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Breaking up is hard to do

RalA, mitochondrial fission and cancer

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The small GTPases RalA and RalB are activated downstream of oncogenic Ras. While activation of RalA is critically important for tumor initiation and growth of Ras-driven cancers, the highly similar small GTPase RalB is implicated in cell survival and metastasis. This difference in function between these two related proteins maps to the C-terminus, a 30 amino acid region that regulates subcellular localization and contains several potential phosphorylation sites. Here we discuss our recent evidence that phosphorylation by the mitotic kinase Aurora A promotes RalA relocalization to mitochondrial membranes, where it recruits the effector RalBP1 and the large dynamin-related GTPase Drp1 to promote mitochondrial fission. As upregulation of both RalA and Aurora A have been observed in human tumors, and phosphorylation of RalA at the site targeted by Aurora A promotes tumorigenesis, it is possible that regulation of mitochondrial fission is one mechanism by which RalA promotes cancer.

Keywords: RalA, RalBP1, mitochondrial fission, Aurora A, cell cycle

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Mutations in the *RAS* gene are present in nearly one third of all human cancers and up to 90% of pancreatic cancers.[1] Constitutively active Ras proteins promote tumorigenesis through the engagement of downstream effector proteins, including phosphatidylinositol-3 kinase (PI3K),[2] the Raf family of serine/threonine kinases,[3-6] and the RalGEF family of guanine nucleotide exchange factors.[7-9] Of these three, the RalGEF pathway is the least well understood, although mounting evidence points to a role for this pathway in multiple aspects of tumorigenesis.[10-18] RalGEFs comprise a family of six guanine nucleotide exchange factors (RalGDS, RGL, RGL2/Rlf, RGL3, RalGPS1 and RalGPS2) of which all but RalGPS1 and RalGPS2 are directly activated by Ras.[19] These proteins promote the activation of two nearly identical small GTPases, RalA and RalB, which in turn engage a diverse set of downstream effector molecules, including the exocyst components Sec5[20,21] and Exo84,[22] the large multifunctional RalBP1 (RLIP76/RLIP1/RIP1),[23] the actin cross-linking protein filamin,[24] and the transcription factor ZONAB.[25] Despite 80% sequence identity, and near 100% identity in their effector binding domains,[26] RalA and RalB promote distinct aspects of tumor growth downstream of oncogenic Ras. While RalA has been shown to be important for tumor initiation and growth, RalB seems to play more of a role in promoting survival and metastasis.[13,17,27,28] This difference in function between RalA and RalB maps to the 30 amino acid hypervariable C-terminus of the protein, as replacing this region in RalB with that of RalA is sufficient to impart both the polarized membrane delivery function, as well as transforming activity to RalB.[13,29]

The hypervariable regions of RalA and RalB are both highly basic and have a conserved CAAX motif that targets the protein for prenylation, but they otherwise share little sequence identity.[26] Both sequence analysis and targeted mutational analysis of these regions have identified several putative phosphorylation sites that are unique to either RalA or RalB.[12,13,30,31] These sites are of particular interest, as analysis of the related small GTPase KRas suggests that phosphorylation of the hypervariable domain can have a profound

impact on subcellular localization and protein function.[32] Of the two serine residues in the RalA C-terminus (S183 and S194) and five threonine or serine residues in the RalB C-terminus (T178, S182, S192, S193 and S198), several have been shown to be targets of phosphorylation,[12,30,31] with the best-characterized being S194 of RalA. S194 is phosphorylated by Aurora A, a kinase activated in mitosis that is known to be important for mitotic entry, centrosome duplication and maturation, bipolar spindle assembly and cytokinesis,[31,33] and can be dephosphorylated by the phosphatase PP2A.[30] Phosphorylation of S194 leads to an increase in active, GTP-bound RalA and redistributes the protein from the plasma membrane to internal membranes.[30,31,34] Furthermore, Aurora A cooperates with RalA to promote tumorigenesis, and mutating S194 to alanine or dephosphorylating S194 by PP2A inhibits RalA transforming ability.[30,34]

In order to better understand the role of this phosphorylation, we examined the specific subcellular localization of RalA in the presence and absence of ectopic Aurora A. Similar to what was found for KRas following its phosphorylation by PKC,[32] immunofluorescence analysis of GFP-tagged RalA and immunoblot analysis of biochemically fractionated cells revealed that a portion of RalA localizes at mitochondria or membranes tightly associated with this organelle. Furthermore, expression of an active (T288D) mutant of Aurora A or mutation of RalA-S194 to Aspartic acid to mimic phosphorylation increased the amount of RalA in the mitochondria while inhibition of Aurora A, or mutation of RalA-S194 to Alanine had the opposite effect.[35]

Analysis of the localization of RalA at mitochondria also revealed a striking difference in the morphology of mitochondria in cells with activated Aurora A. While the majority of cells expressing a vector control exhibited a mixture of long interconnected mitochondria and short punctate mitochondria, cells expressing active Aurora A^{T288D} exhibited a large percentage of small punctate mitochondria. Cells expressing the kinase-inactive $Aurora^{K162R}$, on the other hand, exhibited a large percentage of long interconnected mitochondria.

Mitochondrial morphology is maintained through a balance of the opposing processes of fusion and fission and it has become clear in recent years that maintaining this balance is critical to a number of cellular processes and implicated in a number of human diseases.[36-38] Given that Aurora A activity promoted the mitochondrial localization of RalA and affected mitochondrial morphology, we performed both loss-of-function and gain-of-function analysis and found that RalA was required for normal mitochondrial dynamics. Specifically, Aurora A promoted mitochondrial division in a RalA and S194 dependent fashion, and expression of the phosphomimetic $RalA^{S194D}$ was sufficient to induce mitochondrial fragmentation. Furthermore, phosphorylation of RalA by Aurora A promoted mitochondrial fission, as opposed to blocking fusion, and was accompanied by an increase in the levels of the large, fission-promoting GTPase Drp1 at the mitochondria.[35]

Phosphorylation of RalA on S194 was previously shown to promote the binding of RalA to RalBP1 over Sec5 and Exo84.[34] Consistent with this, shRNA-mediated knockdown of RalBP1 phenocopied the effects of RalA knockdown on mitochondrial morphology, promoting long interconnected networks. Additionally, expression of active Aurora A^{T288D} resulted in an increase in the levels of RalBP1 associated with the mitochondrial fraction. This increase in mitochondrial RalBP1 was abrogated by knockdown of RalA, suggesting that RalA recruits RalBP1 to mitochondria to promote mitochondrial fission. Indeed fusion of the C-terminus of RalA in the S194D phosphomimetic configuration to RalBP1 was sufficient to promote both mitochondrial localization and mitochondrial fission.[35]

Mitochondrial fission increases during mitosis, and is dependent on both the mitochondrial recruitment and phosphorylation of Drp1.[39-41] As Aurora A is active during mitosis,[42] we tested and found that the levels of S194-phosphorylated RalA were increased in mitotic HeLa cell extracts when compared with unsynchronized cells and that this phosphorylation was coincident with increased recruitment of RalA, RalBP1, and Drp1 to mitochondrial membranes at mitotis. Importantly, knockdown of RalA, RalBP1 or Aurora A led to a decrease in Drp1 recruitment to mitochondria during mitosis, suggesting that this pathway is critical for the mitotic mitochondrial recruitment of Drp1.

During mitosis, Drp1 is also phosphorylated on S616 by the mitotic kinase Cyclin B/CDK1.[31] Previous studies have shown that RalBP1 binds to Cyclin B/CDK1 and promotes the phosphorylation of the protein Epsin to suppress receptor-mediated endocytosis.[43] We confirmed that RalBP1 bound to Cyclin B and associated with CDK1 kinase activity in mitotic extracts. Furthermore, similar to its role with Epsin, RalBP1 promoted CDK1 phosphorylation of Drp1 in vitro, and loss of RalBP1, but not RalA, decreased the levels of S616 phosphorylated Drp1 in mitotic extracts. Importantly, we detected a complex including RalA, RalBP1, Drp1 and Cyclin B on biochemically enriched mitochondrial membranes during mitosis, suggesting a model whereby RalBP1 forms a complex with Drp1 and Cyclin B/CDK1 to promote Drp1 phosphorylation and this complex is recruited to mitochondrial membranes through the binding to S194-phosphorylated RalA (**Fig. 1**).

This model predicts that mitotic mitochondrial fission will be disrupted in cells lacking either RalA or RalBP1. Indeed, while the mitochondria of scramble control cells underwent fission during metaphase, mitochondria remained interconnected in cells in which either RalA or RalBP1 had been knocked down by shRNA. The effects of RalA knockdown were rescued by expression of an shRNA-resistant wild-type RalA, but not $RalA^{S194A}$, underscoring the importance of this phosphorylation site for mitochondrial fission.

The loss of mitotic mitochondrial fission in RalA and RalBP1 knockdown cells often resulted in mitochondrial bridges between daughter cells during cytokinesis, which was associated with a failure to complete cytokinesis and unequal distribution of mitochondria to the two daughter cells. Additionally, knockdown cells exhibited signs of mitochondrial dysfunction, with decreased levels of ATP and reduced cell growth over time.[35]

Notably, upregulation of Aurora A protein levels[44,45] and RalA activity[10,11,13,46,47]

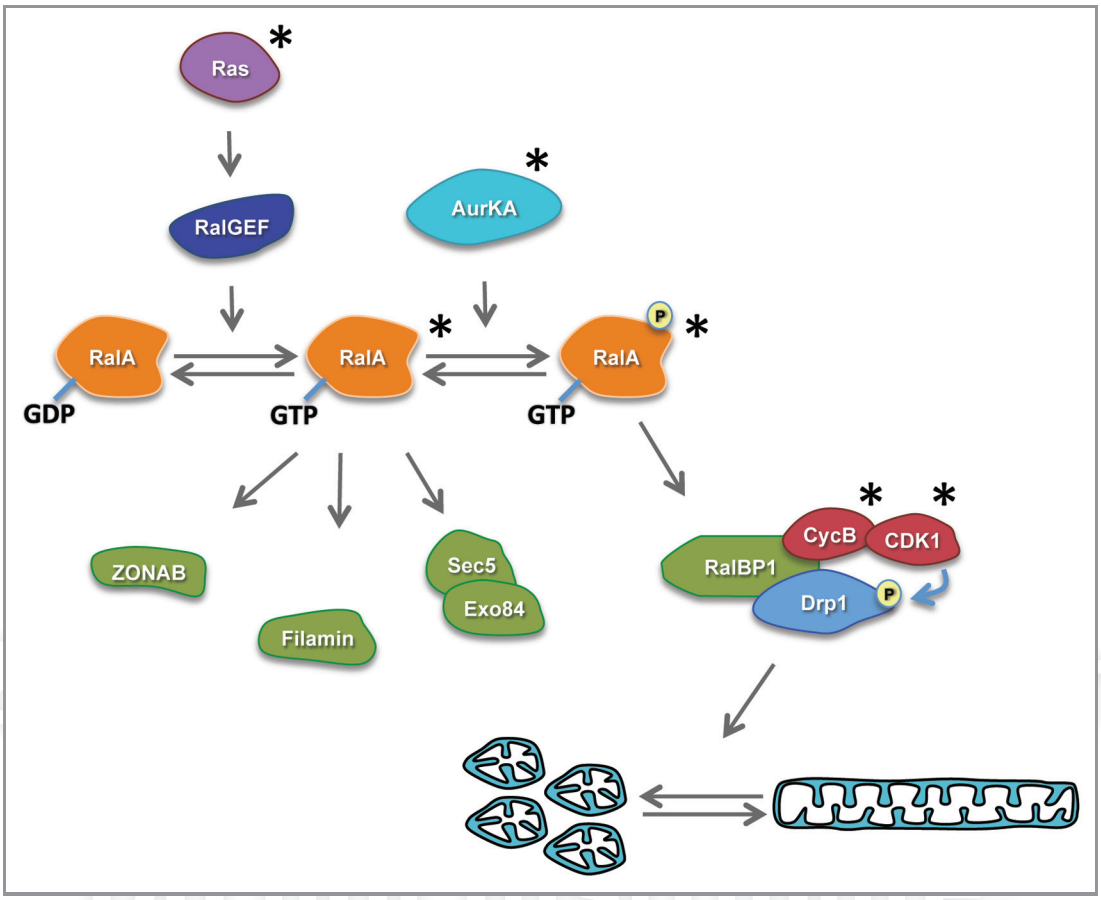

Figure 1. Ras activation of RalA leads to engagement of multiple effector pathways. The RalGEF-Ral signaling pathway is a key effector pathway downstream of activated Ras. Activation of RalA in turn leads to activation of several downstream signaling pathways affecting diverse cellular processes, including exocytosis, endocytosis, actin dynamics and transcription. New evidence also indicates that following phosphorylation by Aurora A, RalA and its effector RalBP1 play a key role in the regulation of mitochondrial fission during mitosis by regulating the phosphorylation of Drp1 and its recruitment to mitochondrial membranes. "*" denotes proteins whose expression or activity have been reported to be elevated in human cancer.

has been observed in human cancer cells. Furthermore, knockdown or inhibition of Aurora A kinase activity impairs tumor growth,[48,49] and knockdown or inhibition of RalA reduces Ras-induced transformation and tumor growth of multiple cancer cell lines derived from a diverse set of cancers.[10,11,13,30,46,47,50-52] Importantly, the loss of Ras-induced tumor formation following RalA knockdown can be rescued in most cells by wildtype, but not an S194A mutant of RalA, confirming the importance of Aurora A phosphorylation for this process.[30,31,34] Maintenance of a dynamic mitochondrial network has been shown to be important for several cellular processes that are also known to be critical for tumorigenesis, including autophagy and the cargo-specific clearance of mitochondria known as mitophagy,[53-56] apoptosis[57-59] and maintenance of metabolic function.[37,60,61] We have shown that metabolic function is compromised in cells lacking RalA and RalBP1, ultimately leading to diminished cellular growth.[35] Taken together, these data suggest the intriguing possibility that aberrant activation and phosphorylation of RalA promote tumor growth, at least in part, through the regulation of mitochondrial fission. The finding that the tumor suppressor p53 regulates mitochondrial fission through down-regulation of miR-499, which negatively regulates Drp1 expression,[62] suggests that dysregulated mitochondrial dynamics might be a general feature of cancer cells and indeed, analysis of cancer cell lines by electron microscopy has revealed abnormal mitochondrial morphologies.[63]

It will be interesting to test whether, in addition to metabolic function, autophagy and apoptosis are also affected by loss of RalA and RalBP1, and whether their restoration can rescue the loss of tumorigenesis phenotype characteristic of RalA inhibition. Intriguingly, RalB has been shown to directly impact both autophagy[64] and apoptosis,[28] through engagement of Exo84 and Sec5, respectively, however it is not known how these two related small GTPases use different effector pathways to potentially converge on the same cellular processes and what impact this convergence may have on Ras-driven tumorigenesis.

If the control of mitochondrial dynamics proves to be important for Ras-driven tumorigenesis, it could represent an important step towards discovering a more effective way to treat cancer. While inhibition of Ras or other small GTPases has proven to be problematic as a therapeutic strategy,[65] perhaps targeted disruption of mitochondrial dynamics will prove

more pharmacologically feasible. Small molecule inhibitors of the mitochondrial fusion and fission machinery, including the recently described Drp1 inhibitor Mdivi-1,[66] are under development,[67] and it will be enlightening to test them in mouse tumor models to see if they have a clinical effect on tumor growth.

In conclusion, the finding that phosphorylation of RalA promotes mitochondrial fission through the recruitment of RalBP1 and Drp1 to mitochondria represents an important step forward in our understanding of RalA function, in the functional differences between RalA and RalB, and provides potential insight into how RalA regulates Ras-mediated tumorigenesis.

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