Sumoylation of LIN-1 promotes transcriptional repression and inhibition of vulval cell fates

Elizabeth R. Leight, Danielle Glossip and Kerry Kornfeld*

Department of Molecular Biology and Pharmacology Washington University School of Medicine, St Louis, MO 63110, USA
*Author for correspondence (e-mail: kornfeld@molecool.wustl.edu)

Accepted 23 December 2004

Development 132, 1047-1056
Published by The Company of Biologists 2005
doi:10.1242/dev.01664

Summary

The LIN-1 ETS transcription factor inhibits vulval cell fates during *Caenorhabditis elegans* development. We demonstrate that LIN-1 interacts with UBC-9, a small ubiquitin-related modifier (SUMO) conjugating enzyme. This interaction is mediated by two consensus sumoylation motifs in LIN-1. Biochemical studies showed that LIN-1 is covalently modified by SUMO-1. *ubc-9* and *smo-1*, the gene encoding SUMO-1, inhibit vulval cell fates and function at the level of *lin-1*, indicating that sumoylation promotes LIN-1 inhibition of vulval cell fates. Sumoylation of LIN-1 promoted transcriptional repression and mediated an interaction with MEP-1, a protein previously shown to associate with the nucleosome remodeling and histone deacetylation (NuRD) transcriptional repression complex. Genetic studies showed that *mep-1* inhibits vulval cell fates and functions at the level of *lin-1*. We propose that sumoylation of LIN-1 mediates an interaction with MEP-1 that contributes to transcriptional repression of genes that promote vulval cell fates. These studies identify a molecular mechanism for SUMO-mediated transcriptional repression.

Key words: LIN-1, SUMO, ETS, Chromatin, Vulval development, Transcription

Introduction

Caenorhabditis elegans LIN-1 is a DNA-binding transcription factor that is a member of the Elk subfamily of ETS proteins (Beitel et al., 1995; Treisman, 1994). The function of *lin-1* during the development of the hermaphrodite vulva has been characterized extensively. The vulva is a specialized epidermal structure that is formed by the descendents of three ectodermal blast cells, P5.p, P6.p and P7.p (Horvitz and Sternberg, 1991). In wild-type hermaphrodites, the anchor cell of the somatic gonad signals to P6.p using the LIN-3 epidermal growth factor-like ligand (Greenwald, 1997; Kornfeld, 1997; Sternberg and Han, 1998). LIN-3 binds to the LET-23 receptor tyrosine kinase (RTK), activating a signal transduction pathway that includes the SEM-5 adaptor protein, the LET-341 Ras guanine nucleotide exchange factor, LET-60 Ras, LIN-45 RAF, MEK-2 mitogen-activated protein (MAP) kinase kinase and MPK-1 extracellular signal-regulated kinase (ERK) MAP kinase. The activation of this pathway causes P6.p to adopt the 1° vulval cell fate (eight descendants). When P6.p adopts the 1° vulval cell fate it signals to P5.p and P7.p through the LIN-12 Notch receptor, causing these cells to adopt the 2° vulval cell fate (seven descendants). Although P3.p, P4.p and P8.p are capable of adopting vulval fates, they receive neither of these signals and thus adopt the nonvulval 3° cell fate (two descendants). In hermaphrodites with a loss-of-function mutation in any of the core signaling genes, P5.p, P6.p and P7.p adopt nonvulval 3° fates, resulting in a worm with a vulvaless (Vul) phenotype.

Genetic analysis indicates that *lin-1* is a crucial target of the RTK/Ras/ERK signaling pathway. *lin-1(lf)* mutations cause a strong multivulva (Muv) phenotype; P3.p, P4.p and P8.p inappropriately adopt vulval cell fates, and the resulting ectopic tissue forms a series of ventral protrusions. Thus, *lin-1* activity inhibits the 1° vulval cell fate and/or promotes the 3° cell fate. The Muv phenotype caused by *lin-1(lf)* mutations is epistatic to the Vul phenotype caused by loss-of-function mutations in *mpk-1* and other upstream signaling genes, indicating that *lin-1* functions downstream of MPK-1 (Ferguson et al., 1987; Lackner et al., 1994; Wu and Han, 1994).

The *lin-1* gene encodes a 441 amino acid protein that contains a conserved ETS DNA-binding domain (Beitel et al., 1995). Mutations in the ETS domain that abrogate DNA binding cause a strong Muv phenotype, demonstrating that DNA binding is necessary for LIN-1 to inhibit the 1° vulval cell fate (Miley et al., 2004). LIN-1 contains two docking sites for ERK, the D domain and FQFP motif, and 17 S/TP motifs that are potential ERK phosphorylation sites (Fig. 1A) (Fantz et al., 2001; Jacobs et al., 1999; Tan et al., 1998). Mutations of the FQFP motif that decrease phosphorylation of LIN-1 by ERK cause a gain-of-function Vul phenotype (Jacobs et al., 1998). Thus, phosphorylation of LIN-1 by MPK-1 ERK prevents LIN-1 from functioning as a constitutive inhibitor of the 1° cell fate. The mechanisms that enable LIN-1 to inhibit vulval cell fates and phosphorylation of LIN-1 to relieve this inhibition are not well defined.

To characterize the function of LIN-1, we used the yeast two-hybrid system to identify proteins that bind LIN-1. Here, we show that LIN-1 binds the SUMO-conjugating enzyme UBC-9 and is covalently modified by SUMO. Sumoylation of LIN-1 mediated transcriptional repression and promoted binding to MEP-1, a protein associated with the NuRD

transcriptional repression complex (Unhavaithaya et al., 2002). These studies identify a new post-translational modification of LIN-1, characterize the function of LIN-1 sumoylation, and identify a mechanism for SUMO-mediated transcriptional repression.

Materials and methods

Yeast two-hybrid screen and reporter gene assays

The YEL3 strain was generated by transforming the L40 strain (Vojtek et al., 1993) with a bait plasmid containing the *lin-1* cDNA encoding amino acids 1-252 cloned into pBTM116 (Bartel et al., 1993). A random-primed cDNA library from mixed-stage hermaphrodites (kindly provided by R. Barstead), containing cDNAs fused to the GAL4 AD, was transformed into YEL3 (Schiestl and Gietz, 1989). Prey plasmids were isolated from positive colonies, and the cDNAs were sequenced using standard techniques.

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 Fig. 5, yeast transformants were grown in selective media at 30°C to an optical density of ~1.0 before analysis.

Monitoring sumoylation of LIN-1 in *S. cerevisiae* and cultured cells

The YEL15 strain that contains the LA:LIN-1(1-252) expression plasmid and a plasmid that encodes 6xHis- and FLAG-tagged mature SUMO1/SMT3 driven by the GAL10 promoter (kindly provided by E. Johnson) (Johnson and Blobel, 1999) and the YEL3 strain were grown at 30°C in YPG media to an optical density of ~1.3. Cells were resuspended in 2 ml lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% NP-40, 50 mM N-ethylmaleimide (NEM), 2 mM PMSF and complete EDTA-free protease inhibitor cocktail (Roche)]. His-tagged proteins were purified using Ni-NTA agarose beads according to the manufacturer's protocol (Qiagen), separated by SDS-PAGE and immunoblotted with α-FLAG M2 antibody (Sigma) or α-LexA DBD antibody (Upstate Biotechnology) using standard procedures (Sambrook et al., 1989).

The plasmid pFastBac DUAL (Invitrogen) was modified to encode GST:LIN-1(1-64), GST:LIN-1(1-64; 9-16A) or GST:LIN-1(1-64; K10A) with or without 6xHis- and FLAG-tagged *C. elegans* SMO-1. Proteins were expressed in Sf9 cells using the baculovirus system (Invitrogen, Bac-to-Bac Baculovirus Expression Systems manual). Infected cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NEM, 1.5 mM DTT, 1.5 mM PMSF and complete protease inhibitor cocktail (Roche). The GST:LIN-1 fusion proteins were purified using glutathione sepharose as described (Jacobs et al., 1998), separated by SDS-PAGE and immunoblotted with α-GST antibody (Santa Cruz Biotechnology) or α-FLAG M2 antibody.

Cell culture and reporter gene assays

The 293 human embryonic kidney (HEK) cell line (ATCC CRL-1573) was transfected using Ca^{2+} phosphate precipitation (Sambrook et al., 1989) so that each well received 100 ng L8G5-luciferase reporter plasmid (kindly provided by Dr Khochbin) (Lemercier et al., 2000), 200 ng LexA-VP16 expression plasmid (Lemercier et al., 2000), 100 ng CMV-β-galactosidase expression plasmid and 200 ng GAL4 DNA-binding domain (G4) fusion protein expression plasmid. Cells were harvested after ~19 hours, and luciferase activity was measured according to the manufacturer's techniques (Promega).

To generate SMO-1:LIN-1 fusion proteins that would be resistant to isopeptidase cleavage, we designed G4 and LA plasmids (Figs 3, 5) that expressed SMO-1 residues 1-88; this fragment lacks the C-terminal di-glycine isopeptidase cleavage site. Fusion proteins were confirmed to be the predicted size for the intact protein by western blotting.

Analysis of *C. elegans* strains

C. elegans strains were cultured at 20°C as described by Brenner (Brenner, 1974). *smo-1(ok359)* was generated by the International *C. elegans* Gene Knockout Consortium by screening animals mutagenized by TMP/UV for a deletion of the K12C11.2 gene that encodes SMO-1. We outcrossed *ok359* to the N2 wild-type strain twice and constructed double mutants using standard techniques.

HT115(DE3) *E. coli* transformed with a plasmid that expresses double-stranded RNA from the *smo-1*, *ubc-9* or *mep-1* gene were produced by Fraser et al. (Fraser et al., 2000) and Kamath et al. (Kamath et al., 2003), and distributed by MRC geneservice. Generally, genomic fragments were PCR amplified using the indicated primers and cloned into the L4440 control plasmid (Timmons and Fire, 1998) between copies of the bacteriophage T7 promoter (*smo-1*, 5′-GAGAAACC-GAGTATCTCAGTGGA-3′ and 5′-GCGATGCGTTTAATTAAGT-TTTG-3′; *ubc-9*, 5′-CTTATCGATCGGATTTCTGTTTG-3′ and 5′-CTACCACGAAGCAAGCATCTACT-3′; *mep-1*, 5′-CCTCTTCTG-GAACGCTTGTC-3′ and 5′-CTGGTTCTCTTGTGCGTTCA-3′). Cells were grown overnight at 37°C in LB media containing 50 μg/ml ampicillin, diluted 1:100 in 2×YT media containing 50 μg/ml ampicillin, grown at 37°C for 6 hours and seeded onto a Petri dish containing NGM agar, 50 μg/ml ampicillin and 100 μM IPTG. The following day (day 1), L4 hermaphrodites were placed on the Petri dish. These hermaphrodites were transferred to a new Petri dish daily, and progeny were scored for the Muv phenotype. We defined limited and extensive exposure to RNAi as progeny laid on day 1 and day 2 plates or day 3 and day 4 plates, respectively. We determined the number of descendants of P3.p, P4.p and P8.p for hermaphrodites at the 'Christmas tree' stage of vulval development based on cell position and morphology using DIC microscopy for limited exposure *smo-1* RNAi and extensive exposure *mep-1* RNAi.

Results

LIN-1 binds the E2 SUMO conjugating enzyme UBC-9

To identify proteins that associate with LIN-1 and contribute to the regulation of vulval cell fates, we conducted a yeast two-hybrid screen using as bait a fusion protein containing the LexA DNA-binding domain (LA) and the N terminus of LIN-1 [LIN-1(1-252)]. We screened 4×10^6 cDNAs from a mixed-stage *C. elegans* cDNA library and identified 233 cDNAs that encode proteins that bind LIN-1 specifically. Eight cDNAs encode UBC-9, a protein that shares 53% identity over 156 amino acids with *S. cerevisiae* Ubc9, an E2 SUMO-conjugating enzyme. The covalent attachment of SUMO to protein substrates involves a heterodimeric E1 SUMO-activating enzyme and the E2 SUMO-conjugating enzyme Ubc9 (Desterro et al., 1999; Johnson and Blobel, 1997; Johnson et al., 1997). Ubc9 binds a consensus sumoylation motif in the substrate (ψKxE, where ψ is a large hydrophobic amino acid and K is the SUMO acceptor) and catalyzes the formation of an isopeptide bond between the C-terminal glycine of SUMO and the ε amino group of lysine (Desterro et al., 1997; Sampson et al., 2001; Schwarz et al., 1998).

To define regions of LIN-1 that are necessary and sufficient to bind UBC-9, we analyzed fragments of LIN-1 containing amino acids 1-64, 65-145 and 146-252. LA:LIN-1(1-64) and LA:LIN-1(146-252) were sufficient to mediate robust binding to UBC-9, indicating that LIN-1 contains two separable binding sites for UBC-9 (Fig. 1B, lines 2, 9). We noted that the LIN-1(1-

64) fragment contains the sequence $VK_{10}KE$ that matches the ψKxE consensus sumoylation motif. To determine if this motif is necessary for LIN-1 to bind UBC-9, we mutated residues 9-16 to alanine. The binding of UBC-9 to the LA:LIN-1(1-64; 9-16A) mutant was reduced 28-fold relative to the binding of LA:LIN-1(1-64) (Fig. 1B, line 3). This motif was further characterized by mutating each of the four residues individually. A substitution of the predicted SUMO acceptor lysine (K10A) or the highly-conserved glutamic acid (E12A) dramatically reduced binding of UBC-9 (Fig. 1B, lines 5, 7). A substitution of the moderately conserved valine (V9A) partially decreased binding of UBC-9, whereas a substitution of the non-conserved lysine (K11A) had no significant effect (Fig. 1B, lines 4, 6). These results demonstrate a correlation between the function of each residue in the ψKxE consensus sumoylation motif in promoting sumoylation (Sampson et al., 2001) and the function of each residue in the $VK_{10}KE$ motif in promoting binding of UBC-9. In particular, residues predicted to be crucial for sumoylation were crucial for the binding of UBC-9.

We noted that the LIN-1(146-252) fragment that was sufficient to bind UBC-9 contains the sequence $VK_{169}DE$ that matches the consensus sumoylation motif. A 25 amino acid segment of LIN-1 that contains this motif, LIN-1(156-180), also bound robustly to UBC-9 (Fig. 1B, line 10). To determine if this motif is necessary for binding, we mutated the entire motif (168-171A) or the predicted SUMO acceptor lysine (K169A). The binding of UBC-9 to the LA:LIN-1(156-180; 168-171A) mutant was decreased by 60-fold relative to the binding of LA:LIN-1(156-180) (Fig. 1B, line 11). Mutation of the predicted SUMO acceptor lysine also significantly reduced binding of UBC-9 (Fig. 1B, line 12).

LIN-1 is covalently modified by SUMO-1

Because SUMO and the sumoylation enzymes are highly conserved from *S. cerevisiae* to *H. sapiens*, we monitored sumoylation of LIN-1 in yeast and cultured cells. We co-expressed LIN-1 and yeast SUMO1/Smt3 with a His- and FLAG-tag (HF-SUMO) in yeast cells and purified proteins covalently modified by HF-SUMO by metal affinity chromatography. Western blotting revealed species of LA:LIN-1(1-252) with retarded mobility in cells that express HF-SUMO but not control cells lacking HF-SUMO (Fig. 2A, lane 4 versus lane 3). The calculated molecular weight of these proteins suggests that LIN-1 was covalently modified by multiple SUMO proteins. These results demonstrate that LIN-1 is sumoylated in yeast.

To monitor sumoylation of LIN-1 in Sf9 insect cells, we expressed GST:LIN-1(1-64), purified the protein by glutathione-sepharose affinity chromatography, and analyzed the protein by SDS-PAGE and western blotting. The majority of GST:LIN-1(1-64) protein had the predicted molecular weight of 32 kDa, but a small fraction displayed a higher molecular weight (Fig. 2B, lane 2). To determine if this species is sumoylated LIN-1, we co-expressed GST:LIN-1(1-64) and a His- and FLAG-tagged *C. elegans* SMO-1 (HF-SUMO). The high molecular weight LIN-1 species reacted with the anti-FLAG antibody, indicating that it contains HF-SUMO (Fig. 2B, lane 7). Furthermore, the higher molecular weight LIN-1 species were eliminated by mutating the entire consensus sumoylation motif (Fig. 2B, lane 8) or the predicted SUMO acceptor lysine (Fig. 2C, lane 6). Together, these studies demonstrate that LIN-1 is covalently modified by SUMO and the consensus sumoylation motif is required for sumoylation. Only a small fraction of steady-state LIN-1 was post-translationally modified by SUMO. It is possible that sumoylation is a stable modification of a small fraction of LIN-

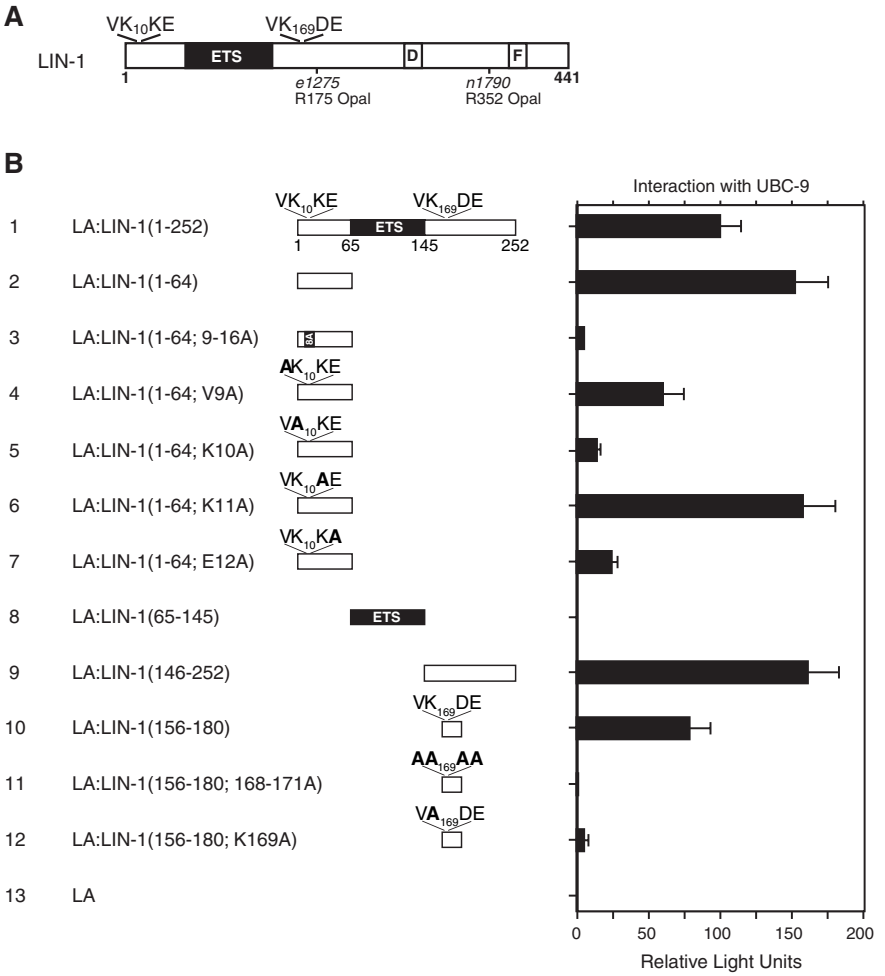


Fig. 1. UBC-9 binds two consensus sumoylation motifs of LIN-1. (A) Schematic of LIN-1: ETS DNA-binding domain (black) and consensus sumoylation motifs (above); the D domain (D) and the FQFP motif (F) are docking sites for ERK. The positions of the *e1275* and *n1790* mutations and amino acid numbers are shown below. (B) The interaction of GAL4AD:UBC-9(1-166) with the indicated LexA DNA-binding domain (LA):LIN-1 fusion protein was monitored using the two-hybrid system. Bars represent the average LexA-dependent β-galactosidase activity from at least five independent yeast transformants and lines indicate the standard deviation. The values were normalized by setting the interaction with LA:LIN-1(1-252) equal to 100 RLU.

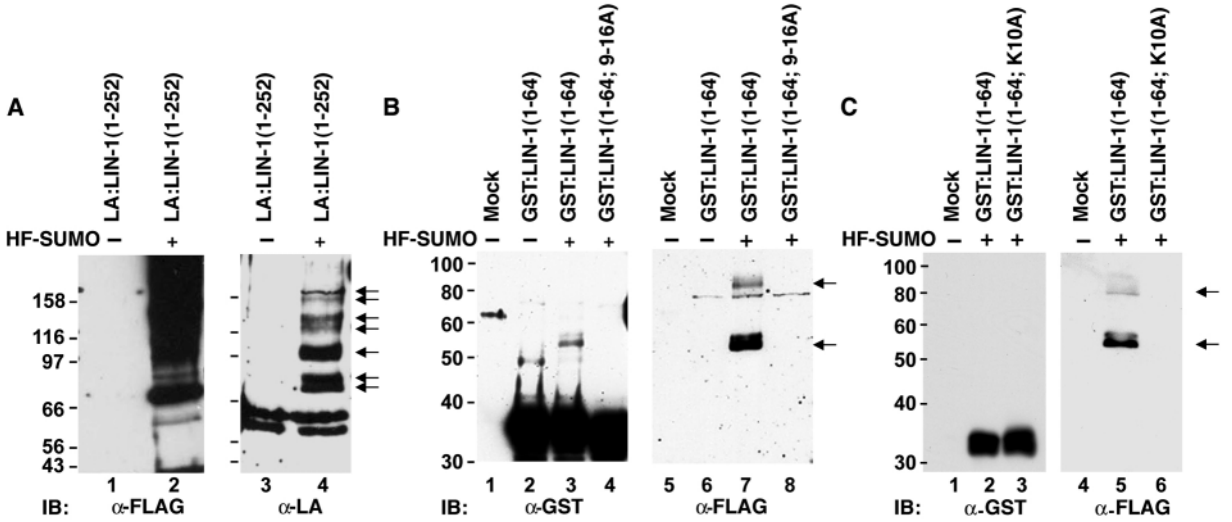


Fig. 2. LIN-1 is covalently-modified by SUMO-1. (A) Extracts from yeast expressing LA:LIN-1(1-252) alone (–) or with His- and FLAG-tagged SUMO1/Smt3 (HF-SUMO) (+) were subjected to metal affinity chromatography. Bound proteins were separated by SDS-PAGE and immunoblotted (IB). An anti-FLAG antibody detected all proteins modified by HF-SUMO; an anti-LA antibody detected LA:LIN-1(1-252) complexes. The arrows indicate high molecular weight forms of LA:LIN-1(1-252) that appear to be covalently modified by one or multiple HF-SUMO moieties (14 kDa), and may also contain endogenous SUMO1/Smt3 (11 kDa). Bands present in lanes 3 and 4 are a cross-reactive endogenous yeast protein that was present in strains lacking LA:LIN-1 (data not shown) and 60 kDa unmodified LA:LIN-1(1-252) that bound the affinity matrix in a Ni^{2+}-independent manner (data not shown). Molecular weight markers (in kDa) are indicated. (B) Extracts from Sf9 cells that were not infected (Mock) or infected with viruses that express GST:LIN-1 alone (–) or with His- and FLAG-tagged *C. elegans* SMO-1 (HF-SUMO) (+) were subjected to glutathione sepharose affinity chromatography to purify the GST fusion proteins. Bound proteins were separated by SDS-PAGE and immunoblotted. An anti-GST antibody detected all LIN-1 species; an anti-FLAG antibody detected LIN-1 that was covalently modified by HF-SUMO. Arrows indicate sumoylated isoforms of LIN-1 (lanes 3 and 7) that were absent in extracts containing mutant GST:LIN-1(1-64; 9-16A) (lanes 4 and 8). (C) Extracts from Sf9 cells were analyzed as in B. Arrows indicate sumoylated isoforms of LIN-1 (lane 5) that were absent in extracts containing mutant GST:LIN-1(1-64; K10A) (lane 6).

1, a transient modification of a large fraction of LIN-1, or sumoylated LIN-1 is cleaved by isopeptidases during purification.

smo-1 and *ubc-9* negatively regulate vulval cell fates and function at the level of *lin-1*

If sumoylation is important for LIN-1 function, then mutations that reduce sumoylation might affect cell fate determination and result in a Vul or Muv phenotype. *C. elegans* contains a single gene that encodes SUMO, designated *smo-1*. We used two methods to reduce the function of *smo-1*. First, we analyzed the *smo-1(ok359)* null allele that contains a deletion of the entire *smo-1* locus. *smo-1(ok359)* homozygous mutants were sterile. To analyze vulval development, we derived *smo-1(ok359)* homozygotes from *smo-1(ok359)*/+ hermaphrodites. These mutants displayed a completely penetrant protruding vulva (Pvl) phenotype. Broday et al. (Broday et al., 2004) have attributed the Pvl phenotype to the presence of an abnormal vulE cell and impaired formation of the uterine-seam cell and demonstrated that the LIM domain transcription factor LIN-11 is sumoylated. In addition, we observed that nine percent of *smo-1(ok359)* mutants displayed a Muv phenotype, defined as one or more ventral protrusions displaced from the position of the vulva when viewed with a dissecting microscope (Table 1, line 2). Broday et al. (Broday et al., 2004) observed a similar defect. Second, we used RNA interference (RNAi) to reduce the levels of *smo-1* RNA by feeding hermaphrodites *E. coli* that express double-stranded *smo-1* RNA. Consistent with a previous report (Fraser et al., 2000), extensive exposure to *smo-1*(RNAi) resulted in a highly penetrant embryonic lethal phenotype; the few surviving adults displayed a Pvl phenotype. However, limited exposure to *smo-1*(RNAi) allowed most animals to survive to adulthood, and 9% of these adult hermaphrodites displayed a Muv phenotype (Table 1, line 7). To characterize the cellular basis for this Muv phenotype, we used DIC microscopy to examine *smo-1*(RNAi) hermaphrodites. P3.p, P4.p and P8.p generated three or more descendants, indicating that the cell adopted a partial vulval fate, with a frequency of 0%, 18% and 18%, respectively (n=11). These results indicate that *smo-1* has multiple functions during development and is necessary for embryonic viability, fertility, vulval morphogenesis and inhibition of vulval cell fates.

To determine the position of *smo-1* in the genetic pathways that specify vulval cell fates, we analyzed the interactions of *smo-1* with *mek-2*, *mpk-1* and *lin-1*. *mek-2(n2678)* is a probable null allele that causes a completely penetrant Vul phenotype (Kornfeld et al., 1995). *mek-2(lf)* alleles suppress the Muv phenotype caused by activated *let-60 ras*, but do not suppress the Muv phenotype caused by *lin-1(lf)* mutations (Kornfeld et al., 1995). *mpk-1(n2521)* is a partial loss-of-function mutation that likewise strongly suppresses the Muv phenotype caused by activated *let-60 ras*, but does not suppress the Muv phenotype caused by *lin-1(lf)* mutations (Lackner et al., 1994). If *smo-1* is necessary for sumoylation of LIN-1 and the *smo-1(lf)* Muv phenotype is caused by a loss of *lin-1* activity, then these mutations are predicted to not suppress the *smo-1* Muv phenotype. *mek-2(n2678)* did not suppress the Muv phenotype caused by *smo-1(ok359)* (Table 1, line 5). *mek-2(n2678)* and *mpk-1(n2521)* did not suppress the Muv phenotype caused by

Table 1. *smo-1* and *ubc-9* inhibit vulval cell fates and interact with *lin-1*

Genotype	RNAi*	% Muv†	*n*‡
Wild type	NA	0	306
smo-1(ok359)§	NA	9	94
*lin-1(n1790*gf)¶	NA	14	283
*smo-1(ok359); lin-1(n1790*gf)**	NA	82	120
smo-1(ok359); mek-2(n2678)††	NA	17	299
Wild type	Control	0	3233
Wild type	*smo-1*	9	617
mek-2(n2678)‡‡	Control	0	237
mek-2(n2678) ‡‡	*smo-1*	20	55
mpk-1(n2521)§§	Control	0	676
mpk-1(n2521)§§	*smo-1*	5	558
smg-1(r861); lin-1(e1275)¶¶	Control	2	3143
smg-1(r861); lin-1(e1275) ¶¶	*smo-1*	78	404
*lin-1(n1790*gf)	Control	6	1399
*lin-1(n1790*gf)	*smo-1*	51	376
Wild type	*ubc-9*	0.4	976
smg-1(r861); lin-1(e1275) ¶¶	*ubc-9*	12	457
*lin-1(n1790*gf)	*ubc-9*	27	971

NA, not applicable.

*L4 hermaphrodites were fed HT115(DE3) *E. coli* transformed with a control plasmid or a plasmid that expresses double-stranded RNA from the indicated gene. Progeny laid on the first and second day of culture on the RNAi bacteria were scored for the Muv phenotype.

†Adult hermaphrodites were scored as multivulval (Muv) if they displayed one or more ventral protrusions displaced from the site of the vulva when examined using a dissecting microscope.

‡*n*, number of hermaphrodites examined.

§*smo-1(ok359)* homozygous hermaphrodites were sterile, protruding vulva (Pvl), non-blister (non-Bli), non-egg laying defective (non-Egl) self-progeny of *smo-1(ok359)/bli-3(e767) egl-30(n686)* hermaphrodites.

¶Complete genotype: *smo-1(ok359)/bli-3(e767) egl-30(n686); lin-1(n1790)*.

**These hermaphrodites were sterile, Pvl, non-Bli, non-Egl self-progeny of *smo-1 (ok359)/bli-3(e767) egl-30(n686); lin-1(n1790)* hermaphrodites.

†† These hermophrodites were sterile, non-GFP positive self-progeny of *smo-1 (ok359)mek-2(n2678)/hT2g* hermaphrodites.

‡‡*mek-2(n2678)* homozygous hermaphrodites were sterile, non-GFP-positive self-progeny of *mek-2(n2678)/ hT2g* hermaphrodites.

§§Complete genotype: *mpk-1(n2521) unc-79(e1068)*.

¶¶Complete genotype: *smg-1(r861) unc-54(r293); lin-1(e1275)*.

smo-1(RNAi) (Table 1, lines 9, 11). These results support the model that *smo-1* functions at the level of *lin-1*. However, the *mpk-1* mutant has some *mpk-1* activity, and the *mek-2* mutant is derived from a *mek-2/+* hermaphrodite and may have residual *mek-2* activity. Therefore, these data do not exclude the possibility that the *smo-1(lf)* Muv phenotype requires some *mek-2* or *mpk-1* activity and that *smo-1* functions upstream of *mek-2* or *mpk-1*.

Loss-of-function mutations of *smo-1* and *lin-1* both cause a Muv phenotype. To analyze the interaction between these genes, we generated a partial loss-of-function *lin-1* mutation. The *lin-1(e1275 R175Opal)* mutation causes a Muv phenotype with a penetrance of 91% at 20°C (Beitel et al., 1995). The *lin-1(e1275)* mRNA contains a premature stop codon and is likely to have a short half-life because of nonsense-mediated decay. In a double mutant with *smg-1(r861)*, a gene that is necessary for nonsense-mediated decay, the *lin-1(e1275)* mRNA appears to be stabilized and the penetrance of the Muv phenotype is reduced to 2% (Table 1, line 12). If *smo-1* is necessary for the sumoylation and function of LIN-1, then these mutants are predicted to be highly sensitive to a reduction of *smo-1* activity. *smo-1*(RNAi) caused the penetrance of the Muv phenotype to increase to 78% in this *smg-1(r861); lin-1(e1275)* genetic background (Table 1, line 13). These results demonstrate a strong interaction between partial loss-of-function mutations in *smo-1* and *lin-1*.

We previously described gain-of-function mutations of *lin-1* (Jacobs et al., 1998). The strongest gain-of-function mutation is *lin-1(n1790*gf *R352Opal)* (Fig. 1A). The *lin-1(n1790)* mutation 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 MAPK docking site and is partially resistant to negative regulation by MPK-1 (Jacobs et al., 1999). The *lin-1(n1790*gf*)* 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 *smo-1* is necessary for the sumoylation and function of LIN-1(1-351), then the double mutant is predicted to lack functional LIN-1 and display a strong Muv phenotype. The *smo-1(ok359); lin-1(n1790*gf*)* double mutants displayed a Muv phenotype that was 82% penetrant, significantly greater than the Muv phenotype of *ok359* and *n1790* single mutants (Table 1, line 4). *lin-1(n1790*gf*)* hermaphrodites fed *smo-1*(RNAi) likewise displayed a highly penetrant Muv phenotype (Table 1, line 15). These data support the model that *smo-1* functions at the level of *lin-1* and that sumoylation of LIN-1 is necessary for inhibition of vulval cell fates.

To investigate the function of *ubc-9*, we fed hermaphrodites *E. coli* that expressed double-stranded *ubc-9* RNA. Wild-type hermaphrodites exposed to *ubc-9*(RNAi) occasionally displayed a Muv phenotype, although the penetrance was only 0.4% (Table 1, line 16). *ubc-9*(RNAi) caused a significant Muv phenotype of 12% and 27% in *smg-1(r861); lin-1(e1275)* and *lin-1(n1790)* hermaphrodites, respectively (Table 1, lines 17, 18). These results indicate that *ubc-9* functions to repress vulval cell fates and interacts genetically with *lin-1*.

Sumoylation of LIN-1 promotes transcriptional repression

To investigate the mechanism by which sumoylation of LIN-1 inhibits vulval cell fates, we monitored the transcriptional activity of LIN-1 in 293 human embryonic kidney cells. We used a reporter plasmid that contains eight LexA-binding sites and five GAL4-binding sites upstream of an E1A promoter that regulates expression of luciferase (Fig. 3A). A LexA DNA-binding domain:VP16 (LexA:VP16) fusion protein was used to robustly activate this reporter (Fig. 3B, lines 1, 2). The ability of fusion proteins containing the GAL4 DNA binding domain (G4) to activate or repress transcription was monitored. G4:LIN-1(1-64) repressed transcription sevenfold relative to G4 alone (Fig. 3B, lines 2, 3). Thus, LIN-1 residues 1 to 64 are sufficient to repress transcription. Substitutions of the entire consensus sumoylation motif (9-16A) or the SUMO acceptor lysine (K10A) resulted in LIN-1 mutants that failed to repress transcription (Fig. 3B, lines 5, 7). Thus, the $VK_{10}KE$ consensus sumoylation motif is necessary for transcriptional repression by LIN-1(1-64). The same assay system was used to show that the $VK_{169}DE$ consensus sumoylation motif is necessary for transcriptional repression mediated by LIN-1(156-180) (data not shown).

The $VK_{10}KE$ motif might be necessary for transcriptional repression because it mediates sumoylation of LIN-1 or because it has an additional activity. To distinguish between these models, we determined if sumoylation of LIN-1 is

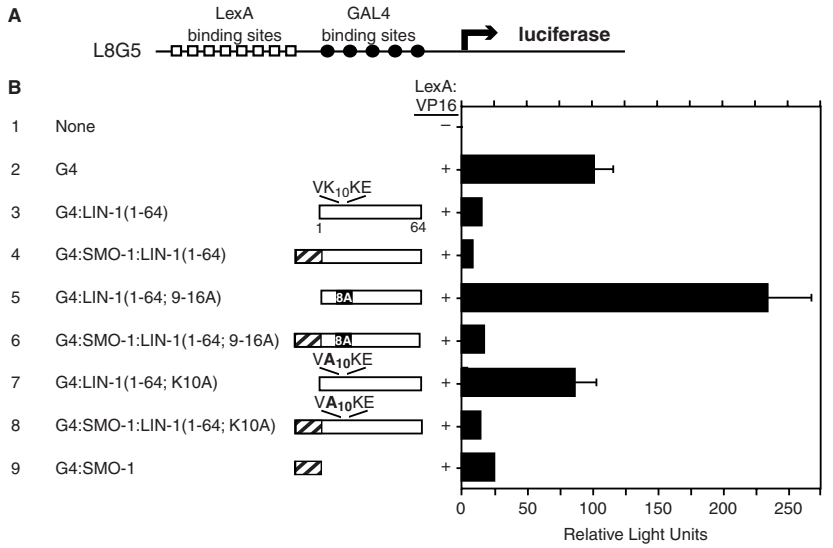


Fig. 3. Sumoylation promotes transcriptional repression by LIN-1. (A) The promoter region of the L8G5 reporter plasmid: eight LexA-binding sites (white boxes), five GAL4-binding sites (black circles), an E1A promoter (arrow) and a luciferase-coding region. (B) 293 HEK cells were transiently transfected with: (1) the L8G5 reporter plasmid; (2) an expression plasmid that encodes the GAL4 DNA-binding domain (G4) alone or fused to the indicated fragments of LIN-1 and/or *C. elegans* SMO-1; (3) an expression plasmid that encodes the LexA:VP16 fusion protein (+ or –); and (4) a reporter plasmid that encodes β-galactosidase to measure transfection efficiency. Bars indicate luciferase activity divided by β-galactosidase activity. Values are the average and standard deviation of three to four independent transfections conducted in parallel. Values were normalized by setting the value for G4 alone equal to 100 RLU. Western blotting demonstrated that the LexA:VP16 fusion protein was expressed at equivalent levels independent of the co-expressed G4 fusion protein, and that each G4:LIN-1 fusion protein was expressed, although the levels could not be estimated because of a crossreactive protein of similar size (data not shown).

sufficient to mediate transcriptional repression. *C. elegans* SMO-1(1-88) was fused to LIN-1(1-64) or the sumoylation-defective LIN-1 mutants. Addition of SMO-1 to the sumoylation-defective LIN-1 mutants restored transcriptional repression; G4:SMO-1:LIN-1(1-64, 9-16A) repressed transcription 13-fold relative to G4:LIN-1(1-64, 9-16A) and G4:SMO-1:LIN-1(1-64, K10A) repressed transcription sixfold relative to G4:LIN-1(1-64, K10A) (Fig. 3B, lines 6, 8). Translational fusion of SMO-1 to LIN-1(1-64) resulted in a twofold repression relative to LIN-1(1-64) (Fig. 3B, line 4). Interestingly, SMO-1 fused to G4 in the absence of LIN-1 repressed transcription by fourfold relative to G4 alone (Fig. 3B, line 9). These results demonstrate that SMO-1 is sufficient to restore transcriptional repression to LIN-1 mutants that lack the $VK_{10}KE$ motif, indicating that sumoylation of this motif mediates transcriptional repression.

LIN-1 binds MEP-1, and the interaction is mediated by two consensus sumoylation motifs

To characterize the mechanisms by which sumoylation of LIN-1 mediates transcriptional repression, we analyzed proteins that were identified in the two-hybrid screen using LIN-1(1-252) as bait and have been implicated in transcriptional regulation. One-hundred and twenty-three out of 233 cDNAs identified encode MEP-1. MEP-1 is a zinc finger protein that associates with *C. elegans* LET-418/CHD-4 and HDA-1, homologs of the vertebrate Mi-2β and HDAC-1, respectively (Fig. 4A) (Belfiore et al., 2002; Unhavaithaya et al., 2002). These proteins are core components of the NuRD transcriptional repression complex.

To define regions of LIN-1 that are necessary and sufficient to bind MEP-1, we analyzed fragments of LIN-1 containing amino acids 1-64, 65-145 and 146-252. LA:LIN-1(1-64) and LA:LIN-1(146-252) were sufficient to mediate robust binding to MEP-1, indicating that LIN-1 contains two separable binding sites for MEP-1 (Fig. 4B). To identify amino acids of LIN-1(1-64) that mediate binding, we expressed eight LIN-1(1-64) mutants that have eight consecutive amino acids changed to alanine and measured their interaction with MEP-1 quantitatively (Fig. 4C). The striking result of this experiment was that the substitution of LIN-1 residues 9-16 dramatically reduced binding of MEP-1 75-fold relative to the binding of wild-type LIN-1(1-64) (Fig. 4C, lines 1, 3). To characterize the role of the LIN-1 sumoylation motif $VK_{10}KE$, we mutated each residue to alanine. Substitution of the SUMO acceptor lysine (K10A) or the highly conserved glutamic acid (E12A) dramatically reduced binding of MEP-1 by 53-fold and 41-fold, respectively (Fig. 4C, lines 11, 13). Substitution of the moderately-conserved valine (V9A) and the non-conserved lysine (K11A) reduced binding of MEP-1 by sevenfold and 1.3-fold, respectively (Fig. 4C, lines 10, 12). These results demonstrate a correlation between the function of each residue in the ψKxE motif in promoting sumoylation (Sampson et al., 2001) and the function of each residue in the $VK_{10}KE$ motif in promoting binding of MEP-1.

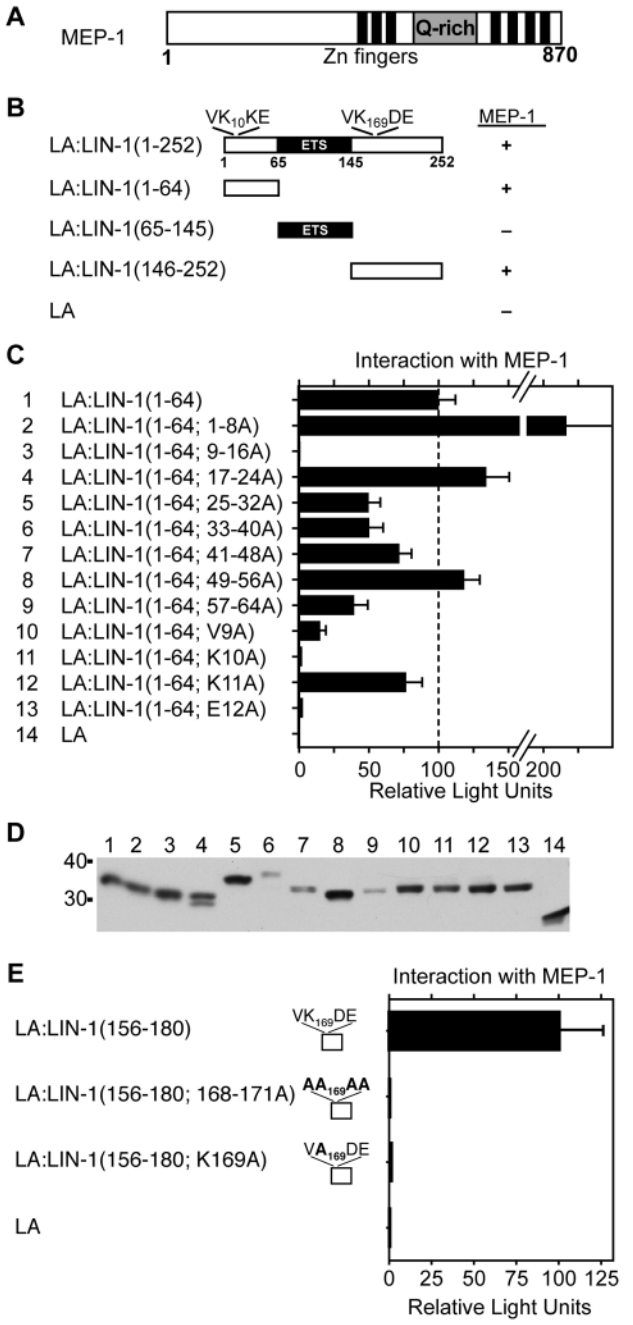
To investigate MEP-1 binding to LIN-1 residues 146-252, we analyzed the LIN-1(156-180) fragment that contains the $VK_{169}DE$ sumoylation motif. MEP-1 strongly interacted with LIN-1(156-180) (Fig. 4E). Mutations of the entire motif (168-171A) or the predicted SUMO acceptor lysine (K169A) markedly reduced binding of MEP-1 to LIN-1 (Fig. 4E). These studies demonstrate that a 64 amino acid fragment of LIN-1 containing the consensus sumoylation motif $VK_{10}KE$ and a 25 amino acid fragment of LIN-1 containing the consensus sumoylation motif $VK_{169}DE$ are sufficient to bind MEP-1, and for both LIN-1 fragments the SUMO acceptor lysine is necessary for binding.

Sumoylation of LIN-1 promotes binding of MEP-1

The ψKxE motifs of LIN-1 may directly interact with MEP-1, or post-translational modification of these motifs by SUMO may promote the binding of MEP-1. To investigate these possibilities, we expressed His-tagged MEP-1 in baculovirus-infected Sf9 cells and partially purified the protein using metal affinity chromatography. GST:LIN-1(1-64) was expressed in *E. coli* and purified by glutathione affinity chromatography. His:MEP-1 did

not detectably interact with GST:LIN-1(1-64) in a GST pull-down assay. Because bacterially expressed LIN-1 is not sumoylated, these data suggest that sumoylation of LIN-1 is necessary for the interaction with MEP-1.

We reasoned that if sumoylation of the LIN-1 ψKxE motifs mediates MEP-1 binding, then the addition of SUMO to a LIN-1 mutant that lacks the ψKxE motif might restore binding of MEP-1. We generated a translational fusion of the *C. elegans* SUMO-1 homolog, SMO-1, and the LIN-1(1-64; 9-16A) mutant that lacks the ψKxE motif and measured its interaction with MEP-1 in yeast. The interaction of MEP-1 with LA:SMO-1:LIN-1(1-64; 9-16A) was increased by eightfold relative to the interaction with LA:LIN-1(1-64; 9-16A) (Fig. 5, lines 2, 3). If sumoylation of LIN-1 mediates the interaction with MEP-1, then MEP-1 might display binding to SUMO in the absence of LIN-1. Consistent with this prediction, MEP-1 displayed a threefold greater interaction with LA:SMO-1 than LA alone (Fig. 5, lines 4, 5). These findings indicate that the ψKxE motif promotes binding by mediating sumoylation of LIN-1 and not by directly interacting with MEP-1.



mep-1 inhibits vulval cell fates and acts at the level of *lin-1*

To test the model that the interaction of LIN-1 and MEP-1 is important for *lin-1* function in vivo, we used genetic analysis to characterize the function of *mep-1* during vulval development. The activity of the *mep-1* gene was reduced by feeding wild-type hermaphrodites bacteria that express double-stranded *mep-1* RNA. Limited exposure of wild-type hermaphrodites to *mep-1*(RNAi) caused 6% of hermaphrodites to display a Muv phenotype (Table 2, line 2), whereas extensive exposure to *mep-1*(RNAi) caused a 58% Muv phenotype (n=326). To characterize how *mep-1* RNAi affects Pn.p cell fates, we examined hermaphrodites using DIC microscopy. P3.p, P4.p and P8.p generated three or more descendants, indicating that the cell adopted a partial vulval fate, with frequencies of 10%, 60% and 50%, respectively (n=10) (Fig. 6). These results indicate that *mep-1* inhibits vulval cell fates in P3.p, P4.p and P8.p.

To determine the position of *mep-1* in the genetic pathways that specify vulval cell fates, we analyzed the interactions of *mep-1* with *mek-2*, *mpk-1* and *lin-1*. *mek-2(n2678)* and *mpk-1(n2521)* did not suppress the Muv phenotype of hermaphrodites fed *mep-1(RNAi)* (Table 2, line 8; data not shown). These results suggest that *mep-1* acts downstream of *mek-2* and *mpk-1* if these genes act in a linear pathway. *mep-1*(RNAi) caused a significant Muv phenotype of 14% in *smg-1(r861); lin-1(e1275)* hermaphrodites (Table 2, line 4). *mep-1*(RNAi) caused a significant Muv phenotype of 23% in the genetic background with the gain-of-function *lin-1(n1790*gf*)* allele (Table 2, line 6). These findings are consistent with the model that *mep-1* functions at the level of *lin-1* to inhibit vulval cell fates.

Fig. 4. The sumoylation motifs of LIN-1 are necessary for the interaction with MEP-1. (A) Schematic of MEP-1 with zinc-finger motifs (black) and glutamine-rich region (gray) (Belfiore et al., 2002). (B) The interactions between LA:LIN-1 fusion proteins and MEP-1(155-859) fused to the GAL4 activation domain (GAL4AD) were measured qualitatively using the yeast two-hybrid system. A (+) indicates robust activation of a LexA-dependent *lacZ* reporter gene. (C) The interactions between wild-type LA:LIN-1(1-64) or the indicated mutant and MEP-1 were measured quantitatively. Bars represent the average LexA-dependent β-galactosidase activity and lines indicate the standard deviation of at least six independent yeast transformants. The signal with LA:LIN-1(1-64) was set to 100 relative light units (RLU); the signals with mutant proteins are proportional. (D) To monitor expression of LA:LIN-1 proteins, we analyzed protein extracts from transformed yeast by western blotting using an anti-LA antibody. Lanes 1-14 correspond to LA fusion proteins listed as 1-14 in C. (E) The interaction of MEP-1 with the indicated LA:LIN-1 fusion protein was measured quantitatively. Bars represent the average of six independent yeast transformants and lines indicate the standard deviation. The signal with LA:LIN-1(158-180) was set to 100 RLU and signals with mutant proteins are proportional.

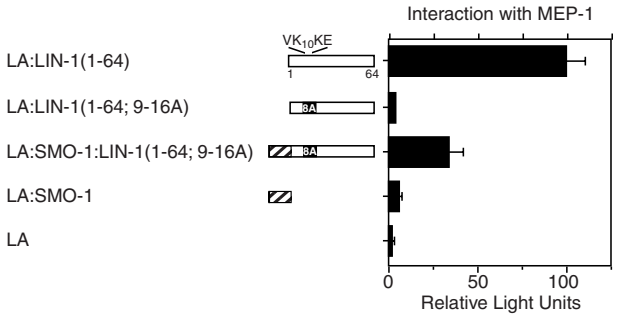


Fig. 5. SUMO is sufficient to increase binding of MEP-1 to LIN-1. The association of MEP-1 with the indicated LA fusion proteins was monitored using the yeast two-hybrid system. Bars represent the average LexA-dependent β-galactosidase activity from three independent yeast transformants grown to logarithmic phase in selective media, and lines indicate the standard deviation. The values were normalized by setting the interaction of each protein with LA:LIN-1(1-64) to 100 RLU. The LA fusion proteins were expressed at similar levels as determined by western blotting (data not shown).

Table 2. *mep-1* inhibits vulval cell fates and interacts with *lin-1*

Genotype	RNAi*	% Muv	*n*
Wild type	Control	0	2775
Wild type	*mep-1*	6	1350
smg-1(r861); lin-1 (e1275)†	Control	2	2762
smg-1(r861); lin-1 (e1275)†	*mep-1*	14	1742
*lin-1(n1790*gf)	Control	6	1311
*lin-1(n1790*gf)	*mep-1*	23	254
mek-2(n2678)‡	Control	0	237
mek-2(n2678)‡	*mep-1*	15	225

*L4 hermaphrodites were fed HT115(DE3) *E. coli* transformed with a control plasmid or a plasmid that expresses double-stranded RNA from *mep-1*. Progeny laid on the first and second day of culture on the RNAi bacteria were scored for the Muv phenotype.

†Complete genotype: *smg-1(r861) unc-54(r293); lin-1(e1275).*

‡*mek-2(n2678)* homozygous hermaphrodites were sterile, non-GFP-positive self-progeny of *mek-2(2678)/hT2g* hermaphrodites.

Discussion

Genetic analyses have demonstrated that *lin-1* inhibits Pn.p cells from adopting the 1° vulval cell fate and that *lin-1* is a critical target of Ras-mediated signaling in the P6.p cell. However, mechanisms of LIN-1 transcriptional regulation have not been well defined. By conducting a screen for proteins that interact with LIN-1, we identified UBC-9, an enzyme that mediates sumoylation, and MEP-1, a protein that has been implicated in transcriptional repression. Our findings elucidate how LIN-1 regulates transcription and cell fate decisions, and suggest a model for SUMO-mediated transcriptional repression that may apply to other transcription factors.

LIN-1 is sumoylated

Here, we present evidence indicating that LIN-1 is sumoylated. First, LIN-1 contains two ψKxE consensus sumoylation motifs, $VK_{10}KE$ and $VK_{169}DE$. Second, UBC-9, the homolog of the *S. cerevisiae* Ubc9 SUMO conjugating enzyme, binds both of the LIN-1 consensus sumoyation motifs. These results suggest that UBC-9 conjugates SUMO to K_{10} and K_{169} of LIN-1. Third, biochemical studies demonstrated that LIN-1 is covalently modified by one or more SUMO moieties, and the consensus sumoylation motif is required for sumoylation. LIN-1 has not been previously reported to be sumoylated, and these findings reveal a new mechanism of LIN-1 regulation.

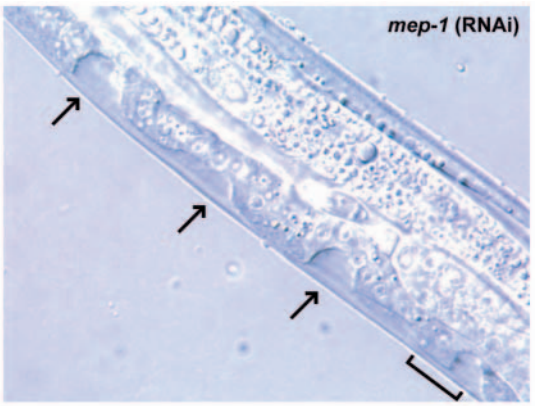


Fig. 6. *mep-1* inhibits vulval cell fates of P3.p, P4.p and P8.p. A wild-type hermaphrodite at the 'Christmas tree' stage of vulval development treated with extensive exposure to *mep-1* RNAi. A bracket indicates the vulval invagination formed by P5.p-P7.p; arrows indicate ectopic invaginations formed by the descendants of P3.p and P4.p.

Sumoylation of LIN-1 promotes inhibition of the 1° vulval cell fate

The function of LIN-1 sumoylation was investigated in animals by reducing the activity of *smo-1* using a deletion allele and RNAi and by reducing the activity of *ubc-9* using RNAi. Because *smo-1* was essential for embryonic viability and fertility, vulval development was examined in adult hermaphrodites with a partial reduction of *smo-1* activity. A reduction of *smo-1* function caused a Muv phenotype, demonstrating that *smo-1* inhibits Pn.p cells from adopting vulval cell fates. The *smo-1* Muv phenotype was partially penetrant; this might be a result of residual *smo-1* activity, or *smo-1* might not always be necessary to inhibit vulval cell fates. The *smo-1(lf)* Muv phenotype was not suppressed by a probable null mutation of *mek-2* or a partial loss-of-function mutation of *mpk-1*. These *mpk-1* and *mek-2* mutations strongly suppress more highly penetrant Muv phenotypes caused by synthetic multivulva genes or upstream genes in the Ras signaling pathway (Kornfeld et al., 1995; Lackner et al., 1994). Thus, *smo-1* probably functions downstream of *mek-2* and *mpk-1* if these genes act in a linear signaling pathway. Furthermore, reducing the activity of *smo-1* and *ubc-9* diminished the activity of a constitutively active LIN-1 mutant, indicating that *smo-1* and *ubc-9* are necessary for LIN-1 to inhibit vulval cell fates. Together, the biochemical studies showing that LIN-1 is sumoylated and the genetic studies showing that SMO-1 and UBC-9 are necessary for LIN-1-mediated inhibition of vulval cell fates support the model that sumoylated LIN-1 inhibits vulval cell fates.

Sumoylation of LIN-1 mediates transcriptional repression

A diverse group of transcription factors are post-translationally

modified by SUMO. For most of these proteins, including Sp3, Myb, Jun, Elk1, p300, C/EBP and CtBP, sumoylation promotes transcriptional repression (Bies et al., 2002; Dahle et al., 2003; Gill, 2003; Girdwood et al., 2003; Kim et al., 2002; Lin et al., 2003; Muller et al., 2000; Ross et al., 2002; Sapetschnig et al., 2002; Subramanian et al., 2003; Yang et al., 2003). However, for a few proteins, including HSF1, sumoylation promotes transcriptional activation (Hong et al., 2001). To characterize how sumoylation affects LIN-1, we monitored the transcriptional activity of LIN-1 in cultured cells. A fragment of LIN-1 containing a consensus sumoylation motif caused transcriptional repression. The consensus sumoylation motif was necessary for transcriptional repression, and fusion of SUMO to the mutant LIN-1 was sufficient to restore repression. These findings demonstrate that sumoylation of LIN-1 mediated this transcriptional repression activity.

Previous studies of *lin-1* did not distinguish between the models that *lin-1* inhibits vulval cell fates by activating transcription of genes that promote the 3° non-vulval cell fate or repressing transcription of genes that promote the 1° vulval cell fate. Based on the results that sumoylation of LIN-1 mediates transcriptional repression and inhibition of vulval cell fates, we infer that LIN-1 inhibits the 1° vulval cell fate by repressing target gene transcription. Therefore, *lin-1* target genes promote the 1° vulval cell fate. Together, these findings suggest that in the six Pn.p cells during larval development, LIN-1 is sumoylated and represses transcription of target genes that promote the 1° fate. When the anchor cell activates the RTK/Ras/ERK pathway in P6.p, MPK-1 ERK phosphorylates LIN-1 and relieves the LIN-1-mediated transcriptional repression, and genes that promote the 1° fate are now transcribed in P6.p. Phosphorylation may disrupt sumoylation of LIN-1 and cause LIN-1 to activate transcription of genes that promote the 1° vulval cell fate, as phosphorylation of human Elk1 by ERK activates transcription (Treisman, 1994; Yang et al., 2003).

Sumoylated LIN-1 binds MEP-1: a molecular mechanism for SUMO-mediated transcriptional repression

Although sumoylation has been shown to affect the activity of several transcription factors, the mechanisms have not been well defined. The most detailed descriptions of the mechanism of SUMO-mediated transcriptional repression are the studies of Girdwood et al. (Girdwood et al., 2003), showing that sumoylated p300 interacts with HDAC6, and of Yang and Sharrocks (Yang and Sharrocks, 2004), showing that sumoylated Elk1 interacts with HDAC-2. These studies indicate that sumoylation mediates recruitment of chromatin remodeling enzymes. However, these HDACs have not been shown to directly bind the SUMO moieties. In our screen for proteins that interact with LIN-1, over 50% of the positives were MEP-1. The Krüppel-type zinc-finger protein MEP-1 was identified as a nuclear protein that associates with the MOG-1, MOG-4 and MOG-5 DEAH box proteins, and the MOG-6 cyclophilin-like protein, suggesting that it functions with these proteins to regulate the *fem-3* RNA (Belfiore et al., 2002; Belfiore et al., 2004). In addition, MEP-1 interacts with LET-418/CHD-4 and HDA-1, homologs of the Mi-2 and HDAC-1 core components of the NuRD complex, respectively (Unhavaithaya et al., 2002). The NuRD complex possesses ATP-dependent nucleosome remodeling activity that is dependent upon Mi-2 and histone deacetylase 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).

mep-1 appears to have multiple functions during *C. elegans* development, because it is necessary for larval viability, gonadogenesis and oocyte production (Belfiore et al., 2002; Unhavaithaya et al., 2002). *mep-1* mutants display abnormal gene expression in larvae, indicating that *mep-1* regulates gene expression. *mep-1* mutants exhibit a partially penetrant Muv phenotype (Belfiore et al., 2002); this phenotype becomes highly penetrant in combination with a synMuv A allele (Unhavaithaya et al., 2002).

Our studies have revealed that the LIN-1 interaction with MEP-1 required the $VK_{10}KE$ and $VK_{169}DE$ consensus sumoylation motifs. Translational fusion of SUMO to LIN-1 mutants lacking these motifs partially restored binding to MEP-1. These findings suggest that sumoylation of LIN-1 allows MEP-1 binding. If MEP-1 is associated with the NuRD complex, then sumoylation of LIN-1 might promote recruitment of the NuRD complex to *lin-1* target genes, resulting in gene silencing.

The genetic analysis of *mep-1* supports this model. Reducing the activity of *mep-1* using RNAi caused a Muv phenotype. The *mep-1(lf)* Muv phenotype was not suppressed by a loss-of-function of *mek-2* or *mpk-1*, indicating that *mep-1* functions downstream or parallel to *mek-2* and *mpk-1*. In addition, reducing *mep-1* function diminished the activity of a constitutively-active LIN-1 mutant, indicating that MEP-1 is necessary for LIN-1 to inhibit vulval cell fates. Thus, *smo-1*, *ubc-9* and *mep-1* all displayed similar genetic properties and function at the level of *lin-1* to inhibit vulval cell fates.

Based on our findings, we propose a model for the inhibition of vulval cell fates by LIN-1. Newly synthesized LIN-1 associates with the E2 SUMO-conjugating enzyme UBC-9 and becomes sumoylated at residues K_{10} and K_{169}. LIN-1 then binds to GGA motifs in target genes that promote the 1° vulval cell fate. The SUMO moieties of LIN-1 interact with MEP-1, leading to recruitment of the NuRD complex. This complex probably induces multiple changes in *lin-1* target genes that promote silencing, including ATP-dependent nucleosome remodeling and histone deacetylation. Sumoylation of LIN-1, even if transient, can cause an enduring change in transcriptional activity by promoting covalent modifications of histones and chromatin restructuring. This may be a general mechanism for SUMO-mediated transcriptional repression, as MEP-1 might interact with the SUMO moieties of additional transcription factors.

smo-1 (ok359) was provided by the International *C. elegans* Gene Knockout Consortium. We thank Ginger Miley for strains, Brooke Lane and Blake Coblentz for technical assistance, and members of the laboratory for reviewing the manuscript. This research was supported by NIH grants R01 CA84271 and F32 GM66605. K.K. is a recipient of a Scholar Award from the Leukemia and Lymphoma Society. E.R.L. was supported by a Keck postdoctoral fellowship.

References

Bartel, P. L., Chien, C.-T., Sternglanz, R. and Fields, S. (1993). In *Cellular Interactions in Development: A Practical Approach* (ed. D. A. Hartley), p. 153. Oxford: Oxford University Press.

Beitel, G. J., Tuck, S., Greenwald, I. and Horvitz, H. R. (1995). The *Caenorhabditis elegans* gene *lin-1* encodes an ETS-domain protein and defines a branch of the vulval induction pathway. *Genes Dev* **9**, 3149-3162.

Belfiore, M., Mathies, L. D., Pugnale, P., Moulder, G., Barstead, R., Kimble, J. and Puoti, A. (2002). The MEP-1 zinc-finger protein acts with MOG DEAH box proteins to control gene expression via the *fem-3* 3′ untranslated region in *Caenorhabditis elegans*. *RNA* **8**, 725-739.

Belfiore, M., Pugnale, P., Saudan, Z. and Puoti, A. (2004). Roles of the *C. elegans* cyclophilin-like protein MOG-6 in MEP-1 binding and germline fates. *Development* **131**, 2935-2945.

Bies, J., Markus, J. and Wolff, L. (2002). Covalent attachment of the SUMO-1 protein to the negative regulatory domain of the c-Myb transcription factor modifies its stability and transactivation capacity. *J. Biol. Chem.* **277**, 8999-9009.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Broday, L., Kolotuev, I., Didier, C., Bhoumik, A., Gupta, B. P., Sternberg, P. W., Podbilewicz, B. and Ronai, Z. (2004). The small ubiquitin-like modifier (SUMO) is required for gonadal and uterine-vulval morphogenesis in *Caenorhabditis elegans*. *Genes Dev.* **18**, 2380-2391.

Dahle, O., Andersen, T. O., Nordgard, O., Matre, V., del Sal, G. and Gabrielsen, O. S. (2003). Transactivation properties of c-Myb are critically dependent on two SUMO-1 acceptor sites that are conjugated in a PIASy enhanced manner. *Eur. J. Biochem.* **270**, 1338-1348.

Desterro, J. M., Rodriguez, M. S., Kemp, G. D. and Hay, R. T. (1999). Identification of the enzyme required for activation of the small ubiquitin-like protein SUMO-1. *J. Biol. Chem.* **274**, 10618-10624.

Desterro, J. M., Thomson, J. and Hay, R. T. (1997). Ubch9 conjugates SUMO but not ubiquitin. *FEBS Lett.* **417**, 297-300.

Fantz, D. A., Jacobs, D., Glossip, D. and Kornfeld, K. (2001). Docking sites on substrate proteins direct extracellular signal-regulated kinase to phosphorylate specific residues. *J. Biol. Chem.* **276**, 27256-27265.

Ferguson, E. L., Sternberg, P. W. and Horvitz, H. R. (1987). A genetic pathway for the specification of the vulval cell lineages of *Caenorhabditis elegans*. *Nature* **326**, 259-267.

Fraser, A. G., Kamath, R. S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M. and Ahringer, J. (2000). Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* **408**, 325-330.

Gill, G. (2003). Post-translational modification by the small ubiquitin-related modifier SUMO has big effects on transcription factor activity. *Curr. Opin. Genet Dev.* **13**, 108-113.

Girdwood, D., Bumpass, D., Vaughan, O. A., Thain, A., Anderson, L. A., Snowden, A. W., Garcia-Wilson, E., Perkins, N. D. and Hay, R. T. (2003). P300 transcriptional repression is mediated by SUMO modification. *Mol. Cell* **11**, 1043-1054.

Greenwald, I. S. (1997). Development of the vulva. In C. elegans *II* (ed. D. L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Preiss), pp. 519-541. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Hong, Y., Rogers, R., Matunis, M. J., Mayhew, C. N., Goodson, M. L., Park-Sarge, O. K., Sarge, K. D. and Goodson, M. (2001). Regulation of heat shock transcription factor 1 by stress-induced SUMO-1 modification. *J. Biol. Chem.* **276**, 40263-40267.

Horvitz, H. R. and Sternberg, P. W. (1991). Multiple intercellular signalling systems control the development of the *Caenorhabditis elegans* vulva. *Nature* **351**, 535-541.

Jacobs, D., Beitel, G. J., Clark, S. G., Horvitz, H. R. and Kornfeld, K. (1998). Gain-of-function mutations in the *Caenorhabditis elegans lin-1* ETS gene identify a C-terminal regulatory domain phosphorylated by ERK MAP kinase. *Genetics* **149**, 1809-1822.

Jacobs, D., Glossip, D., Xing, H., Muslin, A. J. and Kornfeld, K. (1999). Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase. *Genes Dev.* **13**, 163-175.

Johnson, E. S. and Blobel, G. (1997). Ubc9p is the conjugating enzyme for the ubiquitin-like protein Smt3p. *J. Biol. Chem.* **272**, 26799-26802.

Johnson, E. S. and Blobel, G. (1999). Cell cycle-regulated attachment of the ubiquitin-related protein SUMO to the yeast septins. *J. Cell Biol.* **147**, 981-994.

Johnson, E. S., Schwienhorst, I., Dohmen, R. J. and Blobel, G. (1997). The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer. *EMBO J.* **16**, 5509-5519.

Kamath, R. S., Fraser, A. G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., le Bot, N., Moreno, S., Sohrmann, M. et al. (2003). Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* **421**, 231-237.

Kim, J., Cantwell, C. A., Johnson, P. F., Pfarr, C. M. and Williams, S. C. (2002). Transcriptional activity of CCAAT/enhancer-binding proteins is controlled by a conserved inhibitory domain that is a target for sumoylation. *J. Biol. Chem.* **277**, 38037-38044.

Kornfeld, K. (1997). Vulval development in *Caenorhabditis elegans*. *Trends Genet.* **13**, 55-61.

Kornfeld, K., Guan, K. L. and Horvitz, H. R. (1995). The *Caenorhabditis elegans* gene *mek-2* is required for vulval induction and encodes a protein similar to the protein kinase MEK. *Genes Dev.* **9**, 756-768.

Lackner, M. R., Kornfeld, K., Miller, L. M., Horvitz, H. R. and Kim, S. K. (1994). A MAP kinase homolog, *mpk-1,* is involved in ras-mediated induction of vulval cell fates in *Caenorhabditis elegans*. *Genes Dev.* **8**, 160-173.

Lemercier, C., Verdel, A., Galloo, B., Curtet, S., Brocard, M. P. and Khochbin, S. (2000). mHDA1/HDAC5 histone deacetylase interacts with and represses MEF2A transcriptional activity. *J. Biol. Chem.* **275**, 15594-15599.

Lin, X., Sun, B., Liang, M., Liang, Y. Y., Gast, A., Hildebrand, J., Brunicardi, F. C., Melchior, F. and Feng, X. H. (2003). Opposed regulation of corepressor CtBP by SUMOylation and PDZ binding. *Mol. Cell* **11**, 1389-1396.

Miley, G. R., Fantz, D., Glossip, D., Lu, X., Saito, R. M., Palmer, R. E., Inoue, T., van den Heuvel, S., Sternberg, P. W. and Kornfeld, K. (2004). Identification of residues of the *Caenorhabditis elegans* LIN-1 ETS domain that are necessary for DNA binding and regulation of vulval cell fates. *Genetics* **167**, 1697-1709.

Muller, S., Berger, M., Lehembre, F., Seeler, J. S., Haupt, Y. and Dejean, A. (2000). c-Jun and p53 activity is modulated by SUMO-1 modification. *J. Biol. Chem.* **275**, 13321-13329.

Ross, S., Best, J. L., Zon, L. I. and Gill, G. (2002). SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization. *Mol. Cell* **10**, 831-842.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sampson, D. A., Wang, M. and Matunis, M. J. (2001). The small ubiquitin-like modifier-1 (SUMO-1) consensus sequence mediates Ubc9 binding and is essential for SUMO-1 modification. *J. Biol. Chem.* **276**, 21664-21669.

Sapetschnig, A., Rischitor, G., Braun, H., Doll, A., Schergaut, M., Melchior, F. and Suske, G. (2002). Transcription factor Sp3 is silenced through SUMO modification by PIAS1. *EMBO J.* **21**, 5206-5215.

Schiestl, R. H. and Gietz, R. D. (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.* **16**, 339-346.

Schwarz, S. E., Matuschewski, K., Liakopoulos, D., Scheffner, M. and Jentsch, S. (1998). The ubiquitin-like proteins SMT3 and SUMO-1 are conjugated by the UBC9 E2 enzyme. *Proc. Natl. Acad. Sci. USA* **95**, 560-564.

Sternberg, P. W. and Han, M. (1998). Genetics of RAS signaling in *C. elegans*. *Trends Genet.* **14**, 466-472.

Subramanian, L., Benson, M. D. and Iniguez-Lluhi, J. A. (2003). A synergy control motif within the attenuator domain of CCAAT/enhancer-binding protein alpha inhibits transcriptional synergy through its PIASy-enhanced modification by SUMO-1 or SUMO-3. *J. Biol. Chem.* **278**, 9134-9141.

Tan, P. B., Lackner, M. R. and Kim, S. K. (1998). MAP kinase signaling specificity mediated by the LIN-1 Ets/LIN-31 WH transcription factor complex during *C. elegans* vulval induction. *Cell* **93**, 569-580.

Timmons, L. and Fire, A. (1998). Specific interference by ingested dsRNA. *Nature* **395**, 854.

Tong, J. K., Hassig, C. A., Schnitzler, G. R., Kingston, R. E. and Schreiber, S. L. (1998). Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex. *Nature* **395**, 917-921.

Treisman, R. (1994). Ternary complex factors: growth factor regulated transcriptional activators. *Curr. Opin. Genet. Dev.* **4**, 96-101.

Unhavaithaya, Y., Shin, T. H., Miliaras, N., Lee, J., Oyama, T. and Mello, C. C. (2002). MEP-1 and a homolog of the NURD complex component Mi-2 act together to maintain germline-soma distinctions in *C. elegans*. *Cell* **111**, 991-1002.

Vojtek, A. B., Hollenberg, S. M. and Cooper, J. A. (1993). Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* **74**, 205-214.

Wade, P. A., Jones, P. L., Vermaak, D. and Wolffe, A. P. (1998). A multiple subunit Mi-2 histone deacetylase from Xenopus laevis cofractionates with an associated Snf2 superfamily ATPase. *Curr. Biol.* **8**, 843-846.

Wu, Y. and Han, M. (1994). Suppression of activated Let-60 ras protein defines a role of *Caenorhabditis elegans* Sur-1 MAP kinase in vulval differentiation. *Genes Dev.* **8**, 147-159.

Xue, Y., Wong, J., Moreno, G. T., Young, M. K., Cote, J. and Wang, W. (1998). NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. *Mol. Cell* **2**, 851-861.

Yang, S. H., Jaffray, E., Hay, R. T. and Sharrocks, A. D. (2003). Dynamic interplay of the SUMO and ERK pathways in regulating Elk-1 transcriptional activity. *Mol. Cell* **12**, 63-74.

Yang, S. H. and Sharrocks, A. D. (2004). SUMO promotes HDAC-mediated transcriptional repression. *Mol. Cell* **13**, 611-617.

Zhang, Y., LeRoy, G., Seelig, H. P., Lane, W. S. and Reinberg, D. (1998). The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. *Cell* **95**, 279-289.