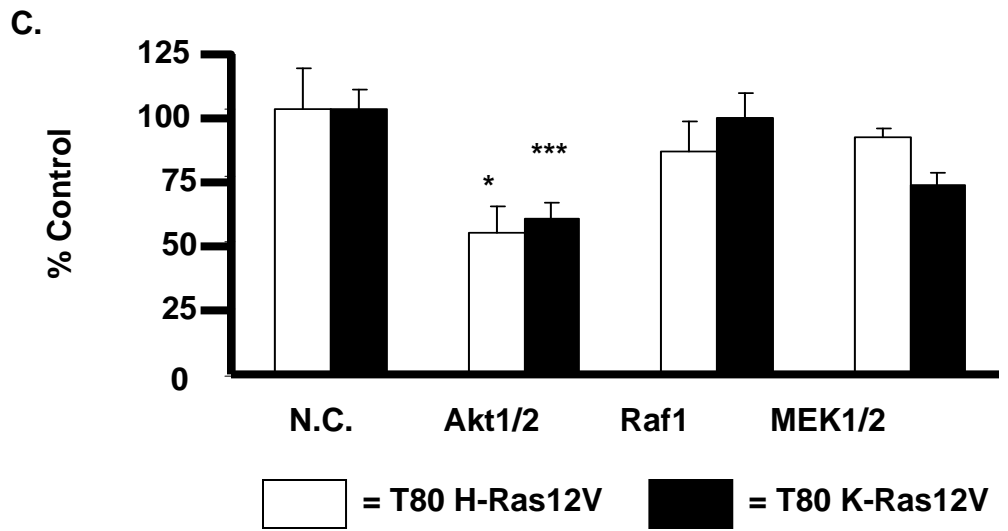


Figure 14. Ras transformation of HOSE cells requires expression of Akt1/2 but not Raf1 or MEK1/2. Small interfering RNA (siRNA) specific to Akt1/2 and Raf1 or a negative control siRNA were transfected for 48 hours into T80, T80H and T80K cells. Cells were collected, counted using trypan blue dye exclusion assay and collected for western blot analysis (A) and an equal number of cells were plated for soft agar assay (B) and scored manually at the end of two weeks; for T80-H N.C. cells 153 \pm 11 cells = 100%, for T80-K N.C. cells 115 \pm 17 cells = 100%; results are representative of three experiments each performed in triplicate.

K-Ras effector loop mutants which preferentially activate RalGDS and/or PI3K, but not Raf1, are capable of transforming T80 HOSE cells to a similar, or greater, extent as compared to fully active K-Ras^{12V}.

To further evaluate the contributions of the Ras effector pathways RalGDS/Ral, PI3K/Akt, and Raf/MEK/Erk to Ras transformation in human ovarian surface epithelial cells we used retroviruses to stably transduce oncogenically active K-Ras^{12V} with effector loop amino acid substitutions (35S, 37G, and 40C) which are well characterized to restrict the interaction of Ras to, respectively, Raf-1, RalGDS and PI3K (22, 23, 32, 39, 45, 46). Figures 15A and 15B (see following pages) show that K-Ras^{12V} stimulated phosphorylation of P-Erk1/2 and Akt as well as induced RalA and Ral GTP loading. K-Ras^{12V35S} (which activates Raf but not PI3K or RalGDS) activated Erk1/2 phosphorylation but did not stimulate RalA or RalB GTP loading and did not activate Akt1/2 phosphorylation (figure 15A and 15B, see following pages). Similarly, K-Ras^{12V37G} (which binds RalGDS but is defective in binding to Raf and PI3K) stimulated GTP-loading of RalA and RalB but did not stimulate phosphorylation of Akt1/2 and Erk1/2 (figure 15A and 15B, see following pages). K-Ras^{12V40C} (which activates PI3K but not RalGDS or Raf) activated Akt but not Erk1/2 phosphorylation and did not stimulate GTP-loading of RalA or RalB (figure 15A and 15B, see following pages). Further evaluation of the transforming potential of these mutational variants relative to fully oncogenic K-Ras revealed that Ras effector loop mutants which preferentially activated RalGDS/Ral (K-Ras^{12V37G}) and PI3K/Akt (K-Ras^{12V40C}), but not Raf/MEK/Erk (K-Ras^{12V35S}), were capable of transforming T80 HOSE cells to a similar or greater extent than K-Ras^{12V} (figure 3C, see following pages). For example, KRas^{12V} and K-

Ras^{12V40C} increased the number of colonies from 62 ± 16 in pBABE (T80 cells) to 195 ± 25 and 201 ± 13 respectively. K-Ras^{12V37G} was more potent and resulted in 282 ± 20 colonies. In contrast, K-Ras^{12V35S} was dramatically reduced in transforming potential, 50% of K-Ras^{12V} or 95.3 ± 10 colonies (p value < 0.005). These findings both confirmed and extended our results by further demonstrating the central importance of PI3K/Akt and RalGDS/Ral, but not Raf-1, in ovarian epithelial cell transformation.

Figure 15. K-Ras mutants which preferentially activate RalGDS and/or PI3K, but not Raf1, are capable of transforming T80 HOSE cells to a similar, or greater, extent as compared to fully active K-Ras^{12V}

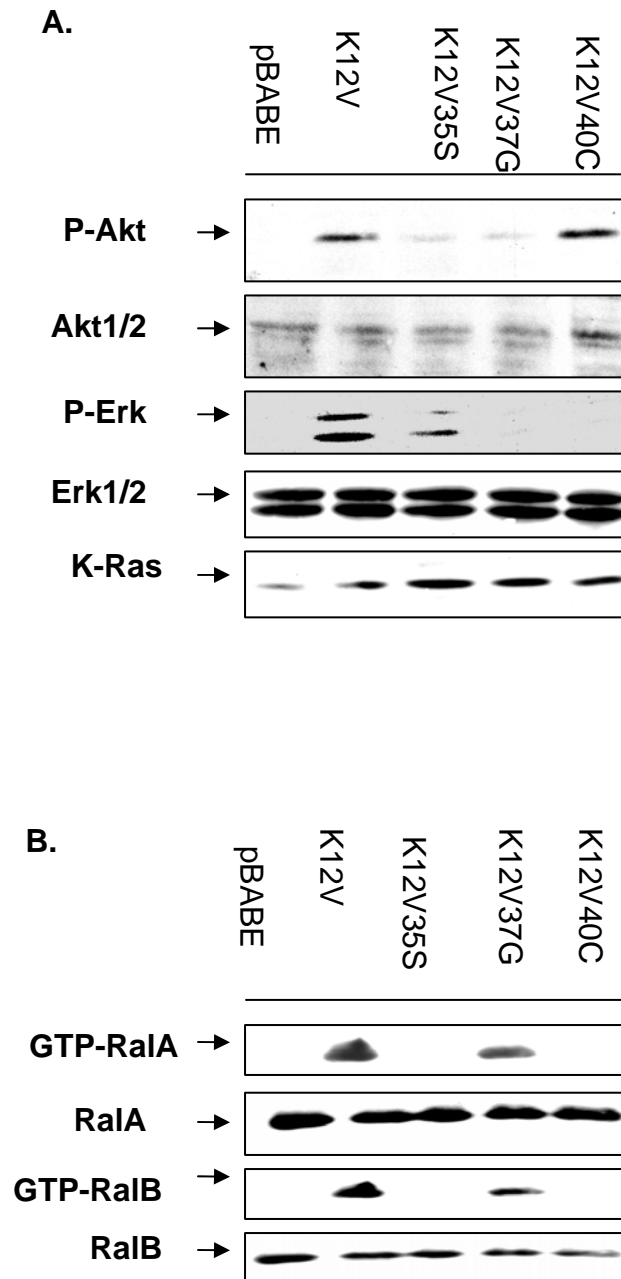


Figure 15. K-Ras mutants which preferentially activate RalGDS and/or PI3K, but not Raf1, are capable of transforming T80 HOSE cells to a similar, or greater, extent as compared to fully active K-Ras^{12V}

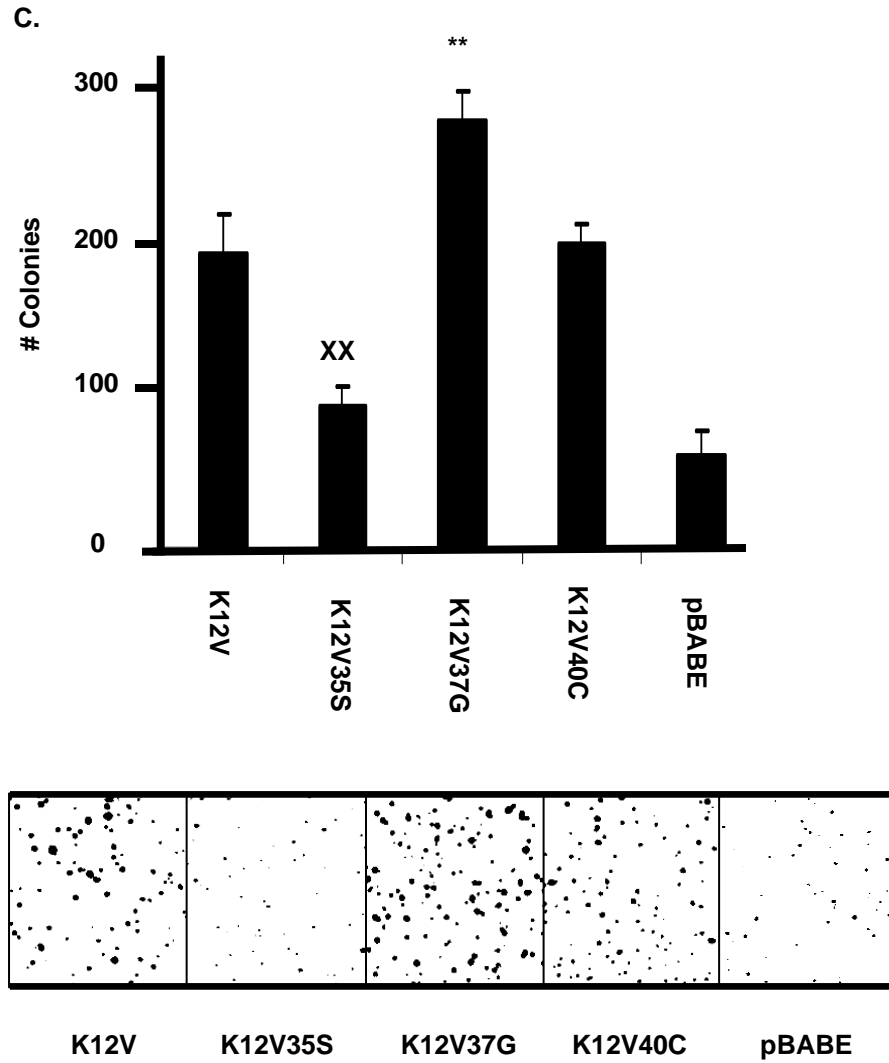


Figure 15. K-Ras mutants which preferentially activate RalGDS and/or PI3K, but not Raf1, are capable of transforming T80 HOSE cells to a similar, or greater, extent as compared to fully active K-Ras^{12V}. T80 cells stably transduced with empty vector, K-Ras^{12V} (T80K), K-Ras^{12V35S} (T80K35S), K-Ras^{12V37G} (T80K37G) or K-Ras^{12V40C} (T80K40C) were collected and lysed. Cell lysates were separated by SDS-PAGE and used for western blot analysis (A) or quantitation of Ral-GTP levels using GST-RalBP pull down assay (B); results are representative of three experiments. T80, T80K and T80K35S, T80K37G and T80K40C cells were seeded into soft agar for two weeks, colonies were manually scored (C); results are representative of three experiments each performed in triplicate. **= $p < 0.01$ (greater than T80K), xx= $p < 0.01$ (less than T80K)

Pharmacological inhibitors of PI3K or Ral, but not MEK, inhibit Ras transformation of HOSE cells.

While we have demonstrated that expression and activation of RalGDS/Ral and PI3K/Akt, but not Raf-1/ Mek1/2 are required for Ras transformation we also wanted to determine the effects that pharmacological perturbation of these pathways would have on Ras transformation. We used well-characterized inhibitors of MEK (U0126) and PI3K (LY294006) to selectively attenuate these pathways. We also used GGTI-2417, a small molecule competitive inhibitor of geranylgeranyltransferase-I (36), to inhibit the prenylation of RalA and RalB. Both RalA and RalB require geranylgeranylation for both localization and biological activity (19) and are necessary components of GGTI anti-neoplastic activities (10). As expected, pharmacological inhibition of PI3K by LY294006 attenuated phosphorylation of Akt (Figure 16A, see following pages). LY294006 also inhibited P-Erk1/2 in both cell lines. GGTI-2417, similar to our previous reports, inhibited the prenylation of both RalA and RalB (see slight mobility shift in Figure 16A, see following pages). The MEK1/2 inhibitor UO1266 partially inhibited Erk1/2 phosphorylation, without any effect on Akt phosphorylation (Figure 16A, see following pages). GGTI-2417 inhibited soft agar clonogenicity by 85.5% (T80K, p value <0.005) and by 54.5% (T80H, p value <0.05) (Figure 16B, see following pages). LY294006 inhibited soft agar clonogenicity by 89.6% (T80H, p value <0.01) and by 90.1% (T80K, p value <0.01). In contrast, UO126 did not attenuate either T80H or T80K soft agar clonogenicity.

Figure 16. Pharmacological inhibitors of PI3K or Ral, but not MEK, inhibit Ras transformation of HOSE cells.

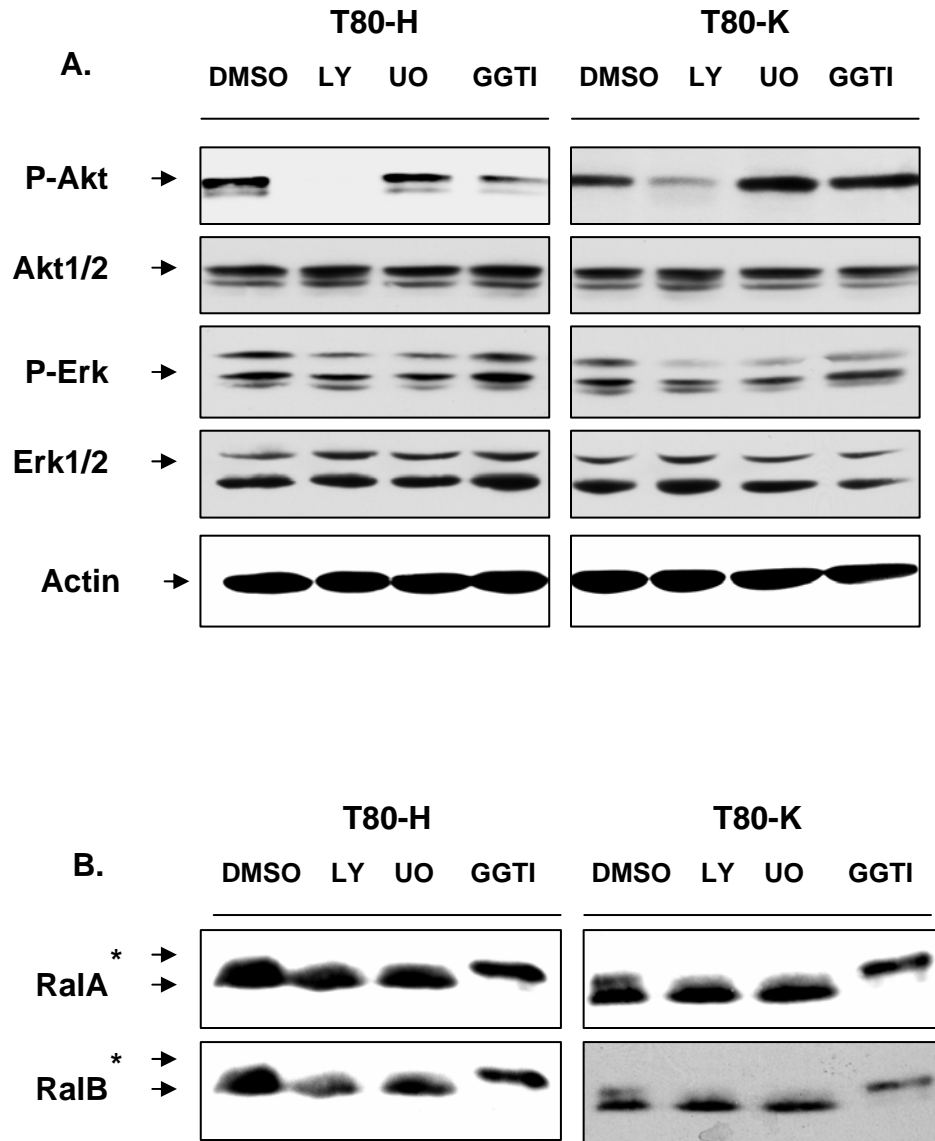


Figure 16. Pharmacological inhibitors of PI3K or Ral, but not MEK, inhibit Ras transformation of HOSE cells.

C.

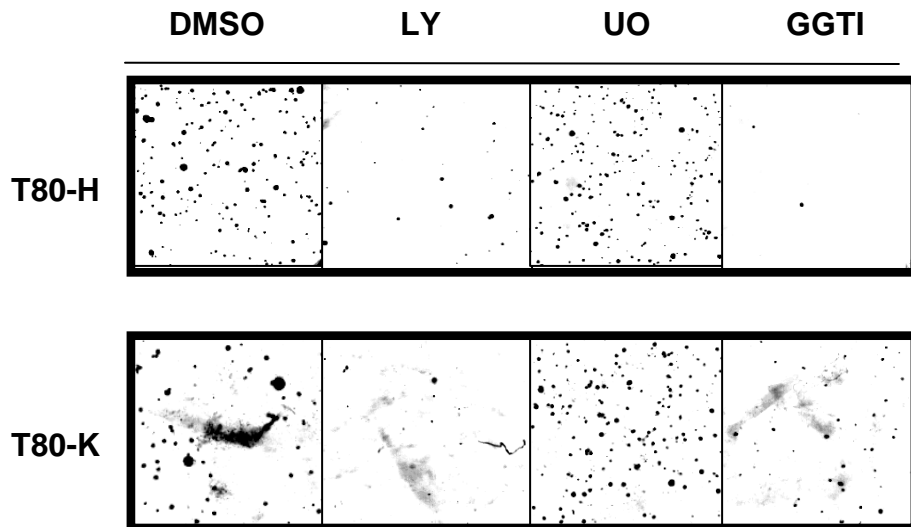
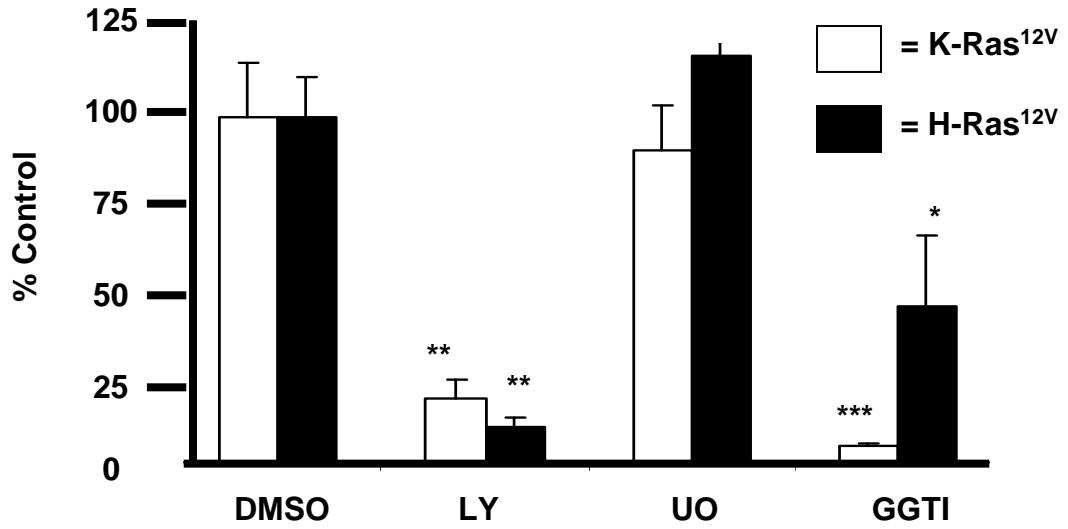


Figure 16. Pharmacological inhibitors of PI3K or Ral, but not MEK, inhibit Ras transformation of HOSE cells. T80, T80H and T80K cells were exposed to full growth media containing either 0.01% DMSO, 20 μ M LY29004, 10 μ M U0126, or 10 μ M GGTI-2417 for 48-hours. Cells were then collected, counted using trypan blue dye exclusion assay and collected for western blot analysis (A) and an equal number of cells were plated for soft agar assay (B) and scored manually at the end of two weeks; results are representative of three experiments each performed in triplicate.

Discussion

Several studies (7, 11, 20, 41, 44) have implicated mutant K-Ras in the development of ovarian cancer, especially in mucinous sub-type malignancies, the second most common form of ovarian neoplasia (7, 33, 44). In this study we examined the roles of the three most thoroughly characterized Ras effector pathways RalGEF-Ral, PI3K-Akt and Raf-MEK-Erk in mediating Ras anchorage independent growth of a genetically defined model of human ovarian cancer. We determined that Ras anchorage independent growth in this tissue type proceeds primarily through the RalGEF-Ral and PI3K-Akt pathways but not through Raf-MEK-Erk, in sharp contrast to previous reports that spontaneously immortalized rat ovarian surface epithelial cells could be transformed by H-Ras^{12V} in a Raf1-dependent manner (43). It is important to note that anchorage independent growth is only one measure of transformation and other aspects of transformation, such as invasion and metastasis may require Raf1 signal transduction.

We first determined that the transforming activity of both H- and K-Ras^{12V} required the expression and activity of RalA, RalB and Akt1/2 but not Raf1 or Mek1/2. We next determined that K-Ras mutants which preferentially activated RalGEF, or PI3K, but not Raf, were capable of transformation to a similar or greater extent than K-Ras capable of activating all three pathways. Importantly, we discerned that K-Ras capable of only activating the Raf-MEK-Erk pathway was incapable of transforming T80 cells. These results suggest that RalGEF or PI3K activation alone is sufficient to promote Ras-mediated anchorage-independent growth. However, it should be emphasized that these effector mutants do retain the ability to bind other Ras effectors that may regulate transformation. For example the 37G mutant retains the ability to activate phospholipase

C epsilon. We have also used pharmacological probes to provide further support for the role of Ral and Akt but not Raf pathways in Ras – mediated ovarian cell transformation.

One of our initial observations was that differences exist in both transforming potency and effector utilization among Ras isoforms. Indeed, K-Ras^{12V} was less potent than H-Ras^{12V} in the ability to both transform and activate Akt and Erk. Interestingly, another independently derived T80K cell line has also exhibited similarly diminished transformative capability in soft agar assays (29), making it unlikely that this is an artifact of viral transduction. It is interesting to speculate on the causes of this differential activity as these results stand in marked contrast to previous studies of H-Ras^{12V} in rat ovarian surface epithelial (ROSE) cells in which stable expression of H-Ras^{12V} did not stimulate Akt activation in ROSE cells (43). However, we find that H-Ras^{12V} potently stimulates Akt activation whereas K-Ras^{12V} was less potent. Similarly, while stable expression of either Ras oncogene stimulated Erk hyper-phosphorylation, K-Ras^{12V} was less potent. However, H- Ras^{12V} and K- Ras^{12V} were equipotent in their ability to stimulate GTP-loading of RalA and RalB. While these results suggest a species-specific difference in Ras isoform effector utilization, at least when compared to the previous studies in ROSE cells (43), there has been no effort to systematically determine Ras isoform tissue-specific differences in effector utilization. However, previous studies in murine fibroblast NIH-3T3 cells indicate that H-Ras preferentially activates Erk whereas K-Ras preferentially activates Akt (48). We next determined which limbs of the Ras pathways are required for its ability to transform HOSE cells.

We found that either specific depletion of RalA or RalB by siRNA or inhibition of Ral prenylation by GGTI-2417 was sufficient to strongly attenuate transformation by

Ras^{12V}. Others have previously demonstrated a central requirement for RalGDS (27), and specifically RalA, in the transformative activity of H-Ras^{12V} in HEK-HT cells.

Furthermore, studies in NIH-3T3 fibroblasts have shown that ectopic expression of dominant negative RalA can partially reverse Ras-dependent transformation (1); and inhibition of RalA by either siRNA mediated depletion or overexpression of dominant negative RalA has been shown to inhibit anchorage independent proliferation, invasion and metastasis of human tumor cell lines of non-ovarian origin (6, 45, 51). While RalB expression was previously described as dispensable for H-Ras^{12V} mediated transformation of human embryonic epithelial kidney cells (27) we have found a central requirement for RalB in both H- and K-Ras^{12V} transformation of ovarian epithelial cells. Indeed, the effects of depletion of RalB by siRNA were equivalent to depletion of RalA in attenuating clonogenicity of T80H and T80K cells. However, we have also previously reported in the human pancreatic cancer cell line MiaPaCa2 biological differences between RalA and RalB, which suggested a role for RalA but not RalB in clonogenicity (10). Using a chemical biology approach we determined that GGTI-insensitive mutants of RalA rescued from GGTI-inhibition of anchorage-independent growth while similar mutants of RalB rescued from divergent processes such as inhibition of anchorage-dependent proliferation, induction of apoptosis, increase in p27^{Kip1} and decrease in survivin in human pancreatic cancer cells (10). Others, such as White, Theoderescu, Der and Counter have used siRNA to RalA and RalB to similarly demonstrate divergence between the Ral isoforms in multiple human pancreatic and renal cancer cell lines, as well as certain human cervical and prostate cancer cell lines, respectively HeLa and SW480 (6, 28, 35). Given the presumed biologically divergent roles of the Ral family it is

interesting to consider whether the ability of RalA and RalB expression to govern Ras transformation is through redundant or divergent pathways.

In support of a role for RalGEF/Ral signaling in the progression of ovarian cancer, expression of K-Ras^{12V37G}, which is deficient for activation of Raf-1 and PI3K, activated GTP-loading of RalA and RalB to a similar extent as K-Ras^{12V} and was able to transform to a similar or greater extent than K-Ras^{12V} in T80 cells. This is similar to the results in the HEK-HT system reported by the Counter lab in which H-Ras^{12V37G} had independent transformative capability (27) but stands in marked contrast to the results of the Weinberg lab where H-Ras^{12V37G} was only transforming when combined with activation of PI3K (15).

We have also identified the PI3K/Akt pathway as being central for Ras-induced ovarian oncogenesis. Indeed, previous studies on the PI3K/Akt signaling pathway in ovarian cancer provide ample evidence that aberrations in this pathway could play an important role in the progression of ovarian neoplasia. Specifically, Akt2 kinase activity is elevated in ovarian carcinoma patient biopsies (52), Akt2 gene amplification occurs in 12-18% of ovarian carcinoma samples analyzed (2, 5) and mutation of PI3K p110 subunit occurs in 20% of serous carcinomas (47). However, none of these observations have been correlated to Ras mutational status. Our results demonstrate that inhibition of Akt1/2 expression by siRNA and inhibition of Akt activation by the PI3K inhibitor LY294006 strongly attenuated both T80H and T80K transformation. These results are in concurrence with previous reports in ROSE cells in which LY294006, an inhibitor of PI3K, reversed H-Ras^{12V} transformation (43). However, in this system since H-Ras^{12V} expression did not result in hyperphosphorylation of Akt1, or Akt2, no role could be

assigned to Akt signaling. In contrast in our present study we found both H-Ras^{12V} and K-Ras^{12V} could activate Akt1/2. This is consistent with previous studies in spontaneously immortalized NIH-3T3 murine fibroblasts and rat intestinal epithelial (RIE) cells that have correlated Ras isoform transformation potential to the activation of PI3K (26). However, it is important to note, despite the lower level of Akt activation by K-Ras^{12V} as opposed to H-Ras^{12V} both T80H and T80K cell lines were equally sensitive to inhibition of Akt activation by LY and expression knockdown by Akt siRNA. Ascribing a causal role for PI3K and Akt in Ras transformation of epithelial cells is particularly important given the wealth of small molecules currently under clinical investigation as anti-neoplastic agents which target PI3K and Akt (18). Indeed, one of these compounds, triciribine monophosphate (TCNP) (50), is currently in phase I trials in patients where tumors (including ovarian) contain persistently activated hyper-phosphorylated Akt (Clinical trial #NCT00363454). Our results suggest inhibition of either PI3K or Akt could be beneficial to patients harboring Ras mutation positive ovarian tumors.

In further support of a role for PI3K/Akt signaling in the ovarian neoplastic processes independent of Ras we have found KRas^{12V40C}, activates Akt to a similar extent as K-Ras^{12V} and is capable of similar transforming activity. However, since this effector domain mutant retains the ability to bind to other Ras effectors, more definitive demonstration of this will require a more thorough examination of the independent transforming activity of constitutively activated Akt and PI3K isoforms. Previous cell line systems, such as HEK-HT and HMEC have not ascribed an independent role for PI3K/Akt signaling in the induction of epithelial oncogenesis however PI3K-Akt signaling was required for maintenance of Ras transformation in HEK-HT cells (6, 15).

Furthermore, given both frequency of PTEN deletions in ovarian cancer (24) and the presence of activating point mutations in PI3K p110 gene (47) as well as Akt1 and Akt2 (4, 34, 42) these results are consistent with epidemiologic observations. To date most studies carried out have examined the overlapping incidence of BRAF and RAS mutations. While some limited studies have focused on understanding the overlapping frequency of RAS and PI3K mutations in ovarian cancer, these results suggest further analysis of the mutational status of the various Ras effectors, such as mutational aberrations in PI3K/Akt signaling and the various RalGEFS, relative to Ras mutation among the various sub-types of ovarian cancer is warranted.

Perhaps most interestingly our results suggest that Raf/Mek/Erk stimulation does not play a substantial role in either H- or K-Ras^{12V} transformation of human ovarian surface epithelial cells. Stable expression of K-Ras^{12V35S}, deficient for activation of PI3K and RalGDS, activated Erk phosphorylation to a similar extent as K-Ras^{12V} but lacked transformative capacity in T80 cells. Furthermore, specific depletion of either Raf1 or MEK1/2 by siRNA or pharmacologic inhibition of MEK by U0126 did not affect Ras mediated transformation. Taken together, these results suggest that ovarian cancer patients with ovarian subtypes typically devoid of BRAF mutations, and whose tumors harbor activating Ras mutations, might not benefit from Raf or MEK inhibitors currently in clinical use.

In summary we have described, for the first time, the essential pathways for Ras transformation in human ovarian surface epithelial cells. We have reduced these signaling requirements to activation of RalA, RalB and Akt1/2 and have further demonstrated that specific inhibition of these proteins by either small interfering RNA or by pharmacologic

inhibition is potentially limiting to Ras transformation of an experimentally derived ovarian model. These results validate the Ral-GDS/Ral and the PI3K/Akt pathways as targets for developing novel anti-cancer drugs to combat ovarian cancer. To this end, we have recently identified a selective Akt activation inhibitor, triciribine monophosphate or API-002, which will soon enter phase II clinical trials (50). Similarly, our GGTase I inhibitor, GGTI-2418, is undergoing advanced preclinical studies and will soon enter phase I clinical trials. Finally, while we have isolated the Ras signal transduction pathways required for ovarian transformation whether activated RalGEFs and/or PI3K isoforms can independently transform ovarian surface epithelial cells remains unknown and could further define the central requirements for human surface ovarian epithelial cell oncogenesis.

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Chapter 4

Discovery of a Proposed Database of Ral Interacting Proteins: RACK1 Binds Ral and is Required for H- and K-Ras Mediated Transformation

By

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Abstract

RalA and B proteins mediate Ras malignant transformation in multiple human epithelial cell types and have been determined to be independently transforming in a tissue specific manner. However, very little is known about the protein-binding partners that govern Ral A and B biological activities. Here, we have used a proteomics approach to describe a proposed database of novel Ral interacting proteins. Despite a greater than 85% sequence similarity RalA and RalB interact primarily with different proteins. However, the proposed database also contains common interacting partners. For example, we have identified and validated RACK1/GBLP as a novel RalA and RalB interacting protein. Specifically, we have determined that RACK1 interacts with ectopically expressed RalA and RalB as well as with endogenous RalA. Furthermore, depletion of RACK1 by siRNA phenocopies the effects of RalA and RalB depletion in reducing the ability of oncogenic H-Ras^{12V} and K- Ras^{12V} to transform human ovarian epithelial cells. These results both expand the number of proposed Ral A and B protein binding partners. They also led us to identify a specific protein interaction with RACK1 and identify RACK1 expression as a central requirement for both H-Ras^{12V} and K- Ras^{12V} transformation of human ovarian surface epithelial cells.

Introduction

One of the best studied mechanisms of oncogenesis in human cancer is mutational activation of members of the Ras GTPase gene family. Mutations in Ras gene family members (H-, K-, and N- Ras) occur in approximately 30% of human cancers (6). Mutation of Ras isoforms has been associated with progression of numerous types of human epithelial cancer such as pancreatic, lung, ovarian and breast (6, 16, 19). Recently, we (Falsetti and Sebti manuscript in submission) and others (18, 37) have used genetically defined models of human epithelial transformation to describe the tissue specific mechanisms of Ras transformation (18, 37). In these models, sequential inactivation of the tumor suppressors p53 and retinoblastoma (Rb) protein by ectopic expression of SV40-T-antigen (SV40-T), coupled with stable expression of human telomerase catalytic subunit (hTERT) in human epithelial cells recapitulates the critical events in the development of human neoplastic disease and creates a minimally transformed model for the study of oncogenic signaling pathways (15-17, 19). Addition of constitutively activated H- or K- Ras^{12V} results in a robust transformation response and mimics many of the pathological events of tumor progression (15-17, 19, 28). Multiple studies have used a combination of pharmacological and genetic approaches to determine the tissue specific requirements for Ras transformation. Interestingly, all of these studies indicate that the Ras/ Ral guanine nucleotide dissociation stimulator (RalGDS)/Ral signaling pathway is required for human epithelial cell transformation by oncogenic Ras. Both Counter and Weinberg's groups have separately reported that in sequentially transformed human embryonic kidney (HEK-HT) cells RalGDS/Ral signaling is required for Ras transformation (18, 37). Counter's group has specifically defined a RalGDS/RalA

signaling axis which is independently transforming in this system while Weinberg's group has determined that Ras requires both RalGDS and phosphatidylinositol 3,4,5-triphosphate (PI3K) signaling for transformation in a similar, independently derived, human embryonic kidney cell line. These proposed mechanisms of Ras transformation are not common to all human epithelial cell lines; for example, in a genetically defined model of human mammary tumorigenesis, the human mammary epithelial cell line (HMEC), Ras transformation requires activation of at least three downstream effector pathways: RalGEF-Ral, PI3K-Akt and Raf-Mek (37). We have previously used a genetically defined model of human ovarian cancer, human ovarian surface epithelial cells (T80), to determine that H- and K-Ras^{12V} transformation of human ovarian cells requires RalGEF-Ral and PI3K-Akt, but not Raf-Mek, signaling. In further confirmation of a role for the Ral small GTPases in oncogenesis both RalA and RalB have been identified as critical mediators of multiple tumorigenic processes, including metastasis, invasion, anchorage-independent growth, survival and cell motility (8, 11, 34, 42, 43, 46). Specifically, depletion of RalA by siRNA has been shown to inhibit anchorage-independent proliferation of multiple human cancer cell lines, such as the cervical and prostate cancer cell lines HeLa and SW680 (8) as well as multiple human pancreatic cancer cell lines (27). Also, RalA has also been shown to be required for anchorage independent growth of sequentially Ras transformed human ovarian (Falsetti and Sebt, manuscript in submission) and kidney cells (26). Similarly, stable depletion of RalA, but not RalB, by shRNA has been shown to inhibit tumor formation and metastasis of multiple human pancreatic cancer cell lines in athymic nude mice (27). Further confirming a role for RalA in metastasis, stable overexpression of constitutively activated

RalA has been shown to promote both standard and experimental metastasis *in vivo* (41, 42) and in human prostate cancer cells stable expression of activated RalA promotes bone, but not brain, metastasis (47). Even in non-epithelial cancers there is emerging evidence that RalA is involved in tumorigenicity; for example, in human HT1080 fibrosarcoma cells stable overexpression of activated RalA promotes anchorage-independent growth (46). In agreement with these findings two cellular processes thought to promote metastasis and anchorage independent growth, cell motility and invasion, are inhibited by siRNA mediated depletion of RalA in multiple human renal cancer cell lines (34).

While RalA has been well validated to play an essential role in anchorage independent processes in multiple tissue types the role of RalB has been determined to be far more tissue specific. For example, while depletion of either RalA or RalB inhibits anchorage independent growth in human ovarian epithelial cells transformed by either H- or K-Ras^{12V} (Falsetti et al, manuscript in submission) only depletion of RalA but not RalB inhibits H-Ras^{12V} transformation of HEK-HT cells (26). We, as well as others have previously determined a role for RalB, but not RalA in the survival of multiple human cancer cell lines (8, 11). Specifically, White's group has found that depletion of RalB by siRNA induces apoptosis in both HeLa and SW680 human cancer cell lines (8). Similarly, we have previously determined using a chemical biology approach that a RalB, but not RalA, survival pathway underlies the apoptotic response to geranylgeranyltransferase I inhibitors (GGTIs) (11). Specifically, by ectopically expressing a GGTI-resistant RalB mutant in human pancreatic carcinoma cells (MiaPaCa2) we have shown that GGTI-induction of apoptosis requires inhibition of

RalB, but not RalA, processing (11). Taken as a whole, these studies strongly suggest a role for RalB tumor survival in a wide array of tissue types as well as a tissue specific role in anchorage independent growth.

Despite the wealth of information regarding the role of Ral small GTPases in transformation very little is known about the pathways through which Ral exerts these effects. For example, while RalA and RalB bear a high similarity to Ras over 20 Ras effectors (6, 39), but only 6 Ral effectors have been described to date. RalA and/or RalB have been previously shown to interact with phospholipase D1 (PLD1), the exocyst components Sec5/ Exo84, Filamin A, ZO-1 N-terminally associated binding protein (ZONAB) and Ral binding protein-1 (RalBP1/RLIP) (12). Precise roles for these proteins in the various transformation specific processes that are regulated by RalA and RalB remain poorly defined. Further underscoring the need for an understanding of Ral protein interactions, while RalA and RalB are commonly thought of as Ras effector proteins both RalA and RalB are found in the hyperactivated state independently of Ras in human pancreatic tumors (26, 27). Thus novel means of Ral activation and inactivation may constitute an important and undescribed mechanism of transformation. However, no Ral GTPase activating proteins (RalGAPs), which would negatively regulate RalA and RalB, have been described to date. Also, RalA and RalB are known to regulate diverse physiological processes such as signaling via STAT3, NF-KB, JNK and AFX (12). However, the intermediate proteins in these signal transduction pathways through which RalA and RalB exert these functions remain unknown.

In an effort to more fully understand the protein interactions that govern the biological activities of RalA and RalB we have used proteomic analysis to describe a

proposed database of Ral interacting proteins. We have uncovered several potential RalA and RalB interacting partners and used a systems biology approach to analyze the themes that emerge from this proposed database. We have further characterized one of these proteins, receptor for activated C-kinase-1 (RACK1, also known as Guanine nucleotide binding protein-1 [GBLP1]), as a novel RalA and RalB interacting protein that is required for both oncogenic H- and K- Ras mediated transformation of human ovarian surface epithelial (T80) cells. In addition to uncovering a critical role for RACK1 expression in both H- and K-Ras mediated transformation this study also constitutes the first large-scale analysis aimed at uncovering the biological differences and similarities between the Ral isoforms and provides an important database of potential proteins through which RalA and RalB may mediate their biological functions.

Materials and Methods

Cells and culture—Human ovarian surface epithelial cells T80 and T80H were cultured as described previously (28). The T80K stably expressing cell lines were maintained in similar culture conditions as the T80 and T80H cells. Human HEK-293T cells were a kind gift from Dr. Gary Reuther and were maintained in Dulbecco's modified minimum essential media (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 10% CO₂ and 37°C.

Immunoprecipitation of FLAG-tagged protein – FLAG-Ral proteins were isolated using FLAG-agarose beads according to the manufacturer's recommendations (Sigma-Aldrich, St. Louis, MO). Briefly, transfected cells were lysed using Cell Lytic-M lysis buffer (Sigma-Aldrich) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and lysates were incubated overnight with FLAG-agarose beads at 4°C while rocking; after incubation the beads were washed four times with an excess of lysis buffer. Subsequently, samples were boiled at 100°C for 10 minutes in 2X Laemmli sample buffer to elute bound proteins.

Immunoprecipitation of RalA protein – Endogenous RalA was isolated using anti-human RalA mouse monoclonal antibody RalA (61022, BD Biosciences Pharmingen, Franklin Lakes, NJ). Briefly, T80, T80H or T80K cells were lysed using Cell Lytic-M lysis buffer (Sigma-Aldrich, St. Louis, MO) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and lysates were incubated overnight with anti-RalA antibody at 4°C while rocking; after incubation protein A anti-IgG agarose beads were added and incubated for 2 hours at 4°C then washed four times with an excess of lysis buffer.

Following this, samples were boiled at 100°C for 10 minutes in 2X Laemmli sample buffer to elute RalA and RalA binding proteins.

Identification of Ral- interacting proteins – Following immunoprecipitation and SDS-PAGE, gel bands were visualized using GelCode Blue (Promega, Madison, WI), excised and washed once with water and twice with 50 mM ammonium bicarbonate in 50% aqueous methanol. Samples were digested overnight with modified sequencing grade trypsin (Promega, Madison, WI). Peptides were extracted from the gel slices and concentrated under vacuum centrifugation. A nanoflow liquid chromatograph (LC Packings/Dionex, Sunnyvale, CA) coupled to an electrospray ion trap mass spectrometer (LTQ Orbitrap, Thermo, San Jose, CA) was used for tandem mass spectrometry peptide sequencing experiments. Peptides were separated with a C18 reverse phase column (LC Packings C18Pepmap, 75 um ID x 15 cm) using a 40 minute gradient from 5% B to 50% B (A: 2% acetonitrile/0.1% formic acid; B: 90% acetonitrile/0.1% formic acid). Five tandem mass spectra were acquired for each MS¹ scan (spray voltage 2.5 kV, 30% normalized collision energy, scanning m/z 450-1,600). Sequences were assigned using Sequest (Thermo) and Mascot (www.matrixscience.com) database searches against NCBI or SwissProt protein entries of the appropriate species. Oxidized methionine, deamidation, and carbamidomethyl cysteine were selected as variable modifications, and as many as 2 missed cleavages were allowed. Assignments were manually verified by inspection of the tandem mass spectra and coalesced into Scaffold reports (www.proteomesoftware.com). RalA and RalB interacting proteins were identified from this initial list by subtractive analysis of the vector transfected cells. Specifically, to qualify as a proposed FLAG-Ral interacting partner the protein had to fulfill one of two

criteria: the protein had to be identified by at least 2 peptides at greater than 94% probability in at least one of two experiments and could not be present in any of the three vector-transfected controls; or the protein had to be identified by at least 2 peptides at greater than 94% probability in both experiments and could not be present in more than one of three vector transfected controls.

Small interfering RNA (siRNA) sequences- SiRNA sequences targeting RalA . RalA(5'-GACAGGUUUCUGUAGAAGAdTdT-3') and RalB (5'-GGUGAUCAUGGUUGGCAGCdTdT-3') were purchased from Dharmacon using previously described sequences(8). Pre-designed chemically synthesized siRNA targeting RACK1 (Cell Signaling Technology Inc., Danvers, MA) were used according to manufacturer's recommendation.

Small interfering RNA (siRNA) transfection procedure- Cells were grown to 50% confluence and transfected with Oligofectamine transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, 5 µl of Oligofectamine reagent was suspended per 1mL of OPTI-MEM medium (Invitrogen, Carlsbad, CA) and allowed to equilibrate at 24-27°C. A 100 nM final concentration of siRNA was suspended and complexed with the liposomes. Cells were briefly washed with OPTI-MEM and the transfection mix was plated on top of the cells and incubated at 37°C. Following an 8 h incubation, 2mL of complete growth medium, without penicillin-streptomycin, was added and the cells were further incubated at 37°C overnight. The media was replaced after 24 h with complete growth media for 24 hours. Experiments were performed in triplicate.

Western blotting—Cells were harvested, and lysed in HEPES lysis buffer as described previously (11). Proteins were then resolved by 11.5% SDS-PAGE gel and immunoblotted with antibodies against phosphorylated-Ser473 Akt1/2 (9217, Cell Signaling Technologies Inc., Danvers, MA), phosphorylated-Thr202/Tyr 204 Erk1/2 (9101, Cell Signaling Technologies Inc., Danvers, MA), Akt1/2 (N-19, Santa Cruz Biotechnology, Santa Cruz, CA), Erk1/2 (p44/p42 MAP Kinase, 9102, Cell Signaling Technologies Inc., Danvers, MA), RalA (61022, BD Biosciences Pharmingen, Franklin Lakes, NJ), H-Ras (C-20, Santa Cruz Biotechnology, Santa Cruz, CA), K-Ras (F234, Santa Cruz Biotechnology, Santa Cruz, CA), RalB (04037, Millipore, Billerika, MA), β -actin (AC15, Sigma-Aldrich, St. Louis, MO) and anti-FLAG M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO). The ECL blotting system (NEN Life Science Products, Boston, MA) was used for detection of positive antibody reactions. Experiments were performed in triplicate.

Soft agar clonogenicity assay- For soft agar growth assays, the cell lines were seeded at a cell density of 5000/well in triplicate in 12-well culture dishes in 0.3% agar over a 0.6% bottom agar layer as previously described(11). Cultures were fed once weekly until colonies grew to a suitable size for observation (approximately 14 days). Colonies were photographed after overnight incubation with 1 mg/ml MTT in cell growth media. The colony number was visually determined and quantified. Experiments were performed in triplicate.

Results

Use of an ectopically expressed FLAG-tagged Ral-Tandem MS system to isolate and identify novel Ral binding partners: Determination of a potential database of Ral interacting proteins

Although RalA and RalB are required for mutant Ras malignant transformation in a variety of human cancers, the mechanism by which they mediate Ras transformation is not known. This prompted us to identify a database of potential RalA and RalB interacting proteins with the ultimate goal of discovering effectors of Ral A and Ral B that mediate Ras transformation. To this end we ectopically expressed N-terminal FLAG-tagged RalA^{72L} and RalB^{72L} in human embryonic kidney cells (HEK-293T) then isolated potential Ral-interacting proteins through subsequent FLAG immunoprecipitation, separation of protein complexes by SDS-PAGE and protein identification in the various gel segments by tandem MS and subsequent SCAFFOLD analysis as described under Material and Methods. Figures 17A and 17B show that transient transfection resulted in high levels of expression of FLAG-RalA and RalB, respectively, as demonstrated in both whole cell lysate as well as FLAG immunoprecipitates as detected by both Ral antibody western blots and GelCode Blue stain

Figure 17. Expression and immunoprecipitation of FLAG-Ral^{72L}

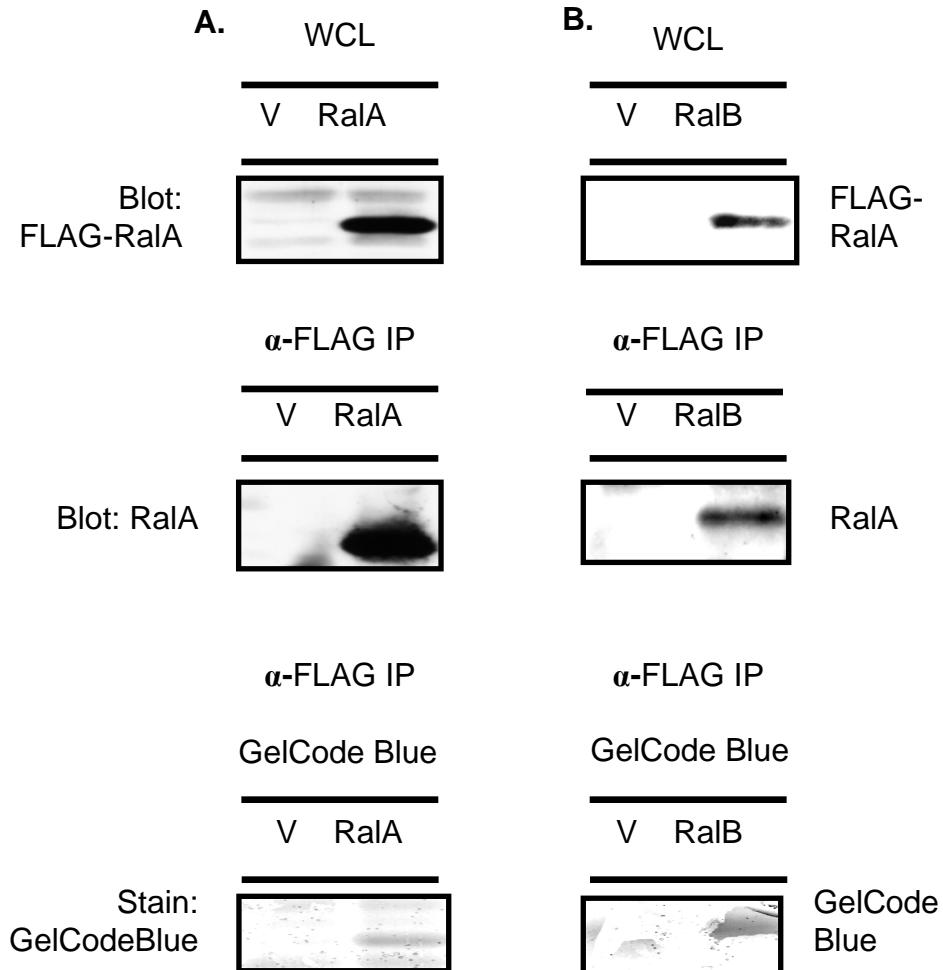


Figure 17. Expression and immunoprecipitation of FLAG-Ral^{72L}. Retroviral pBABE plasmids containing either RalA^{72L} (A) or RalB^{72L} (B) along with an empty vector sequence were transiently transfected into HEK-293T for 48 hours. Cells were collected, lysed, and immunoprecipitated following which protein was analyzed for western blot analysis or GelCode Blue.

Using a proteomic approach we have identified 68 RalA and 28 RalB potential interacting proteins. Of these the known Ral-interacting protein Filamin A, as well as the closely related Filamin C, were identified as potential RalA and RalB interacting partners in this system (see tables 2 and 3, following pages). Additionally, other predicted members of known Ral interacting complexes, such as ZO-1 and SC22B, were also identified as potential Ral-interacting proteins (see tables 2 and 3, following pages). These results provide evidence in support of the overall feasibility of our approach in identifying both proposed direct, as well as indirect, Ral-interacting partners.

Table 2. Proposed RalA interacting proteins (pt1)

RalA SCAFFOLD Results			
Gene name	Protein ID	Peptides Matched (>94%)	
		Experiment 1	Experiment 2
14-3-3 Gamma	1433G	3	
6-phosphogluconate dehydrogenase	6PGD	2	
acyl coenzyme A-thioesterase-9	ACOT9	3	
alpha-centractin	ACTZ	2	
AP-3 complex subunit-Mu-1	AP3M1	2	
actin related protein-10	ARP10	2	
F-actin capping protein	CAPZB	3	
citrate sythase, mitochondrial precursor	CISY	4	
casein kinase II subunit-1	CSK1	3	
uncharacterized protein	CT116	2	
elongation factor 1B	EF1B	2	
protein FAM98B	FA98B	2	
flap endonuclease 1	FEN1	2	
filamin A	FLNA		5
filamin C	FLNC		2
guanine-nucleotide binding protein	GBLP	7	6
GTP-binding protein1	GTPB1		3
60S ribosomal protein S26	RS26		3
40S ribosomal protein S6	RS6	2	6
40S ribosomal protein SA	RSSA	7	2
reticulon-4	RTN4		3
vesicle-trafficking protein	SC22B	2	
spindlin-1	SPIN1	3	1
spindlin-3	SPIN3	2	
vesicle-associated membrane	VAPB	2	
vimentin	VIME		3
60S ribosomal L7A	RL7A	2	
40S ribosomal S16	RS16	1	2
40S ribosomal S17	RS17		2
40S ribosomal S20	RS20		2

Table 2. Proposed RalA interacting proteins (pt2)

RalA SCAFFOLD Results			
Gene name	Protein ID	Peptides Matched (>94%)	
		Experiment 1	Experiment 2
heterogeneous nuclear protein-F	HNRPF	2	3
heterogeneous nuclear protein-G	HNRPG	4	
isocitrate dehydrogenase (NADP)	IDHP	3	
eukaryotic translation initiation factor	IF4H	3	
interleukin enhancer factor 2	ILF2	10	
keratin type 1 cytoskeletal-18	K1C18	7	
keratin type 2 cytoskeletal-8	K2C8	2	
casein kinase I isoform epsilon	KCIE	2	6
creatine kinase mitochondrial precursor	KCRU	2	4
leucine rich repeat protein-47	LRC47		6
putative RNA-binding protein-7L	LUC7L	2	
phosphate carrier protein	MPCP	2	
negative elongation factor	NELFE	3	
negative elongation factor	NELFE	2	
protein NipSnap3	NPS3A	2	
proliferation associated protein-2G4	PA2G4	11	
phosphoglycerate kinase-1	PGK1	4	
prohibitin	PHB	5	
serine-threonine phosphatase-1B	PP1B		2
proteasome subunit alpha	PSA1	2	
26S proteasome regulatory subunit	PSD11	7	
26S proteasome non-ATPase	PSD13		2
26S proteasome regulatory subunit-8	PSMD8	2	
multifunctional protein ADE2	PUR6	5	
ralA	RALA	10	14
ran GTP binding protein	RAN	2	
reticulocalbin-1	RCN1	3	
60S ribosomal protein-10A	RL10A	5	
60S ribosomal protein-17	RL17	2	

Table 3. Proposed RalB interacting proteins

RalB SCAFFOLD Results			
Gene name	Protein ID	Peptides Matched (>94%)	
		Experiment 1	Experiment 2
14-3-3 epsilon	1433E	5	
neuroblast differentiation factor	AHNK		4
actin-like protein 2	ARP2		2
probable ATP-dependent RNA	DDX5		6
protein enabled homologue	ENAH	2	
filamin A	FLNA		3
filamin C	FLNC		3
guanine-nucleotide binding	GBLP	4	5
GTP binding protein	GTPB1		4
heterogeneous nuclear riboprotein	HNRPK	2	2
eukaryotic translation initiation	IF32		12
keratin, Type I cytoskeletal-16	K1C16	3	
creatine kinase	KCRU		4
nucleoporin-like 2	NUPL2		2
pyruvate dehydrogenase E1	ODPA		2
pyrroline-5-carboxylate	P5CR2	3	
poly(rC) binding protein-2	PCBP2		3
peroxiredoxin-1	PRDX1	2	2
RalB	RALB	4	6
60S ribosomal protein-18	RL18		2
heterogeneous nuclear	ROA1	3	1
40S ribosomal protein-S26	RS26		5
40S ribosomal protein-S6	RS6	2	7
splicing factor-S7	SFRS7	3	
tubulin beta-2 chain	TBB2C		2
vimentin	VIME		2
XPA-binding protein	XAB1		3
tight junction protein	ZO1		6

RACK1/GBLP interacts with ectopically expressed RalA and RalB in HEK-293T cells

One interesting protein our analysis revealed to be a potential RalA and RalB interacting proteins was guanine nucleotide-binding protein GBLP, also known as receptor for activated C-kinase1 (RACK1), which is a scaffolding protein able to interact simultaneously with several signaling molecules (32). In order to confirm and validate that RalA and RalB interact with RACK1 we ectopically expressed FLAG-tagged RalA^{72L} and RalB^{72L} in HEK-293T cells and used FLAG-affinity immunoprecipitation to isolate Ral protein complexes. Both RalA^{72L} and RalB^{72L} were expressed at high levels in HEK-293T cells (figs. 18A and 18B, following page). In confirmation of our MS/MS results both ectopically expressed RalA^{72L} and RalB^{72L} interacted with endogenous RACK1 (figs. 18A and 18B). These results demonstrate that a RACK1/Ral complex is formed within HEK-293T cells. While these results are strongly suggestive that Ral proteins interact with a RACK1 containing complex, if not directly with RACK1, we wanted to further validate this interaction using endogenous RalA.

Figure 18. RACK1/ GBLP interacts with ectopically expressed Ral^{72L}

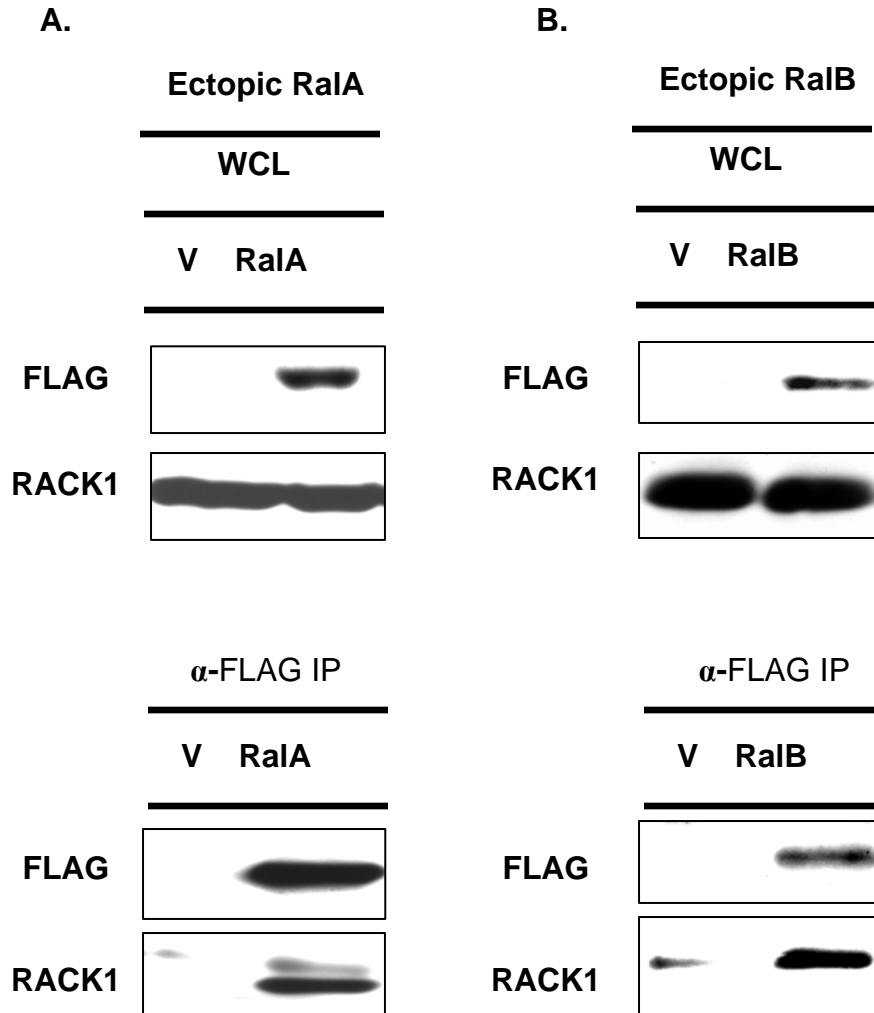


Figure 18. RACK1 interacts with ectopically expressed Ral^{72L}. Retroviral pBABE plasmids containing either RalA^{72L} (A) or RalB^{72L} (B) along with an empty vector sequence were transiently transfected into HEK-293T for 48 hours. Cells were collected, lysed, and immunoprecipitated following which protein was analyzed for western blot analysis or GelCode Blue.

RACK1/GBLP interacts with endogenously expressed RalA in ovarian cancer cells

We next evaluated whether RACK1 binds RalA in human cancer cells where Ral proteins are critical to Ras transformation. We have previously reported (Falsetti and Sebti, manuscript in submission) that RalA and RalB expression is required for both H- and K-Ras^{12V} transformation of human ovarian surface epithelial cells, which are sequentially immortalized by stable expression of human telomerase catalytic subunit (hTERT) and simian virus-40 large T-antigen (T80 cells). Therefore, we used T80 cells transformed with H-Ras12V (T80H) and K-Ras12V (T80K) to evaluate the binding of endogenous RACK1 to endogenous RalA protein and to subsequently determine the importance of these interactions to Ras-mediated transformation in this human ovarian cancer model. Figure 3 shows that in both the non-transformed and Ras-transformed T80 isogenic cell lines RalA strongly interacted with endogenous RACK1 but not with beta-actin (Figure 19, following page). Importantly, expression levels of RalA and RACK1 did not vary between cell lines. Also, since RalA is GTP-bound in both T80H and T80K (Falsetti and Sebti, manuscript in submission) we expected that if RalA binding to RACK1 was GTP-dependent there would be increased association in the Ras transformed cell lines. However, RalA binding to RACK1 appeared to be GTP-independent and only varied according to the levels of RalA isolated in each experiment.

Figure 19. RACK1/GBLP interacts with endogenous RalA protein

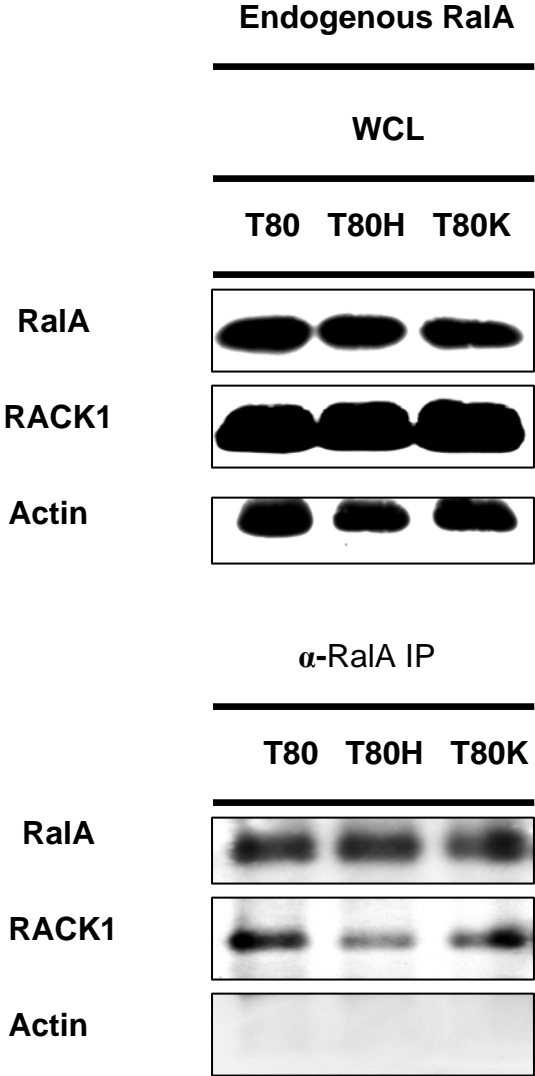


Figure 19. RACK1 interacts with endogenously expressed RalA. Protein lysate was collected from T80, T80H and T80K cells and used for immunoprecipitation of endogenous RalA using anti-RalA monoclonal antibody.

RACK1 depletion phenocopies the effects of Ral depletion in Ras transformed ovarian epithelial cells

Since we have previously characterized both RalA and RalB expression as being required for H- and K- Ras^{12V}-mediated transformation of T80 cells and since RalA and RalB were found to interact with RACK1 we endeavored to further define a role for RACK1 in Ras-mediated transformation of human epithelial cells. One widely used method of determining similar roles for proteins is to determine if the effects of depletion of one gene will phenocopy the effects of the other. To that end, we used siRNA to specifically and potently deplete T80H and T80K cells of RalA, RalB and RACK1 and then examined the effects of single depletion of each gene on Ras transformation. As demonstrated in figures 20A and 20B (following page) we were able to specifically and potently deplete the cells of RalA, RalB or RACK1 through transient transfection with siRNA targeting regions unique to each gene. Furthermore, depletion of RACK1 phenocopies the effects of Ral depletion on Ras transformation in both T80H and T80K cells. Specifically, RalA inhibited T80H clonogenicity in soft agar by 54.9% (p value <0.001) and T80K clonogenicity by 51.7% (p value <0.001). Depletion of RalB by siRNA inhibited T80H clonogenicity by 53.8% (p value <0.001) and T80K clonogenicity by 65.2% (p value <0.001). In support of a role for RACK1 in the Ral-dependent Ras-mediated transformation of T80 cells RACK1 depletion by siRNA inhibited T80H clonogenicity by 59.9% (p value <0.001) and T80K clonogenicity by 34.3% (p value <0.01).

Figure 20. RACK1/ GBLP depletion phenocopies the effects of Ral depletion in Ras-transformed ovarian epithelial cells

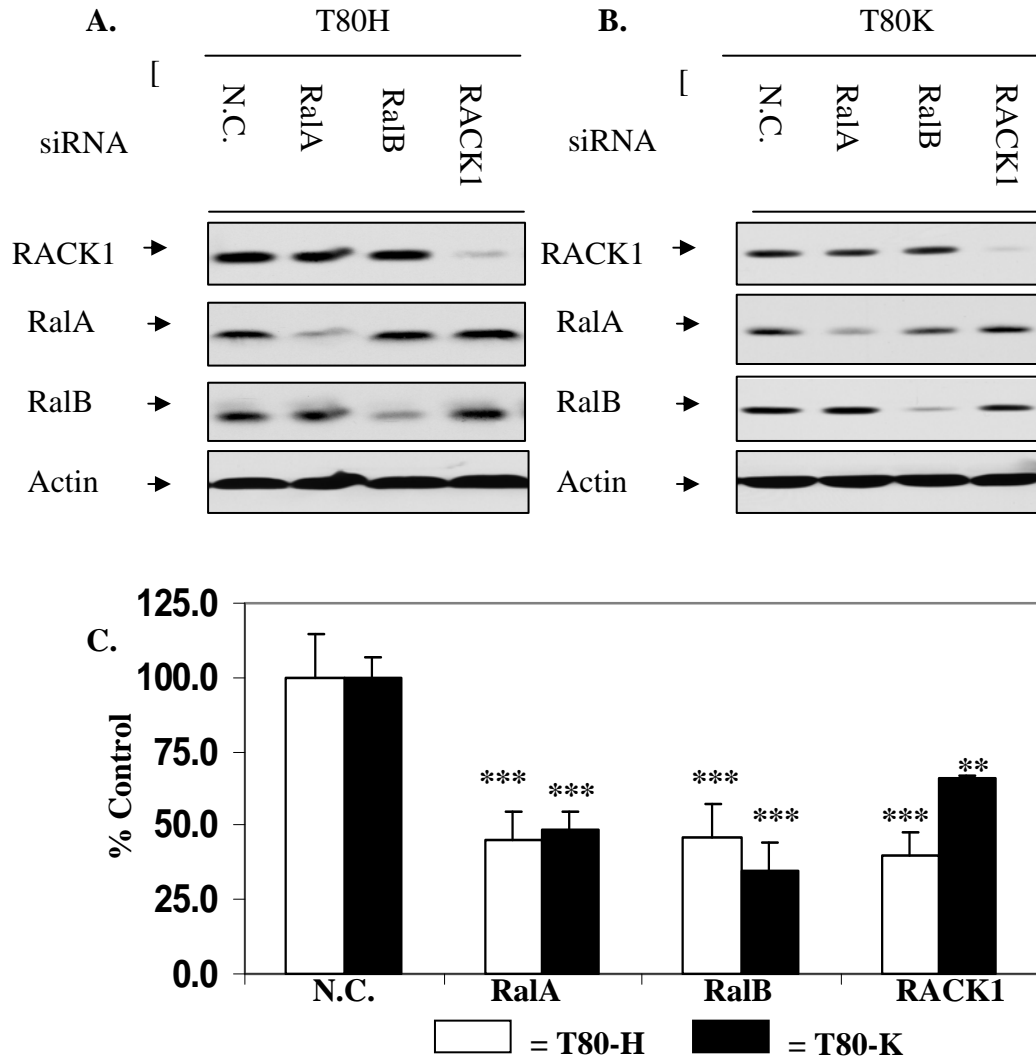


Figure 20 Ras transformation of T80 cells requires expression of RalA/B and Akt1/2 but not Raf1. Small interfering RNA (siRNA) specific to RalA, RalB, and RACK1 or a negative control siRNA were transfected for 48 hours into T80, T80H and T80K cells. Cells were collected, counted using trypan blue dye exclusion assay and collected for western blot analysis (A) and an equal number of cells were plated for soft agar assay (B) and scored manually at the end of two weeks; results are representative of three experiments each performed in triplicate.

Discussion

While Ral proteins are well known to be important components of Ras-mediated transformation of multiple human epithelial cell types very little is known about the protein-binding partners that govern Ral biological activity. Here, we have used a proteomics approach to describe, for the first time, a proposed database of novel Ral-interacting proteins. Despite a greater than 85% sequence identity RalA and RalB interact primarily with different proteins across a wide range of gene ontology sub-groups. Furthermore, we have identified and validated RACK1/ GBLP as a novel RalA, and RalB, interacting protein. Specifically, we have determined that RACK1 interacts with ectopically expressed RalA and RalB as well as with endogenous RalA. Furthermore, depletion of RACK1 by siRNA phenocopies the effects of RalA and RalB depletion in reducing the ability of multiple Ras isoforms to transform human ovarian epithelial cells. These results both dramatically expand the number of proposed Ral protein binding partners and identify a specific protein, RACK1, which is at least partially required for Ras transformative activity.

While use of proteomics technology has allowed us to rapidly identify a multitude of potential individual protein interactions with both RalA and RalB like all systems biology analyses confirmation and validation of these results will require extensive efforts to individually characterize each of these potential partners. Nevertheless, we felt a further analysis of these potential interacting partners by sorting these proteins using SWISS-PROT GO annotation would be helpful in revealing potentially emergent biological themes in RalA and RalB biological function. In particular, these analyses revealed cytoskeletal regulation as a potential biological process that could be governed

by both RalA and RalB. Importantly, both RalA and RalB potentially interacted with multiple critical regulatory proteins with well-described roles in the regulation of this process (9, 13, 24, 40). Our proteomics analysis suggests that both RalA and RalB interact with both Filamin A (FLNA) and Filamin C (FLNC), known regulators of the cytoskeleton. Importantly, RalA has been previously shown to interact with Filamin A in a GTP-dependent manner (33). The filamin family consists of three genes: Filamin-A, -B, and -C; of these Filamin-A is the most well understood. (13). Filamins play a critical role in cross-linking the cortical actin cytoskeleton into orthogonal networks and link these three dimensional actin structures to the inner leaflet of the cellular membrane (40). Furthermore, Filamins modulate the response of cells to their extra-cellular environment by regulating changes in shape and motility (38). Interestingly, our analyses revealed vimentin (VIME, see tables 2 and 3), an important intermediate filament in mesenchymal cells which is commonly overexpressed in cancer cells as part of the epithelial to mesenchymal transition (22), to be a potential RalA and RalB interacting protein. Another potential RalB associated protein. tubulin beta-2 chain (TBB2C, see table 3), is known to form a supramolecular complex with vimentin (9). Certainly, a further analysis of the roles of vimentin and filamin in Ral-driven actin cytoskeletal changes would be warranted given these results.

We validated RACK1 as interacting with both ectopically expressed RalA^{72L} and RalB^{72L} as well as with endogenous RalA in an activation state independent manner. Interestingly, there exists a paradigm for small GTPase interaction with RACK1. Rac2, a small GTPase highly homologous to RalA and RalB, has been previously shown to interact indirectly with RACK1 and this interaction has been shown to regulate natural

killer cell adhesion (30). This is particularly interesting given that Ral negatively regulates Rac proteins via the Rac-GAP protein RalBP1 (23, 35). Ral and Rac proteins regulate similar processes such as cell motility, transformation, invasion, cell-cell adhesion and actin reorganization (4, 5, 12, 44). Perhaps, the ability of RalA and RalB to subvert Rac processes occurs via both negative regulation of Rac GTP levels and through subsequent association with Rac downstream effectors such as RACK1.

We have also demonstrated, using siRNA, that RACK1 expression was at least partially required for both H- and K-Ras^{12V}-mediated transformation of T80 cells and that the effects depletion of RACK1 was similar to the effects of RalA or RalB depletion. This is consistent with previous work showing that a putative dominant negative isoform of GBLP (or RACK1) has been previously shown to reverse K-Ras^{12V} transformation in NIH-3T3 cells (2) and to restore contact inhibition and stress fiber formation (3). In contrast, paradoxically, overexpression of wild-type RACK1 in transformed NIH-3T3 cells reduces both anchorage-independent and –dependent proliferation (2). Nevertheless, other studies support the implication of RACK1 in oncogenesis. For example, studies have used antisense siRNA to inhibit RACK1 expression in NIH-3T3 cells and have demonstrated a requirement for RACK1 in cell spreading and cell proliferation (20). Furthermore, RACK1 expression is dramatically increased in both human carcinomas and during human ovarian morphogenesis (1).

In addition to playing apparently contradictory roles in anchorage independent proliferation, RACK1 is known to play a role in a variety of cell process such as coupling of signal transduction to control of protein transcription and translation (32), recruitment of ribosomes to local sites of translation (such as focal adhesions) (32), regulation of cell

spreading (3), cytoskeletal organization (20), and cell cycle progression (7). RACK1 associates with a variety of signal transduction molecules that may account for some of these biological activities, such as Protein Kinase C- β (PKC- β), β -integrin and c-Src (29).

Most interestingly, multiple mechanisms exist through which RACK1 and RACK1- interacting proteins, such as PKC isoforms, may regulate Ras. RACK1 has been previously shown to associate with p120RasGAP (24), a well-characterized negative regulator of Ras activity (31), though the exact significance of the RACK1 interaction in RasGAP biological function has not yet been established. RACK1 is a well-characterized regulator of multiple PKC isoforms (29), which are known to regulate Ras guanyl nucleotide releasing proteins (RasGRPs), positive regulators of Ras signaling (14). PKC phosphorylation of K-Ras in the hypervariable C-terminal region results in accumulation of K-Ras in the outer mitochondrial membrane and promotes apoptosis (36). Additionally, phorbol esters, which activate PKC isoforms, are known to cooperate with Ras in transformation and deregulation of diacylglycerol (DAG) has been observed in Ras-transformed cells, indicating that Ras and PKC might cooperate during transformation (10, 21, 25, 45). Certainly, our results support a role for RACK1 expression in regulating Ras transformation of human ovarian surface epithelial cells and suggest that further study of both the Ral/RACK1 association and the role of RACK1 in human ovarian epithelial cell transformation will prove to be interesting avenues of future research.

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Chapter 5
Summary and Implications

By

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In this thesis we have determined novel roles for RalA and RalB in human ovarian neoplasia, defined a database of potential Ral interacting proteins, validated RACK1 as a novel Ral interacting protein which is required for Ras ovarian transformation. Furthermore, in the second chapter of this thesis we have used an elegant experimental chemical biology rescue system to determine the roles of inhibition of RalA and RalB prenylation in the GGTI-anti-cancer mechanisms of action. Here we determined that both RalA and RalB are validated targets of GGTIs and that inhibition of RalA prenylation is required for GGTI-inhibition of anchorage independent growth whereas inhibition of RalB prenylation is required for GGTI-mediated suppression of proliferation and induction of apoptosis. Rescue of the anti-proliferative and pro-apoptotic effects of GGTI-2417 by expression of GGTI-resistant RalB also rescued from induction of p27kip1, a well known CDK inhibitor of the cell cycle, and suppression of survivin, a well known inhibitor of apoptosis.

Future work will undoubtedly further define a role for RalB in regulation of the survivin gene expression. Data not presented in this thesis certainly indicate the importance of RalB in regulating survivin expression. Specifically, we have depleted MiaPaCa2 pancreatic cancer cells of survivin by Ral B but not Ral A siRNA. This depletion has dramatically reduced the level of survivin expression. Surprisingly, depletion of RalB by siRNA does not result in an induction of p27kip1. However, this illustrates the complex mechanism of action of GGTIs. GGTIs inhibit phosphorylation of Akt in a non-Ral dependent manner. This is relevant in that Ras governs p27kip1 expression in two synergistic pathways: Akt dependent phosphorylation of the AFX transcription factor and Ral dependent phosphorylation of AFX. These two

phosphorylation events regulate, respectively, the nuclear import and DNA binding activity of AFX, a well characterized regulator of p27kip1 transcription. Further work should establish if dual inhibition of Akt and RalB expression is sufficient to induce p27kip1 expression and if GGTIs affect AFX phosphorylation and transcriptional activity. Certainly, these results suggest new avenues for research in the GGTI anti-tumor mechanism of action.

In Chapter 3 of this thesis, we have both discovered new roles for, and reaffirmed the importance of, both RalA and RalB in human cancer. We have defined Ral, as well as Akt, as central mediators of Ras-driven ovarian oncogenesis. In contrast to previous systems, both RalA and RalB were found to be required for ovarian transformation. Surprisingly, Raf1 and Mek1/2 are not required for Ras-transformation in this system. Indeed, K-Ras mutants incapable of activating Raf/Mek/Erk signaling were still able fully recapitulate the same degree of transformation as fully active oncogenic K-Ras. These results suggest that therapeutic intervention in ovarian cancer may be greatly assisted by future efforts to target RalGEF/Ral and PI3K/Akt signaling.

There remains much work to be done in defining a role for Ral signaling in ovarian cancer development. Of particular interest, the lack of a role for Raf-1 signaling in this system suggests that the T80 cell system is a more appropriate model for ovarian cancer sub-types such as mucinous ovarian and high-grade serous ovarian but not low-grade serous ovarian. Low-grade serous is characterized by a non-overlapping pattern of K-Ras and B-Raf mutation which can be interpreted as clear evidence that these two genes operate in a redundant fashion. However, the incidence of K-Ras mutation in high-grade serous and mucinous coupled with a complete lack of B-Raf mutations and

strikingly different disease etiology, as compared to low-grade serous, indicates non-Raf oncogenesis may determine the progression of these neoplasms. In order to more fully expand on this concept further work will need to determine a role for B-Raf in Ras transformation. Furthermore, further work will need to determine a role for individual RalGEFs and PI3K/Akt genes in promoting transformation of T80 cells. If these cells are capable of forming tumors in intra-ovarian nude mouse xenograft models, it will be of great interest to determine which sub-types of ovarian cancers these cells will most closely resemble. Also, assessing patterns of Ral activation and potential mutation of RalGEFs in human ovarian tumors, divided by subtype, could serve as further evidence of a role for Ral in ovarian cancer.

In the fourth chapter of this thesis we have used a subtractive proteomics method to determine a database of potential Ral interacting proteins. One of these proteins, RACK1, was validated as a Ral interacting protein. The expression of RACK1 was required for both H- and K-Ras oncogenesis in T80 cells, a system where RalA and RalB are also required. These results indicate a role for RACK1 in Ras-driven ovarian oncogenesis and, potentially, in the activity of Ral proteins.

Interestingly, RACK1 is a pleiotropic scaffolding molecule which interacts with other small GTPases, such as Rac1, and serves as a platform for PKC isoforms to interact with target substrates. The provocative question then is does RACK1 serve as a platform for Ral phosphorylation by PKC? If so what is the role of phosphorylation of Ral? These questions are made all the more relevant by the observation that Aurora A phosphorylates RalA and that phosphorylation regulates Ral localization and function. Similarly,

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Samuel C. Falsetti received his bachelor's degree from the University of Tampa in 2002 where he majored in Biochemistry, as well as Marine Biology. Prior to working in the Sebti lab he has worked in the labs of Ping Dou, Ph.D and Gary Litman, Ph.D. While at USF he has published three papers and has two more in various stages of submission. He has served as the chief financial officer for the Association of Medical Science Graduate Students and is currently the Director of Student Recruitment for the Florida Chapter of the American Medical Writing Association. He has received awards for excellence in research from USF Health and the USF-IGERT program. Currently, he is employed as Associate Scientific Director for a medical communications company. He enjoys traveling, spending time at home with his wife and (crazy) pets, as well as reading Dilbert.