Published in final edited form as:
Dev Cell. 2011 January 18; 20(1): 84–96. doi:10.1016/j.devcel.2010.12.004.

Ras Effector Switching Promotes Divergent Cell Fates in *C. elegans* Vulval Patterning

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SUMMARY

The *C. elegans* vulva is patterned by epidermal growth factor (EGF) activation of Ras to control 1° fate, and 1° fate induces antagonistic Notch-dependent 2° fate. Furthermore, a spatial EGF gradient, in addition to inducing 1° fate, directly contributes to 2° fate via an unknown pathway. We find that in addition to its canonical effector, Raf, vulval Ras utilizes an exchange factor for the Ral small GTPase (RalGEF), such that Ras-RalGEF-Ral antagonizes Ras-Raf pro-1° fate activity. Consistent with its restricted expression pattern, Ral participates in EGF pro-2° activity. Thus, we have delineated a Ras effector-switching mechanism whereby position within the morphogen gradient dictates that Ras effector usage is switched to RalGEF from Raf to promote 2° instead of 1° fate. Our observations define the utility of Ras effector switching during normal development, and may provide a possible mechanistic basis for cell and cancer type differences in effector dependency and activation.

INTRODUCTION

An emerging complexity of mammalian Ras signal transduction is the assortment of catalytically diverse effectors that may facilitate the elaborate biological activities of Ras in normal and neoplastic cells. The precise role that each effector serves, dynamic regulation of effector utilization, and interplay between effector networks are issues that remain poorly understood. Analysis of *C. elegans* vulval development has provided key insights into Ras signaling components and concepts conserved in mammalian cells. The vulval precursor cells (VPCs) are a developmental equivalence group of six ventral epithelial cells (P3.p–P8.p) (Sternberg, 2005) (Figure 1A). The nearby anchor cell (AC) induces VPCs to assume a highly reproducible 3°-3°-2°-1°-2°-3° pattern of fates. The AC-proximal VPC is induced to assume the 1° fate, flanking VPCs assume the 2° fate, and distal uninduced VPCs assume the non-vulval 3° fate.

Along with studies in other systems, analyses of the pro-1° AC inductive signal were instrumental in delineating the first signal transduction pathway connecting the cell surface to the nucleus (Egan and Weinberg, 1993). The AC secretes LIN-3/EGF (epidermal growth factor), which promotes LET-60/Ras activation of the LIN-45/Raf-MEK-ERK mitogen-

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activated protein kinase (MAPK) signaling cascade to regulate the LIN-1 (Ets) and LIN-31 (HNF) transcription factors, thereby inducing 1° fate (Sundaram, 2006) (Figure 1B). Analogously to human cancers, mutational activation of LET-60/Ras promotes ERK activation, leading to excess vulval induction (Figures 2A and 2B), while loss of pathway components results in vulval absence. All constituents of this signaling pathway, particularly LET-60/Ras, are strongly conserved (Figure S1C). This degree of conservation argued, prematurely, that our understanding of Ras effector signaling was complete. However, subsequent mammalian cell studies characterized additional Ras effectors, with now at least 10 distinct functional classes identified (Repasky et al., 2004). With many effectors expressed ubiquitously, an unresolved issue is how Ras effector utilization is orchestrated to facilitate the complex biological outputs of Ras.

Recent analyses have implicated the guanine nucleotide exchange factor for the Ral GTPase (RalGEF) as an effector of importance comparable to Raf in Ras-dependent human oncogenesis (Chien and White, 2003; Hamad et al., 2002). Like Ras, Ral functions as a GDP/GTP-regulated switch. Since RalGEF and Ral are conserved in *C. elegans*, EGF activation of Ras could involve the RalGEF-Ral pathway in regulation of vulval cell fate.

Two competing models have been proposed to illustrate the mechanisms of vulval fate patterning. The original “morphogen gradient model” posits that a LIN-3/EGF, AC-maximal concentration gradient differentially patterns VPCs dictated by proximity to the AC (Katz et al., 1995; Katz et al., 1996; Sternberg and Horvitz, 1986, 1989). This model posits that while strong EGF signal induces 1° fate, diminished EGF signal directly promotes 2° fate for more distal VPCs. Appropriately, an ERK-responsive 1° fate reporter was highly expressed in the presumptive 1° VPC, with transient low expression in presumptive 2° VPCs (Yoo et al., 2004), but further mechanistic support is lacking.

In contrast, the “sequential induction model” proposes that EGF induces only the most proximal VPC, which becomes 1°. Subsequently this presumptive 1° cell expresses DSL ligands that, via the LIN-12/Notch receptor, laterally induce neighboring VPCs to assume 2° fate (Chen and Greenwald, 2004). Accordingly, the LET-23/EGF receptor (EGFR) is necessary for 1° but not 2° fate induction (Koga and Ohshima, 1995; Simske and Kim, 1995), and pro-1° EGF and pro-2° Notch pathways together are necessary and sufficient to generate initial commitment to the 2°-1°-2° fate pattern (Greenwald, 2005; Sternberg, 2005). However, the “sequential induction model” and the “morphogen gradient model” have yet to be mechanistically reconciled.

Importantly, 1° and 2° fates are mutually antagonistic; via “quenching” mechanisms inappropriate pathway activities are reduced to minimize conflicting pro-1° and pro-2° signals in the same cell. Presumptive 1° cells enact programs that antagonize pro-2° signaling (Levitan and Greenwald, 1998; Yoo and Greenwald, 2005), and conversely presumptive 2° cells enact programs that antagonize pro-1° signaling (Berset et al., 2001; Berset et al., 2005; Yoo et al., 2004). For example, the LIP-1 ERK protein phosphatase is expressed in presumptive 2° lineages to quench ERK signaling. Thus, the developmental consequences of EGF activation of Ras-Raf signaling in 2° lineages may be minimal, and pro-2° EGF activity mediated through distinct effector pathways.

The most plausible composite model for robust vulval patterning would be one that reconciles these three evidence-based models: graded morphogen signaling, sequential induction, and pathway quenching. Yet nothing is known about the mechanism of the putative pro-2° EGF signal. Despite decades of research into how a single morphogen gradient can induce multiple cell fates, there are few instances in which the mechanism of such differential inductions is understood (Piddini and Vincent, 2009).

In this study, we identify a mechanism for EGF pro-2° signaling and thus reconcile the three features of vulval patterning into a unified model. We show that during vulval patterning Ras through Raf transduces a pro-1° signal, then through the RalGEF-Ral pathway transduces a pro-2° signal. Ral signaling antagonizes Raf and regulates the balance of 1° and 2° fates. Ral is necessary and sufficient to drive maximal Notch pro-2° activity, and the RalGEF-Ral pathway is quenched in presumptive 1° cells by restricted Ral expression. In summary, our study establishes that Ras effector utilization is controlled to signal for distinct cellular outcomes. Analogous mechanisms may therefore contribute to the distinct patterns of effector utilization that occur in different settings of mutant Ras-driven human cancers.

RESULTS

C. elegans Contains Single RalGEF and Ral Orthologs

The *C. elegans* genome contains single RalGEF (*rgl-1*; F28B4.2) and Ral (*ral-1*; Y53G8AR.3) genes. *rgl-1* encodes predicted splice variants producing proteins of 860 and 880 residues that share the identical domain architecture with human Ras-GTP Association (RA) domain-containing RalGEFs: an N-terminal Ras Exchange Motif (REM), a central CDC25 homology (RasGEF) catalytic domain, and a C-terminal RA domain (Figure S1A). *ral-1* encodes a predicted protein of 213 residues consisting of a GTPase domain and C-terminal membrane-targeting sequence sharing strong sequence identity (61–65%) with human RalA and RalB (Figures 1C and S1B). The effector binding regions of human and *C. elegans* Ral GTPases share high identity, suggesting common effector utilization; Ral effector orthologs are also conserved in *C. elegans*. The strong conservation of the RalGEF effector pathway components suggests an important role in *C. elegans* LET-60/Ras function.

RGL-1-RAL-1 Antagonizes Ras-Raf in Ras-mediated Vulval Development

Genetic dissection of LET-60/Ras signaling in vulval development was instrumental in delineating the Raf-MEK-ERK pathway in mammalian cells. Consequently, we used multiple genetic approaches to dissect the role of RGL-1 and RAL-1 in Ras-directed vulval development. In a moderately activating gain-of-function (gf) LET-60 background (*let-60* allele *n1046*; G13E mutation), we introduced *rgl-1(RNAi)* or *ral-1(RNAi)*. Unexpectedly, we found increased, rather than decreased, vulval hyper-induction (Figure 2C). Negative control RNAi targeting *gfp* (green fluorescent protein) and *lin-3* (encoding EGF, acting upstream) had no effect, while positive control RNAi targeting *gap-1* (encoding RasGAP, a negative regulator of LET-60 activity) enhanced the *let-60*(gf) phenotype. *rgl-1* or *ral-1* knockdown in a wild-type (WT) background caused no defect (data not shown), suggesting a modulatory role for RGL-1-RAL-1 signaling.

A caveat is that the RGL-1/RAL-1-dependent phenotypes could be specific to *let-60*(*n1046*gf), for example if the *in situ* activating mutation conferred inappropriate LET-60/Ras utilization of RGL-1. We ruled out this concern by showing that *rgl-1(RNAi)* and *ral-1(RNAi)* enhanced vulval hyper-induction due to LIN-3/EGF over-expression, an activating LET-23/EGFR mutation, and a transgene expressing activated LIN-45/Raf, and suppressed the under-induced phenotype conferred by reduced function mutations (rf) in *lin-3*/EGF or *let-23*/EGFR (Figures S2A–E).

To corroborate these RNAi results with an independent methodology, we also analyzed a *ral-1* deletion (Δ), *tm2760*, which removes a portion of intron 3 including the splice donor site. Presumably intron 3 splicing is blocked, resulting in strong loss of function. *ral-1*(Δ) enhanced the *let-60*(gf) hyper-induced phenotype (Figure 2D).

Dominant negative (dn) RAL-1(S31N), predicted to sequester and inactivate its GEF (Urano et al., 1996), should also enhance activated LET-60-driven pro-1° activity. We generated *let-60*(gf) animals harboring *ral-1*(dn) driven by the VPC-specific *lin-31* promoter (P_{lin-31}) (Tan et al., 1998). The hyper-induced vulval phenotype of *let-60*(gf) animals expressing RAL-1(dn) was enhanced compared to non-transgenic siblings (Figure 2E). In contrast, VPC-specific expression of RAL-1(gf) (gain-of-function, Q75L) suppressed the hyper-induced vulval phenotype of *let-60*(gf) animals relative to their non-transgenic siblings (Figure 2F), while RAL-1(gf) caused no defect in a WT background (data not shown). Control VPC-specific expression of WT RAL-1 did not alter the *let-60*(gf) phenotype, indicating that our VPC expression system is phenotypically neutral (data not shown).

Taken together, these data support four conclusions. First, RGL-1 and RAL-1 antagonize the canonical Ras-Raf-MEK-ERK pro-1° signal. Second, RGL-1 and RAL-1 function comparably in vulval patterning and likely comprise a RGL-1-RAL-1 signaling module. Third, RGL-1 and RAL-1 function cell autonomously in VPCs. Fourth, neither loss nor gain of RGL-1 or RAL-1 function in a WT background perturbed vulval patterning, suggesting that RGL-1 and RAL-1 are not part of core pro-1° or pro-2° induction pathways, but rather are modifiers of LET-60/Ras stimulated vulval patterning signals. Other comparable pathway modifiers, both positive and negative, have been identified in sensitized genetic screens. Perturbation of these genes caused no phenotype alone, but collectively they exert a profound influence on vulval patterning (Berset et al., 2001; Berset et al., 2005; Sundaram, 2006; Yoo et al., 2004; Yoo and Greenwald, 2005).

RAL-1 Contributes to the 1°/2° Fate Decision

To evaluate directly whether disruption of *ral-1* perturbs cell fate specification, we used a P_{egl-17}::*cfp-LacZ* transgene as a reporter of 1° cell fate (Yoo et al., 2004). Notch-dependent lateral signal normally prevents formation of neighboring 1° cells. However, when the 1°–2° signaling balance is genetically disrupted to favor 1° fate, the frequency of neighboring 1° lineages increases (Berset et al., 2001; Berset et al., 2005; Yoo et al., 2004). In a *let-60*(gf) background, *ral-1(RNAi)* increased significantly the number of adjacent CFP-positive lineages (Figures 3A–C and S3A–C). Because *gfp*-directed RNAi inhibits CFP expression, *daf-3(RNAi)* rather than *gfp(RNAi)* was validated and used as a negative control (Figure S3D). We conclude that RAL-1 activity promotes 2° fate at the expense of 1° fate, either indirectly by antagonizing 1° fate, or directly by promoting 2° fate.

LET-60/Ras Mediates Genetically Separable Pro-1° and Antagonistic Signals

RGL-1 was identified previously in a yeast two-hybrid screen with activated LET-60 bait, but further characterization was not pursued (Shibatohge et al., 1998). We hypothesize that LET-60/Ras binds and activates RGL-1 to antagonize the Ras-Raf pro-1° signal, and thus under certain conditions loss of LET-60/Ras should be functionally equivalent to loss of RGL-1 and RAL-1. However, LET-60/Ras activation of LIN-45/Raf is an essential event during vulval induction, so we could not directly assess the necessity of LET-60 for RGL-1 activation. To address this question we used hyper-inducing mutations in downstream genes, thought to be independent of upstream Ras-Raf-MEK-ERK activity. In vulval induction the principal pro-1° transcription factors are inhibited by ERK. Consequently, the *lin-31(n301)* null mutation (“0”) and the *lin-1*(*e1275*ts) reduced function mutation (“rf”) confer moderate hyper-induction. RNAi targeting *lin-45*/Raf and *mpk-1*/ERK had no effect on hyper-induction, indicating that *lin-31*(0) and *lin-1*(rf) are Raf-MEK-ERK independent. *lin-45*/Raf and *mpk-1*/ERK RNAi were validated separately for activity (Figures S3D–F and not shown). In contrast RNAi of *let-60*, *rgl-1* or *ral-1* enhanced the *lin-31*(0) and *lin-1*(rf) hyper-induced phenotypes (Figures 3D and 3E), suggesting that LET-60/Ras functions similarly to RGL-1-RAL-1 in antagonizing Ras-Raf, and that this signal functions parallel to or

downstream of LIN-31/LIN-1 transcription factors. We also repeated this result with mutations; partial loss of *let-60*/Ras but not *lin-45*/Raf enhanced *lin-31*(0) (Figure 3F). These data further suggest that in addition to the canonical pro-1° Ras signal through Raf-MEK-ERK, Ras also signals through RGL-1-RAL-1 to antagonize Ras-Raf.

Ectopic LET-60/Ras Is Sufficient to Induce RGL-1-RAL-1 Activity

LET-60 shares 73–77% identity with human Ras proteins, with 100% identity in the core effector-binding domain (32–40; Figure S1C). To assess whether Ras is sufficient to activate RGL-1, we used mutationally activated LET-60 (*let-60*(*12V*)) with missense mutations that result in differentially impaired effector binding. The E37G mutation retains effective interaction with RalGEF but not Raf or PI3K, whereas the T35S mutation retains Raf but not PI3K or RalGEF binding (Rodriguez-Viciana et al., 1997; White et al., 1995). We generated otherwise WT animals harboring transgenes driving VPC-specific expression of *let-60*(*12V*) (general gf) or *let-60(12V,35S)* (Raf gf). As expected, both transgenes caused a Raf-dependent hyper-induced phenotype (Figures S3E and S3F). In contrast, transgenic VPC-expressed *let-60(12V,37G)* (RalGEF gf) suppressed the hyper-induced phenotype of *let-60*(gf) animals compared to non-transgenic siblings (Figure 3G), equivalent to the effect of *ral-1*(gf) (above) and consistent with RalGEF-selective activity. Control VPC-specific expression of *let-60*(+) in the *let-60*(gf) background caused no phenotype (data not shown).

While the H-Ras(12V,37G) effector-binding mutant is impaired in its ability to activate PI3K and Raf, the mutant protein still retains the ability to bind other Ras-binding proteins in addition to RalGEF (Kelley et al., 2001). However, we showed that the LET-60(12V, 37G) phenotype is entirely RGL-1- and RAL-1-dependent (Figure 3G), suggesting that the LET-60(12V,37G) phenotype is not due to signaling through other effectors. Thus, we conclude that Ras can signal through RalGEF in vulval fate specification.

In mammalian cells Ras is thought to be the exclusive small GTPase activator of RalGEFs, but in *Drosophila* the Rap family of Ras-related small GTPases has been implicated in RalGEF activation (Mirey et al., 2003; Rodriguez-Viciana et al., 2004). Our results argue that in the vulva, it is Ras that signals through RalGEF, but we also tested vulval function of characterized *C. elegans* Rap proteins. Injected *rap-1(RNAi)* or *rap-2(RNAi)* failed to enhance the *let-60*(gf) phenotype (not shown), suggesting that Raps are not required for the RGL-1-RAL-1 signal. Previously, RAL-1 was shown to function redundantly with the Rap ortholog RAP-1 in essential epithelial morphogenesis, while loss of both *rap-1* and *rap-2* is synthetic lethal (Frische et al., 2007; Pellis-van Berkel et al., 2005). For these reasons Raps were not more extensively analyzed, but multiple lines of evidence argue that Ras activates RGL-1-RAL-1 in vulval development as a bona fide vulval signaling module.

RAL-1 Cooperates with Notch to Specify 2° Vulval Fate

1° and 2° fates are mutually antagonistic, such that loss (or gain) of one pathway boosts (or suppresses) the other. In this context there are two interpretations of our data thus far. First, Ras-RalGEF-Ral could be an anti-1° pathway that impinges on the pro-1° pathway downstream of the LIN-1 and LIN-31 transcription factors. Second, Ras-RalGEF-Ral could function as an alternative signaling output of the EGF signal to promote 2° fate, thus finally providing a mechanism for the morphogen gradient model. This second model is consistent with the described mutual 1° vs. 2° antagonism in conjunction with our observations, as follows. First, loss of a pro-2° signal (*e.g.* loss of *rgl-1* and *ral-1*) should enhance the pro-1° activity of *let-60*(gf). Second, activation of a pro-2° signal (*e.g.* ectopic activated RAL-1 or RalGEF-selective Ras), should suppress the pro-1° activity of *let-60*(gf). Third, loss of a pro-2° signal (*e.g.* loss of *let-60*, *rgl-1* and *ral-1*) in the ERK-independent hyper-induced transcription factor mutants should enhance their hyper-induced phenotypes. Therefore, we

hypothesize that Ras-RalGEFRal promotes 2° fate, but our experiments thus far have only examined Ras, RalGEF and Ral function in pro-1° assays.

To evaluate the second model we used sensitized dominant activated (d) LIN-12/Notch backgrounds. *lin-12*(*n302*d) and *lin-12*(*n379*d) mutant animals have two features critical for our study. First, they lack an AC, the source of EGF. Second, their activated pro-2° signal is relatively weak, and thus sensitive to further stimulation (Greenwald et al., 1983). Importantly, since there is no AC, the Notch activity assayed is likely to be LIN-3/EGF-independent. VPC-specific expression of activated *ral-1*(gf) significantly enhanced *lin-12*(d) excess 2° cell phenotypes (Figures 4A–C and S4A). Ectopic *ral-1*(gf) also enhanced *glp-1*(q35d)/+ (Figure S4B); *glp-1* encodes the second *C. elegans* Notch receptor (Mango et al., 1991). RAL-1 is therefore sufficient to promote Notch pro-2° activity.

Additionally, expression of a RalGEF-selective LET-60(12V,37G) transgene, described above, in the *lin-12*(*n302*d) background was sufficient to drive 2° fates, and this activity was entirely *rgl-1*- and *ral-1*-dependent (Figure 4D). We note that as predicted from the 1° vs. 2° mutual antagonism, LET-60(12V,37G) transgenes confer opposite effects in *let-60*(*n1046*gf) pro-1° (Figure 3G) vs. *lin-12*(d) pro-2° backgrounds (Figure 4D). As an alternative test of this same principle, we constructed a strain containing *lin-12*(*n379*d), *let-60*(*n1046*gf) and the *lin-45*/Raf loss-of-function mutation, *n2506*. In this strain LET-60/Ras was active, but Raf pro-1° signaling was abrogated. We observed strong Ras-dependent enhancement of the *lin-12*(d) 2° induction, and this effect was significantly suppressed by *ral-1(RNAi)* (Figure 4E). Thus, Ras is sufficient to drive 2° fate, and Ras requires RalGEF and Ral for this activity.

To determine whether RAL-1 is also necessary for LIN-12/Notch function, we used *ral-1(RNAi)* in weakly activated *lin-12*(*n302*d) and *lin-12*(*n379*d) mutant animals, as well as the moderately activating *lin-12*(*n676*d) and strongly activating *lin-12*(*n950*d) and *lin-12*(*n952*d) mutant animals. In no case did we observe *ral-1(RNAi)* suppression of the *lin-12*(d) excess 2° phenotype (data not shown). This result is expected if RAL-1 is required only for the EGF pro-2° signal, and not the Notch pro-2° activity per se, and thus loss of RAL-1 had no consequences in the absence of EGF signal.

To evaluate this model further we determined RAL-1 necessity for LIN-12/Notch pro-2° function under EGF-dependent conditions. A *lin-12*/Notch hypomorphic allele (rf) confers mildly compromised 2° induction, yet the AC is still present and a single vulval invagination forms (Sundaram and Greenwald, 1993). In this background, *ral-1(RNAi)* caused modest but significant losses of 2° lineages and increased morphogenetic defects (Figure 4F). We propose that *ral-1* is necessary for full LIN-12/Notch pro-2° activity, but only under EGF-dependent conditions. Together, these observations suggest that Ras-RalGEF-Ral mediates an EGF signal that promotes 2° fate, and we tested this hypothesis.

EGF Levels Insufficient for 1° Induction Can Induce 2° Fate through Ras and Ral

EGF was shown previously to be sufficient to induce 2° cells in the absence of neighboring 1° cells, arguing that there exists an EGF pro-2° signal (Katz et al., 1995; Katz et al., 1996; Sternberg and Horvitz, 1986, 1989). To examine a putative RAL-1 function in propagating the pro-2° EGF signal, we used two reagents to develop a robust EGF pro-2° signaling assay. First, *lin-12*(*n379*d), described above, is a weakly activating Notch mutation that abolishes AC development in ~90% of animals and weakly induces an ectopic 2° phenotype (Greenwald et al., 1983). Second, to titrate EGF levels with temperature, we added to the *lin-12*(d) background the temperature sensitive *lin-15*(*n765*ts) mutation, which at 15°C caused no ectopic 1° induction but at 25°C is strongly hyper-induced via ectopic EGF expression. *lin-15* encodes components of a transcriptional regulatory complex that

represses LIN-3/EGF expression outside of the AC, in the epithelia surrounding the VPCs (Cui et al., 2006).

We compared the *lin-12*(d); *lin-15*(ts) double mutant to *lin-12*(d) and *lin-15*(ts) single mutant strains from 15° to 18°C (Figure 5A). *lin-12*(d) is not temperature sensitive; at all temperatures ~10% of animals had a normal AC/vulva and animals averaged ~0.4 ectopic invaginations that we judged to be 2° based on morphological criteria (the distal 2° lineage cells adhere to the cuticle, while the proximal cells invaginate) (Katz et al., 1995). In *lin-15*(ts) single mutant animals, we observed no and rare ectopic vulval induction at 15°C and 16°C respectively; however, since all animals had an AC they formed normal vulvae. But *lin-15*(ts) ectopic induction greatly increased at 17°C and 18°C, and these invaginations contained combined 1° and 2° lineages typical for ectopic pro-1° signaling. To better contrast the *lin-15*(ts) single mutant ectopic pro-1° phenotype with the double mutant *lin-12*(d); *lin-15*(ts) pro-2° phenotype, below, we show only ectopic pseudovulvae induced in the *lin-15*(ts) single-mutant background, and excluded WT vulvae (Figure 5A, red).

Strikingly, in the double mutant strain we observed strong synergy at 15°C and 16°C ($P<0.00001$ for both), temperatures at which *lin-15*(ts) alone was not sufficient to induce ectopic 1° invaginations (Figure 5A). By morphology these excess invaginations were 2°, indicating that sub-threshold EGF activity in a sensitized background induced large numbers of 2° cells. To verify that the observed *lin-15*(ts) effect was EGF-dependent, we targeted *lin-3*/EGF with RNAi and observed suppression of *lin-15*(ts) synergistic phenotypes (Figure 5B). Including the *mgIs21 lin-11* promoter::*GFP* fusion transgene in the *lin-12*(d); *lin-15*(ts) strain as a reporter of 2° fate verified that these ectopically induced cells were mostly 2° cells (Figure 5E and 5F). Thus we demonstrated that the putative EGF pro-2° signal cooperates with the Notch pro-2° signal to specify 2° fate, a property predicted to increase fidelity of vulval patterning. Furthermore, we have precisely controlled EGF input into 2° fate induction.

A parsimonious working model posits that in presumptive 2° cells, EGF-activated Ras signals preferentially through RGL-1 rather than Raf. A prediction of this model is that LET-60/Ras and RAL-1 activity are necessary for full LIN-3/EGF pro-2° signaling output. Our system for studying this EGF signaling property allowed us to analyze sufficient numbers of animals to evaluate our model. We subjected *lin-12*(d); *lin-15*(ts) animals grown at 16°C to *let-60*- (Figure 5B) or *ral-1*-directed RNAi (Figure 5C). Importantly, loss of both *let-60* and *ral-1* suppressed the level of 2° hyper-induction, as did control loss of *lin-12*. As an internal control for *lin-12(RNAi)* efficacy, we observed dramatic suppression of the *lin-12*(d) absent-AC defect (from 13.2% with *gfp*(*RNAi*) to 76.5% with *lin-12(RNAi)*) (data not shown).

We also performed *lin-45(RNAi)* in this background, and found that loss of LIN-45/Raf enhanced 2° fate induction, consistent with the pro-1° function of Ras-Raf (Figure 5B). We hypothesize that naïve presumptive 2° VPCs activate both Raf and RalGEF in response to EGF signal, such that loss of the antagonistic Raf derepresses general pro-2° signaling.

To corroborate the *lin-15* system we reproduced the *lin-12*(d); *lin-15*(ts) phenotype with an activating mutation in LET-23/EGFR. Previously it was shown that *let-23*(sa62*gf*)/+ animals induced 2° and 3° fate in isolated VPCs without an AC, while *sa62*/*sa62* conferred mostly 1° fate (Katz et al., 1996). We found that *let-23*(*sa62*gf)/+; *lin-12*(*n379*d) had more 2° cells than *lin-12*(*n379*d) alone, and this effect was suppressed by *let-60(RNAi)* or *ral-1(RNAi)* (Figure 5D). Together, these data indicate that the Ras-RalGEF-Ral pathway mediates the EGF pro-2° signal.

Regulation of RAL-1 Expression Provides a Mechanism for Effector Switching

Transgenic embryos harboring a *ral-1* promoter-driven *gfp* fusion construct showed broad embryonic GFP expression. Post-embryonically, GFP was observed in excretory canals, a small number of neurons, and was expressed dynamically in vulval lineages. Prior to EGF induction, GFP was expressed in all VPCs, but at the time of induction GFP was restricted to P5.p, P6.p and P7.p, cells receiving the EGF signal (Figures 6A and 6B). Soon thereafter expression was extinguished in the presumptive 1° cell (P6.p), persisted strongly in presumptive 2°s (P5.p and P7.p), and was faintly restored in presumptive 3°s (Figure 6C). Further dynamic expression changes were seen in later vulval development, after fate specification (Figure S5).

We show that LET-60/Ras switches effectors from pro-1° LIN-45/Raf output in presumptive 1° cells to pro-2° RGL-1-RAL-1 output in presumptive 2° cells. We hypothesize that the mechanism of effector switching contains two components. First, RAL-1 expression following initial induction is quickly restricted mainly to presumptive 2°s, and therefore persistent Ras pro-2° signaling is limited to presumptive 2° cells. Second, concordant restriction to presumptive 2° cells of LIP-1/ERK phosphatase quenches the ERK signal. Thus, soon after initial induction the predominant Ras effector output in presumptive 2° cells is RGL-1-RAL-1.

The early vulval RAL-1 expression pattern mirrors that of LIP-1 (Berset et al., 2001), indicating that both RAL-1 and LIP-1 are precociously present to influence interpretation of the initial EGF inductive signal to naïve VPCs. If so, in presumptive 1° cells the Ras-RalGEF-Ral pro-2° response to EGF is expected to conflict with the Ras-Raf pro-1° response. Likewise, in presumptive 1° cells the Ras-Raf pro-1° response should be blunted by early LIP-1/ERK phosphatase expression. Therefore, rapid transcriptional exclusion of both LIP-1 and RAL-1 proteins from the presumptive 1° cell facilitates maximal Ras-Raf pro-1° activity. Supporting this model, *rgl-1(RNAi)* or *ral-1(RNAi)* suppressed the under-induced phenotype conferred by reduced function mutations (rf) in *lin-3*/EGF or *let-23*/EGFR (Figures S2D and S2E), and comparable suppression was observed with loss of LIP-1 (Berset et al., 2001). We argue that loss of RAL-1 or LIP-1 strengthens the initial pro-1° inductive event in presumptive 1° cells, and thus rescues compromised EGF pro-1° signaling. Hence, RAL-1 and LIP-1 cooperate as a programmed switch to toggle Ras output from Raf to RGL-1 in presumptive 2° cells.

DISCUSSION

RGL-1-RAL-1 Provides a Mechanistic Key to Interpretation of the EGF Morphogen Gradient

The continually expanding number of functionally diverse effectors raises the issue of how Ras signaling output is controlled through dynamic spatial and temporal effector utilization to orchestrate its complex biology in normal and neoplastic cells. We describe mechanisms whereby a balance of redirected effector signal output and pathway quenching can bring two antagonistic pathways into harmony, with each faithfully promoting divergent fates in response to the same initial patterning signal. This general patterning reinforcement/fidelity mechanism may prove to be widespread in metazoan development.

The molecular mechanisms of EGF induction of 1° fate and consequent 1°-dependent Notch induction of 2° fate are well characterized. Additionally, a graded EGF receptor signal has been shown to exist, but direct EGF signaling from the AC is sufficient but not necessary for 2° fate induction (Koga and Ohshima, 1995; Simske and Kim, 1995). The mechanism by which the pro-2° EGF receptor signal is propagated was previously unknown.

We incorporate the insights from our study of Ral signaling into a new model in which we reconcile prior models of graded morphogen signaling, sequential induction, and signal quenching (Figure 7). We mechanistically validated the “graded morphogen model” and show that Ras effector switching serves to emphasize the antagonistic relationship between Ras and Notch. In the AC-proximal VPC (P6.p) EGF activates Ras and the ERK MAPK cascade to induce 1° fate, which by stimulating production of Notch ligands in turn induces 2° fate in neighboring VPCs. In presumptive 2° cells the Raf pro-1° signal is rapidly quenched by 2°-specific expression of LIP-1/ERK phosphatase (Berset et al., 2001) and other negative regulators (Berset et al., 2005;Yoo et al., 2004;Yoo and Greenwald, 2005). Instead, Ras signals through RGL-1 to promote 2° fate. Thus, the utilization of the RGL-1-RAL-1 signaling module is a critical feature of the differential response of cells across the EGF gradient. Such pathway interweaving may result in developmental fidelity and robustness of vulval patterning (Braendle and Felix, 2008).

Loss of LET-60/Ras or RAL-1 suppressed the induction of 2° cells by *lin-12*(d); *lin-15*(ts) at 16°C and the induction of 2° cells by *let-23*(*sa62*gf)/+; *lin-12*(d), but only partially. We therefore speculate that Ras-RGL-1-RAL-1 comprises only part of the pro-2° EGF signal. We note that additional EGF pro-2° signaling activity may be Ras-independent, and is perhaps engaged directly by activated EGF receptor.

Morphogen gradients have been studied for decades, yet there are still significant mysteries in differential interpretation of signals across gradients (Lawrence, 2001). In other systems a variety of gradient response mechanisms exist, from differential transcription of target genes to signal-induced reprogramming of signal response (Ibanes and Izpisua Belmonte, 2008; Piddini and Vincent, 2009), but correlation and causation are not always clear in these systems. In cultured human cells exposed to ectopic EGF or heregulin ligand, downstream pathway utilization varies dramatically by cell line, time of exposure, and ligand concentration (Chen et al., 2009). In vulval patterning EGF gradient input is superimposed on sequential EGF and Notch signals, and our results suggest that Ras effector switching comprises a significant portion of EGF gradient interpretation.

Effector Switching Achieves Divergent Developmental Outcomes from the Same Signal

Our results indicate that Ras switches effector utilization between presumptive 1° and 2° cells by restricting RAL-1 expression to presumptive 2° cells. It is unknown how this 2°-specific RAL-1 transcriptional expression is patterned, but we hypothesize a combination of ERK pro-1° and Notch pro-2° transcriptional outputs. However, unlike the promoters of 2°-specific Notch-responsive *lst* genes, the promoter of *ral-1* lacks concentrated conserved Notch-responsive sequence elements (not shown). If past studies are an indicator, there are likely to be multiple overlapping systems that cooperatively reprogram EGF output.

Clearly LIN-45/Raf effector pathway quenching is also critical, since there are significant consequences of loss of LIP-1/ERK phosphatase (Berset et al., 2001). Based on our GFP expression studies we speculate that a similar quenching phenomenon may exist for RAL-1 at the transcriptional level, since a *ral-1* reporter is rapidly excluded from presumptive 1° cells after initial induction. Thus RAL-1 quenching occurs in presumptive 1° cells and is complementary to Ras-Raf-ERK quenching in presumptive 2° cells.

Theoretically, effector switching can also occur at the level of Ras effector binding. Loss of the *C. elegans* SOC-2/SUR-8 (human Shoc2) diminishes Ras-Raf signaling (Selfors et al., 1998; Sieburth et al., 1998). Shoc2 physically scaffolds Ras and Raf, thus regulating Ras-Raf association and pathway activation (Li et al., 2000). Although no such protein has been identified for Ras-RalGEF scaffolding, dynamic developmental regulation of such scaffolds could critically impact effector usage.

Previous studies of PC12 pheochromocytoma cell differentiation suggested that, via differential effector usage, divergent developmental outcomes arise from a particular signal. Nerve growth factor activation of Ras promotes Raf- and PI3K-dependent neuronal differentiation and growth cessation (Jackson et al., 1996; Sano and Kitajima, 1998). Conversely, Ras activation of RalGEF promotes proliferation and not differentiation (Goi et al., 1999). Thus, Ras has the potential to promote both pro-differentiation and anti-differentiation by engaging different effectors in the same cell type. In PC12 cells, RalGEF is speculated to be eventually uncoupled from Ras (Goi et al., 1999). Thus, while the potential for effector switching has been demonstrated in cell culture, mechanisms of pathway interaction are lacking.

The Ras-RalGEF parallelism to Ras-Raf was foreshadowed by a previous *Drosophila* study (Karim and Rubin, 1998). Also in *Drosophila*, activated Ras phenotypes were enhanced by a Ral dominant negative, consistent with the antagonistic Ras-Raf and Ras-RalGEF activities described here (Mirey et al., 2003). The use of multiple Ras effectors in parallel in the same developmental event may explain these puzzling results, or the relationship amongst Ras, Ral and Rap small GTPases may be different in different developmental contexts.

Interplay between Notch and Ras signaling is a common theme in developmental biology, and Notch and Ras interplay is also observed in mouse pancreatic cell differentiation and cancer development (Mysliwiec and Boucher, 2009; Sundaram, 2005). Whether this pancreatic Ras-Notch interplay depends on K-Ras activation of the RalGEF-Ral pathway is not known, but it is intriguing that RalGEF but not Raf is preferentially activated in pancreatic cancer cells and Ral activation is necessary for pancreatic cancer growth (Lim et al., 2005; Lim et al., 2006).

Efforts to develop anti-Ras inhibitors have focused on targeting effector signaling (Yeh and Der, 2007), and have been complicated by cell- and cancer-type differences in effector dependency and activation (Hamad et al., 2002; Lim et al., 2005; Rangarajan et al., 2004; Tuveson et al., 2004). How differential effector utilization and activation is achieved remains an unresolved issue. Our observations establish one mechanism for this phenomenon. Additional mechanisms may involve regulation of the subcellular localization of Ras to distinct membrane compartments, leading to spatial regulation of effector activation (Bivona et al., 2006; Onken et al., 2006).

In conclusion, we demonstrate a patterning role for Ras effector switching that has implications beyond developmental genetics. Studies in model genetic organisms in conjunction with mouse and cell culture studies were instrumental in developing our early understanding of key signal transduction pathways, including canonical EGF signaling through the Ras-Raf-MEK-ERK signaling module to regulate transcription. Recent studies in pathway quenching, and now effector switching, to promote alternative ligand outputs argue that *C. elegans* vulval patterning continues to yield important insights into diverse biological fields.

EXPERIMENTAL PROCEDURES

General *C. elegans* Methods, Strains, Constructs and Transgenic Lines

Strain handling and generation of constructs and transgenic lines used standard methods, see Supplemental Data.

ral-1(tm2760); *let-60*(*n1046*gf) Double Mutant Construction

The *ral-1(tm2760)* deletion, kindly provided by Shohei Mitani, removes nucleotides 418–996 (numbered from position +1 of the *ral-1* initiating ATG codon), deleting part of the

splice donor site. Single worm PCR of *ral-1(tm2760)* was performed as described (Williams et al., 1992). In the initial strain isolate, heterozygous *tm2760* co-segregated with a sterile mutation. Outcrossing *tm2760* and recombination in the *daf-2-dpy-17* interval failed to separate the sterile mutation from *tm2760*. *ral-1(RNAi)* did not impact fertility, even when performed in the *eri-1(mg366)* RNAi hypersensitive background (Kennedy et al., 2004). Therefore, we hypothesize that sterility is conferred by a mutation closely linked to *ral-1(tm2760)*. Sterile animals formed a functional vulva, so in the *let-60*(*n1046*gf) background we balanced *ral-1(tm2760)* with *unc-93*(*e1500*sd) *dpy-17(e164)*. We used the semi-dominant *unc-93*(*e1550*sd) Unc phenotype to distinguish tm2760/tm2760 homozygotes from *tm2760*/*unc-93 dpy-17* heterozygotes, and scored vulvas of non-Unc animals. Single worm PCR (T_m=53°, cycles=35) with the primers TZ23 (CAACAAGTCGTCCATAAAGTG), TZ24 (GGCGAAAAACGAGAAAAGAAC), and TZ25 (GAATTTTTCAGGCTTTCTGACG) confirmed the *tm2760*/*tm2760* genotype of each scored animal.

Bacterially Mediated RNA interference

Bacterially mediated RNAi was performed mostly as described (Fire et al., 1998; Kamath et al., 2001; Timmons et al., 2001). Each feeding RNAi (fRNAi) clone was sequenced to confirm identity. Eighty μl of bacteria was seeded on NGM agar plates containing 1 mM IPTG and 50 μg/ml carbenicillin. L4 larvae were added to the plates the following day. After 24 h, animals were transferred to new plates, and parents were removed after an additional 24 h. We consistently obtained stronger fRNAi phenotypes at 23°C, and thus all fRNAi experiments were performed at 23°C. *gfp(RNAi)* or *daf-3(RNAi)* was used as a control. *pop-1(RNAi)* was included in all experiments as a positive control for RNAi efficacy. Phenotypes were only scored if we observed 100% lethality on the *pop-1(RNAi)* plates.

Vulval Induction Assay

Late L4 hermaphrodites were mounted as described previously in 5 mM sodium azide/M9 buffer on slides with agar pads. L4 vulval invaginations were visualized under DIC Nomarski optics (Sternberg and Horvitz, 1986; Sulston and Horvitz, 1977). Images were captured using a Nikon Eclipse E800 microscope with a Hamamatsu C2400-07 Newvicon camera controlled by Metamorph acquisition software (Molecular Devices). WT animals scored 3.0 (3 induced VPCs). Values greater than 3.0 indicated hyper-induction, less than 3.0 under-induction.

Fluorescence Microscopy

Live animals were mounted in 2 mg/ml tetramisole/M9 buffer on slides with agar pads and visualized using a Nikon Eclipse TE2000U microscope equipped with a DVC-1412 CCD camera (Digital Video Camera Company) controlled by the Hamamatsu SimplePCI acquisition software.

HIGHLIGHTS

- Ras-RalGEF-Ral indirectly antagonizes Ras-Raf pro-1° vulval fate induction.
- The Ras-RalGEF-Ral signaling module cooperates with Notch to specify 2° fate.
- The EGF pro-2° signal is transduced in part by Ras-RalGEF-Ral.
- Induction restricts Ral expression to presumptive 2° vulval precursor cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank V. González-Pérez for pilot 37G experiments, A. Reiner for organization, N. Dudley, A. Fire, S. Kim, Y. Kohara, and M. Maduro for plasmids, K. Caron, A. Fanning, and B. Goldstein at UNC-CH for microscope use, I. Greenwald, A. Hajnal, M. Han, J. Kimble, S. Mitani, P. Sternberg, and the *C. elegans* Genetics Center for worm strains, and A. Cox, R. Dubose, V. González-Pérez, A. Kidd III, K. Monahan, K. Pedone, C. Rocheleau and D. Vigil for discussions and critical reading of the manuscript. This work was supported by NIH grants GM085309 (DJR) and CA42978 (CJD).

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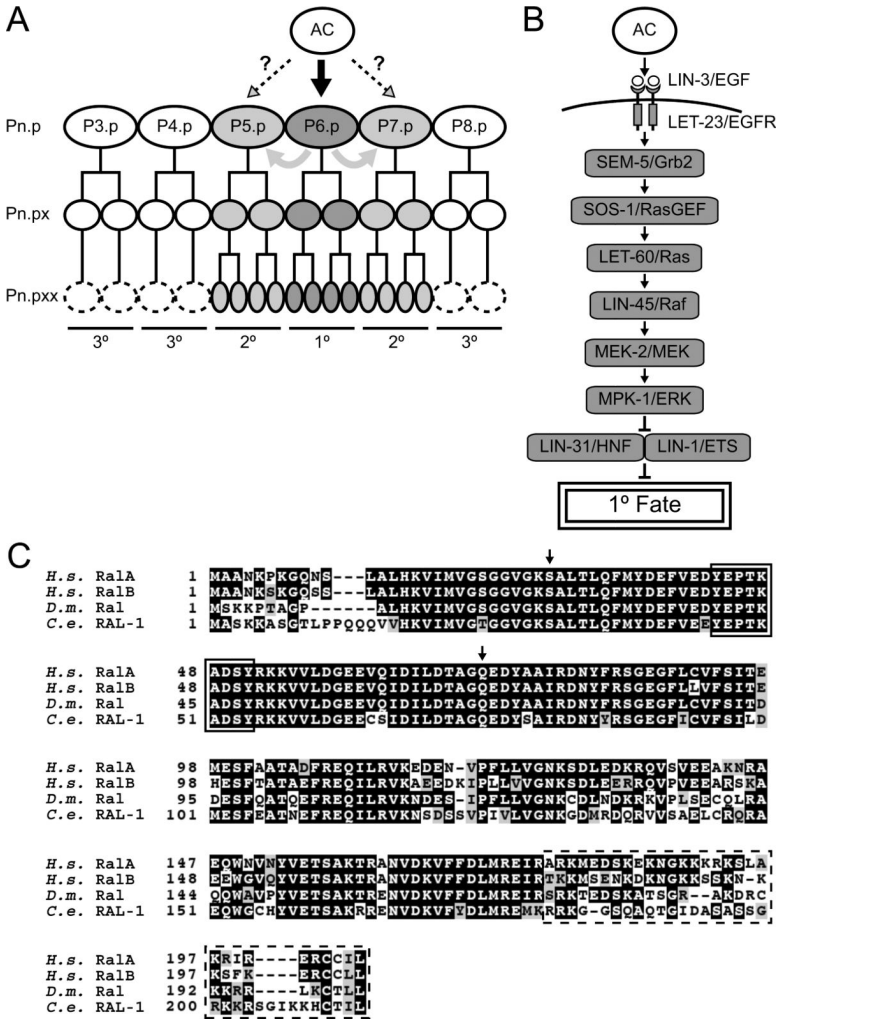


Figure 1. An Overview of VPC Patterning

(A) A graded EGF signal from the anchor cell (AC) induces vulval fates. High EGF levels (black arrow) activate the EGFR-Ras-Raf pathway in P6.p to induce 1° fate. Lateral signal (gray arrows) from the presumptive 1° cell activates LIN-12/Notch in P5.p and P7.p to induce 2° fate. 1° and 2° descendents form the vulva; 3° VPCs are non-vulval. Low EGF levels (dashed arrows) may help pattern P5.p and P7.p.

(B) The EGF-Ras-MAP kinase signal transduction pathway specifies 1° cell fate.

(C) Sequence alignment of Homo sapiens (H.s.) RalA and RalB, Drosophila melanogaster (D.m.) Ral, and Caenorhabditis elegans (C.e.) RAL-1. Identical and similar residues are marked with black and grey shading, respectively. Residues S31 (mutated to N for dn) and Q75 (mutated to L for gf) are shown by arrows. The core effector domain is boxed in solid lines, and the C-terminal hypervariable and CAAX prenylation signal motif region in dashed lines. Accession numbers for RGL-1 and RAL-1 are NP_001123140 and NP_497689, respectively. See also Figure S1.

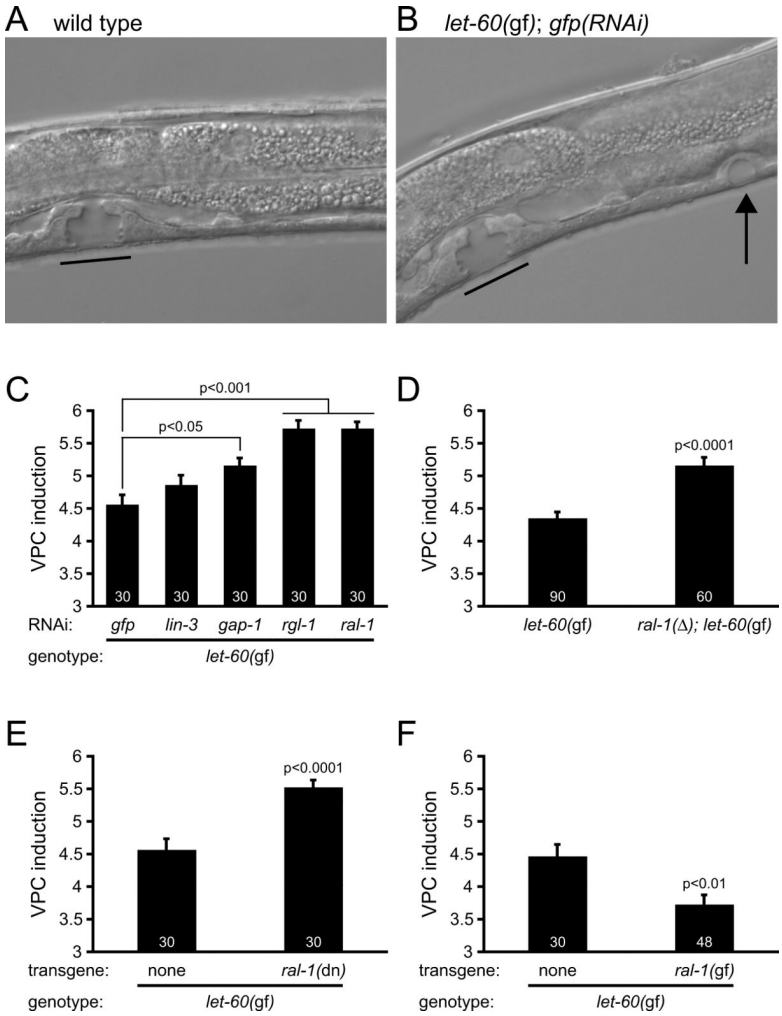


Figure 2. RAL-1 Antagonizes LET-60-dependent Vulval Induction

(A and B) DIC micrographs of (A) wild type and (B) *let-60(n1046*gf); *gfp(RNAi)* animals as late L4 larvae. Bars indicate the typical 2°-1°-2° vulva, and the arrow indicates a pseudovulva. Anterior is left and ventral down.

(C) *rgl-1(RNAi)* or *ral-1(RNAi)* enhanced *let-60(n1046*gf) hyper-induction. Negative controls were *gfp(RNAi)* and *lin-*3/EGF*(RNAi)*, and the positive control was *gap-1*/RasGAP*(RNAi)*. Data shown are representative of six independent assays.

(D) The *ral-1* deletion, *tm2760*, enhanced *let-60(n1046*gf). The *n1046* single mutant was counted in nine assays, the double mutant in four.

(E) Transgenic dominant-negative RAL-1(S31N) enhanced *let-60*(gf). Two transgenes were analyzed; that shown was assayed three times, another four.

(F) Transgenic activated RAL-1(Q75L) suppressed *let-60*(gf). One transgene was assayed seven times.

Y-axis is the number of VPCs induced to vulval (1° and 2°) fates. Data are the mean ± standard error of the mean (SEM). For statistical reasons single, non-pooled assays are shown, and white numbers represent animals scored therein. Statistics were calculated by Kruskal-Wallis, Dunn test (C) or Mann-Whitney test (D–F).

See also Figure S2.

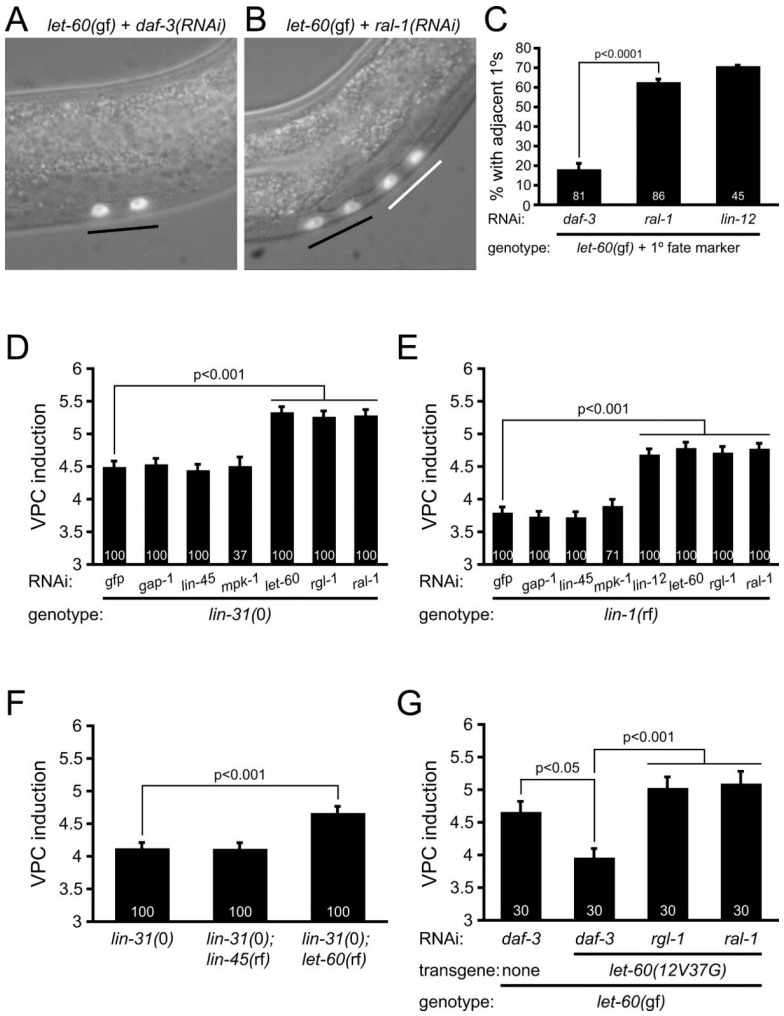


Figure 3. Ras-RGL-1-RAL-1 Bypasses Ras-Raf to Control Cell Fate

(A and B) Expression of P_{egl-17}::*cfp-lac*Z in VPC daughters. Overlaid DIC and CFP fluorescence images of (A) *let-60(n1046*gf); *daf-3(RNAi)* and (B) *let-60*(gf); *ral-1(RNAi)* at the Pn.px stage. The black bar indicates P6.px and white bar indicates P7.px cells.

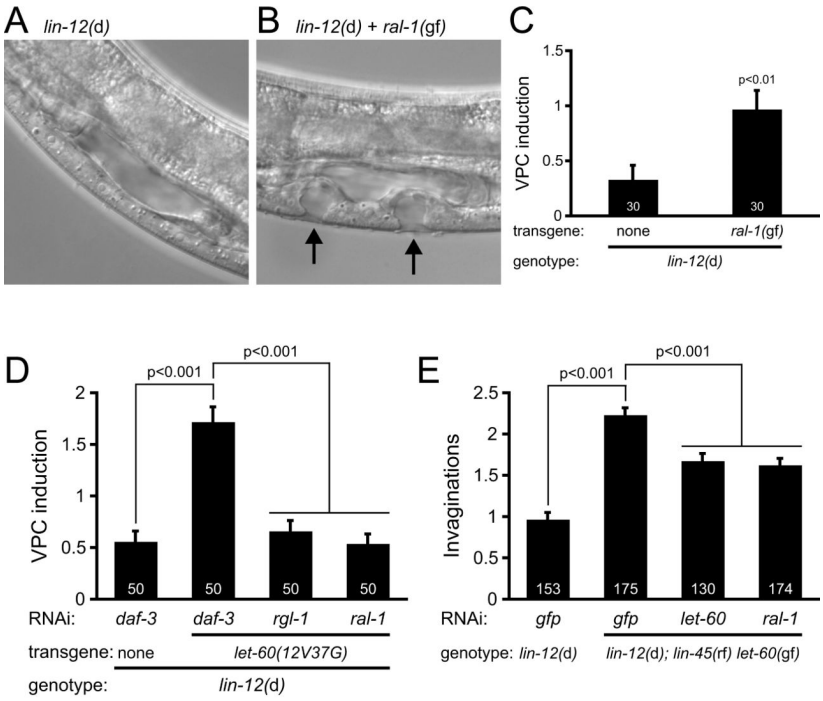
(C) Percent L3 larvae with CFP-positive lineages neighboring the P6.p lineage (P5.p or P7.p derived) at the Pn.px stage in the *let-60*(gf); *arIs92* (P_{egl-17}::*cfp-lac*Z) background. Shown are average percentages of animals with adjacent 1° cell fate from three independent assays ± SEM. The numbers of adjacent 1° cells out of the total per assay were, for *daf-3(RNAi)* 3/25, 6/26, and 6/30, for *ral-1(RNAi)* 17/27, 19/29, and 18/30, and for *lin-12(RNAi)* 12/17 and 20/28. White numbers represent pooled total animals scored.

(D) *let-60-*, *rgl-1-*, and *ral-1*-directed RNAi enhanced the hyper-inducing *lin-31(n301). gfp, gap-1*, *lin-45*, and *mpk-1* RNAi controls were negative. Data shown are representative of three independent assays.

(E) *lin-12-*, *let-60-*, *rgl-1-*, and *ral-1*-directed RNAi enhanced the hyper-inducing *lin-1(e1275*ts) at 23°C. *gfp, gap-1*, *lin-45*, and *mpk-1* RNAi controls were negative. Data shown are representative of three independent assays.

(F) *let-60(n2021*rf) but not *lin-45(sy96*rf) enhanced the hyper-inducing *lin-31(n301)*. Data shown are representative of four independent assays.

(G) Transgenic activated LET-60(12V, 37G) (RalGEF selective) suppressed *let-60(n1046*gf) compared to non-transgene bearing siblings, and was RGL-1 and RAL-1 dependent. Two transgenes were assayed three times each.
Y-axis is the number of VPCs induced to vulval (1° and 2°) fates. Data are the mean ± SEM. For statistical reasons single, non-pooled assays are shown, and white numbers represent animals scored therein. Statistics were calculated by Fisher's Exact Test (C) or Kruskal-Wallis, Dunn test (D–G).
See also Figure S3.



F

Row	Genotype	% abnormal vulva	% loss of 2° fate	n
1	*lin-12*(rf, ts); *gfp(RNAi)*	33.3	1.0	504
2	*lin-12*(rf, ts); *ral-1(RNAi)*	46.1**	4.1*	434
3	*lin-12*(rf, ts); *lin-12(RNAi)*	65.2****	14.8****	310

Figure 4. Ras-RalGEF-Ral Promotes Pro-2° Fate

(A and B) DIC micrographs of late L4 stage (A) *lin-12(n302*d) and (B) *lin-12(n302*d) + *ral-1(Q75L)* animals. Arrows indicate ectopic 2° cells. Anterior is left and ventral is down.

(C) Transgenic activated RAL-1(Q75L) enhanced 2° cell induction of activated *lin-12(n302*d). Two transgenes were assayed four times each.

(D) Transgenic activated LET-60(12V,37G) (RalGEF selective) enhanced *lin-12(n302*d) compared to non-transgene bearing siblings, and was RGL-1 and RAL-1 dependent. Two transgenes were assayed three times each.

(E) Endogenous activated LET-60 (*let-60(n1046*gf)) with blocked LIN-45/Raf (*lin-45(n2506*rf)) enhanced *lin-12(n379*d), and was RAL-1-dependent. Data shown are representative of three independent assays.

Y-axis is the number of VPCs induced to vulval (1° and 2°) fates (C and D) or total vulval invaginations (E). Data are the mean ± SEM. For statistical reasons single non-pooled assays are shown, and white numbers represent animals scored therein.

(F) The *lin-12(n137n460*rf) temperature sensitive hypomorph is sensitive to loss of RAL-1. *ral-1(RNAi)* caused significant loss of 2° lineages and increased incidence of morphologically abnormal vulvae. We observed double 2° loss in *ral-1(RNAi)* but not *gfp(RNAi)* controls, and thus loss of *ral-1* was qualitatively different. Results are from three pooled assays (*p<0.05, **p<0.01, ****p<0.0001).

Statistics were calculated by Mann-Whitney test (C), Kruskal-Wallis, Dunn test (D and E) or Fisher's Exact test (F).

See also Figure S4.

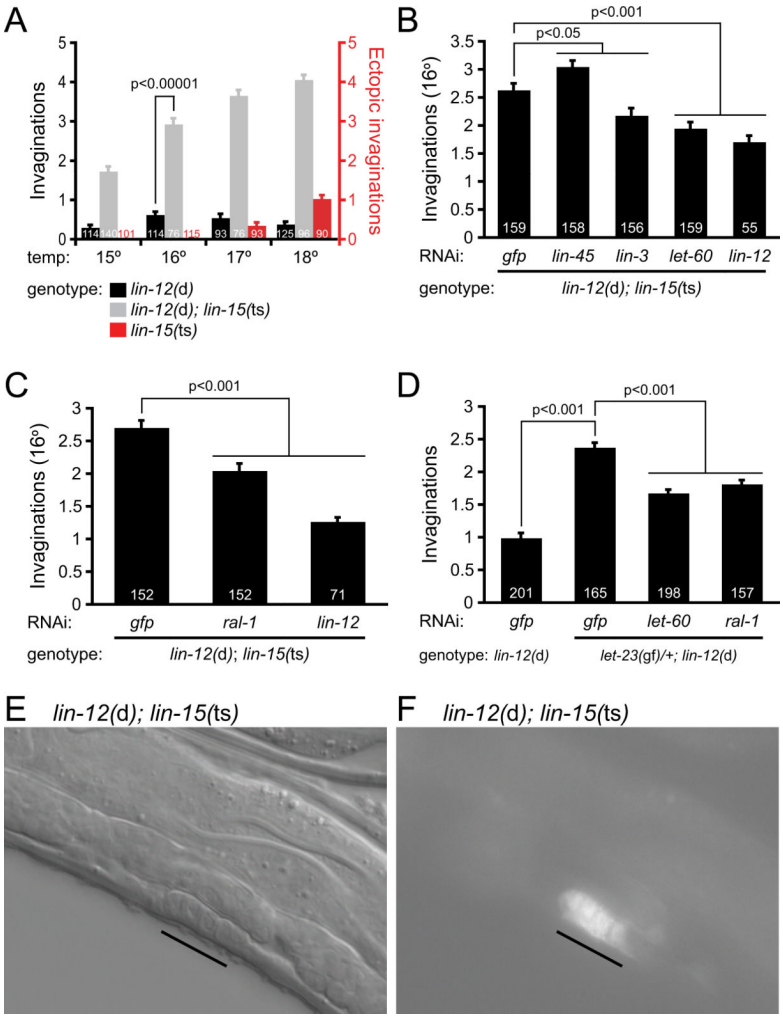


Figure 5. *ral-1*-dependent EGF and EGFR Signaling is Sufficient for Pro-2° Activity

(A) A comparison of *lin-12(n379*d) alone (black bars), *lin-12(n379*d); *lin-15(n765*ts) (gray bars) and *lin-15(n765*ts) alone (red bars), all grown on *gfp(RNAi)*. Animals were grown at 15°, 16°, 17° or 18°C. Total vulval invaginations (left, black Y axis for black and gray columns), or ectopic pseudovulval invaginations (right, red Y axis for red columns) were scored.

(B) RNAi of *lin-3*/EGF, *let-60*/Ras or *lin-12*/Notch suppressed invaginations induced by *lin-12(n379*d*); lin-15(n765*ts) at 16°C, and *lin-45(RNAi)* enhanced. Data shown are representative of three independent assays.

(C) *ral-1(RNAi)* or *lin-12(RNAi)* suppressed invaginations induced by *lin-12(n379*d); *lin-15(n765*ts) at 16°C. Data shown are representative of three independent assays.

(D) Activated *let-23(sa62*gf)/+ (moderately activated EGFR) enhanced invaginations induced by *lin-12(n379*d) in a LET-60- and RAL-1-dependent manner. Data shown are representative of three independent assays.

Y-axis is the number of total vulval invaginations at 16°C (B and C) or 23°C (D). Data are the mean ± SEM. For statistical reasons single, non-pooled assays are shown, and white numbers represent animals scored therein. Statistics were calculated by Mann-Whitney test (A) or Kruskal-Wallis, Dunn test (B–D).

(E and F) Ectopic vulval cells induced at 16°C by *lin-15*(ts) in the *lin-12(n379*d) background are 2°. (E) DIC and (F) epifluorescent images of *mgIs21*(P_{lin-11}::*gfp+rol-6(d)*);

lin-*12(n379*d); *lin-15(n765*ts). 19/20 ectopically induced VPCs expressed the *lin-11* 2° fate marker.

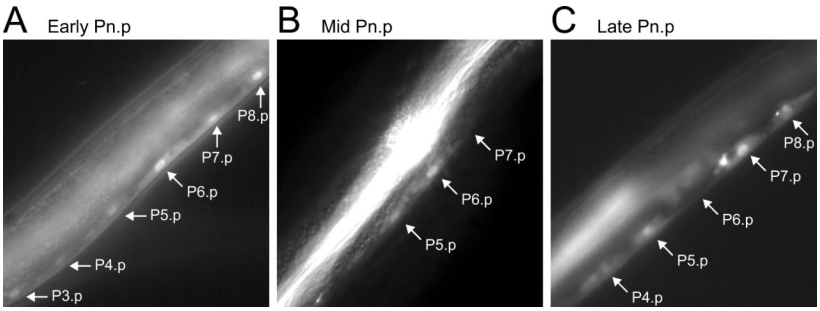


Figure 6. P*ral-1*::*gfp* is Dynamically Expressed During Early Vulval Development

Before induction P$_{ral-1}$::*gfp* (NLS+) is expressed in all VPCs, but first is restricted to EGF-induced presumptive 1° and 2° VPCs, and then to 2° cells only. (A) Early Pn.p stage before induction. (B) Mid Pn.p stage. The background glow is strong excretory canal expression. The P7.p nucleus was GFP-positive, but was out of the plane of focus. (C) Late Pn.p stage with absent P6.p expression.

See also Figure S5.

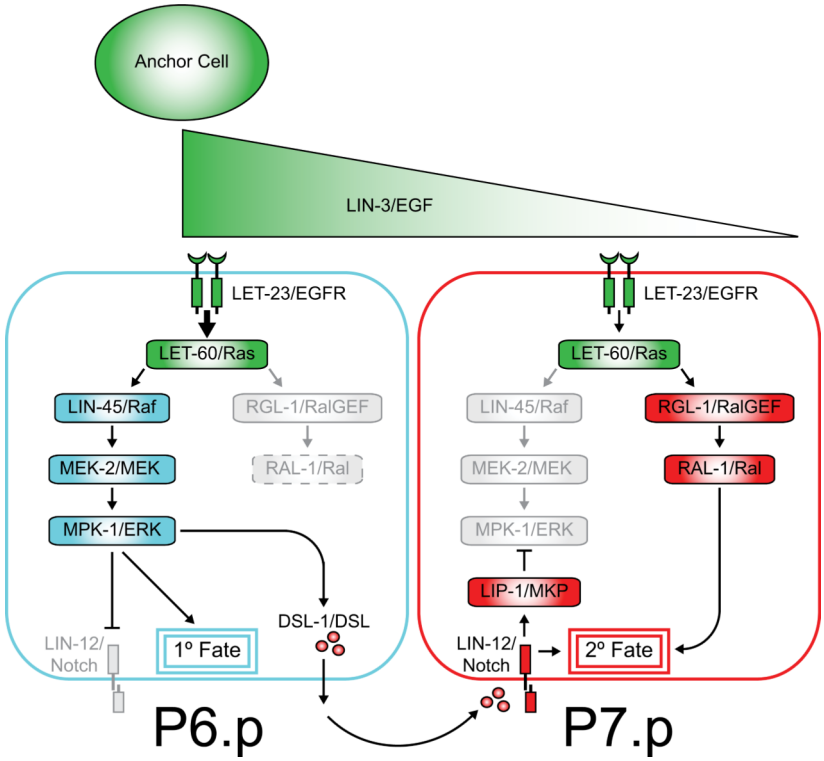


Figure 7. EGF Signaling through Ras Uses Effector Switching to Induce Opposing Vulval Fates

Signal promoting both fates is shown in green, pro-1° signal in blue, pro-2° signal in red, and quenched signals in gray. A putative EGF concentration gradient, in combination with sequential induction, faithfully patterns vulval fates. In presumptive 1° cells EGF activates Ras to utilize Raf to promote 1° cell fate. Pro-2° signaling through Notch is quenched. Putative quenching of RGL-1-RAL-1 pro-1° activity is based on RAL-1 exclusion from presumptive 1° cells. Presumptive 1° cells produce DSL ligands to induce neighboring VPCs via Notch to assume 2° fate. In presumptive 2° cells, Notch induces production of LIP-1/ERK phosphatase and other 2°-specific proteins to quench the Raf pro-1° signal. Also, EGF activates Ras to utilize RGL-1-RAL-1 to promote 2° fate. Thus, the EGF signal toggles its developmental output by Ras effector switching.