SEL-10/Fbw7-dependent negative feedback regulation of LIN-45/Braf signaling in C. elegans via a conserved phosphodegron

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The conserved E3 ubiquitin ligase component named SEL-10 in Caenorhabditis elegans and Fbw7 in mammals targets substrates for ubiquitin-mediated degradation through a high-affinity binding site called a Cdc4 phosphodegron (CPD). As many known substrates of Fbw7 are oncoproteins, the identification of new substrates may offer insight into cancer biology as well as aspects of proteome regulation. Here, we evaluated whether the presence of an evolutionarily conserved CPD would be a feasible complement to proteomics-based approaches for identifying new potential substrates. For functional assessments, we focused on LIN-45, a component of the signal transduction pathway underlying vulval induction and the ortholog of human Braf, an effector of Ras in numerous cancers. Our analysis demonstrates that LIN-45 behaves as a bona fide substrate of SEL-10, with mutation of the CPD or loss of sel-10 resulting in increased activity and protein stability in vivo. Furthermore, during vulval induction, the downstream kinase MPK-1/ERK is also required for LIN-45 protein degradation in a negative feedback loop, resulting in degradation of LIN-45 where ERK is highly active. As the CPD consensus sequence is conserved in human Braf, we propose that Fbw7 may also regulate Braf stability in some cell contexts. We discuss the implications of our findings for vulval development in C. elegans, the potential applicability to human Braf, and the value of a CPD-based predictive approach for human Fbw7 substrates.

[Keywords: vulva; C. elegans; FBXW7; Braf; ERK; Ras; SCF complex]

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Fbw7 is a highly conserved subunit of the multiprotein E3 ubiquitin ligase Skp1/Cul1/F-box (SCF) complex (Feldman et al. 1997; Skowyra et al. 1997). In this complex, Fbw7 interacts with the ubiquitination machinery via its F-box domain and provides substrate specificity by recognizing target proteins via its “WD repeat” domain (Orlicky et al. 2003; Hao et al. 2007). Mutation of the gene encoding Fbw7, FBXW7, is associated with many different cancers in human patients as well as mouse models, implicating it as an important tumor suppressor (Mao et al. 2004; Akhoondi et al. 2007). Consistent with this role, Fbw7 regulates the stability of oncoprotein substrates, including Notch1, Cyclin E1, c-Myc, and c-Jun (Nakayama and Nakayama 2006; Welcker and Clurman 2008; Crusio et al. 2010).

The first member of the Fbw7 family, Cdc4p, was identified in the classic genetic screen for cell cycle regulators in budding yeast (Hartwell et al. 1973; Yochem and Byers 1987); the first animal ortholog, SEL-10, was identified genetically in a screen for negative regulators of the receptor LIN-12/Notch in Caenorhabditis elegans (Sundaram and Greenwald 1993; Hubbard et al. 1997). The functional relationship between SEL-10 and LIN-12/Notch is conserved, as the mammalian ortholog Fbw7 also targets mammalian Notch proteins (Gupta-Rossi et al. 2001; Oberg et al. 2001; Wu et al. 2001). This relationship has demonstrable clinical significance in T-cell acute lymphoblastic leukemia (T-ALL), where NOTCH1-activating mutations are significantly associated with either FBXW7 loss of function or truncations of NOTCH1 that abrogate Fbw7 binding to the Notch protein (Malyukova et al. 2007; O’Neil et al. 2007; Thompson et al. 2007). However, in most cancers where FBXW7 mutations are found, the functionally relevant protein substrates have not been determined.

Notch and other oncoprotein substrates have a high-affinity binding site for Fbw7 termed the Cdc4 phosphodegron (CPD) (Nash et al. 2001; for review, see Welcker and Clurman 2008). Binding of Fbw7 to these substrates requires phosphorylation of two amino acids in the CPD. This requirement for phosphorylation creates the potential for complex regulation, as the Fbw7–substrate interaction could be impacted by differential phosphorylation.
SEL-10/Fbw7 down-regulates LIN-45/Braf
depending on physiological, developmental, or cellular context.
The identification of novel substrates for Fbw7 is of great interest because of its role as a tumor suppressor. Global proteomic strategies have been used to identify substrates affected by all SCF complexes, or specific F-box proteins, based on assays for protein stability and coimmunoprecipitation from cultured cells. However, in two large studies that identified many potential novel substrates, none of the known, validated Fbw7 substrates were identified [Yen and Elledge 2008; Emanuele et al. 2011]. In a recent study, detection of known substrates was substantially different in several cell lines [Yumimoto et al. 2012], suggesting that CPD phosphorylation is highly influenced by cell context and possibly explaining the small number of substrates found in previous studies using only a single cell line. Nevertheless, even when multiple lines were used, not all known substrates were identified.

Here, we explored whether a computational approach based on the presence of an evolutionarily conserved CPD would provide a simple, context-independent approach to identifying potential Fbw7 substrates. We performed an extensive genetic analysis in C. elegans of a predicted substrate of special interest: LIN-45, the ortholog of human Braf, an effector of Ras that is important in cell–cell interactions in development and is commonly activated in human cancers. Much of our functional analysis used the C. elegans vulval induction cell–cell interaction paradigm that has yielded many insights into regulatory mechanisms applicable to mammals. Our results suggest that the computational approach may be an effective complement to proteomics-based approaches. In addition, our analysis uncovered an unexpected feedback mechanism modulating Ras–Raf–ERK signal transduction in vulval induction.

Results

Computational identification of candidate SEL-10/Fbw7 substrates conserved in nematodes and vertebrates

All CPDs, whether low- or high-affinity binding sites, contain two residues that are essential for Fbw7 binding: a phosphorylated “central threonine” at position 0 and a proline at the +4 position [Nash et al. 2001]. In addition, mutational analyses of high-affinity CPDs in mammalian substrates and C. elegans LIN-12 (Fig. 1A; Supplemental Fig. 1) indicate that a second phospho-residue, a serine or threonine at the +4 position, is also required for high-affinity binding [Welcker et al. 2004; Ye et al. 2004; Sundqvist et al. 2005; Wei et al. 2005; Thompson et al. 2007; Olson et al. 2008]. Beyond these essential elements, a second proline, variably at the +2 and +3 positions, may ensure proper spacing of the dual phospho-residues [Orlicky et al. 2003; Hao et al. 2007], and hydrophobic residues are favored at positions −1 and −2 [Nash et al. 2001]. We used this information to generate a collection of CPD consensus sequences for our database searches [Materials and Methods], which led to two lists: a “nem-

Figure 1. C. elegans LIN-45 has a conserved CPD and is negatively regulated by SEL-10. [A] Alignment of high-affinity CPD motifs from validated human substrates and C. elegans LIN-12. Residues essential for Fbw7 binding or Fbw7-mediated degradation are shown in red here and in B. As mutations in the “central threonine” and “+4 serine” residues were incorporated into LIN-45, we note that in all cases shown here, mutation of these residues to alanine has been documented to have one or more of the following consequences: stabilization of the substrate, increased activity of the substrate, and/or loss of Fbw7 binding to the substrate (for LIN-12, see Supplemental Fig. 1). (B) Potential conserved high-affinity CPDs identified in C. elegans LIN-45 and human Braf. Conserved Raf functional domains [black] include the Ras-binding and cysteine-rich domains [RB], 14-3-3 binding domain, and kinase domain. LIN-45 has a single predicted high-affinity CPD [black arrow]; residues 431-436 of C. elegans,

conserved in three other nematode species analyzed. Braf has a predicted high-affinity CPD [black arrow; residues 400-405] in the corresponding position, conserved in zebratinh [and other vertebrates]. Human Braf also has a second predicted CPD [gray arrow; residues 332-337 of human] that did not fulfill our conservation criterion. Full alignments of the region between the Ras-binding and kinase domains are shown in Supplemental Figure 2. (C) sel-10(0) suppresses lin-45 partial loss of function. Rod-like larval lethality [Let] associated with lin-45(hypomorphic alleles n2506 and n2018 and suppression by the null sel-10(ar41) as percentage of total progeny. (*) P-value < 0.0001 for comparisons of lin-45 to lin-45, sel-10 strains. The number of larvae scored [n] is indicated at right. The markers unc-24(e138) and him-5(e1490) were also homozygous in strains containing or compared with lin-45(n2506). The markers dpy-20(e1282) and him-5(e1490) were also homozygous in strains containing or compared with lin-45(n2018). [D] sel-10(0) does not bypass the need for lin-45. The sterile [Ste] and vulvales [Vul] phenotype of the null allele lin-45(dx19) in sel-10(+ or sel-10(0) as percentage of total adults. Number of adults scored [n] is indicated at right. All animals scored were also homozygous for lon-3(e2175). lin-45(dx19) genotypes were F1 segregants from lin-45(dx19)/DnT1; lon-3(e2175)/DnT1 or lin-45(dx19)/DnT1; lon-3(e2175) sel-10(ar41)/DnT1 mothers.
atode set” in which a CPD consensus sequence found in a *C. elegans* protein is conserved in three other nematode species (Supplemental Table 1) and a “vertebrate set” in which a CPD consensus sequence found in a human protein is also present in the zebrafish *Danio rerio* (Supplemental Table 2), each case requiring conservation to mitigate against chance occurrence in a single species. We then looked at the intersection of these sets and required that the conserved CPD consensus sequence be contained in a cytosolic protein or the intracellular domain of a transmembrane protein, resulting in a list of 116 potential human Fbw7 substrates conserved in *C. elegans* [Supplemental Table 3].

**Using C. elegans to assess a new candidate substrate: LIN-45/Braf**

From our list of putative SEL-10/Fbw7 substrates conserved in both nematodes and vertebrates, we focused on LIN-45/Braf because human *BRAF* is an oncogene commonly mutated or activated in human cancers, there is functional evidence that LIN-45 is regulated by biochemical mechanisms similar to mammalian Braf (Chong et al. 2001; Sundaram 2006), and LIN-45 and Braf each contain a highly conserved putative CPD in corresponding positions (Fig. 1B). These features suggest that the target relationship and effect on Braf activity inferred from analysis of LIN-45 in *C. elegans* may be conserved. In addition, *lin-45* was especially well suited for functional analysis in vivo, as its involvement has been elucidated in multiple cell fate decisions in *C. elegans*, including in vulval precursor cells [VPCs], a cell type in which SEL-10/Fbw7 is known to function [Hubbard et al. 1997].

**Loss of sel-10 suppresses phenotypes caused by reduced lin-45 activity**

Since a bona fide substrate should be stabilized in the absence of SEL-10 binding, we used several functional assays to assess whether LIN-45 is a substrate of SEL-10. First, a null allele of *sel-10* should increase *lin-45* activity, as assessed by suppression of a mutant phenotype caused by a partial loss-of-function mutation. In contrast, loss of *sel-10* is not expected to suppress a *lin-45*-null mutation, as protein stabilization will have no effect if the target is absent. Loss or reduction of *lin-45* activity has different consequences in different tissues: “rod-like” larval lethality results from a defect in excretory duct cell specification, a vulvaless phenotype results from insufficient transduction of the inductive signal in the VPCs, and sterility results from pachytestane arrest during meiosis in the germline [for review, see Sundaram 2006].

We found that loss of *sel-10* suppresses defects resulting from partial loss of *lin-45* activity. The hypomorphic alleles *lin-45(n2506)* and *lin-45(n2018)* affect conserved residues of the Ras-binding domain and greatly reduce but do not entirely eliminate *lin-45* activity [Hsu et al. 2002]. Although these mutants are not vulvaless or sterile, both were useful because they display an intermediate phenotype: Homozygotes die with the characteristic rod-like morphology, but some survive, and the strains can be propagated and analyzed as homozygotes. The rod-like larval lethality of hypomorphic alleles is efficiently suppressed in a sel-10-null [sel-10(0)] background [Fig. 1C], suggesting that sel-10 acts as a negative regulator of *lin-45* activity.

We also tested whether a null allele of *lin-45* can be suppressed by *sel-10(0)*. Null alleles of *lin-45* must be maintained as heterozygotes, and homozygous segregants do not display much rod-like larval lethality because of maternally supplied *lin-45(+)* activity; however, adult hermaphrodites display vulvaless and sterile phenotypes because maternal activity “runs out” by later larval stages. All adults display these phenotypes, even in a sel-10(0) background [Fig. 1D]. As the genetic interactions below establish that loss of *sel-10* increases *lin-45* activity in the VPCs, the lack of suppression of the vulvaless phenotype of *lin-45*-null mutants by *sel-10(0)* is consistent with a role for SEL-10 in regulating, rather than bypassing, the need for LIN-45.

**Loss of sel-10 enhances phenotypes resulting from activated LIN-45**

*lin-45* is a component of an EGFR-mediated inductive signaling pathway that causes VPCs to generate the vulva [Fig. 2A; Sundaram 2006]. Alterations of VPC fate alone do not compromise viability or fertility, so mutations in endogenous genes or engineered transgenes that alter signal transduction may be readily obtained. Increasing the activity of the EGFR–Ras–Raf–ERK pathway in the VPCs causes additional VPCs that normally are out of the range of the inductive signal to behave as if they have received it. This ectopic induction results in a multivulva phenotype characterized by large pseudovulval protrusions anterior and posterior to the normal vulva [Fig. 2B].

Since substrates should be stabilized by removal of *SEL-10*, a *sel-10*-null mutant is expected to enhance the activity of mutant alleles of *lin-45* in which signal transduction is constitutive. In human *BRAF*, the amino acid substitution V600E is frequently found in cancers and renders the kinase constitutively active and independent of Ras activity. In addition, in wild-type Braf, the residues T599 and S602 are phosphorylated upon activation by Ras [Zhang and Guan 2000], and LIN-45 constitutive activity has been achieved by phosphomimetic mutations at these sites [Chong et al. 2001]. In the LIN-45 protein, the substitution V627E is analogous to Braf[V600E], and substitutions T626E/T629D [“LIN-45(ED)”] mimic phosphorylation of the activation loop.

We expressed LIN-45[V627E] and LIN-45[ED] in VPCs using a tissue-restricted promoter and found that both transgenes caused a multivulva phenotype, with variable penetrance and expressivity in three independent lines [Fig. 2E]. We crossed LIN-45[V627E] and LIN-45[ED]-expressing transgenes into a sel-10(0) background and saw a significant increase in the penetrance and expressivity of the multivulva phenotype, particularly in the number of large pseudovulvae [Fig. 2C]. Expression of LIN-45[V627E] and LIN-45[ED] occasionally cause the
SEL-10/Fbw7 down-regulates LIN-45/Braf

Figure 2. Activated LIN-45 is negatively regulated by SEL-10 and the LIN-45 CPD. (A) Normal pattern of VPC fates. The VPCs (P3.p–P8.p) are initially equivalent. In the L3 stage, the EGF-like inductive signal produced by the anchor cell (AC) of the gonad results in high EGFR–Ras–Raf–ERK activity, causing P6.p to adopt the 1° vulval fate and produce lateral signal ligands such as LAG-2 (for review, see Sternberg 2005). This lateral signal results in high LIN-12/Notch activity in P5.p and P7.p, which adopt the 2° vulval fate. P3.p, P4.p, and P8.p have the potential to adopt vulval fates but do not because they lie outside the range of the spatial patterning signals. (B) Photomicrographs of the hemaphrodite mid-body. (Top) A wild-type hemaphrodite has a normal vulva. [(Bottom) Constitutive Ras–Raf–ERK activity causes outer VPCs to adopt vulval fates and produce pseudovulvae (arrows), resulting in a multi-vulva phenotype, as shown in a representative sel-10(ar41); arEx1292[lin-45(ED)]/him-5(e1490) hermaphrodite. (C) sel-10(0) enhances lin-45 constitutive activity but not lin-45(+) The penetrance and expressivity of the multi-vulva phenotype caused by constitutively active forms of LIN-45 [LIN-45(ED) or LIN-45(V627E)] is enhanced by sel-10(ar41). Shown here is the percentage of adult hemaphrodites carrying the transgenes arEx1300[lin-45(+)], arEx1474[lin-45(V627E)], or arEx1292[lin-45(ED)] displaying one, two, or three or more large ectopic pseudovulvae. All strains also were homozygous for him-5(e1490). (*) P-value < 0.0001 for comparisons of control and sel-10 strains. The number of adults scored (n) is indicated at right. (D) sel-10(0) enhances the ectopic 1° fate specification caused by LIN-45[ED]. The percentage of L3 hemaphrodites carrying arEx1292[lin-45(ED)] that display ectopic expression of the reporter arIs131[lag-2p::xia-2::yfp] in Pn.px and Pn.ppx stage cells. Ectopic expression of lag-2p::yfp was categorized as “alternating 1°” when VPCs adjacent to the ectopic expression were lag-2p::yfp-negative and as “adjacent 1°” when the neighboring VPCs were lag-2p::yfp-positive. When two adjacent VPCs adopt the 1° fate, Raf activity is not only high enough to induce a vulval fate, it must be sufficiently high to override LIN-12/Notch activity, which acts to oppose Ras–Raf–ERK activity [Sternberg 2005]. Both strains also were homozygous for him-5(e1490). (*) P-value < 0.0001 for comparisons of all ectopic 1° fate as well as adjacent 1° fate observed in control and sel-10 strains. The number of L3 larvae scored (n) is indicated at right. (E) CPD mutations enhance lin-45 constitutive activity but not lin-45+. The penetrance and expressivity of the multi-vulva phenotype caused by constitutively active forms of LIN-45 [LIN-45(ED) or LIN-45(V627E)] is enhanced by mutating the central threonine and +4 serine of the CPD to alanine, i.e., T432A and S436A [AA]. Shown here is the percentage of adult hemaphrodites carrying transgenes expressing the indicated forms of LIN-45 displaying one, two, or three or more large ectopic pseudovulvae. Individual bars represent independent strains isolated for each transgene. Transgenes scored were arEx1300 and arEx1301 for (+), arEx1294 and arEx1296 for (AA), arEx1474, arEx1475, and arEx1476 for [V627E], arEx1479, arEx1483, and arEx1485 for [AA, V627E], arEx1291, arEx1292, and arEx1335 for [ED], and arEx1287, arEx1288, and arEx1337 for [AAED]. The number of adults scored (n) is indicated at right.

vulva to burst at the L4-to-adult molt, which may reflect defects in VPC specification or the resulting vulval cells. We saw a dramatic increase in bursting in the sel-10(0) background: Whereas none of the control LIN-45(V627E) (n = 59) and LIN-45[ED] (n = 67) hemaphrodites burst, 58% of sel-10(0); LIN-45(V627E) (n = 51) and 43% of sel-10(0); LIN-45[ED] (n = 73) hemaphrodites died after bursting.

In wild-type vulval fate patterning, the EGFR–Ras–Raf–ERK inductive signal specifies a vulval cell fate termed “1°,” and this signal is coordinated with LIN-12/Notch-mediated cell–cell interactions to ensure that only one VPC adopts the 1° fate [Fig. 2A]. We used the 1° fate-specific marker lag-2p::yfp, which is directly regulated by Ras signaling via the ERK substrate LIN-1 (Zhang and Greenwald 2011), to examine VPC fate directly. Using lag-2p::yfp, we confirmed that the low level of ectopic 1° fate caused by expression of LIN-45[ED] was significantly enhanced by loss of sel-10 [Fig. 2D]. In addition, we saw a dramatic increase in the frequency of adjacent VPCs expressing the 1° fate marker [Fig. 2D], consistent with loss of sel-10 causing a sufficiently high level of Ras–Raf–ERK activity to override the lateral interactions that normally prevent adjacent VPCs from adopting the 1° fate.

All of the genetic interactions that we observed imply sel-10 as a negative regulator of lin-45 activity. Since these genetic interactions do not distinguish whether the effect of sel-10 on lin-45 is direct or indirect, we next examined the effect of mutations made in cis in lin-45.

Mutation of the CPD increases lin-45 activity

The CPD has been well characterized structurally, functionally, and biochemically as a motif that binds Fbw7 [Orlicky et al. 2003; Hao et al. 2007]. Mutation of essential residues in the CPD has been established as a functional test for Fbw7-dependent degradation of several different substrates; these mutations act in cis to prevent a substrate from being targeted [Welcker et al. 2004; Ye et al. 2004; Sundqvist et al. 2005; Wei et al. 2005;
Thompson et al. 2007; Olson et al. 2008). To assess whether loss of sel-10 increases lin-45 activity through a direct effect on LIN-45, we introduced alanine substitutions into the “central threonine” and “+4 serine” residues (T432A/S436A, called “AA” here) of constitutively active LIN-45[V627E] and LIN-45[ED] and found that these mutations caused a dramatic enhancement of their constitutive activity, as assessed by increased penetrance and expressivity of the multivulva phenotype (Fig. 2E). These results indicate that the phenotypic effect of loss of sel-10 is likely to be a direct consequence of stabilizing LIN-45. As described below, mutation of the CPD leads to the stabilization of YFP-tagged LIN-45, further supporting this conclusion.

We also expressed LIN-45[+] and LIN-45[AA] in the same manner. Expressing “extra” LIN-45[+] or LIN-45[AA] in VPCs did not cause large pseudovulvae in a sel-10(+) or sel-10(0) background [Fig. 2C,E, data not shown]. These observations indicate that loss of sel-10 or mutation of the LIN-45 CPD does not in itself activate LIN-45, consistent with LIN-45 remaining dependent on ubiquitination and protein degradation, removing because substrate binding by SEL-10/Fbw7 results in the VPC where Ras–Raf–ERK signaling is active.

LIN-45 is post-translationally down-regulated in the VPC where Ras–Raf–ERK signaling is active

Because substrate binding by SEL-10/Fbw7 results in ubiquitination and protein degradation, removing sel-10 or mutating the substrate CPD may produce a detectable elevation of the target protein level. To test whether SEL-10 regulates LIN-45 protein accumulation in vivo, we created a fosmid-based reporter transgene, fosmid[yfp-lin-45] [Materials and Methods]. Since our results indicate that sel-10 is capable of regulating lin-45 activity during vulval development, we examined YFP-LIN-45 expression in VPCs.

Induction and patterning of VPC fates occurs by two sequential signaling events involving both EGF and Delta/Serrate/LAG-2 (DSL) [Fig. 2A]. Initially, the six VPCs, named P3.p–P8.p, are unspeciﬁed and competent to take on vulval fates. During the early L3 stage, LIN-3/EGF activates LET-23/EGFR and the Ras–Raf–ERK cascade in P6.p [Sundaram 2006]. As a result, P6.p adopts the 1° cell fate, and the C. elegans Elk1 ortholog LIN-1 is phosphorylated, abrogating its repression of lag-2 [Zhang and Greenwald 2011]. The resulting production of ligands, including LAG-2 in P6.p, activates LIN-12/Notch in the neighboring cells, P5.p and P7.p, causing them to adopt the 2° cell fate. These cell–cell interactions as well as multiple positive and negative feedback loops ensure that full Ras–Raf–ERK activation only occurs in P6.p (Bers et al. 2001, 2005; Yoo et al. 2004).

The VPCs are specified during the L3 stage, prior to their division, by the time they divide, they have been specified to the 1° or 2° vulval fates or the 3° nonvulval fate [Greenwald et al. 1983; Sternberg and Horvitz 1986]. Prior to division, it is not readily apparent whether the spatial patterning signaling events have occurred or been fully resolved by feedback mechanisms. However, the daughters of the VPCs, “Pn.px,” are fully committed; thus, we score cell fate markers at the Pn.px or later stages to be confident that the specification process has been completed. We observed that, prior to division, YFP-LIN-45 expressed from fosmid[yfp-lin-45] was evident in the cytoplasm of all VPCs [Fig. 3B]. In contrast, at the Pn.px and later stages, YFP-LIN-45 was markedly reduced in the descendants of P6.p compared with the descendants of P5.p and P7.p [Fig. 3B]. This result was surprising.

![Figure 3](image_url)

**Figure 3.** LIN-45 is down-regulated post-translationally in P6.p, the VPC where Ras–Raf–ERK signaling is activated. (A) Normal pattern of P5.p, P6.p, and P7.p fates and LIN-45/Raf activity. Ras–Raf–ERK activity is high in P6.p, which adopts the 1° vulval fate, and low in other VPCs, including P5.p and P7.p, which adopt the 2° vulval fate. After fate specification, VPCs divide, and the daughter “Pn px” cells are fully committed. Except when noted, all quantification for lin-45 reporters was done by scoring hermaphrodites at Pn.px and Pn.pxx stages to ensure that the specification process has been completed. (B) The fosmid[yfp-lin-45] reporter. (Top) Photomicrograph of YFP-LIN-45 expressed from arEx1482 in VPCs. (AC) Anchor cell of the gonad. (Bottom) YFP-LIN-45 in a Pn.px stage hermaphrodite, showing that YFP-LIN-45 has been down-regulated in the daughters of P6.p but is evident in neighboring cells. (C) The lin-31p::yfp-lin-45 tissue-specific reporter. To assess post-translational LIN-45 stability, YFP-LIN-45 was expressed in VPCs and their daughters using heterologous regulatory sequences, the lin-31 promoter, and the unc-54 3′ UTR. Shown here is YFP-LIN-45 accumulation in a Pn.px stage hermaphrodite carrying arEx1528. YFP-LIN-45 accumulation was scored for three independent transgenes. (D) The lin-31p::2xNLS-yfp control reporter. The lin-31 promoter and the unc-54 3′ UTR used in C results in uniform expression of 2xNLS-YFP [open arrow]. Shown here is 2xNLS-YFP in a Pn.px stage hermaphrodite carrying arEx1541[lin-31p::2xNLS-yfp]. 2xNLS-YFP accumulation was scored for three independent transgenes.
because the YFP-LIN-45 level appeared lowest where lin-45 activity is highest.

To test whether down-regulation of LIN-45 occurs post-translationally, we expressed YFP-LIN-45 using heterologous sequences that drive expression in VPCs: the lin-31 promoter and the neutral 3’ untranslated region (UTR) from unc-54 [lin-31p::yfp-lin-45] (Fig. 3C). YFP-tagged LIN-45 protein is still absent in P6.p descendants (Fig. 3C), while the same heterologous sequences drive uniform expression of 2xNLS-YFP in VPCs (Fig. 3D), indicating that down-regulation of YFP-LIN-45 is indeed post-translational. A fosmid-based reporter expressing mCherry-SEL-10 [Materials and Methods] is expressed uniformly in VPCs [Supplemental Fig. 3], indicating that down-regulation of YFP-LIN-45 is not due to patterned post-translational stabilization in a P6.p descendant. We note that we verified that YFP-LIN-45 expressed from the fosmid or tissue-restricted construct is functional in a rescue assay [Supplemental Material, Supplemental Fig. 4].

Taken together, our results indicate that LIN-45 is specifically down-regulated post-translationally when EGFR–Ras–Raf–ERK signaling is activated in P6.p.

**SEL-10 and the CPD are required for LIN-45 down-regulation**

The down-regulation of LIN-45 that we observe in P6.p requires sel-10 activity. YFP-LIN-45 expressed from fosmid[yfp-lin-45] or the lin-31p::yfp-lin-45 tissue-specific reporter stably accumulated in P6.p descendants in a sel-10(0)-null mutant [Fig. 4A,B]. Transcription driven by the tissue-specific promoter remains uniform in the sel-10(0) background (Fig. 4C). Expression of YFP-LIN-45 was also stabilized in P6.p in a second null mutant, sel-10(ok1632), ruling out genetic background differences [Supplemental Fig. 5].

To test whether the CPD mediates the post-translational stability of YFP-LIN-45, we introduced the alanine substitutions into the CPD (T432A and S436A) of the YFP-LIN-45 protein reporter [Fig. 4D]. As described above, these mutations increase the signal-transducing activity of LIN-45(V627E) and LIN-45(ED). We found that either single mutation, T432A or S436A, is sufficient to stabilize YFP-LIN-45 expression in P6.p descendants [Fig. 4E,F]. Furthermore, the pattern of YFP-LIN-45(T432A,S436A) protein accumulation is not altered by loss of sel-10 activity [Supplemental Fig. 6].

These observations that LIN-45 protein down-regulation is dependent on both sel-10 in trans and the CPD in cis are consistent with the well-characterized mechanism by which SEL-10/Fbw7 directly targets the CPDs of known substrates.

An ERK docking site and MKP-1/ERK are necessary for LIN-45 down-regulation

To account for why LIN-45 is down-regulated in P6.p, the cell that requires its activity, but not in the other VPCs, which do not, we considered the possibility that a negative regulatory loop comes into play.

One possible way that such a negative regulatory loop could be achieved is that activation of LIN-45 per se
results in a conformational change that allows CPD phosphorylation and recruitment of SEL-10. Activation of Raf proceeds by several steps, including Ras-binding, dimerization, and phosphorylation of the kinase activation loop, and finally results in catalytic activity of the kinase domain ([for review, see Matallanas et al. 2011]). To test whether these steps are required in cis, we generated LIN-45 reporters containing mutations to disrupt side-to-side dimerization [R533H], phosphorylation of the activation loop [T626A/T629A], and kinase catalytic activity [K507M] [Zhang and Guan 2000, Rajakulendran et al. 2009]. These mutant forms of YFP-LIN-45 were expressed in an otherwise lin-45(+) background and were downregulated normally [Supplemental Fig. 7]. Finally, we found that LIN-45(218–480), a fragment that contains the CPD but lacks the main functional domains [Fig. 5A], is sufficient for down-regulation in P6.p descendants [Fig. 5B]. These results argue against a requirement for an activated conformation or complex in ultimately leading to LIN-45 regulation by SEL-10.

Another possibility is that MEK or ERK, the downstream kinases integral to Raf–MEK–ERK signal transduction, phosphorylates the LIN-45 CPD. In considering this possibility, we focused on ERK, as LIN-45 contains three sites that conform to a class of ERK docking domains termed the D domain. The substrate specificity of MAP kinases such as ERK, JNK, and p38 is determined by modular “docking sites” distinct from the phosphoacceptor, and prediction of docking sites has been remarkably successful in identifying new ERK targets in C. elegans [Arur et al. 2009]. One of the predicted D domains within LIN-45 is located directly adjacent to the CPD and conforms well to the D-domain consensus H/K/R-X(0,5)-L/I-X-L/I established in C. elegans and humans [Fig. 5A; Jacobs et al. 1999; Biondi and Nebreda 2003]. ERK is proline-directed, requiring proline at the “+1 position” and favoring proline at the “−2 position” [Songyang et al. 1996], making the CPD “central threonine” residue T432 an ideal phospho-acceptor. To test whether this predicted docking site is important for LIN-45 down-regulation, we generated alanine substitutions in YFP-LIN-45 at two leucine residues in positions that are essential for ERK binding to other D domains [Fantz et al. 2001]. We found that these mutations, L429A/L431A, stabilized LIN-45 in P6.p after cell divisions [Fig. 5C], indicating that direct ERK binding to LIN-45 is required for down-regulation.

To test whether ERK activity is required for LIN-45 down-regulation, we crossed the lin-31p::yfp-lin-45 transgene to a mutant of the C. elegans ERK ortholog mpk-1. mpk-1 activity is essential for vulval cell fate induction; neither the 1° fate nor the 2° fate is specified in mpk-1-null mutants [Lackner and Kim 1998]. We used a temperature-sensitive allele of mpk-1 to reduce activity by shifting mutants to the restrictive temperature, and in animals in which there was sufficient mpk-1 activity to support vulval induction, the reduced mpk-1 activity led to stabilization of YFP-LIN-45 protein in descendants of P6.p [Fig. 5D]. Expression of the reporter lin-31p::2xNLS-yfp was unaffected in the mpk-1(ts) background [Fig. 5E]. These observations indicate that mpk-1/ERK activity is required for YFP-LIN-45 down-regulation.

We also tested two transcription factor genes that act downstream from MPK-1. lin-1, a C. elegans Elk1 ortholog that is directly phosphorylated by ERK [Jacobs et al. 1998], and sur-2/MED23, a Mediator component [Singh and Han 1995], are not required for LIN-45 down-regulation [Supplemental Fig. 8], placing the down-regulation step upstream of known ERK effectors in P6.p.

These results indicate that LIN-45 protein stability is regulated by the downstream kinase MPK-1/ERK and vulval induction. To ensure that P6.p had adopted the 1° fate, mpk-1(+) and mpk-1(ts) strains carrying arEx1528[lin-31p::yfp-lin-45] were examined in the P6.pxx stage (i.e., after two rounds of cell division); if P6.p had not been induced to the 1° fate, it would have divided only once, as is characteristic for the 3° nonvulval fate. YFP-LIN-45 accumulation persists in induced P6.p descendants. Both strains were also homozygous for him-5(e1490). (1) P-value < 0.0001 for comparison of P6.p in control and mpk-1 strains. (E) The reporter lin-31p::2xNLS-yfp is unaffected by mpk-1. 2xNLS-YFP accumulation was scored at the P6.pxx stage for arEx1541[lin-31p::2xNLS-yfp] in mpk-1(+) and mpk-1(ts) strains at 25°C. Both strains also were homozygous for him-5(e1490).
that MPK-1 acts by directly binding and phosphorylating LIN-45.

**Functional relevance of LIN-45 down-regulation by sel-10 to VPC fate patterning**

In many cases, loss of an individual negative regulator of EGFR–Ras–ERK signaling in the VPCs does not cause aberrant vulval development, but concomitant loss of two regulators can cause ectopic vulval induction, resulting in pseudovulvae. In particular, gap-1(0), a null mutation in the gap-1/GTPase-activating protein, is a sensitive background for assessing negative regulators of Ras signal transduction [Hubbard et al. 2000; Berset et al. 2001; Yoo et al. 2004]. Whereas sel-10(0) mutants have normal vulval induction [Hubbard et al. 1997], sel-10(0); gap-1(0) adults displayed ectopic pseudovulvae (nine out of 186, 4.8%) more frequently than either gap-1(0) [one out of 180, 0.6%] or sel-10(0) adults (0 out of 119) [P-value = 0.02, Fisher’s exact test]. Ectopic induction was also observed at the level of individual VPCs by looking for the presence of an invagination in the early L4 stage: sel-10(0); gap-1(0) L4 larvae had an ectopic invagination (seven out of 55, 12.7%) more often than gap-1(0) L4 larvae [one out of 66, 1.5%] [P-value = 0.02, Fisher’s exact test]. These results indicate that sel-10 activity is functionally relevant in the regulation of lin-45 activity and redundant with other negative regulatory mechanisms of the Ras pathway.

**Discussion**

The E3 ubiquitin ligase component Fbw7 is important in development and disease. As several validated substrates contain a “CPD,” a phosphorylation-dependent binding site for Fbw7, we hypothesized that the presence of a high-affinity CPD consensus sequence may be an effective strategy for identifying potential new substrates of Fbw7. We chose LIN-45/Braf from our list of candidates for an extensive functional analysis in C. elegans and validated it as a new substrate of SEL-10/Fbw7. In addition, in the VPCs, an important paradigm for elucidating conserved principles and regulatory mechanisms acting in signal transduction during development, we showed that SEL-10/Fbw7-mediated degradation of LIN-45/Braf requires ERK activity in a negative feedback loop. We discuss here the potential predictive value of the conserved CPD of human Braf as well as general applicability of the CPD prediction approach and the role and mechanism of negative feedback of LIN-45 and human Braf.

**The potential predictive value of a CPD**

The relationship between SEL-10 and LIN-45 in C. elegans seems likely to hold true for Fbw7 and Braf in humans in certain cell contexts. Human Braf contains a CPD consensus sequence that is conserved in other vertebrates in a position equivalent to the CPD in LIN-45 in an otherwise poorly conserved region before the kinase domain. The homology with LIN-45 suggests that this potential CPD of Braf is accessible to phosphorylation and subsequent binding by Fbw7. Indeed, phosphorylation of T401, the “central threonine” of the predicted CPD, has been observed in human Braf after growth factor stimulation of NIH3T3 and mouse embryonic fibroblast [MEF] cells [McKay et al. 2009; Ritt et al. 2010], indicating that the potential CPD of Braf is accessible. Thus, in a cell context in which phosphorylation of the putative “+4 serine” S405 also occurs, we expect that Braf could become a substrate for Fbw7-promoted degradation. Such a relationship could be relevant to cancer if somatic mutation or loss of FBXW7 increases Braf activity in primary tumors or perhaps in relapsed BRAF(V600E) tumors that are resistant to Raf and MEK inhibitors, by analogy with what has been observed for FBXW7 mutations in relapsed T-ALL [O’Neil et al. 2007; Thompson et al. 2007].

Because we identified LIN-45 as a potential SEL-10 substrate solely on the basis of its conserved CPD, we suggest that other novel substrates may be predicted on the basis of containing a conserved CPD. We believe that our predictive approach will indeed be useful because it does not depend on biochemical properties or cellular context, particularly in contexts that cannot be directly assayed easily using proteomics approaches. Specifically, we suggest that our list of predicted “vertebrate-conserved” substrates [Supplemental Table 2] may contain proto-oncogenes that are deregulated in Fbw7 mutant tumors or may be found to be mutated as more tumor genome sequence information becomes available.

**Negative feedback modulation of Raf activity**

In the VPC developmental patterning paradigm, we found that SEL-10 mediates negative feedback of LIN-45/Braf stability and activity such that activation of the downstream kinase ERK results in LIN-45 protein degradation [Fig. 6]. This negative feedback loop accounts for the initially paradoxical observation that sel-10 promotes the instability of LIN-45 in P6.p, the VPC in which activity of the Ras–Raf–ERK cascade is at a maximum during vulval induction, whereas LIN-45 is stable in other VPCs where activity of the Ras–Raf–ERK cascade is low.

A different mode of negative feedback regulation has been described for human Braf. Ritt et al. [2010] identified sites on human Braf that are phosphorylated in NIH3T3 cells, likely by ERK, leading to inactivation of Braf within minutes after growth factor stimulation. However, in NIH3T3 cells, the identified mechanism does not involve degradation of Braf; rather, phosphorylation of three of the sites curtails activity by causing dissociation of Braf from activated Ras and the Braf/Raf1 heterodimer. Curiously, in NIH3T3 cells, feedback phosphorylation of the fourth site, T401, which lies within the predicted CPD, does not have a strong negative effect on Braf activity [Ritt et al. 2010], raising the possibility that NIH3T3 cells do not provide a relevant context for assaying its effect—as might be the case if these cells lack a kinase that could phosphorylate the “+4 serine” of the putative CPD. Furthermore, C. elegans LIN-45 has potential phospho-
VPC specification: Although loss of negative regulation of LIN-45 activity is meaningful in against spurious activation of Ras–Raf–ERK signal transduction in cells other than P6.p, helping to ensure that the 1° fate is specified in only one VPC during spatial patterning. The high degree of redundancy in negative regulation of Ras–Raf–ERK signaling may contribute to the precise and robust patterning of VPC fate that has been well documented under many different developmental and environmental conditions [Braendle and Félix 2008].

Materials and methods

CPD prediction in nematode and vertebrate proteins

The consensus CPD and Notch1-like CPD patterns were as follows [in Prosite syntax, \{\} indicates amino acids not allowed, [\] indicates amino acids allowed, and X indicates any amino acid]: consensus: [HKRDE]-[ST]-P-X-[ST] and [HKRDE]-[ST]-P-X-P-[ST]. Protein accessions in Swiss-Prot and TrEMBL databases were scanned using ScanProsite [http://ca.expasy.org/tools/scanprosite] [de Castro et al. 2006], and matches were filtered for accessions from C. elegans, Caenorhabditis briggsae, Caenorhabditis remanei, D. rerio, and Homo sapiens. To compare across species, protein sequence hits from individual species were uploaded as new proteomes in OrthoMCL [http://orthomcl.org/cgi-bin/OorthoMclWeb.cgi?r=index] [Chen et al. 2006], producing an ortholog group assignment for each sequence. Ortholog group assignments were compared between species using the list manipulation functions in Galaxy [https://main.g2.bx.psu.edu]. BioMart [http://useast.ensembl.org/biomart/martview] was used to retrieve Ensembl gene information for conserved hits.

C. elegans genetics

All strain names and full genotypes are listed in the Supplemental Material. The following mutations were used: LGI: sur-2(ky9); LGII: mtk-1(ga111ts), pha-1(e2123ts); LGIV: lin-1(e1275ts), the hypomorphic alleles lin-45(n2018) and lin-45(n2506), and the null alleles lin-45(dx19), unc-24(e138), dpy-20(e1282); LGV: lon-3(e2175), the null alleles sel-10(ar41) and sel-10(ok1632), and him-5(e1490); and LGX: the null allele gap-1(e1993). The transgene arl131[yag-2p::2xnl5-yfp] was described by Zhang and Greenwald [2011].

Fosmid-based reporter transgenes

Fosmid-based reporters were generated using λ Red-mediated recombination as described by Tursun et al. [2009]. The fosmid[yfp-lin-45] reporter was produced from fosmid WRM0613cC02 (Source BioScience) and contains a yfp cDNA amplified from pBALU2 and inserted at the ATG of lin-45 isoform Y73B6A.5a. The resulting gene fusion encodes N-terminally tagged YFP-LIN-45. The fosmid[sel-10-mCherry] reporter was produced from fosmid WRM0632B09 and contains an mCherry cDNA amplified from pBALU12 and inserted at the ATG of sel-10. The resulting gene fusion encodes N-terminally tagged mCherry-SEL-10. Primers used in the generation of fosmid reporters are listed in the Supplemental Material.

Plasmids used for expression in C. elegans

C. elegans lin-45 cDNA isoform Y73B6A.5a was amplified from the ProQuest C. elegans cDNA library [Invitrogen] and ligated by TOPO TA cloning into pCR-XL [Invitrogen], producing pCC18.

Figure 6. Model for negative feedback leading to LIN-45 degradation mediated by SEL-10 and MPK-1. In P6.p, LIN-45 is activated in an EGFR and Ras-dependent manner involving Ras binding, dimerization, and phosphorylation of the LIN-45 activation loop. Active LIN-45 leads to activation of the downstream kinase MPK-1/ERK, resulting in phosphorylation of the LIN-45 CPD as well as MPK-1 substrates that promote the 1° cell fate. The “central threonine” CPD residue of LIN-45 has the hallmark of an ERK phospho-acceptor site, while another unknown kinase is expected to phosphorylate the “+4 serine.” The “+4 serine kinase” may but need not be dependent on Ras–Raf–ERK. When phosphorylated at both critical CPD residues, LIN-45 is recognized by SEL-10 for ubiquitination, leading to degradation. We note that dephosphorylation of LIN-45 and mammalian Raf proteins also occurs [Kao et al. 2004; Ritt et al. 2010], so a “recycling” step may be another fate of phosphorylated LIN-45. In other VPCs, such as P5.p and P7.p, LIN-45 and downstream kinase MPK-1 are inactive, so LIN-45 protein is stable.

acceptor sites in positions similar to the three phospho-acceptor sites in human Braf that govern association with the signaling complex [data not shown], suggesting that this mode of negative feedback might also occur in LIN-45.

While it remains to be seen whether both modes of negative feedback operate in both nematodes and mammals, the existence of a form of ERK-mediated negative feedback in both systems suggests that such modulation of Braf activity is important in vivo. In mammalian PC12 cells, the duration of ERK signaling has different outcomes, with transient signaling promoting growth and sustained signaling promoting differentiation [for review, see Marshall 1995], suggesting that the rapid inactivation of Braf observed by Ritt et al. (2010), so a “recycling” step may be another fate of phosphorylated LIN-45. In other VPCs, such as P5.p and P7.p, LIN-45 and downstream kinase MPK-1 are inactive, so LIN-45 protein is stable.

In C. elegans, we found that the contribution of sel-10 to negative regulation of LIN-45 activity is meaningful in VPC specification: Although loss of sel-10 or the GTPase gap-1 individually does not alter vulval fate patterning, ectopic induction indicative of elevated Ras–Raf–ERK signal transduction emerged in the double mutant. Thus, we propose that SEL-10 modulates the level of activated LIN-45/Raf in the VPCs in conjunction with other negative regulators to set a threshold for Ras–Raf–ERK signaling. We expect that this threshold would guard against spurious activation of Ras–Raf–ERK signal transduction in cells other than P6.p, helping to ensure that the 1° fate is specified in only one VPC during spatial patterning. The high degree of redundancy in negative regulation of Ras–Raf–ERK signaling may contribute to the precise and robust patterning of VPC fate that has been well documented under many different developmental and environmental conditions [Braendle and Félix 2008].
Generation of transgenic C. elegans

Transgenic strains used for the assessment of lin-45 and lin-12 gain-of-function phenotypes were generated in the N2 background by germline injection of hermaphrodites with a mixture of 1 ng/µL linear plasmid DNA, 1 ng/µL myo-3p:mCherry, and 50 ng/µL Puv1-digested N2 genomic DNA.

Strains carrying fluorescent reporter transgenes were generated in the pba-1(e2123) background, and rescue of the temperature-sensitive pba-1(+) lethal phenotype was used as a selective transgene marker [Granato et al. 1994]. Fosmid-based reporters were injected as a mixture of 15 ng/µL linear fosmid DNA, 1 ng/µL pBX[pha-1(+)], 1 ng/µL PCW2.1[ceh-22p::gfp], and 50 ng/µL Puv1-digested N2 genomic DNA. Strains carrying pB253-based YFP reporters were generated by injection of a mixture of 1 ng/µL linear plasmid DNA, 1 ng/µL pBX[pha-1(+)], 1 ng/µL PCW2.1[ceh-22p::gfp], and 50 ng/µL Puv1-digested N2 genomic DNA.

Assessment of L1 lethality and multivulva phenotypes

To assess L1 lethality of lin-45(h) [hypomorphic] mutants, L4 hermaphrodites were picked individually and transferred to fresh plates daily. At 48 h after egg laying, progeny were examined at the dissecting microscope and scored as live larvae, dead larvae, or unhatched eggs. To assess vulvalues and sterile phenotypes of the lin-45(0) [null] mutant, L4 hermaphrodites were picked individually, and adults were scored 24 h later for the presence of a vulva and visible eggs inside the germline. lin-45(h) mutants did not display vulvalues and sterile phenotypes. To assess the multivulva phenotype caused by lin-45 transgenes, L4 hermaphrodites from uncrowded cultures were picked to fresh plates; transgenic adults were examined 24 h later at the dissecting microscope and scored for the presence of a normal vulva and pseudovulvae. All lin-45 mutant and control strains scored for L1 lethality and multivulva phenotypes were cultured at 20°C using standard procedures. gap-1 mutant and control strains scored for the multivulva phenotype were cultured at 25°C.

We note that we mainly scored large pseudovulvae, the canonical phenotype of excessive Ras-Raf-ERK activity. We also observed a weak, low-penetration phenotype characterized by much smaller pseudovulvae when expressing excess LIN-45(+) in the sel-10(0) background. As we did not see this phenotype in strains expressing LIN-45[AA] in a sel-10(+/-) background, we infer that it may result from deregulation of a different SEL-10 substrate, not LIN-45.

Scoring of fluorescent reporter expression

Except when noted, pba-1(e2123) strains carrying pba-1(+), YFP-expressing transgenes were cultured at 22°C to select for rescue of the pba-1(e2123) temperature-sensitive lethal phenotype. To obtain L3 larvae, adult hermaphrodites were allowed to lay eggs for 12–16 h overnight. When strains used were egg-laying-defective, gravid adult hermaphrodites were bleached, and the eggs were plated. At ~36 h after egg collection, L3 larvae were mounted in M9 buffer containing 12 mM Levamisole. Except where otherwise noted, cell fate markers were scored at the Pn.pxxx stage to be confident that the specification process has been completed. YFP fluorescence was scored at 40× magnification using a Zeiss Axio Imager Z1 microscope with an ApoTome system and a Hamamatsu ORCA-ER camera. For all yfp-lin-45 transgenes, a 1.0 sec exposure time was used to capture the YFP image and score VPCs as positive or negative.

Because the mpk-1(ga111) mutant is sterile at higher temperatures, transgenic strains containing mpk-1(ga111) were maintained at 20°C. In mpk-1(ga111) temperature shift experiments, eggs were plated and cultured for 36–38 h at 20°C, then shifted for 6–8 h to 25°C before L3 larvae were scored. Approximately half of mpk-1(ga111) pha-1(e2123); him-5(e1490); arf1528[lin-31p::yfp-lin-45] hermaphrodites were vulvaless after this temperature shift [25 out of 47, 53% of adults], while none of the mpk-1(+) pha-1(e2123); him-5(e1490); arf1528 hermaphrodites were vulvaless. To avoid analyzing VPCs that had failed to execute a vulval fate, we scored expression of YFP transgenes at the Pn.pxxx stage in strains containing or compared with mpk-1(ga111).

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[PCR-XL-lin-45]. C. elegans lin-12 cDNA originated from GS8p270. Plasmids used for expression of lin-45 and lin-12 in C. elegans were constructed in pB253 [Tan et al. 1998], a vector containing promoter elements from lin-31 and a 3’ UTR from unc-54. For cloning purposes, an internal NotI restriction site in lin-45 was abolished by the generation of a silent point mutation (g2394a). The yfp-lin-45 fusion gene was created by PCR and encodes YFP followed directly by the initiator methionine of lin-45. All lin-45 and yfp-lin-45 cDNAs were ligated into the NotI site of pB253. lin-12[intrachromosomal cut] encodes an initiator methionine followed by the intrachromosomal domain of LIN-12, residues 931–1429. cDNAs were ligated into the BglII and NotI sites of pB253. Primers used for cDNA cloning, gene fusions, and site-directed mutagenesis are listed in the Supplemental Material.

Scoring of fluorescent reporter expression

Except when noted, pba-1(e2123) strains carrying pba-1(+), YFP-expressing transgenes were cultured at 22°C to select for rescue of the pba-1(e2123) temperature-sensitive lethal phenotype. To

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