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COMMENTARY

A signalling cascade for Ral

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ABSTRACT

Ras is the most mutated oncoprotein in cancer. Among the three oncogenic effectors of Ras – Raf, PI3 Kinase and RalGEF>Ral – signalling through RalGEF>Ral (Ras-like) is by far the least well understood. A variety of signals and binding partners have been defined for Ral, yet we know little of how Ral functions *in vivo*. This review focuses on previous research in *Drosophila* that defined a function for Ral in apoptosis and established indirect relationships among Ral, the CNH-domain MAP4 Kinase *misshapen*, and the JNK MAP kinase *basket*. Most of the described signalling components are not essential in *C. elegans*, facilitating subsequent analysis using developmental patterning of the *C. elegans* vulval precursor cells (VPCs). The functions of two paralogous CNH-domain MAP4 Kinases were defined relative to Ras>Raf, Notch and Ras>RalGEF>Ral signalling in VPCs. MIG-15, the nematode ortholog of *misshapen*, antagonizes both the Ral-dependent and Ras>Raf-dependent developmental outcomes. In contrast, paralogous GCK-2, the *C. elegans* ortholog of *Drosophila happyhour*, propagates the 2°-promoting signal of Ral. Manipulations via CRISPR of Ral signalling through GCK-2 coupled with genetic epistasis delineated a -Ras>RalGEF>Ral>Exo84>GCK-2>MAP3K^{MLK-1}> p38^{PMK-1} cascade. Thus, genetic analysis using invertebrate experimental organisms defined a cascade from Ras to p38 MAP kinase.

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Introduction

Ras, a small GTPase, is the most mutated oncoprotein: 19% of human cancers are estimated to harbour a mutationally activating Ras allele [1]. Unfortunately, with a few exceptions, oncogenic Ras cannot be targeted therapeutically [2]. Therefore, in recent decades researchers have focused on targeting oncogenic signalling effectors of Ras.

RAS has three main oncogenic effectors: Raf, PI3 Kinase, and RalGEF>Ral. Ras signalling through different effectors, or combinations of effectors, occurs even in the same Ras-positive tumour type [3]. Raf, a Serine/Threonine kinase, mediates the canonical ERK MAP Kinase output of Ras [4]. Ras signalling through the PI3K>PDK>Akt cascade, which can also be activated directly through receptor tyrosine kinases, has also been well studied [5]. However, even when effective small molecule inhibitors impose tumour regression, this temporary success is frequently followed by recurrence, often through re-wiring of the Ras signalling network [6]. RalGEF>Ral and its downstream signalling is much less well understood than for Raf and PI3K. Consequently, combinatorial targeting of Ras oncogenic effectors cascades is an important goal in cancer treatment.

RalGEF is directly bound by activated Ras, and functions as an exchange factor ('GEF') to promote GTP-loading and hence activation of its substrates, Ral (Ras like; RalA and RalB in mammals, with partially distinct functions [7]). Activated Ral, like activated Ras, engages with an array of binding partners, including PLC-D1, PLD, filamin, and ZONAB [8–11]. Oncogenic Ras>RalGEF>Ral signalling uses three effectors different than those used by Ras: RalBP1, Exo84 and Sec5. RalBP1 regulates diverse cellular processes, including the actin cytoskeleton and endocytosis [reviewed in 12, 13]. Exo84 and Sec5 are subunits of the exocyst, a heterooctameric protein complex that regulates exocytosis [14]. In addition to signalling through the exocyst, Ral may be an integral part of the exocyst, perhaps providing essential subcellular targeting functions [15].

Of particular note, in cancer RalB activates the kinase TBK1 to promote tumour survival [16]. RalB signalling through the exocyst also promotes autophagy and catabolic metabolism [17] and activation of TORC1 in tumour invasion [18]. The latter study also revealed a role for Ral and its heterodimeric inhibitory GAP in control of lifespan in *C. elegans*.

In *C. elegans*, Ral (RAL-1) has been implicated as an essential regulator of function of the exocyst and thereby

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Table 1. A table of conserved signalling molecules in mammals, *Drosophila* and *C. elegans*.

Mammals	*Drosophila*	*C. elegans*
HGK/MAP4K (4,6–8)	*misshapen (msn)*	MIG-15
MAP4K (1–3,5)	*happyhour (hppy)*	GCK-2
JNK (1–3)	*basket (bsk)*	JNK-1
MAP3K9/MLK	*slipper (slpr)*	MLK-1
p38 (α,β,γ,δ)	*p38*	PMK-1
Ras (H,N,K)	*ras*	LET-60
Ral (A/B)	*ral*	RAL-1
Exo84	*exo84*	EXOC-8
Notch (1,2,3)	*Notch*	LIN-12

directs polarized vesicle fusion events, and may intersect with the PAR system to control cell polarity [19–22]. With potentially hundreds of biochemical interactions with the exocyst complex, identification of Ral signalling activities downstream of exocyst signalling partners Sec5 and Exo84 has proved refractory to conventional biochemistry-based discovery approaches.

Core components of the Ras signalling network are conserved in the invertebrate model organisms *Drosophila melanogaster* and *C. elegans*. Components of Ral signals discussed in this review are shown in Table 1. Compared to multiple genes encoding orthologs in mammals (three Ras-, three Raf-, three PI3K-, four RalGEF- and two Ral-encoding genes), the genomes of *Drosophila* and *C. elegans* each harbour single genes for each [23,24]. Thus, in the absence of paralog redundancy, genetic analysis in these invertebrate systems provides an excellent way to explore Ral signalling cascades downstream of the exocyst. An additional advantage is that the use of multiple effectors by small GTPases is difficult to untangle using biochemical or cell biological approaches, but is amenable to genetic approaches, where functional sufficiency and necessity can be determined. Here, we will summarize combined efforts in using yeast two hybrid, cell culture, *Drosophila* and *C. elegans* that identified a Ral signalling cascade downstream of the exocyst, utilizing a CNH (citron N-terminal homology) domain-containing MAP4 Kinase that triggers a p38 MAP kinase cascade.

Ras-dependent development of the *C. elegans* vulva

The *C. elegans* vulva is a reproductive organ that connects the uterus to the outside environment, thus allowing sperm to enter and fertilized embryos to be extruded. Development of the vulva is an excellent model for studying developmental processes, as mutants show simple, consistent and easily observed phenotypes.

The six vulva precursor cells (VPCs), a developmental equivalence group along the ventral midline named – anterior to posterior – P3.p, P4.p, P5.p, P6.p, P7.p, and P8.p, are induced to form the vulva (Figure 1). The Anchor Cell (AC) in the ventral developing gonad emits the EGF signal that induces three of these VPCs to assume 1° and 2° cell fates in the highly reproducible 2°-1°-2° pattern. P6.p, located closest to the AC source of EGF, receives the highest dose of EGF ligand from the AC and typically assumes the central 1° fate. These cells then undergo three rounds of cell division in stereotyped lineages to generate the vulva. The remaining six VPCs more distal from the AC assume the ground, uninduced and non-vulval 3° cell fate, to yield a final pattern of 3°-3°-2°-1°-2°-3° VPC fates with 99.8% accuracy [25,26]. The presumptive 1° cell receiving EGF signal activates EGFR signalling to trigger a Ras>Raf>MEK>ERK cascade [27]. Two models describe induction of 2° VPC fate: sequential induction and graded signal. In the sequential induction model, induced P6.p both assumes 1° fate and secretes a lateral signal of DSL (Delta-Serrate-Notch) ligands to induce neighbouring P5.p and P7.p VPCs via $Notch^{Lin-12}$, which is necessary and sufficient to promote 2° fate [28–30]. In contrast, the presence of a graded signal was inferred by the ability of isolated VPCs to assume 1°, 2°, or 3° fates based on their distance from the inducing AC or dose of EGF^{LIN-3} activation of $EGFR^{LET-23}$ [27,31,32].

We [24] reconciled the sequential induction and graded signal models: both are true. Ras switches effectors during VPC fate patterning, from Ras>Raf promoting 1° fate to Ras>RalGEF>Ral promoting 2° fate in support of the major Notch/LIN-12 signal. Ras>RalGEF>Ral is necessary to interpret the lower dose 2°-promoting EGF>EGFR signal from the AC [24]. Therefore, sequential induction plays a central role in 2° VPC patterning, and a graded morphogen signal helps modulate 2° signalling by layering over the fundamental sequential induction process. The observation that Ras>RalGEF>Ral promotes 2° VPC fate led to further study of the Ral signalling cascade in the VPC model. But the next direction was unclear, because the phenotype conferred by loss of RalGEF>Ral signalling is too subtle to be identified by unbiased forward genetic screens.

A helpful hint from *Drosophila*

A key breakthrough in defining this cascade came from a study of Ral signalling using yeast two-hybrid screening of a human cDNA library with human Sec5 as bait, validation of Sec5-partner binding using human cell culture, and genetic analysis in the fruit fly, *Drosophila*, to

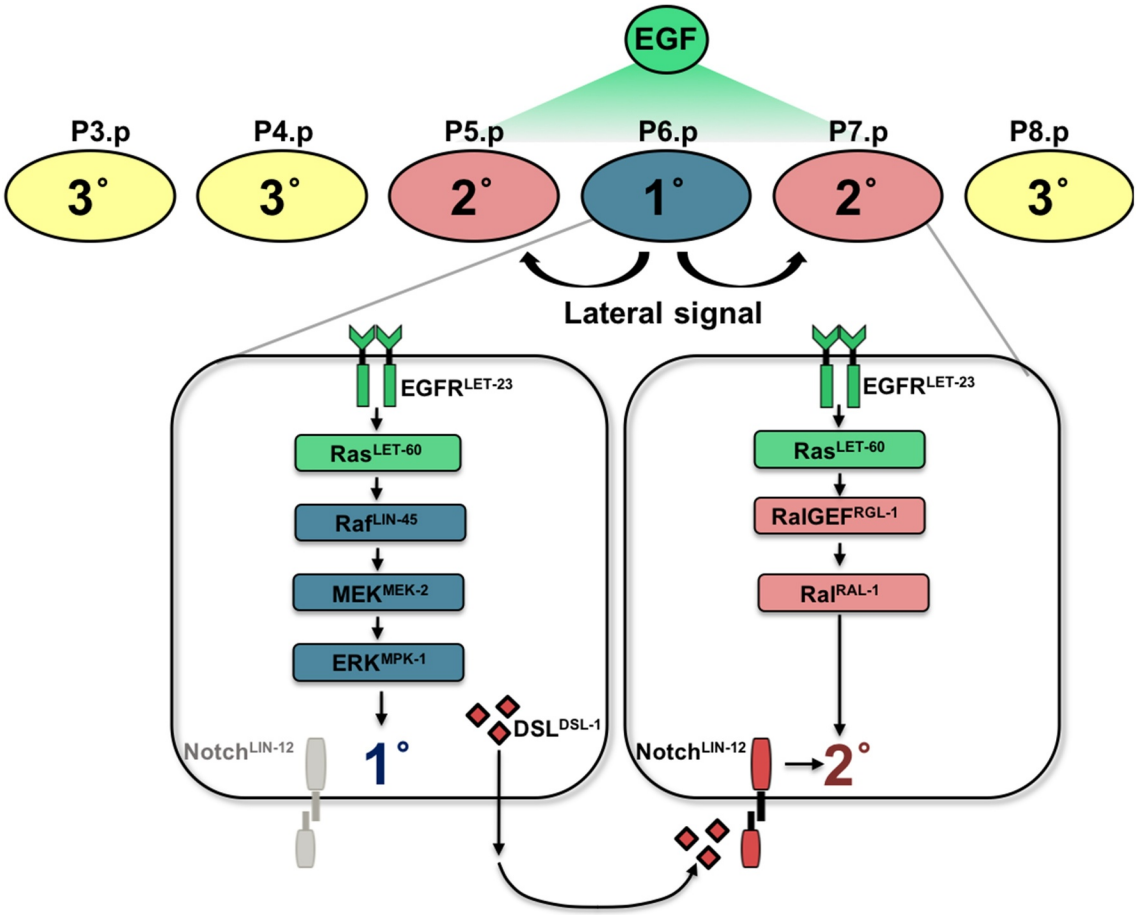

Figure 1. *C. elegans* VPC fate patterning and signalling cascades in the Ras and Notch signalling network. A) Developmental patterning of VPC fates. The anchor cell (AC) induces six equipotent VPCs to form the 3°-3°-2°-1°-2°-3° pattern of cell fates. EGFR>Ras>Raf>MEK>ERK MAP kinase cascade induces 1° fate. Through sequential induction, 1° cells induce immediate neighbours to assume 2° fate by secreting DSL ligands for the Notch receptor. The graded EGF signal also triggers activation of Ras>RalGEF>Ral to induce 2° fate in neighbouring VPCs.

determine whether candidate proteins function in the same biological process. *Drosophila* Ral was identified as an essential gene, with putative reduced function alleles identified by imprecise P element excision, which left transposon sequences in the promoter of Ral, thus decreasing detectable protein expression [33]. Reduction of Ral function and transgenic dominant-negative Ral conferred defects in development of sensory bristles, a result of excess apoptosis.

To identify potential signalling cascades downstream of the Ral-Sec5 signal [33] used yeast two hybrid (Y2H) screening with human Sec5 as bait and a human cDNA library as prey. They identified a kinase, HGK/NIK, whose binding to Sec5 was validated by reciprocal co-immunoprecipitation in cultured cells [33]. HGK/NIK is a member of the Ste20 Group of Ser/Thr kinases, specifically the GCK family, which function as MAP4 Kinases (MAP kinase kinase kinase kinases). The GCK family have distinctive N-terminal S/T Kinase and C-terminal CNH (citron N-terminal homology) domains linked by a central region containing PXXP motifs associated with binding by SH3 domains [34–36]. The GCK family has two subfamilies, GCK-I and GCK-IV. The HGK/NIK kinase identified via Y2H, also known as MAP4K4, is part of the GCK-IV group, which also includes mammalian MAP4K6/MINK, MAP4K7/TNIK, MAP4K8/NRK/NESK, *Drosophila misshapen* (*msn*) and *C. elegans* MIG-15. The paralogous GCK-I subfamily includes mammalian MAP4K1/HPK1, MAP4K2/GCK, MAP4K3/GLK, MAP4K5/KHS1/GCKR, *Drosophila* happyhour (*hppy*) and *C. elegans* GCK-2 [37].

With an apoptosis phenotype caused by reduced function alleles of Ral, double mutant strains were constructed to assess increase or decrease in apoptosis. These experiments determined that *msn*, JNK and p38 all participate in regulating bristle cell apoptosis, as do Ral-interacting exocyst components Sec5 and Exo84,

Ral non-interacting component Exo70, and non-exocyst Ral effector, RalBP1. While Ral and p38 appeared to inhibit apoptosis, *msn* and JNK promoted it (Figure 2). However, technical limitations in genetic analysis of epistasis and colinearity hampered understanding of what cascade was defined by this analysis, if any. Many alleles used were reduction of function in essential *Drosophila* genes, while others were over-expression and/or dominant negative constructs, with attendant potential artefacts. Additionally, the GCK-I subfamily member in *Drosophila, hppy*, was only identified in 2009 [38] and thus was not included in this analysis of apoptotic phenotypes [33]. Reduction of *msn* (MAP4K) and *basket* (JNK) functions caused opposite effects on apoptosis. Yet in another study, reduced function of *hppy*, the paralog of *msn*, was associated with JNK in apoptosis [39], a potential contradiction between the two analyses that may depend more on difference contexts of tissue and development than on intermolecular relationships. Therefore, this study was unable to delineate the potentially opposing relationship between MAP4Ks *hppy* and *msn* in this system, nor their relationship to Ral. Such detailed genetic analysis is possible in *C. elegans*.

Connecting the dots in *C. elegans*

As noted above, *C. elegans* also encodes MAP4 kinases in the GCK family: GCK-2 (GCK-I subfamily, fly *hppy*) and MIG-15 (GCK-IV subfamily, fly *msn*) (Figure 1). Gene knockouts of most of the *C. elegans* genes in this analysis are viable, which greatly facilitated genetic epistasis analysis. Additionally, we had identified mutant alleles of RalGEF and Ral that selectively ablated signalling activity without altering non-canonical functions of these proteins (scaffolding PDK-Akt signalling for RalGEF and exocyst functions for Ral [37,40]. Finally, the advent of CRISPR allowed us to make selective activating mutations in endogenous genes and to fluorescently tag endogenous proteins. With these advantages, we were able to delineate the Ral signalling pathway and its relationship with GCK family CNH domain-containing MAP4Ks: to promote 2° vulval fate, Ral signals through Exo84 and a cascade consisting of GCK-2/MAP4K>MLK-1/MAP3K and PMK-1/p38 MAP kinase (Figure 3).

The promotion of 2° vulval fate by Ral is a modulatory signal. As expected, none of these genes is essential to induce 2° VPCs: we did not observe any significant difference in 2° VPC patterning among single mutants for Ral, Ral effectors (Exo84, RalBP1 and Sec5), *gck-2* or *mig-15* versus wild type animals. Using CRISPR/Cas9-mediated genome editing, we created constitutively activated endogenous Ral and GCK-2, which were both sufficient to increase induction of 2° cells in support of Notch. The latter consists of an in-frame deletion of the central PXXP-containing linker in the endogenous GCK-2, which in *Drosophila* was found to constitutively activate this family of proteins [41]. Together with other *ral-1, gck-2* and *mig-15* reduced function mutations, these tools allowed us to order their encoded proteins genetically into a linear signalling cascade, but required use of sensitized genetic backgrounds to measure biological impacts: genetic perturbation of these modulatory signalling

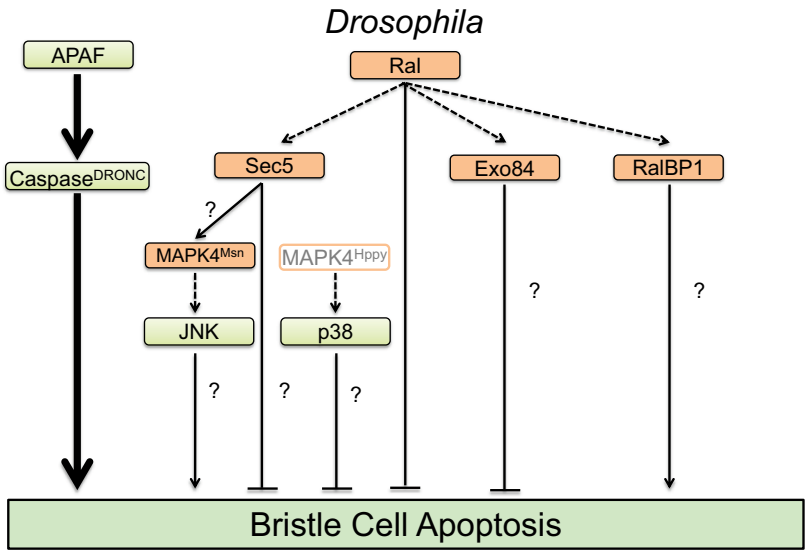

Figure 2. Potential components of the Ral signalling network. Regulatory relationships of Ral and functionally related proteins in apoptosis during bristle development in *Drosophila* as inferred by mutant interactions with hypomorphic mutations in Ral [33].

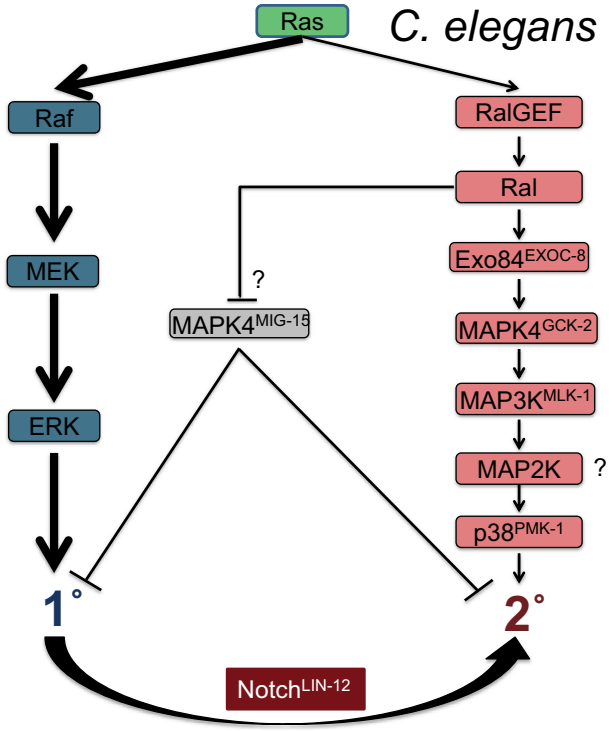

Figure 3. Regulatory relationships of the Ras>RalGEF>Ral>Exo84>GCK-2>MLK-1>PMK-1 cascade that promotes 2° VPC fate in support of Notch during *C. elegans* VPC fate patterning. The Ral>GCK-2 cascade was ordered through epistatic interactions, while MIG-15 appears to function in parallel to inhibit both 1°- and 2°-promoting signals [37].

components in an otherwise wild-type mutant background did not perturb VPC patterning.

In sensitized genetic backgrounds, mutations make animals more responsive to perturbation of modulatory genes that contribute to VPC patterning. Two main backgrounds are used for investigating signals promoting 1° and 2° fates. *let-60(n1046*gf) causes a G13E change in Ras that is constitutively activating and induces ectopic 1° cells. *lin-12(n379*d) causes a E889K change in the extracellular domain of Notch that is weakly activating: this mutation both constitutively activates Notch to induce ectopic 2° cells. *lin-12 (n379*d) also abrogates development of the AC, and thus abolishes EGF signal and simplifies the system [24,29].

In the *let-60(n1046gf)* background, both RNAi-dependent depletion and mutations reducing activation of Ral caused elevated levels of ectopic 1° cells, while constitutively activated Ral induced fewer ectopic 1° cells. In the same background, loss of Exo84 but not Sec5 or RalBP1 phenocopied reduced function of Ral, and induced more ectopic 1° cells, arguing that Ral 2°-promoting signal is propagated through Exo84.

RNAi targeting both of the MAP4Ks, GCK-2 and MIG-15, showed significantly increased ectopic 1° VPCs in *let-60(n1046*gf) background, which phenocopied reduced function of *ral-1*. But in the *lin-12(n379*d) background, loss of *mig-15* significantly increased formation of ectopic 2° cells, while reduction of Ral or GCK-2 function did not. Hence, loss of GCK-2 phenocopies loss of Ral and is consistent with Ral activating GCK-2; loss of MIG-15 is inconsistent with functioning in a positive regulatory cascade downstream of Ral. (We speculate that MIG-15 functions in yet another signal that antagonizes both 1° and 2° cell fates, perhaps through promoting 3° fate; R. Fakieh and D. Reiner, unpublished). Also, in the *lin-12(n379*d) background constitutively activated endogenous Ral and GCK-2 were both sufficient to increase induction of ectopic 2° cells. This increased induction conferred by activated Ral depends on the activity of GCK-2. VPC-specific expression of GCK-2 rescues this effect, and endogenously tagged GCK-2 is expressed throughout the animal, including in VPCs. Thus, five lines of reasoning suggest that GCK-2 fits the criteria of a molecule that propagates the 2°-promoting Ral signal: 1) loss of GCK-2 phenocopies loss of Ral; 2) loss of GCK-2 is epistatic to activated Ral; 3) constitutively activated GCK-2 phenocopies activated Ral; 4) ectopic VPC-specific expression of GCK-2 rescues the mutant defect; 5) GCK-2 is

expressed in the VPCs. Additionally, genetic analysis of $MAP3K^{MLK-1}$ and $p38^{PMK-1}$ MAP Kinase are also consistent with function in the Ral cascade. Endogenously tagged $p38^{PMK-1}$ is also expressed ubiquitously.

Taken together, these results indicate that the modulatory Ras>RalGEF>Ral 2°-promoting signal is propagated via an Exo84>GCK-2>$MAP3K^{MLK-1}$>$p38^{PMK-1}$ signalling cascade. This cascade mediates the lower dose EGF signal that promotes 2° fate, providing a mechanistic view of graded signalling: a central ERK MAP Kinase cascade downstream of Ras is necessary and sufficient for induction of 1° fate, while a p38 MAP kinase cascade downstream of Ras>RalGEF>Ral promotes 2° signalling in a modulatory role (Figure 3).

What we have learned

Looking back at the work of Balakireva *et al.*(33),we conjecture that in that apoptotic model in *Drosophila*, Ral may signal through *hppy* (GCK-2-like) and not *msn* (MIG-15-like), but the authors did not know of *hppy* at that time and so it was not tested [33]. Perhaps Sec5-*msn* signalling promotes apoptosis in opposition to Ral-*hppy*. Similarly, it is tempting to speculate that the activation of *Drosophila hppy*, originally identified in a *Drosophila* behavioural model by reduction of function mutations that increase resistance to ethanol treatment [38], also functions downstream of Ral, though that concept has not been tested. However, it is unclear whether the relationships of these MAP4Ks with cognate small GTPases like Ral are conserved throughout different developmental contexts.

The same caveat also extends to kinases functioning downstream of GCK-2-like and MIG-15-like kinases: is p38 associated with GCK-2-like (GCK-I subfamily) and JNK with MIG-15-like (GCK-IV subfamily) in every biological context in which they operate? We doubt it. A plethora of studies in cell culture of GCK family MAP4Ks of both subfamilies are contradictory on their associations with p38 and JNK, yet many of these rely on over-expression of the relevant kinase. In *Drosophila, msn* controls dorsal enclosure of the embryo upstream of the *basket* ortholog of JNK [42,43]. Perhaps, CNH domain-containing MAP4Ks are 'mix and match' with p38 and JNK signalling, similarly to general observations with MAP3Ks and MAP2Ks and p38 and JNK signalling. This question remains to be determined in systems that permit exacting analysis of pathway relationships.

We also observed an unexpected property of endogenous p38 (*C. elegans* PMK-1) *in vivo*. Using tagged endogenous PMK-1, we explored the possibility of cytosol-to-nuclear translocation of PMK-1 in presumptive 2° cells as a function of activation by upstream Ral and GCK-2. Such behaviour was described for p38 in other systems [44]. The C-terminal CRISPR tag of endogenous PMK-1 did not alter 2°-promoting activity [40]. Yet we observed that a subset of the cytosolic pool of tagged endogenous PMK-1 was tonically translocated to nuclei in every somatic cell in the animal. We also expressed single-copy PMK-1, tagged at the N-terminus of the protein and with a different fluorescent tag, only in VPCs. Still we observed tonic nuclear localization of a subset of the cellular protein pool in all six VPCs. We posit that PMK-1, a MAP kinase typically associated with anti-inflammatory functions, is constitutively activated at low levels throughout the animal, at least under standard laboratory growth conditions for *C. elegans*.

At an upstream level in the signalling network, in the *Drosophila* model for the impact of ethanol on animal behaviour, the effect of mutated *hppy* is in opposition to the effect of mutated EGFR signalling through Ras>Raf [38]. Analogously, the Ral>GCK-2 2°-promoting signal in VPC patterning also acts in opposition to the EGFR 1°-promoting signal. It is unclear whether such relationships are conserved throughout biological events within the same animal or across evolution. Frequently in signal transduction, signalling cascades or modules are conserved through evolution, but relationships between cascades are not conserved. Instead, the needs of each system are likely satisfied through assembly of signalling modules to perform the needed function. Consequently, we imagine that status of certain kinases downstream of Ral would need to be validated in different tumour types with elevated levels of activated Ral. But at least now we have candidate molecules to assay, which could define biomarkers and druggable targets for Ral-driven tumours.

The main era of discovery of novel signal transduction pathways has passed. Yet the described studies in invertebrate genetic model systems, coupled with yeast two-hybrid screening and biochemical validation in cell culture, illustrate that we are still able to delineate novel associations and signalling cascades. Modern biochemical discovery methodologies, like immunoprecipitation-mass spectrometry or proximity labelling-mass spectrometry, may discover further identify molecules whose signalling functions downstream of Ras and Ral can be validated *in vivo* using *C. elegans* or *Drosophila* genetics, thus distinguishing those binding partners in the Ras network that have biological importance [45]. Conversely, Y2H-interactome studies coupled with similar biochemical discovery modalities in *C. elegans*, including tissue-specific approaches, may facilitate identification of novel players in Ral signalling [46,

47, reviewed in 48]. Additionally, the described use of gain-of-function mutations in endogenous Ral [37] may facilitate novel Ral functions than can be further explored via genetics.

Our research has established a signal transduction cascade from Ras to Ral to CNH-domain MAP4 kinase to p38 MAP kinase during the development of the *C. elegans* vulva. These results also illuminate the signalling mechanism by which different cell fates in a gradient of ligand are promoted by distinct signal transduction cascades, of great interest to developmental biologists. Additional novel mechanisms by which such signals are propagated remain to be discovered. A single facet of Ral signalling has been illuminated. With the use of invertebrate genetic model organisms, many additional insights into Ral biology are likely to be gained.

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Disclosure statement

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