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MINI-REVIEW

Targeting Ras signaling in AML: RALB is a small GTPase with big potential

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ABSTRACT

Acute myeloid leukemia (AML) is a devastating malignancy for which novel treatment approaches are desperately needed. Ras signaling is an attractive therapeutic target for AML because a large proportion of AMLs have mutations in *NRAS, KRAS*, or genes that activate Ras signaling, and key Ras effectors are activated in virtually all AML patient samples. This has inspired efforts to develop Ras-targeted treatment strategies for AML. Due to the inherent difficulty and disappointing efficacy of targeting Ras proteins directly, many have focused on inhibiting Ras effector pathways. Inhibiting the major oncogenic Ras effectors, the mitogen-activated protein kinase (MAPK) and/or phosphatidylinositiol-3-kinase (PI3K) pathways, has generally demonstrated modest efficacy for AML. While this may be in part related to functional redundancy between these pathways, it is now clear that other Ras effectors have key oncogenic roles. Specifically, the Ras-like (Ral) GTPases have emerged as critical mediators of Ras-driven transformation and AML cell survival. Our group recently uncovered a critical role for RALB signaling in leukemic cell survival and a potential mediator of relapse following Ras-targeted therapy in AML. Furthermore, we found that RALB signaling is hyperactivated in AML patient samples, and inhibiting RALB has potent anti-leukemic activity in preclinical AML models. While key questions remain regarding the importance of RALB signaling across the genetically diverse spectrum of AML, the specific mechanism(s) that promotes leukemic cell survival downstream of RALB, and how to pharmacologically target RALB signaling effectively – RALB has emerged as a critical Ras effector and potential therapeutic target for AML.

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Acute myeloid leukemia (AML) is an aggressive hematologic malignancy characterized by genetic mutations that promote proliferation and prevent differentiation of myeloid progenitors. Despite aggressive cytotoxic chemotherapy, the majority of adults with AML die of relapsed or treatment refractory disease.[1] Furthermore, a large proportion of older adults with AML are not fit for intensive treatment approaches, and have only palliative treatment options. While the genetic landscape of AML has been extensively characterized,[2,3] effective genetically based therapies have yet to be realized. The toxicity and disappointing outcomes associated with conventional approaches have driven interest in developing safer and more effective targeted treatments.

The *RAS* proto-oncogenes – HRAS, NRAS, and KRAS – are among the most frequently mutated genes in human cancer. Ras small GTPases act as molecular switches to modulate signal transduction by cycling between active guanine triphosphate (GTP)-bound and inactive guanine diphosphate (GDP)-bound states.[4] Ras activation is catalyzed by guanine exchange factors (GEFs) that promote the exchange of GDP for GTP in response to growth factor receptor activation, and negatively regulated by the effects of GTPase activating proteins (GAPs) to greatly enhance the inefficient intrinsic Ras GTPase activity.[5] Oncogenic mutations in *RAS* genes most commonly involve amino acid substitutions at codons 12, 13, or 61 that disrupt the coordination of the catalytic glutamine residue at codon 61 and impair GTP hydrolysis, thereby leading to constitutive activation of Ras effector pathways and cellular transformation.[6] Ras-GTP regulates diverse cellular processes including proliferation, motility, and survival by interacting with a complex array of effector enzymes (Fig. 1).[7]

The mitogen-activated protein kinase (MAPK) and phosphatidylinositiol-3-kinase (PI3K) signaling pathways are the Ras effector pathways with the most well established roles in cancer. Activation of MAPK signaling involves Ras-GTP binding of RAF kinases resulting in plasma membrane localization and activation of their serine/threonine kinase activity.[8,9] Subsequently, active RAF phosphorylates and activates the mitogen-activated

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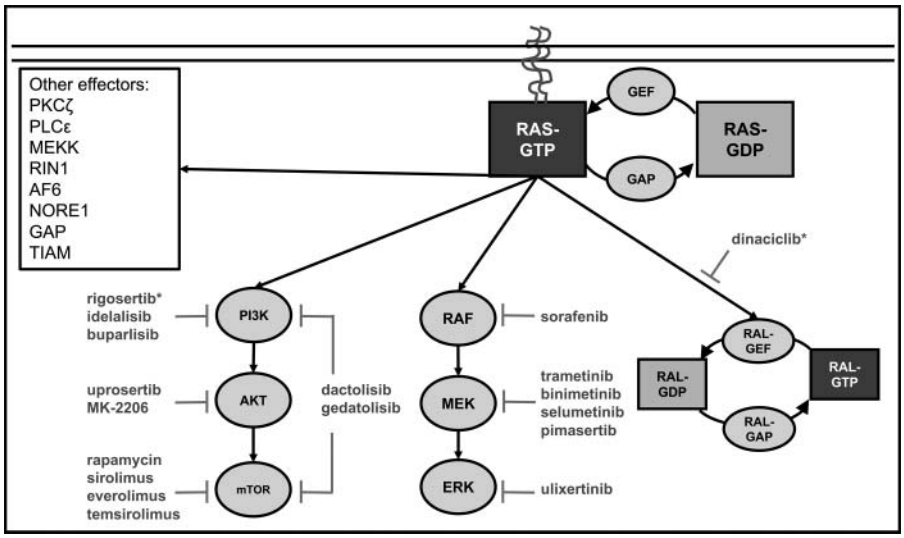

Figure 1. Canonical Ras signaling. Ras acts as a molecular switch that transduces signals from growth factor receptors to a variety of effector enzymes. Ras proteins are activated by guanine-exchange factors (GEFs) that promote the exchange of GDP for GTP leading to membrane localization and activation of effector enzymes. Ras proteins are negatively regulated by GTPase activating proteins (GAPs) that catalyze Ras's intrinsic GTPase activity resulting in the hydrolysis of GTP to GDP. The major oncogenic Ras effector pathways include the phosphatidylinositiol-3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and Ras-like (Ral) small GPTase signaling pathways. The role of other Ras effectors in oncogenesis remains unclear. Selected inhibitors of Ras effector signaling that have been evaluated in clinical trials for AML are included. A complete list of clinical trials can be found at ClinicalTrials.gov. * Dinaciclib also inhibits CDK1, CDK2, CDK5, CDK9 and rigosertib also inhibits polio-like kinase 1 (PLK1).

kinase kinases, MEK1 and MEK2, that phosphorylate and activate the mitogen-activated kinases, ERK1 and ERK2. Primary ERK targets include the ETS family transcription factors, JUN, and ultimately drive AP1-mediated proliferation.[10] Similarly, Ras-GTP induces PI3K signaling through interactions with type I PI3K catalytic subunits resulting in localization to the membrane and kinase activation leading to phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2) to produce phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3 then acts as a second messenger activating AKT-dependent and AKT-independent signaling pathways that modulate diverse cellular processes including proliferation, survival, motility, and metabolism.[11,12]

Approximately 15–25% of AMLs harbor activating mutations in *NRAS* or *KRAS*.[3,13,14] Unlike many solid tumors, both *NRAS* and *KRAS* are mutated in AML, although *NRAS* mutations predominate. While *RAS* mutations are seen across the spectrum of genetically heterogeneous AMLs, they are more common in specific AML sub-sets. For example, *NRAS* mutations occur in approximately 40% of AML with inv(16) or t(16;16) and 20% of AML with t(8;21), t(9;11), inv(3), or t(3;3).[13,15,16] Similarly, *KRAS* mutations are found in approximately 15% of AML with inv(16) or t(16;16) and 20% of AML with t(6;9).[13,15,16] *NRAS* mutations are also found in about 30% of AML with biallelic mutation of *CEPBA* and 20% of AML with mutated *NPM1*.[13,15] While *RAS* mutations do not have a clear impact on clinical outcomes for AML patients, there is a suggestion that AML with oncogenic *RAS* mutations benefit more from cytarabine containing chemotherapy regimens than AML with wild-type *RAS*.[17,18] In addition to AML with mutant *RAS*, proteins that regulate the activation of Ras (e.g. *PTPN11* and *NF1*) and signaling receptors that rely on Ras for their oncogenic effects (e.g., *FLT3* and *KIT*) are also frequently mutated in AML.[3,13,14,19,20] While oncogenic *RAS* mutations occur at similar frequencies across the age spectrum of AML, pediatric AMLs exhibit a distinct pattern of mutations in upstream regulators of *RAS* with an increased frequency of *KIT* mutations and fewer FLT3-ITD mutations than adult AML, reflective of the distinct pathogenesis of AML in children compared with adults.[14] Together with the prevalence of *RAS*-associated mutations described above, the almost ubiquitous activation of MAPK and PI3K signaling in AML further supports a key role for Ras signaling in the growth and survival of leukemic cells. Together, these observations have fueled intense interest in the development of Ras-targeted AML therapy.[21,22]

Ras's picomolar affinity for GTP and the challenge of designing a small molecules capable of restoring mutant Ras's defective GTPase activity have thwarted the successful development of direct inhibitors of oncogenic Ras.[23] Although the recent development of a specific small molecule inhibitor of KRAS(G12C) suggests that

these hurdles may not be insurmountable.[24] An alternative approach to overcome the inherent difficulty of targeting Ras directly is targeting the post-translational processing and localization of Ras. Unfortunately, farnasyltransferase inhibitors (FTIs), such as tipifarnib, demonstrated impressive preclinical activity, but subsequent clinical studies yielded disappointing results due to resistance driven by alternative prenylation pathways for Ras.[5] Alternatively, targeting the palmitoylation/depalmitoylation cycle of Ras with the acyl protein thioesterase (APT) inhibitor palmostatin B can disrupt the localization of oncogenic Ras and inhibit the growth and clonogenicity of murine haematopoietic cells expressing oncogenic Nras, but the translational potential of this strategy remains to be determined.[25] While renewed efforts fueled by the National Cancer Institute's Ras Initiative are challenging the paradigm that Ras is an undruggable cancer target, drugs that directly target Ras have yet to make their way into clinical practice.

The struggle to directly inhibit Ras has motivated intense efforts to target Ras effector pathways in AML. These efforts have largely focused on the MAPK and/or PI3K pathways, and have generally demonstrated modest and predominately cytostatic effects in a variety of AML models.[26-30] For example, inhibition of MEK alone or in combination with PI3K in a mouse model of *Nras* mutant AML inhibited proliferation but failed to induce leukemic cell death, suggesting that MAPK and PI3K pathways drive AML proliferation but may be dispensable for AML survival.[30] Similarly, our group found that inhibition of MAPK and/or PI3K signaling led to G0/G1 cell cycle arrest of human AML cell lines with negligible effects on apoptosis, and led to predominately static effects *in vivo* in a murine *NRAS(G12V)*-driven AML model.[26] The clinical experience targeting MAPK and PI3K have been similar. Inhibition of MEK with selumetinib had modest and transient activity for patients with relapsed/refractory AML, and inhibition of AKT with MK-2206 had essentially no activity against AML in phase II clinical trials.[31,32] Strategies that combined inhibitors of MEK and MDM2 or MEK, mTOR, and BCL2 have demonstrated synergistic anti-leukemic activity and induced leukemic cell apoptosis *in vitro*, suggesting that combined inhibition of Ras signaling together with key survival pathways may be advantageous.[28,29] The modest efficacy of targeting the MAPK or PI3K pathways alone is likely related to functional redundancy and/or feedback loops that compensate for the loss of a single effector pathway. Indeed, biopsy specimens from patients with advanced solid tumors that were treated with the mTOR inhibitor everolimus exhibited higher levels of MAPK signaling.[33] Another potential explanation for the lack of efficacy of MAPK and/or PI3K inhibitors is that alternate Ras effectors may play important roles in cancer growth and survival. Supporting the later, elegant studies investigating the essential oncogenic signals downstream of Ras revealed that activation of MAPK and PI3K signaling are not sufficient to transform human fibroblasts.[34] Similarly, we found that combined inhibition of MAPK and PI3K could not reproduce the apoptotic effects of *NRAS* oncogene withdrawal in an *NRAS(G12V)*-addicted AML mouse model, indicating that other Ras effector(s) provide critical support to leukemic cells.[26]

Other clues to the key mediators and specific vulnerabilities of Ras-driven cancer cells come from several large-scale synthetic lethality screens. These screens are based on the premise that specific mutations can have insignificant or beneficial effects in isolation, but can be lethal when combined. Transcriptome-scale loss of function screens have identified genes and pathways that exhibit synthetic lethality with mutant Ras.[35] For example, a recent CRISPR/Cas9-based screen identified synthetic lethal interactions between genes involved in Ras processing and MAPK signaling and oncogenic *RAS* mutations in human and murine leukemia cells.[36] While such screens have uncovered putative Ras-associated cancer genes and pathways, comparisons between these studies are complicated by the differences in technology, conditions, and model systems used. These differences undoubtedly contribute to the lack of overlap observed across studies, but may also indicate that Ras's vulnerabilities are greatly influenced by the cellular and molecular context. Furthermore, functional validation in relevant and robust model systems including primary patient-derived cancer cells will be essential to validate candidate genes and pathways identified in large-scale synthetic lethal screens to determine their true translational potential.

There is mounting evidence that Ras-like (Ral) proteins are critical effectors of Ras in cancer (Fig. 2). Like Ras, the Ral proteins, RALA and RALB, are small GTPases that are activated by Ral guanine exchange factors (RalGEFs) that promote the exhange of GDP for GTP and are inactivated by Ral GTPase activating proteins (RalGAPs) that catalyze their instrinsic GTPase activity. Ral-GTP, and in some cases Ral-GDP, interacts with various effectors to regulate diverse cellular processes. The best characterized effectors of Ral signaling include RALBP1/RLIP and the SEC5 and EXO84 subunits of the hetero-octomeric exocyst complex, which has exocytic and non-exocytic cellular functions.[37] Seminal studies uncovered an essential role for Ral proteins in the transformation of murine fibroblasts downstream of Ras.[38,39] Subsequent studies confirmed that Ral activation downstream of Ras was sufficient for transformation of human cells.[34] RALA and RALB appear to have unique roles in anchorage-

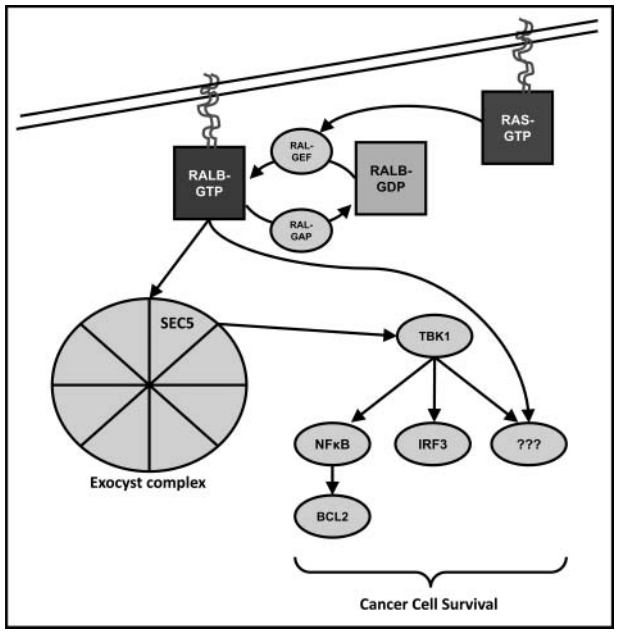

Figure 2. Oncogenic RALB signaling. RALB transduces signals to effector enzymes downstream of Ras. Like Ras, RALB activation is regulated by Ral-specific guanine-exchange factors (Ral GEFs) and GTPase activating proteins (Ral GAPs). The specific oncogenic mechanism(s) of RALB are not well understood, but is thought to involve interaction with the SEC5 subunit of the exocyts complex to recruit and activate the non-canonical IκB kinase TANK-binding kinase 1 (TBK1) that promotes cancer cell survival through NFκB and interferon response factor 3 (IRF3).

independent growth and survival, respectively;[40] however, either RALA or RALB, but not both, are required for proliferation of murine *Kras* oncogene-driven non-small cell lung cancer cells, suggesting some degree of functional redundancy.[40,41] The divergent functional roles of RALA and RALB, which can interact with similar effectors *in vitro*, are primarily mediated by their unique subcellular localization. While the functional roles for specific Ral effectors in cancer are not well understood, a synthetic lethal screen identified an essential role for the RALB-SEC5-TBK1 signaling axis in the maintenance of several *KRAS* oncogene-driven human epithelial cancers.[42] RALB appears to be required for the survival of malignant cells, but not normal cells, making it an attractive therapeutic target.[40] Our group uncovered a critical role for RALB in human AML cell survival, and confirmed that RALB-TBK1 signaling is hyperactivated in AML patient samples.[26] We also demonstrated that the clinically relevant drug, dinaciclib, has RALB-dependent anti-leukemic effects in murine and human preclinical AML models including patient derived AML mouse xenografts (PDX mice) with negligible effects on normal blood progenitor cells.[43] A central role for RALB signaling in AML was corroborated by other work by our group that discovered that Ras oncogene-independent activation of RALB signaling is a targetable mechanism of relapse after suppression of oncogenic Ras expression in a mouse model of *NRAS(G12V)*-addicted AML.[43] The specific mechanism that drives Ras oncogene-independent activation of RALB signaling in this model and the role of RALB in human AML relapse are areas of active investigation. These studies support a central role for RALB in the pathophysiology of AML and as a promising therapeutic target.

While Ral GTPases and their effectors have emerged as important drivers of cancer and RALB appears to play a key role in AML, several unanswered questions remain. Our findings demonstrate that RALB signaling is hyperactivated in several diverse primary AML patient samples, but whether RALB-dependence is a general feature of AMLs or is limited to specific genetic subsets (e.g., AML with oncogenic Ras mutations) has not been systematically evaluated.[26] From a translational perspective, this has important implications for identifying specific AML patients that might benefit from RALB-based therapy. Another challenge is to develop clinically relevant strategies to target RALB. Similar to Ras, there are technical hurdles to directly targeting Ral GTPases related to its structure and affinity for GTP. While the clinically relevant cyclin dependent kinase (CDK) inhibitor dinaciclib has RALB-dependent effects against AML, it also has RALB-independent effects through inhibition of CDK1, CDK2, CDK5, and CDK9.[43] Ral specific small molecule inhibitors have recently been developed with encouraging preclinical results, but have not yet made the leap into clinical development.[44,45] Pharmacologic targeting of Ral effector pathways represents another potential therapeutic strategy, but the detailed mechanisms that support AML survival downstream of RALB have not been characterized. We have shown that knockdown of RALB leads to decreased expression of BCL2 in leukemic cells, but the mechanism and functional consequence of this association remains to be seen.[26] Given the virtually ubiquitous development of treatment resistance seen with clinical targeting of single oncogenic pathways, it seems likely that potent and durable anti-leukemic responses will require combined targeting of multiple signaling nodes. A more comprehensive evaluation of RALB survival signaling will be critical to understand its pathophysiology, and will likely uncover novel drug targets. A better understanding of Ras and key effectors like RALB will be essential to guide the rational development of safer and more effective targeted cancer treatments.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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