

Conversion of the LIN-1 ETS Protein of *Caenorhabditis elegans* from a SUMOylated Transcriptional Repressor to a Phosphorylated Transcriptional Activator

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ABSTRACT The LIN-1 ETS transcription factor plays a pivotal role in controlling cell fate decisions during development of the *Caenorhabditis elegans* vulva. Prior to activation of the RTK/Ras/ERK-signaling pathway, LIN-1 functions as a SUMOylated transcriptional repressor that inhibits vulval cell fate. Here we demonstrate using the yeast two-hybrid system that SUMOylation of LIN-1 mediates interactions with a protein predicted to be involved in transcriptional repression: the RAD-26 Mi-2 β /CHD4 component of the nucleosome remodeling and histone deacetylation (NuRD) transcriptional repression complex. Genetic studies indicated that *rad-26* functions to inhibit vulval cell fates in worms. Using the yeast two-hybrid system, we showed that the EGL-27/MTA1 component of the NuRD complex binds the carboxy-terminus of LIN-1 independently of LIN-1 SUMOylation. EGL-27 also binds UBC-9, an enzyme involved in SUMOylation, and MEP-1, a zinc-finger protein previously shown to bind LIN-1. Genetic studies indicate that *egl-27* inhibits vulval cell fates in worms. These results suggest that LIN-1 recruits multiple proteins that repress transcription via both the SUMOylated amino-terminus and the unSUMOylated carboxy-terminus. Assays in cultured cells showed that the carboxy-terminus of LIN-1 was converted to a potent transcriptional activator in response to active ERK. We propose a model in which LIN-1 recruits multiple transcriptional repressors to inhibit the 1^o vulval cell fate, and phosphorylation by ERK converts LIN-1 to a transcriptional activator that promotes the 1^o vulval cell fate.

KEYWORDS LIN-1; SUMO; ETS; chromatin; vulval development; transcription

CELL fates are established during development by spatially controlled activation of signal transduction pathways that regulate transcription factors. The Elk subfamily of ETS transcription factors are critical targets of the receptor tyrosine kinase (RTK)/Ras/extracellular-regulated kinase (ERK)-signaling pathway (Treisman 1994). This subfamily includes extensively characterized proteins from three divergent animals: vertebrate Elk-1, *Drosophila* Aop/Yan, and *Caenorhabditis elegans* LIN-1. The mechanisms that enable ETS proteins to switch cell fates in response to signaling pathway activation are not fully defined, and to address this

important issue it is critical to elucidate the function of these proteins both prior to ERK activation and following phosphorylation by ERK. These issues have significant implications for human health, since ETS proteins contribute to human diseases including cancer (Dittmer 2003).

lin-1 has been extensively characterized during development of the hermaphrodite vulva, an epidermal structure used for egg laying and sperm entry (reviewed by Horvitz and Sternberg 1991; Greenwald 1997; Kornfeld 1997; Sternberg 2005; Sundaram 2013). In third-larval-stage hermaphrodites, six ventral epidermal blast cells called P3.p–P8.p (Pn.p cells) lie along the anterior–posterior axis. These Pn.p cells are an equivalence group, since each cell can adopt the 1^o vulval cell fate (eight descendants), the 2^o vulval cell fate (seven descendants), or the nonvulval 3^o cell fate (two descendants). During vulval induction, the anchor cell of the somatic gonad secretes the LIN-3 ligand, an epidermal growth factor (EGF) homolog, thereby activating the

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LET-23/RTK on the adjacent P6.p cell (Aroian *et al.* 1990; Hill and Sternberg 1992). Activated LET-23 recruits the SEM-5 adaptor (GRB2) and the LET-341/SOS-1 guanine nucleotide exchange factor (SOS), followed by sequential activation of the GTPase LET-60 (RAS) and the protein kinases LIN-45 (RAF), MEK-2 (MAPK kinase, or MEK), and MPK-1 (ERK) (Beitel *et al.* 1990; Han and Sternberg 1990; Clark *et al.* 1992; Han *et al.* 1993; Lackner *et al.* 1994; Wu and Han 1994; Kornfeld *et al.* 1995; Wu *et al.* 1995; Chang *et al.* 2000; Hsu *et al.* 2002). Activated MPK-1 phosphorylates substrates including the LIN-1 ETS transcription factor, thereby promoting the 1° fate in P6.p. P6.p signals P5.p and P7.p to adopt 2° fates by activating a lateral signal involving LIN-12/Notch. The 22 descendants of P5.p, P6.p, and P7.p invaginate and differentiate to form the vulval structure. P3.p, P4.p, and P8.p receive neither signal and therefore adopt 3° fates. Mutations that reduce activation of RTK/Ras/MAPK signaling cause a vulvaless (*Vul*) phenotype, whereas mutations that constitutively activate this pathway cause more than three Pn.p cells to adopt the 1° or 2° vulval cell fate, resulting in a multivulval (*Muv*) phenotype characterized by ectopic patches of vulval tissue.

LIN-1 is a transcription factor that plays a critical role in establishing vulval cell fates. Loss-of-function mutations that abrogate sequence-specific DNA-binding activity cause a strong *Muv* phenotype, demonstrating that DNA binding is necessary for *lin-1* function and that *lin-1* function is necessary to inhibit the 1° fate (Sulston and Horvitz 1981; Beitel *et al.* 1995; Miley *et al.* 2004). Genetic epistasis studies established that *lin-1* functions downstream of *mpk-1* ERK (Ferguson *et al.* 1987; Lackner *et al.* 1994; Wu and Han 1994). LIN-1 contains 17 S/TP motifs that are potential ERK phosphorylation sites and two docking sites for ERK, the D-domain and the FQFP motif (Jacobs *et al.* 1998, 1999; Tan *et al.* 1998). Mutations of LIN-1 that decrease phosphorylation by ERK cause a gain-of-function *Vul* phenotype, indicating that phosphorylation is necessary to stop LIN-1 from inhibiting the 1° fate (Jacobs *et al.* 1998). These studies are consistent with two possible models. Phosphorylation by ERK may abrogate LIN-1 activity in P6.p. Alternatively, phosphorylation may convert LIN-1 from an inhibitor to an activator of the 1° cell fate. Here we address these models by analyzing how phosphorylation by ERK affects the transcriptional activity of LIN-1. Our results indicate that the carboxy-terminus of LIN-1 is converted from a transcriptional repression domain to a potent transcriptional activation domain by ERK phosphorylation.

LIN-1 contains two consensus SUMOylation motifs. The E2 small ubiquitin-related modifier (SUMO) conjugating enzyme, UBC-9, binds these motifs and mediates the covalent attachment of SUMO to LIN-1 (Leight *et al.* 2005). *C. elegans smo-1*, the gene encoding SUMO, and *ubc-9* inhibit vulval cell fates (Poulin *et al.* 2005) and genetically interact with *lin-1* (Leight *et al.* 2005), indicating that SUMOylation of LIN-1 mediates inhibition of vulval cell fates. SUMOylation of LIN-1 promotes transcriptional repression (Leight *et al.* 2005), but the mechanism has not been well defined. We

previously showed that SUMOylated LIN-1 interacts with MEP-1, a component of the NuRD transcriptional repression complex, suggesting that this interaction is one mechanism of LIN-1 transcriptional repression (Leight *et al.* 2005). The HOX gene *lin-39* is a positive regulator of vulval cell fates and a direct transcriptional target of LIN-1 (Wagmaister *et al.* 2006). Guerry *et al.* (2007) showed that LIN-1 interacts with the Mi-2 nucleosome-remodeling protein LET-418 on the *lin-39* promoter.

Here we identify proteins that bind LIN-1 and may contribute to transcriptional repression by LIN-1. SUMOylation of LIN-1 mediated recruitment of two chromatin regulatory enzymes in yeast cells: SET-6 and RAD-26. SET-6 is homologous to SUV39H1, a histone methyltransferase that mediates transcriptional repression (Bannister *et al.* 2001; Lachner *et al.* 2001; Nielsen *et al.* 2001). RAD-26 shares homology with the Mi-2 β /CHD4 component of the NuRD transcriptional repression complex. In addition, the EGL-27/MTA1 component of the NuRD complex binds the carboxy-terminus of LIN-1 in yeast cells independently of SUMOylation. These findings suggest that LIN-1 recruits cofactors via both its SUMOylated amino-terminus and its unSUMOylated carboxy-terminus to inhibit vulval cell fates. *rad-26* and *egl-27* function to inhibit vulval cell fates in worms. These data support a model in which SUMOylated LIN-1 recruits multiple chromatin regulatory proteins to repress transcription of vulval cell-fate genes, and phosphorylation by ERK MAP kinase in the P6.p cell converts LIN-1 to a transcriptional activator that promotes vulval cell fates.

Materials and Methods

Yeast two-hybrid screens and reporter gene assays

The YEL3 strain encoding a LexA_{DBD}:LIN-1(1–252) fusion protein was described by Leight *et al.* (2005). The YEL2 strain was generated by transforming the L40 strain (Vojtek *et al.* 1993) with a bait plasmid encoding LIN-1(253–441) cloned into pBTM116 (Bartel *et al.* 1993). A random-primed complementary DNA (cDNA) library from mixed-stage hermaphrodites (kindly provided by R. Barstead), containing cDNAs fused to the GAL4AD, was transformed into YEL2 and YEL3 (Schiestl and Gietz 1989). Prey plasmids were isolated from positive colonies. cDNAs that failed to PCR-amplify with MEP-1 or MPK-1 gene-specific primers were sequenced using standard techniques.

Bait plasmids encoding EGL-27(1–1129), which corresponds to the largest predicted *egl-27* transcript, EGL-27(1–637), and EGL-27(629–1129) (Herman *et al.* 1999; Solari *et al.* 1999), were cloned into pAS2-1 (CLONTECH, Palo Alto, CA) and transfected into *Saccharomyces cerevisiae* strain Hf7c (Feilotter *et al.* 1994). Positive clones growing on His dropout media were assayed for β -galactosidase activity.

To monitor activation of the LexA-dependent *lacZ* reporter, we prepared lysates from at least six independent yeast transformants of equivalent size and measured β -galactosidase

activity using the Galacto-Light Plus System (Applied Biosystems). For Figure 2, yeast transformants were grown in selective media at 30° to an OD of ~1.0 before analysis.

Analysis of *C. elegans* strains

C. elegans strains were cultured at 20° as described by Brenner (1974) unless otherwise noted. The following mutations were analyzed: *let-60(n1046)* (Beitel *et al.* 1990), *lin-1(e1275 R175Opal)* (Beitel *et al.* 1995), *lin-1(n1790 R352Opal)* (Jacobs *et al.* 1998), *egl-27(n170)* (Herman *et al.* 1999), *egl-27(we3)* (Solari *et al.* 1999), *lin-15A(n767)* and *lin-15B(n744)* (Ferguson and Horvitz 1989), *mpk-1(oz140)* (Lee *et al.* 2007), and *set-6(tm1611)* (Andersen and Horvitz 2007). *rad-26(tm1991)* was generated by the National Bioresource Project by screening animals mutagenized by trimethyl psoralen (TMP)/UV for a deletion of the C27B7.4 gene. We outcrossed *tm1991* to the N2 wild-type strain twice and constructed double mutants using standard techniques. *set-6(ok2195)* was obtained from the *Caenorhabditis* Genetics Center (CGC) and outcrossed multiple times to the N2 wild-type strain.

cDNAs encoding RAD-26(17–655), SET-6(1–708) and EGL-27(691–1119) were cloned into the L4440 plasmid (Timmons and Fire 1998) for expression of double-stranded RNA (dsRNA). Plasmids were transformed into HT115(DE3) *Escherichia coli*. L4 hermaphrodites were placed on petri dishes containing bacteria that expressed dsRNA or control bacteria with the L4440 plasmid and transferred to new petri dishes daily; progeny were scored for the Muv phenotype.

Protein purification and GST pull-down assays

6xHis- and FLAG-tagged RAD-26(17–655) (HF:RAD-26) and SET-6(22–538) (HF:SET-6) were expressed in Sf9 cells using a baculovirus expression system (Invitrogen, Bac-to-Bac Baculovirus Expression Systems manual). DNA encoding EGL-27(691–1119) was cloned in a pET-28a(+) plasmid (EMD Biosciences) with the 6xHis- and T7 tag, and the protein was expressed in *E. coli*. HF:RAD-26, HF:SET-6, and T7:EGL-27 were purified using native conditions as described in Protocol 16 of the QIAexpressionist handbook (Qiagen, August 2002) with minor modifications. DNAs encoding LIN-1(1–64) and the SMO-1(1–88) were cloned in-frame with GST into the plasmid pGEX-4T-1 (Amersham Pharmacia Biotech). DNA encoding LIN-1(281–441) was cloned in-frame with GST in the plasmid pGEX-2T (Amersham Pharmacia Biotech). GST and GST fusion proteins were expressed in *E. coli* and partially purified under native conditions using glutathione sepharose as described (Jacobs *et al.* 1998).

For pull-down assays in Figure 3, equimolar amounts of HF:RAD-26 and HF:SET-6 were incubated with ~5 µg of glutathione sepharose-bound GST, GST:LIN-1(1–64), or GST:SMO-1 for 1 hr at 4° in binding buffer (PBS + 0.1% NP-40). The glutathione sepharose was washed twice; bound proteins were eluted by boiling in sample buffer and separated by SDS-PAGE (Sambrook *et al.* 1989).

HF:RAD-26 and HF:SET-6 were detected by immunoblotting using the α-FLAG M2 antibody (Sigma). Pull-down assays in Figure 5C were performed similarly, except T7:EGL-27 was incubated with ~1 µg of glutathione sepharose-bound GST, GST:LIN-1(1–64), or GST:LIN-1(281–441). Bound T7:EGL-27 was detected by immunoblotting using the α-T7 Tag monoclonal antibody (EMD Biosciences). For pull-down assays in Figure 5D, the *egl-27* cDNA was cloned into pGEX-4T-1 to encode GST:EGL-27(1–1129), and a full-length *mep-1* cDNA was cloned into pTrcHis (Invitrogen) to encode 6xHis:MEP-1. Fusion proteins were expressed in *E. coli* strain BL21(DE3) and purified by the method of Frangioni and Neel (1993) with minor modifications. An equimolar amount of 6xHis:MEP-1 was added to GST and GST:EGL-27(1–1129) bound to glutathione sepharose beads. 6xHis:MEP-1 was detected with HRP-conjugated anti-histidine (anti-HIS) antibody (Santa Cruz) using the West Pico Super Signal Chemiluminescent detection system (Pierce).

Reporter gene assays in mammalian cultured cells

The MC3T3 mouse fibroblast cell line was grown in alpha-minimum essential medium (α-MEM) with 10% fetal calf serum and transfected as described by Fantz *et al.* (2001) with (1) the pFR-LUC reporter plasmid (Stratagene), (2) an expression plasmid that encodes GAL4 DNA-binding domain (G4) alone or fused to fragments of LIN-1 or Elk-1 (pFA-Elk, Stratagene), and (3) the CMV-β-galactosidase control plasmid. Cells were treated with 5% glycerol 1 day after transfection, incubated an additional 2 days, and harvested for cell lysates; luciferase and β-galactosidase activity were quantified. Cells were exposed to 50 µg/ml recombinant basic fibroblast growth factor (bFGF) (Upstate Biotechnology) for 20 hr beginning 2 days post-transfection. Site-directed mutagenesis was used to generate mut. D (K280A, K283A, L287A, L289A), mut. F (F381A, Q382A, F383A), and the double mutant (mut. D/F).

Results

LIN-1 interacted with RAD-26, SET-6, and EGL-27 in yeast cells

To identify proteins that associate with LIN-1 (Figure 1A), we conducted yeast two-hybrid screens. In the first screen, the bait was the LexA DNA-binding domain (LA) fused to the amino-terminal 252 residues of LIN-1, which includes the ETS DNA-binding domain and two consensus SUMOylation motifs. A total of 233 cDNAs that encode proteins that bind LIN-1(1–252) specifically were identified by screening 4 × 10⁶ cDNAs from a mixed-stage *C. elegans* cDNA library. A total of 123 cDNAs encoded the zinc-finger protein MEP-1 that we described previously (Leight *et al.* 2005). Four cDNAs derived from the C27B7.4/*rad-26* gene. The predicted RAD-26 protein contains a DNA-dependent ATPase domain of the SNF2 family N-terminal class and a helicase C-terminal domain (Figure 1B). RAD-26 shares 23% identity over 883 amino

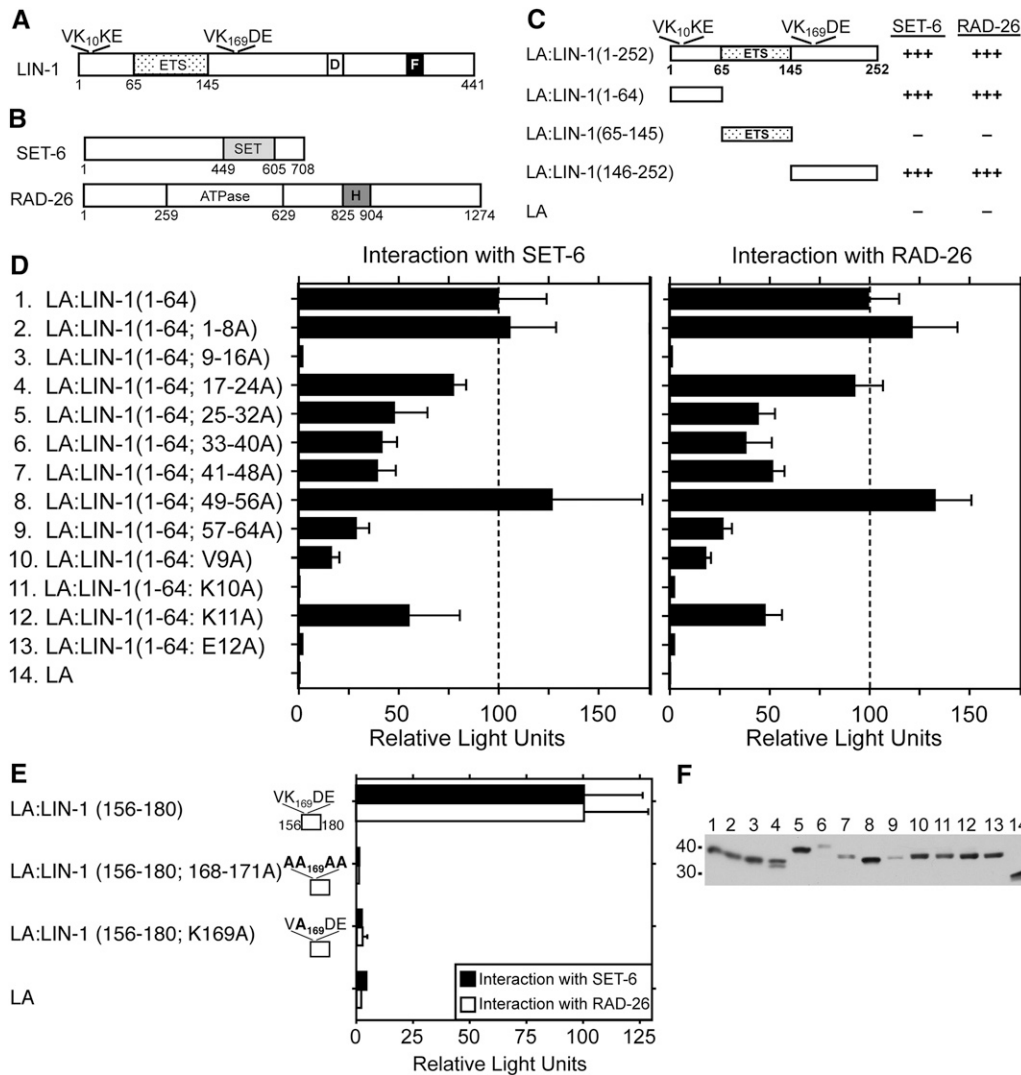


Figure 1 LIN-1 binds RAD-26 and SET-6 in yeast cells. (A) Schematic of LIN-1 showing the ETS DNA-binding domain (dotted), consensus SUMOylation motifs (ψ KxE), and docking sites for ERK MAP kinase [D-domain (open) and FQFP motif (solid)]. Amino acid numbers are shown below. (B) SET-6 contains a SET lysine methyltransferase domain (light shading). RAD-26 contains a DNA-dependent ATPase domain of the SNF2 family N-terminal class (open) and a helicase C-terminal domain (dark shading). (C) Interactions between LA DNA-binding domain:LIN-1 fusion proteins and SET-6(22–538) or RAD-26(17–655) fused to the GAL4 activation domain (GAL4AD) were measured qualitatively. Symbols indicate robust activation (+++) and no detectable activation (–) of a LexA-dependent *lacZ* reporter gene. (D and E) Interactions were measured quantitatively by assaying LexA-dependent β -galactosidase activity. Bars represent the average and standard deviation of three to six independent yeast transformants. Values were normalized by setting the signal with wild-type LA:LIN-1 to 100. (F) Expression of LA:LIN-1 proteins was analyzed by Western blotting using an anti-LA antibody. Lanes 1–14 correspond to proteins in columns 1–14 of D.

acids with LET-418/CHD-4, a component of the NuRD transcriptional repression complex. Interestingly, LET-418/CHD-4 binds MEP-1 (Unhavaithaya *et al.* 2002). Two cDNAs derived from the *C49F5.2/set-6* gene. The predicted SET-6 protein contains a SET lysine methyltransferase domain and is predicted to function as a histone H3 lysine 9 methyltransferase based upon its homology to the transcriptional repressor SUV39H1 (Bannister *et al.* 2001; Lachner *et al.* 2001; Nielsen *et al.* 2001) (Figure 1B).

In the second screen, the bait was the LexA DNA-binding domain fused to the LIN-1 carboxy-terminus (residues 253–441), which includes the D-domain and FQFP docking sites for ERK MAP kinase. A total of 143 cDNAs that encode proteins that bind LIN-1(253–441) specifically were identified by screening 1.3×10^6 cDNAs. Thirty-seven cDNAs encoded MPK-1 ERK MAP kinase. Seven cDNAs encoded EGL-27. EGL-27 shares 24% identity and 41% similarity over 467 amino acids with the MTA-1 component of the NuRD transcriptional repression complex (Herman *et al.* 1999). EGL-27 contains an ELM1 domain that is predicted to mediate protein–protein interactions, an ELM2 domain

that is predicted to mediate DNA binding or protein–protein interactions, a SANT DNA-binding domain, and a GATA zinc-finger domain.

Two SUMOylation motifs of LIN-1 are necessary to promote binding of RAD-26 and SET-6 in yeast cells

To define regions of LIN-1 that are sufficient to bind SET-6 and RAD-26, we analyzed fragments of LIN-1. LA:LIN-1(1–64) and LA:LIN-1(146–252) mediated robust binding of SET-6 and RAD-26, indicating that LIN-1 contains two separable binding sites for SET-6 and RAD-26 (Figure 1C). To identify amino acids necessary for binding, we analyzed mutants of LIN-1(1–64) with eight consecutive amino acids changed to alanine. Substitution of LIN-1 residues 9–16 dramatically reduced binding of SET-6 and RAD-26 by 106- and 44-fold, respectively (Figure 1D, line 3, and Figure 1F). LIN-1 contains a consensus SUMOylation motif in this region, VK₁₀KE (Sampson *et al.* 2001). Substitution of the SUMO acceptor lysine (K10A) dramatically reduced binding of SET-6 and RAD-26 by 62- and 37-fold, respectively (Figure 1D, line 11). Substitution of the highly conserved glutamic

acid (E12A) reduced binding of *SET-6* and *RAD-26* by 40- and 30-fold, respectively (Figure 1D, line 13). Substitution of the moderately conserved valine (V9A) decreased binding of *SET-6* and *RAD-26* by 6- and 5-fold, respectively, whereas mutation of the nonconserved lysine (K11A) had minimal effects (Figure 1D, lines 10 and 12). These results demonstrate a correlation between the function of each residue in the ψ KxE motif in promoting SUMOylation and in promoting binding of *SET-6* and *RAD-26*.

To investigate *SET-6* and *RAD-26* binding to *LIN-1* residues 146–252, we analyzed the *LIN-1*(156–180) fragment that contains the VK₁₆₉DE SUMOylation motif. *SET-6* and *RAD-26* robustly bound *LIN-1*(156–180) (Figure 1E). Mutations of the entire motif (168–171A) or the predicted SUMO acceptor lysine (K169A) markedly reduced binding of *LIN-1* to *SET-6* and *RAD-26* (Figure 1E). Thus, 64 residues of *LIN-1* containing the SUMOylation motif VK₁₀KE and 25 residues of *LIN-1* containing the SUMOylation motif VK₁₆₉DE were sufficient to bind *SET-6* and *RAD-26*, and for both *LIN-1* fragments the SUMO acceptor lysine was necessary for binding.

SUMOylation of *LIN-1* promotes binding of *RAD-26* and *SET-6* in yeast cells

The ψ KxE consensus SUMOylation motifs of *LIN-1* are post-translationally modified by SUMO-1 in yeast cells (Leight *et al.* 2005). If SUMOylation of the ψ KxE motifs promotes binding of *SET-6* and *RAD-26*, then the addition of SUMO-1 to a SUMOylation-defective *LIN-1* mutant might restore binding. To mimic the SUMOylated isoform of *LIN-1*, we generated a translational fusion of *C. elegans* *SMO-1* and the *LIN-1*(1–64; 9–16A) SUMOylation-defective mutant. This fusion protein functioned as efficiently as SUMOylated *LIN-1*(1–64) to repress transcription in cultured cells, indicating that this translational fusion of *SMO-1* can be functional (Leight *et al.* 2005). The interaction of *SET-6* and *RAD-26* with LA:*SMO-1*:*LIN-1*(1–64; 9–16A) was increased by 24- and 3-fold, respectively (Figure 2, lines 2 and 3; data not shown). These results indicate that *SET-6* and *RAD-26* bind SUMOylated *LIN-1*.

RAD-26* directly binds *SUMO* and *LIN-1

To determine if *LIN-1* directly binds *SET-6* and *RAD-26*, we expressed histidine- and FLAG-tagged *SET-6*(22–538) (HF:*SET-6*) and *RAD-26*(17–655) (HF:*RAD-26*) proteins in insect cells and purified these proteins by metal affinity chromatography. GST-tagged *C. elegans* *SMO-1* and *LIN-1*(1–64) were expressed in *E. coli* and purified by glutathione affinity chromatography. Binding was monitored by the GST pull-down method. HF:*SET-6* did not bind GST:*SMO-1* nor GST:*LIN-1*(1–64), indicating that additional proteins may facilitate the interaction between *SET-6* and *LIN-1* in yeast cells or the appropriate pull-down conditions were not identified. HF:*RAD-26* did bind GST:*SMO-1* and GST:*LIN-1*(1–64) specifically (Figure 3). These findings demonstrate that *RAD-26* can directly bind both *SMO-1* and *LIN-1*.

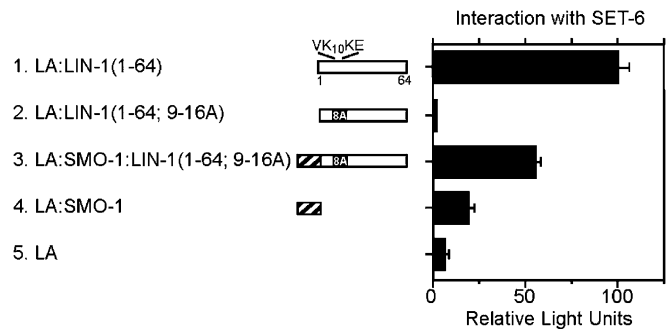


Figure 2 SUMO was sufficient to increase binding of *SET-6* to *LIN-1* in yeast cells. Associations of *SET-6* with LA fusion proteins were monitored using the yeast two-hybrid system. Bars represent the average and standard deviation of LexA-dependent β -galactosidase activity from three independent yeast transformants. Values were normalized by setting the interaction of *SET-6* with LA:*LIN-1*(1–64) to 100. The LA fusion proteins were expressed at similar levels as determined by Western blotting (data not shown).

***rad-26* functions in worms to inhibit vulval cell fates**

To investigate the possibility that these protein interactions are functionally significant in worms, we reduced the activity of *rad-26* and *set-6* and monitored vulval development in wild-type and sensitized genetic backgrounds. *rad-26* and *set-6* have been analyzed in genome-wide screens using RNA interference (RNAi), but phenotypes have not been reported (Piano *et al.* 2002; Kamath *et al.* 2003; Sonnichsen *et al.* 2005). Wild-type hermaphrodites exposed to *rad-26* dsRNA did not display a *Muv* phenotype (Table 1, line 15). The *rad-26(tm1991)* allele is deleted for exon 6 and a portion of exon 7 and is predicted to encode a truncated protein of 412 amino acids that lacks half of the ATPase domain and the entire helicase domain, suggesting that the mutant protein has reduced activity. *rad-26(tm1991)* mutants grown at 15° and 20° did not display a *Muv* phenotype (Table 1, lines 2 and 6).

To analyze interactions of *rad-26* with the EGFR/Ras/MAPK pathway, we utilized sensitized genetic backgrounds. *let-60(n1046gf)* is a gain-of-function mutation that causes a temperature-sensitive *Muv* phenotype (Beitel *et al.* 1990). When *let-60(n1046gf)* hermaphrodites were fed *rad-26* dsRNA, the penetrance of the *Muv* phenotype increased significantly from 16 to 85% (Table 1, line 17), indicating that *rad-26* inhibits vulval cell fates. *mpk-1(oz140)* is a partial loss-of-function mutation that affects ERK MAP kinase and inhibits vulval induction: *rad-26(tm1991)* did not cause a *Muv* phenotype in this genetic background (Table 1, line 11). *lin-1(e1275 R175Opal)* is a partial loss-of-function mutation that causes a temperature-sensitive *Muv* phenotype. A double mutant with *rad-26(tm1991)* increased the penetrance of the *lin-1(e1275)* *Muv* phenotype significantly from 41 to 89% (Table 1, line 4). The *lin-1* *Muv* phenotype is caused by a transformation of the cell fates of P3.p, P4.p, and P8.p from nonvulval to vulval (Ferguson *et al.* 1987). Nomarski optics were used to confirm that the *lin-1(e1275)*

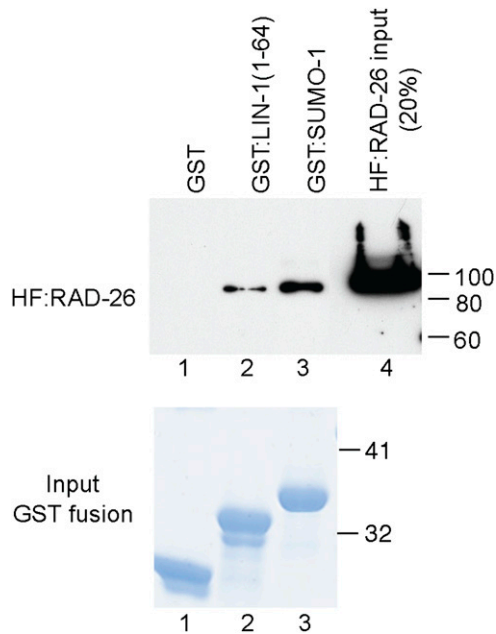


Figure 3 RAD-26 binds SMO-1 and LIN-1 in purified extracts. HF:RAD-26 (17–655) was incubated with equivalent amounts of glutathione agarose-bound GST, GST:LIN-1(1–64), or GST:SMO-1. Bound HF:RAD-26 was visualized by immunoblotting using α -FLAG antibody (top). Lane 4 shows 20% of the HF:RAD-26 input. HF:RAD-26 did not bind GST alone as assessed by prolonged exposure of the immunoblot. Input GST fusion proteins were visualized by SDS-PAGE and Simply Blue staining (bottom). Molecular weight markers (in kDa) are indicated.

rad-26(tm1991) double-mutant animals displayed ectopic vulval cell fates (Figure 4, A and B). These results demonstrate that *rad-26* functions to inhibit vulval cell fates and are consistent with the model that *rad-26* is positioned in the EGFR/Ras/MAPK pathway or a parallel signaling pathway.

To investigate the position of the *rad-26* gene in the Ras pathway, we analyzed a *lin-1* gain-of-function mutation. *lin-1(n1790gf R352Opa1)* causes a weak vulvaless phenotype and partially suppresses the *Muv* phenotype caused by activated *let-60 ras*; the LIN-1(1–351) protein lacks the FQFP ERK docking site and is partially resistant to negative regulation by MPK-1 ERK (Jacobs *et al.* 1999). The *lin-1(n1790gf)* allele also causes a low penetrance *Muv* phenotype because the *lin-1* mRNA contains a premature stop codon and is subject to nonsense-mediated decay. If *rad-26* functions upstream of *lin-1* in a linear signaling pathway, then we predict that the *rad-26* mutation will not significantly affect the *lin-1(n1790)* phenotype. By contrast, if *rad-26* functions downstream or at the level of *lin-1*, then we predict that the *rad-26* mutation will promote a *Muv* phenotype in the *lin-1(n1790)* background. Specifically, if RAD-26 is recruited to SUMOylated LIN-1(1–351) and contributes to LIN-1 function, then removing the RAD-26 protein is predicted to impair the function of LIN-1(1–351) as a constitutive inhibitor of vulval cell fates, resulting in a *Muv* phenotype. The *lin-1(n1790gf rad-26(tm1991))* double-mutant hermaphrodites displayed a *Muv* phenotype that was 29% penetrant, significantly

Table 1 Genetic analysis of *rad-26*

Genotype ^a	RNAi ^b	% <i>Muv</i> ^c	<i>n</i> ^d
1. Wild type ^e	NA	0	1702
2. <i>rad-26(tm1991)</i> ^e	NA	0.1	1424
3. <i>lin-1(e1275)</i> ^e	NA	41	2271
4. <i>lin-1(e1275 rad-26(tm1991))</i> ^e	NA	89	1238
5. Wild type	NA	0	1002
6. <i>rad-26(tm1991)</i>	NA	0	308
7. <i>lin-1(n1790)</i>	NA	8	840
8. <i>lin-1(n1790 rad-26(tm1991))</i>	NA	29	1592
9. <i>rad-26(tm1991); set-6(ok2195)</i>	NA	0	2345
10. <i>egl-27(we3); rad-26(tm1991)</i>	NA	0	1678
11. <i>mpk-1(oz140); rad-26(tm1991)</i>	NA	0	1596
12. <i>rad-26(tm1911); lin15A(n767)</i>	NA	0	1843
13. <i>rad-26(tm1911); lin-15B(n744)</i>	NA	0	1688
14. Wild type	Control	0	1556
15. Wild type	<i>rad-26</i>	0	1787
16. <i>let-60(n1046)</i> ^e	Control	16	375
17. <i>let-60(n1046)</i> ^e	<i>rad-26</i>	85	932
18. <i>lin-1(n1790)</i>	Control	3	153
19. <i>lin-1(n1790)</i>	<i>rad-26</i>	11	262

^a To facilitate comparisons, the following experiments (indicated by line numbers) were conducted in parallel: 1–4, 5–8, 14–15, 16–17, and 18–19. The difference between the percentage of the *lin-1(n1790)* *Muv* phenotype in line 7 and line 18 might reflect day-to-day variability or the difference between control RNAi bacteria and standard OP50 bacteria as a food source.

^b L4 hermaphrodites were fed *E. coli* containing a control plasmid or a plasmid that expresses dsRNA from the *rad-26* gene, and the vulval phenotype of the progeny that grew on these dishes was scored. NA, not applicable.

^c Adult hermaphrodites were scored as *Muv* if they displayed one or more ventral protrusions displaced from the site of the vulva. Hermaphrodites were propagated at 20° unless otherwise indicated. The χ^2 test was used to compare two values. The following comparisons (indicated by line numbers) were significantly different ($P < 0.05$): 1 and 3, 2 and 4, 3 and 4, 5 and 7, 6 and 8, 7 and 8, 16 and 17, 18 and 19.

^d *n*, number of hermaphrodites examined.

^e Hermaphrodites were propagated at 15° because *lin-1(e1275)* and *let-60(n1046)* cause heat-sensitive *Muv* phenotypes.

greater than the *Muv* phenotype of *n1790* and *tm1991* single-mutant animals (Table 1, line 8). Consistent with this observation, *lin-1(n1790gf)* hermaphrodites fed *rad-26* dsRNA displayed a weak but significant enhancement of the *Muv* phenotype (Table 1, line 19). These findings indicate that the LIN-1(1–351) protein may require *rad-26* function to inhibit vulval cell fates efficiently.

To analyze the function of *set-6*, we reduced the activity of *set-6* by feeding dsRNA. Wild-type hermaphrodites exposed to *set-6* dsRNA did not display obvious vulval defects (data not shown). The *set-6(tm1611)* and *set-6(ok2195)* deletion mutations did not cause an independent *Muv* phenotype (data not shown). By contrast to *rad-26*, *set-6(lf)* mutations did not appear to interact with the EGFR/Ras/MAPK pathway. The *Muv* phenotype of *lin-1(e1275); set-6(ok2195)* double-mutant animals was similar to single-mutant *lin-1(e1275)* animals (data not shown). The *Muv* phenotype of *let-60(n1046); set-6(tm1611)* and *let-60(n1046); set-6(ok2195)* double-mutant animals was similar to that of *let-60(n1046)* single-mutant animals (data not shown). These findings indicate that *set-6(lf)* mutations do not interact strongly with mutations that affect the EGFR/Ras/MAPK pathway. *set-6(ok2195)* did not cause a *Muv* phenotype in combination with *rad-26(tm1991)*

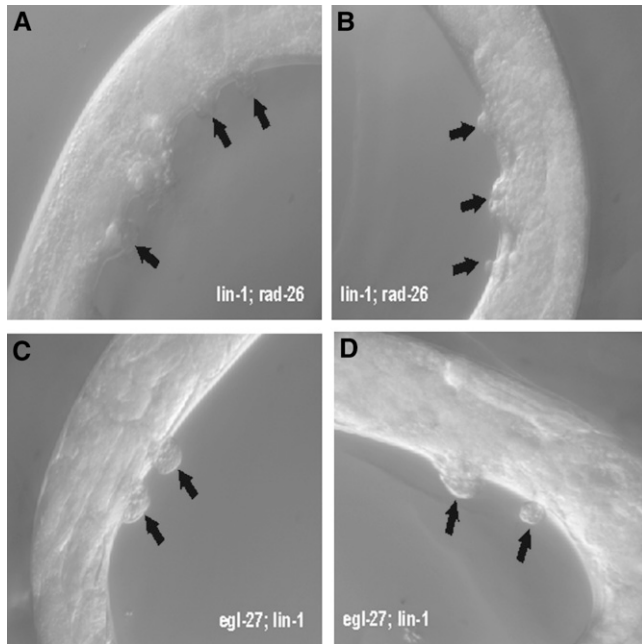


Figure 4 Ectopic vulval protrusions in *rad-26* and *egl-27* mutant animals. (A–D) DIC images of the mid-body region. The genotypes are *lin-1(e1275) rad-26(tm1991)* (A and B) and *egl-27(we3); lin-1(n1790)* (C and D). Arrows mark protruding ectopic vulval tissue. Magnification is $\times 400$.

(Table 1, line 9) or *egl-27(we3)* (Table 2, line 8). These results do not provide evidence that *set-6* plays a significant functional role in *lin-1*-mediated inhibition of vulval cell fates and raise the possibility that the interaction between *SET-6* and *LIN-1* proteins may reflect a role in a different tissue.

The synthetic multivulva (synMuv) pathway is composed of two major groups of genes called class A and class B (Ferguson and Horvitz 1989). Loss-of-function mutations in a single class A gene or a single class B gene do not cause an independent *Muv* phenotype, but a double-mutant animal containing any class A mutation and any class B mutation displays a strong *Muv* phenotype. Thus, the class A and class B synMuv genes appear to function redundantly to inhibit vulval cell fates. The synMuv genes appear to function non-cell-autonomously in the *hyp7* hypodermal cell to inhibit the expression and secretion of the *lin-3* ligand; in class AB double-mutant animals, ectopic secretion of *lin-3* causes the *Muv* phenotype (Herman and Hedgecock 1990; Cui *et al.* 2006). Several genes associated with the NURD complex have been proposed to function in the synMuv pathway, including *let-418* and *egr-1/lin-40* (Solari and Ahringer 2000; von Zelewsky *et al.* 2000). Andersen and Horvitz (2007) analyzed *set-6* for a role in the synMuv pathway; the *set-6(tm1611)* deletion allele did not cause a *Muv* phenotype in combination the class A synMuv gene *lin-15A* or the class B synMuv gene *lin-15B*, indicating that *set-6* is not a synMuv gene. To investigate the possibility that *rad-26* is a synMuv gene, we generated double-mutant animals with *lin-15A* or *lin-15B* mutations. *rad-26(tm1991)* did not cause a *Muv* phenotype in combination with either class A or class

Table 2 Genetic analysis of *egl-27*

Genotype ^a	RNAi	% <i>Muv</i> ^b	<i>n</i>
1. Wild type	NA	0	1002
2. <i>egl-27(n170)</i>	NA	1	954
3. <i>egl-27(we3)</i>	NA	1	594
4. <i>lin-1(n1790)</i>	NA	8	840
5. <i>egl-27(we3); lin-1(n1790)</i>	NA	21	438
6. <i>egl-27(we3); lin-15A(n767)</i>	NA	0	1831
7. <i>egl-27(we3); lin-15B(n744)</i>	NA	0	1639
8. <i>egl-27(we3); set-6(ok2195)</i>	NA	0	1522
9. Wild type	Control	0	1134
10. Wild type	<i>egl-27</i>	1	851
11. <i>let-60(n1046)</i> ^c	Control	27	1466
12. <i>let-60(n1046)</i> ^c	<i>egl-27</i>	72	363
13. <i>lin-1(n1790)</i>	Control	3	524
14. <i>lin-1(n1790)</i>	<i>egl-27</i>	28	200

Columns are as described in Table 1.

^a To facilitate comparisons, the following experiments (indicated by line numbers) were conducted in parallel; 4–5, 9–10, 11–12, and 13–14. The difference between the percentage of the *let-60(n1046)* *Muv* phenotype shown in line 11 compared to that in line 16 of Table 1 may reflect day-to-day variability in temperature or humidity.

^b The χ^2 test was used to compare two values. The following comparisons were significantly different ($P < 0.05$): 1 and 2, 1 and 3, 1 and 4, 3 and 5, 4 and 5, 9 and 10, 11 and 12, and 13 and 14.

^c Hermaphrodites were propagated and scored at 15°.

B synMuv mutations, indicating that *rad-26* is not a synMuv gene (Table 1, lines 12 and 13).

EGL-27 binds two domains of LIN-1 that are separable from the SUMOylation motifs and ERK-docking sites

EGL-27 was identified based on an interaction with *LIN-1* (253–441), which contains the carboxy-terminus. To characterize the interaction with *EGL-27*, we used the yeast two-hybrid system to define domains of *LIN-1* that are necessary and sufficient to mediate binding. We mutagenized one or both of the ERK-docking sites and monitored the association of these *LIN-1* derivatives with *EGL-27* and *MPK-1*. *MPK-1* displayed reduced binding to LA:*LIN-1* (253–441; mut. D) and LA:*LIN-1* (253–441; mut. F) and failed to bind LA:*LIN-1* (253–441; mut. D/F) (Figure 5B, lines 2–4). By contrast, *EGL-27* efficiently bound each of these *LIN-1* derivatives, indicating that the ERK-docking sites in *LIN-1* are not necessary for *EGL-27* binding.

To define regions of *LIN-1* that are sufficient to bind *EGL-27*, we monitored the association of *EGL-27* to fragments of *LIN-1*. These studies demonstrated that *EGL-27* binds two domains in *LIN-1* contained in residues 296–341 and residues 386–441 (Figure 5B, lines 6 and 8).

To determine if *EGL-27* can directly bind *LIN-1*, histidine- and T7-tagged *EGL-27* (691–1119) were expressed in *E. coli*, purified using metal affinity chromatography, and monitored for binding to purified GST:*LIN-1* (281–441) using a GST pull-down assay. *EGL-27* reproducibly bound GST:*LIN-1* (281–441) more efficiently than GST:*LIN-1* (1–64) or GST alone (Figure 5C), demonstrating that *EGL-27* can directly bind the carboxy-terminus of *LIN-1*.

To further characterize the function of *EGL-27*, we used the yeast two-hybrid system to identify *C. elegans* proteins

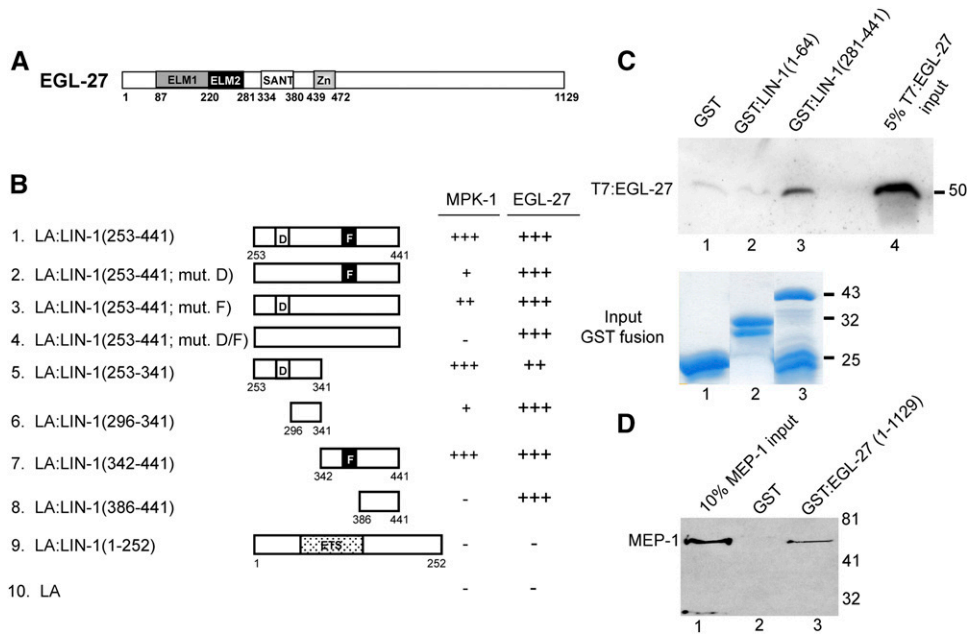


Figure 5 LIN-1 binds EGL-27 in yeast cells and purified extracts. (A) EGL-27 protein (Solari *et al.* 1999) contains ELM1 (dark shading), ELM2 (solid), SANT (open), and GATA-like zinc-finger (light shading) domains. (B) The interactions between LA:LIN-1 proteins and MPK-1(1-444) and EGL-27(691-1119) fused to the GAL4 activation domain were measured. Symbols indicate robust (+++), moderate (++), weak (+), and no detectable (-) activation of a LexA-dependent *lacZ* reporter gene. (C) 6xHis- and T7-tagged EGL-27(691-1119) (T7:EGL-27) was incubated with equivalent amounts of glutathione agarose-bound GST, GST:LIN-1(1-64) or GST:LIN-1(281-441). Bound T7:EGL-27 was visualized by immunoblotting using α -T7 antibody (top). Lane 4 shows 5% of the T7:EGL-27 input. Input GST fusion proteins were visualized by SDS-PAGE and Simply Blue staining (bottom). Lower-molecular-weight proteins in lanes 2 and 3 represent degradation

products. (D) 6xHis-tagged MEP-1 (MEP-1) was incubated with equivalent amounts of glutathione agarose-bound GST or GST:EGL-27(1-1129). Bound MEP-1 was visualized by immunoblotting using anti-HIS antibody. Lane 1 shows 10% of the MEP-1 input.

that interact with EGL-27. EGL-27(1-1129) was used to screen 3.4×10^6 cDNAs, and eight positive cDNAs were identified; three of these cDNAs corresponded to MEP-1. EGL-27(629-1129) was used to screen 6.9×10^4 cDNAs, and 49 positive cDNAs were identified; 2 of these cDNAs corresponded to UBC-9. MEP-1 and UBC-9 were previously demonstrated to interact with LIN-1 and to function to inhibit vulval cell fates during development (Leight *et al.* 2005). A fragment of MEP-1-containing residues 364-870, which includes all seven zinc-finger domains, was sufficient to interact with full-length EGL-27 in yeast. In addition, MEP-1 residues 330-870 were sufficient to interact with the amino-terminal 637 residues of EGL-27 in yeast (data not shown). The interaction of MEP-1 with full-length EGL-27 was demonstrated to be a direct interaction by a GST-pull-down assay (Figure 5D).

***egl-27* inhibits vulval cell fates**

egl-27 loss-of-function mutations were first identified based on egg-laying defects (Trent *et al.* 1983). *egl-27* mutations also cause defects in TL and TR cell polarity, migration of HSN cells and QL neuroblast descendants (Herman *et al.* 1999), and embryonic patterning (Solari *et al.* 1999). *egl-27* has been demonstrated to inhibit vulval cell fates since *egl-27* RNAi was reported to enhance the *Muv* phenotype of *lin-37(n758) egr-1(RNAi)* animals from 21 to 50% (Solari and Ahringer 2000). *egl-27(n170)* was reported to enhance the *let-60(n1046) Muv* phenotype, increasing the percentage of induced P3.p cells from 47 to 79%, induced P4.p cells from 46 to 74%, and induced P8.p cells from 28 to 62% (Chen and Han 2001a). We hypothesized that LIN-1 recruits EGL-27 to repress transcription of vulval cell-fate genes. To test this

prediction, we reduced the function of *egl-27* using loss-of-function mutations or RNAi and monitored vulval development in different genetic backgrounds. *egl-27(we3)* is a nonsense mutation that results in a truncated protein of 811 amino acids (Solari *et al.* 1999). *egl-27(n170)* is a deletion of at least the first eight exons of *egl-27* and affects the full-length transcript, but not the smaller transcripts (Herman *et al.* 1999). Both *egl-27(n170)* and *egl-27(we3)* hermaphrodites displayed a very low penetrance *Muv* phenotype of 1% (Table 2, lines 2 and 3). Likewise, 1% of wild-type hermaphrodites exposed to *egl-27* RNAi displayed a *Muv* phenotype (Table 2, line 10). These results indicate that *egl-27* functions to inhibit vulval cell fates, but the effect is difficult to measure in an otherwise wild-type background.

To investigate the possibility that *egl-27* functions in the synMuv pathway, we generated double-mutant animals containing *egl-27(we3)* and a mutation of the class A synMuv gene *lin-15A* or the class B synMuv gene *lin-15B*. These double-mutant animals did not display a *Muv* phenotype, indicating that *egl-27* is not a synMuv gene (Table 2, lines 6 and 7). This is similar to a previous report showing that *egl-27* RNAi did not cause a *Muv* phenotype in combination with *lin-15A* and caused only a low-penetrance *Muv* phenotype (5%) in combination with the synMuv B gene *lin-37* (Solari and Ahringer 2000).

To investigate the possibility that *egl-27* functions in the EGFR/Ras/MAPK pathway, we examined *egl-27* function in sensitized genetic backgrounds. The penetrance of the *let-60(n1046gf) Muv* phenotype was significantly increased from 27 to 72% in hermaphrodites fed *egl-27* dsRNA (Table 2, line 12), consistent with previous studies demonstrating that *egl-27(n170)* enhanced the frequency of vulval cell

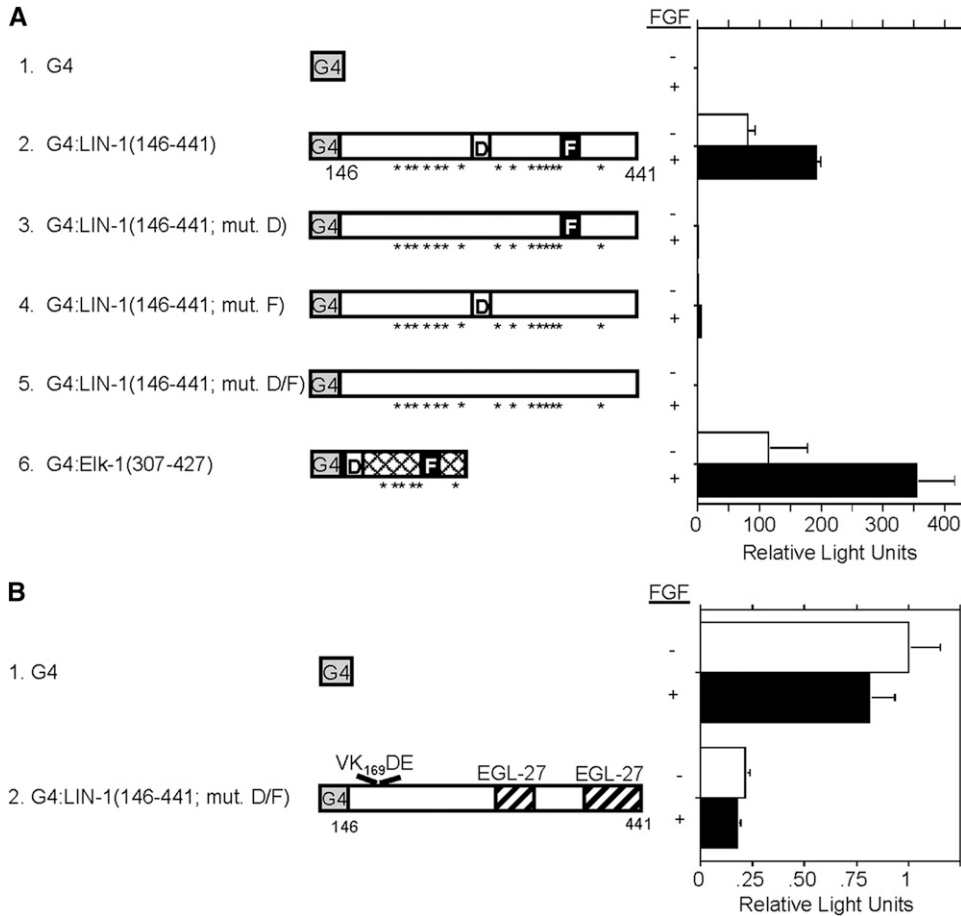


Figure 6 The carboxy-terminus of LIN-1 contains an ERK-dependent transcriptional activation domain and an ERK-independent repression domain. MC3T3 murine fibroblast cells were transfected with the following: the pFR-LUC reporter plasmid that contains five tandem GAL4-binding sites upstream of a basal promoter that regulates expression of luciferase; an expression plasmid that encodes GAL4 DNA-binding domain (G4) alone or fused to the indicated fragment of LIN-1 or Elk-1; and a reporter plasmid that encodes β -galactosidase to measure transfection efficiency. Cells were untreated (-) or treated with basic fibroblast growth factor (FGF) to activate ERK (+). (A) The schematics illustrate the ERK MAP kinase docking sites, the D-domain (D) and the FQFP motif (F). Asterisks indicate 15 S/TP motifs in LIN-1 that are potential ERK phosphorylation sites and 6 S/TP motifs in Elk-1 that are phosphorylated by ERK (Cruzalegui *et al.* 1999). Bars indicate luciferase activity divided by β -galactosidase activity. Values were normalized by setting the value for G4 alone in the absence of FGF to 1.0. Values represent the average and standard deviation of two to five independent transfections conducted in parallel. (B) The schematic of LIN-1 illustrates the VK₁₆₉DE consensus SUMOylation motif (top) and two domains that are sufficient to bind EGL-27 (hatched).

fates of P3.p, P4.p, and P8.p (Chen and Han 2001a). The *egl-27(we3); lin-1(n1790gf)* double-mutant animals displayed a *Muv* phenotype that was 21% penetrant, significantly greater than the *Muv* phenotype of *n1790* and *we3* single mutants (Table 2, line 5). An analysis using DIC optics confirmed that these double-mutant animals displayed ectopic protrusions of vulval tissue (Figure 4, C and D). Likewise, 28% of *lin-1(n1790gf)* hermaphrodites fed *egl-27* dsRNA displayed a *Muv* phenotype, which was significantly higher than the 3% of *lin-1(n1790gf)* hermaphrodites fed control bacteria that displayed a *Muv* phenotype (Table 2, line 14). Thus, two independent methods of reducing *egl-27* function promoted vulval cell fates in the presence of *lin-1(n1790)*, indicating that this is a robust result although the effect is not fully penetrant. These results are consistent with the model that EGL-27 may promote the ability of gain-of-function LIN-1(1–351) protein to inhibit vulval cell fates. *egl-27(we3)* did not cause a *Muv* phenotype in combination with *rad-26(tm1991)* (Table 1, line 10) or *set-6(ok2195)* (Table 2, line 8).

Carboxy-terminus of LIN-1 functions as an ERK-dependent transcriptional activation domain and an ERK-independent transcriptional repression domain

To analyze how phosphorylation by ERK regulates LIN-1, we analyzed transcriptional activity of the carboxy-terminus of

LIN-1. Fragments of LIN-1 fused to the GAL4 DNA-binding domain were expressed in the MC3T3 murine fibroblast cell line, and transcription of a GAL4-dependent reporter gene was monitored. To increase ERK activity, we treated cells with bFGF. To decrease the ability of ERK to phosphorylate LIN-1, we mutated the ERK-docking sites (Jacobs *et al.* 1998, 1999). LIN-1(146–441) functioned as a potent activator of transcription, activating transcription by 81-fold relative to the G4 only control (Figure 6A, line 2). Treatment of cells with bFGF resulted in a twofold increase in the transcriptional activation potential of G4:LIN-1(146–441), suggesting that phosphorylation of LIN-1 by ERK promotes transcriptional activation. The activity of the LIN-1 carboxy-terminus in this assay is similar to the activity of the Elk-1 carboxy-terminus (Figure 6A, line 6). One possible explanation for the relatively small effect of adding bFGF is that ERK is already highly active under the standard culture conditions. This is likely since the cells were cultured with 10% fetal calf serum that contains a variety of growth factors that activate ERK.

To address the possibility that LIN-1 is highly phosphorylated by ERK under standard culture conditions, we investigated the effect of reducing phosphorylation of LIN-1 by mutating the docking sites for ERK. LIN-1 contains two docking sites for ERK, and we mutated only the D-domain,

G4:LIN-1(146–441, mut. D); only the FQFP motif, G4:LIN-1(146–441, mut. F); and both the D-domain and the FQFP motif, G4:LIN-1(146–441, mut. D/F). Compared to wild-type LIN-1, these mutations reduced transcriptional activity by 251-, 75-, and 380-fold, respectively. These results indicate that, under standard culture conditions, binding of ERK to the D-domain and FQFP motif of LIN-1 and phosphorylation of LIN-1 dramatically increases the transcriptional activation potential of the carboxy-terminus of LIN-1.

These experiments allowed us to measure the transcriptional activity of the LIN-1 carboxy-terminus in the absence of ERK phosphorylation. The LIN-1(146–441) fragment contains one consensus SUMOylation motif and two binding sites for EGL-27. In the absence of ERK-docking sites, this fragment of LIN-1 repressed transcription by four- to sevenfold relative to G4 alone (Figure 6B, line 2). These results are consistent with the model that SUMOylation of LIN-1 and/or EGL-27 binding to the carboxy-terminus of LIN-1 mediates transcriptional repression and that phosphorylation by ERK converts LIN-1 to a transcriptional activator (Figure 7).

Discussion

SUMOylated LIN-1 recruits a nucleosome-remodeling enzyme to inhibit vulval cell fates

Genetic analyses demonstrate that *lin-1* inhibits Pn.p cells from adopting vulval cell fates (Sulston and Horvitz 1981; Beitel *et al.* 1995; Jacobs *et al.* 1998). DNA binding and SUMOylation are necessary for this LIN-1 activity, suggesting that SUMOylated LIN-1 represses transcription of vulval cell-fate genes (Miley *et al.* 2004; Leight *et al.* 2005). Here we define a novel mechanism for transcriptional repression by SUMOylated LIN-1, recruitment of RAD-26.

We discovered RAD-26 as a LIN-1-interacting protein in yeast cells. SUMOylation of LIN-1 was necessary and sufficient to promote binding of RAD-26 in yeast cells. Furthermore, RAD-26 directly binds both LIN-1 and SUMO in purified extracts. These observations suggest that RAD-26 binds SUMOylated LIN-1 in worms; however, this biochemical interaction in worms has not been tested directly. Our genetic analyses demonstrated that *rad-26* inhibits vulval cell fates. *rad-26* interacted genetically with multiple genes in the Ras-signaling pathway that function in P6.p, and *rad-26* did not cause a *Muv* phenotype in combination with class A or class B synMuv genes that function in *hyp7*. These genetic results are consistent with the model that *rad-26* functions in the EGFR/Ras/MAPK-signaling pathway or that *rad-26* functions in parallel to this signaling pathway, and they do not establish the cellular site of action of *rad-26*. Together, these biochemical and genetic results are consistent with the model that RAD-26 binding to LIN-1 promotes the ability of LIN-1 to repress primary vulval cell fates. Although reducing the activity of *rad-26* or *lin-1* causes ectopic vulval cell fates, the frequency of ectopic vulval cell fates is

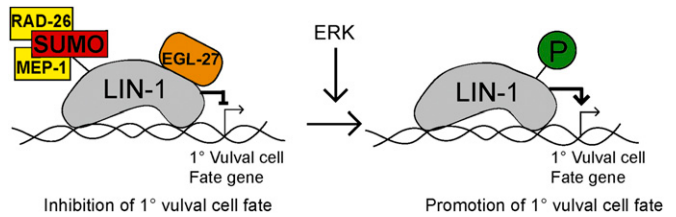


Figure 7 ERK converts LIN-1 from a SUMOylated transcriptional repressor to a phosphorylated transcriptional activator. A model for the regulation of vulval cell fates by LIN-1. LIN-1 represses 1° vulval cell-fate genes by recruiting RAD-26 and MEP-1 to its SUMOylated amino-terminus and EGL-27 to its unSUMOylated carboxy-terminus. RAD-26 remodels nucleosomes, and EGL-27 and MEP-1 facilitate histone deacetylation through their association with HDAC-1 and HDAC-2. Following activation of the EGFR/Ras/MAPK pathway in P6.p, ERK converts LIN-1 to a phosphorylated transcriptional activator of 1° vulval cell-fate genes.

higher when *lin-1* activity is reduced. This observation suggests that *lin-1* retains some ability to inhibit vulval cell fates in the absence of *rad-26*.

RAD-26 is a homolog of vertebrate Mi-2 β /CHD4, a core component of the NuRD transcriptional repression complex. This complex possesses ATP-dependent nucleosome-remodeling activity that is dependent upon Mi-2 β /CHD4 and histone deacetylase (HDAC) activity provided by HDAC-1 and HDAC-2; both of these activities promote transcriptional silencing (Tong *et al.* 1998; Wade *et al.* 1998; Xue *et al.* 1998; Zhang *et al.* 1998; Wang and Zhang 2001). Multiple components of the *C. elegans* NuRD complex have been shown to inhibit vulval cell fates, including CHD-3 and LET-418 that are homologs of vertebrate Mi-2 β /CHD4 (Lu and Horvitz 1998; Solari and Ahringer 2000; von Zelewsky *et al.* 2000; Chen and Han 2001a,b). Guerry *et al.* (2007) showed that LET-418 interacts with LIN-1 to repress transcription of the *lin-39* target gene during vulval induction, although the role of SUMOylation was not described. Thus, LIN-1 interacts with two different homologs of vertebrate Mi-2: RAD-26 and LET-418. One possible explanation for the relatively mild vulval defects caused by *rad-26(lf)* mutations is that one or more genes function redundantly with *rad-26*. In particular, *chd-3* and *let-418* are highly similar to *rad-26*, and von Zelewsky *et al.* (2000) showed that *let-418* and *chd-3* function redundantly during vulval development. An important goal of future studies is to determine the unique and overlapping activities of *rad-26*, *let-418*, and *chd-3*. Together, these studies indicate that recruitment of the NuRD complex by LIN-1, and perhaps by additional transcription factors, is critical for inhibition of vulval cell fates.

The identification of RAD-26 provides important new insights into the mechanisms by which SUMOylated LIN-1 mediates transcriptional repression, since this protein has not been previously reported to interact with LIN-1 or contribute to vulval cell-fate specification. Furthermore, these findings have implications for other transcription factors. Over 30 transcription factors, including 7 ETS proteins, have been demonstrated to be post-translationally modified by SUMO-1 (Gill 2003; Hay 2005). In the majority of these cases, SUMOylation promotes repression of transcription.

A critical question in this field is: How does SUMOylation mediate transcriptional repression? SUMOylation of p300 and Elk-1 promotes an association with histone HDACs that repress transcription (Girdwood *et al.* 2003; Yang and Sharrocks 2004). However, these HDACs were not shown to directly bind the SUMO moieties. Furthermore, the HDAC inhibitor trichostatin A (TSA) blocks repression at some, but not all, promoters (Yang *et al.* 2002). Thus, SUMO is likely to mediate transcriptional repression by additional, undefined mechanisms. In *Drosophila* cells, Mi-2, MEP-1, and a polycomb protein are important for SUMO-dependent transcriptional repression (Stielow *et al.* 2007). Our studies define a new mechanism by which SUMO mediates transcriptional repression: recruitment of a nucleosome-remodeling enzyme. Given that the NuRD complex possesses both nucleosome-remodeling and histone deacetylase activities, the direct binding of SUMO-1 to RAD-26/Mi-2 β may account for the SUMO-1-mediated recruitment of HDAC activity observed in vertebrates (Girdwood *et al.* 2003; Yang and Sharrocks 2004).

EGL-27/MTA1 component of the NuRD complex binds LIN-1 independently of LIN-1 SUMOylation and inhibits vulval cell fates

We discovered EGL-27/MTA1 as a LIN-1-interacting protein in yeast cells. EGL-27 binds two domains in the carboxy-terminus of LIN-1 in yeast cells, and binding is independent of SUMOylation. Furthermore, EGL-27 directly binds LIN-1 in purified extracts. These results suggest that EGL-27 binds LIN-1 in worms; however, this biochemical interaction in worms has not been analyzed directly. The EGL-27 expression pattern was determined using an EGL-27::GFP reporter gene by Solari *et al.* (1999) and Herman *et al.* (1999). EGL-27 is expressed in somatic nuclei of most or all cells from the 50-cell stage of embryogenesis through adulthood. The results indicate that EGL-27 is expressed in the vulval precursor cells, which is consistent with the model that it interacts with LIN-1. EGL-27 is a homolog of the MTA1 component of the NuRD transcriptional repression complex, and we hypothesize that recruitment of EGL-27 to LIN-1 mediates transcriptional repression of vulval cell-fate genes. Consistent with this hypothesis, previous genetic studies showed that *egl-27* inhibits vulval cell fates (Solari and Ahringer 2000; Chen and Han, 2001a). Our genetic analyses extend these findings by providing evidence that *egl-27* interacts with multiple genes in the EGFR/Ras/MAPK-signaling pathway that function in Pn.p cells, including *lin-1*. *egl-27* did not cause a *Muv* phenotype in combination with class A or class B synMuv genes that function in *hyp7*. These genetic results are consistent with the model that *egl-27* functions in the Ras-signaling pathway or that *egl-27* functions in parallel to the Ras-signaling pathway, and they do not establish the cellular site of action of *egl-27*. The *Muv* phenotype caused by reducing the activity of *egl-27* is not as strong as the *Muv* phenotype caused by reducing the activity of *lin-1*, suggesting that *lin-1* retains some function to inhibit vulval cell fates in the absence of *egl-27*. One possible explanation for the relatively

mild vulval defects caused by *egl-27(lf)* mutations is that one or more genes function redundantly with *egl-27*. In particular, *lin-40/egr-1* is highly similar to *egl-27*. Notably, Chen and Han (2001a,b) showed that *lin-40* negatively regulates vulval cell-fate specification, and Solari *et al.* (1999) showed that *egl-27* and *lin-40/egr-1* function redundantly during embryonic patterning. An important goal of future studies is to determine the unique and overlapping activities of *egl-27* and *lin-40*.

By searching for proteins that interact with EGL-27, we demonstrated that EGL-27 could interact with UBC-9 and MEP-1. UBC-9 is an E2 SUMO-conjugating enzyme that binds consensus SUMOylation motifs and mediates the covalent attachment of SUMO-1. EGL-27 contains five consensus SUMOylation motifs, including four in the EGL-27(629–1129) fragment that binds UBC-9, suggesting that EGL-27 may be SUMOylated. MEP-1 is a component of the NURD transcriptional repression complex (Unhavaithaya *et al.* 2002). We previously demonstrated that MEP-1 interacts with the SUMOylated N terminus of LIN-1 and functions to inhibit vulval cell fates (Leight *et al.* 2005). This interaction of MEP-1 with SUMO may be conserved since *Drosophila* MEP-1 mediates SUMO-dependent transcriptional repression (Stielow *et al.* 2007). These observations suggest that MEP-1 may be recruited by two mechanisms: directly by binding to the SUMOylated N terminus of LIN-1 and indirectly by binding to EGL-27.

Our studies demonstrate that LIN-1 binds multiple proteins that are predicted to function to repress transcription: RAD-26 and MEP-1 bind two SUMOylation motifs in the amino-terminus of LIN-1, and EGL-27 binds two domains in the carboxy-terminus of LIN-1. LIN-1 also binds the LIN-31 transcription factor (Tan *et al.* 1998) and LET-418 (Guerry *et al.* 2007). If these proteins function independently to modify chromatin and repress transcription, then elimination of one component might impair, but not abrogate, the function of the complex, resulting in a weak *Muv* phenotype. Mutations of *rad-26*, *egl-27*, and *mep-1* all cause weak *Muv* phenotypes, consistent with the model that LIN-1 employs multiple independent mechanisms to repress transcription.

Phosphorylation of LIN-1 by ERK converts LIN-1 from a transcriptional repressor to a transcriptional activator

We examined how ERK affects the transcriptional activity of LIN-1 to determine if phosphorylation by ERK abrogates LIN-1 activity or converts LIN-1 to a transcriptional activator. If LIN-1 cannot be phosphorylated by ERK because the docking sites for ERK are mutated, then the carboxy-terminus of LIN-1 repressed transcription by about fivefold. By contrast, the carboxy-terminus of LIN-1 activated transcription by ~80-fold when ERK-docking sites were intact. Stimulating ERK activity with bFGF caused a further 2-fold increase in transcriptional activation. Overall, the difference between transcriptional activation by LIN-1 that cannot be phosphorylated and LIN-1 that is maximally phosphorylated is ~900-fold. The dramatic ERK-responsive transcriptional activation

potential of the carboxy-terminus of LIN-1 strongly supports the model that ERK switches LIN-1 from a transcriptional repressor to a phosphorylated transcriptional activator.

These results extend the similarities between LIN-1 and vertebrate Elk-1, documenting the relevance of LIN-1 as a model for understanding vertebrate biology. Elk-1 was initially characterized as a ternary complex factor that binds the serum response element present in the promoters of immediate early genes, such as *c-fos* (Treisman 1994). Upon activation of the RTK/Ras/ERK pathway, ERK phosphorylates multiple sites in the carboxy-terminus of Elk-1, resulting in potent transcriptional activation of immediate early genes (Janknecht *et al.* 1993; Marais *et al.* 1993; Cruzalegui *et al.* 1999). By contrast, studies of the *Drosophila* ETS protein Aop/Yan indicated that, in the absence of activated ERK, Aop/Yan functions as an inhibitor of neuronal cell fates by mediating transcriptional repression (O'Neill *et al.* 1994). ERK-mediated phosphorylation of Aop/Yan results in its nuclear export and degradation, thus abrogating the repressor activity of Aop/Yan and allowing cells to adopt neuronal cell fates (Rebay and Rubin 1995). Our results indicate that LIN-1 regulation is different from that of Aop/Yan.

These molecular studies of LIN-1 provide a unifying hypothesis that explains diverse genetic observations of *lin-1*. In two instances where the EGFR/Ras/MAPK pathway functions during early development, *lin-1* appears to be a positive mediator of this pathway. Howard and Sundaram (2002) showed that *lin-1(lf)* mutations could cause a larval lethal phenotype similar to loss-of-function mutations in positive regulators of EGFR/Ras/MAPK signaling, suggesting that *lin-1* is a positive mediator of this signaling pathway during larval development. Jiang and Wu (2014) recently reported that the *lin-3*, *let-23*, *let-60*, *mpk-1*, *lin-1* pathway promotes specific programmed cell-death events; phosphorylated LIN-1 directly binds the *egl-1* promoter and activates transcription of *egl-1*, consistent with a transcriptional activation function of phosphorylated LIN-1. The function of *lin-1* during vulval development is more complex. *lin-1* null mutations cause P3.p, P4.p, and P8.p to adopt vulval cell fates, suggesting that *lin-1* is necessary to inhibit vulval cell fates and may not be necessary to promote vulval cell fates (Sulston and Horvitz 1981; Ferguson *et al.* 1987). However, Tiensuu *et al.* (2005) showed that *egl-17* expression in P6.p is abnormal in *lin-1(lf)* mutants, indicating that *lin-1* function is required in P6.p for normal gene expression. Wagmaister *et al.* (2006) identified *lin-39* as a directly regulated LIN-1 target gene. In *lin-1(lf)* mutants, *lin-39* gene expression is upregulated in P3.p, P4.p, and P8.p, consistent with LIN-1 functioning as a transcriptional repressor in these cells. In addition, *lin-39* gene expression is down-regulated in P6.p, consistent with LIN-1 functioning as a transcriptional activator in P6.p. Zhang and Greenwald (2011) demonstrated that *lag-2*, which encodes the ligand for Notch, is transcriptionally repressed in all six Pn.p cells prior to ERK activation, and that this repression requires *lin-1*. Farooqui *et al.* (2012) proposed that nonphosphorylated LIN-1 directly binds and

activates the *let-502* promoter in the secondary cells P5.p and P7.p, suggesting that LIN-1 may have different regulatory mechanisms in the primary cell P6.p, the secondary cells P5.p and P7.p, and the tertiary cells P3.p, P4.p, and P8.p. We propose that SUMOylated LIN-1 represses transcription of target genes in P3.p, P4.p, and P8.p and that phosphorylated LIN-1 activates transcription of target genes in P6.p (Figure 7). In *lin-1(lf)* mutants, the absence of SUMOylated LIN-1 causes derepression of target genes in P3.p, P4.p, and P8.p, and these derepressed target genes are sufficient to promote a vulval cell fate. Although the absence of phosphorylated LIN-1 in P6.p results in diminished activation of target genes, P6.p can still adopt the proliferative vulval cell fate.

An important question that is raised by these findings is: How does phosphorylation by ERK convert LIN-1 from a transcriptional repressor to a transcriptional activator? Activation of ERK might result in decreased SUMOylation of LIN-1. For example, the steady-state level of SUMOylated Elk-1 decreases when ERK is activated in vertebrate cells (Yang *et al.* 2003). Phosphorylation of LIN-1 by ERK might disrupt the binding interaction of LIN-1 with transcriptional repressors. In particular, the EGL-27 transcriptional repressor binds two domains in the carboxy-terminus of LIN-1 that contain multiple consensus phosphorylation sites for ERK. Phosphorylated LIN-1 might recruit transcriptional activators. Phosphorylation of Ets-1 and Ets-2 by ERK mediates recruitment of the transcriptional coactivators CREB-binding protein and p300 (Foulds *et al.* 2004). Phosphorylated Elk-1 interacts with the mediator subunit Sur2 to promote transcriptional activation (Stevens *et al.* 2002; Cantin *et al.* 2003), and the *C. elegans* *sur-2* gene promotes vulval cell fates (Singh and Han 1995), suggesting that phosphorylated LIN-1 might recruit SUR-2 to promote transcriptional activation. Proteins recruited by phosphorylated LIN-1 might reverse the chromatin modifications caused by transcriptional repressors, such as the histone deacetylation and nucleosome remodeling caused by the NuRD complex. These mechanisms are not mutually exclusive, and important goals of future studies are to determine the relative importance of these mechanisms and identify transcriptional activators recruited to LIN-1.

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