EVALUATION OF CHRONIC RALGTPASE ACTIVATION AS A CORE SPECIFIER OF ONCOGENIC TRANSFORMATION

APPROVED BY SUPERVISORY COMMITTEE

Michael A. White, Ph.D.

Lily Huang, Ph. D.

John D. Minna, M.D.

Lawrence Lum, Ph.D.

DEDICATION

This work is dedicated to my family, who give their endless support and encouragement across the Pacific Ocean to me. I also want to thank my husband Jen-Chieh, for always being there for me, and share, discuss every aspect in our life with me.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Michael White, for his inspiring guidance and advice through the entire study. I also very appreciate his admirable patience and generous support that allow me to develop the story and finally have some progression. I would also like to thank my committee members, Dr. Huang, Dr. Lum, and Dr. Minna, for their precious time and suggestions to this work. I am grateful to all the white lab members and my friends in cell biology department for sharing thoughts, experiences, discussions as well as reagents; particularly Yu-Chen, Yi-Hung and Pei-Ling, who helped me with countless problem solving and shared useful techniques with me. I am also very thankful to Jen-Shuan, Jess, Wan-Jin, Ron, René, John, and Sungsoo for their support and friendship. I would also like to specially thank my previous mentor, Dr. Tzuu-Shuh Jou, who is formidably patient and disciplined. Without his mentoring, I could not have gone so far. Lastly, I owe my deepest appreciation to my husband and my parents, who never rest a single day to deal with my caprice and love me unconditionally. It is them to make my life complete.

EVALUATION OF CHRONIC RALGTPASE ACTIVATION AS A CORE SPECIFIER OF ONCOGENIC TRANSFORMATION

by

TZULING CHENG

DISSERTATION / THESIS

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

December, 2008

EVALUATION OF CHRONIC RALGTPASE ACTIVATION AS A CORE SPECIFIER OF ONCOGENIC TRANSFORMATION

Tzuling Cheng, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2008

Michael A. White, Ph.D.

Ral (RAS-Like) GTPases, RalA and RalB, were originally identified based on sequence similarity to Ras and are directly activated via the Ras effector family Ral guanine nucleotide exchange factors (RalGEFs). Previous studies have demonstrated that RalA and RalB collaborate to maintain tumorigenicity through regulating both proliferation and survival. Remarkably, RalB is specifically required for survival in Ras-dependent tumor cells rather than normal cells, while RalA is required for anchorage-independent proliferation but dispensable for survival. However, the spectrum of cancer cell lineages dependent upon Ral functions for tumor formation is currently unknown. We examined whether Ral pathway activation is required for proliferation of cancer cells

with activated Ras, Raf, or PI3K. Our data indicate that the Ral pathway is aberrantly activated and required for maintaining tumorigenicity of cancers that are driven by oncogenes other than Ras. In order to begin to understand how the Ral pathway may be chronically engaged in diverse oncogenic backgrounds, we further examined the expression of RalGEFs in a variety of cells derived from different tissue origin. Our results showed a divergent and complex distribution of RalGEFs among different cell types. In addition, through examination of historical tumor resequenceing efforts, we found several somatic mutations in RalGEFs, including RalGDS and RGL1. Through biochemical and cell biological studies, we find that the RGL1 mutations identified in human breast cancers are gain-of -function mutations, and found the mutations contribute to tumor cell survival through RalB pathway. Furthermore, we showed that chronic activation of RGL1 is sufficient to transform immortalized human mammary epithelial cells. Together, our data suggest RGL1 is a *bona fide* oncogene. These studies broaden our knowledge about RalGEF-Ral contributions in tumorigenicity, and provide a potential target for cancer therapeutic interventions.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Kim SC, Sprung R, Chen Y, Xu Y, Ball H, Pei J, **Cheng T**, Kho Y, Xiao H, Xiao L, Grishin NV, White M, Yang XJ, Zhao Y. Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. Mol Cell. 2006 Aug;23(4):607-18.

Cheng T, Ou Y and White M. Evaluation of chronic RalGTPases activation as a core specifier of oncogenic transformation. In progress.

Komurov, K., **Cheng, T.**, Rosenblatt, K., and White, M. A. 2008. Integrating functional genomics, proteomics and computational analysis for the characterization of cellular signaling networks. In preparation.

LIST OF FIGURES

LIST OF DEFINITIONS

AF-6 – ALL1 fused gene from chromosome 6

AKT – v-akt murine thymoma viral oncogene homolog 1

AP-1 – activator protein 1

ARF – ADP ribosylation factor

ATF-2 – activating transcription factor 2

BCAR3 – breast cancer anti-estrogen resistance 3

BrdU – 5-Bromo-2'-deoxy-uridine

CREB – cAMP responsive element binding protein 1

DAPI – 4',6'-diamidino-2-phenylindole

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

Elk-1 – member of ETS oncogene family

ERK – extra-cellular signal-regulated kinases

EWS – Ewing sarcoma

FACS – fluorescence-activated cell sorting

FOS – v-fos FBJ murine osteosarcoma viral oncogene homolog

FOXO1 – forkhead box O transcription factor 1

GAP – GTPase activating protein

GDP – guanosine 5'-diphosphate

GEF – guanine nucleotide exchange factor

GFP – green fluorescence protein

GSK3β – synthase kinase-3β

GTP – guanosine 5'-triphosphate

Grb2 – growth factor receptor bound protein 2

HA – hemaglutinin

HBEC – human bronchial epithelial cells

HMEC – human mammary epithelial cells

hTERT – human telomerase reverse transcriptase

JIP1 – JNK interacting protein 1

JNK – Jun NH_2-terminal kinase

MAPK – mitogen-activated protein kinase

MEK – mitogen-activated protein kinase kinase

Nck – non-catalytic region of tyrosine kinase adaptor protein 1

NF1 – neurofibromin

NF-kB – nuclear factor of kB

PDK1 – Phosphoinositide dependent kinase 1

PI3K – phosphatidylinositol 3-kinase

PIP_2 – phosphatidylinositol biphosphate

PIP_3 – phosphatidylinositol triphosphate

PH – pleckstrin homology

PLD – phospholipase D

PTEN – phosphatase and tensin homolog deleted on chromosome ten

qPCR – quantitative real-time PCR

RasGRP/RasGRF – Ras guanyl nucleotide-releasing proteins/factors

RalBP1 – Ral binding protein 1

RalGDS – ral guanine nucleotide dissociation stimulator

RalGPS – Ral GEF with PH domain and SH3 binding motif

RAD/RID – Ras association domain/Ras interacting domain

RASSF – Ras association (RalGDS/AF-6) domain family member

RBD – Ral-binding domain

RasGEF_N/REM – RasGEF N-terminal domain/Ras exchanger motif

RGL –ral guanine nucleotide dissociation stimulator-like

Rgr – RalGDS related protein

RNAi – RNA interference

siRNA – short interfering RNA

$SCF^{\beta TrCP}$ – ubiquitin protein ligase complex SCFs (SKP1-cullin-F-box) beta-transducin repeat containing

SH3 – Src homology 3 domain

SOS – son of sevenless

STAT3 – signal transducers and activators of transcription 3

ZO-1 – tight junction protein 1 (zona occludens 1)

ZONAB – ZO-1-associated nucleic acid binding protein

CHAPTER ONE

INTRODUCTION

Cancer and oncogenes

The homeostasis of cells reflects a dynamic balance of proliferation, differentiation and apoptosis. Cancers, however, arise from aberrant growth of cells that escape from the surveillance system. The possible causes and mechanisms of cancers have raised much debate and controversy, but finally converge to one widely accepted principle: the process of carcinogenesis is extremely complicated. The accumulated efforts from 70s to present have revealed that the development of cancer is a multistep process of acquired capability, including reduced dependency on exogenous growth stimuli, desensitized to growth inhibition, escape from apoptosis, infinite replication, vasculaturization, and finally invasion of adjacent tissue for metastasis (Hanahan and Weinberg 2000).

The onset of cancer requires mutations in genes controlling cell proliferation and survival. The mutated genes were soon identified as oncogenes, tumor-suppressor genes, and genes responsible for maintaining genome stability. Oncogenes encode proteins that control proliferation, cell survival or both. They can be activated by structure alteration resulting from mutation, truncation or gene fusion, e.g. B-Raf (Davies *et al.* 2002), EGFR (Arteaga 2002) and EWS gene in Ewing sarcoma (Riggi and Stamenkovic 2007). Activation of oncogenes can also occur by gene amplification, e.g. Myc, cyclin D1 and Ras (Hardisson 2003; Jilong Yang 2008) are frequently amplified in lung cancers, gastrointestinal cancers and head and neck cancers. However, while oncoproteins like

Myc or Ras accelerate cell proliferation, they simultaneously trigger or sensitize cells to apoptosis (Lowe 1999; Sherr 2001). This leads to the hypothesis that apoptosis might be a built-in failsafe mechanism to prevent inappropriate cell expansion (Evan and Littlewood 1998). Among these intricate regulations governing human tumorigenesis, it was further proposed by Douglas Green and Gerard Evan that "deregulation of cell proliferation together with suppression in apoptosis, create a platform that is both necessary and can be sufficient for cancers to arise" (Green and Evan 2002).

Ras oncoprotein and effector pathways

The first human oncogene was isolated from bladder carcinoma (Goldfarb *et al.* 1982; Pulciani 1982; Shih and Weinberg 1982) and identified as homologue of Harvey sarcoma virus *ras* gene (H-Ras) (Parada *et al.* 1982). Soon after that, K-Ras and N-Ras were found from lung carcinomas, neuroblastoma and leukaemia cell lines (Der *et al.* 1982; Hall *et al.* 1983). Sequence analysis revealed that the oncogenic alleles result from point mutations that affect the reading frame of various *ras* oncogenes (often causing substitutions in residue 12, 13,or 61) (Reddy *et al.* 1982; Taparowsky 1982). To date, activated *ras* is associated with around 30% of human cancers; moreover, the specific association between the various ras oncogenes and particular types of cancers were found. For instance, H-Ras mutations were predominantly found in bladder cancers while K-Ras mutations were frequent in pancreatic, colon and lung cancers and N-Ras mutations were linked primarily to lymphoid malignancy and melanomas (Karnoub and Weinberg 2008).

The activation of Ras proteins contributes to not only oncogenic transformation but also normal cell proliferation and differentiation. Ras proteins are members of the

superfamily of low molecular mass GTPases, which possess an intrinsic GTP hydrolysis activity. However, their activity is mainly regulated by GTPase-activating proteins (GAPs), which accelerate the GTP hydrolysis to a GDP-bound form (inactive state); and guanine nucleotide exchange factors (GEFs), which release the bound GDP allowing GTP binding (to an active state, figure 1.1). The activated mutations of Ras proteins mimic the GTP-bound form of Ras (Tong 1989) resulting in sustained stimulation of Ras pathway that promote cell proliferation and survival. Additionally, post-translational lipid modifications acting on the C-terminal CAAX motif of Ras was also found to be another key determinant for its activation (Hancock *et al.* 1990). This lipid processing is required for Ras compartmentalization by anchoring Ras proteins to the proper membranes (Chiu 2002; Matallanas 2006).

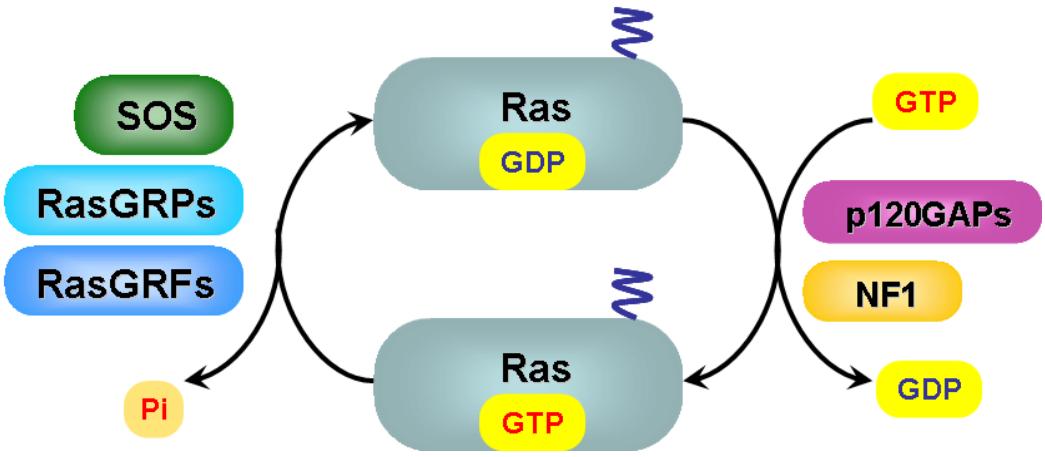


Fig. 1.1 The Ras GTPase cycle. The on-off cycle of Ras GTPase is tightly controlled by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). GAPs, such as p120GAP or neurofibromin (NF1); GEFs, such as son of sevenless (SOS) and Ras guanyl nucleotide-releasing proteins/factors (RasGRPs and RasGRFs) .

The downstream effector signaling of Ras mediating oncogenic transformation are most well known as the following (also see figure 1.2):

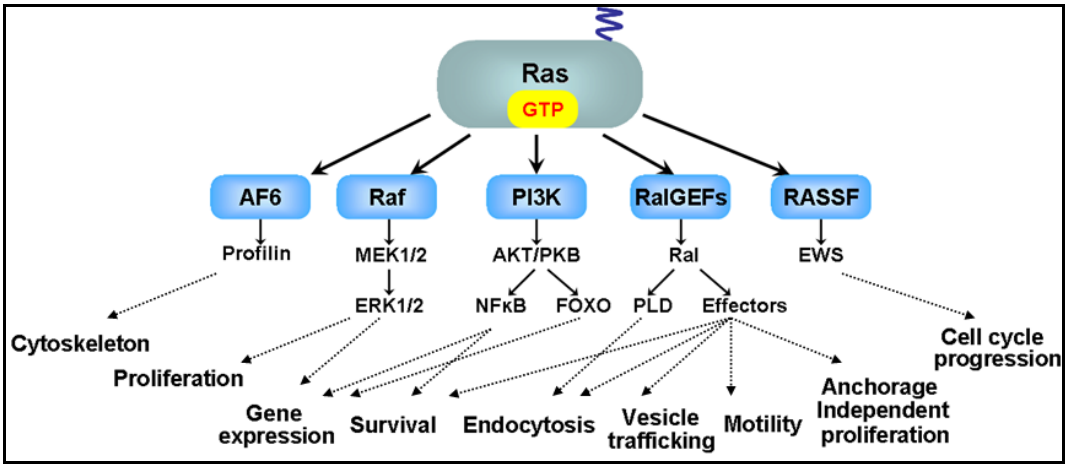


Fig. 1.2 Ras signaling networks. Ras proteins function as nucleotide driven switches that relay extracellular cues to cytoplasmic signaling cascades.

Raf-MEK-ERK pathway

Raf1 Ser/Thr kinase was the first identified *bona fide* mammalian Ras effector. It is also the most intensively studied. Raf can be activated by Ras through direct binding of its N-terminal regulatory domain and subsequently relocated to the plasma membrane for further phosphorylations (Zhang 1993; Marais *et al.* 1995). The interaction with Ras and the activating phosphorylations relieves the auto-inhibitory conformation of Raf (Chong and Guan 2003; Tran *et al.* 2005), whereupon activated Raf phosphorylates and activates mitogen-activated protein kinase kinase 1and 2 (MEK1 and MEK2). MEKs are dual-specific kinases that relay the signals from Raf by further phosphorylating and activating mitogen-activated protein kinases (MAPKs)/extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2). ERKs can activate numerous cytoplasmic and nuclear targets by phosphorylation, including kinases, cytoskeletal proteins and transcription factors such as myc, Elk-1, Fos and AP-1. These downstream signals are usually responsible for multiple cellular events. For example, the 90 KDa ribosomal S6 kinase ($p90^{Rsk}$) can be

phosphorylated by ERK and subsequently activates transcription factor CREB, which plays a pivotal role in cell proliferation, survival and development. Cyclin D1 expression is also triggered by ERK, which in turn promotes the cell cycle progression from G1 to S phase (McCubrey *et al.* 2007). The Raf-MEK-ERK signaling cascade has been shown to be both sufficient and necessary for Ras-induced transformation of murine cells (Khosravi-Far *et al.* 1995; White 1995). However, the requirement of Ras for Raf is overcome by targeting Raf to the plasma membrane (Leevers *et al.* 1994); furthermore, the subsequent identification of Raf mutations, in non-overlapping frequencies with Ras mutations in cancers (Rajagopalan 2002), highlight the Raf-MEK-ERK signaling in oncogenesis.

PI3K and RalGEFs pathway

The p110 catalytic subunit of class I phosphoinositide 3-kinases (PI3Ks) and the guanine nucleotide-exchange factors for Ras-like (RalA and RalB) small GTPases (RalGEFs) were soon identified as Ras effectors after Raf (Rodriguez-Viciana 1994; Spaargaren and Bischoff 1994). Class I PI3Ks are dimeric enzymes composed of a catalytic (p110) and a regulatory (p85 or p101) subunit. Activated Ras directly binds to the catalytic subunit of PI3Ks and recruits it to plasma membrane, where activated PI3Ks convert phosphatidylinositol 4,5 biphosphate (PIP_2) into phosphatidylinositol 3,4,5 triphosphate (PIP_3), a crucial secondary messenger that controls cell survival, motility and morphology by recruiting proteins containing pleckstrin homology (PH) domains to cellular membranes (Zhao and Vogt 2008). Among these downstream proteins, the serine/threonine kinase AKT/protein kinase B is the most extensively studied which plays an essential role in preventing apoptosis and growth inhibition by regulation of

cytoplasmic and nuclear components ,such as glycogen synthase kinase-3β (GSK3β) and forkhead box O transcription factor 1 (FOXO1) (Zhao and Vogt 2008). As with Raf, PI3K activity was also shown to be required for Ras transformation of murine cells (Rodriguez-Viciana 1997). Importantly, cells also possess an intrinsic antagonistic mechanism to prevent over activation of PI3K pathway by converting PIP_3 to PIP_2, which is conducted by the phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome ten), a tumor suppressor. Activation of PI3Ks by mutations or gene amplification and the mutations of PTEN results in enhanced PI3Ks signaling, which is associated with oncogenic transformation and cancers (Yuan and Cantley 2008; Zhao and Vogt 2008).

The RalGEFs are specific guanine nucleotide exchange factors for Ral GTPase (Albright *et al.* 1993; Kikuchi *et al.* 1994), they were first identified as Ras effectors by yeast two hybrid screening (Hofer *et al.* 1994; Kikuchi *et al.* 1994). Early studies showed that the main function of RalGEFs is to activate the downstream substrates RalA and RalB upon Ras activation, which also participate in Ras-mediating transformation. However, activation of RalGEFs alone is not sufficient to transform murine fibroblasts (Urano *et al.* 1996; White *et al.* 1996), suggesting this pathway only plays an auxiliary role in oncogenesis. Intriguingly, more recent studies in human cancers indicate contradictory observations. First, the Chris Counter group showed that activation of RalGEF pathway alone, but not PI3Ks or Raf pathways, was sufficient for Ras transformation of human cells (Hamad 2002). Later on, Chris Marshall group found that RalGDS (a member of RalGEFs) is required for Ras-dependent carcinogenesis *in vivo* (Gonzalez-Garcia 2005). Additionally, the finding from our lab suggests that RalA and

RalB have distinct function in tumor formation (Chien and White 2003). Together, emphasize the importance of RalGEFs in oncogenesis.

Beside the above-mentioned, Ras also plays diverse roles in regulating normal cell functions through some less characterized effectors, such as AF-6 and RASSF proteins (Kuriyama 1996; Vavvas *et al.* 1998). AF-6 contains microtubule- and actin-binding motifs, which suggests that Ras can modulate cytoskeleton in response to exogenous stimuli (Ponting and Benjamin 1996). Lately, AF-6 was found to associate with profilin (Boettner *et al.* 2000). In addition, overexpression of AF-6 inhibits RAP1-induced cell adhesion (Zhang *et al.* 2005). Together, these results suggest that Ras can utilize AF-6 to regulate not only normal function but also promote metastasis. RASSF proteins were reported as potential tumor suppressors (Vos 2003; Vos *et al.* 2003); indeed, it was found that RASSF1A is epigenetically inactivated at high frequency in a variety of solid tumors (Dammann *et al.* 2000; Agathanggelou *et al.* 2001). In addition, RASSF1A knockout mice are more susceptible to spontaneous and induced tumorigenesis (Tommasi *et al.* 2005). However, the detailed mechanism of RASSF proteins as tumor suppressors has not been fully understood. Recent studies found that re-introduction of RASSF1A expression causes growth arrest in lung and breast tumor-derived epithelial cells (Shivakumar *et al.* 2002). Mechanistically, RASFF1A associates with EWS (Ewing Sarcoma breakpoint protein) resulting in inhibition of cyclin D1 accumulation; meanwhile, it is also required to restrict $SCF^{\beta TrCP}$ activity to allow G/S phase transition. The paradox observation implies that the function of RASSF1A may be context dependent (Whitehurst *et al.* 2008).

RalGEF family

Among the three well-known effector pathways of Ras, the RalGEFs pathway is the latest identified and also the least studied. However, the significant contribution of RalGEFs pathway to human oncogenesis has caught more and more attentions. The first identified member of RalGEF family is RalGDS (Ral guanine dissociation stimulator) (Albright *et al.* 1993); shortly, others were found and designated as RGL1 (RalGDS like-1), RGL2/RAB2L/Rlf, and RGL3 (Kikuchi *et al.* 1994; Peterson *et al.* 1996; Shao and Andres 2000). They all possess a RasGEF catalytic domain in the center, a RasGEF N-terminal domain (RasGEF_N, or Ras exchanger motif, REM) at N-terminus, and a Ras association domain (RAD, or Ras interacting domain, RID) at C-terminus (Figure 1.3). The RAD binds to active Ras resulting in recruitment of RalGDS to the plasma membrane, which has been shown to be required for Ras-dependent Ral activation (Matsubara *et al.* 1999). However, activation of PKC suppresses Ras-mediated RalGDS activation without affecting their protein interaction, suggesting that Ras binding may not be sufficient to fully activate RalGEFs (Rusanescu *et al.* 2001). Indeed, later studies showed that 3-phosphoinositide-dependent protein kinase-1 (PDK1) binds to the N-terminal REM domain of RalGDS in a kinase independent way, and mediates growth factor-induced RalGEF activation (Tian *et al.* 2002) (also see figure 1.4 (a)). This specific RalGEF activation is at least responsible for the EGF-induced membrane protrusion through activating Ral (Yoshizaki *et al.* 2007); furthermore, RalGDS also reciprocally activates AKT under EGF and insulin stimulation through the RalGDS-PDK1 and RalGDS-JIP1 (JNK interacting protein 1)-AKT complexes (Hao *et al.* 2008).

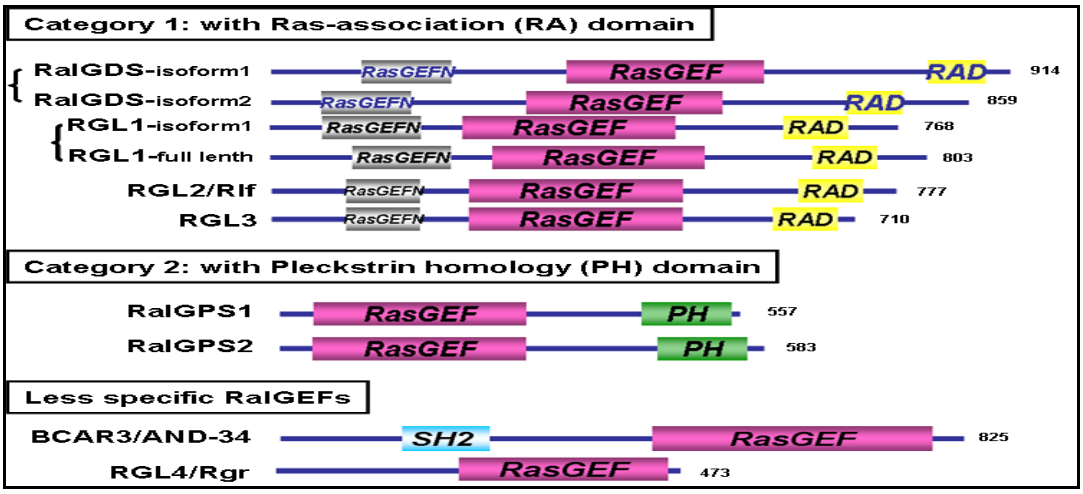


Fig. 1.3 RalGEF family.

Not long ago, an additional family of RalGEFs was identified by sequence homology searching for Ras family GEFs, including RalGEF2/RalGPS1 (RalGEFs with PH domain and SH3 binding motif) and RalGPS2 (de Bruyn *et al.* 2000; Rebhun *et al.* 2000). The unique features of these RalGPS include a PXXP central motif, and a pleckstrin homology (PH) domain instead of a RA domain in the C-terminus, which recognizes PIP_3 and $PI_{3,4}P_2$ and causes translocation of RalGPS from cytoplasm to membranes. Though this membrane targeting is essential for GEF activity, administration of insulin or the PI3K inhibitor LY294002 did not change RalGPS1 activity toward Ral in murine fibroblasts or human embryonic kidney cells respectively, suggesting RalGPS1 activity is not induced by the PI3Ks. Thus, the PH domain may serve to constitutively anchor RalGPS1 to membranes (de Bruyn *et al.* 2000; Rebhun *et al.* 2000). However, a recent study using murine RalGPS2 expressed in human embryonic kidney cells found that Ral activity was dampened by wortmannin treatment, arguing with the previous finding (Ceriani *et al.* 2007). On the other hand, the PXXP motif of RalGPS1 was shown to associate with the SH3 domain containing proteins Grb2 and Nck in vivo. Importantly,

overexpression of Grb2 enhanced Ral activation through RalGPS1-ΔPH, but not full length RalGPS1 (Rebhun *et al.* 2000), indicating either the PH domain of RalGPS1 serves as an auto-inhibitory domain, or there exists some mechanisms which leads to the translocation and activation of RalGPS1. Interestingly, a series of observations from two less specific GEFs: RGL4/Rgr (RalGDS-related) and BCAR3 (breast cancer anti-estrogen resistance 3, also named AND-34), suggest the possible mechanism for RalGEFs activation. Both Rgr and BCAR3 possess RasGEF domain but lacking of REM and RA domain (D'Adamo *et al.* 1997; Gotoh *et al.* 2000), which result in constitutive activation of Ral and other small GTPase like Ras or Rac (Hernandez-Munoz *et al.* 2000; Cai *et al.* 2003). This observation indicates the auto-inhibitory interaction may come from the association of either N-terminal REM or C-terminal RA or PH domain with central GEF catalytic domain (figure 1.4). Nevertheless, how RalGPS are activated and what are the physiological insults for them will require further investigation.

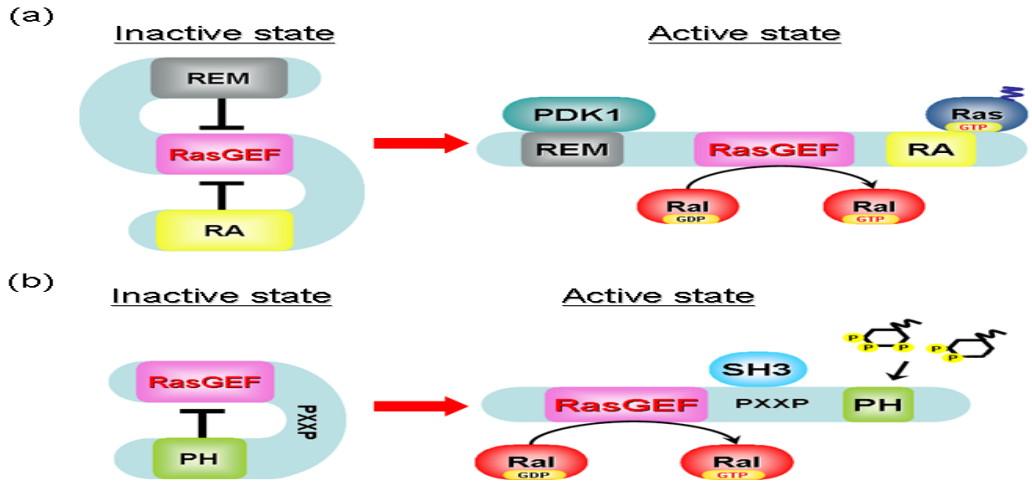


Fig. 1.4 The possible mechanism of different RalGEFs activation. (a) The possible regulatory mechanism for RalGDS and RalGDS like RalGEF family, and for RalGPS family (b).

RalGTPase and signaling pathways

Ral (Ras-like) protein is a member of the Ras family of small GTPases, which function through cycling between active GTP-bound and inactive GDP-bound conformations. There are two Ral genes in humans, RalA and RalB, which encoded proteins sharing 82% sequence identity (Figure 1.5). Ral proteins function as a crucial node in regulatory networks that selectively couple numerous extracellular and intracellular cues to appropriate cellular responses, including proliferation, migration, vesicle trafficking, gene transcription and survival. For instance, oxidative stress induces Ral activation, which results in phosphorylation and activation of JNK (Jun NH_2-terminal kinase) and JNK-mediated phosphorylation of FOXO4 (Forkhead transcription factors box 04) on Thr477 and Thr451. Phosphorylation of both residues is critical for FOXO4 transcriptional activity (Essers *et al.* 2004). Ral was also found to activate c-Src upon EGF stimuli, which further phosphorylates and activates STAT3 (signal transducers and activators of transcription 3) in PC12 cells (Goi *et al.* 2000), or activates p38 to transmit a signal to ATF2 (activating transcription factor 2) by phosphorylation on Thr69 in A14 fibroblasts (Ouwens *et al.* 2002). Additionally, there are some evidences suggesting that Ral contributes to cell proliferation through regulating NF-kB activation and subsequent cyclin D1 accumulation (Henry *et al.* 2000). Together, these data suggest that Ral is a biological switch that couples diverse stimuli to appropriate cellular responses.

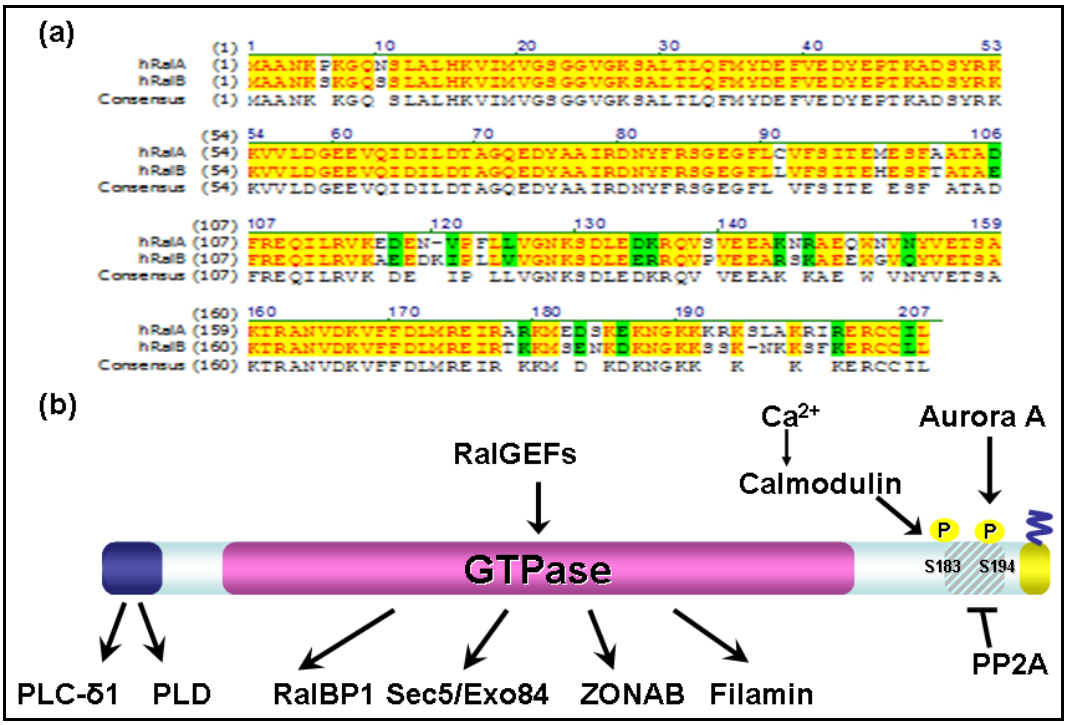


Fig. 1.5 The Ral GTPase. (a) RalA and RalB amino acid alignment. The alignment was performed using AlignX from VectorNTI software (Invitrogen). Identical amino acid are shown in yellow shadow, similar amino acid are shown in green shadow. (b) RalA domain structure and its regulatory network.

A growing body of literature suggests that RalGEF-Ral pathway is more important in Ras-dependent oncogenesis in human cancer than other Ras effector pathways (Hamad *et al.* 2002; Rangarajan *et al.* 2004; Lim *et al.* 2005; Lim *et al.* 2006; Omidvar *et al.* 2006; Falsetti *et al.* 2007; Sablina *et al.* 2007). Remarkably, regardless of the high similarity between RalA and RalB, they seem to function differently. Studies employing transient or stable RNA interference of RalA or RalB expression implied that RalA is dispensable for proliferation or survival of normal or tumorigenic cells under adherent conditions, but is required for the anchorage-independent proliferation of cancer cell lines (Chien and White 2003; Lim *et al.* 2005; Lim *et al.* 2006; Falsetti *et al.* 2007);

whereas RalB is required for the survival of a variety of tumor-derived cell lines but not non-cancerous proliferating epithelial cells (Chien and White 2003; Falsetti *et al.* 2007). Moreover, RalB is also implied in regulating cell migration and cell invasion (Oxford *et al.* 2005; Lim *et al.* 2006; de Gorter *et al.* 2008).

The regulation of Ral GTPase and the biological outcomes can be addressed from various aspects; from upstream RalGEFs to Ral proteins itself, and finally to downstream signaling pathways. Several Post-translational modifications of Ral proteins have been reported to correlate with their functions, including prenylation and phosphorylation. Both RalA and RalB possess C-terminal CAAX motif and are geranylgeranylated (Kinsella *et al.* 1991), which is required for their proper localization and interaction with the effector RalBP1 (will discuss in next page) and calmodulin (Matsubara *et al.* 1997; Sidhu *et al.* 2005). A recent study also showed that treatment of human pancreatic cancer with geranylgeranyltransferase I inhibitors (GGTIs) led to similar phenotype as depletion of RalA and RalB (Falsetti *et al.* 2007). RalA but not RalB has been found to be phosphorylated at Ser11, Ser183 by unknown kinase (Sablina *et al.* 2007), and Ser194 by Aurora-A (Wu *et al.* 2005). The phosphorylation on Ser183 and Ser194 of RalA were further confirmed to act in concert with PP2A-Aβ in regulation of tumor progression (Sablina *et al.* 2007).

The occupation of small GTPases is to activate downstream signaling pathway through specific effectors that associate with GTP-bound status. Several Ral effectors were identified in cells, including Ral-binding protein 1 (RalBP1, also known as RLIP76), exocyst subunit Sec5 and Exo84, filamin, and ZO-1-associated nucleic acid-

binding protein (ZONAB). In addition, there also GTP-independent Ral binding proteins that may conduct downstream signals, including PLD1, PLC-δ1 and calmodulin.

RalBP1

RalBP1 was identified as a Ral GTP-dependent interacting protein (Cantor *et al.* 1995; Jullien-Flores *et al.* 1995; Park and Weinberg 1995) and mutation of the Ral effector domain impairs its interaction with RalBP1 suggest that RalBP1 is an effector protein of Ral (Hinoi et al., 1996). Although the function is presently unclear, it contains a GTPase activating (GAP) domain against Rac/CDC42 in its central region. Recent studies imply that both Ral and RalBP1 are involved in regulating receptor endocytosis (Nakashima et al., 1999; Jullien-Flores et al., 2000).

Exocyst

The exocyst is a conserved multi-protein complex originally identified in the budding yeast *Saccharomyces cerevisiae* (TerBush *et al.* 1996). It is composed of eight subunits including Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 (Lipschutz and Mostov 2002). The mammalian exocyst is localized to the areas of active exocytosis, such as growth cones of neurons and the junctions of polarized epithelial cells (Novick and Guo 2002). The observations that Ral is required for the exocyst complex assembly and regulates exocyst-dependent basolateral membrane protein targeting suggest that the exocyst can function as an effector of Ral proteins (Moskalenko *et al.* 2002; Moskalenko *et al.* 2003). Ral proteins are enriched in secretory granules and synaptic vesicles, and expression of dominant negative Ral mutant in mouse central nervous system suppresses refilling of the readily releasable pool of synaptic vesicles (Polzin *et al.* 2002). This result

may reflect the regulation of the vesicle tethering function of the exocyst by Ral GTPases in the physiological condition (Novick and Guo 2002).

Filamin

Filamin is an actin-binding protein that polymerizes the actin cytoskeleton (Stossel *et al.* 2001). One study has suggested that filamin can associate with RalA in a GTP-dependent manner (Ohta *et al.* 1999). Notably, expression of the constitutively active RalA mutant induces filopodia formation in Swiss 3T3 cells, but not in filamin-deficient cells (Ohta *et al.* 1999). In fact, several observations have suggested the activation of RalA in regulating cytoskeletal reorganization and cell motility (Gildea *et al.* 2002; Takaya *et al.* 2004). It remains to be determined whether filamin is involved in mediating these cellular processes in response to RalA activation.

ZONAB

The Y-box transcription factor ZONAB is a tight junction-associated protein that binds to SH3 domain of ZO-1 (Balda and Matter 2000) and regulates cell proliferation at the G1/S phase transition by shuttling between nucleus and plasma membrane (Balda *et al.* 2003). At low cell density, ZONAB localizes to the nucleus and regulates the expression of genes with inverted CCAAT boxes such as the proto-oncogene ErbB-2, proliferating cell nuclear antigen (PCNA) and cyclin D1; while at high cell density, the formation of tight junctions between neighboring epithelial cells allows ZO-1 to recruit ZONAB to the plasma membrane, thus constraining its transcriptional regulation (Balda and Matter 2000; Sourisseau *et al.* 2006). ZONAB was identified as a Ral effector in a cell density-dependent manner in canine kidney epithelial cells (Frankel *et al.* 2005). The RalA–ZONAB interaction occurs at high cell density resulting in a relief of

transcriptional repression of a ZONAB-regulated promoter. Additionally, expression of oncogenic Ras alleviates transcriptional repression by ZONAB in a RalA-dependent manner (Frankel *et al.* 2005). These studies present ZONAB as a key component that couples cell density to proliferation control, as well as provide a possible mechanism for Ral pathway contributions in Ras-mediated transformation.

PLD1

Phospholipase D enzymes (PLDs) hydrolyze phosphatidylcholine to choline and phosphatidic acid (PA), a potent lipid second messenger implicated in numerous physiological processes such as vesicle budding and transport (Jenkins and Frohman 2005). One of the two mammalian PLD proteins, PLD1, was found constitutively associated with the N-terminal 11 amino acid of Ral. Expression of constitutively active Ral mutant potentates Ras- and EGF-induced PLD1 activation (Jiang et al. 1995), one of the possible mechanisms is through cooperation with another small GTPase, ADP ribosylation factor (ARF), to activate PLD1 (Kim et al. 1998). Conversely, though there is no evidence supporting direct interaction between PLD2 and Ral, one study showed that Ral, together with RalGDS and PLD2 are constitutively associated with class 1 mGluRs (metabotropic glutamate receptors) and regulate constitutive mGluRs endocytosis (Bhattacharya *et al.* 2004). Additionally, later studies also implied that activation of PLD1 may contribute to Ras and Ral-mediated cell proliferation and transformation (Lu *et al.* 2000; Xu *et al.* 2003). For example, expression of either Ral or PLD1 in EGF receptor (EGFR) over-expressing rat 3Y1 fibroblasts increases their colony-forming efficiency in the absence of EGF stimulation (Lu *et al.* 2000). Moreover, PLD1 activity is elevated in H-Ras transformed NIH 3T3 cells, presumably results from

synergistic action of RalA and ARF6 (Xu *et al.* 2003). These results implicate that the regulation of PLD1 activity by Ral may be responsible for a part of Ras-mediated transformation.

Objectives

Previous studies have focused on the role of Ral activation in Ras-mediated transformation; however, how Ral activation contributes to tumorigenesis in diverse genetic backgrounds remains to be answered. In this study, we examine the contribution of Ral activation to oncogenic transformation in the context of diverse oncogenic backgrounds, and evaluate RalGEF family members as candidate human oncogenes.

CHAPTER TWO

RAL ACTIVATION IS REQUIRED FOR ANCHORAGE INDEPENDENT PROLIFERATION DRIVEN BY DIVERSE ONCOGENIC STIMULI

Introduction

Ral (Ras-like) GTPases were originally identified on the basis of their sequence similarity to the Ras family of small GTPases (Chardin and Tavitian 1986). Two Ral genes, RalA and RalB, are ubiquitously expressed in human tissues, with protein products sharing 82% identity. The role of Ral proteins in cell regulation was highlighted by the observation that Ral proteins lay on a Ras effector pathway. Ral GTPases are activated in response to numerous mitogenic regulatory cascades, and Ral activation has been implicated as a key contributing factor to Ras-induced oncogenic transformation (Feig *et al.* 1996; White *et al.* 1996; Reuther and Der 2000). Although the detailed mechanism of Ral contribution to cell proliferation and transformation is not fully understood, accumulating evidence suggests that RalA may be dispensable for proliferation of human epithelial cells and tumor-derived cell lines in adherent culture, but required for anchorage-independent proliferation of transformed cells (Chien and White 2003; Lim *et al.* 2005; Lim *et al.* 2006; Sablina *et al.* 2007). In contrast, RalB is required for survival of transformed cells (Chien and White 2003; Falsetti *et al.* 2007). Additionally, RalA and RalB seem to play antagonistic roles in the regulation of cell survival and transformation (Lim *et al.* 2005; Oxford *et al.* 2005; Lim *et al.* 2006). Here, we found that Ral activation is required for maintaining anchorage-independent proliferation in a broad-spectrum of cancer cell lines, regardless of their diverse genetic

backgrounds. Notably, we find that Ral activity is maintained or elevated in suspension cultures of all the cancer cells we examined, including those without oncogenic Ras activation, but not in the immortalized human epithelial cells. We also showed that both Raf-MEK-ERK and PI3K pathway are down-regulated upon suspension even though their Ras activity still maintained. Together, our data suggest that when cancer cells encounter matrix dissociation challenge (a necessary process for metastasis), they maintain RalGEF-Ral pathway activity to support tumorigenicity.

Results

Previous work has shown that inhibition of Ral function by expression of a minimal Ral-binding domain to block Ral-effector interactions impairs anchorage-independent proliferation of several tumor cell lines, such as H1299 and SW480 (Chien and White 2003). We wondered whether this requirement also exists in cancer cell lines carrying oncogenic Raf mutation or with chronic PI3K activations. We first examined Ral contribution to proliferation of these cell lines in adherent culture using the same strategy. Expression of the minimal Ral-binding domain of RalBP1 (RBD), a candidate Ral effector, inhibits Ral function in cells presumably through inhibition of association of endogenous Ral effectors with activated RalA and RalB (Jullien-Flores *et al.* 2000; De Ruiter *et al.* 2001; Rosario *et al.* 2001; Moskalenko *et al.* 2002). As shown in figure 2.1A, RLIP-RBD expression can obstruct proliferation of most cell lines in adherent culture, except HeLa and DU145. However, the proliferative capacity of RLIP-RBD expressing cells was drastically impaired in all cell lines under suspension condition (figure 2.1B).

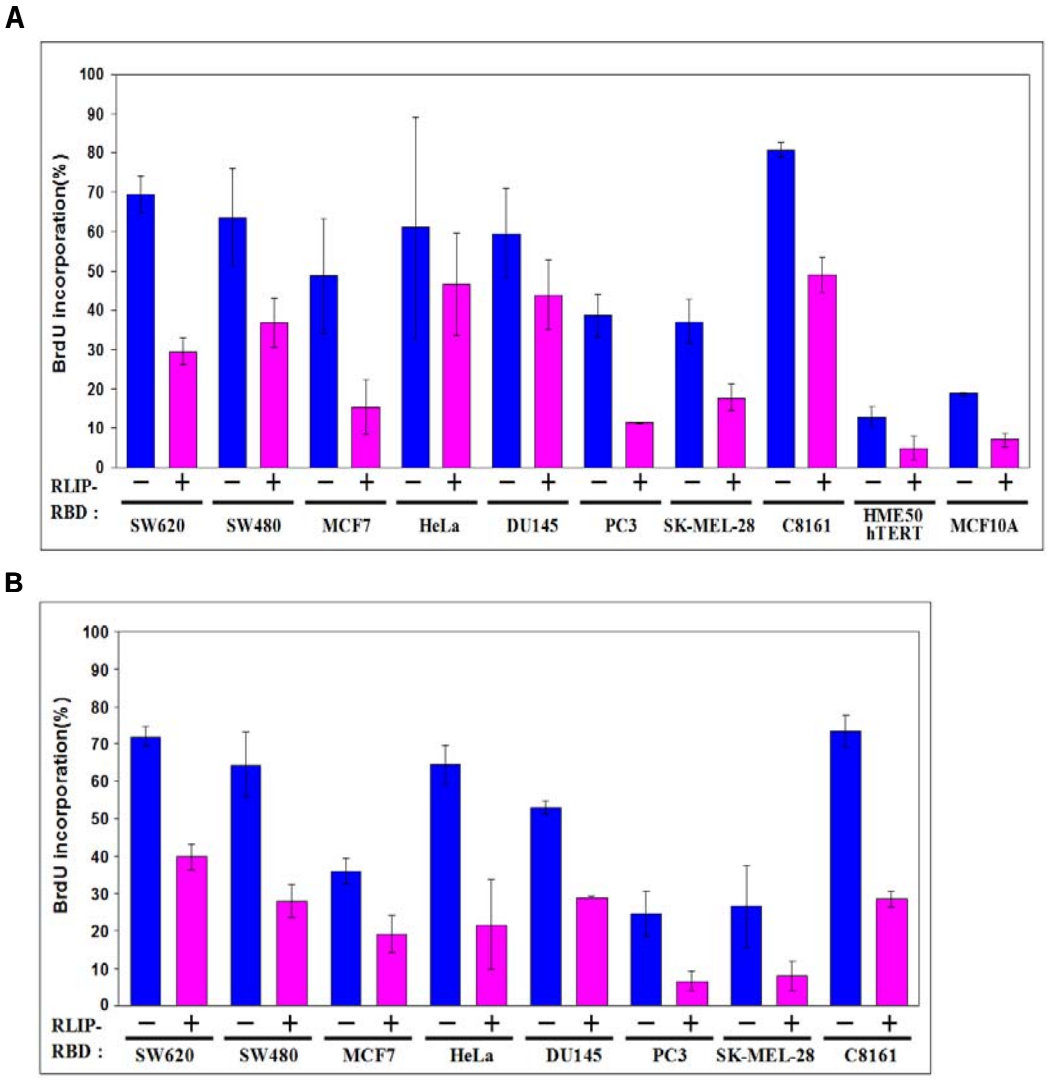


Fig. 2.1 Ral is required for anchorage-independent proliferation of broad-spectrum of cancer cells. (A) The indicated cell lines were transiently transfected with myc-RLIP-RBD or GFP. 56 hours following transfection, cells were incubated in BrdU (30μM) for an additional 16 hours in adherent (attached) culture. (B) The indicated cell lines were transiently transfected with myc-RLIP-RBD or GFP. 48 hours following transfection, cells were suspended for an additional 24 hours. BrdU were added 8 hours after suspension and incubate for an additional 16 hours. The percentage of RBD expressing cells that incorporated BrdU is shown. Over 100 transfected cells were analyzed for each experimental group. Bars represent standard error from the mean for three independent experiments.

The similar results were also observed by using Sec5-RBD, the minimal Ral-binding domain of another Ral effector Sec5 (data not shown), indicating this effect is Ral specific. The BrdU negative RBD-expressing cells showed no signs of blebbing, or pyknotic nuclei suggesting that the proliferative defect was a consequence of cell cycle arrest rather than apoptosis.

Since cancer cells can proliferate in an anchorage-independent manner, we then examined whether Ral pathway is activated in suspension culture. Interestingly, we found both RalA and RalB activity are elevated or maintained in cancer cells upon suspension, whereas in immortalized epithelial cells, the activity of RalA and RalB are suppressed (figure 2.2, MCF10A and HME). This observation correlates with the proliferative capacity of cancer cells and immortalized normal epithelial cells in suspension cultures, implicating Ral function in supporting matrix-independent proliferation.

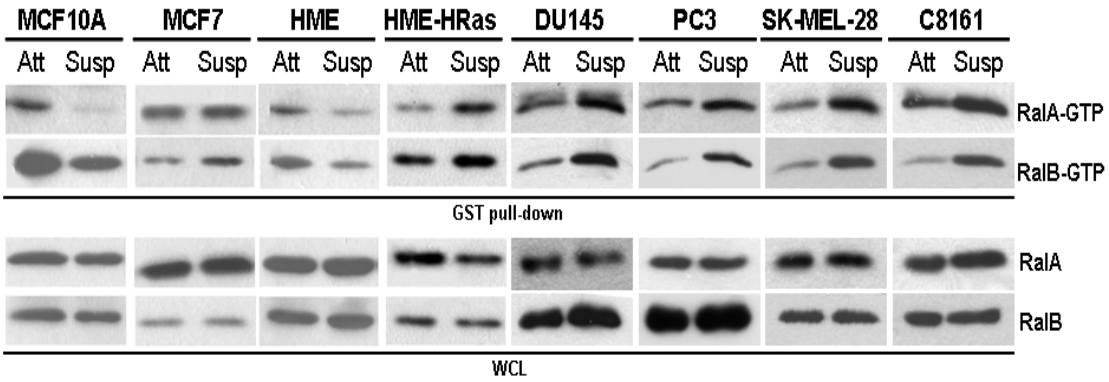


Fig. 2.2 Ral activation is maintained in cancer cells but not normal cells upon suspension. The indicated cells were plated on plastic (Att) or suspension (Susp) for 24 hours and subjected to Ral activation assay, using GST-Sec5-RBD to immobilize endogenous GTP-bound form of Ral. The whole cell lysates and GTP-bound form Ral were resolved and analyzed by SDS-PAGE using isoform specific antibody.

To our surprise, the Raf-MEK-ERK and PI3K pathway were maintained in cancer cells under suspension condition, even in those that have chronic Ras, Raf, or PI3K activation (figure 2.3A, HME-HRas, SK-MEL-28 and PC3, respectively). We then directly tested Ras activity in those cells, as shown in figure 2.3B. Ras activity was maintained in those cells in which Raf-MEK-ERK and PI3K pathway activation was repressed. These results suggest that in the absence of matrix association, Raf and PI3K effector pathways are uncoupled from Ras. Whether the maintenance of Ral activation is resulting from Ras or other stimuli or both remains to be determined.

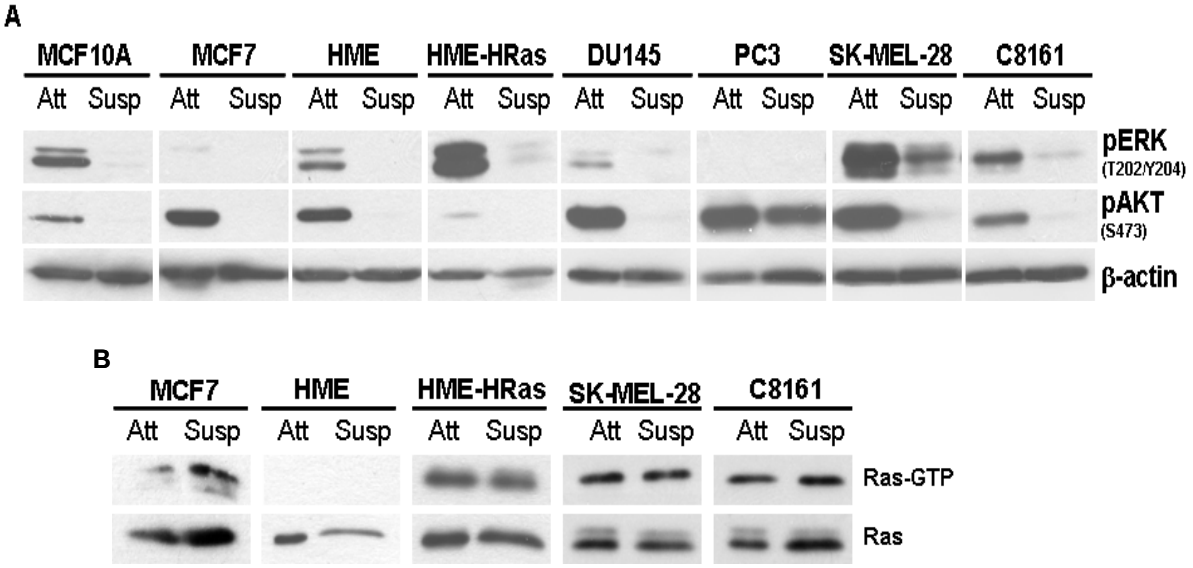


Fig. 2.3 Ras activation is uncoupled from Raf-MEK-ERK and PI3K activation upon suspension. (A) The whole cell lysates from indicated cells were acquired from figure 2.2. The Raf-MEK-ERK and PI3K activation were analyzed by using phospho-ERK1/2 and phosphor-AKT antibody as described. β-actin was used as loading control. (B) The indicated cells were plated on plastic (Att) or suspension (Susp) for 24 hours and subjected to Ras activation assay, using GST-Raf-1-RBD to immobilize endogenous GTP-bound form of Ras. The whole cell lysates and GTP-bound form Ras were resolved and analyzed by SDS-PAGE.

Discussion

The RalGEF-Ral pathway has been shown to play a pivotal role in human oncogenesis (Chien and White 2003; Rangarajan *et al.* 2004; Lim *et al.* 2006; Bodemann and White 2008). In this study, we found that Ral activity is elevated or maintained in cancer cells but not normal cells in the absence of matrix association; moreover, inhibition of Ral activation by expressing a minimal effector Ral-binding domain diminished matrix-independent proliferation of cancer cells with diverse genetic backgrounds. We also found that cancer cells with no activated Ras mutation may still employ wild type Ras to support proliferation in suspension culture, and this event is uncoupled from Raf-MEK-ERK and PI3K pathway activation. These data suggest that RalGEF-Ral pathway is crucial for cancer cells to proliferate under absence of extracellular matrix (ECM), and this process could be mediated via Ras-dependent or -independent regulation.

The evasion from tethered surrounding tissue and maintenance of matrix-independent proliferation is a hallmark of aggressive tumors (Hanahan and Weinberg 2000). Activation of Ral has been shown to be a common prerequisite for tumorigenic transformation of various human primary cells (Rangarajan *et al.* 2004). Notably, inhibition of RalA reduced tumorigenic growth of Ras transformed human kidney epithelial cells and pancreatic cancer lines (Lim *et al.* 2005; Lim *et al.* 2006; Sablina *et al.* 2007). In addition, loss of RalA in metastastatic prostate cancer cells (PC3) inhibits bone metastasis but did not affect subcutaneous tumor growth (Yin *et al.* 2007). Whereas inhibition of RalB showed disparate influence on tumor progression, including survival of cancer cells, migration and metastasis (Chien and White 2003; Oxford *et al.* 2005;

Chien *et al.* 2006; Lim *et al.* 2006; Rosse *et al.* 2006; Falsetti *et al.* 2007; Yin *et al.* 2007). Here, we found that inhibition of Ral activation restrained anchorage-independent proliferation of cancer cells with diverse genetic backgrounds, including oncogenic Raf mutation (SK-MEL-28), tumor suppressor PTEN deletion (PC3) and KRas mutation(SW480,SW620). This suggests Ral activation is also required for tumorigenic growth of cancer cells derived from oncogenic lesions other than Ras. We also noticed that in adherent culture, proliferation of some cancer cells and two human immortalized epithelial cells was suppressed when Ral activation is obstructed. Interestingly, we saw a high frequency of morphological changes with long dendrite or binucleated in those non-cancerous epithelial cells which Ral activation is repressed; whereas in cancer cells, no obvious changes were found. The former is consistant with previous studies which found Ral is required for the completion of cytokinesis (Chien *et al.* 2006; Cascone *et al.* 2008); while the latter remains unclear. It has been shown that Ral activation attenuates DNA damage induced inactivation of cyclinB1/Cdc2 and G2 cell cycle arrest, which may contribute to genetic instability of cancer cells (Agapova *et al.* 2004). Therefore, inhibition of Ral may reverse the tolerance of genetic instability and causes cell arrest.

Elevated Ral activation upon suspension in cancer cells but not non-cancerous epithelial cells correlates with their ability to proliferate in a matrix-independent fashion (data not shown). As opposed to Ral, Raf-MEK-ERK and PI3K activation were unable to sustain in suspension even in those cancer cells with chronic Ras, Raf or PI3K activation (figure 2.3). These results are in agreement with previous reports, which persistent activation of ERK and Akt was not consistently seen in pancreatic tumors from patient despite of oncogenic KRas expression, while RalA and RalB are consistently activated

(Yip-Schneider *et al.* 1999; Lim *et al.* 2006). This suggests that upon suspension, Ras signaling is uncoupled from Raf-MEK-ERK and PI3K pathways. Furthermore, the sustained activation of RalA and RalB in prostate cancer cells (DU145, with normal expression of PTEN, and PC3, PTEN null cell line) and melanoma cells (SK-MEL-28, which harbors $BRaf^{V599E}$ homozygous mutation, and C8161, with wild type NRas and BRaf) suggests that there are stimuli other than Ras that can directly activate RalGEF-Ral pathway in suspension. Indeed, several stimuli has been shown to activate Ral in a Ras-independent manner, such as EGF, LPA and calcium (Hofer *et al.* 1998). Recent studies also showed that Akt-PDK1 complex can mediate growth factor induced Ral activation (Yoshizaki *et al.* 2007). Our data imply that the RalGEF-Ral pathway maybe universally employed by broad-spectrum of cancers in supporting tumorigenic growth, and targeting RalGEF-Ral pathway in cancer therapy could be an effective approach.

Materials and Methods

Cell culture

HeLa and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals). DU145, PC3, SK-MEL-28, and C8161 cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum. SW480 and SW620 were grown in 1x L-15 medium (Cellgro) supplemented with 10% fetal bovine serum. HME50-hTERT (HME) cells, a human diploid mammary epithelial cell line immortalized by hTERT expression (a gift from J. Shay, UT Southwestern Medical Center) and HRasV12 transformed HME50-hTERT (HME-HRas), were grown

in MEGM mammary epithelial cell growth medium (Cambrex Bio Science Walkersville, Inc.) supplemented with 0.4% bovine pituitary extract, human epidermal growth factor (hEGF) 5 ng/ml, insulin 5 mg/ml, hydrocortisone 0.5 mg/ml, gentamicin 0.5 ml/l.

Reagents and antibodies

psGFP, pRK5myc-RLIP76-RBD, pGEX5X3-Sec5-RBD and pGEX4T-Raf1-RBD are as previously described (Moskalenko *et al.* 2002; Chien *et al.* 2006). The following primary antibodies were used: mouse monoclonal anti-RalA (BD, Transduction Laboratories); mouse monoclonal anti-RalB (a gift from Larry Feig, Tufts University School of Medicine, Boston, MA); mouse monoclonal anti-phospho-ERK1/2 (T202/Y204), rabbit polyclonal anti-phospho-AKT(S473)(Cell Signaling); mouse monoclonal anti-BrdU (BD Biosciences); rabbit polyclonal anti-Myc A14 (Santa Cruz Biotechnology); and mouse monoclonal anti-β-actin (Sigma-Aldrich). The following secondary antibodies were used for either immunofluorescence or immunoblotting: Alexa fluor 594 goat anti-mouse IgG (H+L), Alexa fluor 488 goat anti-rabbit IgG (H+L) (Molecular probe, Eugene, OR); peroxidase-conjugated goat anti-mouse IgG (H+L) and peroxidase-conjugated goat anti-rabbit IgG (H+L) (Jackson Immunoresearch, West Groves, PA).

Transfection

Before transfection, cells were seeded into 12 well cluster plates with (for attachment condition) or without (for suspension condition) coverslips to ~50% confluence on next day. Plasmids were transfected using Fugene HD transfection reagent (Roche Applied

Science) according to the manufacturer's instructions. For HME cells, ExGen 500 tranfection reagent (Fermentas Inc.) was used instead of Fugene HD.

Proliferation assay

48 hours post-transfection, the cells for suspension condition were replated onto 1% agarose-coated plates while the cells for attachment condition were also fed with fresh medium. Bromodeoxyuridine (BrdU) was then added 8hr later to a final concentration of 30μM. Following additional 14-16 hour incubation, cells were fixed with 3.7% formaldehyde, permeabilized with CSK extraction buffer (50 mM NaCl, 300 mM Sucrose, 10 mM Pipes, pH6.8, 3 mM MgCl2, 0.5% (v/v) Triton X-100) for 5 min, and then treated with 2 M HCl for 10 min. Cells were then blocked with blocking solution (10% goat serum, 1% BSA, 50mM NH_4Cl in PBS) for 1 hr. BrdU incorporation was visualized with mouse monoclonal anti-BrdU and Alexa fluor 594-conjugated anti-mouse IgG. Expression of the Myc-tagged Ral-binding domain was visualized with rabbit anti-Myc (A14) polyclonal antibodies and Alexa fluor 488-conjugated anti-rabbit IgG.

Expression, purification of GST fusion proteins

Overnight cultures of E. coli transformed with recombinant pGEX plasmids were diluted 1:50 in LB with ampicillin and incubated with shaking at 37℃ until the OD_{600} reached 0.6. Protein expression was then induced with 0.5 mM isopropyl-β-$_D$-thioglactopyranoside (IPTG) for 4 hr. Bacteria pellet was collected by centrifugation at 3000 rpm for 10 min at 4℃, resuspended in 1:25 (v/v) cold lysis buffer (20 mM Tris, pH 8.0, 1% Triton X-100, 500 mM NaCl, 1mM EDTA, 0.1mg/ml lysozyme with Complete proteinase inhibitor tablet (Roche)), and incubate with rocking in 4℃ for 30 min .The

bacteria suspension was then sonicated on ice with 90% power (Vibra Cells, Sonics and Materials Inc.) for 8 times, 15 sec each. The lysate was clarified by centrifugation at 10K g for 20 min at 4℃ and subjected to incubate with glutathione-Sepharose beads (GE Healthcare Life Sciences) at 4℃ for overnight. The beads were then washed 4-5 times with washing buffer (20 mM Tris, pH 8.0, 50 mM NaCl, 1mM EDTA, 1mM DTT, 20% glycerol with proteinase inhibitor), aliquoted and stored at -20℃.

Ral and Ras activation assay

The cells for suspension condition were replated into Ultra-low attachment surface plates (Corning, Lowell, MA), while the cells for attachment condition was fed with fresh medium 24hr before assay. Cells were lysed with lysis buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 200 mM NaCl, 10 mM $MgCl_2$, 0.5mM DTT, 10% glycerol with Complete proteinase inhibitor and PhosSTOP Phosphatase inhibitor cocktail tablet (Roche)) for 5 min at 4℃ with rocking. The lysates were then clarified by centrifugation at 13K rpm, 5 min at 4℃ and measured protein concentration by Precision Red Advanced Protein Assay Reagent (Cytoskeleton, Inc.). The lysates were incubated with GST-Sec5 RBD or GST-Raf1-RBD immobilized on glutathione-Sepharose beads for 45-60 min at 4℃ and washed 3 times with lysis buffer prior to SDS-PAGE analysis.

CHAPTER THREE

THE RALGEFS PROFILE IN DIVERSE CELL TYPES

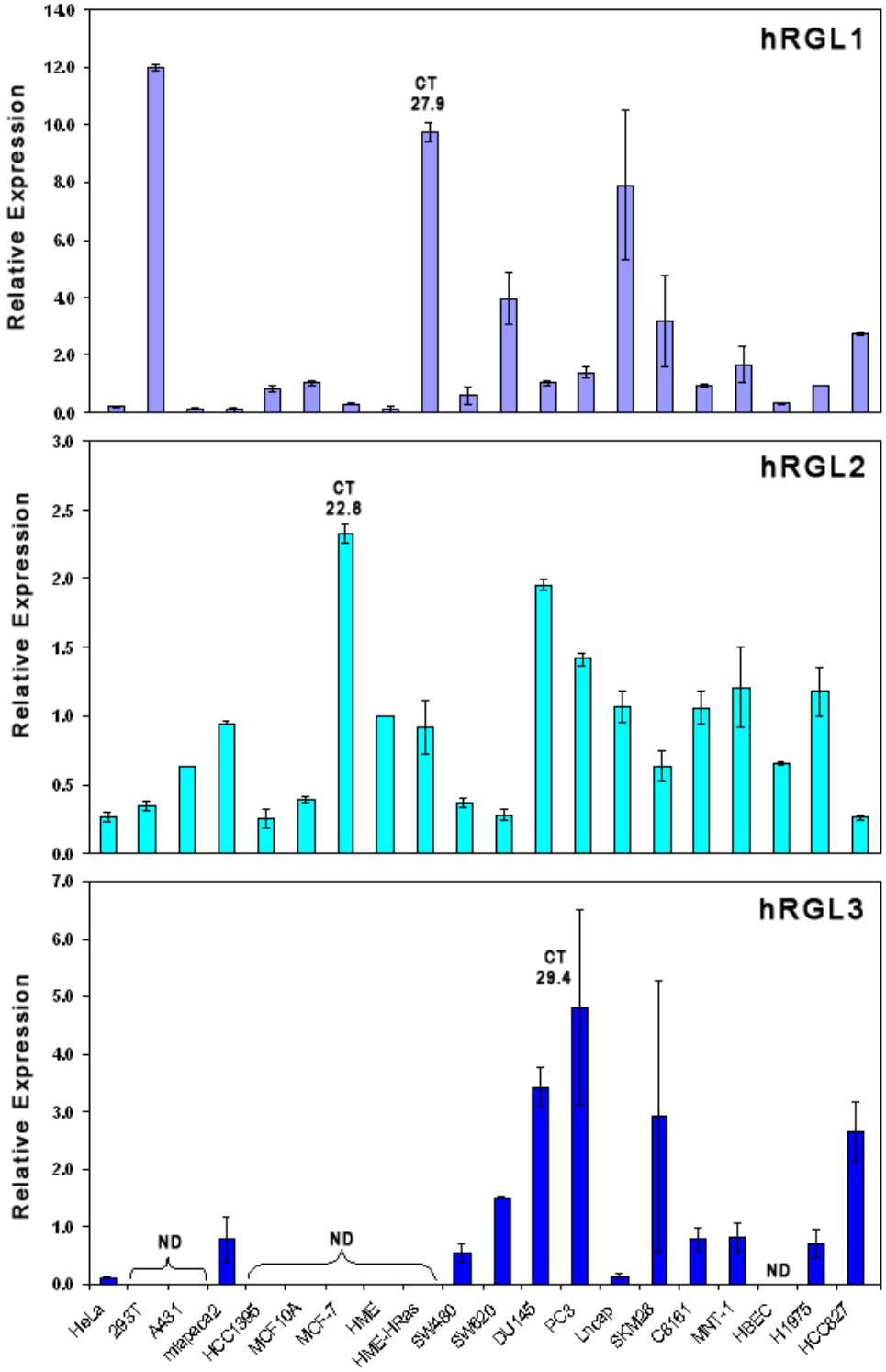
Introduction

The RalGEFs are specific guanine nucleotide exchange factors for Ral GTPase , which bind to nucleotide-free Ral and facilitate the GTP loading (Pan and Wessling-Resnick 1998). The first identified member is RalGDS (Ral guanine dissociation stimulator), by yeast two hybrid screening for Ras effectors (Albright *et al.* 1993; Kikuchi *et al.* 1994). Shortly, others were found and designated as RGL1 (RalGDS like-1), RGL2/RAB2L/Rlf, and RGL3 (Kikuchi *et al.* 1994; Peterson *et al.* 1996; Shao and Andres 2000). They all possess a RasGEF catalytic domain in the center, a RasGEF N-terminal domain (RasGEF_N, or Ras exchanger motif, REM) at the N-terminus, and a Ras association domain (RAD, or Ras interacting domain, RID) at the C-terminus (Figure 1.3). Lately, an additional family of RalGEFs was identified by sequence homology searching for Ras family GEFs, including RalGEF2/RalGPS1 (RalGEFs with PH domain and SH3 binding motif) and RalGPS2 (de Bruyn *et al.* 2000; Rebhun *et al.* 2000). The unique features of these RalGPS include a PXXP central motif, and a pleckstrin homology (PH) domain instead of a RA domain in the C-terminus, which recognizes PIP_3 and $PI_{3,4}P_2$ and causes translocation of RalGPS from cytoplasm to membranes. The contribution of RalGEFs in tumor progression attributes to the activation of its substrates-RalA and RalB. Little is known about endogenous RalGEFs function in human cells; from their expression level to their specificity. Here, we employed an unbiased, quantitative measurement of endogenous RalGEFs mRNA expression in a wide range of

cancer and non-cancerous cell lines. Surprisingly, our survey showed a diverse expression pattern of RalGEFs in various cell lines; unlike RalA and RalB, which are abundantly and ubiquitously expressed. Remarkably, we found that in 14 pairs of normal and tumor kidney tissues from patients, RalGDS expression is significantly elevated. Our results suggest that there might be a variety of preferential usage of RalGEFs to activate Ral pathway in cell lines derived from different tissues; in addition, the elevated RalGDS expression in kidney tumor indicates that RalGEFs family may directly associate with tumor progression.

Results

In order to understand the functions of endogenous RalGEFs in human oncogenesis, we asked whether they are expressed differently in cancer cells versus non-cancerous cells. Since effective RalGEFs antibodies are not currently available, we applied quantitative real-time PCR (qPCR) to detect the mRNA expression in 20 cell lines derived from cervix, kidney, skin, pancreas, colon, breast, prostate, bronchia and lung (figure 3.1). Surprisingly, RalGEFs expression levels are very different. For example, RGL2 and RalGPS2 are abundantly and evenly expressed in all cell lines (cyclo number to threshold (CT) around 20-24), whereas RalGDS, RGL1, RGL3 and RalGPS1 expressions are less abundant and much more diverse (CT various from 25 to less than 34). Additionally, we found that RGL3 expression is exclusively silent in breast derived cell lines, regardless of their tumorigenicity; while RalGDS is profoundly expressed in 293T and Lncap. Moreover, in an oncogenic HRas transformed HME-hTERT cell line, RGL1 expression level is 68.4 fold higher than the parental HME-hTERT line, whereas


hRGL1
Relative Expression
CT
27.9
hRGL2
CT
22.8
hRGL3
CT
29.4
ND
ND
ND
HeLa
293T
A431
mapaca2
HCC1395
MCF10A
MCF-7
HME
HME-HRas
SW480
SW620
DU145
PC3
Lncap
SKM28
C8161
MNT-1
HBEC
H1975
HCC827

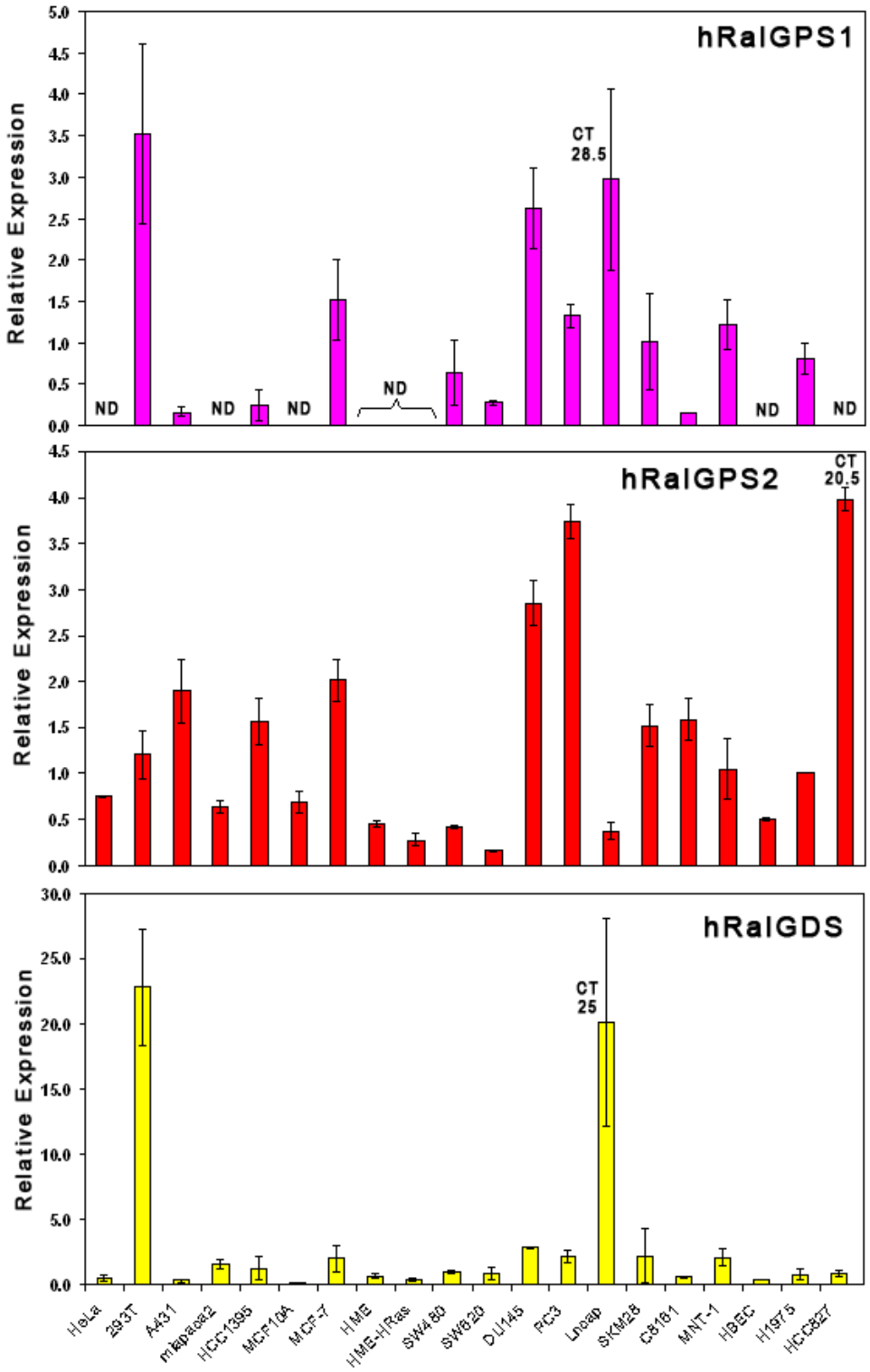

hRalGPS1
Relative Expression
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ND
ND
ND
ND
ND
CT
28.5
ND
ND
hRalGPS2
Relative Expression
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CT
20.5
hRalGDS
Relative Expression
30.0
25.0
20.0
15.0
10.0
5.0
0.0
CT
25
HeLa
293T
A431
mkpasa2
HCC1395
MCF10A
MCF-7
HME
HME-HRas
SW480
SW620
DU145
PC3
Lncap
SKM28
C8161
MNT-1
HBEC
H1975
HCC827

Fig. 3.1 Ral guanidyl exchange factors (RalGEFs) mRNA expression profile in various cell lines. The relative mRNA levels of RalGEFs in 20 cell lines derived from cervix (HeLa), kidney (293T), skin (A431, SKM28, C8161, MNT-1), pancreas (miapaca2), colon (SW480, SW620), breast (HCC1395, MCF7, MCF10A, HME, HME-HRas), prostate (DU145, PC3, Lncap), bronchia (HBEC) and lung (H1975, HCC827). All values are expressed relative to cyclophilin, and arithmetically adjusted to the medium-expressing sample as a unit of 1. Values represent the means and SD of two independent samples for each cell line. Note that as these data are portrayed, comparisons can only be made between different cell lines for a single GEF, not between various GEFs. Setting arbitrary cut-offs at $C_T < 25$ (abundant); $25 < C_T < 30$ (moderate); $C_T > 35$ (non detectable (ND)).

expression of other RalGEFs are quite comparable. This result suggests that cells derived from different origin may use different RalGEFs to activate Ral pathway.

The RalGEF-Ral pathway is known to be exploited during Ras-mediating tumorigenesis; however, how the RalGEF-Ral pathway contributes to oncogenesis in general remains unclear. Previously, we have shown that Ral activation is required for tumorigenic growth in a broad-spectrum of cancer cells. Here, we acquired mRNA from 14 pairs of normal and tumorous human kidney tissue from patients through cancer center, which have been analyzed by microarray. Interestingly, RalA, RalB, and several RalGEFs members are significantly elevated in tumorous but not normal samples. We therefore validated the gene expression of RalA, RalB, RalGDS and RGL1 in those samples, as shown in figure 3.2 (according to our pre-test result from two pairs of samples, no obvious difference in expression of RGL2, RGL3, RalGPS1, and RalGPS2. So we only measure RalGDS and RGL1 here.). We find that RalGDS mRNA expression is significantly elevated in tumorous tissues suggesting that RalGEFs expression may correlate with kidney cancer.

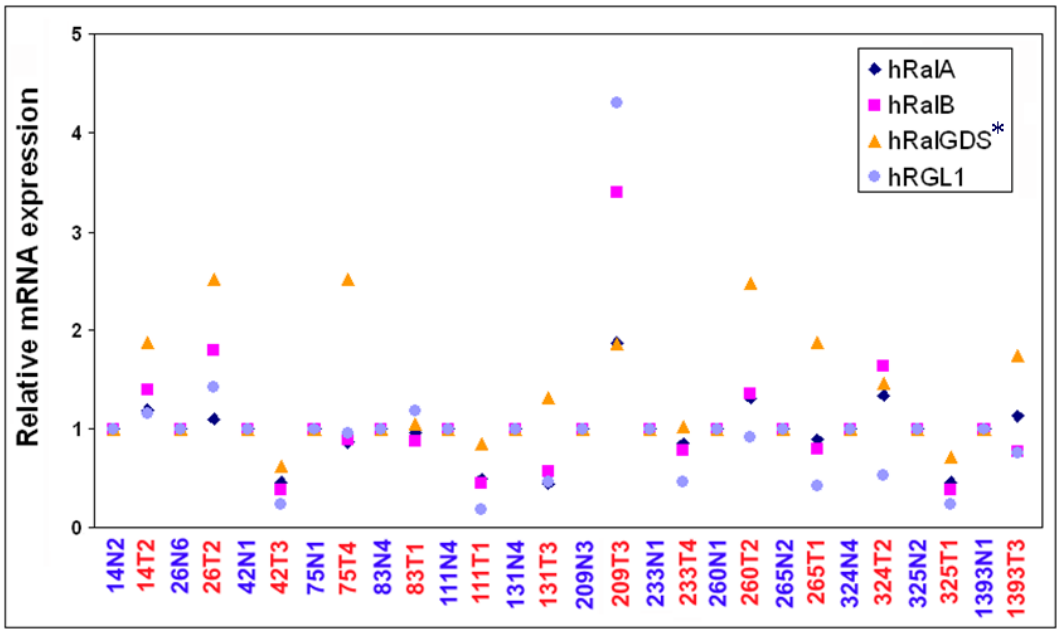


Fig. 3.2 Ral A, RalB, RalGDS and RGL1 mRNA expression in 14 pairs of normal and tumorous kidney tissue from patients. The relative mRNA expression levels of RalA (blue diamond), RalB (pink square), RalGDS (orange triangle), and RGL1 (light blue circle) were measured by qPCR. Each pair represents normal (blue) and tumorous (red) tissue obtained from the same individual (numerical labeled). All values are expressed relative to β-actin, and arithmetically adjusted to the normal tissue of each pair as a unit of 1. Statistical significance was evaluated by Student's *t* test (*P<0.01).

Discussion

Despite observations that the RalGEF-Ral pathway can impact human oncogenesis and tumor progression, little is known about how RalGEFs are regulated. Recent studies revealed that RalGEFs could activate the Ral pathway downstream of a Ras-independent mechanism. For example, RalGDS can be activated by PDK1 through physical interaction with its N-terminal REM domain (Tian *et al.* 2002; Yoshizaki *et al.* 2007); furthermore, it can also response to calcium presumably through association with calmodulin (Rondaij *et al.* 2008), a major calcium response sensor (Crivici and Ikura

1995), and activate Ral. Importantly, the identification of another RalGEF family lacking of RA domain (de Bruyn *et al.* 2000; Rebhun *et al.* 2000) further indicates that the RalGEF-Ral pathway in human oncogenesis may not only contribute to Ras associated tumors but also to a broader variety of cancers.

Here we employed a sensitive, quantitative way to detect endogenous RalGEFs expression in various cell lines. Our results pointed out several interesting observations: first, RalGEF family member expression is not ubiquitous like RalA and RalB, indicating the usage of any distinct RalGEF to activate Ral may vary depending on cellular origins. RGL3 expression is non-detectable in all breast derived cell lines tested, while RalGDS is significantly up-regulated in 293T and Lncap (figure 3.1). Second, in the oncogenic HRas transformed human mammary epithelial cells, RGL1 is selectively up-regulated compared to other RalGEFs, suggesting that RGL1 expression may be regulated by activated HRas (further examination of this hypothesis is described in next chapter). Third, RGL2 and RalGPS2 are abundantly and more evenly expressed in all the cell lines we examined, suggesting their role in regulating Ral might be more static. Since the Ral pathway is also involved in maintaining normal cellular function like endocytosis and exocytosis (Nakashima *et al.* 1999; Feig 2003; Camonis and White 2005; van Dam and Robinson 2006; Li *et al.* 2007), which occur constantly in cells, it is possible that RGL2 and RalGPS2 are exploited for the maintenance of these routine events. Furthermore, our results showed that RalGDS but not RGL1 is significantly up-regulated in human kidney tumors (12 out of 14 samples).

Together, our studies suggest that RalGEFs may be selectively utilized for different cellular events and for different cell types. The further investigation of

endogenous RalGEFs function could help in understanding the Ral pathway contribution in not only normal cell functions but also oncogenic transformation.

Materials and Methods

Cell culture

HeLa, A431, miapaca2, MCF-7 and MNT-1 cells were maintained in Dulbecco's modified Eagle's medium(DMEM) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals).293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) without sodium pyruvate supplemented with 10% fetal bovine serum. HCC1395, DU145, PC3, Lncap, SK-MEL-28, C8161, H1975, and HCC827 cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum. SW480 and SW620 were grown in 1x L-15 medium (Cellgro) supplemented with 10% fetal bovine serum. MCF10A, HME, and HME-HRas cells were grown in MEGM mammary epithelial cell growth medium (Cambrex Bio Science Walkersville, Inc.) supplemented with 0.4% bovine pituitary extract, human epidermal growth factor (hEGF) 5 ng/ml, insulin 5 μg/ml, hydrocortisone 0.5 μg/ml, gentamicin 0.5 ml/l. HBEC cells, a human bronchial epithelial cell line immortalized by hTERT expression (a gift from J. Shay, UT Southwestern Medical Center), were grown in defined Keratinocyte-SFM medium (GIBCO) supplemented with human epidermal growth factor (hEGF) 5 ng/ml and bovine pituitary extract 50 μg/ml .

RNA measurement and real-time PCR

RNA from cultured cells was isolated using High Pure RNA Isolation Kit **(**Roche Applied Science) following the manufacturer’s user manual. 4 μg of total RNA was

treated with RNase-free DNase (Roche), then reverse-transcribed with oligo-dT primer using SuperScript II (Invitrogen). Quantitative real-time PCR (qPCR) was performed using an Applied Biosystem Prism 7900HT sequence detection system and SYBR-green chemistry(Sood *et al.* 2000). Gene-specific primers were designed using using the on-line program D-LUX™ Designer Software from Invitrogen, and validated by analysis of template titration and dissociation curves. Primer sequences are provided as below:

Gene Name	Accession Number	Sequence of Primers (5' to 3')
hRGL1	NM_015149.3	F: ATTGAGAAGTGGATCAACATCG
		R: AAACGATGGCCCTCAAGGAG
hRGL2	NM_004761.2	F: AGGCTCCAAGTGTCATCAGTCG
		R: AGCAGTAGAGGACCTTCGCCG
hRGL3	NM_001035223.1	F: TGTGCTGGGTTCCGTGCT
		R: GGGTTAGATTGCAGGGCG
hRalGDS	NM_006266.2	F: GTTTCCAGGGACAGTTTCCG
		R: CAGCTCCCGGCTCAATGAGTA
hRalGPS1	NM_014636.1	F: CCTGTCCTGACACATCTGTTGC
		R: GTAGGTGCCTTGCTTTCTCCG
hRalGPS2	NM_018037.1	F: GGTTACACCAGAAGAATATGCG
		R: CCATCCACAACTTGAAAGCTCA
hRalA	NM_005402.2	F: TGTACGATGAGTTTGTGGAGGA
		R: CCATCTAGCACTACCTTCTTCCG
hRalB	NM_002881.2	F: AGGCCAGGAGTAAAGCCG
		R: TCAAAGAACACCTTGTCCACGTT
hβ-Actin	NM_001101	F: GCACCCAGCACAATGAAGAT
		R: GCCGATCCACACGGAGTAC
hcyclophilin B	NM_000942	F: TGCCATCGCCAAGGAGTAG
		R: TGCACAGACGGTCACTCAAA

10 µl qPCR reactions contained 62.5 ng of reverse-transcribed RNA, each primer (150 nM) and 5 µl of 2X SYBR Green PCR master mix (Applied Biosystems). Multiple housekeeping genes were evaluated in each assay to insure that their RNA levels were invariant under the experimental conditions of each study. Results of qPCR were evaluated by the comparative Ct method (user bulletin No.2, Perkin Elmer Life Sciences)

using cyclophilin as the invariant control gene. For RalA, RalB, RalGDS, RGL1 profiles in 14 human kidney tumor and normal tissue pairs (cDNA samples were gift from James Brugarolas, UT Southwestern Medical Center), 10 μl qPCR reactions contained 3.8 ng of cDNA, each primer (150 nM) and 5 μl of 2X SYBR Green PCR master mix (Applied Biosystems). Results of qPCR were evaluated by the comparative Ct method using β-Actin as the invariant control gene.

CHAPTER FOUR

THE NOVEL ONCOPROTEIN RGL1

Introduction

Previous studies have shown the RalGEF-Ral pathway plays a pivotal role in Ras-mediated transformation (Hamad *et al.* 2002; Rangarajan *et al.* 2004; Gonzalez-Garcia *et al.* 2005; Yin *et al.* 2007), in addition, our studies also showed that RalGEF-Ral pathway is also required for tumorigenic growth of cancer cells derived from oncogenic lesions other than Ras. However, unlike Ras, which was found mutated in around 30% of all human cancers, RalGEF members or Ral proteins themselves were not found mutated in tumors until recently (Sjoblom *et al.* 2006; Ding *et al.* 2008). Through examination of historical data sets, we found that mutations in RGL1 and RalGDS had been identified in 3 out of 11 breast and 11 colon cancers respectively by sequencing human protein coding genes (13,023 genes) (Sjoblom *et al.* 2006); whereas RalA and RalB were found mutated in lung carcinomas (Ding *et al.* 2008). Remarkably, the loci of point mutations in RalGEFs or Ral proteins were in those regions that potentially related to their functions. The mutation in RalGDS (1/11 incidence) occurred in GEF (R496L) domain, while mutations in RGL1 (2/11 incidence) happened in Ras exchanger motif (Y209S) and Ras association domain (V734M); moreover, mutations in RalA (G59W) and RalB (G23V) were found in the switch I, II region. The later mutation mimics the classic gain-of-function Ras mutations found in over 30% of human tumors. These observations potentially highlight a key role of RalGEF-Ral pathway in human tumorigenesis.

Here, we examined the potential role of RGL1 as a proto-oncoprotein. We found that depletion of RGL1 by siRNA decreased the GTP-bound form RalA and RalB, and induced programmed cell death in HCC1395 (with heterozygous Y209S mutation of RGL1) but not in other cell lines (with wild type RGL1). Over expression of tagged RGL1 activated both RalA and RalB, but the mutant form of RGL1 drives more RalB activation compared to wild type, while RalA activation remains the same. These results suggest that Y209S mutation of RGL1 is a gain of function mutation, and provide survival signal through activation of RalB. Significantly, we found that expression of RGL1 alone is sufficient to transform human telomerase-immortalized mammary epithelial cells, indicating RGL1 may be a *bona fide* oncogene. Lastly, we showed that unlike wild type RGL1, both RGL1 mutants failed to dimerize and associate with oncogenic Ras, indicating the mechanism of hyper-active RGL1 mutants may result from relief of auto-inhibition by conformational changes.

Results

The identification of RalGEFs mutations in human cancers implied a direct association of RalGEF-Ral pathway activation and cancer. Interestingly, Ras mutations were not found in those cancer cell lines or xenografts in which RalGDS or RGL1 mutations were found (Sjoblom *et al.* 2006). To test whether RGL1 is a potential oncoprotein, we first verified the genomic mutation is indeed resulting in a mutated mRNA product (figure 4.1A). The PCR products from genomic DNA or mRNA showed double peak at the depicted position (the mutation sites on RGL1 is Y209S (c.626A>C) in the breast cancer cell line HCC1395 and V734M (c.2200G>A) in xenografts BB12T), indicating a heterozygous mutation in genome and a mixture of wild type and mutant

mRNA product. We then examined if RGL1 is required for survival or proliferation of HCC1395. The RNAi-mediated RGL1 depletion in HCC1395 caused the appearance of condensed pyknotic nuclei and hypodiploid apoptotic bodies and correlates with the accumulation of cleaved PARP, indicating that the inhibition of

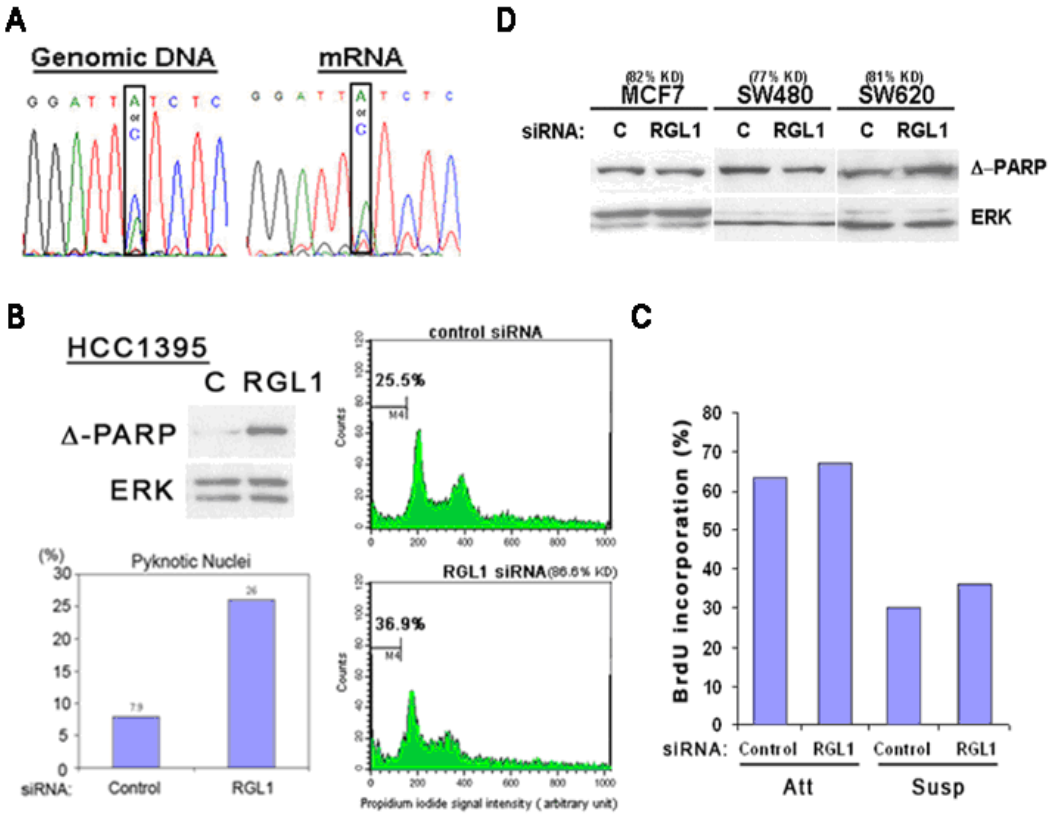


Fig. 4.1 RGL1 is required for survival of HCC1395 but not for related cancer cells lacking RGL1 mutation. (A) Electropherograms of the DNA and mRNA sequences of HCC1395 cell line. (B) HCC1395 cells were transiently transfected with non-targeting control siRNA pool or RGL1 siRNA pool for 48 hours in adherent and subjected to suspension culture for another 24 hours. The cells were directly lysed in Laemmili sample buffer following by SDS-PAGE analysis. The presence of cleaved poly (adenosine diphosphate-ribose) polymerase (Δ-PARP) indicates of relative caspase activity. Total ERK is shown as loading control. An aliquot of cells was stained with propidium iodide and followed by microscopic examination for pyknotic nuclei and flow cytometry for DNA content analysis. Apoptotic cells appear as a population with sub-G1 signal intensities as a consequence of DNA fragmentation. RGL1 knockdown efficiency

was validated by qPCR. (C) HCC1395 cells were transient transfected with siRNA as indicated. For adherent culture, 56 hours post-transfection, cells were incubated in BrdU (30μM) for an additional 16 hours. For suspension culture, 48 hours following transfection, cells were suspended for an additional 24 hours. BrdU were added 8 hours after suspension and incubate for an additional 16 hours. The percentage of RBD expressing cells that incorporated BrdU is shown. Over 100 transfected cells were analyzed for each experimental group. (D) MCF7, SW480 and Sw620 cells were transient transfected with siRNA as indicated and followed by similar procedures as (A), except the cells were cultured in adherent for 72 hours before subjected to suspension.

RGL1 engages programmed cell death. This phenotype is even more prominent in suspension culture (figure 4.1B). Interestingly, we did not see any change in proliferation upon depletion of RGL1, whether in adherent or suspension culture (figure 4.1C). The inhibition of RGL1 phenotypically mimics the inhibition of RalB but not RalA (Chien and White 2003; Chien *et al.* 2006; Falsetti *et al.* 2007), suggesting RGL1 may selectively activate RalB in HCC1395. Since the RGL1 siRNA oligos knockdown both wild type and Y209S mutant RGL1, it is possible that either wild type or Y209S mutant are required for survival of HCC1395. Therefore, we picked MCF7, SW480, and SW620 cell lines in which RGL1 expression varies from low (close to HCC1395 expression, figure 3.1) to high to examine whether RGL1 depletion will cause apoptosis in those cell lines (figure 4.1D). Equivalent efficiencies of RGL1 depletion RGL1 in these lines did not result in any obvious cell death. This suggests that the Y209S mutant but not wild type RGL1 provides the survival signal to HCC1395. As RGL1 depletion in HCC1395 phenotypically mimics RalB depletion, we then examined the direct effect on Ral activation. As shown is figure 4.2A, inhibiting RGL1 expression results in reduction of the GTP-bound form of both RalA and RalB, indicating the endogenous RGL1 drives both RalA and RalB activation in HCC1395. In contrast, we only observed a decreased RalA activation in SW620 and no obvious changes in RalA or RalB activation in MCF7

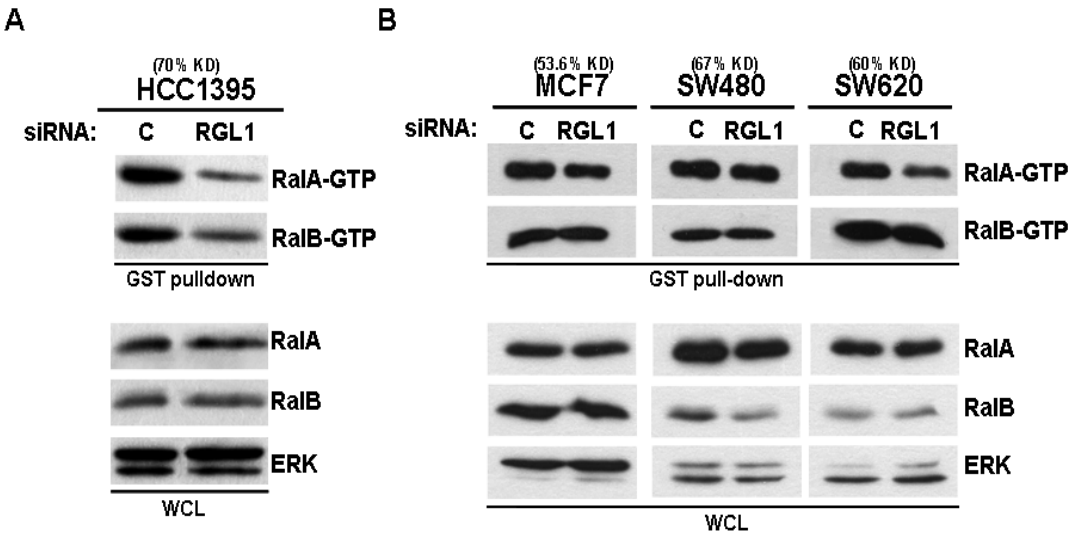


Fig. 4.2 RGL1 is required for maintaining RalA and RalB activation in HCC1395, but only required for maintaining RalA activation in SW620. (A) HCC1395 cells were transiently transfected with indicated siRNA for 48 hours followed by suspension culture and serum starved for another 24 hours. The cells were then subjected to Ral activation assay as described before. The whole cell lysates and GTP-bound form Ral were resolved and analyzed by SDS-PAGE using isoform specific antibody, total ERK is shown as loading control. RGL1 knockdown efficiency was validated by qPCR. (B) MCF7, SW480 and SW620 cells were transfected with indicated siRNA and followed similar process as (A), except the cells were cultured in adherent for 72 hours before subjected to suspension.

and SW480. These data together suggest that the wild type RGL1 drives RalA activation whereas the Y209S mutant drives RalB activation, though the selective activation of Ral family members by RGL1 may be cell context dependent.

We wondered if the cell death phenotype upon loss of RGL1 in HCC1395 is indeed RGL1 specific (i.e. the effect is caused by RGL1 but not other RalGEFs). We therefore examined the effect of two other RalGEF members on cell survival as well as Ral activation. As shown in figure 4.3, inhibiting of RalGDS expression by siRNA causes decrease in RalA activation but not RalB; while inhibiting RalGPS1 shows no apparent effect on both in HCC1395. Notably, unlike the result we found with RGL1 depletion,

none of them are required for survival in HCC1395 as shown by cleaved PARP. This result strengthens our hypothesis which Y209S mutant RGL1 activates the RalB pathway to support HCC1395 survival in a gain of function manner.

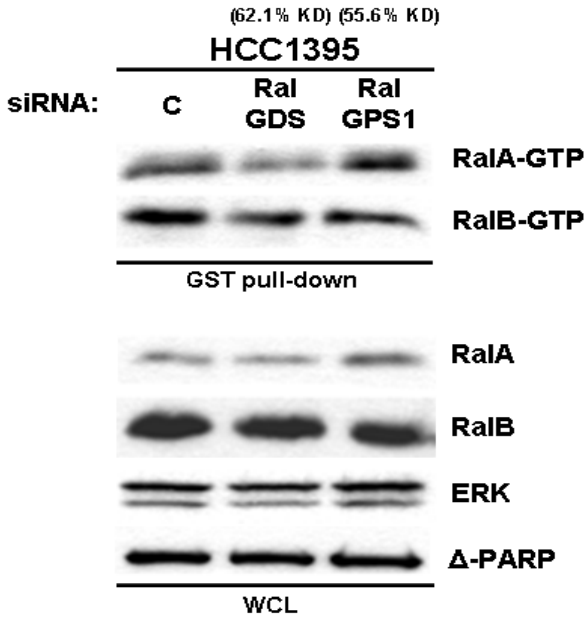


Fig. 4.3 RalGDS is required for maintaining RalA activation in HCC1395, but only required for survival. (A) HCC1395 cells were transiently transfected with indicated siRNA for 48 hours followed by suspension culture and serum starved for another 24 hours. The cells were then subjected to Ral activation assay as described before. The whole cell lysates and GTP-bound form Ral were resolved and analyzed by SDS-PAGE using isoform specific antibody. The presence of Δ-PARP indicates of relative caspase activity, total ERK is shown as loading control. RalGDS and RalGPS1 knockdown efficiency was validated by qPCR.

By loss of function studies, we identified the Y209S mutant RGL1 as a crucial protein for survival by activating RalB pathway. To further characterize this mutant, we examined the GEF activity of wild type and Y209S mutant by overexpression in HeLa and 293T cells. We found that both wild type and Y209S mutant can activate RalA and RalB; remarkably, the Y209S mutant drives more RalB activation than wild type

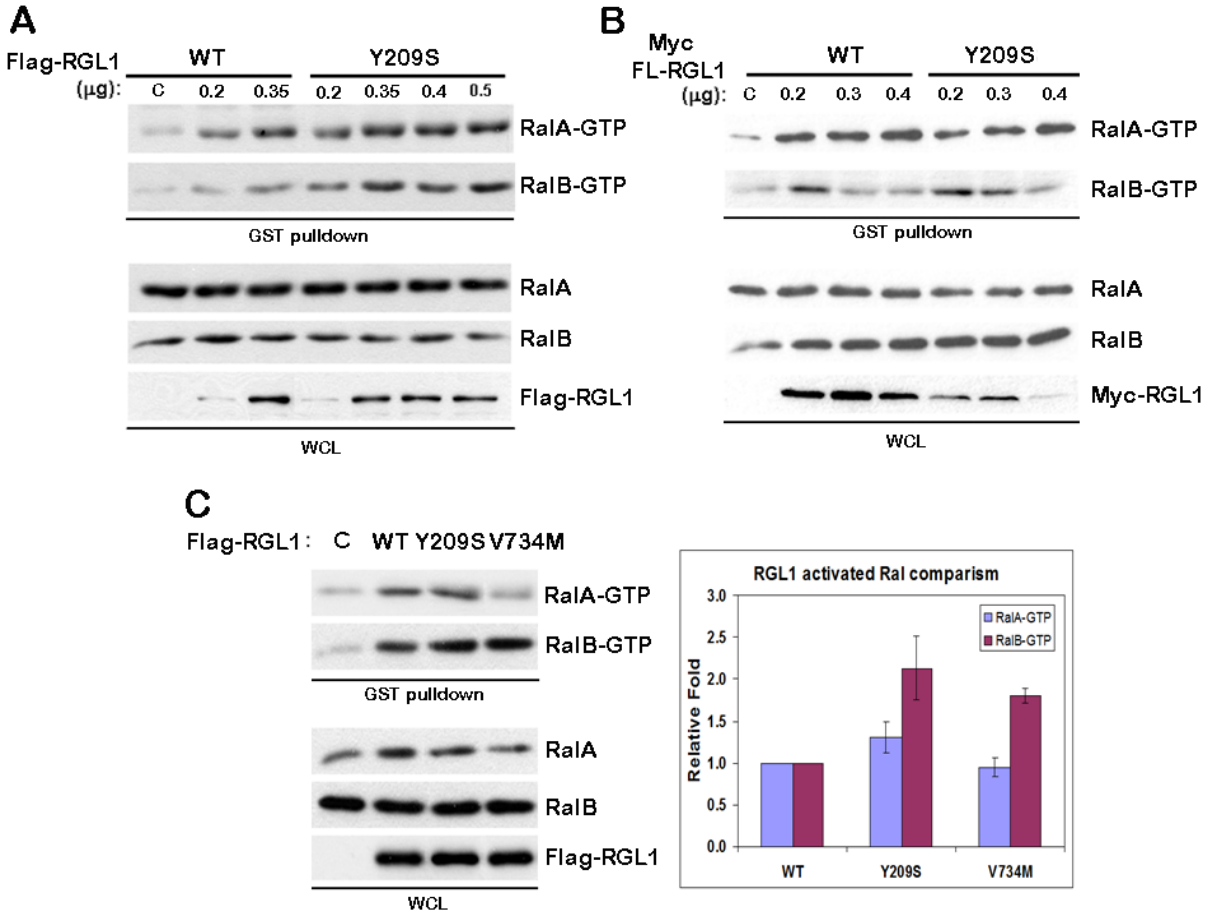


Fig. 4.4 RGL1 mutants drive more RalB activation than wild type RGL1. (A) HeLa cells were transfected with indicated constructs for 48 hours following by serum starvation for an additional 24 hours, and subjected to Ral activation assay as described before. The whole cell lysates and GTP-bound form Ral were resolved and analyzed by SDS-PAGE for the indicated proteins. (B) 293Tcells were transfected with indicated constructs for 24 and subjected to Ral activation assay as described in (A). (C) 293Tcells transfected with indicated constructs for 48 hours following by serum starvation for an additional 24 hours, and subjected to Ral activation assay as described in (A). A quantification chart was shown, the SEM were from two independent experiments.

(figure 4.4A, comparison between equal-amount of wild type and Y209S mutant expressions, e.g. lane 2 and 4, lane 3 and 5.), while RalA activation remains the same. Besides, the activation of RalB driven by Y209S mutant reached plateau earlier than driven by wild type RGL1 (figure 4.4A and data not shown). Interestingly, two isoform

of human RGL1 has been reported; one is specifically expressed in brain (full-lentgh, FL), whereas another is ubiquitous expressed through all tissues (Sood *et al.* 2000). The brain specific isoform has a substitution of 45 amino acids for the first 9 amino acids of the ubiquitous isoform, and so far, it still unclear whether this altered N-terminus of brain isoform alters the function of RGL1 or simply reflects usage of a brain specific promoter that coincidentally use a different initiation codon. In addition, there is no evidence that cancer cells preferentially express either isoform. To test which isoform is expressed by HCC1395, we designed isoform specific primer and analyzed by qPCR (data not shown). In HCC1395, both isoforms are expressed at relative equal levels. To examine whether the brain isoform also has the same specificity toward RalB, we made the full-length RGL1 constructs (both wild type and Y209S mutants) and tested their GEF activity as shown in figure 4.4B. The full-length RGL1 proteins behave similarly as the ubiquitous isoform, except the expression of full-length Y209S mutant is fewer in compare to Y209S mutant of ubiquitous isoform. Our results suggest that both RGL1 isoform function similarly in terms of their GEF activity and specificity.

Another RGL1 mutant found in breast tumor tissue (BB12T) with a heterozygous point mutation results in amino acid change in 734 from valine to methionine (V734M). To test whether this mutant also has elevated activity toward RalB, we carried out the same experiment to examine wild type and two RGL1 mutants at the same time (figure 4.4C). When expression levels of wild type and mutants are comparable, both mutants show higher activity with RalB. This result implies that both RGL1 mutants are gain of function mutants. However, what is the molecular mechanism of that remains unknown.

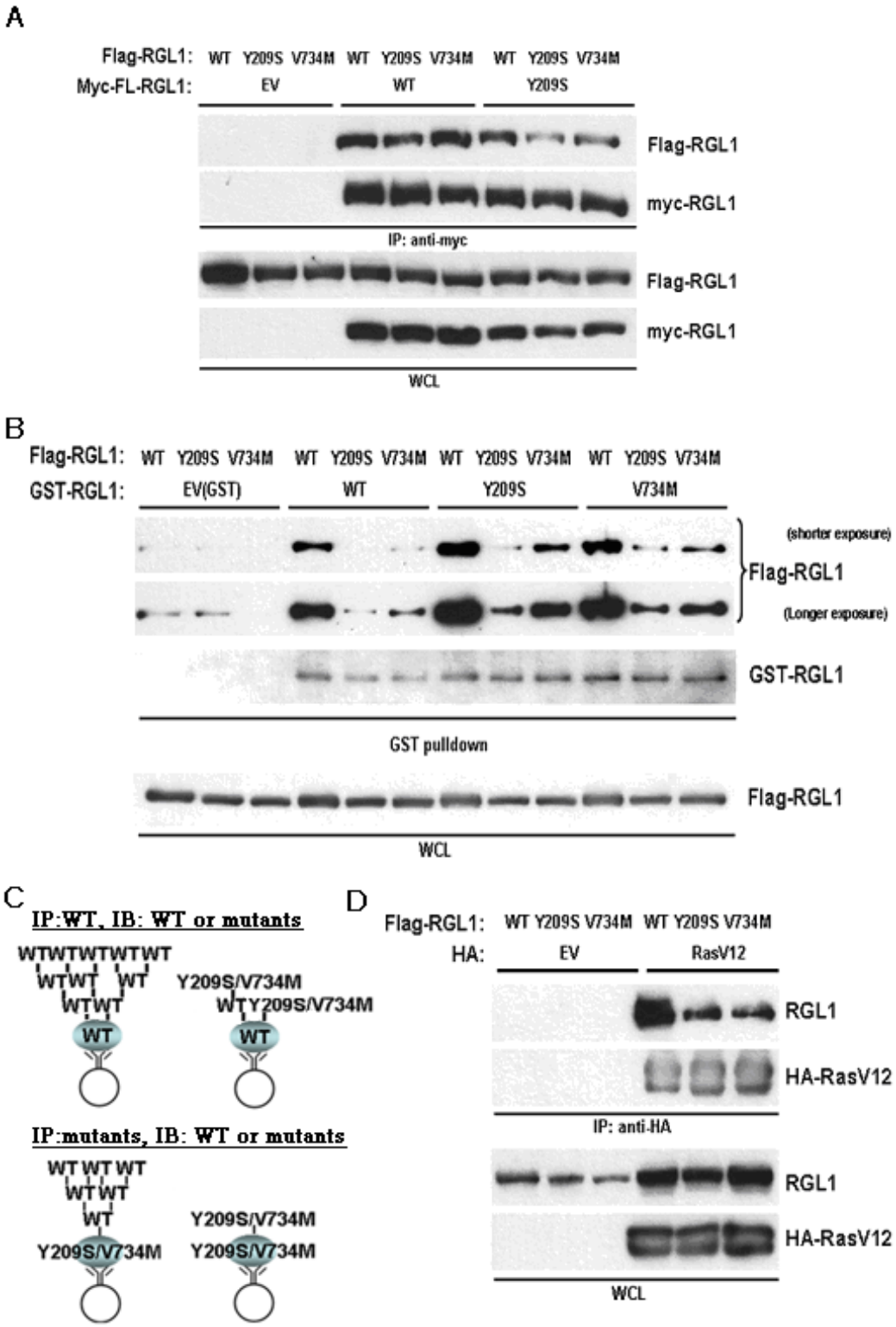

A
Flag-RGL1: WT Y209S V734M WT Y209S V734M WT Y209S V734M
Myc-FL-RGL1: EV WT Y209S
Flag-RGL1
myc-RGL1
IP: anti-myc
Flag-RGL1
myc-RGL1
WCL
B
Flag-RGL1: WT Y209S V734M WT Y209S V734M WT Y209S V734M WT Y209S V734M
GST-RGL1: EV(GST) WT Y209S V734M
(shorter exposure)
Flag-RGL1
(Longer exposure)
GST-RGL1
GST pulldown
Flag-RGL1
WCL
C
IP:WT, IB: WT or mutants
WT
Y209S/V734M
WT
IP:mutants, IB: WT or mutants
WT
Y209S/V734M
Y209S/V734M
D
Flag-RGL1: WT Y209S V734M WT Y209S V734M
HA: EV RasV12
RGL1
HA-RasV12
IP: anti-HA
RGL1
HA-RasV12
WCL

Fig. 4.5 RGL1 mutants have reduced homo- or hetero-dimerization, and decreased association with oncogenic Ras. (A) 293T cells were co-transfected with indicated constructs for 24 hours and subjected to immunoprecipitation (IP) with anti-myc antibody. The whole cell lysates and IP were resolved and analyzed by SDS-PAGE for the indicated proteins. (B) 293T cells were co-transfected with indicated constructs for 48 hours and subjected to GST pulldown with glutathione sepharose. The whole cell lysates and pulldown were resolved and analyzed by SDS-PAGE for the indicated proteins. (C) A proposed model for RGL1 dimerization. (D) 293T cells were co-transfected with indicated constructs for 24 hours and subjected to immunoprecipitation (IP) with anti-HA antibody. The whole cell lysates and IP were resolved and analyzed by SDS-PAGE for the indicated proteins.

The studies of RalGDS and a less specific RalGEF member-Rgr revealed one possible mechanism of RalGEF activation is through release of an auto-inhibitory conformation by removing the N-terminal REM domain or C-terminal RA domain (Leonardi *et al.* 2002). To test whether the RGL1 mutants may be activated through that mechanism, we examined the ability of RGL1 in dimerization. As shown in figure 4.5A and B, wild type RGL1 dimerization is stronger with itself than either mutant, while both mutants have weaker dimerization with wild type and barely form homo-dimers. This was seen by expressing either full-length isoform (Myc-FL-RGL1) or ubiquitous isoform (Flag-RGL1 or GST-RGL1). A note has been taken that when we performed reciprocal immunoprecipitation or pulldown of wild type and mutants (figure 4.5A, lane5 and 7; figure 4.5B, lane5 and 7, lane 6 and 10)), the stoichiometry of the pulldown RGL1 is not 1:1 ratio. We reason that might result from oligomerization of wild type as shown in figure 4.5C. Another possible mechanism for RGL1 activation is through association with activated Ras via its C-terminal RA domain. We therefore examined if RGL1 mutant can interact with oncogenic Ras by co-transfection of HRasV12 construct with either wild type or mutants RGL1 (Figure 4.5D). We found that neither mutant has the same strong interaction as wild type, indicating the mutants are likely less responsive to

oncogenic Ras stimulation. Together, our data suggest that the molecular mechanism for hyperactive RGL1 mutants is through mutational inactivation of auto-inhibitory associations; moreover, this active conformational status of RGL1 mutants can lead to a Ras-independent hyper-activation of Ral pathway (figure 4.7). However, how their Ral family specificity is achieved remains an enigma.

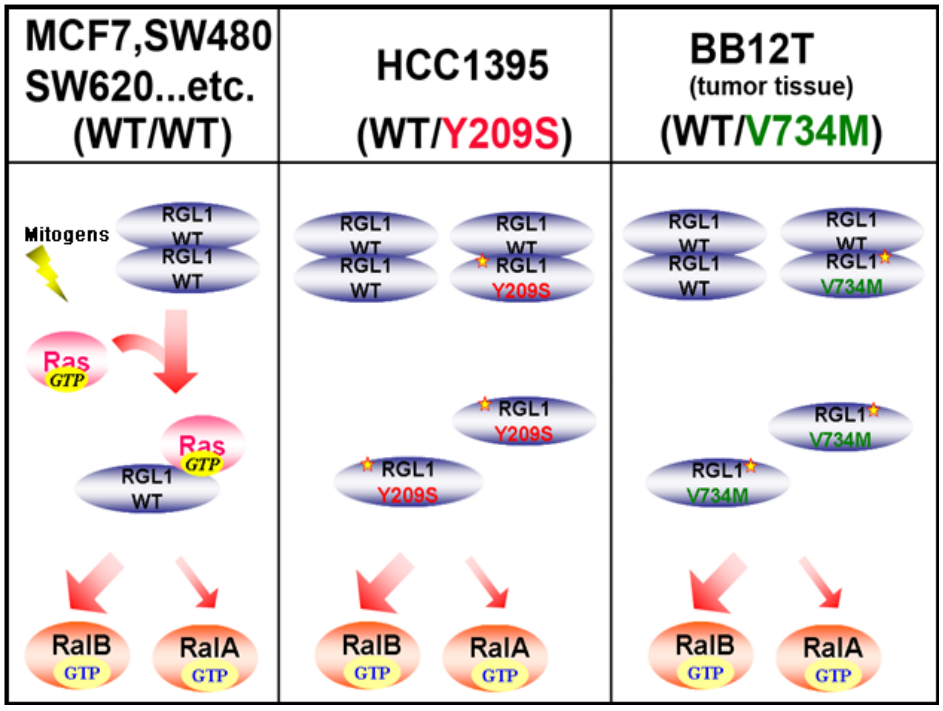


Fig. 4.6 A proposed model for molecular mechanism of RGL1 acitivation. In the cell lines without RGL1 mutation, the wild type RGL1 forms homo-dimer, and restricts downstream Ral activation. Upon receiving activated Ras signal, RGL1 switches to "open" conformation through interaction with Ras and further conducts the signal to RalA and RalB. In the case of RGL1 mutant expressing breast cancer cells (wither HCC1395 or BB12T), the mutants already in a less restrained status (by auto-inhibitory relief), which can active Ral pathway without Ras activation.

Previous studies have shown that persistent RalGEF-Ral activation is sufficient to transform immortalized human kidney epithelial cells (by hTERT and both small and large T antigen) (Hamad *et al.* 2002). Here, we found the RGL1 mutations identified in

human breast cancers are gain of function mutations, which amplify RalB activation for supporting cancer cell survival. Furthermore, both mutations are uncoupled from Ras activation and found in cancer cells that have no Ras mutation. Together, these observations suggest that RGL1 may be a potential oncogene. To examine that, we performed transformation assay by introducing tagged RGL1 wild type or mutant proteins into HME-hTERT cells mediated via retroviruses infection, and subsequently seeded them in semisolid agar to measure anchorage-independent growth. The infection efficacy were above 90%, as visualized by mRFP control (figure 4.7), and the RGL1 expression were lower but more even distributed than liposome based transfection (figure 4.7, lower panel and data not shown). Remarkably, expression of either wild type or mutant RGL1 is sufficient to transform HME-hTERT cells, as well as to deflect anoikis

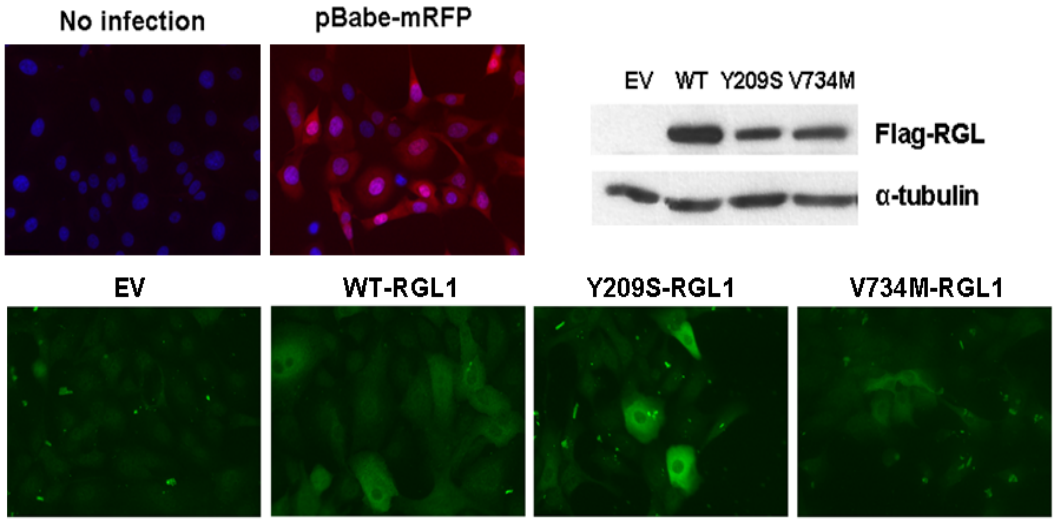


Fig. 4.7 The infection efficacy of HME-hTERT cells by RGL1 expressing retroviruses. The HME-hTERT cells were infected with replication-defective retroviruses expressing indicated constructs for 4 days, and fixed for immuno-fluorescence (no staining for pBabe-mRFP). pBabe-mRFP was used as a quality control of infection. The corresponding protein expressions were shown by western blot, using α-tubulin as loading control.

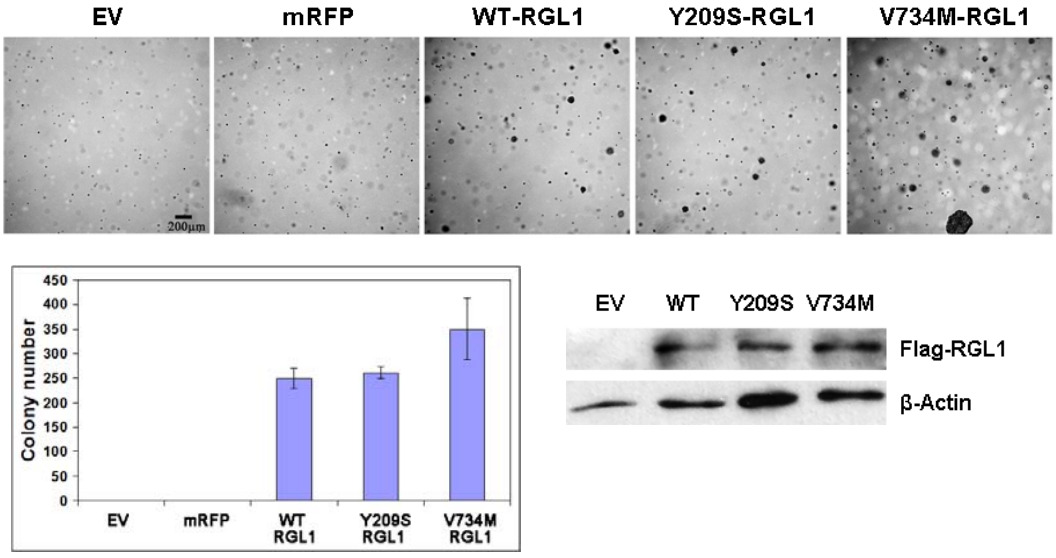


Fig. 4.8 RGL1 expression is sufficient to transform HME-hTERT cells. HEM-hTERT cells expressing indicated constructs for 3 days were seeded (2×10^4) in quadruplicates in semisolid agar. Representative photographs of anchorage-independent growth were taken 3 weeks later. Bars represent the means and SD of quadruplicates; the corresponding expression of RGL1 wild-type and mutants when seeded in soft agar were analysis by western blot using β-actin as loading control.

(figure 4.8 and data not shown).These data together suggest RGL1 may be a *bona fide* oncogene. Additionally, we noticed that in an oncogenic HRas transformed human immortalized mammary cells (HME-HRas), RGL1 mRNA expression level is 68.4 fold increased in comparison to the parental cells (HME). This correlates with our hypothesis that RGL1 may be an oncogene and support tumorigenesis. To test that, we examined whether RGL1 is required for HME-HRas survival, as shown in figure 4.9. The inhibition of RGL1 expression phenotypically mimics HRas and RalB depletion but not RalA, resulting in apoptosis of HME-HRas cells. Furthermore, inhibition of Sec5, a know RalB effector that mediated survival of cancer cells (Chien *et al.* 2006), also led to apoptosis of HME-HRas. This result suggests that the survival signal in HME-HRas is supported by HRas-RGL1-RalB-Sec5 signaling cascade.

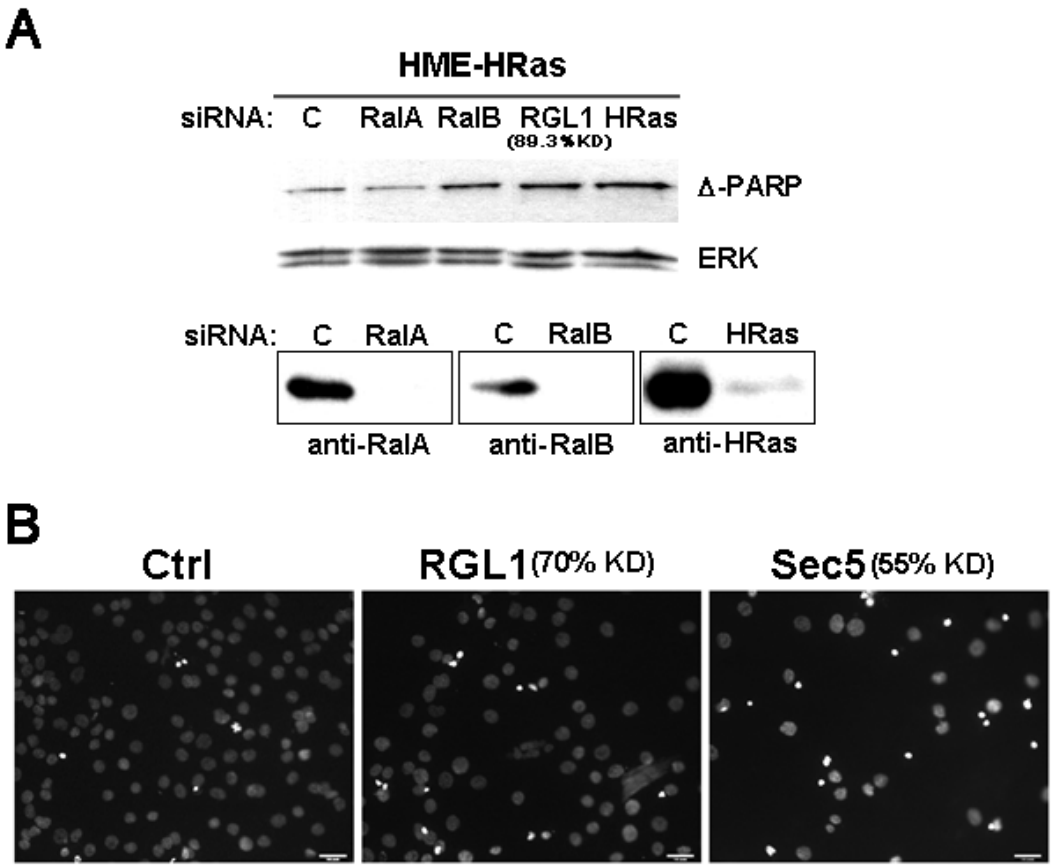


Fig. 4.9 HRas, RGL1 and RalB pathway are both required for survival of HME-HRas. (A) HME-HRas cells were transiently transfected with indicated siRNA. 72 hours post-tranfection, whole cell lysates were analyzed by SDS-PAGE for the indicated proteins. RNAi efficacy was validated by western blot or qPCR. (B) HME-HRas cells were transiently transfected with indicated siRNA. 72 hours later, cells were fixed and stained with DAPI to visualize chromatin structure.

It is known that Ral activation is relatively elevated in tumorigenic cells than related non-tumorigenic cells (Chien *et al.* 2006; Lim *et al.* 2006), however, how Ral is activated remains unclear. Here, our results suggest one possible mechanism is through up-regulating RalGEFs, which in this case is RGL1. We further examine if the elevated RGL1 expression results from oncogenic HRas expression. We did not observe any significant changes in RGL1 expression either by depleting HRas in HME-HRas cells or

by overexpression of HRasV12 in HME or HeLa cells (data not shown); indicating oncogenic HRas does not regulate RGL1 mRNA expression directly, or at least, it is not an acute response. We then speculate that chronic activation of oncogenic Ras may lead to or select for up-regulation of RalGEFs. By comparison between two sets of immortalized human bronchial epithelial cells with its corresponding tumorigenic lines (HBEC3KT vs. HBEC3KT-KRas; HBEC15KT53 vs. HBEC15KT53-KRas), we did not see any apparent alteration in RalGEFs expression upon KRas expression (figure 4.10).

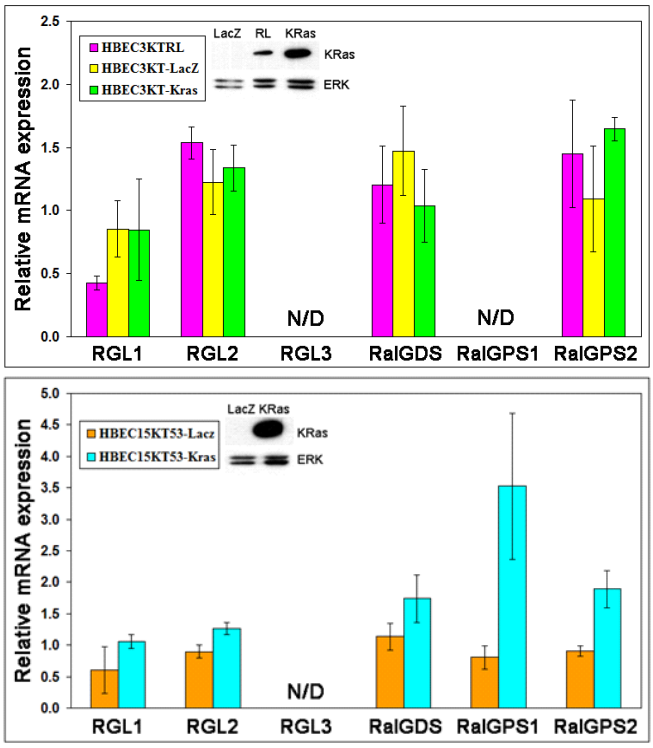


Fig. 4.10 None of RalGEFs mRNA expression is elevating in KRas transformed HBECs. The relative mRNA levels of RalGEFs in HBEC3KTs and HBEC15KT53s. RL: retrovirus transduced low KRas expression, LacZ: lentivirus transduced lacZ expression, KRas: lentivirus transduced KRas expression. All values are expressed relative to cyclophilin, and arithmetically adjusted to the medium-expressing sample as a unit of 1. Values represent the means and SD of three independent samples for each cell line. Note that as these data are portrayed, comparisons can only be made between different cell lines for a single GEF, not between various GEFs. Setting arbitrary cut-offs at CT < 25 (abundant); 25 < CT < 30 (moderate); CT > 35 (non detectable (ND)).

This finding disagrees with our previous observation in HME and HME-HRas pair, suggesting the elevated RGL1 expression may result from clonal selection. Perhaps mammary epithelial cells require up-regulation of RGL1-RalB survival signaling to tolerate oncogenic HRas; while bronchial epithelial cells it may employ other pathways to support tumorigenic growth.

In order to further understand the RGL1 function in complex regulatory networks, we generated RGL1 antibody and tested the specificity by depletion of endogenous RGL1 in HME-HRas cells (figure 4.11). The antibody recognizes a specific band around 90 KDa, which disappears in RGL1 siRNA sample, indicating this antibody can recognize endogenous RGL1. In addition, the efficacy of RGL1 siRNA validated by mRNA level correlates with the protein level at 72 or 96 hours post-transfection, indicating the protein expression level can be reflected by the mRNA level. The antibody could serve as a useful tool for future studies.

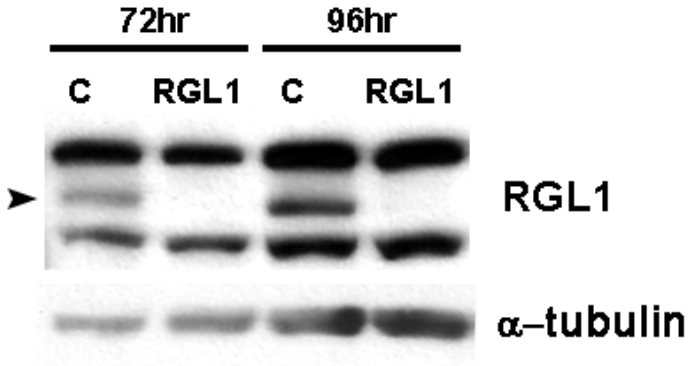


Fig. 4.11 The purified RGL1 rabbit polyclonal antibody can recognize endogenous RGL1 in HME-HRas cell line. HME-HRas cells were transiently transfected with indicated siRNA. 72 or 96 hours post-transfection, whole cell lysates were analyzed by SDS-PAGE with purified rabbit polyclonal anti-RGL1 antibody. Arrow head indicates

the endogenous RGL1 band, knockdown efficiency were also validated by qPCR (79% and 84% of knockdown, respectively).

Discussion

The necessity and sufficiency of RalGEF-Ral pathway in Ras-dependent transformation has been recognized and well accepted (Hamad *et al.* 2002; Rangarajan *et al.* 2004; Bodemann and White 2008); however, unlike Ras or other Ras effector pathways, the mutations of RalGEFs or Ral are rarely characterized until recently (Sjoblom *et al.* 2006; Ding *et al.* 2008). Here, we have validated the RGL1 mutation in breast cancel cell line HCC1395 is a gain of function mutation which provides survival signal through RalB activation. We examined another mutation identified in breast tumor tissue under culture conditions and found it to activate RalB as well. Notably, in an oncogenic HRas transformed mammary cell line (HME-HRas), RGL1 mRNA expression is specifically elevated. By loss of function studies we showed that HRas-RGL1-RalB pathway is required for survival of HME-HRas. Remarkably, chronic activation of RGL1 alone is sufficient to transform immortalized human mammary cells. Together, our results suggest that RGL1 is a *bona fide* oncogene and may contribute to tumorigenesis in breast cancer.

Despite RalGEFs contribution to diverse cellular functions through Ral activation, little is known about how their specificity toward Ral family members is achieved. A recent study showed that different RalGEFs may have distinct specificity toward RalA or RalB. During cytokinesis process in HeLa cells, depletion of RalGDS or RalGPS2 phenocopied depletion of RalA, while depletion of RGL1 or RalGPS1 phenocopied depletion of RalB (Cascone *et al.* 2008). Our results are consistent with

their finding, in which depletion of RalGDS phenotypically mimics depletion of RalA, whereas RGL1 phenotypically mimics depletion of RalB in HCC1395; moreover, we saw that the increase in GTP-bound RalB is more prominent than GTP-bound RalA under low RGL1 expression level in HeLa and 293T (data not shown), indicating RGL1 has some specificity toward RalB. However, the specificity may be cell context dependent since we observed decreased RalA activity in SW620 and no changes in RalA or RalB activity in MCF7 and SW480 upon depletion of RGL1 (figure4.2). It is also possible that we did not deplete RGL1 enough to see that phenotype since we used siRNA to transiently knockdown RGL1 but not a knockout.

Here, we showed that both RGL1 mutants are hyper-active in activating RalB which results in amplification of survival signaling and leads to oncogene-driven addiction to the RGL1-RalB pathway. Although we can not test that hypothesis in breast tumor tissue (because of accessibility), our observations in HCC1395 and an epigenetically transformed human mammary cell line (HME-HRas) support our model. Furthermore, we found that both RGL1 mutants reduce association with oncogenic HRas, indicating their hyper-active status is Ras independent; additionally, the mutants fail to dimerize suggesting the less restrained conformation. These data are not only consistent with previous findings in SOS1 (RasGEF) and Rgr (Leonardi *et al.* 2002; Martello and Pellicer 2006; Tartaglia *et al.* 2007), but also strengthen our proposed model which RGL1 mutations are gain of functions.

The most important evaluation of candidate oncogene is to test their capacity in driving tumorigenic growth. RalGEF-Ral pathway has been showed to be either necessary or sufficient to transform normal cells in conjunction with various genetic

elements (Hamad *et al.* 2002; Lim *et al.* 2005; Sablina *et al.* 2007). Most of the time, it was under the condition together with Ras, small and large T antigen in immortalized human kidney embryonic epithelial cells. Here, we showed that chronic activation of RGL1 (either wild type or mutants) is sufficient to transform immortalized human mammary cells (HME-hTERT), indicating RGL1 may be a *bona fide* oncogene. Though it might be only oncogenic in mammary cells, since the specified requirements for transformation of cells derived from different tissue origin may apply (Rangarajan *et al.* 2004). This remains to be tested. Our studies reveal the crucial role of the RalGEF-Ral pathway in human breast cancers and provide an example for oncogenic transformation mediated by Ras-independent Ral pathway activation.

Materials and Methods

Cell culture

HeLa and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals).293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) without sodium pyruvate supplemented with 10% fetal bovine serum. HCC1395 cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum. SW480 and SW620 were grown in 1x L-15 medium (Cellgro) supplemented with 10% fetal bovine serum. HME and HME-HRas cells were grown in MEGM mammary epithelial cell growth medium (Cambrex Bio Science Walkersville, Inc.) supplemented with 0.4% bovine pituitary extract, human epidermal growth factor (hEGF) 5 ng/ml, insulin 5 μg/ml, hydrocortisone 0.5 μg/ml, gentamicin 0.5 ml/l. HBEC3KT and HBEC15KT53 cells are human bronchial epithelial cell line immortalized by hTERT and CDK4 expression

(HBEC15KT53: with p53 knockdown. A gift from John Minna, UT Southwestern Medical Center), were grown in defined Keratinocyte-SFM medium (GIBCO) supplemented with human epidermal growth factor (hEGF) 5 ng/ml and bovine pituitary extract 50 μg/ml .

Reagents and antibodies

The following primary antibodies were used: mouse monoclonal anti-RalA, mouse monoclonal anti-cleaved PARP (Asp214), and mouse monoclonal anti-BrdU (BD Biosciences); mouse monoclonal anti-RalB (a gift from Larry Feig, Tufts University School of Medicine, Boston, MA); rabbit polyclonal anti-ERK1/2, mouse monoclonal anti-GST (26H1), and rabbit monoclonal anti-Myc (71D10) (Cell Signaling); rabbit polyclonal anti-Flag (Sigma-Aldrich); rabbit polyclonal anti-HA (Y11), mouse monoclonal anti-Myc (9E10), mouse monoclonal anti-Myc (9E10) agarose, and mouse monoclonal anti-HA (F7) agarose (Santa Cruz Biotechnology). The following secondary antibodies were used for either immunofluorescence or immunoblotting: Alexa fluor 594 goat anti-mouse IgG (H+L), Alexa fluor 594 goat anti-rabbit IgG (H+L), and Alexa fluor 488 goat anti-rabbit IgG (H+L) (Molecular probe, Eugene, OR); peroxidase-conjugated goat anti-mouse IgG (H+L) and peroxidase-conjugated goat anti-rabbit IgG (H+L) (Jackson Immunoresearch, West Groves, PA).

Plasmids construction

The coding region of full-length human RGL1 transcripts (NM_015149.3) (Sood *et al.* 2000) were PCR amplified from HME-HRas cDNA together with pV3-Flag-RGL1 ,a shorter form of RGL1 transcripts (AF186779.1, constructs were made with

sequence of identical region of both isoform, a gift from Jacques Camonis, Institute Curie, Paris, France) using the following primers, which were designed to incorporate BamHI and XbaI site and cloned into pRK5-myc vector:
Forward (BamHI site): 5'- CGCGGATCCATGGAGGTGA AACCTGTGGG-3',
Reverse (XbaI site): 5'- TGCTCTAGATCAGAGGGTGATTTTGC-3'.
The full-length pRK5-myc-RGL1-Y209S mutant was made by using primer-specific mutagenesis with following primers: Forward: 5'- CTGCTGGATTCTCTCACACGGA-3', Reverse: 5'- TCCGTGTGAGAGAATCCAGCAG-3'. Retroviral plasmid pBabe-puro-mRFP was made by PCR amplification monomeric red fluorescence protein from pCMV-mRFP-LC3 (a gift from Tamotsu Yoshimori, Osaka University, Japan) with following primers: Forward (BamHI site): 5'- CGCGGATCCATGGCCTCCTCCG-3', Reverse (EcoRI site): 5'-CCCGAATTCTTAGGCGCCGGTGG-3', and cloned into BamHI-EcoRI site. pBabe-puro-Flag-RGL1-WT and mutants were made (thanks Yi-Hung Ou, a great graduate student in White lab) by PCR amplification from pV3-Flag-RGL1 constructs using following primers: Forward (BamHI site): 5'- CGCGGATCC GCCACCATGGATTAC-3', Reverse (SalI-XhoI site): 5'- TTTGTCGACCTCGAGT CAGAGGGTGATTTTGCTGTGT-3', and cloned into BamHI-SalI site (SalI site is abolished after ligation). The fidelity of all PCR-generated constructs was confirmed by DNA sequencing.

DNA and siRNA Transfection

For DNA transfection, cells were seeded into 6 well cluster plates or 60 mm dishes to ~50% confluence on next day. Plasmids were transfected using Fugene HD transfection reagent (Roche Applied Science) according to the manufacturer's instructions. For siRNA

transfection, HCC1395 were reverse transfected (Whitehurst and White 2006) with 100nM siRNA by using oligofectamine (Invitrogen); MCF-7, SW620, SW480 were reverse transfected with 100nM siRNA by using DharmaFECT1 (Thermo Fisher Scientific Inc.); HME-HRas were transfected with 100nM siRNA by using DharmaFECT1. siRNA were designed by standard methods and use the following sense sequences to target genes of interests: Non-targeting control I, 5'-AUGAACGUG AAUUGCUCAAUU-3', Non-targeting control II, 5'- ACUCUAUCGCCAGCGUGA CUU-3', luciferase (as control), 5'- CUUACGCUGAGUACUUCGAdTdT-3', hRalA 5'-AGCUAAUGUUGACAAGGUAdTdT-3', hRalB 5'- GAAGAAGUUCAGAUAGA UAdTdT-3'. For RalGDS, RGL1, and RalGPS1 silencing, the SMARTpool (siGENOME) were obtained from Dharmacon (Thermo Fisher Scientific Inc.).

Virus production and Infection

Amphotropic retroviruses were produced by transfection of the 293FT cells (a gift from William Hahn, Dana-Farber Cancer Institute, Boston, Massachusetts) with pBabe-puro constructs as described above and pCL-Ampho (Imgenex, San Diego, Calif.), a vector encoding a replication-defective helper virus, by Fegene HD (Roche Applied Science). The virus supernatant was collected at 24-48 hr post-transfection, and used to infect HME cells with 8μg/ml polybrene. Lentivirus were produced by transfection of 293FT cells with pLKO.1 lentiviral shRNA constructs generated by TRC (Root *et al.* 2006), pCMVΔR8.91 (a packaging plasmid containing *gag*, *pol* and *rev* genes), and pMD2.G (VSV-G expressing plasmid) by Fugene HD. The virus supernatant was collected at 24-48 hr post-transfection, and used to infect HCC1395 and HME-HRas cells with 8μg/ml polybrene.

RGL1 Antibody production and purification

The recombinant antigens: GST-RGL1-full-lehgth and GST-RGL1-88-505 recombinant proteins (pGEX-2T-RGL1 constructs were gift from Akira Kikuchi, Hiroshima University School of Medicine, Tokyo, Japan) were expressed in *E. coli*. Briefly, the transformed *E. coli* were initially grown at 37 °C to an absorbance of 0.5~0.6 (optical density at 600 nm) and subsequently transferred to 20 °C for induction with isopropyl-β-$_D$-thioglactopyranoside (IPTG) 0.2mM for 20 hr. The bacteria pellet was then resuspended in lysis buffer (20 mM Tris, pH 8.0, 1% Triton X-100, 500 mM NaCl, 1mM EDTA with Complete proteinase inhibitor tablet (Roche)) and passed through EmulsiFlex-05 high pressure cell homogenizer (Avestin) 5-6 times to crack the cells. The supernatant were clarified by centrifugation at 10K g for 30 min at 4 °C and subjected to incubate with glutathione-Sepharose beads (GE Healthcare Life Sciences) at 4℃ for overnight. The purified GST-RGL1 proteins were then eluted by adding 50mM glutathion (in 50mM Tris-HCl, pH 8.0). Two rabbit polyclonal antibody were generated (Invitrogen) according to manufactory premium protocol. The crude sera were affinity purified by western strip technique, and tested by RGL1 knockdown cell lysates.

RNA measurement and real-time PCR

RNA from cultured cells was isolated using High Pure RNA Isolation Kit **(**Roche Applied Science) following the manufacturer's user manual. 1-2 μg of total RNA was reverse-transcribed with oligo-dT primer or random hexamers using SuperScript II (Invitrogen). Quantitative real-time PCR (qPCR) was performed using an Applied Biosystem Prism 7900HT sequence detection system and SYBR-green chemistry. Gene-

specific primers were designed using using the on-line program D-LUX™ Designer Software from Invitrogen, and validated by analysis of template titration and dissociation curves. Primer sequences are provided as below:

Gene Name	Accession Number	Sequence of Primers (5' to 3')
hRGL1	NM_015149.3	F: ATTGAGAAGTGGATCAACATCG R: AAACGATGGCCCTCAAGGAG
hRGL1 short form	AF186779	F: ACATTGCGTTGGCCTCCG R: GGTGACATGGTAAACAGCTCCTTC
hRGL1 full-length	AF186780	F: GCATCGTTCTAATTGCCG R: CTCAGGAACGCAGCCTCTAGC
hRalGDS	NM_006266.2	F: GTTTCCAGGGACAGTTTCCG R: CAGCTCCCGGCTCAATGAGTA
hRalGPS1	NM_014636.1	F: CCTGTCCTGACACATCTGTTGC R: GTAGGTGCCTTGCTTTCTCCG
hβ-Actin	NM_001101	F: GCACCCAGCACAATGAAGAT R: GCCGATCCACACGGAGTAC
hcyclophilin B	NM_000942	F: TGCCATCGCCAAGGAGTAG R: TGCACAGACGGTCACTCAAA

Results of qPCR were evaluated by the comparative Ct method (user bulletin No.2, Perkin Elmer Life Sciences) using cyclophilin or β-Actin as the invariant control gene.

Apoptosis assays

Apoptosis was quantitatively evaluated by flow cytometry or counting pyknotic nuclei under microscopy. Cells were collected, fixed with 70% ethanol at 4°C for 30 min, and stained with propidium iodide at 37°C for 30 minutes. Approximately 10,000 events were collected for each assay, and analyzed using Cell Quest software (Becton Dickinson).

Immunofluorescence

Cells were rinse with PBS and fixed in 3.7% formaldehyde at room temperature for 30 minutes. After fixation, cells were permeabilized for 5 min in CSK extraction buffer (50 mM NaCl, 300 mM Sucrose, 10 mM Pipes, pH6.8, 3 mM $MgCl_2$, 0.5% (v/v) Triton X-

100) and blocked in blocking solution (10% goat serum, 1% BSA, 50mM NH_4Cl in PBS) for 1 hr. Cells were then stained with primary and secondary antibodies for 1 hr at room temperature for each procedure and mounted on VECTASHIELD mounting medium (Vector Laboratories, Inc.).

Immunoprecipitation

293T cells were lysed with lysis buffer (1% NP-40, 20mM Tris-HCl, pH7.5, 0.5% sodium deoxycholate, 10mM MgCl2, 2mM EGTA, 10% Glycerol, 137mM NaCl, 1mM DTT with Complete proteinase inhibitor and PhosSTOP Phosphatase inhibitor cocktail tablet (Roche)) for 20min and clarified by centrifugation at 13K rpm for 5 min at 4˚C.The supernatant was collected and incubated with antibody-conjugated agarose or glutathione-Sepharose beads (GE Healthcare Life Sciences) for 3 hr then washed before subjected to SDS-PAGE analysis. Monoclonal anti-HA (F7) antibody-conjugated agarose (Sigma) and monoclonal anti-Myc (9E10) antibody-conjugated agarose (Santa Cruz Biotechnology) were used for immunoprecipitation.

Proliferation assay

48 hours post-transfection, the cells for suspension condition were replated onto 1% agarose-coated plates while the cells for attachment condition were also fed with fresh medium. Bromodeoxyuridine (BrdU) was then added 8hr later to a final concentration of 30μM. Following additional 14-16 hour incubation, cells were fixed with 3.7% formaldehyde, permeabilized with CSK extraction buffer (50 mM NaCl, 300 mM Sucrose, 10 mM Pipes, pH6.8, 3 mM MgCl2, 0.5% (v/v) Triton X-100) for 5 min, and then treated with 2 M HCl for 10 min. Cells were then blocked with blocking solution (10% goat serum, 1% BSA, 50mM NH_4Cl in PBS) for 1 hr. BrdU incorporation was

visualized with mouse monoclonal anti-BrdU and Alexa fluor 594-conjugated anti-mouse IgG.

Ral activation assay

48 hr or 72hr post transfection, cells were serum starved for 24hr prior assay. Cells were lysed with lysis buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 200 mM NaCl, 10 mM $MgCl_2$, 0.5mM DTT, 10% glycerol with Complete proteinase inhibitor and PhosSTOP Phosphatase inhibitor cocktail tablet (Roche)) for 5 min at 4℃ with rocking. The lysates were then clarified by centrifugation at 13K rpm, 5 min at 4℃ and measured protein concentration by Precision Red Advanced Protein Assay Reagent (Cytoskeleton, Inc.). The lysates were incubated with GST-Sec5 RBD immobilized on glutathione-Sepharose beads for 45-60 min at 4℃ and washed 3 times with lysis buffer prior to SDS-PAGE analysis.

Soft agar assay

Growth of cells in soft agar was determined by plating 2 x 10^4 cells in 12 well cluster plate in quadruplicates in 0.33% SeaKem GTG agar (Cambrex Bio Science Rockland, Inc., Rockland, ME). Colonies were took picture 3 weeks later, and stained with 0.05% crystal violet (in 20% Methanol) for 30 minutes and counted under dissecting microscopy.

CHAPTER FIVE

CONCLUSIONS AND DISCUSSION

Conclusions

Our initial study revealed the crucial role of Ral activation in supporting tumorigenic growth of Ras-dependent and –independent cancer cells. We showed that inhibition of Ral activation impairs anchorage-independent proliferation of cancer cells with diverse genetic backgrounds. Meanwhile, Ral activation is elevated or maintained in cancer cells but not normal cells in the absence of matrix association, which correlates with their ability to proliferate in matrix-independent manner. In addition, only Ral activation is concordant with the proliferation status of cancer cells and normal cells upon suspension, whereas ERK and AKT signals were both reduced. These data suggest that the aberrant proliferation of cancer cells under matrix disassociation is supported by RalGEF-Ral pathway regardless of oncogenic Ras activation.

In the second part of our studies, we questioned how RalGEFs distribute in a wide range of cancerous and non-cancerous cells in order to better understand how Ral is regulated better. We applied quantitative real-time PCR to acquire RalGEFs expression profiles. Surprisingly, the RalGEFs distribution is much more complex than we expected. RGL2 and RalGPS2 are abundantly and ubiquitously expressed in every cell line we examined, while RalGDS, RGL1, RGL3 and RalGPS2 display more distinct distribution. This study implied that cells derived from different origin may engage different RalGEFs (or combination) to activate Ral and execute various functions. Additionally, we found that RalGDS mRNA expression is significantly elevated in tumorous tissue by analysis of

Ral and RalGEFs in 14 pairs of normal versus tumor kidney tissues, suggesting the existence of direct correlation between RalGEFs expression and cancers.

Lastly, we validated mutations found in one of the RalGEFs—RGL1, in human tumors, as gain of function mutations, and our results suggest RGL1 as a novel oncogene that can transform an immortalized human mammary cell line. Two mutations were found in RGL1 from sequencing human protein coding genes of 11 breast cancers; one was found in the N-terminal Ras exchange motif (Y209S) from HCC1395 breast cancer cell line, another was found in C-terminal Ras association domain (V734M) from BB12T breast tumor tissue. We showed that RGL1 is required for survival of HCC1395 but not other related cancer cell lines which do not harbor RGL1 mutation. RGL1 depletion in HCC1395 results in reduced RalA and RalB activation, while RGL1 depletion only decreases RalA activation in SW620 and has no effect in SW480 and MCF7. Epigenetic overexpression of RGL1 wild type or mutants activate both RalA and RalB, surprisingly, RGL1 mutants drive more RalB activation compare to wild type, while RalA activation remains equivalent to wild-type. Together, these data suggest that RGL1 mutations are gain of function mutations and provide survival signal through RalB activation in HCC1395. The molecular mechanism of the hyper-active RGL1 mutants may result from relief of auto-inhibition, since we observed that RGL1 mutants were impaired in dimerization as well as association with activated Ras. The discordant observations in Ral activation between different cell lines upon RGL1 depletion implies that the usage and specificity of RalGEFs in Ral activation may differ from cell line to cell line.

Additionally, we found that oncogenic HRas transformed human mammary epithelial cell line (HME-HRas) has an elevated RGL1 expression status compare to its

parental line (HME); depletion of RGL1 leads to apoptosis of HME-HRas, which phenotypically mimics depletion of HRas and RalB. This result suggests an oncogenic addiction of HRas-RGL1-RalB pathway in HME-HRas. Furthermore, we showed that chronic activation of RGL1 is sufficient to transform immortalized human mammary epithelial cells. Together, our studies identified RGL1 as the first *bona fide* oncogene found in RalGEFs family which may relate to breast cancer.

Discussion

The RalGEF-Ral pathway has been shown to make enormous contributions to tumorigenesis and metastasis (Joffe and Adam 2001; Gonzalez-Garcia *et al.* 2005; Lim *et al.* 2005; Chien *et al.* 2006; Lim *et al.* 2006; Smith *et al.* 2006; Sablina *et al.* 2007; Yin *et al.* 2007); however, most efforts were done from the viewpoint of Ras-mediated oncogenesis. Our previous observations and recent studies reveal the possibility that RalGEF-Ral pathway activation may also contribute to Ras-independent transformation. It was known that PDK1 physically interacts with N-terminus of RalGDS and enhances its activity upon EGF stimulation (Tian *et al.* 2002); moreover, a PDK1-AKT complex mediates EGF-induced membrane protrusion through activating Ral (Yoshizaki *et al.* 2007). These findings suggest that the PI3K effector pathway can facilitate RalGEF-Ral pathway activation. Additionally, the finding of another RalGEF family with PXXP motif and PH domain instead of RA domain (de Bruyn *et al.* 2000; Rebhun *et al.* 2000), provide direct evidence that Ras is not the only activator for RalGEF-Ral activation. As shown by our results, we found that Ral activation is required for tumorigenic growth in suspension culture of a variety of cancer cells with diverse genetic backgrounds, including oncogenic KRas, HRas, BRaf and chronic PI3K expression. Importantly, we

found that among the three well-known effector pathways mediating transformation, only Ral activation is consistent with the tumorigenic growth, indicating upon suspension, the Raf-MEK-ERK or PI3K pathway is uncoupled from proliferation signals. This is consistent with previous studies (Lim *et al.* 2005; Lim *et al.* 2006), and suggests approaches targeting RalGEF-Ral pathway in cancer therapy may be more effective.

The mechanisms of how RalGEFs get activated remain poorly understood. Most research has used overexpression, which sometimes overwhelms biological systems and obscures the nature of RalGEF functions. Owing to poor antibody availability, we examined the RalGEFs mRNA expression in a wide panel of cell lines. Surprisingly, the RalGEFs expression is divergent and complicated, unlike RalGTPase which are ubiquitously expressed. This implies that the RalGEF crew may be selectively engaged in cells derived from different tissue origin. The redundancy and specificity of RalGEFs are other issues waiting to be answered; however, that may also be cell type dependent. A recent study in collaboration with our group, using loss of function methods revealed the specificity of RalGEFs during cytokinesis. Their data suggested that RalGDS and RalGPS2 specifically activate RalA while RGL1 and RalGPS1 activate RalB during cytokinesis (Cascone *et al.* 2008),and it was the first time that RalGEFs distinct specificity was shown. It still requires antibody development and further studies to get a better understanding in RalGEFs diversity and specificity.

In the last part of our study, we validated gain of function mutations of RGL1 which amplify survival signaling through RalB activation to support tumor cell survival. A possible mechanism of the hyper-active RGL1 mutations is through relief of auto-inhibitory interactions by reducing the N-terminus or C-terminus constraints. Similar

observations were also reported in Rgr and SOS1, which are another RalGDS related GEF and RasGEF respectively. Though Rgr also activates Ral, because the specificity of Rgr is not restricted to Ral (Rgr also activates Ras), we did not include it as a RalGEF in our study. The Rgr was originally identified as an oncogene in rabbit squamous cell carcinoma (D'Adamo *et al.* 1997), lately, a human orthologue was also found (Leonardi *et al.* 2002). Interestingly, this group found mutant Rgr transcripts from lymphoid malignant cell lines and tissues were truncated and oncogenic. The truncated transcripts only contain the C-terminal GEF domain indicating the N-terminus may serve as a self-inhibitory domain. Meanwhile, SOS1, a RasGEF was also found mutated in patients with Noonan syndrome (a developmental disorder characterized by short stature, facial dysmorphia, congenital heart defects and skeletal anomalies). The mutation clusters were scattering mostly on the N-terminus PH and REM domain, resulting in hyper-active Ras (Tartaglia *et al.* 2007). Together with our studies, this highlights aberrant guanine nucleotide exchange factors as another pathological cause of human disease.

Our study broadens knowledge of the participation of the RalGEF-Ral pathway in tumorigenicity. Further investigation will focus on finding the specificity of individual RalGEFs and the related cellular functions and will hopefully help us understand the whole picture of the RalGTPase contributions in tumorigenesis.

BIBLIOGRAPHY

AGAPOVA, L. S., J. L. VOLODINA, ET AL. (2004). "ACTIVATION OF RAS-RAL PATHWAY ATTENUATES P53-INDEPENDENT DNA DAMAGE G2 CHECKPOINT." J BIOL CHEM 279(35): 36382-9.

AGATHANGGELOU, A., S. HONORIO, ET AL. (2001). "METHYLATION ASSOCIATED INACTIVATION OF RASSF1A FROM REGION 3P21.3 IN LUNG, BREAST AND OVARIAN TUMOURS." ONCOGENE 20(12): 1509-18.

ALBRIGHT, C. F., B. W. GIDDINGS, ET AL. (1993). "CHARACTERIZATION OF A GUANINE NUCLEOTIDE DISSOCIATION STIMULATOR FOR A RAS-RELATED GTPASE." EMBO J 12(1): 339-47.

ARTEAGA, C. L. (2002). "EPIDERMAL GROWTH FACTOR RECEPTOR DEPENDENCE IN HUMAN TUMORS: MORE THAN JUST EXPRESSION?" ONCOLOGIST 7(90004): 31-39.

BALDA, M. S., M. D. GARRETT, ET AL. (2003). "THE ZO-1-ASSOCIATED Y-BOX FACTOR ZONAB REGULATES EPITHELIAL CELL PROLIFERATION AND CELL DENSITY." J CELL BIOL 160(3): 423-32.

BALDA, M. S. AND K. MATTER (2000). "THE TIGHT JUNCTION PROTEIN ZO-1 AND AN INTERACTING TRANSCRIPTION FACTOR REGULATE ERBB-2 EXPRESSION." EMBO J 19(9): 2024-33.

BHATTACHARYA, M., A. V. BABWAH, ET AL. (2004). "RAL AND PHOSPHOLIPASE D2-DEPENDENT PATHWAY FOR CONSTITUTIVE METABOTROPIC GLUTAMATE RECEPTOR ENDOCYTOSIS." J NEUROSCI 24(40): 8752-61.

BODEMANN, B. O. AND M. A. WHITE (2008). "RAL GTPASES AND CANCER: LINCHPIN SUPPORT OF THE TUMORIGENIC PLATFORM." NAT REV CANCER 8(2): 133-40.

BOETTNER, B., E. E. GOVEK, ET AL. (2000). "THE JUNCTIONAL MULTIDOMAIN PROTEIN AF-6 IS A BINDING PARTNER OF THE RAP1A GTPASE AND ASSOCIATES WITH THE ACTIN CYTOSKELETAL REGULATOR PROFILIN." PROC NATL ACAD SCI U S A 97(16): 9064-9.

CAI, D., A. IYER, ET AL. (2003). "AND-34/BCAR3, A GDP EXCHANGE FACTOR WHOSE OVEREXPRESSION CONFERS ANTIESTROGEN

RESISTANCE, ACTIVATES RAC, PAK1, AND THE CYCLIN D1 PROMOTER." CANCER RES 63(20): 6802-8.

CAMONIS, J. H. AND M. A. WHITE (2005). "RAL GTPASES: CORRUPTING THE EXOCYST IN CANCER CELLS." TRENDS CELL BIOL 15(6): 327-32.

CANTOR, S. B., T. URANO, ET AL. (1995). "IDENTIFICATION AND CHARACTERIZATION OF RAL-BINDING PROTEIN 1, A POTENTIAL DOWNSTREAM TARGET OF RAL GTPASES." MOLECULAR & CELLULAR BIOLOGY 15(8): 4578-84.

CASCONE, I., R. SELIMOGLU, ET AL. (2008). "DISTINCT ROLES OF RALA AND RALB IN THE PROGRESSION OF CYTOKINESIS ARE SUPPORTED BY DISTINCT RALGEFS." EMBO J 27(18): 2375-87.

CERIANI, M., C. SCANDIUZZI, ET AL. (2007). "FUNCTIONAL ANALYSIS OF RALGPS2, A MURINE GUANINE NUCLEOTIDE EXCHANGE FACTOR FOR RALA GTPASE." EXP CELL RES 313(11): 2293-307.

CHARDIN, P. AND A. TAVITIAN (1986). "THE RAL GENE: A NEW RAS RELATED GENE ISOLATED BY THE USE OF A SYNTHETIC PROBE." EMBO J 5(9): 2203-8.

CHIEN, Y., S. KIM, ET AL. (2006). "RALB GTPASE-MEDIATED ACTIVATION OF THE IKAPPAB FAMILY KINASE TBK1 COUPLES INNATE IMMUNE SIGNALING TO TUMOR CELL SURVIVAL." CELL 127(1): 157-70.

CHIEN, Y. AND M. A. WHITE (2003). "RAL GTPASES ARE LINCHPIN MODULATORS OF HUMAN TUMOUR-CELL PROLIFERATION AND SURVIVAL." EMBO REP 4(8): 800-6.

CHIEN, Y. AND M. A. WHITE (2003). "RAL GTPASES ARE LINCHPIN MODULATORS OF HUMAN TUMOUR-CELL PROLIFERATION AND SURVIVAL." EMBO REP. 4: 800.

CHIU, V. K. (2002). "RAS SIGNALLING ON THE ENDOPLASMIC RETICULUM AND THE GOLGI." NATURE CELL BIOL. 4: 343.

CHONG, H. AND K. L. GUAN (2003). "REGULATION OF RAF THROUGH PHOSPHORYLATION AND N TERMINUS-C TERMINUS INTERACTION." J BIOL CHEM 278(38): 36269-76.

CRIVICI, A. AND M. IKURA (1995). "MOLECULAR AND STRUCTURAL BASIS OF TARGET RECOGNITION BY CALMODULIN." ANNU REV BIOPHYS BIOMOL STRUCT 24: 85-116.

D'ADAMO, D. R., S. NOVICK, ET AL. (1997). "RSC: A NOVEL ONCOGENE WITH STRUCTURAL AND FUNCTIONAL HOMOLOGY WITH THE GENE FAMILY OF EXCHANGE FACTORS FOR RAL." ONCOGENE 14(11): 1295-305.

DAMMANN, R., C. LI, ET AL. (2000). "EPIGENETIC INACTIVATION OF A RAS ASSOCIATION DOMAIN FAMILY PROTEIN FROM THE LUNG TUMOUR SUPPRESSOR LOCUS 3P21.3." NAT GENET 25(3): 315-9.

DAVIES, H., G. R. BIGNELL, ET AL. (2002). "MUTATIONS OF THE BRAF GENE IN HUMAN CANCER." NATURE 417(6892): 949-54.

DE BRUYN, K. M., J. DE ROOIJ, ET AL. (2000). "RALGEF2, A PLECKSTRIN HOMOLOGY DOMAIN CONTAINING GUANINE NUCLEOTIDE EXCHANGE FACTOR FOR RAL." J BIOL CHEM 275(38): 29761-6.

DE GORTER, D. J., R. M. REIJMERS, ET AL. (2008). "THE SMALL GTPASE RAL MEDIATES SDF-1-INDUCED MIGRATION OF B CELLS AND MULTIPLE MYELOMA CELLS." BLOOD 111(7): 3364-72.

DE RUITER, N. D., B. M. BURGERING, ET AL. (2001). "REGULATION OF THE FORKHEAD TRANSCRIPTION FACTOR AFX BY RAL-DEPENDENT PHOSPHORYLATION OF THREONINES 447 AND 451." MOL CELL BIOL 21(23): 8225-35.

DER, C. J., T. G. KRONTIRIS, ET AL. (1982). "TRANSFORMING GENES OF HUMAN BLADDER AND LUNG CARCINOMA CELL LINES ARE HOMOLOGOUS TO THE RAS GENES OF HARVEY AND KIRSTEN SARCOMA VIRUSES." PROC. NATL ACAD. SCI. USA 79: 3637.

DING, L., G. GETZ, ET AL. (2008). "SOMATIC MUTATIONS AFFECT KEY PATHWAYS IN LUNG ADENOCARCINOMA." NATURE 455(7216): 1069-75.

ESSERS, M. A., S. WEIJZEN, ET AL. (2004). "FOXO TRANSCRIPTION FACTOR ACTIVATION BY OXIDATIVE STRESS MEDIATED BY THE SMALL GTPASE RAL AND JNK." EMBO J 23(24): 4802-12.

EVAN, G. AND T. LITTLEWOOD (1998). "A MATTER OF LIFE AND CELL DEATH." SCIENCE 281(5381): 1317-22.

FALSETTI, S. C., D. A. WANG, ET AL. (2007). "GERANYLGERANYLTRANSFERASE I INHIBITORS TARGET RALB TO INHIBIT ANCHORAGE-DEPENDENT GROWTH AND INDUCE APOPTOSIS AND RALA TO INHIBIT ANCHORAGE-INDEPENDENT GROWTH." MOL CELL BIOL 27(22): 8003-14.

FEIG, L. A. (2003). "RAL-GTPASES: APPROACHING THEIR 15 MINUTES OF FAME." TRENDS CELL BIOL 13(8): 419-25.

FEIG, L. A., T. URANO, ET AL. (1996). "EVIDENCE FOR A RAS/RAL SIGNALING CASCADE." TRENDS BIOCHEM SCI 21(11): 438-41.

FRANKEL, P., A. ARONHEIM, ET AL. (2005). "RALA INTERACTS WITH ZONAB IN A CELL DENSITY-DEPENDENT MANNER AND REGULATES ITS TRANSCRIPTIONAL ACTIVITY." EMBO J 24(1): 54-62.

GILDEA, J. J., M. A. HARDING, ET AL. (2002). "THE ROLE OF RAL A IN EPIDERMAL GROWTH FACTOR RECEPTOR-REGULATED CELL MOTILITY." CANCER RES 62(4): 982-5.

GOI, T., M. SHIPITSIN, ET AL. (2000). "AN EGF RECEPTOR/RAL-GTPASE SIGNALING CASCADE REGULATES C-SRC ACTIVITY AND SUBSTRATE SPECIFICITY." EMBO J 19(4): 623-30.

GOLDFARB, M., K. SHIMIZU, ET AL. (1982). "ISOLATION AND PRELIMINARY CHARACTERIZATION OF A HUMAN TRANSFORMING GENE FROM T24 BLADDER CARCINOMA CELLS." NATURE 296: 404-409.

GONZALEZ-GARCIA, A. (2005). "RALGDS IS REQUIRED FOR TUMOR FORMATION IN A MODEL OF SKIN CARCINOGENESIS." CANCER CELL 7: 219.

GONZALEZ-GARCIA, A., C. A. PRITCHARD, ET AL. (2005). "RALGDS IS REQUIRED FOR TUMOR FORMATION IN A MODEL OF SKIN CARCINOGENESIS." CANCER CELL 7(3): 219-26.

GOTOH, T., D. CAI, ET AL. (2000). "P130CAS REGULATES THE ACTIVITY OF AND-34, A NOVEL RAL, RAP1, AND R-RAS GUANINE NUCLEOTIDE EXCHANGE FACTOR." J BIOL CHEM 275(39): 30118-23.

GREEN, D. R. AND G. I. EVAN (2002). "A MATTER OF LIFE AND DEATH." CANCER CELL 1(1): 19-30.

HALL, A., C. J. MARSHALL, ET AL. (1983). "IDENTIFICATION OF TRANSFORMING GENE IN TWO HUMAN SARCOMA CELL LINES AS A NEW MEMBER OF THE RAS GENE FAMILY LOCATED ON CHROMOSOME 1." NATURE 303: 396.

HAMAD, N. M. (2002). "DISTINCT REQUIREMENTS FOR RAS ONCOGENESIS IN HUMAN VERSUS MOUSE CELLS." GENES DEV. 16: 2045.

HAMAD, N. M., J. H. ELCONIN, ET AL. (2002). "DISTINCT REQUIREMENTS FOR RAS ONCOGENESIS IN HUMAN VERSUS MOUSE CELLS." GENES DEV 16(16): 2045-57.

HANAHAN, D. AND R. A. WEINBERG (2000). "THE HALLMARKS OF CANCER." CELL 100(1): 57-70.

HANCOCK, J. F., H. PATERSON, ET AL. (1990). "A POLYBASIC DOMAIN OR PALMITOYLATION IS REQUIRED IN ADDITION TO THE CAAX MOTIF TO LOCALIZE P21RAS TO THE PLASMA MEMBRANE." CELL 63: 133.

HAO, Y., R. WONG, ET AL. (2008). "RALGDS COUPLES GROWTH FACTOR SIGNALING TO AKT ACTIVATION." MOL CELL BIOL 28(9): 2851-9.

HARDISSON, D. (2003). "MOLECULAR PATHOGENESIS OF HEAD AND NECK SQUAMOUS CELL CARCINOMA." EUROPEAN ARCHIVES OF OTO-RHINO-LARYNGOLOGY 260(9): 502.

HENRY, D. O., S. A. MOSKALENKO, ET AL. (2000). "RAL GTPASES CONTRIBUTE TO REGULATION OF CYCLIN D1 THROUGH ACTIVATION OF NF-KAPPAB." MOL CELL BIOL 20(21): 8084-92.

HERNANDEZ-MUNOZ, I., M. MALUMBRES, ET AL. (2000). "THE RGR ONCOGENE (HOMOLOGOUS TO RALGDS) INDUCES TRANSFORMATION AND GENE EXPRESSION BY ACTIVATING RAS, RAL AND RHO MEDIATED PATHWAYS." ONCOGENE 19(23): 2745-57.

HOFER, F., R. BERDEAUX, ET AL. (1998). "RAS-INDEPENDENT ACTIVATION OF RAL BY A CA(2+)-DEPENDENT PATHWAY." CURR BIOL 8(14): 839-42.

HOFER, F., S. FIELDS, ET AL. (1994). "ACTIVATED RAS INTERACTS WITH THE RAL GUANINE NUCLEOTIDE DISSOCIATION STIMULATOR." PROC. NATL ACAD. SCI. USA 91: 11089.

JENKINS, G. M. AND M. A. FROHMAN (2005). "PHOSPHOLIPASE D: A LIPID CENTRIC REVIEW." CELL MOL LIFE SCI 62(19-20): 2305-16.

JIANG, H., J. Q. LUO, ET AL. (1995). "INVOLVEMENT OF RAL GTPASE IN V-SRC-INDUCED PHOSPHOLIPASE D ACTIVATION." NATURE 378(6555): 409-12.

JILONG YANG, X. D. A. J. F. L. R. P. K. H. K. C. X. H. J. T. W. Z. (2008). "GENETIC ABERRATIONS OF GASTROINTESTINAL STROMAL TUMORS." CANCER 113(7): 1532-1543.

JOFFE, E. B. AND A. ADAM (2001). "[CELL TRANSFORMATION AND SURVIVAL MECHANISMS REGULATED BY GTPASE RAL]." MEDICINA (B AIRES) 61(5 PT 2): 658-63.

JULLIEN-FLORES, V., O. DORSEUIL, ET AL. (1995). "BRIDGING RAL GTPASE TO RHO PATHWAYS. RLIP76, A RAL EFFECTOR WITH CDC42/RAC GTPASE-ACTIVATING PROTEIN ACTIVITY." JOURNAL OF BIOLOGICAL CHEMISTRY 270(38): 22473-7.

JULLIEN-FLORES, V., Y. MAHE, ET AL. (2000). "RLIP76, AN EFFECTOR OF THE GTPASE RAL, INTERACTS WITH THE AP2 COMPLEX: INVOLVEMENT OF THE RAL PATHWAY IN RECEPTOR ENDOCYTOSIS." J CELL SCI 113(PT 16): 2837-44.

KARNOUB, A. E. AND R. A. WEINBERG (2008). "RAS ONCOGENES: SPLIT PERSONALITIES." NAT REV MOL CELL BIOL 9(7): 517-31.

KHOSRAVI-FAR, R., P. A. SOLSKI, ET AL. (1995). "ACTIVATION OF RAC1, RHOA, AND MITOGEN-ACTIVATED PROTEIN KINASES IS REQUIRED FOR RAS TRANSFORMATION." MOL. CELL. BIOL. 15: 6443.

KIKUCHI, A., S. D. DEMO, ET AL. (1994). "RALGDS FAMILY MEMBERS INTERACT WITH THE EFFECTOR LOOP OF RAS P21." MOL. CELL. BIOL. 14: 7483.

KIKUCHI, A., S. D. DEMO, ET AL. (1994). "RALGDS FAMILY MEMBERS INTERACT WITH THE EFFECTOR LOOP OF RAS P21." MOL CELL BIOL 14(11): 7483-91.

KIM, J. H., S. D. LEE, ET AL. (1998). "ACTIVATION OF PHOSPHOLIPASE D1 BY DIRECT INTERACTION WITH ADP- RIBOSYLATION FACTOR 1 AND RALA." FEBS LETT 430(3): 231-5.

KINSELLA, B. T., R. A. ERDMAN, ET AL. (1991). "CARBOXYL-TERMINAL ISOPRENYLATION OF RAS-RELATED GTP-BINDING PROTEINS ENCODED BY RAC1, RAC2, AND RALA." J BIOL CHEM 266(15): 9786-94.

KURIYAMA, M. (1996). "IDENTIFICATION OF AF-6 AND CANOE AS PUTATIVE TARGETS FOR RAS." J. BIOL. CHEM. 271: 607.

LEEVERS, S. J., H. F. PATERSON, ET AL. (1994). "REQUIREMENT FOR RAS IN RAF ACTIVATION IS OVERCOME BY TARGETING RAF TO THE PLASMA MEMBRANE." NATURE 369: 411.

LEONARDI, P., E. KASSIN, ET AL. (2002). "HUMAN RGR: TRANSFORMING ACTIVITY AND ALTERATION IN T-CELL MALIGNANCIES." ONCOGENE 21(33): 5108-16.

LI, G., L. HAN, ET AL. (2007). "RALA AND RALB FUNCTION AS THE CRITICAL GTP SENSORS FOR GTP-DEPENDENT EXOCYTOSIS." J NEUROSCI 27(1): 190-202.

LIM, K. H., A. T. BAINES, ET AL. (2005). "ACTIVATION OF RALA IS CRITICAL FOR RAS-INDUCED TUMORIGENESIS OF HUMAN CELLS." CANCER CELL 7(6): 533-45.

LIM, K. H., K. O'HAYER, ET AL. (2006). "DIVERGENT ROLES FOR RALA AND RALB IN MALIGNANT GROWTH OF HUMAN PANCREATIC CARCINOMA CELLS." CURR BIOL 16(24): 2385-94.

LIPSCHUTZ, J. H. AND K. E. MOSTOV (2002). "EXOCYTOSIS: THE MANY MASTERS OF THE EXOCYST." CURR BIOL 12(6): R212-4.

LOWE, S. W. (1999). "ACTIVATION OF P53 BY ONCOGENES." ENDOCR RELAT CANCER 6(1): 45-8.

LU, Z., A. HORNIA, ET AL. (2000). "PHOSPHOLIPASE D AND RALA COOPERATE WITH THE EPIDERMAL GROWTH FACTOR RECEPTOR TO TRANSFORM 3Y1 RAT FIBROBLASTS." MOL CELL BIOL 20(2): 462-7.

MARAIS, R., Y. LIGHT, ET AL. (1995). "RAS RECRUITS RAF-1 TO THE PLASMA MEMBRANE FOR ACTIVATION BY TYROSINE PHOSPHORYLATION." EMBO J 14(13): 3136-45.

MARTELLO, L. A. AND A. PELLICER (2006). "BIOCHEMICAL AND BIOLOGICAL ANALYSES OF RGR RALGEF ONCOGENE." METHODS ENZYMOL 407: 115-28.

MATALLANAS, D. (2006). "DISTINCT UTILIZATION OF EFFECTORS AND BIOLOGICAL OUTCOMES RESULTING FROM SITE-SPECIFIC RAS ACTIVATION: RAS FUNCTIONS IN LIPID RAFTS AND GOLGI COMPLEX ARE DISPENSABLE FOR PROLIFERATION AND TRANSFORMATION." MOL. CELL. BIOL. 26: 100.

MATSUBARA, K., T. HINOI, ET AL. (1997). "THE POST-TRANSLATIONAL MODIFICATIONS OF RAL AND RAC1 ARE IMPORTANT FOR THE ACTION OF RAL-BINDING PROTEIN 1, A PUTATIVE EFFECTOR PROTEIN OF RAL." FEBS LETT 410(2-3): 169-74.

MATSUBARA, K., S. KISHIDA, ET AL. (1999). "PLASMA MEMBRANE RECRUITMENT OF RALGDS IS CRITICAL FOR RAS-DEPENDENT RAL ACTIVATION." ONCOGENE 18(6): 1303-12.

MCCUBREY, J. A., L. S. STEELMAN, ET AL. (2007). "ROLES OF THE RAF/MEK/ERK PATHWAY IN CELL GROWTH, MALIGNANT TRANSFORMATION AND DRUG RESISTANCE." BIOCHIMICA ET BIOPHYSICA ACTA (BBA) - MOLECULAR CELL RESEARCH 1773(8): 1263-1284.

MOSKALENKO, S., D. O. HENRY, ET AL. (2002). "THE EXOCYST IS A RAL EFFECTOR COMPLEX." NAT CELL BIOL 4(1): 66-72.

MOSKALENKO, S., C. TONG, ET AL. (2003). "RAL GTPASES REGULATE EXOCYST ASSEMBLY THROUGH DUAL SUBUNIT INTERACTIONS." J BIOL CHEM 278(51): 51743-8.

NAKASHIMA, S., K. MORINAKA, ET AL. (1999). "SMALL G PROTEIN RAL AND ITS DOWNSTREAM MOLECULES REGULATE ENDOCYTOSIS OF EGF AND INSULIN RECEPTORS." EMBO J 18(13): 3629-42.

NOVICK, P. AND W. GUO (2002). "RAS FAMILY THERAPY: RAB, RHO AND RAL TALK TO THE EXOCYST." TRENDS CELL BIOL 12(6): 247-9.

OHTA, Y., N. SUZUKI, ET AL. (1999). "THE SMALL GTPASE RALA TARGETS FILAMIN TO INDUCE FILOPODIA." PROC NATL ACAD SCI U S A 96(5): 2122-8.

OMIDVAR, N., L. PEARN, ET AL. (2006). "RAL IS BOTH NECESSARY AND SUFFICIENT FOR THE INHIBITION OF MYELOID DIFFERENTIATION MEDIATED BY RAS." MOL CELL BIOL 26(10): 3966-75.

OUWENS, D. M., N. D. DE RUITER, ET AL. (2002). "GROWTH FACTORS CAN ACTIVATE ATF2 VIA A TWO-STEP MECHANISM:

PHOSPHORYLATION OF THR71 THROUGH THE RAS-MEK-ERK PATHWAY AND OF THR69 THROUGH RALGDS-SRC-P38." EMBO J 21(14): 3782-93.

OXFORD, G., C. R. OWENS, ET AL. (2005). "RALA AND RALB: ANTAGONISTIC RELATIVES IN CANCER CELL MIGRATION." CANCER RES 65(16): 7111-20.

PAN, J. Y. AND M. WESSLING-RESNICK (1998). "GEF-MEDIATED GDP/GTP EXCHANGE BY MONOMERIC GTPASES: A REGULATORY ROLE FOR MG2+?" BIOESSAYS 20(6): 516-21.

PARADA, L. F., C. J. TABIN, ET AL. (1982). "HUMAN EJ BLADDER CARCINOMA ONCOGENE IS HOMOLOGUE OF HARVEY SARCOMA VIRUS RAS." NATURE 297: 474-478.

PARK, S. H. AND R. A. WEINBERG (1995). "A PUTATIVE EFFECTOR OF RAL HAS HOMOLOGY TO RHO/RAC GTPASE ACTIVATING PROTEINS." ONCOGENE 11(11): 2349-55.

PETERSON, S. N., L. TRABALZINI, ET AL. (1996). "IDENTIFICATION OF A NOVEL RALGDS-RELATED PROTEIN AS A CANDIDATE EFFECTOR FOR RAS AND RAP1." J BIOL CHEM 271(47): 29903-8.

POLZIN, A., M. SHIPITSIN, ET AL. (2002). "RAL-GTPASE INFLUENCES THE REGULATION OF THE READILY RELEASABLE POOL OF SYNAPTIC VESICLES." MOL CELL BIOL 22(6): 1714-22.

PONTING, C. P. AND D. R. BENJAMIN (1996). "A NOVEL FAMILY OF RAS-BINDING DOMAINS. TRENDS." BIOCHEM. SCI. 21: 422.

PULCIANI, S. (1982). "ONCOGENES IN HUMAN TUMOR CELL LINES: MOLECULAR CLONING OF A TRANSFORMING GENE FROM HUMAN BLADDER CARCINOMA CELLS." PROC. NATL ACAD. SCI. USA 79: 2845-2849.

RAJAGOPALAN, H. (2002). "TUMORIGENESIS: RAF/RAS ONCOGENES AND MISMATCH-REPAIR STATUS." NATURE 418: 934.

RANGARAJAN, A., S. J. HONG, ET AL. (2004). "SPECIES- AND CELL TYPE-SPECIFIC REQUIREMENTS FOR CELLULAR TRANSFORMATION." CANCER CELL 6(2): 171-83.

REBHUN, J. F., H. CHEN, ET AL. (2000). "IDENTIFICATION AND CHARACTERIZATION OF A NEW FAMILY OF GUANINE

NUCLEOTIDE EXCHANGE FACTORS FOR THE RAS-RELATED GTPASE RAL." J BIOL CHEM 275(18): 13406-10.

REDDY, E. P., R. K. REYNOLDS, ET AL. (1982). "A POINT MUTATION IS RESPONSIBLE FOR THE ACQUISITION OF TRANSFORMING PROPERTIES BY THE T24 HUMAN BLADDER CARCINOMA ONCOGENE." NATURE 300: 149.

REUTHER, G. W. AND C. J. DER (2000). "THE RAS BRANCH OF SMALL GTPASES: RAS FAMILY MEMBERS DON'T FALL FAR FROM THE TREE." CURR OPIN CELL BIOL 12(2): 157-65.

RIGGI, N. AND I. STAMENKOVIC (2007). "THE BIOLOGY OF EWING SARCOMA." CANCER LETTERS 254(1): 1.

RODRIGUEZ-VICIANA, P. (1994). "PHOSPHATIDYLINOSITOL-3-OH KINASE AS A DIRECT TARGET FOR RAS." NATURE 370: 527-532.

RODRIGUEZ-VICIANA, P. (1997). "ROLE OF PHOSPHOINOSITIDE 3-OH KINASE IN CELL TRANSFORMATION AND CONTROL OF THE ACTIN CYTOSKELETON BY RAS." CELL 89: 457.

RONDAIJ, M. G., R. BIERINGS, ET AL. (2008). "GUANINE EXCHANGE FACTOR RALGDS MEDIATES EXOCYTOSIS OF WEIBEL-PALADE BODIES FROM ENDOTHELIAL CELLS." BLOOD 112(1): 56-63.

ROOT, D. E., N. HACOHEN, ET AL. (2006). "GENOME-SCALE LOSS-OF-FUNCTION SCREENING WITH A LENTIVIRAL RNAI LIBRARY." NAT METHODS 3(9): 715-9.

ROSARIO, M., H. F. PATERSON, ET AL. (2001). "ACTIVATION OF THE RAL AND PHOSPHATIDYLINOSITOL 3' KINASE SIGNALING PATHWAYS BY THE RAS-RELATED PROTEIN TC21." MOL CELL BIOL 21(11): 3750-62.

ROSSE, C., A. HATZOGLOU, ET AL. (2006). "RALB MOBILIZES THE EXOCYST TO DRIVE CELL MIGRATION." MOL CELL BIOL 26(2): 727-34.

RUSANESCU, G., T. GOTOH, ET AL. (2001). "REGULATION OF RAS SIGNALING SPECIFICITY BY PROTEIN KINASE C." MOL CELL BIOL 21(8): 2650-8.

SABLINA, A. A., W. CHEN, ET AL. (2007). "THE TUMOR SUPPRESSOR PP2A ABETA REGULATES THE RALA GTPASE." CELL 129(5): 969-82.

SHAO, H. AND D. A. ANDRES (2000). "A NOVEL RALGEF-LIKE PROTEIN, RGL3, AS A CANDIDATE EFFECTOR FOR RIT AND RAS." J BIOL CHEM 275(35): 26914-24.

SHERR, C. J. (2001). "THE INK4A/ARF NETWORK IN TUMOUR SUPPRESSION." NAT REV MOL CELL BIOL 2(10): 731.

SHIH, C. AND R. A. WEINBERG (1982). "ISOLATION OF A TRANSFORMING SEQUENCE FROM A HUMAN BLADDER SARCOMA CELL LINE." CELL 29: 161-169.

SHIVAKUMAR, L., J. MINNA, ET AL. (2002). "THE RASSF1A TUMOR SUPPRESSOR BLOCKS CELL CYCLE PROGRESSION AND INHIBITS CYCLIN D1 ACCUMULATION." MOL CELL BIOL 22(12): 4309-18.

SIDHU, R. S., S. M. ELSARAJ, ET AL. (2005). "CALMODULIN BINDING TO THE SMALL GTPASE RAL REQUIRES ISOPRENYLATED RAL." BIOCHEM BIOPHYS RES COMMUN 336(1): 105-9.

SJOBLOM, T., S. JONES, ET AL. (2006). "THE CONSENSUS CODING SEQUENCES OF HUMAN BREAST AND COLORECTAL CANCERS." SCIENCE 314(5797): 268-74.

SMITH, S. C., G. OXFORD, ET AL. (2006). "THE METASTASIS-ASSOCIATED GENE CD24 IS REGULATED BY RAL GTPASE AND IS A MEDIATOR OF CELL PROLIFERATION AND SURVIVAL IN HUMAN CANCER." CANCER RES 66(4): 1917-22.

SOOD, R., I. MAKALOWSKA, ET AL. (2000). "THE HUMAN RGL (RALGDS-LIKE) GENE: CLONING, EXPRESSION ANALYSIS AND GENOMIC ORGANIZATION." BIOCHIM BIOPHYS ACTA 1491(1-3): 285-8.

SOURISSEAU, T., A. GEORGIADIS, ET AL. (2006). "REGULATION OF PCNA AND CYCLIN D1 EXPRESSION AND EPITHELIAL MORPHOGENESIS BY THE ZO-1-REGULATED TRANSCRIPTION FACTOR ZONAB/DBPA." MOL CELL BIOL 26(6): 2387-98.

SPAARGAREN, M. AND J. R. BISCHOFF (1994). "IDENTIFICATION OF THE GUANINE NUCLEOTIDE DISSOCIATION STIMULATOR FOR RAL AS A PUTATIVE EFFECTOR MOLECULE OF R-RAS, H-RAS, K-RAS, AND RAP." PROC. NATL ACAD. SCI. USA 91: 12609.

STOSSEL, T. P., J. CONDEELIS, ET AL. (2001). "FILAMINS AS INTEGRATORS OF CELL MECHANICS AND SIGNALLING." NAT REV MOL CELL BIOL 2(2): 138-45.

TAKAYA, A., Y. OHBA, ET AL. (2004). "RALA ACTIVATION AT NASCENT LAMELLIPODIA OF EPIDERMAL GROWTH FACTOR-STIMULATED COS7 CELLS AND MIGRATING MADIN-DARBY CANINE KIDNEY CELLS." MOL BIOL CELL 15(6): 2549-57.

TAPAROWSKY, E. (1982). "ACTIVATION OF THE T24 BLADDER CARCINOMA TRANSFORMING GENE IS LINKED TO A SINGLE AMINO ACID CHANGE." NATURE 300: 762.

TARTAGLIA, M., L. A. PENNACCHIO, ET AL. (2007). "GAIN-OF-FUNCTION SOS1 MUTATIONS CAUSE A DISTINCTIVE FORM OF NOONAN SYNDROME." NAT GENET 39(1): 75-9.

TERBUSH, D. R., T. MAURICE, ET AL. (1996). "THE EXOCYST IS A MULTIPROTEIN COMPLEX REQUIRED FOR EXOCYTOSIS IN SACCHAROMYCES CEREVISIAE." EMBO J 15(23): 6483-94.

TIAN, X., G. RUSANESCU, ET AL. (2002). "PDK1 MEDIATES GROWTH FACTOR-INDUCED RAL-GEF ACTIVATION BY A KINASE-INDEPENDENT MECHANISM." EMBO J 21(6): 1327-38.

TOMMASI, S., R. DAMMANN, ET AL. (2005). "TUMOR SUSCEPTIBILITY OF RASSF1A KNOCKOUT MICE." CANCER RES 65(1): 92-8.

TONG, L. A. (1989). "STRUCTURAL DIFFERENCES BETWEEN A RAS ONCOGENE PROTEIN AND THE NORMAL PROTEIN." NATURE 337: 90.

TRAN, N. H., X. WU, ET AL. (2005). "B-RAF AND RAF-1 ARE REGULATED BY DISTINCT AUTOREGULATORY MECHANISMS." J BIOL CHEM 280(16): 16244-53.

URANO, T., R. EMKEY, ET AL. (1996). "RAL-GTPASES MEDIATE A DISTINCT DOWNSTREAM SIGNALING PATHWAY FROM RAS THAT FACILITATES CELLULAR TRANSFORMATION." EMBO J. 15: 810.

VAN DAM, E. M. AND P. J. ROBINSON (2006). "RAL: MEDIATOR OF MEMBRANE TRAFFICKING." INT J BIOCHEM CELL BIOL 38(11): 1841-7.

VAVVAS, D., X. LI, ET AL. (1998). "IDENTIFICATION OF NORE1 AS A POTENTIAL RAS EFFECTOR." J. BIOL. CHEM. 273: 5439.

VOS, M. D. (2003). "RASSF2 IS A NOVEL K-RAS-SPECIFIC EFFECTOR AND POTENTIAL TUMOR SUPPRESSOR." J. BIOL. CHEM. 278: 28045.

VOS, M. D., A. MARTINEZ, ET AL. (2003). "THE PRO-APOPTOTIC RAS EFFECTOR NORE1 MAY SERVE AS A RAS-REGULATED TUMOR SUPPRESSOR IN THE LUNG." J. BIOL. CHEM. 278: 21938.

WHITE, M. A. (1995). "MULTIPLE RAS FUNCTIONS CAN CONTRIBUTE TO MAMMALIAN CELL TRANSFORMATION." CELL 80: 533.

WHITE, M. A., T. VALE, ET AL. (1996). "A ROLE FOR THE RAL GUANINE NUCLEOTIDE DISSOCIATION STIMULATOR IN MEDIATING RAS-INDUCED TRANSFORMATION." J. BIOL. CHEM. 271: 16439.

WHITE, M. A., T. VALE, ET AL. (1996). "A ROLE FOR THE RAL GUANINE NUCLEOTIDE DISSOCIATION STIMULATOR IN MEDIATING RAS-INDUCED TRANSFORMATION." J BIOL CHEM 271(28): 16439-42.

WHITEHURST, A. W., R. RAM, ET AL. (2008). "THE RASSF1A TUMOR SUPPRESSOR RESTRAINS ANAPHASE-PROMOTING COMPLEX/CYCLOSOME ACTIVITY DURING THE G1/S PHASE TRANSITION TO PROMOTE CELL CYCLE PROGRESSION IN HUMAN EPITHELIAL CELLS." MOL CELL BIOL 28(10): 3190-7.

WHITEHURST, A. W. AND M. A. WHITE (2006). "HARNESSING RNAI FOR ANALYSES OF RAS SIGNALING AND TRANSFORMATION." METHODS ENZYMOL 407: 259-68.

WU, J. C., T. Y. CHEN, ET AL. (2005). "IDENTIFICATION OF V23RALA-SER194 AS A CRITICAL MEDIATOR FOR AURORA-A-INDUCED CELLULAR MOTILITY AND TRANSFORMATION BY SMALL POOL EXPRESSION SCREENING." J BIOL CHEM 280(10): 9013-22.

XU, L., P. FRANKEL, ET AL. (2003). "ELEVATED PHOSPHOLIPASE D ACTIVITY IN H-RAS- BUT NOT K-RAS-TRANSFORMED CELLS BY THE SYNERGISTIC ACTION OF RALA AND ARF6." MOL CELL BIOL 23(2): 645-54.

YIN, J., C. POLLOCK, ET AL. (2007). "ACTIVATION OF THE RALGEF/RAL PATHWAY PROMOTES PROSTATE CANCER METASTASIS TO BONE." MOL CELL BIOL 27(21): 7538-50.

YIP-SCHNEIDER, M. T., A. LIN, ET AL. (1999). "LACK OF ELEVATED MAP KINASE (ERK) ACTIVITY IN PANCREATIC CARCINOMAS DESPITE ONCOGENIC K-RAS EXPRESSION." INT J ONCOL 15(2): 271-9.

YOSHIZAKI, H., N. MOCHIZUKI, ET AL. (2007). "AKT-PDK1 COMPLEX MEDIATES EPIDERMAL GROWTH FACTOR-INDUCED MEMBRANE

PROTRUSION THROUGH RAL ACTIVATION." MOL BIOL CELL 18(1): 119-28.

YUAN, T. L. AND L. C. CANTLEY (2008). "PI3K PATHWAY ALTERATIONS IN CANCER: VARIATIONS ON A THEME." ONCOGENE 27(41): 5497-510.

ZHANG, X. F. (1993). "NORMAL AND ONCOGENIC P21RAS PROTEINS BIND TO THE AMINO-TERMINAL REGULATORY DOMAIN OF C-RAF-1." NATURE 364: 308.

ZHANG, Z., H. REHMANN, ET AL. (2005). "AF6 NEGATIVELY REGULATES RAP1-INDUCED CELL ADHESION." J BIOL CHEM 280(39): 33200-5.

ZHAO, L. AND P. K. VOGT (2008). "CLASS I PI3K IN ONCOGENIC CELLULAR TRANSFORMATION." ONCOGENE 27(41): 5486-96.