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Role of *C. elegans lin-40* MTA in vulval fate specification and morphogenesis

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SUMMARY

Vulval differentiation in *Caenorhabditis elegans* involves several fundamental cellular events, including cell fusion, division and migration. We have characterized the role of the *lin-40* (also known as *egr-1*) gene in these cellular processes. LIN-40 is homologous to the metastasis-associated factor 1 (MTA1) in mammals, which has been identified as a component of the nucleosome remodeling and histone deacetylation (NuRD) complex that functions as a transcriptional co-repressor. We show here that *lin-40* negatively regulates vulval fate specification at least partly by promoting cell fusion between the vulval precursor cells and the hypodermal syncytium at an early larval stage. This inhibitory function of *lin-40* might be carried out by downregulating *lin-39* Hox expression. We also show that *lin-40* is specifically required for cell divisions along the transverse orientation during vulval morphogenesis.

Key words: *lin-40* MTA, *lin-39* Hox, VPCs competence, Vulval induction, Morphogenesis, Division orientation, *C. elegans*

INTRODUCTION

The NuRD complex has been found to be involved in transcriptional repression by regulating chromatin structure in mammalian cells (Knoepfler and Eisenman, 1999). This complex possesses both histone deacetylation and nucleosome remodeling activities (Xue et al., 1998) and functions together with other transcriptional regulators, including the Rb/E2F complex (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998), in some specific developmental processes (Ahringer, 2000). Metastasis-associated factor 1 (MTA1) and MTA2 have been identified as components of the NuRD complex (Xue et al., 1998; Zhang et al., 1999), although their function in transcriptional repression is not yet well understood. The MTA proteins have also been shown to be present at high levels in metastatic tumor cell lines (Toh et al., 1994; Zhang et al., 1999), but little is known about their contribution to tumorogenesis and metastasis. Other components of the NuRD complex include HDAC1/2 (histone deacetylases), RbAp46/48 (histone-binding proteins), Mi2 (ATPase and helicase) and MBD3 (methyl binding protein) (Xue et al., 1998; Zhang et al., 1999). Recently, several *C. elegans* homologs of the NuRD complex components have been identified; they function in the class B synthetic Multivulva (synMuv) pathway and negatively regulate vulval differentiation (Lu and Horvitz, 1998; Solari and Ahringer, 2000; von Zelewsky et al., 2000). *C. elegans* counterparts of the tumor suppressor Rb and the E2F family transcription factors were found to function similarly and possibly together with the NuRD complex components in the class B synMuv pathway to repress transcription during vulval fate specification (Ceol and Horvitz, 2001; Lu and Horvitz, 1998). These class B synMuv gene products include LIN-35 Rb, EFL-1 E2F, DPL-1 DP, LIN-53 RbAp46/48, HDA-1 HDAC and LET-418 Mi-2 (Ceol and Horvitz, 2001; Lu and Horvitz, 1998; Solari and Ahringer, 2000; von Zelewsky et al., 2000). Another group of genes, termed the class A synMuv genes, act redundantly with the class B genes in repressing vulval fate specification. However, the biochemical function of the class A genes remains unclear (Clark et al., 1994; Huang et al., 1994). Loss-of-function mutations in either class of the synMuv genes do not generate any vulval phenotype, whereas double mutations that disrupt the activities of both pathways lead to a Multivulva (Muv) phenotype, which is caused by ectopic vulval fate specification (Fay and Han, 2000).

Vulval differentiation in *C. elegans* is also controlled by several other regulatory pathways, including the RTK/Ras/MAPK (Kornfeld, 1997; Sternberg and Han, 1998), *lin-39* Hox (Wang et al., 1993; Clark et al., 1993) and LIN-12/Notch signaling pathways (Greenwald, 1998). During the first and second larval stages (L1 and L2), six out of the twelve ectodermal Pn.p cells, P3.p to P8.p, have the potential to adopt the vulval fate, whereas the other Pn.p cells fuse with the surrounding hypodermal syncytium and lose their competence for vulval induction (Fig. 1). The *lin-39* Hox gene, which is expressed in P(3-8).p (referred to as the vulval precursor cells or VPCs), is essential for preventing them from fusing with the hypodermis and maintaining their identity as the VPCs (Fig. 1) (Clark et al., 1993; Wang et al., 1993). Later during the third larval stage (L3), the vulval fate is induced in three (P5.p, P6.p, and P7.p) of the VPCs, while the remaining three fuse with the hypodermal syncytium, hyp7 (Fig. 1). *lin-39* activity is also required at this stage of vulval development (Clandinin et al., 1997; Maloof and Kenyon, 1998), possibly to prevent the

progeny of the VPCs from fusing to hyp7 and keep them responsive to other signaling events. The RTK/Ras/mitogen-activated protein kinase (MAPK) pathway is activated during L3 in P(5-7).p by an inductive signal from the anchor cell in the neighboring gonad and actively promotes the vulval fate in the VPCs (Sternberg and Han, 1998).

After the generation of the VPCs during L1/L2 and vulval induction in P(5-7).p during L3, which we collectively refer to as a vulval fate specification process, a subsequent series of morphogenetic events take place, which include cell divisions along specific axes, cell migrations, and cell fusions (Fig. 1) (Sharma-Kishore et al., 1999). First, the three induced VPCs divide twice in a longitudinal orientation, giving rise to 12 granddaughter cells. Then, each granddaughter cell divides in a characteristic way (Fig. 1): the four granddaughters of P5.p undergo longitudinal, longitudinal, transverse or no division, respectively, anterior to posterior (abbreviated as LLTN); P7.p divides in a mirror-symmetrical way to P5.p (abbreviated as NTLL); P6.p derivatives all undergo transverse divisions (abbreviated as TTTT). After the second round of divisions, the progeny of the VPCs start to delaminate from the cuticle at the ventral surface and migrate dorsally. The progeny of the outer two VPCs, P5.p and P7.p, also migrate towards the position of P6.p, where the vulva will be located (Fig. 1). The migrating cells send out cellular processes to contact the corresponding cells from the other side and fuse with them to form a toroidal syncytium. A stack of seven concentric toroids are generated by the 22 vulval cells and they surround the lumen of the vulva (Sharma-Kishore et al., 1999). The mechanisms that underlie the morphogenesis of the vulva, as well as the regulatory factors involved in this developmental event, remain largely unknown.

We have identified *lin-40* MTA as a regulator of the cell-specific division pattern during vulval morphogenesis, and further studies reveal its involvement in vulval fate specification. Our genetic analyses of *lin-40* mutations disagree with a previous conclusion that *lin-40* (also known as *egr-1*) acts as a class A synMuv gene in repressing vulval induction (Solari and Ahringer, 2000). We report our studies of the genetic interactions between *lin-40* and the synMuv genes, the Ras/MAPK pathway and the *lin-39* Hox gene.

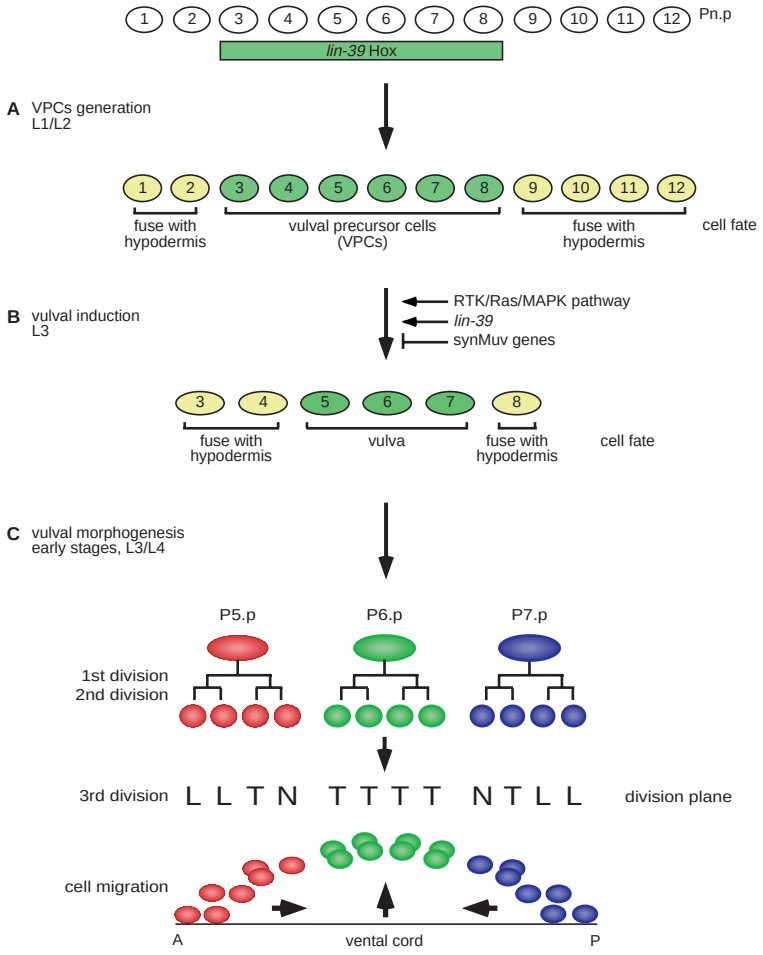

Fig. 1. Major events and regulators in generation of the VPCs (A), vulval induction (B) and early stages of vulval morphogenesis (C). (A) During the L1/L2 stages, the fusion between P(3-8).p and hyp7 is blocked by the action of *lin-39* Hox, which is expressed in these six cells (Clark et al., 1993; Wang et al., 1993). P3.p through P8.p (VPCs) have the potential to adopt the vulval fate, whereas other Pn.ps fuse with the hypodermis. (B) During vulval induction at the L3 stage, the RTK/Ras/MAPK pathway transduces a vulval inductive signal from the anchor cell and the synMuv genes prevent the vulval fate in the VPCs (Riddle et al., 1997). *lin-39* is also required at this stage to maintain the competence of the VPCs and their progeny for vulval induction (Maloof and Kenyon, 1998; Clandinin et al., 1997). In wild-type animals, three VPCs are induced to become vulval cells and the other three divide once and then fuse with the hypodermis. (C) During the early stages of vulval morphogenesis, the induced VPCs first divide twice in a longitudinal orientation, each giving rise to four granddaughters. All but two of the granddaughter cells undergo one more round of division, and each has a specific division plane. L stands for longitudinal division, T for transverse division and N for no division. The dividing cells also migrate dorsally as well as towards the center of the vulva (where the inner cell P6.p is located).

MATERIALS AND METHODS

C. elegans strains and phenotypic analysis

Strains were maintained according to the standard protocol (Brenner, 1974). All genetic analyses were performed at 20°C, unless otherwise noted. The *ku285* allele was isolated in a screen for vulval morphogenesis defective mutants after ethyl methanesulfonate (EMS) mutagenesis (Hanna-Rose and Han, 1999).

The *lin-40* alleles used in the non-complementation tests with *ku285* were *e2173*, *s1053*, *s1345*, *s1351*, *s1352*, *s1358*, *s1360*, *s1373*, *s1506*, *s1593*, *s1611*, *s1634*, *s1669* and *s1675* (Clark et al., 1990; Johnsen and Baillie, 1991). These *lin-40* alleles are associated with a lethal or sterile phenotype and thus were maintained in heterozygous strains (*dpy-18(e364)/eT1* III; *lin-40 unc-46(e177)/eT1* V) (Clark et al., 1990; Johnsen and Baillie, 1991; Rosenbluth and Baillie, 1981). *ku285* failed to complement 10 of these alleles, *s1053*, *s1345*, *s1351*, *s1352*, *s1358*, *s1360*, *s1373*, *s1593*, *s1669* and *s1675*.

In double mutants between *ku285* and the synMuv alleles, the actual genotypes of the synMuv mutations (Riddle et al., 1997) were *dpy-10(e128) lin-8(n111)* II, *rol-1(e91) lin-38(n751) unc-52(e444)* II, *lin15(n433)* X (Ferguson and Horvitz, 1989), *unc-3(e151) lin-15(n767)* X, *dpy-17(e164) lin-9(n112)* III, *unc-13(e1091) lin-*

35(n745) I, *unc-32(e189) lin-36(n766)* III, and *lon-1(e185) lin-37(n758)* III. Some class A synMuv mutations, such as *lin-8(n111), lin-38(n751)* and *lin-15(n433)*, were linked with no or remote phenotypic markers. To confirm the presence of these class A mutations in the double mutants between them and *ku285*, a class B synMuv mutation was introduced into the double mutants to test the segregation of a Muv phenotype.

Other alleles used in this study (Riddle et al., 1997) included *egl-27(n170)* II, *lin-39(n1760)* III (Clark et al., 1993), *let-60(n1046)* IV, *unc-60(m35)* V (McKim et al., 1994), *lag-2(q393)* V (Henderson et al., 1994), *unc-46(e177)* V and *sDf27* V (Rosenbluth et al., 1985).

Cloning and sequencing of *lin-40*

Standard three-point mapping techniques were used to locate the mutation in the *ku285* allele of *lin-40*. Among 27 Unc non-Egl recombinant progeny from *unc-60 ku285/lag-2* heterozygotes, 21 animals segregated the Let phenotype of *lag-2*. Among 17 Unc non-Let progeny from *lag-2 unc-46/ku285* heterozygous animals, 13 recombinants segregated the Egl phenotype of *ku285*. We thus mapped *ku285* to a genetic location of –8.0 map unit between *lag-2* and *unc-46* on chromosome V.

In DNA-mediated germline transformation experiments (Mello and Fire, 1995) using a *sur-5::gfp* reporter pTG96 (Gu et al., 1998), two overlapping cosmids, W08A12 and T27C4, fully rescued the mutant phenotypes of *ku285*, including late larval lethality, sterility, Muv and abnormal vulval morphogenesis. A *Eco*RI to *Kpn*I subclone of these two cosmids, pZC78, retains the rescuing activity of *ku285* and three other *lin-40* alleles, *s1593*, *s1669* and *s1675*. pZC78 contains a single open reading frame, T27C4.4, as predicted by the Genome Sequencing Consortium, as well as 5 kb of upstream sequence and 1.5 kb of sequence downstream of the stop codon. However, the early-to-mid larval lethality associated with several other *lin-40* alleles was not rescued by this genomic fragment. As three of the rescued *lin-40* alleles are likely to be null mutations and most of the other non-rescuable alleles were generated from Tc1 transposon mutagenesis, it is likely that the non-rescuable alleles contain lesions in other loci besides *lin-40*. The molecular lesions in these four rescuable alleles were determined by sequencing genomic DNA from mutant animals. *s1669* is a 128 bp deletion from nucleotide 1383 to 1510 after the first ATG in *lin-40* cDNA sequence. *s1675* is a 5bp deletion from nucleotide 458 to 462 after the first ATG. Lesions of *ku285* and *s1593* are indicated in Fig. 2.

Three cDNA clones, yk394g5, yk79e4 and yk117a12, were obtained from Yuji Kohara. The yk394g5 clone contains 17 bp sequence upstream of the predicted start codon of *lin-40* and 507 bp into the coding sequence. The 17 bp upstream sequence contains an in-frame stop codon. yk79e4 contains the sequence from nucleotide 1311 after the predicted start codon to nucleotide 188 after the stop codon of a shorter transcript, which is referred to as *lin-40a*. yk117a12 starts at nucleotide 1297 after the first ATG and extends to 58 bp after the stop codon of a longer alternatively spliced transcript, referred to as *lin-40b*. The latter two cDNA clones contain a poly(A) tail at their 3′ ends. None of these three cDNA clones contains the sequence in the middle (from nucleotide 507 to 1297) of the coding region. Thus, using PCR, we isolated the cDNA fragment missing from these Kohara cDNA clones from a *C. elegans* mixed-staged cDNA library (a gift from R. Barstead). Only one cDNA clone was isolated and it was ligated with the Kohara clones to generate full-length cDNA constructs for both *lin-40a* and *lin-40b*.

An independent study by Solari and Ahringer (Solari and Ahringer, 2000) also identified the *lin-40b* transcript and lesions in two *lin-40* alleles, *s1593* and *s1669*. The *lin-40* gene has also been named *egr-1* (for *egl-27*-related gene) in previous reports (Solari and Ahringer, 2000; Solari et al., 1999). As *egr-1* is the previously published *lin-40* locus and the name ‘egr’ does not directly reflect the mutant phenotype or protein property, we thus refer to the gene as *lin-40* or *lin-40* MTA.

Double-stranded RNA interference (RNAi)

PCR primers that each contain a T7 promoter sequence were used to generate a cDNA fragment that corresponds to the first 1.4 kb of the *lin-40* coding sequence. Double-stranded RNA (dsRNA) was generated from the 1.4 kb template using a large-scale T7 transcription kit (Novagen, Madison, WI). RNAi was carried out as described previously (Fire et al., 1998). The dsRNA was injected into worms at 25 ng/μl. Injected animals were transferred to individual fresh plates after 16 hours and their progeny were scored for mutant phenotype. A higher concentration of dsRNA (100 ng/μl) was also tested and it did not cause additional or more severe defects.

GFP reporter constructs

The coding sequence of the *gfp* reporter gene (Chalfie et al., 1994) was excised from the plasmid pPD102.33 (a gift from A. Fire, S. Xu, J. Ahnn and G. Seydoux) and was inserted in-frame after the first methionine residue of the predicted *lin-40*-coding sequence at a *Sac*I site. Three different GFP reporter constructs were generated. One of them contains the same genomic sequence as in pZC78. In the other two reporter constructs, the genomic sequence after intron II was replaced with either the *lin-40a* or *lin-40b* cDNA sequence. All three constructs were injected into *unc-119(ed3)* animals at 10 ng/μl, together with an *unc-119*(+) plasmid pDP#MM016B (Maduro and Pilgrim, 1995) at 40 ng/μl.

jam-1::gfp reporter

The *jam-1* (junction associated molecule 1) gene product is present in cell adhesion junctions (Mohler et al., 1998). A *jam-1::gfp* reporter (a gift from J. Simske and J. Harding) was injected into *unc-119(ed3)* animals together with pDP#MM016B (Maduro and Pilgrim, 1995), and was integrated into the worm genome using gamma irradiation (W. Hanna-Rose, unpublished). A resulting integrated transgene *kuIs46[jam-1::gfp+ u119(+)]* X was used in this study.

Antibody staining

A *lin-39::lacZ* reporter integrated on chromosome IV, *muIs6[lin-39::lacZ+pRF4(rol-6d)]* (Wang et al., 1993), was introduced into *lin-40(ku285)* by mating. As other *lin-40* alleles, all associated with lethality or sterility, were maintained as heterozygotes, and the maternal *lin-40* gene product might interfere with the testing result, these alleles were not examined in this experiment. Animals were fixed according to the protocol of Bettinger et al. (Bettinger et al., 1996), and then stained overnight with an anti-β-galactosidase antibody (1:500 dilution) from Promega (Madison, WI). After three washes in the buffer B (Bettinger et al., 1996) for several hours, animals were incubated with a Cy3-conjugated goat anti-mouse antibody (Jackson Immunoresearch, West Grove, PA) for several hours in the dark. All animals were also stained with DAPI and a monoclonal antibody MH27 (a gift from R. Waterston), which recognizes an epitope in the *jam-1* gene product in cell adhesion junctions. The MH27 antibody served as a positive control for the antibody staining and the DAPI staining helped in identifying Pn.ps. Only animals that had positive MH27 antibody staining and had at least one Pn.p cell stained with anti-β-galactosidase were counted in the experiment.

RESULTS

lin-40 mutations disrupt specific aspects of vulval morphogenesis as well as vulval induction

We initiated our studies of *lin-40* by isolating the *ku285* mutation in a screen for mutants defective in vulval morphogenesis (Hanna-Rose and Han, 1999). Animals homozygous for *ku285* were found to have normal vulval induction but be egg-laying defective, owing to

Table 1. *lin-40* mutations specifically disrupt the transverse divisions during vulval morphogenesis

Genotype	P5.p	P6.p*	P7.p
Wild type	LLTN	TTTT	NTLL
lin-40(ku285)	LL**L**N	TTTT	NTLL
	LLTN	TTT**L**	N**L**LL
	LLTN	TTT**L**	N**L**LL
	LLTN	TTTT	NTLL
	LLTN	**L**TT**L**	N**O**LL
	LL**L**N	TTTT	N**O**LL
	LLTN	TTT**L**	NTLL
	LL**L**N	**L**TT**L**	NTLL
	LLTN	TTTT	NTLL
	LL**L**N	TTTT	NTLL
	LLTN	TTT**L**	NTLL
	LLTN	TTTT	NTLL
	LLTN	TTT**L**	NTLL
	LLTN	TTT**L**	N**L**LL‡
	LL**L**N	TTTT	NTLL
lin-40(RNAi)	LLTN	TTT**L**	NTLL
	LL**L**N	TTTT	N**L**LL
	LLTN	TTTT	NTLL
	LL**L**N	TTTT	N**L**LL
	LLTN	TTTT	NTLL
	LLTN	TTTT	NTLL
	LL**L**N	TTTT	N**L**LL
	LLTN	TTTT	NTLL
	LL**L**N	TTTT	NTLL
	LLTN	TTTT	NTLL
	LLTN‡	TTTT	N**L**LL
	LLTN	TTT**L**	N**L**LL

Lineage analyses were carried out on a DIC microscope using Nomarski optics. RNAi was performed in animals heterozygous for *lin-40(ku285)*.

L, longitudinal division axis; T, transverse division axis; O, oblique division axis; N, no division. The abnormal division axes are in bold. Each row represents the lineage from a single animal.

*The two most inner granddaughter cells of P6.p were observed to undergo longitudinal divisions at a very low frequency and this defect is not represented by lineages shown here.

‡These cells did not migrate to the center of the vulva and instead formed a separate vulval invagination.

morphogenetic abnormalities in the vulva (Table 1). Lineage analyses showed that in *ku285* animals, the first two rounds of vulval cell divisions appeared to be normal; each induced VPC divided twice in a longitudinal orientation (data not shown). However, in the third and final division, vulval cells that normally change the division plane to a transverse orientation sometimes failed to do so and instead continued to divide along the longitudinal axis (Table 1). Other vulval cells that normally undergo longitudinal divisions or no divisions were not affected by the *ku285* mutation (Table 1), suggesting that *lin-40* is specifically required for preventing the longitudinal division or promoting the transverse division during the final round of vulval cell divisions. In addition, in *ku285* mutants, the progeny of the induced P5.p or P7.p cell sometimes failed to migrate towards P6.p or failed to fuse with other vulval cells and instead formed a separate vulval invagination (Table 1). This defect was more severe in double mutants between a class B synMuv mutation and *lin-40(ku285)* (Fig. 3B). *ku285* animals also displayed a partial sterile phenotype (data not shown).

Genetic mapping of *ku285* located the mutation near the *lin-40* gene, defined by several previously isolated alleles (Clark et al., 1990; Johnsen and Baillie, 1991). Non-complementation tests revealed that *ku285* failed to complement 10 of these alleles (see Materials and Methods), suggesting that *ku285* is a mutation in the *lin-40* locus. These previously isolated *lin-40* alleles cause sterility and lethality at various developmental stages (Johnsen and Baillie, 1991), and three of them, *s1593*, *s1669* and *s1675*, exhibited an additional Muv phenotype (Table 2; Fig. 3A; data not shown).

To determine if the mutant phenotypes of these *lin-40* alleles result from a reduction or complete loss of *lin-40* gene activity, we performed a deficiency test and double-stranded RNA interference (RNAi) experiments (Fire et al., 1998). Trans-heterozygous animals carrying the *ku285* mutation and a deletion of the genomic sequence around the *lin-40* locus, *sDf27* (Rosenbluth et al., 1985), displayed lethal and sterile phenotypes (data not shown), similar to those of *lin-40* alleles. In addition, hermaphrodites heterozygous for *ku285*, *s1669* or *s1675* that were injected with dsRNA of *lin-40* (see Materials and Methods) produced progeny that also showed similar defects to those of *lin-40* alleles, including sterility, lethality, Muv and abnormal vulval morphogenesis (Table 1; data not shown). Taken together, these analyses indicate that *ku285* and 10 other alleles of *lin-40* are loss-of-function mutations. The pleiotropic phenotypes of *lin-40* mutants suggest that this gene is involved in multiple developmental processes and the Muv phenotype indicates that *lin-40* negatively regulates vulval fate specification.

We further investigated the effect of the *lin-40(ku285)* mutation on vulval induction in a *let-60* Ras*(n1046gf)* background, where P3.p, P4.p and P8.p are often ectopically induced, resulting in a Multivulva (Muv) phenotype (Beitel et al., 1990; Han et al., 1990). Although *lin-40(ku285)* did not cause any vulval induction phenotype in an otherwise wild-type background, it led to an elevated level of vulval induction in combination with the *let-60(n1046gf)* mutation in P3.p, P4.p and P8.p (see Table 4). This is in support of the above conclusion that *lin-40* represses the vulval fate in the VPCs.

lin-40 encodes a homologue of mammalian MTA proteins

We cloned the *lin-40* gene by DNA-mediated rescuing experiments and by determining molecular lesions associated with *lin-40* mutant alleles (see Materials and Methods). Two transcripts that differ only in their 3′ regions were found to be produced from the *lin-40*-coding sequence by alternative splicing (Fig. 2A; Materials and Methods). We refer to the shorter transcript that encodes an 870 amino acid protein as *lin-40a*, and the longer one that encodes a 1022 amino acid protein as *lin-40b*. The full-length cDNAs for both transcripts were able to rescue the *lin-40* mutant phenotypes (data not shown), suggesting that the extra sequence in the longer transcript is not required for *lin-40* function. DNA lesions in the four rescued *lin-40* alleles have been identified (Fig. 2A,B). The *ku285* mutation is defined by a G to A transition in the splice acceptor site in front of exon II. Sequencing of RT-PCR products revealed that three different transcripts were produced in the *ku285* mutant. Two of the transcripts contain premature stop codons shortly after the mutation site, and the third is wild type. The presence of a wild-type transcript suggests that *ku285* is a partial loss-of-function mutation (Han et al., 1993). In *s1669* and *s1675*, deletions of genomic sequence result in reading frame shifts and

premature stop codons in exon III (Fig. 2A,B; Materials and Methods) (Solari and Ahringer, 2000). A point mutation was identified in *s1593*, which also generates a premature stop codon in exon III (Fig. 2A,B) (Solari and Ahringer, 2000). The latter three alleles are likely to be null mutations.

As it was also shown in a previous report (Solari and Ahringer, 2000), the *lin-40* gene encodes a protein that is homologous to MTA1 and MTA2 in mammals, which were identified as components of the NuRD complex (Xue et al., 1998; Zhang et al., 1999). The similarity between MTA and its *C. elegans* homologs, LIN-40 and EGL-27 (Ch'ng and Kenyon, 1999; Herman et al., 1999; Solari et al., 1999), is mostly restricted to several conserved peptide motifs, including a leucine zipper, a SANT domain (a DNA-binding domain first identified in the oncogene *myb*), and a zinc-finger motif (Fig. 2B) (Nawa et al., 2000; Solari et al., 1999; Toh et al., 2000). Another conserved domain, the SH3-binding domain, is only present in mammalian MTA1 (Fig. 2B) (Toh et al., 1994; Toh et al., 1995). The similarity between LIN-40, MTA1 and MTA2 suggests that *lin-40* might function as a transcriptional regulator.

We have examined the expression pattern of *lin-40* using a *gfp* translational fusion reporter, which contains the genomic sequence of *lin-40* and fully rescued the four *lin-40* alleles. The fusion protein was predominantly localized to the nuclei of most, if not all, somatic cells (data not shown). A similar observation was reported previously by Solari and Ahringer (Solari and Ahringer, 2000). We further analyzed the expression of *lin-40a* and *lin-40b* isoforms using *gfp* reporters fused to the respective cDNA sequences and revealed that LIN-40A was mostly localized to the nucleus (Fig. 2C,D), whereas LIN-40B was present at a lower level in both the cytoplasm and the nucleus (Fig. 2E,F).

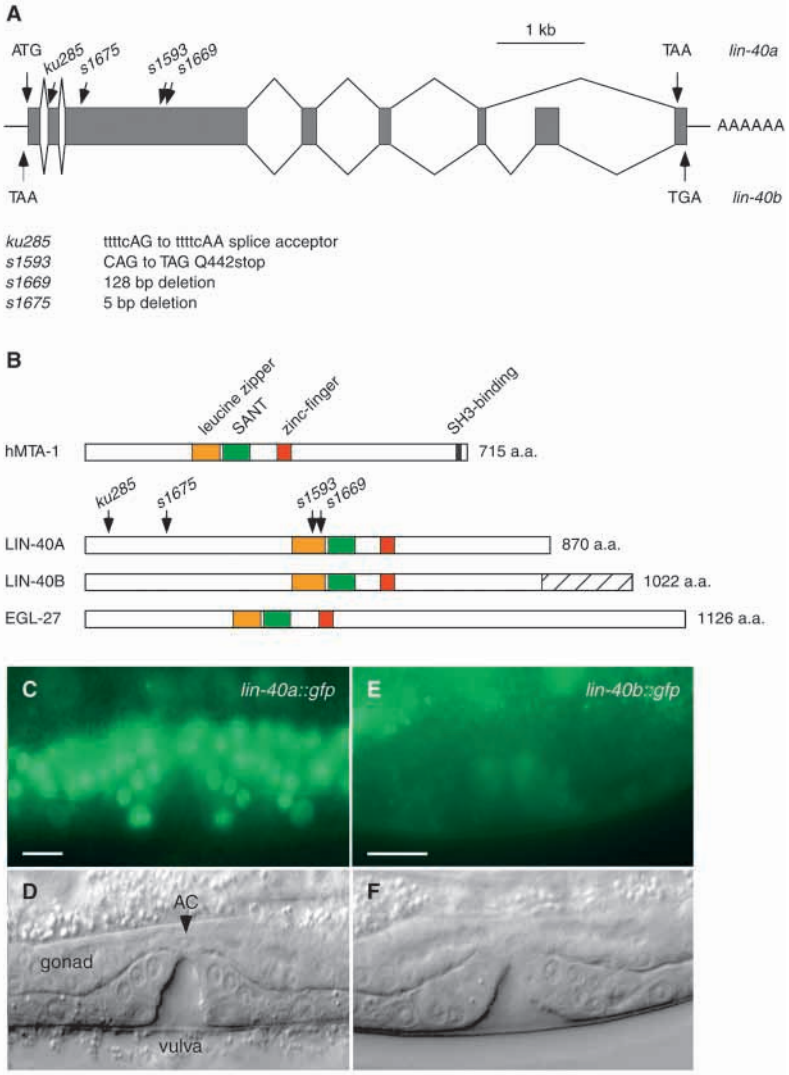

Fig. 2. (A) Two transcripts, *lin-40a* and *lin-40b*, are generated from the *lin-40* locus by alternative splicing. cDNA clones that represent both transcripts have a polyA tail at their 3′ ends and an in-frame stop codon in front of the start codon. Arrows on top of the genomic sequence and protein products in B indicate the positions of mutations in *lin-40* alleles. (B) The protein structure of MTA1 proteins. Human MTA1 (hMTA-1) and its homologues in *C. elegans*, LIN-40A, LIN-40B and EGL-27, share some consensus peptide motifs, including a leucine zipper, a SANT domain and a zinc-finger motif. The SH3-binding domain is only present in mammalian MTA1. The hatched domain in LIN-40B represents the sequence that interacts with LIN-36 and LIN-53 in a two-hybrid screen. (C,D) The expression pattern of a *lin-40a::gfp* reporter gene. A high level of GFP fusion protein was detected predominantly in the nuclei of most, if not all, somatic cells. Shown here is the presence of LIN-40A::GFP in vulval cells, the anchor cell (AC) and many other gonadal cells. (E,F) The expression pattern of a *lin-40b::gfp* reporter. LIN-40B::GFP was found to be present at a lower level than LIN-40A::GFP in both the cytoplasm and the nucleus. Ventral is downwards and anterior is towards the left. Scale bars: 10 μm.

lin-40 does not function as a typical class A or class B synMuv gene

Given that mammalian MTA1 and MTA2 were found to be associated with the NuRD complex and that several class B synMuv genes encode proteins similar to components of the complex, we hypothesized that *lin-40* acts as a class B synMuv gene. To test this model, we constructed double mutants between *lin-40(ku285)* and several class A synMuv mutations, including *lin-8(n111)*, *lin-38(n751)* and *lin-15A(n433)*. None of these class A synMuv mutations exhibited a Muv phenotype in combination with *lin-40(ku285)* (Table 2). Replacing *lin-40(ku285)* with RNAi against *lin-40* generated similar results in these class A mutants (Table 2), suggesting that *lin-40* is not a class B synMuv gene. However, another class A mutation, *lin-15(n767)*, showed an allele-specific interaction with *lin-40(ku285)* as it caused a Muv phenotype in the *ku285* background (an average of 3.3 VPCs were induced in the *lin-40(ku285); lin-15(n767)* double mutant versus three VPCs being induced in wild-type animals) (Table 2). This allele also behaved differently from other class A synMuv mutations under other assay conditions (Z. C. and M. H., unpublished).

A previous study by Solari and Ahringer (Solari and Ahringer, 2000) led to the conclusion that *lin-40* acts as a class

Table 2. Vulval induction phenotype caused by *lin-40* mutations and genetic interactions between *lin-40* and synMuv genes

	Genotype	Number of induced VPCs	*n*
	Wild type	3.0	18
	lin-40(ku285)	3.0	51
	*lin-40(s1669)**	3.5	59
	*lin-40(s1675)**	3.4	44
	ku285/+; lin-40(RNAi)‡	3.1	36
	s1669/+; lin-40(RNAi)‡	3.6	10
	s1675/+; lin-40(RNAi)‡	3.5	6
	lin-40(RNAi)§	3.0	42
class A; *lin-40*	*lin-8(n111); lin-40(ku285)*	3.0	48
	lin-8(n111); lin-40(RNAi)	2.98	54
	lin-38(n751); lin-40(ku285)	3.0	41
	lin-38(n751); lin-40(RNAi)	3.0	20
	lin-15(n433); lin-40(ku285)	3.0	25
	lin-15(n767); lin-40(ku285)	3.3	37
	lin-15(n767); lin-40(RNAi)	3.1	24
class B; *lin-40*	*lin-9(n112); lin-40(ku285)*	3.04	55
	lin-9(n112); lin-40(RNAi)	2.95	54
	lin-35(n745); lin-40(ku285)	3.0	75
	lin-35(n745); lin-40(RNAi)	2.8	19
	lin-36(n766); lin-40(ku285)	3.01	134
	lin-36(n766); lin-40(RNAi)	2.9	57
	lin-37(n758); lin-40(ku285)	2.7	56
	lin-37(n758); lin-40(RNAi)	2.8	21
class A; B	*lin-8(n111); lin-35(n745)*	5.8	15

*The actual genotypes of these lin-40 mutants are dpy-18(e364) III; lin-40(s1669) unc-46(e177) V and dpy-18(e364) III; lin-40(s1675) unc-46(e177) V.

‡RNAi experiments were carried out in strains heterozygous for the three corresponding lin-40 alleles and only homozygous lin-40 mutant progeny were scored for phenotype.

§lin-40(RNAi) indicates that the experiments were performed in animals that are homozygous wild type at the lin-40 locus. Note that RNAi of lin-40 in wild-type background does not cause any vulval phenotype.

See Materials and Methods for the actual genotypes of synMuv mutations.

A synMuv gene. They showed that in two class B synMuv mutants, *lin-9(n112)* and *lin-37(n758*), which were subjected to *lin-40*(RNAi), multiple vulva-like protrusions were observed in the animals (in an average of 61% of *lin-37* mutants) under dissecting microscopes. This has led to the suggestion that P3.p, P4.p or P8.p. is ectopically induced into vulval cells. Such a defect was not seen in class A synMuv or wild-type animals subjected to *lin-40*(RNAi) (Solari and Ahringer, 2000). To test this possibility of *lin-40* being a class A synMuv gene, we constructed double mutants between *lin-40(ku285)* and four class B synMuv mutations, *lin-9(n112)*, *lin-35(n745)*, *lin-36(n766)* and *lin-37(n758)*. Examination of vulval induction under Nomarski optics showed that the average number of VPCs being induced in these double mutants was not significantly higher than that in wild-type animals (Table 2). Interestingly, in these double mutants, P(5-7).p sometimes failed to be induced to adopt the vulval fate, whereas the other three VPCs were, at a low frequency, ectopically induced (Table 3). In addition, an interesting vulval morphogenetic defect was often observed. The induced vulval cells sometimes did not migrate or integrate properly with other vulval cells so that the progeny of P(5-7).p often formed individual vulval invaginations (Fig. 3B). In these *lin-40(ku285); synMuvB* double mutants, up to three vulva-like protrusions could be formed from the progeny of P(5-7).p, whereas in wild-type animals, these cells form only a single vulval invagination. We also applied RNAi against *lin-40* to the same class B synMuv mutants as did Solari and Ahringer (Solari and Ahringer, 2000) and observed a similar morphogenetic defect as in *lin-40(ku285); synMuvB* animals. The phenotype of underinduction in P(5-7).p and overinduction in P(3,4,8).p was also seen in *lin-40*(RNAi)*; synMuvB* double mutants (Table 3). Such a phenotype in vulval induction is clearly different from the synthetic Muv phenotype caused by a combination between class A and class B genes (Ferguson and Horvitz, 1989; Lu and Horvitz, 1998). Overall, these results indicate that *lin-40* does not function as a typical class A synMuv gene and that *lin-40* and class B genes have weak synergistic effect on vulval induction in both negative and positive directions. Although we observed a similar percentage of animals showing the 'multiprotrusion' phenotype in *lin-40*; synMuvB animals (62% of *lin-37(n745); lin-40*(RNAi) animals, *n*=21 and 36% of *lin-37(n745); lin-40(ku285)* animals, *n*=56) as did Solari and Ahringer (Solari and Ahringer, 2000), we concluded that such a phenotype was mostly due to the defect in vulval morphogenesis. instead of ectopic vulval induction.

A systematic two-hybrid interaction screen using genes involved in vulval development identified LIN-40 as an interacting protein with two class B synMuv gene products, LIN-36 and LIN-53 (Walhout et al., 2000). Mammalian homologs of LIN-53, RbAp46 and RbAp48, are core components of the NuRD complex. We sequenced the *lin-40* construct used in the two-hybrid screen (Walhout et al., 2000) and found that it includes a 524 bp sequence that encodes only the C-terminal fragment of LIN-40B (Fig. 2B). We also found that in GAL4 transcription-based two-hybrid assays, this

Table 3. Genetic interactions between *lin-40* and class B synMuv genes in regulating vulval induction

	Induction (%)						
Genotype	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	*n*
Wild type	0	0	100	100	100	0	18
lin-9(n112); lin-40(ku285)	0	1.8	100	100	98.0	4.1	55
lin-9(n112); lin-40(RNAi)	1.9	0.9	94.4	100	92.6	5.6	54
lin-35(n745); lin-40(ku285)	0	0	100	100	100	0	75
lin-35(n745); lin-40(RNAi)	0	0	89.5	100	94.7	0	19
lin-36(n766); lin-40(ku285)	0	0	99.3	100	100	1.5	134
lin-36(n766); lin-40(RNAi)	0	0	96.5	100	91.2	0	57
lin-37(n758); lin-40(ku285)	0	0	87.5	100	75.0	6.3	56
lin-37(n758); lin-40(RNAi)	0	4.8	80.9	100	95.2	0	21

RNAi experiments were performed in animals homozygous wild type at the *lin-40* locus.

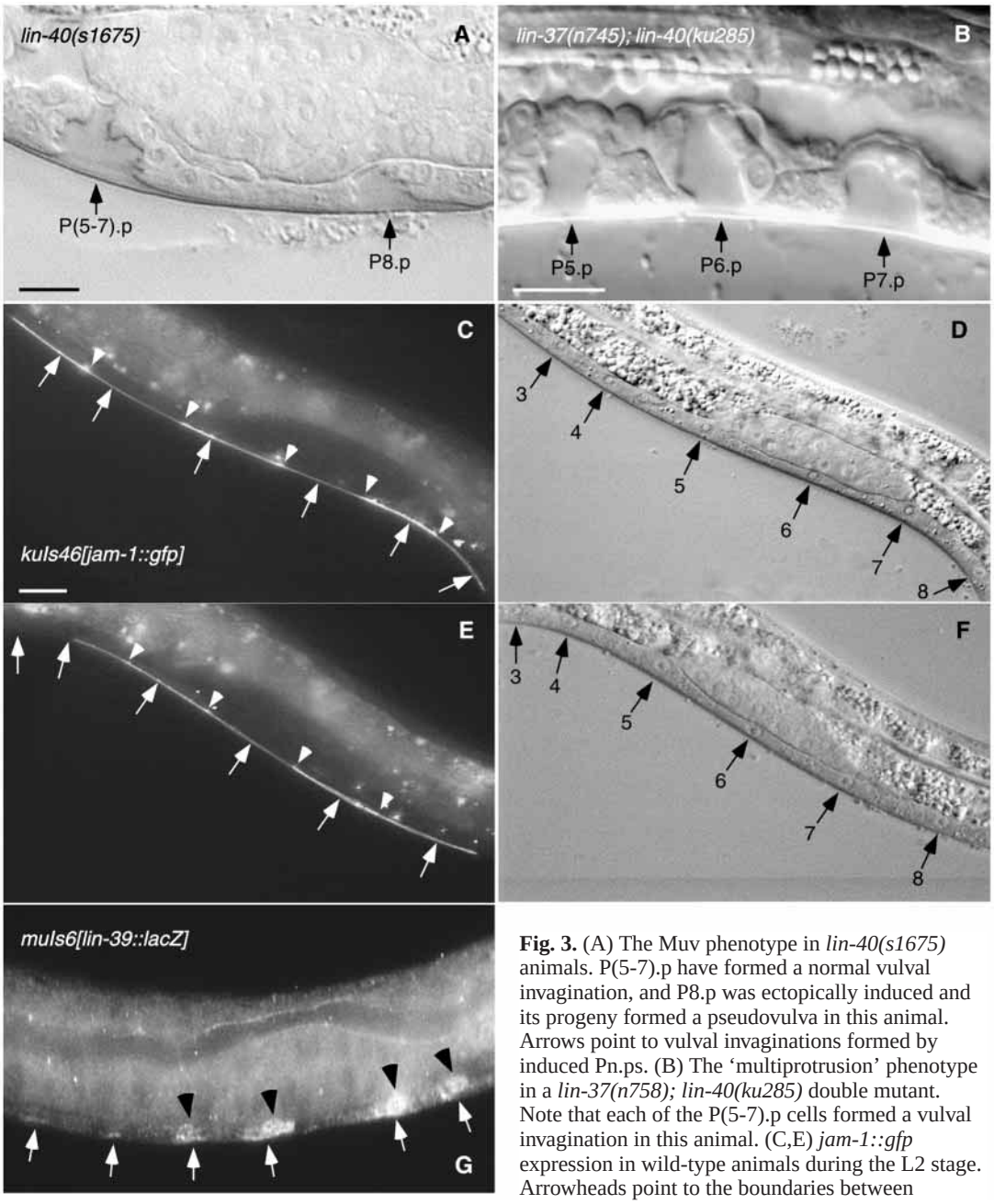

Fig. 3. (A) The Muv phenotype in *lin-40(s1675)* animals. P(5-7).p have formed a normal vulval invagination, and P8.p was ectopically induced and its progeny formed a pseudovulva in this animal. Arrows point to vulval invaginations formed by induced Pn.ps. (B) The 'multiprotrusion' phenotype in a *lin-37(n758); lin-40(ku285)* double mutant. Note that each of the P(5-7).p cells formed a vulval invagination in this animal. (C,E) *jam-1::gfp* expression in wild-type animals during the L2 stage. Arrowheads point to the boundaries between neighboring Pn.ps and arrows indicate the nuclei of Pn.ps. JAM-1::GFP can be detected on the apical surface of Pn.ps where they adjoin the hypodermal syncytium. Note that in E, but not in C, P3.p is already fused to hyp7. (D,F) DIC Nomarski images of the worms shown in C,E. Numbers next to arrows represent the identity of Pn.ps. (G) *lin-39::lacZ* expression in wild-type animals. Arrows point to the MH27 staining on the surface of Pn.ps and arrowheads indicate the nuclei with positive anti-β-galactosidase staining. Ventral is downwards and anterior is towards the left. Scale bars: 10 μm.

protein fragment interacted with a class A synMuv protein, LIN-15A, but did not interact with two other class B synMuv proteins, HDA-1 HDAC and LIN-35 Rb (data not shown). However, under the same condition, neither LIN-40B nor LIN-40A full-length protein showed any interaction with any of the synMuv proteins that bind to the LIN-40B C-terminal fragment (data not shown). As both *lin-40a* and *lin-40b* can rescue *lin-40* mutant phenotypes, the functional significance of this C-terminal fragment and its interactions with some synMuv proteins remain unclear.

lin-40 promotes the fusion of the Pn.p cells with hyp7

The fusion between Pn.ps and the hypodermal syncytium, hyp7, directly affects vulval induction. Blocking this fusion in P(3-8).p during L1/L2 is essential to maintain their

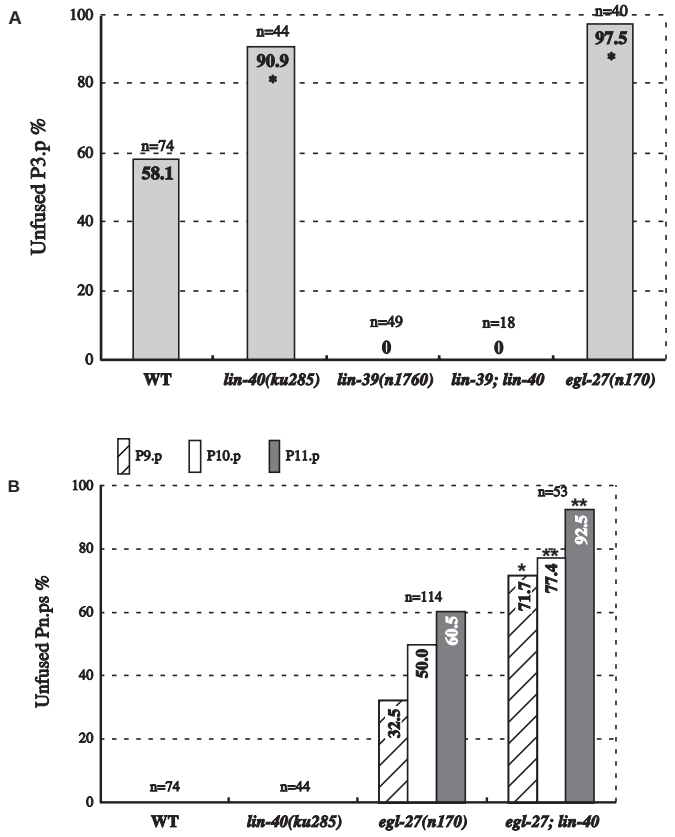

Fig. 4. Mutations in *lin-40* and *egl-27* affect the fusion between Pn.ps and hyp7 during the L2 stage. *y*-axis represents the percentage of Pn.ps that remain unfused. (A) A partial loss-of-function mutation in *lin-40* caused an elevated percentage of the unfused cell fate in P3.p. This effect was suppressed by a mutation in *lin-39(n1760)*. The *egl-27(n170)* null allele also enhanced the unfused cell fate in P3.p. **P*<0.001, ***P*<0.05. *P* values were derived from comparing data from the mutants to that from the wild-type animals. (B) Although *lin-40(ku285)* alone did not affect the fusion in P(9-11).p, it dramatically increased the abnormal unfused fate in these cells in an *egl-27(n170)* background. *P* values were derived from comparing the fusion frequencies of P9.p, P10.p and P11.p between *egl-27* and *egl-27; lin-40*.

responsiveness to the vulval inductive signal at the later stage. *lin-39* Hox activity is required to prevent this fusion as *lin-39* loss-of-function mutations cause abnormal fusion of P(3-8).p with hyp7 and eliminate vulval induction in the fused cells (Clark et al., 1993; Wang et al., 1993). It is thus conceivable that *lin-40* may negatively regulate vulval induction by acting early in the fusion process to promote cell fusion and thus repress the competence of the VPCs for vulval induction.

In wild-type hermaphrodites, P4.p to P8.p remain unfused with hyp7 100% of the time at the L2 stage, whereas P3.p fuses with hyp7 and loses its competence for vulval induction about 50% of the time (Sternberg and Horvitz, 1986; Sulston and White, 1980) (Fig. 4A). Such variability in the P3.p cell fusion provides us a sensitive assay to test if *lin-40* affects the cell fusion process. In this test, the frequency of the P3.p cell fusion served as an indicator of the competence status of the VPCs. A JAM-1::GFP fusion protein, which is present in cell adhesion junctions (Mohler et al., 1998), was used to score the fusion event (Fig. 3C-F; Materials and Methods). In a VPC that has fused with hyp7, this fusion protein can no longer be seen at the apical surface of the cell (Fig. 3E). We observed that *lin-40(ku285)* significantly increased the frequency of P3.p being unfused to about 91% (Fig. 4A), suggesting that the normal function of *lin-40* is to promote the fusion between the VPCs, at least P3.p, and the hyp7 cell.

Furthermore, a null mutation in *lin-39(n1760)* suppressed the elevated percentage of unfused P3.ps imposed by *lin-40(ku285)* (Fig. 4A). In addition, the competence of the other VPCs was also reduced in *lin-39(n1760); lin-40(ku285)* double mutants, as in *lin-39(n1760)* animals, where P4.p through P8.p abnormally fused to hyp7 and failed be induced to become vulval cells (data not shown). Together these results suggest that the *lin-39* Hox gene is most likely to function downstream of *lin-40* in controlling vulval cell competence.

We also tested if the other MTA1 homolog in *C. elegans*, *egl-27*, is also involved in controlling the competence of the VPCs. We observed that P3.p cells remained unfused at a higher frequency in *egl-27(n170)* null mutants (Fig. 4A). In addition, although *egl-27(n170)* did not cause a Muv phenotype in an otherwise wild-type background (data not shown), it increased the vulval induction level in a *let-60(n1046gf)* mutant background (Table 4). These results indicate that *egl-27* also promotes cell fusion and therefore represses the competence of the vulval precursor cells.

lin-40 also regulates the cell fusion decision in posterior Pn.ps

In *egl-27* mutants, the posterior P9.p though P11.p cells sometimes fail to fuse with hyp7 (Ch'ng and Kenyon, 1999). We found that *lin-40(ku285)* was not able to affect cell fusion in these posterior Pn.ps in otherwise wild-type animals (Fig. 4B). However, *lin-40(ku285)* significantly exacerbated the

Table 4. A *lin-40* mutation enhances the competence of the VPCs for vulval induction

	Induction (%)						
Genotype	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	*n*
Wild type	0	0	100	100	100	0	23
lin-40(ku285)	0	0	100	100	100	0	51
egl-27(n170)	0	0	100	100	100	0	18
let-60 Ras(n1046gf)	46.9	46.3	100	100	100	27.8	81
lin-40(ku285); let-60(n1046)	74.1‡	87.9*	100	100	100	84.5*	29
egl-27(n170); let-60(n1046)	79.4‡	73.5‡	100	100	100	61.8‡	17

**P*<0.001.
‡*P*<0.05.
P values were derived from comparing data from *let-60(n1046)* double mutants with those from *let-60*.

fusion defect in an *egl-27(n170)* mutant background; these posterior Pn.ps remained unfused at much higher frequencies in *egl-27; lin-40* double mutants (Fig. 4B). In addition, *lin-40(ku285)* weakly induced the vulval fate in the unfused Pn.ps in *egl-27(n170)* mutants, so that these cells sometimes underwent cell divisions and other morphogenetic events to form pseudo-vulval structures (data not shown).

Although we were unable to test the competence of P(4-8).p using the JAM-1::GFP reporter, as these cells always remain unfused with hyp7 in wild-type animals, we speculate that *lin-40* also promotes cell fusion in these Pn.ps, based on the fact it functions similarly in both P3.p and P(9-11).p. This hypothesis is supported by the observation that a *lin-40* mutation increased the competence of not only P3.p, but also P4.p and P8.p, in a *let-60(n1046)* background (Table 4).

lin-40 negatively regulates *lin-39* expression

Given the fact that *lin-39* encodes a Hox family transcription factor, and the fact that LIN-40 is likely to be involved in transcriptional repression like mammalian MTA proteins, it is conceivable that *lin-40* controls vulval cell competence partly by regulating the *lin-39* gene, most probably in a negative manner. This inhibition of *lin-39* might be accomplished by either downregulating *lin-39* expression or acting as a transcriptional co-repressor to inhibit the downstream targets of *lin-39*. Because the vulva-specific targets of *lin-39* remain unknown, we tested only the first hypothesis by examining *lin-39* expression in a *lin-40* mutant background.

To analyze the vulva-specific expression of *lin-39*, we made use of an integrated *lin-39::lacZ* reporter transgene (Wang et al., 1993) (Materials and Methods). This reporter is weakly but consistently expressed in P5.p through P8.p during the L2 stage, and is present at very low levels in P3.p and P4.p (Wang et al., 1993) (Fig. 3G). Using an anti-β-galactosidase antibody, the fusion gene product was assayed in the VPCs during the L2 stage, as *lin-39* expression is upregulated during the later L3 stage by the Ras/MAPK pathway (Maloof and Kenyon, 1998). The frequency of visible LIN-39::lacZ reporter in each VPC was used as a measure of *lin-39* expression and a MH27 antibody was used as a positive control for antibody staining (see Materials and Methods). As indicated in Table 5, *lin-39::lacZ* expression was significantly increased in *lin-40(ku285)* mutants. For example, 67.1% of P5.p cells had visible *lin-39::lacZ* expression in *lin-40(ku285)* as opposed to 43.7% in wild-type animals. This result suggests that *lin-40* negatively regulates *lin-39* expression, which is consistent with the above genetic results showing that a *lin-40* mutation increased the unfused cell fate in Pn.ps and enhanced vulval induction in the VPCs.

Table 5. *lin-40* regulates *lin-39* expression

	lin-39::lacZ expression (% stained cells)				
Genotype	P5.p	P6.p	P7.p	P8.p	n
Wild type	43.7	48.1	81.0	92.5	158
lin-40(ku285)	67.1*	82.3*	96.2‡	100‡	79

Both anti-β-galactosidase and MH27 antibodies were used at the same time (see Materials and Methods). Worms with positive MH27 staining and with at least one cell stained for anti-β-galactosidase were scored. *lin-39::lacZ* expression in P3.p and P4.p is extremely weak (Wang et al., 1993), and results in these two cells were thus excluded from this study.

*$P<0.001$

‡$P<0.05$.

P values were derived from comparing data from *lin-40(ku285)* mutants with those from wild-type animals.

DISCUSSION

Our genetic studies of *lin-40* indicate that it plays multiple roles in regulating vulval fate specification and vulval morphogenesis. *lin-40* represses vulval induction in the VPCs, possibly by promoting the fusion between these cells and hyp7; this function is likely to be carried out by negatively regulating the *lin-39* Hox gene. Later during vulval morphogenesis, *lin-40* function is required for cell divisions along the transverse axis and some cell migration and fusion processes.

Our result indicates that *lin-40* has an opposite effect to that of the Ras/MAPK pathway in controlling *lin-39* expression. Previously it has been shown that the Ras/MAPK pathway positively regulates *lin-39* expression and that the level of LIN-39 correlates with the strength of the inductive signaling mediated by the Ras/MAPK pathway (Maloof and Kenyon, 1998). Constitutive activation of the Ras/MAPK pathway leads to an elevated level of LIN-39, whereas impairment of the activity of this pathway results in a reduced level of LIN-39 in all VPCs (Maloof and Kenyon, 1998). In contrast, we found that the *lin-40(ku285)* loss-of-function mutation caused an upregulation of *lin-39* expression (Table 5), suggesting that *lin-40* plays an inhibitory role in controlling *lin-39* expression. Consistently, in this *lin-40* mutant, P3.p remained unfused at a higher frequency during the L2 stage (Fig. 4A) and was induced by the Ras/MAPK pathway to adopt the vulval fate at a higher level during the L3/L4 stages (Table 4). Furthermore, reduction of *lin-39* activity by a *lin-39(n1760)* mutation was able to overcome the effect caused by *lin-40(ku285)* (Fig. 4A). Taken together, these results indicate that *lin-40* functions upstream of *lin-39* to repress its expression and this inhibition of *lin-39* allows the VPCs to fuse with hyp7 and therefore represses vulval induction (Fig. 5). Further analyses on

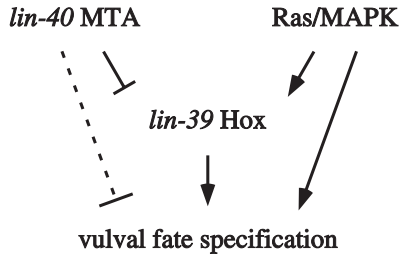

Fig. 5. How *lin-40* regulates vulval fate specification. *lin-39* Hox is a pivotal regulator in maintaining the competence of the VPCs at both the L1/L2 and later stages and therefore allowing vulval induction to occur. *lin-40* MTA represses vulval fate specification by inhibiting *lin-39* expression during the L2 stage and thus reducing the potential of the VPCs to be induced. By contrast, the Ras/MAPK pathway positively regulates *lin-39* expression during the L3 stage (Maloof and Kenyon, 1998). Thus, *lin-40* antagonizes the Ras/MAPK pathway at least partly at the level of *lin-39* expression. *lin-40* might also function in a *lin-39*-independent manner to repress downstream targets required for vulval fate specification, as indicated by a broken line.

synMuv genes in regulating this *lin-39*-mediated cell fusion process revealed a highly related, although somewhat different from that of *lin-40*, function of these genes (Z. C. and M. H., unpublished).

Although the molecular identity of *lin-40* MTA suggests a role similar to that of some class B synMuv genes, we did not identify the typical synergistic effect that would define *lin-40* as either a class A or a class B synMuv gene (Table 2, Table 3). Since several other *C. elegans* homologs the NuRD complex components have been implicated in the class B synMuv pathway (e.g. HDA-1 HDAC, LET-418 Mi-2 and LIN-53 RbAp46/48) (Lu and Horvitz, 1998; Solari and Ahringer, 2000; von Zelewsky et al., 2000), the lack of the synMuv gene activity in *lin-40* suggests that it might not function within the *C. elegans* NuRD complex. Alternatively, the *C. elegans* NuRD complex might regulate vulval induction in both the LIN-35 RB-related class B synMuv pathway and the LIN-40-mediated pathway. This hypothesis is supported by the fact that a mutation in *let-418* Mi-2 (another component of NuRD) causes a weak Muv phenotype (P8.p is sometimes induced) by itself and a more severe Muv phenotype in combination with a class A synMuv mutation (von Zelewsky et al., 2000). A weak Muv phenotype was also observed in single mutants of another class B gene, *lin-13*, which codes for a zinc-finger protein with a putative Rb-binding domain (Melendez and Greenwald, 2000). In addition, *lin-40* and *let-418* Mi-2 mutants share some other phenotypes that were not observed in *lin-35* Rb and some other class B synMuv mutants, which include lethality, sterility and abnormalities in vulval cell divisions (von Zelewsky et al., 2000) (this study). This fact suggests that these two genes, perhaps together with other components of the *C. elegans* NuRD complex, are also involved in other developmental events that are independent of *lin-35* Rb or some other class B synMuv activities.

The observation of a 'multiprotrusion' phenotype in *lin-40* and in *lin-40; synMuvB* double mutants, which mostly resulted from a failure in vulval cell migration or fusion during vulval morphogenesis (Fig. 3B), suggests a previously uncharacterized function for *lin-40* and some class B synMuv genes in controlling these cellular processes. We further showed that *lin-40* also regulates cell divisions during vulval morphogenesis, as the divisions along the transverse axis during the third round of vulval cell divisions were specifically affected by *lin-40* mutations (Table 1). *lin-40* appears to prevent the longitudinal division in cells where the division plane will be changed to an angle perpendicular to the previous one. This transition of division orientation requires a pause in the cell division cycle because the transverse divisions usually occur later than the longitudinal divisions. In *lin-40* mutants, when cells abnormally divided longitudinally, the delay before the third cell division was also eliminated. Thus, it is possible that *lin-40* functions to impose a brief break in the cell cycle to allow the rearrangement of the cytoskeleton for the next cytokinesis.

We are indebted to David Baillie for providing several *lin-40* alleles. We thank Weiming Zhang for isolating the *ku285* allele, Jeff Simske and Jeff Harding for the *jam-1::gfp* construct, Cynthia Kenyon for the *lin-39::lacZ* integrated strain, Yuji Kohara for cDNA clones, Alan Coulson for cosmids, Andy Fire for *gfp* vectors, Marian Walhout and Marc Vidal for two-hybrid constructs, and the *C. elegans* Genetic Center (University of Minnesota) for some strains used in this work. We also thank David Fay, Wade Johnson, Dan Starr, Bill Wood and Kimberly Van Auken for critically reading the manuscript, and members of the Han laboratory for discussions. This work was supported by a RO1 grant from NIH (GM47869) to M. H., who is an Assistant Investigator of the Howard Hughes Medical Institute.

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