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LIN-61, One of Two *Caenorhabditis elegans* Malignant-Brain-Tumor-Repeat-Containing Proteins, Acts With the DRM and NuRD-Like Protein Complexes in Vulval Development but Not in Certain Other Biological Processes

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ABSTRACT

Vulval development in *Caenorhabiditis elegans* is inhibited by the redundant functions of the synthetic multivulva (synMuv) genes. At least 26 synMuv genes have been identified, many of which appear to act via transcriptional repression. Here we report the molecular identification of the class B synMuv gene *lin-61*, which encodes a protein composed of four *m*alignant *b*rain *t*umor (MBT) repeats. MBT repeats, domains of ~100 amino acids, 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 cases have been shown to bind modified histones. *C. elegans* contains one other MBT-repeat-containing protein, MBTR-1. We demonstrate that a deletion allele of *mbtr-1* does not cause a synMuv phenotype nor does *mbtr-1* appear to act redundantly with or in opposition to *lin-61*. We further show that *lin-61* is phenotypically and biochemically distinct from other class B synMuv genes. Our data indicate that while the class B synMuv genes act together to regulate vulval development, *lin-61* functions separately from some class B synMuv proteins in other biological processes.

As cells divide during development, their descendants become increasingly restricted in their capacities to adopt different cell fates. These restrictions in cell fate involve the modulation of gene expression, frequently through modifications of the surrounding chromatin. Mutations in factors that control chromatin structure can lead to developmental defects in numerous organisms (reviewed by Margueron *et al.* 2005).

In *Caenorhabiditis elegans*, the regulation of vulval development involves evolutionarily conserved proteins important for signal transduction, chromatin remodeling, and transcriptional repression. The vulva of the *C. elegans* hermaphrodite is formed from three of six equipotent blast cells, P3.p–P8.p (Sulston and Horvitz 1977; Sulston and White 1980; Sternberg and Horvitz 1986). Although all six cells are competent in adopting 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 nonvulval syncytial hypodermis. A number of signaling pathways specify vulval development, including a receptor tyrosine kinase/Ras pathway, a Wnt pathway, and a Notch pathway (Greenwald *et al.* 1983; Yochem *et al.* 1988; Beitel *et al.* 1990; Han *et al.* 1990; Eisenmann *et al.* 1998; Gleason *et al.* 2002). Mutations affecting these pathways either can cause P3.p, P4.p, and P8.p aberrantly to adopt vulval cell fates and thereby generate a multivulva (Muv) phenotype or can cause none of the P*n*.p cells to adopt a vulval cell fate, resulting in a vulvaless (Vul) phenotype (Greenwald *et al.* 1983; Sternberg and Horvitz 1989; Eisenmann *et al.* 1998).

The ectopic induction of P3.p, P4.p, and P8.p can also be caused by mutations in the synthetic multivulva (synMuv) genes, which have been placed into three classes, A, B, and C, on the basis of 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 highly penetrant Muv phenotype, whereas animals with a loss-of-function mutation in a single class are predominantly not Muv. Many of the synMuv genes encode proteins implicated in chromatin remodeling and transcriptional repression (Lu and Horvitz 1998; von Zelewsky *et al.* 2000; Ceol and Horvitz 2001, 2004; Couteau *et al.* 2002; Dufourcq *et al.* 2002; Poulin *et al.* 2005). The gene *lin-3*, which encodes an EGF-like ligand that promotes vulval

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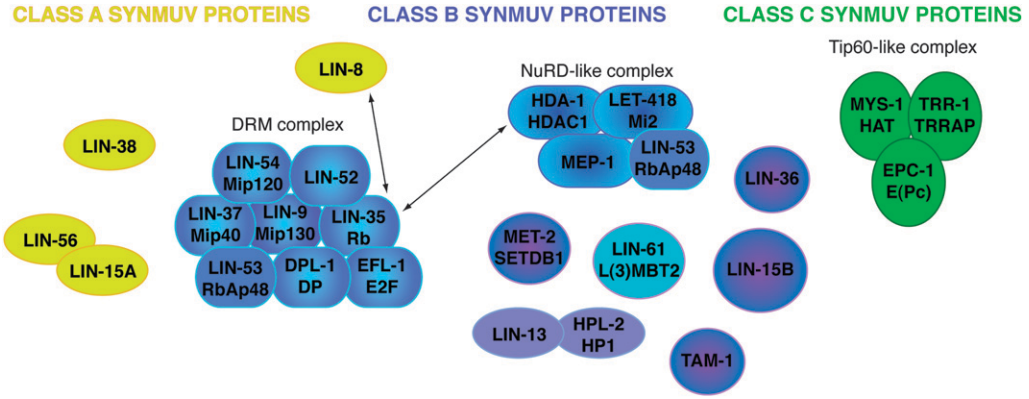


Figure 1.—A synMuv protein interaction map. Class A synMuv proteins are shown in yellow, Class B synMuv proteins in shades of blue, and Class C synMuv proteins in green. This assignment of proteins to specific classes is based on published classifications. The synMuv protein complexes indicated have been demonstrated directly in co-immunoprecipitation experiments, have been suggested by studies of protein stability, or are based on homology to complexes identified in other organisms. Interactions that have been suggested by yeast two-hybrid and GST pull-down experiments but not demonstrated in co-immunoprecipitation experiments are shown by double-headed arrows. References for the interactions shown are as follows: LIN-15A–LIN-56 (E. Davison and H. R. Horvitz, unpublished observations); LIN-8–LIN-35 (Davison *et al.* 2005); DRM complex (Harrison *et al.* 2006); NuRD-like complex (Unhavaithaya *et al.* 2002; Harrison *et al.* 2006); HDA-1–LIN-35 (Lu and Horvitz 1998); LIN-13—HPL-2 (Coustham *et al.* 2006); Tip60-like complex (Ceol and Horvitz 2004).

induction (Hill and Sternberg 1992), appears to be transcriptionally repressed by at least some of the synMuv genes, and it has been proposed that loss of synMuv gene activity results in the ectopic expression of *lin-3* and the consequent activation of the receptor tyrosine kinase/Ras pathway that induces vulval formation (Cui *et al.* 2006a).

The synMuv proteins likely form a number of distinct transcriptional regulatory complexes (Figure 1). EFL-1 E2F, DPL-1 DP, and LIN-54, which likely bind directly to DNA and repress transcription, are components of the evolutionarily conserved *D*P, *R*b, and class B syn*M*uv (DRM) complex (Ceol and Horvitz 2001; Harrison *et al.* 2006). The DRM complex also includes the class B synMuv proteins LIN-35 Rb, LIN-53 RbAp48, LIN-9, LIN-37, and LIN-52 (Harrison *et al.* 2006) and is nearly identical to two complexes shown to repress transcription in Drosophila, the Myb-MuvB and dREAM complexes (Korenjak *et al.* 2004; Lewis *et al.* 2004). The synMuv proteins LET-418 Mi2, LIN-53 RbAp48, and HDA-1 HDAC1 (*h*istone *de*a*c*etylase I) are homologous to components of the mammalian *nu*cleosome *r*emodeling and *d*eacetylase (NuRD) complex (Lu and Horvitz 1998; von Zelewsky *et al.* 2000; Dufourcq *et al.* 2002). These three *C. elegans* proteins form a complex *in vivo* and associate with the zinc-finger-containing synMuv protein MEP-1 (Unhavaithaya *et al.* 2002). The synMuv proteins MET-2 and HPL-2 are homolgous to SETDB1 and HP1, respectively (Couteau *et al.* 2002; Poulin *et al.* 2005); SETDB1 is a methyltransferase that can methylate lysine 9 of histone H3, and HP1 binds this modified residue (Bannister *et al.* 2001; Schultz *et al.* 2002). HPL-2 associates with another class B synMuv protein, LIN-13, and the two proteins might act together in transcriptional repression (Coustham *et al.* 2006). The class C synMuv genes encode homologs of a Tip60/NuA4-like histone acetyltransferase complex, which might act in either transcriptional repression or activation (Ceol and Horvitz 2004). Additional synMuv proteins have been identified, including LIN-8, LIN-15A, LIN-15B, LIN-36, LIN-38, LIN-56, and TAM-1 (Clark *et al.* 1994; Huang *et al.* 1994; Hsieh *et al.* 1999; Thomas and Horvitz 1999; Thomas *et al.* 2003; Davison *et al.* 2005; A. Saffer, E. Davison and H. R. Horvitz, unpublished observations). While it is likely that these proteins also function in transcriptional repression, whether they interact with other identified synMuv proteins remains to be determined.

Here we report the molecular identification and characterization of the class B synMuv gene *lin-61* and the finding that *lin-61* encodes a protein similar to Polycomb-group (PcG) proteins. PcG proteins were initially identified by their abilities to repress the transcription of Hox genes and have since been found to repress additional targets, including genes regulated by E2F transcription factors (Dahiya *et al.* 2001; Ogawa *et al.* 2002). PcG proteins include histone methyltransferases and proteins that bind to the histones methylated by such transferases. The Drosophila PcG proteins Sex Comb on Midleg (SCM) and Sfmbt each contain MBT repeats, which are motifs of ~100 amino acids. MBT repeats have been found in many transcriptional repressors, including human L(3)MBT (Bornemann *et al.* 1996; Ogawa *et al.* 2002; Boccuni *et al.* 2003), which is in a complex with multiple other PcG-group proteins and with E2F6 (Ogawa *et al.* 2002).

We report that *lin-61* encodes a protein that contains four MBT repeats and that localizes to chromatin. LIN-61 does not associate with either of the two known synMuv protein complexes, the pocket-protein-containing DRM complex and the NuRD-like complex, and can act separately from members of these complexes. We propose that MBT-repeat-containing proteins, such as Polycomb-group proteins, cooperate with Rb-containing

complexes and histone deacetylase complexes to repress certain genes but act independently of these complexes to regulate expression of other genes.

MATERIALS AND METHODS

Strains: Unless otherwise specified, all *C. elegans* strains were cultured at 20° on NGM agar seeded with *Escherichia coli* strain OP50 as described by Brenner (1974). The wild-type strain was N2 (Bristol). 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, n3446, n3447, n3624, n3687, n3736, n3807, n3809, n3922)* (this study), *mbtr-1(n4775)* (this study), *lin-65(n3441)* (Ceol *et al.* 2006), *lin-53(n3368)* (Andersen *et al.* 2006), *ccEx6188* [*rol-6(su1006); myo-3::Ngfp-lacZ*] (Hsieh *et al.* 1999); LGII—*lin-8(n2731)* (Thomas *et al.* 2003), *lin-38(n751), lin-56(n2728)* (Thomas *et al.* 2003), *trr-1(n3712)* (Ceol and Horvitz 2004), *dpl-1(n3316)* (Ceol and Horvitz 2001), *mnCI*[*dpy-10(e128) unc-52(e444)*] (Herman 1978); LGIII—*lin-13(n770)* (Ferguson and Horvitz 1989), *lin-37(n758), mat-3(ku233)* (Garbe *et al.* 2004), *hpl-2(n4274)* (E. Andersen and H. R. Horvitz, personal communication), *lin-52(n3718)* (Ceol *et al.* 2006), *qCI*[*dpy-19(e1259) glp-1(q339)*]; LGIV—*ark-1(n3701)* (Ceol *et al.* 2006); LGV—*hda-1(e1795)* (Dufourcq *et al.* 2002), *tam-1(cc567)* (Hsieh *et al.* 1999), *let-418(n3719)* (Ceol *et al.* 2006), *mep-1(n3703)* (Ceol *et al.* 2006), *lin-54(n2231, n3423)* (Thomas *et al.* 2003; Harrison *et al.* 2006), *qIs56 [lag-2::gfp; unc-119(+)]* (Siegfried and Kimble 2002); LGX—*lin-15A(n433, n767, sy197), lin-15B(n744), mys-1(n3681, n4075)* (Ceol and Horvitz 2004), *sli-1(n3538)* (Ceol *et al.* 2006), *gap-1(ga133)* (Hajnal *et al.* 1997), *lin(n3542)* (Ceol *et al.* 2006), *pkIs1605* [*rol-6*(*su1006*); *hsp16/2::gfp-lacZ*(out of frame)] (Pothof *et al.* 2003). The translocations nT1[*unc(n754)*] (LGIV and LGV), nT1 [*qIs51*] (LGIV and LGV) and *hT2* [*qIs48*] (LGI and LGIII) and the chromosomal inversion *mIn1* [*dpy-10(e128) mIs14*] were used as balancers; each contains an integrated *gfp* transgene linked to the balancer (Edgley and Riddle 2001; Mathies *et al.* 2003).

Isolation of the *mbtr-1(n4775)* deletion allele: Genomic DNA pools from EMS-mutagenized animals were screened for a deletion using PCR, as described by Ceol and Horvitz (2001). Deletion mutant animals were isolated from a frozen stock and backcrossed to the wild type at least twice. *mbtr-1(n4775)* removes nucleotides 30,255–32,134 of cosmid Y48G1A. The sequence of the deletion junction is ATTT TAAAAATTGAG/AATTTTGTTGAA, with the slash indicating the deletion breakpoint.

Transgenic strains: For rescue of the *lin-61(sy223); lin-15A(n767)* and *lin-61(n3624); lin-15A(n767)* synMuv phenotypes, cosmid or subclone DNA (5 or 10 ng/μl) was coinjected with a dominant *rol-6* marker plasmid (pRF4) (80 ng/μl) as described in Mello *et al.* (1991). pMMH15, which was constructed by subcloning a *Stu*I–*Sac*II fragment of the cosmid R06C7 corresponding to bases 15,366–19,753 into pBluescript, was injected for subclone rescue. For expression of *mbtr-1* and *lin-61* driven by the *dpy-7* promoter, constructs were injected at 25 ng/μl with *sur-5::gfp* (pTG96; kindly provided by M. Han) at 20 ng/μl and a 1-kb ladder (Invitrogen, Carlsbad, CA) at 80 ng/μl.

RNA interference analysis of *lin-61* and *mbtr-1*: Templates for *in vitro* transcription reactions were made by PCR amplification of cDNAs yk732e5 or yk268b4 (kindly provided by Y. Kohara), including flanking T3 and T7 promoter regions. RNA was transcribed *in vitro* using T3 and T7 polymerases and 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). This region corresponds to the amino acids likely to be absent in the protein produced in *lin-61(n3809)* animals, allowing these animals to provide a control for antibody specificity. The antiserum was affinity purified against full-length MBP-LIN-61. The rabbits and guinea pigs were immunized and maintained by Covance (Denver, PA). Anti-LIN-61, anti-LIN-9 (Harrison *et al.* 2006), anti-LIN-35 (Harrison *et al.* 2006), anti-LIN-37 (Harrison *et al.* 2006), anti-LIN-52 (Harrison *et al.* 2006), anti-HDA-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-HPL-2 (Coustham *et al.* 2006), anti-LIN-56 (E. Davison and H. R. Horvitz, personal communication), and antitubulin DM1A (Millipore, Bedford, MA) antibodies were used at a 1:1000 dilution for Western blots. Anti-LIN-8 (Davison *et al.* 2005), anti-LIN-53 (Harrison *et al.* 2006), anti-LIN-54 (Harrison *et al.* 2006), and anti-DPL-1 (Ceol and Horvitz 2001) were used at a 1:500 dilution for Western blots. 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 RNA interference (RNAi) hypersensitivity, we assessed the sensitivity of worms to bacteria expressing *hmr-1* or *cel-1* dsRNAs, which previously have been used to characterize the RNAi hypersensitivity of synMuv mutants (Wang *et al.* 2005). Wild-type animals are only mildly affected by the dsRNA produced by these bacterial strains, but animals that are hypersensitive to RNAi are severely affected (Wang *et al.* 2005). L4 larvae were grown on *E. coli* strain HT115 expressing either *hmr-1* or *cel-1* dsRNA, and 24 hr later the young adult hermaphrodites were transferred to fresh plates with *E. coli* expressing the same dsRNA (Kamath and Ahringer 2003). The hermaphrodites were allowed to lay eggs for 24 hr and were then removed. The progeny of hermaphrodites grown on *E. coli*-expressing *cel-1* dsRNA were scored for developmental arrest at the L2 larval stage. The progeny of hermaphrodites grown on *E. coli*-expressing *hmr-1* dsRNA were scored for embryonic lethality. To score 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 1-hr incubation with Alexa Fluor 594 goat anti-mouse IgM (Invitrogen, Carlsbad, CA) at a dilution of 1:25. To score for the Tam phenotype (Hsieh *et al.* 1999), hermaphrodites homozygous for the extrachromosomal array *ccEx6188 [myo-3::Ngfp-lacZ]* were grown for at least two generations at 25°. Using a dissecting microscope equipped with fluorescence optics, we scored animals homozygous for the extrachromosomal array and either for *lin-61(n3809)*, *lin-61(n3992)*, *lin-35(n745)*, and *mbtr-1(n4775)* or for both *lin-61(n3809)* and *mbtr-1(n4775)* for reduced GFP expression as compared to animals carrying only the *ccEx6188* transgene. Hermaphrodites homozygous for the transgene *pkIs1605* [*rol-6*(*su1006*); *hsp16/2::gfp/lacZ*(out-of-frame)] were scored for expression of LacZ after being grown at 20°, heat-shocked at 31° for 2 hr, and allowed to recover at 20° for 1 hr. The hermaphrodites were then fixed and stained with X-gal for the presence of β-galactosidase. Vulval defects in a *mat-3(ku233)* mutant background were scored using Nomarski differential interference contrast microscopy to observe mid-L4

hermaphrodites. Vulval development was scored as abnormal if the invagination was asymmetric or if the developing vulva contained fewer than the 22 nuclei found in wild-type animals (Garbe *et al.* 2004).

Embryo lysates: Embryos were harvested from liquid cultures, 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, Basel, Switzerland) 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. Lysates were clarified by two 15-min 16,000 g centrifugations in a microcentrifuge at 4°. Protein concentration was determined using the Pierce Coomassie Plus Protein Assay Reagent (Pierce Biotechnology, Rockford, IL). Lysate was diluted to 5–10 mg/ml and was used immediately or stored at −80°.

Immunoprecipitation experiments: Antibodies were cross-linked to protein A Dynabeads (Invitrogen, Carlsbad, CA) using dimethyl pimelimidate (Pierce Biotechnology) essentially as described by Harlow and Lane (1999), with the two following exceptions: reactions were stopped with 0.1 m Tris, pH 8.0, and beads were washed three times for 1 min in 100 mm glycine, pH 2.5, followed by a single wash with lysis buffer. The beads were then resuspended in PBS. A total of 500 μl of precleared lysate (2.5–5 mg of total protein) was incubated with 25 μl of affinity-purified antibody bound to 25 μl of beads at 4° for 1–2 hr for each immunoprecipitation reaction and were then washed three times for 5 min each at 4° in lysis buffer. The beads were then resuspended in 20 μl of 2× protein sample buffer, boiled for 5 min, and loaded on an SDS–polyacrylamide gel. HRP-conjugated protein A (Bio-Rad, Hercules, CA) was used for detection of antigens on Western blots following co-immunoprecipitation experiments.

RESULTS

***lin-61* is a class B synMuv gene:** Ten *lin-61* alleles have been isolated in five different screens. The original *lin-61* allele, *sy223*, was isolated in the laboratory of P. Sternberg (personal communication) on the basis of its synMuv phenotype in combination with a loss-of-function mutation in *lin-15A*, *sy197*, and *sy223* and was mapped to linkage group I (LGI). We found that *sy223* complemented *lin-53(n833)*, an allele of the only identified class B synMuv gene on LGI at the time. These data suggested that *sy223* defined a new class B synMuv gene. Five additional 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). Four more alleles, *n3687*, *n3807*, *n3809*, and *n3922*, were isolated in screens for mutants altered in transgene expression (H. T. Schwartz, D. M. Wendell and H. R. Horvitz, personal communication). These alleles were all mapped to LGI and shown to be allelic with *sy223* in complementation tests (C. J. Ceol, H. T. Schwartz, D. M. Wendell and H. R. Horvitz, personal communication; data not shown).

None of the 10 *lin-61* alleles caused a Muv phenotype in the absence of other mutations (Table 1). Each caused a synMuv phenotype in combination with a loss-of-function mutation of the class A synMuv gene *lin-56* (Table 2). A putative *lin-61* null allele, *n3809* (see below), caused a synMuv phenotype in combination with loss of function of each of the four class A synMuv genes (Table 1) but not in combination with mutations in the class B synMuv genes *lin-15B*, *lin-35*, or *lin-37* (Table 1). Loss of function of *lin-61* also did not increase the Muv phenotype caused by *hda-1(e1795)* (Table 1). Whereas null mutations in classically defined class B synMuv genes such as *lin-35* and *lin-15B* enhance the

TABLE 1

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

Genotype	% 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)
lin-15B(n744)	0 (272)
lin-35(n745)	0 (104)
lin-37(n758)	0 (318)
hda-1(e1795)	31 (143)
trr-1(n3712)[a]	18 (39)
mys-1(n3681)[a,b]	8 (36)
mys-1(n4075)[a,b]	15 (20)
lin-61 + 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)
lin-61 + class B synMuv double mutants	
lin-61(n3809); lin-15B(n744)	0 (153)
lin-61(n3809); lin-35(n745)	0 (178)
lin-61(n3809); lin-37(n758)	0 (165)
lin-61(n3809); hda-1(e1795)	20 (96)
lin-61 + class C synMuv double mutants	
lin-61(n3809); trr-1(n3712)[a]	17 (111)
lin-61(n3809); mys-1(n3681)[a]	7 (45)

All animals were raised at 20°. The Muv phenotype was scored using a dissecting microscope, except in the cases noted. *trr-1(n3712)* mutant homozygotes were recognized as the non-GFP progeny of *trr-1(n3712)/mIn1[dpy-10 mIs14]* heterozygous parents. *hda-1(e1795)* homozygotes were recognized as the non-GFP progeny of *hda-1(e1795)/nT1[qIs51]; +/nT1[qIs51]* heterozygous parents. *lin-61(n3687)* and *lin-61(n3922)* were also homozygous for the linked integrated transgene *nIs133*, which carries *pkd-2:gfp* and a rescuing *lin-15AB* construct.

[a] Muv, more than three P*n*.p cells were induced as scored using Nomarski optics.

[b] These data are from Ceol and Horvitz (2004). *mys-1(n4075)* is a deletion mutation.

TABLE 2

Sequences of *lin-61* alleles and allele strengths

lin-61 allele	Wild-type sequence	Mutant sequence	Mutation effect	% Muv (*n*) With *lin-56(n2728)*	% Muv (*n*) With *lin-15A(n433)*
+	—	—	—	0 (many)	0 (many)
lin-61(n3442)	agAAT	aaAAT	Exon 4 splice acceptor	98 (154)	14 (220)
lin-61(n3446)	CAA	TAA	Q412ochre	96 (87)	13 (241)
lin-61(n3809)	CAA	TAA	Q159ochre	92 (176)	14 (261)
lin-61(sy223)	agCTC	aaCTC	Exon 6 splice acceptor	89 (255)	11 (129)
lin-61(n3624)	CCG	TCG	P132S	85 (220)	5.6 (251)
lin-61(n3807)	GGA	GAA	G250E	83 (136)	6.9 (246)
lin-61(n3736)	TTT	TCT	F247S	80 (313)	1.3 (232)
lin-61(n3922)	GGA	GAA	G445R	52 (167)	NA[a]
lin-61(n3447)	AGT	AAT	S354N	47 (237)	1.1 (278)
lin-61(n3687)	CAA	TAA	Q322ochre	23 (305)	NA[a]

Amino acid substitutions are indicated as wild-type residue, residue number, and mutant residue. Coding bases are shown as uppercase letters. Intronic bases are shown as lowercase letters. All animals were raised at 20°. The Muv phenotype was scored using a dissecting microscope.

[a] NA, not applicable because *lin-61(n3687)* and *lin-61(n3922)* were also homozygous for the linked integrated transgene *nIs133*, which carries *pkd-2::gfp* and a rescuing *lin-15AB* construct.

weak Muv phenotype of loss-of-function mutations of class C synMuv genes (Ceol and Horvitz 2004), *lin-61(n3809)* did not enhance the weak Muv phenotype caused by loss of function in either of the class C genes *trr-1* or *mys-1* (Table 1). This result might suggest that *lin-61* has class C synMuv activity (Ceol and Horvitz 2004). However, whereas null mutations in class C synMuv genes cause a P8.p induction as single mutants at a penetrance of ~15% (Table 1; Ceol and Horvitz 2004), 0/24 *lin-61(n3809)* animals showed P8.p induction. Furthermore, mutations in class C synMuv genes cause a Muv phenotype in combination with loss of function of class B synMuv genes, but, in combination with mutations in any of a number of class B synMuv genes, *lin-61(n3809)* did not cause a Muv phenotype (Table 1). We therefore suggest that *lin-61* is not likely to have class C synMuv activity. The failure to enhance the Muv phenotype of animals mutant for class C synMuv genes could instead result from the fact that putative null mutations in *lin-61* cause a weaker synMuv phenotype than do null mutations in other class B synMuv genes. For these reasons, 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 four cosmids (C01H6, C12E8, C12C7, and C33F11) 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 region, R06C7, rescued the synMuv phenotype of *lin-61(n3624); lin-15A(n767)* animals. A subcloned *Stu*I–*Sac*II 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 2A). As reported elsewhere, RNAi directed against *R06C7.7* caused a synMuv phenotype in animals mutant for the class A synMuv gene *lin-15A* but not in wild-type animals (Poulin *et al.* 2005; our unpublished data). To confirm that *R06C7.7* is *lin-61*, we determined the sequence of *R06C7.7* from *lin-61(sy223)* animals. *sy223* encodes a G-to-A transition at the splice-acceptor site of the last predicted exon of *R06C7.7* (Figure 2B and Table 2). Mutations affecting the coding region of *R06C7.7* were found for all other *lin-61* alleles, including three nonsense mutations, five missense mutations, and one mutation in a splice-acceptor site (Figure 2B, Table 2).

We determined the sequence of a full-length *lin-61* cDNA, yk732e5, and determined that the *lin-61* transcript is SL1 spliced and comprises six exons (Figure 2B). *lin-61* transcripts contain no 5′-UTR, as the SL1 leader sequence is spliced directly to the predicted ATG start codon. The next in-frame methionine codon is 373 nucleotides downstream and would produce a protein product inconsistent with the observed mobility of LIN-61 by SDS–PAGE (see below). *lin-61* encodes a predicted protein of 491 amino acids composed almost exclusively of four MBT repeats (Figure 2C) as recognized by SMART and PSI-BLAST databases (Altschul *et al.* 1997; Schultz *et al.* 2000). MBT repeats were initially identified in the Drosophila protein lethal (3) malignant brain tumor [l(3)mbt] (Wismar *et al.* 1995) and are present in many other metazoan proteins but not in proteins from other kingdoms (Bornemann *et al.* 1996; Tomotsune *et al.* 1999; Usui *et al.* 2000; Boccuni *et al.* 2003; Markus *et al.* 2003; Arai and Miyazaki 2005; Klymenko *et al.* 2006). MBT-repeat-containing proteins include the Drosophila Polycomb-group proteins SCM and Sfmbt. In addition to their MBT repeats, l(3)mbt, SCM, and Sfmbt each contain atypical zinc fingers and a single sterile α-motif (SAM) domain (Wismar *et al.*

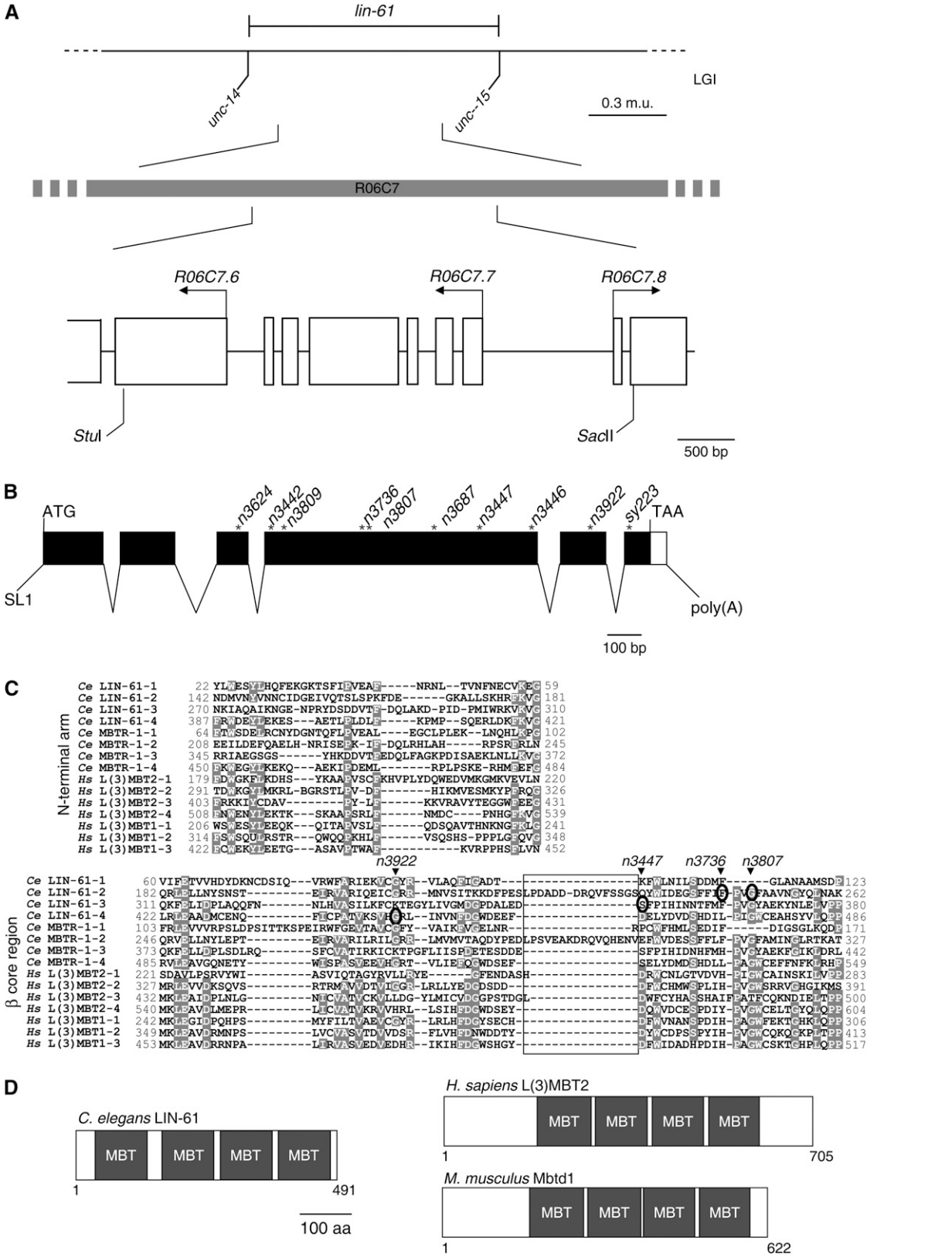


FIGURE 2.—Molecular cloning of *lin-61*. (A) *lin-61* maps between *unc-14* and *unc-15* on LGI. Part of cosmid R06C7 is shown below as a shaded bar. The rescuing *Stu*I–*Sac*II fragment of R06C7 is shown below the cosmid. Open boxes represent the exons of the predicted genes within the subclone. Arrows indicate the direction of transcription. (B) *lin-61* gene structure as determined from cDNA and genomic sequences. Solid boxes indicate coding sequence. Open box indicates the 3′ untranslated region. Predicted translation initiation (ATG) and termination (TAA) codons are shown along with the site of polyadenylation [poly(A)] and

(*Continued*)

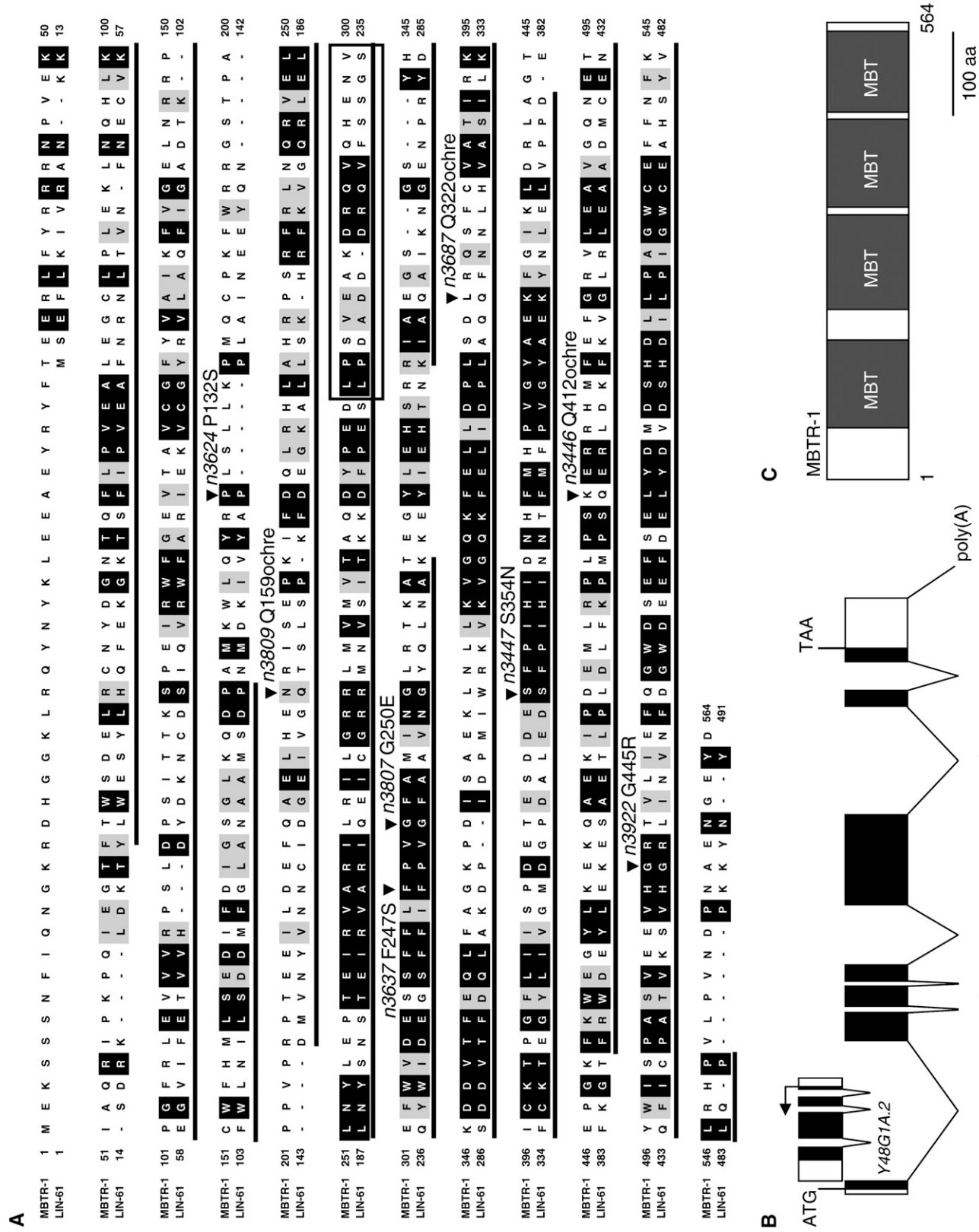
1995; Bornemann *et al.* 1996; Klymenko *et al.* 2006). The SAM domain of SCM mediates homodimerization and interaction with the Polycomb protein Polyhomeotic (Peterson *et al.* 1997; Kim *et al.* 2005). While these Drosophila proteins contain functional domains in addition to MBT repeats, proteins in other species, including human and mouse L(3)MBT2 and Mbtd1, are composed almost exclusively of four MBT repeats, similar to LIN-61 (Figure 2D). Given that LIN-61 does not contain any recognized domain apart from the MBT repeats 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, 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 one other *lin-61* nonsense mutation, *n3446*, and by either of the splice-acceptor mutations, *n3442* and *sy223* (Table 2). Although *n3687* causes an ochre mutation at amino acid 322, animals homozygous for this *lin-61* allele in combination with a loss-of-function mutation in a class A synMuv gene have a weak synMuv phenotype. The low penetrance of this synMuv phenotype suggests that a partially functional LIN-61 protein product might be made in *lin-61(n3687)* animals. If such a product exists, it is either reduced in abundance or not recognized by anti-LIN-61 polyclonal antibodies, as LIN-61 is not detected by immunoblotting (see below). Alternatively, the strain containing *lin-61(n3687)* might contain an additional mutation that partially suppresses the synMuv phenotype. As *n3867* was isolated in a screen for altered transgene expression, this strain contains a closely linked integrated transgene that suppresses recombination on part of LGI, including *lin-61*, and drives the overexpressesion of both *lin-15A* and *lin-15B*. It is possible that this transgene suppresses the synMuv phenotype. *lin-61(n3922)* was isolated in the same screen and contains the same linked transgene. This transgene therefore might reduce the penetrance of the synMuv phenotype in this strain as well. As *n3922* results in a missense rather than in a nonsense mutation, we cannot predict the expected penetrance of the synMuv phenotype in animals homozygous for *n3922* and a mutation in a class A synMuv gene.

Four of the five missense mutations in *lin-61, n3447*, *n3736*, *n3807*, and *n3922* alter residues in 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 the putative null alleles based upon double mutants with the class A synMuv mutations *lin-15A(n433)* and *lin-56(n2728)*. The missense mutations form an allelic series. Specifically, *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 had a phenotype intermediate to the phenotypes caused by other missense mutations, as evident from the fact that *lin-61(n3736)* in combination with *lin-15A(n433)* caused a lower penetrance synMuv phenotype than did *lin-61(n3807)* or *lin-61(n3624)* at 20°. However, when these strains were raised at 23° or when the *lin-61* alleles were combined with a mutation in the class A synMuv gene, *lin-56*, *lin-61(n3676)* caused 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 had 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 a protein containing MBT repeats, we searched the *C. elegans* genome for additional proteins containing MBT repeats. Using BLAST (Altschul *et al.* 1997), Pfam (Bateman *et al.* 2002), and SMART (Schultz *et al.* 2000), we identified a single additional MBT-repeat-containing protein in the *C. elegans* genome, encoded by the predicted gene *Y48G1A.6* (Figure 3A). We determined the sequence of a full-length cDNA for *Y48G1A.6*, yk268b4 (accession no. DQ904352). The GENEFINDER (WormBase at http://wormbase.org, release WS 160) 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

of SL1 *trans*-splicing (SL1). The positions of the mutations found in the 10 *lin-61* alleles are indicated above the gene structure. (C) Alignment of the MBT repeats from the *C. elegans* (*Ce*) proteins LIN-61 and MBTR-1 and the *Homo sapiens* (*Hs*) proteins L(3)MBT2 and L(3)MBT1, accession nos. Q969R5 and Q9Y468, respectively. Each repeat is shown separately with the repeat number indicated next to the protein name. The top portion corresponds to the N-terminal arm and the bottom portion corresponds to the contiguous β-core region of the MBT repeat, as defined by structural analysis (Sathyamurthy *et al.* 2003; Wang *et al.* 2003). Shaded residues indicate identities among >8 of the 15 MBT repeats. Circled residues indicate positions of missense mutations in LIN-61. The corresponding allele is indicated above the residue. The missense mutation *n3624* is located between the first and second repeats. The boxed region indicates the 15 amino acids inserted in the second MBT repeats of LIN-61 and MBTR-1. (D) Schematic of *C. elegans* LIN-61, *H. sapiens* L(3)MBT2, and *Mus musculus* Mbtd1 proteins, accession nos. NP_492050, Q969R5, and AAH62907, respectively. Shaded boxes indicate the positions and relative sizes of the four MBT repeats.


A
MBTR-1
LIN-61
n3624 P132S
n3809 Q159ochre
n3637 F247S
n3807 G250E
n3687 Q322ochre
n3447 S354N
n3446 Q412ochre
n3922 G445R
B
ATG
Y48G1A.2
TAA
poly(A)
500 bp
n4775
C
MBTR-1
MBT
MBT
MBT
MBT
1
564
100 aa

frame of the predicted protein. Furthermore, the incorporation of this predicted intron results in a protein product that is similar to the analogous region of LIN-61 and is internal to the third MBT repeat, suggesting that this intron is likely to be an important part of the protein product. Because the protein encoded by *Y48G1A.6* contains MBT repeats, we named this gene *mbtr-1*, for *m*alignant *b*rain *t*umor *r*epeats.

The primary structure of MBTR-1 is similar to that of LIN-61 (Figure 3A). Like LIN-61, MBTR-1 is composed almost exclusively of four MBT repeats and lacks the SAM domain and zinc fingers found in many MBT-repeat-containing proteins in other organisms (Figure 3). MBTR-1 is 36% identical to LIN-61 and is more similar to LIN-61 than to proteins in any other organisms. However, according to BLAST searches (Altschul *et al.* 1997), LIN-61 is more similar to the five homologs in *C. briggsae* than it is to MBTR-1. LIN-61, MBTR-1, and the five *C. briggsae* homologs of these two genes share an insertion of ~15–16 amino acids in their second MBT repeat not found in other MBT-repeat-containing proteins (Wang *et al.* 2003; Figure 2C and Figure 3A). This observation suggests that Caenorhabditis MBT-repeat-containing proteins might have diverged from a single ancestral protein rather than arising from multiple different MBT-repeat-containing ancestral proteins. It is unclear how these additional amino acids might alter the structure of the MBT repeat or contribute to the function of the protein.

GENEFINDER (WormBase at http://wormbase.org, release WS 160) predicts and the identification of cDNAs corresponding to *Y48G1A.2* (WormBase at http://wormbase.org, release WS 160) confirm the existence of the open reading frame Y48G1A.2 within the first intron of *mbtr-1* (Figure 3B). *Y48G1A.2* and *mbtr-1* are transcribed from different strands.

To analyze 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 frameshift after amino acid 165. The *n4775* deletion does not remove any of the coding sequence for Y48G1A.2. It remains possible that *n4775* could effect the expression of *Y48G1A.2*, because the deletion removes upstream sequences >1 kb from the translational start site for Y48G1A.2; these sequences might be necessary for proper expression of *Y48G1A.2*.

We have not identified a mutant phenotype associated with *mbtr-1(n4775)*. *mbtr-1(n4775)* did not cause a synMuv phenotype in combination with either the strong class A synMuv mutant *lin-15A(n767)* or the strong class B synMuv mutant *lin-15B(n744)* (0% $n > 100$). Animals mutant for both *mbtr-1* and *lin-61* were not Muv and did not display any other obvious phenotypic defects (0% $n > 100$). *mbtr-1* and *lin-61* do not redundantly provide class A synMuv activity, as *mbtr-1(n4775) lin-61(n3809); lin-15B(n744)* animals were not Muv. Additionally, the deletion allele of *mbtr-1* did not enhance or suppress the synMuv phenotype of *lin-61(n3809); lin-15A(n767)* animals: 97% of *lin-61(n3809); lin-15A(n767)* ($n = 166$) animals were Muv at 20° and 24% were Muv at 15° ($n = 118$), and, similarly, 94% of *mbtr-1(n4775) lin-61(n3809); lin-15A(n767)* ($n = 157$) animals were Muv at 20° and 27% were Muv at 15° ($n = 145$).

Expression of a *lin-61* cDNA in the hypodermis under the control of the *dpy-7* promoter (Myers and Greenwald 2005) rescued the synMuv phenotype of *lin-61(n3809); lin-15A(n767)* animals. Specifically, in seven independent lines, expression of *dpy-7p::lin-61* reduced the penetrance of the synMuv phenotype of *lin-61(n3809); lin-15A(n767)* animals from 97% to 27, 29, 31, 47, 48, 48, or 52% (supplemental Table 1 at http://www.genetics.org/supplemental/). However, when we used the *dpy-7* promoter to drive expression of a full-length *mbtr-1* cDNA in *lin-61(n3809); lin-15A(n767)* animals, little if any rescue of the synMuv phenotype was observed in eight independent lines. The penetrance of the synMuv phenotype for the eight lines was 78, 86, 88, 91, 93, 93, 94, and 100% (supplemental Table 1 at http://www.genetics.org/supplemental/). While we cannot be certain that in these experiments MBTR-1 was expressed at the same level and time as LIN-61was, these data suggest that, although the two proteins are closely related, MBTR-1 is unable to provide the function normally provided by LIN-61.

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. Affinity-purified antibodies recognized a band corresponding to a protein of ~60 kDa on Western blots of protein extracts from wild type but not *lin-61(n3809)* animals (Figure 4A). This molecular weight is similar to the size of the predicted LIN-61 product, 57 kDa.

We used both the guinea pig and rabbit polyclonal antibodies to analyze the localization of LIN-61 by immunostaining embryos, larvae, and adult hermaphrodites.

Figure 3.—MBTR-1 sequence and structure. (A) Alignment of MBTR-1 and LIN-61. Solid boxes indicate identities between LIN-61 and MBTR-1, and shaded boxes indicate similarities between the two proteins. Underlined regions correspond to the four MBT repeats. The solid box indicates the 15–16 amino acid insertions in the second MBT repeats of LIN-61 and MBTR-1. (B) *mbtr-1* gene structure as determined from cDNA and genomic sequences. Solid boxes indicate coding sequence. Open boxes indicate 5′ and 3′ untranslated regions. Predicted translation initiation (ATG) and termination (TAA) codons are shown along with the site of polyadenylation [poly(A)]. The predicted gene within the first intron of *mbtr-1*, *Y48G1A.2*, is shown. The arrow depicts the direction of transcription. The genomic region deleted in *n4775* is indicated by brackets. (C) Schematic of the MBTR-1 protein. Shaded boxes indicate the positions and relative sizes of the four MBT repeats.

Similarly to all synMuv proteins studied to date (Melendez and Greenwald 2000; Ceol and Horvitz 2001; Couteau *et al.* 2002; Ceol and Horvitz 2004; Davison *et al.* 2005; Harrison *et al.* 2006), LIN-61 was localized to all or almost all nuclei throughout development from the one-cell embryo to the adult (Figure 4, B and H, and data not shown). In the embryo, LIN-61 appeared to localize to discrete foci in the nucleus (Figure 4, B and D). Both HPL-2 and LIN-13 have been reported to 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 was 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 4, H and J). No anti-LIN-61 staining was seen in *lin-61(n3809)* mutant embryos, larvae, or adults (Figure 4, F and L, and data not shown).

To understand better how LIN-61 might act with other synMuv proteins to regulate vulval development, we analyzed the localization of LIN-61 in animals mutant for any of 26 genes that regulate vulval development, including four class A synMuv genes, 17 class B synMuv genes, two class C synMuv genes, and genes encoding three Ras-pathway modifiers that function to regulate vulval development (supplemental Table 2 at http://www.genetics.org/supplemental/). No 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: Crystal structures have been solved for both a peptide containing two MBT repeats and a peptide containing three MBT repeats (Sathyamurthy *et al.* 2003; Wang *et al.* 2003). Both structures show that individual MBT 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 a stabilized tertiary structure (Sathyamurthy *et al.* 2003; Wang *et al.* 2003). The N-terminal arm of the first repeat interacts with the core region of the last repeat, forming, in the case of three repeats, a propeller-like structure (Wang *et al.* 2003).

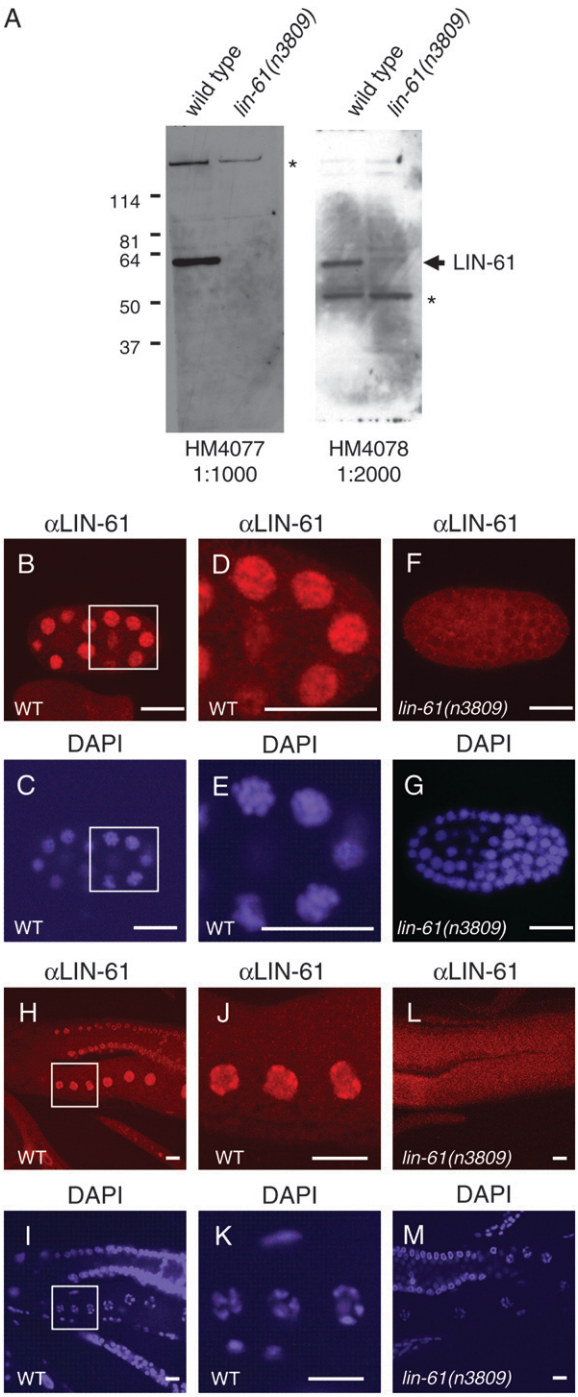


Figure 4.—LIN-61 is an ubiquitously expressed nuclear protein with punctate localization. (A) Affinity-purified antibodies raised against recombinant LIN-61 were used to blot extracts from both wild-type and *lin-61(n3809)* mutant animals. Asterisks denote nonspecific immunoreactivity. HM4077 antibodies were raised in a guinea pig. HM4078 antibodies were raised in a rabbit. (B, D, F, H, J, and L) Whole-mount staining with anti-LIN-61 antisera. HM4077 was used for whole-mount staining of embryos, and HM4078 was used for whole-mount staining of adults. (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) Enlargement of the boxed portion of B. (E) Enlargement of the boxed portion of C. (F) LIN-61 was absent in *lin-61(n3809)* embryos. (G) DAPI staining of the embryo shown in F. (H) LIN-61 was broadly expressed in the adult hermaphrodite germline and was localized to condensed chromosomes. (I) DAPI staining of the germline shown in H. (J) Enlargement of the boxed portion of H. (K) Enlargement of the boxed portion of I. (L) LIN-61 staining was absent from the germline of *lin-61(n3809)* adult hermaphrodites. (M) DAPI staining of the germline shown in K. WT, wild type. Bars, 10 μm.

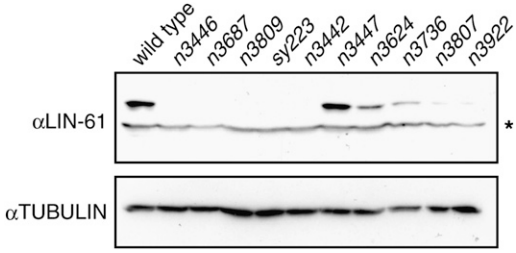


Figure 5.—Residues within the β-core region MBT repeats of LIN-61 are likely to be important for protein folding and stability. LIN-61 levels are reduced in many *lin-61* mutant animals. Equivalent amounts of protein from mixed-stage cultures of each of the genotypes indicated above the lanes were loaded in each lane. Proteins were separated by SDS–PAGE and immunoblotted with the antibodies indicated at the left. Antitubulin antibodies were used to assess protein loading and transfer. The asterisk denotes nonspecific immunoreactivity.

We analyzed LIN-61 protein levels in strains carrying each of the 10 mutant alleles of *lin-61* to determine whether 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 5; data not shown). (It is conceivable, but unlikely, that a stable truncated protein product that does not contain the epitope recognized by either of the polyclonal antibodies was present.) *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)* animals had wild-type or nearly wild-type LIN-61 protein levels. These data were verified by analyzing LIN-61 protein levels in animals with any of the five missense mutations using both Western blots and immunocytochemistry (Figure 5 and data not shown).

Analysis of pleiotropies associated with loss of function of *lin-61* or *mbtr-1*: The class B synMuv genes have roles in many processes in addition to the regulation of vulval development, including the regulation of RNAi sensitivity, restriction of the domains of expression of PGL-1 and *lag-2::gfp*, regulation of transgene expression, protection of the genome from mutations, and suppression of vulval defects caused by the mutation *mat-3(ku233)* (Hsieh *et al.* 1999; Dufourcq *et al.* 2002; Unhavaithaya *et al.* 2002; Garbe *et al.* 2004; Poulin *et al.* 2005; Wang *et al.* 2005; Cui *et al.* 2006b). While class B synMuv genes act similarly to each other in vulval development, they often do not function similarly in the aforementioned processes (Hsieh *et al.* 1999; Garbe *et al.* 2004; Poulin *et al.* 2005; Wang *et al.* 2005; Cui *et al.* 2006b). To understand better the biological roles of *lin-61* and *mbtr-1* and to compare these genes to the previously described class B synMuv genes, we investigated whether putative null mutations in either or both of these genes results in specific pleiotropies known to be affected by the synMuv genes (Table 3).

Mutations in a number of class B synMuv genes have been reported to cause hypersensitivity to RNAi (Wang *et al.* 2005; Cui *et al.* 2006b). We found in multiple experiments that *lin-61(n3809)*, *mbtr-1(n4775)*, and *mbtr-1(n4775) lin-61(n3809)* animals did not show enhanced sensitivity to either *hmr-1* or *cel-1* RNAi as compared to the 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).

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
Ectopic PGL-1 staining	No	No	No	Yes[d]	Yes
Ectopic *lag-2::gfp* expression[e]	No	No	No	Yes	Yes
Mutator	Yes	No	Yes	No	No
Tam	No	No	No	Yes[f]	Yes[f]
Suppressor of *mat-3(ku233)*	Yes	No	Yes	Yes[g]	Yes[g]

For details concerning how each phenotype was scored, see materials and methods. Data for quantitative assays are presented in the text or in the references cited.

[a] *mbtr-1(n4775)* did not enhance or suppress the synMuv phenotype of *lin-61(n3809); lin-15A(n767)* animals.
[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.
[f] Hseih *et al.* (1999).
[g] Garbe *et al.* (2004).

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). Using antibody staining, we did not observe any PGL-1 misexpression in *lin-61(n3809)*, *mbtr-1(n4775)*, or *mbtr-1(n4775) lin-61(n3809)* animals. Staining of *lin-15B(n744)* animals with the same antibody reliably showed misexpresion of PGL-1 in the soma, as previously reported (Wang *et al.* 2005).

In addition to repression of PGL-1 expression, class B synMuv genes also restrict the domain of *lag-2::gfp* expression. A *lag-2::gfp* reporter that is expressed in the distal tip cells and vulvas of wild-type hermaphrodites is misexpressed in the gut of *hda-1* mutant animals (Dufourcq *et al.* 2002). RNAi against any of several other synMuv genes also causes *lag-2::gfp* misexpression (Poulin *et al.* 2005). In a *lin-61(n3809)*, *mbtr-1(n4775)*, or *mbtr-1(n4775) lin-61(n3809)* genetic background, *lag-2::gfp* did not display similar misexpression in the gut.

Most class B synMuv genes can prevent the silencing of repetitive transgene arrays in the soma, such as the *myo-3::gfp* transgene (Hsieh *et al.* 1999), and loss of function of any of these genes results in the transgene array modifier (Tam) phenotype. Neither *lin-61(n3809)*, *lin-61(n3922)*, *mbtr-1(n4775)*, nor *mbtr-1(n4775) lin-61(n3809)* resulted in silencing of an extrachromosomal array carrying *myo-3::gfp*. Animals homozygous for *lin-35(n745)* and the same *myo-3::gfp* extrachromosomal array had a Tam phenotype, as had previously been reported (Hsieh *et al.* 1999).

A role for *lin-61* in protecting the genome from DNA instability was identified in a genomewide RNAi screen (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* or any of 60 other genes could cause mosaic expression of the transgene, suggesting that loss of function of these genes can result in insertion or deletion mutations that cause the *LacZ* open reading frame to be in frame with the translational start site. In addition to *lin-61*, the only other class B gene that was identified in this screen was *hda-1*. We used the same out-of-frame *LacZ* reporter to test whether a loss-of-function allele of *lin-61* caused mosaic expression of *LacZ* similar to that caused by RNAi. Mosaic *LacZ* staining was evident in a significant proportion of *lin-61(n3809)* animals. Mosaic *LacZ* staining was also observed in *mbtr-1(n4775) lin-61(n3809)* animals but not in singly mutant *mbtr-1(n4775)* animals. In the same experiment, animals homozygous for the transgene alone showed no *LacZ* staining. In addition, animals homozygous for *lin-35(n745)* or *lin-15B(n744)* and the transgene did not show any *LacZ* staining. Thus, *mbtr-1*, *lin-35*, and *lin-15B* do not share with *lin-61* a role in maintaining genome stability.

The partial loss-of-function allele *ku233* of the gene *mat-3*, which encodes a member of the anaphase-promoting complex, causes a vulval defect that can be suppressed by loss of function of the class B synMuv genes *lin-35*, *lin-15B*, *lin-53*, *dpl-1*, and *efl-1* (Garbe *et al.* 2004). No coding mutations have been reported in *mat-3* animals homozygous for the *ku233* allele. The vulval defect of *mat-3(ku233)* animals is likely caused by two adjacent nucleotide changes 400 bp upstream of the *mat-3* translational start site, resulting in a 5- to 10-fold reduction in *mat-3* RNA levels (Garbe *et al.* 2004). A likely null allele of *lin-35* restores expression of *mat-3* to wild-type levels, suggesting that LIN-35 represses transcription of *mat-3* (Garbe *et al.* 2004). As reported for loss-of-function mutations in other class B synMuv genes, *lin-61(n3809)* suppressed the *mat-3(ku233)* vulval defect: 58% of *mat-3(ku233)* ($n = 55$) animals had abnormal vulvas, as compared to only 3.5% of *lin-61(n3809); mat-3(ku233)* ($n = 57$) animals. Thus, LIN-61 might act with LIN-35 to repress transcription of *mat-3*. Loss of *mbtr-1* function did not suppress the *mat-3(ku233)* vulval defects and did not significantly modify the *lin-61(n3809)* suppression: 59% of *mbtr-1(n4775); mat-3(ku233)* ($n = 51$) had abnormal vulvas and 12% of *mbtr-1(n4775) lin-61(n3809); mat-3(ku233)* ($n = 43$) had abnormal vulvas. (In this case, 12% is not statistically different from 3.5% as determined by chi square test; $P > 0.1$.)

Double mutants between *mbtr-1(n4775)* and *lin-61(n3809)* appeared indistinguishable from the *lin-61(n3809)* single-mutant animals in all of these assays (Table 3). The inability to detect a role for *lin-61* in a number of synMuv-regulated pleiotropies therefore is not the result of redundant function with the only other MBT-repeat-containing protein.

LIN-61 is not a core member of the DRM or NuRD-like complexes of synMuv proteins: We have recently identified two complexes 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 (Unhavaithaya *et al.* 2002; Harrison *et al.* 2006). We used co-immunoprecipitation experiments to test whether LIN-61 associates with members of either of these two complexes. We demonstrated that although LIN-61 could be precipitated from wild-type but not *lin-61(n3809)* embryonic extracts using anti-LIN-61 antibodies, DRM complex members failed to co-immunoprecipitate with LIN-61 (Figure 6A). Reciprocally, LIN-61 failed to co-immunoprecipitate with the DRM complex members LIN-37 and LIN-9 (Figure 6B). These data indicate that LIN-61 is not a core component of the DRM complex, although LIN-61 could be weakly associated with the complex or associate at only certain stages of development or in only specific cell types. HDA-1, a component of the NuRD-like complex, similarly failed to co-immunoprecipitate with LIN-61 (Figure 6A), suggesting that LIN-61 also is not a member of the

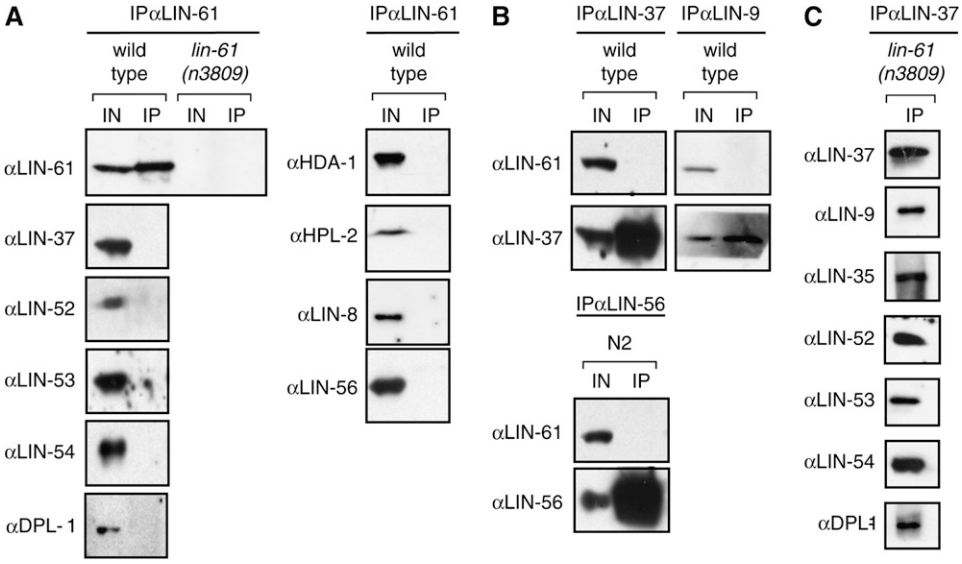


Figure 6.—LIN-61 is not a core component of the DRM or NuRD-like complexes. (A) LIN-61 does not immunoprecipitate a number of synMuv proteins, including members of the DRM and NuRD-like complexes. Extracts from either wild-type or *lin-61(n3809)* mutant embryos (as indicated above the lanes) were precipitated using antibodies against LIN-61 (HM4077). Immunoprecipitates were analyzed using immunoblots with antibodies specific to the antigen indicated at the left. (B) DRM complex members LIN-37 and LIN-9 co-immunoprecipitate a number of class B synMuv proteins (Harrison *et al.* 2006), but fail to co-immunoprecipitate LIN-61. Extracts from wild-type embryos were precipitated using antibodies indicated above the lanes and immunoblotted with antibodies specific to the antigens indicated at the left. (C) DRM complex assembly and stability is not perturbed in animals lacking *lin-61* function. Extracts from *lin-61(n3809)* mutant embryos were precipitated using antibodies against LIN-37 and immunoblotted with antibodies specific to the antigen indicated at the left. IN, 2% of the input; IP, 100% of the immunoprecipitate.

NuRD-like complex. Two class A synMuv proteins, LIN-8 and LIN-56, and the class B synMuv protein HPL-2 also did not co-immunoprecipitate with LIN-61 (Figure 6A).

While our data demonstrate that LIN-61 is not a core component of the DRM complex, it remained possible that LIN-61 could act to modify the formation of this complex. We therefore used co-immunoprecipitation experiments to determine whether the DRM complex is properly formed in *lin-61(n3809)* mutant animals. Seven members of the DRM complex that co-immunopreciptate with LIN-37 in extracts from wild-type animals also co-immunoprecipitated with LIN-37 in extracts from *lin-61(n3809)* mutant embryos (Figure 6C), suggesting that LIN-61 function is not required for proper DRM complex formation. However, we cannot preclude the possibility that LIN-61 might affect the activity of the DRM complex through a mechanism distinct from altering complex formation or stability.

DISCUSSION

At least 26 synMuv genes have been identified, 19 of which have been categorized as class B synMuv genes (Ferguson and Horvitz 1985; Hsieh *et al.* 1999; Thomas *et al.* 2003; Ceol and Horvitz 2004; Davison *et al.* 2005; Poulin *et al.* 2005; Ceol *et al.* 2006). Genetic and biochemical studies suggest that these class B synMuv genes are not all likely to regulate vulval cell-fate specification together through a single mechanism. For example, the genes *hda-1*, *let-418*, and *lin-13* have been categorized as class B synMuv genes because loss-of-function mutations in these genes cause a strong synMuv phenotype in combination with loss of function in a class A synMuv gene. However, these loss-of-function mutations cause a weak Muv phenotype as single mutants (Ferguson and Horvitz 1985; 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 second mutation in a class A or C synMuv gene. We have shown that some synMuv proteins likely function together in a NuRD-like complex that is biochemically distinct from the DRM complex, which contains at least 8 other class B synMuv proteins (Harrison *et al.* 2006).

In this article, we demonstrate that one of the two *C. elegans* MBT-repeat-containing proteins, LIN-61, acts with other class B synMuv proteins to regulate vulval development. We further show that the LIN-61 does not share with other class B synMuv proteins a role in RNAi hypersensitivity, *pgl-1* and *lag-2::gfp* repression, or modification of transgene silencing. Furthermore, LIN-61 has a role in maintaining genome stability not evident for LIN-35 or LIN-15B. These data in combination with the observation that LIN-61 does not co-immunoprecipitate with a large number of synMuv proteins suggest that LIN-61 can function independently of other class B synMuv proteins.

MBT-repeat-containing proteins are not required for *C.elegans* viability: *C. elegans* contains only two predicted proteins containing MBT repeats, LIN-61 and MBTR-1. Both are composed almost exclusively of MBT repeats and lack both the atypical zinc fingers and the SAM domain found in many MBT-repeat-containing proteins in other organisms, including Sex Comb on Midleg, Sfmbt, and lethal (3) malignant brain tumor, proteins required for the viability of Drosophila (Wismar *et al.* 1995; Bornemann *et al.* 1996; Klymenko *et al.* 2006).

By contrast, the *C. elegans* MBT-repeat-containing proteins are not required for viability. Mutant animals that lack both LIN-61 and MBTR-1 appear superficially wild type. We showed that while LIN-61 is important for the proper regulation of vulval development in sensitized genetic backgrounds, 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.

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 proteins Sfmbt and SCM (Bornemann *et al.* 1996; Klymenko *et al.* 2006), that latter of which is a substoichiometric component of the Polycomb Repression Complex 1 (PRC1) (Shao *et al.* 1999). 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, 1998). Human L(3)MBT1 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 analyses demonstrate 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, MBT domains from the human proteins L(3)MBT1 and CGI-72 and the Drosophila protein Sfmbt have been shown to bind histones methylated on specific residues (Kim *et al.* 2006; Klymenko *et al.* 2006). Together, these findings suggest that MBT repeats might bind to modified histones and repress transcription. Our observations—that LIN-61 is composed almost exclusively of MBT repeats, that LIN-61 functions with chromatin modifiers in vulval development, and that LIN-61 localizes to condensed chromosomes—suggest that LIN-61 functions in transcriptional repression possibly via direct interaction with modified histones.

***lin-61* missense mutations identify residues of MBT repeats likely important for structure and function:** We showed that LIN-61 levels are severely reduced in *n3736*, *n3807*, and *n3922* mutant animals, suggesting that the residues mutated in these animals might be necessary for proper protein folding and/or stability. Consistent with this hypothesis, we examined the crystal structures of peptides containing MBT repeats (Sathyamurthy *et al.* 2003; Wang *et al.* 2003) and observed that the residues mutated by both *n3736* and *n3807* are likely to be in the β-barrel core region and to be important for interaction with the N-terminal arm of the preceding repeat.

We determined a possible structure for the fourth MBT repeat of LIN-61 using homology modeling based on the crystal structure for the MBT repeats from human L(3)MBT1 (Wang *et al.* 2003). This structure revealed that *n3922* is located in the turn between β-sheets 2 and 3 of the β-barrel core region of MBT repeat 4. In other MBT repeats, this residue is most often a neutrally charged glycine or a positively charged aspartate. The mutation in *n3922* changes a glycine to a negatively charged arginine. Perhaps the protein structure can accommodate a neutral or positive charge at this turn, but is disrupted by the incorporation of a negative charge.

The missense mutations *n3447* and *n3624* 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 rather are important for LIN-61 function. The missense mutation in *n3447* is in the third MBT repeat and changes a serine to an asparigine. 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. Because the mutation in *n3624* is in the region between the first and second MBT repeats, this portion of LIN-61 is also likely to be important for function.

***lin-61* functions separately from the DRM and NuRD-like complexes:** In Drosophila, the MBT-repeat-containing protein l(3)mbt was identified as a substoichiometric component of the Myb–MuvB complex, named to reflect the fact that it contains both Myb and a number of proteins homologous to the *C. elegans* class B synMuv proteins (Lewis *et al.* 2004). The Myb–MuvB complex includes a fly Rb protein as well as dE2F2 and dDP and represses transcription of many E2F-responsive genes. 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 function with the Myb–MuvB complex only at specific promoters.

The Myb–MuvB complex and another Drosophila complex, the dREAM complex (Korenjak *et al.* 2004; Lewis *et al.* 2004), are very similar to the DRM complex that we identified in *C. elegans* (Harrison *et al.* 2006). Our co-immunoprecipitation data demonstrate that LIN-61 is not a core component of either the DRM or the NuRD-like complexes. However, since the immunoprecipitates were from embryonic protein extracts, it is possible 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, such as vulval development. Nonetheless, 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.

hda-1, which encodes a histone deacetylase component of the NuRD-like complex, was the only synMuv gene that was identified along with *lin-61* in the RNAi screen for genes with a role in protecting the genome from instability (Pothof *et al.* 2003). However, we detected no association between HDA-1 and LIN-61 in co-immunoprecipitation experiments. It is possible, as discussed above, that these proteins interact transiently or interact only in a subset of cells. Alternatively, HDA-1 and LIN-61 might have distinct roles in maintaining the stability of the genome. An additional 59 genes were identified in the RNAi screen (Pothof *et al.* 2003) and might function with HDA-1, LIN-61, or both in this capacity.

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. A loss-of-function mutation in *lin-61* causes a class B synMuv phenotype and genomic instability 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 cause a class B synMuv phenotype and to suppress *mat-3(ku233)*. However, both *lin-35(n745)* and *lin-15B(n744)* share a number of other pleiotropies that do not appear to involve the function of either MBT-repeat-containing protein, including RNAi hypersensitivity, PGL-1 somatic misexpression, the Tam phenotype, and ectopic *lag-2::gfp* expression in the gut (Table 3). These data suggest that in many biological functions *lin-61* does not act with *lin-35* or *lin-15B*, although *lin-35* and *lin-15B* might act together in these functions.

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 does not share roles in many other biological processes with other class B synMuv genes. LIN-61 is also distinguished from other class B synMuv proteins by co-immunoprecipitation experiments, which show that LIN-61 is not a core component of the DRM or NuRD-like complexes. On the basis of these observations, we suggest that other MBT-repeat-containing proteins, such as Polycomb-group proteins, act with Rb-containing and HDAC-containing complexes to control certain biological processes but act independently of these complexes to regulate other biological processes.

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