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The Mi-2 nucleosome-remodeling protein LET-418 is targeted via LIN-1/ETS to the promoter of *lin-39*/Hox during vulval development in *C. elegans*

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Abstract

The fate of the vulval cells in *Caenorhabditis elegans* is specified, at least in part, through a highly conserved RTK/Ras mediated signaling cascade that negatively regulates the activity of the ETS-like transcription factor LIN-1. The Hox gene *lin-39* functions downstream of both, the LIN-3/RTK/Ras pathway and LIN-1 and plays a pivotal role in controlling vulva cell competence and induction. Here we show that LET-418, a *C. elegans* ortholog of the human NuRD component Mi-2, negatively modulates the activity of *lin-39*. LET-418 interacts in vivo with specific regions in the promoter of *lin-39* and this interaction depends on LIN-1. Our data provide evidence for a model in which LIN-1 recruits LET-418/Mi-2 as co-repressor to the promoter of *lin-39*, thereby restricting its activity to the basal levels required in the vulva precursor cells (VPCs) for normal vulval development. Thus, our data suggest that the interaction between LIN-1 and LET-418/Mi-2 may link RTK/Ras signaling with chromatin remodeling and gene expression.

Keywords: *C. elegans*; Vulva; LET-418/Mi-2; NuRD; RTK/Ras; *lin-39*/Hox; LIN-1/ETS

Introduction

The vulva of *Caenorhabditis elegans* is made from the descendants of the three hypodermal blast cells P(5–7).p. These cells are members of the vulval equivalence group P(3–8).p, a set of six cells with the potential to adopt either a vulval or a non-vulval fate. They are referred to as the vulval precursor cells (VPCs). The process of vulval cell specification can be divided into two major sequential steps (for review see Sternberg, 2005). During the first and second larval stages (L1 and L2), the six VPCs are rendered competent to acquire a vulval fate by remaining unfused with the surrounding hypodermal syncytium (hyp7). The other Pn.p cells fuse to the hypodermis and can no longer become vulval cells (Beitel et al., 1995; Sulston and Horvitz, 1977).

The second step of vulval fate specification, termed vulval induction, occurs during the third larval stage (L3). Three out of the six unfused VPCs, through the interaction of Ras, Notch and Wnt signaling pathways, adopt either a 1° (P6.p) or a 2° (P5.p and P7.p) vulval cell fate and undergo series of cell divisions (Sternberg, 2005). The remaining three VPCs (P3.p, P4.p and P8.p) adopt a non-vulval 3° cell fate (Sternberg and Horvitz, 1986). At the beginning of vulval induction, a highly conserved RTK/Ras signaling cascade is activated in P6.p by a LIN-3/EGF signal from the neighboring gonadal anchor cell (AC) through the receptor tyrosine kinase LET-23/RTK, leading to the induction of the 1° vulval fate (Sternberg and Han, 1998). Induction of the 1° vulval fate is negatively regulated by the transcription factor LIN-1/ETS (Beitel et al., 1995). A *lin-1* loss-of-function (lf) mutation causes the ectopic induction of all six Pn.p cells, resulting in a multivulva (Muv) phenotype (Beitel et al., 1995; Ferguson et al., 1987; Lackner et al., 1994). Recently, it was proposed that, in the absence of Ras signaling, sumoylated LIN-1 represses genes required for adoption of induced vulval

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cell fates via recruitment of a chromatin remodeling complex, and that phosphorylation of LIN-1 by MPK-1 relieves this repression and converts LIN-1 into a transcriptional activator (Leight et al., 2005; Wagmaister et al., 2006b).

Another important player for vulval development is the Hox gene *lin-39*. It functions downstream of both, the RTK/Ras signaling pathway and *lin-1*, and plays a pivotal role in controlling vulval cell competence and induction (Chen and Han, 2001; Eisenmann et al., 1998; Maloof and Kenyon, 1998; Shemer and Podbilewicz, 2002). At the L1/L2 larval stages its activity prevents fusion of the VPCs with the hyp7, and loss of *lin-39* at this time causes the VPCs to fuse with hyp7 (Clark et al., 1993; Shemer and Podbilewicz, 2002; Sternberg and Horvitz, 1986; Wang et al., 1993). During vulval induction in the L3 stage, *lin-39* transcription is upregulated in a RTK/Ras-dependent manner (Maloof and Kenyon, 1998; Wagmaister et al., 2006a). Loss of *lin-39* activity at this time causes the VPCs to adopt incorrect vulval fates (Clandinin et al., 1997; Wagmaister et al., 2006a). Several transcription factors regulate *lin-39* expression during vulval development, among them LIN-1. In *lin-1* mutants, *lin-39* expression is upregulated in all VPCs, suggesting that LIN-1 acts as a repressor of *lin-39* (Maloof and Kenyon, 1998; Wagmaister et al., 2006a). Recently, it was shown that LIN-1 binds to the promoter of *lin-39* and acts both negatively and positively on *lin-39* transcription in different VPCs (Wagmaister et al., 2006b).

Vulval induction is also negatively regulated by the SynMuv (Synthetic Multivulva) genes. They encode the components of at least three functionally redundant pathways (SynMuvA, SynMuvB and SynMuvC) that repress the vulval fate in all VPCs (Ceol and Horvitz, 2004). Single lf mutations in either class do not cause any obvious vulval phenotype, whereas the combination between two mutations from different classes (e.g., a class A and a class B mutation) results in an increase of vulval specification of the Pn.p cells and leads to a Muv phenotype (Ceol and Horvitz, 2004; Fay and Han, 2000; Ferguson and Horvitz, 1989). Many SynMuv genes encode transcription and chromatin associated factors, including proteins of the NuRD (nucleosome remodeling and histone deacetylase)-like and Rb-like complexes, suggesting that these complexes may be involved in repression of gene activity. It was shown that at least some SynMuv genes act in the hyp7 to promote vulval fates (Hedgecock and Herman, 1995; Herman and Hedgecock, 1990; Myers and Greenwald, 2005). Recently, Cui et al. (2006) found that the SynMuv A and SynMuv B gene classes are functionally redundant for transcriptional repression of the key target gene *lin-3*, and that ectopic expression of *lin-3* in the hypodermis is the main cause of the SynMuv phenotype.

Here we show that the class B SynMuv gene *let-418* acts as a co-repressor of LIN-1 to negatively regulate the activity of the Hox gene *lin-39*. *let-418* encodes an ortholog of the mammalian Mi-2 protein, a SNF2-like ATP-dependent chromatin remodeler, which has been identified as a specific component of the NuRD complex from human cell lines and *Xenopus* egg extracts (Tong et al., 1998; Wade et al., 1998, 1999; Xue et al., 1998; Zhang et al., 1998). *let-418* is an essential gene that is expressed in most nuclei of the worm and depletion of its activity results in a pleiotropic phenotype that includes vulval defects, sterility and, without the maternal contribution, L1 larval arrest (von Zelewsky et al., 2000). Moreover, *let-418* is required for the maintenance of somatic differentiation (Unhavaithaya et al., 2002).

We found that LET-418 associates in vivo with the promoter of *lin-39*, suggesting that it represses its transcription through chromatin remodeling. LET-418 interacts physically with the transcription factor LIN-1, and its association with the *lin-39* promoter depends on the activity of LIN-1. Upregulation of *lin-39*::*lacZ* expression in the VPCs of *let-418* mutants is independent of *lin-3*, strongly suggesting a cell-autonomous function of *let-418* in the VPCs. Based on our results we propose a model in which LIN-1 recruits LET-418 to the promoter of *lin-39* in the VPCs to keep its transcriptional activity to the basal level required for normal vulva development.

Materials and methods

Nematode strains and culture conditions

Nematodes were grown at 20 °C under standard conditions, unless otherwise indicated. Wild-type strain and parent of all mutant strains was *C. elegans* Bristol N2. Mutations and balancer chromosomes used in this study were: LGIII: *lin-39(n709ts)*, *lin-39(n1760)*, *dpy-18(e364)*, eT1(III;V); LGIV: *lin-1 (e1275ts)*, *lin-1(sy254)*, *muIs6*[*lin-39*::*lacZ*+*pRF4(rol-6d)*]; LGV: *unc-46 (e177)*, *let-418(s1617)*, *let-418(n3536ts)*; LGX: *lin-15A(n767)*, *lin-1(e1275ts)*, *swEx582[lin-1*::*gfp]*.

RNAi by feeding

Primers used for PCR amplification of the cDNAs were 5′-GTATC-CATGGGCTGCACACGCCAATCGTCA-3′ and 5′-AGTTCCATGGTCCAT-TTTCAGACATGAATT-3′ for *let-418*, 5′-CATCATCCGTCGACTCAATC-3′ and 5′-ACGATGGGAACTTGAACGAG-3′ for *lin-1* and 5′-CATCTCGGA-GACGGAAAATC-3′ and 5′-GTTGGCGGAATATGTTTTGG-3′ for *lin-15A*. The PCR amplified cDNA sequences of *let-418* (440 bp) and *lin-1* (1206 bp) were inserted into the vector pPD129.36 (gift from A. Fire), those of *lin-15A* (930 bp) in the vector pYZT. *let-418(RNAi)* and *lin-1(RNAi)* strongly phenocopied the mutant phenotype.

Construction of pEXPR/LIN-1::*GFP*

The vector pEXPR/LIN-1::GFP was constructed based on a previous report by Beitel et al. (1995). For cloning we used the Invitrogen Gateway Technology. A 15 kb long *lin-1* genomic DNA fragment was amplified with the Expand Long template PCR System from Roche, using the primers lin-1attB1 left and lin-1attB2 right. These primers contained a B1 or B2 Att site for the generation of a pENTRlin-1 entry vector by BP recombination of the PCR product and pDONR201 donor vector. We then generated a pDEST-GFP destination vector using the Gateway Vector Conversion System by inserting a RfA cassette in the *Sma*I site of the pPD95.77 gfp reporter plasmid from the Fire Lab Vector Kit. Finally the pEXPR/LIN-1::GFP vector was obtained by LR recombination of the pENTRlin-1 and the pDEST-GFP vectors. Primers were lin-1attB1left: 5′ ggggacaagtttgtacaaaaaagcaggctagttttcttgtttcgggaag 3′, lin-1attB2right: 5′ ggggaccactttgtacaagaaagctgggtccaaagttggcatttttatgg 3′.

Generation of LET-418 antibodies

Rat and rabbit anti-LET-418 antibodies were generated using LET-418 specific peptides. The antibodies recognized a protein of the expected size in extracts from wild-type worms, that was absent in extracts from *let-418(s1617)* animals (data not shown).

Protein co-immunoprecipitation

Co-immunoprecipitation experiments were made as previously described (Chuang et al., 1996; Rocheleau et al., 1999; Takacs-Vellai et al., 2005). Wild-type, *lin-1(e1275ts)*, *lin- 1(e1275ts);swEx582*[*lin-1*::*gfp*], mixed stage worms were homogenized in lysis buffer (25 mM HEPES NaOH, pH 7.4, 150 mM NaCl, 1 mM DTT, 0.5% Triton X-100, 1 mM EDTA NaOH and protease inhibitor cocktail, Roche) using a 5 mm POLYTRON stainless steel homogenizer (Kinematica AG). After centrifugation of the worm carcasses for 15 min at 4 °C, approximately 1 mg of protein extract from the supernatant was used for immunoprecipitation in combination with 1 μg of antibodies or serum and 10% protein G sepharose beads (Zymed). Following overnight incubation at 4 °C on a rotating wheel, several washes in PBS were performed. The proteins in the incubated lysates (beads) were separated by gel electrophoresis, immunoblotted as described (Sambrook and Maniatis, 1989) and revealed using either rat or rabbit anti-LET-418 antibodies at a dilution of 1:500. Co-immunoprecipitation of LIN-1::GFP was done using mouse anti-GFP native antibodies (Obiogene).

Antibody staining

Synchronized L1 larvae containing a *lin-39*::*lacZ* fusion construct integrated on Chr. IV (*muIs6*[*lin-39*::*lacZ*+*pRF4(rol-6d)*], Wang et al., 1993) were grown at 25 °C on different RNAi feeding plates. Late L2 animals were fixed and co-stained with anti-β-galactosidase antibody (dilution 1/500, Promega), anti-MH27 mouse antibody (dilution 1/1000, DSHB, University of Iowa) and DAPI as described in Chen and Han (2001).

Real-time RT PCR analyses

The quantitative analysis of *lin-39* expression was performed by real-time RT-PCR. For each experiment, total RNA was isolated from 30 wild-type, *let-418 (s1617)* or *lin-1(sy254)* tightly synchronized late L2 stage larvae. Each RNA sample was split into 3 identical RT-PCR reactions. The *lin-39* mRNA levels were determined using the Qiagen SybrGreen RT-PCR system on Rotorgene 2000 (Menzel et al., 2004). For each genotype, the mRNA levels of the reference gene *gpd-1* were determined as an internal standard by using the same protocol. The mean values from two independent experiments were calculated and normalized using *gpd-1* as internal standard. Primers were: 5′-cctggaaggagacgatgatg-3′ and 5′-cgcgtgaacctcctgtagtt-3′ for *lin-39* and 5′-aaaggacacggttcaagtgg-3′ and 5′-acaacgaaatcggctttgac-3′ for *gpd-1*.

Chromatin immunoprecipitation (ChIP) and quantitative PCR

ChIP experiments were adapted from Chu et al. (2002). For all experiments, mixed stage worms were used to be sure that age and tissue distribution of the animals from which the extracts were done is identical. Wild-type worms were grown at 25 °C. *let-418*(*n3536ts*) or *lin-1(n1275ts)* worms were cultivated at 15 °C, then shifted to 25 °C for 24 h prior to harvest them. As controls, *let-418*(*n3536ts*) or *lin-1(n1275ts)* worms were grown in parallel at 15 °C. The worms were fixed in M9 buffer containing 2% formaldehyde at room temperature for 30 min. Excess formaldehyde was quenched and removed with a 0.1 M Tris–HCl (pH 7.5) wash followed by two M9 washes. Worm lysates were prepared by sonication in ChIP lysis buffer and protease inhibitor cocktail (Roche). Cellular debris were cleared by centrifugation. The average sonicated chromatin size of the fragments tested each time was around 500 bp (data not shown). The sonified chromatin was centrifuged at 14,000 rpm for 20 min and the supernatants, containing soluble chromatin fragments were saved. The chromatin fractions were precleared twice against Protein G Sepharose (ZyMed) and kept for 4 h at 4 °C on a rotating plate. The suspensions were then centrifuged at 14,000 rpm for 30 s to discard nonspecifically-bound chromatin fragments. Aliquots from the supernatant (equivalent to 50 μg DNA) were taken in equal portions for each ChIP reaction and an additional 1% volume of such a portion was saved as input control. Each reaction was incubated 4 h/overnight with either 4 μg of the corresponding affinity-purified antibody except 2 μg of acetylated histone 3 antibody or without antibody as control. After clearing non-specific aggregates by centrifugation at 14,000 rpm, the immunocomplexes were captured with Protein G Sepharose, subjected to two 1 ml ChIP low salt buffer washes, two 1 ml ChIP high salt buffer washes and two 1 ml TE buffer washes and finally eluted with 1% SDS, 0.01 M Tris–HCl (pH 8.0). For ChIP analysis, formaldehyde crosslinks were reversed by incubation at 65 °C overnight in 0.2 M NaCl. Proteins were removed by proteinase K digestion and the DNA was purified with QIAquick PCR products purification kit (QIAGEN). For input DNA control, the total DNA was extracted from 1% of starting lysates as described above. As negative control we performed ChIP with no antibody (mock-IP), which gave the same negative results as ChIP performed with normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, California; 4 mg/ml) as serum control (results not shown). ChIP experiments with the anti-acetylated histone 3 antibody (Upstate, 06-599, 2 mg/ml) were performed as positive control. For each set of experiments, the number of PCR cycles was adjusted until no or only a very weak signal was detected for the mock-IP DNA (no antibody). PCR products resolved on a 2% agarose gel were visualized by ethidium bromide staining and ranged from 30 to 35 cycles for different primer pairs. ChIP performed on the unrelated gene *ntl-1* gave no DNA signals. ChIP experiments and PCR amplifications were performed at least twice for each sample.

Promoter sequence analysis

The alignment of the *lin-39* promoter sequences from *C. elegans* and *C. briggsae* was performed using the online softwares LALIGN (Huang and Miller, 1991) (http://www.ch.embnet.org/software/LALIGN_form.html) and VISTA (Mayor et al., 2000) (http://www-gsd.lbl.gov/vista/index.shtml). Sequences conserved between the two species were identified with the Matinspector software (Quandt et al., 1995) (http://www.gene-regulation.com/; http://www-gsd.lbl.gov/vista/index.shtml) using the Transfac database (Wingender et al., 2000).

Results

LET-418 promotes Pn.p cell fusion and represses vulval induction by negatively controlling lin-39 activity

In wild-type animals, all VPCs P(4–8).p and about 50% of the P3.p cells remain unfused (Sternberg and Horvitz, 1986). In *let-418(RNAi)* animals, however, we observed a significant increase of the number of unfused P3.p cells. Whereas in wild-type animals 54% of the P3.p cells remained unfused (n=24), *let-418(RNAi)*-depleted animals showed 85% (n=20) and *let-418(n3536ts)* mutants 90% (n=41) of unfused P3.p cells. This suggested that *let-418* might negatively regulate the activity of *lin-39*. To further investigate this issue, we used *lin-39* mutants carrying the weak temperature-sensitive allele *n709ts*. At the restrictive temperature of 25 °C, *lin-39(n709ts)* activity was reduced, resulting in an increased level of P(3–8).p fusion (Table 1). We observed a significant rescue of the fusion phenotype in all VPCs P(3–8).p in *lin-39(n709ts)*;*let-418 (RNAi)* worms (Table 1). Remarkably, *let-418(RNAi)* was not able to rescue the fusion phenotype of *lin-39(n1760)* null mutants (Table 1), indicating that minimal levels of *lin-39* activity are required. *lin-1(RNAi)* also rescued the fusion phenotype of *lin-39(n709ts)* mutant animals (Table 1 and Chen and Han, 2001), suggesting that *lin-1*, like *let-418*, negatively controls *lin-39* activity.

During the L3 larval stage of wild-type animals, *lin-39* activity is upregulated in P(5–7).p by EGF/RTK/Ras signaling from the gonadal AC (Maloof and Kenyon, 1998; Wagmaister

Table 1
Depletion of *let-418* rescues the fusion phenotype of lin-*39(rf)* mutants

Genotype	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	Number
Wild-type	54	100	100	100	100	100	24
RNAi control	52.5	100	100	100	100	100	40
lin-39(n709ts)	30	75	92	98	84	57	63
lin-39(n709ts); RNAi control	34	74	91	97	77	54	35
lin-39(n709ts); let-418(RNAi)	56*	90*	100*	100	93	76*	70
lin-39(n709ts); lin-1(RNAi)	59*	91*	100	100	95	77*	44
lin-39(n1760)	0	9	11	11	11	7	46
lin-39(n1760); let-418(RNAi)	0	10	10	10	10	7	20

L2 larvae, grown at 25 °C, were stained with anti-MH27 antibodies that specifically mark the adherens junction of unfused cells. Numbers indicate the percentage of unfused Pn.p cells. *$p < 0.05$. p values were derived from comparing data from *lin-39(n709ts)* animals to those of *lin-39(n709ts);let-418 (RNAi)* or *lin-39(n709ts);lin-1(RNAi)* animals using Fisher's Exact Test. As RNAi control, the *E. coli* strain HT115 transformed with the empty vector pPD129.36 was used.

et al., 2006a). High levels of LIN-39 are required for the induction of the vulval fates in these cells. At restrictive temperature, the reduced activity of *lin-39(n709ts)* results in an incomplete induction of the P(5–7).p cells (Clandinin et al., 1997). We found that depletion of *let-418* by RNAi partially restored the induction defects of the P(5–7).p descendants in *lin-39(n709ts)* mutants (Table 2). In summary, our data suggested that the rescue of the *lin-39(n709ts)* phenotype through loss of *let-418* function is due to an increase of *lin-39* activity, indicating that *let-418* may function as a negative regulator of *lin-39* activity in the VPCs or act upstream of *lin-39*.

Table 2
Depletion of *let-418* partially rescues *lin-39(rf)* vulval induction defects

Genotype	P5.p	P6.p	P7.p	*n*
Wild-type	0.0	100.0	0.0	
	100.0	0.0	100.0	
	0.0	0.0	0.0	
				120
lin-39(n709)	0.0	93.2	0.0	
	69.9**	0.0*	65.0**	
	30.1**	6.8*	35.0**	
				103
lin-39(n709); RNAi control	0.0	91.7	0.0	
	71.7	0.0	65.0	
	28.3	8.3	35.0	
				60
lin-39(n709); let-418(RNAi)	0.0	97.6	0.0	
	90.6**	0.0	81.2*	
	9.4**	2.4	18.8*	
				85

The numbers indicate % of induction. Animals were grown at 25 °C. The vulval induction levels of the P5.p, P6.p and P7.p cells and there descendents were scored by observing mid-L4 larvae under DIC Nomarski optics as described in Chen and Han (2001).
lin-39(n709), p values were derived from comparing data from *lin-39(n709)* to those from wild-type animals, the others were derived from comparing data from *RNAi*-depleted animals to those from *lin-39(n709)* animals in a Fisher's Exact Test. As RNAi control, the *E. coli* strain HT115 transformed with the empty vector pPD129.36 was used.
* $p < 0.05$.
** $p < 0.001$.

LET-418 regulates lin-39 expression

To test the influence of depletion of *let-418* on *lin-39* expression in the VPCs, we analyzed the vulval specific expression pattern of an integrated *lin-39*::*lacZ* reporter transgene (Wang et al., 1993). This construct contained about 12 kb of the upstream *lin-39* promoter region, and was frequently expressed in P6.p to P8.p and at lower levels also in P3.p to P5.p of late L2 stage animals (see Table 3). Using anti-β-galactosidase antibodies, we determined the frequency of visible *lin-39*::*lacZ* expression in each VPC of wild-type and *let-418(RNAi)* late L2 larvae. The percentage of *lin-39*::*lacZ* expressing VPCs was significantly higher in *let-418(RNAi)* animals than in wild-type worms or in control worms treated with the empty RNAi vector (Table 3). RNAi depletion in the SynMuv A gene *lin-15A*, however, caused no significant change in *lin-39*::*lacZ* expression as compared to wild-type animals (Table 3). Moreover, *lin-1(RNAi)* animals had a similar increase in the *lin-39*::*lacZ* expression frequency as *let-418-*

Table 3
let-418 negatively regulates *lin-39* expression

Genotype	*lin-39*::*lacZ* expression (% expressing cells)						
	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	*n*
lin-39::*lacZ*							
Wild-type	7.4	22.2	33.3	66.7	74.1	66.7	27
RNAi control	10.5	21.1	31.6	57.9	84.2	68.4	19
let-418/(RNAi)	33.3*	55.6*	88.9**	94.4*	83.3	89	18
lin-1(RNAi)	13	52.2*	69.6*	91.3*	78.3	87	23
lin-15A(RNAi)	14.3	19.1	38.1	61.9	76.2	76.2	22
lin-39::*lacZ;let-418ts*							
RNAi control at 15 °C	9.1	27.3	36.4	54.6	72.7	77.3	22
lin-3(RNAi) at 15 °C	4.4	21.7	43.5	52.2*	69.6	73.9	23
RNAi control at 25 °C	34.8*	60.9*	87.0*	95.7*	82.6	91.3	23
lin-3(RNAi) at 25 °C	30.4	65.2*	82.6**	91.3**	87	87	23

An integrated *lin-39*::*lacZ* strain (see Materials and methods) was grown at 25 °C on different RNAi feeding plates. *lin-39*::*lacZ;let-418ts* worms were grown, either at 15 °C or 25 °C, on empty vector or *lin-3(RNAi)* feeding plates. *lin-3* has no effect on the *lin-39*::*lacZ* expression. However, control worms grown to adulthood at 15 °C or 25 °C on *lin-3(RNAi)* feeding plates showed 100% of Vul phenotype (each case $n > 100$), indicating that *lin-3(RNAi)* was effective.
To determine the levels of reporter gene expression in the VPCs, late L2 larvae were co-immunostained with an anti-β-galactosidase antibody and the MH27 monoclonal antibody. Only animals that had positive MH27 antibody staining in VPCs and had at least one VPC stained with anti-β-galactosidase were counted. p values were derived from comparing data from RNAi-depleted animals to those from animals treated with the empty RNAi vector using Fisher's Exact Test. For the *lin-3* experiment, p values were derived from comparing data from empty or *lin-3(RNAi)* depleted animals to those from empty vector RNAi treated animals at 15 °C.
* p value < 0.05.
** p value < 0.001.

depleted worms in the VPCs (Table 3). Corresponding results were obtained using a shorter, partially rescuing *lin-39*::*gfp* fusion construct carrying about 5 kb of upstream sequences (results not shown). Altogether, these results suggested that LET-418, like LIN-1, negatively controls *lin-39* expression in the VPCs.

To further assess *lin-39* expression in the *let-418* mutant background, we performed a real-time RT-PCR experiment with total RNA isolated from wild-type, *let-418* and *lin-1* late L2 larvae before vulval induction. As an internal standard the housekeeping gene *gpd-1* was used. In agreement with the previous results, we found a significant increase of *lin-39* mRNA in *let-418(s1617)* and *lin-1(sy254)* mutants as compared to wild-type animals (4.2±0.9SE fold for *let-418* and 5.5±2.0SE fold for *lin-1*, Fig. 1). A *lin-39*::*gfp* reporter gene was not only expressed in the VPCs, but also in other cells of the central body region, including cells of the ventral nerve cord, the myoblasts and their descendents, a few neurons in the region of the pharynx and some intestinal cells (data not shown). Consistent with the RT-PCR results, we noticed an increase of the reporter gene expression in many of these cells in *let-418* and *lin-1*-depleted animals as compared to wild type worms (data not shown). However, no obvious difference in cell type expression between WT and *let-418* animals was observed. We have also compared wild-type and *let-418* mutant animals stained with anti-LIN-39 antibodies whose staining pattern corresponded to that of the *lin-39*::*gfp* reporter. In agreement with the results from the *lin-39*::*gfp* expression study and the real-time RT-PCR experiment, we noted an increased antibody staining in the VPCs and in many of the *lin-39* expressing cells of both, *let-418* and *lin-1*-depleted animals as compared to WT animals (data not shown). An increase of LIN-39 expression in *lin-1* mutant animals stained with anti-LIN-39 antibodies was previously reported (Maloof and Kenyon, 1998). In summary, our results suggested that LET-418 and LIN-1 control the level of *lin-39* expression not only in the VPCs, but also in many other *lin-39* expressing cells during development.

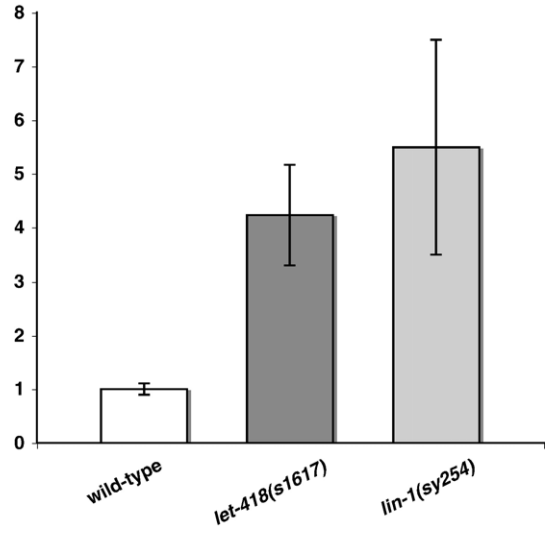

Fig. 1. *lin-39* expression is increased in *let-418(s1617)* and *lin-1(sy254)* mutants. Quantitative RT-PCR experiments revealed a significant increase of the *lin-39* mRNA levels in *let-418(s1617)* (4.2±0.9SE) and *lin-1(sy254)* (5.5±2SE) mutants as compared to wild-type animals. The mean values for each genotype were obtained from two independent experiments and normalized against those of the housekeeping gene *gpd-1* used as internal standard (see Materials and methods). Bars represent fold enrichment relative to the wild-type value that has been set to 1 for clarity.

lin-39 is a direct target of LET-418

Mi-2 proteins from vertebrate and flies have been proposed to exhibit a transcriptional repressor activity through chromatin remodeling (Marhold et al., 2004; Xue et al., 1998; Zhang et al., 1999). In order to test whether LET-418 directly regulates *lin-39* expression by binding to its promoter, we performed chromatin immunoprecipitation (ChIP) experiments. First we searched for putative regulatory regions in the promoter of *lin-39* by comparing the genomic sequences of the *C. elegans lin-39* gene and its ortholog in the related species *C. briggsae* (Genome Sequencing Centre, Washington University, St. Louis, MO, USA). Whereas intergenic sequences have diverged considerably between the two nematode species, conservation of both sequence and function of regulatory elements has been shown for a number of genes (Gilleard et al., 1997; Kennedy et al., 1993; Krause et al., 1994; Xue et al., 1992). Alignment of the *C. elegans* and *C. briggsae* genomic sequences using the LALIGN (Huang and Miller, 1991) and the VISTA (Mayor et al., 2000) online software revealed several significantly conserved regions with sequence identities of over 50% (not shown) that were located within the first 6 kb of upstream promoter sequences of *lin-39*. Regions III (positions −5636 and −5438 upstream of the START codon) and IV (positions −3445 to −3154) were chosen to test for LET-418 binding (Fig. 2A). Region IV contained the most conserved sequences in the promoter of *lin-39* and region III was part of a promoter fragment recently identified to contain a RTK/Ras responsive element necessary for LIN-39::GFP expression in P6.p (Wagmaister et al., 2006b). As negative controls we used two DNA fragments located about 12.5 kb (region I: positions −12,673 to −12,524) and 8.5 kb (region II: positions −8729 to −8468) upstream of the *lin-39* coding region that did not contain conserved sequences (Fig. 2A) and a fragment of the unrelated gene *ntl-1* (Collart and Struhl, 1994; Tucker et al., 2002) (see Fig. 2C).

The ChIP experiments were performed by immunoprecipitating total sonicated chromatin with anti-LET-418 antibodies. We found an association of LET-418 with the two conserved regions III and IV in wild-type animals (see Fig. 2B). This binding was specific, since PCR amplifications of the *lin-39* promoter regions I and II and the control fragment of the gene *ntl-1* revealed no DNA bands (Fig. 2B). To further confirm the specificity of the association of LET-418 with fragments III and IV, we performed ChIP experiments with *let-418(ts)* animals. As expected, PCR bands were present only at the permissive temperature of 15 °C but not at the restrictive temperature of 25 °C (Fig. 2B). Altogether, our data show that LET-418 associates specifically with

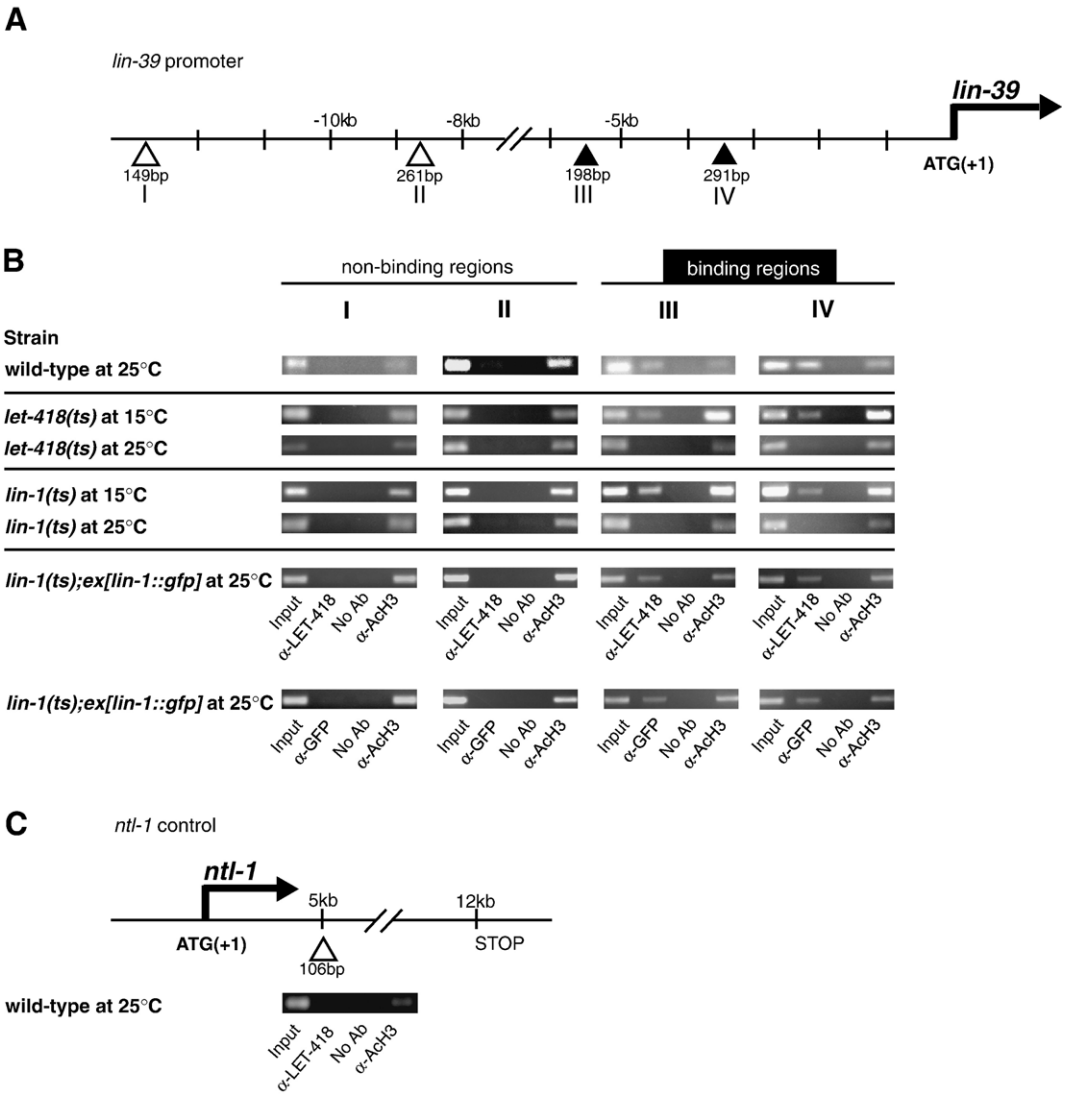

Fig. 2. The *C. elegans* protein LET-418 associates specifically with at least two conserved regions of the *lin-39* promoter. (A) The promoter sequences of the Hox gene *lin-39* from *C. elegans* and *C. briggsae* share several conserved regions with over 50% similarity that are likely to represent important regulatory elements. Two of them (regions III and IV) were chosen to test for LET-418 binding. Regions I and II, located about 12 and 8 kb upstream of the *lin-39* START codon, contain no obvious conserved sequences and were therefore used as negative controls for the ChIP experiments. Localization and length of the *lin-39* promoter fragments used for the ChIP experiments are indicated. (B) The *C. elegans* protein LET-418 associates specifically with the *lin-39* promoter regions III and IV, and this association depends on LIN-1. DNA from chromatin immunoprecipitated with rabbit anti-LET-418 was amplified by PCR and analyzed on agarose gels. Chromatin precipitated with anti-acetyl-histone H3 antibodies, that recognized acetylated K9 and 14, was used as positive control for the ChIP experiments. LET-418 binds *lin-39* promoter regions III and IV, but not the non-conserved control regions I and II. In *let-418(n3536ts)* animals, association of LET-418 with regions III and IV was observed only at the permissive temperature of 15 °C, but not at the restrictive temperature of 25 °C. Binding of LET-418 depended on the activity of LIN-1, since in *lin-1(e1275ts)* animals LET-418 associated with the fragments III and IV only at the permissive temperature of 15 °C but not at the restrictive temperature of 25 °C. The rescuing construct *lin-1*∷*gfp*, however, was able to restore LET-418 binding in these animals at restrictive temperature. Consistently, the LIN-1∷GFP fusion protein bound to promoter fragments III and IV, but not to the control fragments I and II. (C) LET-418 does not bind randomly to genomic DNA as shown by the coding region of the gene *ntl-1*.

regions III and IV of the *lin-39* promoter, suggesting a direct transcriptional control.

LET-418 is targeted to the lin-39 promoter by the transcription factor LIN-1/ETS

It has been proposed that the vertebrate and insect Mi-2 complexes are brought to their sites of repression by the action of specific transcription factors (Bowen et al., 2004; Ng and Bird, 2000; Struhl, 1998). Since the transcription factor LIN-1 and LET-418 are both required for the negative control of *lin-39* expression, and since depletion of either of them results in de-repression of *lin-39* transcription (Fig. 1 and Table 3), we hypothesized that a LET-418 containing repressor complex could be recruited to the promoter of *lin-39* by the action of LIN-1. To test this hypothesis, we first checked whether LET-418 interacts with LIN-1. Since we were unable to obtain reliable anti-LIN-1 antibodies, we generated a translational

lin-1::*gfp* fusion construct that contains a 15 kb long genomic *lin-1* fragment (see Materials and methods). On Western blots loaded with total protein extracts from *lin-1(n1275ts);swEx582* [*lin-1*::*gfp*] animals, anti-GFP-antibodies detected a faint band corresponding to the predicted length of the LIN-1::GFP fusion protein (data not shown). Furthermore, the *lin-1*::*gfp* transgene was able to rescue the Muv phenotype of *lin-1 (e1275ts)* animals, suggesting that the LIN-1::GFP fusion protein is functional. To eliminate endogenous LIN-1, we shifted the *lin-1(n1275ts);swEx582*[*lin-1*::*gfp*] animals for two generations at the restrictive temperature of 25 °C before performing the co-immunoprecipitation experiments. Using anti-GFP antibodies we were able to co-immunoprecipitate LET-418, demonstrating that the two proteins interact in vivo (Fig. 3). If LIN-1 recruits LET-418 to the promoter of *lin-39*, we expected that LET-418 binding should be abolished in *lin-1 (n1275ts)* mutants. We tested this assumption by performing ChIP experiments using *lin-1(ts)* animals at both, permissive and restrictive temperature. Whereas LET-418 bound the *lin-39* promoter regions III and IV in *lin-1(ts)* extracts at 15 °C, this association was abolished in *lin-1(ts)* animals at 25 °C (Fig. 2B). The rescuing translational fusion construct *lin-1*::*gfp* was able to restore LET-418 binding in the *lin-1(ts)* background at 25 °C, and as expected, LIN-1::GFP bound to the promoter regions III and IV (Fig. 2B). Altogether, the results suggested that a complex containing LET-418 is targeted by the transcription factor LIN-1 to the promoter of *lin-39*.

Control of lin-39 represents a cell-autonomous function of let-418 in the VPCs

Recently it was shown that at least some of the SynMuv genes are functionally redundant for transcriptional repression of the key target gene *lin-3* in the hypodermis, and that ectopic expression of *lin-3* in the hypodermis is the main cause of the SynMuv phenotype (Cui et al., 2006). To test whether the SynMuv phenotype of *let-418* also depends on *lin-3* we have depleted *let-418(ts);lin-15A* mutant animals with *lin-3(RNAi)*. We observed a significant reduction of the SynMuv phenotype, similar to that of *lin-15AB* mutants treated with *lin-3(RNAi)* as controls (Fig. 4). These results suggest that *let-418* is acting upstream of the inductive signaling pathway to produce the SynMuv phenotype.

To test, whether the upregulation of *lin-39*::*lacZ* in the VPCs of *let-418* mutants is also *lin-3* dependant, we crossed the *lin-39*::*lacZ* reporter into a *let-418(ts)* mutant background.

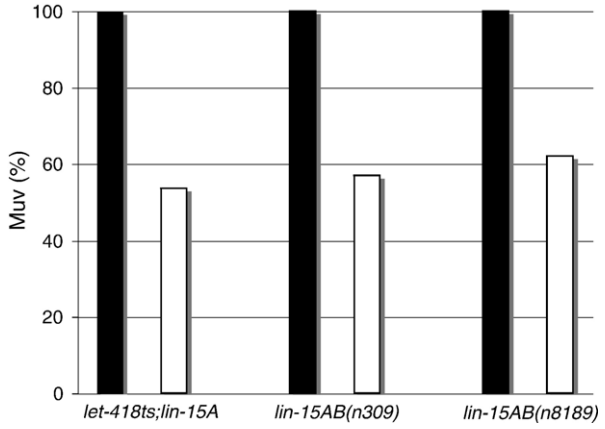

Fig. 4. The SynMuv phenotype of *let-418;lin-15A* mutants is reduced in *lin-3(RNAi)*-depleted animals. *N2*, *let-418;lin-15A* or *lin-15AB* animals were fed either with bacteria containing an empty vector (black filled columns) or bacteria expressing *lin-3* dsRNA (white columns). Worms showing two or more prominent ventral protrusions were scored as Muv. *p* values for all *lin-3(RNAi)* data are <0.001 (derived from comparing data from *lin-3(RNAi)*-depleted animals to those from control animals using Fisher's Exact Test).

At the restrictive temperature of 25 °C the expression levels of *lin-39*::*lacZ* in the VPCs were elevated as previously seen in *let-418(RNAi)* animals (Table 3). Depletion of these worms with *lin-3(RNAi)* did not change the expression levels of the reporter gene, suggesting that the increase of *lin-39*::*lacZ* expression is independent of *lin-3*. Altogether, these experiments provide strong evidence for a cell-autonomous function of *let-418* in the VPCs.

Discussion

Here we show that the Hox gene *lin-39*, a key regulator for vulval development, is a direct target of LET-418, a *C. elegans* ortholog of the mammalian chromatin remodeling protein Mi-2. LET-418 interacts with the promoter of *lin-39*, suggesting that it directly controls its transcription in the VPCs and in other cells during development. Interaction of LET-418 with the *lin-39* promoter depends on the transcription factor LIN-1/ETS, a direct downstream target of the inductive RTK/Ras signaling pathway during vulva development. Based on these findings, we propose a model in which LET-418 functions as co-repressor of LIN-1 to negatively regulate the expression of *lin-39*. This model links RTK/Ras signaling to chromatin remodeling via a LIN-1 and LET-418 containing NuRD-like complex.

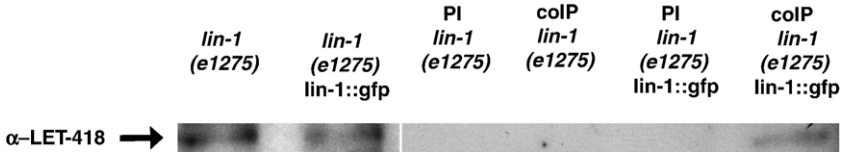

Fig. 3. LET-418 forms a complex with LIN-1. LET-418 co-immunoprecipitates with LIN-1::GFP. Western blot probed with rabbit anti-LET-418 antibodies. Lane 1: total extract from *lin-1(e1275ts)* animals (2% input); lane 2: total extract from *lin-1(e1275ts);lin-1*::*gfp* animals (2% input); lane 3: total *lin-1(e1275ts)* worm extracts immunoprecipitated with non-immune total mouse IgG as control; lane 4: total *lin-1(e1275ts)* mutant extracts immunoprecipitated with mouse anti-GFP antibodies; lane 5: total *lin-1(e1275ts);lin-1*::*gfp* worm extracts immunoprecipitated with non-immune total mouse IgG as control; lane 6: total *lin-1(e1275ts);lin-1*::*gfp* worm extracts immunoprecipitated with mouse anti-GFP antibodies.

LET-418 and LIN-1 negatively control the expression of lin-39

RNAi depletion of *let-418* partially rescued the VPC fusion defects and the vulval induction phenotype of the hypomorphic *lin-39* allele *n709ts*, indicating that *let-418* suppresses the *lin-39* activity in the VPCs during normal vulval development. To further investigate this issue, we analyzed the expression patterns of two integrated *lin-39* reporter constructs that were variably expressed in Pn.p cells during the late L2 stage of wild-type worms. We found a significant increase of the number of VPCs expressing these reporter genes in *let-418*-depleted animals (Table 3 and results not shown). Upregulation of endogenous *lin-39* expression in the VPCs of *let-418* animals was confirmed by using anti-LIN-39 antibody staining (not shown). Increased expression levels were also observed in other *lin-39* expressing cells of *let-418*-depleted animals, but interestingly no obvious ectopic expression was found. A general increase of *lin-39* expression in *let-418* mutant animals was confirmed by performing whole animal real-time RT-PCR experiments (Fig. 1). In summary, we found that *let-418* negatively controls the *lin-39* expression levels in the VPCs and in many other cells normally expressing *lin-39* during development.

LET-418 suppresses lin-39 transcription by LIN-1/ETS mediated promoter binding

By performing ChIP experiments we found that LET-418 specifically associated with the *lin-39* promoter fragments III and IV (see Fig. 2B). This binding depended on the transcription factor LIN-1, which in vivo forms a complex with LET-418. No LET-418 association with the *lin-39* promoter was observed in *lin-1(ts)* mutants at the restrictive temperature of 25 °C, but the rescuing fusion construct *lin-1*::*gfp* was able to restore LET-418 binding in these animals. Furthermore, ChIP experiments confirmed in vivo binding of LIN-1::GFP to the *lin-39* promoter fragments III and IV (Fig. 2B). In agreement with our data, it was recently shown that LIN-1 binds in vitro to several sites within a 1.3 kb long *lin-39* promoter fragment surrounding our promoter fragment III (fragment pMJ5 in Wagmaister et al., 2006b). Both proteins, LET-418 and LIN-1, are required for the negative control of the *lin-39* activity, and depletion of either of them results in de-repression of *lin-39* transcription (Fig. 1, Table 3 and Maloof and Kenyon, 1998). Altogether, these data provide evidence for a model in which LIN-1 and LET-418 form an inhibitory complex that binds to specific regions in the promoter of *lin-39* and negatively controls its transcriptional activity.

The vertebrate protein Mi-2 exerts its inhibitory function on gene expression as a member of the NuRD complex (Xue et al., 1998; Zhang et al., 1999). Since the genome of *C. elegans* encodes several homologs of vertebrate NuRD components, the existence of a worm NuRD-like complex was suggested. We found that one of these homologs, HDA-1, co-immunoprecipitated with LET-418 (unpublished data) and also repressed the expression of an integrated *lin-39*::*gfp* fusion construct in the VPCs (unpublished data and Chen and Han, 2001). Moreover, the class B SynMuv gene *lin-53*/RbAp48, that encodes another *C. elegans* ortholog of a mammalian NuRD component, also negatively regulates *lin-39* expression (Chen and Han, 2001). Taken together, these findings provide indirect evidence for a *C. elegans* NuRD-like complex that binds to the promoter of *lin-39* and negatively controls its transcription in the non-induced P(3–8).p cells, likely through histone modification and chromatin remodeling. Besides LET-418, HDA-1 and LIN-53/RbAp48, this putative NuRD-like complex is likely to involve other proteins encoded by the SynMuvB pathway, however its exact composition remains to be determined.

Regulation of lin-39 transcription in the VPCs by LET-418 is not redundant with the SynMuvA genes

Recently it was shown that some of the SynMuv A and SynMuv B genes are functionally redundant for transcriptional repression of the key target gene *lin-3/EGF*, and that ectopic expression of *lin-3* in the hypodermis is the main cause of the SynMuv phenotype (Cui et al., 2006). We observed a significant reduction of the SynMuv phenotype of *let-418;lin-15A* mutant animals depleted with *lin-3(RNAi)*, suggesting that *let-418* is also acting upstream of the inductive signaling pathway, probably in the hypodermis, to produce the SynMuv phenotype. In contrast, the upregulation of *lin-39*::*lacZ* in the VPCs of *let-418*-depleted animals was not dependent on LIN-3, suggesting that the transcriptional control of *lin-39* by LET-418 in the VPCs represents a cell-autonomous function. Neither a SynMuv A nor a SynMuv B mutation alone caused a significant increase in *lin-3* transcription, suggesting that the SynMuv A and SynMuv B genes may encode independent transcriptional regulatory complexes that repress *lin-3* directly or act on direct regulators of *lin-3* (Cui et al., 2006). Upregulation of *lin-39*, however, occurs in *let-418* single mutants, and *lin-15(RNAi)* depletion alone had no effect. Furthermore, *lin-15A(n767);let-418(RNAi)* double mutants did not display a further increase of the *lin-39* expression levels (data not shown). These data demonstrate that the regulation of *lin-39* by *let-418* is not redundant with the SynMuvA class of genes.

LET-418 associates in an LIN-1-dependent manner to two different promoter regions of *lin-39* (III and IV in Fig. 2B). This suggests that a LET-418 containing NuRD-like complex mediates silencing by binding to multiple sites within the *lin-39* promoter. Repression of *lin-39* transcription by the LIN-1/LET-418 complex, however, is not complete, and non-induced VPCs still have basic levels of *lin-39* expression that are required for normal vulval development. Based on these data we propose that LET-418 acts as part of a control system in the VPCs that prevents *lin-39* (and perhaps other key genes) from being ectopically induced in the absence of an inducing RTK/Ras signal. Activation of the Ras pathway in P6.p leads to phosphorylation of LIN-1, which relieves its repressor activity (Leight et al., 2005; Tan et al., 1998). This may result in the release of the LET-418 containing NuRD-like repressor complex, thereby allowing de-condensation of the chromatin and upregulation of *lin-39* transcription (see model in Fig. 5). Interestingly, not all SynMuv B genes act in the same way on

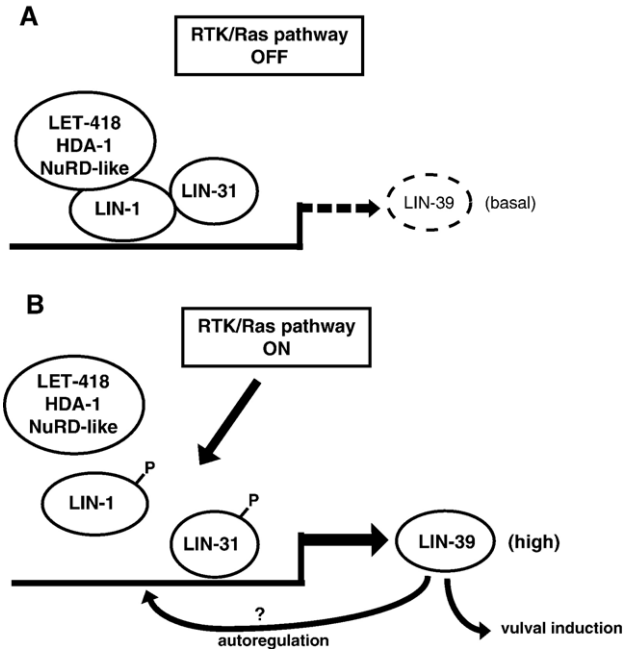

Fig. 5. A model for the transcriptional regulation of *lin-39* by the LET-418/NuRD-like complex. (A) In absence of RTK/Ras signaling in the VPCs, a LIN-1 binds to the promoter of *lin-39* and recruits a LET-418 and HDA-1 containing NuRD-like complex. This complex acts as co-repressor of LIN-1 to repress *lin-39* transcription by affecting histones and condensing the chromatin. The repression, however, is not complete, since basal levels of LIN-39 are required for normal vulval development. (B) Upon RTK/Ras signaling in the cells P(5–7).p, LIN-1 is phosphorylated and releases the repressive NuRD-like complex. This allows de-condensation of the chromatin and upregulation of *lin-39* transcription required for vulval cell fate induction.

the expression of *lin-39* in the VPCs. Depletion of *lin-35/Rb*, *efl-1* and *lin-37*, e.g., leads to a downregulation of the *lin-39* transcription in the VPCs, suggesting that these class B genes are required to upregulate or maintain *lin-39* expression (Chen and Han, 2001). Although all SynMuv B genes cause a common phenotype (Muv in the presence of a class A or class C SynMuv mutation), they seem to function in different regulatory mechanisms.

Transcriptional repression by ETS family proteins through chromatin structure and DNA accessibility has previously been shown to occur in a considerable number of different processes and organisms (Hsu and Schulz, 2000; Li et al., 2000; Mavrothalassitis and Ghysdael, 2000). The human LIN-1 ortholog ELK-1, e.g., recruits the HDAC containing chromatin-remodeling complex mSin3 to silence specific target genes (Kukushkin et al., 2002; Yang et al., 2001). ELK-1 is, as LIN-1, a direct target of the RTK/Ras pathway, and phosphorylation of ELK-1 disrupts its repressive activity (Yang et al., 2003a,b; Yang and Sharrocks, 2004).

Besides their vulval phenotype, mutations in *let-418* and other NuRD-like SynMuv B genes display a variety of additional defects throughout development, suggesting that the NuRD-like complex (either alone or in combination with other complexes) may target many genes that are not directly related to the SynMuv phenotype. One important function of the NuRD-like complex may be to link signaling pathways and developmental decision steps to chromatin structure, thereby promoting switch-like behavior and ensuring robustness of cell-cell signaling. This could explain, e.g., why mutations in human NuRD components can lead to the development of cancer and other diseases.

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