

Differential involvement of RalA and RalB in colorectal cancer

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Mutationally activated K-Ras can utilize a multitude of downstream effector proteins to promote oncogenesis. While the Raf and phosphoinositol-3-kinase effector pathways are the best-studied and validated, recent studies have established the critical importance of Ral guanine nucleotide exchange factor (RalGEF) activation of the RalA and RalB small GTPases in cancer biology. Due to recent evidence that the RalGEF-Ral pathway is necessary for the tumorigenic and metastatic potential of *KRAS* mutant pancreatic ductal adenocarcinoma (PDAC) tumor cells, we investigated whether or not Ral signaling was necessary for *KRAS* mutant colorectal cancer (CRC) tumor cell growth. As in PDAC, we found upregulated RalA and RalB activation in CRC tumor cell lines and tumors. Surprisingly we found antagonistic roles for RalA and RalB in the regulation of CRC tumor cell anchorage-independent growth. This observation contrasts with PDAC, where RalA but not RalB is necessary for PDAC tumor cell anchorage-independent growth. Our results emphasize cancer cell type differences in Ral function and hence the need for distinct Ral targeted therapeutic approaches in the treatment of CRC vs. PDAC.

Mutations in the three *RAS* oncogenes (*HRAS*, *KRAS* and *NRAS*) occur in approximately 32% of all human cancers (COSMIC), with highest frequencies seen in pancreatic ductal adenocarcinoma (PDAC; 90%), colorectal carcinoma (CRC; 40%) and non-small cell lung carcinoma (NSCLC; 25%). Experimental studies with cell culture and mouse models

of cancer have provided strong validation for the importance of mutationally activated *RAS* in primary tumor progression, invasion and metastasis. Consequently, there has been tremendous interest and effort in the development of anti-Ras drugs for cancer treatment. The direct targeting of the mutated Ras protein has proved difficult so most efforts to target Ras have instead revolved around inhibition of Ras post-translational processing and downstream effector signaling.¹ It has long been appreciated that Ras downstream signaling through the Raf-MEK-ERK mitogen-activated protein kinase cascade and phosphatidylinositol-3-kinase (PI3K)-Akt-mTOR effector pathways is necessary for mutant *RAS*-dependent cancer growth. This has prompted the development and clinical evaluation of >40 small molecule inhibitors of these two pathways. However, there is also strong evidence that other effector pathways must also contribute to mutant *RAS*-dependent tumor growth.²⁻⁴ Among the other nine classes of candidate Ras effectors, guanine nucleotide exchange factors for the Ral small GTPases (RalGEFs) have emerged as perhaps the third best validated effector critical for cancer growth.

The Ral small GTPases (RalA and RalB) are members of the Ras superfamily.⁵ Like Ras, Ral cycles between inactive GDP-bound and active GTP-bound states (Fig. 1). This cycle is regulated by Ral-selective GEFs and GAPs. Of the six known RalGEFs, four contain Ras association domains and can function as effectors of Ras. Although RalA and RalB share significant sequence identity (82%) and both domain structural and biochemical similarity (Fig. 2),

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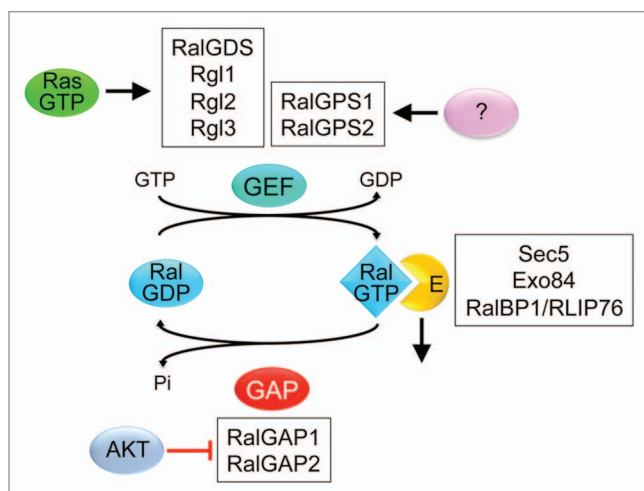


Figure 1. Regulators of the Ral GDP-GTP cycle. RalA and RalB are activated by six known RalGEF proteins, four of which contain a Ras-association (RA) domain and are activated by Ras. A second class of RalGEF is characterized by a pleckstrin homology domain and their mechanism of regulation is not known. Ral-GTP deactivation is catalyzed by two structurally-related RalGAP heterodimeric α - β complexes that return Ral to its inactive GDP-bound conformation.⁶ AKT phosphorylation can inactivate RalGAP2.⁷ Active GTP-bound Ral can interact with a number of effector proteins including two components of the exocyst, Sec5 and Exo84, as well as RalBP1/RLIP76, a GAP for the Cdc42 and Rac small GTPases.

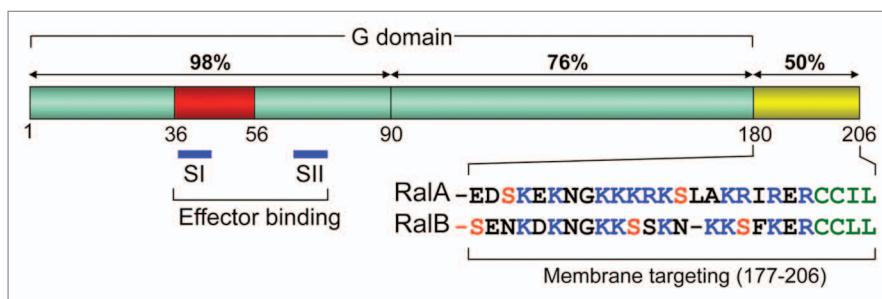


Figure 2. Ral isoform structure. RalA and RalB share overall 82% sequence identity. The N-terminal 180 residues comprise the G domain involved in GTP binding and hydrolysis. The strongest sequence identity is seen in the N-terminal 90 residues (98%) that include sequences involved in effector interaction.⁸ Residues 36–56 correspond to Ras residues 25–45 involved in effector interaction. The switch I (residues 41–51) and II (69–81) sequences change in conformation during the GDP-GTP cycle and one or both sequences are involved in binding to specific effectors. The effector interaction sequences are conserved 100% in RalA and RalB. The greatest divergence (50%) is in the C-terminal membrane-targeting sequences. This membrane targeting sequence terminates with a CAAX tetrapeptide sequence that signals for posttranslational modification by addition of a geranylgeranyl isoprenoid lipid to the cysteine residue. Point mutations in the effector interaction sequences (36–56) cause differential impairment in effector binding.

their functions in cancer cells have been found to be highly distinct. Their distinct functions are due, in part, to their divergent C-terminal sequences, which dictate distinct subcellular membrane localization and differential effector utilization. While Ral GTPases have been implicated in the growth of a diverse spectrum of cancer types, in light of the existence of RalGEFs that can serve as effectors of

Ras, and that *KRAS* is the most frequently mutated *RAS* gene (85% of all *RAS* mutations; COSMIC), our studies have focused on cancers with the highest frequency of *KRAS* mutations, PDAC and CRC.

In our previous studies with PDAC, we determined that RalA and RalB activation is elevated in PDAC tumor cell lines and tumors.⁹ Using RNA interference to selectively ablate RalA or RalB expression, we

determined that RalA but surprisingly not RalB was required for anchorage-independent growth in vitro as well as tumorigenic growth in vivo.¹⁰ In contrast, RalB was critical for Matrigel invasion in vitro and lung colonization metastasis in vivo. Our observations that the highly related Ral isoforms can exhibit such striking functional differences in PDAC adds to similar findings described in other cancer types. However, while RalA and/or RalB have been implicated in the aberrant growth properties of cancers, including *RAS* wild type cancers, there are also strong indications of cancer type differences in their specific roles.¹¹

To extend our studies beyond PDAC, we wanted to determine if Ral GTPases played similar roles in other *KRAS* mutant human cancers. We therefore focused our studies on *KRAS* mutant CRC. We showed unexpectedly that CRC tumor cells have a surprising difference in their requirement for either RalA or RalB.¹² RalA signaling was found to be required for the anchorage-independent growth of CRC tumor cells while RalB was found to antagonize their anchorage-independent growth (Fig. 3). This finding adds to the increasing evidence that RalA and RalB play distinct roles in human oncogenesis, and additionally, that these roles can vary strikingly in different cancer types.

Ral GTPases Differentially Regulate the Anchorage-Independent Growth of CRC cells

Previous studies of other cancers of various tissue origins including bladder,¹³ melanomas,¹⁴ and pancreatic ductal adenocarcinoma (PDAC),¹⁰ indicated that the active pool of the Ral GTPases was upregulated in tumors. Due to the high frequency of activating *KRAS* mutations found in CRC we hypothesized that the oncogenic K-Ras protein could lead to an enhancement in Ral GTPase activation through enhanced K-Ras-RalGEF signaling. We found that human patient derived CRC tumor cell lines displayed high levels of active RalA and RalB and that the patterns of activation for RalA and RalB were similar. This indicates that both Ral proteins are overactivated in concert in CRC perhaps due to their sharing of common

RalGEF proteins. Whether or not different RalGEF proteins can lead to changes in RalA vs. RalB activation downstream of K-Ras remains an open question. Further analyses of human patient CRC tissue showed that both Ral proteins were similarly hyperactivated in tumor tissue as compared with normal tissue indicating a potential role for Ral activation in CRC oncogenesis.

Next we posed the question of whether or not the Ral proteins were necessary for the anchorage-independent growth of CRC tumor cells. Anchorage-independent growth is a hallmark of transformed cells¹⁵ and is a reliable *in vitro* measure of tumorigenic growth *in vivo*. Ral proteins have previously been identified as being necessary for PDAC anchorage-independent growth in soft agar.¹⁰ Using stable shRNA suppression of RalA protein we found that RalA was necessary for the anchorage-independent growth of CRC cells consistent with results from other tumor cell types. Surprisingly, we saw a dramatic increase in the ability of CRC cells stably expressing RalB shRNA to form colonies in soft agar indicating that RalB was necessary to suppress anchorage-independent growth. Perhaps even more interesting was the fact that dual suppression of RalA and RalB resulted in no change in soft agar growth as compared with control cells. These results indicate that there is an inherent balance found in CRC cells between RalA and RalB and that this balance is critically important in controlling the anchorage-independent growth properties of these cells.

Ral GTPases Utilize Common and Distinct Effectors to Control CRC Anchorage-Independence

RalA and RalB are identical in their core effector interaction and switch I and II sequences. Hence, they share the ability to interact with a common set of effector proteins. The best characterized effectors include two components of the exocyst complex, Sec5 and Exo84, as well as RalBP1/RLIP76.⁵ RalBP1 has two distinct functions, first as a GAP for Rho small GTPases, second as a glutathione-conjugate transporter.⁸ Previous studies have established roles for all three of

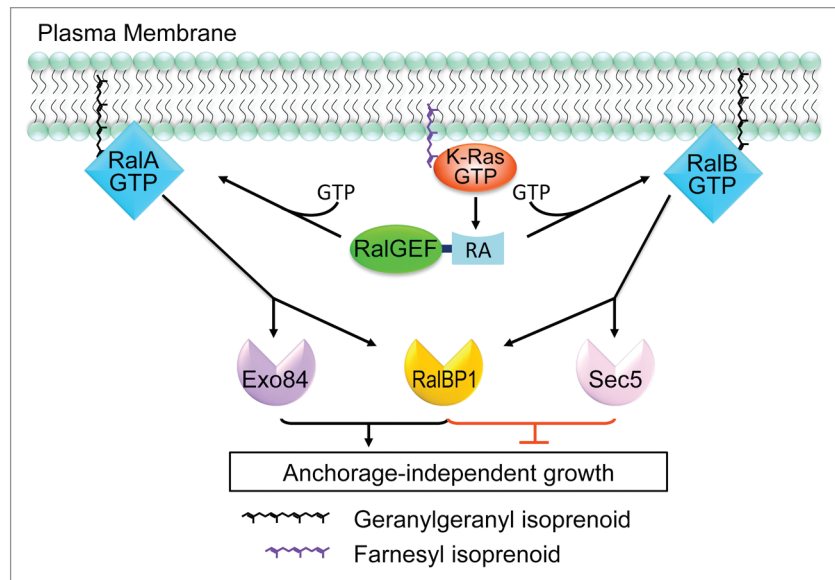


Figure 3. Divergent roles for RalA and RalB in the regulation of CRC anchorage-independent growth. Oncogenic K-Ras can activate RalGEFs through association with the RA domain of RalGEFs, leading to their recruitment to the plasma membrane, facilitating the activation of plasma membrane-bound RalA and RalB. Active RalA interacts with Exo84 and RalBP1 to promote anchorage-independent growth while RalB interacts with Sec5 and RalBP1 to suppress the anchorage-independent growth of CRC cells. Additionally, active RalA can negatively regulate the activation of RalB resulting in an enhancement in the anchorage-independent growth of CRC cells. Ral and K-Ras plasma membrane association is mediated in part by posttranslational modification by a C20 geranylgeranyl or C15 farnesylisoprenoid, respectively.

these proteins in the oncogenic growth of cancer cells.^{16,17} Due to the ability of the Ral GTPases to activate common effector proteins we hypothesized that the differences seen between RalA and RalB in their contribution to anchorage-independence could be due to differential effector engagement. Established effector binding mutants of Ral that are differentially perturbed in their binding of individual effectors allowed us to delineate the downstream effector interactions that are necessary for different Ral phenotypes.¹⁸ Upon re-expression of RNAi-insensitive cDNA sequences encoding RalA or RalB proteins containing effector-uncoupling mutations we found that RalA and RalB both required RalBP1 engagement to mediate their opposing effects on soft agar growth. This utilization of the same effector to mediate opposing activities may seem surprising. However, RalA and RalB exhibit similar but distinct subcellular localizations. Whereas both RalA and RalB each exhibit plasma membrane localization, active RalA but not RalB was found to be present on intracellular membranes including recycling endosomes.¹⁹ This raises the

possibility that due to their different localizations, their engagement of RalBP1 may not result in the same cellular outcome. Interestingly, both Ral proteins required association with exocyst subunits, but they utilized distinct components of this octameric complex. RalA required Exo84 but not Sec5 binding while RalB required Sec5 but not Exo84 association. Why RalA and RalB use different components of the same protein complex is unclear. Exocyst proteins have been reported to exist in different intracellular pools and distinct engagement of these pools specifically by either RalA or RalB has been shown.^{20,21} Perhaps these different pools of exocyst subcomplexes that are distinct from the canonical exocyst complex are being utilized differently by RalA and RalB to control the anchorage-independent growth of CRC cells. Additionally, exocyst-independent functions for some subunits have been reported previously in reference 21, and a role for RalB and Sec5 to activate the TBK1 kinase in the innate immune response²⁰ indicates that there are roles for Ral-exocyst engagement outside of normal exocytic regulation.

RalA and RalB with Opposing Roles in CRC Anchorage-independent Growth

Since RalA and RalB displayed opposing phenotypes in their regulation of CRC anchorage-independent growth we hypothesized that perhaps RalA could actually antagonize RalB activity and vice versa. This stemmed from our initial finding that upon shRNA depletion of RalA, the GTP-loading of RalB was dramatically enhanced. Re-expression of RalA resulted in a return to basal activity for RalB. The exact same was seen for RalA when RalB was stably depleted, suggesting that the activity levels of each Ral isoform may influence the activity level of the other isoform. This may also provide mechanistic insight into why depletion of RalA or RalB could lead to the observed changes in soft agar growth. Since we found that RalB suppressed anchorage-independent growth and that loss of RalA resulted in decreased anchorage-independent growth, perhaps the reason for diminished soft agar colony formation observed with RalA shRNA was due in part to enhanced RalB signaling. The same could also be true in the case of RalB depletion. Namely, loss of RalB is associated with activation of RalA that can drive the anchorage-independent growth of CRC cells.

Consistent with these observations we recently found that overexpression of ectopic constitutively active Ral results in the diminished activation of the other Ral isoform. For example, ectopic expression of a GTPase-deficient RalA Q72L mutant that is locked in a GTP-bound state and thus constitutively active leads to decreased endogenous RalB activation. This indicates that it may not merely be accessibility to RalGEF proteins that is leading to changes in Ral activation and instead argues that downstream Ral signaling could have built-in feedback mechanisms to control total cellular Ral activity levels. Experiments involving constitutively active but effector-binding deficient mutants of the Ral proteins would address this possibility. This could provide novel insight into whether Ral signaling through the exocyst or RalBP1 or even unknown effector proteins helps to control total cellular Ral-GTP levels.

Implications for Potential Ral-targeted Therapeutics for CRC Treatment

Due to the potential value in inhibiting Ral signaling as a means to target oncogenic K-Ras in human cancer treatment, an important open question is whether or not Ral-isoform specific inhibitors would be more effective than pan-Ral therapies. Our studies suggest that the answer will depend on the cancer type. For PDAC, since RalA promotes tumorigenic growth and RalB promotes invasion and metastasis, a pan-Ral inhibitor will be advantageous over RalA or RalB selective inhibition. In contrast, since we opposing functions for RalA and RalB in CRC growth, with concurrent silencing having no net impact on growth, a RalA-selective inhibitor would seem to be preferred. However, one major caveat is that we have not rigorously assessed the role of RalA and RalB for CRC invasion and metastasis. These analyses will be needed before a clear determination of whether RalA-selective inhibition is desired for CRC.

Like Ras, Ral GTPases themselves are not considered “druggable”. Instead, as in the case with Ras, indirect approaches to target Ral have been considered. One way to target Ral would be to target the post-translational modifications of the Ral proteins, a similar approach that has been used to target the Ras proteins. A previous study utilized a geranylgeranyltransferase inhibitor (GGTI) that blocked the lipid modification necessary for proper subcellular localization of RalA and RalB.²² By blocking the geranylgeranyl moiety from being added to the Ral proteins, Sebti and colleagues showed that this prevented RalA from promoting anchorage-independent growth while also preventing RalB from promoting the anchorage-dependent growth and apoptosis avoidance of MiaPaCa-2 PDAC cells. Whether or not GGTIs would be effective in targeting Ral signaling in CRC is an important question to answer. Since we found that simultaneous depletion of RalA and RalB resulted in no change to CRC anchorage-independent growth we anticipate that GGTI treatment will impair the functions of both Ral isoforms, and consequently,

would be ineffective in inhibiting primary CRC tumor growth.

How might Ral isoform-selective inhibition be achieved? One attractive approach may lie in inhibiting a post-translational modification of Ral. A recent trend in small GTPase signaling is that phosphorylation near the C-terminal membrane targeting region can regulate function.¹⁹ Both RalA and RalB are phosphorylated near their C-terminus by different protein kinases and phosphorylation is critical for their roles in supporting tumor growth.^{23,24} This raises the possibility that specifically inhibiting Aurora-A which phosphorylates RalA but not RalB could selectively block RalA. Indeed there are currently clinical trials to evaluate the efficacy of Aurora-A inhibitors in a variety of tumor types.²⁵ One can imagine that in CRC a RalA-specific inhibitor may be more beneficial than pan-Ral or RalB-specific therapies due to the growth suppressive properties of RalB. Whether or not Aurora-A kinase inhibitors will be effective in limiting the anchorage-independent or tumorigenic growth promoting properties of RalA in CRC remains to be studied.

Conclusions

As we continue to discover more of the influence of aberrant Ral signaling on human cancer growth, it is becoming increasingly clear that RalA and RalB have distinct contributions. In the context of Ras-driven PDAC tumor cells RalA is critical for supporting the anchorage-independent and tumorigenic growth. Evidence that RalB signaling is necessary for metastasis necessitates a better understanding of just how RalA and RalB contribute to malignancy. Interestingly we found opposing roles for RalA and RalB in promoting the anchorage-independent growth of CRC tumor cells further indicating cancer type specificity of Ral signaling. The downstream signaling of the two Ral proteins is distinct despite the two proteins sharing common effector proteins, perhaps due to spatially-distinct subcellular localization.¹⁹ Another possibility is that distinct upstream stimuli that impinge on Ral activation by influencing GTP-loading or post-translational

modifications may bifurcate RalA and RalB signaling. Aside from the four RalGEFs that can serve as Ras effectors, there are two RalGEFs regulated by non-Ras mechanisms and additionally two Ral GTPase activating proteins, and their roles in Ral activation in *KRAS* mutant cancers remain to be studied. Future investigation into the mechanisms that drive the functional differences of the two Ral isoforms in both PDAC and CRC may also identify new directions for isoform-selective pharmacologic inhibitors for cancer therapy.

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