Integration of EGFR and LIN-12/Notch Signaling by LIN-1/Elk1, the Cdk8 Kinase Module, and SUR-2/Med23 in Vulval Precursor Cell Fate Patterning in *Caenorhabditis elegans*

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ABSTRACT Six initially equivalent, multipotential Vulval Precursor Cells (VPCs) in *Caenorhabditis elegans* adopt distinct cell fates in a precise spatial pattern, with each fate associated with transcription of different target genes. The pattern is centered on a cell that adopts the “1°” fate through Epidermal Growth Factor Receptor (EGFR) activity, and produces a lateral signal composed of ligands that activate LIN-12/Notch in the two flanking VPCs to cause them to adopt “2°” fate. Here, we investigate orthologs of a transcription complex that acts in mammalian EGFR signaling—*lin-1/Elk1*, *sur-2/Med23*, and the Cdk8 Kinase module (CKM)—previously implicated in aspects of 1° fate in *C. elegans* and show they act in different combinations for different processes for 2° fate. When EGFR is inactive, the CKM, but not SUR-2, helps to set a threshold for LIN-12/Notch activity in all VPCs. When EGFR is active, all three factors act to resist LIN-12/Notch, as revealed by the reduced ability of ectopically-activated LIN-12/Notch to activate target gene reporters. We show that overcoming this resistance in the 1° VPC leads to repression of lateral signal gene reporters, suggesting that resistance to LIN-12/Notch helps ensure that P6.p becomes a robust source of the lateral signal. In addition, we show that *sur-2/Med23* and *lin-1/Elk1*, and not the CKM, are required to promote endocytic downregulation of LIN-12-GFP in the 1° VPC. Finally, our analysis using cell fate reporters reveals that both EGFR and LIN-12/Notch signal transduction pathways are active in all VPCs in *lin-1/Elk1* mutants, and that *lin-1/Elk1* is important for integrating EGFR and *lin-12/Notch* signaling inputs in the VPCs so that the proper gene complement is transcribed.

KEYWORDS *C. elegans*; vulva; EGF receptor; Notch; Elk1; LIN-1; Cdk8; CDK-8; SUR-2; Med23

The development of the *Caenorhabditis elegans* vulva is a valuable paradigm for studying signal transduction and how cells integrate multiple signaling inputs to regulate the expression of specific gene complements. Six Vulval Precursor Cells (VPCs), numbered P3.p-P8.p, each have the potential to adopt one of three fates, termed 1°, 2°, or 3° (Figure 1A). These fates are specified in the L3 larval stage and can be distinguished by division pattern, marker expression, and the terminal cell types produced after the lineage is completed (Figure

1B). Patterning is initiated by an “inductive signal,” the Epidermal Growth Factor (EGF)-like LIN-3 ligand produced by the anchor cell (AC) of the gonad. LIN-3/EGF activates EGF receptor (EGFR) and a canonical Ras-Raf-ERK cascade in P6.p, the VPC nearest the AC, which causes P6.p to adopt the 1° fate. The EGFR-Ras-ERK cascade also promotes the transcription of genes encoding Delta/Serrate/LAG-2 (DSL) family ligands for LIN-12/Notch in P6.p. These ligands constitute a “lateral signal” that activates LIN-12/Notch in the flanking VPCs, P5.p and P7.p, and causes these cells to adopt the 2° fate. EGFR-Ras may also support LIN-12/Notch activity in P5.p and P7.p via an alternate effector pathway, RalGEF-Ral (Zand *et al.* 2011). The remaining VPCs do not receive either the EGF or DSL signals and adopt the 3° fate. In addition to positive regulatory modes, like ligand production, the precise patterning of VPCs depends on negative regulatory modes as well. These include mechanisms that set high thresholds for response in all

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VPCs, as well as mutually inhibitory mechanisms for cross-talk between EGFR and LIN-12/Notch in the presumptive 1° and 2° VPCs [reviewed in Sundaram (2006)].

Activating the EGFR signal transduction pathway modulates the activity of transcription factors that change target gene expression. The transcription factor LIN-1 is a member of the Elk1 subfamily of ETS transcription factors (Beitel *et al.* 1995; Hart *et al.* 2000; Shaye and Greenwald 2011), and is a key target of EGFR-Ras-ERK activation in VPCs (Beitel *et al.* 1995). LIN-1 (Jacobs *et al.* 1998) and Elk1 (Gille *et al.* 1992; Marais *et al.* 1993) are phosphorylated by ERK. For both LIN-1 (Leight *et al.* 2005, 2015) and Elk1 (Marais *et al.* 1993; Gille *et al.* 1995; Yang *et al.* 2003), the unphosphorylated, sumoylated transcription factor acts as a repressor, and the phosphorylated form acts as an activator. In mammalian cells, ERK-phosphorylated Elk1 associates with the Med23 component of the large multiprotein complex called Mediator, which links DNA-bound transcription factors to the basal transcription machinery (Allen and Taatjes 2015), to promote transcription of its targets. It has been proposed that the *C. elegans* ortholog SUR-2/Med23 (Singh and Han 1995), as well as another Mediator component with which SUR-2 interacts, LIN-25/Med24 (Tuck and Greenwald 1995; Nilsson *et al.* 1998), work together with LIN-1/Elk1 to promote 1° fate in P6.p (Leight *et al.* 2015). Indeed, there are clear roles for these genes in positively regulating 1° fate (Howard and Sundaram 2002; Tiensuu *et al.* 2005); however, the mechanisms by which SUR-2/Med23 and LIN-1/Elk1 regulate lateral signal gene transcription in VPCs do not conform to this model. SUR-2/Med23 is required for transcriptional activation of lateral signal genes in P6.p, likely via association with the Hox protein LIN-39, whereas LIN-1 is not required to activate transcription of lateral signal genes, only to repress transcription in all other VPCs (Zhang and Greenwald 2011).

Along with Elk1, Med23 also associates reversibly with the Cdk8 Kinase Module (CKM) (Boyer *et al.* 1999), a protein complex that modulates Mediator activity and consists of four subunits: Cdk8, Cyclin C, Med12, and Med13 [reviewed in Allen and Taatjes (2015)]. When associated with the Mediator core complex, the CKM can sterically prevent RNA Pol II binding to cause transcriptional repression of target genes, or can promote transcriptional activation via the kinase activity of Cdk8. In *C. elegans*, the CKM has been implicated in the control of cell cycle quiescence of VPCs (Clayton *et al.* 2008) and, when combined with mutations that activate EGFR pathway components or may have general effects on chromatin structure, in promoting ectopic vulval fate in VPCs that would normally adopt the 3° fate (Moghal and Sternberg 2003; Clayton *et al.* 2008; Grants *et al.* 2016). However, as discussed further herein, *cdk-8* and *cic-1*/Cyclin C null mutants are homozygous viable and have overtly normal vulval development, suggesting that they are not required for normal VPC fate patterning; in contrast, *dpy-22*/Med12 and *let-19*/Med13 null mutants are not viable, complicating the interpretation of their requirements in VPC fate patterning.

In this study, we analyze different combinations of *lin-1*, *sur-2*, and the CKM, and observe that they act in parallel to mediate different processes during VPC fate specification. Our analysis suggests that in the absence of EGFR signaling, the CKM, but not SUR-2, helps set a threshold to LIN-12/Notch activity in all VPCs. We also find that in the presence of EGFR signaling, all three factors are required to resist the response to ectopic LIN-12/Notch activity in P6.p, but only *sur-2*/Med23 and *lin-1*/Elk1 are required for endocytic downregulation of LIN-12-GFP in P6.p. Our further investigation of the role of *lin-1*/Elk1 in VPC patterning using cell fate reporters revealed that VPCs have characteristics of both 1° and 2° fate, suggesting that *lin-1*/Elk1 is important for integrating EGFR and *lin-12*/Notch signaling inputs in the VPCs so that the gene complement for a specific cell fate is transcribed.

Materials and Methods

C. elegans genetics

Full genotypes are listed in Supplemental Material, Table S1. The following mutations were used and are described fully in WormBase unless otherwise indicated: LGI: *cdk-8(tm1238)*, *sur-2(ku9)*; LGII: *let-19(os33)*, and a marked derivative of *mIn1[mls14 dpy-10(e128)]*; LGIII: *lin-12(n302)*, *lin-12(n941)*, a marked derivative of *qC1[dpy-19(e1259) glp-1(q339) qIs26]*, *cic-1(tm3740)*, *pha-1(e2123)*; LGIV: *lin-1(n304)*; and LGV: *lin-25(ga67)*. LGX: *dpy-22(e652)*, *nre-1(hd20)* *lin-15b(hd126)* [isolated as a double mutant in Schmitz *et al.* (2007)].

The following transgenes were used: *arIs107[mir-61p::2xnl-yfp]* (Yoo and Greenwald 2005); *arEx1080[lin-31p::lin-12(intraΔP)]* (Li and Greenwald 2010); *arIs131[lag-2p::2xnl-yfp]* (Zhang and Greenwald 2011); *arEx1575[lin-12::gfp]* (Karp and Greenwald 2013); *arIs222[lag-2p::tagrfp]* (Sallee and Greenwald 2015; Sallee *et al.* 2017); and *arTi31[lin-31p::lin-45(AA, V627E)]* (de la Cova *et al.* 2017).

The *arIs116[lst-5p::2xnl-yfp]* transgene was described by Choi (2009), and has been used as a LIN-12 target gene reporter and 2° fate marker in other studies [e.g., Li and Greenwald (2010), Karp and Greenwald (2013), and Keil *et al.* (2017)]. It is robustly expressed in the L3 stage, when 2° fate specification occurs, suggesting that LIN-12 activity is the main input into its expression in VPCs, in contrast to most other LIN-12 target gene reporters, which have a dynamic pattern of expression starting in the L2 stage (Yoo *et al.* 2004). We note that *arIs107[mir-61p::2xnl-yfp]* (Yoo and Greenwald 2005), also used in this study, is specific to the L3 stage but is harder to visualize than *arIs116*.

Generation of single-copy insertion miniMos-based transgenes

The *arTi102*, *arTi117*, *arTi120*, *arTi121*, and *arTi190* transgenes drive expression using regulatory elements from the *lin-31* promoter (Tan *et al.* 1998) and the *unc-54* 3'-UTR. Each were cloned in the miniMos transgenesis vector pCFJ910 (Frøkjær-Jensen *et al.* 2014) (Addgene, #44481). Single-copy

transgenes were generated by germline injection into N2 animals and insertions were isolated as described by Frøkjær-Jensen *et al.* (2014).

The transgenes *arTi102* and *arTi190* were modeled after *arEx1080* (Li and Greenwald 2010). They encode LIN-12 (intra Δ P), the intracellular domain of LIN-12 protein (WormBase sequence R107.8), from residues G931 to R1340. In *arTi102*, LIN-12(intra Δ P) is untagged; in *arTi190*, it is tagged at the C-terminus with mKate2.

The transgenes *arTi117*, *arTi120*, and *arTi121* encode CDK-8 protein (WormBase sequence F39H11.3) in frame with the T2A peptide (Ahier and Jarriault 2014) and mCherry-H2B. *arTi117* encodes CDK-8(+), and *arTi120* and *arTi121* encode CDK-8(D182A).

RNA interference (RNAi)

Feeding RNAi was performed as described, using HT115-derived bacterial strains expressing *C. elegans* gene sequences (Kamath and Ahringer 2003) (Source BioScience) or mCherry. Briefly, eggs were prepared from *lin-12(n302)*; *nre-1(hd20)* *lin-15b(hd126)* hermaphrodites maintained at 20° using a bleach/sodium hydroxide protocol and placed on RNAi plates containing the appropriate bacterial strain. RNAi experiments were conducted at 25°.

Assessment of “Multivulva” phenotype in the *lin-12(n302)* enhancement assay

Strains containing *lin-12(n302)* were grown at 20°. In RNAi experiments, eggs were placed on RNAi plates and the Multivulva phenotype of adults was assessed 3–4 days after egg preparation. Animals with three or more pseudovulvae were scored as Multivulva. When scoring conventional genotypes, L4 hermaphrodites were picked and the Multivulva phenotype of adults was assessed 24 hr later. Because *let-19(0)* homozygotes arrest during larval development, homozygous progeny of a balanced *let-19(os33)/mIn1* strain were identified by loss of the *mIn1[mIs14 dpy-10(e128)]* balancer.

Scoring fluorescent reporter expression

Strains were raised at 20 or 25° and scored at the L3 stage when VPCs were undivided (Pn.p), or had undergone either one cell division (Pn.px), or two cell divisions (Pn.pxx). Animals were immobilized in 10 mM levamisole, mounted on a 2% agarose pad on a glass slide, and imaged at 40 $\times$ on either a Zeiss Axio Imager D1 with an AxioCam MRm or a Zeiss Axio Imager Z1 with a Hamamatsu Orca-ER camera. Illumination was provided by an X-Cite 120Q light source (EXFO photonics solutions). Exposure times and scoring thresholds were established for individual reporters based on brightness of expression in control strains.

Data availability

All strains and reagents are available upon request. Please refer to Table S1 for full genotypes and strain names.

Results

The CKM negatively regulates *lin-12* activity in uninduced VPCs

lin-12(d) missense mutations cause ligand-independent constitutive activity (Greenwald and Seydoux 1990). All *lin-12(d)* mutants lack an AC and therefore lack the cellular source of the LIN-3/EGF inductive signal. These mutants can be ranked in an allelic series (Greenwald *et al.* 1983) based on their vulval phenotype: in a “weak” *lin-12(d)* mutant, exemplified by *lin-12(n302)*, all VPCs adopt the 3° fate, as in wild-type hermaphrodites when the AC is ablated; in a “strong” *lin-12(d)* mutant, higher constitutive *lin-12* activity causes all VPCs to adopt the 2° fate, causing a characteristic Multivulva phenotype (Figure 2A). Loss of a negative regulator such as *sel-10/Fbw7* boosts the activity of the weak *lin-12(d)* allele, such that all VPCs adopt the 2° fate instead of the 3° fate and the animals become Multivulva (Sundaram and Greenwald 1993; Hubbard *et al.* 1997).

Null alleles of *cdk-8* are homozygous viable and fertile, and have normal VPC fate specification based on marker gene expression and vulval anatomy (Figure 1C). We find that the null allele *cdk-8(tm1238)* enhances *lin-12(n302)* activity based on the characteristic Multivulva phenotype of adults (Figure 2, B and C) and expression of *arIs116[lst-5p::2xnl5-yfp]*, a direct transcriptional target of LIN-12/Notch (Li and Greenwald 2010) (Figure 2D). RNAi against *cdk-8* in a *lin-12(n302)* background sensitized for RNAi also enhances the Multivulva phenotype (Figure 2E). Thus, *cdk-8* behaves as a negative regulator of *lin-12* activity in this assay.

We evaluated the three other components of the CKM, *cic-1/Cyclin C*, *dpy-22/Med12*, and *let-19/Med13*, in the same assay. Null alleles of *cic-1* are homozygous viable and fertile, as is the hypomorphic allele *dpy-22(e652)*. Homozygous *let-19* null mutants can be obtained as sterile segregants from heterozygotes. Loss of activity of each of these genes enhances *lin-12(n302)*, based on the Multivulva phenotype or *arIs116[lst-5p::2xnl5-yfp]* expression (Figure 2, C–E). Thus, all four components of the CKM are required for negative regulation of *lin-12* activity in VPCs and are likely to work together in this process.

In human cells, Cdk8 kinase activity is dispensable when the CKM is associated with the Mediator core complex in repressing gene expression, implying that the CKM primarily plays a structural role in repressor mode (Knuesel *et al.* 2009a). In contrast, kinase activity appears to be essential when the CKM–Mediator complex promotes activation of target gene expression (Knuesel *et al.* 2009b). The mutation of a catalytic aspartate residue in the kinase domain to alanine (D173A) completely inactivates the kinase activity of mammalian Cdk8 (Akoulitchchev *et al.* 2000). Sequence alignment of *C. elegans* CDK-8 protein with the human Cdk8 protein shows that this catalytic residue and its context are conserved in *C. elegans* (Figure 2F), so we infer that the corresponding D182A mutation should lack kinase activity as well.

Figure 1 VPC fate specification. (A) Schematic of VPC fate specification. The EGF-like inductive signal produced by the AC of the gonad activates a canonical EGFR-Ras-ERK cascade in P6.p, causing it to adopt the 1° vulval fate and transcribe lateral signal genes, including the DSL-type ligand *lag-2*. The lateral signal activates LIN-12 in P5.p and P7.p, which adopt the 2° vulval fate. The fates are represented as 1° vulval fate (red), 2° vulval fate (blue), and 3° nonvulval fate (gray). (B) Photomicrographs of VPCs and their descendants in the L3 stage. The red 1° fate reporter, *arls222[lag-2p::tagrfp]*, is a direct target of the EGFR-Ras-ERK pathway. The yellow 2° fate reporter, *arls116[st-5p::2xnl-yfp]*, is a direct target of LIN-12/Notch. Top, marker expression in VPCs (the “Pn.p” stage); middle, marker expression in the daughters of P5.p-P7.p (the “Pn.px” stage); bottom, marker expression in the granddaughters of P5.p-P7.p (the “Pn.pxx” stage). (C) *cdk-8* null mutants have normal expression of cell fate markers. For each genotype, 14/14 individuals scored in a single experiment showed normal marker expression. Left, photomicrograph of a *cdk-8(tm1238); arls222[lag-2p::tagrfp]* hermaphrodite at the Pn.px stage, showing normal *lag-2* reporter expression in the anchor cell (arrowhead) and in the two daughters of P6.p (bracket). Right, photomicrograph of a *cdk-8(tm1238); arls116[st-5p::2xnl-yfp]* hermaphrodite at the Pn.px stage, showing normal 2° fate marker expression in the daughters of P5.p and P7.p (brackets). AC, anchor cell; DSL, Delta/Serrate/LAG-2; EGF, Epidermal Growth Factor; EGFR, EGF receptor; Pn.p, the L3 stage when VPCs were undivided; Pn.px, the L3 stage when VPCs had undergone one cell division; Pn.pxx, the L3 stage when VPCs had undergone two cell divisions; VPC, Vulval Precursor Cell.

We therefore investigated the requirement for kinase activity by examining the ability of wild-type CDK-8 or CDK-8(D173A), expressed in VPCs, to rescue the enhancement of *lin-12(n302)* by *cdk-8(0)*. To do so, we constructed single-copy insertion transgenes in which CDK-8(+) or CDK-8(D173A) is expressed in VPCs as part of a bicistronic transcript, made by fusing mCherry-H2B via the viral T2A peptide, which causes ribosome skipping and works efficiently in *C. elegans* (Ahier and Jarriault 2014). The visualization of mCherry-H2B gives confidence that the upstream CDK-8 protein is expressed in the VPCs even when it has no rescuing activity. We find that CDK-8(+) efficiently rescues the Multivulva phenotype of *cdk-8(0); lin-12(n302)*, while two independent lines carrying transgenes that express the CDK-8(D173A) mutant are not rescued (Figure 2G). The inability of CDK-8(D173A) to rescue the

enhancement of *lin-12(n302)* by *cdk-8(0)* supports the inference that the mutation abrogates kinase activity and suggests that kinase activity is essential for this role of the CKM.

We tested the requirement for *sur-2*/Med23 or *lin-25*/Med24 using null alleles, and did not observe enhancement of *lin-12(n302)* (Figure 2C). This observation contrasts with the requirement for *sur-2* in the negative regulation of *lin-12* activity when EGFR-Ras-ERK is active, as described below.

The CKM is not required for EGFR- and SUR-2-promoted transcription of the lateral signal gene *lag-2* in P6.p

Ligands for LIN-12 constitute the lateral signal, and the genes encoding these ligands are transcribed in P6.p in response to EGFR-Ras-ERK signaling (Chen and Greenwald 2004). Characterization of the upstream region of *lag-2* identified a

Figure 2 The CKM acts in a kinase-dependent manner to negatively regulate *lin-12* activity. (A) Schematic showing VPC fates adopted depending on the degree of elevated *lin-12* activity. *lin-12(d)* mutations prevent the development of the anchor cell. In *lin-12(n302)*, a relatively weak *lin-12(d)* allele, all VPCs adopt the 3° nonvulval fate, as would wild-type VPCs in the absence of an anchor cell. *lin-12(n302)* activity can be enhanced in VPCs by removal of negative regulators, resulting in strong activity and causing all VPCs to adopt the 2° fate. (B) Photomicrographs showing that *cdk-8(0)* enhances *lin-12(n302)* activity to cause the distinctive Muv phenotype associated with all VPCs adopting the 2° fate. (C) Loss of individual CKM components, but not the core Mediator components *sur-2* or *lin-25*, enhances *lin-12(n302)* activity, as assessed by the Muv phenotype. Graph shows percentage of adult hermaphrodites that are Muv. ** $P < 0.001$ compared to *lin-12(n302)* (Fisher's exact test). † Scored homozygous *let-19(os33)* progeny from heterozygous *let-19(os33)/mIn1* mothers. (D) Loss of individual CKM components enhances *lin-12(n302)* activity, as assessed by expression of the LIN-12 target reporter *arls116[lst-5p::2xnl5-yfp]*. Schematic representation of the percentage of individual VPCs that display YFP fluorescence. (E) Reduction of individual CKM components by RNAi enhances *lin-12(n302)* activity. Graph shows percentage of adult hermaphrodites that are Muv. ** $P < 0.001$ and * $P < 0.01$ compared to RNAi against mCherry (Fisher's exact test). (F) ClustalW2 alignment (Larkin et al. 2007) of human Cdk8 and *C. elegans* CDK-8 showing conservation of the region required for kinase activity. The aspartate residue essential for Cdk8 kinase activity, D182, and the corresponding *C. elegans* residue, D173, mutated to alanine for the analysis in G are highlighted in red. (G) CDK-8 kinase activity is necessary for negative regulation of *lin-12(n302)* activity. Graph shows percentage of adult hermaphrodites that are Muv. Transgene *arTi117* expresses CDK-8(+) in the VPCs, restoring activity and rescuing enhancement of *lin-12(n302)* by *cdk-8(0)*. Two independent transgenes (*arTi120* and *arTi121*) expressing the putative kinase-dead CDK-8(D182A) mutant in the VPCs do not rescue *cdk-8(0)*. All three transgenes express a bicistronic transcript in which CDK-8 is

cis-regulatory module composed of VPCrep, an element for repression in all VPCs, adjacent to VPCact, an element that is required for activation in all VPCs (Zhang and Greenwald 2011). The current model is that VPCrep is a binding site for LIN-1/Elk1 and VPCact is a binding site for a Hox protein, likely LIN-39. Both LIN-1 and LIN-39 are present in all VPCs (Wagmaister *et al.* 2006; Zhang and Greenwald 2011). When the inductive signal activates EGFR-Ras-ERK in P6.p, phosphorylation of LIN-1 by ERK relieves repression so that LIN-39 can promote transcription of *lag-2*. The Mediator components SUR-2/Med23 and LIN-25/Med24 are required for *lag-2* transcription even when VPCrep is deleted, consistent with Mediator acting in conjunction with LIN-39 to promote *lag-2* transcription through VPCact rather than acting with LIN-1/Elk1 to promote repression through VPCrep (Zhang and Greenwald 2011).

We assayed the effect of the CKM using null alleles for *cdk-8* and *cic-1* on the expression of the *lag-2* transcriptional reporter *arIs222[lag-2p::tagrfp]*, which contains 7.2 kb of 5' flanking region (Sallee and Greenwald 2015; Sallee *et al.*, 2017). This reporter is strongly expressed in P6.p and its descendants in otherwise wild-type worms (Figure 1B), and is expressed normally in mutants lacking the CKM components *cdk-8* or *cic-1* (Figure 1C). Furthermore, the 2° fate marker *arIs116[lst-5p::2xnl-yfp]* is expressed normally in CKM mutants, confirming that the lateral signal is produced (Figure 1C). These results suggest that *sur-2* and *lin-25* may promote transcription of lateral signal genes independently of the CKM, consistent with their distinctive abnormal vulval phenotypes. We also find that *arIs222[lag-2p::tagrfp]* expression in P6.p, as well as the AC, is greatly reduced in *let-19* animals, but such animals typically arrest prior to or during the L3 stage, so we cannot conclude that *arIs222[lag-2p::tagrfp]* transcription requires LET-19.

Resistance to activated LIN-12 in P6.p depends on the relative balance of EGFR and LIN-12 activity, and allows for robust expression of lateral signal gene reporters

As described above, constitutive activation of LIN-12 in *lin-12(d)* mutants eliminates the AC. When constitutively active forms of LIN-12 are expressed specifically in VPCs, and therefore do not prevent formation of the AC, P6.p adopts and maintains the 1° fate (Shaye and Greenwald 2005; Li and Greenwald 2010), suggesting the existence of a mechanism for countering *lin-12* activity associated with 1° fate.

To ascertain if the CKM, SUR-2, and LIN-1 play a role in this mechanism, we first needed to characterize it further. To do so, we utilized transgenes that drive expression in VPCs using regulatory sequences from the *lin-31* gene (*lin-31p*) (Tan *et al.* 1998) and the constitutively active form LIN-12(*intraΔP*). This derivative of the intracellular domain (“intra”) mimics the natural signal-transducing cleavage product (Struhl *et al.* 1993), but

is stabilized by removal of a degron (“ΔP”), resulting in more potent constitutive activity (Li and Greenwald 2010; Deng and Greenwald 2016).

In the presence of the extrachromosomal array *arEx1080[lin-31p::lin-12(intraΔP)]*, all VPCs other than P6.p adopt the 2° fate, while P6.p still adopts the 1° fate (Figure 3A). The LIN-12 target reporter *arIs116[lst-5p::2xnl-yfp]* is generally not transcribed in P6.p, consistent with implementation of a mechanism that resists constitutive *lin-12* activity (Figure 3A). In contrast, two independent, single-copy integrated transgenes that encode LIN-12(*intraΔP*) lead to transcription of *arIs116[lst-5p::2xnl-yfp]* in P6.p (Figure 4, A–C). We interpret this observation as indicating that these transgenes result in sufficient ectopic LIN-12 activity in P6.p to overcome resistance to LIN-12 activity in P6.p, thereby allowing us to examine the consequences of constitutive LIN-12 activity in a 1° VPC. To do so, we simultaneously scored *arIs116[lst-5p::2xnl-yfp]* and *arIs222[lag-2p::tagrfp]* on a VPC-by-VPC basis in the presence of the single-copy insertion transgene *arTi102[lin-31p::lin-12(intraΔP)]*. We observed that not only was *arIs116[lst-5p::2xnl-yfp]* expressed in P6.p, indicating that the resistance was overcome, but also that *arIs222[lag-2p::tagrfp]* expression was concomitantly reduced in P6.p, suggesting that resistance is important for ensuring strong lateral signal gene expression (Figure 4A).

To test if this effect may be an artifact of the *arTi102[lin-31p::lin-12(intraΔP)]* insertion site or *arIs222[lag-2p::tagrfp]* reporter, we combined a different single-copy insertion transgene, *arTi190[lin-31p::lin-12(intraΔP)-mkate2]* and a different *lag-2* reporter, *arIs131[lag-2p::2xnl-yfp]* (Zhang and Greenwald 2011). We observed a similar reduction of *lag-2* reporter expression in this independent combination (Figure 4C), suggesting that reduced lateral signaling is a *bona fide* effect of ectopic LIN-12 activation in a 1° VPC.

We also tested whether LIN-12 activity from the *arEx1080[lin-31p::lin-12(intraΔP)]* transgene, which appears to have lower constitutive activity, is sufficient to reduce transcription of *arIs131[lag-2p::2xnl-yfp]* in P6.p. We observed that *lag-2* reporter expression levels were initially reduced during the Pn.p stage (Figure 4C), consistent with the inference that lateral signaling in a 1° VPC is reduced by increased LIN-12 activity and further supporting the existence of a mechanism for countering *lin-12* activity in 1° VPCs. However, in this genotype, expression returned to wild-type levels by the Pn.px stage. We interpret the difference in the timing of the restoration of *lag-2* reporter expression between the two strains as reflecting the fact that expression driven by *lin-31p* regulatory sequences diminishes over the course of the lineage in induced VPCs, while the AC continues to produce LIN-3/EGF, so that the strength of *lin-12* activity and the number of copies of *lag-2*

fused to T2A-mCherry-H2B to confirm transgene expression in VPCs (see *Materials and Methods*). ** $P < 0.001$ compared to *cdk-8(0)*; *lin-12(n302)* (Fisher's exact test). CKM, Cdk8 Kinase module; Muv, Multivulva; RNAi, RNA interference; VPC, Vulval Precursor Cell; YFP, yellow fluorescent protein.

Figure 3 Resistance to constitutively active LIN-12 signal transduction in P6.p. (A) Left, schematic representing the resistance to LIN-12 activation in P6.p in the presence of the AC. The *arEx1080* transgene expresses constitutively active LIN-12(*intraΔP*) in all VPCs. However, the LIN-12 target reporter *arls116* is not expressed in P6.p, allowing genes to be assessed for roles in resistance to activated LIN-12 associated with 1° fate. Right, photomicrograph showing expression of the LIN-12 target reporter *arls116[lst-5p::2xnl::yfp]*; *arEx1080[lin-31p::lin-12(intraΔP)]* in daughters of all VPCs except P6.p, where LIN-12 signal transduction is resisted. (B) Loss of resistance to LIN-12 activation in P6.p in *cdk-8* null mutants. Left, schematic in which P6.p fate is denoted as 1°~2°, since it now expresses *arls116[lst-5p::2xnl::yfp]* but will form a functional vulva. Figure 4 considers the state of P6.p further. Right, photomicrograph of *cdk-8(tm1238); arls116[lst-5p::2xnl::yfp]; arEx1080[lin-31p::lin-12(intraΔP)]* showing resistance in P6.p is lost and YFP is present in daughters of all VPCs. (C) Control for D, showing that *arls116[lst-5p::2xnl::yfp]* is not ectopically expressed in P6.p in the absence of individual CKM components or *sur-2*. Graph shows percentage of animals with YFP fluorescence in P6.p. N.S. denotes not significant compared to wild-type (Fisher's exact test). (D) Loss of individual CKM or *sur-2* relieve resistance to expression of *arls116[lst-5p::2xnl::yfp]* in P6.p in the presence of *arEx1080[lin-31p::lin-12(intraΔP)]*. Graph shows percentage of animals with YFP fluorescence in P6.p. ** $P < 0.001$ compared to wild-type (Fisher's exact test). AC, anchor cell; CKM, Cdk8 Kinase module; VPC, Vulval Precursor Cell; YFP, yellow fluorescent protein.

regulatory sequences in the reporters may come into play later in development.

In sum, our results suggest that 1° fate-associated resistance to activated LIN-12 depends on the relative balance of EGFR and LIN-12 activity, and that this resistance helps ensure that P6.p becomes the robust source of lateral signal (Figure 4D). In the next section, we examine if the CKM and SUR-2 play a role in this resistance, and in the following section describe how our investigation of a potential role for *lin-1* in this resistance led to novel observations about the role of *lin-1* in VPC patterning.

The CKM and SUR-2/Med23 are required for resistance of P6.p to signal transduction by expression of constitutively active LIN-12/ Notch

As described above, analysis of the role of the CKM in the absence of the inductive signal suggests that the CKM plays a role in setting a threshold by opposing LIN-12 activity in the VPCs. We therefore assessed whether the CKM also mediates the resistance to activated LIN-12 in P6.p in the presence of the inductive signal when EGFR is activated. Additionally, we asked if *sur-2*, which acts in P6.p to promote lateral signal gene expression but does not enhance *lin-12(n302)* activity,

Figure 4 Activated LIN-12 expressed from single-copy transgenes overcomes resistance in P6.p and leads to repression of lateral signal gene expression. (A) Constitutive LIN-12 activity provided by *arTi102[lin-31p::lin-12(intraΔP)]* overcomes resistance to *lin-12* activity in P6.p, the 1° VPC. Each row in the chart represents an individual with *arls222[lag-2p::tagrfp]* and *arls116[lst-5p::2xnl5-yfp]* reporters. On the left, the reporters are in an otherwise wt background; on the right, they are in the background of *arTi102*, which expresses LIN-12(intraΔP). In each hermaphrodite, each marker was scored on a per VPC basis. Red, only tagRFP fluorescence was present; blue, only YFP fluorescence was present; purple, both tagRFP and YFP were present; and gray, no fluorescence was observed. (B) *arls131[lag-2p::2xnl5-yfp]* expression is inhibited by LIN-12(intraΔP) expressed from *arTi190*. Photomicrographs of a

is required for resistance to activated LIN-12 in P6.p. To do so, we removed the activity of individual genes using null alleles in the presence of *arEx1080[lin-31p::lin-12(intraΔP)]*, the weak transgene that does not overwhelm the resistance in P6.p, and assessed transcription of the LIN-12 target *arIs116[lst-5p::2xnl-yfp]*. We found that removal of any of these genes allowed *arIs116[lst-5p::2xnl-yfp]* transcription in P6.p, indicating that resistance to activated LIN-12 due to EGFR activation is relieved (Figure 3, B–D).

We then assessed whether removal of *cdk-8* reduces *arIs131[lag-2p::2xnl-yfp]* expression in the presence of *arEx1080[lin-31p::lin-12(intraΔP)]*, as might be expected if loss of *cdk-8* increases constitutive *lin-12* activity. Although we could only assay a limited number of Pn.px-stage hermaphrodites due to the low brood size of the strain, we observed reduction of *lag-2* reporter expression in 4/8 *cdk-8(0)*; *arIs131[lag-2p::2xnl-yfp]*; *arEx1080[lin-31p::lin-12(intraΔP)]* hermaphrodites as compared to *arIs131[lag-2p::2xnl-yfp]*; *arEx1080[lin-31p::lin-12(intraΔP)]* hermaphrodites (13/14), which is statistically significant by Fisher's exact test ($P < 0.04$).

The requirement for the CKM for resistance to nuclear LIN-12 contrasts with the lack of a requirement for the CKM to promote *lag-2* transcription in the same VPC. These observations are consistent with independent function of different subcomplexes, e.g., a subcomplex containing both the CKM and SUR-2 for resistance to nuclear LIN-12, and a subcomplex that lacks the CKM (but may have LET-19/Med13) for expression of *lag-2*. We could not test if CDK-8 kinase activity is required for resistance to nuclear LIN-12 in P6.p because of synthetic lethality when we tried to construct strains containing *cdk-8(0)*, *arIs116*, *arEx1080*, and any of the single-copy CDK-8-expressing transgenes, for reasons we did not investigate further.

Loss of LIN-1 leads to ectopic LIN-12 signal transduction in all VPCs

Previous studies suggest that loss of *lin-1* does not affect production of the inductive signal by the AC or activation of the EGFR-Ras-ERK phosphorylation cascade *per se* (Beitel *et al.* 1995; de la Cova and Greenwald 2012). We began to test if *lin-1* is required for resistance to activated LIN-12 in P6.p by constructing a control strain in which *lin-1(n304)*, a null mutant, and the LIN-12 target gene reporter *arIs116*

[lst-5p::2xnl-yfp], were combined. We observed that the reporter is expressed in all VPCs in this strain, even though it lacks any transgenic source of constitutively active LIN-12 (Figure 5, A and B). To determine whether this unexpected expression of *arIs116[lst-5p::2xnl-yfp]* in all VPCs is a property of the reporter transgene, we tested a second LIN-12 target gene reporter, *arIs107[mir-61p::2xnl-yfp]* (Yoo and Greenwald 2005). Again, we observed ectopic expression in all VPCs in *lin-1(n304)* (Figure 5C), indicating that LIN-12 target genes may be generally derepressed in the absence of *lin-1* activity. We note that precocious derepression of *arIs116[lst-5p::2xnl-yfp]* is not observed during the L2 stage (Figure 5D), indicating that the temporal mechanism that normally restricts induction of this target to the L3 stage is not abrogated by loss of *lin-1*, and suggesting that the observed expression in all VPCs reflects a function of *lin-1* relevant to spatial patterning.

Due to the above observations, and because loss of *lin-1* leads to derepression of lateral signal gene expression (Zhang and Greenwald 2011), we hypothesized that LIN-12 signal transduction is ectopically activated in all VPCs in a *lin-1(0)* mutant background. We tested this hypothesis genetically by examining the effect of removing *lin-12* activity in a *lin-1(0)* background. If LIN-12 signal transduction occurs in all VPCs in a *lin-1(0)* background, then *lst-5* expression in the absence of LIN-1 would still require *lin-12* activity. When we examined *lin-12(0)*; *lin-1(0)*; *arIs116[lst-5p::2xnl-yfp]* hermaphrodites, we observed that *arIs116[lst-5p::2xnl-yfp]* was no longer expressed (Figure 5E), consistent with the possibility that LIN-12 signal transduction is ectopically activated.

Normally the EGFR-mediated inductive signal causes LIN-12 protein to be endocytosed and degraded in P6.p (Levitani and Greenwald 1998; Shaye and Greenwald 2002) (Figure 5, F and G), yet P6.p expresses LIN-12 target gene reporters in *lin-1(0)*. Thus, if loss of *lin-1* indeed causes ectopic LIN-12 signal transduction, we would predict that loss of *lin-1* also prevents LIN-12 downregulation in P6.p despite its 1° fate characteristics, such as *lag-2* expression and formation of a functional vulva. Indeed, when we assessed the presence of LIN-12-GFP in a *lin-1(0)* background, we observed that it is visible at the apical membrane of P6.p, as in the other VPCs, indicating that it has not been downregulated (Figure 5, F

hermaphrodite of genotype *arTi102[lin-31p::lin-12(intraΔP)]*; *arIs222[lag-2p::tagRFP]*; *arIs116[lst-5p::2xnl-yfp]* showing expression of the *lst-5* reporter in daughters of P6.p (top), and, inhibition of the *lag-2* reporter expression in P6.p in the same individual (middle). In the merge (bottom), tagRFP can be seen in the AC (arrowhead) and neurons, but not in Pn.px cells. (C) Constitutive LIN-12 activity from *arTi190[lin-31p::lin-12(intraΔP)-mkate2]* overcomes resistance to *lin-12* activity in P6.p, leading to reduced expression of *arIs131[lag-2p::2xnl-yfp]* and to ectopic transcription of *arIs116[lst-5p::2xnl-yfp]*. In this panel, the two markers were scored in separate strains. The graphs show percentage of animals with fluorescent protein expression in P6.p, its daughters, and granddaughters. At the Pn.p stage, * $P < 0.015$ and ** $P < 0.0005$ when wt and transgene-containing strains were compared (Fisher's exact test). When *arEx1080* and *arTi190* are compared in the Pn.px and Pn.pxx stages, * $P < 0.0005$. In the bottom graph, the "+" value represents the same data shown in (A). (D) Model illustrating that resistance to activated LIN-12 in P6.p depends on the relative balance of EGFR and LIN-12 activity. Left, resistance to weak activity of LIN-12(intraΔP) allows for expression of *lag-2* reporters and lack of expression of LIN-12 target reporters. Right, resistance can be overcome by strong activity of LIN-12(intraΔP), resulting in expression of LIN-12 target gene reporters and diminished expression of *lag-2* reporters. EGFR, Epidermal Growth Factor receptor; Pn.px, the L3 stage when VPCs had undergone one cell division; Pn.pxx, the L3 stage when VPCs had undergone two cell divisions; RFP, red fluorescent protein; VPC, Vulval Precursor Cell; wt, wild-type; YFP, yellow fluorescent protein.

Figure 5 Loss of LIN-1 results in ectopic LIN-12 activity in all VPCs, and abrogates LIN-12-GFP endocytic downregulation in P6.p. (A) Photomicrograph of *arls116[lst-5p::2xnl5-yfp]* expression in *lin-1(+)* (left) or *lin-1(n304)* (null, right). (B) Loss of LIN-1 results in ectopic expression of the LIN-12 target gene reporter *arls116[lst-5p::2xnl5-yfp]* in all VPCs. Here and in (C), the graph compares percentage of VPCs in *lin-1(+)* to *lin-1(0)* that express YFP. ** $P < 0.001$ and * $P < 0.016$ for *lin-1(+)* compared to *lin-1(0)* (Fisher's exact test). (C) Loss of LIN-1 results in ectopic expression of a different LIN-12 target gene reporter, *arls107[mir-61p::2xnl5-yfp]* in all VPCs. (D) Loss of LIN-1 does not cause precocious expression of *arls116[lst-5p::2xnl5-yfp]*. Graph shows percentage of animals having more than one VPC expressing YFP. N.S. denotes not significant compared to wild-type (Fisher's exact test). (E) Photomicrograph of a *lin-12(n941); lin-1(n304); arls116[lst-5p::2xnl5-yfp]* hermaphrodite. Expression of YFP is not observed, indicating that ectopic expression of LIN-12 target reporters in *lin-1(0)* requires *lin-12* activity. Animals homozygous for both mutations were isolated from heterozygous strain

and G). Furthermore, as described above, the 1° fate is normally associated with resistance to activated LIN-12, yet two different targets of LIN-12 are transcribed in P6.p and in all other VPCs in a *lin-1(0)* background, indicating that resistance is abrogated or overcome.

LIN-1 coordinates cross-talk between the inductive and lateral signaling pathways

Cell lineage analysis suggested that in *lin-1(0)* mutants, VPCs commit to either the 1 or 2° fate and that VPCs are able to resolve the relative strength of the EGFR and LIN-12/Notch signaling inputs (Beitel *et al.* 1995, discussed further below). However, using direct targets of the inductive and lateral signaling pathways to assess the state of signal transduction in the VPCs, we instead observe highly penetrant expression of direct targets for both EGFR and LIN-12/Notch pathways in all VPCs (Figure 6, A and B). We interpret this observation as indicating that loss of *lin-1* activity compromises at least some aspects of the ability of VPCs to integrate the signaling inputs from these pathways.

The simultaneous activation of EGFR and LIN-12/Notch signal transduction observed in *lin-1(0)* mutants contrasts with the mutually exclusive expression of the same targets when the Ras cascade *per se* is activated. We scored expression of the same signaling pathway reporters on a VPC-by-VPC basis in the presence of a transgene that expresses a constitutively active and stable form of LIN-45/Braf in all VPCs *arTi31[lin-31p::lin-45(AA, V627E)]* (de la Cova and Greenwald 2012; de la Cova *et al.* 2017). In contrast to *lin-1(0)*, we found that when the Ras cascade is activated, each VPC generally expresses one marker or the other, not both (Figure 6, C and D). The innermost VPCs adopt their normal fates, indicating that the AC inductive signal continues to center the pattern on P6.p. In addition, the outermost VPCs, which normally would adopt the 3° fate but instead form ectopic pseudovulvae away from the AC, are patterned by their neighbors: P8.p always expresses only the *lag-2* reporter, because its neighbor P7.p always adopts its normal 2° fate; and P3.p and P4.p generally express one or the other reporter, but not both, implying that they retain the ability to resolve their fates through interactions between them. Thus, we interpret the difference in marker pattern as indicating that loss of *lin-1* not only causes all VPCs to behave as if they have been induced, but also abrogates coordination between the inductive and lateral signaling pathways.

Discussion

In the *C. elegans* VPCs, multiple mechanisms coordinate LIN-12/Notch and EGFR signaling inputs to ensure a precise and robust spatial pattern of distinct cell fates. The pattern is

centered on P6.p, in which EGFR-Ras-ERK is activated by an EGF-like ligand produced by the gonad, and subsequently produces a lateral signal composed of ligands that activate LIN-12/Notch. We examined how several parameters impacting the activity of *lin-12/Notch* in VPC fate patterning are affected by loss of three components that have biochemical relationships: LIN-1, the ortholog of the Elk1 subfamily of transcription factors, which regulates lateral signal gene transcription in response to the inductive signal; SUR-2, the ortholog of Med23, which in mammalian cells links Elk1 to the core Mediator complex; and the facultative modulator of the Mediator complex, the CKM.

We discuss here our three main conclusions and their implications. (i) The CKM, but not SUR-2/Med23, helps set a threshold for LIN-12/Notch activity in all VPCs. (ii) In P6.p, where EGFR is active and LIN-12/Notch is normally inactive, we find that the CKM, SUR-2 and LIN-1 are all required for EGFR-associated resistance to ectopically activated LIN-12/Notch, while only SUR-2 is required for lateral signal gene transcription. (iii) We extend the understanding of the integration of inductive and lateral signaling in view of our new insights into how loss of *lin-1* impacts *lin-12* activity, and how sufficient ectopic LIN-12 activity in P6.p can overcome EGF-promoted resistance and oppose 1° fate. Our results suggest that different configurations of the CKM, SUR-2, and LIN-1 operate simultaneously in VPCs to coordinate EGFR and LIN-12/Notch signaling to specify the 2° VPC fate, and emphasize the crucial role of *lin-1* in VPC fate patterning (Figure 7, A–C).

The CKM and basal activity of LIN-12/Notch in VPCs

We identified a requirement for the CKM and its associated kinase activity in negative regulation of *lin-12* activity. Loss of *cdk-8* strongly enhanced the mildly activated mutation *lin-12(n302)*, an allele that has two useful properties: (i) it affords a sensitized background for observing increased *lin-12* activity (Hubbard *et al.* 1997; de Souza *et al.* 2007), and (ii) it removes the AC of the gonad, which serves as the cellular source of the EGF signal, and hence removes input from EGFR-mediated induction into VPC fate. Thus, the genetic interactions we observed with Mediator CKM components indicate that the CKM exerts negative regulation on basal *lin-12* activity independent of EGFR-mediated input. We suggest that the CKM is important for setting a threshold in VPCs for response to *lin-12* activity.

In mammalian cells, Fryer *et al.* (2004) found that Cdk8 and Cyclin C associate with the Notch nuclear complex, and that Cdk8 phosphorylates the Notch intracellular domain to promote its targeting by Fbw7 for ubiquitination and proteasome-mediated degradation. Based on these observations and other

of genotype *lin-12(n941)/qC1*; *lin-1(n304)/oxTi915*; *arIs116[ist-5p::2xnl5-yfp]*. (F) Photomicrographs showing endocytic downregulation of LIN-12-GFP accumulation in the apical membrane of *lin-1(+)* hermaphrodite (left), and loss of downregulation in a *lin-1(0)* hermaphrodite (right). Each image is a maximum projection of a z-stack taken on a Zeiss spinning disk confocal system. Images were processed using Fiji/ImageJ (Schindelin *et al.* 2012, 2015). (G) Graphs show percentage of VPCs with GFP evident in the apical membrane of the genotypes shown in (F): *pha-1*; *arEx1575[lin-12-gfp]* (left) and *pha-1*; *lin-1(n304)*; *arEx1575[lin-12-gfp]* (right). ** $P < 0.001$ for *lin-1(+)* compared to *lin-1(0)* (Fisher's exact test). VPC, Vulval Precursor Cell; YFP, yellow fluorescent protein.

Figure 6 Resolution of cell fate in different genotypes. All strains contain *arls222*[*lag-2p*::*tagRFP*] and *arls116*[*lst-5p*::*2xnl5-yfp*], scored simultaneously on a per-VPC basis, except for *lin-1(0)*, which is based on the complete penetrance of *lag-2* expression in Zhang and Greenwald (2011) and data in Figure 5. Red, only the tagRFP marker was observed; blue, only YFP was observed; purple, both markers were observed; and gray, neither marker was observed. (A) Typical expression pattern for wt hermaphrodites; refer to Figure 1B for images. (B) Typical expression pattern for *lin-1(0)* hermaphrodites. (C) Chart depicting reporter expression on a per-VPC basis of *arT31*[*lin-31p*::*lin-45*(AA, V627E)]; *arls222*[*lag-2p*::*tagRFP*]; *arls116*[*lst-5p*::*2xnl5-yfp*] hermaphrodites. (D) Photomicrograph of *arT31*[*lin-31p*::*lin-45*(AA, V627E)]; *arls222*[*lag-2p*::*tagRFP*]; *arls116*[*lst-5p*::*2xnl5-yfp*]. Top, tagRFP channel; middle, YFP channel; and bottom, images merged with DIC. RFP, red fluorescent protein; VPC, Vulval Precursor Cell; wt, wild-type; YFP, yellow fluorescent protein

supporting data, they proposed that Cdk8 activity promotes degradation of the Notch enhancer complex at target genes. Their biochemical observations were corroborated in a subsequent study, which further showed that *in vivo* Cyclin C is a haploinsufficient tumor suppressor for the *NOTCH1*-driven cancer T cell acute lymphoblastic leukemia (Li *et al.* 2014). Furthermore, a recent study of another *NOTCH1*-driven cancer, chronic lymphocytic leukemia, found that mutations in *MED12* correlate with increased levels of NOTCH1 intracellular domain, and that Cdk8 kinase activity negatively regulates its level in this cancer context as well (Wu *et al.* 2017). These findings are consistent with our conclusions that individual components of the CKM all contribute to the negative regulation of *lin-12* activity in a CDK-8 kinase-dependent fashion.

sel-10, the *C. elegans* ortholog of Fbw7, was first implicated in negative regulation of Notch through genetic analysis in *C. elegans* (Sundaram and Greenwald 1993; Hubbard *et al.* 1997). The functional relationship of *sel-10* and *lin-12* parallels the functional relationship of *FBXW7* (the gene that encodes Fbw7) and *NOTCH1*; mutations that inactivate *sel-10* increase the activity of *lin-12*(*n302*) and mutations that inactivate *FBXW7* increase the activity of oncogenic forms of *NOTCH1* with similar lesions (Gupta-Rossi *et al.* 2001; Öberg *et al.* 2001; Wu *et al.* 2001). However, in *C. elegans*, it is not clear that negative regulation of *LIN-12* by *CDK-8* is direct; although the intracellular domain of *LIN-12*/Notch must be able to assemble into the nuclear complex to be degraded by *SEL-10*/Fbw7 (Deng and Greenwald 2016), the Cdk8-dependent phosphorylation of the mammalian Notch1 intracellular domain occurs at serines that are not conserved in *C. elegans* *LIN-12*. It is possible that the CKM phosphorylates *LIN-12* directly, but at a different site than in mammalian Notch1. Alternatively, the negative regulation of *lin-12* activity by the CKM in VPCs may occur by a different mechanism, such as phosphorylation of another component of the nuclear complex or more indirectly.

Different requirements for *SUR-2*, the CKM, and *LIN-1* in *P6.p* for different functions relevant to *LIN-12*/Notch and VPC patterning

The roles of *SUR-2*, the CKM, and *LIN-1* are different in *P6.p* for three functions that are relevant to *LIN-12*/Notch signaling and VPC patterning: transcription of the lateral signal genes (Figure 7A), downregulation of the receptor form of *LIN-12* (Figure 7B), and resistance to the nuclear form of *LIN-12* (Figure 7C). Although the physical interactions cannot be known from this genetic analysis, we can make some hypotheses about complexes and mechanisms that mediate the different functions.

With respect to transcription of the lateral signal genes in *P6.p*, *SUR-2*/Med23 is required for expression (Chen and Greenwald 2004) and *LIN-1*/Elk1 is not, although *lin-1* plays an important role in VPC patterning by repressing the lateral signal genes in other VPCs (Zhang and Greenwald 2011). Our analysis of *cdk-8* and *cic-1* indicates that the CKM is not required for expression of the lateral signal genes. Thus, the *LIN-39*/Hox activator complex that promotes lateral signal gene transcription in *P6.p* may contain *SUR-2* but not the CKM, and the *LIN-1* repressor complex that prevents expression in other VPCs may not contain either *SUR-2* or the CKM. We note that a small difference in penetrance of *lag-2* expression reported for a complex background that differed in *cdk-8* activity, interpreted as evidence that *cdk-8* partially contributes to *LIN-1*-mediated repression of lateral signal genes (Grants *et al.* 2016), is not statistically significant, so at present there is no unequivocal evidence for a partial contribution of the CKM to *LIN-1*-mediated repression of lateral signal genes.

The endocytic downregulation of a GFP-tagged receptor form of *LIN-12* in *P6.p* was previously shown to require *SUR-2* (Shaye and Greenwald 2002); we now find that *LIN-1* is also required for this process, but that the CKM is

A The CKM is not required for LIN-1-mediated repression or SUR-2-mediated activation of lateral signal gene transcription

B LIN-1 and SUR-2, but not the CKM, are required for transcription of a factor that targets LIN-12 for endocytic downregulation

C LIN-1, SUR-2 and the CKM are required to resist LIN-12 activity in P6.p

Figure 7 Summary and models for the roles of LIN-1, SUR-2, and the CKM in P6.p. Black lines connecting components are hypothetical protein–protein interactions based on known interactions in mammalian cells that are consistent with our genetic data, but more complex models in which the various components act in parallel are also possible. (A) Regulation of *lag-2* transcription in VPCs. We show herein that the CKM is not required for either repression of a *lag-2* reporter in uninduced VPCs or transcriptional activation in P6.p. Prior analysis of a *cis*-regulatory module in this reporter (Zhang and Greenwald 2011) suggested that LIN-3/EGF activation of EGFR-Ras-ERK leads to phosphorylation of LIN-1 and relief of repression of *lag-2* via VPCrep, allowing a Hox gene, likely LIN-39 (Niu *et al.* 2011), to promote its transcription via VPCact. SUR-2/Med23 is required for *lag-2* transcription even when VPCrep is deleted, consistent with SUR-2 acting in conjunction with LIN-39 to promote *lag-2* transcription through VPCact rather than functioning with LIN-1/Elk1 to promote repression through VPCrep. (B) Endocytic downregulation of LIN-12. We present results herein indicating that *lin-1*, but not the CKM, is required for endocytic downregulation of LIN-12 in P6.p. Since *sur-2* is also required for this process (Shaye and Greenwald 2002), and phosphorylated Elk1 interacts with Med23 in mammalian cells, we propose that a complex between LIN-1 and SUR-2 promotes expression of one or more target genes that promote endocytic downregulation of LIN-12. (C) Resistance to LIN-12 activity in P6.p. Our analysis suggests that P6.p is able to resist constitutively active LIN-12, and that overcoming this resistance by higher constitutive activity has deleterious consequences for the expression of *lag-2*. We also report that LIN-1, SUR-2, and the CKM are required for this resistance. Because all three components have the ability to form a complex in mammalian cells, we propose that they do so for this function. We envisage that resistance could be achieved if the complex acts directly to repress key LIN-12 target gene(s) or indirectly through transcription of a factor that opposes LIN-12 nuclear complex activity, assembly, or stability. We note that the 1 kb regulatory region present in the *arls116[*lts-5p::2xnl5-yfp*]* reporter does not contain a canonical Elk1 binding site, but there are numerous short GGA sequences that could in principle be Ets factor binding sites. CKM, Cdk8 Kinase module; EGF, Epidermal Growth Factor; EGFR, EGF receptor; VPC, Vulval Precursor Cell; VPCact, an element that is required for LIN-39/Hox-mediated activation in VPCs; VPCrep, an element for LIN-1-mediated repression in VPCs; LBS, LAG-1 Binding Site.

not. In addition, analysis of *cis*-acting sequences in the intracellular domain of LIN-12 suggested that kinases and ubiquitin ligases promote its internalization and degradation, leading to the hypothesis that one or more of these factors are under the transcriptional control of the EGFR-Ras-ERK pathway (Shaye and Greenwald 2005). Our analysis here suggests the further hypothesis that one or more direct transcriptional targets are activated by ERK-phosphorylated LIN-1 recruitment of Mediator via SUR-2, as in mammals, although no direct targets regulated in this way have been identified in *C. elegans* as yet.

Finally, the CKM, SUR-2, and LIN-1 are all required for EGFR-associated resistance to a stabilized, nuclear form of LIN-12/Notch that mimics the natural signal-transducing form after ligand-induced cleavage. Thus, we envisage that a complex containing all three components may mediate this function. In such complexes, a requirement for Cdk8 kinase activity is associated with transcriptional activation (Knuesel *et al.* 2009b), and the lack of a requirement for Cdk8 kinase activity with repression (Knuesel *et al.* 2009a). However, we were unable to test if CDK-8 kinase activity was required for resistance because of synthetic lethality of the necessary

genotype. It is possible that this complex functions in repressor mode to directly repress *LIN-12* target genes; alternatively, it may function in activator mode to promote expression of one or more targets of EGFR-Ras-ERK that mediate resistance.

We note that there are differences in the contributions of the EGFR-Ras-ERK pathway to vulval development in other species when compared to *C. elegans* (Felix 2007; Sommer 2012). There also may be differences in the contributions of the components we have studied here to vulval development in the related species *C. briggsae*; the ortholog of *lin-1* is critical for inhibiting ectopic vulval fate (Sharanya *et al.* 2015), but the ortholog of *sur-2* may not be essential for production of the lateral signal (Mahalak *et al.* 2017). Finally, these components function in different configurations and activate or repress different target genes in other developmental contexts in *C. elegans*, such as the excretory system (Howard and Sundaram 2002; Rocheleau *et al.* 2002; Sundaram and Buechner 2016).

Integrating the EGFR-Ras-ERK inductive signaling and LIN-12/Notch lateral signaling pathways

Our observations on the phenotype of *lin-1(0)* mutants and the resistance to activated *LIN-12* in P6.p are relevant to how signaling inputs are integrated during VPC specification.

lin-1 has been viewed as an “inhibitor of vulval fate,” because loss of *lin-1* causes ectopic vulval induction (Ferguson *et al.* 1987; Beitel *et al.* 1995), while missense mutations near phospho-acceptor sites prevent the generation of additional vulval cells even in the presence of activated Ras (Jacobs *et al.* 1998). In terms of molecular mechanism, it has been proposed that sumoylated *LIN-1* recruits transcriptional repressors to inhibit the 1° fate, and ERK-mediated phosphorylation of *LIN-1* converts it into a transcriptional activator to promote 1° fate in P6.p (Leight *et al.* 2015). This model accounts for a positive role for *lin-1* in promoting 1° fate as inferred from a requirement for expression of *egl-17*, a marker for 1° fate in VPCs (Howard and Sundaram 2002; Tiensuu *et al.* 2005), and is compatible with the regulation of *lag-2* expression by loss of *LIN-1* repression in response to the inductive signal. The later role of *lin-1* in descendants of 2° VPCs during vulval morphogenesis (Farooqui *et al.* 2012) is distinct from the role in VPC fate specification considered here.

In classic cell lineage analysis, VPC fates were assigned using the best criteria available at the time: the plane of the final division in the lineage and adhesive properties of the terminal cells (Sternberg and Horvitz 1986). Such analysis suggested that in a *lin-1(0)* mutant, each VPC adopts a 1 or 2° fate, depending on the relative input of competing inductive and lateral signaling pathway activity (Beitel *et al.* 1995); however, it was also noted that many of the lineages were abnormal. Here, using markers that directly report the activities of the EGFR and *LIN-12*/Notch pathways, we observed that both EGFR-Ras-ERK and *LIN-12*/Notch signal transduction is active in VPCs in *lin-1(0)* mutants, likely accounting for the abnormal lineages previously observed. We observed

that expression of these reporters persists throughout the lineage in *lin-1(0)* mutants, in contrast to their clean resolution by the Pn.px stage when *LIN-45*/Braf, the first kinase in the Ras cascade, is constitutively activated. We interpret these findings as indicating that *lin-1* is required for cross-talk between the EGFR and *LIN-12* signal transduction pathways, such that loss of *lin-1* not only leads to ectopic 1° fate, but also abrogates the normal mechanisms that mediate cross-talk between the EGFR-Ras and *LIN-12*/Notch, such as downregulation of *LIN-12*.

In previous reports it has been briefly noted that, in response to EGFR activity, P6.p appears to be refractory to an activated form of *LIN-12* lacking the extracellular domain (Shaye and Greenwald 2005; Li and Greenwald 2010). We have now characterized this phenomenon further using a stabilized form of the untethered intracellular domain, which mimics the ultimate cleavage product after ligand binding, and transcriptional reporters for EGFR-Ras-ERK and *LIN-12* target genes. Our observation that *lag-2* expression can be repressed when *lin-12* activity is sufficiently high in the presence of the inductive signal is evidence that “high” *LIN-12* can oppose EGFR-Ras-ERK in presumptive 1° VPCs, suggesting that EGFR-promoted resistance to activated *LIN-12* is a mechanism for preventing inappropriate activation of 2° fate genes that would otherwise inhibit the adoption of 1° fate. How high constitutive *lin-12* activity overcomes resistance may be mechanistically related to what normally occurs in 2° VPCs, where activation of *LIN-12* leads to expression of negative regulators of EGFR-Ras-ERK activity (Berset *et al.* 2001; Yoo *et al.* 2004) and positively reinforces *lin-12* activity through a microRNA-mediated double negative feedback loop (Yoo and Greenwald 2005). We speculate that *LIN-1*, *SUR-2*, and the CKM work together to promote the expression of one or more target genes that implement resistance to *LIN-12*.

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