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lin-35 and *lin-53*, Two Genes that Antagonize a *C. elegans* Ras Pathway, Encode Proteins Similar to Rb and Its Binding Protein RbAp48

Xiaowei Lu and H. Robert Horvitz*
Howard Hughes Medical Institute
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

The Ras signaling pathway for vulval induction in *Caenorhabditis elegans* is antagonized by the activity of the synthetic multivulva (synMuv) genes, which define two functionally redundant pathways. We have characterized two genes in one of these pathways. *lin-35* encodes a protein similar to the tumor suppressor Rb and the closely related proteins p107 and p130. *lin-53* encodes a protein similar to RbAp48, a mammalian protein that binds Rb. In mammals, Rb and related proteins act as regulators of E2F transcription factors, and RbAp48 may act with such proteins as a transcriptional corepressor. We propose that LIN-35 and LIN-53 antagonize the Ras signaling pathway in *C. elegans* by repressing transcription in the vulval precursor cells of genes required for the expression of vulval cell fates.

Introduction

Extracellular signals can promote or inhibit cell growth and differentiation via distinct signaling systems. The proper integration of multiple signals allows a cell to respond appropriately to its environment and is vital for homeostasis. Inappropriate activity of one or more signaling pathways can lead to tumorigenesis. Receptor tyrosine kinase (RTK)/Ras pathways define one class of oncogene signaling pathways (reviewed by Cantley et al., 1991; Hunter, 1997). RTK/Ras pathways function during the normal development of many organisms, including the nematode *Caenorhabditis elegans* (reviewed by Dickson and Hafen, 1994).

In *C. elegans*, the formation of the hermaphrodite vulva is induced by an RTK/Ras signaling pathway. The vulva is generated from six multipotent ventral ectodermal blast cells, P3.p–P8.p (Sulston and Horvitz, 1977; Sulston and White, 1980). Each of these six P(3–8).p cells can potentially adopt either the 1° vulval cell fate, the 2° vulval cell fate, or the 3° nonvulval cell fate (Sulston and White, 1980; Kimble, 1981; Sternberg and Horvitz, 1986). During wild-type development, a signal from the gonadal anchor cell induces the nearest P(3–8).p cell, P6.p, to adopt the 1° fate and the adjacent P5.p and P7.p cells to adopt the 2° fate. The cells furthest from the anchor cell, P3.p, P4.p, and P8.p, adopt the uninduced 3° fate. Vulval induction acts through a signaling pathway, which includes the *lin-3* EGF-like ligand, the *let-23* RTK, the *sem-5* adaptor, *let-60* Ras, the *ksr-1* kinase, *lin-45* Raf, *mek-2* MEK, and *mpk-1* MAP kinase, to regulate the activities of the ETS transcription factor LIN-1 and the winged-helix transcription factor LIN-31 (reviewed by Horvitz and Sternberg, 1991; Sundaram and Han, 1996; Tan et al., 1998).

Vulval induction is negatively regulated by the synthetic multivulva (synMuv) genes (Horvitz and Sulston, 1980; Ferguson and Horvitz, 1989). Loss-of-function mutations in these genes result in a multivulva (Muv) phenotype as a consequence of the expression of vulval cell fates by the P3.p, P4.p, and P8.p cells. The Muv phenotype of these mutants requires mutations in two genes. Specifically, these synMuv mutations fall into two classes, referred to as A and B. Animals carrying a class A and a class B mutation have a Muv phenotype, while animals carrying one or more mutations of the same class have a wild-type vulval phenotype. These mutations appear to define two functionally redundant pathways that negatively regulate the expression of vulval cell fates.

Four class A genes (*lin-8*, *lin-15*A, *lin-38*, and *lin-56*) and ten class B genes (*lin-9*, *lin-15*B, *lin-35*, *lin-36*, *lin-37*, *lin-51*, *lin-52*, *lin-53*, *lin-54*, and *lin-55*) have been identified (Horvitz and Sulston, 1980; Ferguson and Horvitz, 1989; J. H. Thomas and H. R. H., unpublished data). *lin-15* encodes both A and B activities in two nonoverlapping transcripts (Ferguson and Horvitz, 1989; Clark et al., 1994; Huang et al., 1994). *lin-15*A, *lin-15*B, *lin-9*, and *lin-36* encode novel proteins (Beitel, 1994; Clark et al., 1994; Huang et al., 1994; J. H. Thomas and H. R. H., unpublished data).

To elucidate the molecular mechanism by which the synMuv genes inhibit vulval induction, we have characterized two class B synMuv genes, *lin-35* and *lin-53*. Our findings indicate that in *C. elegans* vulval development an Rb-mediated pathway antagonizes the RTK/Ras pathway of vulval induction.

Results

Molecular Identification of *lin-35*

We mapped *lin-35* between *unc-40* and the Tc1 polymorphism *stP124* (Williams et al., 1992) on LGI. We identified two overlapping cosmids from this interval, C03E6 and C32F10, each of which rescued the Muv phenotype of a *lin-35; lin-15*A strain in germline transformation experiments (Figure 1A). The smallest subclone that retained rescuing activity was a 9.3 kb XhoI-EcoRV fragment from the region of overlap between C03E6 and C32F10. The *C. elegans* genome consortium (Wilson et al., 1994) had determined the DNA sequence of this fragment. Using the 9.3 kb minimal rescuing fragment as a probe, we detected a single 3.2 kb transcript in both embryonic and mixed-stage poly(A)$^+$ RNA on a Northern blot (Figure 1C), and we isolated cDNA clones from an embryonic cDNA library and determined the complete sequence of the longest cDNA (3.2 kb). We deduced the gene structure by comparing the genomic and the cDNA sequences (Figure 1B). The cDNA contains a single open reading frame (ORF) of 961 amino

*To whom correspondence should be addressed (e-mail: horvitz@mit.edu).

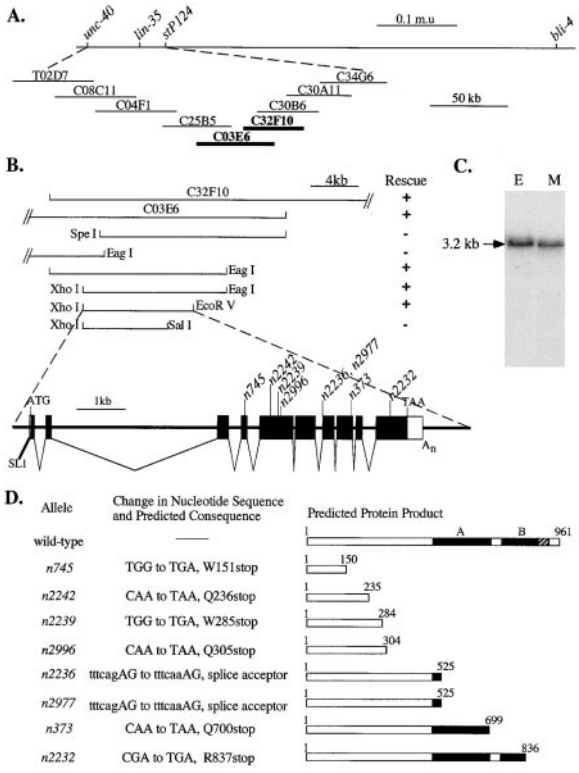

Figure 1. Cloning of *lin-35*

(A) Genetic and physical maps of the *lin-35* region (from the ACEDB database; e.g., Wilson et al., 1994). Dashed lines indicate alignments between the genetic and physical maps. Short horizontal lines represent cosmid clones assayed in germline transformation experiments. Cosmids that rescued the *lin-35* mutant phenotype are shown in bold (see text).

(B) Deletions and subclones derived from cosmids C32F10 and C03E6 were tested for *lin-35* rescuing activity. The penetrance of rescued lines was less than 20% Muv on average, as compared to more than 90% Muv in nonrescued lines and the starting mutant strain. +, rescue; −, no rescue. At the bottom is the structure of the *lin-35* gene deduced from the genomic and cDNA sequences. The *trans*-spliced leader SL1, the initiation and stop codons, and the poly(A) tail are indicated. Solid boxes indicate coding sequences, and open boxes indicate noncoding sequences. The positions of the eight *lin-35* mutations are indicated by the vertical lines above the boxes.

(C) Northern blot analysis of *lin-35*. *lin-35* message is present in both embryonic (E) and mixed-staged (M) poly(A)+ RNA (3 μg in each lane) from wild-type *C. elegans* as detected using the 9.3 kb XhoI-EcoRV minimal rescuing fragment as a probe.

(D) *lin-35* mutations. Each predicted mutant protein is represented schematically by a box labeled with its length in amino acids. Wild-type LIN-35 is shown on top. The A/B pockets are indicated by solid boxes. The region required for E2F binding includes the A and B pockets and the hatched box.

acids and appears to be full-length by three criteria: its size matches that of the transcript detected on the Northern blot, it contains the last 11 nucleotides of the SL1 *trans*-spliced leader sequence (Krause and Hirsh, 1987) at its 5′ end and a poly(A) tail at its 3′ end, and expression of this cDNA under control of the *C. elegans* heat shock promoters (Stringham et al., 1992) rescued the *lin-35* mutant phenotype (data not shown). To confirm that the rescuing activity observed was indeed *lin-35* activity, we identified the molecular lesions associated with the eight existing *lin-35* alleles (Figure 1D). Six alleles are nonsense mutations, and two alleles have an identical splice-acceptor mutation in the fourth intron despite their independent isolation. The allele *n745* contains an early nonsense mutation predicted to eliminate the C-terminal 84% of LIN-35. This allele may completely eliminate *lin-35* function.

lin-35 Encodes a Protein Similar to the Tumor Suppressor Rb

The predicted LIN-35 protein shares significant sequence similarity with the mammalian pocket proteins, which include the tumor suppressor Rb (Friend et al., 1986), p107 (Ewen et al., 1991), and p130 (Hannon et al., 1993; Li et al., 1993) (Figure 2). The similarity extends across the entire lengths of the proteins, including the "A/B pocket" domains, which mediate interactions with proteins important for cell cycle regulation, such as viral oncoproteins and E2F (reviewed by Taya, 1997). Overall, LIN-35 is 20% identical to p130, 19% to p107, 15% to Rb, and 16% to RBF, an Rb-related protein in *Drosophila* (Du et al., 1996). Regions important for pocket protein function are more conserved. For example, the N-terminal region of the B pocket domain of LIN-35 (amino acid residues 744–839) is 34% identical to p130, 34% to p107, 29% to Rb, and 30% to RBF. The spacer region that separates the A and B pockets of LIN-35 is not related in sequence to those of the pocket proteins. This spacer is short in length, like that of Rb. By contrast, p130 and p107 have much longer spacer regions, which are conserved between them and mediate their stable association with cyclin/cyclin-dependent kinase (CDK) complexes (reviewed by Zhu et al., 1994). Because LIN-35 is not particularly similar in sequence to any one of the three mammalian pocket proteins, *lin-35* may have diverged from an ancestor of the three mammalian genes before these three genes diverged from each other.

LIN-35 Protein Is Present in Vulval Cells

We raised polyclonal antibodies against a peptide from the N-terminal region of LIN-35. Affinity-purified antisera recognized a single protein of about 110 kDa present in wild-type protein extracts but absent in *lin-35(n745)* extracts on Western blots (Figure 3A). Thus, the antisera appeared to recognize specifically the LIN-35 protein product.

We stained wild-type and *lin-35* mutant worms with the purified anti-LIN-35 peptide antisera. In wild-type animals, the nuclei of most if not all cells in embryos and newly hatched L1 larvae stained (Figures 3B and 3C). In older larvae and adults, staining appeared diminished and became restricted to the nuclei of certain cells in the head and tail regions (data not shown) and of the P(3–8).p cells and their descendants (Figures 3D and 3E). We did not observe any staining during these stages in hyp7, the hypodermal syncytium that surrounds the P(3–8).p cells; hpy7 is the proposed site of action of *lin-15* and *lin-37* (Herman and Hedgecock, 1990; Hedgecock and Herman, 1995). The presence of LIN-35 protein in the P(3–8).p cells and their descendants is consistent with the hypothesis that *lin-35* acts cell autonomously to regulate vulval development. The distribution of LIN-35 protein suggests that *lin-35* may have other functions. However, mutations in *lin-35* do not have any obvious

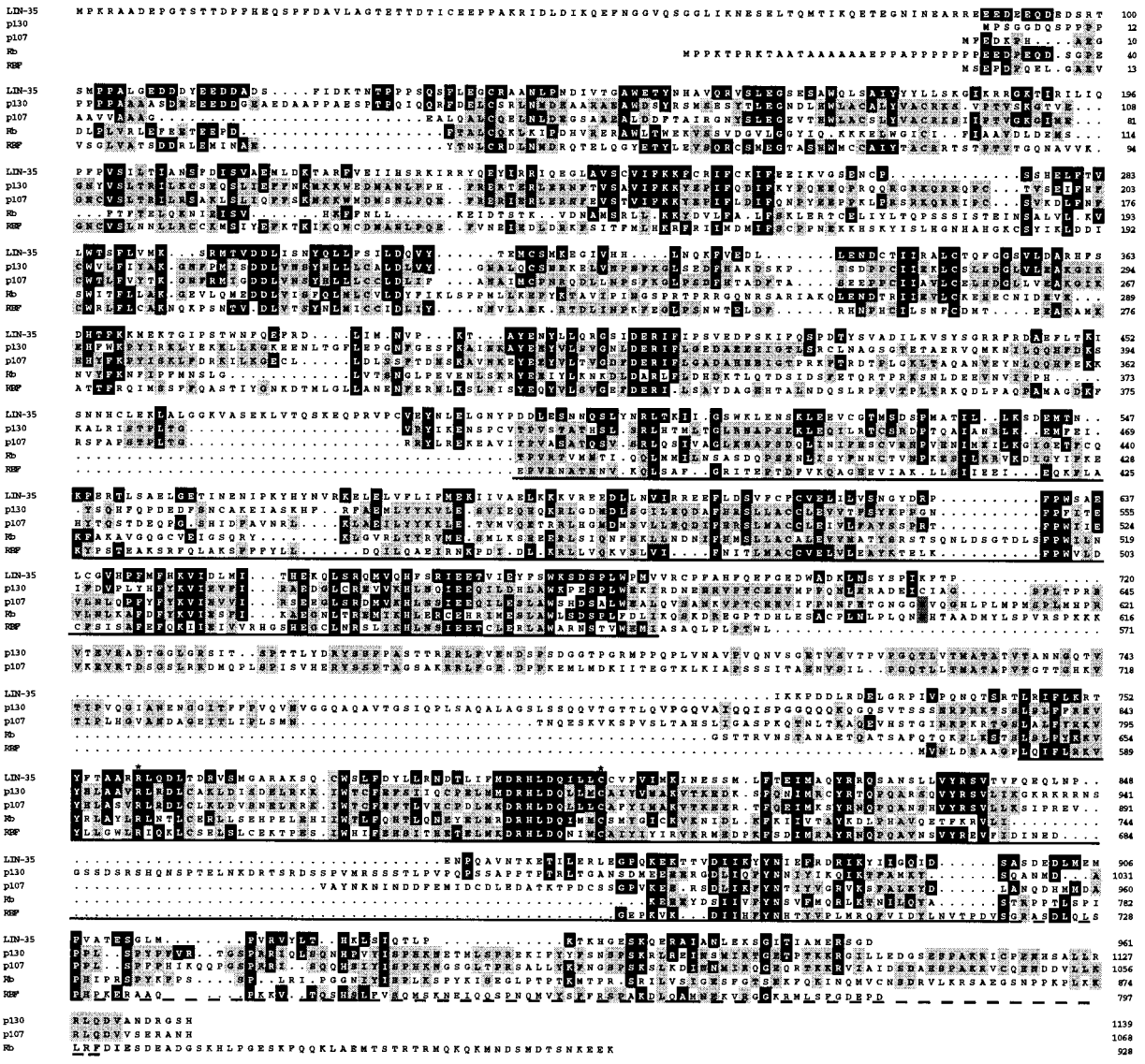

Figure 2. Sequence Alignment of LIN-35 and Other Pocket Proteins

The numbers on the right indicate amino acid positions. Identities with LIN-35 are shaded in black, and identities among other pocket proteins are shaded in gray. Two amino acid residues known to be mutated in Rb in human cancers are labeled with asterisks. The A and B pockets are underlined, and the region at the C terminus also required for E2F binding is indicated by the dashed line.

pleiotropic effects (data not shown). It is possible that other functions of *lin-35*, like those involved in vulval development, are redundant.

lin-53 Encodes a Protein Similar to RbAp48

Based on the finding that *lin-35* encodes a protein similar to Rb, we reasoned that other genes in the class B synMuv pathway might also be evolutionarily conserved. We identified from the *C. elegans* sequence database genomic sequences and expressed sequence tags (ESTs) (Wilson et al., 1994) predicted to encode proteins similar to proteins known to interact with Rb, and we compared their map positions with those of known class B synMuv genes. The cosmid K07A1 contained two predicted genes, named K07A1.12 and K07A1.11, both similar to RbAp48 (p48) and RbAp46 (p46), two closely related proteins that bind to Rb in vivo (Huang et al., 1991; Qian et al., 1993; Qian and Lee, 1995). This cosmid mapped near the class B synMuv gene *lin-53* (Ferguson and Horvitz, 1989; J. H. Thomas and H. R. H., unpublished data) between *unc-29* and *lin-11* on LGI. K07A1.11 lies about 100 bp 3′ to K07A1.12 in the same orientation. To investigate whether *lin-53* corresponded to either of these genes, we tested to see if cosmid K07A1 would rescue the Muv phenotype of a *lin-53(n833); lin-15*A strain. Since the two existing alleles of *lin-53* are semidominant (see below), we looked for a partial rescue. Transgenic animals carrying K07A1 exhibited a Muv phenotype of reduced expressivity and penetrance compared to nontransgenic animals, indicating partial rescue (data not shown).

To confirm that K07A1 indeed contained *lin-53* activity, we determined the sequence of K07A1.12 and K07A1.11 from the two independently isolated *lin-53*

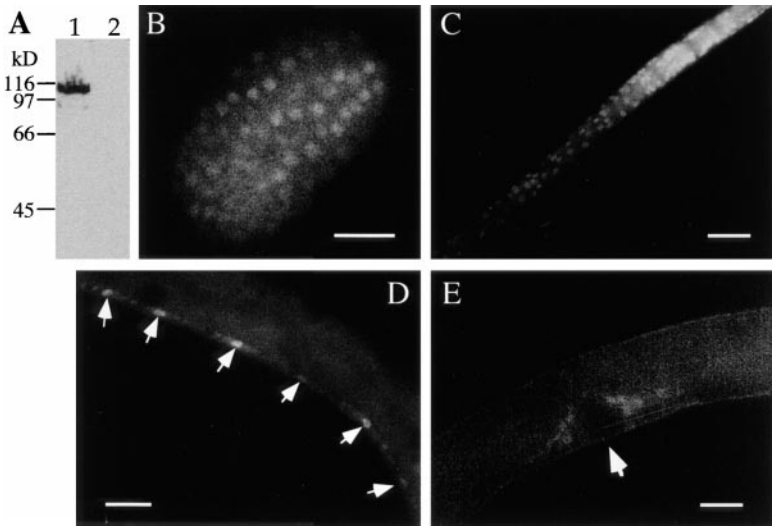

Figure 3. LIN-35 Protein Expression

(A) Anti-LIN-35 peptide antibodies detected a protein of expected size (110 kDa) in wild-type (lane 1) but not in *lin-35(n745)* mutant (lane 2) extracts. The molecular weights of marker proteins are indicated.

(B–E) Whole-mount staining of wild-type embryos and larvae using anti-LIN-35 peptide antibodies. Scale bar, 10 μm.

(B and C) LIN-35 is broadly expressed in embryos and in newly hatched L1s.

(D) LIN-35 staining in nuclei of P(3–8).p cells (arrows) in an L3 animal.

(E) LIN-35 staining in nuclei of the developing L4 vulva. Ventral is down; anterior is to the left. Arrow points to the invagination located at the center of the developing vulva.

alleles, *n833* and *n2978*, and found they carried an identical mutation in K07A1.12 (see below) and had no mutation in K07A1.11. We therefore conclude that K07A1.12 is *lin-53*. We used EST clones (Kohara et al., unpublished results) that correspond to each gene as probes to screen for additional cDNAs. The longest *lin-53* cDNA contained at its 5′ end the last seven nucleotides from the *C. elegans trans*-spliced leader SL1 followed by three nucleotides upstream of the first ATG and at its 3′ end a poly(A) tail. The longest K07A1.11 cDNA contained at its 5′ end the last ten nucleotides from the *C. elegans trans*-spliced leader SL2 (Huang and Hirsh, 1989) followed by six nucleotides upstream of the first ATG and at its 3′ end a poly(A) tail (Figure 4A). The tandem arrangement of these two genes in close proximity, with the message of the 3′ gene SL2 *trans*-spliced, suggests that they form a complex locus and are cotranscribed (Spieth et al., 1993).

The LIN-53 protein is 72% identical to p48 and 70% to p46, while K07A1.11 is 53% identical to p48 and 52% to p46. p48 and p46 are 7 WD-repeat proteins, which are regulatory proteins that mediate protein–protein interactions (Neer et al., 1994; Neer and Smith, 1996). Several p48-related proteins have been identified in different organisms, including the p55 subunit of the *Drosophila melanogaster* chromatin assembly factor 1 (Tyler et al., 1996) and the *Saccharomyces cerevesiae* proteins Msi1p (Ruggieri et al., 1989) and Hat2p (Parthun et al., 1996). LIN-53 is 72%, 27%, and 25% identical to these proteins, respectively. The mutation in *lin-53(n833)* and *lin-53(n2978)* animals causes a leucine-to-phenylalanine change at a conserved leucine in the fifth WD domain (Figure 4B).

Since K07A1.11 is 54% identical to LIN-53, we tested whether expression of K07A1.11 could rescue the *lin-53* mutant phenotype. Driven by the *col-10* gene promoter, which is strongly expressed in hypodermal and hypodermal blast cells including the P(3–8).p cells during larval development (P. Olsen and V. Ambros, personal communication), expression of the *lin-53* cDNA but not of the K07A1.11 cDNA partially rescued the Muv phenotype. This result suggests that K07A1.11 cannot substitute for wild-type *lin-53* function.

Wild-Type *lin-53* Activity Is Required for the Class B synMuv Pathway

To investigate further the role of *lin-53* in the class B synMuv pathway, we used RNA-mediated interference (RNAi), which has been shown to produce a specific phenocopy of the loss-of-function phenotype of a targeted gene by an unknown mechanism (Fire et al., 1998). We assayed the phenotypes of the progeny of wild-type, *lin-15*A, or *lin-15*B mothers injected with antisense RNA derived from a *lin-53* cDNA clone. In all cases, antisense RNA injection caused embryonic lethality, suggesting that *lin-53* is required during embryogenesis. Similar observations have been reported by Shi and Mello (1998), who studied the role of the gene we have now identified as *lin-53* in embryonic development.

Because of this lethality, we were unable to use the RNAi technique to address the role of *lin-53* during vulval induction. Instead, we used the *col-10* promoter to drive expression of the antisense strand of a *lin-53* cDNA in hypodermal and hypodermal blast cells. About 18% of *lin-15*A animals carrying the P*col-10* antisense *lin-53* construct were Muv, and this Muv phenotype was dependent on the presence of the *lin-15*A mutation (Table 1). Neither antisense expression of the K07A1.11 cDNA nor sense expression of the *lin-53* or K07A1.11 cDNAs had any effect in similar experiments. These experiments suggest that wild-type *lin-53* activity is required for the class B synMuv pathway.

lin-53(n833) Is Likely to Have a Dominant-Negative Effect

Unlike other synMuv mutations, *lin-53(n833)* and *lin-53 (n2978)* cause a semidominant class B synMuv phenotype (e.g., *lin-53(n833)/+; lin-15*A animals have an incompletely penetrant Muv phenotype [Table 2]). Since loss of *lin-53* function causes a class B synMuv phenotype, as indicated by our antisense experiments, *lin-53* might be a haplo-insufficient locus; alternatively, *lin-53(n833)* might be a dominant-negative mutation. To distinguish between these two possibilities, we examined the phenotypes of animals of genotype *+/Df; lin-15*A (the *Df* chromosome was deleted for the *lin-53* locus, see Table 2 for details). Five of 413 *+/Df; lin-15*A

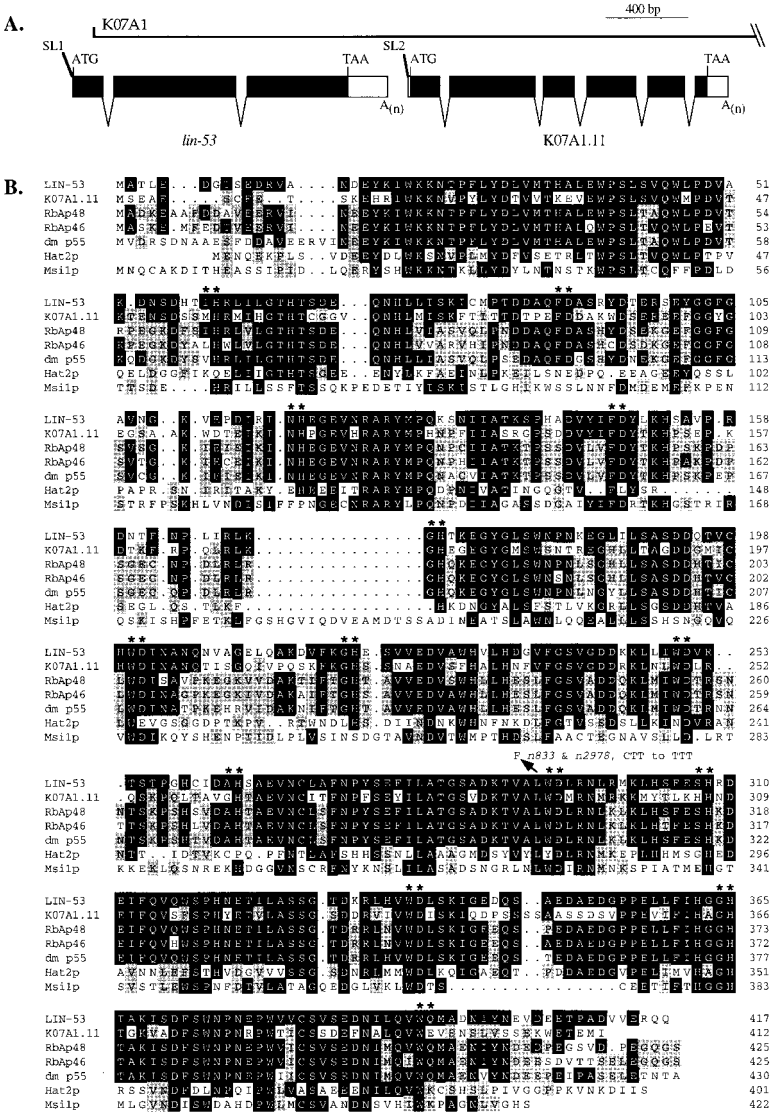

Figure 4. *lin-53* Structure and Similarity to Human p48

(A) Genomic organization of the *lin-53*/K07A1.11 locus. The top horizontal line indicates cosmid K07A1, which is missing the presumptive 5′ regulatory region and part of the first exon of *lin-53*. Solid boxes indicate coding sequences, and open boxes indicate noncoding sequences. SL1 and SL2 *trans*-spliced leaders, initiation and stop codons, and poly(A) tails are indicated.

(B) Sequence alignment of LIN-53, K07A1.11, human p48 and p46, *Drosophila* p55, and *S. cerevisiae* Hat2p and Msi1p. Identities with LIN-53 are shaded in black, and identities among other proteins are shaded in gray. The signature residues of each WD repeat are indicated by asterisks. The two identical *lin-53* L292F mutations (*n833* and *n2978*) are indicated by the arrow.

animals examined were Muv (Table 2). This penetrance of the Muv phenotype is much lower than in a *lin-53(n833)/+; lin-15*A strain, indicating that a 2-fold reduction in wild-type *lin-53* activity only occasionally causes a synMuv phenotype and that *lin-53(n833)* is unlikely to simply reduce or eliminate *lin-53* function. Rather, *lin-53(n833)* is probably a dominant-negative mutation.

Table 1. *lin-53* Tissue-Specific Antisense Expression Causes a synMuv Phenotype

Construct	Percent Muv (n)	
	*unc-76; lin-15*A	*unc-76*
$P_{col\text{-}10}$ *lin-53* antisense	18 ± 5% (672)[a]	0 (many)
$P_{col\text{-}10}$ *lin-53*	0 (many)	ND
$P_{col\text{-}10}$ K07A1.11	0 (many)	ND
$P_{col\text{-}10}$ K07A1.11 antisense	0 (many)	ND

ND, not determined.
[a] Number obtained from three transgenic lines.

Table 2. *lin-53(n833)* May Act as a Dominant-Negative Allele

lin-53 Genotype[a]	Percent Muv (n)
n833/n833	100 (many)
n833/+	12 (500)[b]
+/qDf9	1 (413)[c]
+/+; Ex[n833]	17 ± 6 (504)[d]

All strains tested were homozygous for *lin-15*A. Muv. animals with at least one pseudovulva on their ventral sides; n, number of animals scored.
[a] In heterozygous strains, *lin-53(n833)* chromosomes were marked with *dpy-5*, and *lin-53*(+) chromosomes with *unc-29* and *lin-11*.
[b] Number indicates penetrance of Muv phenotype among *n833/+* progeny from *n833/+* hermaphrodites.
[c] Number indicates penetrance of Muv phenotype among *+/qDf9* progeny from *+/qDf9* hermaphrodites. *qDf9* deletes *lin-53* as confirmed by PCR of homozygous deficiency embryos.
[d] Number obtained from three transgenic lines.

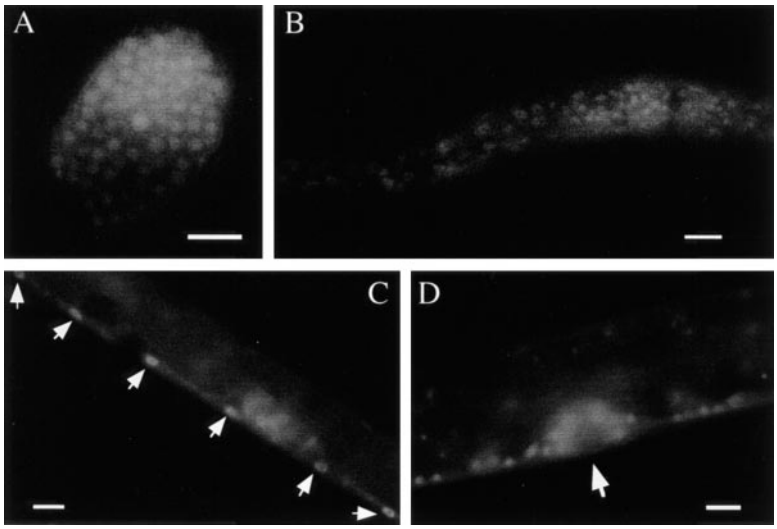

Figure 5. A GFP::LIN-53 Transgene Is Expressed in Many Nuclei

(A and B) GFP is expressed in most nuclei in embryos and newly hatched L1s.
(C) GFP is expressed in the nuclei of P(3–8).p cells (arrows) in an L3 animal.
(D) GFP is expressed in the nuclei of the developing L4 vulva. Ventral is down; anterior is to the left. Arrowhead points to the invagination located at the center of the developing vulva. Scale bar, 10 μm.

Consistent with this hypothesis, expression of a *lin-53* cDNA carrying the *n833* mutation (L292F) driven by the *col-10* promoter caused a partially penetrant Muv phenotype in *lin-53(+); lin-15*A animals (Table 2). The *lin-53(n833)* mutation appears to affect only vulval development, while the *lin-53* null phenotype may be embryonic lethality. One explanation for the tissue-specific effect of the *lin-53(n833)* allele could be that vulval development is particularly sensitive to a decreased dosage of wild-type *lin-53*. Alternatively, the dominant-negative mutant LIN-53 protein may be titrating a factor that becomes limiting specifically in vulval tissue.

A GFP::LIN-53 Transgene Is Expressed in the Vulval Cell Nuclei

A transgene containing the *lin-53* cDNA tagged with GFP (Chalfie et al., 1994) at its N terminus and under the control of the endogenous *lin-53* promoter was capable of partially rescuing the Muv phenotype of *lin-53(n833); lin-15*A animals. We examined the GFP expression pattern in animals carrying an integrated array of this transgene and observed GFP expression in most if not all nuclei during embryogenesis and in newly hatched L1s (Figures 5A and 5B). During larval development, we observed fluorescence in many nuclei in the head and tail regions, similar to the LIN-35 staining pattern seen with the anti-LIN-35 peptide antibody. GFP was also present in hypodermal cells throughout development (data not shown). At the time of vulval induction, GFP was visible in all P(3–8).p cells and persisted until after the P(3–8).p cell divisions and vulval morphogenesis were complete (Figures 5C and 5D).

A *C. elegans* Homolog of Histone Deacetylase May Act in the *lin-35* Rb-Mediated synMuv Pathway

Based on recent findings that Rb can recruit a histone deacetylase, HDAC1, to repress transcription from target promoters (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998) and the fact that p48 has been identified as a subunit of histone deacetylase (Taunton et al., 1996), we investigated whether several *C. elegans* histone deacetylase homologs, *hda-1*, *hda-2*, and *hda-3* (Shi and Mello, 1998), might be involved in the synMuv pathway. RNAi of *hda-1* caused embryonic lethality, while RNAi of *hda-2* and *hda-3* produced no obvious phenotypic abnormality in either a *lin-15*A or *lin-15*B background (data not shown). We then tested whether tissue-specific antisense expression of *hda-1* driven by the *col-10* promoter can cause a synMuv phenotype. Similar to *lin-53* antisense expression, *hda-1* antisense expression caused a Muv phenotype in a *lin-15*A background but not in a wild-type background (i.e., a class B synMuv phenotype). Antisense expression of the closely related *hda-3* gene had no effect (Table 3). This result suggests that the histone deacetylase gene *hda-1* activity is required for the *lin-35* Rb-mediated synMuv pathway.

LIN-35 Rb, LIN-53 p48, and HDA-1 Interact In Vitro

To determine whether LIN-35 Rb can directly bind LIN-53 p48 or HDA-1, as predicted by their sequences, we performed GST pull-down experiments. Bacterially produced glutathione S-transferase (GST) or GST::LIN-53 or GST::HDA-1 fusion proteins immobilized on beads were incubated with different in vitro translated ^{35}S-methionine-labeled proteins. GST::LIN-53 interacted with a fragment of LIN-35 Rb that contains the entire A/B pocket (LIN-35BX) but not with LIN-35 fragments that lack the intact

Table 3. *hda-1* Tissue-Specific Antisense Expression Causes a synMuv Phenotype

	Percent Muvs (n)	
Construct	*unc-76; lin-15A*	*unc-76*
$P_{col\text{-}10}$ *hda-1* antisense	21 ± 9% (462)[a]	0 (many)
$P_{col\text{-}10}$ *hda-3* antisense	0 (many)	ND

ND, not determined.
[a] Number obtained from three transgenic lines.

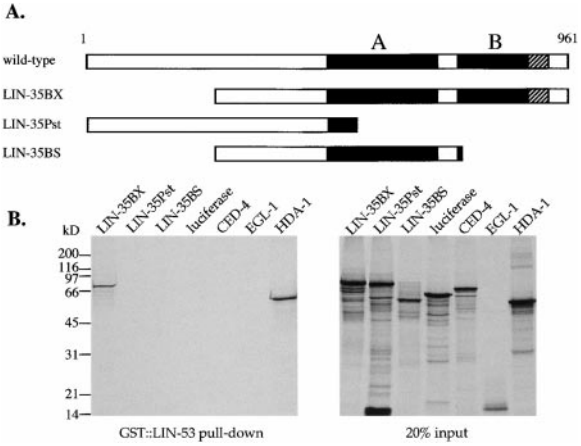

Figure 6. LIN-35 Rb, LIN-53 p48, and HDA-1 In Vitro Interactions

(A) Schematic of LIN-35 Rb fragments used.

(B) GST::LIN-53 interaction with LIN-35 Rb and HDA-1. (Left) GST::LIN-53 beads were incubated with in vitro translated ^{35}S-methionine-labeled LIN-35 fragments, luciferase, CED-4, EGL-1, or HDA-1. (Right) Twenty percent of the input radiolabeled proteins used in the experiments shown in the left panel. The molecular weights of marker proteins are indicated.

A/B pocket or with control proteins (Figure 6B). Interestingly, GST::LIN-53(L292F) and GST::HDA-1 fusion proteins also interacted with the LIN-35BX fragment (data not shown). It is worth noting that the HDA-1 sequence lacks a recognizable LXCXE motif, which is involved in the interaction between human Rb and HDAC1 (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998). Therefore, HDA-1 does not appear to interact with LIN-35 Rb via an LXCXE motif. GST::LIN-53 and GST::LIN-53(L292F) fusion proteins also interacted with HDA-1 (Figure 6B and data not shown). None of the proteins tested was retained by GST beads (data not shown). These results indicate that direct physical interactions can occur between any two of the three proteins LIN-35 Rb, LIN-53 p48, and HDA-1. We have not determined whether a ternary complex containing these three proteins can exist.

The *lin-35* and *lin-53* synMuv Phenotypes Require a Functional RTK/Ras Signaling Pathway

To determine how the *lin-35* and *lin-53* synMuv genes interact with the Ras signaling pathway during vulval development, we analyzed the vulval phenotype of triple mutants carrying either a *lin-35* or a *lin-53* mutation, a mutation in a class A synMuv gene, and a vulvaless (Vul) mutation in a Ras signaling gene (Table 4). The synMuv phenotype was epistatic to the Vul phenotype caused by a mutation in the *lin-3* gene, which encodes the inductive signal (Table 4). This observation suggests that the *lin-35* and *lin-53* genes act downstream of or in parallel to *lin-3* and that the synMuv phenotype does not require the *lin-3* inductive signal.

By contrast, the Vul phenotypes of mutations in *let-23* RTK, *sem-5*, *let-60* Ras, *lin-45* Raf, and *mpk-1* genes were epistatic to the synMuv phenotype (Table 4). These observations suggest that the *lin-35* and *lin-53* genes act upstream of or in parallel to the RTK/Ras signaling genes. In other words, the expression of vulval cell fates by the P3.p, P4.p, and P8.p cells in synMuv mutants requires a functional RTK/Ras signal transduction pathway. These observations concerning *lin-35* and *lin-53* are equivalent to previous observations concerning other synMuv genes (Ferguson et al., 1987; Huang et al., 1994; J. H. Thomas and H. R. H., unpublished data).

Table 4. Epistasis Analysis between synMuv and Vul Mutations

Genotype[a]	Vulval Phenotype
synMuv[a]	Muv
lin-3(n378)	Vul
lin-3(n378); synMuv	Muv
let-23(lf)	Vul
let-23(lf); synMuv	Vul
sem-5(lf)	Vul
sem-5(lf); synMuv	Vul
let-60(lf)[b]	Vul
let-60(lf); synMuv	Vul
lin-45(lf)	Vul
lin-45(lf); synMuv	Vul
mpk-1(lf)[c]	Vul
mpk-1(lf); synMuv	Vul

[a] Two synMuv genotypes were studied: *lin-35(n745); lin-15*A and *lin-53(n833); lin-15*A.

[b] *let-60(lf)* animals were derived from *let-60(lf)*/+ heterozygotes in order to rescue the maternal-effect L1 larval lethality.

[c] Vulval phenotype was assayed at 25°C, since *mpk-1(oz140)* is temperature sensitive.

Since in synMuv mutants P5.p, P6.p, and P7.p generally express normal vulval cell fates, synMuv gene activities are not required in these three cells. To determine whether the synMuv genes can act in the P5.p, P6.p, and P7.p cells, we analyzed the phenotype of triple mutants carrying either a *lin-35* or a *lin-53* mutation, a mutation in a class A synMuv gene, and a Vul mutation in *lin-2*, *lin-7*, or *lin-10*. These three genes act as positive regulators of the LET-23 RTK activity by localizing LET-23 to the basolateral membrane of the P(3–8).p cells, thus allowing better access to the LIN-3 inductive signal from the anchor cell (Kaech et al., 1998). Mutations in *lin-2*, *lin-7*, and *lin-10* in general cause P5.p, P6.p, and P7.p to adopt the uninduced 3° fate and result in a Vul phenotype. By contrast, in the triple mutants, P5.p, P6.p, and P7.p in general adopt a vulval fate (Table 5). Thus, in these three cells in the absence of receptor localization the activity of RTK/Ras pathway is insufficient to induce vulval cell fates unless synMuv gene activity is eliminated. This observation reveals that synMuv gene activity can function in P5.p, P6.p, and P7.p. We conclude that synMuv gene activity acts in all six P(3–8).p cells to antagonize the activity of the RTK/Ras signaling pathway.

Discussion

lin-35 encodes a protein related to Rb, and *lin-53* encodes a protein with striking similarity to an Rb-binding protein, p48 (72% identity). *lin-35*, *lin-53*, and a *C. elegans* histone deacetylase gene act in the same genetic pathway to antagonize a Ras signal transduction pathway in *C. elegans*. We propose that in mammals Rb,

Table 5. Gene Interactions with *lin-2*, *lin-7*, and *lin-10*

Genotype[a]	Vulval Phenotype	Percent Induced						
		P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	n
synMuv[a]	Muv	100	100	100	100	100	100	24
lin-2(lf)	Vul	0	5	48	62	38	0	21
lin-2(lf); synMuv	Muv, Muv/Vul[b]	64	79	100	100	96	71	28
lin-7(lf)	Vul	0	0	22	30	11	0	27
lin-7(lf); synMuv	Muv, Muv/Vul	49	73	92	100	89	57	37
lin-10(lf)	Vul	0	0	14	33	19	0	21
lin-10(lf); synMuv	Muv, Muv/Vul	58	79	96	100	96	50	24

[a] Two synMuv genotypes were studied: *lin-35(n745); lin-15*A (shown) and *lin-53(n833); lin-15*A (data not shown, but similar); comparable observations have been made of *lin-36(n766); lin-15*A and *lin-15(e1763)* animals (J.H. Thomas and H.R.H., unpublished data).
[b] Muv/Vul, animals with both ectopic vulval tissues and nonfunctional vulvae.

p48, and histone deacetylase genes act in a tumor suppressor pathway that involves mechanisms and molecules similar to those of the synMuv pathway in *C. elegans* and that may well antagonize a mammalian Ras pathway.

lin-35 Rb, *lin-53* p48, and *hda-1* May Act to Repress Transcription in the P(3–8).p Cells

Rb, a key regulator of the mammalian cell cycle (reviewed by Weinberg, 1995; Wang, 1997), acts mainly through E2F transcription factors (composed of E2F and DP heterodimers), which regulate the expression of genes required for entry into S phase (reviewed by Dyson, 1998; Nevins, 1998). Rb, p107, and p130 can interact with DNA-bound E2F either to abolish transactivation or to exert active repression of transcription from target promoters. One mechanism by which Rb represses transcription is to bind and recruit the histone deacetylase HDAC1, presumably to remodel chromatin structure on a target promoter and thereby limit access of the transcriptional machinery to the DNA (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). p48 has been found together with HDAC1 in a large corepressor complex required for Mad-Max-mediated transcriptional repression (Hassig et al., 1997; Laherty et al., 1997).

We propose that the class B synMuv genes inhibit vulval induction by a conserved mechanism: LIN-35 Rb forms a complex with a sequence-specific transcription factor (indicated as "TF" in Figure 7), presumably an E2F-like protein, and recruits a corepressor complex containing HDA-1, LIN-53 p48, and other proteins (indicated as "X" in Figure 7) to turn off the transcription of vulval specification genes via E2F-binding sites (Figure 7). In the wild type, in the P3.p, P4.p, and P8.p cells, synMuv gene activity antagonizes the basal activity of the RTK/Ras pathway by repressing transcription of vulval genes. As a result, those cells adopt the nonvulval 3° fate. In P5.p, P6.p, and P7.p, on the other hand, the antagonistic effect of synMuv gene activity is inactivated or can be overcome by the activated RTK/Ras pathway, thereby releasing transcriptional repression and permitting the expression of vulval fates. In the P(3–8).p cells of a synMuv mutant, repression cannot occur and all six P(3–8).p cells express vulval fates, resulting in a Muv phenotype. The synMuv genes do not appear to exert their effects by regulating cell cycle progression of the P(3–8).p cells, since all six of these cells have very similar cell cycle profiles (Sulston and Horvitz, 1977; Euling and Ambros, 1996).

Class B synMuv Genes Act in an Intercellular Signaling Pathway to Regulate Transcription

Both *lin-35* and *lin-53* appear to be expressed in the P(3–8).p cells and their descendants, consistent with the hypothesis that these class B synMuv genes act in P(3–8).p. Some class B synMuv genes, specifically *lin-15* and *lin-37*, appear to act in the hypodermal cell hyp7 to regulate an intercellular signal (Herman and Hedgecock, 1990; Hedgecock and Herman, 1995), while others, specifically *lin-36* (J. H. Thomas and H. R. H., unpublished data), *lin-35*, and *lin-53*, seem to act in the P(3–8).p cells to control the response to that signal. We suggest that a LIN-35 Rb/LIN-53 p48/HDA-1 nuclear complex responds to the intercellular signaling pathway encoded by other class B synMuv genes to regulate transcription during vulval development in *C. elegans*.

Many questions remain to be answered. For example, what are the ligand, receptor, and signal transducers in the class B synMuv pathway? So far, none of the cloned class B genes appears to encode a secreted or transmembrane protein. Also, what are the other components of the proposed repressor complex, and what functions might they serve? It is possible that components of the class B synMuv signal transduction pathway are used in biological processes in addition to vulval development, so that mutations in such components would cause pleiotropic effects and would not be isolated as synMuv mutants. Indeed, the null phenotypes of *lin-53* and *hda-1* appear to be embryonic lethality, as indicated by RNAi experiments (this study, Shi and Mello, 1998), and strong mutations in *lin-9* cause sterility (Ferguson and Horvitz, 1989). The characterization of the remaining class B genes and the identification of genes that interact with the known class B genes should provide more insight into this signaling pathway.

How Might the synMuv Pathway Interface with the Ras Pathway?

Gene interaction experiments both by us and by others indicate that in synMuv mutants anchor cell-independent activity of the Ras pathway is necessary for the expression of vulval cell fates (Ferguson et al., 1987;

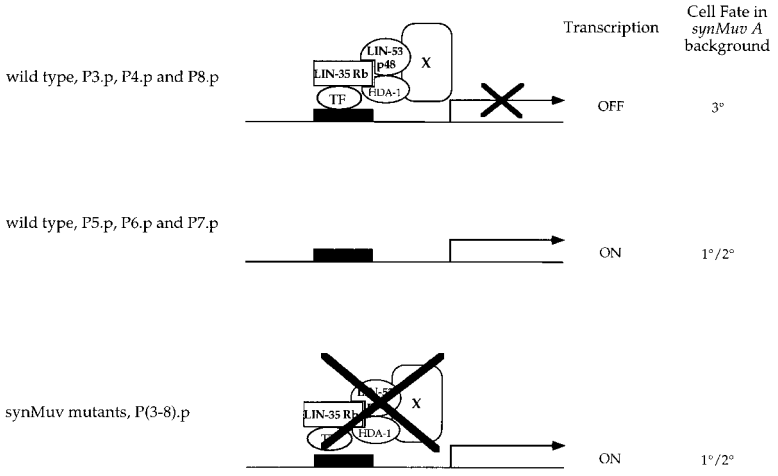

Figure 7. Models for How LIN-35 and LIN-53 May Cooperate to Repress Transcription

"TF" indicates a sequence-specific transcription factor, presumably, an E2F-like protein; the closed box indicates a DNA-binding site for TF in the promoter. In the P3.p, P4.p, and P8.p cells of wild-type animals, the synMuv genes are active, a repressor complex "X" that contains LIN-53 and HDA-1 is recruited by the DNA-bound LIN-35/TF complex to turn OFF transcription, and the 3° fate is expressed. In the P5.p, P6.p, and P7.p cells of wild-type animals, synMuv genes are inactive or their effect is overcome by the signal-dependent activation of the Ras pathway. As a result, the transcription of vulval cell-fate genes is ON, leading to the expression of 1° or 2° fates. By contrast, in the P3–8.p cells of synMuv mutants, repression is relieved, transcription is ON, and the 1° or 2° fates are expressed.

Huang et al., 1994; J. H. Thomas and H. R. H., unpublished data). Thus, the synMuv genes must act genetically upstream of or in parallel to the Ras pathway. Action in parallel would be consistent with recent findings from studies of mammalian cells: dominant-negative Ras and Ras neutralizing antibodies induced an Rb-dependent block in DNA synthesis and G1 arrest (Mittnacht et al., 1997; Peeper et al., 1997), suggesting that Rb functions to inhibit mitogenesis downstream of or in parallel to Ras.

Although we cannot yet assign the precise point of interface between the synMuv and Ras pathways in vulval development, our molecular analyses of the class B synMuv pathway take us one step closer to understanding the nature of the anatagonism between the synMuv genes and the Ras pathway. As we discussed above, we propose that the class B synMuv pathway acts by repressing transcription in the P(3–8).p cells.

What might be the target genes for LIN-35 Rb-mediated transcriptional repression? Candidate genes include those in the *let-23* RTK/*let-60* Ras pathway. However, given the findings of Peeper et al. (1997) cited above, it seems more likely that the class B synMuv genes act to repress genes involved in vulval differentiation. Since the LIN-1 ETS protein also seems likely to act by repressing vulval differentiation genes (Beitel et al., 1995; Tan et al., 1998), LIN-35 Rb and LIN-1 may have at least partially overlapping targets. The identification of target genes for the LIN-35 Rb repressor complex and for the LIN-1 ETS protein may well help establish the nature of the antagonism between the Rb pathway and the Ras pathway both in *C. elegans* and in other organisms.

Experimental Procedures

Strains and Genetics

C. elegans strains were cultured as described by Brenner (1974) and were grown at 20°C unless otherwise noted. Mutations used (Riddle et al., 1997) were as follows: LGI, *unc-40(e271)*, *bli-6(e937)*, *dpy-5(e61)*, *unc-29(e1072)*, *lin-11(n566)*, *lin-10(n299)*, and *qDf9* (Ellis and Kimble, 1995); LGII, *lin-7(e1413)* and *let-23(sy97)*; LGIII, *mpk-1(oz140)*; LGIV, *lin-3(n378)*, *let-60(n1876)*, and *lin-45(sy96)*; LGX, *lin-15(n767)* (a class A mutation), *lin-15(n744)* (a class B mutation), *lin-2(n397)*, and *sem-5(n2030)*. To map *lin-35* with respect to *unc-40* and *stP124*, we isolated Bli non-Unc recombinants from the progeny of *unc-40 stP124 bli-4/lin-35(n2977); lin-15(n433)* hermaphrodites and scored the progeny of these recombinants for *lin-35* and *stP124*. Of 63 recombinants, 19 had *stP124* and were non-Muv, 9 had *stP124* and were Muv, and 35 did not have *stP124* and were Muv.

Transgenic Animals

Germline transformation was performed as described by Mello et al. (1991). DNA (30–100 ng/μl) was coinjected with a *unc-76* rescuing plasmid (100 ng/μl) (Bloom and Horvitz, 1997), and lines of non-Unc-76 transgenic animals were established. Chromosomal integration of extrachromosomal arrays of transgene was accomplished by γ-ray irradiation of transgenic animals.

Identification of cDNAs and Sequence Analysis of cDNAs and Mutant Alleles

To identify *lin-35* and *lin-53* cDNAs, we screened a cDNA library made from *C. elegans* embryonic RNA (Okkema and Fire, 1994). The sequences of the ends of inserts of positive clones were first determined using vector primers, and the complete sequence of the longest cDNA was then determined using primers positioned within the coding sequences. The sequence of at least one additional cDNA clone for each gene was determined to confirm splicing patterns. The sequences of both strands of the coding regions and splice junctions were determined from PCR fragments amplified from the *lin-35* and *lin-53* mutant alleles and purified by gel electrophoresis. DNA sequences were determined using an automated ABI 373A DNA sequencer (Applied Biosystems).

In Vitro Interaction Assays

Wild-type and mutant *lin-53* cDNAs were cloned into vector pGEX4T-3 (Pharmacia), expressed in *E. coli* strain BL21(DE3), and purified with glutathione sepharose beads as recommended by the manufacturer (Pharmacia).

lin-35, *lin-53*, and *hda-1* cDNA fragments were cloned into the vector pCITE4a(+) (Novagen) or pRK5. The resulting constructs were used as templates to synthesize ^{35}S-methionine-labeled proteins in the TNT Coupled Reticulocyte Lysate System (Promega). Labeled proteins were incubated with equal amounts of GST fusion proteins for 2 hr at 4°C. Bound proteins were eluted with 2× SDS sample buffer and analyzed by SDS-PAGE (10%) and autoradiography.

Antibodies

A peptide from the N-terminal region of LIN-35, HSRKIRRYQEYIRR, with a cysteine added to its N terminus was coupled to keyhole limpet hemacyanin (KLH) and used to immunize rabbits and obtain antisera (Zymed). Antibodies were purified over a peptide affinity

column (Pierce). For Western blots, the LIN-35 protein was visualized using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and chemiluminescent detection reagents (Pierce). Immunocytochemistry was as described by Finney and Ruvkun (1990).

RNA and RNAi Analyses

For Northern blot analysis, poly(A)$^+$ RNA was isolated using the FAST TRACK system (Invitrogen). RNA was subjected to electrophoresis and transferred to Nytran. The filter was probed with ^{32}P-radiolabeled *lin-35* genomic DNA. PCR fragments containing cDNA flanked by the T7 and the T3 promoters amplified from the cDNA phage lysates were used for in vitro RNA synthesis as described (Fire et al., 1998). The unmodified RNA was resuspended in H_2O and injected at 1–5 μg/μl. Injected animals were moved to a new plate 12 hr after injection to enrich for progeny that had been subjected to antisense injection.

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