Identification of Conserved Chromatin-Regulatory Complexes among the Class B Synthetic Multivulva Proteins

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Abstract

The class A, B, and C synthetic Multivulva (synMuv) genes act redundantly to antagonize Ras-mediated vulval induction in *C. elegans*. Many of these genes encode proteins that are likely to function in transcriptional repression and chromatin remodeling. The class B synMuv protein LIN-35 is similar to the mammalian tumor suppressor Rb. Studies of mammalian systems have identified many chromatin-remodeling factors that are recruited to promoters through association with Rb or other pocket proteins. We have identified a complex of at least seven class B synMuv proteins, including LIN-35 Rb. While this complex contains proteins such as DPL-1 DP and LIN-53 RbAp48 that were thought to interact with LIN-35 Rb based on similarities to mammalian systems, it also includes four additional proteins that were not previously known to be associated with pocket proteins- LIN-9, LIN-37, LIN-52, and LIN-54. We have named this complex the DRM complex for DP, Rb, and MuvB, complex. As similar protein complexes were simultaneously identified in flies, and these proteins all have mammalian homologs, it is likely that a similar complex exists in humans to regulate gene expression. We have further shown that although pocket proteins are known to interact with histone deacetylases, in *C. elegans* the synMuv protein HDA-1 HDAC1 is not a component of the DRM complex although it is component of a NuRD-like complex. Furthermore, the NuRD-like complex has functions distinct from the DRM complex in vulval development. We have also characterized the class B synMuv protein LIN-61, which is not a core member of either the DRM or NuRD-like complexes. LIN-61 contains four malignant brain tumor (MBT) repeats, which are in multiple transcriptional repressors. Thus like other class B synMuv proteins, LIN-61 likely functions to repress transcription of genes that induce vulval development. Our biochemical and genetic characterizations of the synMuv genes have identified a new pocket protein-containing complex and have demonstrated the existence of multiple complexes among the class B synMuv proteins. Thus the many proteins that have previously been classified as class B synMuv proteins are likely to be functioning in independent complexes to regulate vulval development through transcriptional repression.

Thesis Supervisor: H. Robert Horvitz
Title: David H. Koch Professor of Biology

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INTRODUCTION:

Pocket proteins interact with a diversity of chromatin-remodeling enzymes

Introduction

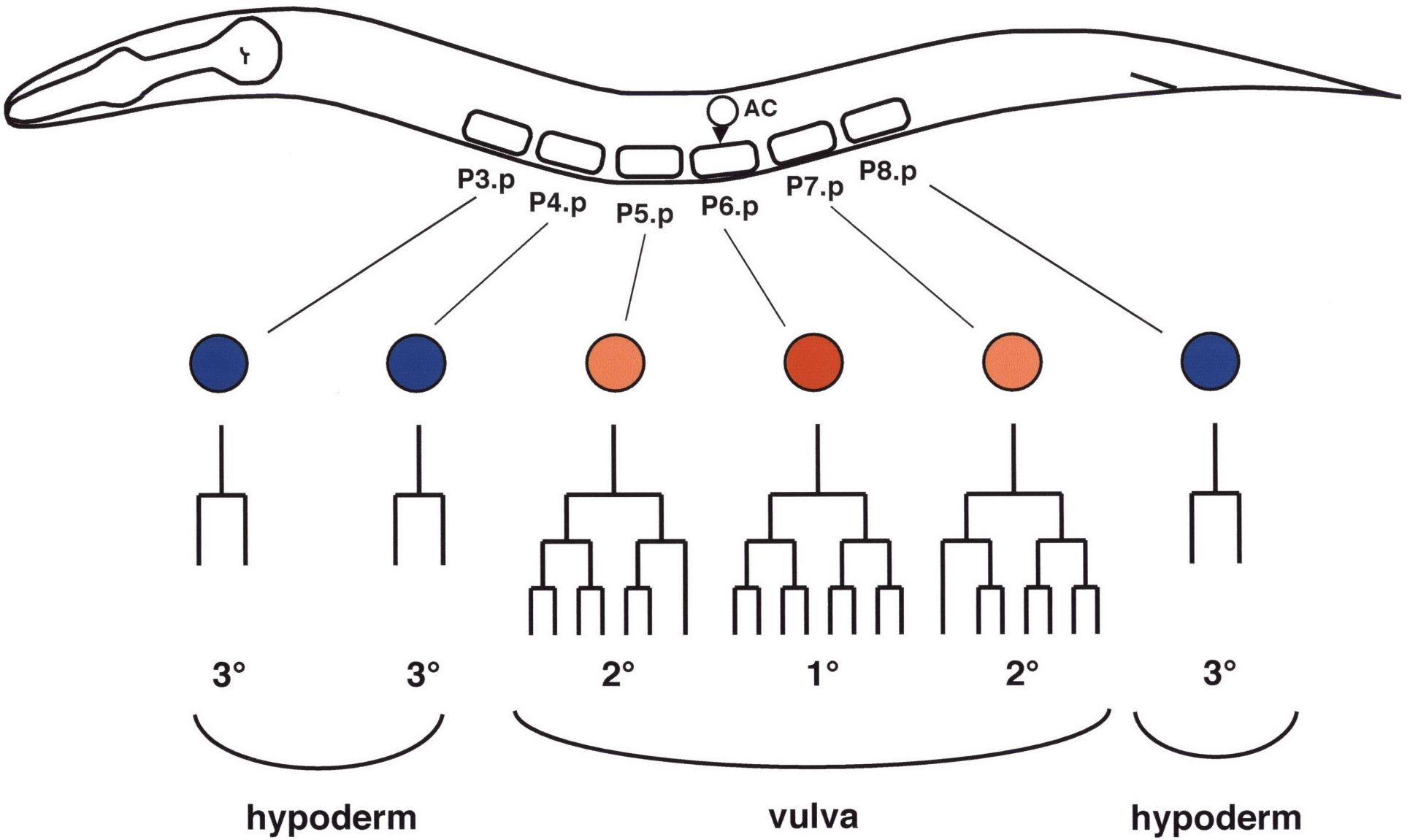
To understand how an entire organism develops requires first an understanding of the coordinated control of cell division and differentiation. These complex processes are in turn regulated by changes in gene expression that can be mediated by modifications to the chromatin state. Biochemical studies have identified many factors that function to regulate chromatin and to control transcription levels, and genetic studies have implicated many of these factors in developmental regulation. Future studies must be directed towards understanding how these changes in gene expression result in cellular differentiation and ultimately in the development of the organism as a whole.

Proper development of the vulva of the nematode *Caenorhabiditis elegans* results from the combined activities of a number of signaling pathways, including a receptor tyrosine kinase (RTK)/ Ras pathway, a Notch-signaling pathway, and a Wnt pathway (YOCHEM *et al.* 1988; BEITEL *et al.* 1990; HAN *et al.* 1990; GLEASON *et al.* 2002). The hermaphrodite vulva is specified to develop during the second larval stage from three of six ectodermal blast cells (P3.p-P8.p) (Figure 1). Although all six blast cells are capable of adopting vulval cell fates, in wild-type development only the descendants of P5.p, P6.p, and P7.p contribute to the vulva. P3.p, P4.p, and P8.p generally divide just once and fuse with the surrounding hypodermis.

Mutations in genes required for vulval specification result in either vulvaless (Vul) mutant animals or multivulva (Muv) mutant animals. Analysis of mutants with these phenotypes demonstrated the importance of an inductive RTK/Ras pathway in properly specifying P5.p, P6.p, and P7.p. Gain-of-function mutations in the Ras homolog *let-60* result in extra cell divisions and a Muv phenotype. By contrast, loss of function in Ras pathway components results in Vul animals (STERNBERG and HAN 1998). Studies of other systems have shown that the components of these pathways are highly conserved in other organisms, as are the roles these proteins play in development. For example, an RTK/Ras pathway is required to properly specify the photoreceptors in the development of the eye in *Drosophila melanogaster* (WASSARMAN *et al.* 1995). In addition to its roles in wild-type development, Ras also has been shown to be an

Figure 1. The *C. elegans* vulva develops from three of six ectodermal blast cells. During the L2 and early L3 stages, an EGF-like signal from the gonadal anchor cell induces P5.p, P6.p, and P7.p to divide and generate the vulva. Although P3.p, P4.p, and P8.p are competent to adopt vulval cell fates, in wild-type development they divide once and fuse with the surrounding hypodermis. The lineages for the descendants of P3.p-P8.p are shown.

Figure1



oncogene in mammals, and gain-of-function mutations have been found in many human cancers (BARBACID 1987).

The activity of the Ras-pathway in *C. elegans* vulval cell-fate specification is antagonized by the activities of the synthetic multivulva (synMuv) genes. Based on genetic interactions, the synMuv genes fall into three classes, A, B, and C. Loss of function in any single class does not result in a Muv animal. However, animals that have loss-of-function mutations in members of any two classes are Muv.

Four class A synMuv genes have been identified, and all encode novel proteins. Two of the class A synMuv proteins have THAP domains (CLARK *et al.* 1994; HUANG *et al.* 1994), which have been shown to bind DNA (CLOUAIRE *et al.* 2005). The presence of THAP domains in LIN-15A and LIN-56 suggests that these proteins might directly interact with DNA. Among the class B and class C synMuv proteins are many proteins that likely are involved in transcriptional repression and chromatin remodeling (Table 1). These include HDA-1, a homolog of a histone deacetylase, and LET-418, a homolog of an ATP-dependent chromatin-remodeling enzyme Mi2 (VON ZELEWSKY *et al.* 2000; DUFOURCQ *et al.* 2002). These proteins along with LIN-53, a homolog of RbAp48, are similar to components of the mammalian Nucleosome Remodeling and Deacetylase (NuRD) complex (LU and HORVITZ 1998; XUE *et al.* 1998; ZHANG *et al.* 1998a; ZHANG *et al.* 1999). The class B synMuv proteins MET-2 and HPL-2 are homolgous to proteins with histone methyltransferase activity and histone methyl binding activity, respectively (COUTEAU *et al.* 2002; POULIN *et al.* 2005). The class C proteins likely form a histone acetyltransferase complex (CEOL and HORVITZ 2004)

Among the class B synMuv proteins also is a protein similar to the mammalian tumor suppressor Rb (LU and HORVITZ 1998). In addition, the class B synMuv proteins EFL-1 and DPL-1 are homologous to the mammalian heterodimeric transcription factor E2F, which is known to recruit Rb to DNA (CEOL and HORVITZ 2001). Extensive studies have been performed in mammals to better understand the function of Rb, and it has been demonstrated that Rb interacts with many proteins to control proper gene expression and ultimately to regulate cell cycle and development. Understanding the

important role of Rb and related proteins will require unifying our knowledge of the function of these proteins in multiple organisms.

Control of transcription levels can be achieved by changes in chromatin structure

In the nucleus, eukaryotic DNA is packaged with proteins to form chromatin. The basic unit of chromatin is the nucleosome, which consists of 147 base pairs of DNA wrapped around a protein octamer consisting of two molecules each of the histone proteins H2A, H2B, H3, and H4. Each histone protein is composed of a globular core region and an N-terminal tail of unstructured amino acids that protrude from the octamer. These histone tails are subject to post-translational modifications including phosphorylation, ubiquitination, acetylation, methylation, and ADP-ribosylation (SIMS *et al.* 2003). These histone modifications and other changes in the chromatin structure can modulate the transcription levels of nearby genes. The two best studied modifications are acetylation and methylation.

Acetylation occurs on lysines on the N-terminal tails of all four histones and has been linked to transcriptional activation. Histone acetyltransferases or proteins that can recruit histone acetyltransferases through protein/protein interactions often act as transcriptional activators. Acetylation of lysines decreases the interactions between histones and the negatively-charged DNA by blocking the positive charge on the lysine residue. This is proposed to open the chromatin structure and thereby allow proteins involved in transcription to access the DNA. Conversely, hypoacetylation of histones has been linked with transcriptional repression, and many transcriptional repressors are associated with histone deacetylase activity (KOUZARIDES 1999).

Methylation, another post-translational modification of histones, occurs on arginine and lysine moieties in the tails of histone H3 and histone H4. Methylation has been linked with both transcriptional repression and transcriptional activation depending on the methylated residue. For example, methylation of lysines 9 and 27 on histone H3 and methylation of lysine 20 on histone H4 are associated with transcriptional repression. By contrast, methylation of lysine 4 of histone H3 has been associated with

transcriptional activation. Amino acids can also be mono-, di-, or tri-methylated, adding a further level of complexity. Until recently, no enzymes capable of removing methyl groups from amino acids had been discovered, leading to the suggestion that methylation could result in a more permanent change in transcriptional activity than changes in the acetylation state. This proposal is supported by the fact that methylation of lysine 9 of histone H3 (H3K9) is highly associated with heterochromatin, a region of the genome that is transcriptionally repressed (SIMS *et al.* 2003). The recent discovery of enzymes that remove methyl groups suggests that, like acetylation, histone methylation is also reversible (SHI *et al.* 2004; TSUKADA *et al.* 2006; WHETSTINE *et al.* 2006). However, the association of H3K9 methylation with heterochromatin implicates this modification in generating or maintaining long-term transcriptional repression.

Chromatin structure also can be modulated through the activities of ATP-dependent chromatin-remodeling enzymes. These enzymes utilize ATP to change the contacts between histones and the DNA and to move nucleosomes along the DNA. Proteins with these activities are involved with depositing nucleosomes on newly replicated DNA as well as in transcriptional control. In transcriptional control, the activities of ATP-dependent chromatin-remodeling enzymes are context dependent and can result in either transcriptional activation or repression (SUDARSANAM and WINSTON 2000).

Chromatin-modifying enzymes do not necessarily act individually. Sometimes these enzymes function in a step-wise fashion. For example, methylation on H3K9 is blocked by prior acetylation of the same residue. Thus H3K9 methyltransferases often require the prior activity of histone deacetylases. In other instances, histone deacetylases depend on the actions of chromatin-remodeling enzymes, which alter the chromatin structure to allow for deacetylation.

Enzymes that can modify chromatin structure are recruited to specific areas of the genome through their interactions with transcription factors that bind sequence-specifically to DNA. Regulation of these protein-protein interactions allow transcription levels of individual genes to be modulated during certain times in development, in specific cell types or through the cell cycle. One well-studied example of such a highly

regulated transcription factor is the E2F family of proteins. They are regulated in large part through their interactions with a family of proteins that are related to the tumor suppressor Rb.

Pocket proteins and their E2F binding partners are conserved across species

The retinoblastoma gene (*Rb*) was the first identified tumor suppressor. The original mutation was discovered because of its causative role in a hereditary form of pediatric retinoblastoma (FRIEND *et al.* 1986; FUNG *et al.* 1987; LEE *et al.* 1987). Mutations in *Rb* have subsequently been found in a large number of human cancers, and the Rb-pathway is probably inactivated in most human cancers. The gene encodes a 928-amino acid protein that has several functional domains. Together, two domains in the C-terminus, the Walker A and B domains, form the central pocket, and it is to this region that many of the proteins that interact with Rb bind. Many proteins bind Rb through an LXCXE motif (where X is any amino acid), which has been shown to associate with domain B (WHYTE *et al.* 1988; DYSON *et al.* 1989; LUDLOW *et al.* 1989; LEE *et al.* 1998). However, proper folding of domain B can only be achieved through interactions with domain A (LEE *et al.* 1998). Additional Rb-binding partners, such as E2F proteins, interact with the Rb pocket region, but do not utilize an LXCXE motif for the interaction (HUANG *et al.* 1992; LEE *et al.* 1998), suggesting these proteins bind at distinct sites, and that Rb could interact with multiple binding partners simultaneously.

Mammals contain two additional pocket-domain-containing proteins, p107 and p130 (EWEN *et al.* 1991; HANNON *et al.* 1993; LI *et al.* 1993; MAYOL *et al.* 1993). The sequences of these two proteins are more similar to each other than to Rb, but all three proteins contain the pocket domain and have been shown to have many of the same binding partners. p107 and p130, but not Rb, both have a spacer region in domain B that contains binding sites for cell-cycle regulated kinases, allowing p107 and p130 to specifically interact with these cyclin-dependent kinases (EWEN *et al.* 1992; FAHA *et al.* 1992; LEES *et al.* 1992). In addition, the N-termini of p107 and p130 differ significantly from Rb. Despite the conserved pocket domains and the many shared binding partners, p107 and p130 are functionally distinct from Rb, as demonstrated by the fact that

mutations in *p107* and *p130* have not been found in a large number of cancers. However, studies of knockout mice demonstrated that functional redundancies exist among these three proteins and that p107 and p130 might have roles as tumor suppressors (COBRINIK *et al.* 1996; LEE *et al.* 1996).

Rb-/- embryos die between day 13 and day 15 (CLARKE *et al.* 1992; JACKS *et al.* 1992; LEE *et al.* 1992). By contrast, *p107* and *p130* null mice in the same genetic background, 129/sv, develop normally (COBRINIK *et al.* 1996; MULLIGAN and JACKS 1998). Mice mutant for both *p107* and *p130* die shortly after birth, suggesting that p107 and p130 have redundant functions important for development (COBRINIK *et al.* 1996). Characterization of mice mutant for *Rb* and either *p107* or *p130* also show that functional overlap exists between these proteins, as doubly mutant embryos die earlier than *Rb-/-* embryos alone (LEE *et al.* 1996). As highlighted by the fact that in a Balb/c genetic background rather than in a 129/sv background *p130-/-* causes embryonic lethality and *p107-/-* causes severe developmental defects (LECOUTER *et al.* 1998a; LECOUTER *et al.* 1998b), it is important only to compare phenotypes for mutations made in the same genetic backgrounds.

While there are published reports of Rb interacting with over 100 different proteins (MORRIS and DYSON 2001), the function of Rb is understood best in relation to the E2F family of sequence-specific DNA-binding transcription factors. E2F DNA-binding activity is generally attributed to heterodimeric complexes that contain one member of the E2F family and one member of the DP family. There are eight identified mammalian E2F proteins, five of which (E2F1-5) have been shown to interact with pocket proteins. Six of the E2F proteins have been shown to form heterodimers with either one of the two mammalian DP-family members (TRIMARCHI and LEES 2002). The recently identified E2F7 and E2F8 have two DNA-binding domains and do not require dimerization with a DP-family member to bind DNA (DE BRUIN *et al.* 2003; DI STEFANO *et al.* 2003; LOGAN *et al.* 2004; CHRISTENSEN *et al.* 2005; LOGAN *et al.* 2005; MAITI *et al.* 2005).

E2F-family members bind to DNA and, when not bound by pocket proteins, can activate transcription of nearby genes. By contrast, interactions of pocket proteins with

their E2F-binding partners generally result in transcriptional repression of E2F-responsive genes. Characterization of the different E2F-family members suggests that some are generally found bound to DNA without an associated pocket protein and are predominantly involved in transcriptional activation, E2F1-3, while others are more often associated with transcriptional repression and are likely found bound to DNA and a pocket protein simultaneously, E2F4-5. Overexpression of E2F1, E2F2, and E2F3 results in transcriptional activation (HELIN *et al.* 1992; JOHNSON *et al.* 1993; LEES *et al.* 1993; QIN *et al.* 1994; LUKAS *et al.* 1996), while E2F4, E2F5, and E2F6 have been associated with transcriptional repression (LUKAS *et al.* 1996; MANN and JONES 1996; TRIMARCHI *et al.* 1998).

The different results that have been observed in the overexpression studies might result from distinct subcellular localizations of the two classes of E2F family members. E2F1, E2F2, and E2F3, which have been associated with transcriptional activation, have a nuclear localization signal and are consistently localized to the nucleus. By contrast, the repressor E2Fs, E2F4 and E2F5, each have a nuclear export signal and are found in the nucleus only during G0 and G1. Binding to their partner pocket proteins can induce nuclear localization of E2F4 and E2F5 (TRIMARCHI and LEES 2002).

Individual pocket proteins associate with subsets of the E2F-family members. Rb has been found to specifically associate with E2F1, E2F2, and E2F3, and to a lesser extent E2F4. Conversely, p107 and p130 interact with the repressor E2F proteins, E24 and E2F5 (TRIMARCHI and LEES 2002).

Homologs of these mammalian pocket proteins can be found in a large number of species. *Drosophila melanogaster* contains two Rb homologs, RBF1 and RBF2, two E2F family members, dE2F1 and dE2F2, and a single DP family member, dDP (DYNLACHT *et al.* 1994; OHTANI and NEVINS 1994; HAO *et al.* 1995; DU *et al.* 1996; SAWADO *et al.* 1998; FROLOV *et al.* 2001; STEVAUX *et al.* 2002). The amino acid sequences of RBF1 and RBF2 are both more similar to the p107 and p130 than they are to mammalian Rb. Similar to the characterization of "activator" E2F proteins and "repressor" E2F proteins found in mammals, dE2F1 has been linked with transcriptional

activation and dE2F2 has been associated with transcriptional repression (FROLOV *et al.* 2001). As discussed above, *Caenorhabditis elegans* contains a single pocket protein, LIN-35 (LU and HORVITZ 1998). However, LIN-35 is more similar in sequence to the mammalian pocket proteins p107 and p130. *C. elegans* also contain two E2F-like proteins, EFL-1 and EFL-2 and a single DP family member, DPL-1 (CEOL and HORVITZ 2001; PAGE *et al.* 2001). The reduced complexity of these organisms makes studies of the functions of these proteins simpler than in mammalian systems; however there are obvious functional differences among the homologs. For example, animals that are null for the only worm pocket protein are viable, whereas mice lacking multiple pocket proteins die as fetuses (CLARKE *et al.* 1992; JACKS *et al.* 1992; LEE *et al.* 1992; COBRINIK *et al.* 1996; LEE *et al.* 1996; LU and HORVITZ 1998).

The role of Rb in cell cycle control

The initial identification of a potential role for Rb in controlling cell-cycle progression was through the observation that DNA tumor viruses transform cells by the ability of viral proteins, such as adenovirus E1A, SV40 large tumor antigen, and human papillomavirus (HPV) E7, to bind and inactivate Rb (DECAPRIO *et al.* 1988; WHYTE *et al.* 1988; DYSON *et al.* 1989). It was shown that these proteins bind to Rb through their LXCXE motifs and induce inappropriate entry into the cell cycle. Subsequent studies have resulted in a detailed understanding of how Rb, through its interactions with E2F, might control cell cycle progression.

The cell cycle is divided up into four distinct phases: G1, S, G2, and M. G1 and G2 are gap phases and allow time for the cell to grow and monitor its metabolic state. During S phase, the cell replicates its DNA, and during M phase, the cell divides. G0 refers to the state of cells that have exited the cell cycle. When stimulated, cells in G0 reenter the cell cycle in G1. The progression of the cell through the cell cycle is regulated by cyclins and cyclin-dependent kinases (cdks) (Figure 2A). Cyclin-dependent kinases are a set of proteins whose ability to phosphorylate their substrates varies through the cell cycle and whose activities are controlled by binding with specific cyclins.

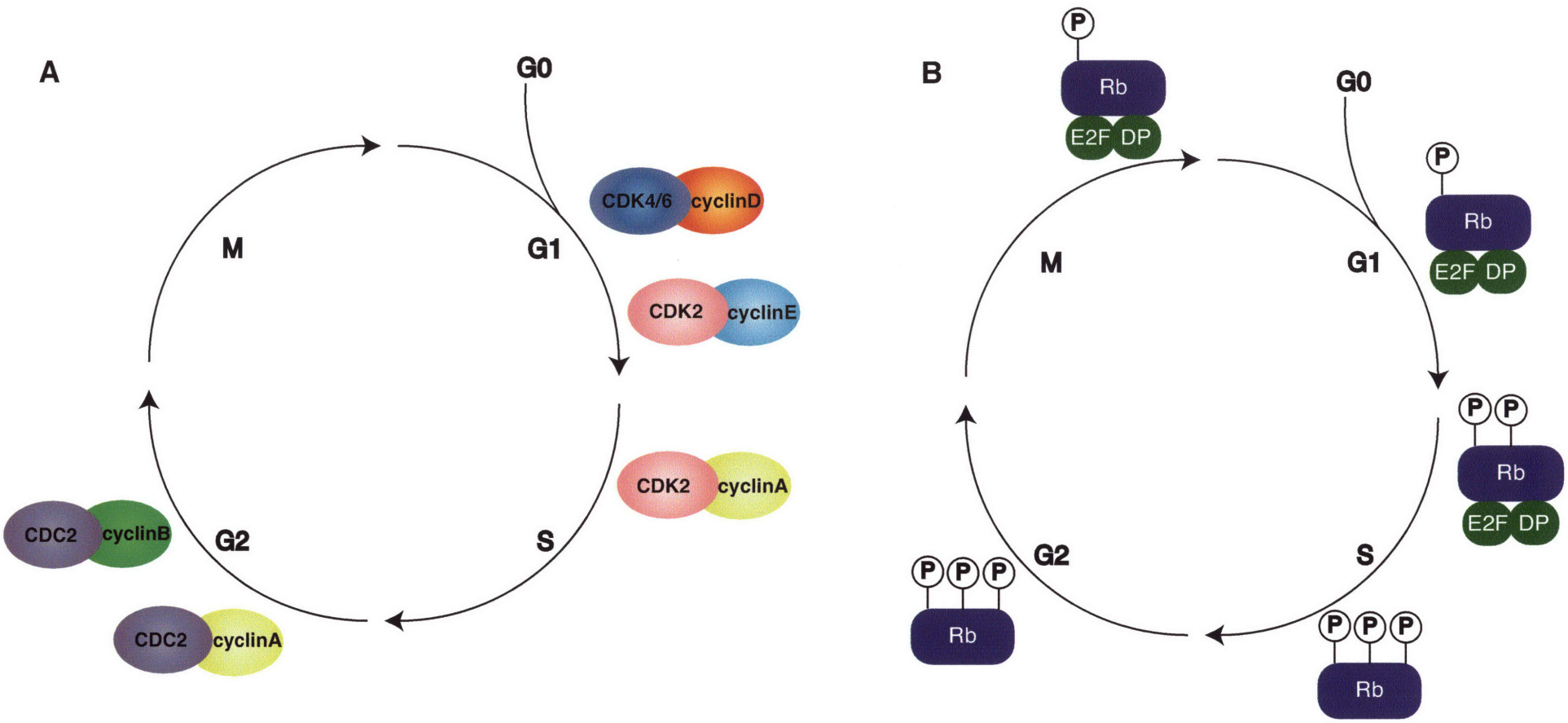
Figure 2: Cell-cycle progression is regulated by the sequential activation of multiple proteins.

A) Different cyclin-dependent kinases are sequentially activated by expression of their cyclin binding partners.

B) Rb is phosphorylated as cells progress through the cell-cycle. Phosphorylation state is indicated by the circled P.

C) The chromatin factors associated with pocket proteins are controlled by the level of phosphorylation. In G0, pocket proteins are hypophosphorylated and can interact with both HDAC and BRG1. This complex can repress expression from both the *cyclinE* and *cyclinA* promoters. As the cell progresses through G1, cyclinD/cdk4/6 can phosphorylate the pocket proteins and inhibit interaction with HDAC. *cyclinE* expression is activated. By contrast, the association of pocket proteins with BRG1 can maintain repression of *cyclinA*. cdk2 can now associate with cyclinE and further phosphorylate the pocket proteins, releasing BRG1 and allowing for cyclinA expression. Activation of cyclinA/cdk2 permits the cell to progress through S phase.

Figure 2


A
G0
G1
S
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M
CDK4/6
cyclinD
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cyclinE
CDK2
cyclinA
CDC2
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E2F
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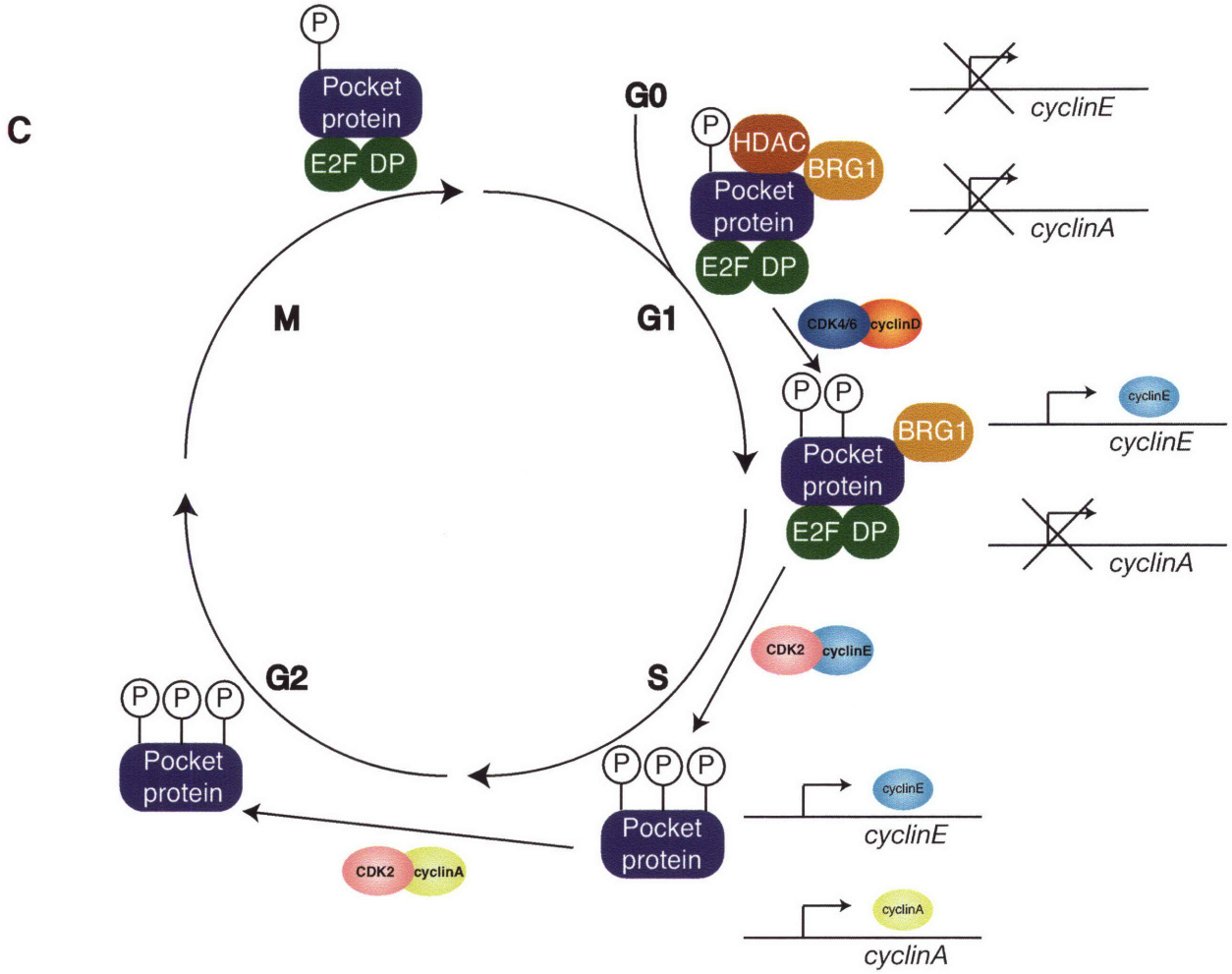
Figure 2


C
Pocket protein
E2F DP
G0
HDAC
BRG1
Pocket protein
E2F DP
cyclinE
cyclinA
M
G1
CDK4/6 cyclinD
Pocket protein
BRG1
E2F DP
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cyclinE
cyclinA
CDK2 cyclinE
G2
S
Pocket protein
Pocket protein
cyclinE
cyclinE
CDK2 cyclinA
cyclinA
cyclinA

While the levels of cdks are relatively constant throughout the cell cycle, cyclin levels vary substantially.

Rb contains 16 potential cyclin dependent kinase (cdk) phosphorylation sites. As a consequence of cdk regulation, Rb alternates between hypophosphorylated and hyperphosphorylated states as cells progress through the cell cycle. In G0, Rb is hypophosphorylated, and this form of the protein binds tightly to E2F proteins. Cyclin D-cdk4/6 phosphorylates Rb during early G1, cyclin E/cdk2 phophorylates Rb near the end of G1, and cyclin A/cdk2 might maintain phosphorylation of Rb during S phase. As Rb becomes increasingly phosphorylated contacts with different binding partners are interrupted and, in the simplest model, hyperphosphorylated Rb can no longer bind to E2F and repress its function as a transcriptional activator, allowing expression of E2F-regulated cell cycle genes (TRIMARCHI and LEES 2002).

The details above clearly are simplified. Rb is not the only pocket protein that can regulate transcription of genes necessary for progression through the cell cycle. In addition, levels of each pocket protein change as the cell progresses through the cell cycle. p130 levels are highest in non-cycling quiescent and differentiated cells. Conversely, p107 levels rise as the cell is stimulated to proliferate (CLASSON and DYSON 2001). In addition, studies have shown that the spectrum of interactions between pocket proteins and the E2F family of proteins are not static throughout the cell cycle. During G0, the most predominant E2F/pocket-protein complexes are repressor complexes, either E2F4 or E2F5 bound by p130. As the cell progresses into G1 these complexes are replaced by pocket-protein complexes containing either E2F1, E2F2, E2F3, or E2F4. Finally, during S phase many of the activator E2F proteins are free and able to activate transcription (TAKAHASHI *et al.* 2000; RAYMAN *et al.* 2002).

Pocket proteins repress E2F-responsive genes by two distinct mechanisms

E2F proteins positively regulate transcription of many genes required for cell cycle progression. While E2F1-5 all contain a potential transactivation domain, overexpression of only E2F1, E2F2, and E2F3 can drive cells into the cell cycle (JOHNSON *et al.* 1993; QIN *et al.* 1994; LUKAS *et al.* 1996), suggesting that these three

proteins, but not E2F4 and E2F5, cause potent transcriptional activation of genes required for cell cycle entry. In addition, recruitment of E2F to promoters can lead to activation of transgenes containing upstream E2F binding sites (HELIN *et al.* 1992; SHAN *et al.* 1992).

E2F might activate transcription by a number of different mechanisms. E2F binds directly to the TATA-binding protein, a key component of the transcriptional machinery (HAGEMEIER *et al.* 1993; EMILI and INGLES 1995). This interaction could allow E2F to recruit the transcriptional machinery to the promoters of specific genes, and this, in turn, can lead to transcriptional activation. Alternatively, E2F recruits transcriptional coactivators, like CREB-binding protein (CBP), to promoter regions. CBP is able to interact with known transcriptional activators, such as histone acetyltransferase complexes, and can bridge an interaction between these activators and transcription factors, like E2F, that are bound to DNA (TROUCHE *et al.* 1996). Finally, E2F binding can alter the DNA structure at the promoter, and this bending of the DNA can cause transcriptional activation even in the absence of a functional E2F activation domain (CRESS and NEVINS 1996).

Experiments in which Rb is overexpressed suggest that the binding of pocket proteins to E2F causes transcriptional repression (HAMEL *et al.* 1992). Pocket proteins bind close to the transactivation domain of E2F, allowing them to repress transcription by blocking the ability of E2F to interact with proteins required for activation (FLEMINGTON *et al.* 1993; HELIN *et al.* 1993).

Rb also has an active role in transcriptional repression (BREMNER *et al.* 1995; SELLERS *et al.* 1995; WEINTRAUB *et al.* 1995). Artificial recruitment of Rb to promoters in the absence of E2F can result in transcriptional repression, suggesting that some function of Rb itself is capable of mediating transcriptional repression (BREMNER *et al.* 1995; SELLERS *et al.* 1995). The ability to actively repress transcription is shared by the other pocket proteins and this function is mediated by the ability of the pocket proteins to interact with a large number of enzymes that modify chromatin structure.

Pocket proteins interact with histone deacetylases

Pocket proteins can actively repress transcription through the recruitment of histone deacetylase activity to the promoters of E2F-repsonsive genes (BREHM *et al.* 1998; FERREIRA *et al.* 1998; LUO *et al.* 1998; MAGNAGHI-JAULIN *et al.* 1998). The pocket domains of Rb, p107, and p130 are essential for the interaction of these proteins with histone deacetylase 1 (HDAC1) (BREHM *et al.* 1998; FERREIRA *et al.* 1998; LUO *et al.* 1998; MAGNAGHI-JAULIN *et al.* 1998; RAYMAN *et al.* 2002). Despite the fact that both E2F proteins and HDAC1 interact with pocket proteins through the pocket domain, ternary complexes can be formed containing an E2F, a pocket protein, and HDAC1 (BREHM *et al.* 1998; MAGNAGHI-JAULIN *et al.* 1998; FERREIRA *et al.* 2001). The existence of such ternary complexes suggests that pocket proteins can actively repress transcription by interacting with E2F and recruiting histone deacetylase activity to promoters.

HDAC1 contains an IACEE motif, which is similar to the LXCXE motif that mediates the interactions of viral proteins with Rb. This IACEE motif might be important for the interaction between pocket proteins and HDAC1 (BREHM *et al.* 1998; FERREIRA *et al.* 1998; MAGNAGHI-JAULIN *et al.* 1998), since HDAC1 lacking the IACEE motif no longer strongly associates with pocket proteins. Because viral proteins interrupt the interaction between HDAC1 and pocket proteins, it is likely that both viral proteins and HDAC1 interact with pocket proteins through the same domain (BREHM *et al.* 1998; FERREIRA *et al.* 1998; MAGNAGHI-JAULIN *et al.* 1998). However, it remains unclear whether the interactions between HDAC1 and pocket proteins are directly mediated by the IACEE motif or whether other proteins are involved in the interaction (KENNEDY *et al.* 2001; LAI *et al.* 2001).

The interaction of Rb with histone deacetylases is not limited to HDAC1. Rb can also interact with HDAC2 and with HDAC3. HDAC2, but not HDAC3 contains an IACEE motif that might be important for its interactions with pocket proteins (DAHIYA *et al.* 2000). Histone deacetylases have been categorized into three distinct classes based on sequence similarity. HDAC1, HDAC2, and HDAC3 are all class I histone deacetylases that are most similar to the yeast histone deacetylase Rpd3. Class II histone deacetylases are more similar to yeast Hda1 but share similarity to class I histone

deacetylases in their active sites. Class III histone deacetylases require an NAD cofactor and are more similar to yeast Sir2 (EKWALL 2005). No interaction was detected between Rb and the class II histone deacetylases HDAC4-6 (DAHIYA *et al.* 2000).

The interaction between Rb and histone deacetylases controls the acetylation state of histones at certain promoters, and the acetylation state is correlated with changes in transcription levels (FERREIRA *et al.* 2001; MORRISON *et al.* 2002; SIDDIQUI *et al.* 2003). At the *cyclin E* promoter, which is specifically regulated by Rb through its interaction with E2F, Rb recruitment of HDAC activity can result in the deacetylation of histones H3 and H4 of a single nucleosome located at the transcriptional start site. It is to this nucleosome that Rb and HDAC1 are localized (MORRISON *et al.* 2002). Thus, the recruitment of Rb to the *cyclin E* promoter by E2F proteins might result in transcriptional repression, at least in part, through the deacetylation of this nucleosome. Rb also mediates H4 deacetylation at the *cyclin A*, *cdc2*, *topoisomerase IIa,* and *thymidylate synthase* promoters. This deacetylation likely causes transcriptional repression as Trichostatin A (TSA), an HDAC inhibitor, prevented repression of many of these genes (SIDDIQUI *et al.* 2003). Additionally, *DHFR* is an E2F target gene that is silent in early G1 and is expressed at the G1- S transition. HDAC1 is specifically associated with the *DHFR* promoter during G0 and G1, during which time the promoter is hypoacetylated and transcriptionally inactive. During the transition into S phase, HDAC1 is no longer associated with the promoter, and the promoter becomes acetylated at lysines 5 and 12 of histone H4 (FERREIRA *et al.* 2001). These data suggest that the association of pocket proteins with histone deacetylase activity is likely to be functionally relevant in controlling the expression levels of at least some E2F-responsive genes.

Studies of histone deacetylases from a number of organisms have demonstrated that they are often found in large, multimeric complexes. HDAC1 and HDAC2 have been found to be components of both the mammalian Nucleosome Remodeling and Deacetylase (NuRD) complex and the Sin3 complex (XUE *et al.* 1998; ZHANG *et al.* 1998a; ZHANG *et al.* 1998b). In addition to HDAC1 and HDAC2, these complexes both contain RbAp48 and RbAp46 but differ in a number of other components (QIAN *et al.* 1993; XUE *et al.* 1998; ZHANG *et al.* 1998a; ZHANG *et al.* 1998b. RbAp48 and RbAp46

were initially identified as proteins that could bind to Rb *in vitro* {Qian, 1995 #22). They were later shown to be components of a number of chromatin-remodeling complexes and are capable of binding histone H4 (VERREAULT *et al.* 1996; XUE *et al.* 1998; ZHANG *et al.* 1998a; ZHANG *et al.* 1999). Pocket proteins specifically interact with the Sin3 complex, but not with the NuRD complex (LAI *et al.* 2001; RAYMAN *et al.* 2002).

The Sin3 complex is recruited by the LXCXE-containing protein RBP1 to pocket proteins rather than through direct interaction of HDACs with pocket proteins (LAI *et al.* 2001). Despite the requirement of RBP1 for Sin3 recruitment, some Rb-associated HDAC activity remains in the absence of RBP1 (LAI *et al.* 2001). This activity could come from a number of different sources. The HDAC activity could result from interactions between Rb and HDAC3, which is not a member of the Sin3 complex. Alternatively, the residual activity could be accounted for by direct interaction between Rb and HDAC1 or HDAC2 that is not associated with the Sin3 complex as has been proposed (BREHM *et al.* 1998; MAGNAGHI-JAULIN *et al.* 1998). Finally, HDAC activity could be recruited to Rb by a protein distinct from RBP1. For example, RbAp48 is a protein that can mediate interactions between Rb and HDAC1, HDAC2, and HDAC3 (NICOLAS *et al.* 2000; NICOLAS *et al.* 2001).

Studies of murine cells have demonstrated that Sin3 and HDAC1 are associated with the promoters of some endogenous genes in primary cells during quiescence but that these proteins dissociate during G_1 and S phase when gene expression is initiated (RAYMAN *et al.* 2002). The recruitment of HDAC1 to specific promoters in quiescent cells depended on the presence of p107 and p130 but not Rb (RAYMAN *et al.* 2002). Thus, at least in quiescent cells, HDAC-mediated repression of E2F-responsive genes might depend primarily on the pocket proteins p107 and p130. This is in agreement with data showing that during quiescence specific E2F promoters are predominantly occupied by a repressor E2F, E2F4, bound by p130 and are hypoacetylated. By late G_1, these promoters are bound by the activator E2F proteins, E2F1 and E2F3, and are acetylated (TAKAHASHI *et al.* 2000).

The recruitment of histone deacetylase activity to promoters of E2F-responsive genes is an important means for pocket proteins to repress transcription whether

HDACs are recruited individually or as components of the larger Sin3 complex. However, histone deacetylation is not the only means of Rb-mediated transcriptional repression. All pocket proteins retain the ability to partially repress a reporter construct with upstream E2F binding sites even in the presence of the HDAC inhibitor Trichostatin A, indicating that these proteins can repress these transgenes through an HDAC-independent pathway (FERREIRA *et al.* 1998). In addition, the repression of the SV40 enhancer or the herpes virus thymidine kinase promoter by artificial recruitment of Rb cannot be alleviated by the addition of TSA, further demonstrating that this repression is not dependent on HDAC activity (LUO *et al.* 1998). The expression of a constitutively active form of Rb results in cell cycle arrest, and this arrest cannot be alleviated by the addition of TSA, showing that even without HDAC function the constitutively active form of Rb is likely to be repressing genes necessary for progression through the cell cycle (SIDDIQUI *et al.* 2003). These data demonstrate that pocket proteins do not repress transcription only through the recruitment of histone deacetylase activity. Consistent with these data, pocket proteins have been found to interact with a number of other enzymes that modify chromatin structure.

Pocket proteins interact with ATP-dependent chromatin-remodeling enzymes

BRG1 (brahma/SWI2-related gene 1) is in a class of proteins related to the yeast SWI2/SNF2 ATP-dependent chromatin-remodeling enzyme (KHAVARI *et al.* 1993). Murine BRG1 was initially shown to bind to Rb in a yeast two-hybrid assay (DUNAIEF *et al.* 1994). Given the yeast two-hybrid interaction, that BRG1 contains an LXCXE motif, that BRG1 interacts with the pocket region of Rb and that the interaction between BRG1 and Rb can be disrupted by viral oncoproteins, it is likely that the interaction between BRG1 and Rb is direct (DUNAIEF *et al.* 1994). Experiments using SW13 cells that lack BRG1 demonstrated a functional role for BRG1 in Rb-mediated cell-cycle arrest (DUNAIEF *et al.* 1994; STROBECK *et al.* 2000; ZHANG *et al.* 2000). Overexpression of BRG1 in SW13 cells can cause growth arrest and flattened cell morphology, indicating the cells can no longer properly progress through the cell cycle. This morphology and growth arrest is dependent on interaction with pocket proteins (DUNAIEF *et al.* 1994).

BRM (brahma), another mammalian ATP-dependent chromatin-remodeling enzyme similar to yeast SWI2/SNF2, is also capable of binding to pocket proteins in the yeast two-hybrid system (STROBER *et al.* 1996). Expression of BRM, like BRG1, in SW13 cells induces flattened, growth-arrested cells, suggesting BRM might also function with pocket proteins in mediating cell-cycle arrest (STROBECK *et al.* 2000). BRM and BRG1 are functionally redundant in cooperating with Rb to mediate cell-cycle arrest, since only cell lines lacking both proteins are resistant to the cell-cycle arrest caused by constitutive activation of Rb, while cell lines lacking only BRG1 are not resistant (STROBECK *et al.* 2002). These data show that BRM and BRG1 both bind Rb and mediate the ability of Rb to block cell-cycle progression.

SWI2/SNF2 was initially identified in yeast as a transcriptional activator (LAURENT *et al.* 1991; LAURENT *et al.* 1993). However, BRG1 binds to the hypophosphorylated form of Rb (DUNAIEF *et al.* 1994), suggesting that BRG1 might cooperate with Rb to mediate transcriptional repression. hBRM forms a complex with E2F1 and Rb, and this interaction represses E2F1-driven transcription in transient transfection assays (TROUCHE *et al.* 1997). By contrast to this role in transcriptional repression, there are also data to suggest that BRM cooperates with Rb to activate E2F-independent transcription by the glucocorticoid receptor (SINGH *et al.* 1995). Together these results demonstrate that the functional consequence of the interaction between Rb and BRM likely depends on the promoter to which these proteins are recruited.

An *in vivo* role for SWI/SNF-like proteins in regulating E2F-responsive genes also was shown in *Drosophila*. Loss-of-function mutations in the fly homologs of SWI1, SWI2, and SWI3, *brahma, moira*, and *osa,* respectively, enhanced the rough eye phenotype caused by overexpression of dE2F1 and dDP. A similar phenotype was caused by loss-of-function in a *Drosophila* Rb homolog RBF1, suggesting that *Drosophila* SWI/SNF proteins function together with Rb in the fly eye (STAEHLING-HAMPTON *et al.* 1999).

The role of SWI/SNF proteins in Rb-mediated transcriptional repression was demonstrated for the *cyclin A* promoter. Neither the HDAC inhibitor TSA nor the DNA-methylation inhibitor 5-aza-2-deoxycytidine were able to block the transcriptional

repression of the *cyclin A* promoter caused by constitutive activation of Rb (ZHANG *et al.* 2000; SIDDIQUI *et al.* 2003). By contrast, overexpression of a dominant negative form of BRG1 could alleviate some of the repression (SIDDIQUI *et al.* 2003). These data suggest that HDAC activity and ATP-dependent chromatin remodeling might separately mediate repression by pocket proteins by acting at different promoters. By contrast, data for repression of *Plk1*, another E2F-regulated gene, demonstrate that in some cases HDAC activity could be dependent on prior chromatin-remodeling (GUNAWARDENA *et al.* 2004). Repression of *Plk1* expression by pocket proteins requires functional ATP-dependent chromatin-remodeling enzymes, since activation of pocket proteins only repressed *Plk1* in cells that expressed BRG1 and BRM1. E2F proteins and pocket proteins still associated with the *Plk1* promoter in the absence of these two proteins. However, deacetylation of the *Plk1* promoter required functional BRG1 and BRM1 (GUNAWARDENA *et al.* 2004). Thus for the *Plk1* promoter prior chromatin remodeling by BRG1 or BRM1 might be required for histone deacetylation.

Pocket proteins interact with histone methyltransferases

In addition to histone deacetylases and ATP-dependent chromatin-remodeling proteins, pocket proteins have also been shown to interact with the histone methyltransferase SUV39H1, possibly through its LXCXE motif (NIELSEN *et al.* 2001; VANDEL *et al.* 2001; NICOLAS *et al.* 2003). SUV39H1 is the human homolog of the *Drosophila* protein *Su(var)3-9* that was initially identified in screens for mutations that could suppress the silencing of genes located in heterochromatic regions. It was later shown that *Su(var)3-9* is a methyltansferase that methylates lysine 9 of histone H3 (H3K9), a mark often associated with heterochromatin. Further research demonstrated that the H3K9 methyl mark can be bound by the chromodomain protein HP1 (BANNISTER *et al.* 2001), and that HP1 is also associated with silenced chromatin (JAMES *et al.* 1989).

A domain shared between *Su(var)3-9* and other methyltransferases, the SET domain, is known to be necessary for the methyltransferase activity. Expression of SUV39H1 can repress the *cyclin E* promoter when coexpressed with Rb only when the

SET domain is intact, indicating that the histone methyltransferase activity of SUV39H1 is necessary to mediate this repression. In agreement with these data, cyclin E expression is upregulated in cells lacking SUV39H1 and the closely related histone methyltransferase SUV39H2 (NIELSEN *et al.* 2001). The interaction between Rb and SUV39H1 might be cell-cycle regulated, as phosphorylation of Rb by cyclin E/cdk2 interrupts the interaction between the two proteins (VANDEL *et al.* 2001). Rb is capable of interacting with both SUV39H1 and E2F, so it is possible that Rb is recruiting SUV39H1 to E2F-responisve promoters (VANDEL *et al.* 2001).

Rb also binds HP1 with the yeast two-hybrid assay (WILLIAMS and GRAFI 2000), and HP1 can coimmunoprecipitate Rb from nuclear extract (NIELSEN *et al.* 2001). Furthermore, HP1, Rb, and SUV39H1 can simultaneously bind to a methylated H3 peptide. Thus these three proteins might form a complex when bound to modified H3 that is incorporated into chromatin (NIELSEN *et al.* 2001).

The ability of Rb to recruit a histone methyltransferase to promoters was demonstrated to be important for the *cyclin E* promoter. A single nuceleosome is required for the regulation of *cyclin E* transcription (MORRISON *et al.* 2002). This nucleosome is methylated at lysine 9 of histone H3 and is bound by HP1 only in *Rb+/+* and not *Rb-/-* cells, demonstrating that methylation and binding of HP1 might be a mechanism to ensure repression of the *cyclin E* gene (NIELSEN *et al.* 2001).

Studies of the endogenous *DHFR* promoter indicate that methylation also is likely to be important in regulating expression of this gene. During G0, histones in the promoter are methylated at lysine 9 of histone H3, but as the cells progress to the G_1-S transition the methyl mark disappears and the H3 tails are primarily acetylated (NICOLAS *et al.* 2003). These data combined with the fact that HDAC activity is associated with the *DHFR* promoter during G0 (FERREIRA *et al.* 2001) suggest that there might be a cooperative interaction between HDAC activity and histone methyltransferase activity to properly control the expression of some E2F-responsive genes. Because histone H3 acetylation of lysine 9 can block methylation of the same residue, such cooperation between histone deacetylases and methyltransferases has been proposed. In some cases methyltransferases might require HDAC activity to first remove the acetyl group

from the lysine. This model is supported by the fact that SUV39H1 has been shown to interact with histone deacetylases as well as with RbAP48 and RbAp46, components of a number of histone deacetylase-containing complexes (ZHANG *et al.* 1997; XUE *et al.* 1998; ZHANG *et al.* 1998a; VAUTE *et al.* 2002).

Given the association between the H3K9 methyl mark and heterochromatin, methylation on lysine 9 of histone H3 is correlated with long term repression of gene expression. Pocket proteins are known to have roles both in cell cycle control and in differentiation. The transcriptional repression of cell cycle-regulated genes must be alleviated as the cell progresses through the cell cycle. By contrast, as cells differentiate and exit the cell cycle these genes often become stably repressed. It has been proposed that the more transient repression of cell-cycle genes in cycling cells might be regulated by histone deacetylation that is easily reversed. In differentiating cells, the more permanent repression of the cell-cycle genes might be achieved through methylation and the subsequent binding of HP1.

For the *DHFR*, *B-Myb*, *cyclin E*, and *cyclin D1* promoters, histone acetylation increases in cells as they progress through the cell cycle. Acetylation levels are low in both G0 of cycling cells and in differentiated cells (AIT-SI-ALI *et al.* 2004). Levels of H3K9 methylation at the *DHFR* promoter do not change as cells progress through the cell cycle. Conversely, in differentiating cells H3K9 methylation levels at the *DHFR*, *B-Myb*, *cyclin E,* and *cyclin D1* promoters increase dramatically as the cells differentiate (AIT-SI-ALI *et al.* 2004). The transcriptional repression of *cyclin D1* in differentiating cells, but not in cycling cells is dependent on the histone methyltransferase SUV39H1 (AIT-SI-ALI *et al.* 2004). These data suggest that in differentiating cells Rb might utilize interactions with SUV39H1 to repress transcription, while in cycling cells Rb recruits histone deacetylases.

Rb also has a role in the formation of heterochromatic bodies during senescence and is found at the promoters of many E2F responsive genes in senescent, but not quiescent cells where p107 and p130 appear to be the predominant pocket proteins (RAYMAN *et al.* 2002; NARITA *et al.* 2003). The interaction between Rb and SUV39H1

might be important for the role of Rb in the formation of this heterochromatic region and for long-term repression of E2F-responsive genes.

Pocket proteins interact with additional potential chromatin modifying enzymes

The best characterized chromatin modifying proteins associated with pocket proteins are those discussed above, histone deacetylases, ATP-dependent chromatin-remodeling proteins, and histone methyltransferases. However, pocket proteins are known to associate with over 100 different proteins, and these include additional proteins that might be involved in chromatin remodeling.

Rb also interacts with the Jumonji domain 2-containing protein JMJD2A (GRAY *et al.* 2005), which has recently been shown to be a demethylase for trimethylated lysine 9 and trimethylated lysine 36 of histone H3 (WHETSTINE *et al.* 2006). JMJD2A demethylation results in dimethylated lysines, which are likely to have functions distinct from the trimethylated form of H3K9 (WHETSTINE *et al.* 2006). In addition to interacting with Rb, JMJD2A can interact with class I histone deacetylases and the coreceptor N-CoR and when recruited to promoters can repress transcription (YOON *et al.* 2003; GRAY *et al.* 2005; ZHANG *et al.* 2005).

Rb is associated also with the DNA methyltransferase DNMT1 (ROBERTSON *et al.* 2000; PRADHAN and KIM 2002), which is the major methyltransferase acting to maintain DNA methylation through the cell cycle (LEONHARDT *et al.* 1992). DNA methylation has been linked with many biological processes, including gene silencing (BIRD and WOLFFE 1999), suggesting that recruitment of DNMT1 could function in transcriptional repression. DNMT1 interacts with Rb, E2F, and HDAC1 and acts as a transcriptional repressor when recruited to promoters (ROBERTSON *et al.* 2000). However, other data demonstrate that Rb instead might act by binding to DNMT1 and inhibiting the interaction between DNMT1 and its substrate DNA. This interaction interferes with the ability of DNMT1 to methylate DNA and could possibly lead to transcriptional activation (PRADHAN and KIM 2002).

Pocket proteins likely control different sets of genes by different mechanisms

Given that pocket proteins interact with a large number of proteins involved in gene regulation and the fact that a large number of genes are regulated by these proteins, it is likely that various protein combinations will control transcription at different gene targets (Figure 3A). Studies of expression of the *cyclin A* and *cyclin E* genes have demonstrated that transcriptional repression at these promoters, while both mediated by Rb and its interaction with E2F, is controlled by different chromatin modifying enzymes. Furthermore, this difference in transcriptional control can help to explain how Rb, through its interactions with E2F proteins and chromatin-remodeling enzymes, can control cell-cycle progression.

Overexpression of Rb and BRG1 in SW13 cells arrested them in S phase and not in G1. Thus Rb and BRG1 likely function together to control the transition between S and M phase rather than in the earlier G1-S transition (ZHANG *et al.* 2000). Rb and BRG1 might retain the ability to repress transcription during G1. Indeed, the *cyclin A* gene, which has been shown to require BRG1 but not HDAC for Rb-mediated repression, is repressed through the G1 phase, showing that a functional Rb-BRG1 repressor complex likely exists during G1. By contrast, the *cyclin E* gene, which is normally activated during G1, is repressed by a histone deacetylase activity (ZHANG *et al.* 2000; MORRISON *et al.* 2002), suggesting that the Rb-HDAC repressor complex is inactivated during G1. Phosphorylation of Rb by cyclin D/cdk4 during G1 is capable of interrupting the interaction between Rb and HDAC1 (HARBOUR *et al.* 1999). However, Rb remains associated with BRG1, even when cdk4 is activated by cyclin D (ZHANG *et al.* 2000). Activation of cdk2 by cyclin E can disrupt the association between Rb and BRG1 (ZHANG *et al.* 2000). Along with data showing that Rb, HDAC1, and BRG1 can form a single complex, these data are most easily explained by a model for the sequential role of Rb in controlling the expression of cyclin genes. Initially a single complex including Rb, HDAC1, and BRG1 can repress transcription. The expression of cyclin D in G1 activates cdk4, which can phosphorylate Rb and disrupt the association of Rb and HDAC, but leaves a functional Rb-BRG1 complex. Due to the disruption of

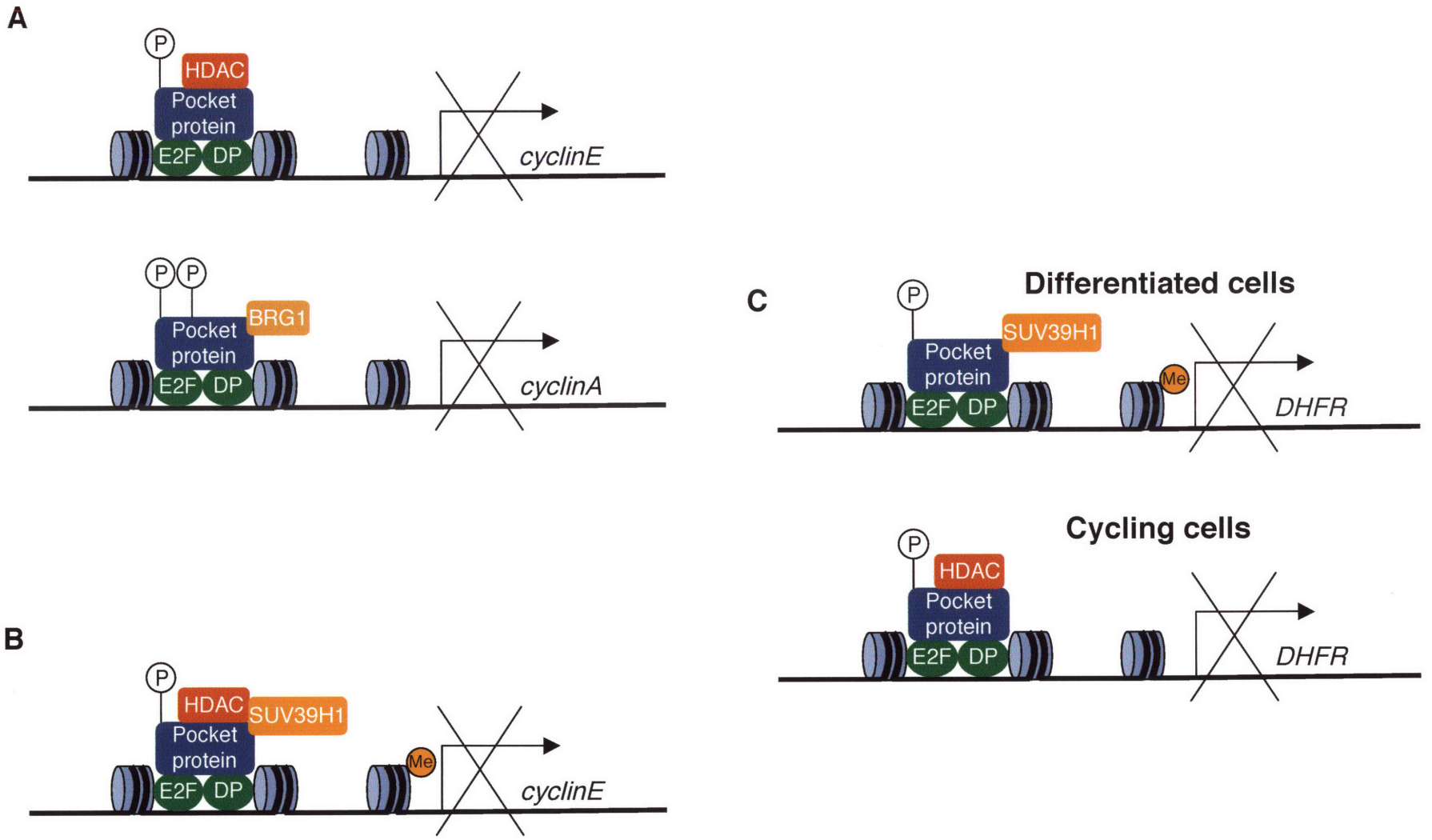
Figure 3: Pocket proteins regulate transcription of target genes by association with different transcription factors.

A) Pocket proteins repress transcription of different target genes by association with different chromatin-remodeling factors. Pocket protein association with HDACs, but not BRG1, is required to repress *cyclin E* expression. By contrast, repression of *cyclinA* requires pocket proteins to recruit BRG1, but not HDACs, to the promoter.

B) Pocket-protein repression of some genes utilizes multiple chromatin-remodeling activities in a sequential fashion. A single nucleosome in the *cyclinE* promoter is regulated by both histone deaceytlase activity and histone methyltransferase activity. Histone methyltransferase activity provided by SUV39H1 might require prior deacetylation of the histone by HDAC.

C) Pocket proteins might utilize association with different chromatin factors to regulate the same gene in different cellular contexts. The *DHFR* promoter is methylated in differentiated cells, presumably from an association of pocket proteins with histone methyltransferases like SUV39H1. By contrast, in cycling cells the *DHFR* promoter is regulated by histone acetylation and is repressed by pocket proteins associating with HDAC.

Figure 3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Pocket protein
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the Rb-HDAC complex, cyclin E is now expressed. Cyclin E/ cdk2 can now phosphorylate Rb resulting in the interruption of the Rb-BRG1 complex permitting expression of cyclin A and allowing the cells to progress through S phase (ZHANG *et al.* 2000).

Chromatin-remodeling enzymes do not only work alone at different promoters but can cooperate to regulate the transcriptional profile of a gene through the cell cycle or development (Figure 3B). For example, a single nucleosome in the *cyclin E* promoter can be modified by both methylation and acetylation, suggesting a cooperation between pocket protein-associated histone deacetylase activity and histone methyltransferase activity (NIELSEN *et al.* 2001; MORRISON *et al.* 2002). Perhaps the histone methyltransferase requires deacetylation of the histone before the histone can be methylated. In another example, histone deacetylation of the *Plk1* promoter required activity of ATP-dependent-chromatin-remodeling factors (GUNAWARDENA *et al.* 2004).

Finally, the same promoters might be regulated by different transcriptional regulators at different stages of development or in different cell types (Figure 3C). Histone methylation of a number of promoters is correlated with differentiation and permanent exit from the cell cycle (AIT-SI-ALI *et al.* 2004). HDAC activity or chromatin-remodeling activity might be more important for regulation of these genes in cycling cells. Because different pocket proteins can be found associated with the same promoter under varying circumstances, chromatin-modifying enzymes could be recruited to the promoter by one specific pocket protein in cycling cells and a different pocket protein in differentiating cells. For example, p107 was not found associated with the *E2F* and *cyclin A* promoters during quiescence. However once the cells progressed through a round of the cell cycle, p107 could be detected readily on the same promoters (TAKAHASHI *et al.* 2000).

Pocket proteins have roles beyond cell-cycle control

Studies of E2F-responsive genes in *Drosophila* suggest that, as discussed above, not all genes regulated by E2F are controlled in the same manner. Microarray experiments using RNAi to reduce levels of either the activator E2F, dE2F1, the

repressor E2F, dE2F2, the single DP homolog, dDP, or the two pocket proteins, RBF1 and RBF2, either singly or together, in *Drosophila* SL2 cells demonstrated that the different E2F proteins did not regulate the same sets of genes (DIMOVA *et al.* 2003). Genes activated by dE2F1 included many homologs of genes involved in cell-cycle regulation that are known to be regulated by E2F in mammalian cells. Loss of dE2F2 did not greatly affect transcription of these genes. Conversely a set of genes that are repressed by dE2F2 and that were not affected by loss of dE2F1 included many genes that are sex or cell-type specific and might be involved in developmental regulation (DIMOVA *et al.* 2003). The microarray data also show significant redundancy among the two pocket proteins in *Drosophila* (DIMOVA *et al.* 2003), as has been suggested for mammals from the phenotypes of knockout mice (COBRINIK *et al.* 1996; LEE *et al.* 1996).

Recently, two complexes have been identified in *Drosophila* that contain the fly pocket proteins, RBF1 and RBF2, the Myb-MuvB and dREAM complexes (KORENJAK *et al.* 2004; LEWIS *et al.* 2004), and Chapter 2 of this thesis contains the characterization of a similar complex from *C. elegans*, the DRM complex. These three complexes contain, in addition to pocket proteins, a number of other proteins that have been shown previously to interact with Rb, including dE2F2/ EFL-1, dDP/DPL-1, and p55/LIN-53, a homolog of RpAp48. In addition to these previously identified Rb-associated proteins, these complexes contain many proteins that have not been previously implicated in pocket protein function such as Mip40/LIN-37, Mip130/LIN-9, Mip120/LIN-54, and dLin52/LIN-52. In *Drosophila*, these complexes repress genes that might be important for differentiation, but do not seem to be required for repression of cell-cycle regulated genes (KORENJAK *et al.* 2004; LEWIS *et al.* 2004). In *C. elegans,* all members of this complex are all class B synMuv genes, suggesting that this complex is likely involved in the repression of genes that induce vulval specification.

The data from *Drosophila* suggest that a large number of genes regulated by pocket proteins might be involved in differentiation and development (DIMOVA *et al.* 2003). There is also evidence in mammalian systems that pocket proteins have roles in developmental regulation (LIPINSKI and JACKS 1999). For example, Rb is required for neuronal and muscle differentiaton. Some of these genes might be regulated by

interaction of pocket proteins with E2F family members. However it is important to recognize that E2F proteins are only a subset of the transcription factors bound by Rb (MORRIS and DYSON 2001). For example, the role of Rb in muscle differentiation is best understood in its regulation of transcription factors such as MyoD and not E2F. Interestingly, Rb might function to increase transcriptional activity from promoters regulated by these transcription factors (LIPINSKI and JACKS 1999).

Conclusions

Pocket proteins interact with a diverse number of transcription factors and chromatin-remodeling enzymes, and while they have been most well studied as transcriptional corepressors, they can also lead to gene activation (SINGH *et al.* 1995). Through these many protein-protein interactions, pocket proteins can regulate transcription of a large number of genes. Further complexity is found in mammals, which contain three pocket proteins, eight E2F family members, and two DP proteins. Genetic background can further complicate studies of these proteins at the organismal level. This is exemplified by the embryonic lethality caused by a *p130* null allele in a Balb/c background, but the similar null allele is viable in a 129/sv genetic background (COBRINIK *et al.* 1996; LECOUTER *et al.* 1998a; LECOUTER *et al.* 1998b; MULLIGAN and JACKS 1998). Studies of protein function in cell culture systems should be performed with the knowledge that different cell types might give different results.

Most studies of the role of pocket proteins in transcriptional control have focused on E2F-regulated genes that are involved in cell-cycle control. More recently it is has become clear that pocket proteins also have central roles in developmental regulation. Studies of how these developmentally-controlled genes are regulated by the association of pocket proteins with different chromatin modifying enzymes are important to get a better understanding for the role these associations play *in vivo*.

Studies of non-mammalian systems, such as flies and nematodes, are important for understanding how pocket proteins and the many proteins with which they interact function in an organism as a whole. Given both the reduced complexity of these

systems and their amenability to genetic manipulation, studies of flies and nematodes will aid in resolving the ever-broadening role of pocket proteins.

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Table 1
synMuv genes encode proteins with homologs in other species

Gene name	Homolog or domains	synMuv class
lin-8		Class A
lin-15A	THAP domain	Class A
lin-38	Zinc finger	Class A
lin-56	THAP domain	Class A
mcd-1	Zinc finger	Class A
lin-9	Mip130	Class B
lin-13	Zinc fingers	Class B
lin-15B	THAP domain	Class B
lin-35	p107, p130	Class B
lin-36	THAP domain	Class B
lin-37	Mip40	Class B
lin-52	dLin-52	Class B
lin-53	RbAp48, p55	Class B
lin-54	Mip120	Class B
lin-61	h l(3)mbt-like2	Class B
lin-65		Class B
dpl-1	DP	Class B
efl-1	E2F4, 5	Class B
hpl-2	HP1	Class B
met-2	SETDB1	Class B
hda-1	HDAC1	Class B
let-418	Mi2	Class B
mep-1	Zinc fingers	Class B
tam-1	RING finger/B-box	Class B
sli-1	cCbl	Class B
gap-1	GTPase activating protein	Class B
ark-1	Ack-related tyrosine kinase	Class B
epc-1	Enhancer of Polycomb	Class C
mys-1	Tip60/ Esa1p	Class C
trr-1	TRRAP	Class C
ssl-1	SWI/SNF	Class C

CHAPTER 2

A subset of *C. elegans* class B synMuv proteins encodes a conserved LIN-35 Rb-containing complex distinct from a NuRD-like complex

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Craig Ceol cloned *lin-54*, generated and purified the anti-LIN-54 antiserum and characterized the LIN-54 expression pattern. Xiaowei Lu cloned *lin-37*, generated the anti-LIN-37 antiserum and generated and purified the LIN-53 antiserum.

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ABSTRACT

The synthetic multivulva (synMuv) genes act redundantly to antagonize the specification of *Caenorhabditis elegans* vulval cell fates, which are promoted by an RTK/Ras pathway. At least 26 synMuv genes have been genetically identified, several of which encode proteins with homologs that act in chromatin remodeling or transcriptional repression. Here we report the molecular characterization of two synMuv genes, *lin-37* and *lin-54.* We show that *lin-37* and *lin-54* encode proteins in a complex with at least seven synMuv proteins, including LIN-35, the only *C. elegans* homolog of the mammalian tumor suppressor Rb. Three components of this complex are likely to be required for complex stability. This complex is distinct from a second complex of synMuv proteins with a composition similar to that of the mammalian Nucleosome Remodeling and Deacetylase (NuRD) complex. The class B synMuv complex we identified is evolutionarily conserved and is likely to function in transcriptional repression and developmental regulation.

INTRODUCTION

Cell differentiation requires coordinated changes in gene regulation. Such changes in gene expression levels are controlled by transcription factors and often are mediated through the modification of chromatin states. Biochemical techniques have identified many protein complexes that function in transcriptional repression and chromatin remodeling (AYER 1999; BECKER and HORZ 2002). However, the *in vivo* functions of these complexes in organismal development have been unclear. Conversely, genetic studies of the nematode *Caenorhabditis elegans* have implicated genes likely to be involved in chromatin-mediated transcriptional repression as important in the development of the hermaphrodite vulva (see below), but the biochemical properties of the products of these genes have been largely unknown.

The *C. elegans* vulva arises from three of six ectodermal blast cells that form the vulval equivalence group (SULSTON and HORVITZ 1977; SULSTON and WHITE 1980). These six cells, P3.p-P8.p, are generated during the first of four larval stages (L1). In the second larval stage (L2) these cells are specified to adopt one of three distinct cell fates, called primary, secondary, or tertiary, which are distinguished by their patterns of cell divisions and the descendant cell types they generate. P6.p adopts a primary fate, dividing to form eight nuclei, while P5.p and P7.p adopt secondary fates, dividing to form seven nuclei each. These 22 descendants subsequently generate the vulva, through which sperm can enter and eggs can exit. Although P3.p, P4.p, and P8.p are competent to adopt vulval fates, instead they adopt a tertiary non-vulval fate, usually dividing once and fusing with the adjacent syncytial hypodermis. The specification of the primary and secondary cell fates (as opposed to the tertiary non-vulval cell fate) requires the activities of several conserved pathways, including receptor tyrosine kinase (RTK)-Ras, Notch, and Wnt signaling cascades (YOCHEM *et al.* 1988; BEITEL *et al.* 1990; HAN *et al.* 1990; GLEASON *et al.* 2002). Mutations affecting these signaling pathways can cause animals to lack a vulva and express a vulvaless or Vul phenotype, or to have ectopic vulval protrusions and express a multivulva or Muv phenotype (YOCHEM *et al.* 1988; STERNBERG and HORVITZ 1989; HAN *et al.* 1990; BEITEL *et al.* 1995; GLEASON *et al.* 2002).

The specification of the primary and secondary vulval cell fates promoted by these pathways is antagonized by the synthetic multivulva (synMuv) genes (HORVITZ and SULSTON 1980; FERGUSON and HORVITZ 1989). The synMuv genes have been grouped into classes A, B, and C on the basis of genetic interactions (FERGUSON and HORVITZ 1989; CEOL and HORVITZ 2004). Animals homozygous for loss-of-function mutations in any single synMuv gene class are not Muv, whereas animals homozygous for mutations in any two classes are Muv. Some class B synMuv genes encode proteins with homologs in other species that function in chromatin remodeling and transcriptional repression. These genes include *lin-35*, which encodes the only *C. elegans* homolog of the mammalian Rb tumor-suppressor protein, and *efl-1* and *dpl-1*, which encode proteins homologous to the E2F and DP subunits of the heterodimeric transcription factor E2F (LU and HORVITZ 1998; CEOL and HORVITZ 2001). The class B synMuv proteins LIN-53 RbAp48, HDA-1 HDAC1 and LET-418 Mi2 are homologous to components of the mammalian Nucleosome Remodeling and Deacetylase (NuRD) complex (LU and HORVITZ 1998; XUE *et al.* 1998; ZHANG *et al.* 1998a; ZHANG *et al.* 1999; SOLARI and AHRINGER 2000; VON ZELEWSKY *et al.* 2000; CEOL and HORVITZ 2001; DUFOURCQ *et al.* 2002; UNHAVAITHAYA *et al.* 2002).

At least 12 additional class B synMuv genes have products that might act with LIN-35 Rb or components of a *C. elegans* NuRD-like complex in determining vulval cell fates (HORVITZ and SULSTON 1980; FERGUSON and HORVITZ 1989; UNHAVAITHAYA *et al.* 2002; OWEN *et al.* 2003; THOMAS *et al.* 2003; POULIN *et al.* 2005). However, genetic techniques are insufficient to determine how the protein products of the synMuv genes interact. In this study, we report the molecular identification of two class B synMuv genes, *lin-37* and *lin-54,* and identify a new complex of class B synMuv proteins that likely controls cell-fate specification through transcriptional repression.

RESULTS

***lin-37* and *lin-54* are class B synMuv genes:** Two alleles of *lin-37*, *n758* and *n2234*, were previously isolated in independent screens for synMuv mutants (FERGUSON and HORVITZ 1989; THOMAS *et al.* 2003). Two *lin-54* alleles, *n2231* and *n2990*, were isolated in a screen for synMuv mutants in the *lin-8(n111)* and *lin-15A(n433)* class A mutant backgrounds, respectively (THOMAS *et al.* 2003). *lin-37* and *lin-54* are class B synMuv genes, as alleles in each gene cause a Muv phenotype with mutations in any of the four identified class A synMuv genes or with mutations in the class C synMuv gene *trr-1* (Table 1; FERGUSON and HORVITZ 1989; THOMAS *et al.* 2003; data not shown). Like the synMuv phenotype caused by loss-of-function mutations in other synMuv genes, the synMuv phenotype caused by *lin-37* or *lin-54* loss of function in a synMuv class A mutant background requires *let-60* Ras pathway activity, but not the upstream *lin-3* EGF signal (FERGUSON *et al.* 1987; HUANG *et al.* 1994; LU and HORVITZ 1998; THOMAS and HORVITZ 1999; CEOL and HORVITZ 2001; CEOL and HORVITZ 2004; POULIN *et al.* 2005; data not shown).

LIN-37 encodes a small hydrophilic protein with weakly conserved homologs in other species: We mapped *lin-37* to a region of LGIII and performed transformation rescue of the synMuv phenotype of *lin-8(n111); lin-37(n758)* with cosmids and subcloned portions of cosmids in the region (Supplemental Results and Supplemental Figure 1). Frameshift mutations of the predicted genes in the minimal rescuing fragment demonstrated that *lin-37* rescuing activity was encoded by *ZK418.4* (Supplemental Results and Supplemental Figure 1). In addition, RNAi targeted against *ZK418.4* caused a highly penetrant synMuv phenotype in a *lin-15A(n767)* background but not in a wild-type or a *lin-15B(n744)* background. To confirm that *ZK418.4* is *lin-37*, we determined the sequence of *ZK418.4* in the two *lin-37* mutants, *n758* and *n2234*. *n758* contains a G-to-A transition at the splice donor site after the first exon probably generating a truncated LIN-37 protein. *n758* therefore is likely to be a null allele. *n2234* contains an A-to-T transversion resulting in an amber mutation that should eliminate more than half of the predicted protein product (Figure 1A).

We screened a cDNA library to obtain clones for the mRNA that encodes the LIN-37 protein. The intron-exon structure of the *lin-37* locus was determined by comparing the complete sequences of the longest *lin-37* cDNA clones with genomic sequence (Figure 1A and Supplemental Results). We constructed a full-length *lin-37* cDNA and showed that it was able to provide rescuing activity. Conceptual translation of the reconstructed *lin-37* cDNA resulted in a hydrophilic protein of 275 amino acids with weak similarity to the *Drosophila melanogaster* protein Mip40 and related vertebrate proteins (BEALL *et al.* 2002; KORENJAK *et al.* 2004).

LIN-54 encodes a protein with two copies of a conserved cysteine-rich motif: *lin-54* had previously been mapped to an interval of LGIV between *unc-30* and *lev-1* (THOMAS *et al.* 2003). We performed germline-transformation experiments using cosmids and subcloned portions of cosmids from this region and found the predicted gene *JC8.6* could rescue the synMuv phenotype of *lin-54(n2231); lin-15A(n433)* mutants (Supplemental Results and Supplemental Figure 2). DNA sequence analysis of *JC8.6* in strains containing *lin-54(n2231)* or *lin-54(n2990)* identified missense mutations in each (Figure 2A). RNAi targeted against *JC8.6* caused a synMuv phenotype in either a *lin-8(n111)* or a *lin-15A(n767)* background (OWEN *et al.* 2003; KORENJAK *et al.* 2004; POULIN *et al.* 2005; data not shown), consistent with our identification of *JC8.6* as *lin-54*. *JC8.6(RNAi)* did not cause a Muv phenotype in wild-type or *lin-15B* mutant backgrounds, thus showing the same genetic interactions as *lin-54* mutations and other class B synMuv mutations. The recessive nature of the effects of *lin-54(n2231)* and *lin-54(n2990)* and the similarity of their phenotypes to that caused by *lin-54(RNAi)* suggest that these alleles result in a reduction of *lin-54* function.

To establish the null phenotype of *lin-54* we isolated two deletions affecting *JC8.6*, *n3423* and *n3424* (Supplemental Results). In addition to causing a class B synMuv phenotype (Table 1), both deletions also caused a fully penetrant sterile phenotype, as do null mutations in some other class B synMuv genes (FERGUSON and HORVITZ 1989; THOMAS and HORVITZ 1999; BEITEL *et al.* 2000; MELENDEZ and GREENWALD 2000; VON ZELEWSKY *et al.* 2000; CEOL and HORVITZ 2001; THOMAS *et al.* 2003). Because of this sterility the synMuv phenotypes of *lin-54(n3423)* and

lin-54(n3424) mutants can be scored only in *lin-54* homozygous progeny of heterozygous mothers. This experimental design likely explains why the penetrances of the synMuv phenotypes of the *lin-54* deletion mutants we observed are weaker than those of *lin-54* missense mutants, as the *lin-54* animals probably displayed a maternal rescue (Table 1). Similar maternal effects have been noted for other synMuv phenotypes (THOMAS *et al.* 2003).

The DNA sequences of *lin-54* cDNAs revealed alternatively spliced transcripts that would generate predicted proteins of 435 (LIN-54L) and 429 (LIN-54S) amino acids in length (for details see Supplemental Materials and Methods). These proteins are identical except for six additional amino acids found in LIN-54L. The missense allele *n2231* is predicted to cause an alanine-to-threonine substitution of amino acid 422 in LIN-54L or amino acid 416 of LIN-54S. *n2990* is predicted to cause a glycine-to-glutamic acid substitution at amino acid 252 in LIN-54L and amino acid 246 in LIN-54S (Figure 2B). Both predicted LIN-54 proteins are rich in cysteines, which are clustered in two domains that share a nearly identical pattern and spacing. Domains with this signature $CXCX_4CX_4CXCX_6CX_{2-3}CXCX_2C$ sequence are found in proteins from plants and animals, including insects and mammals (CVITANICH *et al.* 2000). Some of these proteins have similarity to LIN-54 outside the cysteine-rich domains. Notable among these LIN-54 homologs are the fruit fly *Drosophila melanogaster* protein Mip120 and the soybean *Glycine max* protein CPP1, which have sequence-specific DNA-binding activities (CVITANICH *et al.* 2000; BEALL *et al.* 2002). The DNA-binding activity of CPP-1 was mapped to the cysteine-rich domains. Recently, yeast-one hybrid and chomatin immunoprecipitation (ChIP) studies have verified that LIN-54 can bind to the promoters of multiple genes expressed in the digestive tract (DEPLANCKE *et al.* 2006). In addition, *Drosophila* Mip120 interacts with homologs of other class B synMuv proteins (BEALL *et al.* 2002; BEALL *et al.* 2004; KORENJAK *et al.* 2004; LEWIS *et al.* 2004). Both human and mouse Tesmin (SUGIHARA *et al.* 1999) share with LIN-54 and Mip120 an extended region of similarity from the cysteine-rich motifs through their C-termini. This region includes the A425T mutation found in *n2231*, but this residue is not conserved among the other proteins. The missense mutation in *n2990* converts a conserved glutamine

that immediately precedes the second cysteine motif to a glutamic acid, possibly interrupting the function of this cysteine-rich motif (Figure 2B).

LIN-37 and LIN-54 are broadly expressed in nuclei throughout development: We generated rabbit and guinea pig polyclonal antibodies that specifically recognize LIN-37 and LIN-54, respectively, in western blots and immunostaining. Antibodies raised against LIN-37 recognized a protein of approximately 35 kD on western blots of wild-type protein extracts but not of protein extracts from *lin-37(n758)* mutant animals (Figure 1B). In immunostainings, LIN-37 was detected in most if not all nuclei of wild-type animals from the one-cell embryo through the adult (Figure 1C- D, 1G and data not shown) and was absent in *lin-37(n758)* mutant animals (Figure 1E-F).

Antibodies raised against LIN-54 recognized a protein of 50 kDa on western blots of wild-type protein extracts; this protein was absent in *lin-54(n3423)* mutant extracts (Figure 2C). Immunostainings of wild-type animals demonstrated that, like LIN-37, LIN-54 was present in the nuclei of all or almost all cells from the embryo through the adult (Figure 2D-I and data not shown). In the hermaphrodite germline, LIN-54 was localized to condensed chromosomes during the diakinesis phase of meiosis (Figure 2F-G). LIN-54 was not detected in *lin-54(n3423)* mutant animals (Figure 2J-K).

LIN-37, LIN-54 and other class B synMuv proteins form a large-molecular-weight- protein complex: Previous studies have demonstrated that some class B synMuv proteins can interact *in vitro* and *in vivo* (LU and HORVITZ 1998; WALHOUT *et al.* 2000; CEOL and HORVITZ 2001; UNHAVAITHAYA *et al.* 2002). We performed gel-filtration experiments and detected LIN-37 and LIN-54 in fractions corresponding to an apparent molecular weight of between 440 and 669 kDa. Because both LIN-37 and LIN-54 coelute well above their monomeric molecular weight, it is likely that these proteins are members of a multisubunit complex or complexes (Figure 3A). To identify components of this complex we performed coimmunoprecipitation experiments.

We generated antibodies against the class B synMuv proteins LIN-9, LIN-35, LIN-52, and LIN-53 (for details see Supplemental Materials and Methods). These antibodies specifically recognize proteins of approximately 70 kD, 110 kD, 22 kD, and

50 kD, respectively, in wild-type but not in corresponding mutant protein extracts (Supplemental Figure 3). Antibodies that recognize the synMuv proteins DPL-1, HDA-1, HPL-2, LET-418, and LIN-56 were previously generated (CEOL and HORVITZ 2001; COUTEAU *et al.* 2002; DUFOURCQ *et al.* 2002; UNHAVAITHAYA *et al.* 2002; E. Davison and H.R.H. unpublished results).

We used anti-LIN-37 antibodies to immunoprecipitate proteins from both wild-type and *lin-37(n758)* mutant embryo extracts. We determined the presence of synMuv proteins that coimmunoprecipitated with LIN-37 using western blots. The class B synMuv proteins LIN-9, LIN-35 Rb, LIN-52, LIN-53 RbAp48, LIN-54, and DPL-1 DP were coimmunoprecipitated by anti-LIN-37 antiserum specifically from wild-type but not from *lin-37(n758)* extracts (Figure 3B). The class B synMuv protein HPL-2, the class A synMuv protein LIN-56 and tubulin did not coimmunoprecipitate with LIN-37 from either wild-type or mutant protein extracts, demonstrating that the identified interactions are specific (Figure 3B). The same set of proteins also coimmunoprecipitated with LIN-37 in the presence of 50 μg/ml ethidium bromide (EtBr). EtBr can disrupt complexes dependent on DNA structure and DNA binding (LAI and HERR 1992), thereby suggesting that these interactions are not DNA-dependent (Figure 3C). These class B synMuv proteins were also precipitated by antibodies that recognize LIN-9, LIN-52, or LIN-54 (Figure 3D) but not by other rabbit polyclonal antibodies that recognize LIN-61 or LIN-56 (data not shown). We failed to detect DPL-1 in some of the coimmunoprecipitations, but because we also failed to detect DPL-1 in the input we believe this result reflects poor recognition by the antibody and not the absence of DPL-1. When DPL-1 was detected in the input, DPL-1 was also seen in the immunoprecipitated proteins. Tubulin did not coimmunoprecipitate with LIN-9, LIN-52, or LIN-54. Our analyses of immunoprecipitates from extracts from synchronized cultures showed that these proteins remain associated throughout larval development, including the late L2 and early L3 stages when the fates of the Pn.p cells are specified (Figure 3E). The gel filtration chromatography and coimmunoprecipitation experiments demonstrate that at least seven class B synMuv proteins form a complex *in vivo*. Since this complex contains homologs of the known transcriptional regulators Rb and DP and mutations in members of the complex cause a

Muv phenotype we refer to this complex as the DRM (DP, Rb, and MuvB) complex. This complex includes only a subset of class B synMuv proteins as LIN-37 fails to coimmunoprecipitate with LIN-36::GFP (Supplemental Figure 4) and HPL-2 does not coimmunoprecipitate with LIN-37 or LIN-9.

Antibodies that recognize the class B synMuv protein EFL-1 recognize EFL-1 in immunostaining (PAGE *et al.* 2001) but did not work in western blots, so we could not assess precipitates for the presence of EFL-1. All DP-like proteins characterized to date exist as heterodimers with E2F proteins (DYSON 1998). For this reason, we suggest that at least one of the two *C. elegans* members of the E2F family, EFL-1 or EFL-2 (CEOL and HORVITZ 2001; PAGE *et al.* 2001), associates with the precipitated proteins *in vivo*. EFL-1 seems a likely candidate, since *efl-1* is a class B synMuv gene (CEOL and HORVITZ 2001).

The DRM complex is distinct from a C. elegans NuRD-like complex: The mammalian homologs of the synMuv proteins HDA-1 HDAC1 (DUFOURCQ *et al.* 2002), LET-418 Mi2 (VON ZELEWSKY *et al.* 2000), and LIN-53 RbAp48 (LU and HORVITZ 1998) are components of the mammalian NuRD (Nucleosome Remodeling and Deacetylase) complex (TONG *et al.* 1998; XUE *et al.* 1998; ZHANG *et al.* 1999). It has been proposed that, by analogy to mammalian systems, these proteins form a NuRD-like complex in C. elegans and are recruited to promoters of vulval genes by the class B synMuv proteins LIN-35 Rb, EFL-1 E2F, and DPL-1 DP (SOLARI and AHRINGER 2000; VON ZELEWSKY *et al.* 2000; CEOL and HORVITZ 2001; DUFOURCQ *et al.* 2002; UNHAVAITHAYA *et al.* 2002). Since we identified LIN-35 Rb and DPL-1 DP as components of the DRM complex, we tested whether this complex also included the chromatin-modifying enzymes HDA-1 HDAC1 or LET-418 Mi2 by testing if these proteins coimmunoprecipitate with LIN-37.

LET-418 did not coimmunoprecipitate with LIN-37 from wild-type protein extracts (Figure 4A). A small amount of HDA-1 was present in the precipitate generated from extracts from wild-type embryos after coimmunoprecipitation with anti-LIN-37 antisera, but a similar low amount was detected in precipitates from *lin-37* null mutant embryos. Low levels of nonspecific precipitation of HDA-1 were also noted by Unhavaithaya *et al.* (2002). We conclude that HDA-1 did not specifically coimmunoprecipitate with LIN-37.

HDA-1 was not observed in coimmunoprecipitates with LIN-37 in extracts from L3 larvae (Figure 4B). In addition, HDA-1 did not precipitate with LIN-9, LIN-52, or LIN-54 (Figure 4B-C). Reciprocally, LIN-37 did not coimmunoprecipitate with HDA-1 (Figure 4A). By contrast, we found that LIN-53 did coimmunoprecipitate with HDA-1 (Figure 4A). LET-418 had previously been shown to coimmunoprecipitate with HDA-1 (UNHAVAITHAYA *et al.* 2002), and we observed low levels of LET-418 in HDA-1 immunoprecipitates (Figure 4A).

These results suggest that HDA-1, LET-418, and LIN-53 are found in a complex distinct from the DRM complex. We propose that LIN-53 is present in both the DRM complex and a NuRD-like complex. RbAp48, the mammalian homolog of LIN-53, has similarly been found in multiple chromatin-modifying complexes, including the chromatin assembly complex Caf1, the NuRD complex, and the Sin3 complex (VERREAULT *et al.* 1996; XUE *et al.* 1998; ZHANG *et al.* 1998a; ZHANG *et al.* 1998b).

Vulval development of animals with mutations in NuRD-like complex components is phenotypically distinguishable from that of animals mutant for DRM complex components: All of the proteins identified in the DRM complex, as well as HDA-1 and LET-418, have previously been considered to be class B synMuv proteins (LU and HORVITZ 1998; BEITEL *et al.* 2000; VON ZELEWSKY *et al.* 2000; CEOL and HORVITZ 2001; DUFOURCQ *et al.* 2002; THOMAS *et al.* 2003). Our data suggest that these proteins can be found in two distinct complexes. Interestingly, mutations that disrupt these two complexes seem to have distinct phenotypic consequences. Whereas most class B synMuv mutants are completely non-Muv in the absence of mutations in class A or class C synMuv genes, *hda-1* and *let-418* single mutants display a weak Muv phenotype. Specifically, *hda-1* mutants are 20-31% Muv (DUFOURCQ *et al.* 2002; data not shown), and when cultured at 22.5°C about 1.5% of *let-418(n3536)* mutants are Muv (our unpublished results). In addition, in 7% of *let-418(ar114)* animals P8.p is induced (VON ZELEWSKY *et al.* 2000); P8.p induction is a Muv phenotype (Ceol and Horvitz 2004). Furthermore, *let-418* is unlike most class B synMuv genes in that the level of P8.p induction of *let-418(ar114)* animals is enhanced to 13-20% by a typical class B mutation

(VON ZELEWSKY *et al.* 2000), whereas a second class B mutation does not alter the vulval phenotype of most class B mutants.

In concert with our biochemical data, these genetic data support the hypothesis that the members of the NuRD-like complex have functions distinct from those of the DRM complex in the regulation of vulval development.

Some DRM complex components are absent in mutants lacking certain other complex components: We investigated whether a DRM complex can form properly when specific components are either absent or mutated. We analyzed extracts from presumptive *lin-35* and *lin-37* null mutants, as these mutants can be maintained as homozygotes and therefore can be grown in sufficient quantities for biochemical analyses. For the genes *lin-9, lin-52, lin-53*, and *lin-54*, which have sterile null phenotypes (BEITEL *et al.* 2000; CEOL and HORVITZ 2001; THOMAS *et al.* 2003), we isolated protein extracts from embryos with viable missense mutations that are known to confer synMuv phenotypes. In general, we used anti-LIN-37 anitbodies to isolate the DRM complex. To analyze extracts from *lin-37(n758)* embryos, which lack detectable LIN-37, we used anti-LIN-9 or anti-LIN-52 antibodies to perform coimmunoprecipitations.

In all but one of the immunoprecipitates from extracts of DRM complex mutants, the presence of the unmutated complex members was unaffected (Figure 5A). The absence of neither LIN-35 Rb nor LIN-37 significantly affected the ability of the remaining complex components to coprecipitate. The missense mutations in *lin-52(n771), lin-53(n833),* and *lin-54(n2231)* also did not strongly affect the associations among complex members (data not shown). Only a missense mutation in *lin-9, n112*, which is predicted to change glycine 341 to glutamic acid (BEITEL *et al.* 2000), altered the composition of the complex by reducing the presence of another protein: LIN-53 RbAp48 failed to strongly associate with the complex when coimmunoprecipitated with either anti-LIN-37 antiserum or anti-LIN-9 antiserum (Figure 5A). The *n112* mutation affects a conserved residue in a region of LIN-9 that is highly conserved in the homologous human protein hLin9 and the homologous *Drosophila* proteins ALY and Mip130/TWIT (WHITE-COOPER *et al.* 2000; BEALL *et al.* 2002; GAGRICA *et al.* 2004). The

only other identified missense mutation of *lin-9* known to cause a homozygous viable class B synMuv phenotype is *n3675* (C.J.C., F. Stegmeier, M.M.H, and H.R.H., unpublished results), which changes the poorly conserved aspartic acid 305 to asparagine. *lin-9(n3675)* did not reduce the levels of LIN-53 RbAp48 in the complex (Figure 5A). The *lin-9(n3675)* mutation is in the same highly conserved region as the *lin-9(n112)* G341E mutation, but the altered residue is not conserved in *Drosophila* ALY or human Lin-9.

These data suggest that an interaction between LIN-9 and LIN-53 might be important for the incorporation of LIN-53 into the DRM complex, but that complex assembly is not the sole function of LIN-9 in vulval development. Specifically another function of LIN-9 might be modified in the *lin-9(n3675)* mutant. Alternatively, both of the *lin-9* mutations as well as the other missense mutations we tested might cause defects in the formation of the complex too subtle to identify by assessing the presence of known members by immunoprecipitation.

Because we could obtain quantities of lysate sufficient to analyze the association of complex components by coimmunoprecipitation only from fertile mutants, we could not directly ask whether sterile null mutants might affect the composition of the complex. Instead, we analyzed the total protein levels of DRM complex components when each member was removed by mutation. Since a null allele of *lin-53* had not previously been isolated, we screened a chemical-mutagenesis library and identified a deletion allele, *n3368*, which starts after codon 52, removes more than half of the coding sequence, and, is likely to be a null allele. Animals homozygous for *lin-53(n3368)* are sterile and are synMuv in combination with the class A synMuv mutant *lin-15A(n767)*. To compare sterile mutants to the wild type, we examined L4 larvae since adult wild-type hermaphrodites contain developing embryos that are absent in sterile mutants.

Null mutants in *lin-9*, *lin-53,* and *lin-54* decreased the protein levels of many other complex components, including LIN-9, LIN-37, LIN-52, and LIN-54 (Figure 5B). In *lin-9* null mutants, LIN-37, LIN-52, and LIN-54 levels were decreased as compared to wild-type levels. Null mutants in *lin-53* showed decreased levels of LIN-9, LIN-37, LIN-52, and LIN-54. In *lin-54* null mutants levels of LIN-9 and LIN-52 were decreased. These

decreases could reflect decreases in either protein synthesis or stability. Loss of LIN-35, LIN-37, or DPL-1 proteins did not significantly affect the protein levels of any other DRM complex components. LIN-35 and LIN-53 levels remained unchanged in all genotypes examined, except as expected *lin-35* or *lin-53* mutants, respectively (Figure 5B). The mammalian homologs of these two proteins- Rb and RbAp48 - are found in multiple complexes, and thus their overall levels may not be dependent on the presence of any one protein complex. Our data suggest that a complete loss of LIN-9, LIN-53, or LIN-54 can disrupt DRM complex formation and result in the degradation of other complex members. Alternatively, it is possible that the loss of any of these three proteins causes a decrease in transcription of some but not all of the genes that encode DRM complex members.

DISCUSSION

***lin-37* and *lin-54* likely act in the DRM complex:** The synMuv genes act to antagonize vulval cell-fate specification. At least 26 synMuv genes define at least three distinguishable classes, A, B, and C (HORVITZ and SULSTON 1980; FERGUSON and HORVITZ 1989; HSIEH *et al.* 1999; BEITEL *et al.* 2000; MELENDEZ and GREENWALD 2000; THOMAS *et al.* 2003; CEOL and HORVITZ 2004), and it has been suggested that the synMuv proteins form a transcriptional repressor complex or complexes (LU and HORVITZ 1998; SOLARI and AHRINGER 2000; VON ZELEWSKY *et al.* 2000; CEOL and HORVITZ 2001; UNHAVAITHAYA *et al.* 2002; POULIN *et al.* 2005). The synMuv proteins DPL-1 DP and EFL-1 E2F have been proposed to act through LIN-35 Rb to recruit a NuRD-like complex to the promoters of genes that control vulval cell-fate specification (SOLARI and AHRINGER 2000; CEOL and HORVITZ 2001).

In this paper we describe the molecular identification and characterization of two class B synMuv genes, *lin-37* and *lin-54*. We show that LIN-37 and LIN-54 form a multisubunit protein complex together with at least five other class B synMuv proteins: LIN-9, LIN-35 Rb, LIN-52, LIN-53 RbAp48, and DPL-1 DP. This DRM complex is biochemically and genetically distinct from a NuRD-like complex that includes HDA-1 HDAC1, LET-418 Mi2, and LIN-53 RbAp48. Based on these observations we propose that LIN-35 Rb and DPL-1 DP likely have a function in vulval development distinct from recruitment of the NuRD complex. However, mammalian Rb has been shown to bind HDAC1 (BREHM *et al.* 1998; LUO *et al.* 1998; MAGNAGHI-JAULIN *et al.* 1998), and our data do not preclude the possibility that LIN-35 Rb and DPL-1 DP function both in the context of the DRM complex and in recruiting the NuRD complex.

The DRM complex might act in vulval development: Although the DRM complex is composed of synMuv proteins, the complex was identified in embryos and larvae, not specifically in the cells involved in vulval development. Therefore it is possible that *lin-37* and *lin-54* do not regulate vulval cell fates as components of the DRM complex. Three observations lead us to suggest otherwise. First, that seven class B synMuv proteins with similar roles in vulval development form a complex in vivo is most simply explained if these proteins act together in the context of the DRM complex

to control vulval development; furthermore, as described below, these genes each act in a variety of biological processes, but the only known normal biological process they all share is a role in vulval development. Second, certain mutations that cause a synMuv phenotype disrupt complex formation, suggesting that the synMuv phenotype associated with these alleles could be caused by lack of DRM complex activity. Third, the DRM complex exists during all developmental stages, including the stage when the Pn.p cells are specified. The simplest hypothesis is that LIN-9, LIN-35, LIN-37, LIN-52, LIN-53, LIN-54, and DPL-1 act as components of the DRM complex to antagonize the specification of vulval cell fates. Given that homologs of DPL-1 are found as heterodimers with E2F family members (DYSON 1998), it is likely that one of the two C. elegans E2F family members is also in the DRM complex. We suggest that this E2F family member is EFL-1, since loss of function of *efl-1* but not *efl-2* cause a class B synMuv phenotype (CEOL and HORVITZ 2001).

Vulval specification is unlikely to be the only function for the DRM complex or at least of the components of the DRM complex, as these proteins are ubiquitously expressed and mutations in genes that encode complex members are pleiotropic. A subset of DRM complex members act in G_1 regulation (BOXEM and VAN DEN HEUVEL 2002), are needed for fertility (Beitel et al 2000; Ceol and Horvitz 2001; Thomas et al 2003) and cause hypersensitivity to RNAi (WANG *et al.* 2005). All complex members modify expression of transgene arrays (HSIEH *et al.* 1999; E. Andersen and H.R.H. unpublished results).

The DRM complex probably functions in transcriptional repression: The DRM complex we have characterized is similar to two recently described and highly similar complexes that contain *Drosophila* homologs of a number of class B synMuv proteins (KORENJAK *et al.* 2004; LEWIS *et al.* 2004). First, the dREAM complex was identified by biochemical purification of *Drosophila* Rb-containing complexes from embryo extracts followed by mass spectrometry and western blot analysis. This complex contains homologs of LIN-9, LIN-35, LIN-37, LIN-54, LIN-53, DPL-1, and EFL-1; these homologs are called Mip130, RBF, Mip40, Mip120, p55, dDP, and dE2F2, respectively. All of these proteins except the LIN-54 homolog, Mip120, were identified

by mass spectrometry following biochemical purification. Mip120 was found to copurify with this complex based upon western blots, but was not identified using mass spectrometry (KORENJAK *et al.* 2004).

Second, the Myb-MuvB complex was purified by immunoprecipitation of the LIN-54 homolog Mip120 or the LIN-9 homolog Mip130 from *Drosophila* tissue-culture cells and the identification of coimmunoprecipitating proteins by mass spectrometry. The Myb-MuvB complex contains all of the components in the dREAM complex as well as stoichiometric amounts of dLin-52, the fly homolog of LIN-52, and substoichiometric amounts of Rpd3, the fly homolog of HDA-1, and L(3)MBT, a protein similar to the class B synMuv protein LIN-61 (LEWIS *et al.* 2004; M.M.H., X. Lu. and H.R.H. unpublished results). The differences between the dREAM and Myb-MuvB complexes might be a consequence of the different methods used for purification or might reflect the existence in different tissues or during different developmental stages of multiple subcomplexes with overlapping constituencies. Both the dREAM and Myb-MuvB complexes can mediate transcriptional repression of many E2F-target genes (KORENJAK *et al.* 2004; LEWIS *et al.* 2004). The high degree of similarity between the DRM complex and the dREAM and Myb-MuvB complexes indicates that the DRM complex might, like the two *Drosophila* complexes, act in transcriptional repression.

The DRM complex we have identified differs slightly from both the dREAM and the Myb-MuvB complexes. Unlike the dREAM complex (KORENJAK *et al.* 2004), the DRM complex contains a LIN-52 dLin52-like protein. Unlike the Myb-MuvB complex (LEWIS *et al.* 2004), the DRM complex does not contain HDA-1 Rpd3 or LIN-61 L(3)MBT (M.M.H. and H.R.H., unpublished results). In addition to LIN-61, one other L(3)MBT-like protein exists in *C. elegans* (M.M.H. and H.R.H., unpublished results), and it is possible that this protein associates with the DRM complex. It is also possible that like the LIN-54 homolog Mip120, the LIN-52 homolog dLin-52 is present in the dREAM complex but failed to be detected by mass spectroscopy. Given the similarities of the DRM, dREAM and Myb-MuvB complexes, we propose that there is a core complex that consists of LIN-35 RBF, EFL-1 E2F2, DPL-1 DP, LIN-9 Mip130, LIN-37 Mip40, LIN-52 dLin-52, LIN-

53 p55, and LIN-54 Mip120 and that this complex might associate with other proteins during specific stages of development or in certain cell types (Figure 6B).

The dREAM and Myb-MuvB complexes both contain the DNA-binding protein Myb. In these complexes, Myb acts in site-specific DNA replication but apparently not in transcriptional repression (KORENJAK *et al.* 2004; LEWIS *et al.* 2004). There is no clear Myb homolog in *C. elegans* (KORENJAK *et al.* 2004; LIPSICK 2004). Two predicted *C. elegans* proteins F32H2.1 and D1081.8 have weak similarity to Myb restricted to their SANT domains, which mediate the binding of Myb to DNA (PETERS *et al.* 1987). We and others have found that RNAi targeting either of these genes did not cause a synMuv phenotype in the background of the class A synMuv mutant *lin-15A(n767)* (KORENJAK *et al.* 2004; data not shown). It is possible that F32H2.1 or D1081.8 associate with the DRM complex *in vivo* but are not required for the function the DRM complex might have in vulval development. Alternatively, the *C. elegans* functional ortholog of the *Drosophila* Myb protein found in the dREAM and Myb-MuvB complexes might not be readily identifiable by comparisons of primary sequence. It is also possible that the *C. elegans* DRM complex does not contain a Myb ortholog.

The dREAM complex is required for the repression of dE2F-regulated genes with sex-specific or tissue-specific expression patterns, but not for the repression of dE2F-regulated genes involved in cell-cycle regulation (DIMOVA *et al.* 2003; KORENJAK *et al.* 2004). synMuv proteins are known to control transgene expression, cell-cycle regulation, viability, and RNAi sensitivity in addition to vulval development (HSIEH *et al.* 1999; BEITEL *et al.* 2000; MELENDEZ and GREENWALD 2000; VON ZELEWSKY *et al.* 2000; CEOL and HORVITZ 2001; BOXEM and VAN DEN HEUVEL 2002; UNHAVAITHAYA *et al.* 2002; CEOL and HORVITZ 2004; WANG *et al.* 2005). Given the broad expression patterns of the synMuv genes and the numerous different phenotypic abnormalities caused by the loss of individual synMuv proteins, including members of the DRM complex, we propose that, similar to their *Drosophila* counterparts, the DRM complex proteins are involved in the repression of many targets important for diverse biological functions, including the specification of the vulval-cell fate.

The DRM complex likely contains at least two sequence-specific DNA-binding activities. Both the EFL-1 E2F and DPL-1 DP heterodimer and LIN-54 Mip120 are likely to bind DNA in a sequence-specific manner, since their homologs in other species function as DNA-binding proteins. These proteins might target the DRM complex specifically to loci containing sequence motifs recognized by both proteins or to loci containing sequence motifs recognized by either protein.

We propose the following two alternative models for how the DRM complex might act in transcriptional repression (Figure 6C, D). First (Figure 6C), the DRM complex could be recruited to DNA by multiple DNA-binding factors including LIN-54 and the heterodimeric transcription factor formed by EFL-1 and DPL-1. The DRM complex could then act with the NuRD complex to repress transcription. Alternatively (Figure 6D), the DRM complex could bind DNA and subsequently bind unmodified histone tails and prevent their acetylation; similar activities have been proposed for the dREAM complex by Korenjak *et al.* (2004), who demonstrated that the dREAM complex can bind unmodified histone H4 tails. This binding might be mediated by LIN-53, since the mammalian homolog RbAp48 can bind histone H4 (VERREAULT *et al.* 1996). At some target genes the histone deacetylase activity might be provided by a NuRD-like complex, containing HDA-1 HDAC1, LET-418 Mi2 and LIN-53 RbAp48; alternatively the NuRD-like complex might act on other target genes.

The DRM complex and a NuRD-like complex have separable functions during vulval development: Although neither the DRM nor the dREAM complexes contains any known chromatin-modifying or chromatin-remodeling enzymes, these complexes might require the activity of a histone deacetylase to mediate transcriptional repression, as noted above. Mutations in genes encoding components of either the DRM or the NuRD-like complex require an additional class A or class C synMuv mutation to produce a highly penetrant Muv phenotype. However, we found that mutations in at least two of the NuRD-like complex components, *hda-1* and *let-418*, alone can cause low penetrance Muv phenotypes, suggesting that the chromatin-remodeling and chromatin-modifying activities of this complex might be required more broadly for the transcriptional repression of genes necessary for proper vulval

development than is the activity of the DRM complex. Perhaps other class B synMuv proteins not associated with the DRM complex, for example LIN-61, act redundantly with the DRM complex to maintain the repressed state formed by the activity of the NuRD-like complex.

Does Rb function within the context of a DRM-like complex to control development in mammals? The high degree of conservation shared by the DRM/dREAM/Myb-MuvB complexes in *C. elegans* and *Drosophila* and the important roles that the components of DRM complex play in *C. elegans* development suggest that a similar complex exists in other organisms, including humans. Indeed the human homolog of LIN-9, hLin-9 can interact with Rb and might act with Rb to specifically promote differentiation (but not to inhibit cell-cycle progression) (GAGRICA *et al.* 2004). Perhaps Rb or other Rb-family proteins act within the context of a DRM-like complex to control development. Rb could act as a tumor suppressor through such a role in developmental regulation in addition to its role in cell-cycle regulation. In *C. elegans* vulval development a mutation in *lin-35* Rb can, in combination with an additional mutation in a class A or C synMuv gene, cause extra cell divisions. Further biochemical and genetic studies of nematodes, insects, and mammals should elucidate the role that this complex plays in development and in carcinogenesis.

MATERIALS AND METHODS

Culture conditions and strains: All strains were cultured at 20°C, unless otherwise specified, on NGM agar seeded with *E. coli* strain OP50, as described by Brenner (1974). N2 (Bristol) was the wild-type strain. For a list of the mutant alleles used see Supplemental Material and Methods.

Transgenic Strains: Germline transformations were performed as described by Mello et al. (1991). For details see Supplementary Materials and Methods.

RNAi Analyses: Templates for in vitro transcription reactions were made by PCR amplification of either cDNAs including flanking T3 and T7 promoter regions or coding exons from genomic DNA using T3-and T7-tagged oligonucleotides. RNA was transcribed in vitro using T3 and T7 polymerases. In vitro transcribed RNA was denatured for 10 min and annealed prior to injection.

Antibody Preparation, Immunocytochemistry and Western Blots: Animals were injected with recombinant protein corresponding to all or part of the protein of interest. For details see Supplemental Material and Methods. All rabbits and guinea pigs were maintained by Covance (Denver, PA), except the guinea pigs used to generate the LIN-54 antibodies were maintained at MIT. All antibodies were used at a 1:1000 dilution for western blots, except for anti-LIN-53, anti-LIN-54, and anti-DPL-1, which were used at 1:500. Affinity purified antibodies were used in all cases except for LIN-35 antibodies. For LIN-35 western blots we used unpurified serum from the third production bleed. For details on affinity purification see Supplemental Material and Methods. Larvae and adults for immunostaining were fixed in 1% paraformaldehyde for 30 min, as described by Finney and Ruvkun (1990). Embryos were fixed for 20 minutes in 0.8% paraformaldehyde, as described by Guenther and Garriga (GUENTHER and GARRIGA 1996). Affinity-purified anti-LIN-37 and anti-LIN-54 antibodies were used at a 1:100 dilution for immunocytochemistry.

Embryo lysates: Embryos were harvested from liquid cultures and bleached to kill larvae and adults in a solution containing 5N NaOH and 20% hypochlorite. The embryos were resuspended in 1 ml of lysis buffer (25 mM HEPES pH 7.6, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.5 mM EGTA, 0.1% NP-40, 10% glycerol with

Complete EDTA-free protease inhibitors (Roche Diagnostics)) for each gram of embryos and frozen in liquid nitrogen. The embryos were thawed at room temperature and sonicated 15 times for 10 seconds using a Branson sonifier 450 at setting 5. The homogenate was then centrifuged at top speed in a microcentrifuge for 15 min at 4°C. The supernatant was removed to a new tube and was spun for 15 min at top speed in a microcentrifuge. The remaining supernatant was pooled and the protein concentration was determined using the Pierce Coomassie Plus Protein Assay Reagent (Pierce Biotechnology). The lysate was diluted to 5-10 mg/ml and was used immediately or stored at -80°C and thawed no more than once before use.

Immunoprecipitation experiments: Antibodies were crosslinked to Protein A Dynabeads (Invitrogen) using dimethyl pimelimidate (Pierce) essentially as described by Harlow and Lane (1999), with the following exceptions: reactions were stopped with 0.1M Tris pH 8.0, and beads were washed three times for 1 min in 100 mM glycine pH 2.5 and then in lysis buffer. The beads were resuspended in PBS. 500 μl of lysate, corresponding to 2.5-5 mg of total protein, were incubated with 25 μl of affinity-purified antibody bound to 25 μl of beads for each immunoprecipitation reaction. Prior to incubation with antibody-bound beads, lysates were initially precleared with 25 μl of beads for between 1 hr and overnight at 4°C. The precleared lysates were recirculated over the antibody-bound beads at 4°C for 1-2 hr and were then washed three times for 5 min each at 4°C in lysis buffer. Following the final wash, the beads were resuspended in 20 μl of 2X protein sample buffer, boiled for 5 min, and loaded on an SDS-polyacrylamide gel. HRP conjugated protein A (Bio-Rad) was used for detection of protein by western blot following coimmunoprecipitation experiments, except for detection of tubulin in which HRP conjugated goat anti-mouse (Bio-Rad) was used.

Gel filtration chromatography: Embryo extract in buffer (15 mM HEPES pH7.6, 0.1 M KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol with Complete EDTA-free protease inhibitors (Roche Diagnostic)) was precipitated with solid ammonium sulfate added to a final concentration of 20%. The supernatant from this precipitation was then precipitated with a concentration of 30% ammonium sulfate. The pellets following

protein precipitation were resuspended in elution buffer (EB) (25 mM HEPES pH 7.6, 150 mM KCl, 50 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1% NP-40, 10% glycerol), combined, and 50 μl were loaded onto a pre-equlibrated Superose 6 PC 3.2/30 column (Amersham). The sample was eluted using EB at a flow rate of 0.02 ml/min. 75 □l fractions were collected. Proteins of known molecular weights (158 kD aldolase, 232 kD catalase, 440 kD ferritin, 669 kD thyroglobulin; Amersham) were used as standards and were detected by measuring the absorbance of each collected fraction at 280 nm.

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Table 1. lin-37 *and* lin-54 *Mutations Cause a Class B synMuv Phenotype*

	Genotype	Percent Muv (n)
Single mutants	*lin-15A(n433)*	0 (302)
	lin-15A(n767)	0 (350)
	lin-15B(n744)	0 (272)
	lin-35(n745)	0 (104)
	lin-37(n758)	0 (318)
	lin-37(n2234)	0 (170)
	lin-54(n2231)	0 (368)
	lin-54(n2990)	0 (327)
	lin-54(n3423)	0 (541)
	lin-54(n3424)	0 (242)
	trr-1(n3712)[a]	13 (89)[b]
Double mutants with class A mutations	*lin-37(n758); lin-15A(n433)*	100 (111)
	lin-37(n758); lin-15A(n767)	100 (102)
	lin-37(n2234); lin-15A(n433)	100 (104)
	lin-37(n2234); lin-15A(n767)	100 (124)
	lin-54(n2231); lin-15A(n433)	99 (227)
	lin-54(n2231); lin-15A(n767)	100 (221)
	lin-54(n2990); lin-15A(n433)	100 (291)
	lin-54(n3423); lin-15A(n433)	92 (96)
	lin-54(n3423); lin-15A(n767)	100 (314)
	lin-54(n3424); lin-15A(n767)	99 (87)
Double mutants with class B mutations	*lin-37(n758); lin-15B(n744)*	0 (94)
	lin-54(n3423); lin-15B(n744)	0 (88)
	lin-35(n745); lin-37(n758)	0 (115)
	lin-35(n745); lin-54(n3423)	0 (108)
	lin-37(n758); lin-54(n3423)	0 (329)
Double mutants with class C mutations	*trr-1(n3712); lin-37(n758)*[a]	55 (20)[b]
	trr-1(n3712); lin-54(n2231)[a]	42 (33)

All animals were raised at 20°C. The Muv phenotype was scored using a dissecting microscope except in the cases noted. *trr-1* mutant homozygotes were recognized as the non-GFP progeny of *ttr-1/mIn1[dpy-10 mIs14]* heterozygous parents. *lin-54(n3423)* and *lin-54(n3424)* homozygotes were recognized as the non-GFP progeny of *lin-54/nT1[qIs51]; +/nT1[qIs51]* heterozygous parents. *lin-54(n3423); lin-15A(n433), lin-54(n3423); lin-15A(n767), lin-54(n3424); lin-15A(n767),* and *lin-54(n3423); lin-15B(n744)* were also homozygous for *unc-30(e191).*

[a] Muv, greater than three Pn.p cells induced.

[b] From Ceol and Horvitz (2004).

Figure 1. Cloning and expression of *lin-37*

(A) *lin-37* maps between *sma-3* and *mec-14* on LGIII. *lin-37* gene structure as determined from cDNA and genomic sequences is indicated. Shaded boxes indicate coding sequence, and open boxes indicate 5' and 3' untranslated regions. Predicted translation initiation and termination codons are shown along with the poly(A) tail and the SL2 leader sequence. Mutations found in the two *lin-37* alleles are indicated above the gene structure. The cosmid ZK418 is shown below as a gray bar. (B) Affinity-purified antibodies raised against recombinant LIN-37 were used to blot extracts from both wild-type and *lin-37(n758)* mutant animals. The asterisk denotes non-specific immunoreactivity. (C, E, G) Whole-mount staining with anti-LIN-37 antisera. (C) LIN-37 is expressed in all nuclei of the developing embryo. (D) 4,6-Diamidino-2-phenylindole (DAPI) staining of the embryo shown in (C). (E) LIN-37 is absent from *lin-37(n758)* embryos. (F) DAPI staining of the embryo shown in (E). (G) LIN-37 expression in the mid body of an L1 larvae. (H) DAPI staining of the larvae shown in (G). WT, wild type. Scale bars, 10 μm.

Figure 1

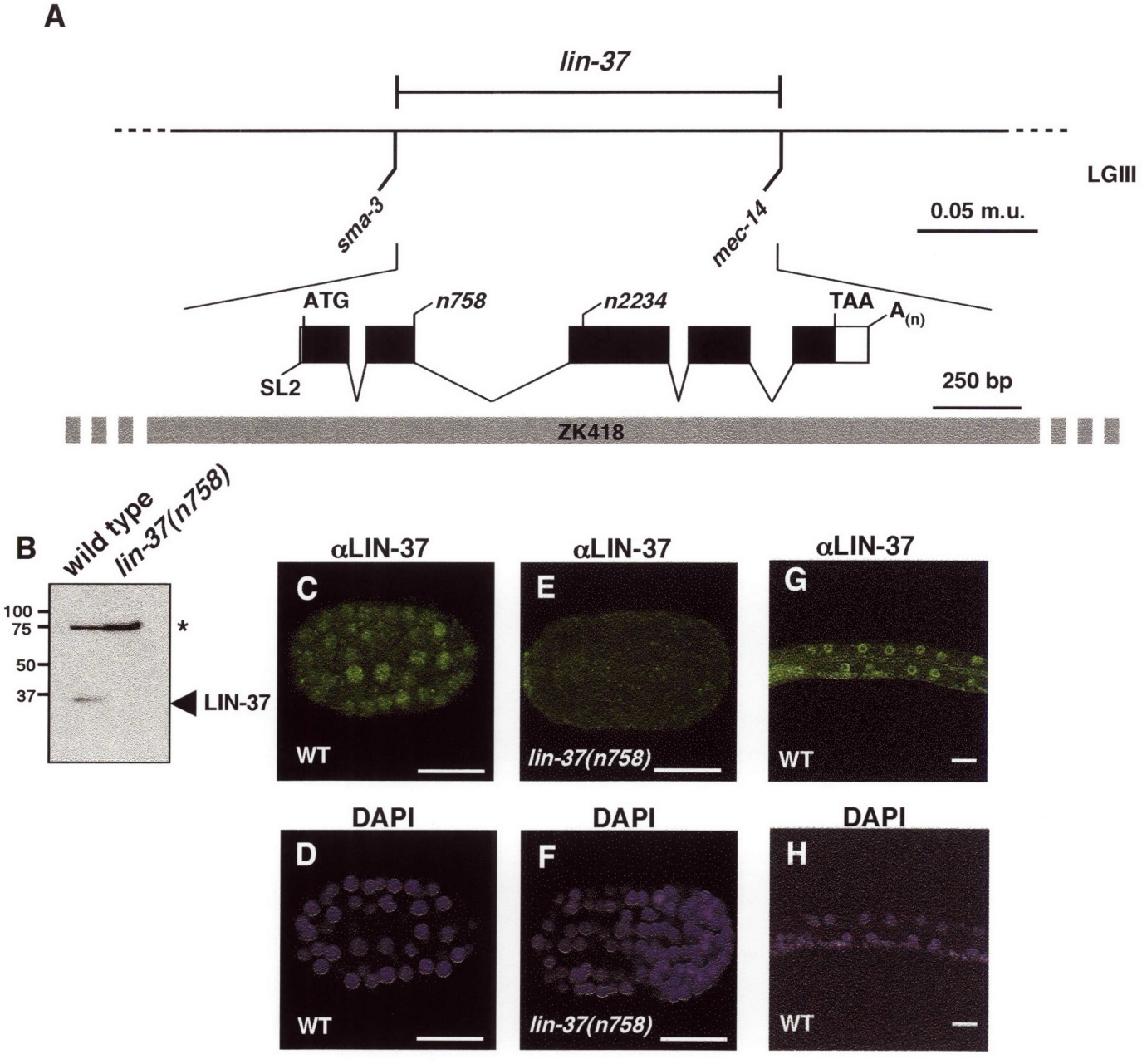
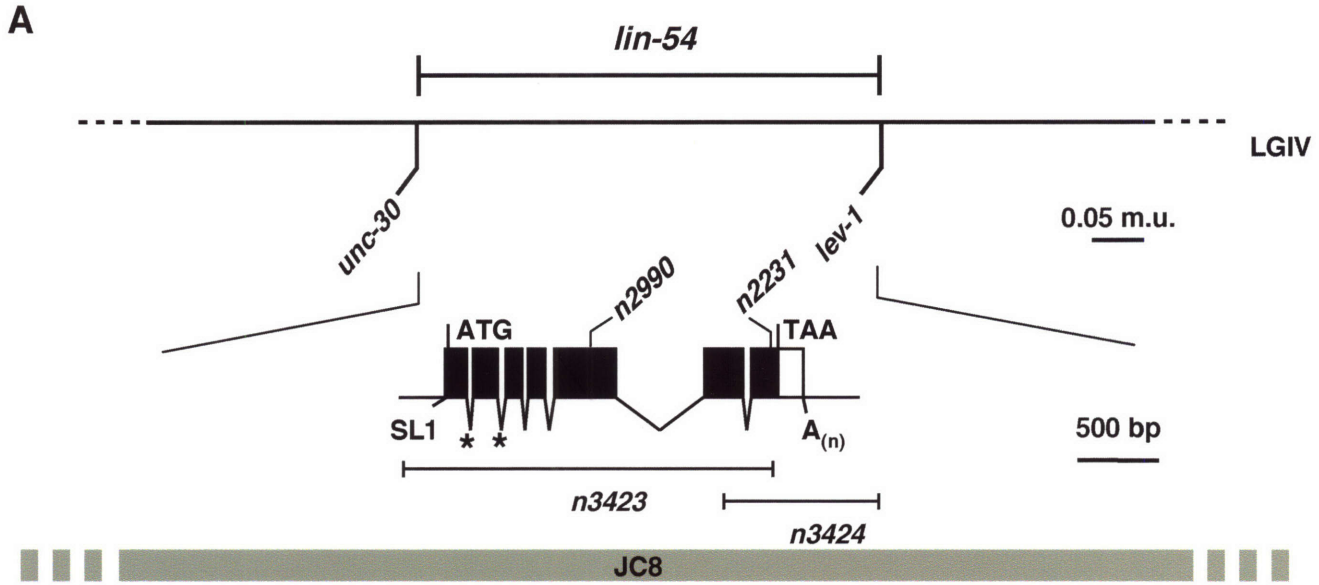

A
lin-37
LGIII
sma-3
mec-14
0.05 m.u.
ATG
n758
n2234
TAA
A(n)
SL2
250 bp
ZK418
B
wild type
lin-37(n758)
100
75
50
37
*
LIN-37
αLIN-37
αLIN-37
αLIN-37
C
E
G
WT
lin-37(n758)
WT
DAPI
DAPI
DAPI
D
F
H
WT
lin-37(n758)
WT

Figure 2. Cloning and expression of *lin-54*

(A) *lin-54* maps between *unc-30* and *lev-1* on LGIV. *lin-54* gene structure as derived from cDNA and genomic sequences is indicated as in Figure 1. The positions of the mutations in the four *lin-54* alleles are indicated. Positions of the alternative splice acceptors for exon 2 or 3 are shown by asterisks. In both cases, the use of alternative splice acceptors creates three amino acid differences in the *lin-54* coding sequence (see Supplemental Materials and Methods). As described in Supplemental Materials and Methods, the SL1 leader is also alternatively spliced. The cosmid JC8 is shown below as a gray bar. (B) Alignment of the cysteine-rich motifs of LIN-54 with proteins with sequences from *Drosophila melanogaster, Glycine max, Homo sapiens,* and *Mus musculus*. Solid boxes indicate identity with LIN-54. The conserved cysteine residues of the two cysteine-rich domains are shown by black dots. The arrowhead indicates the site of the *n2990* missense mutation. (C) Affinity-purified antibodies raised against recombinant LIN-54 were used to blot extracts from both wild-type and *lin-54(n3423)* mutant animals, which were derived from heterozygous mothers. (D, F, H, J) Whole-mount staining with anti-LIN-54 antisera. (D) LIN-54 is broadly expressed in the adult hermaphrodite germline. (E) DAPI staining of the animal shown in (D). (F) Enlargement of the boxed portion of (D). LIN-54 is localized to condensed chromosomes (arrow). (G) DAPI staining of the enlarged portion shown in (F). (H) LIN-54 is broadly expressed in the embryo. (I) DAPI staining of the embryo shown in (F). (J) LIN-54 is absent in *lin-54(n3423)* hermaphrodites. (K) DAPI staining of the normal germline shown in (J). WT, wild type. Scale bars, 10 μm.

Figure 2



B

C.e. LIN-54	174	RKPCNCTKSQCLKLYCDCFANGEFCRD-CNCKDCHNN	210
D.m. Mip120	738	RKHCNCSKSQCLKLYCDCFANGEFCQD-CTCKDCFNN	773
G.m. CPP1	473	CKRCNCKKSKCLKLYCDCFAAGTYCTDPCACQGCLNR	509
H.s. tesmin	84	GSTLPGPPKITLAGYCDCFASGDFCNN-CNCNNCCNN	120
M.m. tesmin	264	GPALQGPPKITLSGYCDCFSSGDFCNS-CSCNNLRHE	300
C.e. LIN-54	250	QKGCHCKKSGCLKNYCECYEAKVPCTDRCKCKGCQNT	286
D.m. Mip120	811	NKGCNCKRSGCLKNYCECYEAKIPCSSICKCVGCRNM	847
G.m. CPP1	560	KRGCNCKRSMCLKKYCECYQANVGCSSGCTCEGCKNV	596
H.s. tesmin	158	NKGCNCRRSGCLKNYCECYEAQIMCSSICKCIGCKNY	194
M.m. tesmin	334	SKGCNCKRSGCLKNYCECYEAKIMCSSICKCIACKNY	370

n2990
G252E

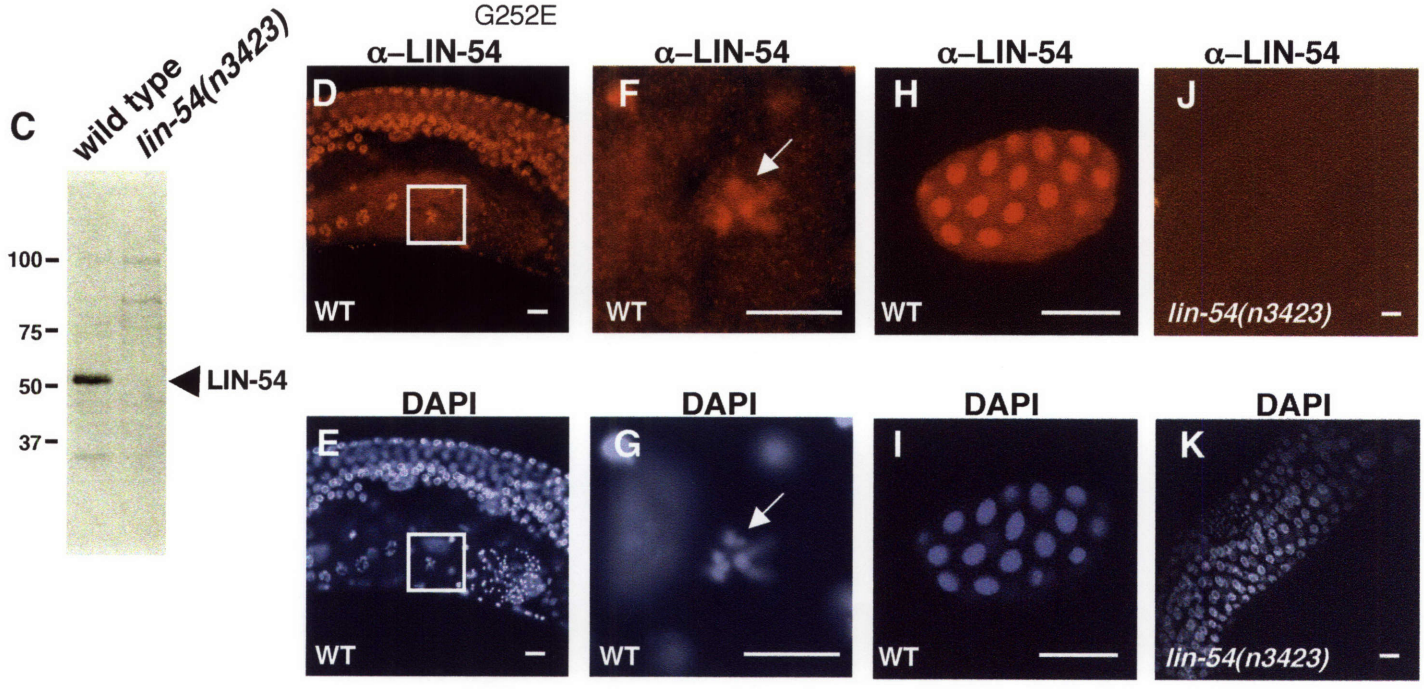


Figure 3. A subset of class B synMuv proteins form a complex *in vivo*.
(A) Gel filtration chromatography fractions immunoblotted with antibodies specific to the antigen indicated on the left. LIN-37 and LIN-54 coeluted in early fractions. Numbers beneath correspond to the fraction in which a protein of the size indicated (in kilodaltons) eluted from the column. (B) Embryo extracts from both wild-type and *lin-37(n758)* mutant animals were precipitated with anti-LIN-37 antibodies. Proteins were separated by SDS-PAGE and immunoblotted with antibodies specific to the antigen indicated on the left. (C) Immunoprecipitations were performed using wild-type embryonic extracts in the presence of 50 μg/ml ethidium bromide (EtBr). (D) Immunoprecipitations were performed using embryonic extracts from the wild type and antibodies that specifically recognize LIN-9, LIN-52, or LIN-54. Coimmunoprecipitation of LIN-52 with anti-LIN-54 antibodies was from L1 larval extracts. (E) Extracts from wild-type L1 or late L2/ early L3 larvae were precipitated as in (B). WT, wild type. IN, 2% of input for immunoprecipitations. 10% of input for gel filtration chromatography. V_0 void volume. V_t total volume.

Figure 3

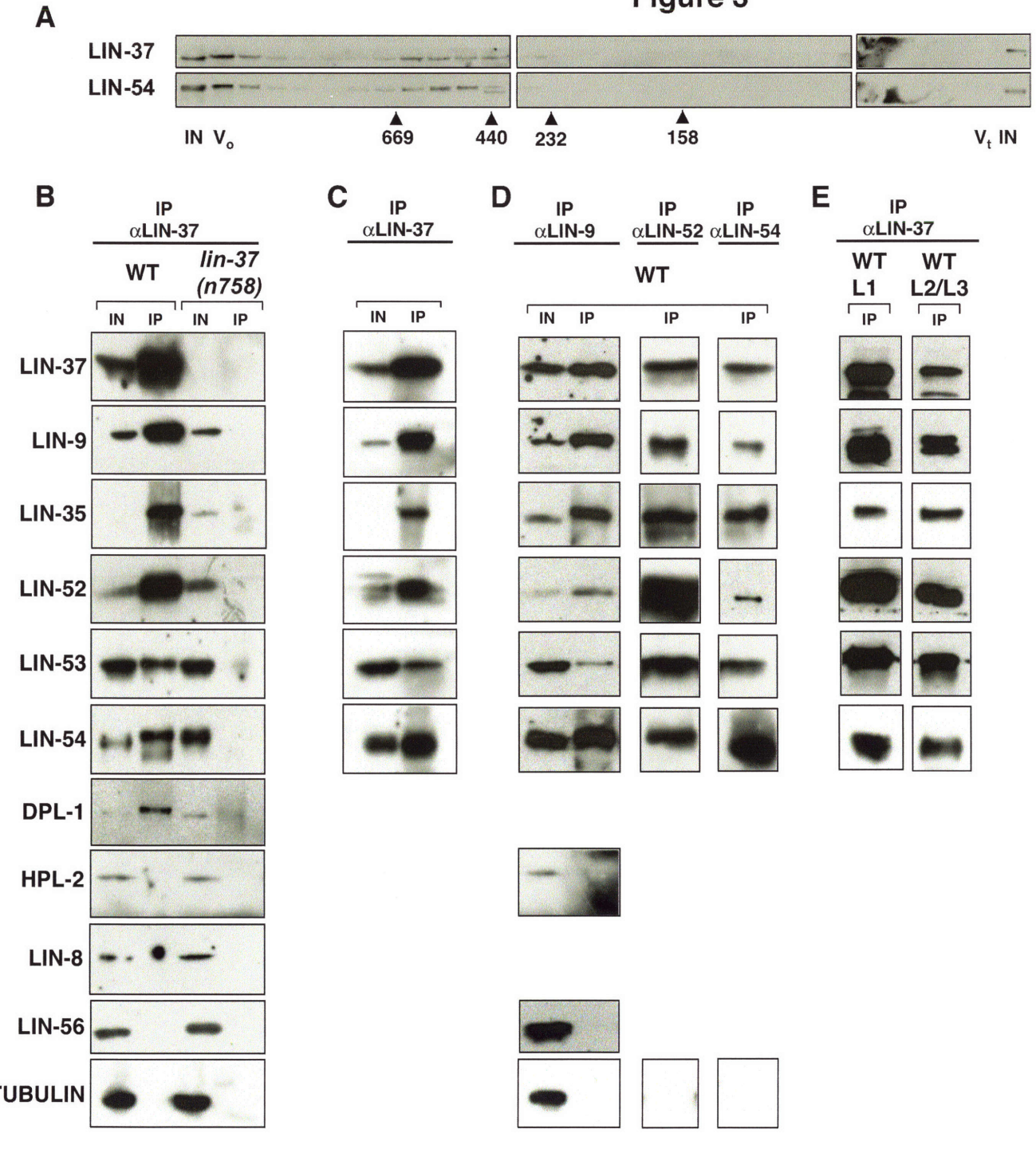

A
LIN-37
LIN-54
IN
V_o
669
440
232
158
V_t
IN
B
IP
αLIN-37
WT
lin-37 (n758)
IN
IP
IN
IP
LIN-37
LIN-9
LIN-35
LIN-52
LIN-53
LIN-54
DPL-1
HPL-2
LIN-8
LIN-56
TUBULIN
C
IP
αLIN-37
IN
IP
D
IP
αLIN-9
IP
αLIN-52
IP
αLIN-54
WT
IN
IP
IP
IP
E
IP
αLIN-37
WT
L1
WT
L2/L3
IP
IP

Figure 4. NuRD-like complex components do not precipitate with the DRM complex (A) Extracts from either wild-type or *lin-37(n758)* mutant animals (as indicated above the lanes) were precipitated with antibodies directed against either HDA-1 or LIN-37 or with non-specific anti-rabbit IgG. Immunoprecipitations were analyzed using antibodies specific to the antigens indicated to the left. (B) Extracts from either wild-type or *lin-37(n758)* mutant L3 larvae were precipitated with antibodies directed against LIN-37, LIN-9, or LIN-52. Immunoprecipitations were analyzed using antibodies specific to the antigens indicated to the left. (B) Wild-type embryo extracts were precipitated with antibodies that recognize LIN-9, LIN-52, or LIN-54 and were analyzed by western blots with antibodies that recognize HDA-1. WT, wild type. IN, 2% of the input. IP, 100% of the immunoprecipitate.

Figure 4

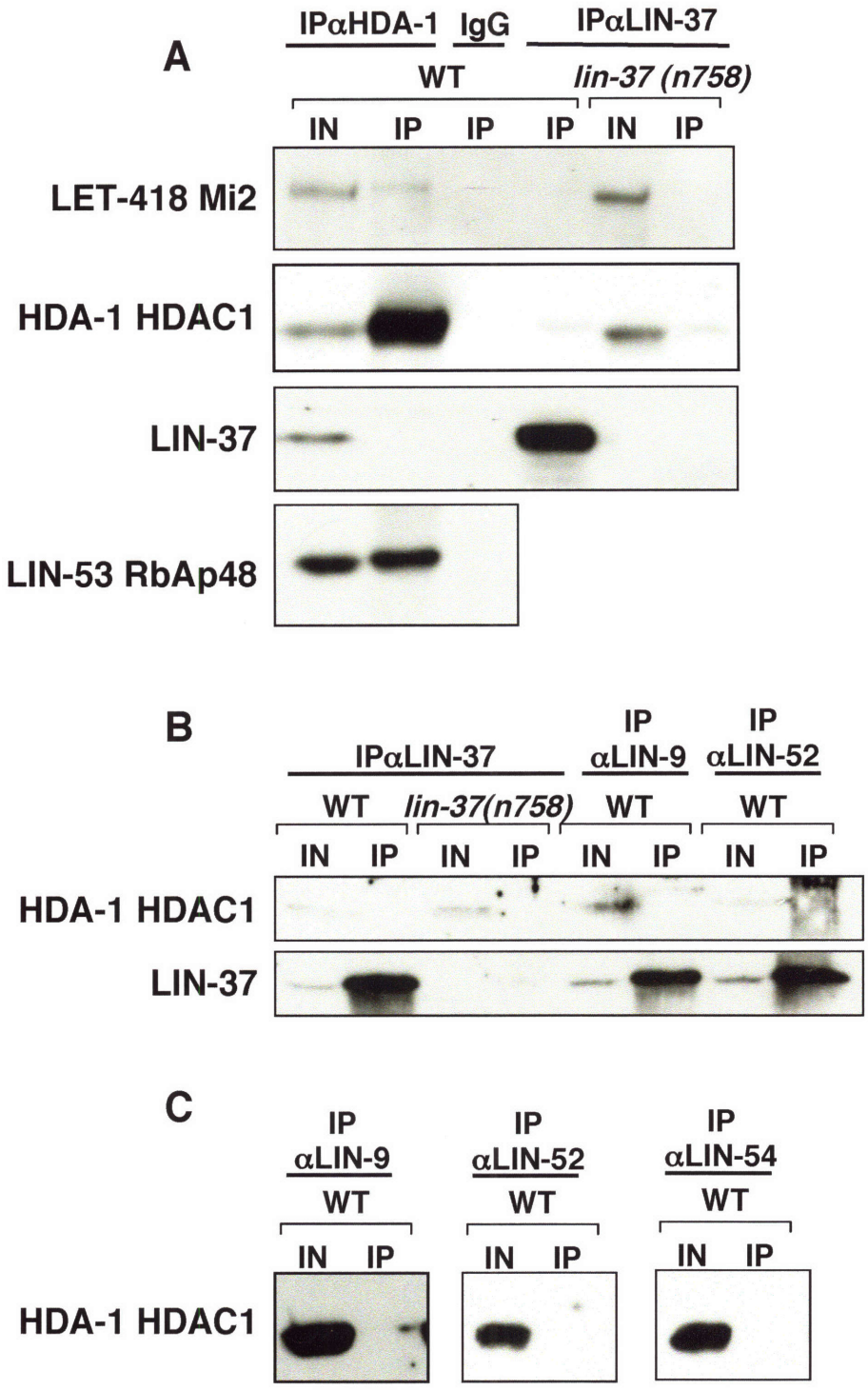

A
IPαHDA-1
IgG
IPαLIN-37
WT
lin-37 (n758)
IN
IP
IP
IP
IN
IP
LET-418 Mi2
HDA-1 HDAC1
LIN-37
LIN-53 RbAp48
B
IPαLIN-37
IP αLIN-9
IP αLIN-52
WT
lin-37(n758)
WT
WT
IN
IP
IN
IP
IN
IP
IN
IP
HDA-1 HDAC1
LIN-37
C
IP αLIN-9
IP αLIN-52
IP αLIN-54
WT
WT
WT
IN
IP
IN
IP
IN
IP
HDA-1 HDAC1

Figure 5. Analysis of the DRM complex and its components in mutant backgrounds. (A) Anti-LIN-37 antibodies were used for immunoprecipitation of proteins from embryo extracts from animals of the indicated genotype, except in the case of extracts from *lin-37(n758)* animals, when anti-LIN-52 antibodies were used. For immunuoprecipitations from *lin-9(n112)* embryo extract, both anti-LIN-9 and anti-LIN-37 antibodies were used. Following immunoprecipitation, proteins were separated by SDS-PAGE and immunoblotted using the antibodies specific to the antigens listed to the left. IN, 2% of the input. IP, 100% of the immunoprecipitate. (B) Protein from 100 L4 larvae of the genotype noted above each column was loaded per lane. The antibodies used on each blot are indicated on the left. Anti-tubulin antibodies were used to assess protein loading and transfer. Asterisks denote non-specific immunoreactivity.

Figure 5

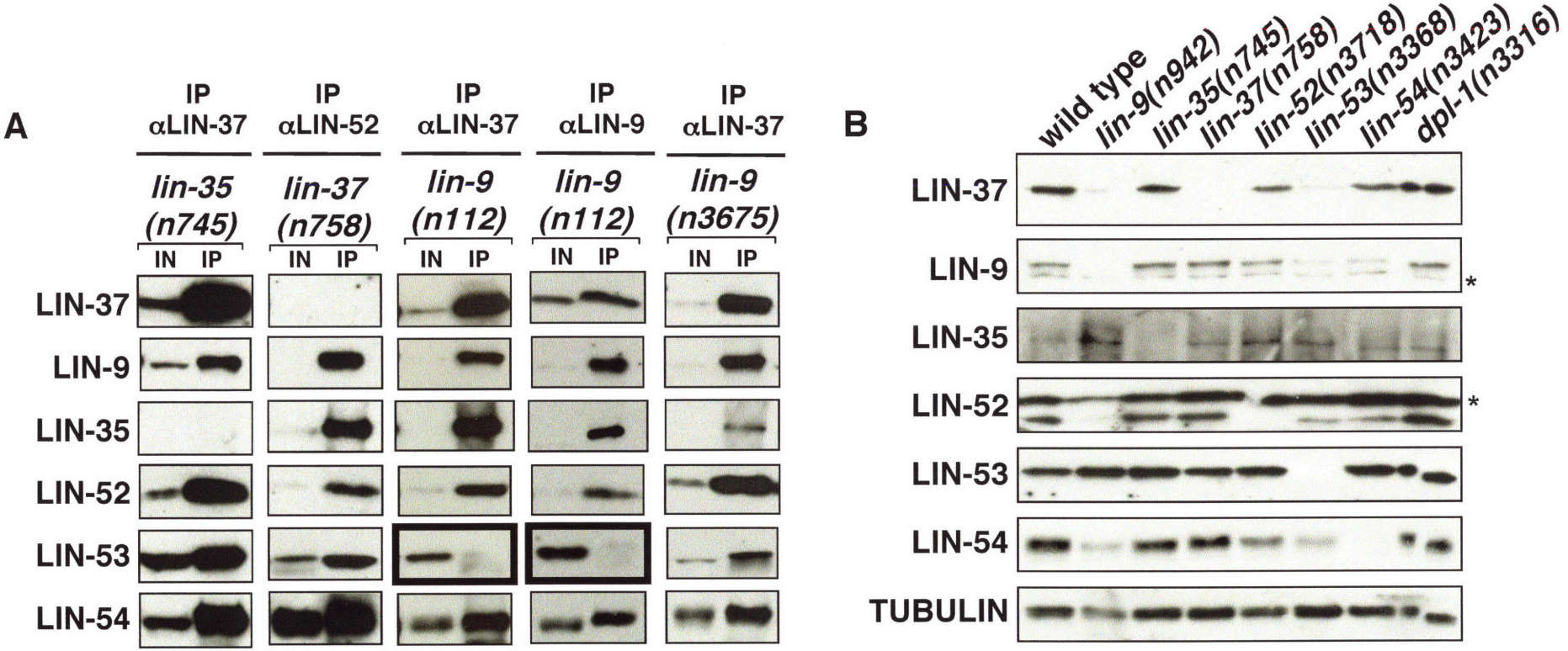

A
IP αLIN-37
IP αLIN-52
IP αLIN-37
IP αLIN-9
IP αLIN-37
lin-35 (n745)
lin-37 (n758)
lin-9 (n112)
lin-9 (n112)
lin-9 (n3675)
IN IP
LIN-37
LIN-9
LIN-35
LIN-52
LIN-53
LIN-54
B
wild type
lin-9(n942)
lin-35(n745)
lin-37(n758)
lin-52(n3718)
lin-53(n3368)
lin-54(n3423)
dpl-1(n3316)
LIN-37
LIN-9
LIN-35
LIN-52
LIN-53
LIN-54
TUBULIN
*
*

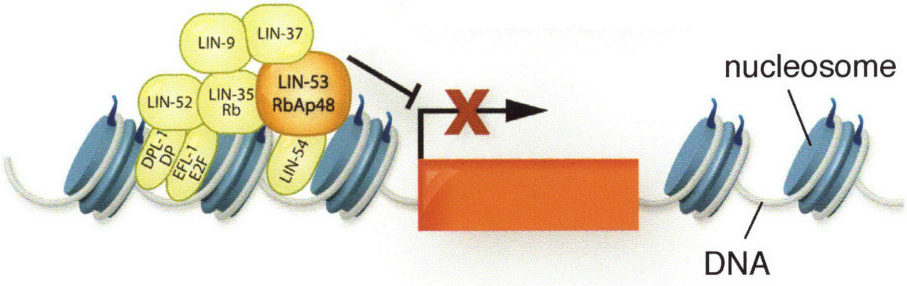
Figure 6. Schematic indicating the components of the DRM and NuRD-like complexes and a model for how these complexes act in vulval development. (A) A list of DRM complex members and the homologs found in *Drosophila melanogaster* and *Homo sapiens*. (B) The DRM complex contains at least eight proteins: LIN-9, LIN-35 Rb, LIN-37, LIN-52, LIN-53 RbAp48, LIN-54, DPL-1 DP, and EFL-1 E2F. Model: This complex binds chromatin to repress the transcription of genes that promote the expression of vulval cell fates. red box, a gene that promotes the expression of vulval cell fates. (C) Model for joint action of the DRM and NuRD-like complexes. The DRM complex could act with the NuRD-like complex to repress transcription. (D) Model for sequential roles of the NuRD-like and DRM complexes. Genes that promote the expression of vulval cell fates are transcriptionally active when the histones in the region of their promoters are acetylated. The NuRD-like complex (which contains at least HDA-1 HDAC1, LET-418 Mi2 and LIN-53 RbAp48) deacetylates these histones, causing transcriptional repression. This transcriptional repression is maintained by the binding of the DRM complex to the deacetylated histones. Note that is not known if the NuRD-like complex and the DRM complex act on the same set of target genes. LIN-53 RbAp48 is a component of both the NuRD-like and the DRM complexes. See text for references

Figure 6

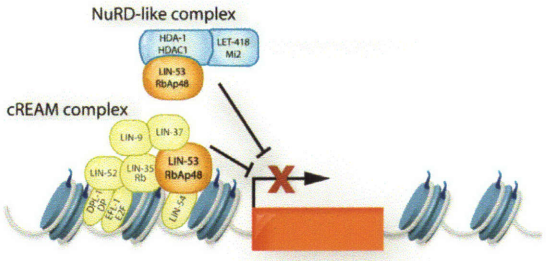
A

C. elegans	*D. melanogaster*	*H. sapiens*
LIN-9	Mip130	hLin-9
LIN-35	RBF	Rb, p107, p130
LIN-37	Mip40	F25965
LIN-52	dLin-52	LOC91750
LIN-53	p55	RbAp48
LIN-54	Mip120	tesmin
DPL-1	dDP	DP-1
EFL-1	dE2F2	E2F-4, E2F-5

B



C



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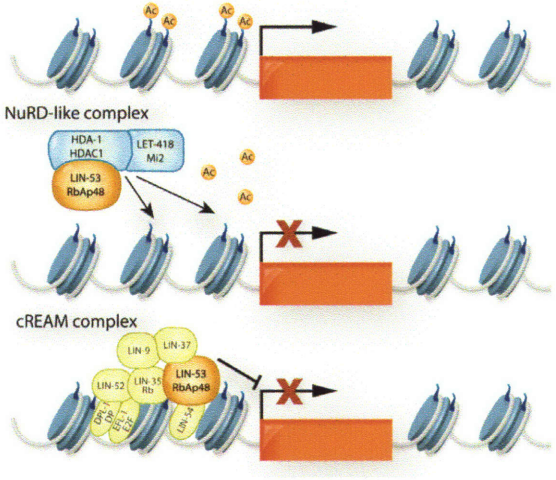

Supplemental Information

SUPPLEMENTAL RESULTS

Cloning of *lin-37*: We mapped *lin-37* between *sma-3* and *mec-14* on LGIII and performed transformation rescue of the synMuv phenotype of *lin-8(n111); lin-37(n758)* with cosmids in this region. A 13.8 kb fragment containing five predicted open reading frames (ORFs) present in both of the rescuing cosmids F31H1 and C49B6, was able to rescue the synMuv phenotype of *lin-8(n111); lin-37(n758)* animals (Supplemental Figure 1). We introduced frameshift mutations into the minimal rescuing fragment to disrupt each of the five complete open reading frames and tested each construct for rescue. Only disruption of *ZK418.4* eliminated the rescuing activity of the 13.8 kb fragment, suggesting that *ZK418.4* encodes *lin-37* (Supplemental Figure 1).

By screening a cDNA library we obtained clones for mRNAs that encode *lin-37.* Multiple cDNAs had the last four nucleotides of the *C. elegans trans*-spliced leader SL2 immediately preceding the first ATG; this SL2 sequence is not found in the genomic sequence. That the immediately upstream gene *ZK418.5* is transcribed in the same direction as *lin-37*, the two genes are separated by only 170 base pairs and *lin-37* is SL2 trans-spliced suggest that *ZK418.5* and *lin-37* are co-transcribed as an operon (BLUMENTHAL 1995).

Cloning of *lin-54*: Germline-transformation experiments using genomic DNA clones from the interval of LGIV to which *lin-54* was mapped identified two overlapping cosmids, MMMC1 and JC8, each of which could rescue the synMuv phenotype of *lin-54(n2231); lin-15A(n433)* mutants (Supplemental Figure 2). DNA subclones of the overlapping region that contained the predicted ORF *JC8.6*, including a subclone that contained *JC8.6* as the only complete predicted ORF, rescued the *lin-54(n2231); lin-15A(n433)* synMuv phenotype, whereas subclones that lacked a complete *JC8.6* sequence failed to rescue (Supplemental Figure 2).

The deletion allele *lin-54(n3423)* removes 2338 base pairs beginning 280 base pairs upstream of the predicted *lin-54* initiator codon and leaves only the final ten *lin-54* codons intact (Figure 2A). The deletion allele *lin-54(n3424)* removes 1140 base pairs, including the last exon and half of the preceding exon (Figure 2A).

SUPPLEMENTAL MATERIAL AND METHODS

Culture conditions and strains: Mutant alleles used are listed below and are described by Riddle et al. (1997) unless otherwise noted: LGI: *lin-35(n745), lin-53(n833, n3368)* (Lu and Horvitz, 1998; this study) ; LGII: *dpl-1(n3316)* (CEOL and HORVITZ 2001), *lin-8(n111), trr-1(n3712)* (CEOL and HORVITZ 2004); LGIII: *lin-9(n112, n942, n3675)* (C.J.C., F. Stegmeier, M.M.H. and H.R.H unpublished results), *lin-37(n758, n2234)* (FERGUSON and HORVITZ 1989; THOMAS *et al.* 2003), *lin-52(n771, n3718)* (THOMAS *et al.* 2003); LGIV: *lin-54(n2231, n2990, n3423, n3424)* (THOMAS *et al.*, 2003; this study), *unc-30(e191)*; LGV: *hda-1(e1795)* (DUFOURCQ *et al.* 2002), *let-418(ar114, n3536)* (SEYDOUX *et al.* 1993; VON ZELEWSKY *et al.* 2000); LGX: *lin-15A(n433, n767), lin-15B(n744)* (FERGUSON and HORVITZ 1989). The translocations *nT1[qIs51]* (IV;V) and *hT2[qIs48]* (I;III) and the chromosomal inversion *mIn1[dpy-10(e128) mIs14]* were also used. *qIs48, qIs51* and *mIs14* are integrated transgenes that express GFP and are linked to the translocation or balancer (EDGLEY and RIDDLE 2001; MATHIES *et al.* 2003). *nIs93* is an integrated transgene that expresses LIN-36::GFP and is capable of rescuing the synMuv phenotype of *lin-36(n766); lin-15A(n767)* (THOMAS and HORVITZ 1999).

Transgenic Strains: For rescue of the *lin-8(n111); lin-37(n758)* Muv phenotype, cosmid or subclone DNA (20 ng/μl) was coinjected with a dominant rol-6 marker plasmid (pRF4) (100 ng/μl). For rescue of the *lin-54(n2231); lin-15A(n767)* synMuv phenotype cosmid (5-10 ng/μl) or plasmid (50-80 ng/μl) was injected along with the pRF4 plasmid (50-80 ng/μl).

Determination of *lin-54* cDNA sequence: *lin-54* cDNA sequence was determined from the EST clones yk414a8 and yk454f3 (kindly provided by Y. Kohara). The 5' ends of *lin-54* transcripts were determined using the 5' RACE system v2.0 (Invitrogen) according to the manufacturer's recommendations. We found SL1 splice-leader sequence on 5' RACE isolates and detected two alternative SL1 splice sites: gtttaattacccaagtttgagtattttcagtgttgacaATG and gtttaattacccaagtttgagtgttgacaATG, where SL1 sequence is underlined and the predicted translational start site is in uppercase. Two alternative splice acceptors exist for the first (ag/AACTTACAGGC versus agaacttacag/GC) and second (ag/CTTTTTCAGCC versus agctttttcag/CC) introns. For

each intron, the use of the alternative splice acceptors only results in a nine nucleotide variance in the length of the cDNA. The intronic sequence is shown in lowercase, and the exonic sequence in uppercase.

Antibody Preparation: Anti-LIN-9 antiserum was generated by immunizing rabbits with purified MBP-LIN-9 (full-length) fusion protein. The antiserum was affinity purified against GST-LIN-9 (full-length) as described by Koelle and Horvitz (1996). Anti-LIN-35 antiserum was generated by immunizing guinea pigs with purified MBP-LIN-35 (amino acids 270-961) fusion protein. Serum from the third production bleed was used for western blot experiments. (Other anti-LIN-35 antiserum was previously reported (LU and HORVITZ 1998), but we were unable to establish specificity of this antiserum for LIN-35 in the studies reported in this manuscript.) Anti-LIN-37 antiserum was generated by immunizing rabbits with LIN-37 (full-length):His fusion protein. Anti-LIN-37 antiserum was affinity purified against GST-LIN-37 (full-length). Anti-LIN-52 antiserum was generated by immunizing rabbits with purified MBP-LIN-52 (full-length) fusion protein and was affinity purified against GST-LIN-52 (full-length). Anti-LIN-53 rabbit antiserum was generated by immunization with the peptide CNEVDEETPADVVERQQ (amino acids 402-417) and was affinity purified using this peptide. This peptide is unique to LIN-53 and is not contained in the related C. elegans protein RBA-1. Anti-LIN-54 antiserum was generated by immunizing guinea pigs with GST-LIN-54 (full-length) fusion protein and was affinity purified against MBP-LIN-54 (full-length).

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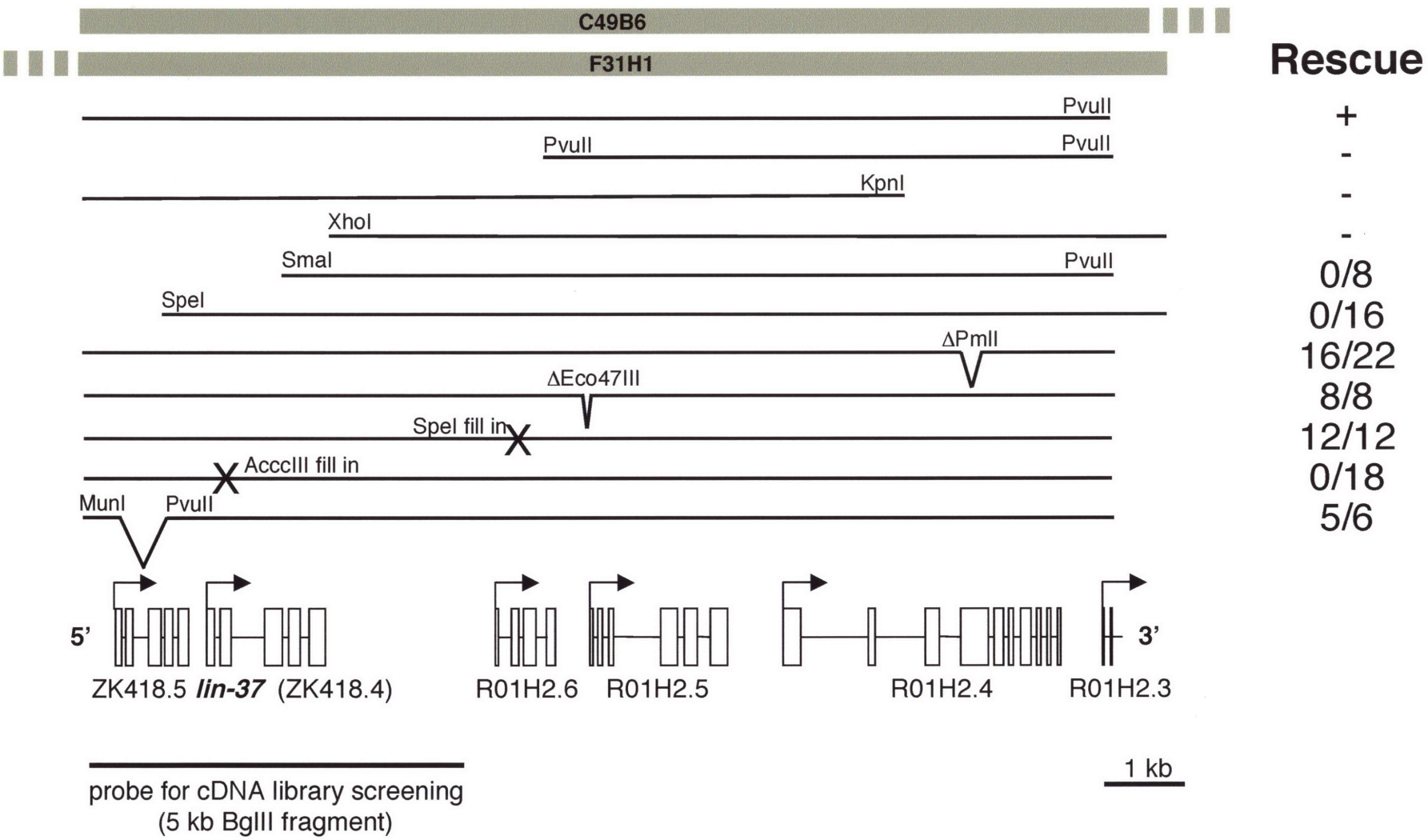
Supplemental Figure 1. Molecular cloning of *lin-37*.

Rescuing cosmids C49B6 and F31H1 are shown as gray bars. Dotted bars indicate that the cosmid continues in the direction shown. The subcloned portions of the cosmids injected for rescue are shown below. The five predicted genes within the 13.8 kb rescuing fragment are indicated on the bottom. The arrows indicate the direction of transcription. The fraction of transgenic lines rescued for the *lin-8(n111); lin-37(n758)* Muv phenotype is shown on the right. +, rescue; -, no rescue.

Supplemental Figure 1

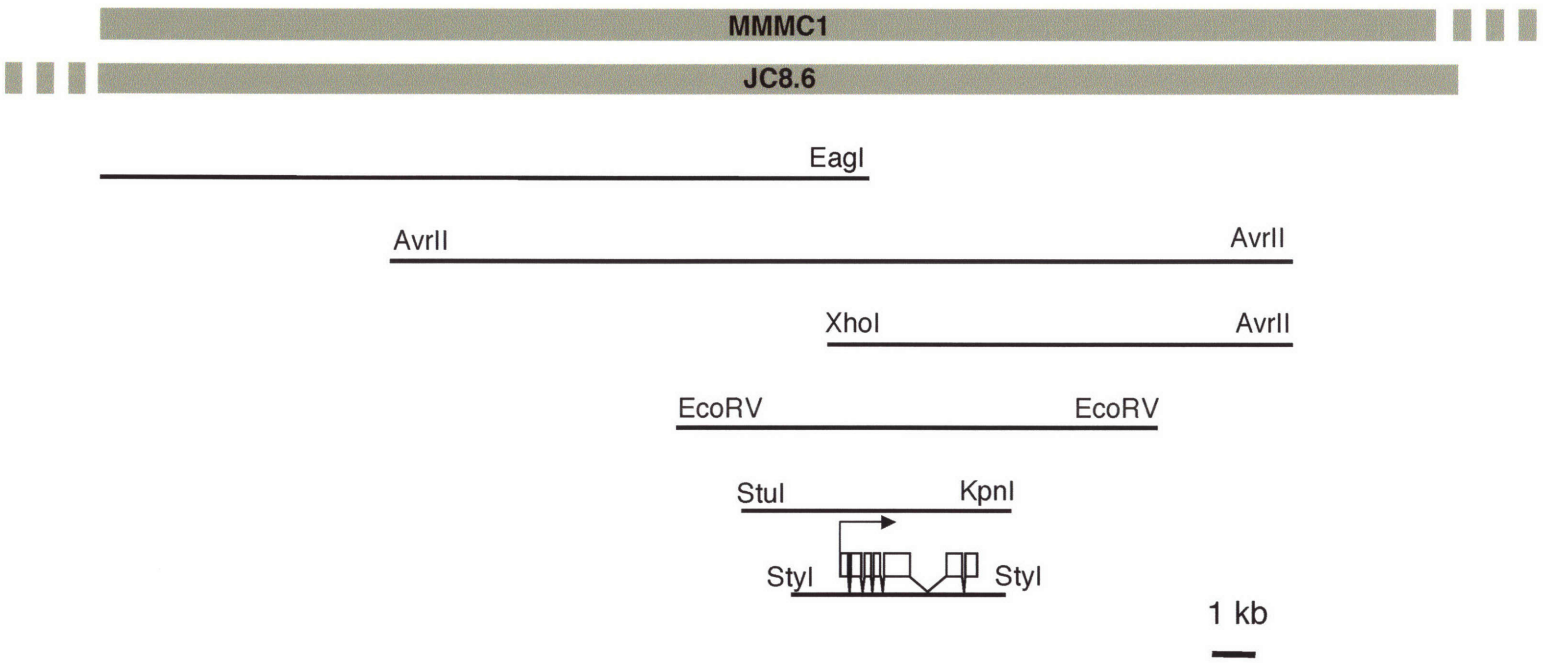


Rescue
+
-
-
-
0/8
0/16
16/22
8/8
12/12
0/18
5/6

Supplemental Figure 2. Molecular cloning of *lin-54*.

Rescuing cosmids MMMC1 and JC8.6 are shown as gray bars. Dotted bars indicate that the cosmid continues in the direction shown. The subcloned portions of the cosmids injected for rescue are shown below. The fraction of transgenic lines rescued for the *lin-54(n2231); lin-15A(n233)* Muv phenotype is shown on the right. The *lin-54* gene is indicated on the minimal rescuing fragment. The arrow indicates the direction of transcription.

Supplemental Figure 2

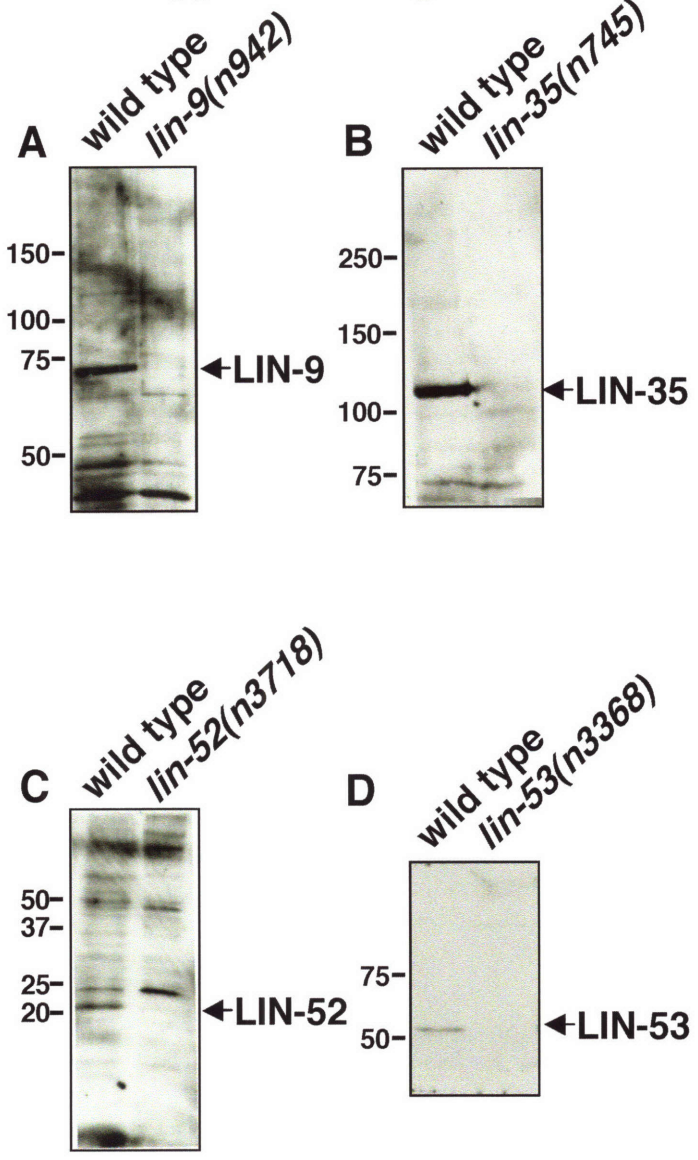


Rescue
0/8
7/7
0/13
9/10
3/4
4/4

Supplemental Figure 3. Antibodies specifically recognize LIN-9, LIN-35, LIN-52 and LIN-53

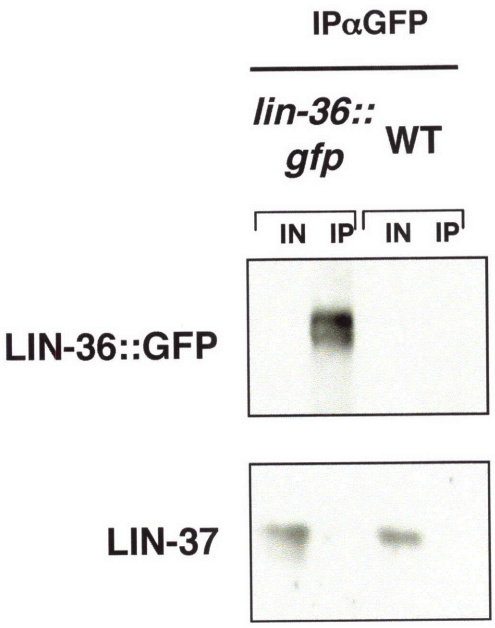
(A) Affinity-purified antibodies raised against recombinant LIN-9 were used to blot extracts from 50 young adult wild-type or *lin-9(n942)* mutant animals. (B) Affinity-purified antibodies raised against recombinant LIN-35 were used to blot extracts from a mixed stage population of wild-type or *lin-35(n745)* mutant animals. (C) Affinity-purified antibodies raised against recombinant LIN-52 were used to blot extracts from 50 young adult wild-type or *lin-52(n3718)* mutant animals. (D) Affinity-purified antibodies raised against a peptide specific to LIN-53 were used to blot extracts from 50 young adult wild-type or *lin-53(n3368)* mutant animals.

Supplemental Figure 3



Supplemental Figure 4. LIN-37 does not coimmunoprecipitate with LIN-36::GFP. Extracts from both wild-type embryos and embryos carrying *nIs93*, a rescuing *lin-36::gfp* transgene, were immunoprecipitated with anti-GFP antibodies (mAb3E6, Qbiogen). Proteins were separated by SDS-PAGE and immunoblotted with antibodies specific to the antigen indicated on the left.

Supplemental Figure 4



CHAPTER 3

LIN-61, one of two *C. elegans* MBT-repeat-containing proteins, acts separately from the DRM and NuRD-like complexes in developmental regulation

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This manuscript is being prepared for publication.
Xiaowei Lu mapped and cloned *lin-61*. The original *lin-61* allele, *sy223*, was isolated in Paul Sternberg's laboratory.

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ABSTRACT

Vulval development in *Caenorhabiditis elegans* is inhibited by the redundant functions of the class A, B, and C synthetic multivulva (synMuv) genes. At least 31 synMuv genes have been identified, many of which appear to act via transcriptional repression. Here we report the identification of the class B synMuv gene *lin-61*, which encodes a protein composed of four malignant brain tumor (MBT) repeats. MBT repeats are domains of approximately 100 amino acids, and have been found in multiple copies in a number of transcriptional repressors, including Polycomb group proteins. MBT repeats are important for the transcriptional repression mediated by these proteins and in some proteins act by binding to modified histones. *C. elegans* contains one other MBT-repeat-containing protein, MBTR-1. We demonstrated that a deletion allele of *mbtr-1* does not cause a class B synMuv phenotype nor does it enhance or suppress the abnormalities caused by loss of function in *lin-61.* We further showed that *lin-61* is biochemically and genetically separable from other class B synMuv genes in a number of biological processes. These data demonstrate that while the class B synMuv genes cooperate to regulate vulval development, they have distinct functions in other processes.

INTRODUCTION

Development requires cells that are initially equivalent to differentiate into organs and tissues. These changes in cell fate often are mediated by modulating gene expression, which can be controlled through modifications to the surrounding chromatin. Mutations in factors that control chromatin structure can lead to developmental defects in numerous organisms reviewed in (reviewed in MARGUERON *et al.* 2005). In *Caenorhabiditis elegans,* the regulation of vulval development have involves evolutionarily conserved factors important for signal transduction, chromatin remodeling and transcriptional repression. Genetic and biochemical studies of these factors should help to elucidate how proteins that modify chromatin structure can cooperate during development.

The *C. elegans* hermphrodite vulva is formed from three of six equipotent blast cells, P3.p-P8.p (SULSTON and HORVITZ 1977; SULSTON and WHITE 1980). Although all six cells are competent to adopt a vuval cell fate, in wild-type development only P5.p, P6.p and P7.p divide to generate the vulva. P3.p, P4.p, and P8.p normally divide once and fuse with the surrounding syncytial hypodermis. Many signaling pathways are required to properly specify vulval development, including a receptor tyrosine kinase/Ras pathway, a Wnt signaling pathway, and a Notch signaling pathway (YOCHEM *et al.* 1988; BEITEL *et al.* 1990; HAN *et al.* 1990; GLEASON *et al.* 2002). Mutations affecting these pathways either can result in P3.p, P4.p, and P8.p aberrantly adopting vulval cell fates, a multivulva (Muv) phenotype, or none of the Pn.p cells adopting a vulval cell fate, a vulvaless (Vul) phenotype (YOCHEM *et al.* 1988; STERNBERG and HORVITZ 1989; BEITEL *et al.* 1990; HAN *et al.* 1990; GLEASON *et al.* 2002).

Vulval induction is antagonized by the synthetic multivulva (synMuv) genes, which have been placed into three classes, A, B, and C, based on their genetic interactions (FERGUSON and HORVITZ 1989; CEOL and HORVITZ 2004). Because of redundancy among the three classes, only animals with loss-of-function mutations in two synMuv classes have a multivulva phenotype, whereas animals with a loss-of-function mutation in a single class are not Muv. Many of the synMuv genes encode proteins that have been implicated in chromatin remodeling and transcriptional

repression. These include proteins such as EFL-1 E2F, DPL-1 DP, and LIN-54, which likely bind directly to DNA and repress transcription as components of the evolutionarily-conserved DP, Rb and MuvB (DRM) complex (CEOL and HORVITZ 2001; M.M.H, C.J. Ceol and H.R.H unpublished results.) The synMuv proteins LET-418, LIN-53, and HDA-1 are homologous to components of the mammalian Nucleosome Remodeling and Deacetylase (NuRD) complex (LU and HORVITZ 1998; VON ZELEWSKY *et al.* 2000; DUFOURCQ *et al.* 2002). The synMuv proteins HPL-2 and MET-2 are homolgous to HP1 and SETDB1, respectively (COUTEAU *et al.* 2002; POULIN *et al.* 2005). HP1 binds to methylated lysine 9 of histone H3, and SETDB1 is a methylatransferase that can methylate this lysine residue (BANNISTER *et al.* 2001; SCHULTZ *et al.* 2002). In other organisms all of these proteins function in transcriptional repression, and recently in *C. elegans*, a number of class B synMuv genes have been implicated in transcriptional repression of *lin-3* EGF (XUE *et al.* 1998; ZHANG *et al.* 1998; ZHANG *et al.* 1999; BANNISTER *et al.* 2001; CUI *et al.* 2006a). The class C synMuv genes encode homologs of a Tip60/NuA4-like histone acetyltransferase complex, which might act in transcriptional repression or activation (CEOL and HORVITZ 2004).

Polycomb-group (PcG) proteins were initially identified by their ability to transcriptionally repress Hox genes but have since been shown to repress additional targets including genes regulated by E2F transcription factors (DAHIYA *et al.* 2001; OGAWA *et al.* 2002). PcG proteins include enzymes with histone methyltransferase activity and proteins that bind to these histone modifications as well as many other proteins, including Sex Comb on Midleg (SCM). SCM contains two malignant brain tumor (MBT) repeats, which are motifs of approximately 100 amino acids. MBT repeats have been found in many transcriptional repressors, including l(3)mbt, which is in a complex with multiple other PcG group proteins and with E2F6 in human cells (OGAWA *et al.* 2002).

Here we report the characterization of the class B synMuv gene, *lin-61*. *lin-61* encodes a protein composed of four MBT repeats that is localized to chromatin. LIN-61 is not a core component of the pocket-protein-containing DRM complex or the NuRD-like complex and acts separately from members of these complexes to regulate some

aspects of development. Thus MBT-repeat-containing proteins, such as Polycomb-group proteins, might cooperate with Rb-containing complexes and histone deacetylase complexes to repress a subset of genes, but likely act independently of these complexes to regulate expression of other genes.

MATERIALS AND METHODS

Strains: Mutant alleles used are listed below and are described by Riddle et al. (1997) unless otherwise noted: LGI: *unc-14(e57), unc-15(e73); lin-61(sy223, n3442, 3446, n3447, n3624, n3687, n3736, n3807, n3809, n3922* (this study), *mbtr-1(n4775)* (this study), *nIs133* (H. Schwartz and H.R.H unpublished results); LGII: *lin-8(n2731)* (THOMAS *et al.* 2003), *lin-38(n751)* (FERGUSON and HORVITZ 1989), *lin-56(n2728)* (THOMAS *et al.* 2003), *trr-1(n3712)* (CEOL and HORVITZ 2004); LGIII: *hpl-2(n4274)* (E. Andersen and H.R.H. unpublished results), *lin-13(n770)* (FERGUSON and HORVITZ 1989), *lin-37(n758)* (FERGUSON and HORVITZ 1989), *mat-3(ku233)* (GARBE *et al.* 2004); LGV: *hda-1(e1795)* (Dufourcq et al. 2002); LGX: *lin-15A(n433, n767), lin-15B(n744)* (Ferguson and Horvitz 1989), *mys-1(n3681)* (CEOL and HORVITZ 2004), *qIs56 [lag-2::gfp; unc-119(+)]* (SIEGFRIED and KIMBLE 2002), *pkIs1605* [*rol-6*(*su1006*) *hsp16/2::gfp/lacZ*(out of frame)] (POTHOF *et al.* 2003), *ccEx6188* [*myo-3::Ngfp-lacZ rol-6(su1006)*] (HSIEH *et al.* 1999). The translocations *nT1[qIs51]* (IV;V) and *hT2[qIs48]* (I;III) and the chromosomal inversion *mIn1[dpy-10(e128) mIs14]* were also used. *qIs48, qIs51* and *mIs14* are integrated transgenes that express GFP and are linked to the translocation or balancer (EDGLEY and RIDDLE 2001; MATHIES *et al.* 2003).

Transgenic strains: For rescue of the *lin-61(sy223); lin-15A(n767)* or *lin-61(n3624); lin-15A(n767)* Muv phenotype, cosmid or subclone DNA (5 or 10 ng/μl) was coinjected with a dominant *rol-6* marker plasmid (pRF4) (80 ng/μl).

RNAi analysis: Templates for *in vitro* transcription reactions were made by PCR amplification of cDNAs including flanking T3 and T7 promoter regions. RNA was transcribed *in vitro* using T3 and T7 polymerases. *In vitro* transcribed RNA was denatured for 10 min and annealed prior to injection.

Antibody preparation, immunocytochemistry and western blots: Anti-LIN-61 antiserum was generated by immunizing rabbits and guinea pigs with purified GST-LIN-61(amino acids 159-491). The antiserum was affinity purified against full-length MBP-LIN-61. The rabbits and guinea pigs were maintained by Covance (Denver, PA). All antibodies were used at a 1:1000 dilution for western blots, except for anti-LIN-8, anti-LIN-53, anti-LIN-54, and anti-DPL-1, which were used at 1:500. Affinity purified

antibodies were used in all cases except for anti-LIN-35 antibodies, for which we used unpurified serum from the third production bleed. Larvae and adults for immunostaining were fixed in 1% paraformaldehyde for 30 min, as described by Finney and Ruvkun (1990). Embryos were fixed for 20 min in 0.8% paraformaldehyde, as described by Guenther and Garriga (1996). Affinity-purified anti-LIN-61 antisera were used at a 1:100 dilution for immunocytochemistry.

Phenotypic characterization: To score RNAi hypersensitivity, L4 larvae were placed on *E. coli* expressing either *hmr-1* or *cel-1* dsRNA. 24 hours later the young adult hermphrodites were transferred to a fresh plate with *E. coli* expressing dsRNA. The hermphrodites were allowed to lay eggs for 24 hr and were then removed. The progeny of hermphrodites grown on *E. coli* expressing *cel-1* dsRNA were scored for arrest at the L2 larval stage. The progeny of hermphrodites grown on *E. coli* expressing *hmr-1* dsRNA were scored for embryonic lethality. For scoring ectopic PGL-1 expression, L1 larvae were permeabilized using a freeze-crack method followed by a methanol-acetone fixation as described in Wang *et al.* (2005). Permeabilized larvae were incubated with OIC1D4 monoclonal anti-PGL-1 antibodies (Developmental Studies Hybridoma Bank, University of Iowa) at a dilution of 1:20 overnight followed by a one hour incubation with Alexa Fluor 594 goat anti-mouse IgM (Invitrogen) at a dilution of 1:25. To score for the Tam phenotype, animals carrying the extrachromosomal array *ccEx6188 [myo-3::Ngfp-lacZ],* hermphrodites were grown for at least two generations at 25°C. Relative GFP expression was then scored under the fluorescence dissecting microscope. Hermaphrodites carrying the transgene *pkIs1605* [*rol-6*(*su1006*) *hsp16/2*::*gfp/lacZ*(out of frame)] were scored for somatic repeat instability after being grown at 20°C for five days, heat-shocked at 31°C two hr, and allowed to recover at 20°C for one hour. The hermaphrodites were then fixed and stained for the presence of β-galactosidase with X-gal. Vulval defects in *mat-3(ku233)* mutants were scored by Nomarski differential interference contrast (DIC) microscopy of mid-L4 hermaphrodites. Vulval development was scored as abnormal if the invagination was asymmetric or contained fewer than the 22 cells found in wild-type animals.

Embyro lysates: Embryos were harvested from liquid cultures and bleached to kill larvae and adults in a solution containing 5N NaOH and 20% hypochlorite. The embryos were resuspended in 1 ml of lysis buffer (25 mM HEPES pH 7.6, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.5 mM EGTA, 0.1% NP-40, 10% glycerol with Complete EDTA-free protease inhibitors (Roche Diagnostics)) for each gram of embryos and frozen in liquid nitrogen. The embryos were thawed at room temperature and sonicated 15 times for 10 sec using a Branson sonifier 450 at setting 5. The homogenate was then centrifuged at top speed in a microcentrifuge for 15 min at 4°C. The supernatant was removed to a new tube and was spun for 15 min at top speed in a microcentrifuge. The remaining supernatant was pooled and the protein concentration was determined using the Pierce Coomassie Plus Protein Assay Reagent (Pierce Biotechnology). The lysate was diluted to 5-10 mg/ml and was used immediately or stored at -80°C and thawed no more than once before use.

Immunoprecipitation experiments: Antibodies were crosslinked to Protein A Dynabeads (Invitrogen) using dimethyl pimelimidate (Pierce) essentially as described by Harlow and Lane (1999), with the two following exceptions: reactions were stopped with 0.1M Tris pH 8.0 and beads were washed three times for 1 min in 100 mM glycine pH 2.5 followed by lysis buffer. The beads were then resuspended in PBS. 500 μl of lysate, corresponding to 2.5-5 mg of total protein, were incubated with 25 μl of affinity-purified antibody bound to 25 μl of beads for each immunoprecipitation reaction. Prior to incubation with antibody-bound beads, lysates were initially precleared with 25 μl of beads for between 1 hr and overnight at 4°C. The precleared lysates were recirculated over the antibody-bound beads at 4°C for 1-2 hr and were then washed three times for 5 min each at 4°C in lysis buffer. Following the final wash, the beads were resuspended in 20 μl of 2X protein sample buffer, boiled for 5 min, and loaded on an SDS-polyacrylamide gel. HRP-conjugated protein A (Bio-Rad) was used for detection of protein by western blot following coimmunoprecipitation experiments, except for detection of tubulin in which HRP-conjugated goat anti-mouse (Bio-Rad) was used.

RESULTS

***lin-61* is a class B synMuv gene:** Ten *lin-61* alleles have been isolated in three different screens. The original *lin-61* allele, *sy223*, was isolated in the laboratory of Paul Sternberg based on its synMuv phenotype in combination with a loss-of-function mutation in *lin-15A*. Four alleles, *n3687, n3807, n3809,* and *n3922*, were isolated in a screen for transgene misexpression (H. Schwartz, D. Wendell, and H.R.H. unpublished results). Five alleles, *n3442, n3446, n3447, n3624*, and *n3736* were isolated in a screen for mutations that cause a synMuv phenotype with *lin-15A(n767)* (CEOL *et al.* 2006).

As single mutants none of the ten *lin-61* alleles caused a Muv phenotype (Table1). All ten alleles caused a synMuv phenotype in combination with loss of function of the class A synMuv gene *lin-56* (Table 2). *n3809* caused a synMuv phenotype in combination with loss of function in all four class A synMuv genes but not in combination with mutations in the class B synMuv genes *lin-37* and *lin-15B* (Table 1; data not shown). Loss of function of *lin-61* also failed to enhance the Muv phenotype associated with *hda-1(e1795)* (Table 1). Similar to previous reports for reduction of *lin-9, lin-15B, lin-35* and *lin-37* by RNAi (COUTEAU *et al.* 2002), loss of function of *lin-61* caused a Muv phenotype in combination with a mutation in *hpl-2* (Table 1). These data suggest that *lin-61* has a function in vulval development that is redundant with that of *hpl-2* but not with that of *hda-1*. The *lin-61* allele *n3809* did not enhance the weak Muv phenotype associated with mutations in either of the class C genes *trr-1* or *mys-1* (Table 1). Because *lin-61* caused a synMuv phenotype in combination with mutations in class A but not class B synMuv genes and because it did not cause the P8.p induction observed in animals mutant for class C synMuv genes (CEOL and HORVITZ 2004), we consider *lin-61* to be a class B synMuv gene.

***lin-61* encodes an MBT-repeat-containing protein:** We mapped *sy223* to an interval between *unc-14* and *unc-15* on LGI. A pool of cosmids covering the central portion of this region rescued the synMuv phenotype of *lin-61(sy223); lin-15A(n767)* animals, and a single cosmid from this pool, R06C7, rescued the synMuv phenotype of *lin-61(n3624); lin-15A(n767)* animals. A subcloned StuI-SacII fragment containing *R06C7.7* as the only complete predicted open-reading frame was capable of rescuing

the *lin-61(n3624); lin-15A(n767)* synMuv phenotype (Figure 1A). In addition, RNAi directed against *R06C7.7* caused a synMuv phenotype in combination with *lin-15A(n767)* (POULIN *et al.* 2005). To confirm that *R06C7.7* is *lin-61*, we determined the sequence of *R06C7.7* from *lin-61(sy223)* animals. *sy223* contains a G-to-A transition at the splice acceptor site before the last predicted exon (Figure 1B and Table 2). Mutations affecting the coding region of *lin-61* were also found in the nine other *lin-61* alleles, including three nonsense mutations, five missense mutations and one mutation in a splice acceptor (Table 2).

We determined the sequence of *lin-61* cDNAs and determined that the *lin-61* transcript is SL1-spliced and comprises six exons. *lin-61* transcripts contain no 5' UTR as the SL1 leader sequence is spliced directly to the ATG start codon. *lin-61* encodes a predicted protein of 491 amino acids that is composed almost exclusively of four malignant brain tumor (MBT) repeats. MBT repeats are domains of approximately 100 amino acids and have been found in up to four copies per protein. MBT repeats were initially identified in the *D. melanagaster* protein lethal (3) malignant brain tumor (l(3)mbt) (WISMAR *et al.* 1995) and are present in many other metazoan proteins, including the Polycomb group protein Sex Comb on Midleg (SCM) (BORNEMANN *et al.* 1996; TOMOTSUNE *et al.* 1999; USUI *et al.* 2000; BOCCUNI *et al.* 2003; MARKUS *et al.* 2003; ARAI and MIYAZAKI 2005). In addition to their MBT repeats, l(3)mbt and SCM both contain atypical zinc fingers and a single sterile alpha motif (SAM) domain (WISMAR *et al.* 1995; BORNEMANN *et al.* 1996). The SAM domain of SCM mediates homodimerization and interaction with the Polycomb protein Polyhomeotic (PETERSON *et al.* 1997; KIM *et al.* 2005). Given that LIN-61 lacks both of these additional domains and that it is composed almost exclusively of the four MBT repeats, the functionality of LIN-61 is likely provided by the MBT repeats.

Characterization of *lin-61* alleles: The mutation in *lin-61(n3809)* results in an ochre stop codon at amino acid 159 that is predicted to result in a truncated LIN-61 protein and is likely to be a null allele. *lin-61(n3809); lin-56(n2728)* animals have a highly penetrant synMuv phenotype (Table 2). Similarly penetrant synMuv phenotypes are caused by an additional nonsense mutation and both of the splice acceptor

mutations (Table 2). Although *n3687* causes an ochre mutation at amino acid 322, animals carrying this *lin-61* allele have a weak synMuv phenotype. The low penetrance of the synMuv phenotype suggests that a functional protein product might be made in *lin-61(n3687)* animals. Alternatively, the strain containing *n3687* could contain an additional mutation that suppresses the synMuv phenotype. This strain contains a closely linked integrated transgene that overexpresses both *lin-15A* and *lin-15B*. However, it is unlikely that this linked transgene is causing a reduction in the penetrance of the synMuv phenotype, as overexpression of *lin-15A* is incapable of rescuing synMuv defects caused by mutation in *lin-56* (E. Davison and H.R.H. unpublished results).

Four of the five missense mutations in *lin-61*, *n3447, n3736, n3807,* and *n3922*, are in one of the four MBT repeats. The fifth missense mutation, *n3624*, causes a proline-to-serine change in a residue between the first and second MBT repeats. The five missense mutations cause weaker synMuv phenotypes than most of the putative null alleles and form an allelic series. *n3624* and *n3807*, the two strongest missense mutants, cause a less penetrant synMuv phenotype than the nonsense and splice-acceptor mutations, suggesting that some LIN-61 activity might remain in these mutants (Table 2). Animals homozygous for *lin-61(n3736)* and a class A synMuv mutation have an intermediate phenotype, as evident from the fact that *lin-61(n3736)* in combination with *lin-15A(n433)* causes a lower penetrance synMuv phenotype than do *lin-61(n3807)* or *lin-61(n3624)*. However, when these strains are raised at 23°C or when the *lin-61* alleles are combined with a mutation in the class A synMuv gene *lin-56*, *lin-61(n3676)* causes a synMuv phenotype with a penetrance similar to the penetrance of the synMuv phenotypes caused by *lin-61(n3624)* and *lin-61(n3807)* (Table 2 and data not shown). *lin-61(n3447)* and *lin-61(n3922)* mutant animals have the least penetrant synMuv phenotypes (Table 2).

The *C. elegans* genome encodes one additional MBT-repeat-containing protein: Given the molecular identification of LIN-61 as containing four MBT repeats we searched the *C. elegans* genome for additional MBT-repeat-containing proteins. Using BLAST (ALTSCHUL *et al.* 1997), Pfam (BATEMAN *et al.* 2002), and SMART (SCHULTZ *et al.* 2000) algorithms we identified a single additional MBT-repeat-containing protein

encoded by the predicted gene *Y48G1A.6* (Figure 2A). We determined the sequence of a full-length cDNA. The GENEFINDER prediction for the cDNA is predominantly correct, except that the predicted fourth intron is not removed in this cDNA. The incorporation of this predicted intron into the open-reading frame results in a larger fourth exon than predicted but does not alter the frame of the predicted protein (Figure 2B). Given that the protein encoded by *Y48G1A.6* contains MBT-repeats, we named this gene *mbtr-1* for malignant brain tumor repeats.

The structure of MBTR-1 is similar to that of LIN-61 (Figure 2A). Like LIN-61, MBTR-1 is composed almost exclusively of four MBT repeats and lacks the SAM domain and zinc fingers found in other MBT-repeat-containing proteins (Figure 2). MBTR-1 is 36% identical to LIN-61 and is more similar to LIN-61 than to proteins in any other organisms. LIN-61 and MBTR-1 share an insertion of approximately 15 amino acids in their second MBT repeat not found in their human homolog h-l(3)mbt-like II or in other MBT-repeat-containing proteins (Figure 2A, Figure 4A, and data not shown). It is unclear how these additional amino acids might alter the structure of the MBT repeat or contribute to the function of the protein.

To determine the function of *mbtr-1* we identified a deletion allele, *n4775,* which removes exons 4 and 5 of *mbtr-1* and is predicted to result in a frame shift after amino acid 165. GENEFINDER predicts and cDNAs confirm the existence of an open-reading frame *Y48G1A.2* in the first intron of *mbtr-1* (Figure 2B). *Y48G1A.2* and *mbtr-1* are transcribed from the opposite strands. While the *n4775* deletion begins 1 kb upstream of the Y48G1A.2 translational start site and does not remove coding sequences, it remains possible that *n4775* could remove sequences necessary for proper expression of *Y48G1A.2*.

We have not identified a mutant phenotype associated with *mbtr-1(n4775)*. *mbtr-1(n4775)* does not cause a synMuv phenotype in combination with either *lin-15A(n767)* or *lin-15B(n744)*. Animals mutant for both *mbtr-1* and *lin-61* are not Muv. *mbtr-1* and *lin-61* are not redundantly required for a class A synMuv activity as *mbtr-1(n4775) lin-61(n3809); lin-15B(n744)* animals are not Muv. Additionally, loss of function in *mbtr-1* does not enhance or suppress the synMuv phenotype of

lin-61(n3809); lin-15A(n767) animals. 97% of *lin-61(n3809); lin-15A(n767)* are Muv at 20°C and 24% are Muv at 15°C. Similarly, 94% of *mbtr-1(n4775) lin-61(n3809); lin-15A(n767)* are Muv at 20°C and 27% are Muv at 15°C.

LIN-61 is broadly expressed in nuclei throughout development: To determine the expression pattern and localization of LIN-61, we generated guinea pig and rabbit polyclonal antibodies against the C-terminal 332 amino acids of LIN-61 fused with maltose-binding protein. The affinity-purified antibodies recognize a band corresponding to a protein of approximately 60 kD on western blots of wild-type but not *lin-61(n3809)* protein extracts (Figure 3A). This molecular weight is similar to the predicted size of LIN-61 of 57 kD.

We used both the guinea pig and rabbit polyclonal antibodies to analyze the localization of LIN-61 by immunostaining of embryos, larvae, and adult hermaphrodites. Similar to all synMuv proteins studied to date, LIN-61 is localized to all or almost all nuclei throughout development from the one-cell embryo to the adult (Figure 3B, F). In the embryo, LIN-61 appears to localize to discrete foci in the nucleus (Figure 3B). Both HPL-2 and LIN-13 similarly localize to foci in the nucleus (MELENDEZ and GREENWALD 2000; COUSTHAM *et al.* 2006). In addition, the human MBT-repeat-containing protein l(3)mbt and Polycomb group proteins localize to foci in the nucleus (BUCHENAU *et al.* 1998; SAURIN *et al.* 1998; KOGA *et al.* 1999). In the adult hermaphrodite germline, LIN-61 is localized, at least in part, to condensed chromosomes during the diakinesis phase of meiosis, suggesting that some LIN-61 might be localized to chromatin (Figure 3F). No LIN-61 staining was seen in *lin-61(n3809)* mutant embryos, larvae, or adults (Figure 3C, G).

To better understand how LIN-61 might be acting with other synMuv proteins to regulate vulval development we analyzed the localization of LIN-61 in animals mutant for any of 28 genes that regulate vulval development, including four class A synMuv genes, 19 class B synMuv genes, two class C synMuv genes and genes encoding three Ras-pathway modifiers. No detectable change in LIN-61 localization was noted in any of these mutant backgrounds (data not shown), suggesting that these genes do not regulate vulval development by modifying LIN-61 expression or subcellular localization.

Missense mutations in LIN-61 might disrupt protein stability: The crystal structures for peptides containing two or three MBT repeats have been solved (SATHYAMURTHY *et al.* 2003; WANG *et al.* 2003). Both structures show that individual repeats consist of an N-terminal arm and a C-terminal β-barrel core region. The N-terminal arm of one repeat interacts with the β -barrel core region of the preceding repeat, resulting in the stabilized tertiary structure (SATHYAMURTHY *et al.* 2003; WANG *et al.* 2003).

Given that the crystal structures demonstrate residues within the MBT repeats interact to form a stabilized structure, we analyzed LIN-61 protein levels in strains carrying each of the ten mutant alleles to determine if any of the mutations might result in protein misfolding and subsequent degradation. Full-length LIN-61 was absent or levels were greatly reduced in animals with any of the three nonsense mutations or two splice-acceptor mutations (Figure 4B). Alternatively, a truncated protein product could be expressed that does not contain the epitope recognized by either of the polyclonal antibodies. *lin-61(n3736), lin-61(n3807),* and *lin-61(n3922)* animals also showed decreases in LIN-61 protein levels as compared to the wild type, while *lin-61(n3447)* and *lin-61(n3624)* had wild-type or nearly wild-type protein levels. Protein levels in animals with any of the five missense mutations were analyzed by both western blots and by immunocytochemistry (Figure 4B and data not shown). These data suggest that the residues mutated in *n3736, n3807* and *n3922* might be necessary for proper protein folding and stability. Indeed, the mutations in both *n3736* and *n3807* are in residues of the β-barrel core region likely to be important for interaction with the N-terminal arm of the preceding repeat based on the crystal structures (SATHYAMURTHY *et al.* 2003; WANG *et al.* 2003).

The missense mutations in *n3447* and *n3624* mutant animals interrupt the ability of LIN-61 to properly regulate vulval development despite having wild-type or nearly wild-type protein levels. These residues are not required for protein stability but are important for LIN-61 function. While the missense mutation in *n3624* is located between two MBT repeats, the missense mutation in *n3447* is in the third MBT repeat and changes a serine residue to an asparigine residue. Thus it is likely that at least the third

MBT repeat is important for the function of LIN-61 in regulating vulval development and that, more specifically, the serine at residue 354 is important for function.

Analysis of pleiotropies associated loss of function of *lin-61* or *mbtr-1*: The class B synMuv genes have roles in many processes apart from the regulation of vulval development, including the regulation of transgene expression, RNAi sensitivity, cell-cycle control and expression of PGL-1 and LAG-2 (HSIEH *et al.* 1999; BOXEM and VAN DEN HEUVEL 2002; DUFOURCQ *et al.* 2002; UNHAVAITHAYA *et al.* 2002; POULIN *et al.* 2005; WANG *et al.* 2005). While class B synMuv genes act similarly in vulval development, these genes do not all function similarly in the aforementioned processes. A more complete analysis of these pleiotropies should help to elucidate which of the more than 19 identified class B synMuv genes act in similar processes and which have distinct roles in development.

To better understand the role of *lin-61* and *mbtr-1* in development and to compare these genes to the known class B synMuv genes, we investigated whether loss-of-function mutations in either gene or in both genes result in specific synMuv-regulated pleiotropies (Table 3). We found in multiple experiments that *lin-61(n3809)* did not show enhanced sensitivity to either *hmr-1* or *cel-1* RNAi as compared to wild type (data not shown). In the same experiments, *lin-15B(n744), rrf-3(pk1426)* and *eri-1(mg366)* were RNAi hypersensitive, as has previously been reported (SIMMER *et al.* 2002; KENNEDY *et al.* 2004; WANG *et al.* 2005).

Using antibody staining, we did not observe any PGL-1 misexpression in *lin-61(n3809)* animals. PGL-1 is expressed specifically in the germline of wild-type animals (KAWASAKI *et al.* 1998) and is misexpressed in the soma of animals with loss-of-function mutations in a number of class B synMuv genes, including *lin-9, lin-13, lin-15B, lin-35, hpl-2,* and *dpl-1* (UNHAVAITHAYA *et al.* 2002; WANG *et al.* 2005; CUI *et al.* 2006b). Staining of *lin-15B(n744)* animals with the same antibody reliably showed misexpresion of PGL-1 in the soma.

In addition to repression of PGL-1 expression, class B synMuv genes also have a role in *lag-2* repression. A *lag-2::gfp* reporter that is expressed in the distal tip cells and vulva of wild-type hermaphrodites was misexpressed in the gut and epithelia of *hda-1*

mutant animals (DUFOURCQ *et al.* 2002). RNAi against many other synMuv genes also caused *lag-2::gfp* misexpression (POULIN *et al.* 2005). In *lin-61(n3809)* animals, however, *lag-2::gfp* was not expressed in the gut or epithelia.

However, in *lin-61(n3809)* animals carrying the *lag-2::gfp* reporter we observed strong pharyngeal GFP expression that is not seen in wild-type animals. This "green pharynx" phenotype has been previously observed for a CEM-specific GFP reporter (H. Schwartz and H.R.H, unpublished results). The transgene misexpression was seen in animals mutant for two additional class B synMuv genes, *hpl-2* and *lin-13,* and one class A synMuv gene, *lin-8*, but not other synMuv genes (H. Schwartz and H.R.H). These data suggest that while *lin-61* might not act to repress transgene expression in the gut and epithelia like many other class B synMuv genes, it has a role in repression of some transgenes in the pharynx.

By contrast to the transgene misexpression noted for the *lag-2::gfp* transgene, loss of function of most class B synMuv genes results in the silencing of a simple array of the *myo-3::gfp* transgene (HSIEH *et al.* 1999), termed the transgene array modifier (Tam) phenotype. Neither *lin-61(n3809)* nor *lin-61(n3922)* resulted in silencing of an extrachromasomal array carrying *myo-3::gfp (ccEx6188).*

lin-61 was identified in a genome-wide RNAi screen for genes that were involved in protecting the genome from DNA instability (POTHOF *et al.* 2003). Using an out-of-frame *LacZ* reporter that is not expressed in wild-type animals, Pothof *et al.* (2003) demonstrated that RNAi directed against *lin-61* and 60 other genes could cause expression of the transgene, suggesting that loss-of-function of these genes can result in insertion or deletion mutations that result in an in-frame *LacZ*. Besides *lin-61*, the only other class B gene that was identified in this screen was *hda-1*. We used the *LacZ* reporter used in the initial screen to test if a loss-of-function allele in *lin-61* could phenocopy the effects of RNAi. *LacZ* staining was evident in a significant proportion of *lin-61(n3809)* animals. In the same experiment, wild-type animals showed no *LacZ* staining. We further tested animals with mutations in the class B synMuv genes *hpl-2* and *lin-13* and showed that in these animals LacZ was expressed. Therefore these genes might function with *lin-61* in maintaining genome stability.

The vulval defect caused by the loss-of-function mutation in *ku233*, an allele of the gene *mat-3* that encodes a member of the anaphase promoting complex (APC), can be suppressed by loss-of-function in the class B synMuv genes *lin-35*, *lin-15B*, *lin-53*, *dpl-1,* and *efl-1* (GARBE *et al.* 2004). No coding mutations were found in the *ku233* allele. The vulval defect is likely caused by two adjacent base-pair changes 400 base pairs upstream of the *mat-3* start site, which results in a 5-10 fold reduction in *mat-3* RNA levels (GARBE *et al.* 2004). Loss of function of *lin-35* restored expression of *mat-3* to wild-type levels, suggesting that LIN-35 might repress transcription of *mat-3* (GARBE *et al.* 2004). *lin-61(n3809)* could, like loss-of-function mutations in other class B synMuv genes, suppress the *mat-3(ku233)* vulval defect. 58% of *mat-3(ku233)* animals had abnormal vulvas, as compared to only 3.5% of *lin-61(n3809); mat-3(ku233)* animals. Thus, LIN-61 might act to repress transcription of *mat-3*.

Loss of function of *mbtr-1* alone did not cause any of the pleiotropies discussed above. In addition, *mbtr-1(n4775)* did not modify the abnormalities associated with loss of function in *lin-61* nor is *mbtr-1* redundantly required to cause any of the above mentioned abnormalities. Thus, the inability to detect a role for *lin-61* in a number of synMuv-regulated pleiotropies is not the result of compensation by another MBT-repeat-containing protein.

LIN-61 is not a core member of the DRM or NuRD-like complexes: We have recently identified two independent complexes that are composed of class B synMuv proteins, the DRM complex, containing eight class B synMuv proteins including LIN-35 Rb and DPL-1 DP, and a NuRD-like complex, containing at least LET-418 Mi2, HDA-1 HDAC1, and LIN-53 RbAp48 (M.M.H and H.R.H. unpublished results). We used coimmunoprecipitation experiments to test whether LIN-61 associates with members of either of these two complexes. We demonstrated that although LIN-61 could be immunoprecipitated with anti-LIN-61 antibodies from wild-type but not *lin-61(n3809)* embryonic extracts, none of the DRM complex members coimmunoprecipitated with LIN-61 (Figure 5A). Reciprocally, LIN-61 failed to coimmunoprecipitate with the DRM complex members LIN-37 and LIN-9 (Figure 5B). These data demonstrate that LIN-61 is not a core component of the DRM complex, although it could be weakly associated with

the complex or associate only at certain stages of development or in specific cell types. Additionally, HDA-1, a component of the NuRD-like complex, failed to coimmunoprecipitate with LIN-61 (Figure 5A), suggesting that LIN-61 also is not a member of the NuRD-like complex. Two class A synMuv proteins, LIN-8 and LIN-56, and the class B synMuv protein HPL-2 also do not coimmunoprecipitate with LIN-61 (Figure 5A).

While our data demonstrate that LIN-61 is not a core component of the DRM complex, LIN-61 could act to modify the activity of this complex. To partially test this hypothesis, we used coimmunoprecipitation experiments to determine whether the DRM complex is properly formed in a *lin-61(n3809)* mutant animal. All seven members of the DRM complex coimmunoprecipitated with LIN-37 in extracts from *lin-61(n3809)* mutant embryos, suggesting that LIN-61 function is not required for proper complex formation. However, we cannot rule out that LIN-61 might be acting to control the activity of the DRM complex through a mechanism distinct from altering complex formation.

DISCUSSION

Genetic screens have identified at least 31 synMuv genes, 19 of which have been categorized as class B synMuv genes. Genetic and biochemical studies suggest that these class B synMuv genes are not likely to regulate vulval cell-fate specification through the same function. For example, loss-of-function mutations in the class B synMuv genes *hda-1, let-418,* and *lin-13* cause a strong synMuv phenotype in combination with loss of function in a class A synMuv gene but also cause a weak Muv phenotype as single mutants (MELENDEZ and GREENWALD 2000; VON ZELEWSKY *et al.* 2000; DUFOURCQ *et al.* 2002). By contrast, loss of function of other class B synMuv genes does not cause a Muv phenotype in the absence of a mutation in a class A or C synMuv gene. Furthermore, we have shown that some of these proteins likely function together in a NuRD-like complex that is biochemically distinct from the DRM complex, which contains at least eight other class B synMuv proteins (M.M.H. and H.R.H unpublished results).

We demonstrate that only one of the two MBT-repeat-containing proteins in *C. elegans*, LIN-61, acts with other class B synMuv proteins to regulate vulval development. We further show that the function of LIN-61 in other aspects of development is likely to be distinct from a number of other class B synMuv proteins, suggesting it can function independently from these proteins.

MBT-repeat-containing proteins are not required for *C. elegans* viability: *C. elegans* contains only two MBT-repeat-containing proteins, MBTR-1 and LIN-61. Both of these proteins are composed almost exclusively of MBT repeats and lack either atypical zinc fingers or the SPM domain that are found in many MBT-repeat-containing proteins in other organisms, including Sex Comb on Midleg and lethal(3) malignant brain tumor. These proteins are required for the viability of *Drosophila* (WISMAR *et al.* 1995; BORNEMANN *et al.* 1996).

By contast, the *C. elegans* MBT-repeat-containing proteins are not required for viability. In fact, mutant animals that lack both MBTR-1 and LIN-61 appear superficially wild-type. We have shown that while LIN-61 is required to regulate vulval development, MBTR-1 does not have a similar function. We further demonstrated that in a number of

other processes there is no detectable redundancy between MBTR-1 and LIN-61. This suggests that although these two proteins share similar domain structures they do not function redundantly. It remains possible that *mbtr-1* could complement for the loss of *lin-61* function, but it is not expressed in the necessary tissues or at the correct time.

LIN-61 is likely involved in transcriptional repression: Studies of MBT-repeat-containing proteins in other organisms suggest that MBT repeats function in transcriptional repression. Notably, MBT repeats are found in the *Drosophila* Polycomb group protein Sex Comb on Midleg (SCM), which is a substoichiometric component of the Polycomb Repression Complex 1 (PRC1). PRC1 maintains transcriptional repression of genes by binding to methylated histones (CAO *et al.* 2002; CZERMIN *et al.* 2002; MULLER *et al.* 2002). Genetic analysis of SCM suggests that the MBT domains are likely to be important for protein function (BORNEMANN *et al.* 1996; BORNEMANN *et al.* 1998). Human l(3)mbt can repress transcription when artificially recruited to promoters, and this transcriptional activity requires the MBT repeats, but not the zinc fingers or the SAM domain (BOCCUNI *et al.* 2003). In addition, *Drosophila* l(3)mbt is required for transcriptional repression of a number of endogenous genes (LEWIS *et al.* 2004). Sequence and structural analysis demonstrates that MBT repeats are similar to Tudor, PWWP, and chromo domains, suggesting that like these domains, MBT repeats might also bind to modified histones (MAURER-STROH *et al.* 2003; SATHYAMURTHY *et al.* 2003; WANG *et al.* 2003). More recently, the MBT repeats of human l(3)mbt and CGI-72 have been shown to bind histones methylated on specific residues (KIM *et al.* 2006). Together these data suggest that MBT repeats in other proteins might bind to modified histones and repress transcription. The localization of LIN-61 to condensed chromosomes, that it functions with other transcription factors in vulval development and that it is composed almost exclusively of MBT repeats suggest that LIN-61 functions in transcriptional repression via the direct interaction with histones.

***lin-61* functions separately from the DRM and NuRD-like complexes:** In *Drosophila*, the MBT-repeat-containing protein l(3)mbt associates at substoichiometric levels with the Myb-MuvB complex, which includes both fly pocket-proteins RBF1 and RBF2 as well as dE2F2 and dDP (LEWIS *et al.* 2004). The Myb-MuvB complex

represses transcription of many E2F-responsive genes. However, l(3)mbt is required to mediate transcriptional repression of only a subset of these targets (LEWIS *et al.* 2004). Thus l(3)mbt might only function with the Myb-MuvB complex at specific promoters.

The Myb-MuvB complex is very similar to the DRM complex we identified in *C. elegans* (M.M.H. and H.R.H. unpublished results). Our coimmunoprecipitation data demonstrate that LIN-61 is not a core componenent of either the DRM or NuRD-like complexes. Since the immunoprecipitations were from embryonic protein extracts we cannot exclude the possibility that LIN-61 associates with these complexes at different stages in development or in specific cell types. It remains possible that, like l(3)mbt, LIN-61 functions with the DRM complex to control specific processes, like vulval development. However, while both l(3)mbt and LIN-61 contain MBT repeats, LIN-61 does not contain the atypical zinc fingers or the SAM domain found in l(3)mbt. The SAM domain is important for protein-protein interaction and might help to mediate the interaction of l(3)mbt with components of the Myb-MuvB complex.

Our analysis of the pleiotropies associated with loss-of-function mutations in *lin-61* further suggests that in some biological processes *lin-61* functions separately from other class B synMuv genes, including components of the DRM complex. Most notably, loss-of-function mutations in *lin-61* cause genomic instability, result in ectopic pharyngeal expression of a *lag-2::gfp* reporter and suppresses the vulval defect of *mat-3(ku233)* mutant animals. Putative null alleles of *lin-35* and *lin-15B* share with *lin-61* mutants only the ability to suppress *mat-3(ku233).* However, *lin-35(n745)* and *lin-15B(n744)* both share a number of other pleiotropies, including RNAi hypersensitivity, PGL-1 somatic misexpression, the Tam phenotype, and ectopic *lag-2::gfp* expression in the gut and epithelia (Table 3). These data suggest that in many biological functions, *lin-61* is not functioning with *lin-35* or *lin-15B*, although *lin-35* and *lin-15B* might act together for these functions. It is interesting to note that loss of function of all three genes both can suppress the defects in *mat-3(ku233)* mutant animals and can act redundantly with the class A synMuv genes to regulate vulval development. Perhaps, as has been proposed for LIN-35, LIN-61 is also acting to repress transcription of *mat-3*.

We have shown that while *lin-61* shares with other class B genes the ability to regulate vulval development redundantly with the class A synMuv genes, it functions separately from class B genes in many other biological processes. LIN-61 is also distinguished from other class B synMuv proteins by coimmunoprecipitation experiments, which show that LIN-61 is not a core component of the DRM complex. While it is unclear whether LIN-61 functions alone or in a complex with other synMuv proteins, comprehensive biochemical and genetic analyses have the potential to define its functional partners. Future studies should help our understanding of not only how LIN-61 functions but also how other synMuv proteins act to regulate vulval development and more broadly, how transcriptional regulators, like Polycomb group proteins and pocket proteins, might act separately in some biological processes and cooperate in others.

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TABLE 1

***lin-61* mutations cause a class B synMuv phenotype**

	Genotype	Percent Muv (n)
Single mutants	*lin-61(sy223)*	0 (260)
	lin-61(n3442)	0 (327)
	lin-61(n3446)	0 (217)
	lin-61(n3447)	0 (334)
	lin-61(n3624)	0 (242)
	lin-61(n3687)	0 (252)
	lin-61(n3736)	0 (234)
	lin-61(n3807)	0 (278)
	lin-61(n3809)	0 (269)
	lin-61(n3922)	0 (82)
	hda-1(e1795)	31 (143)
	hpl-2(n4274)	0 (>50)
	trr-1(n3712)	18[a] (39)
	mys-1(n3681)[b]	8[a] (36)
Class A synMuv double mutants	*lin-61(n3809); lin-8(n2731)*	72 (414)
	lin-61(n3809); lin-38(n751)	93 (175)
	lin-61(n3809); lin-56(n2728)	100 (180)
	lin-61(n3809); lin-15A(n433)	14 (261)
	lin-61(n3809); lin-15A(n767)	97 (166)
Class B synMuv double mutants	*lin-61(n3809); lin-37(n758)*	0 (165)
	lin-61(n3809); lin-15B(n744)	0 (153)
	lin-61(n3809); hda-1(e1795)	20 (96)
	lin-61(n3809); hpl-2(n4274)	3 (172)
Class C synMuv double mutants	*lin-61(n3809); trr-1(n3712)*	17[a] (111)
	lin-61(n3809); mys-1(n3681)	7[a] (45)

All animals were raised at 20°C. The Muv phenotype was scored using a dissecting microscope except in the cases noted. *trr-1* mutant homozygotes were recognized as the non-GFP progeny of *ttr-1/mIn1[dpy-10 mIs14]* heterozygous parents. *hda-1(e1795)* homozygotes were recognixed as the non-GFP progeny of *hda-1/nT1[qIs51]; +/nT1[qIs51]* heterozygous parents. *lin-61(n3687)* and *lin-61(n3922)* were also homozygous for the linked integrated transgene *nIs133* that includes *pkd-2:gfp* and a rescuing *lin-15AB* construct.

[a] Muv, greater than three Pn.p cells induced.

[b] These data are from Ceol and Horvitz (2004).

TABLE 2
Sequences of *lin-61* alleles and allele strengths

lin-61 allele	Wild-type sequence	Mutant sequence	Subsititution	Percent Muv (n) with *lin-56(n2728)*		Percent Muv (n) with *lin-15A(n433)*	
+				0	(many)	0	(many)
lin-61(n3442)	ag**A**AT	aa**A**AT	exon 4 splice acceptor	98	(154)	14	(220)
lin-61(n3446)	**C**AA	**T**AA	Q412ochre	96	(87)	13	(241)
lin-61(n3809)	**C**AA	**T**AA	Q159ochre	92	(176)	14	(261)
lin-61(sy223)	ag**C**TC	aa**C**TC	exon 6 splice acceptor	89	(255)	11	(129)
lin-61(n3624)	**C**CG	**T**CG	P132S	85	(220)	5.6	(251)
lin-61(n3807)	G**G**A	G**A**A	G250E	83	(136)	6.9	(246)
lin-61(n3736)	T**T**T	T**C**T	F247S	80	(313)	1.3	(232)
lin-61(n3922)	G**G**A	G**A**A	G445R	52	(167)	ND[a]	ND[a]
lin-61(n3447)	A**G**T	A**A**T	S354N	47	(237)	1.1	(278)
lin-61(n3687)	**C**AA	**T**AA	Q322ochre	23	(305)	ND[a]	ND[a]

Amino acid substitutions are indicated as wild-type residue, residue number, and mutant residue. Coding residues are shown as capital letters. Non-coding residues are show as lowercase letters.
All animals were raised at 20°C. The Muv phenotype was scored using a dissecting microscope.
[a]*lin-61(n3687)* and *lin-61(n3922)* were also homozygous for the linked-integrated transgene *nIs133*, which includes *pkd-2::gfp* and a rescuing *lin-15AB* construct.

TABLE 3
Phenotypic characterization of *lin-61(n3809)* and *mbtr-1(n4775)*

Phenotype	*lin-61(n3809)*	*mbtr-1(n4775)*	*mbtr-1(n4775) lin-61(n3809)*	*lin-35(n745)*	*lin-15B(n744)*
class A synMuv	No	No	No	No	No
class B synMuv	Yes	No	Yes[a]	Yes[b]	Yes[c]
RNAi hypersensitive	No	No	No	Yes[d]	Yes[d]
ectopic PGL-1 staining	No	No	No	Yes[d]	Yes[d]
ectopic *lag-2::gfp* expression[e]	No	No	No	Yes	Yes
Mutator	Yes	No	Yes	ND	ND
Tam	No	No	No	Yes[f]	Yes[f]
suppression of *mat-3(ku233)*	Yes	No	Yes	Yes[g]	Yes[g]
Green Pharynx	Yes	No	Yes	No[h]	No[h]

For details on how each phenotype was scored see Materials and Methods.
[a] *mbtr-1(n4775)* does not enhance or suppress the synMuv phenotype of *lin-61(n3809); lin-15A(n767).*
[b] Lu and Horvitz 1998.
[c] Clark et al. 1994. Huang et al. 1994.
[d] Wang et al. 2005.
[e] Ectopic expression refers to misexpression of GFP in the gut or epithelia.
[f] E. Andersen and H.R.H unpublished results.
[g] Garbe 2004.
[h] H. Schwartz and H.R.H. unpublished results.

Figure 1. Molecular cloning of *lin-61*

A) *lin-61* maps between *unc-14* and *unc-15* on LGI. The cosmid R06C7 is shown below as a grey bar. The rescuing StuI-SacII fragment of R06C7 is shown below the cosmid. Open boxes represent the exons of the predicted genes within the subclone. Arrows indicate the direction of transcription.

B) *lin-61* gene structure as determined from cDNA and genomic sequences. Shaded boxes indicate coding sequence. Open boxes indicate the 3' untranslated region. Predicted translation intiation and termination codons are shown along with the poly(A) tail and the SL1 leader sequence. The positions of the mutations found in the ten *lin-61* alleles are indicated above the gene structure.

C) Schematic depiction of the LIN-61 protein. Shaded boxes indicate the positions and relative sizes of the four MBT repeats.

Figure 1

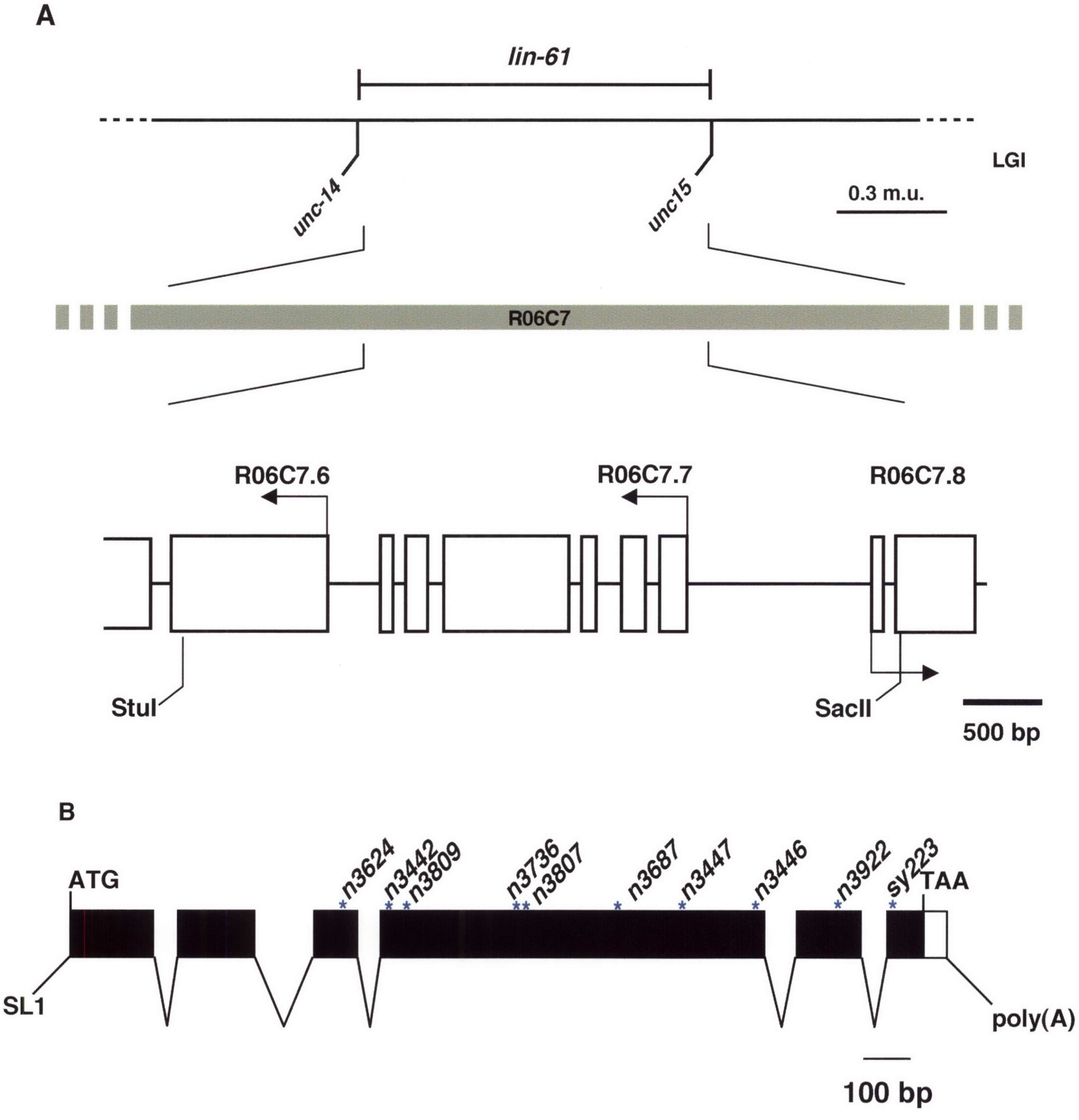

A
lin-61
unc-14
unc15
LGI
0.3 m.u.
R06C7
R06C7.6
R06C7.7
R06C7.8
StuI
SacII
500 bp
B
ATG
n3624
n3442
n3809
n3736
n3807
n3687
n3447
n3446
n3922
sy223
TAA
SL1
poly(A)
100 bp
C
MBT
MBT
MBT
MBT

Figure 2. Identifcation of *mbtr-1*

A) Alignment of MBTR-1 and LIN-61. Solid boxes indicate identities between LIN-61 and MBTR-1. Shaded boxes indicate similarities between the two proteins. Underlined regions correspond to the four MBT repeats. Red box indicates the 15 amino acids inserted into the second repeats of both LIN-61 and MBTR-1.

B) *mbtr-1* gene structure as determined from cDNA and genomic sequences. Shaded boxes indicate coding sequence. Open boxes indicate 5' and 3'untranslated regions. Predicted tranlation initiation and termination codons are shown along with the poly(A) tail. The position of the predicted gene *Y48G1A.2* that is located within the first intron of *mbtr-1* is shown. The arrow depicts the direction of transcription. The genomic region deleted in *n4775* is indicated by brackets.

C) Schematic depiction of the MBTR-1 protein. Shaded boxes indicate the positions and relative sizes of the four MBT repeats.

Figure 2

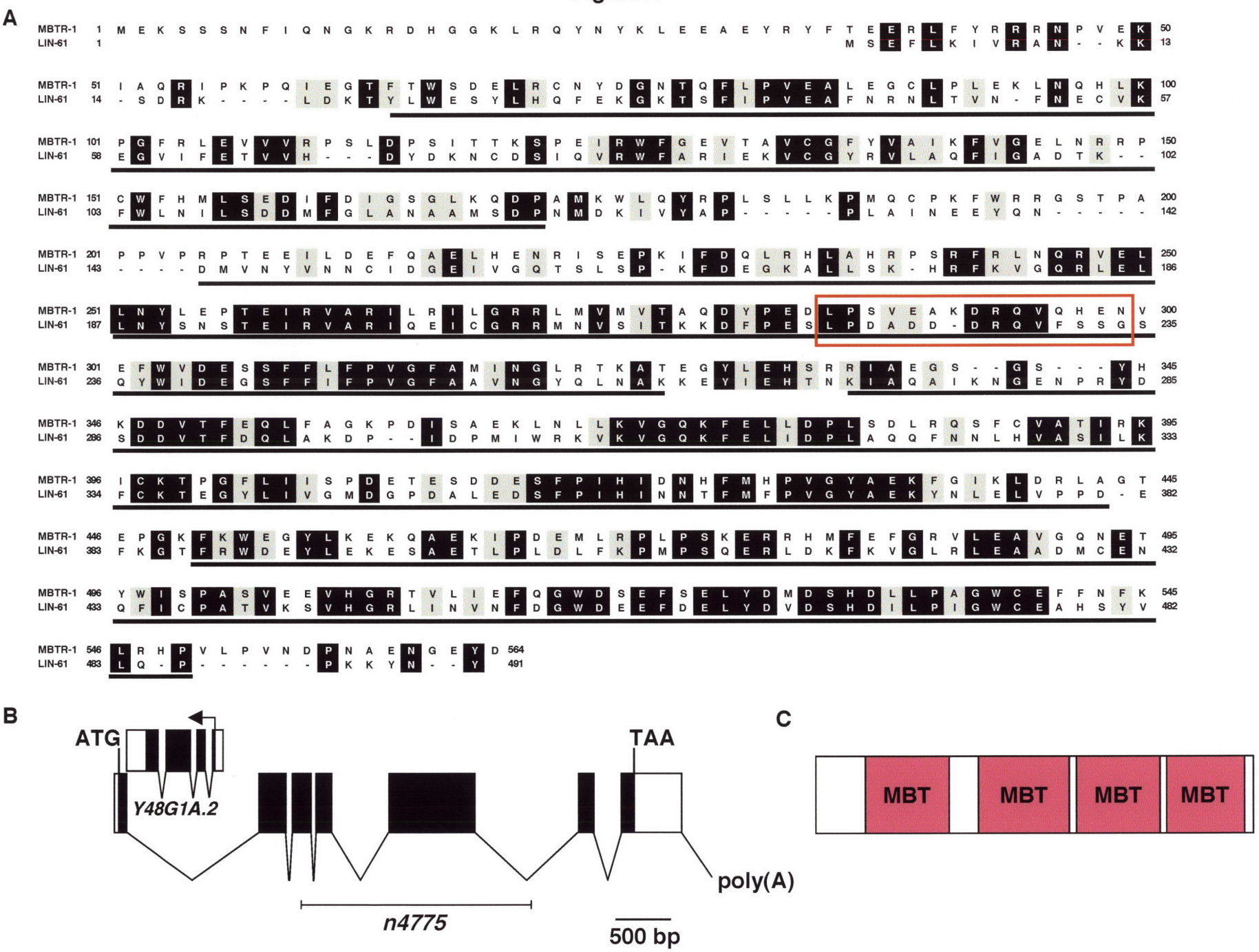

A
MBTR-1 1 M E K S S S N F I Q N G K R D H G G K L R Q Y N Y K L E E A E Y R Y F T E E R L F Y R R R N P V E K 50
LIN-61 1 M S E F L K I V R A N - - K K 13
MBTR-1 51 I A Q R I P K P Q I E G T F T W S D E L R C N Y D G N T Q F L P V E A L E G C L P L E K L N Q H L K 100
LIN-61 14 - S D R K - - - - L D K T Y L W E S Y L H Q F E K G K T S F I P V E A F N R N L T V N - F N E C V K 57
MBTR-1 101 P G F R L E V V V R P S L D P S I T T K S P E I R W F G E V T A V C G F Y V A I K F V G E L N R R P 150
LIN-61 58 E G V I F E T V V H - - - D Y D K N C D S I Q V R W F A R I E K V C G Y R V L A Q F I G A D T K - - 102
MBTR-1 151 C W F H M L S E D I F D I G S G L K Q D P A M K W L Q Y R P L S L L K P M Q C P K F W R R G S T P A 200
LIN-61 103 F W L N I L S D D M F G L A N A A M S D P N M D K I V Y A P - - - - - P L A I N E E Y Q N - - - - - 142
MBTR-1 201 P P V P R P T E E I L D E F Q A E L H E N R I S E P K I F D Q L R H L A H R P S R F R L N Q R V E L 250
LIN-61 143 - - - - D M V N Y V N N C I D G E I V G Q T S L S P - K F D E G K A L L S K - H R F K V G Q R L E L 186
MBTR-1 251 L N Y L E P T E I R V A R I L R I L G R R L M V M V T A Q D Y P E D L P S V E A K D R Q V Q H E N V 300
LIN-61 187 L N Y S N S T E I R V A R I Q E I C G R R M N V S I T K K D F P E S L P D A D D - D R Q V F S S G S 235
MBTR-1 301 E F W V D E S S F F L F P V G F A M I N G L R T K A T E G Y L E H S R R I A E G S - - G S - - - Y H 345
LIN-61 236 Q Y W I D E G S F F I F P V G F A A V N G Y Q L N A K K E Y I E H T N K I A Q A I K N G E N P R Y D 285
MBTR-1 346 K D D V T F E Q L F A G K P D I S A E K L N L L K V G Q K F E L L D P L S D L R Q S F C V A T I R K 395
LIN-61 286 S D D V T F D Q L A K D P - - I D P M I W R K V K V G Q K F E L I D P L A Q Q F N N L H V A S I L K 333
MBTR-1 396 I C K T P G F L I I S P D E T E S D D E S F P I H I D N H F M H P V G Y A E K F G I K L D R L A G T 445
LIN-61 334 F C K T E G Y L I V G M D G P D A L E D S F P I H I N N T F M F P V G Y A E K Y N L E L V P P D - E 382
MBTR-1 446 E P G K F K W E G Y L K E K Q A E K I P D E M L R P L P S K E R R H M F E F G R V L E A V G Q N E T 495
LIN-61 383 F K G T F R W D E Y L E K E S A E T L P L D L F K P M P S Q E R L D K F K V G L R L E A A D M C E N 432
MBTR-1 496 Y W I S P A S V E E V H G R T V L I E F Q G W D S E F S E L Y D M D S H D L L P A G W C E F F N F K 545
LIN-61 433 Q F I C P A T V K S V H G R L I N V N F D D G W D E E F D E L Y D V D S H D I L P I G W C E A H S Y V 482
MBTR-1 546 L R H P V L P V N D P N A E N G E Y D 564
LIN-61 483 L Q - P - - - - - - - P K K Y N - - Y 491
B
ATG
TAA
Y48G1A.2
poly(A)
n4775
500 bp
C
MBT
MBT
MBT
MBT

Figure 3. Expression of LIN-61

A) Affinity-purified antibodies raised against recombinant LIN-61 were used to blot extracts from both wild-type and *lin-61(n3809)* mutant animals. The asterisks denotes non-specific immunoreactivity. HM4077 antibodies were raised in a guinea pig. HM4078 antibodies were raised in a rabbit.

B, D, F, H) Whole-mount staining with anti-LIN-61 antisera.

B) LIN-61 is expressed in discrete foci in the nuclei of the developing embryo.

C) 4,6-Diamidino-2-phenylindole (DAPI) staining of the embryo shown in (B).

D) LIN-61 staining is absent from *lin-61(n3809)* embryos.

E) DAPI staining of the embryo shown in (D).

F) LIN-61 is broadly expressed in the adult hermphrodite germline and is localized to condensed chromosomes.

G) DAPI staining of the germline shown in (F)

H) LIN-61 staining is absent from the germline of *lin-61(n3809)* adult hermaphrodites.

I) DAPI staining of the germline shown in (H).

WT, wild type. Scale bars, 10 μm.

Figure 3

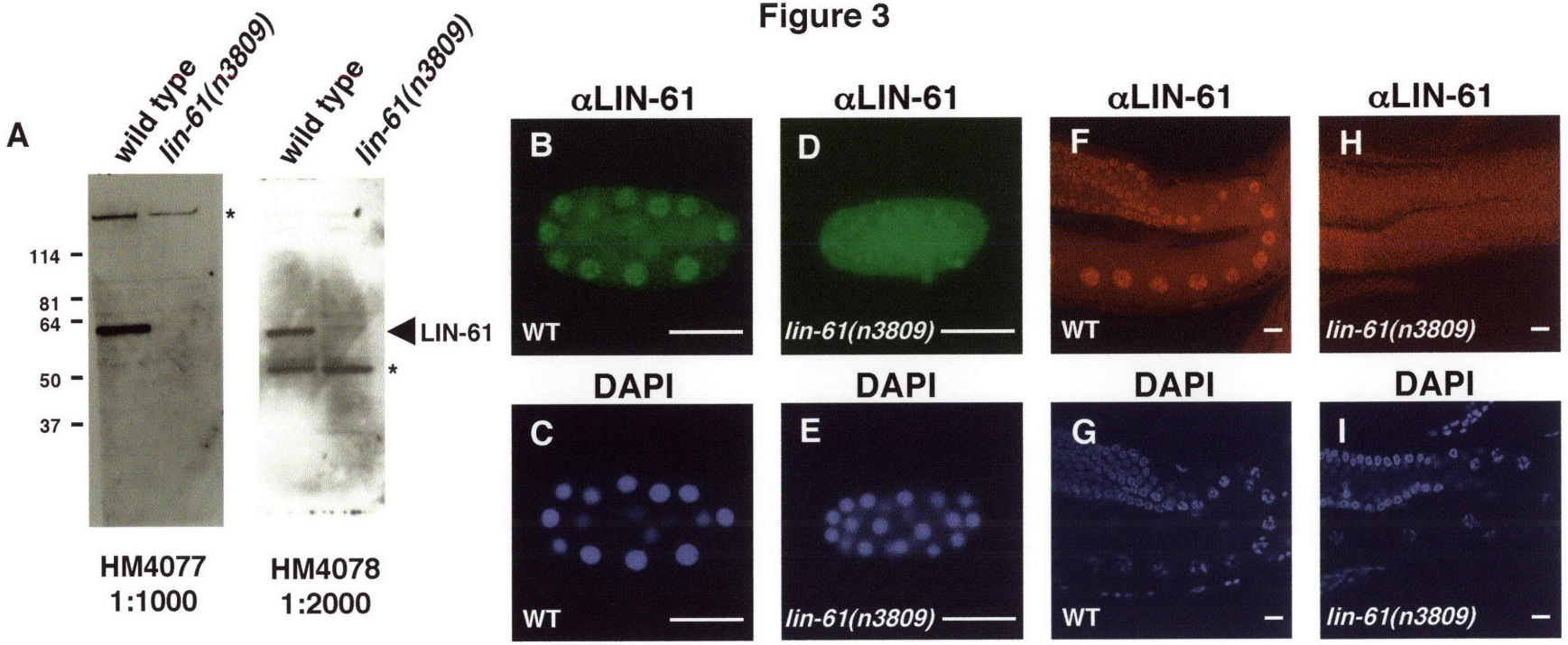

A
wild type
lin-61(n3809)
wild type
lin-61(n3809)
114
81
64
50
37
*
LIN-61
*
HM4077
1:1000
HM4078
1:2000
αLIN-61
αLIN-61
αLIN-61
αLIN-61
B
D
F
H
WT
lin-61(n3809)
WT
lin-61(n3809)
DAPI
DAPI
DAPI
DAPI
C
E
G
I
WT
lin-61(n3809)
WT
lin-61(n3809)

Figure 4. LIN-61 levels are reduced in many *lin-61* mutant animals

A) Alignment of the MBT repeats from *Caenorhabditis elegans* proteins LIN-61 and MBTR-1 and *Homo sapiens* proteins L(3)MBT2 and L(3)MBT1. The top portion corresponds to the N-terminal arm and the bottom portion corresponds to the b-core region. Shaded residues indicate identities between more than eight MBT repeats. Circled residues indicate postions of missense mutations in *lin-61.* The corresponding allele is indicated above the residue. The missense mutation in *n3624* is located between the first and second repeats. Boxed region indicates the 15 amino acids inserted in the second repeats of MBTR-1 and LIN-61.

B) Equivalent amounts of protein from each of the genotypes indicated above were loaded per lane. Proteins were separated by SDS-PAGE and immunoblotted with the antibodies indicated to the left. Anti-tubulin antibodies were used to asses protein loading and transfer. The asterisk denotes non-specific immunoreactivity.

Figure 4

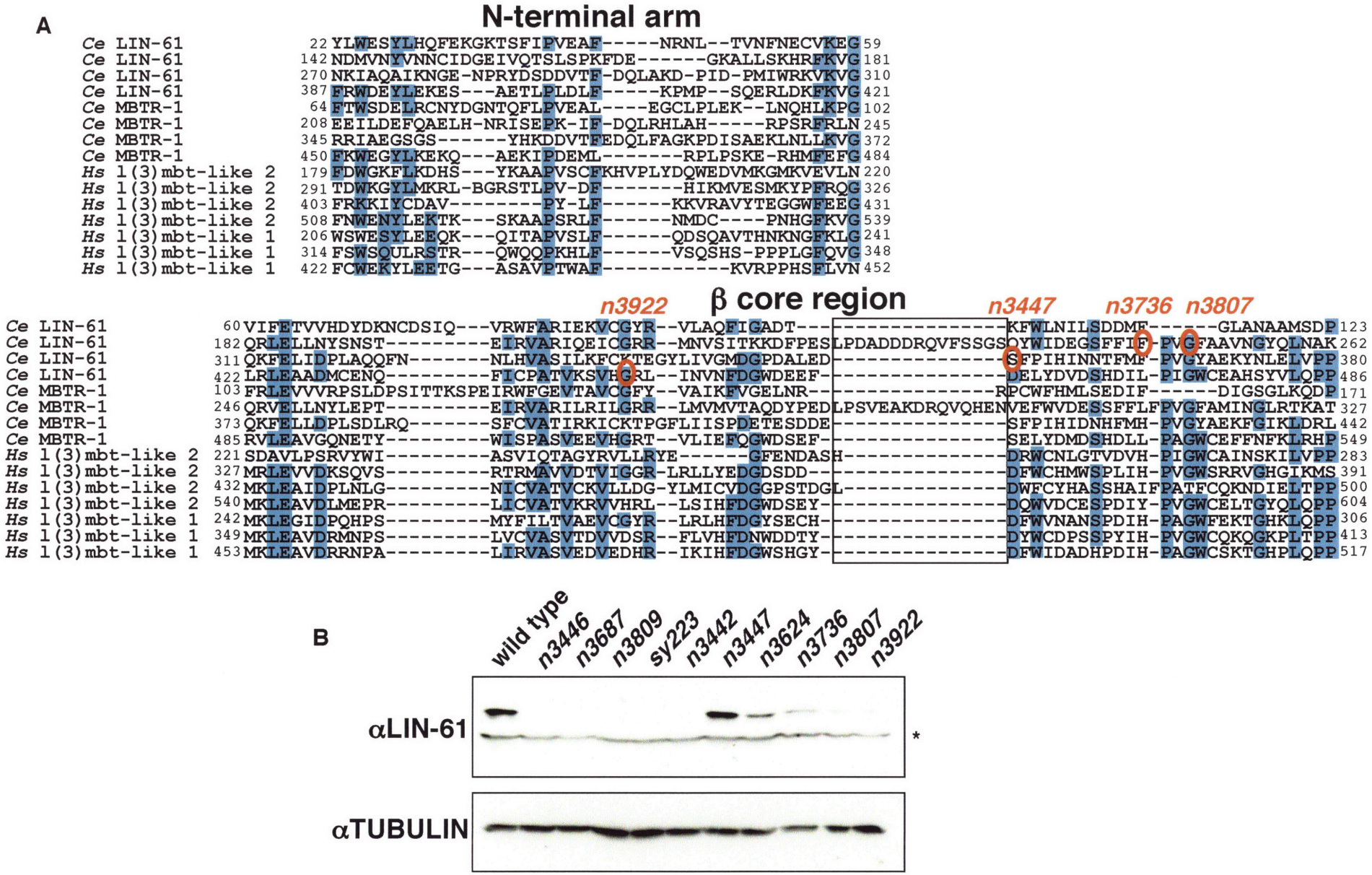

A
N-terminal arm
β core region
n3922
n3447
n3736
n3807
Ce LIN-61
Ce MBTR-1
Hs l(3)mbt-like 2
Hs l(3)mbt-like 1
B
wild type
n3446
n3687
n3809
sy223
n3442
n3447
n3624
n3736
n3807
n3922
αLIN-61
αTUBULIN
*

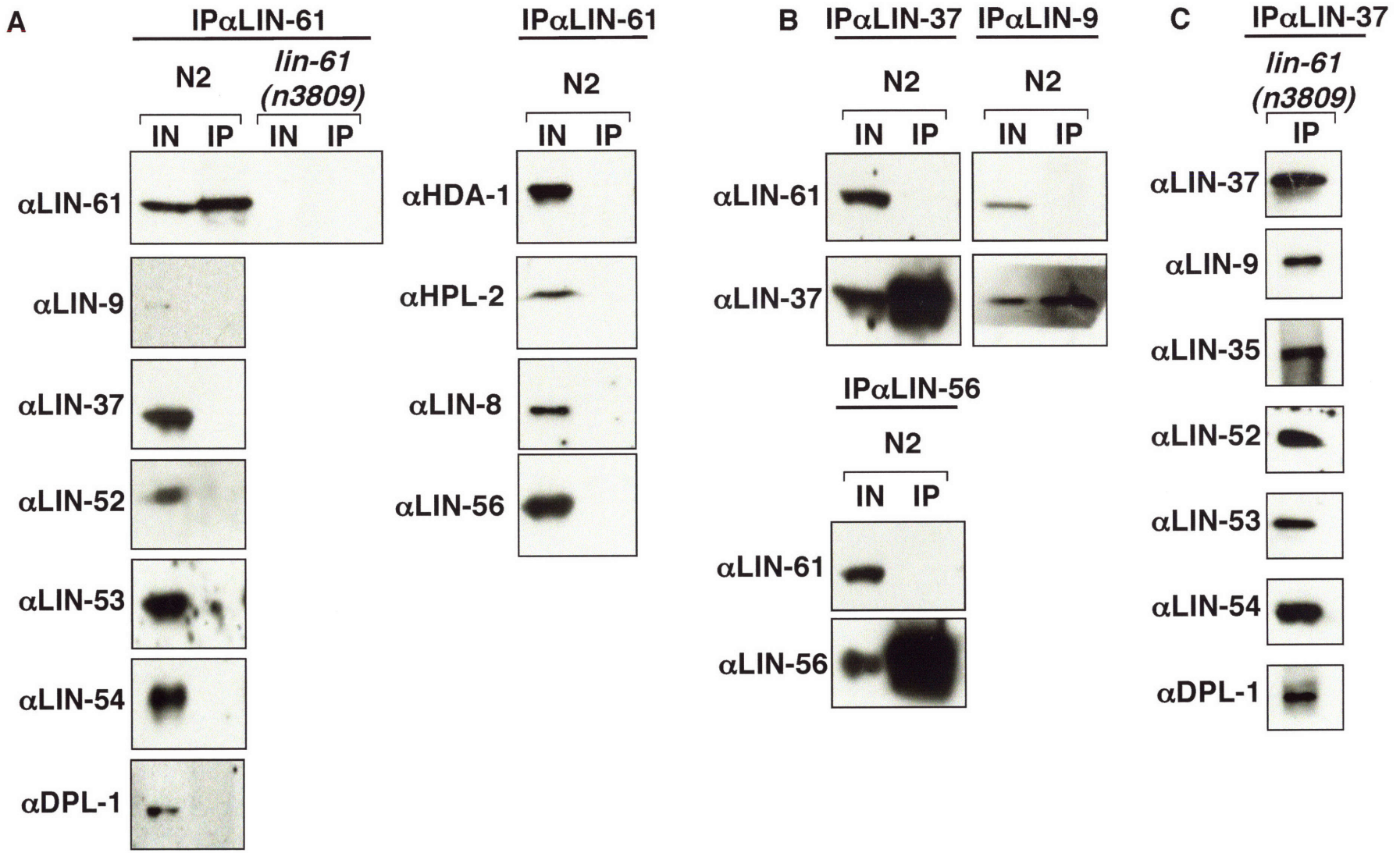
Figure 5. LIN-61 is not a core component of the DRM complex

A) Extract from either wild-type or *lin-61(n3809)* mutant embryos (as indicated above the lanes) were precipitated with antibodies against LIN-61. Immunoprecipitations were analyzed by immunoblots with antibodies specific to the antigen indicated to the left. IN, 2% of the input. IP, 100% of the immunoprecipitate.

B) Extract from wild-type embryos was precipitated with antibodies indicated above the lanes and immunoblotted with antibodies specific to the antigens indicated to the left. IN, 2% of the input. IP, 100% of the immunoprecipitate.

C) Extract from *lin-61(n3809)* mutant embryos were precipitated with antibodies against LIN-37 and immunoblotted with antibodies specific to the antigen indicated to the left. IP, 100% of the immunoprecipitate.

Figure 5


A
IPαLIN-61
N2
lin-61 (n3809)
IN IP IN IP
αLIN-61
αLIN-9
αLIN-37
αLIN-52
αLIN-53
αLIN-54
αDPL-1
IPαLIN-61
N2
IN IP
αHDA-1
αHPL-2
αLIN-8
αLIN-56
B
IPαLIN-37
IPαLIN-9
N2
N2
IN IP
IN IP
αLIN-61
αLIN-37
IPαLIN-56
N2
IN IP
αLIN-61
αLIN-56
C
IPαLIN-37
lin-61 (n3809)
IP
αLIN-37
αLIN-9
αLIN-35
αLIN-52
αLIN-53
αLIN-54
αDPL-1

CHAPTER 4

lin-8, which antagonizes *C. elegans* Ras-mediated vulval induction, encodes a novel nuclear protein that interacts with the LIN-35 Rb protein

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My contribution to this chapter includes LIN-8/ LIN-35 *in vitro* binding studies shown in Figure 4.

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ABSTRACT

Ras-mediated vulval development in *C. elegans* is inhibited by the functionally redundant sets of class A, B, and C synthetic Multivulva (synMuv) genes. Three of the class B synMuv genes encode an Rb/DP/E2F complex that, by analogy with its mammalian and *Drosophila* counterparts, has been proposed to silence genes required for vulval specification through chromatin modification and remodeling. Two class A synMuv genes, *lin-15A* and *lin-56*, encode novel nuclear proteins that appear to function as a complex. We show that a third class A synMuv gene, *lin-8*, is the defining member of a novel *C. elegans* gene family. The LIN-8 protein is nuclear and can interact physically with the product of the class B synMuv gene *lin-35*, the *C. elegans* homolog of mammalian Rb. LIN-8 likely acts with the synMuv A proteins LIN-15A and LIN-56 in the nucleus, possibly in a protein complex with the synMuv B protein LIN-35 Rb. Other LIN-8 family members may function in similar complexes in different cells or at different stages. The nuclear localization of LIN-15A, LIN-56, and LIN-8, as well as our observation of a direct physical interaction between class A and class B synMuv proteins support the hypothesis that the class A synMuv genes control vulval induction through the transcriptional regulation of gene expression.

INTRODUCTION

The Retinoblastoma (Rb) gene was the first tumor suppressor gene to be cloned, and the Rb pathway has been found to be a frequent target of inactivation in many human cancers (NEVINS 2001). The nematode *Caenorhabditis elegans* possesses a single homolog of Rb, *lin-35*, which functions in the inhibition of both cellular proliferation and differentiation (LU and HORVITZ 1998; BOXEM and VAN DEN HEUVEL 2001). The class A synthetic Multivulva (synMuv) genes function redundantly with the *lin-35* Rb gene to inhibit Ras-mediated vulval induction. The analysis of the class A synMuv genes may further our understanding of activities that interact with the Rb pathway in the regulation of cell-fate determination and in the prevention of oncogenic transformation.

The vulva of the *C. elegans* hermaphrodite is the conduit through which embryos are expelled and is also the point of entry for sperm after mating with a male. The vulva is formed by the descendants of three of six equipotent cells, P(3-8).p. These six cells are all able to express any one of three fates: the 1° vulval fate, the 2° vulval fate, and the 3° non-vulval fate. Vulval development is induced by activation of a receptor tyrosine kinase (RTK)/Ras pathway (KORNFELD 1997; STERNBERG and HAN 1998). During wild-type larval development, signaling from the anchor cell of the somatic gonad activates an RTK/Ras pathway, causing P6.p to adopt the 1° vulval fate and directly or indirectly causing P5.p and P7.p to adopt the 2° vulval fate. P(5-7).p then divide to produce 22 cells that migrate and fuse to form the toroidal vulva. P3.p, P4.p, and P8.p normally express the 3° non-vulval fate, dividing once and fusing with the hypodermis. Loss of RTK/Ras pathway signaling results in the expression of the non-vulval 3° fate by P(5-7).p and thus in a Vulvaless (Vul) phenotype. Vul animals lack a functional vulva and are consumed by their internally hatched progeny. Ectopic activation of RTK/Ras pathway signaling results in the expression of 1° or 2° vulval fates by P3.p, P4.p, and P8.p, causing a Multivulva (Muv) phenotype. The extra vulval tissue produced in Muv animals forms ectopic ventral protrusions.

The class A, B, and C synMuv genes act to antagonize RTK/Ras function in potential vulval cells (FERGUSON and HORVITZ 1989; CEOL and HORVITZ 2004). As a result of a functional redundancy among these three classes of synMuv genes, only

hermaphrodites mutant in two sets of genes exhibit the synMuv phenotype. Genetic screens and targeted studies have identified at least four class A synMuv genes – *lin-8*, *lin-15A*, *lin-38*, and *lin-56* – at least 17 class B synMuv genes – *lin-9*, *lin-13*, *lin-15B*, *lin-35*, *lin-36*, *lin-37*, *lin-52*, *lin-53*, *lin-54*, *lin-61*, *dpl-1*, *efl-1*, *hda-1*, *hpl-2*, *let-418*, *mep-1*, and *tam-1* – and four class C synMuv genes – *trr-1*, *mys-1*, *epc-1*, and *ssl-1* (HORVITZ and SULSTON 1980; FERGUSON and HORVITZ 1985, 1989; LU and HORVITZ 1998; HSIEH *et al.* 1999; SOLARI and AHRINGER 2000; VON ZELEWSKY *et al.* 2000; CEOL and HORVITZ 2001; COUTEAU *et al.* 2002; UNHAVAITHAYA *et al.* 2002; THOMAS *et al.* 2003; CEOL and HORVITZ 2004; X. LU, M. M. HARRISON, P. W. STERNBERG and H. R. HORVITZ, unpublished results). A subset of the class B synMuv genes encode proteins that mediate histone modification, chromatin remodeling, and transcriptional repression. In particular, *efl-1*, *dpl-1*, *lin-35*, *lin-53*, *hda-1*, *let-418*, and *hpl-2* encode *C. elegans* homologs of E2F, DP, Rb, the Rb-associated protein RbAp48, a class I histone deacetylase (HDAC), the Mi-2 chromatin remodeling ATPase, and heterochromatin protein 1 (HP1), respectively (LU and HORVITZ 1998; SOLARI and AHRINGER 2000; VON ZELEWSKY *et al.* 2000; CEOL and HORVITZ 2001; COUTEAU *et al.* 2002). The mammalian homologs of LIN-53 RbAp48, HDA-1 HDAC, and LET-418 Mi-2 are components of the histone deacetylase and chromatin remodeling NuRD complex, while HP1 has been shown to function as a histone H3 methyl-lysine-9 binding protein (KNOEPFLER and EISENMAN 1999; RICHARDS and ELGIN 2002). Because of their molecular identities, the synMuv B genes are thought to antagonize RTK/Ras function in the vulva by silencing transcription of vulval specification genes through chromatin modification and remodeling. LIN-35 Rb is likely to play a pivotal role in this process, as evidence suggests that mammalian pRb mediates the association of the sequence-specific heterodimeric transcription factor DP/E2F with the NuRD complex components HDAC1 and RbAp48 as well as with a histone H3 K9 methyltransferase (NICOLAS *et al.* 2000; NIELSEN *et al.* 2001; ZHANG and DEAN 2001). The resultant likely recruitment of Mi-2 and HP1 may induce a facultative heterochromatic state around the targeted genes, preventing transcription. The class C synMuv genes encode a putative *C. elegans* Tip60/NuA4-like histone acetyltransferase complex; it has not yet been determined if

this putative complex acts in transcriptional activation or repression (CEOL and HORVITZ 2004).

The class A synMuv genes may inhibit vulval development through the regulation of transcription. Of the four known class A synMuv genes, two – *lin-56* and *lin-15A* – have been cloned and encode novel nuclear proteins that likely associate in a functional complex *in vivo* (CLARK *et al.* 1994; HUANG *et al.* 1994; E. M. DAVISON, A. M. SAFFER, L. S. HUANG, J. DEMODENA, P. W. STERNBERG and H. R. HORVITZ, manuscript in preparation). Furthermore, LIN-56 and LIN-15A share a novel C2CH motif related to the THAP domain, shown in the human protein THAP1 to possess zinc-dependent sequence-specific DNA-binding activity *in vitro* (CLOUAIRE *et al.* 2005; E. M. DAVISON, A. M. SAFFER, L. S. HUANG, J. DEMODENA, P. W. STERNBERG and H. R. HORVITZ, manuscript in preparation). Here we report our characterization of a third class A synMuv gene, *lin-8*.

MATERIALS AND METHODS

Strains and general techniques: *C. elegans* strains were cultivated on NGM agar seeded with *E. coli* strain OP50 as described by BRENNER (1974) and were grown at 20° unless otherwise indicated. Bristol strain N2 was used as the wild-type strain. The mutant alleles used in this study are listed below, and a description of each can be found in RIDDLE *et al.* (1997) unless noted otherwise:

LG I: *lin-35(n745).*
LG II: *lin-8(n111, n2376, n2378, n2403, n2731, n2738, n2739, n2741)* (THOMAS *et al.* 2003), *dpy-10(e128)*.
LG III: *lin-52(n771)* (FERGUSON and HORVITZ 1989; THOMAS *et al.* 2003).
LG X: *lin-15B(n744, n2245).*

pPK5363 is a Tc1-transposon insertion polymorphism on LG II found in the NL7000 but not the N2 strain (KORSWAGEN *et al.* 1996). *nIs128* contains a gfp transgene integrated into LG II (H. T. SCHWARTZ and H. R. HORVITZ, unpublished results). In addition, the following deficiencies were used: *ccDf1*, *ccDf2*, and *ccDf11* (CHEN *et al.* 1992).

Deletion and Polymorphism Mapping: To test complementation of *lin-8* with the deficiencies *ccDf1*, *ccDf2*, and *ccDf11*, *lin-8(n111) dpy-10(e128); lin-15B(n744)* hermaphrodites were mated with *ccDf1*+ males. The Muv phenotype of the non-Dpy male progeny, half of which should possess *lin-8* in *trans* to the relevant deficiency, was scored. 0/88 and 0/84 male offspring of the *ccDf1* and *ccDf2* crosses, respectively, exhibited ventral protrusions, whereas 20/63 male offspring of the *ccDf11* cross exhibited ventral protrusions.

The left endpoint of *ccDf1* was defined relative to the physical map by determining if cosmid sequences from the interval between *sup-9* and *lin-31* could be amplified by PCR from *ccDf1* homozygous inviable embryos. A drop of chitinase solution (20 mg/ml chitinase, 50 mM NaCl, 70 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$) was placed over single inviable embryos, which were promptly transferred to 10 μL of lysis buffer (50 mM KCl, 10 mM Tris, pH 8.2, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45%

Tween-20, 0.01% gelatin, 60 μg/ml proteinase K) and subsequently frozen at -80°. Embryos were thawed and lysed by incubation at 60° for 1 hr. Proteinase K was inactivated by incubation at 95° for 15 min. The lysate from each inviable embryo was used for three PCR reactions: the test amplification, amplification of a sequence from cosmid F34D6, which served as a positive control for successful lysis, and amplification of *lin-31*, which served as a negative control to confirm identification of each egg as a *ccDf1* homozygote. Sequences from cosmids B0454, ZC239, F39E9, and W10G11 but not from cosmids M151 or F19B10, were successfully amplified from *ccDf1* homozygous inviable embryos, placing the left endpoint of *ccDf1* between W10G11 and M151. The primers used were as follows: F34D6.7, 5'-CACCTGAAGATTCAAGTTTAG-3'; F34D6.11, 5'-GTGTGAGCTCAGCAGCTTC-3'; B0454 Fwd, 5'-GGTTCTCGTTAGCT-GAGTGG-3'; B0454 Rev, 5'-GTACGGAGCCAAGATCATACG-3'; ZC239 Fwd, 5'-GCAG-AGACGTTGGATCCTAGC; ZC239 Rev, 5'-CTTCAGGAGTCGGTGAACTCG-3'; F39E9 Fwd, 5'-CAGTCTCAGGCTAGACTTGG-3'; F39E9 Rev, 5'-GCTGAGCAGATCTCGAAT-GG-3'; W10G11 Fwd1, 5'-GCTTCCACATTCAGTGAAGG-3'; W10G11 Rev1, 5'-CAAGC-CAGAAGAGCAAGTCG-3'; W10G11 N1, 5'-CGAGATGTAAGCTCAGTATGG-3'; M151 Fwd, 5'-CATCGGTCTCCCATAGTTACC-3'; M151 Rev, 5'-GCTCTGGCTGCTCGAGTT-CC-3'; F19B10 Fwd, 5'-CTGAAGCATTGGCTCAGAGG-3'; F19B10 Rev, 5'-CGTCATT-GATGGACCATGTGC-3'; *lin-31* Fwd1, 5'-GCTATTCAGGACTCTGACG-3'; *lin-31* Rev1, 5'-CCTTCCCAGGACGATCG-3'.

The *pPK5363* polymorphism is an insertion of the Tc1 transposon into cosmid C17F4 present in the NL7000 but not in the N2 strain (KORSWAGEN *et al.* 1996). To map *lin-8* against *pPK5363*, *lin-8(n111) dpy-10(e128) / NL7000; lin-15B(n744)* males were crossed with *lin-8(n111) dpy-10(e128); lin-15B(n744)* hermaphrodites, and resulting Muv non-Dpy and Dpy non-Muv hermaphrodite progeny were picked and used to establish homozygous recombinant lines. PCR employing Tc1-specific (5'-GCTGATCG-ACTCGATGCCACGTCG-3') and C17F4-specific (5'-CCATCAACGAGTACGATACG-3') primers was used to determine if polymorphism *pPK5363* was present. Of the Muv non-Dpy chromosomes, 4/13 carried *pPK5363*, and of the Dpy non-Muv chromosomes, 2/14

carried *pPK5363*. These results placed *lin-8* to the left of *pPK5363*, which is itself to the left of *dpy-10*.

Transgenic Animals: Germline transformation by microinjection was performed as described by MELLO *et al.* (1991). The coinjection marker pRF4 was injected at a concentration of 80 ng/μl. Transgenic animals were identified using the Roller phenotype generated by expression of the *rol-6(su1006)* dominant allele from pRF4. Experimental constructs were injected at a concentration of 20 ng/μl or 50 ng/μl.

Antibody Preparation, Immunoblotting, and Immunocytochemistry: Anti-LIN-8 antibodies were generated using purified MBP-LIN-8(aa 1-386) fusion protein. The crude antisera were subsequently affinity purified against GST-LIN-8(aa 1-386) fusion protein as described by KOELLE and HORVITZ (1996) and then pre-adsorbed against an acetone precipitate of proteins prepared from *lin-8(n2731)* mixed-stage worms, essentially as described by HARLOW and LANE (1988). Affinity-purified and pre-adsorbed anti-LIN-8 antibodies HM2247 were used at a dilution of 1:1000 for western blots. Samples for western analysis were prepared by Dounce homogenization of mixed-stage worms in hypotonic lysis buffer (10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM sucrose) containing 1X Protease Inhibitor Cocktail (800 μg/ml benzamidine HCl, 500 μg/ml phenanthroline, 500 μg/ml aprotinin, 500 μg/ml leupeptin, 500 μg/ml pepstatin A, 50 mM phenylmethylsulfonyl fluoride, BD Biosciences, Franklin Lakes, NJ) as well as phosphatase inhibitors (0.2 mM sodium orthovanadate, 50 mM sodium fluoride, 1 uM microcystin-LR).

Affinity-purified and pre-adsorbed anti-LIN-8 antibodies HM2247 were used at a dilution of 1:100 for immunocytochemistry. Anti-a-tubulin mouse monoclonal antibody DM1A (Sigma, St. Louis, MO) and MH27 (FRANCIS and WATERSTON 1991), which recognizes the apical borders of *C. elegans* epithelial cells, were used as positive controls for immunocytochemistry at 1:100 and 1:1000 dilutions, respectively. Embryos were fixed in 0.8% paraformaldehyde for 20 min as described by GUENTHER and GARRIGA (1996). Larvae and adults were fixed in 2% paraformaldehyde for 15 min, essentially as described by FINNEY and RUVKUN (1990). Images were obtained using a

Zeiss LSM510 laser confocal microscope and software, and processed using Adobe Photoshop software.

Two-Hybrid and *In Vitro* Binding Experiments: The yeast two-hybrid screen of a *C. elegans* cDNA library was performed as described by WALHOUT and VIDAL (2001). Full-length *lin-35* Rb was used as bait. 1.4 x 10^6 colonies of the *C. elegans* AD-wrmcDNA library (WALHOUT *et al.* 2000b) were screened. Interaction of LIN-35 Rb and LIN-8 could not be tested in the reverse orientation, as LIN-8 was found to self-activate when fused to the Gal4 DNA-binding domain.

The full-length and partial MBP-LIN-8 constructs were made by subcloning appropriate portions of the *lin-8* cDNA into vector pMAL-c2 (NEB, Beverly, MA). The GST-LIN-8 (aa 175-285) construct was made by subcloning the appropriate portion of the *lin-8* cDNA into vector pGEX-2T (Amersham Biosciences, Piscataway, NJ). MBP and GST fusion constructs were expressed in *E. coli* BL21(DE3) cells (STUDIER *et al.* 1990) and purified using amylose resin (NEB) or Glutathione Sepharose 4B (Amersham Biosciences), respectively, as recommended by the manufacturers. The constructs encoding LIN-35 Rb (aa 1-555) and LIN-35 Rb (aa 270-961) have been described previously (LU and HORVITZ 1998) and were used as templates for *in vitro* synthesis of ^{35}S-labeled protein (TNT Couple Reticulocyte Lysate System, Promega, Madison, WI). *In vitro* binding experiments were otherwise performed as described by REDDIEN and HORVITZ (2000), and formation of protein complexes was analyzed by SDS-PAGE and autoradiography.

RESULTS

LIN-8 defines a family of novel *C. elegans* proteins: The class A synMuv gene *lin-8* was originally identified through the chance recovery of a *lin-8(lf); lin-9(lf)* double mutant in a screen by S. BRENNER (personal communication) for animals abnormal in morphology or behavior; the Muv phenotype of this strain was later shown to be synthetic, as it required the presence of two unlinked mutations, *lin-8(n111)* and *lin-9(n112)* (HORVITZ and SULSTON 1980). An additional eight alleles of *lin-8* have since been identified in two independent screens for synMuv A genes (THOMAS *et al.* 2003). *lin-8* was previously mapped to the 7 map unit interval between *sup-9* and *lin-31* on chromosome II (FERGUSON and HORVITZ 1985). We used the deficiencies *ccDf1*, *ccDf2*, and *ccDf11*, each of which deletes *lin-31* (CHEN *et al.* 1992), to more precisely locate *lin-8* on the physical map. We performed complementation tests and found that of these three deficiencies, only *ccDf11* deletes the *lin-8* locus. As the *sup-9* locus resides in cosmid F34D6 (PEREZ DE LA CRUZ *et al.* 2003) and the left endpoints of *ccDf1*, *ccDf2*, and *ccDf11* had been defined approximately by experiments using the polymerase chain reaction (PCR) (data concerning the left endpoints of *ccDf2* and *ccDf11* were generously provided to us by C. A. SPIKE and R. K. HERMAN), we placed *lin-8* between the cosmids F34D6 and M151 (Figure 1A). Further mapping using the polymorphism *pPK5363* placed *lin-8* between cosmids F34D6 and C17F4. We injected cosmids from this interval into *lin-8(n111); lin-15B(n744)* animals and obtained rescue of their synMuv phenotype with cosmid C03E12 as well as with a 7.5 kb subclone of C03E12 (Figure 1B). This minimal rescuing fragment contains a single intact predicted gene, B0454.1 (*C. ELEGANS* SEQUENCING CONSORTIUM 1998). We determined the sequences of the nine alleles of *lin-8* and found all to contain mutations within the B0454.1 open reading frame (ORF) (Table 1). Furthermore, RNA-mediated interference (RNAi) of B0454.1 in *lin-15B(n744)* animals resulted in a synMuv phenotype, and expression of the B0454.1 ORF under the control of the two *C. elegans* heat-shock promoters (STRINGHAM *et al.* 1992) shortly after L1 lethargus efficiently rescued the synMuv phenotype of *lin-8(n2731); lin-15B(n744)* animals (data not shown). We conclude that *lin-8* and B0454.1 are equivalent.

lin-8 encodes a novel, acidic protein of 386 amino acids (Figure 1C). Sequencing of six *lin-8* cDNA clones (courtesy of Yuji Kohara) verified the exon/intron junctions predicted by GENEFINDER (EDGLEY *et al.* 1997). Extensive database searches (using PSI-BLAST (ALTSCHUL *et al.* 1997), PROSITE (FALQUET *et al.* 2002), Pfam (BATEMAN *et al.* 2002) and SMART (SCHULTZ *et al.* 2000)) with the LIN-8 protein sequence have revealed no significant canonical motifs and no apparent sequence homologs in other species. LIN-8 is, however, a member of a family of 17 *C. elegans* proteins (Figure 2). This family was independently detected by the Pfam protein families database, and is there referred to as DUF278 (BATEMAN *et al.* 2002). While the scores are very weak, LIN-8 identifies the most distant family member in BLAST searches, and vice versa. The biological roles of the remaining 16 family members are not known. Several of the family members, including LIN-8, possess an N-terminal proline-rich region (Figure 2) containing at least one PxxP motif, the core sequence to which SH3 domains can bind (KAY *et al.* 2000).

Characterization of *lin-8* alleles: To identify null alleles of *lin-8* as well as residues important for LIN-8 function, we characterized all nine independently isolated *lin-8* alleles (Tables 1 and 2). Three *lin-8* alleles – *n2731*, *n2738*, *n2739* – contain nonsense mutations (Table 1). The remaining six *lin-8* alleles – *n111*, *n2376*, *n2378*, *n2403*, *n2724*, *n2741* – contain missense mutations (Table 1). Two of the missense alleles, *n2403* and *n2724* (THOMAS *et al.* 2003), contain the identical nucleotide change; only *n2403* was subsequently used for quantitative studies. Four of the five amino-acid residues altered in the missense alleles are conserved in several LIN-8 family members (Figure 2).

The nine *lin-8* alleles are not easily distinguishable in combination with the strong canonical synMuv B allele *lin-15B(n744)* (data not shown). The synMuv phenotype is inherently temperature-sensitive – both its penetrance and expressivity are usually greater in mutants raised at 20° than at 15° (FERGUSON and HORVITZ 1989). We therefore quantitated the penetrance of the synMuv phenotype associated with each *lin-8* allele in combination with two weak synMuv B alleles, *lin-15B(n2245)* (THOMAS *et al.* 2003) and *lin-52(n771)* (FERGUSON and HORVITZ 1989), at both 15° and 20° (Table 2).

The missense alleles can be placed in three categories on the basis of phenotypic strength: weak (*n2741*), intermediate (*n111*), and strong (*n2376*, *n2378*, *n2403*). The three strong missense alleles all mutate charged amino acids in a cluster of residues conserved in many members of the LIN-8 family. Of the nonsense mutations, *n2731* and *n2738* appeared to be substantially stronger than *n2739* when tested in combination with *lin-52(n771)*. Both *n2731* and *n2738* are predicted to truncate more than two-thirds of the wild-type LIN-8 protein, whereas *n2739* is predicted to leave more than two-thirds of the wild-type LIN-8 protein intact.

On the basis of their molecular lesions, we considered *n2731* and *n2738* to be candidate null alleles of the *lin-8* locus. Neither nonsense allele, however, inactivated the synMuv A pathway to the same extent as loss of the class A synMuv gene *lin-56* in the *lin-15B(n2245)* mutant background (Table 2). We also observed that the strong missense mutations *n2376*, *n2378*, and *n2403* appeared more penetrant for the synMuv phenotype than either *n2731* or *n2738* in combination with *lin-15B(n2245)* (Table 2). To more stringently determine if *n2731* and *n2738* were null alleles of the *lin-8* locus, we compared the penetrances of the synMuv phenotype of hermaphrodites homozygous for each allele with hermaphrodites heterozygous for each allele and for *ccDf11*, a deficiency that deletes the *lin-8* locus (see Figure 1A). The penetrances of the synMuv phenotype for both *lin-8(n2731); lin-15B(n2245)* and *lin-8(n2738); lin-15B(n2245)* homozygotes were weaker than those of *lin-8(n2731)/ccDf11; lin-15B(n2245)* and *lin-8(n2738)/ccDf11; lin-15B(n2245)* heterozygotes, respectively (Table 2). One possible interpretation of these observations is that neither *n2731* nor *n2738* completely eliminates *lin-8* function. However, the *ccDf11* deficiency also eliminates several other LIN-8 family members – B0454.9, C41H7.3, C41H7.4, C41H7.5, C41H7.6, F14D2.2, F54D10.3, K08A2.1, and K08A2.4. We suspect that *n2731* and/or *n2738* are null alleles of *lin-8* and that the penetrances of their synMuv phenotypes are enhanced by a decrease in the dosage of one or more of the *lin-8* family genes deleted by *ccDf11*. This latter hypothesis is supported by the observation that no LIN-8 protein is detected in *n2738* protein extracts (see below).

Unlike mutants carrying the nonsense mutations *n2731* and *n2738*, mutants carrying the missense mutation *n2376* had an equally penetrant phenotype when homozygous as when heterozygous over the *ccDf11* deficiency (Table 2), suggesting that *n2376* may be a null allele of *lin-8*. However, *n2376* results in the production of stable LIN-8 protein, at least in extracts from mixed-stage animals (see below). Furthermore, *lin-8(n2376)* retains wild-type *lin-8* function in another assay (H. T. SCHWARTZ and H. R. HORVITZ, unpublished results). Thus, it is likely that the *n2376* allele is not a null allele of *lin-8* but is instead specifically defective for *lin-8* synMuv A function.

LIN-8 is a nuclear protein expressed in many cells: To determine the expression pattern and localization of the LIN-8 protein, we generated a rabbit polyclonal antibody against a fusion of full-length LIN-8 with maltose-binding protein (MBP-LIN-8). The affinity-purified and pre-adsorbed antibody recognized an apparent doublet of approximately 50 kD in wild-type but not in *lin-8(n2731)* or *lin-8(n2738)* protein extracts analyzed by western blots (Figure 3A); the predicted size of the LIN-8 protein is 44 kD. As the two LIN-8 proteins are approximately equal in their levels and the six *lin-8* cDNA clones (courtesy of Yuji Kohara) that we analyzed are identical in sequence, we suspect that the LIN-8 protein may be post-translationally modified. The *n2376* missense mutation does not destabilize the full-length LIN-8 protein (Figure 3A).

Since *lin-8* functions with *lin-15A*, *lin-38*, and *lin-56* to inhibit vulval development (FERGUSON and HORVITZ 1989; THOMAS *et al.* 2003), we analyzed the impact of loss-of-function mutations in these class A synMuv genes on the LIN-8 protein. By western blot analysis, neither the levels nor the electrophoretic mobility of LIN-8 appears to be altered in *lin-56(lf)*, *lin-15A(lf)*, or *lin-38(lf)* mutants (Figure 3A). This result contrasts with that for the class A synMuv proteins LIN-15A and LIN-56, which are dependent on each other, but not on *lin-8* or *lin-38*, for wild-type levels (E. M. DAVISON, A. M. SAFFER, L. S. HUANG, J. DEMODENA, P. W. STERNBERG and H. R. HORVITZ, manuscript in preparation).

We used the same anti-LIN-8 antibody for whole-mount staining of worms. LIN-8 appeared to be predominantly localized to nuclei (Figure 3B-D). We observed LIN-8 expression in many cells in embryos, larvae, and adults, and LIN-8 staining was

particularly prominent in the germline as well as in neuronal nuclei of the head (Figure 3B-D). Although diffuse within the syncytium of the distal gonad arms, LIN-8 was specifically associated with germ cell nuclei during the pachytene stage and was also localized to oocyte nuclei (Figure 3C). No anti-LIN-8 staining was observed in any of these somatic or germ cell nuclei in *lin-8(n2731)* animals of any stage (Figure 3B,C and data not shown). Background staining in the larval midbody of *lin-8(n2731)* animals was too high to examine LIN-8 expression in vulval cells.

LIN-8 interacts with LIN-35 Rb *in vitro*: We performed a yeast two-hybrid analysis of a *C. elegans* cDNA library using full-length *lin-35* Rb as bait and identified LIN-8 as a potential LIN-35 Rb interactor (data not shown). The Gal4-based screen made use of three reporter genes – *GAL1::HIS3*, *GAL1::lacZ*, and *SPAL10::URA3* (FIELDS and SONG 1989; WALHOUT and VIDAL 2001). Of 1.4 x 10^6 transformants, we identified 11 clones that grew on selective medium in the presence of 3-aminotriazole (3-AT). Further analysis revealed that six of these 11 clones also expressed β-galactosidase (β-gal) and were able to grow in the absence of uracil. The six clones that tested positive for expression of all three reporter genes were all found to contain the B0454.1 open reading frame encoding LIN-8. Neither LIN-35 nor LIN-8 interacted in the yeast two-hybrid system with any of 29 other vulval proteins tested (WALHOUT *et al.* 2000a and data not shown). Western blot analyses (data not shown) indicate that LIN-35 is expressed at wild-type levels in *lin-8(n2731)* worms and that LIN-8 is expressed at wild-type levels in *lin-35(n745)* worms, which lack LIN-35 protein (LU and HORVITZ 1998).

To test the hypothesis that LIN-8 and LIN-35 Rb can interact and, if so, to identify the region of LIN-35 Rb required for the interaction, we sought to determine if LIN-8 could associate *in vitro* with two different fragments of LIN-35 Rb. The A/B pocket domain of mammalian pRb, p107, and p130 mediates association with many interacting proteins (MORRIS and DYSON 2001), and in *C. elegans* is contained within a portion of LIN-35 Rb sufficient for interaction in *in vitro* pull-down experiments with LIN-53 RbAp48, HDA-1 HDAC, DPL-1 DP, and EFL-1 E2F (LU and HORVITZ 1998; CEOL and HORVITZ 2001). An [^{35}S]methionine-labeled N-terminal LIN-35 Rb fragment (aa 1-555),

which lacks an intact pocket domain, failed to show any significant association with a full-length MBP-LIN-8 fusion protein in *in vitro* pull-down assays (Figure 4A). By contrast, an [^{35}S]methionine-labeled C-terminal LIN-35 Rb fragment (aa 270-961), which contains the pocket domain, interacted with MBP-LIN-8 (Figure 4A). LIN-8 and LIN-35 Rb are thus capable of interacting in both yeast two-hybrid and *in vitro* pull-down assays. Furthermore, these observations suggest that, as with its other *C. elegans* interactors (LU and HORVITZ 1998; CEOL and HORVITZ 2001), LIN-35 Rb associates with LIN-8 through its C-terminus, possibly via the pocket domain.

We next identified the domain of LIN-8 required for interaction with the C-terminal LIN-35 Rb fragment using an *in vitro* pull-down assay. Progressive deletions of N- and C-terminal LIN-8 residues revealed that amino acids 170-359 of LIN-8 were necessary for interaction with LIN-35 Rb (data not shown; Figure 4C). Furthermore, amino acids 175-285 of LIN-8 were sufficient for interaction with the C-terminal LIN-35 Rb fragment (Figure 4B,C). Several viral proteins interact with the pocket domain of pRb via an LXCXE motif (HARBOUR and DEAN 2000); this sequence is not contained within amino acids 175-285 of LIN-8. Amino acids 175-285 of LIN-8 share a small region of similarity with other LIN-8 family members (Figure 2). None of the *lin-8* missense mutations resides within the interaction domain, and thus no existing mutation compromises the predicted association between LIN-35 Rb and LIN-8 by directly affecting the interaction domain.

DISCUSSION

The class A synMuv genes function redundantly with the *C. elegans* homolog of the mammalian tumor suppressor pRb to inhibit Ras-mediated vulval development. We have shown that the class A synMuv gene *lin-8* encodes a novel nuclear protein that not only functions redundantly but also physically interacts with *C. elegans* Rb. Given these observations, we propose that the class A synMuv genes act in transcriptional regulation. Further characterization of the mechanism by which the proteins of the class A synMuv pathway act may well reveal molecular processes that interact with the mammalian Rb tumor suppressor pathway both in the regulation of cell fate and in the prevention of tumorigenesis.

Class A synMuv genes may regulate transcription: The two previously cloned synMuv A genes – *lin-15A* and *lin-56* – encode novel nuclear proteins that share a novel C2CH motif also found in the synMuv B proteins LIN-15B and LIN-36, as well as in HIM-17, a protein required for meiotic recombination and histone H3 lysine-9 methylation in the germline (REDDY and VILLENEUVE, 2004; E. M. DAVISON, A. M. SAFFER, L. S. HUANG, J. DEMODENA, P. W. STERNBERG and H. R. HORVITZ, manuscript in preparation). This C2CH motif is likely related to the THAP domain (CLOUAIRE *et al.* 2005; ROUSSIGNE *et al.* 2003), and has been proposed to mediate interaction with chromatin or chromatin-associated proteins (REDDY and VILLENEUVE, 2004; E. M. DAVISON, A. M. SAFFER, L. S. HUANG, J. DEMODENA, P. W. STERNBERG and H. R. HORVITZ, manuscript in preparation). The THAP domain of the human protein THAP1 has been shown to possess zinc-dependent sequence-specific DNA-binding activity *in vitro* (CLOUAIRE *et al.* 2005). It has therefore been proposed that the synMuv A proteins inhibit vulval development through the regulation of transcription (E. M. DAVISON, A. M. SAFFER, L. S. HUANG, J. DEMODENA, P. W. STERNBERG and H. R. HORVITZ, manuscript in preparation).

The nuclear localization of LIN-8 and the physical association between LIN-8 and LIN-35 Rb is consistent with the hypothesis that LIN-8 is present at the sites of transcriptional repressor complexes. How might the proteins encoded by these three synMuv A genes modulate transcriptional activity? Three general mechanisms seem

possible. First, like some synMuv B proteins (LU and HORVITZ 1998; SOLARI and AHRINGER 2000; VON ZELEWSKY *et al.* 2000; CEOL and HORVITZ 2001; COUTEAU *et al.* 2002), the synMuv A proteins may impact chromatin structure. Second, the synMuv A proteins may have more direct functions in regulating the initiation, elongation, or termination of transcription. Third, the synMuv A proteins may mediate the localization of target genes to nuclear subdomains where their transcription could be coordinately and efficiently regulated. The localization of genes to the vicinity of centromeric heterochromatin, for example, may contribute to transcriptional repression in *Drosophila* and mammals (GASSER 2001).

Physical interaction between synMuv A and B proteins: Indirect physical interaction between the class A and class B proteins was proposed by WALHOUT *et al.* (2000a), who found that the synMuv A protein LIN-15A shared interactors with the synMuv B proteins LIN-36 and LIN-37 in the yeast two-hybrid system. As RNAi analysis has thus far not revealed a role for any of these shared interactors in the class A or class B synMuv pathways or in antagonism of these pathways (C. J. CEOL and H. R. HORVITZ, unpublished results), the significance of this observation remains unclear.

The direct interaction described in this manuscript between LIN-8 and LIN-35 Rb *in vitro* suggests that LIN-8 and LIN-35 Rb may associate *in vivo*. The biological role of such an interaction is unclear. If the putative interaction between LIN-8 and LIN-35 Rb were to facilitate *lin-8* and/or *lin-35* Rb function in vulval development, then one might expect to observe both synMuv A and B activity associated with one or both of these genes. However, a reduction of *lin-8* function does not result in a synMuv phenotype in combination with a loss of the function of the synMuv A genes *lin-15A*, *lin-38*, or *lin-56* (FERGUSON and HORVITZ 1989; THOMAS *et al.* 2003), suggesting that *lin-8* does not possess class B synMuv activity. Similarly, a loss of *lin-35* Rb function does not result in a synMuv phenotype in combination with a loss of the function of the synMuv B genes *lin-36*, *lin-37*, or *lin-15B* (FERGUSON and HORVITZ 1989), suggesting that *lin-35* Rb does not possess class A synMuv activity.

Proteins that physically interact often work together directly in the same biological process. By contrast, synthetic genetic interactions between null alleles of two genes

usually indicate that the genes affect a biological process through separate mechanisms. Although *lin-8* and *lin-35* Rb function in the parallel synMuv A and synMuv B pathways, respectively, the proteins they encode physically interact *in vitro.* If the interaction between LIN-8 and LIN-35 Rb is biologically important, then three models could explain why neither *lin-8* nor *lin-35* Rb appears to possess both synMuv A and synMuv B activity. First, the functional consequence of the LIN-8/LIN-35 Rb interaction may be redundant with another process in vulval development. For example, LIN-8 may be independently localized to the promoters of vulval specification genes by both LIN-35 Rb and by another protein. Second, LIN-8 and LIN-35 Rb may function together in the vulva, but in some process not required for vulval development. Third, *lin-8* and *lin-35* Rb may act together but not in the vulva. The widespread expression of *lin-8* and *lin-35* Rb (LU and HORVITZ 1998) indicates that they could function together in other tissues. Mutation of *lin-35* Rb has indeed been shown to result not only in the synMuv B phenotype but also in defects in cell cycle progression (BOXEM and VAN DEN HEUVEL 2001; FAY *et al.* 2002; GARBE *et al.* 2004), in defects in pharyngeal morphogenesis (FAY *et al.* 2003), and in severely reduced expression of a muscle cell-specific *gfp* reporter gene from repetitive transgene arrays (the Tam phenotype) (HSIEH *et al.* 1999). Although current evidence suggests that *lin-8* does not function with *lin-35* Rb in the regulation of either cell cycle progression or transgene expression (HSIEH *et al.* 1999; BOXEM and VAN DEN HEUVEL 2002; GARBE *et al.* 2004; E. C. ANDERSEN and H. R. HORVITZ, unpublished observations), the possibility remains that *lin-8* and *lin-35* Rb act together in the developing pharynx or in processes not yet analyzed.

Partial redundancy in the LIN-8 family: The nonsense alleles *n2731* and *n2738* appeared to be null alleles on the basis of their molecular lesions and lack of LIN-8 protein, yet by comparison to loss of the class A synMuv gene *lin-56* and by comparison to a deficiency that removes the *lin-8* locus did not appear to have lost all synMuv A pathway activity. By contrast, three of the *lin-8* missense alleles impaired synMuv A activity almost to the extent seen upon loss of *lin-56.* One of these three *lin-8* missense alleles, *n2376*, acted like a null by deficiency analysis but did not destabilize full-length LIN-8 protein (at least in extracts from mixed-stage animals). One hypothesis

to account for these observations is that LIN-8 normally functions as part of a protein complex and that other family members can partially replace LIN-8 activity within the complex in its absence. Specifically, in mutants that lack LIN-8 protein, closely related family members may partially substitute for LIN-8. *ccDf11*, the deletion used for deficiency analysis, removes both *lin-8* and several *lin-8* family members. The predicted partial replacement of LIN-8 by other LIN-8 family members may therefore be reduced in *lin-8(null)/ccDf11* heterozygotes as compared to *lin-8(null)* homozygotes. The strong missense alleles may encode stable LIN-8 proteins that inactivate other family members either by direct interaction or by competition with a partner. A similar phenomenon has been observed in *S. cerevisiae* for the MAP kinases Fus3 and Kss1, which in wild-type yeast regulate the mating pheromone response and filamentation pathways, respectively (MADHANI *et al.* 1997). In the complete absence of Fus3, Kss1 provides substitute MAPK activity for the mating pheromone response pathway and thus only a slight reduction in mating efficiency is observed. By contrast, when Fus3 is rendered catalytically inactive by a missense mutation, Kss1 cannot substitute in the mating pheromone response pathway and a much stronger mating defect results. A comparable model has been proposed for the histone deacetylase family in *Drosophila*, as missense but not null mutations of the histone deacetylase HDAC1 dominantly suppress silencing caused by position effect variegation (MOTTUS *et al.* 2000).

The biological roles of the remaining 16 *lin-8* family members are not known. Their similarity to *lin-8* suggests that these genes are also likely to have roles in transcriptional regulation, perhaps with other components or in other cells. The high degree of similarity shared by the *lin-8* family members also suggests that redundancy may have prevented their identification in genetic screens: more than one family member may have to be inactivated for a mutant phenotype to be apparent.

Interactions within the class A synMuv pathway: LIN-56 and LIN-15A are dependent on each other for wild-type protein levels and likely form a functional complex *in vivo* (E. M. DAVISON, A. M. SAFFER, L. S. HUANG, J. DEMODENA, P. W. STERNBERG and H. R. HORVITZ, manuscript in preparation). Mutation of *lin-8* or *lin-38* does not perturb the expression or localization of either LIN-56 or LIN-15A, indicating

that neither *lin-8* nor *lin-38* is normally required for formation or stability of the putative LIN-56/LIN-15A complex. In this manuscript, we demonstrate that *lin-56*, *lin-15A*, and *lin-38* do not appear to be required for expression of LIN-8. These results form a basis upon which our understanding both of the roles of individual components of the class A synMuv pathway and of the interactions among these components can be further expanded.

Implications for human cancer: Mammalian tumorigenesis requires deregulation of cell proliferation, cell differentiation, and apoptosis and is thus an inherently synthetic process requiring multiple mutations in the proto-oncogene and tumor suppressor pathways controlling these biological activities (HANAHAN and WEINBERG 2000). The Rb tumor suppressor pathway likely plays a critical role in preventing oncogenic transformation, as its inactivation is observed in many human cancers (NEVINS 2001). That the class A synMuv genes function redundantly with the *C. elegans* Rb pathway suggests that mammalian counterparts of the synMuv A genes may well possess tumor suppressor activity. We hope that characterization of the mechanism by which the class A synMuv genes function will lead to greater understanding of processes that act with the mammalian Rb pathway both in cell-fate determination and in protection from oncogenic transformation and that the redundancy of the class A, B, and C synMuv genes in regulating *C. elegans* vulval cell fates will serve as a model for the etiology of other synthetic processes, such as tumorigenesis, whose manifestation requires multiple mutations.

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TABLE 1

Sequences of *lin-8* mutations

Allele	Wild-type codon	Mutant codon	Substitution
n111	CTG	CCG	L20P
n2741	GTG	ATG	V68M
n2376	GAG	AAG	E148K
n2378	CGC	TGC	R154C
n2403	GAG	AAG	E164K
n2724	GAG	AAG	E164K
n2738	TGG	TAG	W79amber
n2731	CAA	TAA	Q113ochre
n2739	AGA	TGA	R304opal

Amino acid substitutions are indicated as wild-type residue, residue number, and mutant residue.

TABLE 2

lin-8 allele strengths

	Penetrance of Muv phenotype (%)			
	lin-15B(n2245)		*lin-52(n771)*	
Genotype[a,b]	**15° (n)**	**20° (n)**	**15° (n)**	**20° (n)**
lin-8(n111)	29 (94)	100 (109)	49 (98)	99 (143)
lin-8(n2741)	1 (98)	100 (101)	15 (96)	99 (146)
lin-8(n2376)	98 (99)	100 (103)	80 (96)	100 (155)
lin-8(n2378)	99 (98)	100 (101)	88 (97)	100 (142)
lin-8(n2403)[c]	92 (101)	100 (104)	98 (100)	100 (107)
lin-8(n2738)	68 (96)	100 (103)	96 (97)	100 (139)
lin-8(n2731)	77 (74)	100 (104)	99 (97)	100 (146)
lin-8(n2739)	76 (100)	100 (102)	63 (95)	97 (119)
lin-8(n2376) dpy-10(e128)/ccDf11	96 (135)	nd	nd	nd
lin-8(n2738) dpy-10(e128)/ccDf11	95 (127)	nd	nd	nd
lin-8(n2731) dpy-10(e128)/ccDf11	94 (183)	nd	nd	nd
lin-56(n2728)[d]	100 (127)	100 (127)	nd	nd
lin-56(n3355)[d]	100 (124)	100 (100)	nd	nd

[a] Animals homozygous for either a *lin-8(lf)* or *lin-56(lf)* allele were raised at either 15° or 20° for at least three generations before scoring.

[b] To generate *lin-8(lf)/ccDf11; lin-15B(n2245)* animals, *dpy-10(e128); lin-15B(n2245)* hermaphrodites were mated with *ccDf11/nIs128* males. The resulting non-Dpy non-GFP *ccDf11/dpy-10(e128); lin-15B(n2245)* male offspring were then crossed with *lin-8(lf) dpy-10(e128); lin-15B(n2245)* hermaphrodites, and the Muv phenotype of any non-Dpy cross progeny of this mating was scored. All crosses were carried out at 15°.

[c] Since *n2403* and *n2724* contain the same amino acid change, only *n2403* was analyzed.

[d] The *n2728* allele contains a deletion of the entire *lin-56* locus. The *n3355* allele contains an early nonsense mutation within the *lin-56* coding sequence. (E. M. DAVISON, A. M. SAFFER, L. S. HUANG, J. DEMODENA, P. W. STERNBERG and H. R. HORVITZ, manuscript in preparation)

% Muv, percent animals with at least one pseudovulva on their ventral sides

n, number of animals scored

nd, not determined

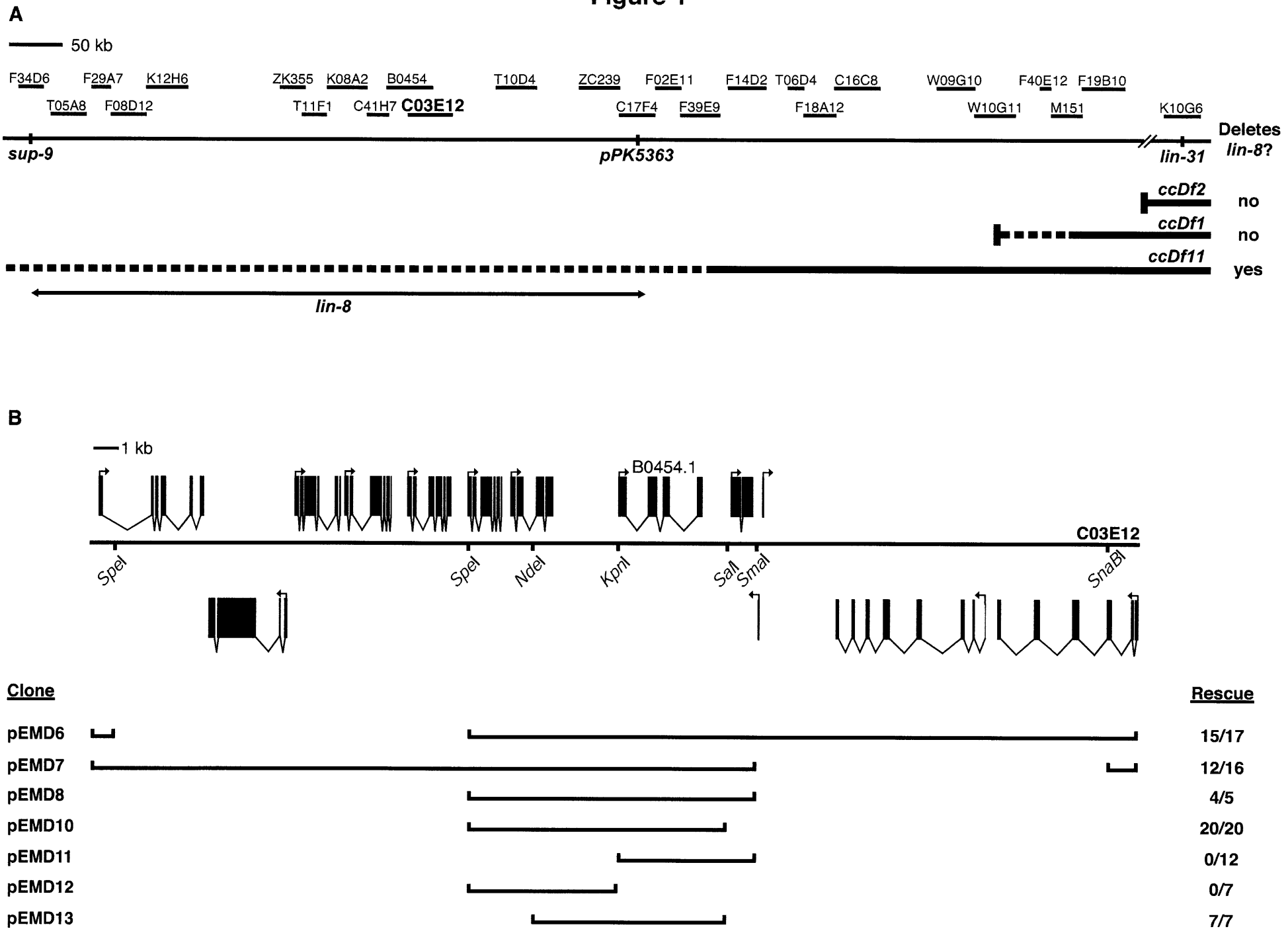
Figure 1: Cloning of *lin-8*.

(A) Physical map of the genomic region containing the *lin-8* locus. Deficiency and polymorphism mapping placed *lin-8* between cosmids F34D6 and C17F4. *lin-8* rescuing cosmid C03E12 is shown in bold. Solid lines indicate regions known to be deleted by the deficiencies; broken lines indicate regions that may be deleted by the deficiencies.

(B) Transformation rescue of *lin-8*. Shown are the predicted open reading frames within rescuing cosmid C03E12, top, as well as the subclones derived from this cosmid, bottom.

(C) Sequence of the LIN-8 protein. The region sufficient for interaction with LIN-35 Rb is underlined.

Figure 1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F40E12
F19B10
W10G11
M151
K10G6
sup-9
pPK5363
lin-31
Deletes lin-8?
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no
ccDf1
no
ccDf11
yes
lin-8
B
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C03E12
SpeI
SpeI
NdeI
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15/17
pEMD7
12/16
pEMD8
4/5
pEMD10
20/20
pEMD11
0/12
pEMD12
0/7
pEMD13
7/7

Figure 1

C

MSKIKTHSTGSKRTVPFYKLPPPVPLPPLPPPDPTRYFSTEKYIALSKDE 50
KFKFDDYDVNDETLKKVVLNEIGKCPDIWSSRSQAAIMEHYPIVATETYR 100
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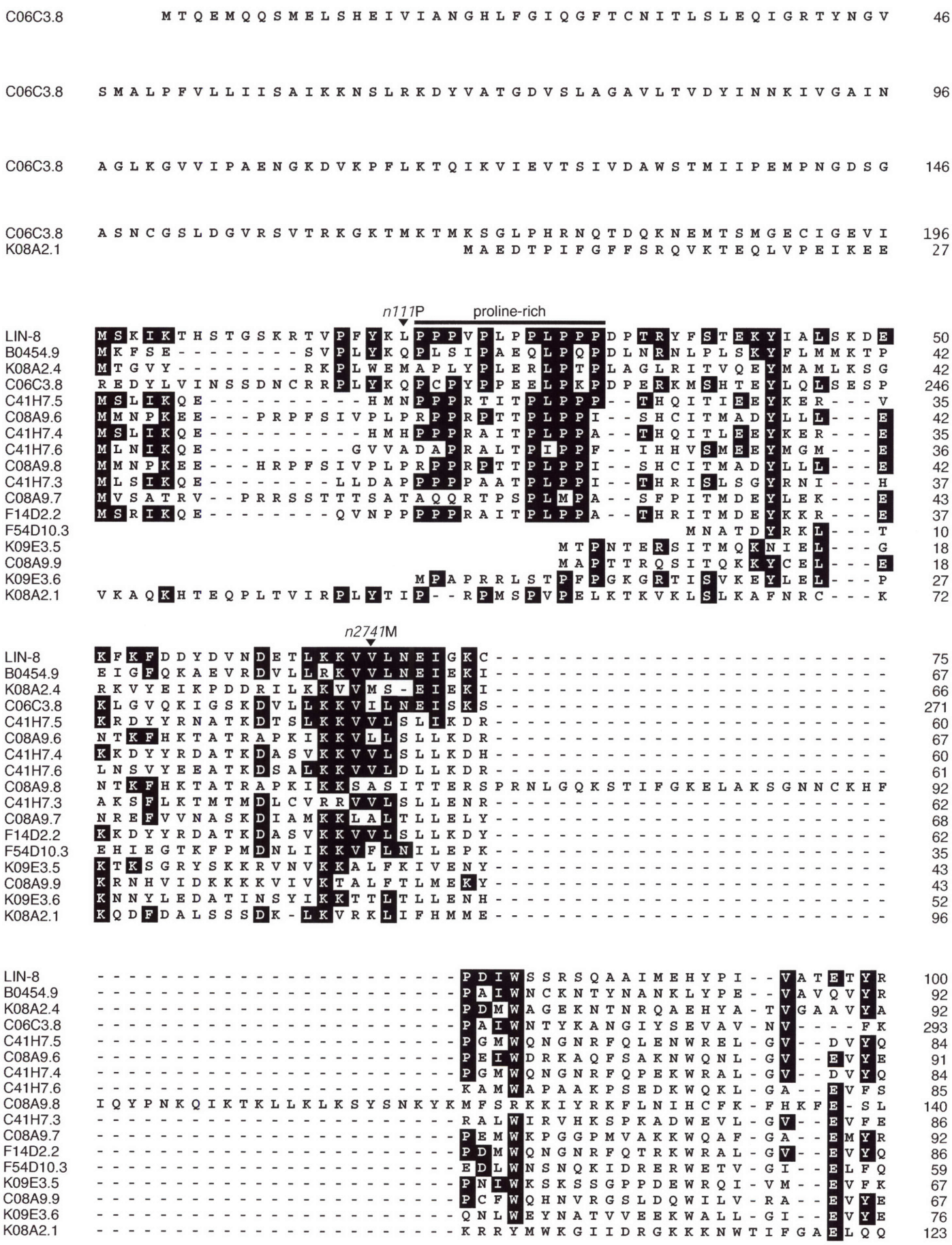
APVREAPSPVVENVSSSSFTPKPPAMINNFGEEMNQITYQAIRIAREQPE 350
RLKLLRKALFDVVLAFDQKEYADVGDLYRDLAQKNS 386

Figure 2. LIN-8 defines a family of *C. elegans* proteins.

Alignment of LIN-8 with the other 16 members of the LIN-8 family; all of these proteins are from *C. elegans*. Solid boxes indicate identity with LIN-8 in at least three additional family members. Arrowheads indicate positions of missense mutations found in the indicated *lin-8* alleles. The proline-rich N-terminal motif and the region of LIN-8 sufficient for interaction with LIN-35 Rb are indicated with solid lines.

Figure 2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n111P
proline-rich
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Figure 2

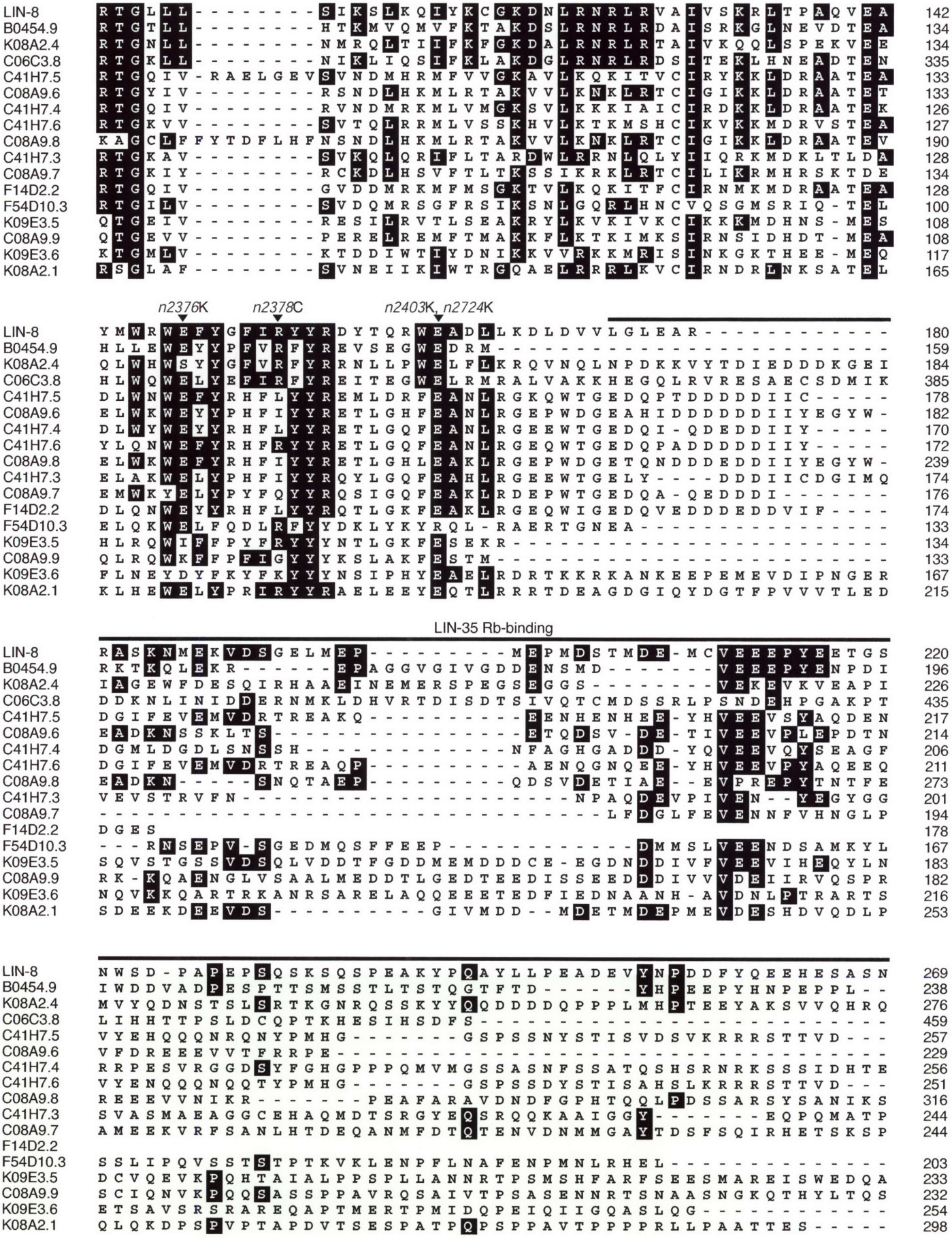
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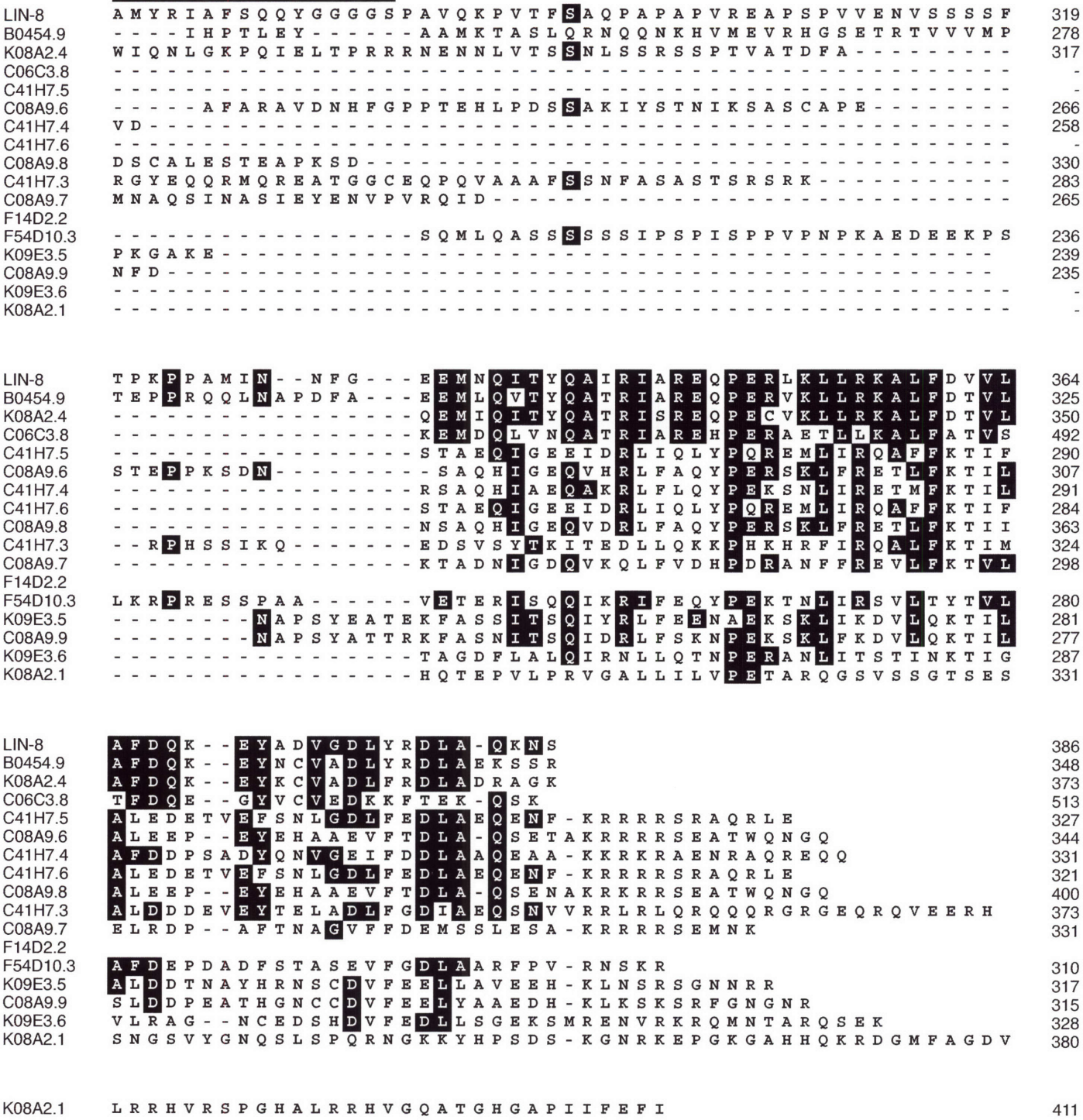
Figure 2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C41H7.3 RGYEQQRMQREATGGCEQPQVAAAFSSNFASASTSRSRK---------- 283
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K08A2.1 ----------HQTEPVLPRVGALLILVPETARQGSVSSGTSES 331
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K09E3.5 ALDDTNAYHRNSCDVFEELLAVEEH-KLNSRSGNNRR 317
C08A9.9 SLDDPEATHGNCCDVFEELYAAEDH-KLKSKSRFGNGNR 315
K09E3.6 VLRAG--NCEDSHDVFEDLLSGEKSMRENVRKRQMNTARQSEK 328
K08A2.1 SNGSVYGNQSLSPQRNGKKYHPSDS-KGNRKEPGKGAHHQKRDGMFAGDV 380
K08A2.1 LRRHVRSPGHALRRHVGQATGHGAPIIFEFI 411

Figue 3. LIN-8 protein is expressed broadly and localized in nuclei.

(A) Western analysis of protein extracts from wild-type, *lin-8(lf)*, *lin-56(n2728)*, *lin-38(n751)*, *lin-15A(n767)*, and *lin-15AB(e1763)* mixed-stage worms probed with affinity-purified and pre-adsorbed anti-LIN-8 antibody. The position of the apparent LIN-8 protein doublet is indicated by the arrow. The molecular weights of marker proteins are indicated at left in kD.

(B-D) Whole-mount staining of wild-type animals with affinity-purified and pre-adsorbed anti-LIN-8 antibodies (green), as well as DAPI (blue) to visualize DNA. Staining with anti-tubulin antibody (red) is shown as a fixation control in embryos. Staining with the MH27 antibody (red), which recognizes the apical borders of *C. elegans* epithelial cells, is shown as a fixation control in adults. Scale bars, 5 μm.

(B) LIN-8 staining is observed in multiple nuclei in the wild-type but not *lin-8(n2731)* embryo.

(C) LIN-8 staining is present in the wild-type but not *lin-8(n2731)* gonad in pachytene nuclei and in oocytes.

(D) LIN-8 staining is observed in multiple nuclei in the wild-type adult head.

Figure 3

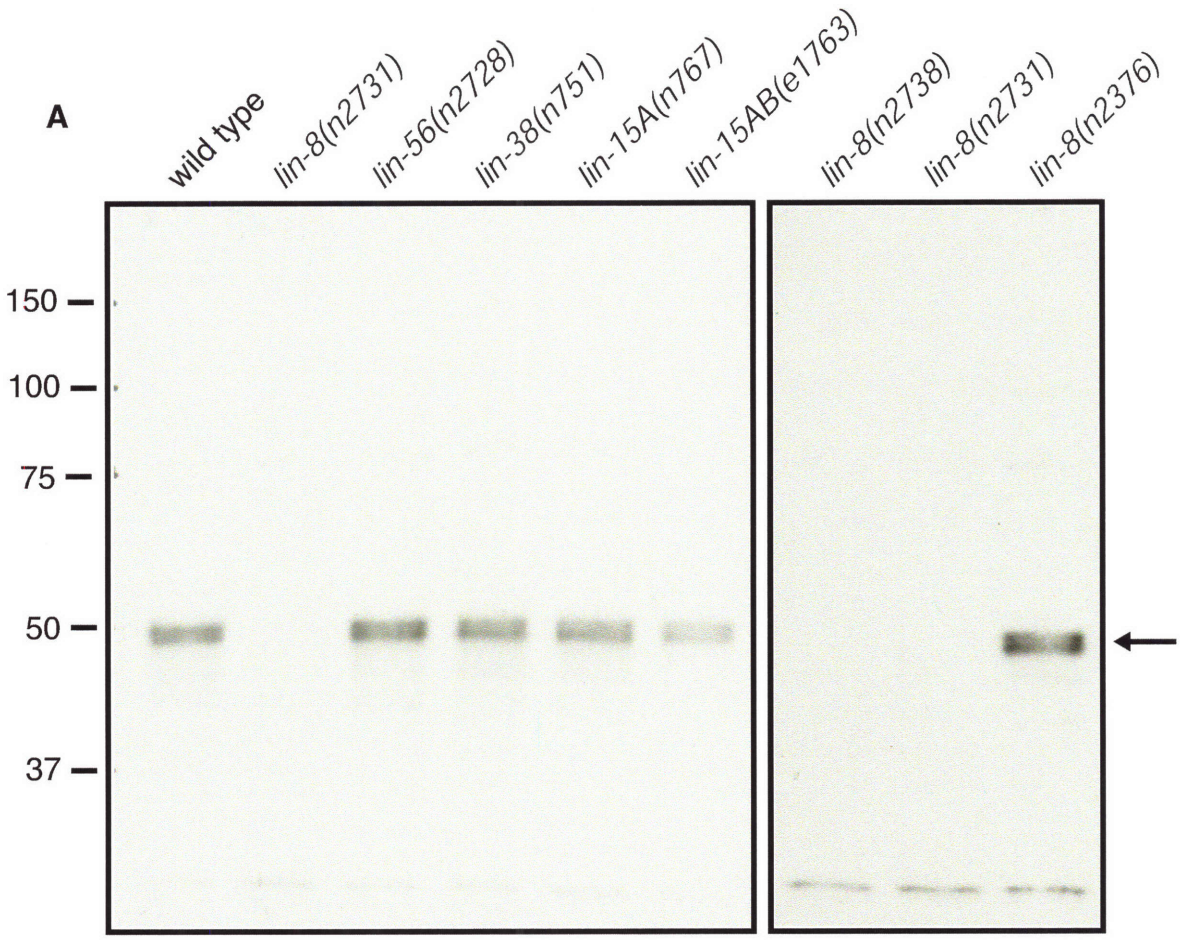

A
wild type
lin-8(n2731)
lin-56(n2728)
lin-38(n751)
lin-15A(n767)
lin-15AB(e1763)
lin-8(n2738)
lin-8(n2731)
lin-8(n2376)
150
100
75
50
37

Figure 3

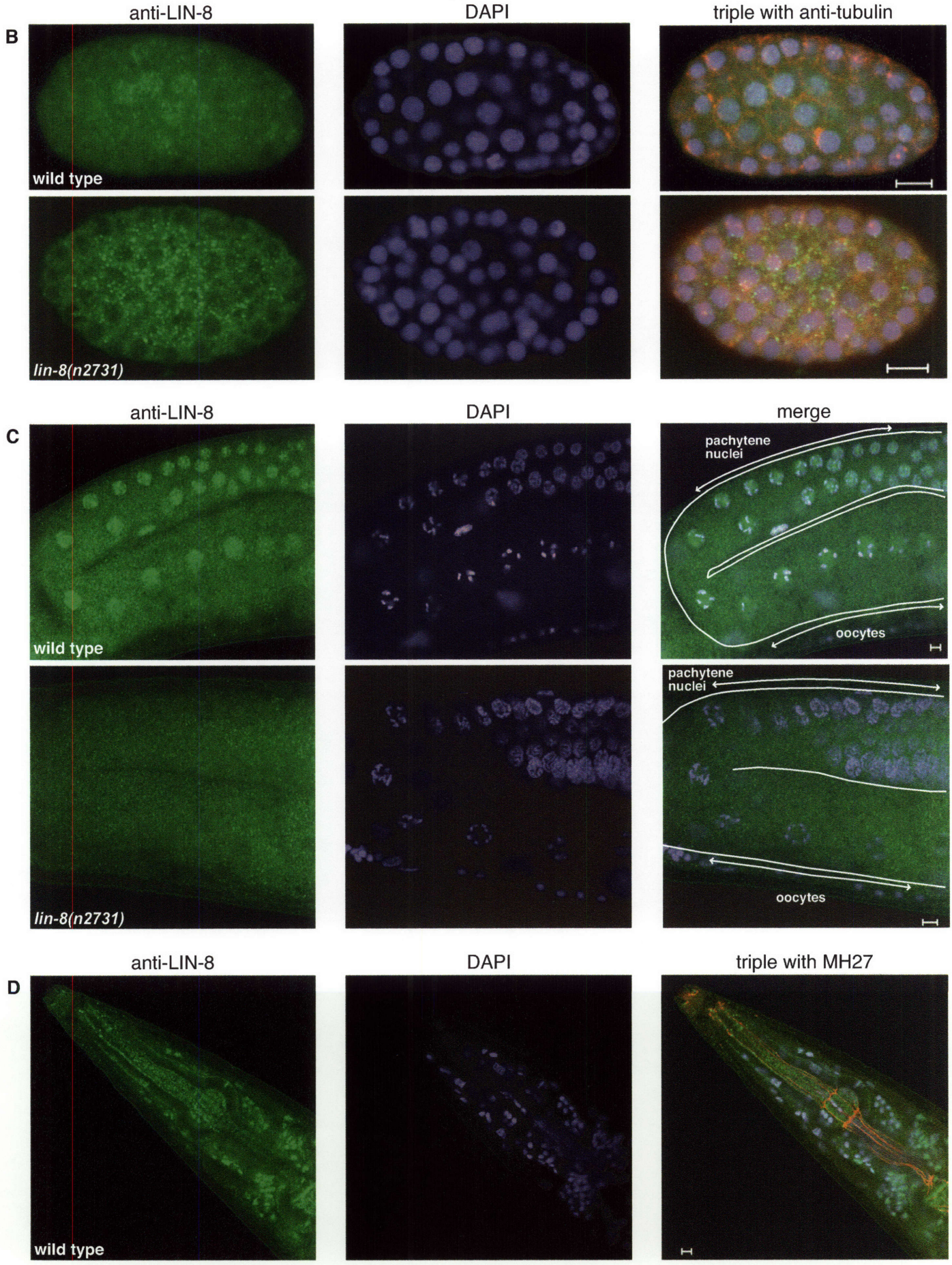

B
anti-LIN-8
DAPI
triple with anti-tubulin
wild type
lin-8(n2731)
C
anti-LIN-8
DAPI
merge
pachytene nuclei
oocytes
wild type
lin-8(n2731)
D
anti-LIN-8
DAPI
triple with MH27
wild type

Figure 4. LIN-8 interacts with LIN-35 Rb *in vitro*.

(A) LIN-35 Rb (aa 270-961) but neither LIN-35 Rb (aa 1-155) nor luciferase interacts with full-length MBP-LIN-8 fusion protein. None of the constructs interacts with MBP alone. 20% of the ^{35}S-labeled proteins used in the binding reactions are shown. Coomassie blue staining indicates that approximately equal amounts of full-length MBP-LIN-8 and MBP were used in the binding reactions. The molecular weights of marker proteins are indicated at the left in kD.

(B) LIN-35 Rb (aa 270-961) but not luciferase interacts with GST-LIN-8 (aa 175-285) and GST-EFL-1. Neither construct binds to GST alone. 20% of the ^{35}S-labeled protein used in the binding reactions are shown. Coomassie blue staining indicates that approximately equal amounts of MBP, GST, and fusion proteins were used in the binding reactions. The molecular weights of marker proteins are indicated at left in kD.

(C) Summary of the LIN-8 fragments used for *in vitro* pull-down experiments and of their ability to interact with LIN-35 Rb (aa 270-961). +, wild-type interaction; +/-, interaction detected but weaker than wild-type; -, no interaction.

Figure 4

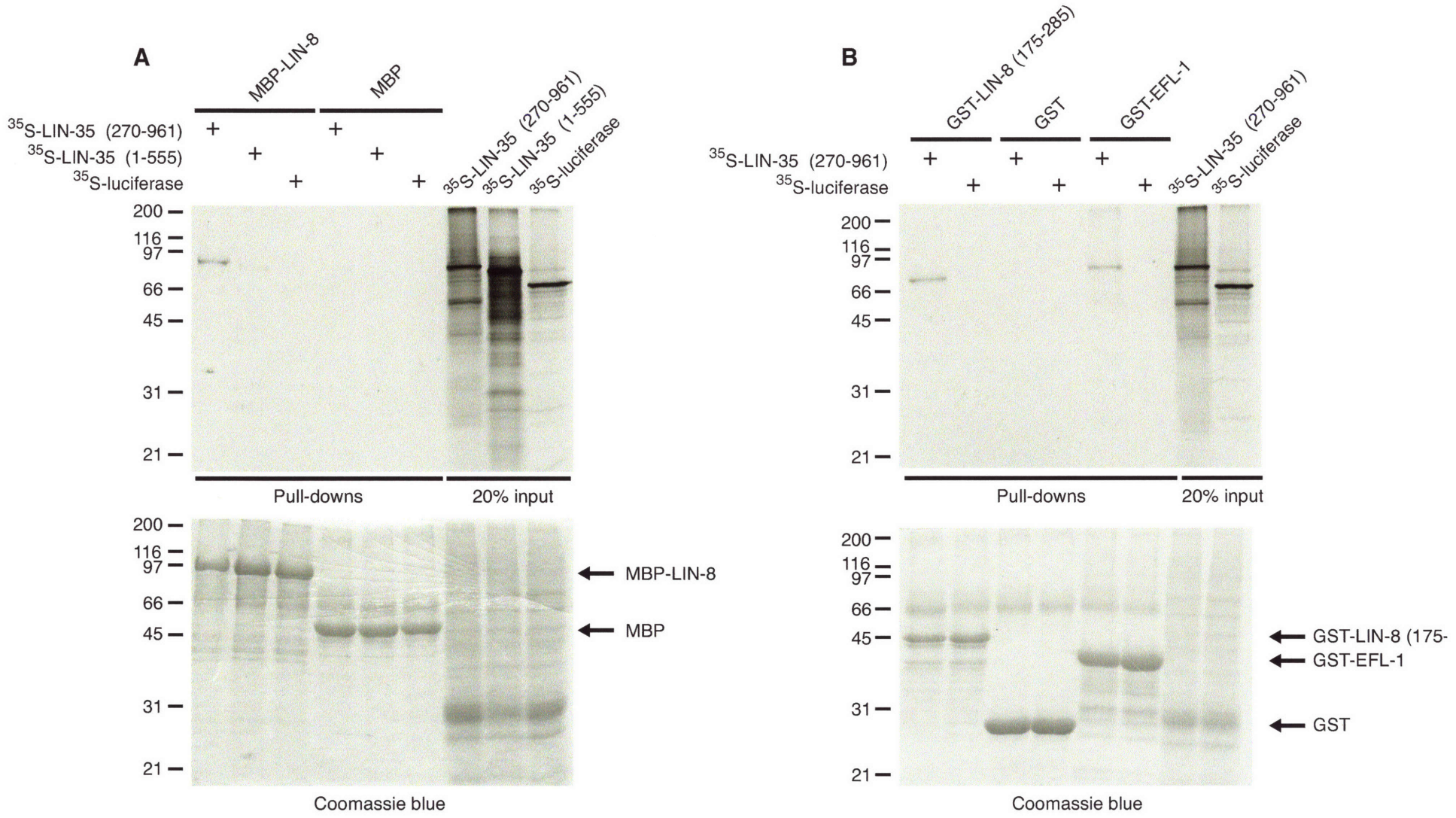
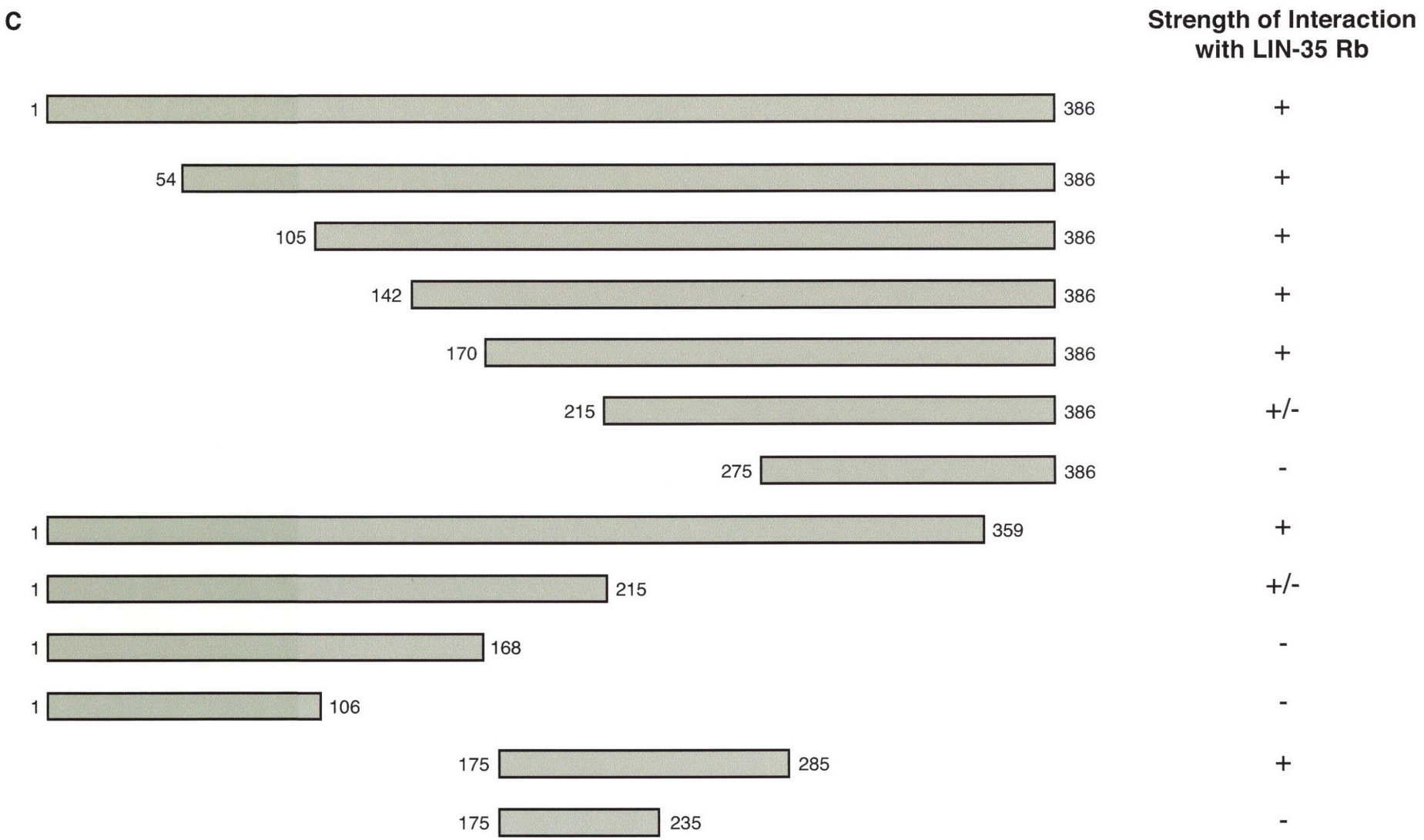

A
MBP-LIN-8
MBP
35S-LIN-35 (270-961)
35S-LIN-35 (1-555)
35S-luciferase
35S-LIN-35 (270-961)
35S-LIN-35 (1-555)
35S-luciferase
200
116
97
66
45
31
21
Pull-downs
20% input
MBP-LIN-8
MBP
Coomassie blue
B
GST-LIN-8 (175-285)
GST
GST-EFL-1
35S-LIN-35 (270-961)
35S-luciferase
GST-LIN-8 (175-
GST-EFL-1
GST
Pull-downs
20% input
Coomassie blue

Figure 4


C
Strength of Interaction with LIN-35 Rb
1
386
+
54
386
+
105
386
+
142
386
+
170
386
+
215
386
+/-
275
386
-
1
359
+
1
215
+/-
1
168
-
1
106
-
175
285
+
175
235
-

CHAPTER 5

Identification and classification of genes that act antagonistically to *let-60* Ras signaling in *Caenorhabditis elegans* vulval development

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My contributions to this chapter include helping to isolate new synMuv mutants and cloning *sli-1* by mapping and transformation rescue.

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ABSTRACT

The synthetic multivulva (synMuv) genes negatively regulate Ras-mediated vulval induction in the nematode *Caenorhabditis elegans.* The synMuv genes define three classes, A, B and C, such that double mutants carrying mutations in genes of any two classes are multivulva. The class B synMuv genes include *lin-35*, a homolog of the retinoblastoma (Rb) tumor suppressor gene, as well as homologs of genes that function with Rb in transcriptional regulation. We screened for additional synMuv mutations using a strategy different from that of previous synMuv genetic screens. Some of the mutations we recovered affect new synMuv genes. We present criteria for assigning synMuv mutations into different genetic classes. We also describe the molecular characterization of the class B synMuv gene *lin-65*.

INTRODUCTION

A fundamental issue in developmental biology is how cells that are initially equivalent in developmental potential ultimately adopt different fates. Genetic studies have indicated that cells within a developmental equivalence group often adopt different fates in response to the combined action of multiple and sometimes competing signals (reviewed by FREEMAN and GURDON 2002). For example, the initial step of R8 photoreceptor specification in ommatidial development in *Drosophila melanogaster* uses both positive and negative signals to properly select presumptive R8 photoreceptors from a field of developmentally equivalent cells in the eye imaginal disc (reviewed by FRANKFORT and MARDON 2002). An overlay of such signals can make a response in binary cell-fate decisions more precise or can increase the number of fates available to a particular cell.

Vulval development in the nematode *Caenorhabditis elegans* involves a set of ectodermal Pn.p cells that initially have similar developmental potentials but ultimately adopt different fates (KIMBLE 1981; STERNBERG and HORVITZ 1986). The specification of Pn.p cells that eventually make vulval tissue occurs in two steps, each of which involves the selection of a subset of Pn.p cells from a larger Pn.p field (SULSTON and HORVITZ 1977). First, in the L1 larval stage shortly after the 12 Pn.p cells are generated, the P1.p and P2.p anterior and P(9-11).p posterior cells fuse with the syncytial hypodermis. Each of the six remaining unfused midbody cells P(3-8).p has the capacity to adopt a vulval cell fate (STERNBERG and HORVITZ 1986). Second, three of these six cells, P(5-7).p, adopt vulval fates and undergo three rounds of division to generate seven (P5.p and P7.p) or eight (P6.p) descendants. P3.p, P4.p and P8.p adopt non-vulval fates, typically dividing only once to generate two descendants that eventually fuse with the syncytial hypodermis. The decision to adopt vulval cell fates occurs during the L2 and early L3 larval stages and is followed by cell divisions and differentiation in the L3 and L4 larval stages, respectively (KIMBLE 1981; STERNBERG and HORVITZ 1986; FERGUSON *et al.* 1987).

Many genes that control the specification of Pn.p fates have been identified. Some of these genes act in a spatially-restricted fashion to select Pn.p cells for vulval

development. The homeobox gene *lin-39* is expressed in the midbody and regulates the sequential steps of fusion and vulval cell-fate specification of the Pn.p cells in this region (CLARK *et al.* 1993; WANG *et al.* 1993; MALOOF and KENYON 1998). Strong loss-of-function *lin-39* mutations result in ectopic P(3-8).p cell fusion during the L1 stage. In partial loss-of-function *lin-39* mutants, unfused P(5-7).p cells are sometimes observed and often show vulval-to-non-vulval cell-fate transformations (CLARK *et al.* 1993). *lin-39* activity therefore promotes unfused cell fates in the L1 stage and vulval cell fates in the L2 and early L3 stages. Genes in the *let-60* Ras signaling pathway also regulate the specification of Pn.p fates (BEITEL *et al.* 1990; HAN and STERNBERG 1990). In addition to *let-60* Ras, this pathway includes the receptor tyrosine kinase *let-23*, the SH2/SH3 adaptor *sem-5* and the MAP kinase *mpk-1*, all of which are broadly conserved in Ras signaling systems (reviewed by MOGHAL and STERNBERG 2003). The role of *let-60* Ras signaling in the specification of vulval cell fates is well characterized. In wild-type animals, the *let-60* Ras pathway is specifically activated in P(5-7).p in response to an EGF-like signal, encoded by *lin-3*, that is produced by the neighboring gonadal anchor cell (HILL and STERNBERG 1992). Mutations that reduce *let-60* Ras pathway activity prevent P(5-7).p vulval cell-fate specification, resulting in a vulvaless (Vul) phenotype. Mutations that abnormally activate this pathway cause P(3-8).p all to adopt vulval cell fates, resulting in a multivulva (Muv) phenotype (BEITEL *et al.* 1990; HAN and STERNBERG 1990; EISENMANN and KIM 1997). Increases in *let-60* Ras pathway activity may promote vulval cell fates in part by upregulating *lin-39* expression (MALOOF and KENYON 1998).

The activities of *lin-39* and genes in the *let-60* Ras pathway are antagonized by the synthetic multivulva (synMuv) genes. The synMuv genes define three redundant classes, A, B and C (FERGUSON and HORVITZ 1989; THOMAS *et al.* 2003; CEOL and HORVITZ 2004). Animals carrying mutations affecting any two classes of synMuv genes are Muv, but animals with a mutation in one synMuv gene or in multiple synMuv genes of a single class undergo wild-type vulval development. All three classes of genes promote the expression of non-vulval cell fates by P(3-8).p. At present it is unknown whether the synMuv mutations cause an increase of *let-60* Ras pathway activity in these cells or cause these cells to be more sensitive to normal levels of *let-60* Ras pathway

activity. Roles for synMuv genes in regulating Pn.p fusion have also been described. Some class B genes, but no class A genes, antagonize *lin-39*-mediated cell fusion of at least one Pn.p cell, P3.p (CHEN and HAN 2001).

Many synMuv genes have been molecularly characterized. The class B synMuv protein LIN-35 is similar to the mammalian tumor suppressor pRb (LU and HORVITZ 1998). Other class B synMuv proteins include DPL-1 and EFL-1, which are similar to mammalian DP and E2F proteins and, by analogy to their mammalian counterparts, likely function to target LIN-35 Rb to DNA (CEOL and HORVITZ 2001). The class B synMuv protein HDA-1 is similar to class I histone deacetylases (LU and HORVITZ 1998) and may be targeted to specific genes by a DPL-1/EFL-1/LIN-35-containing protein complex.

As *lin-35* Rb can act in the surrounding hypodermis to regulate P(3-8).p fates, the genes targeted by a DPL-1/EFL-1/LIN-35-containing complex may function non-cell autonomously to regulate the specification of vulval cell fates (MYERS and GREENWALD 2005). Other class B synMuv proteins also are components of this complex (M.M.H. and H.R.H., unpublished observations), and complexes purified from *Drosophila* extracts containing DP, E2F and Rb homologs contain homologs of the synMuv proteins LIN-9, LIN-37, LIN-52, LIN-53 RbAp48, LIN-54 and LIN-61 (KORENJAK, *et al.* 2004; LEWIS *et al.* 2004). These *Drosophila* complexes can repress transcription of DP/E2F target genes and can inhibit genome-wide DNA replication in ovarian somatic follicle cells.

The class C synMuv genes encode components of a putative histone acetyltransferase complex similar to the human Tip60 and yeast NuA4 histone acetyltransferase complexes (CEOL and HORVITZ 2004). The molecular identities of class B and class C synMuv genes suggest that chromatin remodeling and modification are important in specifying P(3-8).p fates. The class A synMuv gene *lin-15A* encodes a novel protein (CLARK *et al.* 1994; HUANG *et al.* 1994). Little is known about the mechanism of action of the class A synMuv genes.

Previous synMuv genetic screens required that mutant isolates be fertile for the recovery of synMuv mutations. We used a screening approach that allowed the recovery of synMuv mutations that cause recessive sterility. We describe the

characterization of new synMuv mutations and criteria used to distinguish new and previously described classes of synMuv genes.

MATERIALS AND METHODS

Strains and general techniques: Strains were cultured as described by BRENNER (1974) and grown at 20°C unless otherwise indicated. The wild-type parent of all *Caenorhabditis elegans* strains described in this study was the Bristol strain N2, except that some multi-factor mapping experiments used the polymorphic wild-type strains RW7000 (WILLIAMS *et al.* 1992) and CB4856 (WICKS *et al.* 2001). We also used strains containing the following mutations:

LGI: *bli-3(e767)*, *lin-17(n677)*, *unc-11(e47)*, *unc-73(e936)*, *lin-44(n1792)*, *unc-38(x20)*, *dpy-5(e61)*, *lin-35(n745)*, *unc-13(e1091)*, *lin-53(n833)* (FERGUSON and HORVITZ 1989), *unc-54(e1092)* (DIBB *et al.* 1985).

LGII: *lin-31(n301)*, *dpy-10(e128)*, *tra-2(q276)*, *rol-6(e187)*, *dpl-1(n2994)* (CEOL and HORVITZ 2001; THOMAS *et al.* 2003), *let-23(sy10, sy97)*, *unc-4(e120)*, *unc-53(n569)*, *mex-1(it9)*, *rol-1(e91)*, *lin-38(n751)*.

LGIII: *dpy-17(e164)*, *lon-1(e185)*, *lin-13(n770)* (FERGUSON and HORVITZ 1989), *lin-37(n758)*, *lin-36(n766)*, *unc-36(e251)*, *lin-9(n112)*, *unc-32(e189)*, *lin-52(n771)* (FERGUSON and HORVITZ 1989), *dpy-18(e364)*.

LGIV: *lin-1(e1275)*, *unc-5(e53)*, *unc-24(e138)*, *mec-3(e1338)*, *lin-3(n378)*, *sem-3(n1900)* (M.J. STERN and H.R.H., unpublished results), *dpy-20(e1282)*, *unc-22(e66)*, *dpy-26(n198)*, *ark-1(sy247)* (HOPPER *et al.* 2000), *unc-31(e169)*, *unc-30(e191)*, *lin-54(n2231)* (THOMAS *et al.* 2003), *dpy-4(e1166)*.

LGV: *tam-1(cc567)* (HSIEH *et al.* 1999), *unc-46(e177)*, *let-418(s1617)*, *dpy-11(e224)*, *rol-4(sc8)*, *unc-76(e911)*, *efl-1(n3318)* (CEOL and HORVITZ 2001), *dpy-21(e428)*.

LGX: *egl-17(e1313)*, *sli-1(sy143)*, *aex-3(ad418)*, *unc-1(e1598 n1201)* (PARK and H.R.H., unpublished results), *dpy-3(e27)*, *gap-1(ga133)* (HAJNAL *et al.* 1997), *unc-2(e55)*, *lon-2(e678)*, *unc-10(e102)*, *dpy-6(e14)*, *unc-9(e101)*, *unc-3(e151)*, *lin-15B(n744)*, *lin-15A(n767)*, *lin-15AB(n765)*.

Unless otherwise noted, the mutations used are described by RIDDLE (1997). In addition, we used strains containing the following chromosomal aberrations: *mnDf57 II* (SIGURDSON *et al.* 1984), *mnDf90 II* (SIGURDSON *et al.* 1984), *mnDf29 II* (SIGURDSON *et al.* 1984), *mnDf87 II* (SIGURDSON *et al.* 1984), *mIn1[dpy-10(e128) mIs14] II* (EDGLEY and

RIDDLE 2001), *mnC1[dpy-10(e128) unc-52(e444)] II* (HERMAN 1978), *nDf40 III* (HENGARTNER *et al.* 1992), *qC1[dpy-19(e1259) glp-1(q339)] III* (AUSTIN and KIMBLE, 1989), *sDf63 IV* (CLARK and BAILLIE 1992), *sDf62 IV* (CLARK and BAILLIE 1992), *sDf10 IV* (ROGALSKI *et al.* 1982), *hT2[qIs48] (I;III)* (L. MATHIES and J. KIMBLE, personal communication), *eT1(III;V)* (ROSENBLUTH and BAILLIE 1981), *nT1(IV;V)* (FERGUSON and HORVITZ 1985), *nT1(n754) (IV;V)*, *nT1[qIs51] (IV;V)* (L. MATHIES and J. KIMBLE, personal communication). *n754* causes a dominant Unc phenotype, allowing *nT1(n754)*-containing larvae and adults to be scored (E.L. FERGUSON and H.R.H., unpublished results). *mIs14*, an integrated transgene linked to the chromosomal inversion *mIn1* (EDGLEY and RIDDLE 2001), and *qIs48* and *qIs51*, integrated transgenes linked to the reciprocal translocations *hT2(I;III)* and *nT1(IV;V)*, respectively (L. MATHIES and J. KIMBLE, personal communication), consist of GFP-expressing transgenes that allow *mIs14*, *qIs48* or *qIs51*-containing animals to be scored beginning at the 4-cell stage of embryogenesis.

Isolation of new alleles: We mutagenized *lin-15A(n767)* hermaphrodites with ethyl methanesulfonate (EMS) as described by BRENNER (1974). We allowed these animals to recover on food for between 15 minutes and one hour and then transferred individual P_0 larvae in L4 lethargus to 50 mm Petri plates. After three to five days, 20 F_1 L4 larvae per P_0 were individually transferred to 50 mm plates, and F_2 animals on these plates were subsequently screened for a Muv phenotype. We screened the progeny of 3380 F_1 animals using this procedure.

Linkage group assignment: We mapped newly isolated synMuv mutations to linkage groups using standard methods (BRENNER 1974), except for some mutations that we mapped using the polymorphisms present in the wild-type strain RW7000 (WILLIAMS *et al.* 1992).

Complementation tests: We performed complementation tests as described by FERGUSON and HORVITZ (1989). Hemizygous *lin-15B(n3711) lin-15A(n767)* males could not mate. To perform complementation tests with this mutation, we mated *tra-2(q276); lin-15B(n3711) lin-15A(n767)*/++ XX males with marked *lin-15AB* hermaphrodites and scored cross progeny.

Construction of deficiency heterozygotes: To construct *trr-1(n3712)* heterozygotes with the *mnDf57*, *mnDf90* and *mnDf29* deletions, *Df/mIn1; lin-15A(n767)* males were generated. These males were mated with *rol-6 trr-1(n3712)/mIn1; lin-15A(n767)* hermaphrodites, and non-Rol, non-Gfp cross-progeny were scored. *mnDf87* heterozygous males do not mate, so in this case we generated *trr-1(n3712)/mnDf87; lin-15A(n767)* animals by mating *trr-1(n3712)/mIn1; lin-15A(n767)* males with *unc-4 mnDf87/mIn1; lin-15A(n767)* hermaphrodites. *mep-1/Df* animals were constructed by mating *Df/nT1; +/nT1* males with *dpy-20 mep-1; lin-15A(n767)* hermaphrodites and scoring non-Dpy cross-progeny.

Construction of single mutant and unlinked double mutant strains: The synMuv mutations listed below were balanced in *trans* by the specified double mutant combinations or chromosomal aberrations in constructing strains with a single synMuv mutation or strains carrying two unlinked synMuv mutations:
lin-65(n3441): *bli-3(e767) lin-17(n677), hT2[qIs48] (I;III)*
lin(n3628): *unc-11(e47) dpy-5(e61), hT2[qIs48] (I;III)*
lin-35(n745): *dpy-5(e61) unc-13(e1091), hT2[qIs48] (I;III)*
trr-1(n3712): *mIn1[dpy-10(e128) mIs14]*
lin-38(n751): *mnC1[dpy-10(e128) unc-52(e444)]*
mep-1(n3703): *dpy-20(e1282) unc-30(e191), nT1 n754 (IV;V), nT1[qIs51] (IV;V)*
ark-1(n3701): *dpy-20(e1282) unc-30(e191), nT1 n754 (IV;V), nT1[qIs51] (IV;V)*
mys-1(n3681): *unc-46(e177) dpy-11(e224), nT1 n754 (IV;V), nT1[qIs51] (IV;V)*
let-23(sy97) was balanced with *mIn1[dpy-10(e128) mIs14]*.

A *sli-1* single mutant was constucted by generating + *sli-1* + *lin-15A / egl-17* + *unc-1* + hermaphrodites and identifying non-mutant progeny that segregated only Egl Unc non-Muv and non-Egl non-Unc non-Muv animals. Non-Egl non-Unc non-Muv animals were isolated, and *sli-1* homozygotes were identified as those that did not segregate Egl Unc non-Muv progeny. Double mutant strains containing an X-linked mutation in either *sli-1*, *gap-1*, *lin-15A* or *lin-15B* and an autosomal mutation were constructed essentially as described by FERGUSON and HORVITZ (1989).

To ensure that mutations were not lost by recombination, several independent lines were isolated for each strain. Some double mutant strains that exhibited a strong synMuv phenotype were constructed based on their Muv phenotype without the use of balancers.

Construction of linked double mutant strains: To construct an *n3628 lin-35* double mutant, hermaphrodites of genotype *n3628 dpy-5* + + /+ + *lin-35 unc-13; lin-15A* were generated. Muv non-Dpy non-Unc progeny that segregated only Muv non-Dpy non-Unc, Muv Dpy non-Unc and Muv Unc non-Dpy animals were selected. Muv Unc non-Dpy animals of the genotype *n3628 lin-35 unc-13; lin-15A* were isolated, and the *lin-15A* mutation was crossed out using *unc-11 dpy-5* as a balancer.

To construct a *sli-1 lin-15B* double mutant, + *sli-1* + *lin-15A / egl-17* + *unc-1 lin-15AB* hermaphrodites were generated. Muv non-Egl non-Unc progeny that segregated only Muv non-Egl non-Unc and Muv Egl Unc animals were selected. Muv non-Egl non-Unc animals of the genotype *sli-1 lin-15AB* were isolated. From these animals, + *sli-1* + *lin-15AB / egl-17* + *unc-1 lin-15B* animals were generated. non-Muv non-Egl non-Unc progeny that segregated only non-Muv non-Egl non-Unc and Egl Unc non-Muv animals were identified, and non-Muv non-Egl non-Unc animals of the genotype *sli-1 lin-15B* were isolated. A *gap-1 lin-15B* double mutant was similarly constructed using *dpy-3 unc-2* as a balancer.

A *sli-1 gap-1* double mutant was constructed by generating *sli-1* + *dpy-3* + /+ *unc-1* + *gap-1* hermaphrodites and individually isolating non-Dpy non-Unc progeny. Progeny that segregated only non-Dpy non-Unc and Dpy non-Unc animals were identified, and non-Dpy non-Unc animals of the genotype *sli-1 gap-1* were subsequently isolated.

Because *trr-1(n3712)* and *let-23(sy97)* cause recessive sterility and highly penetrant larval lethality, respectively, we could not isolate *trr-1* or *let-23* homozygotes in our construction of a *trr-1 let-23* double mutant. For this reason, we built this double mutant by first generating + *rol-6* + *trr-1 / let-23* + *unc-4* +; *lin-15A* males and mating them with *mIn1[dpy-10(e128) mIs14]; lin-15A* hermaphrodites. non-Dpy cross progeny were individually isolated. non-Dpy progeny with broods consisting of dead larvae, Vul

Unc Gro non-Muv non-Rol non-Gfp and Gfp non-Vul non-Unc non-Gro non-Rol animals were identified. The presence of *trr-1* in these broods, as judged by the *trr-1*-associated growth-rate abnormality (Gro), was later confirmed by complementation testing. *lin-15A* was crossed out to generate a *let-23 unc-4 trr-1 / mIn1[dpy-10(e128) mIs14]* strain.

Assay for P(3-8).p vulval cell fates: Cell fates were scored in L4 hermaphrodites using Nomarski microscopy by counting the number of descendants that had been produced by individual P(3-8).p cells. Scores of 1, 0.5 and 0, were assigned to cells that fully, partially, or not at all adopted vulval cell fates, respectively. P(3-8).p cells that partially adopt a vulval cell fate have one daughter that divides to produce two to four descendants and another daughter that remains undivided (AROIAN and STERNBERG 1991)

RNA-mediated interference: Templates for *in vitro* transcription reactions were made by PCR amplification of cDNAs and their flanking T3 and T7 promoter sequences. *In vitro* transcribed RNA was denatured for 10 minutes and subsequently annealed prior to injection.

***lin-65* rescue:** Using Gateway *in vitro* recombination technology (Invitrogen), we cloned the open reading frame encoding the 728 amino acid LIN-65 protein from a pENTR201*lin-65* entry clone into the pMB1 and pMB7 destination vectors. pMB1 and pMB7 (kindly provided by M. Boxem and S. van den Heuvel) are designed to express inserted sequences under the control of the *C. elegans* heat-shock protein promoters P_{hsp}16-2 and P_{hsp}16-41, respectively. We performed transformation rescue (MELLO *et al.* 1991) using the green fluorescent protein-expressing plasmid pTG96 (kindly provided by Min Han) as a coinjection marker. Transgenic animals were heat-shocked as L1 and L2 larvae for one hour at 33°C and scored as adults. Control transgenic animals were not heat-shocked.

Allele sequence: We used PCR-amplified regions of genomic DNA as templates in determining gene sequences. For each gene investigated, we determined the sequences of all exons and splice junctions. Whenever observed, the sequence of a mutation was confirmed using an independently-derived PCR product. All sequences were determined using an automated ABI 373 DNA sequencer (Applied Biosystems).

RESULTS

Isolation of new synMuv mutants: A severe reduction of class B synMuv gene function is often associated with sterility: 1) In a genetic screen for alleles that did not complement the synMuv phenotype of *lin-9(n112)*, FERGUSON and HORVITZ (1989) recovered two *lin-9* alleles, *n942* and *n943*, that caused recessive sterility. 2) Gene-dosage studies indicate that, in comparison to the wild type, *lin-52(n771)/Df* and *dpl-1(n2994)/Df* heterozygotes have markedly reduced brood sizes (CEOL and HORVITZ 2001; THOMAS *et al.* 2003). 3) Deletion mutations of some synMuv genes recovered using a PCR-based screening approach show recessive sterility, *e.g.*, mutations of *lin-53* (LU 1999), *efl-1* and *dpl-1* (CEOL and HORVITZ 2001).

Previous genetic screens for synMuv mutants (FERGUSON and HORVITZ 1989; THOMAS *et al.* 2003) were performed before a connection between loss of synMuv gene function and sterility was well established. These screens required that isolates be fertile and viable for the recovery of mutant alleles and failed to recover mutations of the class B synMuv genes *efl-1* and *let-418*, both of which can mutate to cause a sterile phenotype (VON ZELEWSKY *et al.* 2000; CEOL and HORVITZ 2001). These results suggested that additional synMuv genes might be identified in a screen that allowed the recovery of homozygous sterile mutations.

To screen for new synMuv mutants, we examined the progeny of individual F_1 animals after EMS mutagenesis of their *lin-15A(n767)* parents. We screened the progeny of 3380 F_1 animals (6760 haploid genomes) for mutations that either alone or in combination with *lin-15A(n767)* caused a recessive Muv phenotype. Mutations that caused recessive sterility in addition to a Muv phenotype were recovered from their heterozygous wild-type siblings present on the same Petri plate. Using this strategy we identified 95 Muv mutations, 24 of which we maintained as heterozygotes because of a recessive sterility that cosegregated with the Muv phenotype. Three mutations caused a Muv phenotype in the absence of *lin-15A(n767)* and were found to affect the previously studied genes *lin-1* and *lin-31*, both of which function downstream of *let-60* Ras in vulval induction (FERGUSON *et al.* 1987). These mutations, *lin-1(n3443)*, *lin-1(n3522)* and *lin-31(n3440)*, were not characterized further. Thirty mutations when in

combination with *lin-15A(n767)* caused a weakly penetrant (< 30%) Muv phenotype. We were unable to convincingly map these mutations to linkage groups. The remaining 62 mutations were assigned to 20 complementation groups (see below). Five of these mutations affect the synMuv gene *lin-61* and will be described elsewhere (M.M.H., X. Lu and H.R.H., manuscript in preparation).

Phenotypes of new mutants: We characterized the penetrance of the Muv phenotype of each strain at 15°C and 20°C (Table 1). At 25°C the penetrance of each strain was between 98 and 100% (n≥25), except for *gap-1(n3535); lin-15A(n767)* (91%, n=111) and *lin(n3542) lin-15A(n767)* (90%, n=42). Since a heat-sensitive Muv phenotype is characteristic of most synMuv strains, including those with null mutations in synMuv genes, it is likely that many individual synMuv mutations are not temperature-sensitive but rather that the synMuv genes regulate a temperature-sensitive process (FERGUSON and HORVITZ 1989).

As described in Table 1, many of these synMuv strains also exhibited a sterile phenotype. In these strains, the sterile phenotype cosegregated with the Muv phenotype during backcrosses and two- and three-factor mapping experiments. For *efl-1*, *let-418* and certain *lin-9* and *lin-53* mutations, we found that our new mutations did not complement the sterile phenotypes caused by previously isolated allelic synMuv mutations (data not shown). Mutations defining new synMuv loci likewise failed to complement each other for the sterile phenotype: *mep-1(n3702)* did not complement *mep-1(n3703)*, and none of the other five *trr-1* mutations complemented *trr-1(n3712)* for the sterile phenotype. These observations indicate that the sterile and Muv phenotypes of these strains were caused by the same mutation.

New synMuv genes: Using two-factor crosses and X chromosome transmission tests (see MATERIALS AND METHODS), we mapped the new mutations to linkage groups. We then determined if each mutation failed to complement mutations in known synMuv genes on the same linkage group. In these tests we identified 41 alleles of known synMuv genes: one *dpl-1*, one *efl-1*, seven *let-418*, three *lin-9*, four *lin-13*, ten *lin-15B*, two *lin-35*, three *lin-36*, one *lin-52*, four *lin-53* and five *lin-61* mutations. We isolated one mutation in *gap-1* and three in *sli-1*, two genes that were originally identified in screens

for mutations that suppress the Vul phenotype caused by a reduction in *let-60* Ras pathway signaling (JONGEWARD *et al.* 1995; HAJNAL *et al.* 1997). We also identified two mutations in *ark-1*, a gene first identified in a screen for mutations that cause ectopic vulval cell fates in a *sli-1* mutant background (HOPPER *et al.* 2000). *gap-1*, *sli-1* and *ark-1* single mutants were previously found to have no (*sli-1*, *gap-1*) or subtle (*ark-1*) defects in vulval development. Our results indicate that *sli-1*, *gap-1* and *ark-1* act redundantly with *lin-15A* to negatively regulate *let-60* Ras signaling.

Mutations that were not assigned to known synMuv complementation groups were tested against unassigned mutations on the same linkage group for complementation. These tests defined five new synMuv loci: *lin-65*, *lin(n3628)*, *mep-1*, *mys-1* and *trr-1*. (*lin(n3542)* may define another new synMuv locus, but since we have not separated *lin(n3542)* from *lin-15A(n767)*, we do not know whether *lin(n3542)* is a synMuv mutation or whether it causes a Muv phenotype on its own.) We used multi-factor crosses (Table 2) and deficiency heterozygotes (Table 3) to map these new synMuv genes on their respective linkage groups. While our studies were in progress, *mep-1* and *lin-65* were independently identified and reported to have a loss-of-function synMuv phenotype (UNHAVAITHAYA *et al.* 2002; POULIN *et al.* 2005). Our detailed characterization of the class C synMuv genes *mys-1* and *trr-1* is presented elsewhere (CEOL and HORVITZ 2004). We separated *lin-65*, *lin(n3628)* and *mep-1* mutations from the parental *lin-15A(n767)* mutation and found that these mutations alone do not cause extra vulval cells to be produced (Table 4). Thus, these mutations synergize with *lin-15A(n767)* and are synMuv mutations.

Interactions with other synMuv mutations: Since mutations affecting *lin-65*, *lin(n3628)*, *mep-1*, *gap-1*, *sli-1* and *ark-1* interact synthetically with a class A synMuv mutation, *lin-15A(n767)*, these genes may either be class B or class C synMuv genes or they may define a new synMuv gene class that shares some but not all properties with class B or class C genes. To distinguish between these possiblities, we built double mutant strains and measured synthetic interactions with *lin-65*, *lin(n3628)*, *mep-1*, *gap-1*, *sli-1* and *ark-1* mutations. We used the strongest available mutation for each of these genes in these strain constructions. *ga133* rather than *gap-1(n3535)* was used as the

gap-1 mutation, because *ga133* is a deletion and is considered a null mutation (HAJNAL *et al.* 1997). For the sake of brevity, *gap-1(ga133)* will be referred to as a "new" synMuv mutation hereafter. We quantified synthetic interactions by directly examining the fates of individual P(3-8).p cells (see MATERIALS AND METHODS). In wild-type animals three cells invariably adopt vulval fates, whereas in Muv mutants more than three cells adopt vulval fates.

We first measured synthetic interactions with the class A mutation *lin-38(n751)* and the class B mutations *lin-15B(n744)* and *lin-35(n745)* (Table 4). The new synMuv mutations interacted synthetically not only with *lin-15A(n767)* but also with *lin-38(n751)*, suggesting a general redundancy with the class A synMuv genes. With *lin-15B(n744)* and *lin-35(n745)* the new mutations showed weak to no synthetic interaction.

We also investigated whether the new mutations interacted synthetically with the class C mutation *trr-1(n3712)* (Table 5). In *trr-1(n3712)* single mutants, P8.p adopts a vulval cell fate at a low but detectable penetrance (CEOL and HORVITZ 2004). We monitored synthetic interactions with *trr-1(n3712)* for P(3-8).p but report synthetic effects only for P8.p, as this cell is particularly sensitive to cell-fate transformation. *lin-65(n3441)*, *mep-1(n3703)*, *gap-1(ga133)* and *sli-1(n3538)* but not *lin(n3628)* and *ark-1(n3701)* showed a strong synthetic interaction with *trr-1(n3712)*. In further tests, *ark-1(n3701)* but not *lin(n3628)* interacted synthetically with the class C mutation *mys-1(n3681)*: *ark-1(n3701); mys-1(n3681)* double mutants had a strong synthetic P8.p vulval fate defect (80%, n=41) as compared to *ark-1(n3701)* (0%, n=33) and *mys-1(n3681)* (8.3%, n=36) single mutants, whereas the P8.p vulval-fate defect of *lin(n3628); mys-1(n3681)* (6.7%, n=30) double mutants was low, like that of *lin(n3628)* (0%, n=37) and *mys-1(n3681)* single mutants. Why *ark-1(n3701)* interacted with one class C mutation but not another is unclear. It is possible that the synthetic interaction with *ark-1(n3701)* is sensitive to maternally-provided levels of class C synMuv activity and *mys-1(n3681)*, which can be maintained in homozygous strains, provided less maternal activity than did *trr-1(n3712)*, which because of its recessive sterility requires that homozygotes be generated from heterozygous parents.

Most of the new mutations interacted synthetically with class A and class C but not with class B mutations, which indicates that these new mutations are neither class A nor class C mutations. The synthetic interaction of *lin(n3628)* with class A but not class B or class C mutations is unusual and will be discussed below.

Suppression of *let-23* mutations: Are *lin-65*, *lin(n3628)*, *mep-1*, *gap-1*, *sli-1* and *ark-1* class B synMuv genes? Neither in combination with class A mutations (FERGUSON *et al.* 1987; LU and HORVITZ 1998; THOMAS and HORVITZ 1999; CEOL and HORVITZ 2001) nor on their own (Table 6) do class B mutations suppress the Vul phenotype caused by strong loss-of-function *let-23* receptor tyrosine kinase mutations. However, previous studies showed that *gap-1* or *sli-1* mutations alone can suppress the *let-23* Vul phenotype (JONGEWARD *et al.* 1995; HAJNAL *et al.* 1997). Together these findings distinguish *gap-1* and *sli-1* from class B synMuv genes and indicate that *let-23* suppression may be used as a criterion in classifying synMuv mutations. We found that mutations affecting *lin-65*, *lin(n3628)*, *mep-1* and *ark-1* did not suppress the *let-23* Vul phenotype (Table 6), suggesting that these genes are not in the same class as *gap-1* and *sli-1*.

Interactions with *ark-1*, *gap-1* and *sli-1* mutations: *gap-1* and *sli-1* mutations interact synthetically to produce extra vulval cells (Table 7). Furthermore, an *ark-1* mutation interacts synthetically with these *gap-1* and *sli-1* mutations, suggesting that all three genes act in parallel in regulating vulval cell fates. Similar synergism of an *ark-1* mutation with *gap-1* and *sli-1* mutations was observed previously (HOPPER *et al.* 2000). By contrast, we observed that the class B synMuv mutations *lin-15B(n744)* and *lin-35(n745)* did not interact synthetically with *gap-1* or *sli-1* mutations (Table 4). This lack of synergism is likely not the result of using weak alleles, as the *lin-15B(n744)*, *lin-35(n745)*, *gap-1(ga133)* and *sli-1(n3538)* mutations used in these studies are strong loss-of-function, and possibly null, mutations of their corresponding genes. These results distinguish *ark-1* from the class B genes *lin-15B* and *lin-35* Rb and suggest that these class B genes do not act with *ark-1* in antagonizing Ras pathway activity. *lin-65*, *lin(n3628)* and *mep-1* mutations also did not interact synthetically with *gap-1(ga133)* or

sli-1(n3538) (Table 7), revealing a further similarity between *lin-65*, *lin(n3628)* and *mep-1* mutations and *lin-15B* and *lin-35* Rb class B synMuv mutations.

Molecular identification of *lin-65*: We mapped the synMuv gene *lin-65* to a small interval between the *C. elegans* strain CB4856 polymorphisms *Y71G12B.17* and *Y71G12B.18* (Figure 1A). This interval contains four complete predicted genes, one of which is a microRNA gene, and portions of three other genes, two of which overlap (*C. ELEGANS* SEQUENCING CONSORTIUM 1998). We performed RNA-mediated interference (RNAi) to determine if inactivation of any of the three complete, protein-encoding genes would result in a synMuv phenotype. RNAi of Y71G12B.9 caused a Muv phenotype in a *lin-15A(n767)* but not in a wild-type or *lin-15B(n744)* background (data not shown). POULIN *et al.* independently found that RNAi of Y71G12B.9 caused a synMuv phenotype (2005). We obtained six cDNAs (kindly provided by Yuji Kohara and coworkers) and compared the sequences of these clones with genomic sequence to determine a gene structure for Y71G12B.9 (Figure 1B). One clone had an SL1 and two had an SL2 splice-leader sequence. The presence of an SL2 splice leader suggests that Y71G12B.9 is a downstream gene in an operon (ZORIO *et al.* 1994). The predicted initiator methionine codon of the SL2-spliced Y71G12B.9 cDNAs lies just downstream of the *trans*-splice site. The open reading frame beginning with this initiator methionine encodes a 728 amino acid protein (Figure 2). The SL1 *trans*-splice site is downstream from that of SL2, and the single SL1-spliced cDNA lacks the initiator methionine corresponding to the 728 amino acid predicted protein. The open reading frames defined by the first three potential initiator methionine codons of the SL1-spliced cDNA are all short (≤16 codons). If the fourth potential initiator methionine codon were used, a 691 amino acid protein lacking the first 37 amino acids of the 728 amino acid protein described above would be synthesized. Expression under the control of the *C. elegans* heat-shock promoters of a cDNA predicted to encode the 728 amino acid protein rescued the Muv phenotype of *lin-65* mutants: two transgenic lines of *lin-65(n3441); lin-15A(n767)* mutants containing P_{hs}:*lin-65* transgenes were 0% (n=73) and 2.0% (n=49) non-Muv without heat shock but were 71% (n=68) and 67% (n=30) non-Muv, respectively, following heat-shock treatment.

We determined the sequence of Y71G12B.9 in *lin-65(n3441)*, *lin-65(n3541)* and *lin-65(n3543)* mutants. *lin-65(n3441)* and *lin-65(n3541)* contain identical nonsense mutations predicted to truncate the Y71G12B.9 protein after 533 of the 728 amino acids. It is unlikely that *lin-65(n3441)* and *lin-65(n3541)* were caused by the same mutational event, since they were isolated from independently mutagenized and screened P_0 animals. *lin-65(n3543)* contains a missense mutation that changes a polar serine residue to a non-polar leucine (S720L). The map position, RNAi phenocopy, cDNA rescue data as well as the mutant allele sequences indicate that Y71G12B.9 is *lin-65*.

The 728 amino acid LIN-65 protein is rich in acidic amino acids (Figure 2). Over 7% and 10% of the total number of amino acids are aspartates and glutamates, respectively, and these acidic amino acids are found both in clusters and dispersed throughout LIN-65. BLAST searches (ALTSCHUL *et al.* 1990) with LIN-65 identified proteins from mammalian and other species that are similarly acid-rich. Because the similarity between LIN-65 and these proteins is primarily limited to acidic residues and not to specific protein domains (data not shown), it is difficult to predict whether these proteins are functional orthologs of LIN-65. As described above, mutations in *lin-65* and *lin-35* Rb show similar genetic interactions, suggesting that *lin-65* is a class B synMuv gene that acts in the *lin-35* Rb pathway. Protein complexes purified from *Drosophila* extracts and analogous to a class B synMuv complex (M.M.H. and H.R.H., unpublished observations) have not been reported to contain LIN-65-like proteins (KORENJAK *et al.* 2004; LEWIS *et al.* 2004). It is possible that LIN-65 and LIN-65 orthologs act upstream of class B synMuv and analogous complexes to promote complex activity or act downstream as effectors of these complexes.

Sequences of synMuv mutations: We determined DNA sequences of 41 mutant synMuv genes identified in our screen; four of these mutant genes had two distinct mutations (Table 8). The 41 include all of the *dpl-1*, *efl-1*, *let-418*, *lin-9*, *lin-13*, *lin-36*, *lin-52*, *lin-53*, *lin-65*, *mep-1*, *mys-1*, *sli-1* and *trr-1* alleles and one of two *lin-35* alleles identified in our screen. Forty of 45 mutations are GC-to-AT transitions, which are characteristic of EMS mutagenesis (ANDERSON 1995). Many of these mutations are predicted to truncate the corresponding synMuv proteins. The truncations predicted by

efl-1(n3639), *let-418(n3719)*, *lin-52(n3718)* and *trr-1(n3704)* are particularly severe, and the synMuv and sterile abnormalities caused by these mutations likely represent the null phenotypes of these genes. In addition, we found missense mutations that disrupt predicted functional domains of synMuv proteins. For example, *n3536*, *n3626*, *n3629* and one of the two mutations of *n3636* affect the ATPase/helicase domain of LET-418. LET-418 is a member of the Mi-2 family of ATP-dependent chromatin remodeling enzymes (SOLARI and AHRINGER 2000; VON ZELEWSKY *et al.* 2000), and the LET-418 missense mutations suggest that LET-418 function is dependent on ATP hydrolysis. At least one mutation affecting the LIN-13 protein, *n3642*, is predicted to disrupt a canonical zinc-finger motif. This missense mutation, along with those isolated previously (THOMAS *et al.* 2003), indicate that at least some of the 24 LIN-13 zinc fingers are important for LIN-13 synMuv activity. Missense mutations affecting other synMuv proteins are not as easily linked to the disruption of predicted functional domains. These mutations may provide useful starting points for identifying functional motifs within synMuv proteins that are not predicted by sequence comparisons.

DISCUSSION

Frequency of mutant isolation: The rate at which we isolated synMuv mutations was much higher than that observed in previous screens. Considering screens that were conducted in class A synMuv mutant backgrounds, we recovered one synMuv mutation per 109 haploid genomes screened as compared with one per 750 (FERGUSON and HORVITZ 1989), one per 400 (THOMAS *et al.* 2003) and one per 667 (THOMAS *et al.* 2003) in previous screens. We believe the reasons for this difference are three-fold. First, our screen design allowed the isolation of synMuv mutations that also caused sterility. Numerous sterile synMuv mutants had been observed in previous screens, but in general the mutations responsible were not recovered. Second, our parental strain carried a strong class A mutation, *lin-15A(n767)*. The penetrance of the Muv phenotype of a synMuv strain is dependent on the combined strengths of the individual synMuv mutations (C.J.C. and H.R.H., unpublished observations). Therefore, even weak mutations could be identified in a strong synMuv background such as *lin-15A(n767)*. Such weak mutations may not have been recovered in the three previous screens described above, all of which were performed in partial loss-of-function synMuv backgrounds. Third, by screening Petri plates with many F_2 progeny derived from a single F_1 animal, we observed many genotypically identical animals for each haploid genome screened. Such screening can efficiently recover partially penetrant synMuv mutations.

Of the 62 mutations described in this study, 24 caused recessive sterility. The 38 mutations that did not cause sterility were recovered at one mutation per 178 haploid genomes screened, a frequency higher than that of previous screens. The difference in the rate of recovery of non-sterile mutants is likely a consequence of the second and third differences in screening described above.

Given that the average gene mutates to loss of function at a rate of about $5x10^{-4}$ under the conditions of EMS mutagenesis we used (BRENNER 1974; MENEELY and HERMAN 1979; GREENWALD and HORVITZ, 1980), our observed rate of 10^{-2} suggests that about 20 genes can mutate by loss of function to cause a synMuv phenotype in combination with a class A synMuv mutation. Including the genes we identified in this

study, a total of 25 such genes have been described to date. Three or fewer alleles of 15 of these genes have been recovered in synMuv screens, indicating that screens for such genes are not saturated.

Different synMuv gene classes likely act in parallel to antagonize *let-60* Ras pathway activity: Class A synMuv mutations synergize with class B mutations but not with other class A mutations, whereas Class B synMuv mutations synergize with class A synMuv mutations but not with other class B synMuv mutations. Such genetic behavior led to the hypothesis that the A and B classes of synMuv genes encode components of two functionally redundant pathways that negatively regulate vulval development (FERGUSON and HORVITZ 1989). Consistent with this hypothesis, a subset of class B synMuv gene products have been shown to physically interact and their homologs are known function together in other organisms (LU and HORVITZ 1998; CEOL and HORVITZ 2001; UNHAVAITHAYA *et al.* 2002; KORENJAK *et al.* 2004; LEWIS *et al.* 2004).

Because we conducted our screen using a class A synMuv background, we anticipated recovering mutations that affected class B synMuv genes. Indeed, 47 of the 62 mutations we isolated affected previously known and newly described class B synMuv genes. However, we discovered that some new mutations define new classes of synMuv genes. synMuv mutations previously were categorized by testing for synergism with class A and class B mutations. From such tests we discovered that some of our new mutations synthetically interacted with both class A and class B mutations; such mutations defined the class C genes *trr-1* and *mys-1* (THIS STUDY and CEOL and HORVITZ 2004). Other new mutations interacted like class B mutations in these standard tests but were distinguished from class B mutations by additional tests. For example, like class B mutations *sli-1(n3538)* synthetically interacted with class A but not class B mutations yet, unlike class B mutations synthetically interacted with *ark-1* and *gap-1* and suppresses the *let-23* Vul phenotype. These results led us to adopt two criteria when classifying synMuv mutations: (1) if two mutations synthetically interact to cause a Muv phenotype, then they are in different classes, (2) if two mutations do not synthetically interact but interact differently with other classes of synMuv mutations or with *let-23*, then they are in different classes. Since we have found that interaction tests

with only class A and class B mutations are insufficient to classify some synMuv genes, we suggest that previously described synMuv genes should be tested more extensively to establish their classifications.

Using more extensive genetic interaction tests and additional criteria to interpret these interactions, we define six classes of genes, synMuv A, synMuv B, synMuv C, *gap-1*, *sli-1* and *ark-1*, that seem to act in parallel to each other to negatively regulate Ras-mediated vulval development (Table 9). Some of these classes, such as *gap-1*, *sli-1* and *ark-1*, likely interface directly with Ras pathway components (see below). The point at which the synMuv A, synMuv B and synMuv C classes interface with Ras signaling is unknown.

Different synMuv gene classes control distinct biochemical activities: A synthetic genetic interaction implies functional redundancy between two sets of genes. There are many possible mechanisms by which two sets of genes might appear redundant. These possibilities include: (1) two sets of genes encode similar sets of proteins with corresponding proteins of each set controlling the same biochemical activity, and hence each set controls the same biological process; (2) two sets of genes encode distinct sets of proteins with each set controlling distinct biochemical activities but the same biological process; and (3) two sets of genes encode distinct sets of proteins with each set regulating distinct but redundant biological processes.

The first of these mechanisms likely does not apply to the different classes of synMuv genes, as no cloned gene in one synMuv class is similar to any gene of another class. Furthermore, many of the cloned synMuv genes, including the class A gene *lin-15A*, the class B gene *lin-35* Rb, the class C genes *trr-1* and *epc-1*, and *ark-1* and *sli-1*, encode the sole *C. elegans* member of their respective gene families.

The redundancy exhibited among *sli-1*, *gap-1* and *ark-1* likely exemplifies the second mechanism. *sli-1*, *ark-1* and *gap-1* are thought to directly down-regulate Ras pathway activity, and, as might be predicted based on their synthetic interactions, each is proposed to act upon a different Ras pathway component. *sli-1* encodes a homolog of the c-Cbl proto-oncoprotein, which is thought to down-regulate receptor tyrosine kinase levels though ubiquitin-mediated degradation (YOON *et al.* 1995; LEVKOWITZ *et al.*

1999). *ark-1* encodes a protein that interacts with the SEM-5 SH2/SH3 adaptor protein and is predicted to be a cytoplasmic tyrosine kinase (HOPPER *et al.* 2000). Since *sem-5* acts downstream of the *let-23* receptor tyrosine kinase, *ark-1* is proposed to inhibit *let-60* Ras signaling downstream of *let-23*. *gap-1* is a member of the GTPase-activating protein (GAP) family (HAJNAL *et al.* 1997). GAPs enhance the catalytic function of Ras family GTPases such as *let-60* Ras, thereby facilitating the switch from active GTP-bound to inactive GDP-bound Ras. The genetic suppression of *let-23(sy97)* by and the molecular identities of *sli-1* and *gap-1* support their action downstream of the *let-23* RTK. Although *ark-1* mutations do not suppress *let-23(sy97)*, HOPPER *et al.* (2000) found that an *ark-1* mutation suppressed the Vul phenotypes caused by weaker *let-23* mutations and by *sem-5* mutations. Based on these suppression data and on the molecular data described above, these authors argued that *ark-1* acts downstream of *let-23*, although its negative regulation of the *let-60* pathway may not be as great as that of *sli-1* or *gap-1*. The redundancy displayed by *sli-1*, *gap-1* and *ark-1* suggests that a mutation affecting one of these genes only mildly affects Ras pathway activity whereas mutations affecting two genes elevate pathway activity to a level that inappropriately transforms vulval cell fates. That these genes converge on the same signaling pathway implies that they regulate the same biological process.

The class A, B and C synMuv genes may or may not act similarly. It is possible that these classes act on components of the *let-60* Ras pathway. Since at least some class A and B synMuv genes are thought to act in the hypodermis, an effect on *let-60* Ras signaling is likely indirect (HERMAN and HEDGECOCK 1990; HEDGECOCK and HERMAN 1995; MYERS and GREENWALD 2005). Alternatively, as in the case of the third mechanism, these classes may regulate entirely distinct biological processes. For example, the class B genes, some of which encode components of a putative histone deacetylase complex, may repress transcription of genes that indirectly promote P(3-8).p cell division. By contrast, the class C genes, which encode components of a putative histone acetyltransferase complex, may activate the transcription of genes, different from those targeted by class B genes, that promote differentiation of P(3-8).p descendants into hypodermal and not vulval cells. A better understanding of synMuv

target genes should help to resolve whether different synMuv classes regulate the same or distinct biological activities.

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TABLE 1

Phenotypes of synMuv mutant strains

Genotype	Percent Muv (n)		Additional abnormalities
	15° C	20° C	
ark-1(n3524); lin-15A(n767)	0 (251)	80 (171)	
ark-1(n3701); lin-15A(n767)	12 (190)	95 (160)	
dpl-1(n3643); lin-15A(n767)[a]	99 (154)	100 (252)	
efl-1(n3639); lin-15A(n767)[a]	93 (74)	100 (78)	Ste
gap-1(n3535) lin-15A(n767)	1 (143)	50 (236)	
let-418(n3536); lin-15A(n767)	0 (201)	55 (183)	hs Ste
let-418(n3626); lin-15A(n767)	2 (62)	97 (76)	Ste
let-418(n3629); lin-15A(n767)	0 (52)	86 (58)	Ste
let-418(n3634); lin-15A(n767)	0 (87)	92 (48)	Ste
let-418(n3635); lin-15A(n767)	0 (76)	71 (70)	Ste
let-418(n3636); lin-15A(n767)	0 (77)	92 (78)	Ste
let-418(n3719); lin-15A(n767)	0 (101)	100 (60)	Ste
lin-9(n3631); lin-15A(n767)	100 (42)	100 (72)	Ste
lin-9(n3675); lin-15A(n767)	43 (166)	100 (105)	

lin-9(n3767); lin-15A(n767)	100 (67)	100 (56)	Ste
lin-13(n3642); lin-15A(n767)	3 (60)	100 (63)	Ste
lin-13(n3673); lin-15A(n767)	61 (145)	97 (129)	
lin-13(n3674); lin-15A(n767)	78 (131)	100 (191)	hs Ste
lin-13(n3726); lin-15A(n767)	31 (225)	99 (149)	hs Ste
lin-15B(n3436) lin-15A(n767)	100 (193)	100 (212)	
lin-15B(n3676) lin-15A(n767)	18 (167)	72 (130)	
lin-15B(n3677) lin-15A(n767)	99 (111)	100 (122)	
lin-15B(n3711) lin-15A(n767)	100 (186)	100 (156)	
lin-15B(n3760) lin-15A(n767)	32 (171)	100 (150)	
lin-15B(n3762) lin-15A(n767)	63 (113)	97 (116)	
lin-15B(n3764) lin-15A(n767)	96 (232)	100 (199)	
lin-15B(n3766) lin-15A(n767)	55 (132)	100 (173)	
lin-15B(n3768) lin-15A(n767)	80 (159)	100 (302)	
lin-15B(n3772) lin-15A(n767)	100 (220)	100 (191)	
lin-35(n3438); lin-15A(n767)	100 (153)	100 (126)	partial Ste at 20°C, Rup
lin-35(n3763); lin-15A(n767)	100 (108)	100 (160)	partial Ste at 20°C, Rup
lin-36(n3671); lin-15A(n767)	65 (191)	100 (151)	

lin-36(n3672); lin-15A(n767)	98 (198)	100 (178)	
lin-36(n3765); lin-15A(n767)	0 (184)	37 (202)	
lin-52(n3718); lin-15A(n767)[b]	100 (41)	100 (82)	Ste
lin-53(n3448); lin-15A(n767)	67 (130)	100 (211)	partial Ste at 20°C
lin-53(n3521); lin-15A(n767)	100 (34)	100 (125)	partial Ste at 20°C
lin-53(n3622); lin-15A(n767)	85 (61)	100 (66)	Ste
lin-53(n3623); lin-15A(n767)	24 (55)	100 (51)	Ste
lin-65(n3441); lin-15A(n767)	80 (165)	99 (195)	
lin-65(n3541); lin-15A(n767)	79 (242)	98 (137)	
lin-65(n3543); lin-15A(n767)	85 (177)	100 (121)	
lin(n3628); lin-15A(n767)	3 (103)	84 (188)	
lin(n3542) lin-15A(n767)	0 (127)	35 (218)	
mep-1(n3680); lin-15A(n767)	5 (122)	97 (105)	hs Ste
mep-1(n3702); lin-15A(n767)	30 (61)	100 (141)	Ste
mep-1(n3703); lin-15A(n767)	25 (72)	100 (107)	Ste
mys-1(n3681); lin-15A(n767)[c]	0 (214)	72 (192)	
sli-1(n3538) lin-15A(n767)	4 (138)	90 (173)	
sli-1(n3544) lin-15A(n767)	5 (153)	80 (265)	cs embryonic lethality

sli-1(n3683) lin-15A(n767)	5 (80)	88 (148)	cs embryonic lethality
trr-1(n3630); lin-15A(n767)[c]	3 (131)	85 (212)	Ste, Gro
trr-1(n3637); lin-15A(n767)[c]	1 (92)	80 (200)	Ste, Gro
trr-1(n3704); lin-15A(n767)[c]	3 (96)	79 (244)	Ste, Gro
trr-1(n3708); lin-15A(n767)[c]	2 (151)	84 (228)	Ste, Gro
trr-1(n3709); lin-15A(n767)[c]	1 (97)	77 (154)	Ste, Gro
trr-1(n3712); lin-15A(n767)[c]	6 (121)	77 (192)	Ste, Gro

The penetrance of the Muv phenotype was determined after synMuv mutant strains grew at the indicated temperature for two or more generations. For most strains for which a fully penetrant sterile phenotype was associated with the Muv phenotype, we scored the penetrance of the Muv phenotype by examining sterile progeny of heterozygous mutant parents. For *trr-1* mutant strains, we scored the penetrance of the Muv phenotype by examining non-Gfp progeny of *trr-1 / mIn1[dpy-10(e128)mIs14]; lin-15A(n767)* heterozygous parents. All strains were backcrossed to *lin-15A(n767)* twice prior to phenotypic characterization. In addition to the phenotypes described above, many of the strains exhibited heat-sensitive inviability as a consequence of rupture and/or general sickness. Ste, sterile; Gro, growth rate abnormal; Rup, rupture at the vulva; cs, cold-sensitive; hs, heat-sensitive. The characterization of some of these strains was previously

described by: [a](CEOL and HORVITZ 2001); [b](THOMAS *et al.* 2003); [c](CEOL and HORVITZ 2004).

TABLE 2

Three- and four-factor crosses

Gene	Genotype of heterozygote	Phenotype of selected recombinants	Genotype of selected recombinants (with respect to unselected markers)
lin-65	+ *lin-65* + / *bli-3* + *lin-17; lin-15A(n767)*	Lin-17	9/19 *lin-65* /+
	bli-3 + *lin-65* /+ *spe-15* +; *lin-15A(n767)*	Muv	10/18 *spe-15* /+
	+ *lin-65 lin-17* / *spe-15* + +; *lin-15A(n767)*	Lin-17	11/11 *spe-15* /+
	bli-3 + + + *lin-65* + /+ *Y73E7.2 Y71G12B.2 Y71G12B.17* + *Y71G12B.18; lin-15A(n767)*	Muv	4/30 *Y73E7.2* /+
		Muv	2/30 *Y71G12B.2* /+
		Muv	1/30 *Y71G12B.17* /+
		Muv	0/30 *Y71G12B.18* /+
	+ *lin-65* + + + *lin-17* / *Y71G12B.17* + *Y71G12B.18* *Y71G12B.27 M01D7.2* +; *lin-15A(n767)*	Lin-17	17/23 *M01D7.2* /+
		Lin-17	18/23 *Y71G12B.27* /+
		Lin-17	21/23 *Y71G12B.18* /+
		Lin-17	23/23 *Y71G12B.17* /+
lin(n3542)	+ + *lin(n3542) lin-15A(n767)* / *unc-10 dpy-6* + *lin-15A(n767)*	Unc	8/8 *lin(n3542)* /+
	+ *lin(n3542)* + *lin-15A(n767)* / *dpy-6* + *unc-9 lin-15A(n767)*	Unc	4/40 *lin(n3542)* /+
lin(n3628)	*lin(n3628)* + + /+ *dpy-5 unc-13; lin-15A(n767)*	Dpy	0/6 *lin(n3628)* /+
		Unc	6/6 *lin(n3628)* /+

	+ *lin(n3628)* + / *unc-11* + *dpy-5; lin-15A(n767)*	Unc	1/11 *lin(n3628)* /+
		Dpy	5/11 *lin(n3628)* /+
	unc-11 + + *lin(n3628)* /+ *unc-73 lin-44* +; *lin-15A(n767)*	Muv	3/9 *unc-73 lin-44* /+ +
	+ + *lin(n3628) dpy-5* / *unc-73 lin-44* + +; *lin-15A(n767)*	Muv	0/21 *unc-73 lin-44* /+ +
	lin(n3628) + *dpy-5* /+ *unc-38* +; *lin-15A(n767)*	Muv	3/7 *unc-38* /+
	unc-11 lin(n3628) + /+ + *unc-38; lin-15A(n767)*	Muv	0/9 *unc-38* /+
mep-1	+ *mep-1* + / *unc-5* + *dpy-20; lin-15A(n767)*	Unc	56/57 *mep-1* /+
		Dpy	2/61 *mep-1* /+
	mep-1 + + /+ *dpy-20 unc-30; lin-15A(n767)*	Dpy	0/51 *mep-1* /+
		Unc	58/58 *mep-1* /+
	+ + *mep-1* + / *unc-24 mec-3* + *dpy-20; lin-15A(n767)*	Unc Mec	10/12 *mep-1* /+
		Unc	17/17 *mep-1* /+
		Mec Dpy	0/8 *mep-1* /+
		Dpy	2/8 *mep-1* /+
	+ *mep-1 dpy-20* + / *lin-3* + + *unc-22; lin-15A(n767)*	Dpy	5/5 *lin-3* /+
		Vul	3/10 *mep-1* /+
	+ + *mep-1*+ / *mec-3 sem-3* + *dpy-20; lin-15A(n767)*	Mec	17/17 *mep-1* /+
		Dpy	6/13 *mep-1* /+

mys-1	+ *mys-1* + / *unc-46* + *dpy-11; lin-15A*	Unc	3/7 *mys-1* /+
		Dpy	7/11 *mys-1* /+
trr-1	+ *rol-6* + *trr-1* / *dpy-10* + *unc-4* +; *lin-15A(n767)*	Rol	3/14 *unc-4* /+
		Dpy	3/3 *trr-1* /+
		Unc	0/8 *trr-1* /+
	+ *trr-1* + / *dpy-10* + *rol-1; lin-15A(n767)*	Rol	9/20 *trr-1* /+
	+ + *trr-1* / *dpy-10 unc-53* +; *lin-15A(n767)*	Unc	0/17 *trr-1* /+
	+ *trr-1* + / *unc-53* + *rol-1; lin-15A(n767)*	Unc	7/10 *trr-1* /+
		Rol	7/10 *trr-1* /+
	+ *trr-1* + *rol-1* / *unc-4* + *mex-1* +; *lin-15A(n767)*	Rol	12/14 *mex-1* /+

Three- and four-factor crosses were performed using standard methods (BRENNER 1974). We mapped *lin-65* using the *Y73E7.2*, *Y71G12B.2*, *Y71G12B.17*, *Y71G12B.18*, *Y71G12B.27*, *M01D7.2* DNA sequence polymorphisms present in the CB4856 strain.

TABLE 3

Deficiency heterozygote mapping

Gene	Genotype of heterozygote	Phenotype of heterozygote
mep-1	*mep-1 / sDf63 unc-31; lin-15A(n767) /+*	Pvl Ste
	mep-1 / sDf62 unc-31; lin-15A(n767) /+	Pvl Ste
	mep-1 / sDf10; lin-15A(n767) /+	WT
trr-1	*rol-6 trr-1 / mnDf57; lin-15A(n767)*	WT
	rol-6 trr-1 / unc-4 mnDf90; lin-15A(n767)	WT
	rol-6 trr-1 / mnDf29; lin-15A(n767)	WT
	trr-1 / unc-4 mnDf87; lin-15A(n767)	Muv

Deficiency heterozygotes were constructed as described in MATERIALS AND METHODS.

WT, wild type; Pvl, protruding vulva; Ste, sterile.

TABLE 4

Interactions of new mutations with class A and B synMuv mutations

		Single mutant		Double mutant with class A				Double mutant with class B			
				lin-15A(n767)		*lin-38 (n751)*		*lin-15B(n744)*		*lin-35(n745)*	
		% >3 vulval fates (n)	Ave. no. vulval fates (±SE)	% >3 vulval fates (n)	Ave. no. vulval fates (±SE)	% >3 vulval fates (n)	Ave. no. vulval fates (±SE)	% >3 vulval fates (n)	Ave. no. vulval fates (±SE)	% >3 vulval fates (n)	Ave. no. fates (±
New mutation	*lin-65(n3441)*	0 (35)	3.0 (±0)	100 (36)	5.9 (±0.04)	97 (37)	5.3 (±0.13)	4.3 (23)	3.02 (±0.02)	ND	ND
	lin(n3628)	0 (37)	3.0 (±0)	71 (41)	3.9 (±0.14)	92 (24)	4.4 (±0.15)	2.7 (37)	3.01 (±0.01)	0 (31)	3.0 (±
	mep-1(n3703)	2.5 (40)	3.01 (±0.01)	100 (29)	6.0 (±0.19)	100 (36)	5.9 (±0.29)	0 (21)	3.0 (±0)	0 (25)	3.0 (±
	ark-1(n3701)	0 (33)	3.0 (±0)	77 (30)	4.5 (±0.20)	56 (34)	3.8 (±0.14)	7.8 (26)	3.06 (±0.04)	7.4 (27)	3.07 (±
	gap-1(ga133)	3.1 (32)	3.02 (±0.02)	58 (38)	3.6 (±0.11)	76 (37)	4.4 (±0.17)	0 (29)	3.0 (±0)	0 (30)	3.0 (±
	sli-1(n3538)	0 (25)	3.0 (±0)	93 (28)	4.6 (±0.16)	30 (27)	3.3 (±0.11)	0 (36)	3.0 (±0)	4.5 (22)	3.02 (±

Class A	*lin-15A(n767)*	0 (24)[a]	3.0 (±0)[a]			*[c]	*[c]	ND	ND	*[c]	*[c]
	lin-38 (n751)	0 (27)[a]	3.0 (±0)[a]	0 (32)	3.0 (±0)			*[c]	*[c]	*[c]	*[c]
Class B	*lin-15B(n744)*	0 (20)	3.0 (±0)	ND	ND	100 (33)	6.0 (±0)			*[c]	*[c]
	lin-35(n745)	0 (48)	3.0 (±0)	100 (22)	6.0 (±0)	100 (27)	6.0 (±0)	0 (26)	3.0 (±0)		
Class C	*mys-1(n3681)*	8.3 (36)[b]	3.06 (±0.03)[b]	100 (26)[b]	5.04 (±0.14)[b]	91 (45)[b]	4.40 (±0.13)[b]	46 (37)	3.38 (±0.08)	ND	ND
	trr-1(n3712)	13 (89)[a]	3.10 (±0.03)[a]	74 (54)[a]	4.07 (±0.12)[a]	79 (14)[a]	4.14 (±0.23)[a]	50 (38)	3.38 (±0.07)	63 (41)	3.43 (±

New synMuv mutations were separated from *lin-15A(n767)*, and double mutant strains were constructed as described in MATERIALS AND METHODS. Because these mutations cause recessive sterility, *mep-1(n3703)* and *trr-1(n3712)* homozygotes were derived from heterozygous parents. *mep-1(n3703)* homozygotes were recognized as the non-Unc progeny of *mep-1(n3703) / nT1 n754* heterozygous parents or the non-Gfp progeny of *mep-1(n3703) / nT1[qIs51]* heterozygous parents.

trr-1(n3712) homozygotes were recognized as the non-Gfp progeny of *trr-1(n3712) / mIn1[dpy-10(e128) mIs14]* heterozygous parents. The *lin(n3628) lin-35* strain was marked with *unc-13*. ND, not determined.

[a] These data are from Table 1 of CEOL and HORVITZ (2004).

[b] These data are from Table 3 of CEOL and HORVITZ (2004).

[c] These data are found elsewhere in this table.

TABLE 5

Interactions of new mutations with the class C synMuv mutation *trr-1(n3712)*

		Percentage of animals with P8.p vulval fate	
		trr-1(+)	*trr-1(n3712)*
	+	0 (many)	13 (89)[a]
New mutation	*lin-65(n3441)*	0 (35)	45 (31)
	lin(n3628)	0 (37)	4.2 (24)
	mep-1(n3703)	2.5 (40)	Let[b]
	ark-1(n3701)	0 (33)	13 (24)
	gap-1(ga133)	3.1 (32)	37 (38)
	sli-1(n3538)	0 (25)	32 (37)
Class A	*lin-15A(n767)*	0 (24)	28 (54)
	lin-38(n751)	0 (27)	36 (14)
Class B	*lin-15B(n744)*	0 (20)[a]	50 (38)[a]
	lin-35(n745)	0 (48)[a]	64 (41)[a]

Double mutant strains were constructed as described in MATERIALS AND METHODS. *mep-1(n3703)* homozygotes were recognized as the non-Unc progeny of *mep-1(n3703)/ nT1 n754* heterozygous parents or the sterile progeny of *mep-1(n3703) / dpy-20(e1282) unc-30(e191)* heterozygous parents. *trr-1(n3712)* homozygotes were recognized as the non-Gfp progeny of *trr-1(n3712) / mIn1[dpy-10(e128) mIs14]* heterozygous parents.

[a] These data are from Table 1 of CEOL and HORVITZ (2004).

[b] We interpret this synthetic lethality as indicating redundancy between *mep-1* and *trr-1*.

TABLE 6

Suppression of the *let-23* vulvaless phenotype

		Ave. no. vulval fates (±SE, n)	
		let-23(+)	*let-23(sy97)*
	+	3.0 (many)	0 (±0, 36)
New mutation	*lin-65(n3441)*	3.0 (±0, 35)[a]	0 (±0, 30)
	lin(n3628)	3.0 (±0, 37)[a]	Let[d]
	mep-1(n3703)	3.01 (±0.01, 40)[a]	Let[d]
	ark-1(n3701)	3.0 (±0, 33)[a]	0.10 (±0.05, 34)
	gap-1(ga133)	3.02 (±0.02, 32)[a]	3.0 (±0, 26)
	sli-1(n3538)	3.0 (±0, 25)[a]	3.0 (±0, 31)
Class A	*lin-15A(n767)*	3.0 (±0, 24)[b]	0 (±0, 21)
	lin-38(n751)	3.0 (±0, 27)[b]	ND
Class B	*lin-15B(n744)*	3.0 (±0, 20)[a]	0.23 (±0.08, 26)
	lin-35(n745)	3.0 (±0, 48)[a]	0.20 (±0.06, 38)

Class C	*mys-1(n3681)*	3.06 (±0.03, 36)[c]	1.47 (±0.15, 31)
	trr-1(n3712)	3.10 (±0.03, 89)[b]	0.28 (±0.07, 46)

mep-1(n3703) homozygotes were recognized as the non-Unc progeny of *mep-1(n3703)/ nT1 n754* heterozygous parents or the sterile progeny of *mep-1(n3703) / dpy-20(e1282) unc-30(e191)* heterozygous parents. *trr-1(n3712)* homozygotes were recognized as the non-Gfp progeny of *trr-1(n3712) / mIn1[dpy-10(e128) mIs14]* heterozygous parents. *let-23(sy97)* was marked with *unc-4(e120)*, and *let-23(sy97)* homozygotes were recognized as the Unc non-Gfp progeny of *let-23(sy97) unc-4(e120) / mIn1[dpy-10(e128) mIs14]* heterozygous parents. ND, not determined because of linkage of these mutations.

[a] These data are from TABLE 1.

[b] These data are from Table 1 of CEOL and HORVITZ (2004).

[c] These data are from Table 3 of CEOL and HORVITZ (2004).

[d] Because of the lethality of these animals, we measured the abilities of *lin(n3628)* and *mep-1(n3703)* to suppress the Vul phenotype caused by *sy10*, a *let-23* allele that is weaker than *sy97*. *lin(n3628)* and *mep-1(n3703)* were unable to suppress the Vul phenotype of *let-23(sy10)*: *lin(n3628); let-23(sy10)* double mutants averaged 0.11 vulval fates (n=27), *let-23(sy10); mep-1(n3703)* double mutants averaged 0.06 vulval fates (n=24) and *let-23(sy10)* single mutants averaged 0.14 vulval fates (n=21).

TABLE 7

Interactions of new mutations with *gap-1* and *sli-1* mutations

		Double mutant with *gap-1(ga133)*		Double mutant with *sli-1(n3538)*	
		% >3 vulval fates (n)	Ave. no. vulval fates (±SE)	% >3 vulval fates (n)	Ave. no. vulval fates (±SE)
New mutation	*lin-65(n3441)*	0 (31)	3.0 (±0)	0 (34)	3.0 (±0)
	lin(n3628)	6.1 (33)	3.05 (±0.03)	0 (36)	3.0 (±0)
	mep-1(n3703)	8.3 (36)	3.07 (±0.04)	5.6 (36)	3.03 (±0.02)
	ark-1(n3701)	83 (40)	4.18 (±0.14)	48 (29)	3.48 (±0.11)
	gap-1(ga133)			46 (35)	3.51 (±0.11)

Double mutant strains were constructed as described in MATERIALS AND METHODS. *mep-1(n3703)* homozygotes were recognized as the sterile progeny of *mep-1(n3703)/ dpy-20(e1282) unc-30(e191)* heterozygous parents. *trr-1(n3712)* homozygotes were recognized as the non-Gfp progeny of *trr-1(n3712) / mIn1[dpy-10(e128) mIs14]* heterozygous parents.

TABLE 8

Sequences of new mutations of class B and C synMuv proteins [a]

A. Features of synMuv proteins

Protein	Class	No. amino acids	Protein similarities and domains[b]
DPL-1	B	598	Similar to DP family transcription factors; contains DNA- and E2F-binding domains
EFL-1	B	342	Similar to E2F family transcription factors; contains DNA-, DP- and Rb-binding domains
LET-418	B	1829	Similar to Mi-2 family ATP-dependent chromatin remodeling enzymes; contains chromodomains, PHD finger motifs and a helicase domain[c]
LIN-9	B	LIN-9L: 644 LIN-9S: 642	Similar to *Drosophila* Mip130 DNA replication and Aly cell cycle regulators and mammalian proteins of unknown function
LIN-13	B	2248	24 predicted Zn-finger motifs
LIN-35	B	961	Similar to Retinoblastoma (pRb) family transcriptional regulators; contains "pocket" interaction domain
LIN-36	B	962	THAP domain, C/H-rich and Q-rich regions
LIN-52	B	161	Similar to *Drosophila* and mammalian proteins of unknown function
LIN-53	B	417	Similar to *Drosophila* p55, mammalian RbAp48 subunits of chromatin remodeling and histone deacetylase complexes; contains WD repeats
LIN-65	B	728	Acid-rich
MEP-1	B	853	Six Zn finger motifs
MYS-1	C	458	Similar to MYST family histone acetyltransferases; contains chromodomain and acetyltransferase domain
SLI-1	*sli-1*	582	Similar to Cbl family ubiquitination-promoting proteins; Contains SH2 domain and RING finger motif
TRR-1	C	4064[d]	Similar to mammalian TRRAP transcriptional regulator

B. Allele sequences

Mutation	Wild-type sequence	Mutant sequence	Substitution, splice site change or aberration	Domain affected by missense mutation
dpl-1(n3643)[e]	TAT	TAA	Y341ochre	-
	GGC	CGC	G533R	unknown
efl-1(n3639)[f]	CAA	TAA	Q175ochre	-
let-418(n3536)	CCT	CTT	P675L	helicase/ATPase
let-418(n3626)	GGT	AGT	G1006S	helicase/ATPase
let-418(n3629)	TCC	TTC	S925F	helicase/ATPase
let-418(n3634)	TGG	TAG	W1128amber	-
let-418(n3635)	CAG	TAG	Q1594amber	-
let-418(n3636)	ACT	TCT	T807S	helicase/ATPase
	TGG	TGA	W1329opal	-
let-418(n3719)	TGG	TAG	W295amber	-
lin-9(n3631)	CAA	TAA	LIN-9L: Q594ochre	-
			LIN-9S: Q592ochre	-
lin-9(n3675)	GAT	AAT	LIN-9L: D305N	unknown
			LIN-9S: D303N	unknown

lin-9(n3767)	CAG	TAG	LIN-9L: Q509amber	-
			LIN-9S: Q507amber	-
lin-13(n3642)	CAT	TAT	H832Y	Zn finger
lin-13(n3673)	CAG	TAG	Q1988amber	-
lin-13(n3674)	CGA	TGA	R1250opal	-
lin-13(n3726)	GGA	GAA	G229E	unknown
lin-35(n3763)[g]	GCA	GTA	A555V	Pocket
	TTG AAA AAG	TTG AAA AAA G	K594frameshift and truncation after 611a.a.	-
lin-36(n3671)	CAT	CCT	H284P	C/H-rich region
	GAA	AAA	E424K	unknown
lin-36(n3672)	CAG	TAG	Q467amber	-
lin-36(n3765)[h]	GCT	GTT	A242V	C/H-rich region
lin-52(n3718)[i]	CAG	TAG	Q31amber	-
lin-53(n3448)	AGT	ATT	S384I	WD repeat
lin-53(n3521)	GAA	AAA	E174K	WD repeat
lin-53(n3622)	AAG/gtatgtgt	AAG/atatgtgt	Exon 1 donor	-
lin-53(n3623)	TGG	TAG	W337amber	-

lin-65(n3441)	TGG	TGA	W534amber	-
lin-65(n3541)	TGG	TGA	W534amber	-
lin-65(n3543)	TCG	TTG	S720L	unknown
mep-1(n3680)	AGT	AAT	S309N	unknown
mep-1(n3702)	CAG	TAG	Q706amber	-
mep-1(n3703)	CTT/gtaagttt	CTT/ataagttt	Exon 3 donor	-
mys-1(n3681)[j]	GGA	AGA	G341R	acetyltransferase
sli-1(n3538)	TCA	TTA	S305L	SH2
sli-1(n3544)	ttttccag/AAA	ttttccaa/AAA	Exon 6 acceptor	-
sli-1(n3683)	ttttttag/GAT	tttttaa/GAT	Exon 4 acceptor	-
trr-1(n3630)[k]	TGG	TAG	W2064amber	-
trr-1(n3637)[k]	CAG	TAG	Q3444amber	-
trr-1(n3704)[k]	CAA	TAA	Q694ochre	-
trr-1(n3708)[k]	CGA	TGA	R1248opal	-
trr-1(n3709)[k]	CGA	TGA	R2550opal	-
trr-1(n3712)[k]	TGG	TAG	W2505amber	-

In the "Wild-type sequence" and "Mutant sequence" columns, exon and intron sequences are denoted by uppercase and lowercase script, respectively. Nucleotides altered by the mutation are underlined.

[a] The synMuv proteins described in this table are limited to those for which we obtained mutant allele sequence; this table is not a comprehensive listing of synMuv proteins.

[b] Molecular descriptions of the proteins listed were obtained from the following sources: DPL-1, EFL-1 (CEOL and HORVITZ 2001; PAGE *et al.* 2001); LET-418 (SOLARI and AHRINGER 2000; VON ZELEWSKY *et al.* 2000); LIN-9 (BEITEL *et al.* 2000); LIN-13 (MELENDEZ and GREENWALD 2000); LIN-35, LIN-53 (LU and HORVITZ 1998); LIN-36 (THOMAS and HORVITZ 1999; REDDY and VILLENEUVE 2004); LIN-52 (THOMAS *et al.* 2003); MEP-1 (BELFIORE *et al.* 2002); SLI-1 (YOON *et al.* 1995); MYS-1, TRR-1 (CEOL and HORVITZ 2004).

[c] The predicted LET-418 protein contains a sequence that is annotated as a helicase domain (see www.wormbase.org). This domain was originally identified in helicases but has since been found in non-helicase proteins. Many of these proteins share a common ATPase activity, and this domain contains residues that are important for ATP binding and hydrolysis.

[d] Because of alternative splicing, *trr-1* encodes proteins that may range in length between 4054 and 4064 amino acids (CEOL and HORVITZ 2004).

[e] These data are from Figure 1 of CEOL and HORVITZ (2001).

[f] These data are from Figure 4 of CEOL and HORVITZ (2001).

[g] The adenosine inserted by the *lin-35(n3763)* frameshift mutation is not underlined, because it is unclear which adenosine in the adenosine repeat was inserted.

[h] In addition to the missense mutation described, we found an additional mutation associated with *lin-36(n3765)*. This mutation, AG/gtaagaagaaaagc to AG/gtaagaagaaaagt, is present in the third intron of *lin-36* and creates a possible splice-donor sequence. If this splice-donor were used, an in-frame ochre (TAA) stop codon would be encountered, truncating the LIN-36 protein after 261 amino acids.

[i] These data are from Figure 3 of THOMAS *et al.* (2003).

[j] These data are from Figure 2 of CEOL and HORVITZ (2004).

[k] These data are from Figure 1 of CEOL and HORVITZ (2004).

TABLE 9

Summary of synMuv genetic interactions

Mutation	Phenotype of double mutant with mutation of specified class						Phenotype of double mutant with *let-23*	Inferred synMuv class of mutant gene
	class A	class B	class C	*gap-1*	*sli-1*	*ark-1*		
lin-15A(n767) lin-38(n751)	non-Muv	Muv	Muv	Muv	Muv	Muv	Vul	A
*lin-15B(n744) lin-35(n745) lin-65(n3441) mep-1(n3703)**	Muv	non-Muv	Muv	non-Muv	non-Muv	non-Muv	Vul	B
lin(n3628)	Muv	non-Muv	non-Muv	non-Muv	non-Muv	non-Muv	Vul	B or *lin(n3628)* †
trr-1(n3712)	Muv	Muv	non-Muv	Muv	Muv	Muv	Vul	C
gap-1(ga133)	Muv	non-Muv	Muv	NA	Muv	Muv	non-Vul	*gap-1*
sli-1(n3538)	Muv	non-Muv	Muv	Muv	NA	Muv	non-Vul	*sli-1*
ark-1(n3701)	Muv	non-Muv	Muv	Muv	Muv	NA	Vul	*ark-1*

We provisionally assign 29 genes to six synMuv classes. The assignments of 11 of these genes (shown above and underlined below) are based on extensive genetic interaction tests. The remaining 18 genes have not been tested as extensively. However, based on known genetic interactions and molecular identities, we speculate that most of these 18 genes will remain in the classes to which they have previously been assigned.

Class A: *lin-8*, *lin-15A*, *lin-38*, *lin-56*

Class B: *lin-9*, *lin-13*, *lin-15B*, *lin-35*, *lin-36*, *lin-37*, *lin-52*, *lin-53*, *lin-54*, *lin-61*, *lin-65*, *dpl-1*, *efl-1*, *hda-1*, *hpl-2*, *let-418*, *lin(n3628)**, *mep-1*

Class C: *trr-1*, *mys-1*, *epc-1*, *ssl-1*

gap-1: *gap-1*

sli-1: *sli-1*

ark-1: *ark-1*

NA = not applicable; since each of these classes contains only one gene, double mutants within the same class cannot be constructed.

* *mep-1(n3703)* and class C synMuv mutations interact to cause larval lethality at a stage earlier than vulval abnormalities can be determined.

† Like class B synMuv mutations, *lin(n3628)* interacts synthetically with class A mutations, does not interact synthetically with class B, *ark-1*, *gap-1* or *sli-1* mutations, and does not suppress the Vul phenotype of *let-23(sy97)*. However, *lin(n3628)* does not interact synthetically with class C mutations. *lin(n3628)* may define yet another class of synMuv genes. Alternatively, the mutation *n3628* may be a partial loss-of-function mutation

too weak to reveal redundancy with class C genes, in which case *lin(n3628)* may be a class B gene. Determination of the *lin(n3628)* null phenotype and genetic interaction tests with a null mutation of this gene should distinguish between these possibilities.

Figure 1. Molecular cloning of *lin-65*

A) The genetic map location of *lin-65* on linkage group I (top) and the physical map interval between the *C. elegans* strain CB4856 polymorphisms *Y71G12B.17* and *Y71G12B.18* and including *lin-65* (bottom).

B) *lin-65* gene structure as derived from cDNA and genomic sequences. Shaded boxes indicate coding sequence and an open box indicates the 3' untranslated region (*lin-65* transcripts also contain a 5' untranslated region that is too small to be viewed in this representation). Predicted translation initiation and termination codons and the poly(A) tail are shown. Arrows above indicate the positions of the *lin-65(n3441)*, *lin-65(n3541)* and *lin-65(n3543)* mutations. The fourth exon of the cDNA yk1279h11 is smaller than that of the other five *lin-65* cDNAs (the end of the yk1279h11 fourth exon is indicated by a dashed line). The use of an alternative splice donor may have created this shorter fourth exon. However, if the end of the yk1279h11 fourth exon were the site of alternative splicing, a CA and not the typical GT splice donor would have been used. In addition, the end of the yk1279h11-specific fourth exon and the beginning of the fifth exon encode multiple glutamine residues and are highly similar in DNA sequence (see Figure 2). The intervening sequence between two regions of highly similar sequence can be lost because of recombination in bacteria (ROBINETT *et al.* 1996). For these reasons we speculate that the apparent alternative splice site at the end of the fourth exon in yk1279h11 may be artifactual and have resulted from an error during the generation or maintenance of this cDNA clone. In support of this possibility: 1) we failed to amplify a shorter-than-wild-type yk1279h11 product in a high stringency RT-PCR using oligonucleotide primers that flank the putative alternative splice junction, and 2) we failed to amplify any RT-PCR products when an oligonucleotide spanning the putative yk1279h11 alternative splice junction was used in a PCR.

Figure 1

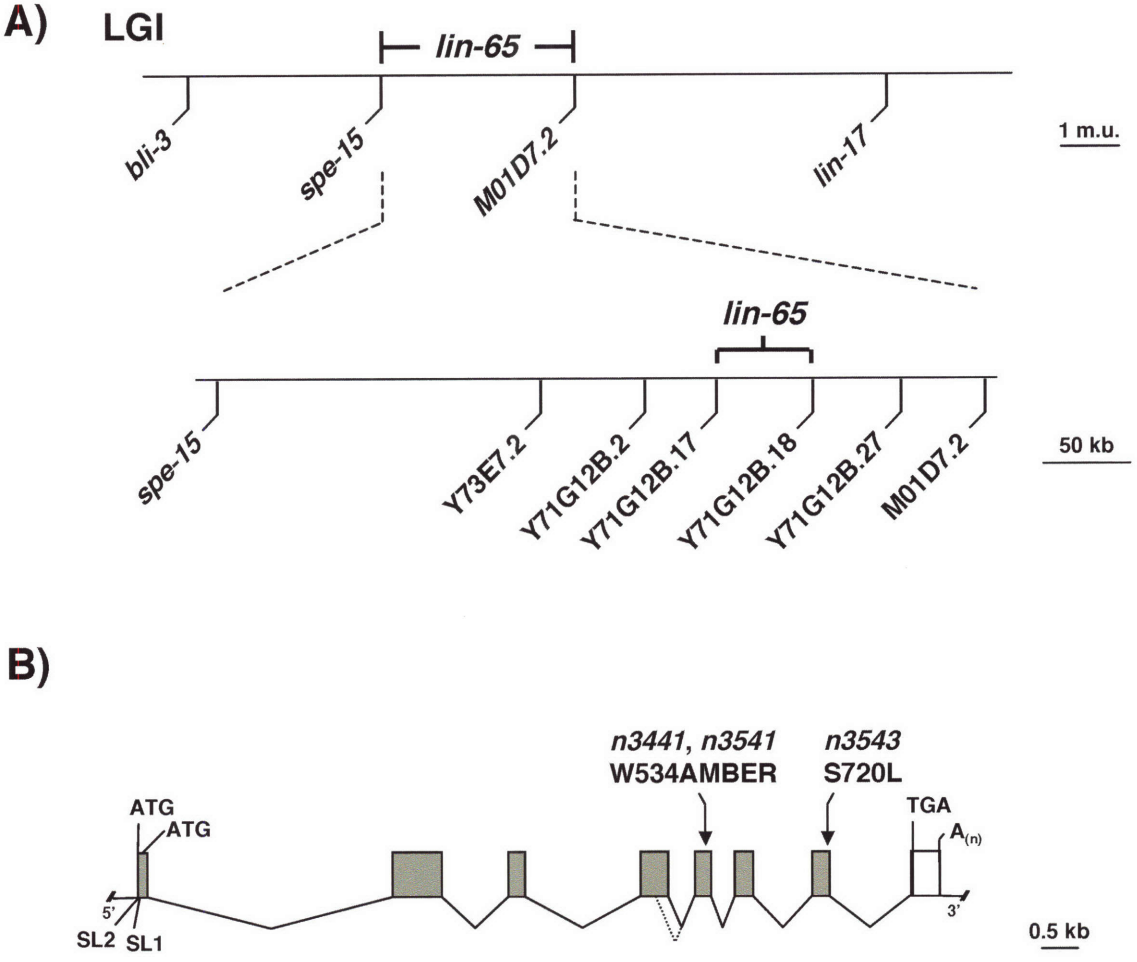
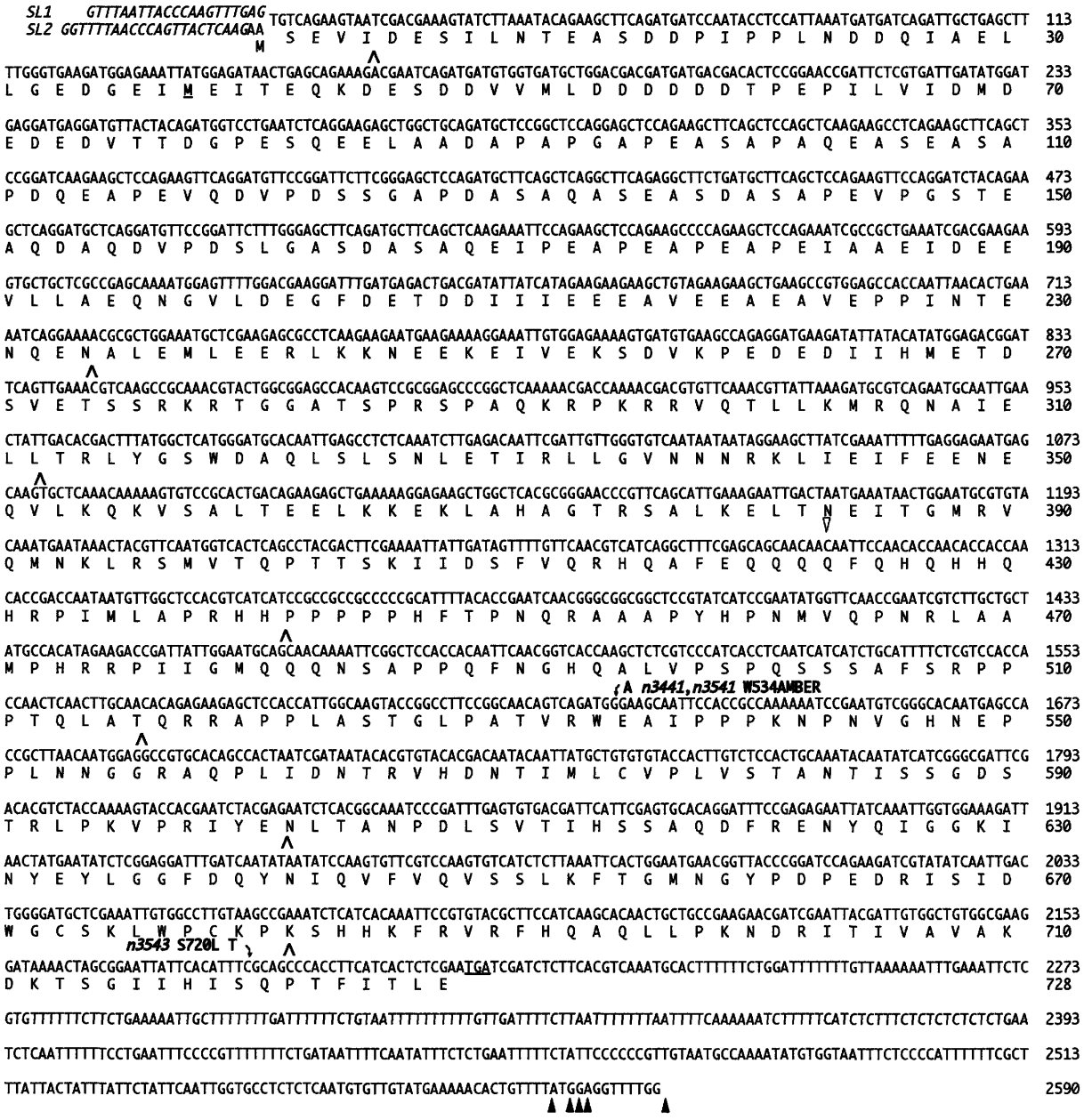

A)
LGI
lin-65
bli-3
spe-15
M01D7.2
lin-17
1 m.u.
lin-65
spe-15
Y73E7.2
Y71G12B.2
Y71G12B.17
Y71G12B.18
Y71G12B.27
M01D7.2
50 kb
B)
n3441, n3541
W534AMBER
n3543
S720L
ATG
ATG
TGA
A(n)
5'
3'
SL2
SL1
0.5 kb

Figure 2. *lin-65* cDNA sequence

lin-65 cDNA sequence indicating differences among individual *lin-65* cDNAs. SL1 and SL2 splice-leader sequences are italicized. The SL1 leader, as observed with one cDNA, is spliced two nucleotides downstream of the site at which the SL2 leader is spliced, as observed with two independently-derived cDNAs. Intron positions are indicated by carats. The translation termination codon is underlined. Sites of alternative polyadenylation are indicated with closed arrowheads. The predicted LIN-65 protein is shown beneath. The SL2-spliced cDNAs are predicted to encode a 728 amino acid protein. The SL1-spliced cDNA cannot encode the predicted initiator methionine of the 728 amino acid protein; it may use the underlined methionine codon to initiate synthesis of a 691 amino acid protein. The alternatively spliced cDNA yk1279h11 is predicted to encode a protein lacking amino acids 421 through 481 of the 728 amino acid protein, although as described in the legend to Figure 1, the alternative splicing of yk1279h11 is likely to be artifactual. The site at which the putative fourth exon in yk1279h11 ends is indicated with an open arrowhead. This end is juxtaposed to the beginning of the fifth exon to give a CAGCAACAA/CAACAAAAT junction sequence.

Figure 2


SL1 GTTTAATTACCCAAGTTTGAG
SL2 GGTTTTAACCCAGTTACTCAAGAA
M S E V I D E S I L N T E A S D D P I P P L N D D Q I A E L 30
TTGGGTGAAGATGGAGAAATTATGGAGATAACTGAGCAGAAAGACGAATCAGATGATGTGGTGATGCTGGACGACGATGATGACGACACTCCGGAACCGATTCTCGTGATTGATATGGAT 233
L G E D G E I M E I T E Q K D E S D D V V M L D D D D D D T P E P I L V I D M D 70
GAGGATGAGGATGTTACTACAGATGGTCCTGAATCTCAGGAAGAGCTGGCTGCAGATGCTCCGGCTCCAGGAGCTCCAGAAGCTTCAGCTCCAGCTCAAGAAGCCTCAGAAGCTTCAGCT 353
E D E D V T T D G P E S Q E E L A A D A P A P G A P E A S A P A Q E A S E A S A 110
CCGGATCAAGAAGCTCCAGAAGTTCAGGATGTTCCGGATTCTTCGGGAGCTCCAGATGCTTCAGCTCAGGCTTCAGAGGCTTCTGATGCTTCAGCTCCAGAAGTTCCAGGATCTACAGAA 473
P D Q E A P E V Q D V P D S S G A P D A S A Q A S E A S D A S A P E V P G S T E 150
GCTCAGGATGCTCAGGATGTTCCGGATTCTTTGGGAGCTTCAGATGCTTCAGCTCAAGAAATTCCAGAAGCTCCAGAAGCCCCAGAAGCTCCAGAAATCGCCGCTGAAATCGACGAAGAA 593
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A n3441,n3541 W534AMBER
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CHAPTER 6

Future Directions

synMuv proteins form multiple complexes that likely regulate transcription levels

At least 31 synMuv genes have been identified and based on their genetic interactions they have been placed into three classes. Among the class B synMuv proteins are homologs of a histone deacetylase and the RB, DP, E2F complex that could recruit the histone deacetylase to DNA. Based on these similarities to mammalian proteins, a model has been proposed suggesting that the class B synMuv proteins act through the recruitment of a histone deacetylase complex to the promoters of vulval genes and regulate cell-fate specification through transcriptional repression. However, both work discussed in this thesis and data from other laboratories have demonstrated that the proteins encoded by the class B synMuv genes are not in a single complex. The DRM complex consists of at least eight class B synMuv proteins: LIN-9, LIN-35, LIN-37, LIN-52, LIN-53, LIN-54, DPL-1, and EFL-1. While the DRM complex includes the only *C. elegans* pocket protein, it is separable from the histone deacetylase-containing NuRD-like complex, which likely consists of at least HDA-1, LET-418, LIN-53, and MEP-1 (UNHAVAITHAYA *et al.* 2002). LIN-61 is not found as a core member of either of these complexes, and LIN-36 and HPL-2 are unlikely to be members of the DRM complex. Studies of the interactions among the synMuv proteins must be extended to other class B synMuv proteins like LIN-15B, LIN-65, MET-2, and TAM-1 for which suitable antibodies do not exist currently.

The identification of these distinct complexes suggests a modified model for how the class B synMuv proteins might act together to maintain transcriptional repression. The NuRD-like complex could be recruited to the promoters of genes that cause vulval induction and deacetylate the histones in that promoter region. Subsequently, the DRM complex could bind to these histones and maintain them in their deacetylated state (Figure 1A). Alternatively, a methyltransferase such as the class B synMuv protein MET-2 could methylate the now deacetylated histone allowing HPL-2 to bind to the methyl mark and recruit LIN-13 to the promoter (Figure 1B). Detailed studies of identified target genes should be able to distinguish between these models. It is possible that each model is correct and that the class B synMuv genes regulate multiple

target genes by different mechanisms. Of course, other models also are possible. Perhaps each complex is acting individually at different target genes.

According to the first model, binding of the DRM complex to promoters would require the histone deacetylase activity of the NuRD-like complex. Thus, in mutants lacking NuRD complex activity DRM would no longer be able to bind to those regions of DNA. If instead MET-2 acts to methylate these histones prior to HPL-2 binding, methylation at specific promoters would be dependent on NuRD complex components. Each of these predctions should be testable using chromatin immunoprecipitation (ChIP) in various mutant backgrounds once genes regulated by the synMuv proteins are identified. ChIP experiments will also allow us to monitor the histone modifications in regions of the genome to which various synMuv proteins have been recruited. For example, it might be expected that at promoters that are repressed by the NuRD-like complex the histones will be hypoacetylated, and that when components of the complex are removed by mutation the histones will become hyperacetylated. Antibodies against many specific histone modifications have been characterized and are commercially available.

Genetic experiments analyzing redundancy among the class B synMuv proteins should also help in refining the proposed models. Loss of function of many class B synMuv genes if combined with a weaker class A synMuv mutant or if double mutants are raised at lower temperatures do not result in a fully penetrant synMuv phenotype. In such backgrounds we can look for enhancement of the weak synMuv phenotype by mutations in other class B synMuv genes. Such enhancement would suggest that the two class B synMuv genes provide redundant functions in regulating vulval development.

synMuv proteins could regulate vulval development through transcriptional repression of *lin-3*

While most class A and class B synMuv proteins are likely functioning to repress transcription, for many of these proteins how they cause this repression is still unknown. While the DRM complex contains at least eight synMuv proteins, none of these complex

components has known chromatin-remodeling activities. Thus it will be important to determine how this protein complex is regulating gene expression. In addition, it is has been unclear whether the redundancy among the different synMuv classes results from functional redundancy in regulating transcription of the same target genes or whether the classes act on different processes. Studies of how the synMuv proteins are cooperating to regulate expression of specific target genes should help us understand how these proteins are functioning.

Recently, transcription of *lin-3*, which encodes an EGF-like signal known to be important in vulval induction, has been shown to be regulated redundantly by the class A and class B synMuv genes (Cui *et al.* 2006a). Loss of function in either the class A synMuv genes *lin-8* or *lin-15A* or the class B synMuv genes *lin-35, lin-36* or *lin-15B* as single mutants did not result in upregulation of *lin-3* mRNA levels. However, in animals mutant for both a class A and class B synMuv gene *lin-3* levels were significantly upregulated. Thus, at least for regulation of *lin-3*, the class A and B synMuv genes are acting redundantly by both repressing transcription of a single locus. It has yet to tested if regulation of *lin-3* levels is directly mediated by binding of synMuv proteins to the *lin-3* promoter. Experiments to determine if this is the case are an obvious next step.

Studies of how the class A and B synMuv proteins are cooperating to repress transcription of specific target genes, like *lin-3,* should give us insight into how these proteins are functioning. ChIP experiments first can be used to determine if the synMuv proteins are directly regulating a given promoter. If this is the case, these experiments should allow us to identify approximately where in the promoter region of specific target genes the synMuv proteins are binding. Given that both class A and B synMuv proteins can repress transcription from the *lin-3* promoter, ChIP experiments could be useful in elucidating if the binding sites for the class A and B synMuv proteins in the promoter are distantly spaced from each other. In addition, it might be possible to show whether all or most of the proteins from a single synMuv class bind to the same region of the promoter.

Binding sites could be verified genetically. If the binding sites for different synMuv proteins are distantly spaced in the promoter, it might be possible to identify deletion

alleles that specifically remove one, but not the other binding site. Animals with these mutations should therefore require loss of function in only one class of synMuv genes to result in a Muv phenotype. For example, a missense allele upstream of *lin-3* causes a strong Muv phenotype when combined with loss of function in class B synMuv genes, but not class A synMuv genes, suggesting that it might target binding of class A synMuv proteins (A. Saffer and H.R.H. unpublished results.) Binding sites could be identified more specifically using transgenic animals. The regions of the genome that have been shown to bind specific synMuv proteins might be able to repress transcription when inserted upstream of a transgene. By systematically removing or mutating portions of this region, it should be possible to narrow down the specific protein-binding site.

Class B synMuv genes do not all function similarly to regulate vulval development

Biochemical data demonstrate that the class B synMuv proteins are not forming a single complex, suggesting that these proteins might not all be functioning together in vulval development. Indeed, genetic analyses of these genes similarly indicate that not all class B synMuv genes are acting identically in the regulation of vulval development. *hda-1, let-418,* and *lin-13* have all been categorized as class B synMuv genes (MELENDEZ and GREENWALD 2000; VON ZELEWSKY *et al.* 2000; DUFOURCQ *et al.* 2002). However, unlike loss of function in other class B synMuv genes, loss of function in these three genes cause Muv phenotypes as single mutants (MELENDEZ and GREENWALD 2000; VON ZELEWSKY *et al.* 2000; DUFOURCQ *et al.* 2002). In addition, mutations in other genes that have been categorized as class B synMuv genes, like *hpl-2*, have synthetic defects when combined with mutations in other class B synMuv genes, suggesting functional redundancy among these genes (COUTEAU *et al.* 2002; COUSTHAM *et al.* 2006). Synthetic interactions between members of the same synMuv class have not been extensively tested and might reveal additional redundancies.

Studies of *lin-3* levels in animals mutant for *let-418, hda-1*, and *lin-13*, which result in a weak Muv phenotype as single mutants (MELENDEZ and GREENWALD 2000; VON ZELEWSKY *et al.* 2000; DUFOURCQ *et al.* 2002), should help in elucidating whether

there are additional genes important for vulval induction regulated by the protein products of these genes. The fact that as single mutants loss of function of these genes causes a Muv phenotype suggests that if *lin-3* is the only target they should cause some derepression of *lin-3* even in the absence of a class A synMuv mutation. If no derepression of *lin-3* is evident, it is likely that these proteins repress additional target genes that contribute to the vulval phenotype.

synMuv proteins regulate many processes in addition to vulval development

Identification of target genes will further our understanding of how the large number of transcriptional regulators that have been identified genetically for their roles in vuval development can act together in some biological processes but not in others. Loss of function of synMuv genes results in a large number of abnormalities in addition to vulval defects, and these defects are not shared by all identified synMuv genes. As discussed in Chapter 3, animals mutant for *lin-35* and *lin-15B* cause RNAi hypersensitivity, LAG-2 misexpression, PGL-1 somatic expression, and modification of expression from simple transgenes, but these pleiotropies are not caused by loss of function of *lin-61*. Thus it is probable that there are genes specifically repressed by LIN-35 and LIN-15B but not LIN-61. Detailed studies of the promoter regions of verified target genes should help us understand why at some promoters, like pro-vulval genes, LIN-61 might be functioning with LIN-35 and LIN-15B while at others these proteins do not function together. Further genetic characterization of the pleiotropies associated with loss of function of additional class B genes along with analysis of the genes they regulate should help in understanding how an array of different transcription factors can coordinately control expression of a large number of genes.

Antibodies exist against a significant number of class B synMuv genes. Antibodies against LIN-9, LIN-37, LIN-52, LIN-53, LIN-54, and LIN-61 are all able to specifically immunoprecipitate the protein they were raised against from *C. elegans* protein extract. These antibodies could be used for ChIP experiments. By hybridizing the genomic fragments that coprecipitate with these proteins to DNA arrays tiled with intragenic sequences, ChIP experiments can identify genomic regions that are bound by

synMuv proteins and, by extension, can find additional target genes. Unfortunately, at the current time intragenic arrays are not available so standard microarrays could be a useful alternative in identifying additional gene targets of the synMuv proteins. In these experiments, mRNA isolated from both single and double synMuv mutant strains would be hybridized to arrays and, by comparison to the wild type, those mRNAs that are more or less abundant in the mutants can be identified. Standard gene arrays, as opposed to intragenic arrays, provide information on whether mRNA levels are upregulated or down regulated. It will be important in analyzing these data to recognize the possibility that the synMuv proteins might not be acting solely as transcriptional repressors but could potentially also have roles in transcriptional activation. By comparison between results obtained with single synMuv mutants and with double synMuv mutants it should be possible to identify genes that are specifically regulated by a single class of synMuv proteins or genes that are regulated by the redundant functions of multiple classes.

In *C. elegans,* the ease of transgenics, RNAi, and screening for targeted deletions, can be used to determine the biological functions of target genes. For example, loss of function of a target gene might result in the suppression specific pleiotropies associated with loss of function in synMuv genes. This would suggest that the increased expression of this gene in a synMuv mutant resulted in the abnormality. Such detailed studies of these target genes could possibly identify additional processes that are regulated by the synMuv genes.

Different target genes are likely to be regulated by different sets of synMuv proteins

Another potential target for repression by the synMuv genes is *mat-3*, which encodes a component of the anaphase-promoting complex. An allele of *mat-3, ku233,* that results in defects in vulval formation contains two adjacent base-pair changes approximately 400 base pairs upstream of the transcriptional start site. These mutations are likely to interfere with transcription of the *mat-3* locus as *mat-3* mRNA levels are 5-10 fold reduced in *ku233* mutant animals. Loss of function of either *lin-35* or *dpl-1*, but not *lin-36* restores *mat-3* mRNA levels in *ku233* mutant animals. In addition, mutations

in the class B synMuv genes *lin-15B, lin-35, lin-53, lin-61, dpl-1,* and *efl-1* can suppress the vulval defects caused by *mat-3(ku233)*, but neither RNAi directed against *lin-36* nor a loss-of-function mutation in *lin-36*, can suppress these defects (GARBE *et al.* 2004) . These data suggest that only a subset of the class B synMuv proteins regulate expression of *mat-3*. This transcriptional regulation might be directly caused by binding of synMuv proteins to the *mat-3* promoter as an E2F-binding site exists 130 base pairs upstream of the transcriptional start site (GARBE *et al.* 2004). It is not known currently whether the class A synMuv proteins act to regulate *mat-3* expression levels. However since loss of function in *lin-15A* and *lin-8* fail to suppress the vulval defects of *mat-3(ku233)* animals, it is unlikely that the class A proteins are regulating *mat-3* expression levels. Thus regulation of *mat-3* appears to be distinct from the regulation of *lin-3* in two ways. First, *mat-3* levels are not redundantly controlled by both class A and class B synMuv proteins, and second, *lin-3,* but not *mat-3*, is likely regulated by LIN-36. Thus individual synMuv target genes are likely to be controlled by distinct combinations of synMuv proteins.

As discussed in the Introduction to this thesis, pocket proteins interact with a diverse array of chromatin-remodeling factors to regulate gene expression. Perhaps in a similar manner different synMuv proteins are cooperating to regulate expression of individual target genes. The NuRD-like complex and the DRM complex could be cooperating to regulate transcription of one set of target genes while the NuRD-like complex could act with MET-2 and HPL-2 at other target genes. Detailed studies of which synMuv proteins are recruited the promoters of specific target genes and which synMuv mutants cause deregulation of these target genes should provide insight into how the different sets of transcription factors are cooperating to control the levels of a diverse array of genes.

Multiple proteins likely act to recruit synMuv complexes to DNA

Given that LIN-36 is unlikely to regulate *mat-3* levels and that each synMuv gene seems to control a different set of pleiotropies, it is unlikely that all synMuv proteins are

regulating the same set of target genes. Perhaps some of this control is mediated by the recruitment of different protein complexes to individual promoters.

How are these various complexes recruited to specific regions of the genome? The DRM complex contains at least three proteins that are involved in sequence-specific DNA binding, EFL-1, DPL-1, and LIN-54, and it is likely that the complex is being recruited to promoters by the activities of these proteins. No DNA-binding protein is a component of the NuRD-like complex. However, MEP-1 binds to the transcription factor LIN-1 when LIN-1 is sumoylated, suggesting that MEP-1 mediates interactions between the NuRD-like complex and transcription factors (LEIGHT *et al.* 2005). LIN-36 contains a THAP domain, and these domains are capable of binding to DNA in a sequence-specific manner (CLOUAIRE *et al.* 2005). Thus LIN-36 might also act to recruit other class B synMuv proteins that are not members of the DRM complex to specific regions of the genome. The class A synMuv proteins LIN-15A and LIN-56 also contain THAP domains.

ChIP experiments should allow us to test, using various single mutant strains, which of the synMuv proteins are required to recruit other synMuv proteins to promoter regions. For example as it is expected that LIN-54 and the heterodimer of EFL-1 and DPL-1 bind DNA and recruit the DRM complex to promoter regions, it is likely that in animals that lack some or all of these proteins other members of the DRM complex will not be recruited to promoters. Furthermore, we can determine if these two sequence-specific DNA-binding activities are cooperating to recruit the DRM complex to promoters containing binding sites for both proteins or whether each activity is required individually to recruit the complex. Finally, we can test whether other proteins, like LIN-36 or LIN-1, are required to recruit class B synMuv proteins to promoters.

How do the coordinated functions of different transcription factors cooperate to regulate development?

More detailed understanding of the functions of the synMuv genes and the genes they target for transcriptional repression will help us to better understand how transcription factors coordinate their functions to control different biological processes.

In addition, a large number of proteins that are predicted to regulate chromatin have been found to suppress the synMuv phenotype (Cui *et al.* 2006b). Loss of function of some of these genes also suppresses abnormalities beyond vulval development that are associated with loss of function of synMuv genes. RT-PCR or northern blots should help to identify whether these suppressors are acting on the same target genes as the synMuv proteins or whether the suppression is a secondary effect from regulation of other gene targets. For example, do mutations in the suppressors reduce *lin-3* mRNA levels to wild type in an animal mutant for both a class A and class B synMuv gene. Furthermore, it will be possible to test whether the suppressors are directly counteracting chromatin modifications by the synMuv proteins. A histone acetyltransferase complex was identified among the suppressors (Cui *et al.* 2006b), and this protein complex could directly counteract the histone deacetylase function of the NuRD-like complex.

Conclusions

The DRM complex is a conserved pocket protein-containing complex that likely acts to repress target genes, possibly including *lin-3* and *mat-3.* As all components of this complex have homologs in mammals, a similar complex might exist in humans and provide yet another mechanism by which pocket proteins can repress their transcriptional targets. As discussed in Chapter 1, pocket proteins can interact with many different chromatin-remodeling enzymes to repress transcription. Detailed studies of individual promoters should help in elucidating how the DRM complex represses transcription and whether chromatin-remodeling enzymes are required for the repression at specific times or in certain tissues. Future studies of the function of pocket proteins in regulating genes necessary for development rather than cell cycle might demonstrate that a DRM-like complex is specifically required for this regulation.

Many additional synMuv genes have been identified that are not components of the DRM complex, but are also likely to act as transcriptional repressors. In the future, studies will need to focus on both identifying the transcriptional targets of the synMuv proteins and on how these proteins function to cause transcriptional repression. Such

analyses should provide insight into the mechanism by which transcriptional regulators are cooperating to regulate the transcription of a large number of target genes.

Acknowlegments

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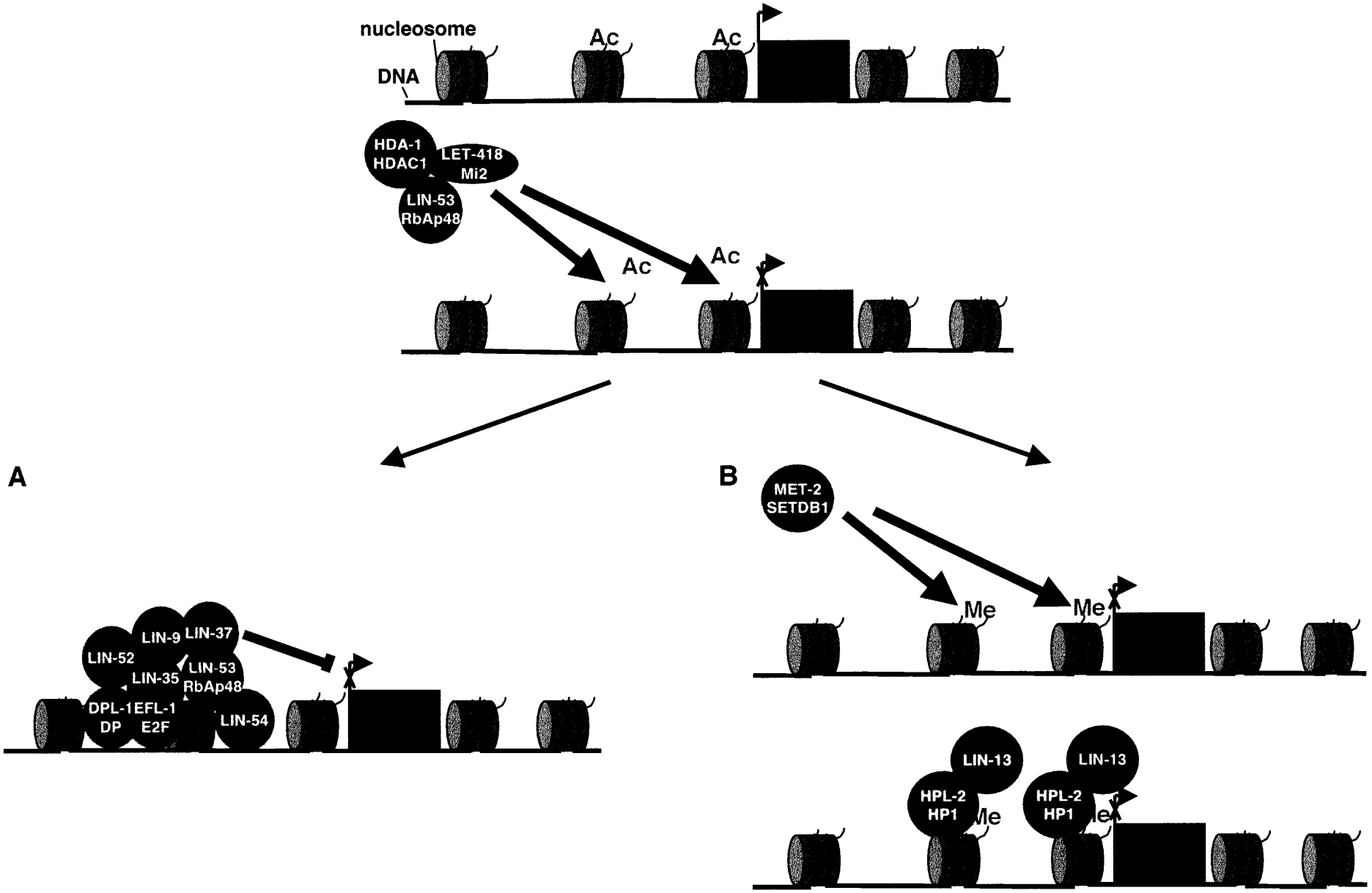
Figure 1. Schematic indicating two possible models for how complexes among the class B synMuv proteins might regulate transcription during vulval development. Genes that promote the expression of vulval cell fates (red box) are transcriptionally active when the histones in the region of their promoters are acetylated. The NuRD-like deacetylates these histones, causing transcriptional repression. (A) The DRM complex maintains transcriptional repression by binding to histones that have been deacetylated by the activity of the NuRD-like complex. (B) The putative methyltransferase MET-2 can methylate deacetylated histones. The methylated histones are subsequently bound by HPL-2, which can recruit LIN-13.

Figure 1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Ac
HDA-1
HDAC1
LET-418
Mi2
LIN-53
RbAp48
Ac
Ac
A
LIN-9
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LIN-52
LIN-53
LIN-35
RbAp48
DPL-1
DP
EFL-1
E2F
LIN-54
B
MET-2
SETDB1
Me
Me
LIN-13
LIN-13
HPL-2
HP1
HPL-2
HP1
Me