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RAL GTPases: Biology and Potential as Therapeutic Targets in Cancer

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Abstract—More than a hundred proteins comprise the RAS superfamily of small GTPases. This family can be divided into RAS, RHO, RAB, RAN, ARF, and RAD subfamilies, with each shown to play distinct roles in human cells in both health and disease. The RAS subfamily has a well-established role in human cancer with the three genes, *HRAS*, *KRAS*, and *NRAS* being the commonly mutated in tumors. These *RAS* mutations, most often functionally activating, are especially common in pancreatic, lung, and colorectal cancers. Efforts to inhibit RAS and related GTPases have produced inhibitors targeting the downstream effectors of RAS

signaling, including inhibitors of the RAF-mitogen-activated protein kinase/extracellular signal-related kinase (ERK)-ERK kinase pathway and the phosphoinositide-3-kinase-AKT-mTOR kinase pathway. A third effector arm of RAS signaling, mediated by RAL (RAS like) has emerged in recent years as a critical driver of RAS oncogenic signaling and has not been targeted until recently. RAL belongs to the RAS branch of the RAS superfamily and shares a high structural similarity with RAS. In human cells, there are two genes, *RALA* and *RALB*, both of which have been shown to play roles in the proliferation, survival, and metastasis of a variety

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of human cancers, including lung, colon, pancreatic, prostate, skin, and bladder cancers. In this review, we summarize the latest knowledge of RAL in the context

of human cancer and the recent advancements in the development of cancer therapeutics targeting RAL small GTPases.

I. Introduction

Two closely related G-proteins, RALA and RALB, constitute the RAL branch of the RAS superfamily of small GTPases (Chardin and Tavitian, 1986). Activating oncogenic RAS mutations have been shown to drive many different types of human cancer (Cox et al., 2014). Since its discovery in 1982, numerous efforts have been made to target RAS in human cancer with little success (Cox et al., 2014). Many different approaches have been tried, including direct targeting of the active site, targeting RAS cell membrane localization, targeting the interaction with its upstream activators, and targeting its downstream effector signaling pathway. To date, the most effective strategy to target RAS oncogenic signaling has been inhibiting the downstream RAF-MEK-ERK and PI3K-AKT-mTOR effector pathways (Roberts and Der, 2007; Yap et al., 2008). In the last decade, however, the RALGEF-RAL signaling pathway has emerged as a third important effector signaling axis downstream of RAS (Gentry et al., 2014). This has prompted the search for ways to manipulate RAL signaling as a potential therapeutic strategy for targeting RAS-driven human cancer.

II. Evolutionary Biology and Structure of RAL

More than 150 small GTPases have been identified in the RAS superfamily (Cox and Der, 2010), which can be divided into five main families (RAS, RHO, RAN, RAB, and ARF) based on differences in sequence, structure, and function (Goitre et al., 2014). The RAS family itself is further divided into six subfamilies: RAS, RAL, RIT, RAP, RHEB, and RAD. RAL is the closest relative of RAS on the phylogenetic tree, sharing a high degree of sequence similarity with RAS (>50%), hence the name RAL (RAS like). RAL gene was initially identified in 1986 during a search for RAS-related genes in a simian cDNA library (Chardin and Tavitian, 1986). The human RALA and RALB genes were identified 3 years later using the simian RAL cDNA as a probe in a human pheochromocytoma cDNA library (Chardin and Tavitian, 1989). The two protein isoforms, RALA and RALB, despite sharing 82% similarity to each other at amino acid level, exhibit distinctive biologic functions especially in human cancer, which will be discussed in section V. Although RAL genes and proteins are highly conserved across species, invertebrates only harbor single RAL gene. Studies in *Caenorhabditis elegans* and *Drosophila* have confirmed the interaction between RAL and its known effectors, like

TANK-binding kinase 1 (TBK1), SEC5, and RALBP1 (Mirey et al., 2003; Chien et al., 2006), and shed light on new effector signaling pathways such as Msn MAP4 kinase and mTOR signaling (Balakireva et al., 2006; Martin et al., 2014).

The protein structure for both RALA and RALB has been resolved, either alone or together with their binding partners (Nicely et al., 2004; Fenwick et al., 2009; Popovic et al., 2016). The tertiary protein structure of RALA and RALB is also very similar; both proteins contain a free-floating N-terminal 11-amino acid sequence, followed by the G-domain, involved in GDP/GTP binding, and the C-terminal membrane targeting sequence. RAL proteins share a similar G-domain architecture with RAS, consisting of six β -sheets connected by loops and five α -helices, which form the highly flexible switch I and switch II regions (Fig. 1). Structural comparison between the GDP- and GTP-bound form of RAL revealed that guanine nucleotide exchange mainly induces conformational changes in the two flexible switch regions, which are responsible for the selective binding to both regulators and effectors of RAL. RALA and RALB share almost identical sequence in the switch regions but differ considerably in the C-terminal hypervariable region. The essential role of the C-terminal hypervariable region in the correct intracellular trafficking and membrane localization of RAS proteins has been extensively studied (Cox et al., 2015). Like RAS, RAL proteins also terminate with the CAAX tetrapeptide motif sequence (C = cysteine, A = aliphatic amino acids, X = any amino acid), which signals for a series of posttranslational modification events that are critical for the membrane association of RAL.

III. RAL Biochemistry and Enzymology

Small GTPases are small (20–25 kDa) G-proteins that can bind to and hydrolyze GTP. A typical G-protein is active when bound to GTP and inactive when bound to GDP and therefore can function as a molecular on and off switch in signaling pathways. RAL activity can be regulated by altering the cellular concentration of GTP and GDP bound states, by posttranslational modifications of the C-terminal end or phosphorylation by Aurora A kinase and protein kinase C (Fig. 2).

A. GTP-GDP Cycling

Like other small GTPases, RAL cycles between the GDP-bound inactive state and GTP-bound active state

ABBREVIATIONS: ERK, extracellular signal-regulated kinase; EXO84, EXO cyst complex 84; FTase, farnesyltransferase; GGTase I, geranylgeranyltransferase I; MEK, mitogen-activated protein kinase/ERK kinase; mTOR, mechanistic target of rapamycin; PI3K, phosphoinositide-3-kinase; RAL, RAS like; RALBP1, RAL binding protein 1; RALGAP, RAL GTPase activating protein; RALGEF, RAL guanine exchange factor; SCC, squamous cell carcinoma; TBK1, TANK-binding kinase 1.

Fig. 1. Structure comparison between human RALA and RALB proteins. Structure models of RALA-GDP, RALA-GNP, RALB-GDP, RALB-GNP are shown in ribbon representation. GDP and GNP (nonhydrolyzable form of GTP) is shown in yellow, Mg is shown as a green sphere, α -helices are shown in red, and β -sheets are shown in cyan. Models were generated with Accelrys Discovery Studio software (San Diego, CA) using published structures.

in cells. The intrinsic GTPase activity of RAL GTPases is very weak, hence they rely on guanine nucleotide exchange factors (RALGEF) and GTPase activating proteins (RALGAP) to catalyze the GDP-GTP exchange. RALGEFs favor formation of RAL-GTP by stimulating exchange of GDP for GTP when intracellular concentrations of GTP are 10 times higher than GDP. RALGAPs catalyze the hydrolysis of GTP to GDP, returning RAL to an inactive state. RALA and RALB have the same enzymology, are regulated by the same set of RALGEFs and RALGAPs, and can bind to the same set of downstream effectors. However, as discussed below, they exhibit distinct roles in cellular function and human cancer.

The seven known RALGEFs, RALGDS, RGL1, RGL2, RGL3, RGL4, RALGPS1, and RALGPS2, can be divided into two groups. The first (RALGDS, RGL1, RGL2, RGL3) can directly bind to the effector binding region of activated RAS and hence relies on RAS activation, while the other group (RALGPS1, RALGPS2, RGL4) can be activated by RAS-independent mechanisms (Gentry et al., 2014). The most characterized RALGEF is RALGDS, which is also the first RALGEF discovered by yeast two-hybrid screening aimed at identifying RAS effectors (Albright et al., 1993). RALGDS was shown to be specific for RALA and RALB but not for other small GTPases. Subsequently, RGL1, RGL2, and RGL3

were also discovered using yeast two-hybrid screening (Kikuchi et al., 1994; Isomura et al., 1996; Wolthuis et al., 1996; Shao and Andres, 2000) and found to share a similar domain structure with RALGDS, consisting of the N-terminal REM (RAS exchanger motif) domain, the CDC25 catalytic domain, and the C-terminal RA (RAS association) domain (Ferro and Trabalzi, 2010). Activated RAS recruits RALGEFs onto the plasma membrane and delivers them to RALA and RALB. The other subset of RALGEFs, RALGPS1, RALGPS2, and RGL4, retain the CDC25 catalytic domain but lack the RAS-interacting domains REM and RA and are therefore activated independent of RAS (D'Adamo et al., 1997; de Bruyn et al., 2000; Rebhun et al., 2000; Ceriani et al., 2007). RALGPS1 and RALGPS2 contain a C-terminal PH (pleckstrin homology) domain that is believed to be important for its membrane targeting and activation of RAL. RGL4 has neither the RA domain nor the PH domain, and its activation mechanism is still poorly understood. Very recently, a chromosomal passenger complex protein, TD-60, was identified as an eighth RALGEF that activates RALA but not RALB during mitosis. However, little is known regarding the structural characteristics or the exact mechanism of Ral activation by TD-60 (Papini et al., 2015). Relatively little is known about RAL specific GAP proteins. Two closely related proteins, RALGAP1 and RALGAP2, were

Fig. 2. (A) The three most characterized effector signaling pathways downstream of RAS. (B) The GDP-GTP cycle of RAL small GTPases. Upstream signals stimulate binding of RALGEF that triggers GDP-GTP exchange, resulting in the activation of RAL. RAL-GTP can then bind to its downstream effector proteins such as RALBP1. Finally, RALGAPs bind to RAL-GTP and hydrolyze GTP to GDP, cycling RAL back to its inactive GDP-bound form.

identified to have GAP activity for RAL (Shirakawa et al., 2009; Chen et al., 2011). RALGAPs are heterodimers containing one α -catalytic subunit and one β -regulatory subunit. Supporting an inhibitory role on RAL activity, knockdown of RALGAP2 α promoted bladder cancer metastasis through enhancement of RAL activity (Saito et al., 2013). RALGAP- α subunit was also shown to be involved in insulin-stimulated RALA activation and GLUT4 trafficking (Chen et al., 2011, 2014). The regulatory subunit RALGAP- β was found to play an essential role in RAL-mediated regulation of spindle formation and chromatin separation during mitosis (Personnic et al., 2014).

B. Posttranslational Modifications

The requirement of posttranslational modifications for correct membrane association of RAS has been widely acknowledged (Gentry et al., 2015). Like RAS, RAL proteins also bear the C-terminal CAAX motif that are subject to similar posttranslational modification. The CAAX motif of RALA is CCIL and of RALB is CCLL (Gentry et al., 2015). The first step of the posttranslational

modifications is the covalent addition of an isoprenoid lipid to the first cysteine residue by the geranylgeranyl-transferase-I (GGTase-I) (Falsetti et al., 2007). The AAX residues were then cleaved by RAS-converting endopeptidase 1; and the terminal lipid-modified cysteine methylated by isoprenylcysteine carboxymethyltransferase-1. This process was shown to be essential for RAL membrane association and subcellular localization.

In addition to prenylation of the CAAX motif, phosphorylation of RAL at the C-terminal region plays an important role in the regulation of RAL activity. Aurora A kinase and protein kinase C were shown to phosphorylate C-terminal serine residues of RALA (S194) and RALB (S198), respectively (Lim et al., 2010; Wang et al., 2010; Martin et al., 2012). Similar to RAS, protein kinase-mediated phosphorylation of the C-terminal of RAL has been shown to regulate the subcellular localization and function of RAL. Phosphorylation of RALA by Aurora A relocates RALA from plasma membrane to endomembranes where RALA interacts with RALBP1 and is required for RALA mediated anchorage-independent growth and tumorigenicity in human cancer cells (Lim et al., 2010). Phosphorylation of RALB by protein kinase C translocated RALB from plasma membrane to the perinuclear region of the cell and is also required for RALB-mediated migration and metastasis (Wang et al., 2010; Martin et al., 2012).

IV. Effectors of RAL

The RAS-RAL signaling axis is involved in various physiologic and pathologic processes, such as membrane trafficking, actin cytoskeletal reorganization, transcription regulation, and kinase cascade signaling. Like RAS, after activation, RAL exerts its biologic function via binding to various effector proteins, most of which were discovered by two-hybrid screening.

A. RAL Binding Protein 1/RLIP76

RAL binding protein 1 (RALBP1, also known as RLIP or RLIP76) was the first identified and most extensively studied effector of RAL. RALBP1 was originally discovered as RAL binding partner in two independent experiments using either a yeast two hybrid library or a cDNA library screen (Cantor et al., 1995; Jullien-Flores et al., 1995). RALBP1 is a 76 kDa protein with a RAL binding domain that binds to both switch I and II regions of RAL-GTP (Fenwick et al., 2010). RALBP1 binds a diverse group of proteins and mediates different cellular functions. RALBP1 bears GAP activity for CDC42/Rac1 small GTPases, which are involved in actin dynamics, filopodia formation, and membrane ruffling (Park and Weinberg, 1995). RALBP1 can also promote invadopodium formation upon activation by RALB (Neel et al., 2012). Furthermore, RALBP1 is necessary for migration of human bladder and prostate

cancers (Wu et al., 2010). Collectively, these results suggest an important role of RALBP1 in cancer cell adhesion, invasion, and migration. RALBP1 is also important in endocytosis, especially receptor-mediated endocytosis of growth factor receptors through direct interaction with proteins known to be involved in receptor-mediated endocytosis such as REPS1, REPS2, and AP2 (Nakashima et al., 1999; Jullien-Flores et al., 2000). RALBP1 functions as a drug transporter in cancer cells and can render cells resistant to certain chemotherapeutic drugs (Awasthi et al., 2000). This function is independent from RAL binding and attributed to the two ATP binding motifs. This allows RALBP1 to function as an ATP-dependent transporter and increase the efflux of glutathione conjugates of electrophilic compounds and therefore protect cancer cells from cytotoxic drugs. RAL-dependent RALBP1 activation is required for mitochondrial fission at mitosis (Kashatus et al., 2011). Mitochondrial fission is prerequisite for the proper distribution of mitochondria to daughter cells during cell division. RALA-RALBP1 signaling plays an essential role in this process by inducing phosphorylation of the enzyme DRP1 GTPase and facilitating recruitment of DRP1 to mitochondrial membranes.

B. SEC5/EXO84 Complex 84

SEC5/EXO84 are critical components of the exocyst complex, which plays an essential role in exosome generation and cell migration. The exocyst complex is a multiprotein complex which is responsible for the appropriate trafficking of various secretory vesicles to specific domains of plasma membrane (He and Guo, 2009). RAL proteins regulate exocyst-dependent vesicle trafficking and are required for exocyst complex assembly (Camonis and White, 2005). SEC5 was first identified as a direct target of RAL and was found to mediate the effects of RALA on both the basolateral delivery of membrane proteins in epithelial cells and the secretion of vesicle contents in neuronal cells (Moskalenko et al., 2002). EXO84 was then identified as a second subunit of the exocyst complex that interacts with RAL and is essential for RAL-mediated assembly of the full octameric exocyst complex (Moskalenko et al., 2003). SEC5 binds to the switch I region (Fukai et al., 2003) while EXO84 binds to both switch I and II regions (Jin et al., 2005) of activated RAL.

C. Transcription and Other Factors

RAL signaling activates transcriptional activity of nuclear factor- κ B through SEC5 and TBK1 (Chien et al., 2006). Active RALB causes the association of SEC5 with TBK1, which then directly phosphorylate and promote the nuclear localization and activation of nuclear factor- κ B. Active RALA activates the ZONAB transcription factor to regulate the cell density of MDCK cells (Frankel et al., 2005). Other less studied examples

include the activation of c-JUN through JNK (de Ruiter et al., 2000), ATF2 and STAT3 through SRC tyrosine kinase (Goi et al., 2000), and FOXO4 (De Ruiter et al., 2001) and NFAT (de Gorter et al., 2007). Filamin, an actin filament crosslinking protein interacts with RALA and is required for RALA-induced filopodia formation (Ohta et al., 1999). RAL proteins directly associate with phospholipase C delta 1 and phospholipase D1, important second messenger proteins of the G-protein coupled receptors (Luo et al., 1997; Sidhu et al., 2005).

V. RAL in Human Cancer

The role of RALGEF-RAL signaling in RAS-driven tumorigenesis was initially overlooked, because early studies in mouse fibroblast cells found the RAF effector pathway, but not PI3K or RALGEF-RAL pathway, to be sufficient to mediate RAS-driven tumor transformation (Urano et al., 1996). The Counter group (Hamad et al., 2002) was the first to report that the RALGEF-RAL pathway, but not RAF or PI3K, was sufficient for RAS transformation of immortalized human cells. In the following study, they further demonstrated that RALA but not RALB is the RALGEF effector protein that plays a critical role for RAS-mediated tumor transformation (Lim et al., 2005). The observation that RALA and RALB might play distinctive roles in tumorigenesis was also supported by a study from the White group (Chien and White, 2003), showing that RALA is required for anchorage-independent growth, whereas RALB is required for survival of human tumor cells. Subsequent studies in the past decade have established the critical role of RAL in both tumorigenesis and growth/metastasis of many types of human cancer.

A. Pancreatic Cancer

Pancreatic cancer is the most popular model for studying the role of RAL in human cancer because of its extremely high frequency of *KRAS* mutations (>90%, COSMIC database) and documented dependency of this tumor type on RAS. To determine which RAS effector signaling pathway mediates the oncogenic signaling, the Counter group examined the activation status of the three canonical effector pathways in panels of human pancreatic cancer cell lines (Lim et al., 2005) and tumor samples (Lim et al., 2006). Both studies showed consistent elevated levels of activated RALA and RALB across the cell lines or tumor samples. Importantly, both RALA and RALB were found to be more commonly activated compared with the other two RAS effector pathways PI3K and RAF, suggesting the critical importance of RAL signaling in *KRAS* driven pancreatic cancer. This central role of RALGEF-RAL signaling in pancreatic cancer was also confirmed by a more recent study showing that the RALGEF protein RGL2 is overexpressed in patient tumors and is necessary for both the anchorage-independent growth and

invasion of tumor cells (Vigil et al., 2010). *RALA* and *RALB* were found to play distinct roles in pancreatic cancer. Stable shRNA suppression of *RALA* but not *RALB* in a panel of 10 genetically diverse human pancreatic cancer cell lines universally reduced their anchorage-independent growth in vitro and subcutaneous growth in vivo. In contrast, *RALB* but not *RALA* knockdown impaired in vitro invasion of these cell lines and metastatic colonization in vivo following tail-vein inoculation (Lim et al., 2006). The distinct roles of RAL in RAS-driven pancreatic cancers was confirmed by another study which showed *RALB* but not *RALA* plays a role in invadopodium formation in human pancreatic cancer cells, supporting a role for *RALB* in metastasis (Neel et al., 2012).

B. Colorectal Cancer

KRAS is the most frequently mutated (45%, COSMIC) oncogene in colorectal cancer. As in pancreatic cancer, upregulated *RALA* and *RALB* activation were found in both colorectal cancer cell lines and patient samples (Martin et al., 2011). *RALA*-responsive gene expression signature generated from three colorectal cancer cell lines was also found to correlate with progression-free survival of colorectal cancer patients in five independent data sets (Györfy et al., 2015). However, *RALA* and *RALB* play antagonistic roles in colon cancer. Stable RNAi suppression of *RALA* reduced anchorage-independent growth of cancer cells, whereas stable suppression of *RALB* enhanced such growth. This discrepancy in isoform function was partially attributed to their differential usage of effector proteins. Both *RALA* and *RALB* required interaction with *RALBP1* but with different components of the exocyst complex. *RALA* interacts with *EXO84*, whereas *RALB* interacts with *SEC5* to mediate their effect on anchorage-independent growth in colorectal cancer cells (Martin et al., 2011). This antagonistic role of *RALA* and *RALB* in colorectal cancer demonstrated cancer cell type differences in RAL function and therefore calls for selective targeting of each RAL isoform in colorectal cancer.

C. Lung Cancer

In contrast to colorectal cancer, *RALA* and *RALB* were shown to have redundant effects on the growth of NSCLC tumor cells. The requirement of RAL in lung cancer tumorigenesis was also established in a genetic knockout mouse model (Peschard et al., 2012). Deletion of both *RALA* and *RALB* genes but not either one alone blocked *KRAS*-driven lung tumor development, suggesting that *RALA* and *RALB* have redundant functions in tumorigenesis in mouse model of lung cancer. By using the human NSCLC cell line A549, the Farassati group (Male et al., 2012) showed that transient knockdown of *RALA* reduced the proliferation and invasiveness of A549 cells in vitro and tumorigenesis in vivo.

More recently, we analyzed *RAL* expression in a panel of 14 human NSCLC cell lines and found that, although both *RALA* and *RALB* had higher expression in *KRAS* mutant cell lines, only *RALA* activity was elevated (Guin et al., 2013), suggesting *RALA* drives tumor growth in NSCLC cell lines. A relationship between *RAL* dependence and *RAS* mutation type was established for NSCLC with cell lines carrying a G12C or G12V *KRAS* mutation found to be more sensitive to *RAL* depletion than those with other *KRAS* mutations or wild-type *KRAS*. *KRAS* mutations are found in about 30% of NSCLC, and G12C and G12V are the most common types of *RAS* mutations. This supported the importance of RAL signaling in driving tumor growth downstream of *RAS* in NSCLC. Immunohistochemical and expression analyses of 337 NSCLC patient tumors demonstrated high expression of both *RALA* and *RALB* proteins was associated with poor survival, supporting the simultaneously targeting of both RAL paralogs in NSCLC. In fact, BQU57 (6-amino-1,3-dimethyl-4-(4-(trifluoromethyl)phenyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile), the first developed RAL inhibitor inhibited growth of NSCLC cell lines both in vitro and in vivo by inhibiting *RALBP1* binding to both RAL paralogs (Yan et al., 2014).

D. Bladder Cancer

Overexpression and increased activation of *RALA* and *RALB* was detected in human bladder cancer cell lines with diverse genetic background and *RAS* mutation status (Smith et al., 2007; Saito et al., 2013). In the *KRAS* mutant UMUC3 human bladder cancer cell line, *RALA* and *RALB* have similar functions in tumor growth but opposite roles in motility (Oxford et al., 2005). Transient RNAi suppression of both *RALA* and *RALB* was required to reduce anchorage-independent growth, whereas *RALA* inhibits and *RALB* promotes the motility of UMUC3 cells. Importantly, this metastasis-promoting function of *RALB* was shown to be dependent on its phosphorylation by protein kinase C in UMUC3 cells (Wang et al., 2010). The importance of RAL signaling in patient bladder tumor samples was also revealed by us using a RAL transcriptional signature that was found to correlate with disease stage, progression to muscle invasion, and survival (Smith et al., 2007, 2012). In addition, overexpression of both the regulators and effectors of RAL was found in human bladder tumor samples. Some examples include RAL activators like *RGL2* and *Aurora A* kinase, as well as RAL effectors like *RALBP1* (Smith et al., 2007). Because *RAS* mutation is very infrequent in bladder cancer (~10%), *RAS*-independent mechanisms have been proposed that mediate RAL activation in bladder cancer. Supporting this notion, work using both human bladder cancer cell lines and a genetic mouse model showed *RAS*-independent activation of RAL might be due to the reduced expression of *RALGAP* in *RAS* wild-type bladder cancer (Saito et al., 2013).

E. Prostate Cancer

The contribution of RAL signaling to prostate cancer growth and progression has been implied in both androgen independence and metastasis (Yin et al., 2007). One study showed that RALA was activated upon androgen deprivation in human prostate cancer cells, which in turn leads to the transcriptional upregulation of vascular endothelial growth factor C (Rinaldo et al., 2007). On the other hand, RALA activation also leads to the interaction of RALBP1 with REPS2, which stimulates the endocytosis of the epidermal growth factor receptor (which has been shown to play a role in androgen independence (Oosterhoff et al., 2003). The RALGEF-RAL pathway was also shown to be both necessary and sufficient for the bone metastasis of prostate cancer. Activation of the RALGEF pathway enhanced bone metastasis of DU145, whereas depletion of either *RALA* or *RALBP1* inhibited bone metastasis of the metastatic PC3 cell line (Yin et al., 2007; Wu et al., 2010). In human prostate tumor samples, RAL transcriptional signature was associated with seminal vesicle invasion, androgen-independent progression, and poor prognostics, supporting the central role of RAL in prostate cancer progression and metastasis (Smith et al., 2012).

F. Melanoma

Approximately one-third of melanomas harbor activating *NRAS* mutations and about 60% harbor *BRAF* mutations (COSMIC database). A role for RALGEF-RAL signaling in *NRAS*-driven melanoma tumorigenesis was first established in an immortalized mouse melanocyte model, where the transforming activities of the three *NRAS* downstream effectors, RAF, PI3K, and RALGEF were evaluated (Mishra et al., 2010). RALGEF signaling was found to have a major contribution to anchorage-independent growth in this model. RALA but not RALB activation was observed in a panel of human melanoma cancer cell lines regardless of their *NRAS* or *BRAF* mutational status (Zipfel et al., 2010). Stable knockdown of *RALA*, and to a lesser extent of *RALB*, inhibited the tumorigenic growth of melanoma cell lines both in vitro and in vivo (Zipfel et al., 2010).

G. Other Tumor Types

Elevated expression and activation of RAL is observed in various types of human cancer. In most models, *RALA* is the dominant isoform driving tumor progression and metastasis, including in hepatocellular carcinoma (Ezzeldin et al., 2014), malignant peripheral nerve sheath tumors (Bodempudi et al., 2009), and ovarian cancer (Wang et al., 2013). One exception, however, is squamous cell carcinoma (SCC), where *RALA* was found to suppress rather than promote tumor progression in SCC cancer cell lines (Sowalsky et al., 2010). This tumor suppressor function of *RALA* was validated by microarray analysis of SCC human tumor samples in which *RALA* gene expression

signature was decreased in cancer tissues compared with normal mucosa (Smith et al., 2012).

In conclusion, in almost all cancer types examined, increased expression and activation of both *RALA* and *RALB* are observed in patient tumor samples compared with normal tissues, regardless of their RAS mutation status. Studies with genetic approaches in human cancer cell lines have established that, in general, *RALA* is important for anchorage-independent growth, whereas *RALB* is important for metastasis and invasion. However, in certain cancer types such as lung cancer and melanoma, both isoforms seem to have overlapping effects. Therefore, targeting both *RALA* and *RALB* simultaneously seems the most appropriate anticancer approach.

VI. Therapeutic Targeting of RAL Signaling

A. Targeting RAL Guanine Exchange Factors

RALGEFs mediate RAS-dependent activation of RAL. Targeting RALGEFs should be as potent as inhibiting RAL itself if the predominant signal activating RAL is RAS mediated. Targeting RALGEFs or other GEFs of the small GTPase family is challenging, because targeting GEF means targeting protein-protein interactions. Nevertheless, inhibitors have been developed that specifically target GEF proteins of the RAS family small GTPases, including ARF, RHO, and RAC1 (Schmidt et al., 2002; Renault et al., 2003; Gao et al., 2004). For RALGEFs, limited structural information is available to understand their binding to RAS or RAL, thus structural studies will be needed before they can be exploited as feasible therapeutic targets of RAL signaling.

B. Targeting RAL Directly

As for RAS, several hurdles hamper the development of small molecule compounds that directly bind to and inhibit RAL activity: 1) the high affinity of RAL small GTPases for guanine nucleotides (picomolar) and the high concentration of GTP/GDP in cells (millimolar) make competing with GTP/GDP binding at the active site of RAL extremely difficult (Cox et al., 2014), 2) the downstream signaling cascade of RAL is relayed through protein-protein interactions, which are difficult to target compared with the enzymatic activity of protein kinases (Arkin et al., 2014), and 3) the surface of activated RAL-GTP protein is very "smooth," with no deep hydrophobic pockets suitable for tight binding of small molecules. Fortunately, unlike RAS, whose oncogenic activity is most commonly due to mutations that keep RAS in constitutively active state, *RAL* mutations in human cancer are extremely rare, allowing for more targeting options (Ostrem et al., 2013). In addition, recent advances in structure-based drug discovery have shed light on both RAS and RAL, allowing inhibitor development.

Structure-based drug discovery relies on the knowledge of the three-dimensional structure of the molecular target, usually determined by either X-ray crystallography or NMR, both of which have their intrinsic limitations for structure-based drug design (Chen et al., 2012). For example, X-ray structure of small GTPases only traps the protein in one low-energy conformation, which might not be physiologically relevant, whereas NMR structures are usually too dynamic for computational modeling. Moreover, the original idea that RAS proteins exist in rigid inactive GDP-bound or active GTP-bound form has been altered when NMR studies of HRAS demonstrated existence of at least two conformation states for the GTP-bound form (Shima et al., 2010; Shima et al., 2013). This new consensus on the dynamic nature of conformational status of GTP-bound RAS and related GTPase, has resulted in several breakthroughs in targeting the RAS family small GTPases. In 2012, two small molecule compounds, DACI and VU0460009, were discovered as KRAS inhibitors using fragment based screening, both of which were shown to bind to the same pocket in the RAS-SOS1 binding surface resulting in the inhibition of RAS activation by the RASGEF SOS1 (Maurer et al., 2012; Sun et al., 2012). Importantly, this binding pocket did not exist on the apoprotein and was in fact formed by compound binding induced conformation changes, suggesting that the structure of RAS protein is dynamic. Fragment-based drug design is based on identifying small chemical fragments that can bind weakly to small pockets on the target, and then growing them or linking them together to produce a lead with a higher affinity (Zhao et al., 2017). This approach is particularly promising for structure-based drug design against RAS family small GTPase including RAL.

Another breakthrough in targeting small GTPases via structure-based drug design is the “stapled peptide” approach. The mimicking of α -helices in protein binding domains has been an emerging area in structure based inhibitor design (Walensky and Bird, 2014). Introduction of synthetic hydrocarbon braces (staples) helps to lock a small peptide in a specific conformation, thereby increasing stability, target affinity, and cell permeability of the peptide. Recently, this technique has been applied to target RAS (Patgiri et al., 2011; Leshchiner et al., 2015). Stapled peptides based on a helix from the RASGEF son of sevenless (SOS) has been used to target RAS. By using the RAL binding domain of RALBP1 as a template, the Mott group designed an α -helical stapled peptide that can bind selectively to active RALB-GTP and block downstream effector signaling of RAL (Thomas et al., 2016). These peptides bind to the switch I and II regions of RAL, which are the sites of interaction with RAL effectors like RALBP1 and regulators like RALGEF. However, because the peptides do not bind to inactive RAL-GDP, they could not inhibit RALGEF binding to RAL and hence could not inhibit RAL activation by RALGEF.

A third breakthrough in the targeting of small GTPases using structure-based approach is the

development of allosteric inhibitors of the inactive GDP-bound form of RAL. Our group identified an allosteric binding site on the surface of RAL-GDP and searched for small molecule ligands that can bind to the allosteric site using a virtual chemical library screening (Yan et al., 2014) (Fig. 3). This generated drug candidates that bound to the allosteric site of RAL-GDP and lock RAL in the GDP-bound inactive state, thereby break up the GDP-GTP cycle and block RAL-mediated signaling. Importantly, these compounds demonstrated biologic activities in human NSCLC both in vitro and in vivo.

C. Targeting RAL Effectors

Another logical approach to interfere with RALGEF-RAL signaling is to inhibit downstream signaling of activated RAL. RAL proteins activate downstream signaling through direct binding with effector proteins. Although specific inhibitors for most RAL effectors do not exist, targeting the protein-protein interaction between RAL-GTP and effector proteins seems to be a reasonable approach that may lead to the discovery of novel inhibitors of RAL signaling.

D. Targeting RAL Localization

Similar to RAS, appropriate localization of RAL proteins to the inner surface of the plasma membrane is required for their biologic functions. As discussed earlier, all RAS family small GTPases have the CAAX C-terminal sequence motif that is subjected to prenylation by either farnesyltransferase (FTase) or geranylgeranyltransferase I (GGTase I) depending on the exact amino acid sequence of AAX. The initial discovery that RAS proteins undergo farnesylation by FTase prompted an intensive effort to develop farnesyltransferase inhibitors with two of them, lonafarnib and tipifarnib, progressing into phase III clinical trials (Berndt et al., 2011). However, both failed to show efficacy in human cancer. One basis for this failure was the alternative prenylation of RAS by GGTase I upon inhibition of FTase. Unlike RAS, which is primarily prenylated by FTase, RAL proteins are substrates for GGTase I (Kinsella et al., 1991) and do not undergo alternative prenylation in response to GGTI treatment (Falsetti et al., 2007). The effect of GGTase I inhibition on RAL signaling has been evaluated in the human Mia PaCa-2 pancreatic cancer cell line (Falsetti et al., 2007). RALA and RALB were shown to be involved in both mediating the inhibition of anchorage-independent growth and cell cycle progression in these cells. The importance of RALB in mediating the growth inhibition effect of GGTIs was also confirmed in other cancer types like glioma and squamous cell carcinoma (Hamada et al., 2011; Song et al., 2015). One potential problem with targeting GGTase is that many other RAS family members are also substrates for GGTase I, targeting of which might result in normal tissue toxicity. However, a recent phase

Fig. 3. Targeting the allosteric site of RAL. Structure of inactive (A) and active (B) RALA, red sphere represents the allosteric site that is only exposed in the GDP-bound form. GNP: nonhydrolyzable form of GTP used in X-ray crystallography. (C) Chemical structure of the first small molecule inhibitor of RAL, BQU57. (D) BQU57 binds to the allosteric site of RALA-GDP.

I clinical trial with GGTI-2418 seemed to be well tolerated (Ullah et al., 2016). Therefore, GGTIs may still be a feasible approach for targeting RAL.

E. Combination Therapy

For a long time, combination therapy at various signaling knots of the RAS pathway has been confined to the RAF-MEK-ERK and PI3K-AKT-mTOR pathways, largely because of the lack of effective inhibitors of other components of RAS signaling. Recent breakthroughs in direct targeting of RAL have provided the conceptual possibility to block all three primary effector pathways downstream of RAS, namely the RAF-MEK-ERK kinase pathway, the PI3K-AKT-mTOR kinase pathway, and the RALGEF-RAL pathway. Potent synergistic activity between PI3K and RAF inhibitors has already been seen in human cell lines and mouse models (Cox et al., 2014). Concurrent inhibition of all three pathways should result in even more potent inhibition of RAS oncogenic signaling. Clinical useful inhibitors have been developed for almost every component of RAF-MEK-ERK and PI3K-AKT-mTOR signaling; for RALGEF-RAL pathway this is only the beginning.

VII. Summary

As the third primary signaling axis that mediates RAS oncogenic signaling, the RALGEF-RAL pathway has been

extensively studied in human cancer and shown to play critical roles in the tumorigenesis, growth, progression, and metastasis of various types of human cancer. Although much remains to be understood about the detailed mechanism of RAL signaling and the distinct roles of *RALA* and *RALB* in human cancer, recent breakthroughs in targeting RAL and RAS have paved the way for the development of more potent and specific RAL-targeted therapies.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Yan, Theodorescu.

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