Ral Signals through a MAP4 Kinase-p38 MAP Kinase Cascade in *C. elegans* Cell Fate Patterning

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SUMMARY

* C. elegans* vulval precursor cell (VPC) fates are patterned by an epidermal growth factor (EGF) gradient. High-dose EGF induces 1o VPC fate, and lower dose EGF contributes to 2o fate in support of LIN-12/Notch. We previously showed that the EGF 2o-promoting signal is mediated by LET-60/ Ras switching effectors, from the canonical Raf- MEK-ERK mitogen-activated protein (MAP) kinase cascade that promotes 1o fate to the non-canonical RalGEF-Ral that promotes 2o fate. Of oncogenic Ras effectors, RalGEF-Ral is by far the least well understood. We use genetic analysis to identify an effector cascade downstream of *C. elegans* RAL-1/ Ral, starting with an established Ral binding partner, Exo84 of the exocyst complex. Additionally, RAL-1 signals through GCCK-2, a citron-N-terminal-homology-domain-containing MAP4 kinase, and PMK-1/ p38 MAP kinase cascade to promote 2o fate. Our study delineates a Ral-dependent developmental signaling cascade in *vivo*, thus providing the mechanism by which lower EGF dose is transduced.

Graphical Abstract
In Brief

Ral is an oncogenic effector of the oncoprotein Ras. Surprisingly, in vivo signals downstream of Ral have mostly defied identification. Shin et al. find that, during developmental patterning of the *C. elegans* VPCs, RAL-1/Ral signals through EXOC-8/Exo84, GCK-2/MAP4K, and PMK1/p38 MAP kinase to promote 2o fate in support of LIN-12/Notch.

INTRODUCTION

Ras is the most mutated oncoprotein. Yet strategies to inhibit oncogenic Ras have failed, so Ras is considered to be mostly “undruggable” (Papke and Der, 2017). Consequently, attention has shifted to oncogenic Ras effectors to identify therapeutic targets. Canonical oncogenic Ras effectors, the Raf-MEK-ERK and phosphatidylinositol 3-kinase (PI3K)-PDK-Akt cascades, are among the best studied and most targeted signaling cascades (Ryan et al., 2015; Wong et al., 2010). Yet even potent small-molecule inhibitors, like the BRAF inhibitor vemurafenib, are subject to multiple bypass mechanisms that permit initially responsive tumors to relapse (Sun et al., 2014). Thus, successful treatment will likely require multi-pronged regimens to simultaneously inhibit multiple Ras effectors.

In addition to the canonical Raf and PI3K cascades, Ras uses RalGEF-Ral to promote tumorigenesis (Feig, 2003). Historically, canonical Ras-Raf and Ras-PI3K signaling was shown to cause cancer transformation of mouse primary fibroblasts (Khosravi-Far et al., 1996; Kyriakis et al., 1992; White et al., 1995). The emergence of immortalized human epithelial cell culture led to the key finding that Ras-RalGEF-Ral is also a critical player in human oncogenesis (Hamad et al., 2002; Urano et al., 1996; White et al., 1996). RalGEF is an exchange factor that promotes guanosine triphosphate (GTP) loading of the Ral (Ras-like) small GTPase (Feig, 2003). Loss of RalGAP (Ral GTPase-activating protein), a putative tumor suppressor, increases tumorigenesis without activated Ras (Oeckinghaus et al., 2014; Saito et al., 2013), further supporting the importance of Ral signaling in cancer.
Three binding partners of Ral have been well validated: RalBP1 (Ral-binding protein 1) and Sec5 and Exo84 subunits of the hetero-octameric exocyst complex (reviewed in Gentry et al., 2014; Figure S1). The exocyst represents an unusual road-block to biochemical bootstrapping of signaling activities: the exocyst is broadly integral to essential cell biological processes (e.g., exocytosis and PAR polarity complex; Wu and Guo, 2015) and potentially binds to hundreds of partners, thus mostly precluding identification of downstream signaling partners via binding studies. Consequently, beyond these immediate binding partners, we know little of downstream functions of Ral signaling through the exocyst in vivo.

Studies in Drosophila provided key hints to the nature of Ral downstream signaling in development. In morphogenetic events, DRal was implicated in antagonizing the JNK MAP kinase (Sawamoto et al., 1999). In bristle apoptosis assays, DRal was found to have negative and positive relationships with JNK and p38 MAP kinase cascades, respectively (Balakir-eva et al., 2006). Importantly, this study also established that exocyst component Sec5 binds to HGK/NIK/MAPK4, a citron N-terminal homology (CNH) domain containing MAP4 kinase. The Drosophila ortholog, Msn (Misshapen), was found to function antagonistically to DRal (Balakireva et al., 2006) and is known to function with JNK in Drosophila embryonic dorsal closure and other morphogenetic events (Su et al., 1998). Yet these studies relied on ectopic overexpression and dominant-negative reagents, which complicated interpretation. Furthermore, direct genetic epistasis could not be assayed because many of the proteins studied are essential for development in Drosophila.

The Ste20 family of mitogen-activated protein kinase kinase kinase kinases (MAP4 kinases or MAP4Ks) is conserved throughout eukaryotes (Dan et al., 2001; Delpire, 2009). Two paralogous subfamilies of this group, GCK-I and GCK-IV (germinal center kinases), are defined by distinctive domain architecture: an N-terminal S/T kinase domain, a C-terminal CNH domain, and an unstructured poly-proline linker region (Figure 1A; Dan et al., 2001). C. elegans GCK-2 (ZC404.9) is an 829-residue protein in the GCK-I subfamily (the ‘‘GCK-2 group’’: Drosophila Hppy [Happyhour], mammalian MAP4K1/HPK1, MAP4K2/GCK, MAP4K3/GLK, and MAP4K5/GCKR/KHS1; Figures S1B and S1C). C. elegans MIG-15 is in the GCK-IV subfamily (the ‘‘MIG-15 group’’: Drosophila Msn, mammalian MAP4K4/NIK/HGK, MAP4K6/MINK, MAP4K7/TNIK, and MAP4K8/NRK/NESK; Figures S1D and S1E).

Critically, Drosophila Hppy was as yet undiscovered at the time of Msn investigation relative to DRal (Balakireva et al., 2006). Hppy antagonizes canonical epidermal growth factor receptor (EGFR) signaling through ERK MAP kinase (MAPK) in ethanol response; its relationship to DRal was not studied (Corl et al., 2009). Thus, we turned to C. elegans vulval precursor cell (VPC) fate patterning to investigate a signaling cascade downstream of Ral relative to these enigmatic MAP4Ks.

During the L3 stage, EGF produced by the gonadal anchor cell (AC) induces six initially equipotent VPCs, P3.p through P8.p, to assume the highly reproducible 3o-3o-2o-1o-2o-3o pattern (Figure 1B). 1o and 2o cells undergo stereotyped divisions and morphogenesis to form the mature vulva, and uninduced 3o cells divide once and fuse with surrounding cells.
Historically, two competing models, the ‘‘morphogen gradient model’’ and the ‘‘sequential induction model,’’ were posited to describe VPC fate patterning. In the morphogen gradient model, graded inductive signal controls fate patterning: the VPC closest to the AC (typically P6.p) receives the highest LIN-3/EGF-LET-23/EGFR signal to induce 1o fate, and neighboring VPCs, P5.p and P7.p, receive lower LIN-3/EGF-LET-23/EGFR signal and thus become 2o (Katz et al., 1995, 1996; Sternberg and Horvitz, 1986, 1989).

Yet identification of key genes in VPC patterning led to the potentially contradictory sequential induction model. LET-23/EGFR and LIN-12/Notch are necessary and sufficient for 1o and 2o induction, respectively (Aroian et al., 1990; Greenwald et al., 1983). Activation of LET-23/EGFR triggers a LIN-45/Raf-MEK-2/MEK-MPK-1/ERK canonical MAPK cascade to induce 1o fate (reviewed in Sundaram, 2013). These presumptive 1o cells in turn secrete Delta/Serrate/Lag-2 (DSL) ligands to induce neighbors to become 2o via the LIN-12/Notch (Chen and Greenwald, 2004). LET-23/EGFR was found to function cell autonomously to induce 1o fate, further supporting the sequential induction model (Koga and Ohshima, 1995; Simske and Kim, 1995). The two models long remained unreconciled, and no mechanism was known by which graded LIN-3/EGF-LET-23/EGFR activity promotes 2o fate (Kenyon, 1995).

Overlaid on this system are ‘‘mutual antagonism’’ mechanisms, by which, after initial induction, presumptive 1o and 2o cells enact programs to exclude potentially contradictory signals. For example, in presumptive 1o cells, LIN-12/Notch receptor is internalized and degraded to prohibit conflicting 2o-promoting signaling (Shaye and Greenwald, 2002, 2005). Conversely, in presumptive 2o cells, LIN-12/Notch-dependent transcription of LIP-1/ERK phosphatase impedes conflicting 1o-promoting MPK-1/ERK signaling (Berset et al., 2001; Yoo et al., 2004). Such antagonistic signals are proposed to act collectively to transition from initial patterning specification to commitment (Sternberg, 2005), thereby avoiding inappropriate and/or ambiguous cell fates that can result from inappropriate signals.

Given the importance of Ras-RalGEF-Ral signaling in cancer, we set out to define a role for this signaling module in VPC fate patterning. We found that LET-60/Ras uses the non-canonical RGL-1/RalGEF-RAL-1/Ral effector to promote 2o fate in support of LIN-12/Notch (Zand et al., 2011). Thus, both the sequential induction and morphogen gradient models are correct: LET-60/Ras switches effectors to interpret the EGF gradient. This mechanism reconciled the two competing models and established a platform for the in vivo study of RAL-1/Ral signaling in VPC fate patterning (Reiner, 2011; Zand et al., 2011; Figure 1B). Yet the downstream output of the LET-60/Ras-RGL-1/RalGEF- RAL-1/Ral 2o-promoting signal remained unknown.

In this study, we determine that EXOC-8/Exo84, a well-validated Ral-binding protein in mammalian cells, is required to propagate the RAL-1 2o-promoting signal. Significantly, we find that RAL-1 requires the CNH-domain-containing GCK-2/ MAP4K and PMK-1/p38 MAPK to promote 2o fate. Genetic perturbation of components of this cascade phenocopied perturbation of RAL-1, and these components are necessary for the 2o-promoting activity of mutationally activated RAL-1. 2o-promoting EGF signal requires GCK-2, putative mutationally activated endogenous GCK-2 is sufficient to increase ectopic 2o cell induction,
and GCK-2 functions cell autonomously in the VPCs. Using CRISPR/Cas9-dependent genome engineering to tag endogenous gene products with fluorescent protein (FP) and epitope, we observed expression and subcellular localization of endogenous RAL-1, GCK-2, and PMK-1 proteins in VPCs. Our in vivo analysis connects Ral to an effector cascade in C. elegans VPC fate patterning.

RESULTS

Criteria for a RAL-1-Dependent 2o-Promoting Signal

Because LIN-12/Notch, but not the LET-60/Ras-RGL-1/RalGEF- RAL-1/Ral signal, is necessary for 2o fate induction, we used a combination of parallelism and epistasis to test the genetic relationships among members of the VPC fate patterning network. Specifically, for this genetic analysis, we used two sensitized genetic backgrounds (Figure S1F). The let-60(n1046gf) G13E-activating mutation confers excess 1o induction, levels of which are sensitive to perturbation of both 1o- and 2o-promoting signals. The weakly activating lin-12(n379d)/Notch mutation both causes ectopic 2o cells and abrogates development of the AC. Consequently, lin-12(n379d) provides a simplified signaling milieu in which EGF is not present (Greenwald et al., 1983). This background is sensitive to perturbation of 2o-promoting, but not 1o-promoting, signals and responds to the EGF-dependent 2o-promoting signal (Zand et al., 2011).

Partly by using these tools, we developed a set of expectations for RAL-1 2o-promoting effectors. (1) Loss of effector function should phenocopy loss of ral-1 function. (2) Constitutively activated effector should phenocopy constitutively activated RAL-1. (3) Loss of effector function should be epistatic to constitutively activated RAL-1. (4) The effector should function cell autonomously in the VPCs. (5) The effector should be expressed in the VPCs. Using these criteria, we systematically evaluated a putative RAL-1 signaling cascade in 2o VPC fate induction.

EXOC-8 Functions in VPC Fate Patterning

We tested whether known Ral binding partners, Sec5 and Exo84 of the exocyst complex and RalBP1/RLIP76 (reviewed in Gentry et al., 2014; Figure S1A), met our first criterion for a RAL-1 effector: loss of effector function should phenocopy loss of ral-1 function. The hetero-octameric exocyst complex generally consists of eight subunits used in different contexts: Sec3; Sec5; Sec6; Sec8; Sec10; Sec15; Exo70; and Exo84 (Wu and Guo, 2015), all of which have single conserved orthologs in C. elegans.

Deletion of ral-1 leads to defects in cell polarity, apparently by disruption of the exocyst complex (Armenti et al., 2014), consistent with previous observations that mammalian Ral functions as a membrane-tethering member of the exocyst (Issaq et al., 2010; Moskalenko et al., 2002, 2003). We previously found that ral-1(RNAi) alone did not confer significant vulval patterning defects, nor did a non-null intronic deletion allele of ral-1 that conferred sterility (Zand et al., 2011). We characterized the ral-1(gk628801rf) R139H mutation, which did not confer visible defects (H.S. et al., unpublished data). In the let-60(n1046gf) background, gk628801rf caused increased 1o induction, consistent with a reduced function.
(rf), but not null allele of ral-1. This result validated our previous findings that reduced ral-1 signaling and hence reduced 2o signaling increased 1o-promoting signals (Figures 2A and S2A).

In the same genetic background, we tested effects of the sec-5(pk2357) strong hypomorph (Frische et al., 2007), exoc8(ok2523), and rlbp-1(tm3665) deletion alleles (exoc-7(ok2006) was included as a negative exocyst control). Among these candidate RAL-1 effectors, only exoc-8(ok2523), a deletion allele, phenocopied ral-1(gk628801rf) (Figures 2B–2D and S2B). As expected, ral-1(gk628801rf) caused no phenotypic changes in the lin-12(n379d) background (Figure S2C). These results are consistent with EXOC-8 mediating RAL-1 2o-promoting signal.

We also tested exoc-8(ok2523) in the lin-12(n379d) background. We observed increased ectopic 2o induction (Figure S2D). This result is inconsistent with our expectation of a RAL-1 effector. Thus, EXOC-8 performs multiple functions in VPC fate patterning, one of which could include mediating a RAL-1 2o-promoting signal.

Loss of GCK-2, but Not MIG-15, Confers the Same Phenotype as Loss of RAL-1

To further explore our first criterion, we used C. elegans genetics to determine relationships among Ral and the paralogous “GCK-2 group” and “MIG-15 group” MAP4Ks.

gck-2(ok2867) is an in-frame 549-bp deletion in exon 5, resulting in deletion of a part of the kinase domain. gck-2(tm2537) is an out-of-frame 565-bp deletion in exon 5, resulting in early stop codons (Khan et al., 2012). Most genetics were done using these alleles and gck-2-directed bacterially mediated RNAi (clone V-4P08; Figure 3A). mig-15(rh148) is a V168E missense mutation predicted to abolish kinase function. mig-15(rh80) is a W898* late nonsense mutation thought to confer strong loss of function (Chapman et al., 2008). We also used mig-15-directed bacterially mediated RNAi (clone X-5G21).

gck-2(ok2867) conferred increased 1o induction in the let-60(n1046gf) background (Figures 3B–3E), similar to ral-1(gk628801rf) or exoc-8(ok2523). gck-2(RNAi) conferred a phenotype similar to that of ral-1(RNAi) (Figure 3F versus Figure S2A). Also, mig-15(RNAi) conferred increased 1o induction in the let-60(n1046gf) background (Figure 3F). We also tested the mig-15(rh148) reduced function allele in the let-60(n1046gf) background. However, we observed severe vulval morphogenesis defects: 2o cells/lineages failed to migrate to join the 1o cell/lineage (Figure S3A). Thus, in double mutant animals, we could not discriminate between ectopic 1os and 2os that had failed to join the 1o of the normal vulva, which precluded interpretation of VPC induction in these strains (Figure S3B). Taken together, these results are consistent with both GCK-2 and MIG-15 functioning as either 2o-promoting or 1o-antagonizing signals.

We assessed the roles of MIG-15 and GCK-2 in the lin-12(n379d) background (AC/EGF absent, mild ectopic 2o induction). Neither ral-1(gk628801rf) nor gck-2(ok2867 or tm2537) altered 2o induction in the lin-12(n379d) background (Figures S2C, S3C, and S3D, respectively). In marked contrast, reduction of mig-15 function robustly elevated 2o induction in the lin-12(n379d) background (Figures 3G and S3E; the strongest mig-15 allele, rh326, was not assayed due to poor viability). This assay was possible with mig-15

Cell Rep. Author manuscript; available in PMC 2019 April 26.
alleles because the isolated 2os in the n379 background could be readily identified, unlike as in the n1046 background, above.

To test background specificity, we evaluated the impact of gck-2(ok2867) in the lin-3/EGF(n378rf) hypo-induced rather than the let-60(n1046gf) hyper-induced background. lin-3(n378rf) supports 20% vulval induction (Hill and Sternberg, 1992). gck-2(ok2867) conferred increased vulval induction (Figure S3F), suggesting that the 2o-promoting signal of GCK-2 is not let-60(n1046gf) background dependent.

Collectively, these genetic results are consistent with the hypothesis that GCK-2 functions as an effector of RAL-1 2o-promoting activity. Meeting our first criterion, GCK-2 antagonizes 1o signal and is neutral in the absence of 2o-promoting EGF. This interpretation is supported below by further genetic analysis.

Conversely, MIG-15 is not consistent with a simple criterion of a RAL-1 2o-promoting effector. Unlike RAL-1, MIG-15 antagonizes both 1o and 2o signals, even in the absence of EGF. We cannot exclude MIG-15 as a second RAL-1 effector, perhaps in a negative regulatory relationship, which we will address in the Discussion. Thus, further analysis of MIG-15 function is outside the scope of this study.

GCK-2 Is Sufficient to Induce 2o Fate in Support of LIN-12/Notch

Our second criterion is that activated effector should phenocopy constitutively activated RAL-1. RAL-1 is sufficient to promote 2o fate: transgenic VPC-expressed ral-1(gf) significantly increased ectopic 2o induction in the lin-12(n379d) background (Zand et al., 2011). This same extrachromosomal array, when integrated as reIs10[ral-1(gf)] (H.S. et al., unpublished data), also increased ectopic 2os in the lin-12(n379d) background (Figure 4A). Using CRISPR/Cas9-mediated genome editing, we generated a gain-of-function mutation (G26V) in the endogenous ral-1 locus, also including an in-frame 50 end mKate2^33Flag tag (see Figures S6I and S6J). The resulting ral-1(re160gf [mKate2^33Flag::ral-1(G26V)]) caused increased 2o fate induction in the lin-12(n379d) background (Figures 4B–4D).

To test whether GCK-2 is sufficient to induce 2o fate, we used CRISPR to generate a putative gck-2(gf). Deletion of the proline-rich linker is thought to constitutively activate Drosophila Msn (MIG-15 group; Su et al., 2000). We deleted the linker in GCK-2 by using the co-CRISPR strategy (Arribere et al., 2014). The gck-2(re113re222) mid-D was generated in gck-2(re113 [mNG^33Flag::gck-2]), which we had already engineered (see below and Figures 4E and S4A). We also generated the gck-2(re113re223) out-of-frame mid-D, which served as a negative control. We assessed protein expression in these alleles by western blot: the out-of-frame re113re223, but not the in-frame re113re222, abolished detectable tagged GCK-2 (Figure 4E). The in-frame re113re222, but not the out-of-frame re113re223, significantly increased ectopic 2o induction in the lin-12(n379d) background (Figure 4F).

We also tested GCK-2 cell autonomy by generating transgenes expressing VPC-specific putative activating gck-2(mid-D) into the lin-12(n379d) background, where we observed weakly increased ectopic 2o induction (p = 0.06; Figure S4B). The same transgene in the lin-12(n379d); gck-2(tm2537) background significantly increased ectopic 2o induction (p
Thus, GCK-2 is sufficient to induce increased 2o induction in support of LIN-12/Notch, consistent with GCK-2 functioning as a 2o-promoting effector of RAL-1.

GCK-2 Functions Downstream of LIN-3/EGF and RAL-1

Our third criterion is that loss of effector function should be epistatic to constitutively activated RAL-1. reIs10[ral-1(gf)] enhanced ectopic 2o induction in the lin-12(n379d) background (Figure 4A) and was blocked by gck-2(ok2867) and gck-2(tm2537) (Figure 5A). Thus, GCK-2 meets our third criterion for a RAL-1 effector.

Critically, in light of complex results from exoc-8(ok2523) in different backgrounds (Figure S2D; see above), reIs10[ral-1(gf)]-dependent ectopic 2o induction was blocked by exoc-8(ok2523) (Figure 5B). That RAL-1 2o-promoting activity depends on EXOC-8 is consistent with EXOC-8 functioning downstream of RAL-1 to transduce the 2o-promoting signal. We therefore speculate that a RAL-1-EXOC-8-GCK-2 cascade transduces a 2o-promoting signal while acknowledging that EXOC-8 may perform other functions (see Discussion).

Multiple lines of evidence indicate that LIN-15 and many other genes in the ‘‘synMuv’’ group cooperate to redundantly restrict LIN-3/EGF expression to the AC (Cui et al., 2006; Fay and Yo-chem, 2007; Herman and Hedgecock, 1990; Huang et al., 1994; Myers and Greenwald, 2005). We previously exploited this feature of the vulval system to titrate LIN-3/EGF ‘‘dose’’ to induce ectopic 2o, but not 1o, VPCs in the lin-12(n379d) background (Zand et al., 2011), consistent with earlier manipulations of LIN-3/EGF and LET-23/EGFR signals to promote 2o fate, which supported the morphogen gradient model (Katz et al., 1995, 1996). At 15oC, a temperature-sensitive mutation in lin-15, n765ts, supports normal vulva induction without inducing ectopic 1o cells. But in the lin-12(n379d) background at 15oC, n765ts strongly increased ectopic 2o induction. We showed that this 2o-promoting activity depends on LIN-3/EGF, LET-60/Ras, RGL-1/Ral-GEF, and RAL-1: RNAi depletion of let-60, rgl-1, and ral-1 blocked the increased ectopic 2os conferred by lin-12(n379d); lin-15(n765ts) at 15oC. We further showed that excess expression of LIN-3/EGF and an activating mutation in LET-23/EGFR conferred similar promotion of 2o fate via activation of the LET-60-RGL-1-RAL-1 module (Zand et al., 2011).

As expected, ral-1(gk628801rf) decreases ectopic 2o induction in the lin-12(n379d); lin-15(n765ts) background at 15o C (Figure 5C), validating our prior results using ral-1(RNAi) (Zand et al., 2011). Similarly, gck-2(RNAi) and gck-2(ok2867) reduced ectopic 2o induction in the lin-12(n379d); lin-15(n765ts) background (Figures 5D and S5A). Thus, we conclude that the 2o-promoting signal of EGF is, at least in part, GCK-2 dependent, consistent with RAL-1 signaling though GCK-2.

Kinase-Dependent GCK-2 Functions Cell Autonomously in VPCs

Our fourth criterion for a RAL-1 effector is that its 2o-promoting activity functions cell autonomously. We generated transgenic extrachromosomal arrays expressing VPC-specific GCK-2(+) and assessed rescue of mutant gck-2 suppression of activated RAL-1. In the reIs10[ral-1(gf)]; lin-12(n379d); gck-2(tm2537) and reIs10[ral-1(gf)]; lin-12(n379d); gck-2(ok2867) backgrounds, VPC-specific expression of wild-type GCK-2 restored the
increased 2o induction phenotype suppressed by gck-2 mutations (Figures 5E, S5B, and S5C). Conversely, in the same backgrounds, VPC-specific expression of putative kinase dead (KD) GCK-2 (for HPK1/MAP4K1, GCK-2 group; Kiefer et al., 1996) failed to rescue mutant gck-2 suppression of rels10 (Figures 5F and S5D). Remember that gck-2(ok2867) enhanced ectopic 1o induction in the let-60(n1046gf) background (see Figure 3E, above). VPC-specific expression of GCK-2(+/-) had no effect in the let-60(n1046gf) background, controlling for effects of VPC-specific GCK-2(+/-) overexpression (Figure S5E). VPC-specific expression of GCK-2(+/-) restored baseline levels of ectopic 1o induction in the let-60(n1046gf); gck-2(ok2867) background (Figure S5E). Taken together, these results suggest that GCK-2 functions cell autonomously in VPCs via its kinase activity.

RAL-1 and GCK-2 Are Expressed in VPCs

Our fifth criterion is that a RAL-1 effector be expressed in the VPCs. When analyzing the sEx10525[p gck-2::gfp+dpy-5(+)]/transcriptional reporter transgene (Hunt-Newbury et al., 2007), we observed no GFP expression in VPCs. Therefore, we used CRISPR-Cas9 genome editing to insert mNG::33Flag into the 50 end of the endogenous gck-2 gene, generating gck-2(re113 [mNG::33Flag::gck-2]) (Figure S6A). We confirmed alleles by western blot (Figure S6B). We observed cytosolic tagged GCK-2 throughout vulval development (Figures 6A, 6B, and S6C–S6F). We observed the expression of GCK-2 in all tissues, including the germline and embryos in the adult hermaphrodite (Figures S6G and S6H).

We also inserted mKate2::33Flag into the 50 end of the endogenous ral-1 gene, with and without the activating G26V mutation (Figures S6I, S6J, S6Q, and S6R). The observed subcellular localization of RAL-1 and RAL-1(G26V) was similar, suggesting that the activating mutation does not alter localization. We observed tagged RAL-1 and RAL-1(G26V) throughout vulval development (Figures 6C, 6D, S6K–S6N, and S6S–S6X). Tagged RAL-1 and RAL-1(G26V) expression in the animal was ubiquitous, including vulva and germline, and localized primarily to plasma membrane and adherens junctions (Figures S6O, S6P, S6Y, and S6Z). To assess co-localization of RAL-1 and GCK-2, we made the ral-1(re160gf[mKate2::33Flag::ral-1(G26V)]); gck-2(re113[mNG::33Flag::gck-2]) strain. We observed strong localization of RAL-1(G26V) to plasma membrane and junctions and GCK-2 to cytosol at Pn.p (1-cell) and Pn.px (2-cell) stages (Figures 6E, 6F, S6AA, and S6AB). Thus, expression of tagged endogenous RAL-1 (wild-type and G26V) and GCK-2 are expressed in VPCs, one of our criteria for a RAL-1-GCK-2 signaling cascade. However, we did not observe evidence of 2o-specific recruitment of GCK-2 to the plasma membrane by activated RAL-1. We will consider this incongruity further in the Discussion.

The PMK-1/p38 MAPK Functions Downstream of RAL-1 and Is Expressed in VPCs

The GCK-2 group is part of the Ste20 family of MAP4Ks and is frequently associated with activation of JNK or p38 MAPK cascades (Dan et al., 2001; Delpire, 2009). Based on our model that RAL-1 signals through GCK-2, we investigated components of MAPK cascades as functioning downstream of RAL-1, identifying MLK-1/MAP3K and PMK-1/p38 as putative components of the RAL-1-GCK-2 2o-promoting signaling cascade.
C. elegans encodes orthologs of MAP3Ks and MAP2Ks (Sakaguchi et al., 2004). The km19 deletion in MLK-1/MLK/MAP3K (Mizuno et al., 2004) enhanced let-60(n1046gf) versus n1046 alone (p = 0.009; N of 90 and 60, respectively), consistent with MLK-1/MAP3K acting in this cascade. In contrast, the ok1382 deletion in MTK-1/MEKK4/MAP3K failed to enhance let-60(n1046gf) versus n1046 alone (p = 0.4; N of 50 and 91, respectively). The km4 deletion in SEK-1/MKK3/6/MAP2K (Tanaka-Hino et al., 2002) and the ok1545 deletion in MKK-4/MKK4/MAP2K failed to enhance let-60(n1046gf) versus n1046 alone (p = 0.6 N of 60 and 60; and p = 0.2 N of 90 and 90, respectively). Several other MAP2Ks were not tested.

C. elegans encodes three p38/MAPK paralogs in an operon, in order: PMK-2; PMK-3; and PMK-1. Of these, PMK-2 and PMK-3 are expressed primarily in intestine and PMK-1 is expressed more broadly. All three are thought to contribute to innate immunity and stress response (Mertensköter et al., 2013). Fitting some of our criteria for a RAL-1 effector, putative null pmk-1(km25) (Mizuno et al., 2004) conferred increased ectopic 1o induction with let-60(n1046gf) and blocked increased ectopic 2o induction with ral-1(gf); lin-12(n379d) (Figures 7A and 7B).

We tagged the endogenous pmk-1 gene with mNG::33Flag at the 30 end (Figures S7A and S7B). We observed PMK-1::mNG expression in vulval lineages and throughout the rest of the animal, with the exception of the germline; germline expression appeared to be silenced, an established phenomenon with certain foreign DNA insertions (Figures 7C, 7D, and S7C–S7F; Dickinson et al., 2015). Thus, expression of PMK-1 meets the criteria of an effector that functions downstream of RAL-1. Using a transgenic pmk-1 promoter translational GFP fusion (Ppmk-1::pmk-1::gfp; Mertensköter et al., 2013), we observed no vulval signal (Figures S7G–S7J). However, because pmk-1 is the last gene in an operon, key regulatory elements may be absent from the construct.

We had hoped to observe activity-dependent cytosol-to-nucleus translocation (Ben-Levy et al., 1998) of PMK-1 as a biomarker for upstream RAL-1 signaling activation. Instead, we observed nuclear and cytosolic localization in all cells, including vulval lineages (Figures 7C, 7D, and S7C–S7F). We considered our C-terminal CRISPR tagging scheme may have altered PMK-1 localization, even though we did not observe phenotypic changes conferred in the sensitized background by PMK-1::mNG (see STAR Methods). Therefore, we N-terminally tagged PMK-1 expressed from lin-31 promoter in VPCs, using the mini-Mos system (de la Cova et al., 2017), and with mKate2 rather than mNG. We observed localization to both nuclei and cytosol of VPCs (Figures S7K–S7N). Consequently, we propose that our tagging strategies do not disrupt PMK-1 function but rather that part of the endogenous PMK-1 population is constitutively targeted to the nucleus. We speculate that overexpression from the extrachromosomal array shows mostly cytosolic localization because only a small subset of the total PMK-1 molecules occupy the nucleus and that the proportion of nuclear PMK-1 to total protein is much higher when looking at endogenous rather than overexpressed protein.
DISCUSSION

We found that EXOC-8/Exo84 contributes to the LET-60/ Ras-RGL-1/RalGEF-RAL-1/Ral 2o-promoting signal during patterning of *C. elegans* VPC fate. By our genetic criteria, GCK-2, a CNH-domain-containing MAP4K orthologous to *Drosophila* Hppy and mammalian MAP4K1, 2, 3, and 5, is a downstream effector of RAL-1, as are MLK-1/MAP3K and PMK-1/p38 MAPK (Figure 7E). The LET-60-RGL-1-RAL-1-EXOC-8-GCK-2-MLK-1-PMK-1 cascade is a non-essential 2o-promoting signal in *C. elegans* VPC fate patterning that supports the essential 2o-promoting signal via LIN-12/Notch. We showed that GCK-2 and PMK-1 function downstream of RAL-1 cell autonomously and that GCK-2 is sufficient to promote 2o fate in support of LIN-12/Notch. We did not observe evidence of ectopic 2o induction by *rels10[rsl-1(gf)]* or *ral-1(re160gf)* alone. Thus, given the modest modulatory role of the RAL-1 2o-promoting cascade, there is no reason to propose that RAL-1 is sufficient to induce 2o fate, though the actual experiment in the absence of *lin-12* is as yet prohibitively difficult (abrogation of LIN-12 function duplicates the anchor cell and thus results in complex VPC induction; Greenwald et al., 1983).

Sec5 and Exo84 are subunits of the exocyst complex and known Ral binding partners in mammals (reviewed in Gentry et al., 2014; Kashatus, 2013). Ral-Exo84 and Ral-Sec5 regulate exocytosis, cancer cell proliferation, and immunity (Chien et al., 2006; Fukai et al., 2003; Issaq et al., 2010; Jin et al., 2005; Moskalenko et al., 2002, 2003; Sugihara et al., 2002). Yet we do not understand how Ral signaling is propagated through the Sec5 and Exo84 exocyst partners. Exo84 and Sec5 also confound biochemical identification of downstream signaling partners, because the exocyst is involved in central cell biological processes and potentially interacts with myriad partners (Tanaka et al., 2017; Wu and Guo, 2015). There are some exceptions: RalB-Sec5 directly recruits and activates the atypical IκB kinase family member TBK1 to contribute to human cancer cell survival (Chien et al., 2006); RalB-Exo84 promotes autophagosome assembly under starvation conditions in human epithelial cells (Bodemann et al., 2011); and under replete conditions, RalB-Sec5 stimulates mTORC1 activation in pancreatic tumor cells to promote cell invasion and inhibit autophagy (Martin et al., 2014). Yet we lack biomarkers for activated Ral and have limited knowledge of effectors downstream of Sec5 and Exo84. Using developmental patterning of the *C. elegans* VPCs as a simple model system, we defined a signaling cascade downstream of RAL-1 in development. Additional signaling cascades may function downstream of RAL-1 in different tissues.

The GCK-2 paralog, MIG-15, also contributes to VPC patterning: depletion of *mig-15* derepressed both 2o- and 1o-inducing backgrounds, respectively, *lin-12(n379d)* and *let-60(n1046gf)*. A 3o-promoting gene might be predicted to similarly antagonize both 1o- and 2o-promoting signals. However, the connection of MIG-15 to 1o-promoting signals and 2o-promoting signals is unclear and is complicated by the role of MIG-15 in vulval morphogenesis. In contrast, we clearly delineated GCK-2 genetically as a component of a positive regulatory cascade downstream of RAL-1-EXOC-8.

Although mutation of neither RLBP-1/RalBP1 nor SEC-5/Sec5 altered vulval patterning in sensitized backgrounds, mutation of EXOC-8/Exo84 conferred phenotypes consistent with
functioning as a signaling intermediary in a RAL-1 2o-promoting cascade. However, mutation of EXOC-8 also conferred defects consistent with other activities in VPC fate patterning: unlike reduced RAL-1 or GCK-2 function, reduced EXOC-8 function conferred increased 2o induction in the lin-12(n379d) 2o-inducing background. We speculate that EXOC-8 performs at least two functions: (1) an intermediary in RAL-1-GCK-2 2o-promoting signaling and (2) in an anti-2o capacity, perhaps with MIG-15. Thus, the roles of EXOC-8 and MIG-15 in VPC fate patterning are enigmatic and will be the subject of future genetic and biochemical studies, particularly as we develop better experimental tools via use of CRISPR.

Activation of the p38 and JNK families of MAPKs has variously been associated with activation of the GCK-I (GCK-2) and GCK-IV (MIG-15) subfamilies of CNH domain MAP4Ks (Delpire, 2009). Neither subfamily has been studied systematically. Our ongoing observation of the literature is consistent with the GCK-2 and MIG-15 subfamilies generally being associated with p38 and JNK activation, respectively. Yet so many of these studies depend on protein overexpression that we hesitate to draw general conclusions. Here, we connect GCK-2 with PMK-1/p38 function, and in Drosophila, dorsal closure of the embryo Msn (MIG-15 subfamily) is associated with JNK function (Su et al., 1998, 2000).

Activation of MAPKs is often associated with cytosol-to-nuclear translocation, initially shown with the canonical ERK MAPK (Gonzalez et al., 1993; Lenormand et al., 1993) but also shown for p38 MAPK (Ben-Levy et al., 1998). Extrachromosomal transgenic C. elegans PMK-1::GFP similarly translocated from cytosol to the nucleus upon stress (Mertensköter et al., 2013). Yet we found that this PMK-1::GFP fusion was not expressed in the VPCs, perhaps because pmk-1 is expressed as part of a multi-gene operon, and so the transgene is missing key regulatory sequences. Further, the transgenic PMK-1::GFP is likely to be overexpressed, as is typical for C. elegans transgenic arrays, and thus may obscure more nuanced regulatory inputs. Consequently, we generated endogenous PMK-1::mNG via CRISPR and also introduced VPC-specific single-to-low copy mK2::PMK-1 (Figures 7 and S7). Although we observed that endogenous PMK-1::mNG was expressed in VPCs, to our surprise, we observed consistent nuclear PMK-1::mNG throughout the animal, including VPCs throughout their fate patterning and nuclear mK2::PMK-1 in VPCs throughout their patterning. One could speculate that we perturbed PMK-1 function both through C- and N-terminal tagging, yet we showed that PMK-1::mNG function appeared normal in the let-23(sa62gf) background. These observations leave us at an impasse. Is the prevailing model for MAPK activation flawed? Is PMK-1/p38, a known stress kinase, tonically activated as a consequence of endogenous stressors or culture conditions? Or is translocation a modest part of the activation process that was previously masked by assay conditions? Our observation may lead to important mechanistic considerations of p38 activation and activation of MAPKs in general and is worth further investigation.

Many small GTPases, including mammalian RalA and RalB, are membrane-targeted through prenylation. Based on its C-terminal CAAX sequence, RAL-1 is inferred to be geranylgeranylated (Reiner and Lundquist, 2016), and our CRISPR tag of endogenous RAL-1 showed strong localization to the plasma membrane in all cells. The canonical mechanism for effector activation, originally defined for Ras-Raf (Block et al., 1996; Chiu
et al., 2002), is recruitment of cytosolic effector to the plasma membrane by activated small GTPase. Although our genetic analysis indicates that GCK-2 functions downstream of the 2o-promoting RAL-1 in VPCs, we did not observe co-localization of tagged endogenous RAL-1 and GCK-2, or activity-dependent enrichment of plasma membrane GCK-2, in presumptive 2o cells. This observation could be explained by activated, mem- brane-tethered RAL-1 recruiting only a small portion of cytosolic GCK-2. Alternatively, perhaps RAL-1 effector activation proceeds through an atypical mechanism, consistent with the non-canonical nature of the exocyst as an effector. The exocyst presents an interesting conundrum in signaling: it is clearly required for much Ral signaling (reviewed in Gentry et al., 2014), yet thwarts conventional biochemical bootstrapping through signaling cascades. Thus, the lack of co-localization of RAL-1 and GCK-2 could also be explained by certain populations of RAL-1 and GCK-2 being constitutively associated, perhaps at the exocyst complex. Though not the same subfamily (GCK-2 versus MIG-15), this model is consistent with co-immunoprecipitation of mammalian Sec5 and HGK/MAP4K4 (Balakireva et al., 2006). We speculate that the complex of RAL-1/EXOC-8/GCK-2 recruits a co-activator when RAL-1 is activated. Such a mechanism was implicated in studies of mammalian MAP4K3 (in the GCK-2 subfamily), in which a putative activating phosphorylation event was detected (Yan et al., 2010). Although shown to be inhibited by PP2AT61ε, the kinase(s) mediating this phosphorylation event remains unknown. Alternatively, perhaps RAL-1 and GCK-2 never physically interact, and thus genetic analysis was required to reveal this cascade.

The advent of CRISPR-based tools permits analysis of endogenous proteins and, hence, potentially improved cell biological and biochemical analysis. For example, all known Ral binding partners were discovered by yeast two-hybrid analysis, an approach with a strong record but also ample false negatives, say, in conditions of activity-dependent interactions or metazoan-specific subcellular localization. Thus, we may be able to use biochemical approaches with tagged endogenous RAL-1 to identify interactors. Yet we also recognize the balance of strengths and weaknesses in the model invertebrate system, so mammalian cell-based studies may complement our genetic analysis to elucidate details of molecular mechanisms.

It is as yet unclear whether our findings of a genetic requirement for PMK-1 downstream of the RAL-1 2o-promoting activity herald a similar use of p38 downstream of mammalian Ral isoforms during development or cancer. An alternative possibility is that Ral in various metazoa signals through an array of effectors, only of some of which are relevant to cancer. Genetic and tissue heterogeneity of tumors, coupled with the historic difficulty of assessing Ral activation levels in tumors, make this question non-trivial to address rigorously. This question is further complicated by the diverging functions of RalA and RalB in cancer and their mostly poorly defined roles in non-pathogenic development and physiology. Yet this study could lead to surveys of phospho-p38 levels in Ras-positive tumors as a function of RalA or RalB, thus potentially satisfying the great demand for cancer biomarkers of Ral activation. Alternatively, establishment of a viable in vivo invertebrate model for RAL-1 function, added to the development of elegant genetic tools, should lead to extensive investigation of RAL-1 in diverse areas of biology, potentially leading us to clinically important biomarkers and “druggable targets” other than p38.
In conclusion, we have demonstrated that the LET-60/Ras- RGL-1/RalGEF-RAL-1/Ral 2o-promoting signal acts through exocyst component EXOC-8/Exo84 to trigger a GCK-2/ MAP4K-PMK-1/p38 MAPK cascade. From a developmental biology standpoint, the mechanism by which the EGF morphogen gradient promotes 2o VPC fate in support of LIN-12/Notch is of long-standing interest (Kenyon, 1995). From a cancer biology standpoint, this study may also contribute to development of diagnostic biomarkers and small-molecule inhibitors for Ras- and Ral-dependent cancers.

STAR+METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, David J. Reiner (dreiner@ibt.tamhsc.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

*C. elegans handling and genetics*—All strains were derived from the N2 wild-type. Nomenclature was as described (Horvitz et al., 1979). Animals were cultured using standard conditions on OP50 bacteria on NGM agar plates at 20o C (Brenner, 1974) except where noted. Strains used are shown in the Key Resources Table.

PCR primers are listed in Table S1. Single animal genotyping PCR reactions used Taq PCR Master Mix (QIAGEN). Deletions in gck-2 were detected by triplex primers REW88/89/90 (Tm: 58oC, 32 cycles), resulting in 298 bp (wild-type), 478 bp (ok2867), and 462 bp (tm2537) amplicons. PCR products were sequenced to confirm reported allele perturbations (Khan et al., 2012). The exoc-8(ok2523) deletion was detected by triplex primers REW109/110/111 (Tm: 58oC, 32 cycles), resulting in 411 bp (wild-type) and 270 bp (ok2523) amplicons. The pmk-1(km25) was detected by PCR using triplex primers, REW85/86/87 (Tm: 55.5oC, 32 cycles), resulting in 345 bp and 591 bp (wild-type) and 216 bp (km25). ral-1(gk628801) was detected by primers DJR778/779 (Tm: 57oC, 32 cycles) to generate a 250 bp amplicon, followed by overnight digestion with HpyCH4IV (NEB) to yield wild-type (121 bp, 51 bp, 48 bp and 30 bp) and gk628801 (151 bp, 51 bp and 48 bp) bands.

The sec-5(pk2357)/+ mutation was maintained as a stable heterozygote by GFP-tagged balancer mIn1mIs14, and homozygotes were obtained by scoring non-green progeny.

METHOD DETAILS

**Vulval induction scoring assay**—To score vulval induction, late L4 animals were mounted on slides with a 3% agar pad in M9 buffer with 5 mM sodium azide. Invaginations of ectopic pseudovulvae were scored under DIC/Nomarski optics (Nikon eclipse Ni). Images were captured using NIS-Elements AR 4.20.00 software. The vulval induction index for ectopic 1o and 2o induction was scored as described elsewhere. Briefly, we counted vulval invaginations, comprising cell lineages of single VPCs undergoing morphogenesis, which were distinct from the composite 2o-1o-2o normal vulva lineages (the ‘‘Christmas tree’’, which we argue more closely resembles the Stanley Cup). As
expected, in the \textit{let-60(n1046gf)} background the normal vulva was oriented on the AC in the center of the gonad. The morphology of ectopic 1o lineages generally conformed with the symmetrical “cap” characteristic of isolated 1o lineages (Katz et al., 1995). In the \textit{lin-12(n379d)} background, the AC and normal vulva were mostly absent, as described (Greenwald et al., 1983). The morphology of ectopic 2o lineages generally conformed with the asymmetric “beret” characteristic of isolated 2o lineages (Green et al., 2008; Katz et al., 1995). When the AC and normal vulva were present in a \textit{lin-12(n379d)} animal, data from that animal were flagged and excluded from the final count of ectopic 2o cells.

As previously described (Zand et al., 2011), the \textit{let-60(n1046gf)} strain is liable to drift, resulting in increased induction of 1o cells. We established many frozen strains of \textit{n1046} single mutants, and \textit{n1046} outcrossed to N2, and established that the typical baseline is 1.2 to 1.5 ectopic 1o cells. We have also consistently observed that the \textit{n1046} baseline is increased by rv0.2 when grown on bacterially mediated RNAi, including \textit{gfp} or \textit{luciferase} control strains (Zand et al., 2011; this study). Consequently, for all strains harboring an \textit{n1046} mutation, we use a stringent protocol to minimize drift: strains are scored and a parafilmed plate established immediately after construction or thawing, strains are refreshed (if necessary) by chunking, and animals are never grown for several generations in culture. Assays in which \textit{n1046} control strains deviate from the expected baseline are discarded. When using this rigorous protocol, we rarely observe significant deviations from expected baselines. Each figure panel with VPC counts is from animals grown together at the same time.

**Plasmids, generation of transgenic lines**—Details of plasmid construction are available upon request. Transgenic lines were generated by microinjection of pB255-derived plasmids (50 ng/ml) with co-injection marker (20 ng/ml; either pPD118.33 [P\textit{myo−2}::\textit{gfp}] or pPD93.97 [P\textit{myo−3}::\textit{gfp}]) into the relevant strain and maintained by selecting for fluorescent animals. \textit{rels10[P\textit{lin-3}::ral-1(Q75L)+ P\textit{myo-2}::\textit{gfp}]} was generated and mapped to position I +5.1 (H.S. et al., unpublished data).

**Bacterially mediated RNA interference**—RNAi plasmids used were: III-7M13 (\textit{ral-1}), V-4P08 (\textit{gck-2}), X-5G21 (\textit{mir-15}), \textit{gfp} (Zand et al., 2011), and \textit{luciferase}. For an RNAi negative control, a luciferase fragment not having sequence overlap with the \textit{C. elegans} genome was amplified from SRE-luciferase plasmid and cloned into the Hind III- and Xho I-cut sites of L4440/pPD129.36. The host of bacterially-mediated RNAi clones was HT115 (Timmons and Fire, 1998). RNAi experiments were performed at 23°C on NGM agar plates supplemented with 1 mM IPTG and 50 mg/ml carbenicillin. Plates were seeded with 80 ml dsRNA-producing bacteria, grown overnight at room temperature, then populated with late \textit{L4} animals. Parents were transferred to another RNAi plate after 1 day, and ectopic pseudovulvae were scored by DIC at the late \textit{L4} stage, 2 days later.

**CRISPR/Cas9-dependent genome editing**—Repair templates were generated by PCR amplification from genomic DNA of rv500 bp homology by Q5 polymerase (NEB), digesting of the target SEC vector, and Gibson Assembly (NEB) directed by homologous ends. The sgRNA targeting sequences were inserted into pJW1236, the Cas9+sgRNA (F+E) plasmid (Ward, 2015), by Q5 site-Directed Mutagenesis (NEB). Plasmids and repair

\textit{Cell Rep.} Author manuscript; available in PMC 2019 April 26.
ssODNs used were listed in the Key Resources Table. mNG^3xFlag::GCK-2 was generated by microinjection of pH521 repair template (20 ng/ml), pH513 sgRNA-Cas9 #1 (25 ng/ml; 50-30 ACTGATGAGTGGGTGGCTCGAGG), pH514 sgRNA-Cas9 #2 (25 ng/ml; 50-30 ATTGATGAGTACTGCGGCGG), and injection marker Pmyo-2::mCherry (2.5 ng/ml) into wild-type (N2) animals. sgRNA targeting sequences are listed.

mKate2^3xFlag::RAL-1(+) and mKate2^3xFlag::RAL-1(G26V) were generated by microinjection of either pTD36 repair template for mKate2^3xFlag::RAL-1(+) or pTD40 repair template for mKate2^3xFlag::RAL-1(G26V) (20 ng/ml), pTD38 sgRNA-Cas9 #1 (25 ng/ml; 50-30 TTCAGAATGGAGGGTTACGGTGG), pHS23 sgRNA-Cas9 #2 (25 ng/ml; 50-30 GCTTCATAAAAAACAAAGGGCGG), and injection marker Pmyo-2::gfp (10 ng/ml) into N2. The ral-1(G26V) mutation was generated by Q5 site-Directed Mutagenesis (NEB) into the homology arm of the repair template used for CRISPR.

PMK-1::mNG^3xFlag was generated by microinjection of pH40 repair template (10 ng/ml), pH526 sgRNA-Cas9 #1 (50 ng/ml; 50-30 TAAGGATGATTCAGTGCGGGGG), pH525 sgRNA-Cas9 #2 (50 ng/ml; 50-30 GCTTCATAAAAAACAAAGGGCGG), and injection marker Pmyo-2::mCherry (2.5 ng/ml) into N2. To minimize steric hindrance, the linker sequence N-SAGGSAGGSAGG-C (Komatsu et al., 2011; 50-30 TCAGCGGAGGTAGCGCCCGAAGTCTGGTTGGA) was inserted between pmk-1 and mNG coding sequences, while RAL-1 and GCK-2 tagging had shorter linker N-SAGG-C (50-30 GGAGCCGGATCT-30) between FP::epitope and the N terminus. Animals were handled and treated with 5 mg/ml hygromycin as described (Dickinson et al., 2015).

The CRISPR knock-in results were confirmed by genotyping PCR using Taq PCR Master Mix (QIAGEN) and sequencing (Genewiz). Using the previously tagged DV3228 gck-2(re113 [mNG^3xFlag::gck-2]) as a starting point, the gck-2(mid-D) was generated by Co-CRISPR using dpy-10(cn64gf) as a Co-CRISPR marker (Arribere et al., 2014). sgRNA-Cas9 constructs were prepared by Q5 site-Directed Mutagenesis (NEB) of pJW1236. The repair ssODN, providing 35 bases of flaking homology arms on each side of the repaired break, was synthesized by IDT. We microinjected DV3228 with pH38 sgRNA-Cas9 #1 (25 ng/ml; 50-30 CTTCGATCTAGGCTGGAGG), pH339 sgRNA-Cas9 #2 (25 ng/ml; 50-30 TATGATATTAAAGTTTCCGAG), repair oligo (10 mM), sgRNA for dpy-10(cn64gf) (pJA58) (25 ng/ml), ssODN repair donor for dpy-10(cn64gf) (600 nM), and injection marker Pmyo-2::mCherry (2.5 ng/ml). Animals were handled and isolated as described (Arribere et al., 2014) by picking Rols and Dpys for PCR genotyping to detect the deletion, followed by sequence analysis of the repaired region and outcrossing to N2.

We assessed CRISPR tagged alleles for possible impacts on function by crossing tagged alleles into the let-60(n1046gf) and the let-23(sa62gf) sensitized backgrounds and comparing ectopic 1o induction of n1046 or sa62 alone versus n1046 or sa62+tagged allele strains. We observed induction indices of 1.4 versus 1.4 for n1046 versus n1046; gck-2(re113), respectively (p = 0.9), 1.4 versus 1.2 for n1046 versus n1046; ral-1(re218), respectively (p = 0.3), and 1.3 versus 1.3 for sa62 versus sa62. pmk-1(re170), respectively (p = 0.8). The functional impact of CRISPR putative gain-of-function mutations are shown in Figure 4B (for the ral-1(re160gf) G26V allele) and Figure 4F (for the gck-2(re113re222) mid-D allele). By visual inspection, none of the CRISPR tags or mutations altered the wild-type development.
Western blotting—Animals were lysed in 4% SDS loading buffer by boiling at 90o C for 2 minutes. Protein samples were run on 4%–15% SDS gel (BIO-RAD). Monoclonal anti-Flag antibody (Sigma-Aldrich F1804) and monoclonal anti-a-tubulin antibody (Sigma-Aldrich T6199) were diluted 1:2000 in blocking solution. Secondary antibody, goat anti-mouse (MilliporeSigma 12–349), diluted in 1:5000 in blocking solution. ECL reaction (Thermo Fisher Scientific) has done for signal generation. Immunoactive proteins were detected by film processor, SRX-101A (Konica Minolta) on X-ray film (Phenix).

MiniMos—reSi6 [P\text{lin-31}::mKate2::linker::pmk-1::unc-54 30UTR] was generated by miniMos (de la Cova et al., 2017; Frøkjær-Jensen et al., 2014). mKate2::linker(SAGG) was tagged to the N-terminal of pmk-1 with lin-31 promoter and unc-54 30UTR. MiniMos-based plasmid pSH41 was generated by subcloning of mKate2::linker::pmk-1 into pCC249 (P\text{lin-31}::unc-54 30 UTR) using Gibson Assembly (NEB). We microinjected N2 wild-type with the P\text{myo-2}::gfp (20 ng/ml), pGH8 (P\text{rab-3}::mCherry::unc-54 30UTR) (10 ng/ml), pCFJ601 (P\text{eft-3}:: mos1 transposase::tbb-2 30 UTR) (65 ng/ml), pMA122 (P\text{hsp16.4}::peel-1::tbb-2 30 UTR) (10 ng/ml), and pHS41 (P\text{lin-31}::mKate2::linker::pmk-1::unc-54 30 UTR) (10 ng/ml). The insertion site is unknown. MiniMos results were tracked by observing mKate2 signal in VPCs.

Image acquisition—L3 animals were mounted in 5 mM sodium azide/M9 buffer on slides with 3% agar pad. pmk-1(re170[pmk-1::mNG^3xFlag]) animals were grown on Comamonas sp. (DA1877) (Avery and Shtonda, 2003) and mounted in 2 mg/mL tetramisole in M9 buffer on slides with 3% agar pad. We hypothesized that stress caused constitutive translocation to the nucleus. However, growth on Comamonas sp. bacteria, which are thought to be non-inflammatory (Avery and Shtonda, 2003), did not alter the degree of nuclear translocation. Similarly, mounting animals on tetramisole rather than sodium azide did not abolish nuclear translocation. All images were captured by A1si Confocal Laser Microscope (Nikon) using NIS Elements Advanced Research, Version 4.40 software (Nikon).

QUANTIFICATION AND STATISTICAL ANALYSIS
In every bar graph panel, animals were scored concurrently to avoid variability, using scoring standards described in the Methods. Values represent either ectopic induction (0–3; 1o or 2o), or, in the case of under-induction, total number of VPCs induced (0–3; both 1o and 2o). Each bar represents mean induction of the cell type indicated, with error bars representing SEM. N equals the animals scored, and is indicated as a white number on each bar. To avoid bias, N was determined randomly, with all prepared animals scored and statistical tests only performed post hoc. General statistical methods are described in each figure legend. Briefly, pairwise tests were performed by t test, multiple tests by ANOVA. P value is shown in each panel, n.s. = not significant. Significance was defined as > 0.05, but in most cases each relationship was tested via multiple assays. For statistical analyses we used GraphPad Prism 5.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank the knockout consortia (Mitani, Barstead, and Moerman labs) plus I. Greenwald and N. Kirienko for strains. We thank members of the Reiner lab for helpful discussions and critical reading of the manuscript. Some strains were provided by the Caenorhabditis Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Wormbase was used constantly. We thank D. Dickinson and B. Goldstein (UNC) for sharing CRISPR SEC vectors prior to publication. We thank Dr. N. Kirienko (Rice) for sharing the AU0038 pmk-1 reporter and Drs. Kirienko and D. Garsin (UT Health) for sharing expertise about inflammatory response and Comamonas sp. bacteria. This work was supported by NIH grants GM085309 and GM121625 to D.J.R.

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• Mutationally activated endogenous RAL-1 and GCK-2 are sufficient to promote 2o fate
• Activated RAL-1 depends on downstream effectors EXOC-8, GCK-2, and PMK-1
• CRISPR-tagged GCK-2 was cytosolic and tagged RAL-1 localized to plasma membranes
• Endogenous CRISPR-tagged PMK-1 localized to both nuclei and cytosol
Figure 1. *C. elegans* CNH Domain Organization MAP4Ks and VPC Fate Patterning

(A) Domain organization and conservation of paralogous *C. elegans* MAP4Ks GCK-2 and MIG-15.

(B) VPCs, P3.p through P8.p, are patterned to assume 3°, 3°, 2°, 1°, 2°, 3° fate by coordinated graded action of EGF secreted from the anchor cell and Notch lateral signal. LET-60/Ras-LIN-45/ Raf-MEK-2/MEK-MPK-1/ERK and LET-60/Ras-RGL-1/RalGEF-RAL-1/Ral promote 1° fate and 2° induction, respectively. Presumptive 1° cells synthesize DSL ligands to induce 2° fate via LIN-12/ Notch.

*Cell Rep.* Author manuscript; available in PMC 2019 April 26.
Figure 2. EXOC-8 Functions in VPC Fate Patterning
The y axis indicates number of ectopic 1° cells. (A and B) The (A) ral-1 (gk628801rf) R139H missense and (B) exoc-8(ok2523) deletion mutations increased ectopic 1° induction in the let-60(n1046gf) background.

(C) The sec-5(pk2357) late nonsense allele (Frische et al., 2007) did not alter ectopic 1° induction in the let-60(n1046gf) background.

(D) The rlbp-1 out-of-frame deletion allele, tm3665, conferred no change in the let-60(n1046gf) background.

N indicated in white on columns. p value calculated by t test. Error bars represent SEM.
Figure 3. Loss of GCK-2, but Not MIG-15, Confers Defects Consistent with a RAL-1 Effector

(A) gck-2 gene structure, domain location, and genetic tools. Light gray: the re113rer222 (312 bp deletion) in-frame and re113rer223 (312 bp deletion; 35 bp insertion) out-of-frame mid-deletions remove the three PxxP sites. Black: tm2537 (565 bp out-of-frame deletion) and ok2867 (549 bp in-frame deletion) are shown. Dark gray: RNAi target sequence is shown.

(B–D) Differential interference contrast (DIC) images of late L4 vulvae and ectopic pseudovulvae. (B) N2 wild-type, (C) let-60(n1046gf), (D) let-60(n1046gf); gck-2(ok2867). Black arrow indicates normal vulva; white arrow indicates ectopic 1° pseudovulvae. The scale bars represent 20 μm.

(E) gck-2(ok2867) enhances ectopic 1° induction in the let-60(n1046gf) background.
(F) gck-2(RNAi) (V-4P08) and mig-15(RNAi) (X-5G21) both increase 1° induction in the let-60(n1046gf) background.
(G) mig-15(rh80) and mig-15(rh148) do alter ectopic 2° induction in the lin-12(n379d) background.

p values calculated by t test (E) or ANOVA (F and G). Error bars represent SEM.
Figure 4. RAL-1 and GCK-2 Are Sufficient to Drive 2° Fate Induction

(A and B) Exogenous (A; rels10[lin-31::ral-1(gf)]) and endogenous (B; ral-1(re160gf[mKate2^3× Flag::ral-1(G26V)]) activated RAL-1 increase 2° induction in the lin-12(n379d) background. Ectopic 2° induction is on the y axis.

(C) Vulvalless (Vul) late L4 lin-12(n379d) animal. Scale bar, 20 μm.

(D) Ectopic 2° pseudovulvae (white arrows) in ral-1(re160gf) lin-12(n379d). The scale bar represents 20 μm.

(E) Western blot detection of GCK-2 from lysates from endogenously tagged wild-type (re113), in-frame mid-deletion (re113re222), and out-of-frame mid-deletion (re113re223) animals, detected by anti-Flag antibody (1:2,000). The mNG::33×Flag:: GCK-2 fusion protein is predicted to be ~124 kDa and the mid-deletion ~110 kDa.

(F) The in-frame mid-deletion gck-2(re113re222), but not the out-of-frame mid-deletion gck-2(re113re223), caused increased 2° induction in the lin-12(n379d) background. Ectopic 2° induction is on the y axis.

p value calculated by t test or ANOVA. Error bars represent SEM.
Figure 5. GCK-2 Functions Cell Autonomously Downstream of LIN-3/EGF and Ral

(A and B) gck-2(ok2867) and gck-2(tm2537) (A) blocked the 2°-promoting activity of rels10[ral-1(gf)] in the lin-12(n379d) background, as does exoc-8(ok2523) (B).

(C and D) Strong enhancement of lin-12(n379d)-dependent 2° induction by lin-15(n765ts) at 15° is reduced by ral-1(gk628801rf) (C) and gck-2(ok2867) (D).

(E and F) Vulva-specific expression of wild-type (reEx176) (E), but not K44E putative kinase dead (reEx181) (F) GCK-2 rescues the suppression of rels10[ral-1(gf)] by gck-2(tm2537) in the lin-12(n379d) background.

Each column pair compares array-bearing versus non-array-bearing siblings. p value was calculated by t test or ANOVA. Error bars represent SEM.
Figure 6. Endogenously Tagged GCK-2 and RAL-1 Are Expressed in VPCs

(A and B) Representative confocal (A) and DIC (B) micrographs of the presumptive 1° (P6.p) and 2° (P5.7.p) VPCs of gck-2(re113[mNeonGreen^{3×Flag::gck-2}] animals.

(C and D) Confocal (C) and DIC (D) micrographs of the presumptive 1° (P6.p) and 2° (P5.7.p) VPCs of ral-1(re160gf[mKate2^{3×Flag::ral-1(G26V)}]) animals.

(E and F) Merged confocal (E) and DIC (F) micrographs of the presumptive 1° and 2° VPCs of the ral-1(re160gf); gck-2(re113) double mutant from a separate animal than the single tags.

“AC” label is placed directly above the anchor cell. The scale bars represent 20 μm.
Figure 7. PMK-1/p38 Function Downstream of RAL-1

Putative null, pmk-1(km25) confers increased ectopic 1°s in the let-60(n1046gf) background.

(B) pmk-1(km25) blocks the increased 2°s in the rels10[lin-31::ral-1(gf)]; lin-12(n379d) background. p values were calculated by t test. Error bars represent SEM.

(C and D) Representative confocal (C) and DIC (D) micrographs of pmk-1(re170[pmk-1::mNG^33 Flag]). Endogenously tagged PMK-1 is expressed in VPCs. Solid arrow, VPC nuclei; open arrow, gut nuclei. The scale bars represent 20 μm.

(E) A signaling transduction model. A LET-60/Ras-RGL-1/RalGEF-RAL-1/Ral-EXOC-8/Exo84-GCK-2/MAP4K-MLK-1/MAP3K-PMK-1/MAPK cascade promotes 2o fate in C. elegans VPC fate patterning.
## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Monoclonal anti-Flag antibody | Sigma-Aldrich | Cat#: F1804; RRID:AB_262044 |
| Monoclonal anti-a-tubulin antibody | Sigma-Aldrich | Cat#: T6199; RRID:AB_477583 |
| Goat anti-mouse antibody | MilliporeSigma | Cat#: 12–349; RRID:AB_390192 |

### Experimental Models: C. elegans Strains

| Strain Description | Source | Identifier |
|--------------------|--------|------------|
| MT12124: let-60(n1046gf) IV | Caenorhabditis Genetics Center | WB Strain: MT12124; WormBase: WBVar00089919 |
| DV2799: ral-1(gk628801) III; let-60(n1046gf) IV | This paper | N/A |
| DV2672: exoc-8(ok2523) I; let-60(n1046gf) IV | This paper | N/A |
| DV2698: sec-5(pk2357)tmIn1[mls14 dpy-10(e128)] II; let-60(n1046gf) IV | This paper | N/A |
| DV2711: exoc-7(oe2006) I; let-60(n1046gf) IV | This paper | N/A |
| DV2682: rlbp-1(tm3665) I; let-60(n1046gf) IV | This paper | N/A |
| DV2443: lin-12(n379d) III; him-8(e1489) IV | This paper | N/A |
| DV3460: ral-1(gk628801) lin-12(n379d) III; him-8(e1489) IV | This paper | N/A |
| DV3298: exoc-8(oe2523) I; lin-12(n379d) III; him-8(e1489) IV | This paper | N/A |
| DV2657: let-60(n1046gf) IV; gck-2(oe2867) V | This paper | N/A |
| DV2710: lin-12(n379d) III; him-8(e1489) IV; gck-2(oe2867) V | This paper | N/A |
| DV2727: lin-12(n379d) III; gck-2(tm2537) V | This paper | N/A |
| DV2965: lin-12(n379d) III; mig-15(ry80) X | This paper | N/A |
| DV2964: lin-12(n379d) III; mig-15(ry148) X | This paper | N/A |
| NJ490: mig-15(ry148) X | Caenorhabditis Genetics Center | WB Strain: NJ490; WormBase: WBVar00241556 |
| DV3498: let-60(n1046gf) IV; mig-15(ry148) X | This paper | N/A |
| MT378: lin-3(n378f) I V | Caenorhabditis Genetics Center | WB Strain: MT378; WormBase: WBVar00089466 |
| DV2833: lin-3(n378f) I V; gck-2(oe2867) V | This paper | N/A |
| DV3326: ral-1(re160gf[mKate2^3xFlag::ral-1(Q75L)]) lin-12(n379d) III; him-8(e1489) IV | This paper | N/A |
| DV2712: rels10[P\_nunc-33::ral-1(Q75L), P\_myo-2::gfp]; lin-12(n379d) III; him-8(e1489) IV | This paper | N/A |
| DV3440: lin-12(n379d) III; him-8(e1489) IV; gck-2(re113re222 [mNGP\_3xFlag::gck-2(mid-D)]) V | This paper | N/A |
| DV3441: lin-12(n379d) III; gck-2(re113re222 [mNGP\_3xFlag::gck-2(mid-D)]) V | This paper | N/A |
| DV3228: gck-2(re113re222[mNGP\_3xFlag::gck-2(mid-D)]) V | This paper | N/A |
| DV3410: gck-2(re113re222[mNGP\_3xFlag::gck-2(mid-D)]) V | This paper | N/A |
| DV3411: gck-2(re113re222[mNGP\_3xFlag::gck-2(mid-D)]) V | This paper | N/A |
| DV3040: lin-12(n379d) III; him-8(e1489) IV; relEx143[P\_nunc-33::gck-2(mid-D), P\_myo-2::gfp] | This paper | N/A |
| DV3125: lin-12(n379d) III; gck-2(tm2537) V; relEx161[P\_nunc-33::gck-2(mid-D), P\_myo-2::gfp] | This paper | N/A |
| DV2725: rels10[P\_nunc-33::ral-1(Q75L), P\_myo-2::gfp]; lin-12(n379d) III; gck-2(oe2867) V | This paper | N/A |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| DV2726: reIs10[P<sup>lin-31::ral-1(Q75L), P<sub>myo-2::gfp</sub>]<sub>1</sub>; lin-12(n379d)<sub>III</sub>; <br>gck-2(tm2537)<sub>V</sub> | This paper | N/A |
| DV2734: reIs10[P<sup>lin-31::ral-1(Q75L), P<sub>myo-2::gfp</sub>]<sub>exoc-8(ok2523)<sub>1</sub>; lin-12(n379d)<sub>III</sub>; him-8(e1489)<sub>IV</sub> | This paper | N/A |
| DV2449: lin-12(n379d)<sub>III</sub>; lin-15(n765ts)<sub>X</sub> | This paper | N/A |
| DV2742: let-60(n1046gf)<sub>IV</sub>; reEx113[P<sup>lin-31::gck-2(+), P<sub>myo-2::gfp</sub></sup>] | This paper | N/A |
| DV3238: raI-1(re160gf[mKate2^3xFlag::ral-1(G26V)])<sub>III</sub> | This paper | N/A |
| DV3195: reIs10[P<sup>lin-31::ral-1(Q75L), P<sub>myo-2::gfp</sub>]<sub>1</sub>; lin-12(n379d)<sub>III</sub>; <br>gck-2(tm2537)<sub>V</sub>; reEx177[P<sup>lin-31::gck-2(+), P<sub>myo-2::gfp</sub></sup>] | This paper | N/A |
| DV3243: reIs10[P<sup>lin-31::ral-1(Q75L), P<sub>myo-2::gfp</sub>]<sub>1</sub>; lin-12(n379d)<sub>III</sub>; <br>gck-2(tm2537)<sub>V</sub>; reEx181[P<sup>lin-31::gck-2(K44E), P<sub>myo-2::gfp</sub></sup>] | This paper | N/A |
| DV3138: reIs10[P<sup>lin-31::ral-1(Q75L), P<sub>myo-2::gfp</sub>]<sub>1</sub>; lin-12(n379d)<sub>III</sub>; <br>him-8(e1489)<sub>IV</sub>; reEx167[P<sup>lin-31::gck-2(+), P<sub>myo-2::gfp</sub></sup>] | This paper | N/A |
| DV3303: ral-1(re160gf[mKate2^3xFlag::ral-1(G26V)])<sub>III</sub>; gck-2(re113[mNG^3xFlag::gck-2<sup>sec</sup>])<sub>V</sub> | This paper | N/A |
| DV3163: gck-2(re113[mNG^SEC^3xFlag::gck-2<sup>sec</sup>])<sub>V</sub> | This paper | N/A |
| DV3496: reSi6[P<sup>lin-31::mKate2::linker::pmk-1::unc-54 30 UTR</sup>] | This paper | N/A |

**Plasmids**

- **pREW2**: RNAi of luciferase was subcloned into L4440
- **pREW18**: P<sup>lin-1::gck-2(+):let-858 3′ UTR</sup>
- **pREW24**: P<sup>lin-1::gck-2(+):unc-54 3′ UTR</sup>
- **pREW25**: P<sup>lin-1::gck-2(mid-deletion):unc-54 3′ UTR</sup>
- **pREW32**: P<sup>lin-1::gck-2(K44E):unc-54 3′ UTR</sup>
- **pHS21**: Homology arms (534 bp upstream and 875 bp downstream) was subcloned into pDD268 mNG<sup>3xFlag::GCK-2 CRISPR knock-in (repair template)</sup>
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| pHS13: Mutagenized pJW1236 using primers, DJR769 and HS130 for mNG^3xFlag::GCK-2 CRISPR knock-in (sgRNA-Cas9 plasmid) (sgRNA #1) | This paper | N/A |
| pHS14: Mutagenized pJW1236 using primers, DJR769 and HS131 for mNG^3xFlag::GCK-2 CRISPR knock-in (sgRNA-Cas9 plasmid) (sgRNA #2) | This paper | N/A |
| pTD36: Homology arms (869 bp upstream and 754 bp downstream) | This paper | N/A |
| pTD40: Homology arms (869 bp upstream and 754 bp downstream) was subcloned into pDD285 for mKate2^3xFlag::RAL-1(G26V) CRISPR knock-in (repair template) | This paper | N/A |
| pTD38: Mutagenized pJW1236 using primers, DJR769 and TD143 for mKate2^3xFlag::RAL-1(+>) and mKate2^3xFlag::RAL-1(G26V) CRISPR knock-in (sgRNA-Cas9 plasmid) (sgRNA #1) | This paper | N/A |
| pHS23: Mutagenized pJW1236 using primers, DJR769 and HS166 for mKate2^3xFlag::RAL-1(+>) and mKate2^3xFlag::RAL-1(G26V) CRISPR knock-in (sgRNA-Cas9 plasmid) (sgRNA #2) | This paper | N/A |
| pHS40: Homology arms (785 bp upstream and 875 bp downstream) was subcloned into pDD268 for PMK-1::mNG^3xFlag CRISPR knock-in (repair template) | This paper | N/A |
| pHS26: Mutagenized pJW1236 using primers, DJR769 and HS177 for PMK-1::mNG^3xFlag CRISPR knock-in (sgRNA-Cas9 plasmid) (sgRNA #1) | This paper | N/A |
| pHS25: Mutagenized pJW1236 using primers, DJR769 and HS178 for PMK-1::mNG^3xFlag CRISPR knock-in (sgRNA-Cas9 plasmid) (sgRNA #2) | This paper | N/A |
| pHS38: Mutagenized pJW1236 using primers, DJR769 and HS264 for GCK-2 mid-deletion CRISPR knock-in (sgRNA-Cas9 plasmid) (sgRNA #1) | This paper | N/A |
| pHS39: Mutagenized pJW1236 using primers, DJR769 and HS265 for GCK-2 mid-deletion CRISPR knock-in (sgRNA-Cas9 plasmid) (sgRNA #2) | This paper | N/A |
| pHS41: Sequence of mKate2::linker::pmk-1 was subcloned into pCC249 (P_{lin-31}::unc-54 30 UTR) for generating reSi6 [P_{lin-31}::mKate2::linker::pmk-1::unc-54 30 UTR] | This paper | N/A |

ssODN (single stranded Oligo Deoxy Nucleotides)

| Repair ssODN for GCK-2 (mid-D) (71 bp): 50 -TATCCCTC ATCGCCGCAACACTTCTGCTAGAAACAGAT CGATGGGAGCAGTTTTCTCAGAAAAATGGATTTGAGAG- 30 | IDT | N/A |
| Repair ssODN for dpy-10 (cn64gf) (101 bp): 50 -CACTTGAGA CTCAATACGGGAGAGAAGAGATGACT GGAACCGTA CGGATAGGTAGCCTACTATAGTTAGGTTGCTAGGAC TTCACATGG CTTCAGACCAACAGCTAT- 30 | Arribere et al., 2014, IDT | N/A |