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14. ABSTRACT

Targeted therapy has proven to be an effective strategy in the treatment of breast cancer. An example of successful targeted therapy is trastuzumab, a humanized monoclonal antibody binds to the extracellular domain of HER2, a receptor which is overexpressed in many breast cancers. The goal of my project is to identify molecular targets for conjunctive therapy that will increase efficacy of trastuzumab therapy. To identify molecular targets necessary for breast cancer cell survival in the presence of trastuzumab, a breast cancer cell line with known cytostatic sensitivity to trastuzumab, will be screened using a high throughput assay using a with a unique small interfering (si)RNA library targeting all 21,125 known genes in the human genome database. This information will help identify new synergistic combinations with existing drugs and novel therapeutic targets that can act synergistically as initial therapy and upon the development of resistance. During the time covered by this annual summary, I have uncovered an important signaling nexus regulating cell survival and drug resistance in breast cancer cells.

15. SUBJECT TERMS
Genomics, breast, cancer, siRNA, screen, Herceptin, trastuzumab

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INTRODUCTION

Targeted therapy has proven to be an effective strategy in the treatment of breast cancer. An example of successful targeted therapy is trastuzumab (Herceptin), a recombinant DNA-derived humanized monoclonal antibody that selectively binds with high affinity to the extracellular domain of HER2, a receptor which is overexpressed in many breast cancers [1,2]. In clinical trials patients who received trastuzumab in combination with standard combination chemotherapy had a significant decrease in disease recurrence compared to patients treated with chemotherapy alone [3,4,5]. Unfortunately, the majority of patients who achieve an initial response to trastuzumab-based regimens generally acquire resistance within 1 year [6,7]. The goal of my project is to identify molecular targets for conjunctive therapy that will increase the cell autonomous efficacy of trastuzumab therapy and potentially switch trastuzumab to a cytocidal drug *in vitro*. To identify molecular targets necessary for breast cancer cell survival in the presence of trastuzumab, the human breast cancer cell line, BT474, with known cytostatic sensitivity to trastuzumab, will be screened using a high throughput assay using a with a unique small interfering (si)RNA library targeting all 21,125 known genes in the human genome database. A screen that looks for much greater reduction in cell number than seen with trastuzumab therapy alone will be conducted in the presence of drug in microtiter plates with a robotic high throughput screening system. We have validated this high throughput approach in a screen of non-small cell lung cancer to identify molecular targets which sensitize cells to the anti-cancer drug paclitaxel [8]. A confluence of observations in concurrent (si)RNA screens has revealed that a RalB-Sec5-TBK1 signaling axis is critical for cancer cell survival. Using a candidate-based approach to evaluate our screen validation platform, we uncovered an important contribution of RalB-Sec5-TBK1 signaling towards the induction and execution of autophagy. We have found by siRNA depletion that RalB and the exocyst are necessary for the engagement of autophagy, and that RalB and the exocyst interact with known components of autophagy induction. Monoallelic losses of the autophagy protein Beclin1 have been detected in 40% of breast cancer cell lines [9]. Studies in mice have revealed Beclin1 to be haploinsufficient tumor suppressor [10]. Paradoxically, inhibition of autophagy sensitizes tamoxifen-resistant breast cancer cells to tamoxifen [11]. We hope that understanding the role of RalB-Sec5-TBK1 signaling in autophagy will provide insight into the paradoxical roles of autophagy in promoting tumor suppression and drug resistance in breast cancer and allow us to better design drug therapy for drug-resistant breast cancers.

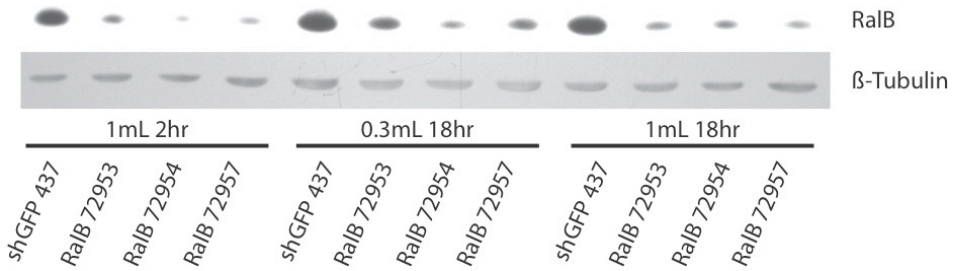
BODY

During the second year of this fellowship I have focused on the following:
1) Establishing a validation platform for the investigation of interesting screen hits.
2) Using a candidate-based approach to test our validation platform, and following up of an exciting connection between cancer cell survival and autophagy. 3) Publication of a manuscript describing a human genome-wide siRNA screen to identify important components of human melanogenesis.

1. My graduate training accomplishments are bulleted below.

2. Work to have been completed:
AIM1, Task 1 has been completed as reported in the last annual report. AIM1 Task 2 (primary screen) was to have been completed, but is awaiting execution in the institutional queue. While the primary screen is in queue, I shifted my focus to AIM2 (validation platform). Using a candidate-based approach, I identified an effective protocol for shRNA-mediated depletion of target proteins:

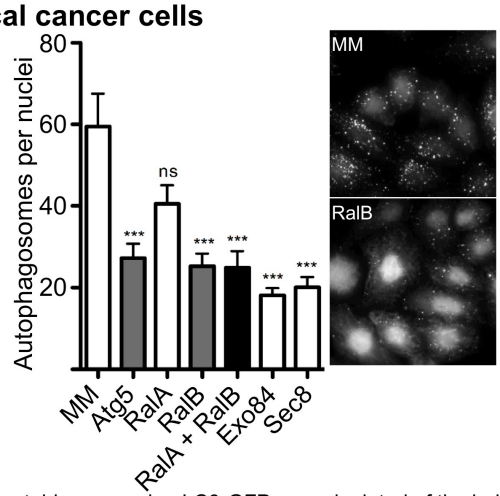
Figure1



Human Bronchial Epithelial Cells (HBEC3-KT) were infected with the indicated lentivirus harboring shRNA expression constructs for the indicated time periods. The level of RalB protein depletion was assayed 72 hours after infection initiation.

In the course of validation experiments, we uncovered an exciting connection between cancer cell survival and (macro)autophagy signaling pathways: RalB and the exocyst are necessary for autophagy. In Figure2, depletion of RalB and the exocyst subunits Exo84 and Sec8 by siRNA lead to similar decreases in autophagy as seen with depletion of the know autophagy protein Atg5.

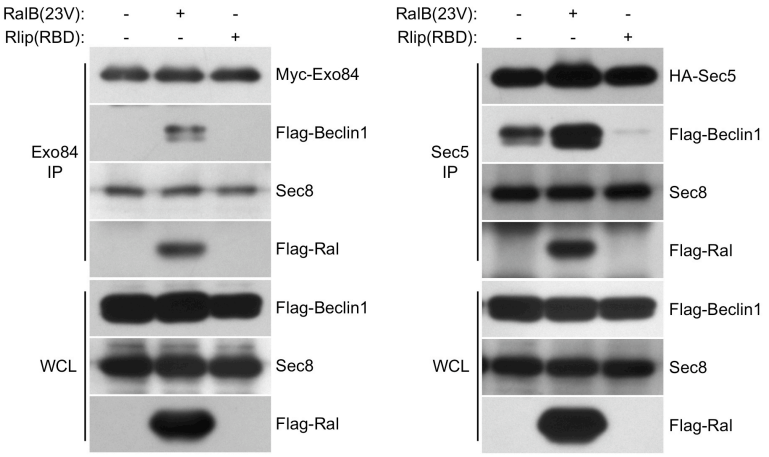
Figure 2
RalB and the Exocyst are necessary for basal autophagy in cervical cancer cells



Hela cells stably expressing LC3-GFP were depleted of the indicated proteins by siRNA for 96 hours before imaging. Statistical significance was evaluated by 1-way ANOVA with Dunnett's post-test comparison for each condition compared to negative control (MM).
*** = $p < 0.0001$; ns = not significant

We decided to follow up this exciting result as it could have major implications in the treatment of drug-resistant breast cancer. I decided to look for an interaction between RalB-Exocyst signaling pathway components with known autophagy signaling components. In Figure3, we found that RalB activation was necessary and sufficient to drive complex assembly between the exocyst and the autophagy protein Beclin1.

Figure3
RalB promotes association between the Exocyst and Beclin1

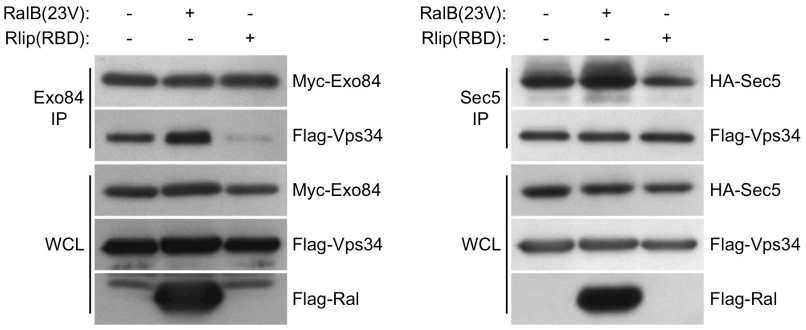


Overexpression of a constitutively active RalB (23V) drives association between the autophagy protein Beclin1 and Ral effectors Sec5 and Exo84. Expression of the minimal Ral binding domain of Rlip blocked assembly of this complex.

The indicated proteins were co-expressed in human embryonal kidney cells (HEK293T) cells for 48 hours and then assayed for interaction by immunoprecipitation of the indicated protein. This experiment is representative of three biological replicates.

To further investigate the interaction of RalB-Exocyst and autophagy signaling components, we tested the interaction of exocyst proteins with Vps34, a phosphatidylinositol-(3)-kinase (PI3K), that is a binding and signaling partner of Beclin1. In Figure 4, we found that co-expression of RalB23V drove association of Exo84 with Vps34.

Figure 4
RalB promotes association between Vps34 and the Exocyst subunit Exo84



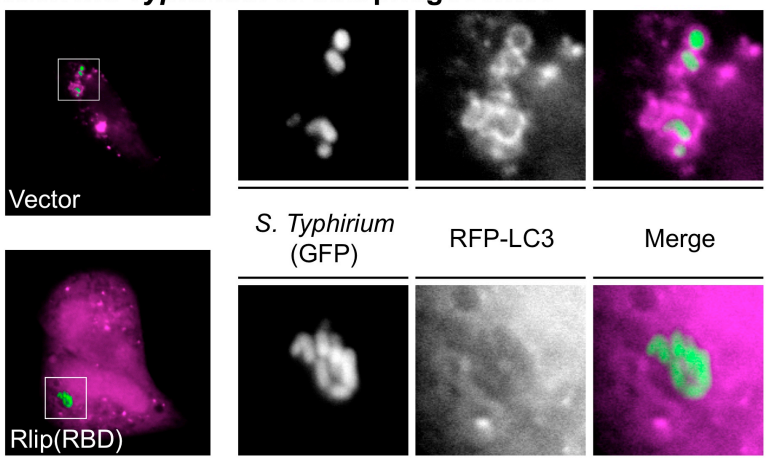
Overexpression of a constitutively active RalB (23V) drives association between the autophagy protein Vps34 and Ral effector Exo84. Expression of the minimal Ral binding domain of Rlip blocked assembly of this complex.The association of Sec5 and Vps34 was unaffected by Ral activity.

The indicated proteins were co-expressed in human embryonal kidney cells (HEK293T) cells for 48 hours and then assayed for interaction by immunoprecipitation of the indicated protein. This experiment is representative of three biological replicates.

Autophagy has been implicated as an important defense pathway in the response to infection with pathogens. We decided to test for the necessity of RalB signaling in response to pathogen infection, as the pathogen would provide a spatially restricted signal for us to investigate the proximity of RalB to the autophagosome. In Figure 5, we found Ral signaling to be necessary for Salmonellae-associated autophagosome induction. In Figure 6, we found that RalB is recruited to the site of Salmonellae and RalB co-localized with a marker of nucleating autophagosomes, Atg5. We also investigated the association of RalB-Exocyst with the autophagosome nucleation machinery in response to Sendai virus (which allowed us a nice, temporally restricted trigger for Ral activation and autophagosome induction). In Figure 7, we found that after infection with Sendai virus, RalB interacted with the “active”conjugated form of Atg5, Atg5-Atg12.

Figure 5

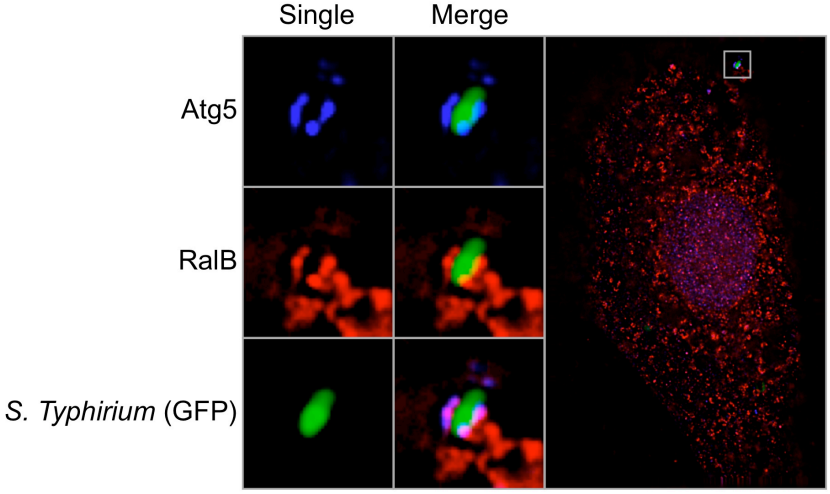
Ral G-protein signaling is necessary for accumulation of *Salmonella Typhirium* in autophagosomes



Overexpression of the minimal Ral binding domain of Rlip blocks accumulation green fluorescent protein (GFP) expressing *S. Typhirium* in autophagosomes as marked by a fusion protein of the red fluorescent protein and LC3. Hela cells were infected for one hour with *S. Typhirium* before treatment with gentamicin for two hours to eliminate extracellular *S. Typhirium*.

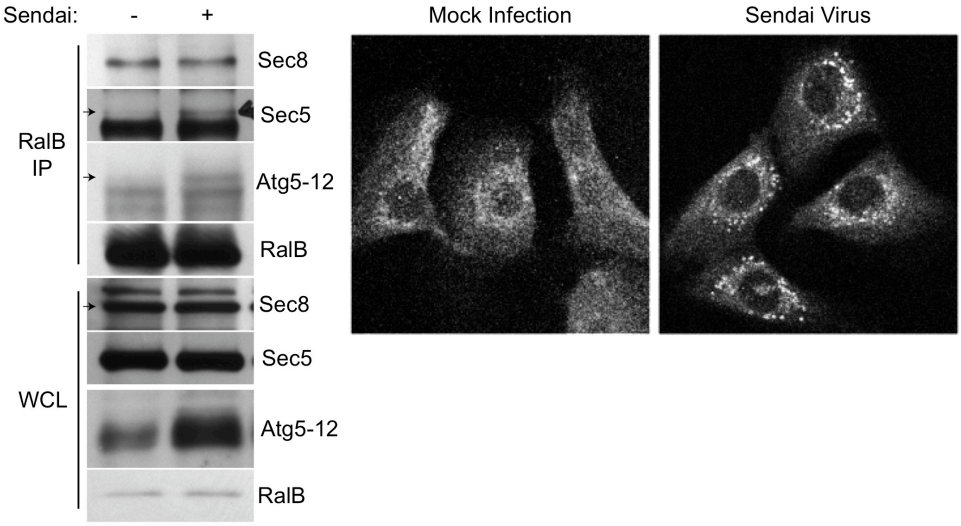
Figure 6

RalB localizes to the site of autophagosome formation in *Salmonella Typhirium* infected cells



RalB is localized to the site of *S. Typhirium*-induced autophagosome formation as marked by co-localization with Atg5. Subcellular localization of endogenous RalB and Atg5 were detected by immuno-fluorescence and imaged by deconvolution microscopy. HBEC3-KT immortalized bronchial epithelial cells were infected for one hour with *S. Typhirium* (GFP) before treatment with gentamicin for one hour to eliminate extracellular *S. Typhirium*.

Figure 7
RalB associates with Atg5-12 upon infection with Sendai Virus



RalB is associated with the conjugated form of autophagy proteins Atg5 and Atg12. HBEC3-KT immortalized bronchial epithelial cells were infected for five hours with Sendai virus before immuno-precipitation with anti-RalB antibody.

The contents of this work concerning the role of the cancer cell survival signaling protein RalB in regulating the drug resistance and tumor suppressor process autophagy are currently being assembled into a manuscript entitled "RalB and the exocyst regulate autophagy through activation of Beclin1/Vps34 and assembly of the Atg5-Atg12—Atg16 complex."

AIM1 SUMMARY
We have completed AIM1 Task1 and are in the institutional queue awaiting execution of primary screen (designated AIM1 Task 2). We anticipate that we will be able to provide the data from the primary screen during the next annual update.

AIM2 SUMMARY
In AIM2, we proposed a scheme, which would allow us to validate important targets identified in the primary screen. I have tested this validation scheme using a candidate-based approach and uncovered an important signaling nexus which connects a key component of cancer cell survival, RalB, with a known drug resistance process in breast cancer, autophagy. Many drug companies are currently developing therapies targeting RalB signaling effector TBK1 and our work shows that these drugs may provide a double-hit by inhibiting a cancer cell survival pathway and blocking a drug resistance process. In addition, our institution has recently recruited an expert in 3D organotypic culture systems, Gray Pearson. I will be able to work very closely with Dr. Pearson to establish my 3D culture system (AIM2, Task 7) when he arrives late in 2008.

KEY TRAINING ACCOMPLISHMENTS

1. I participated in two Journal Clubs in Cell Regulation training program during the Fall and Spring semesters.
2. I presented my research in a Works in Progress seminar series that was attended by faculty and students from Cell Regulation training program.
3. I presented my research in a Works in Progress seminar series that was attended by the directing faculty and students from Medical Scientist Training Program.
4. I presented my research at the annual retreat of the Medical Scientist Training Program (MSTP) at UT Southwestern, which was attended by the directing faculty of the MSTP, students from the MSTP, and invited faculty from UT Southwestern.

KEY RESEARCH ACCOMPLISHMENTS

1. I published a first author review article on the role of Ral proteins in cancer in *Nature Reviews Cancer*. Attached in Appendix.
2. A high-throughput compatible experimental platform has been developed to interrogate BT474 responses to Herceptin and the primary screen is in the institutional queue awaiting execution.
3. I have identified an important pathway regulating autophagy, RalB-Exocyst, which may provide an important link between cancer cell survival signaling and drug resistance in breast cancer.
4. I have participated as a coauthor in the completion and publication a human genome-wide siRNA screen[12].

REPORTABLE OUTCOMES

1. Brian Bodemann and Michael White, Ph.D. "RalB and the Exocyst Regulate Autophagy" 2008 Era of Hope Meeting sponsored by the Department of Defense Breast Cancer Research Program, June 25th – 28th 2008, Baltimore, MD.
2. Brian Bodemann and Michael White, Ph.D. "RalB and the Exocyst Regulate Autophagy" 2008 National MD/PhD conference sponsored by the University of Colorado Medical Scientist Training Program, July 24th – July 26th 2008, Keystone, CO.
3. Brian Bodemann and Michael White, Ph.D. "RalB and the Exocyst Regulate Autophagy" 2008 Sigma Xi Poster Contest sponsored by the University of Texas Southwestern Graduate School Organization, October 1st 2008, Dallas, TX.

CONCLUSIONS

I have completed important work, which may provide an important link cancer cell survival and drug resistance in breast cancer, but also indicates RalB-Exocyst-TBK1 to be an important drug target in breast cancer. We completed AIM1 and have developed an effective high-throughput (si)RNA screening platform for interrogating novel genetic relationships with Herceptin in BT474 breast cancer cells and the primary screen is in the institutional queue awaiting execution.

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APPENDICES

1. To illustrate my development as a scientist I have attached a first author review that I published in *Nature Reviews Cancer*.

Ral GTPases and cancer: linchpin support of the tumorigenic platform

Brian O. Bodemann and Michael A. White*‡*

Abstract | A confluence of recent observations has indicted the Ras-family G-proteins RALA and RALB as key offenders in the subversion of core biological systems driving oncogenic transformation. Here, we will focus on current developments highlighting the pivotal contribution of Ral proteins to the regulatory framework supporting tumorigenesis, and evaluate mechanistic connections between Ral effector activation and generation of this framework.

Geranylgeranyltransferase 1 (GGTase 1). One of the three enzymes in the prenyltransferase group. GGTase 1 adds a 20-carbon isoprenoid called a geranylgeranyl group to proteins bearing a CaaX motif (a four amino-acid sequence at the C terminus of a protein).

Farnesylation
After translation, the Ras proteins undergo four modification steps: isoprenylation, proteolysis, methylation and palmitoylation. Isoprenylation involves the enzyme farnesyltransferase, which transfers a farnesyl group from farnesyl pyrophosphate.

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doi:10.1038/nrc2296

The Ras-like (Ral) guanyl nucleotide-binding proteins, RALA and RALB, first surfaced over 20 years ago when Pierre Chardin isolated their cognate genes from a hybridization screen of B-lymphocyte cDNAs using degenerate probes that contained highly conserved Ras sequences[1]. With 82% identity to each other at the amino-acid level, we now know that RALA and RALB represent the inclusive roster for the Ral branch of the over 170-strong Ras family G-protein tree[2]. Following their initial discovery, the functional relevance of Ral proteins remained enigmatic for some time; however, they gained notoriety after recognition that a class of guanyl nucleotide exchange factors with biochemical specificity for Ral proteins (see BOX 1 for a review of the G-protein cycle) were direct effectors of oncogenic Ras. Importantly, both gain-of-function and loss-of-function studies identified Ral activation as a proximal consequence of Ras expression that could contribute to Ras-induced oncogenic transformation in cell culture model systems[3–8]. As effectively summarized in recent reviews, numerous studies have subsequently explored the participation of Ral proteins in cell regulatory networks, implicating these G-proteins in the seemingly diverse but potentially related processes of cell proliferation, motility, protein sorting and maintenance of cellular architecture[4,5]. Here, we will focus on recent developments that are defining causal relationships between Ral activation and cancer and providing mechanistic accounts of the molecular framework engaged by Ral proteins to support tumorigenic transformation.

Ral and tumorigenicity

Measurements of the relative accumulation of Ral–GTP versus Ral–GDP in cells and tissues have revealed chronic RALA and RALB activation in tumour-derived cell lines versus non-tumorigenic counterparts, and, perhaps more significantly, in tumour samples versus normal tissues[9–11]. Studies employing transient[12] or stable[9] RNA inhibition (RNAi) of *RALA* or *RALB* expression in human cell culture models further supported the functional significance of RALA activation. Depletion of RALA had no overt consequences on the proliferation or survival of normal or tumorigenic cells under adherent conditions, but severely impaired the anchorage-independent proliferation of cancer cell lines[9,12]. This suggested that chronic RALA activation was an obligate component of the pathological regulatory framework that promotes the bypass of normal proliferative restraints. By contrast, RALB was found to be essential for the survival of a variety of tumour-derived cell lines, but was not limiting for the survival of non-cancerous proliferating epithelial cells[9]. Importantly, sensitivity to RALB depletion was conferred on both telomerase-immortalized human mammary and bronchial epithelial cultures upon expression of oncogenic Ras. Thus, RALA and RALB appear to collaborate to promote the bypass of normal restraints on both cell proliferation and survival, at least in a tissue culture setting[12].

This relationship was also revealed by Said Sebti and colleagues through studies of geranylgeranyltransferase 1 inhibitors (GGTI), which are currently undergoing advanced preclinical evaluation as therapeutic agents targeting cancer cell proliferation and survival[13]. Both RALA and RALB are peripheral membrane proteins as a consequence of carboxy-terminal geranylation, and both require membrane association for biological activity[4]. GGTI exposure therefore abrogates Ral function together with that of all other proteins that require this modification for activity[13]. Remarkably, expression of a GGTI-resistant RALB variant, which was modified by replacement of C-terminal geranylated sequences with those that specify farnesylation, conferred resistance to

At a glance

- Chronic activation of the Ras-like (Ral) guanyl nucleotide-binding proteins, RALA and RALB, occurs in tumour-derived cell lines and tumour samples.
- Depletion of RALA severely impairs the anchorage-independent proliferation of cancer cell lines, whereas RALB seems to be essential for the survival of a variety of tumour-derived cell lines.
- RALA is phosphorylated by Aurora kinase A and other, yet to be identified kinases. It is also a substrate of protein phosphatase 2A Aβ. Evidence indicates that dephosphorylation of RALA is a major mechanism by which PP2A Aβ normally restricts tumour progression.
- The effects on tumorigenesis of well-characterised downstream effectors of Ral, such as the Rac-family GTPase-activating protein RLIP, the Y-box transcription factor ZO-1-associated nucleic acid-binding protein (ZONAB) and two subunits of the exocyst complex, SEC5 and EXO84, remain unclear. However, a number of relationships have been identified that might explain Ral-dependent modulation of cell proliferation and survival.
- An important factor that might explain the occurrence of Ral activation in tumorigenesis is the RALB-specific contribution to cancer cell survival through activation of TANK-binding kinase 1 (TBK1). Chronic RALB activation restricts initiation of apoptotic programmes that are normally activated in the context of oncogenic stress.
- Proteins such as TBK1 might prove to be good candidate targets for the development of cancer drugs with a large therapeutic window.

GGTI-induced pancreatic cancer cell apoptosis, but not to GGTI-dependent inhibition of anchorage-independent proliferation. However, a GGTI-resistant farnesylated RALA variant failed to deflect GGTI-induced apoptosis of adherent cell cultures, but rescued anchorage-independent cell proliferation. Thus, the RALA–RALB proliferation and survival axis appears to be a key target of GGTIs that accounts at least in part for the biological consequences of GGTI exposure on cancer cell behaviour[13].

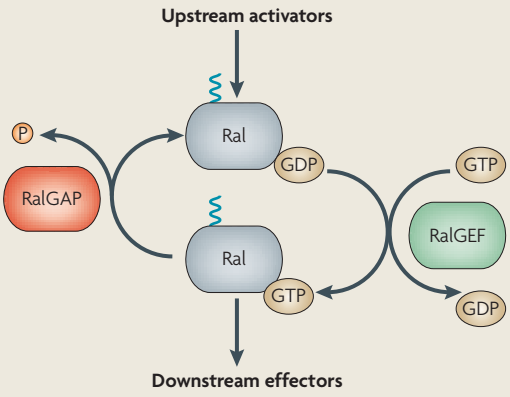
Further indications of a crucial role for Ral G-protein signalling in human cancers comes from the discovery, by Bill Hahn and colleagues, that RALA is a functionally relevant target of the protein phosphatase 2A (PP2A) Aβ (also known as PPP2R2B) tumour suppressor[14]. PP2A Aβ is a structural subunit of the serine–threonine protein phosphatase 2A, and somatic mutations and gene deletions of PP2A Aβ have been found in a significant percentage of human lung, breast and colon cancers[15–19].

RALA is phosphorylated on serines 183 and 194 in cells, potentially by Aurora kinase A together with other, yet to be identified kinases[14,20]. These phosphorylation sites are bona fide PP2A substrates and are associated with a five-fold increase in the GTP-bound active pool of RALA[14]. Remarkably, complementation of RNAi-mediated depletion of endogenous *RALA* with wild-type RALA, but not with RALA variants with alanine substitutions at positions 183 or 194, restored the anchorage-independent growth and tumour-formation properties of human embryonic kidney cells that had been transformed by PP2A Aβ suppression. This strongly suggests that dephosphorylation of RALA is a major mechanism by which PP2A Aβ normally restricts tumour progression[14]. An analogous phosphorylation on serine 181 of KRAS disturbs the association of a stretch of lysine residues within the C terminus of KRAS with the negatively charged inner surface of the plasma membrane, thereby disrupting plasma membrane association and resulting in redistribution of KRAS to endomembrane domains and altered signalling properties[21]. Given that serines 183 and 194 of RALA flank a region of positively charged residues within the sequences that specify membrane targeting, a similar mechanism might also govern the subcellular localization of RALA. Perhaps phosphorylation has a role in regulating the shuttling of RALA between internal membranes and the plasma membrane, which might be crucial for its association with upstream regulators and/or downstream effectors.

***The effects of Ral* in vivo.** A key hallmark of tumorigenic progression is the release from dependence on matrix association for cell-cycle progression and survival. Competency for anchorage-independent proliferation is probably a prerequisite for both primary tumour growth and development of metastatic lesions[22]. Although observations made using traditional monolayer-adherent cultures do not always predict the behaviour of cells within a three-dimensional extracellular matrix, and cannot predict the consequences of epithelial cell–stromal cell communication[23], the studies described above predict that Ral proteins could make obligate contributions to primary tumour formation. This prediction has

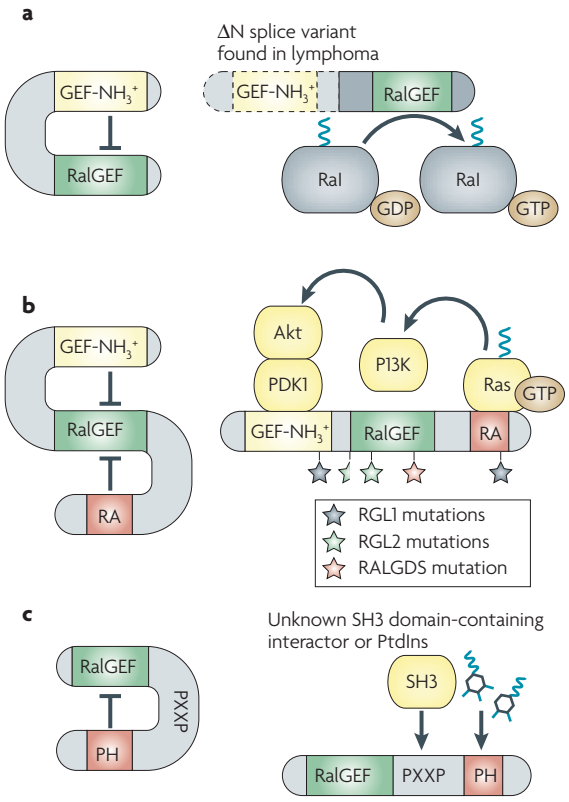
Box 1 | **The G-protein cycle**

Akin to the vast majority of Ras family members, Ral proteins are coupled to the regulation of dynamic cell biological processes by cycling through guanyl nucleotide-dependent conformational transitions. GTP loading, at a 1:1 molar ratio, is required for effector binding and is driven by association with Ral-specific guanyl nucleotide exchange factors (GEFs). Diverse signalling inputs can engage these exchange factors; including growth factors, hormones, membrane depolarization and even pathogen surveillance machinery[4,11]. By extrapolation to Ras proteins, transition to the 'off-state' requires hydrolysis of bound GTP through complementation of Ral's weak intrinsic catalytic activity[71] by GTPase activating proteins (GAPs). Remarkably, although Ral GAP activity was biochemically enriched from brain and testis lysates over 15 years ago, the molecular identity of Ral GAP(s) is still unknown[72].



Endomembrane domains
Internal membranes of the cell such as the nuclear membrane, Golgi and endoplasmic reticulum.

Box 2 | **The RalGEF family and their regulatory cues**



Ral GTPases are engaged in response to a broad variety of mitogenic, trophic and hormonal signals by a diverse group of guanyl nucleotide exchange factors (GEFs) that fall into two major groups: those that can be directly Ras-responsive through a carboxy-terminal Ras-binding domain (RALGDS, RGL1, RGL2), and those that are apparently mobilized by phosphatidylinositol second messengers through a C-terminal pleckstrin homology (PH) domain (RALGPS1, RALGPS2)[4]. Virtual expression profiling, based on UniGene annotation of expressed sequence tags, suggests that all five members are broadly expressed in both normal tissues and tumours. In addition, human retinal G-protein coupled receptor (RGR) has promiscuous exchange factor activity towards Ras G-protein family members, including Ral[73]. The mechanistic basis of RalGEF activation is poorly understood; however, a series of observations suggest that one important aspect involves relief of autoinhibitory interactions of amino-terminal and/or C-terminal regulatory motifs (indicated in the figure in yellow and red, respectively) with the catalytic domain (indicated in green). In the case of RGR (**a**), N-terminal truncation mutants found in lymphoma exhibit cell transformation activity in cell culture[73] and transgenic expression in thymocytes of p15INK4b (also known as *Cdkn2b*)-defective mice induce a high incidence of thymic lymphomas[74]. Consistently with this regulatory motif, 3-phosphoinositide-dependent protein kinase 1 (PDPK1) associates with the N terminus of RALGDS to relieve autoinhibition of catalytic activity in response to epidermal growth factor receptor activation[75,76]. RALGDS is also a direct effector of Ras–GTP through the C-terminal Ras association (RA) domain (**b**). Ras-dependent derepression of intramolecular autoinhibitory interactions is an emerging theme among Ras–effector relationships[2]. Finally, the RALGPS2 PXXP–PH domains have dominant inhibitory activity (**c**), decreasing Ral–GTP accumulation by 50% when expressed in HEK 293 cells. The sensitivity of RalGPS2 activity to wortmannin suggests that this domain is responsive to phosphatidyl inositol kinase activity[77]. Intriguingly, a collection of RalGEF mutations have been identified in human tumours that are distributed among sequences encoding both catalytic and regulatory regions (TABLE 1). Activating mutations in the Ras exchange factor SOS1 cause a distinctive form of Noonan Syndrome, a developmental disorder characterized by facial dysmorphia, short stature, congenital heart defects and skeletal anomalies[78].These mutations cluster in or near the N-terminal GEF domain which is important in maintaining the protein in its autoinhibited form. The functional consequences of the identified RalGEF mutations, if any exist, remain to be determined. PtdIns, phosphatidyl inositol; SH3, SRC homology 3.

been explored using both xenograft models and genetic ablation of the Ral-activating protein and Ras effector RALGDS (BOX 2) in mice.

Chris Counter's group found that chronic depletion of RALA in a large panel of human pancreatic cancer cell lines, through stable integration of shRNA expression constructs, impaired or eliminated the capacity of these cells to form tumours following subcutaneous inoculation of nude mice[9,10]. A similar study from Kathleen Kelly, in the setting of human prostate cancer, found that chronic RALA depletion inhibited bone metastasis in the absence of overt consequences on subcutaneous tumour formation[24]. Neither study implicated RALB in the generation of primary tumours; however, the Counter group found that chronic RALB depletion severely impaired or eliminated the capacity of several pancreatic cancer lines to generate lymph-node metastases following tail vein injection[10]. Interestingly, Ral-dependent gene-expression patterns that might contribute to metastatic behaviour have recently been uncovered by genomic expression profiling[25,26].

Although these studies clearly convict Ral proteins as key offenders in the maintenance of tumorigenicity, further work is needed to determine whether the biological consequences observed reflect distinct contributions of Ral signalling to tumorigenicity in diverse cell types, and/or distinct sensitivities to the tissue microenvironment, and/or differences in phenotypic penetrance as a consequence of residual RALA or RALB protein expression.

Additional genetic evidence for a cancer cell-selective dependency on Ral signalling for survival comes from the generation of mice with a homozygous deletion of *Ralgds* by Chris Marshall's group[27]. *Ralgds*-null mice are viable and overtly normal. However, using a model of topical carcinogen-induced skin papillomas, these investigators found deletion of *Ralgds* resulted in delayed onset and decreased incidence of papillomas that failed to progress to metastatic disease. Histological evaluation

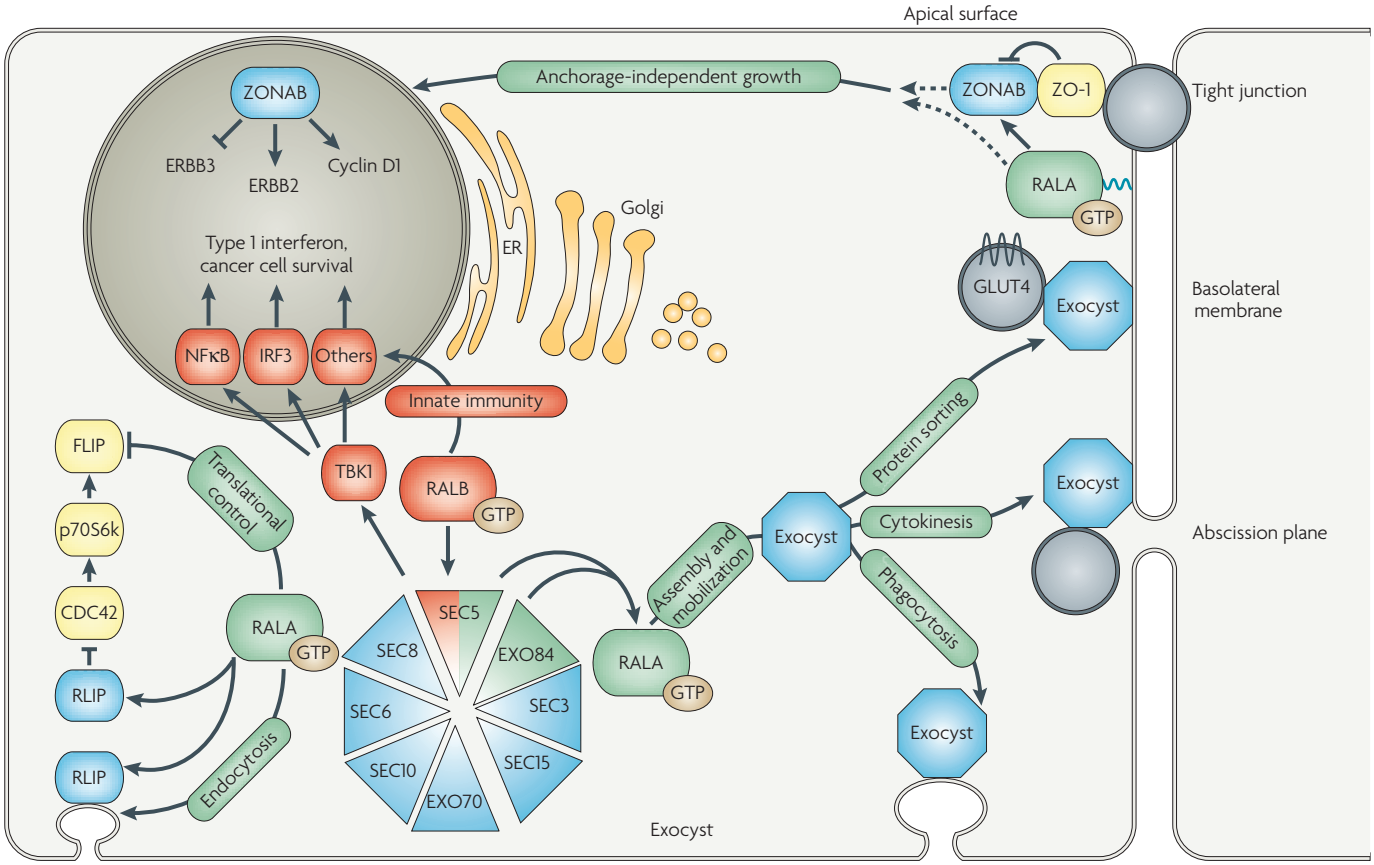


Figure 1 | **Cell biological systems modulated by direct Ral–effector relationships.** Ral proteins engage multiple effectors to direct distinct but perhaps interlocking dynamic biological processes. RALA has a major role in secretory vesicle trafficking through the exocyst, but can also participate in the regulation of gene expression and protein translation through ZO-1-associated nucleic acid-binding protein (ZONAB) and RLIP (also known as ralA binding protein 1), respectively. RALB can directly engage the SEC5 subunit of the exocyst to facilitate host defence responses. Chronic activation of RALA and RALB in cancer probably uncouples the indicated machinery from normal regulatory cues to promote pathological cell proliferation. CDC42, cell division cycle 42; ER, endoplasmic reticulum; IRF3, interferon regulatory factor 3; NFκB, nuclear factor κB; TBK1, TANK-binding kinase 1.

Exocyst complex
The exocyst is a large complex of proteins required for polarised exocytosis in eukaryotic cells. It seems to function primarily as a tether, directing secretory vesicles to specific sites on the plasma membrane.

Coated-pit endocytosis
The coated pit is a specialized region of the membrane that is coated with clathrin (for stability, to aid the transport process). The coated pit forms a coated vesicle and then loses its clathrin coat.

Clathrin adaptor proteins
Proteins that recruit clathrin to membranes and concentrate specific transmembrane proteins in clathrin-coated areas of the membrane.

of carcinogen-induced lesions indicated that proliferation rates were similar but apoptosis rates were higher in papillomas from *Ralgds*-null mice relative to their heterozygous or wild-type litter mates[27]. These observations suggest that RALGDS activity is required to deflect induction of programmed cell death that would otherwise occur in response to aberrant mitogenic signals.

Ral effectors

Despite high sequence similarity to HRAS, KRAS and NRAS, Ral G-proteins engage a distinct cadre of proximal effector proteins compared with the other 35 members of the Ras subclass of small G-proteins. This is probably the consequence of divergence of key residues in the 'effector loop' of Ral proteins, which biochemically specifies G-protein–effector interactions[2]. The most fully characterized effector relationships are outlined in FIG. 1, and include the CDC42 and Rac-family GTPase-activating protein RLIP (also known as RLIP76 and ralA binding protein 1 (RALBP1))[28,29], the Y-box transcription factor ZO-1-associated nucleic acid-binding protein (ZONAB, also known as cold shock domain protein A (CSDA))[30], and two subunits of the exocyst complex, SEC5 (also known as exocyst complex component 2 (EXOC2))[31,32] and EXO84 (also known as EXOC8)[33]. The mechanistic contributions of this functionally diverse group of proteins to RALA- and/or RALB-dependent support of tumorigenic transformation is mostly unknown; however, a number of relationships are coming to light that might begin to account for Ral-dependent modulation of cell proliferation and survival.

RLIP. RLIP is the founding member of the Ral effector family, and has been strongly implicated in the regulation of coated-pit endocytosis through association with clathrin adaptor proteins[34,35]. Short interfering RNAs that target *RLIP* can impair the survival of some human cancer cell lines in culture and can impair xenograft tumour growth[36,37]. Although it is tempting to speculate that aberrant regulation of receptor-mediated endocytosis in the context of chronic Ral activation could have profound consequences on mitogenic signal durations and pathway-specific response coupling, no evidence for this has been reported.

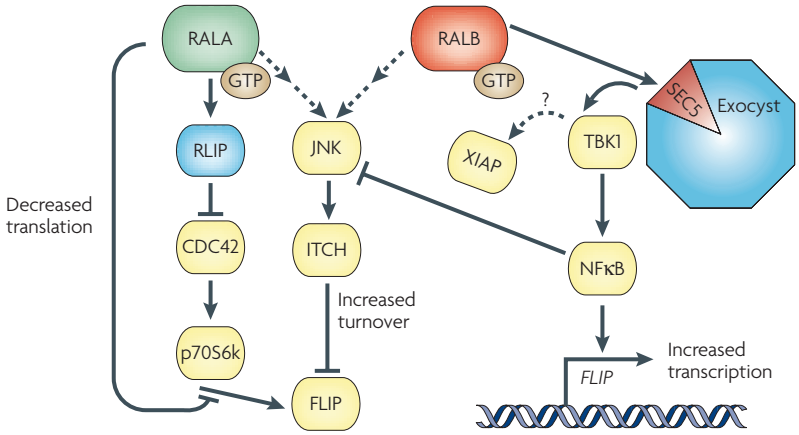


Figure 2 | **Combinatorial integration of Ral activity by FLIP.** Distinct effector use by RALA and RALB may have opposing consequences on the accumulation of the pro-survival factor FLIP. RALA can inhibit FLIP through both increased protein turnover, following activation of the Jun N kinase (JNK)–ITCH pathway, and reduced translation following inhibition of CDC42. By contrast, activation of nuclear factor κB (NFκB) by RALB would be expected to increase *FLIP* mRNA expression. TBK1, TANK-binding kinase 1; XIAP, X-linked inhibitor of apoptosis protein.

By contrast, Russell Peiper's group has recently generated mechanistic evidence that the RALA–RLIP effector pathway functions as a latent tumour-suppressor mechanism that can be engaged by tumour necrosis factor-related apoptosis-inducing ligand (TRAIL, also known as TNFSF10) to suppress translation of the anti-apoptotic protein FLIP (also known as CASP8 and FADD-like apoptosis regulator (CFLAR))[38]. As a consequence, chronic RALA activation can sensitize cells to apoptosis by weakening the brakes that restrain activation of the apoptosome. Intriguingly, two additional proteins engaged on activation of Ral — Jun N-terminal kinase (JNK, also known as mitogen-activated protein kinase 8 (MAPK8))[39] and nuclear factor κB (NFKB)[40] — can modulate FLIP protein accumulation through induction of protein turnover[41] or activation of gene expression, respectively[42] (FIG. 2). This raises the possibility that FLIP is a convergence point for combinatorial signals that couple Ral regulatory network activity to cell-death response thresholds.

ZONAB. The Y-box transcription factor ZONAB is a tight junction-associated protein[43] that was isolated in a RALA–GTP-interaction trap assay[30]. In low-density epithelial cultures ZONAB localizes to the nucleus and participates in the activation of genes that promote cell-cycle progression, including *CCND1* (encoding cyclin D1) and *PCNA* (encoding proliferating cell nuclear antigen) (FIG. 1). The formation of tight junctions between neighbouring epithelial cells allows ZO-1 (also known as tight junction protein 1 (TJP1)) to recruit ZONAB to the plasma membrane, thereby restricting its capacity to engage gene targets in the nucleus[44]. This relationship presents ZONAB as a key component of the molecular framework that couples cell density to proliferation control. Indeed, ZONAB depletion is sufficient to impair proliferation of non-tumorigenic human mammary epithelial cultures, whereas overexpression can deflect cell contact-mediated inhibition of cell-cycle progression[45,46]. Given that the latter is a hallmark of tumorigenic transformation, mobilization of ZONAB upon Ral activation becomes a compelling scenario that may partially account for the capacity of Ral protein activation to relieve proliferative restraints.

Ral and the exocyst. Regulation of the SEC6–8 complex, also known as the exocyst, is a major occupation of Ral proteins in cells, and might represent an overarching context for many of the cell biological systems that are responsive to Ral G-protein activation[5]. The exocyst is a large multiprotein complex responsible for the appropriate targeting and tethering of a subset of secretory vesicles to specific dynamic plasma membrane domains (FIG. 1), including the basolateral surface of epithelial cells, the growth cones of differentiating neuronal cells, sites of synapse formation, the mitotic abscission plane and the leading edge of migrating epithelial cells[47–49]. This has direct consequences on the establishment of appropriate cellular architecture, and the generation and maintenance of functionally specialized plasma-membrane domains. Ral proteins engage the exocyst by direct interaction with two distinct subunits, EXO84 and SEC5, to promote assembly and function of the full hetero-octomeric complex[33]. Beyond physical association, the mechanistic basis for Ral-dependent exocyst assembly and mobilization has yet to be established. However, it is clear that the Ral–excocyst regulatory node is directly employed for the maintenance of epithelial cell polarity[31], cell motility[50] and cytokinesis[51]. Deregulation of these activities would be expected to have acute consequences on tumour cell proliferation and metastasis, but direct functional relationships of the exocyst to tumour initiation or progression remain to be uncovered.

Subverting host defence signalling

Another important factor that might explain the occurrence of Ral activation in tumorigenesis is the RALB-specific contribution to cancer cell survival through activation of TANK-binding kinase 1 (TBK1)[11]. This kinase is a central node in the regulatory network required to trigger host defence gene expression in the face of a virally compromised environment[52–61]. Through a process that is tethered to the exocyst, but perhaps independent of canonical exocyst function, the RALB–SEC5 effector complex directly recruits and activates TBK1 in response to viral exposure. Although dispensable for survival of normal cells in culture, constitutive engagement of this pathway in a variety of cancer cells, through chronic RALB activation, restricts initiation of apoptotic programmes that are normally activated in the context of oncogenic stress[11]. The functional relationship of the RALB–SEC5–TBK1 activation complex with tumour cell survival indicates that oncogenic transformation can commandeer cell-autonomous host defence signalling to deflect cell-death checkpoint activation. This relationship exposes a novel aspect of the aberrant cell regulatory programmes supporting tumorigenicity, and offers the possibility that proteins like TBK1 might be conceptually ideal candidate targets for the development of drugs with a large therapeutic window (FIG. 3).

Apoptosome
A caspase-activating complex that is formed when cytochrome *c* is released from mitochondria. It initiates oligomerization of APAF1, which binds procaspase 9 and thereby initiates the caspase cascade that leads to programmed cell death.

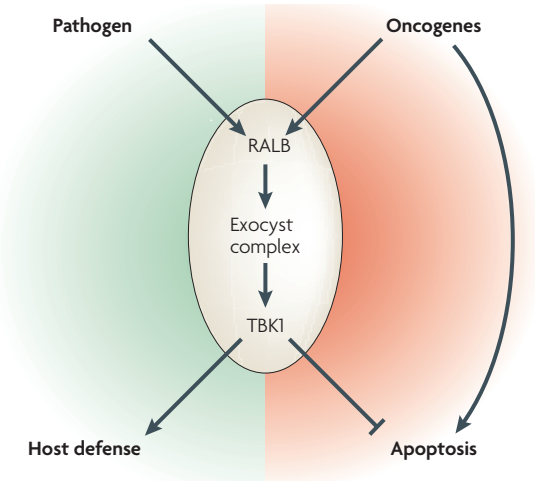


Figure 3 | **Oncogenic subversion of host defence signalling.** In non-tumorigenic epithelia, RALB participates in antiviral surveillance and response signaling by facilitating activation of TANK-binding kinase 1 (TBK1; green). Chronic activation of RALB as a consequence of oncogene expression can engage TBK1 to support cell survival in the face of oncogenic stress (red). The latter represents a conditional dependency on TBK1, loss of which can be synthetically lethal with upstream oncogenic gain-of-function mutations.

TBK1 occupies the non-canonical branch of the IκB kinase family together with IKKε (also known as IKBKE). These kinases are 48% identical at the amino-acid sequence level and share the job of directly mobilizing the interferon regulatory factor 3 (IRF3) and IRF7 transcription factors to drive the interferon response. Whereas TBK1 is constitutively expressed, IKKε is engaged as an immediate early gene product of innate immune signalling[62].

A convergent observation with the study described above was the isolation of IKKε in a screen by Hahn and colleagues for artificially myristoylated kinases that could cooperate with extracellular signal-regulated kinase activation to transform telomerase-immortalized human embryonic kidney cells[63]. Importantly, these investigators found that the genomic locus encoding IKKε is frequently amplified in breast cancers, and IKKε expression is required for breast cancer cell survival.

Innate immune signalling
The innate immune system includes phagocytes, natural killer cells, the complement system and other non-specific components. It protects against infections using mechanisms that exist before infection, providing a rapid response to microbes that is essentially the same regardless of the type of infection.

Myristoylated
Refers to the accession of fatty moieties that allow association with the inner layer of the plasma membrane.

Table 1 | **Mutations in genes encoding proteins proximal to Ral function**

Gene	Mutations in cancer	Refs
RALGDS	Colon (R496L)	79
RGL1	Breast (Y209S, V734M)	79
RGL2	Skin (W272*, G314R)	80
RGR	Lymphoma (ΔN splice variants)	73
SEC6	Breast (Y59N, A514D)	79
SEC8	Colon (S220F, A599T, frameshift)	79
TBK1	Breast (D296H), colon (G410R)	81

A pressing question is the identity of the TBK1 and IKKε substrates that support cancer cell survival and represent the heart of a potential tumour-specific vulnerability. Chronic activation of TBK1 and IKKε in cancer cell lines is correlated with increased transcriptional activity of IRF3, a canonical TBK1 substrate[58], and induction of interferon-response gene (IRG) expression[11,63]. Given the contribution of inflammatory signals in general and TBK1 in particular to a productive tumour microenvironment[64,65], together with the observation that poor patient outcome is associated with increased IRG expression in tumours[66], IRF3 stands out as a logical TBK1 and IKKε effector in cancer. However, at the cell-autonomous level, IRF3 depletion did not mimic the consequences of TBK1 or IKKε depletion on survival, suggesting that distinct substrates are responsible for this phenotype[11,63]. Although possible redundancy with IRF7 has not been explored, the caspase inhibitor X-linked inhibitor of apoptosis protein (XIAP, also known as BIRC4) has been reported as a candidate TBK1 substrate[66], and IKKε can drive chronic NFκB activation in tumour cells to deflect cell death[63,68]. The *Drosophila melanogaster* TBK1 and IKKε orthologue, ik2, drives *D. melanogaster* IAP protein turnover by promoting phosphorylation-dependent polyubiquitylation and degradation. This relationship apparently relieves restraints on non-apoptotic caspase activity rather than driving cell death[67]. The biological relevance of a conserved TBK1–XIAP relationship in mammalian cells is unknown, however it is intriguing to note that loss-of-function XIAP mutations cause X-linked lymphoproliferative syndrome.

Mutations in human cancer

The most compelling indications of the relevance of regulatory systems to the initiation or progression of cancer invariably come from human genetics. For example, numerous somatic mutations associated with disease have been identified in every regulatory node surrounding the core Ras family members, including loss-of-function mutations in GTPase-activating proteins (for example neurofibromin 1 (NF1)), and gain-of-function mutations in Ras guanyl nucleotide exchange factors (such as SOS1), Ras effectors (such as Raf proteins kinases and phosphatidylinositol 3-kinases) and the Ras proteins themselves[69]. Such evidence for the Ral regulatory network, if it exists, remains to be uncovered[70]. Corollaries to the canonical oncogenic Ras mutations that obliterate GTPase activity and therefore freeze Ras proteins in the 'on' conformation might never be forthcoming for Ral proteins owing simply to the biology of Ral effector engagement. For example, multiple observations indicate that Ral proteins must cycle between GDP and GTP conformations for productive mobilization of the exocyst. However recent large-scale cancer genome sequencing efforts have identified a cadre of mutations in genes encoding proteins proximal to Ral function, including Ral guanyl nucleotide exchange factor proteins, components of the exocyst machine and TBK1 (TABLE 1). Whether these somatic cell mutations represent a rich source of functional relevance or are simply passenger mutations that randomly accumulate during tumour evolution remains to be explored.

X-linked lymphoproliferative syndrome
This is a rare immunodeficiency disease characterized by fatal or near-fatal Epstein–Barr virus-induced infectious mononucleosis in childhood, subsequent hypogammaglobulinaemia and a markedly increased risk of lymphoma or other lymphoproliferative diseases.

Future prospects

Although numerous compounds have been identified that kill cancer cells, the difficulty in identifying drugs that are efficacious at concentrations that avoid damage to normal cells and tissues remains an obvious bottleneck in the successful translation of new therapies into the clinic. This circumstance has spurred intense efforts to identify key molecular aberrations that drive tumour initiation and progression as an initial step towards deriving agents with a high therapeutic index together with low toxicity. However, the cell-autonomous events that drive the genesis of human cancers are multifarious and complex. This context makes identification of broadly applicable targeted intervention strategies a seemingly daunting task. However, widespread evidence suggests that a unifying principle governing formation of a 'minimal oncogenic platform' is the co-dependent aberrant regulation of core machinery driving proliferation and suppressing apoptosis. Conditional dependencies on linchpin proteins engaged to drive these pathways during tumorigenesis might represent optimal intervention targets. Recent observations have indicted Ral G-proteins as key offenders in the corruption of the core cell-autonomous machinery driving oncogenic transformation. Further elaboration of the molecular systems engaged by RALA and RALB and their mechanistic relationships is required to help define the utility of targeting this network for cancer therapy.

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DATABASES

Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
Aurora kinase A | BIRC4 | *CCND1* | CDC42 | *Cdkn2b* | CFLAR | CSDA | EXOC2 | EXOC8 | HRAS | ik2 | IKBKE | IRF3 | IRF7 | ITCH | KRAS | MAPK8 | NF1 | NFKB | NRAS | *PCNA* | PDPK1 | PPP2R2B | RALA | RALB | RALBP1 | RALGDS | RALGPS1 | RALGPS2 | RGL1 | RGL2 | RGR | SOS1 | TBK1 | TJP1 | TNFSF10
National Cancer Institute: http://www.cancer.gov/
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FURTHER INFORMATION

Michael A. White's homepage: http://www.utsouthwestern.edu/findfac/research/0,2357,17914,00.html

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