Ral GTPases: crucial mediators of exocytosis and tumourigenesis

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The Ral guanosine triphosphatases (GTPases), RalA and RalB, are members of the Ras superfamily of small GTPases. Research on Ral GTPases and their functions over the past 25 years has revealed the essential involvement of these GTPases in unique and diverse cellular processes including exocyst-mediated exocytosis and related cellular activities. Moreover, it is increasingly appreciated that the aberrant activation of Ral GTPases is one of the major causes of human tumourigenesis induced by oncogenic Ras. Recent evidence suggests that Ral signalling pathways may be potential therapeutic targets for the treatment of human cancers. This review summarizes recent advances in the investigation of Ral GTPases.

Keywords: exocyst/exocytosis/Ral/Ras/tumourigenesis.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CTLs, cytotoxic T lymphocytes; EGF, epidermal growth factor; DRP1, dynamin-related protein 1; EH, epsin homology; GAP, GTPase-activating proteins; GEF, guanine nucleotide exchange factor; GGT1s, GGTase-I inhibitors; GLUT4, glucose transporter 4; GSVs, GLUT4-storage vesicles; GTP, guanosine 5’-triphosphate; GTPases guanosine triphosphatases; MDCK, Madin-Darby canine kidney; MMPs, matrix metalloproteases; mTORC1, mammalian target of rapamycin complex1; NK, natural killer; NMDA, N-methyl-D-aspartate; PLD, phospholipase D; RBD, Ras-binding domain; REM, Ras exchange motif; VEGF, vascular endothelial growth factor; WPBs, Weibel-Palade bodies.

In 1986, a gene closely related to Ras oncogenes was isolated from a cDNA library of simian B-lymphocytes and named Ral (for Ras-like) (1). This gene, now known as RalA, encodes a 206 amino acid protein that shares ~55% sequence identity with Ras proteins. Subsequent screening identified a second Ral gene, RalB, which shares 82% homology with RalA (2). RalA and RalB comprise a family of proteins in the Ras superfamily of small guanosine triphosphatases (GTPases). We now know that Ral GTPases play unique and essential roles in diverse biological processes through their interactions with specific effector proteins. In particular, identification of the exocyst, an octameric protein complex, as a major Ral effector has provided important insights into how Ral GTPases participate in the regulation of exocytosis and other related cellular activities, such as cell motility and actin cytoskeletal rearrangement. Currently, Ral GTPases are attracting considerable research attention because they are activated downstream of oncogenic Ras. Accumulating evidence indicates that Ras-induced Ral activation is essential for tumourigenesis of human cells. Furthermore, genetic studies in mice demonstrate the in vivo involvement of Ral GTPases in tumourigenesis and also in tumour invasion. Ral GTPases are aberrantly activated in human cancer tissues due to deregulation of their regulators. Although the mechanistic details of Ral-mediated tumourigenesis and malignant transformation are not fully understood, recent studies suggest that Ral mediates key signalling pathways important for cancer cell proliferation and survival. Here, we summarize recent findings on the regulation and functions of Ral GTPases in diverse biological processes with particular emphasis on their roles in exocytosis and cancer.

Structure of Ral GTPases

The mammalian Ras superfamily consists of more than 150 members divided into five subfamilies: Ras, Rho, Rab, Arf and Ran subfamilies (3). Ral GTPases belong to the Ras subfamily. Other members in the Ras subfamily are H-Ras, K-Ras, N-Ras, Rap1, Rap2, M/R-Ras, Rheb, Rit and more distantly related GTPases such as Rad, Di-Ras, RasD, Rerg and xB-Ras. The phylogenetic tree of the Ras subfamily members indicates that Ral proteins are one of the closest relatives of the classical Ras proteins (Fig. 1A). Mammals have two highly similar Ral isoforms, RalA and RalB. Invertebrates, such as the fruit fly Drosophila melanogaster and the nematode Caenorhabditis elegans, possess a single Ral gene; there are no Ral orthologues in yeasts, indicating the Ral gene emerged in multicellular organisms during evolution.

Ral GTPases share general structural and biochemical features with Ras and other small GTPases. Ral GTPases consist of a unique N-terminal 11 amino acid extension, followed by a G domain, a universally...
The activity of small GTPases is finely regulated by the action of specific GEFs and GAPs. These regulators often have multiple functional domains and receive diverse upstream signals to control the activity of target small GTPases. Seven GEFs and two GAPs have been identified as regulating Ral GTPases.

**Ral Regulators**

**RalGEFs**

The first Ral-specific GEF was cloned from a mouse fibroblast cDNA using degenerate primers corresponding to a conserved region of the yeast RasGEFs Cdc25 and Ste6 (9). The cloned gene, designated as RalGDS (Ral guanine nucleotide dissociation stimulator), has a Cdc25-like GEF domain, which shows ~25% amino acid identity with that of mammalian RasGEFs such as Sos and RasGRP1. Despite this homology, RalGDS acts specifically on RalA and RalB, but not on Ras, Rap or other Ras family GTPases. RalGDS is a multi-domain protein (Fig. 2A). Notably, RalGDS has a
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RalGDS activity is also regulated by autoinhibition. The N-terminal REM (Ras exchange motif) domain blocks the catalytic domain of RalGDS through intramolecular interaction. It has been shown that binding of PDK1 to the REM domain relieves its autoinhibition and supports Ras-mediated RalGDS activation (19). RalGDS activity is also modulated by Ca$^{2+}$/calmodulin-binding (20) and phosphorylation by protein kinase C (PKC) (21).

Rgr (RalGDS related, also known as RGL4) is a homologue of RalGDS but lacks the RA domain and shows promiscuous GEF activity towards Ras and Ral (22). An N-terminally truncated and constitutively active form of Rgr has been found in lymphoma (23, 24). The function of normal Rgr protein in Ral regulation is unclear.

RalGAP1 (25, 26) and RalGPS2 (27) constitute the second family of Ral-specific GEFs (Fig. 2A). These proteins have a Cdc25 homology domain in their N-terminal region and a pleckstrin homology (PH) domain at their C-terminus. The PH domain of RalGPS preferentially binds phosphatidylinositol (4,5)-bisphosphate (PIP2) and is required for membrane localization of RalGPS. Deletion of the PH domain resulted in impaired RalGEF activity in cells. In addition, RalGPS contains a proline-rich PxxP motif in the central part of the protein, which binds SH3 domain-containing proteins such as Grb2 and Nck. RalGPS proteins are activated by binding to PIP2 and SH3 domain-containing proteins in a Ras-independent manner.

**RalGAPs**

In 1991, Larry Feig’s group demonstrated the existence of Ral-specific GAP activity in the bovine brain cytosol (28). This activity eluted at an apparent molecular weight of over 1,000 kDa in gel filtration chromatography, suggesting that RalGAP is an extremely large protein or exists in a huge protein complex. Following its initial discovery, the molecular identity of RalGAP eluded identification for many years, probably because of protein instability during purification. However, in 2009, we successfully identified RalGAP molecules (29). The RalGAPs were purified from porcine brain and rat lung tissues by using an affinity column containing immobilized RalGDS, a constitutively active form of Rgr has been found in lymphoma (23, 24). The function of normal Rgr protein in Ral regulation is unclear.

RalGAPs are classified into two types according to their domain organization. The RalGDS-type GEFs, RalGDS and RGL1-3, consist of REM, Cdc25 and RA domains. The Cdc25 homology domain exhibits GEF activity. RalGPS-type GEFs, RalGPS1 and RalGPS2 are composed of Cdc25, proline rich, and PH domains. Rgr is a non-selective RalGEF that exhibits promiscuous GEF activity for Ras family GTPases. Sos is also shown for structural comparison. (B) Two RalGAP complexes, RalGAP1 and RalGAP2 exist. These complexes share the same β subunit but contain different α subunits, α1 and α2. RalGAPs are structurally similar to the tuberous sclerosis complex (TSC) that acts as a RhebGAP. REM, Ras-exchange motif; Cdc25 GEF, Cdc25 homology domain; RA, Ras-association domain; PH, pleckstrin homology domain; PxxP, proline rich motif; DH, Dbl homology domain; GAP, GTPase-activating protein domain; aa, amino acids.

### Ras association (RA) domain

The RA domain is conserved in oncogenic Ras proteins and is critical for Ras function. It binds to several effector proteins, including Raf, Rap1, and R-Ras, and regulates their activity. The RA domain is a proline-rich region that interacts with SH3 domains of proteins involved in Ras signaling, such as Grb2 and Nck. The interaction with SH3 domains is mediated by proline-rich motifs, which are common in SH3-binding proteins.

### RalGDS and RalGEFs

RalGDS is a prototype RalGEF and is involved in the regulation of RalGDP and RalGTP levels. RalGDS contains several domains, including the N-terminal REM (Ras exchange motif) domain, which interacts with Ras family GTPases, and the C-terminal Cdc25 homology domain, which functions as a GEF. RalGDS is regulated by autoinhibition, which is mediated by the RA domain at its C-terminus and interferes with Ras-mediated Ral activation. This study revealed a mechanism for RalGEF regulation and provided a possible mechanistic link between Ral activation and inflammation.

### RalGAPs and RalGAPs

RalGAPs are GTPase activating proteins (GAPs) that regulate the GTP hydrolysis of RalGDP by binding to RalGTP. They are essential for the proper localization and function of Ral proteins. RalGAPs are classified into two types: Ral-specific GAPs and RalGAPs. Ral-specific GAPs, such as RalGAP1 and RalGAP2, are highly specific for RalGTP and catalyze the conversion of RalGTP to RalGDP. RalGAPs are structurally similar to the tuberous sclerosis complex (TSC) that acts as a RhebGAP. They contain the N-terminal REM (Ras exchange motif) domain, the Cdc25 homology domain, and the RA domain.

### RalGPS and RalGPSs

RalGPSs are GEFs for Ral family GTPases. They contain a Cdc25 homology domain, a proline-rich PxxP motif, and a PH domain. RalGPSs are activated by binding to PIP2 and SH3 domain-containing proteins, and they act as Ras-independent GEFs for Ral proteins. RalGPSs are involved in various cellular processes, including exocytosis, tumourigenesis, and membrane trafficking.

### Rgl and Rgl homologues

Three paralogues of RalGDS have been reported in mammals and named RGL (RalGDS-like, also known as RGL1) (11), RGL2/Rlf (12, 13) and RGL3 (14). These RGL proteins have the same domain organization as RalGDS and are activated by direct binding of Ras in a similar manner to RalGDS (15), although some studies suggest that Rap1 and R-Ras can activate these RalGEFs in the intracellular compartment (16, 17). It was recently reported that the anti-inflammatory protein TIPE2 interacts with the RA domain of RGL and interferes with Ras-mediated Ral activation (18). This study revealed a mechanism for RalGEF regulation and provided a possible mechanistic link between Ral activation and inflammation.
TSC2 (tuberous sclerosis 2) and, to a lesser extent, that of RapGAPs. TSC2, a tumour suppressor protein, is mutated in tuberous sclerosis, an autosomal dominant genetic disorder characterized by the formation of tumours in multiple organs (32). TSC2 functions in a heterodimeric complex with TSC1 and acts as the GAP for Rheb, a Ras subfamily GTPase implicated in cell growth. RapGAP complexes are structurally similar to the TSC complex, although RapGAPβ and TSC1 have no homology. Despite this structural similarity, RapGAPs exhibit highly selective GAP activity for RalA and RalB and show no GAP activity for Rheb. Interestingly, RapGAPα subunit alone has no GAP activity and requires β subunit binding for GAP activity. The β subunit might allosterically activate the α subunit.

RasGAPs use a conserved arginine residue called the ‘arginine finger’ to catalyse GTP hydrolysis on Ras GTPases (33). In contrast, it has been shown that TSC2 and RapGAPs use an asparagine as an alternative catalytic residue (34, 35). This asparagine, termed the ‘asparagine thumb’, is completely conserved in RapGAPα homologues across species, and mutation of the corresponding asparagine in RapGAPα abolish its GAP activity. Thus RapGAPs, RapGAPs and RhebGAP comprise a family of GAPs that use the asparagine thumb for catalysis of GTP-hydrolysis.

Ral activity is regulated in part by modulation of RapGAP function. In insulin-stimulated skeletal muscle and adipocytes, phosphorylation of RapGAPα (36) and α2 (37) by Akt/PKB induces 14-3-3 binding to RapGAPs and inhibits their activity, which in turn induces Ral activation. A recent study has shown that kB-Ras, a Ras subfamily GTPase implicated in inflammation, interacts with RapGAPs and inhibits Ral activity (38). However, whether phosphorylation or kB-Ras-binding directly affects GAP activity is unknown. These modifications might affect Ral activity through changes in the cellular localization or protein stability of RapGAPs.

RapGAPs are the largest of the 160-plus GAP members of Ras superfamily GTPases (39). However, RapGAPs have no known functional domains except that of the C-terminal GAP domain in the α subunits. The GAP domain region covers only ~7% of the entire amino acid sequence. This suggests that other, as yet unidentified, signalling pathways may converge on RapGAPs. It is not known how RapGAPs sense upstream signals to control the activity of Rap GTPases.

**Ral Effectors and Downstream Pathways**

It is increasingly recognized that a single small GTPase can interact with multiple effectors and can activate multiple downstream pathways. Ral GTPases interact with a wide range of effectors and regulate a variety of downstream pathways including those for membrane trafficking, actin cytoskeletal reorganization, transcription and kinase cascade activation.

**The exocyst complex**

The best-characterized effector of Ral GTPases is an octameric protein complex termed the exocyst (40–42), which is composed of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84. Of these, Ral GTPases interact with the Sec5 and Exo84 subunits of the exocyst. Structural studies of RaLA-Sec5 (43) and RaLA-Exo84 complexes (44) revealed that these effectors have overlapping binding sites on Ralα and thus cannot bind to Ralα simultaneously. The exocyst complex is a molecular tether that catches exocytic vesicles and physically tethers them to the plasma membrane during exocytosis. Ral and the exocyst have been shown to regulate a variety of exocytic pathways in many cell types (Fig. 3).

In platelets, Ral GTPases localize to dense granules that store platelet self-agonists such as ADP and serotonin. Dense granule secretion is essential for platelet activation at the site of thrombus formation. We used in vitro dense granule secretion assay with permeabilized platelets to show that the Ral-binding domain (RBD) of Sec5 or anti-Sec5 RBD antibodies inhibits the induced secretion of dense granules by GppNHp, a non-hydrolysable GTP analogue (45). This result clearly indicates that the Ral-Sec5 interaction mediates the GTP-induced exocytosis of dense granules. Similar roles for Ral and Sec5 have been found in GTP-induced dense granule exocytosis in PC12 rat pheochromocytoma cells (46, 47). Physiologically, platelet dense granule secretion is triggered by an increase in intracellular Ca2+ concentration. The Rab family GTPase Rab27 and its Ca2+-binding effector Munc13-4 mediate this Ca2+-regulated process (48). Interestingly, activation of Ral GTPases greatly reduced the threshold Ca2+ concentration for triggering dense granule secretion. Thus, the physical tethering of dense granules to the plasma membrane by the Ral-exocyst pathway increases the Ca2+-sensitivity of the Ca2+-regulated fusion machinery. It is possible that a mechanistic link exists between the GTP- and Ca2+-dependent pathways. The role of the Exo84 subunit in secretion of platelet dense granules remains unclear.

In endothelial cells, Ralα is associated with Weibel-Palade bodies (WPBs) (49), which contain a multimeric adhesive protein termed von Willebrand factor. Since this protein functions in platelet aggregation and coagulation, exocytosis of WPBs from activated endothelial cells plays an important role in normal haemostasis. Knockdown of RapGDS in endothelial cells results in the inhibition of thrombin- and epinephrine-induced WPB exocytosis, while overexpression of constitutively active RapA promotes the exocytosis of WPBs (50, 51). Thus Ralα and the exocyst mediate WPB exocytosis in endothelial cells.

Ral and the exocyst also regulate insulin secretion in pancreatic β cells. Total internal reflection fluorescence microscopy showed that the exocyst regulates the docking of insulin-containing vesicles at sites of release close to the plasma membrane (52). RapA or exocyst depletion in MIN6 pancreatic β cells (53) or rat islets (54) leads to impaired insulin secretion in response to glucose. Insulin acts on muscle and adipose tissues and...
induces translocation of intracellular vesicles containing glucose transporter 4 (GLUT4) to the plasma membrane, thus enabling efficient glucose uptake in these tissues. RalA is localized to GLUT4-storage vesicles (GSVs) in adipocytes and is activated upon insulin stimulation. Activated RalA recruits the exocyst and mediates targeting of GSVs to the plasma membrane in concert with Myosin V motor protein (55).

Ral and the exocyst also work in immunity. Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells kill virally infected cells by secreting cytotoxic proteins such as perforin and granzymes from specialized secretory compartments called lytic granules. In NK cells, Ral GTPases localize to lytic granules and are activated rapidly after recognition of target cells (56). Knockdown of RalA or Sec5 leads to impaired cytotoxicity due to lytic granule secretion defects. In neutrophils, Ral regulates the secretion of secondary granules that contain antimicrobial agents (57).

In neurons, the exocyst regulates N-methyl-D-aspartate (NMDA) (58) and a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (59) receptor trafficking to the postsynaptic membrane through interaction with postsynaptic density proteins. Expression of activated RalA in mouse hippocampal neurons increased dendritic spine density (60). In Drosophila neuromuscular junctions, constitutively active Ral enhances synaptic activity-driven membrane addition to the postsynapse in a Sec5-dependent manner (60); this membrane addition is required for synaptic plasticity. These studies suggest that Ral and the exocyst regulate postsynapse organization through targeted trafficking of postsynaptic components. Ral also acts at presynapses. In transgenic mice expressing a dominant negative form of RalA, PKC-mediated enhancement of glutamate release is suppressed, while K+-evoked glutamate release occurs at a normal level (61). This study suggests that Ral and the exocyst are dispensable for neurotransmitter release, but important for the replenishment of a readily releasable pool of synaptic vesicles.

In epithelial cells, the exocyst regulates polarization of delivery of basolateral membrane proteins (62). RalA enhances polarized delivery of E-cadherin and epidermal growth factor (EGF) receptor to the basolateral membrane in Madin-Darby canine kidney (MDCK) cells (63). RalA knockdown or the expression of a dominant negative form of RalA caused mislocalization of the basolateral membrane proteins E-cadherin and gp58 to the apical membrane (41). These studies demonstrate that Ral and the exocyst are important for appropriate vesicle targeting in polarized cells. In neurons, Ral and the exocyst regulate the establishment of cell polarity through interaction of Exo84 and Par6 (64).

The exocyst is also required for motility as cell movement requires membrane delivery to actively expanding plasma membrane at the leading edge. RalB has been shown to be required for migration of normal rat kidney cells (65), bladder cancer cells (66) and B-lymphocytes (67). Fluorescence resonance energy transfer analysis revealed that RalA is highly activated at lamellipodia in migrating MDCK cells (68). In addition to membrane lipid components, Ral and the exocyst deliver Sec5-integrin to the focal adhesion at the leading edge through interaction with paxillin (69), which enables adhesion of migrating cells to extracellular matrix proteins such as fibronectin.

During cytokinesis the exocyst localizes to the midbody and mediates the abscission of daughter cells through the fusion of vesicles (70). RalGEFs have been shown to localize to the midbody and regulate

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**Fig. 3 Ral and exocytosis.** Ral GTPases regulate various exocytic pathways in a range of cell types through interaction with the exocyst. Activated Ral on exocytic vesicles binds to the plasma membrane-localized exocyst through an interaction with the Sec5 subunit. During exocytosis, granule contents are released and membrane proteins on exocytic vesicles are exposed to the plasma membrane. These membrane proteins include adhesion molecules, transporters and receptors. In some haematopoietic cells including platelets, cytoytic T lymphocytes (CTLs), and NK cells, the Ral-exocyst pathway functions in concert with the Ca2+-regulated Rab27-Munc13-4 pathway (dashed box) to regulate exocytosis.
cytokinesis through Ral activation (71). Disruption of RapA-Sec5 interaction leads to defective abscission (8).

In addition to functioning in exocytosis, the exocyst regulates actin cytoskeletal reorganization independently of its tethering function. Wei Guo’s group has shown that the Exo70 subunit interacts with the Arp2/3 complex, a key actin polymerization factor, and induces actin-based membrane protrusions at the leading edge of migrating cells (72). Therefore the exocyst coordinates membrane trafficking and actin cytoskeletal dynamics at the actively growing membrane extensions. Although it is unclear whether Ral controls Arp2/3 activity through the exocyst, many studies indicate that Ral and the exocyst control Arp2/3-involved actin cytoskeletal organization, such as filopodia formation (42) and neurite branching (73). Ral also interacts with other actin regulators such as RalBP1 and filamin (see below). Currently, the molecular mechanisms of the complicated patterns of Ral-mediated regulation of actin-based structures remain to be fully elucidated.

**RalBP1/RLIP76**

RalBP1 (also known as RLIP76) was identified in a yeast two-hybrid screen using a constitutively active form of RapA as bait (74, 75). RalBP1 is a multidomain protein that has many functional activities. RalBP1 has a RhoGAP domain and exhibits GAP activity towards the Rho family GTPases Cdc42 and Rac1. Because Cdc42 and Rac1 GTPases are implicated in actin cytoskeletal reorganization, Ral might regulate actin structure through RalBP1-mediated regulation of Cdc42 and Rac1 activities. In addition, RalBP1 engages in receptor-mediated endocytosis through an interaction with the epsin homology (EH) proteins Reps1 (76) and POBI/Reps2 (77, 78). EH domains are found in many proteins engaged in endocytosis. RalBP1 also interacts with the µ2 chain of the clathrin adaptor protein complex AP2 (79). It has been shown that active Rap recruits these endocytic proteins through RalBP1 and controls receptor-mediated endocytosis of EGF receptor and insulin receptor (80). Unexpectedly, RalBP1 knockout mice are viable and grow without any abnormalities under normal conditions. However, these mice exhibit impaired tumour angiogenesis (81). RalBP1-null endothelial cells show migration defects. Since spatial regulation of vascular endothelial growth factor (VEGF) receptor endocytosis is required for normal angiogenesis (82), receptor-mediated endocytosis activity might be impaired in these mice in certain conditions.

Chris Counter’s group showed that RapA and RalBP1 regulate mitochondrial fission (83). Mitochondrial fission is required for equal distribution of mitochondria at mitosis, which is mediated by dynamin-related protein 1 (DRP1) GTPase on the mitochondrial outer membrane. At mitosis, RapA and RalBP1 translocate to mitochondria and facilitate cyclin B-CDK1-mediated phosphorylation of DRP1 to promote mitochondrial fission.

Additionally, RalBP1 has ATP-binding motifs and acts as an ATP-dependent transporter. This activity seems to be important for the cellular export of chemotherapeutic drugs and radiation-induced conjugates in cancer cells (84, 85). The possible role of Ral GTPases in this transporter function remains to be determined.

**Additional effector pathways**

**Phospholipase D.** Ral GTPases interact with PLD (phospholipase D), a lipase that generates phosphatidic acid, in a bound nucleotide-independent manner (86). This interaction is required for PLD activation triggered by tyrosine kinases. Ral GTPases synergistically activate PLD with the Arf GTPases implicated in vesicle budding (87).

M-sec. Certain cells communicate with each other through thin membrane nanotubules called TNTs (tunnelling nanotubules). Ral regulates TNT formation through association with M-sec, a homologue of the exocyst component Sec5 (88).

ZO-1-associated nuclear acid binding protein. Ral directly interacts with ZONAB (ZO-1-associated nuclear acid binding protein), a Y-box transcription factor, in a cell density-dependent manner (89). At low cell density Ral GTPases sequester ZONAB in the cytosol. As cell density increases, ZONAB is released from Ral and enters the nucleus to regulate gene expression.

**Filamin.** RalA interacts directly with filamin, an actin filament-crosslinking protein. A constitutively active form of RalA elicits actin-rich filopod formation in fibroblasts in a filamin dependent manner (90).

**Protein kinase Cη.** In keratinocytes, protein kinase Cη (PKCη) binds to RapA through its C1 domain (91). PKCη and RapA are required for keratinocyte differentiation.

**c-Jun N-terminal kinase.** Ral GTPases are activated by cellular stresses such as reactive oxygen species (92). It has been shown that Ral GTPases activate the transcription factor FOXO4 (forkhead box O4) through c-Jun N-terminal kinase (JNK)-mediated phosphorylation. Mechanistically, RalA associates with the kinase scaffold protein JIP1 and activates JNK by regulating assembly of the kinase cascade complex on the scaffold (93). In *Drosophila* genetic models, however, Ral activity has been found to antagonize the JNK pathway (94, 95).

**NFκB and cyclin D1.** Activated Ral is sufficient to induce NFκB transcription factor activation and NFκB-mediated cyclin D1 expression (96), although the mechanism is unknown.

**Mammalian target of rapamycin complex1.** mTORC1 (mammalian target of rapamycin complex1) is a kinase complex that senses nutrient availability and regulates cell growth by phosphorylating translational factors. mTORC1 activity is regulated by Rheb and Rap GTPases through direct interactions (97). Several studies suggest that Ral GTPases are also involved in the...
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Fig. 4 Ral signalling in cancer. In cancer cells, Ral GTPases are chronically activated by several mechanisms including oncogenic Ras-mediated RalGEF activation, RalGEF upregulation and RalGAP downregulation. Anti-inflammatory proteins TIE2 and x-B-Ras control RalGEFs and RalGAPs, respectively. The tumour suppressor PP2A dephosphorylates RalA and inhibits its activity. Recently characterized chemical compounds, RBC8 and BQU57, have been found to bind directly to Ral and inhibit its activation. Abrerrantly activated Ral GTases induce various downstream signalling pathways that support cancer cell proliferation, survival and invasion. RalA regulates anchorage-independent proliferation through an unknown mechanism. RalB activates cell survival and autophagy through its interactions with the Sec5 and Exo84 exocyst components, respectively. Exocyst-mediated exocytosis of α5-integrin, MMPs and actin cytoskeletal reorganization induce migration and invasion of cancer cells. Ral GTases also regulate other signalling pathways including RalBP1, mTORC1, NFκB and p53. A, RalA-specific pathway; B, RalB-specific pathway.

Ral and Cancer

Oncogenic Ras can activate multiple downstream signalling pathways mediated by distinct effectors, including the well-characterized Raf-MEK-ERK pathway. In addition to the Raf pathway, Ras directly activates the p110 catalytic subunits of phosphatidylinositol 3-kinase (PI3K) and, as described above, the RafGDS-type RalGEFs. Extensive research on the Ras downstream signalling pathways has revealed that the RalGEF pathway is essential for oncogenic Ras-induced tumourigenesis of human cells (Fig. 4).

The RalGEF pathway and tumourigenesis

Robert Weinberg’s group reported that normal human cells can be transformed to the tumourigenic state by introducing three defined factors: oncogenic Ras, telomerase, and SV40 T-antigen (101). Transformed cells can proliferate infinitely and can grow in an anchorage-independent fashion, one of the hallmarks of the tumourigenic state. Subsequent studies dissected the downstream pathways of oncogenic Ras using Ras mutants that can activate single effectors. Initial studies using mouse fibroblasts showed a limited involvement of the RalGEF pathway in oncogenic Ras-mediated transformation. However, subsequent studies revealed that in human cells the RalGEF pathway is essential and even more potent than the other pathways in oncogenic Ras-induced tumourigenesis (102, 103). These surprising results highlighted the importance of Ral functions in human tumourigenesis and the existence of fundamental differences in the behaviour of mouse and human cells in transformation processes (mouse cells can be easily transformed compared with human cells). Chris Counter’s group introduced isoform-specific short hairpin RNA into human cells and found that activation of RalA, but not RalB, is critical for Ras-induced tumourigenesis of human cells (104). RalA dependency in anchorage-independent growth has been observed in many human cancer types. In colon cancer for example, selumetinib, a potent and selective MEK1/2 inhibitor, did not inhibit tumourigenic growth of K-Ras mutant colorectal cancer cells. However, stable RalA knockdown in these cells efficiently blocks anchorage-independent growth more potently than does the mutant B-Raf.
Ligant peripheral nerve sheath tumours (tumourigenesis. RalA functions are also shown in ma-
well-characterized Raf pathway, plays a central role in
suggest that the RalGEF-RalA pathway, as well as the
cellular carcinoma (109) and ovarian cancer (110). Some studies show that RalB is also involved in tu-
mour growth.

The RalGEF pathway and tumour invasion and
metastasis
One intriguing aspect of the RalGEF pathway is that
this pathway promotes tumour invasion and metaesta-
sis. Ward et al. (111) showed that, following tail vain
injection, 3T3 fibroblasts transformed by membrane-
anchored RalGDS formed more invasive, infiltrative
metastasis compared with those transformed with a
constitutively activated form of Raf. They further
showed that in a prostate cancer metastasis model
the constitutive activation of the RalGEF pathway in
non-metastatic prostate cancer cells confers to these
cells the ability to metastasize in bone, the most
common metastatic site for prostate cancer (112). Con-
versely, chronic depletion of RalA in metastatic
prostate cancer cells inhibited the capacity for bone
metastasis. In pancreatic cancer cells, both RalA and
RalB are required for metastatic growth in the lungs
(113). Furthermore, exogenous expression of
RalGAPz2 inhibits lung metastasis of invasive bladder
cancer cells (114). Expression of RalGAPz2(1214), a
GAP activity-deficient mutant, does not repress metas-
tasis. Together, these studies demonstrate that the ac-
tivation of Ral GTpases is essential for the metastatic
growth of tumour cells.

Mechanisms of Ral-mediated tumourigenesis and
malignant transformation
Soft agar colony forming assays of tumour cells
showed that the Raf effectors Sec5 and Exo84, and to a lesser extent RalBP1, are required for the anchor-
age independent growth of tumour cells (115). These
results suggest the involvement of exocyst-mediated
exocytosis in tumourigenesis. However, the role of exo-
cytosis in tumourigenesis is not clearly understood.
Interestingly, it has been shown that oncogenic Ras
induces secretion of the inflammatory cytokine IL-6
to promote tumour growth and tumour angiogenesis
in a paracrine fashion. Enhanced secretion of growth
factors and cytokines may support tumour growth and
modulation of the tumour microenvironment. In addi-
tion, Ral and the exocyst may regulate cell surface
expression of nutrient transporters to meet the high
demand in cancer cells for glucose and amino acids.

Balasubramanian et al. (116) showed that the RalA-
exocyst complex regulates integrin-dependent lipid raft
exocytosis. Loss of integrin-dependent adhesion trig-
gers internalization of the lipid raft microdomain into
recycling endosomes. Because these domains serve
as platforms for growth signalling, their clear-
ance from the plasma membrane inhibits cell prolifer-
ate. Thus chronic activation of RalA keeps these
lipid raft structures on the cell surface, which is essen-
tial for growth factor signalling.

Invadopodia are actin-rich membrane protrusions
formed by invading cells and act to degrade extracel-
lular matrix. The exocyst regulates focal delivery of the
matrix metalloproteases (MMPs) MMP1, MMP9 and
MT1-MMP to support matrix degradation and inva-
sion of cancer cells (117, 118). The highly invasive
phenotype of cancer cells with chronic Ral activation
might reflect this exocyst function.

Michael White’s group has shown the tethering-in-
dependent functions of the exocyst components Sec5
and Exo84 that support cancer cell survival. RalB
binding to Sec5 induces activation of TBK1, an atyp-
ical IkB kinase family member, on the Sec5-containing
exocyst subcomplex and activates the interferon re-
sponsive factor 3 (119). This pathway is necessary for
the innate immune response triggered by viral infec-
tion. In cancer cells, chronic activation of RalB com-
mandeers this machinery and induces an inflammatory
response which is essential for the anti-apoptotic
effect. In contrast, RalB binding to Exo84 induces re-
cruitment of ULK1 and Beclin1-VPS34 complexes on
the Exo84-containing exocyst subcomplex, which initi-
ates autophagosome biogenesis (120). These RalB and
Exo84 functions are critical for the induction of autoph-
yagy by nutrient deprivation. This machinery might
contribute to the survival of growing tumour cells in
nutrient poor conditions. Thus, the exocyst acts as a
platform for kinase signalling cascades in addition to
its role as a vesicle tether. A recent report showed that
ubiquitylation of RalB discriminates these two path-
ways (121).

It is known that acute expression of oncogenic Ras
in normal cells induces cellular senescence through ac-
tivation of tumour suppressors. Recently, it was shown
that depletion of Ral proteins in lung cancer cells har-
bouring mutant K-Ras and wild-type p53 causes upre-
gulation of p53 protein levels through an increase in
protein stability, and results in an increase in the CDK
inhibitor p21WAF (122). Thus, Ral activity seems to
antagonize p53 activity in these cells through destabili-
zation of p53. As loss of p53 activity is critically im-
portant not only for the cell cycle and apoptosis
regulation but also for induction of invasive pheno-
types (123), then Ral-mediated promotion of invasion
and metastasis might depend on the suppression of p53
activity.

As described earlier, Ral activates multiple signal
transduction pathways including NFkB, JNK, and
mTORC1 by as yet undefined mechanisms. These
activities might also be involved in Ral-dependent tu-
mourigenesis and malignant transformation.

Deregulation of Ral activity in human cancers
Activating mutations in Ral genes have not been found
in human cancers. However, growing evidence indi-
cates aberrant activation of Ral GTpases in human
cancer cell lines and more importantly in cancer tis-
sues. Counter’s group showed that RalA and RalB
are specifically activated in pancreatic adenocarcin-
omas but not in normal tissues (113). Interestingly,
Ral and PI3K pathways are not activated in tumour
tissues, suggesting a major role for the RalGEF pathway in pancreatic cancer development. What is the cause of increased Ral activity in pancreatic cancer? Channing Der’s group found that RalGEF RGL2 is aberrantly overexpressed in pancreatic cancer tissues (124). This upregulation of RalGEF is critically important because RGL2 knockdown in a panel of pancreatic cancer cell lines severely impairs anchorage-independent growth and matrigel invasion by these cells. Because K-Ras mutations occur in more than 90% of pancreatic cancers, RalGEF overexpression would further accelerate the activation of Ral GTPases.

In bladder cancers, a relatively low rate of H-Ras mutations (~10%) has been found. However, in invasive human bladder cancer cell lines, Ral is highly activated even in the absence of H-Ras mutation (114). Surprisingly, in these invasive cells, expression of RalGAPα2, the dominant isoform of RalGAP catalytic subunits in the bladder, is strongly suppressed. Because lentivirus-mediated restoration of RalGAPα2 expression in these cells reduces Ral activation to normal levels, the level of RalGAPα2 expression is a key determinant of Ral activity in bladder urothelial cells. Importantly, immunohistochemical analysis of human bladder cancer specimens showed that RalGAPα2 expression is negligible in the most advanced, muscle-invasive cancer tissues, whereas normal urothelial tissues show abundant expression of RalGAPα2. Lower expression levels of RalGAPα2 are strongly correlated with advanced clinical stage and poor survival of patients. These observations demonstrate that downregulation of RalGAPα2 leads to hyperactivation of Ral GTPases. In metastatic prostate cancer, expression of the RasGAP gene DAB2IP is epigenetically silenced by the histone methyltransferase EZH2 (125, 126). Similar epigenetic mechanism may exist for the downregulation of RalGAP expression in advanced human cancers.

Ral activity is also regulated by direct phosphorylation in cancer cells. RalA is phosphorylated at Ser 194 in the C-terminal hypervariable region by Aurora-A kinase (127, 128), whose activity is upregulated in many cancers. Phosphorylation activates RalA by an unknown mechanism and contributes to Aurora-A-induced cellular motility and transformation. Interestingly, the protein phosphatase 2A (PP2A) tumour suppressor dephosphorylates this C-terminal region, and this action is essential for the tumour suppressive function of PP2A (129). RalB is phosphorylated by PKC at Ser 198 (130). In bladder cancer cells, PKC-mediated RalB phosphorylation is essential for anchorage-independent growth and metastasis (131). These studies suggest that deregulation of kinases and phosphatases might result in chronic activation of Ral GTPases in cancer.

Animal models
Several mouse models have been developed to examine the roles of Ral GTPases in tumourigenesis.

RalA and RalB. Chris Marshall’s group investigated a conditional oncogenic K-Ras-driven tumourigenesis model and showed that Ral expression is essential for lung cancer tumourigenesis in mice (132). Unexpectedly, however, either RalA or RalB was sufficient for tumour growth. Thus, although Ral activities, driven by oncogenic K-Ras, are essential for the development of lung tumours, they function in a redundant manner. Ral is also important in embryonic development. RalA-null animals are embryonic lethals due to exencephaly; by contrast, RalB-null animals are viable with no overt phenotype, implying an isoform-specific role for RalA in development. However, as additional loss of one RalB allele exacerbates the exencephalic phenotype, the unique requirement for RalA in embryonic development may reflect the total expression levels of Ral proteins rather than a functional differentiation of the isoforms. Total loss of both Ral isoforms causes lethality in early embryogenesis.

RalGDS. RalGDS KO mice are viable and apparently normal, presumably because of functional redundancy among RalGDS-type RalGEFs. In an experimental tumour model, however, RalGDS-null mice exhibit resistance to the generation and growth of skin tumours induced by topical application of carcinogens, which is dependent on H-Ras activating mutations (133). Thus, RalGDS-mediated Ral activation is important for Ras-induced tumourigenesis in vivo. Histological analysis revealed that apoptosis rates are elevated in skin tumours from RalGDS KO mice, suggesting that Ral activity counters oncogene-induced apoptosis in this model.

RalGAPα2. In human invasive bladder cancer, RalGAPα2 expression is downregulated. To clarify the functional relevance of RalGAPα2 loss in vivo, we generated RalGAPα2 KO mice (114). RalGAPα2 KO mice are viable and grow normally without any abnormalities. However, in bladder tissues of RalGAPα2-null animals, GTP-RalA levels are increased 2.5-fold compared with those of wild-type mice. Intriguingly, the KO mice exhibit severe invasive phenotypes after chemical induction of bladder cancer, indicating that aberrant Ral activity in vivo promotes tumour invasion in bladder cancer.

Therapeutics for the RalGEF Pathway
Extensive efforts have been made to develop drugs that inhibit oncogenic Ras signalling. Notably, numerous compounds targeting Raf or MEK kinases have been developed and tested in clinical trials, but these inhibitors have proved to have limited abilities to produce long-lasting suppression of oncogenic Ras activity in vivo. As Ras signalling plays important roles in Ras-induced oncogenesis, inhibition of Ras signalling may be an alternative approach to suppress oncogenic Ras activity. One possibility is inhibition of Ral prenylation. It has been shown that GGTase-I inhibitors (GGTIs) repress cancer cell proliferation by inducing G1 cell cycle arrest and promoting apoptosis (134). Ral GTPases mediate, at least in part, the effects of GGTIs on cancer cell proliferation and survival (4). Although these studies suggest that inhibition of Ral prenylation...
is an attractive target to treat tumour growth, Ral-specific inhibition might be difficult to achieve since GGTase-I also targets other small GTPases, such as RhoA and Rap1.

Ral proteins have been considered as undruggable targets as they seem to have no drug-binding pockets. However, recent advances in structural analysis and in silico screening have enabled development of an approach for direct targeting of Ras proteins. Several groups have now succeeded in producing direct Ras inhibitors that interfere with Ras-binding to its effectors or GEFs (135–138). Theodorescu’s group used in silico modelling and identified the small compounds RBC8 and BQU57 that could bind to a site in the GDP-bound form of Ras (139). These compounds can lock Ras GTPases in their inactive GDP-bound state, and furthermore, they potently inhibit anchorage-independent and xenograft growth in some, but not all, human cancer cells. Direct Ras inhibitors offer promising lead compounds for future development of novel therapeutics that inhibit oncogenic Ras signalling.

Concluding Remarks

The advances achieved over the past 25 years have revealed the molecular components of Ras signalling pathways and provided basic insights into how Ras GTPases are regulated and how they function in diverse biological and pathophysiological processes. However, many issues remain unresolved. For example, although RalA selective dependency in anchorage-independent growth of tumour cells has been shown, the RalA-specific effector mechanism(s) that supports this proliferation has not yet been identified. Moreover, although it has been suggested that RalB specifically functions in the support of cancer cell survival, the mechanistic details of isoform-specific regulation and effector binding are not fully understood. Because Ras gene KO study clearly showed that RalA or RalB could suffice for K-Ras-induced tumourigenesis, RalA and RalB might have redundant roles at least in the K-Ras-induced tumourigenesis processes in mice. Further elucidation of the molecular mechanisms by which Ral promotes tumourigenesis and malignant transformation is an important issue for future studies.

The development of chemicals that block Ras signalling may offer novel therapeutic approaches for the treatment of tumour growth and metastasis. In this respect, the recent development of direct Ral inhibitors is promising as they effectively inhibit tumourigenic growth of human cancer cells. The combined use of Ras signalling-targeted drugs with existing Ral and PI3K pathway inhibitors may achieve effective suppression of oncogenic Ras activity.

Finally, although much emphasis has been placed on Ras functions in cancer, more attention needs to be focused on its function in exocytosis. There is increasing evidence that exocyst- and Ral-mediated exocytosis is involved in many biologically important processes including platelet activation, immune cell functions, neuronal plasticity and regulation of insulin action. This implies that deregulated Ral activity might lead to pathological conditions such as thrombosis and metabolic syndrome. Interestingly, we showed that Ral GTPases are highly activated in platelets from chronic thromboembolic pulmonary hypertension patients (140). Further investigation on Ral functions will contribute to better understanding of human biology and disease. In particular, genetic engineering approaches in mice using conditional targeting of Ral genes, their regulators, and effectors will clarify the importance of Ral signalling in vivo.

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Conflict of Interest

None declared.

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Ral mediates exocytosis and tumourigenesis


