Ral GTPases: corrupting the exocyst in cancer cells

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The Ras-like small G-proteins RalA and RalB have achieved some notoriety as components of one of a growing variety of candidate Ras effector pathways. Recent work has demonstrated that Ral GTPase activation is required to support both the initiation and maintenance of tumorigenic transformation of human cells. The mechanistic basis for this support remains to be defined. However, the discovery that the exocyst is a direct effector complex for activated Ral proteins suggests that mobilization of polarized exocytosis might be a basic component of the biological framework supporting tumorigenic progression.

As the name implies, the Ras-like GTPases RalA and RalB were first isolated based on sequence similarity to the H-, K- and N-Ras small G-proteins [1]. Human RalA and RalB are 82% identical to each other, and, among the 170-odd small G-proteins present in the genome, they are structurally most related to the Ras subfamily [2]. Ras family GTPases function as crucial nodes in regulatory networks that selectively couple numerous extracellular and intracellular cues to appropriate cell-biological responses, including proliferation, differentiation and survival [3,4]. The importance of appropriately regulated Ras function is highlighted by the high frequency of activating Ras mutations in cancer [5] and by the striking observation that conditional expression of an oncogenic Ras allele is sufficient to drive hyperplasia in multiple tissues within a matter of days post activation [6]. Beyond fundamental biochemical properties, shared by the majority of Ras-like proteins, little was understood about the functional relevance of Ral GTPases until the discovery that a family of Ral-specific GDP–GTP exchange factors (RalGEFs) were direct effectors of Ras oncoproteins. These RalGEFs linked Ras activation to Ral activation, associating Ral proteins with propagation of mitogenic signals and Ras-induced oncogenic transformation [7]. As effectively summarized in recent reviews, numerous studies have subsequently explored the participation of Ral proteins in cell regulatory networks, implicating these GTPases in the control of the seemingly diverse processes of cell proliferation, motility, secretion and maintenance of cellular architecture [8,9]. A mechanistic account of the participation of Ral proteins in the majority of these processes awaits a fuller characterization of the molecular systems directly controlled by Ral activation. However, regulation of the Sec6/8 secretory machine, namely the exocyst, is emerging as a major occupation for Ral proteins in cells [8,9] and might represent an overarching context for many of the biological processes impacted by Ral activation. The exocyst is a 734-kDa protein complex that participates in the intricate secretory vesicle sorting and delivery events required to establish and support functionally and architecturally discrete plasma membrane domains. Here, we focus on current developments highlighting the pivotal contribution of Ral proteins to the regulatory framework supporting tumorigenic transformation and explore mechanistic connections between Ral regulation of the exocyst and the generation of this framework.

Initiation and maintenance of tumorigenic transformation

Exploration of Ras function in the budding and fission yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe generated the first insights that these proteins represent hubs in signal-transduction networks, coupling regulatory cues to the functional activation of multiple downstream effector pathways required to drive an appropriate physiological response [10]. This concept has translated to mammalian cells, where it appears that at least three major effector pathways mediate Ras function: Raf kinase activation, phosphoinositide 3-kinase (PI3K) activation and RalGTPase activation through Ras-interactive RalGEFs [11]. The isolation of Ras effector domain mutations, which can selectively engage distinct members of the Ras effector family, has facilitated exploration of the relative contributions of these effector pathways to Ras function in cells. This analysis has exposed the capacity of Raf activation to contribute to Ras transformation in several model systems [11,12]. However, the relative importance of this pathway in comparison with Raf and PI3K, which have both been demonstrated to be human oncoproteins, has long been enigmatic.

Recent compelling studies, addressing this issue with genetically defined human cell models of tumorigenic progression, suggest that Ral GTPase activation in fact provides essential support to oncogenic Ras-induced tumorigenicity [13,14]. Human fibroblasts, mammary epithelial cells and embryonic kidney cells, isolated from normal tissues, acquire tumorigenic growth properties
upon expression of oncogenic Ras together with telomerase and the SV40 large and small T-antigens. The latter presumably bypass replicative senescence, the p53 and Rb checkpoint proteins, and the phosphatase PP2A, respectively [15,16]. Substitution of oncogenic Ras with the Ras effector mutants selectively coupled to Raf kinases (ras12V,35S), PI3Ks (ras12V,40C) and RalGEFs (ras12V,37G) empirically defined distinct combinations of dominant Ras effector pathways driving tumorigenic transformation of each cell type [14] (Figure 1). The differential sensitivity of human cell types to Ras effector pathway activation could reflect cell-specific differences in signaling environments, pathway connectivity and/or pathway dependency. However, a convergent observation in this and a related study was the apparent universal dependency on forced activation of Ral GTPases by ras12V,37G [13,14]. A caveat to this interpretation is that the capacity of Ras effector mutants to parse out relative contributions of Ras effector pathways is only as good as the selectivity of effector engagement by these mutants in cells. It is important to note that several candidate Ras effectors have been identified that retain association with ras12V,37G, including the junctional protein AF6, the candidate tumor suppressor Nore1, and IMP, an E3 ligase that can modulate the intensity of ERK1/2 activation [17]. These interactions could conceivably partially or fully mediate the consequences of ras12V,37G expression. However, the observation that chronic activation of Ral GTPases through forced expression of an activated Ral GEF could substitute for Ras12V,37G expression in kidney epithelial cells suggests that the Ral pathway plays a dominant role in oncogenic transformation.

The gain-of-function studies described above indicate that Ral activation can make a crucial contribution to tumorigenesis driven by ectopic expression of oncogenic Ras; but how important are Ral GTPases for support of tumorigenicity in authentic human cancer cells? This issue is beginning to be addressed through loss-of-function analysis in human tumor-derived cell lines. Selective depletion of RalA and RalB by RNAi revealed surprisingly discrete but interlocking contributions of these highly similar GTPases to the regulation of cancer cell proliferation and survival [18]. RalB was found to be essential for the survival of a variety of tumor-derived cell lines in culture but was not limiting for the survival of non-cancerous proliferating epithelial cells. By contrast, RalA appeared to be dispensable for survival or proliferation of adherent cultures but was required for the anchorage-independent proliferation of cancer cells. A coupling of RalA and RalB regulatory function was revealed by the observation that inhibition of RalA relieves the sensitivity of tumor cells to loss of RalB [18].

Accumulating evidence suggests that, in 'normal' cells, there is a tight linkage between proliferative and apoptotic machinery, such that increasing the propensity to proliferate increases sensitivity to apoptosis [19]. This is illustrated by activated oncoproteins such as Myc or E1A that promote apoptosis in the absence of accompanying survival signals, potentially through upregulation of regulators and effectors of the apoptosome [20,21]. The distinct consequences of RalA and RalB depletion in normal and tumor cells suggest that Ral isoforms collaborate in the maintenance of oncogenic transformation, mediating both oncogenic proliferation and survival signals. In this scenario, RalA-dependent pathological proliferative pressure must be offset by RalB-dependent inhibition of apoptosis (Box 1). Consistent with this, expression of oncogenic Ras, which is sufficient to support anchorage-independent proliferation of telomerase-immortalized human mammary epithelial cells, sensitizes these cells to RalB-dependent survival signals [18].

Most small GTPases are represented by families of highly structurally similar proteins, as displayed by the founding family members H-Ras, K-Ras and N-Ras. The degree of functional overlap between such family members has largely remained an area of open speculation [2]. The very distinct consequence of RalA versus RalB depletion on cell behavior offers a precedent for discrete biological roles of highly similar GTPases; however, the mechanistic

**Figure 1.** Pivotal contribution of Ral activation to oncogenic Ras-induced tumorigenicity. A variety of primary human cell types isolated from normal tissue can acquire tumorigenic phenotypes upon forced expression of telomerase together with SV40 large and small T-antigen and oncogenic Ras (ras12V). Substitution of ras12V with effector mutations that discriminate between three families of Ras targets – Raf (ras12V,35S), RalGDS (ras12V,37G) and phosphoinositide 3-kinase (PI3K) (ras12V,40C) – reveals selective requirements for activation of these pathways to drive tumorigenic transformation of breast epithelia, fibroblasts and kidney epithelia. While these three cell types display dissimilar sensitivity to Ras-induced activation of Raf family and PI3K family proteins (as indicated by the arrows), activation of Ral GTPases is apparently a common prerequisite to development of tumors in nude mice.
The exocyst is a multisubunit complex that is conserved among eukaryotes and comprises of the core elements Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84. Characterization of exocyst assembly and function in yeasts suggests that the exocyst is a dynamic complex assembled from subunits that form a targeting patch on the plasma membrane and a vesicle-associated subcomplex, which function together to both target and tether secretory vesicles to sites of membrane expansion [26].

**Box 1. The RalA–RalB balancing act in tumor cells**

RalA and RalB collaborate to support cancer cell proliferation and survival. RNAi-mediated depletion of RalA and RalB separately or together revealed discrete but interlocking contributions to promotion of anchorage-independent proliferation and suppression of apoptosis, respectively, in a broad variety of human cancer cell lines. The selective ‘addiction’ of transformed cells to RalB-dependent survival pathways, together with the reversal of this addiction upon depletion of RalA, suggests that a crucial balance of RalA and RalB activity develops in cancer cells. We propose that this balance is reflective of the cell-autonomous coupling of proliferation and apoptosis such that enhanced proliferative propensities translate into increased apoptotic propensity. A conceptual framework for this relationship is represented in the cartoon by the balance of proliferative pressure with suppression of apoptosis (Figure I). Under conditions of oncogene-induced stress, the increased apoptotic propensity driven by RalA is offset by RalB-dependent survival signals (a). Inhibition of RalB function under these conditions therefore leads to apoptosis (b). Reducing proliferative pressure by inhibition of RalA restores balance to the system, relieving dependency on RalB survival signals (c). This relationship, if genuine, suggests that RalB-dependent survival pathways represent conceptually ideal targets for anticancer drugs with high tumor-cell-specific potency.

![Figure I.](image)

(b) (c)
in the PC12 cell model system [23,31] and histamine or thrombin-induced release of von Willebrand factor from endothelial cells [32,33]. Finally, transgenic expression of a dominant-inhibitory RalA mutant in the mouse central nervous system has also revealed a role for Ral activation in neurosecretion, potentially by promoting PKC-dependent amplification of the readily releasable pool of synaptic vesicles [34].

The mechanistic basis of Ral-dependent regulation of exocyst function is only partially understood. Both Sec5 and Exo84 directly bind to the GTP-loaded Ral GTPases in their active conformation [23–25]. The minimally sufficient Ral-binding fragment of Sec5 is an N-terminal domain that adopts an immunoglobulin-like fold [35,36]. By contrast, the Ral-binding domain in Exo84 overlaps with a pleckstrin-homology domain [24]. The functional consequence of this overlap is unknown; however, Ral–GTP can compete with phosphoinositide phosphates for association with Exo84 [24]. Depletion of cellular RalA disrupts the association of exocyst subunits with each other, suggesting that Ral–exocyst interactions are required to promote assembly of the full octameric complex. Density gradient fractionation suggests that Sec5 and Exo84 segregate between presumed exocyst subcomplexes. Therefore, these Ral effectors might represent a regulatable interaction interface for assembly of the full octameric complex during vesicle tethering [23,24] (Figure 2). Further elaboration of this model will require a systematic analysis of the consequences of Ral signaling on exocyst subunit interactions and their subcellular localization.

Several additional small G-proteins have recently been implicated in exocyst biology, perhaps reflecting a highly dynamic regulatory network surrounding the functioning of this secretory machinery. These include the GTP-dependent interactions of Rab11 with Sec15 [37], Arf6 with Sec10 [38], and TC10 with Exo70 [29]. Extrapolation from studies in yeast cells [9] suggests that these interactions might represent regulation of additional discrete steps in secretory vesicle trafficking that include recruitment of exocyst subunits to vesicles and plasma membrane domains, linkage of exocyst subcomplexes to transport machinery, and hand-off of tethered vesicles to the fusion machinery (Figure 2).

**Exocytosis and cancer**

Ostensibly, propagation of growth-regulatory signals and control of the exocyst appear to be rather disparate occupations for Ral GTPases. However, compelling observations from studies of the behavior of normal and cancerous human epithelial cells in organotypic culture models suggest that prominent linkages between these processes support both cell proliferation and survival [39,40]. As described above, the exocyst is required to maintain the molecular polarity of epithelial cell membranes through appropriate delivery of basolateral membrane proteins. In both normal and malignant mammary epithelial cells, a fundamental connection between molecular polarity and cell survival was defined by the observation that ligation of the α6β4 integrin drives tissue polarity and confers resistance to apoptosis [40]. Importantly, auto-activation of this pathway through secretion of laminin-5, the α6β4-integrin ligand, confers resistance to anoikis and is required to support cancer cell survival [39]. The relevance of this relationship is highlighted by the observation that invasive human epidermal neoplasias fail to form in the absence of laminin-5 or α6β4 expression [41]. Although direct connections remain to be explored, one can speculate that the contribution of Ral proteins to tumorigenesis is through exocyst-dependent maintenance of regulatory platforms such as the autocrine laminin-5–α6β4 signaling network. In preliminary support of this notion, tumorigenic epithelial cells appear to be highly sensitive to siRNA-mediated depletion of exocyst subunits (Y. Chien and M.A. White, unpublished).

Recently, components of the exocytic machinery have been directly implicated in human tumorigenesis. Desmoplastic small round cell tumors (DSRCT) often arise as a consequence of chromosomal translocations that generate oncogenic fusions between Ewing's sarcoma (EWS) breakpoint protein and transcription factors. Studies directed at identifying the key target genes of the EWS–WT1 fusion protein resulted in the isolation of the Munc13 family protein BAIAP3 [42]. Munc13 proteins play obligatory roles in a variety of exocytic events by directly promoting
fusión de vesículas secretoras con la membrana plasmática [43,44]. BAIAP3 es un director directo del EWS–WT1 transcription factor, se upregula en DSRCT samples and can promote serum-independence and anchorage-independent proliferation when overexpressed [42]. Interestingly, Munc-13 itself has been identified as a candidate partner of the Exo84 exocyst subunit in Drosophila (see http://pim.hybrigenics.com/pimriderext/droso/exocyst.html). A separate study profiling regions of chromosome amplification in ovarian and breast tumors isolated Rab25 as a gene that is amplified and overexpressed in half of these cancers [45]. Rab25 is a member of the Rab11 family of small GTPases that promote regulated exocytosis and have been implicated in exocytosis function through direct interaction with the Sec15 subunit [37]. Rab25 is necessary and sufficient to promote tumorigenic phenotypes in vitro an in vivo, and increased Rab25 expression is associated with poor prognosis for breast and ovarian cancer patients [45]. Together, these studies strongly suggest that enhanced mobilization of the secretory machinery is an important facet of the pathological regulatory events promoting tumorigenesis.

Concluding remarks

The recent elucidation and manipulation of the Ral GTPase regulatory network in cultured cells has indicted that these proteins act as key offenders in the corruption of the core cell-autonomous machinery driving oncogenic transformation. RalA and RalB are, respectively, required to support anchorage-independent proliferation and suppress apoptosis in human cancer cells. Chronic Ral activation is sufficient in some contexts to induce oncogenic transformation and is a chief mediator of the aberrant growth-regulatory network generated by oncogenic Ras. The major tasks are now to establish the relative involvement of Ral activation in human tumors and to identify the molecular connections between Ral activation and proliferation and survival programs. Facilitating regulated exocytosis, through interaction with the exocyst effector complex, has emerged as a major occupation for Ral proteins in cells. Given the apparent obligatory contribution of enhanced exocytosis to tumorigenesis, the exocyst has emerged as a premier candidate target of Ral-dependent support of oncogenic transformation.

A very recent study has delivered the first clear genetic evidence incriminating Ral GTPase activation in tumor initiation and progression. Mice with a homozygous deletion of RalGDS, a Ral GDP–GTP exchange family member, display reduced incidence and progression of topical carcinoen-induced dermal papillomas [46]. The centrality of exocyt function in the regulatory platform supporting these tumors remains to be defined. However, given that human epidermal neoplasias are dependent upon autocrine-supported laminin-5–p6β4 signaling, it is tempting to speculate that Ral GTPase activation contributes to papilloma formation by generating a permissive tumor microenvironment through exocyst-dependent matrix remodeling. The recent confluence of conceptual advances suggests that a full elaboration of the contribution of Ral GTPases to tumor initiation and progression can be anticipated in the near future.

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