Activation of RalA is critical for Ras-induced tumorigenesis of human cells

Kian-Huat Lim,[1] Antonio T. Baines,[3,4,5] James J. Fiordalisi,[3,4] Michail Shipitsin,[6] Larry A. Feig,[6] Adrienne D. Cox,[3,4,5] Channing J. Der,[3,5] and Christopher M. Counter[1,2,*]

[1]Department of Pharmacology and Cancer Biology
[2]Department of Radiation Oncology
Duke University Medical Center, Durham, North Carolina 27710
[3]Lineberger Comprehensive Cancer Center
[4]Department of Radiation Oncology
[5]Department of Pharmacology
University of North Carolina at Chapel Hill, Chapel Hill, North Carolina
[6]Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts 02111
*Correspondence: count004@mc.duke.edu

Summary

RalGEFs were recently shown to be critical for Ras-mediated transformed and tumorigenic growth of human cells. We now show that the oncogenic activity of these proteins is propagated by activation of one RalGEF substrate, RalA, but blunted by another closely related substrate, RalB, and that the oncogenic signaling requires binding of the RalBP1 and exocyst subunit effector proteins. Knockdown of RalA expression impeded, if not abolished, the ability of human cancer cells to form tumors. RalA was also commonly activated in a panel of cell lines from pancreatic cancers, a disease characterized by activation of Ras. Activation of RalA signaling thus appears to be a critical step in Ras-induced transformation and tumorigenesis of human cells.

Introduction

The genes encoding the small GTPase Ras are mutated to yield proteins that remain in the constitutively active GTP bound state in one-third of all human cancers, where they act as oncoproteins and promote cancer-like growth of mammalian cells (Bos, 1989). At least three primary Ras binding proteins, or effectors, propagate oncogenic Ras signals: the Raf family of serine/threonine kinases that activate the ERK mitogen-activated protein kinase (MAPK) pathway, known to promote cell proliferation; the phosphatidylinositol 3-kinases (PI3Ks), known to promote cell survival; and the Ral guanine nucleotide exchange factors (RalGEFs), activators of the RalA and RalB Ras-related small GTPases (Shields et al., 2000).

Activation of Raf or its downstream targets promotes soft agar growth of rodent fibroblast, a transformed phenotype of cancer cells, and tumor formation (Shields et al., 2000). On the other hand, neither RalGEFs nor PI3Ks are particularly transforming. Activated RalGEF or Ral proteins do not cause potent transformed growth of rodent fibroblast or epithelial cells (Collette et al., 2004; McFall et al., 2001; Ulku and Der, 2003; Urano et al., 1996), although they do enhance transformation of NIH 3T3 cells expressing an activated version of Raf (Urano et al., 1996) or rat fibroblasts expressing the EGF receptor (Lu et al., 2000), and dominant-negative Ral mutants suppressed Ras-induced transformation of NIH 3T3 cells (White et al., 1996). Similarly, activated PI3K alone is not transforming, but enhances the transforming activity of Raf and, when inhibited, blocks Ras-mediated transformed cell growth of NIH 3T3 cells (Rodriguez-Viciana et al., 1997).

Unlike the situation in mice, however, the RalGEF pathway appears to play a more prominent role in transformation of human cells. Specifically, normal human cells from a wide spectrum of cell types are driven to a tumorigenic state upon the expression of four proteins: SV40 T-Ag, which inhibits the function of the tumor suppressors p53 and the Rb pocket proteins; SV40 t-Ag, which indirectly activates c-Myc; the hTERT catalytic subunit of telomerase, which is essential for the cancer phenotype of unlimited growth; and oncogenic (G12V) Ras (Hahn et al., 1999; O'Hayer and Counter, 2005). Using this approach to transform identically matched primary human and mice cells, it was found that murine cells were most potently

SIGNIFICANCE

Ras is activated in one-third of human cancers, making it important to elucidate the oncogenic signals emanating from this protein in order to develop anticancer agents to treat Ras-mediated cancers. While much effort has focused on the MAPK pathway as the key pathway downstream of Ras, based on murine studies, recent evidence points toward another class of Ras effectors, the RalGEFs, as also playing a critical role in human cell transformation and tumorigenesis. We now report that a substrate of RalGEFs, RalA, is critical for Ras-mediated tumor growth of human cancer cells and is commonly activated in human pancreatic cancer cell lines. As such, this protein represents a novel target for therapeutic intervention.

transformed by Raf activation by replacing Ras with effector mutants that preferentially activate only one of the major effector pathways or with activated versions of the effectors themselves, whereas human cell transformation was mediated by activation of the RalGEF family of proteins. Moreover, the tumor growth of the human cells was dependant upon the Ras effector mutant that activates RalGEFs. Impeding RalGEF function via the expression of a dominant-negative version of its substrate also diminished Ras-mediated transformation of the same human cells (Hamad et al., 2002; Rangarajan et al., 2004). Given the importance of RalGEF activation for transformation of human cells, understanding how the RalGEF oncogenic signal is transmitted could provide insight into the mechanism of Ras-induced human tumorigenesis.

Four human RalGEFs that share a common C-terminal Ras association domain have been identified. GTP bound Ras relocates RalGEFs to the membrane fraction where they can promote the exchange of GDP to GTP on their only known substrates, the membrane bound GTP binding proteins RalA and RalB (Kishida et al., 1997; Matsubara et al., 1999). The catalytic activity of RalGEFs is also stimulated upon the association with the protein kinase PDK1 (Tian et al., 2002), and RalGEFs have been reported to have functions independent of the exchange activity (Bhattacharya et al., 2002; Tian et al., 2002). In regard to transformation, overexpression of activated Ral fosters anchorage-independent growth and cell motility in a number of human cancer cell lines (Gildea et al., 2002; Ward et al., 2001; Yamazaki et al., 2001). Additionally, disruption of Ral activation with either small interfering RNAs (siRNA) or inhibition of Ral effector activation impairs the growth of several human cancer cell lines in short-term suspension culture (Chien and White, 2003).

To date, three classes of Ral effectors have been identified: the Cdc42/Rac GTPase-activating protein RalBP/RLIP76; the Sec5 and Exo84 subunits of the exocyst complex; and the actin binding protein filamin (Cantor et al., 1995; Jullien-Flores et al., 1995; Moskalenko et al., 2002; Moskalenko et al., 2003; Ohta et al., 1999; Park and Weinberg, 1995). Both RalA and RalB also bind constitutively to phospholipase D1 (PLD1) (Luo et al., 1997) and stimulate the activation of c-*src*, c-*jun*, AFX, STAT, and NF-κB, as well as cyclin D, through as yet unidentified effectors (de Ruiter et al., 2000; Goi et al., 2000; Henry et al., 2000; Kops et al., 1999). To add to the complexity, despite greater than 85% identity, RalA and RalB appear to perform different functions. For instance, RalA has a much higher affinity for the exocyst complex than does RalB, and only RalA stimulates exocyst function to enhance the rate of delivery of membrane proteins to the basolateral membrane of epithelial cells (Shipitsin and Feig, 2004). It has also been reported that suppression of RalA by siRNA inhibits cancer cell growth in suspension culture, whereas similar suppression of RalB in these cells leads to apoptosis (Chien and White, 2003). As RalGEFs are important for Ras-induced tumorigenesis of human cells and accumulating data suggest that Ral proteins may mediate functions of RalGEFs, we explored whether the RalGEF substrates RalA and RalB play a role in transformed and tumorigenic growth of human cells.

Results and discussion

The GTP exchange activity of a RalGEF is necessary for human cell transformation

Since there is evidence for Ral-independent functions of RalGEFs (Bhattacharya et al., 2002; Tian et al., 2002), as a first step to delineate the signaling pathways through which RalGEF transforms human cells, we tested whether the GTP exchange activity of the RalGEF Rlf was required for transformation. A retrovirus encoding no transgene as a control, or wild-type or catalytically dead R328E mutant version of Rlf fused to the plasma membrane targeting domain of K-Ras (termed CAAX), which is used to mimic the recruitment of Rlf to the plasma membrane during Ras stimulation (de Ruiter et al., 2000; Wolthuis et al., 1996; Wolthuis et al., 1997), were used to infect HEK-HT cells, primary human embryonic kidney cells that ectopically express hTERT, T-Ag and t-Ag (Hahn et al., 1999). These cells provide a genetically defined human cell system to assay Ras oncogenesis (Hamad et al., 2002). Expression of the desired proteins in the resultant polyclonal populations was confirmed by immunoblot analysis (Figure 1A). We also verified that these two forms of Rlf-CAAX did not activate the Raf/MEK/ERK and PI3K/Akt pathways, by measuring the level of phosphorylated forms of ERK1/2 and Akt via immunoblot analysis in serum-starved cells. As a control, we confirmed that Akt and ERK1/2 are highly phosphorylated upon expression of Ras^{G12V} (Figure 1A). The cells were next assayed for anchorage-independent growth when suspended in soft agar, one of the most stringent in vitro measures of the transformed phenotypes typical of cancer cells. Consistent with previous results (Hamad et al., 2002), expression of Rlf-CAAX alone was transforming, to approximately half the level of anchorage-independent growth observed in HEK-HT cells expressing the 37G effector domain mutant of oncogenic Ras (not shown) that is partially defective at activating PI3K and MAPK, but retains the ability to activate RalGEFs (Rodriguez-Viciana et al., 1996; Shields et al., 2000; White et al., 1995; White et al., 1996). However, the catalytically dead Rlf^{R328E}-CAAX failed to transform human cells (Figure 1B), indicating that Ral GTPase activation is likely responsible for RalGEF-mediated transformation.

Activation of RalA, but not of RalB, transforms human cells

We next determined which of the RalA or RalB proteins was responsible for RalGEF-mediated transformation by assaying whether activated forms of RalA or RalB alone could transform human cells. We used RalA and RalB mutated to G23V or Q72L to disrupt their intrinsic and GTPase activating protein (GAP)-stimulated GTPase activity, which leaves the proteins in a constitutively active GTP bound state and insensitive to Ral-GAP activity (Emkey et al., 1991; Frech et al., 1990), or RalA and RalB mutated to F39L, which is analogous to the same mutation in Ras that promotes fast cycling between GDP and GTP bound states (Reinstein et al., 1991), as such a mutation mimics interaction with GEFs and is oncogenic in both Ras and Rho small GTPases (Lin et al., 1997; Reinstein et al., 1991). These three mutants and their wild-type counterparts were stably expressed in HEK-HT cells, as assessed by immunoblot analysis. As expected, these proteins did not activate the PI3K or MAPK pathways (Figure 1A). When the resulting cells were assayed for growth in soft agar, no version of RalB supported anchorage-independent growth, consistent with our previous results that RalB was not transforming (Hamad et al., 2002). Conversely, all RalA proteins transformed HEK-HT cells to some degree, although the most transforming versions were clearly those with the activating (G23V or Q72L) mutations (Figure 1B). The number and size of colonies observed in cells

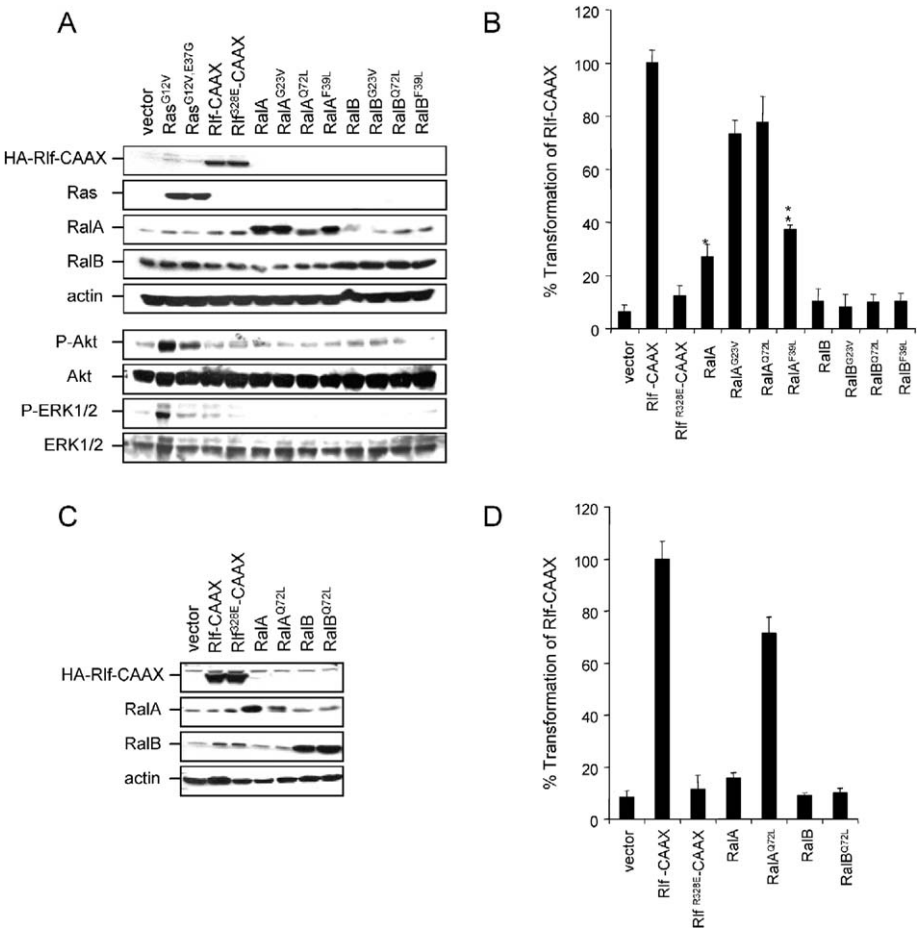

Figure 1. Activated RalA promotes anchorage-independent growth in human kidney and fibroblast cells

Immunoblot analysis of HA-epitope-tagged Rlf-CAAX, Ras, RalA, and RalB in HEK-HT cells (**A**) or BJ-HT (**C**) cells stably infected with retroviruses encoding the described transgenes. MAPK and PI3K activity were measured in the described cells by detecting phosphorylation of ERK1/2 (P-ERK1/2) and Akt (P-Akt), respectively, with phosphospecific antibodies. Actin, total ERK1/2, and total Akt served as loading controls. Anchorage-independent growth of HEK-HT (**B**) and BJ-HT cells (**D**) expressing the described transgenes, as calculated from the average number of colonies ±SD from six plates (two independent experiments conducted in triplicate) and expressed as the percentage of colonies in Rlf-CAAX-transformed cells. *, p = 0.00002 between vector and RalA; **, p = 0.002 between RalA and RalA^{F39L} (two-tailed Student's t test)

expressing activated RalA were smaller than those observed in the positive control, Rlf-CAAX-expressing cells. As such, all cultures were assayed for anchorage-independent growth 1 week later than typically needed to detect soft agar growth. Since Rlf-CAAX was more transforming than activated RalA, it is possible that either RalGEF signaling involves more than the mere activation of RalA, or that expression of constitutively activated RalA mutants fails to recapitulate either the magnitude or proper localization of RalA activated by RalGEFs that is needed to promote full transformation. Nevertheless, as activated forms of RalA, and no variant of RalB, transformed human cells, the signaling events leading to anchorage-independent growth in the RalGEF pathway involve activation of RalA.

We next confirmed the transforming activity of RalA in a completely different cell type. Normal human fibroblasts ectopically expressing hTERT, T-Ag, and t-Ag (BJ-HT cells; Hahn et al., 1999) were stably infected with retroviruses encoding wild-type or the Q72L-activated versions of RalA or RalB, or Rlf-CAAX (as a positive control), or the catalytically dead version of Rlf-CAAX or no transgene (as negative controls). Resultant polyclonal populations were confirmed to express the desired protein by immunoblot analysis (Figure 1C) and assayed for anchorage-independent growth. Although Ras-mediated transformation of human fibroblasts can be weaker than that observed in HEK cells (Hahn et al., 1999; Hamad et al., 2002), constitutively activated RalA (RalA^{Q72L}) and, to a very small degree, wild-type RalA, but not any version of RalB, was also transforming in fibroblasts (Figure 1D). Given the fact that activated RalA alone caused growth transformation of two very different human cell types, we argue that activation of this protein may be broadly sufficient to promote Ras-mediated transformation of human cells.

Inhibition of RalA binding to RalBP1 and Sec5 suppresses transformation

RalA is known to bind to and promote the activation of a number of proteins, including phospholipase D1 (PLD1), the Sec5 and Exo84 subunits of the exocyst complex, and the Cdc42/Rac GTPase-activating protein RalBP1/RLIP76 (Cantor et al., 1995; Jullien-Flores et al., 1995; Moskalenko et al., 2002; Ohta et al., 1999; Park and Weinberg, 1995). PLD1 binding is mediated through the N terminus of RalA, whereas binding to the other proteins is mediated through the core effector loop region within the conserved switch 1 domain. Ral also contains the conserved switch 2 region that, together with the switch 1 region, changes the conformation during GDP/GTP cycling, as well as a C-terminal hypervariable domain that appears to determine the proper subcellular localization of the protein with a highly conserved CAAX tetrapeptide motif, which signals for the posttranslational modification by geranylgeranyl isoprenoid

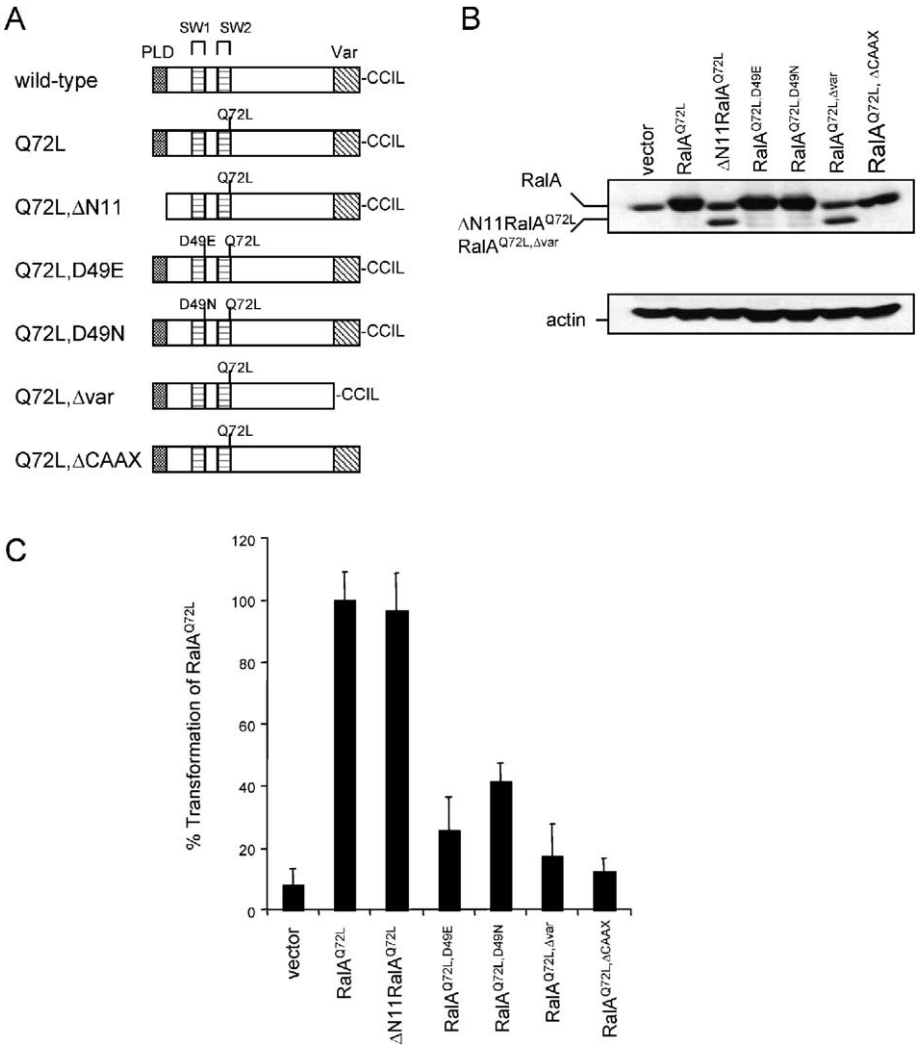

Figure 2. RalA-mediated transformation is dependent on the effector binding and C-terminal localization domains

A: Schematic representation of RalA^{Q72L} effector mutants. PLD, PLD binding domain; SW1and SW2, switch domains 1 and 2; Var, C-terminal hypervariable domain.

B: Detection of RalA protein by immunoblot analysis in HEK-HT cells stably infected with a retroviral vector encoding no transgene (vector) or one of the described RalA^{Q72L} mutants. Actin serves as a loading control.

C: Anchorage-independent growth of polyclonal HEK-HT cells stably expressing the indicated RalA^{Q72L} effector mutants, as calculated from the average number of colonies ± SD from six plates (two independent experiments conducted in triplicate) and expressed as the percentage of colonies detected in RalA^{Q72L}-transformed cells.

lipid essential for membrane association and biological activity (Figure 2A). To address which effectors of RalA are required for transformation, we examined the transforming ability of a panel of activated RalA mutants selectively defective in interacting with the aforementioned binding proteins. Specifically, the ΔN11RalA^{Q72L} mutant is truncated in the first 11 amino acid, reducing PLD1 binding (Jiang et al., 1995); the RalA^{D49E,Q72L} mutant is defective in binding to Sec5 and Exo84 (Moskalenko et al., 2002; Moskalenko et al., 2003); and the RalA^{D49N,Q72L} mutant has diminished affinity for RalBP1 (Cantor et al., 1995).

These different RalA^{Q72L} mutants were stably expressed, as assessed by immunoblot analysis, in HEK-HT cells (Figure 2B) and assayed for growth in soft agar. The ΔN11RalA^{Q72L} retained the transforming potency of RalA^{Q72L}, indicating that binding between RalA and PLD1 is not essential for RalA-mediated cellular transformation (Figure 2C). In agreement, we find that activated RalA is the most transforming (Figures 1B, 1D, and 2C), even though PLD1 can also bind to RalA in the GDP bound inactive state (Jiang et al., 1995). Mutations disrupting RalA binding to RalBP1 or Sec5, however, reduced anchorage-independent growth by up to ~70%, supporting the idea that binding to these two proteins may be important for full RalA-mediated transformation.

RalB inhibits RalGEF-mediated transformation

Activated RalB alone was not transforming, despite being >85% identical to RalA and capable of binding RalA effectors. To further explore the relationship between RalA and RalB in transformation, we tested whether ectopic expression of wild-type RalB could inhibit RalGEF-induced transformation. Wild-type RalA, RalB, or a vector control was stably expressed in HEK-HT cells harboring either Rlf-CAAX, to activate the Ral proteins, or, as a negative control, the catalytically dead Rlf^{R328E}-CAAX protein (Figure 3A). Wild-type RalA, which was extremely weak in transforming in the presence of Rlf^{R328E}-CAAX, slightly enhanced the transforming ability of Rlf-CAAX (Figure 3B), whereas a constitutively active RalA was even more potent (Figures 3C and 3D). Conversely, wild-type RalB reduced the transforming ability of Rlf-CAAX by 40%–50%.

On the basis of these observations, we reasoned that ectopic RalB may compete with endogenous RalA for Rlf-CAAX, as well as for RalA effectors needed for transformation. In sup-

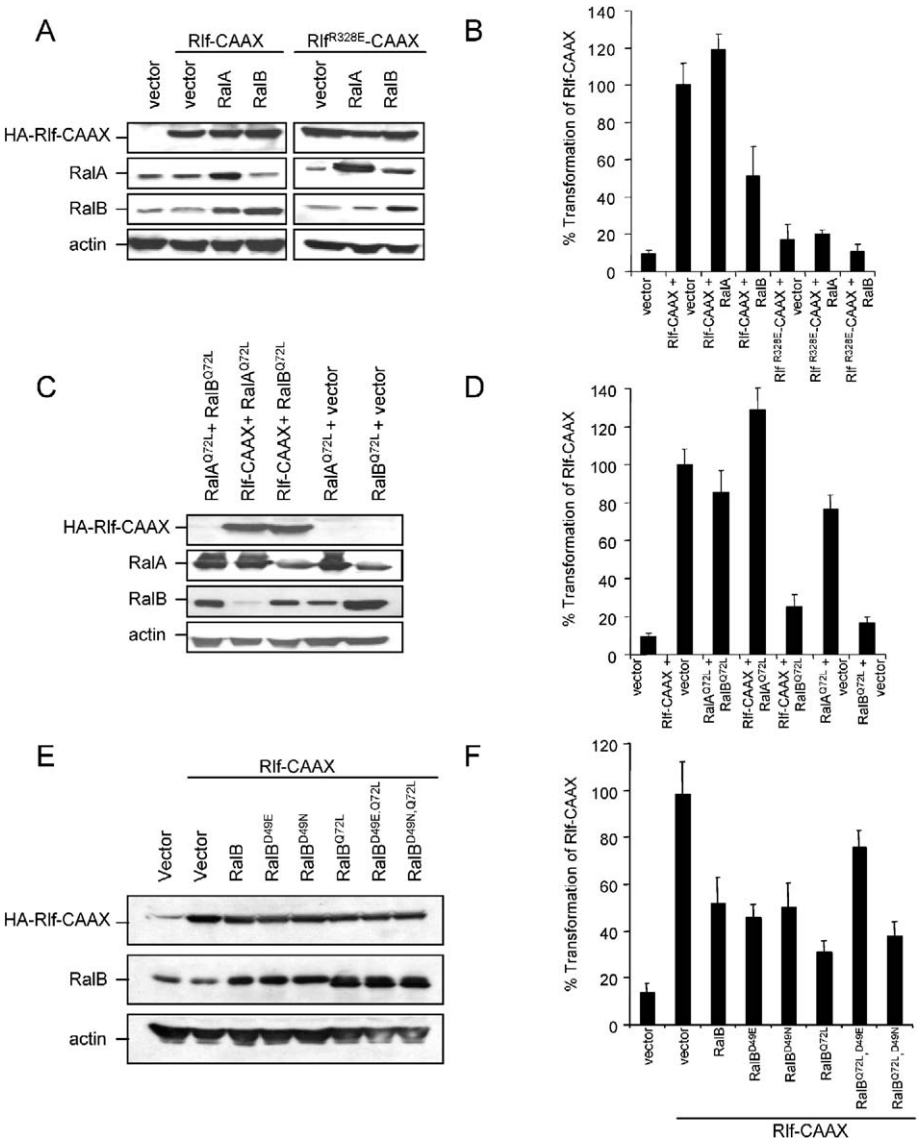

Figure 3. RalB antagonizes RalGEF-mediated transformation

A, C, and E: Detection of HA-epitope-tagged Rlf-CAAX, RalA, and RalB by immunoblot analysis in HEK-HT cells stably infected with retroviruses encoding the described transgenes. Actin serves as a loading control.

B, D, and F: Anchorage-independent growth of polyclonal HEK-HT cells stably expressing the indicated combinations of transgenes calculated from the average number of colonies ± SD from six plates (two independent experiments conducted in triplicate), expressed as the percentage of colonies observed in Rlf-CAAX- transformed cells.

port of this model, expression of RalB mutants defective in binding to either Sec5 and Exo84 (D49E) or RalBP1 (D49N) inhibited Rlf-CAAX-mediated transformation as well as RalB, suggesting that the mode inhibition involves competition for Rlf-CAAX. However, upon making RalB independent of RalGEF activation via the Q72L mutation, RalB^{Q72L} could clearly inhibit Rlf-CAAX-mediated transformation, and this inhibition was reduced 40% to 50% upon disrupting binding to the exocyst complex (RalB^{Q72L,D49E}); in contrast, disruption of RalBP1 binding (RalB^{Q72L,D49N}) did not affect the activity of RalB^{Q72L} in inhibiting Rlf-CAAX-mediated transformation, in agreement with RalB competing for RalA effectors (Figures 3E and 3F). It is worth noting that the inhibition depended upon overexpressing RalB, as ectopic RalB impeded transformation mediated by endogenous RalA, but not in cells overexpressing RalA^{Q72L} (Figures 3C and 3D). Thus, whereas RalA mediates RalGEF-induced transformation, this transformation can be blunted by overexpression of RalB, possibly by competition for RalGEF activation and RalA effectors.

Subcellular localization contributes to the ability of RalA and RalB to affect transformation

Our previous study using the MDCK cell line showed not only that active RalA binds more effectively to the exocyst than does RalB, but also that active RalA, but not RalB, localizes to perinuclear recycling endosomes (Shipitsin and Feig, 2004). In HEK-HT cells, endogenous RalA and RalB were mostly punctuate throughout the cytoplasm and generally did not colocalize, as assessed by immunofluorescence analysis. Upon stimulation of endogenous Ral proteins by ectopic Ras^{G12V}, there was a relatively greater increase in the concentrations of RalA compared to RalB in vesicular-like bodies in the perinuclear region (Figure 4A). We thus postulated that these differences in the subcellular localization of RalA and RalB may contribute to their ability to function in a pro- or antioncogenic fashion.

The C terminus of RalA possesses a hypervariable region terminating in a CAAX motif, both of which are known to be essential for proper localization of active RalA and for its function as a stimulator of secretion (Shipitsin and Feig, 2004). To

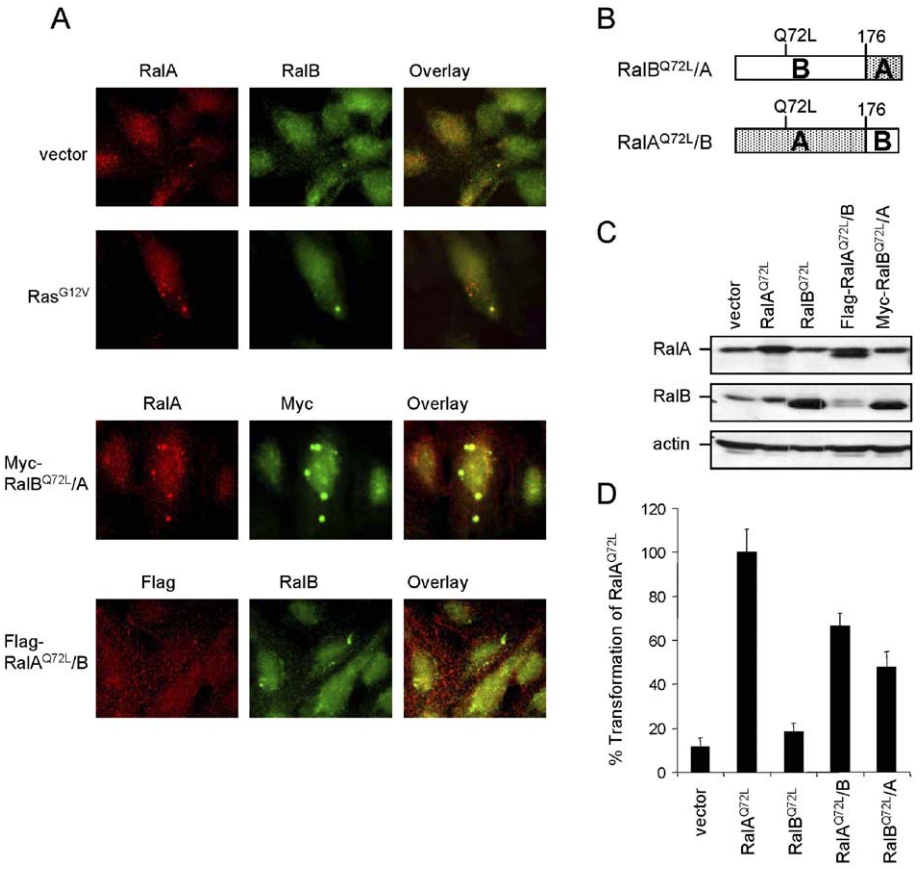

Figure 4. Subcellular localization underlies the ability of RalA and RalB to promote or inhibit transformation, respectively

A: Detection of endogenous RalA and RalB by immunofluorescence analysis in HEK-HT cells stably expressing the empty vector control or Ras^{G12V} (upper 2 rows) or myc epitope-tagged RalB^{Q72L}/A and endogenous RalA or flag epitope-tagged RalA^{Q72L}/B and endogenous RalB (lower 2 rows) in HEK-HT cells stably expressing myc-RalB^{Q72L}/A or flag-RalA^{Q72L}/B, respectively.

B: Schematic representation of Ral^{Q72L} chimeras in which the C terminus of RalA (gray) or RalB (white) is replaced with the C terminus of the opposite Ral protein.

C: Detection of RalA, RalB, or the chimeric versions by immunoblot analysis in the described HEK-HT cells with antibodies that recognize the N-terminal regions of the proteins. Actin serves as a loading control.

D: Anchorage-independent growth of polyclonal HEK-HT cells stably expressing the indicated Ral proteins, as calculated from the average number of colonies ± SD from six plates (two independent experiments conducted in triplicate), expressed as the percentage of colonies observed in RalA^{Q72L}-transformed cells.

determine whether these domains are essential for RalA-mediated transformation, we removed the C-terminal hypervariable region (RalA$^{Q72L,\Delta Var}$) or the CAAX motif (RalA$^{Q72L,\Delta CAAX}$; Figure 2A) from RalA^{Q72L} and expressed these two mutants in HEK-HT cells (Figure 2B). When assayed in soft agar, we found that disrupting either of these C-terminal regions reduced the transforming capability of RalA^{Q72L} almost to the level of negative control vector cells (Figure 2C). Thus, proper subcellular localization is required for Ral-mediated transformation.

Because activated RalA and RalB reside in different sets of membrane compartments and as this localization is mediated by C-terminal regions of the proteins that are required for RalA transformation, we surmised that RalB could be converted, at least partially, to a transforming protein by targeting it to the subcellular domains occupied by activated RalA. We therefore expressed in human HEK-HT cells an activated RalB (RalB^{Q72L}) in which the C-terminal domain was replaced with the corresponding region from RalA (Figures 4B and 4C). As previously described in MDCK cells (Shipitsin and Feig, 2004), the RalB^{Q72L}/A protein associated with perinuclear vesicle-like bodies, which is not normally seen with wild-type RalB, even in Ras^{G12V} overexpressing cells (Figure 4A, second row). Colocalization of RalB^{Q72L}/A with endogenous RalA in these vesicles was also substantially increased (Figure 4A, third row). This altered localization endowed RalB with the ability to transform human cells. HEK-HT cells expressing RalB^{Q72L}/A were able to grow in soft agar, albeit at ~50% of the transformation efficiency of RalA^{Q72L} (Figure 4D). Thus, targeting RalB, a protein that normally inhibits transformation, to sites of activated RalA endowed RalB with transforming functions.

The opposite was also true—namely, targeting RalA to RalB subcellular domains impeded the transforming activity of RalA. When the C-terminal hypervariable region of RalA was switched to that of RalB (Figure 4B) and expressed in HEK-HT cells (Figure 4C), the resultant RalA^{Q72L}/B chimera failed to colocalize with RalA-associated vesicles. Instead, there was an overall increase of colocalization of RalA^{Q72L}/B with endogenous RalB throughout the cytoplasm (Figure 4A, fourth row). Consistent with this mislocalization, the RalA^{Q72L}/B mutant showed a 40% to 50% decrease in its ability to support anchorage-independent growth compared to RalA^{Q72L}.

We surmise that the distinct subcellular localization of activated RalA and RalB may play a role in the effect of these proteins on cell transformation. However, the transforming activity of RalA was not completely abolished by mislocalizing, nor was RalB as efficient at transformation as RalA when targeted to RalA-associated vesicles (Figure 4D). These findings are consistent with our previous observation that RalA and RalB also differ in their ability to bind to at least one effector, the exocyst complex, with RalA binding more strongly to the exocyst than RalB (Shipitsin and Feig, 2004). Thus, in addition to the distinct subcellular localization patterns of RalA and RalB, differential affinities for effectors may also contribute to the disparate transforming potential of these two proteins.

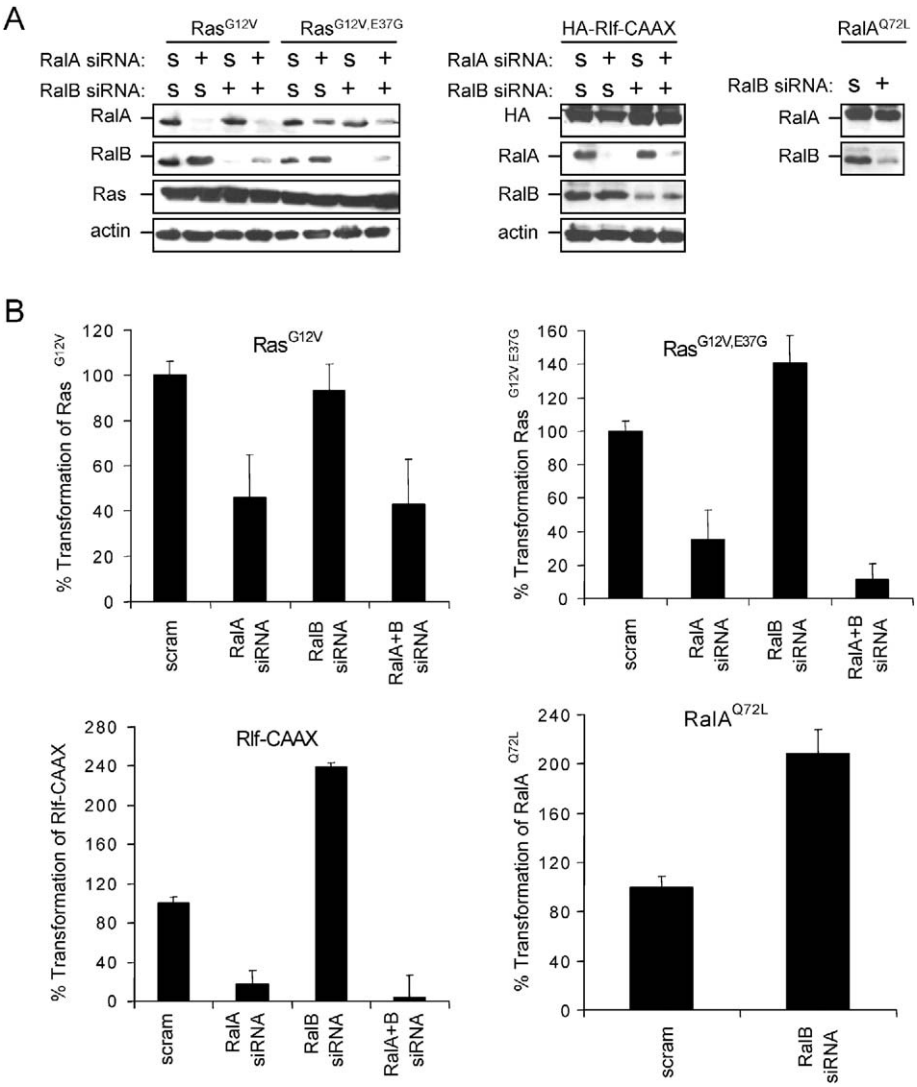

Figure 5. RalA is critical for Ras-induced transformation

A: Detection of RalA, RalB, and Ras by immunoblot analysis in HEK-HT cells transformed by Ras^{G12V}, $Ras^{G12V,E37G}$, Rlf-CAAX, or $RalA^{Q72L}$ and subsequently stably infected with retroviral vectors encoding siRNA (+) targeting RalA, RalB, or both, or as negative control, a scramble sequence (S). Actin level serves as a loading control.

B: Anchorage-independent growth of polyclonal HEK-HT cells transformed by the described transgenes stably expressing the indicated siRNA against RalA and/or RalB, or a scramble sequence (scram) as negative control, as calculated from the average number of colonies ± SD from six plates (two independent experiments conducted in triplicate), expressed as the percentage of colonies observed in scramble control- treated cells.

Loss of RalA inhibits Ras-induced transformation

Ectopically expressed RalA is transforming, whereas RalB impedes transformation. Given the inherent limitations of interpreting phenotypes resulting from overexpressed proteins, we confirmed these results by loss-of-function analysis. Endogenous RalA, RalB, or both, were stably knocked down by siRNAs directed against the mRNAs encoding these proteins in HEK-HT cells expressing Ras^{G12V}, $Ras^{G12V,E37G}$, or Rlf-CAAX (Figure 5A). For negative controls, an empty vector or one encoding scrambled versions of the siRNA sequences were introduced into the transformed HEK-HT cells. All 12 cell lines were then assayed for growth in soft agar. Consistent with RalA activation being critical for Ras-mediated transformation, knockdown of RalA in HEK-HT cells transformed via activation of the RalGEF pathway, by either $Ras^{G12V,E37G}$ or Rlf-CAAX, nearly abolished transformation and reduced oncogenic Ras-transformed cell growth by more than half (Figure 5B). This was not an off-target effect of RNAi, as identical results were obtained with independent siRNA generated against different regions of RalA and RalB (see Figure S1 in the Supplemental Data available with this article online). Perhaps related, transient knockdown of RalA was shown to decrease the proliferation of human cancer cells when suspended in media (Chien and White, 2003).

Conversely, knockdown of RalB in HEK-HT cells transformed via activation of the RalGEF pathway led to an increase in growth in soft agar. This effect was even greater in cells transformed by oncogenic RalA, indicating that the antioncogenic activity of RalB is mediated, at least in part, through antagonizing the transforming activity of RalA (Figure 5B). However, in the more oncogenic situation in Ras^{G12V}- transformed cells, knockdown of RalB had no measurable effect. Although transient knockdown of RalB was previously reported to greatly reduce the proliferative potential of cancer cells by stimulating apoptosis (Chien and White, 2003), this does not appear to be the case with stable expression of the described RalB siRNA in HEK-HT cells or in four human pancreatic cancer cell lines (Figures 5B and 7B). This discrepancy is due to a difference in cell type, the degree of inhibition of RalB expression, or the method by which RNAi is performed. In fact, the increase in transformation upon stable knockdown of RalB that we de-

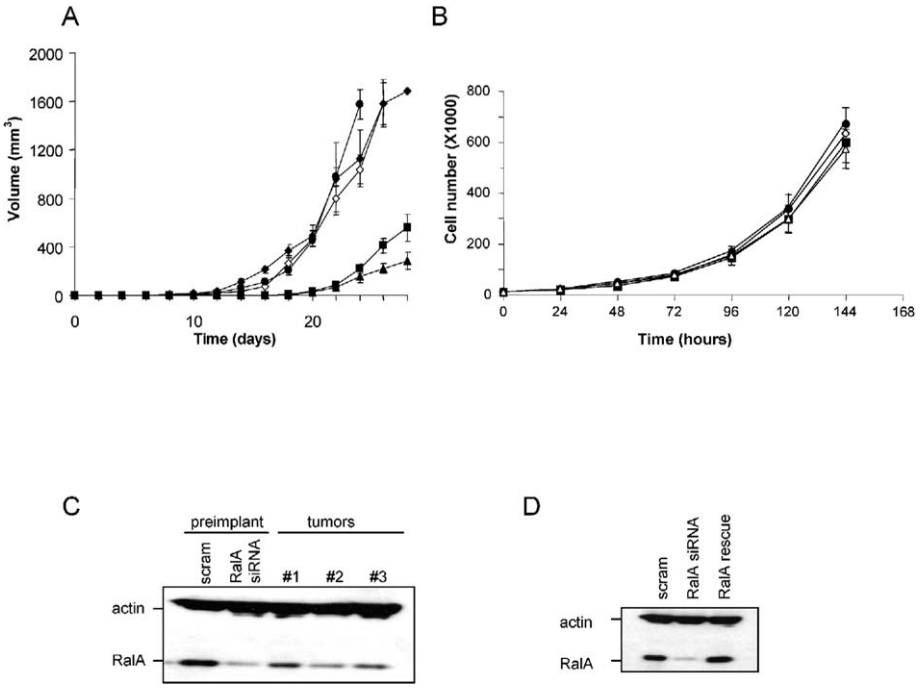

Figure 6. RalA is critical for Ras-induced tumorigenesis

A: Tumor volume (mm^3) ± SD versus time (days) of HEK-HT-Ras^{G12V} cells stably expressing a Ral scramble sequence (◇), RalA-siRNA(■), RalB-siRNA (●), RalA and RalB siRNA (▲) or RalA-siRNA in the presence of siRNA-resistant wild-type RalA (♦) injected into a flank of immunocompromised mice.

B: Cell number ± SD versus time (days) of cultured HEK-HT-Ras^{G12V} cells stably expressing a scramble sequence (□), RalA-siRNA (■), RalB-siRNA (●), or RalA and RalB-siRNA (▲).

C: Detection of RalA by immunoblot analysis in HEK-HT-Ras^{G12V} cells infected with retroviruses encoding a scramble sequence (scram) or RalA-siRNA before implantation (preimplant) into immunocompromised mice, or in cells purified from three resultant tumors (1, 2, 3). Actin serves as a loading control.

D: Detection of RalA by immunoblot analysis in RalA knockdown HEK-HT-Ras^{G12V} cells stably expressing a Ral scramble sequence (scram), RalA siRNA alone (RalA siRNA), or in the presence of an siRNA-resistant wild-type RalA (RalA rescue). Actin serves as a loading control.

tected is in complete agreement with the observation that overexpression of RalB inhibited transformation by Rlf-CAAX (Figures 3B and 3D). Lastly, we noted that simultaneous knockdown of both RalA and RalB was somewhat more potent than was knockdown of RalA alone in inhibiting the transforming ability of $Ras^{G12V,E37G}$ or Rlf-CAAX, perhaps reflecting a general requirement for some level of Ral signaling. In summary, the described loss-of-function studies support the conclusions drawn from analysis of cells overexpressing RalA or RalB: i.e., activated RalA and RalB primarily oppose one another in transformation, with RalA mediating, and RalB inhibiting RalGEF oncogenic signaling.

RalA is critical for Ras-induced tumorigenesis

Given the importance of RalA in Ras-mediated transformation, we next addressed the role of each Ral protein in the most stringent assay: tumorigenesis. RalA and/or RalB were stably knocked down by siRNA in the highly tumorigenic Ras^{G12V}-transformed HEK-HT cells (Figures 5A and 6C), and the resultant cells were injected into immunocompromised mice. As a control, we utilized the same cells engineered to express the corresponding scrambled counterparts. Consistent with the soft agar results, reducing RalB expression had only marginal effects on Ras^{G12V}-mediated tumor growth, perhaps because loss of RalB can do little to enhance the already extremely tumorigenic functions of Ras (Figure 6A). Also consistent with the in vitro experiments, loss of RalA in Ras^{G12V}-transformed HEK-HT caused a 2-fold increase in the latency period and a reduction in the growth kinetics of the outgrowing tumors (Figure 6A). In cases in which tumors eventually did arise, RalA expression was restored, possibly through in vivo selection of cells from the initial polyclonal population that had low levels of siRNA (Figure 6C). Knockdown of RalA had no measurable effect on the ability of the cells to survive and proliferate in vitro; thus, any effect observed must be related to tumorigenesis (Figure 6B). Lastly, the loss of tumorigenic potential by RalA knockdown was completely reversed by expression of a RalA protein engineered to be resistant to RalA siRNA (Figures 6A and 6D). Thus, activation of RalA is critical, if not essential, for Ras-mediated tumorigenic growth of human HEK-HT cells.

Suppression of RalA inhibits the tumorigenic growth of human cancer cell lines

We next addressed whether RalA expression was required for tumorigenic growth of human cancer cell lines harboring an activated *Ras* mutation. To this end, a retrovirus expressing either the RalA siRNA or the scrambled sequence was stably introduced into the human cancer cell line HT1080, a fibrosarcoma cell line, J82, a bladder cell carcinoma cell line, and SW620, a colon adenocarcinoma cell line (Brown et al., 1984; Di Paolo et al., 2001; Fujita et al., 1988), and the resultant cells were shown to have either reduced or unaltered levels of RalA, respectively (Figure 7A). Scramble control cells in all three cases readily formed tumors when injected into immunocompromised mice, whereas suppression of RalA increased the latency period of tumor initiation and decreased the tumor growth kinetics. These decreases were slight in the case of SW620 cells, whereas knockdown of RalA in the J82 cell line abolished the tumorigenic potential of these cells as no tumors were detected even after 100 days, fully 5-fold longer than the average time taken to initiate tumor growth in the presence of RalA (Figure 7B). Thus, RalA is important, and possibly essential in some cases, for the tumorigenic growth of human cancer cell lines with an activated Ras pathway.

RalA is in the GTP bound active state in pancreatic cancer cell lines

To further explore the role of RalA in human cancer, we asked to what extent RalA exists in the GTP bound, active state in

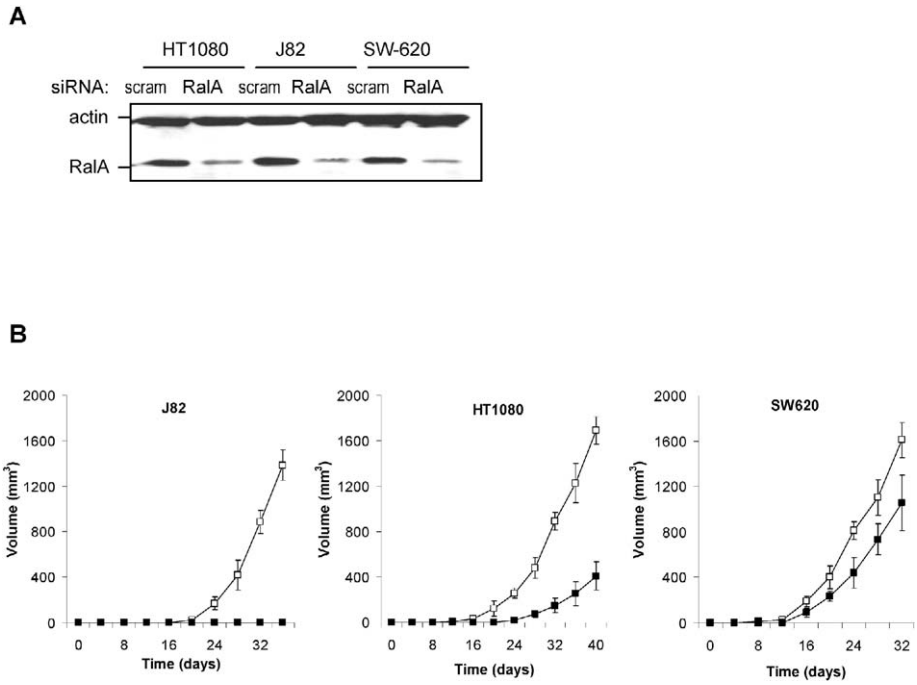

Figure 7. A critical role of RalA in tumorigenic growth of human cancer cell lines

A: Detection of RalA by immunoblot analysis in the described cancer cell lines stably infected with retroviral vectors encoding siRNA against RalA or the scramble control (scram). Actin serves as a loading control.

B: Average tumor volume (mm^3) ± SD versus time (days) of the indicated cancer cell lines expressing siRNA against RalA (■) or scramble control (□) injected into immunocompromised mice.

cells of cancers characterized by oncogenic *Ras* mutations. *K-Ras* is mutated in almost 90% of tumors from patients diagnosed with pancreatic cancer (Bos, 1989). Hence, we measured the levels of GTP bound and total RalA in a panel of 14 pancreatic human cancer cell lines, most of which were known to have an activating mutation in one allele of the *K-Ras* gene (Berrozpe et al., 1994; Calhoun et al., 2003; Iwamura and Hollingsworth, 1998; Kalthoff et al., 1993; Moore et al., 2001; Ryu et al., 2002). Immunoblot analysis revealed that total RalA protein was present, at variable levels, in all of these tumor cell lines. RalA-GTP protein was isolated via an interaction with a recombinant polypeptide encoding the Ral binding domain of the Ral effector protein RalBP1, followed by immunoblot analysis to detect RalA (Wolthuis et al., 1998a; Wolthuis et al., 1998b). While undetectable or low levels of RalA-GTP were seen in a subset of the cell lines (Figure 8A, CFPac-1, Colo 357, and Colo 587), similar to the levels seen in a variety of untransformed epithelial cells (McFall et al., 2001; Ulku and Der, 2003) and in normal human pancreatic tissue (Figure 8B), total RalA-GTP levels were elevated in the remaining majority of cell lines (Figure 8A). This level of RalA-GTP elevation is comparable to that in a variety of Ras-transformed cells (McFall et al., 2001; Ulku and Der, 2003) and was reduced upon the knockdown of oncogenic *K-Ras* in Capan-1 cells (Figure 8B), arguing that this level of RalA-GTP represents an abnormal activation of RalA. In contrast, while RalB protein was detected in these cancer cell lines, RalB-GTP levels were only highly elevated in three of the lines. In summary, the transforming RalA protein, but not the nontransforming RalB protein, is preferentially in its activated state in human pancreatic cancer cells.

Most surprisingly, RalA was more commonly activated compared to the other major Ras effector pathways in these cancer cell lines. While ERK1/2 were phosphorylated to some degree in at least 11 of the lines, high levels of ERK phosphorylation were only seen in one-third of these lines. Similarly, AKT was only weakly activated in the panel of cancer cell lines, with high levels of phosphorylated AKT detected in just three lines (Figure 8A). Intriguingly, we also found that the total *K*-Ras protein and *K*-Ras-GTP levels varied enormously among the cancer cell lines, again despite the fact that RalA-GTP was usually readily detected, suggesting a need to conserve RalA activation, even in the event of diminished Ras protein levels (Figure 8A). In support of this model, independent of the amount of RalA, RalB, PI3K, or MAPK activation, we showed that knockdown of RalA in four of these human pancreatic cancer cell lines almost uniformly reduced their ability to grow in soft agar. Conversely, knockdown of RalB in the same cell lines had little or no effect on the ability of the cells to grow on plastic or in suspension (Figure 8C and data not shown). The finding of RalA being commonly activated in pancreatic cancer cell lines is consistent with a critical role for this effector in tumorigenesis.

RalA and RalB function in Ras oncogenesis

We show that RalA is a principal effector of RalGEFs involved in Ras-mediated transformation and tumorigenesis, as a constitutively active version of RalA promoted anchorage-independent growth whereas inhibition of RalA expression lowered, and, in some cases actually abolished, Ras-mediated transformation and tumorigenesis. While this pathway was not strongly transforming on its own—indicating a collaborative role with other Ras effector pathways—decreasing RalA expression clearly reduced tumorigenic growth. As a decrease of RalA expression had no obvious effect on the cells until they were placed in an oncogenic environment, aberrant RalA activation presumably performs a task required uniquely in the process of tumorigenesis.

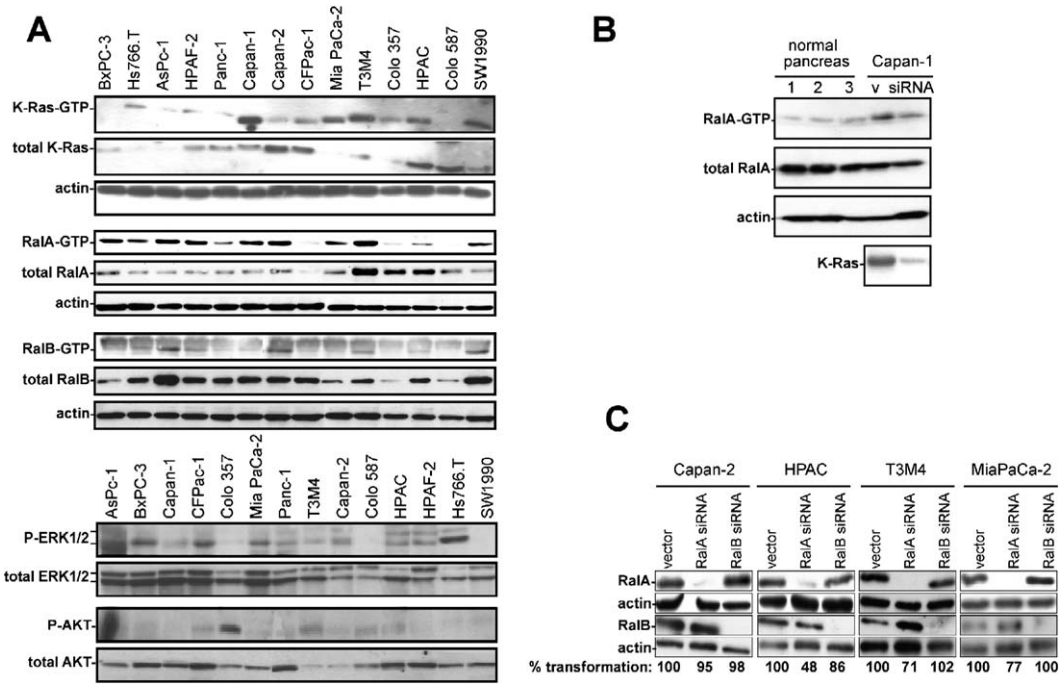

Figure 8. RalA is commonly activated in pancreatic cancer cells to promote transformed cell growth

A: Detection of activated GTP bound K-Ras, RalA, and RalB via association with effector protein domains specific for activated versions of these proteins, followed by immunoblot analysis with antibodies specific for K-Ras, RalA, or RalB in a panel of 14 pancreatic cancer cell lines. Detection of MAPK and PI3K activation in the same cell lines, by immunoblot analysis with phosphospecific antibodies for phosphorylated forms of ERK1/2 (P-ERK1/2) and Akt (P-Akt), respectively. Total K-Ras, RalA, RalB, ERK1/2 and Akt, or actin, serve as loading controls.

B: Detection of activated GTP bound RalA via association with an effector protein domain specific for the activated version of this protein followed by immunoblot analysis with an antibody specific for RalA in normal human pancreatic tissues from three separate donors (1, 2, and 3) or Capan-1 cells stably infected with a control retrovirus (v) or one encoding siRNA specific to the 12V mutant allele of K-Ras that knocks down K-Ras expression, as determined by immunoblot analysis. Total RalA or actin serve as loading controls.

C: Detection of RalA or RalB by immunoblot analysis in the described pancreatic cancer cell lines stably infected with retroviral vector alone (vector) or encoding siRNA targeting RalA or RalB. Actin serves as a loading control. Bottom: anchorage-independent growth of the indicated cell lines, as calculated from the average number of colonies from three plates and expressed as the percentage of colonies observed in vector control cells, except for Capan-2 cultures, which were only counted in one plate because replica plates showed no visible difference in colony formation among the three cell lines.

Ral proteins have been implicated in the regulation of a variety of cellular functions that could conceivably account for their oncogenic activity, including enhancement of gene transcription and regulation of vesicle trafficking. We favor the latter possibility because enhancement of transcription is mediated by both RalA and RalB (Goi et al., 2000; Henry et al., 2000), while in our assays only RalA promoted transformation. Moreover, RalA, but not RalB, promotes basolateral membrane delivery in MDCK cells (Shipitsin and Feig, 2004), as well as transformed and tumorigenic growth of human cells. Both of these activities require proper localization to perinuclear vesicles and depend upon the C-terminal variable domain of RalA. Replacing this domain with that of RalB decreased the transforming and membrane delivery activities of RalA, while replacing the C terminus of RalB with that of RalA endowed RalB with partial transforming and membrane delivery activities. How the effects of RalA on vesicle sorting may be linked to its ability to transform human cells is not yet clear. Perhaps Ral-exocyst interactions are involved in secretion, which could be a factor in autocrine stimulation of cells, similar to the proposed role of Cdc42 in the transformation of cells (Wu et al., 2003). Alternatively, Ral-exocyst interactions are also thought to be critical for filopodia formation (Ohta et al., 1999; Takaya et al., 2004), which may be needed for transformation-associated remodeling of cell structure.

While RalA behaved in an oncogenic fashion, RalB functioned in exactly the opposite manner. RalB overexpression inhibited, while RalB siRNA treatment enhanced, Rlf-CAAX transformation. We suggest that the modus operandi of RalB antioncogenic behavior may be to both compete for binding to RalGEFs, resulting in a decrease in RalA activation, and to sequester Ral effectors to subcellular sites nonproductive for oncogenic signaling, akin to the roles proposed for RalB in inhibiting Ral-mediated basolateral membrane delivery (Shipitsin and Feig, 2004). However, RalB was rather ineffectual at blocking Ras-mediated transformation. This could be because Ras activates pathways that circumvent RalB inhibitory signals, because RalA competes more successfully for effector binding, or because RalA is preferentially activated in cancer cells, as supported by the observation that RalA, and not RalB, was commonly activated in our panel of 14 human pancreatic cancer cell lines. Similarly opposing functions in growth regulation have been described for the highly related RhoA and RhoB small GTPases (Adnane et al., 2002; Jiang et al., 2004). Despite

strong sequence identity and similar effector interaction, RhoA activation has been associated with growth promotion, whereas RhoB exhibits the characteristics of a tumor suppressor (Jiang et al., 2004). The functional differences between RhoA and RhoB have been ascribed to differences in subcellular localization (Wang et al., 2003).

In summary, we show that RalA plays a central role in Ras-mediated tumorigenesis, as loss of RalA function impedes, if not abolishes, the growth of human tumor cells and is widely activated in pancreatic cancer cell lines. Inhibiting RalA function may therefore be an effective anti-Ras strategy for cancer treatment.

Experimental procedures

Cell lines

Human embryonic kidney cells (HEK-HT) and BJ fibroblasts (BJ-HT) stably expressing the early region of SV40 encoding T-Ag and t-Ag and hTERT were previously described (Hahn et al., 1999; Hamad et al., 2002). HT1080 and J82 cell lines were obtained from ATCC; the SW620 cell line or normal human pancreas tissues from three different donors were kind gifts of X.-F. Wang or A.D. Proia and D. Tyler, respectively (Duke University Medical Center, Durham, NC). HT1080 and SW620 cell lines are known to contain a mutant *Ras* allele (Brown et al., 1984; Di Paolo et al., 2001; Fujita et al., 1988). The J82 cell line was confirmed by RT-PCR and direct sequencing to contain a G12V mutation in *H-Ras*. The pancreatic cell lines were obtained from ATCC or are kind gifts of Dr. Bernard E. Weismann (University of North Carolina, Chapel Hill, NC) and Dr. Murray Korc (University of California, Irvine, CA). To measure proliferation rate, each cell line was seeded at 10,000 cells per 6 cm dishes and viable (Trypan blue- negative) cells were counted daily for 6 days. Indicated cell lines were infected with retroviruses generated from the described retroviral vectors encoding no protein, the indicated proteins, or siRNA, followed by appropriate exposure to puromycin, zeocin, or neomycin to select for stably infected polyclonal populations (O'Hayer and Counter, 2005).

Plasmids and cDNAs

Retroviral constructs used were pBabeBleo or pBabepuro with no insert or cDNAs encoding H-Ras^{G12V}, H-Ras^{G12V,E37G} (Hamad et al., 2002), HA-Rlf-CAAX (Wolthuis et al., 1997), RalA, RalB (Guthrie Research Institute), HA-Rlf^{R328E}-CAAX, RalA^{G23V}, RalA^{F39L}, RalA^{Q72L}, RalB^{G23V}, RalB^{Q72L}, RalB^{F39L}, and RalA and RalB effectors mutants (generated by site-directed mutagenesis or by PCR), or N-terminally flag-tagged RalA^{Q72L}/B and Myc-tagged RalB^{Q72L}/A (tagged versions of cDNAs described by (Shipitsin and Feig, 2004)). RalA siRNA sequence AAGACAGGTTTCTGTAGAAGA (and as an alternate: AACAGAGCTGAGCAGTGGAAT), RalB siRNA sequence GACTATGAACCTACCAAAG (and as an alternate: GGTGATCATGGTTGGCAGC), scramble controls in which several mutations were introduced into these sequences, or siRNA specific for K-Ras^{G12V} (Brummelkamp et al., 2002) were cloned into pSUPER-RETRO-PURO or pSUPER-RETRO-GFP/NEO (Oligoengine). siRNA-resistant RalA was generated by introducing silent mutations (bold italics) ***A***GT***AAGC***GT***T***.

Immunoblot

Lysates from the described cell lines were immunoblotted with the antibodies α-Pan-Ras (Oncogene), α-HA (Roche), α-actin (Santa Cruz), α-Raf-1 (Santa Cruz), α-RalA and α-RalB (Transduction Laboratory). Akt and ERK1/2 total protein or phosphorylated forms were detected from cells cultured in medium containing 0.5% fetal bovine serum for 48 hr by immunoblot analysis with the antibodies α-Akt (Cell Signaling Technology), S472 α-phospho(Ser 473)-Akt (New England Biolabs), K-23 α-ERK1/2 (Santa Cruz), and E10 α-phospho(Thr 202/Tyr 204)-p42/44 MAPK (Cell Signaling Technology). Ral-GTP and Ras-GTP levels were detected as previously described (de Rooij and Bos, 1997; Wolthuis et al., 1998a), by capture with bacterially expressed GST-RalBD of RalBP1 or GST-RasBD of Raf1, followed by immunoblot analysis with the aforementioned RalA, RalB, or Ras antibodies.

Immunofluorescence

The described cells were plated on glass microslides the previous day, fixed, permeabilized, and incubated with anti-Flag, anti-Myc (9E10), anti-RalA, and anti-RalB primary antibodies, followed by the appropriate HRP-conjugated secondary antibodies (Jackson Immunoresearch Laboratory) as previously described (Etheridge et al., 2002).

Soft agar

50,000 cells per 3cm plate were suspended in soft agar as described (Cifone and Fidler, 1980; Hamad et al., 2002) and colonies >30 cells scored after 4 weeks. Assays were performed in triplicate and at least twice independently.

Tumorigenesis

According to a protocol approved by the Duke University Institutional Animal Care and Use Committee, 1×10^7 cells mixed with Matrigel were injected subcutaneously into the flanks of 4 SCID/beige mice per cell line, after which tumor volumes were determined at regular intervals as described previously (Hamad et al., 2002). Tumors were harvested, minced, and trypsinized for 2 hr at 37°C and then passed through 18G needles, washed, and plated in DMEM/10% FBS plus puromycin at least 4 days before immunoblot analysis was performed.

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.cancercell.org/cgi/content/full/7/6/533/DC1/.

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