

The LIN-15A and LIN-56 Transcriptional Regulators Interact to Negatively Regulate EGF/Ras Signaling in *Caenorhabditis elegans* Vulval Cell-Fate Determination

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ABSTRACT

The restricted expression of epidermal growth factor (EGF) family ligands is important for proper development and for preventing cancerous growth in mammals. In *Caenorhabditis elegans*, the class A and B synthetic multivulva (synMuv) genes redundantly repress expression of *lin-3* EGF to negatively regulate Ras-mediated vulval development. The class B synMuv genes encode proteins homologous to components of the NuRD and Myb-MuvB/dREAM transcriptional repressor complexes, indicating that they likely silence *lin-3* EGF through chromatin remodeling. The two class A synMuv genes cloned thus far, *lin-8* and *lin-15A*, both encode novel proteins. The LIN-8 protein is nuclear. We have characterized the class A synMuv gene *lin-56* and found it to encode a novel protein that shares a THAP-like C₂CH motif with LIN-15A. Both the LIN-56 and LIN-15A proteins localize to nuclei. Wild-type levels of LIN-56 require LIN-15A, and wild-type levels and/or localization of LIN-15A requires LIN-56. Furthermore, LIN-56 and LIN-15A interact in the yeast two-hybrid system. We propose that LIN-56 and LIN-15A associate in a nuclear complex that inhibits vulval specification by repressing *lin-3* EGF expression.

TUMORIGENESIS requires misregulation of pathways controlling cell proliferation, differentiation, and apoptosis and likely involves multiple mutations that result in the activation of proto-oncogenes and the inactivation of tumor-suppressor genes. A particularly frequent target of misregulation in human cancers is the epidermal growth factor (EGF) and Ras-signaling pathway that controls cell proliferation. The EGF/Ras pathway can be overactivated by misexpression of EGF-like ligands, mutation or overexpression of EGF receptors, or constitutive mutational activation of Ras (reviewed by NORMANNO *et al.* 2001, 2006; DOWNWARD 2003).

In *Caenorhabditis elegans*, an EGF/Ras pathway plays a central role in vulval development (reviewed by KORNFIELD 1997; MOGHAL and STERNBERG 2003). Six multipotent cells, P(3–8).p, of the ventral ectoderm each can express either the 1° or 2° vulval fates or the 3° nonvulval fate. The LIN-3 EGF ligand is expressed in the anchor cell of the somatic gonad and activates the LET-23 EGF receptor (EGFR) in the closest P(3–8).p cells. LET-23 EGFR subsequently signals through a Ras/MAP kinase

pathway to specify vulval fates (AROIAN *et al.* 1990; HAN and STERNBERG 1990; HILL and STERNBERG 1992; LACKNER *et al.* 1994; KORNFIELD *et al.* 1995; WU *et al.* 1995). P6.p assumes the 1° vulval fate, dividing three times to produce eight descendants, and P5.p and P7.p assume the 2° vulval fate, generating seven descendants each. The 22 progeny of P(5–7).p undergo morphogenesis to generate the adult vulva. P3.p, P4.p, and P8.p assume the nonvulval 3° fate and divide once and fuse with a multinucleate hypodermal cell, hyp7. Loss-of-function mutations in components of the EGF/Ras pathway cause P(5–7).p to adopt the nonvulval 3° fate and result in a vulvaless (Vul) phenotype. Gain-of-function mutations in *let-23* EGFR or *let-60* Ras or overexpression of *lin-3* EGF cause P3.p, P4.p, and P8.p to adopt vulval 1° or 2° fates and result in a multivulva (Muv) phenotype (BEITEL *et al.* 1990; HAN and STERNBERG 1990; HILL and STERNBERG 1992; KATZ *et al.* 1996). Muv animals produce extra vulval tissue that forms ectopic ventral protrusions.

The EGF/Ras pathway, which is essential for *C. elegans* vulval induction, is antagonized by the functionally redundant class A and B synthetic multivulva (synMuv) genes (FERGUSON and HORVITZ 1989). Hermaphrodites carrying only a single synMuv mutation generally appear as wild type for vulval induction, while hermaphrodites carrying mutations in both a class A and a class B synMuv gene exhibit a Muv phenotype. Four class A synMuv

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genes and at least 25 class B genes have been identified (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, 2004; COUTEAU *et al.* 2002; UNHAVAITHAYA *et al.* 2002; THOMAS *et al.* 2003; DAVISON *et al.* 2005; POULIN *et al.* 2005; HARRISON *et al.* 2006, 2007; ANDERSEN and HORVITZ 2007). The synMuv genes likely act upstream of EGF/Ras signaling, as loss-of-function of components of the EGF/Ras pathway can suppress the synMuv phenotype (FERGUSON *et al.* 1987; HUANG *et al.* 1994; LU and HORVITZ 1998; CEOL and HORVITZ 2001, 2004; CUI *et al.* 2006). In class A and class B synMuv double mutants, *lin-3* EGF is overexpressed, likely ectopically, indicating that the synMuv genes negatively regulate EGF/Ras signaling by repressing expression of *lin-3* EGF (CUI *et al.* 2006). All synMuv genes tested, including all four class A synMuv genes, repress *lin-3* EGF expression (CUI *et al.* 2006; ANDERSEN *et al.* 2008).

Given their molecular identities, the class B synMuv genes likely repress *lin-3* via chromatin remodeling. *lin-35*, *eft-1*, *dpl-1*, *lin-53*, *hda-1*, *let-418*, *met-2*, and *hpl-2* encode *C. elegans* counterparts of mammalian Rb, E2F, DP, the Rb-associated protein RbAp48, histone deacetylase (HDAC), the Mi-2 chromatin-remodeling enzyme, a histone H3 lysine-9 methyltransferase, and the histone H3 methyl-lysine-9-binding protein HP1, respectively (LU and HORVITZ 1998; SOLARI and AHRINGER 2000; VON ZELEWSKY *et al.* 2000; CEOL and HORVITZ 2001; COUTEAU *et al.* 2002; ANDERSEN and HORVITZ 2007). Studies of these mammalian proteins strongly suggest that an Rb/E2F/DP complex represses transcription of target genes by recruiting HDAC, RbAp48, histone H3 lysine-9 methyltransferase, and HP1 (reviewed by NIELSEN *et al.* 2001; VANDEL *et al.* 2001; ZHANG and DEAN 2001). RbAp48, HDAC, and Mi-2 are components of the histone deacetylase and chromatin-remodeling NuRD complex (reviewed by KNOEPFLER and EISENMAN 1999) and might be involved in transcriptional repression (reviewed by RICHARDS and ELGIN 2002). Furthermore, LIN-35 Rb, LIN-53 RbAp48, DPL-1 DP, and additional class B synMuv proteins form a complex *in vivo* that resembles the Drosophila Myb-MuvB and dREAM and human LINC/DREAM complexes, which regulate the transcription of many E2F-target genes (KORENJAK *et al.* 2004; LEWIS *et al.* 2004; HARRISON *et al.* 2006; LITOVCHICK *et al.* 2007; PILKINTON *et al.* 2007; SCHMIT *et al.* 2007).

Although the class A synMuv genes function redundantly with the class B synMuv genes, the mechanism by which the class A synMuv genes repress *lin-3* EGF expression to inhibit Ras-mediated vulval development is not known. Of the four class A synMuv genes, the *lin-8* and *lin-15A* loci were cloned previously; both encode novel proteins (CLARK *et al.* 1994; HUANG *et al.* 1994; DAVISON *et al.* 2005). Nonetheless, it is likely that the class A synMuv genes also act to regulate gene expres-

sion. First, although novel in sequence, LIN-8 is a nuclear protein that can interact physically with LIN-35 Rb *in vitro*, suggesting that it might be present *in vivo* at the sites of transcriptional repression complexes (DAVISON *et al.* 2005). Second, the class A synMuv genes either directly or indirectly repress the transcription of *lin-3* EGF (CUI *et al.* 2006). We report here the cloning and characterization of the class A synMuv gene *lin-56* and propose that LIN-56 and LIN-15A normally associate in a nuclear complex that affects cell-fate determination by negatively regulating the EGF/Ras pathway.

MATERIALS AND METHODS

Strains: *C. elegans* strains were cultivated as described (BRENNER 1974) and were grown at 20° unless otherwise indicated. Bristol N2 was the wild-type strain. The mutant alleles used in this study are listed below, and a description is presented by RIDDLE *et al.* (1997) unless noted otherwise: LGII: *dpy-10(e128)*, *lin-8(n2731)* (THOMAS *et al.* 2003), *lin-38(n751)*, and *lin-56(n2728)* (THOMAS *et al.* 2003); LGIII: *lin-36(n766)*; LGIV: *let-60(n1876)* (BEITEL *et al.* 1990); and LGX: *lin-15A(n433, n767, n749)*, *lin-15B(n744)*, and *lin-15AB(e1763)*. Also used was the chromosomal rearrangement *mnC1* [*dpy-10(e128) unc-52(e444)*] and the following deficiencies: *eDf21*, *mnDf16*, *mnDf29*, *mnDf57*, *mnDf61*, *mnDf62*, *mnDf71*, and *mnDf90* (SIGURDSON *et al.* 1984). *lin-56(n3355)* was isolated in a noncomplementation screen using *lin-56(n2728)*.

Transgenic animals: Germline transformation by microinjection was performed as previously described (MELLO *et al.* 1991). The transformation markers pRF4 and *myo-3::gfp* were co-injected with experimental constructs at 80 and 50 ng/μl, respectively. Experimental constructs were injected at 10–100 ng/μl. Stable transformants were analyzed.

Molecular biology: Standard molecular biology procedures were followed (SAMBROOK *et al.* 1989). To disrupt *ZK673.3* in pEMD4, an oligonucleotide linker containing an opal stop codon was ligated into an *SphI* site within the second exon. The resulting construct, pEMD5, contains an insertion of the sequence TGAGACTAGTGCATGC and is predicted to produce a truncated protein containing only the first 120 amino acids of the wild-type 322 amino acids of *ZK673.3*. The disrupted *ZK673.3* open reading frame was transferred to pEMD1 by PCR amplification of the region surrounding the engineered insertion from pEMD5, followed by substitution of the wild-type sequence in pEMD1 with the PCR product, generating pEMD14. To ensure that loss of rescuing activity was caused by the disruption of the *ZK673.3* open reading frame, the latter was restored by digestion of pEMD14 with *SphI* followed by religation, generating pEMD15.

RNA interference: RNA interference (RNAi) was performed by feeding animals bacteria expressing double-stranded RNA, essentially as previously described (KAMATH *et al.* 2001; TIMMONS *et al.* 2001). The bacterial strain expressing double-stranded *lin-3* RNA was from KAMATH *et al.* (2003), and the DNA sequence of the insert was determined to ensure that it was correct.

Antibody preparation and immunocytochemistry: Rabbit anti-LIN-56 antisera were generated using purified GST-LIN-56 (aa 1–322) as the immunogen, affinity-purified against MBP-LIN-56 (aa 1–322) as described by KOELLE and HORVITZ (1996), and pre-adsorbed against an acetone precipitate of proteins prepared from *lin-56(n2728)* mixed-stage worms, essentially as described by HARLOW and LANE (1988). Rabbit

anti-LIN-15A antibodies were generated using purified 6His-LIN-15A (aa 77–324) as immunogen and were purified as with the anti-LIN-56 antisera, using 6His-LIN-15A (aa 77–324) protein for affinity purification and *lin-15AB(e1763)* mixed-stage worms for pre-adsorption. Affinity-purified anti-LIN-56 antibodies were used at 1:2000 for immunoblots and, following pre-adsorption, at 1:100 for immunocytochemistry. Affinity-purified and pre-adsorbed anti-LIN-15A antibodies were used at 1:25 for immunocytochemistry. Anti- α -tubulin monoclonal antibody DM1A (Sigma, St. Louis) 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, 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 by using a Zeiss LSM510 laser confocal microscope and software and were processed with Adobe Photoshop.

Subcellular fractionation of embryos: Embryos were collected by bleaching gravid hermaphrodites in a 0.8 N NaOH, 8% hypochlorite solution. Fractionation into nuclear and cytosolic fractions was performed as described by CHEN *et al.* (2000). The protein content of each fraction was determined using Bradford reagent, and 10 μ g of each fraction was used for immunoblots. Rabbit polyclonal antiserum 3930 generated against *C. elegans* lamin (GRUENBAUM *et al.* 2002) was used at 1:5000 on immunoblots as a control for nuclear fractionation quality. Anti-SQV-4 antibodies (HWANG and HORVITZ 2002) were used at 1:3000 on immunoblots as a control for cytosolic fractionation quality.

RT-PCR analysis: Total RNA was isolated from mixed-stage worms using TRIZOL (Invitrogen, Carlsbad, CA). Standard RT-PCR (Titan One Tube RT-PCR System, Roche Applied Science, Indianapolis) was used to qualitatively compare levels of *lin-56* and *lin-15A* RNA in total RNA samples derived from wild-type, *lin-56(n2728)*, *lin-15A(n767)*, and *lin-15AB(e1763)* animals. RT-PCR of hexokinase (H25P06.1) was performed as a control. Primers used were *lin-15A* Fwd9 (5'-CGAATGTCAAGCTTGGC GAACG-3'), *lin-15A* Rev5 (5'-CGGTTTACTGAGAGACCC-3'), *lin-56* Fwd2 (5'-AGACTGGGCAGAATGCG-3'), *lin-56* Rev2 (5'-GCTCCACTTTTTCAGGAAAAC-3'), hexokinase Fwd1A (5'-GAGCTCGGCATTCAATATCG-3'), and hexokinase Rev1B (5'-GCTTCATATGCAGCTGCAACC-3').

Quantitative real-time RT-PCR (HEID *et al.* 1996) was used to measure the amount of *lin-56* mRNA in poly(A)⁺ mRNA samples derived from wild-type, *lin-15A(n767)*, and *lin-15AB(e1763)* animals. Poly(A)⁺ mRNA was purified from total RNA using Oligotex resin (Qiagen, Valencia, CA). cDNA was produced from the purified poly(A)⁺ mRNA using oligo(dT) primer and SuperScript II Rnase H⁻ Reverse Transcriptase (Invitrogen). Using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), absolute *lin-56* and hexokinase mRNA levels were determined relative to a genomic DNA dilution series, and *lin-56* mRNA levels were then normalized to hexokinase mRNA levels. Fifty- and 100-ng mRNA equivalents were analyzed in triplicate for each genotype. *lin-56* and hexokinase reactions were performed in separate tubes. No significant amplification was observed when reverse transcriptase was omitted from the initial RT reaction. Primers and probes used were *lin-56* Fwd (5'-TTGGTGC AAAGTCTACACGATGA-3'), *lin-56* Rev (5'-TTGCGCACATCG AACTTTGT-3'), *lin-56* probe (5'-6-FAM-TCGATCTTCCCTG GGCGAGCAGT-TAMRA-3'), hexokinase Fwd (5'-CGTGGAGC CGCACTCATC-3'), hexokinase Rev (5'-CAGATCCTTCAGCC GCTTCT-3'), and hexokinase probe (5'-VIC-TCGCTTGACTC TCGAAACGATTGCG-TAMRA-3').

Yeast two-hybrid system: Full-length cDNAs for *lin-15A*, *lin-37*, *lin-53*, and *lin-56* were cloned into Gateway entry vector pDONR201 (Invitrogen) and then each transferred into two Gateway two-hybrid destination vectors: pDEST-DB, encoding the Gal4 DNA-binding domain (DB), and pDEST-AD, encoding the Gal4 activation domain (AD) (WALHOUT *et al.* 2000a,b). Yeast strain Y190 (*MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3 leu2-112 URA3::GAL1-lacZ LYS2::GAL1-HIS3 cyh'*) was cotransformed with DB- and AD-fusion constructs. Interaction in the two-hybrid system was assayed by colony formation on SC-Trp-Leu-His medium containing 25 mM 3-aminotriazole (3-AT).

RESULTS

***lin-56* encodes a putative transcription factor containing a THAP-like domain:** *lin-56* was previously mapped close to *unc-4* on chromosome II (THOMAS *et al.* 2003). Using deficiencies, we further mapped *lin-56* to a region of 554 kb between *daf-19* and *bli-1* (Figure 1A). Cosmid ZK673 and a 3.5-kb *SnaBI*-*SnaBI* subclone of ZK673 (pEMD4) rescued the synMuv phenotype when injected into *lin-56(n2728)*; *lin-15B(n744)* animals (Figure 1B). The 3.5-kb minimal rescuing fragment contains a single predicted gene, *ZK673.3*. We found the only existing *lin-56* allele, *n2728*, to contain an 11.2-kb deletion that eliminates not only *ZK673.3* but also its downstream neighbor, *ZK673.4*, and the 3'-end of its upstream neighbor, *ZK673.2* (Figure 1B).

Since *ZK673.3* and *ZK673.4* share a small region of similarity (see below), we were concerned that the class A synMuv phenotype of the *n2728* mutant might be caused not by loss of *ZK673.3*, as the rescue experiments suggest, but either by loss of *ZK673.4* or by loss of both *ZK673.3* and *ZK673.4*. Disruption of *ZK673.3* in a 10.7-kb rescuing subclone of ZK673 (pEMD1) that contains both *ZK673.3* and *ZK673.4* resulted in loss of rescuing activity (pEMD14) (Figure 1B), and expression of a *ZK673.3* cDNA under the control of heat-shock promoters efficiently rescued the synMuv phenotype of *lin-56(n2728)*; *lin-15B(n744)* animals (Table 1). Furthermore, RNAi (FIRE *et al.* 1998) of *ZK673.3* but not of *ZK673.4* resulted in a Muv phenotype in *lin-15B(n744)* animals (data not shown). Finally, we isolated a second *lin-56* allele, *n3355*, in a noncomplementation screen and found *n3355* to correspond to a glutamine-to-ochre nonsense mutation at amino acid 61 of *ZK673.3* (Figure 1C). The strengths of the class A synMuv phenotypes of *lin-56(n2728)* and *lin-56(n3355)* mutants were indistinguishable, as both caused a fully penetrant Muv phenotype in the weak class B synMuv mutant background *lin-15B(n2245)* at 15°. Therefore, we conclude that *ZK673.3* is *lin-56*.

BLUMENTHAL *et al.* (2002) have suggested that *ZK673.2* and *ZK673.3* may be expressed as an operon, because these two genes are separated by <500 bp and because they detected the *C. elegans* SL2 trans-spliced leader sequence at the 5'-end of *ZK673.3* mRNAs.

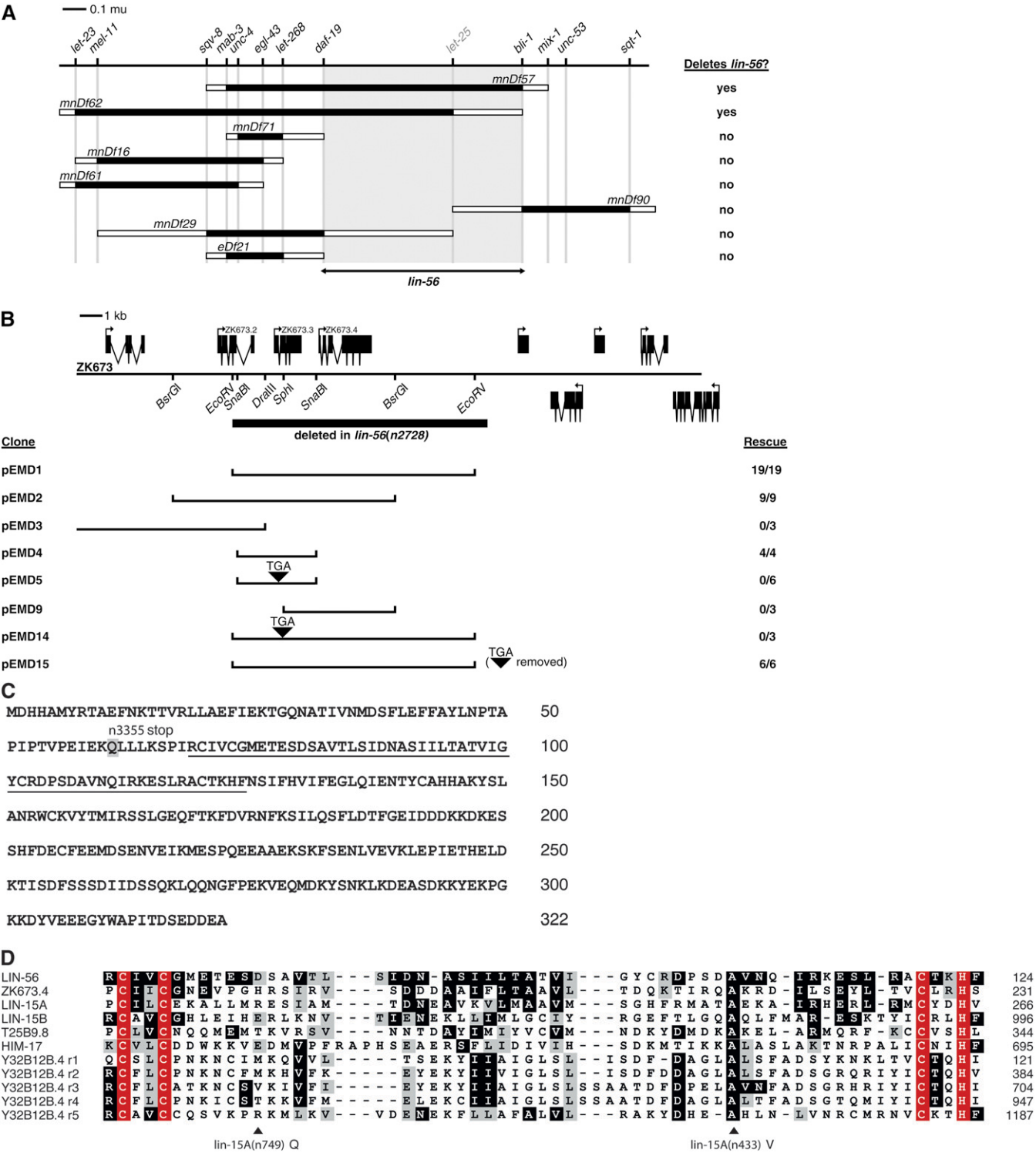


FIGURE 1.—Cloning of *lin-56*. (A) Genetic map of the *lin-56* region. Deficiency mapping placed *lin-56* between the right end-point of *mnDf29* and the left endpoint of *mnDf90*, as defined by the markers *daf-19* and *bli-1*, respectively (shaded region). Cloned genes are in regular type; genes not cloned are in shaded type. Shaded rectangles indicate regions known to be deleted by each deficiency, and open rectangles indicate regions that contain a deficiency endpoint and might be deleted by each deficiency. (B) Transformation rescue of *lin-56*. Predicted genes contained within the rescuing cosmid ZK673 (top) and subclones derived from this cosmid (bottom) are shown. The region deleted in *lin-56*(n2728) mutants is indicated by the solid bar. The sequences flanking the *lin-56*(n2728) deletion are ACCAAAGGAGGAGGTCAGCC [11,190-bp deletion] CCTTGTGGGGGAACAATGCG. Insertion of a TGA stop codon is indicated by a solid arrowhead. (C) Sequence of the LIN-56 protein. The glutamine mutated to an ochre stop codon in the n3355 allele is shaded. The region aligned in D is underlined. (D) Alignment of the novel C₂CH motif found in LIN-56, LIN-15A, LIN-15B, HIM-17, and three additional uncharacterized *C. elegans* proteins. Y32B12B.4 contains five repeats, denoted r1–r5, of the motif. Conserved cysteines and histidine are in red. Solid and shaded boxes indicate identities and similarities, respectively, with LIN-56. Positions of missense mutations found in *lin-15A*(n433) and *lin-15A*(n749) mutants are marked with arrowheads.

TABLE 1

lin-56 overexpression rescues the *lin-56(lf)*; *lin-15B(lf)*, but not the *lin-15AB(lf)*, synMuv phenotype

Genotype	Transgene	Line	Heat shock	% Muv ^a ± SD (n) ^b
<i>lin-56(n2728)</i> ; <i>lin-15B(n744)</i>	<i>P_{hs}-lin-56</i>	1	—	97.9 ± 0.9 (490)
			+	16.5 ± 6.8 (340)
		2	—	99.7 ± 0.3 (608)
			+	17.5 ± 15.3 (244)
<i>lin-56(n2728)</i> ; <i>lin-15B(n744)</i>	<i>P_{hs}-gfp</i>	1	—	100 ± 0 (319)
			+	100 ± 0 (365)
			+	100 ± 0 (280)
<i>lin-15AB(e1763)</i>	<i>P_{hs}-lin-56</i>	1	—	100 ± 0 (211)
			+	100 ± 0 (117)
		2	—	100 ± 0 (112)
			+	100 ± 0 (172)
		3	—	100 ± 0 (319)
			+	100 ± 0 (140)
		4	—	100 ± 0 (226)
			+	100 ± 0 (153)
		5	—	100 ± 0 (107)
			+	100 ± 0 (301)
<i>lin-15AB(e1763)</i>	<i>P_{hs}-gfp</i>	1	—	100 ± 0 (158)
			+	100 ± 0 (158)

lin-56(n2728); *lin-15B(n744)* or *lin-15AB(e1763)* hermaphrodites carrying an extrachromosomal array of either a *lin-56* cDNA (*P_{hs}-lin-56*) or the *gfp*-coding sequence (*P_{hs}-gfp*) fused to the *C. elegans* heat-shock promoters and a dominant Rol marker were synchronized by bleaching and starvation-induced L1 arrest. Expression from the heat-shock promoters was induced at the early L2 stage (24–25 hr post starvation) by incubation at 33° for 1 hr. Animals were assayed in at least three individual batches, with at least 100 Rol worms analyzed in total for each line.

^a % Muv, percentage of Rol animals that were Muv.

^b n, number of animals examined.

However, no sequence upstream of the *ZK673.2* open reading frame was required for rescue by *ZK673.3* of the *lin-56(n2728)* class A synMuv phenotype (Figure 1B), consistent with the hypothesis that *ZK673.2* and *ZK673.3* are at least not always expressed as an operon.

LIN-56, a novel acidic protein of 322 amino acids, contains a domain similar to the THAP domain (Figure 1C), a zinc-finger-like motif that can bind to DNA (CLOUAIRE *et al.* 2005). Specifically, **LIN-56** shares a C-X-[ILV]-C-X(33,38)-A-X(11,13)-C-X(2)-H motif with several *C. elegans* proteins (Figure 1D), including the class A synMuv protein **LIN-15A**, the class B synMuv protein **LIN-15B**, the protein encoded by its downstream genomic neighbor *ZK673.4*, and **HIM-17**, a protein required for meiotic recombination and histone H3 lysine-9 methylation in the germline (REDDY and VILLENEUVE 2004). The conservation of cysteine and histidine residues suggests that this motif may bind a zinc ion and mediate binding to DNA, RNA, protein, or a small molecule (reviewed by LAITY *et al.* 2001). Although the interval between the two internal cysteines is greater than that found in typical zinc fingers (KRISHNA *et al.* 2003), the spacing of the cysteine and histidine residues within the C₂CH motif described here matches the spacing characteristic of the THAP domain, which can mediate sequence-specific DNA binding (ROUSSIGNE *et al.* 2003; CLOUAIRE *et al.* 2005; CAYROL *et al.* 2007; SABOGAL *et al.* 2010). Additional features of the THAP domain, including an invariant tryptophan between the

second and third cysteine residues and a C-terminal AVPTIF motif, are not conserved. This THAP-like motif is likely functionally significant, as two *lin-15A* alleles contain missense mutations therein, and one of these mutations affects a conserved alanine residue (Figure 1D).

The **Muv** phenotypes of many synMuv double mutants are suppressed by RNAi of *lin-3*, suggesting that the synMuv genes act upstream of *lin-3* in vulval development. To determine if *lin-56* also acts upstream of *lin-3*, we inactivated *lin-3* by feeding *lin-56(n2728)*; *lin-15B(n744)* animals bacteria expressing double-stranded *lin-3* RNA. *lin-56(n2728)*; *lin-15B(n744)* animals fed bacteria with an empty vector exhibited a **Muv** phenotype with 100% penetrance (*n* = 209). *lin-56(n2728)*; *lin-15B(n744)* animals fed bacteria expressing *lin-3* double-stranded RNA exhibited a **Muv** phenotype with a lower 85% penetrance (*n* = 179) (*P* < 0.00001), and the expressivity of the **Muv** animals, as determined by the number of ectopic pseudovulvae, was notably lower than animals fed control bacteria. Therefore, *lin-3* is required for the class A synMuv phenotype of *lin-56* mutants, consistent with the hypothesis that *lin-56* acts upstream of *lin-3*.

LIN-56 is a ubiquitous nuclear protein: To determine the expression pattern of the **LIN-56** protein, we generated anti-**LIN-56** antibodies, which recognized a protein of ~40 kDa in wild-type but not in *lin-56(n2728)* protein extracts (Figure 2A); the predicted size of **LIN-56** is 37 kDa. The anti-**LIN-56** antibodies sometimes

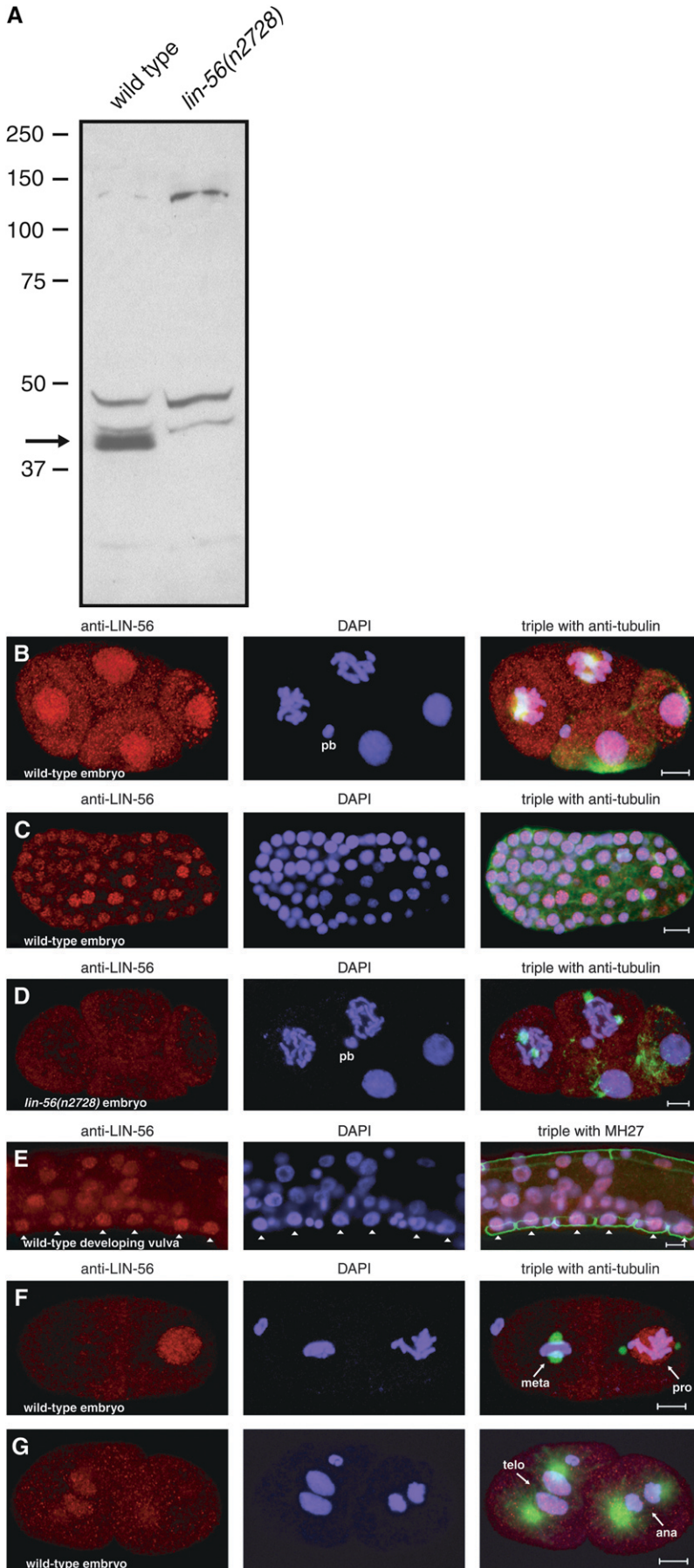


FIGURE 2.—LIN-56 is broadly expressed and localized to nuclei. (A) Immunoblot of wild-type and *lin-56(n2728)* protein extracts probed with anti-LIN-56 antibodies. The arrow indicates the band that corresponds to LIN-56. Molecular weights of marker proteins are indicated at left in kilodaltons. (B–E) Whole-mount staining with anti-LIN-56 antibodies (red), DAPI (blue), and either anti-tubulin antibody (green) in embryos or MH27 antibody (green) in larvae as a fixation control. Scale bars, 5 μ m. Anterior is to the left in all images. pb, polar body. (B and C) LIN-56 staining is observed in the nuclei of most if not all cells throughout embryonic development. Shown are (B) four-cell and (C) late-stage wild-type embryos. (D) Nuclear staining is never observed with anti-LIN-56 antisera in *lin-56(n2728)* animals. Shown is a four-cell *lin-56(n2728)* embryo. (E) LIN-56 is present in the P(3–8).p vulval equivalence group and in their descendants during wild-type vulval development. Shown is the midbody of a wild-type L2 larva. Arrowheads point to nuclei of P(3–8).p. MH27 stains the apical borders between the Pn.p cells. (F and G) LIN-56 does not colocalize with chromatin during metaphase or anaphase. F and G show two-cell wild-type embryos. ana, anaphase; meta, metaphase; pro, prophase; telo, telophase.

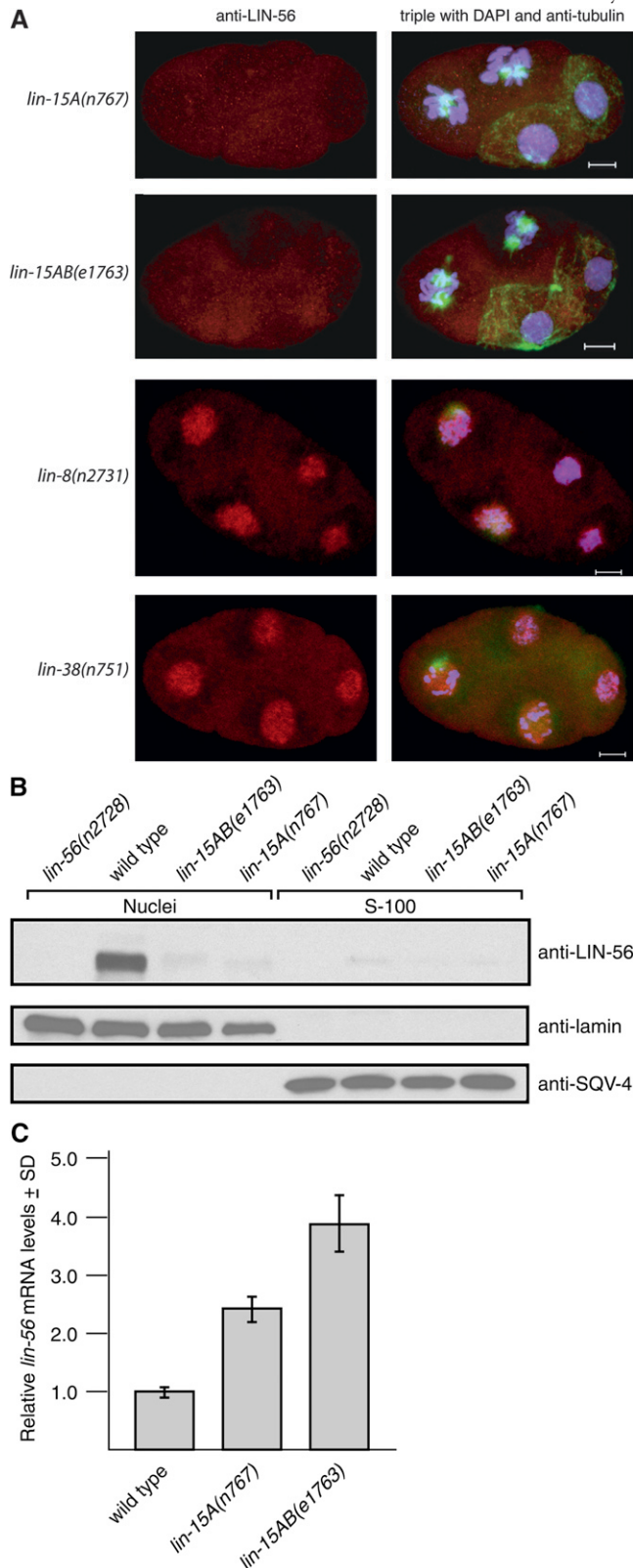


FIGURE 3.—LIN-56 protein but not *lin-56* mRNA levels are greatly reduced in *lin-15A(lf)* mutants. (A) LIN-56 nuclear staining is greatly reduced in *lin-15A(n767)* and *lin-15AB(e1763)* mutants, but not in *lin-8(n2731)* or *lin-38(n751)* mutants. The posterior two cells in the *lin-15A(n767)* and *lin-15AB(e1763)* panels are dividing and therefore would not be expected to display LIN-56 nuclear expression, while the anterior two cells are not dividing. Shown are four-cell embryos stained with anti-

appear to recognize a doublet (data not shown), which could represent alternative splice variants of *lin-56* or products resulting from distinct transcriptional initiation sites. Although the three *lin-56* cDNA clones (courtesy of Y. Kohara) analyzed are identical in sequence and lack any evidence of *trans*-splicing, SL2 *trans*-splicing of the *ZK673.3* mRNA has been observed by others (BLUMENTHAL *et al.* 2002; HWANG *et al.* 2004). Alternatively, LIN-56 may be post-translationally modified. The anti-LIN-56 antibodies also recognized several other proteins (Figure 2A), but those proteins were a different size than predicted for LIN-56 and were present in both wild-type and *lin-56(n2728)* protein extracts and are therefore nonspecific.

The anti-LIN-56 antibodies were used for whole-mount staining of worms. Starting at the late one-cell or early two-cell stage, LIN-56 staining was observed in the nuclei of most if not all somatic cells throughout embryonic and larval development as well as in adulthood (Figure 2, B, C, and E; data not shown). Nuclear staining was never observed with the anti-LIN-56 antibodies in the somatic cells of *lin-56(n2728)* animals of any stage (Figure 2D; data not shown), demonstrating specificity. LIN-56 is present in the P(3–8).p vulval equivalence group and their descendants throughout vulval development (Figure 2E). LIN-56 did not colocalize with chromatin during metaphase or anaphase (Figure 2, F and G). Germline staining was observed in both wild-type and *lin-56(n2728)* animals and thus was not specific.

To confirm that LIN-56 is localized to nuclei, we performed subcellular fractionation of embryo lysates. We used lamin as a marker for nuclear fractions (LIU *et al.* 2000; GRUENBAUM *et al.* 2002) and SQV-4 as a marker for cytosolic material (HWANG and HORVITZ 2002). The majority of the LIN-56 protein cofractionated with nuclear lamin; LIN-56 protein was barely detectable in the SQV-4-containing cytosolic fraction (see Figure 3B). LIN-56 is thus stably associated with nuclei.

LIN-56 protein, but not *lin-56* mRNA, is reduced in *lin-15A(lf)* mutants: We analyzed the impact of mutations in other class A synMuv genes on the expression of LIN-56. After staining with anti-LIN-56 antibodies we observed that the LIN-56 staining pattern of *lin-*

LIN-56 antibody (red), DAPI (blue), and anti-tubulin antibody (green). Scale bars, 5 μ m. Anterior is to the left in all images. (B) Immunoblot of nuclear and S-100 cytosolic fractions derived from wild-type, *lin-56(n2728)*, *lin-15AB(e1763)*, and *lin-15A(n767)* embryos probed with anti-LIN-56 antibody. Shown are the same immunoblot probed with anti-lamin and anti-SQV-4 antibodies as markers for nuclear and cytosolic proteins, respectively. (C) *lin-56* mRNA levels are not reduced in *lin-15A(lf)* mutants as compared to the wild type. Quantitative real-time RT-PCR (HEID *et al.* 1996) was used to measure *lin-56* mRNA levels relative to hexokinase mRNA levels. Results for each genotype are normalized to those for the wild type. Error bars, SD.

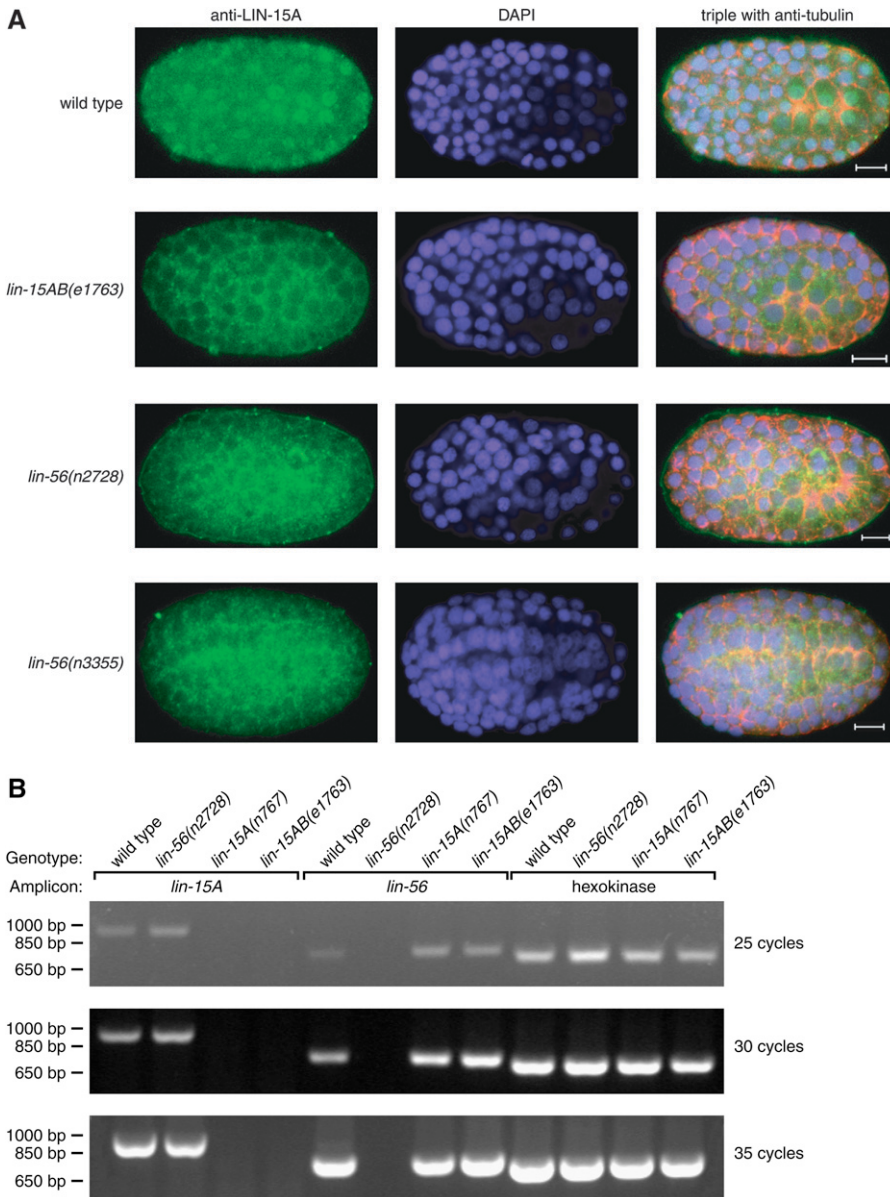


FIGURE 4.—LIN-15A nuclear accumulation but not *lin-15A* mRNA is reduced in *lin-56(lf)* mutants. (A) LIN-15A nuclear staining is greatly reduced in *lin-56(n2728)* and *lin-56(n3355)* mutants. Multicellular embryos were stained with anti-LIN-15A antibodies (green), anti-tubulin antibody (red), and DAPI (blue). The genotype of each embryo is indicated on the left. Scale bars, 5 μ m. Anterior is to the left in all images. (B) *lin-15A* RNA levels are not reduced in *lin-56(n2728)* mutants compared to the wild type. RT-PCR was used to determine levels of *lin-15A*, *lin-56*, and hexokinase RNA in total RNA derived from wild-type, *lin-56(n2728)*, *lin-15A(n767)*, and *lin-15AB(e1763)* animals. Marker DNA fragments are indicated at left. The number of PCR cycles is indicated at right.

8(n2731) and *lin-38(n751)* animals was not different from that of the wild type (Figure 3A; data not shown). *lin-8(n2731)* causes an early nonsense mutation and is likely a protein null allele (DAVISON *et al.* 2005). *lin-38(n751)* is a non-null allele, as null mutations in *lin-38* are inviable (A. M. SAFFER and H. R. HORWITZ, unpublished results). By contrast, LIN-56 nuclear staining was greatly reduced in *lin-15A(n767)* and *lin-15AB(e1763)* individuals at all stages (Figure 3A; data not shown). *lin-15A(n767)* is a small deletion predicted to result in a truncated LIN-15A protein, and *lin-15AB(e1763)* is a large deletion that eliminates *lin-15A* and *lin-15B* (CLARK *et al.* 1994; HUANG *et al.* 1994). Fractionation experiments and immunoblot analyses confirmed that LIN-56 protein was barely detectable in the nuclear fractions of *lin-15A(n767)* and *lin-15AB(e1763)* embryos (Figure 3B). Also, the cytosolic fractions of these *lin-15A(lf)* mutants contained no more

LIN-56 protein than the cytosolic fraction of wild-type embryos (Figure 3B), indicating that the reduced amount of LIN-56 in the nuclei of *lin-15A(lf)* mutants was not the result of mislocalization of this protein to the cytosol. Instead, the total amount of LIN-56 protein in *lin-15A(lf)* animals was greatly reduced in comparison to that in the wild type, suggesting that *lin-15A* is required for the expression or stability of *lin-56* mRNA or LIN-56 protein.

To determine if the low level of LIN-56 protein in *lin-15A(lf)* animals was the result of decreased transcription or stability of *lin-56* mRNA, we used quantitative real-time RT-PCR (HEID *et al.* 1996) to measure *lin-56* mRNA levels in wild-type, *lin-15A(n767)*, and *lin-15AB(e1763)* animals relative to hexokinase mRNA levels. Relative *lin-56* mRNA levels appeared to be somewhat higher in *lin-15A(n767)* and *lin-15AB(e1763)* animals than in wild-type animals (Figure 3C; see also Figure 4B). The reason

TABLE 2

lin-15A overexpression rescues the *lin-36(lf)*; *lin-15A(lf)*, but not the *lin-56(lf)*; *lin-36(lf)*, synMuv phenotype

Genotype	Transgene	Line	Heat shock	% Muv ^a (n) ^b
<i>lin-36(n766)</i> ; <i>lin-15A(n767)</i>	None		—	99.6 (251)
<i>lin-36(n766)</i> ; <i>lin-15A(n767)</i>	<i>P_{hs}-lin-15A</i>	1	—	100 (39)
			+	16 (96)
		2	—	99 (86)
			+	12 (93)
<i>lin-56(n2728)</i> ; <i>lin-36(n766)</i>	None		—	99.6 (234)
<i>lin-56(n2728)</i> ; <i>lin-36(n766)</i>	<i>P_{hs}-lin-15A</i>	1	—	100 (177)
			+	99.8 (605)
		2	—	99.6 (227)
			+	100 (675)

lin-36(n766); *lin-15A(n766)* or *lin-56(n2728)*; *lin-15A(n766)* hermaphrodites with or without an extrachromosomal array of *lin-15A* cDNA (*P_{hs}-lin-15A*) fused to the *C. elegans* heat-shock promoters and a GFP⁺ marker were synchronized by bleaching and starvation-induced L1 arrest. Expression from the heat-shock promoters was induced at the early L2 stage (24–25 hr post starvation) by incubation at 33° for 1 hr.

^a % Muv, percentage of animals that were Muv.

^b n, number of animals examined.

for the apparent increase in *lin-56* mRNA levels in the two *lin-15A(lf)* mutants analyzed was not determined. The decreased amount of LIN-56 protein in *lin-15A(lf)* as compared to wild-type animals thus likely results from a post-transcriptional event, suggesting that LIN-15A is required for either translation or stability of LIN-56 protein.

LIN-15A protein nuclear accumulation, but not *lin-15A* mRNA, is reduced in *lin-56(lf)* mutants: Antisera directed against the LIN-15A protein revealed broad nuclear staining (Figure 4A; data not shown). Cytoplasmic staining was observed in both wild-type animals and in mutants completely lacking LIN-15A and therefore represents nonspecific cross-reactivity and not LIN-15A protein. Since LIN-56 protein levels are reduced in *lin-15A(lf)* mutants, we examined whether LIN-15A protein levels are reduced in *lin-56(lf)* mutants. LIN-15A nuclear

staining was greatly reduced in *lin-56(n2728)* and *lin-56(n3355)* embryos (Figure 4A). This loss of nuclear LIN-15A accumulation indicates that *lin-56* is required for the expression, stability, or nuclear localization of LIN-15A protein. Loss of *lin-56* function reduced LIN-15A nuclear accumulation more in late than in early embryos. LIN-15A nuclear accumulation appeared greater in early *lin-56(n3355)* embryos than in early *lin-56(n2728)* embryos (data not shown), suggesting that *n3355* may not be as strong an allele of *lin-56* as *n2728* or that another gene deleted by *n2728* may also have an effect on LIN-15A levels. LIN-15A expression and localization in *lin-8(n2731)* and *lin-38(n751)* embryos were indistinguishable from those in wild type (data not shown).

We used RT-PCR to measure the amount of *lin-15A* RNA in wild-type, *lin-56(n2728)*, *lin-15A(n767)*, and *lin-15AB(e1763)* animals. *lin-15A* RNA was amplified from

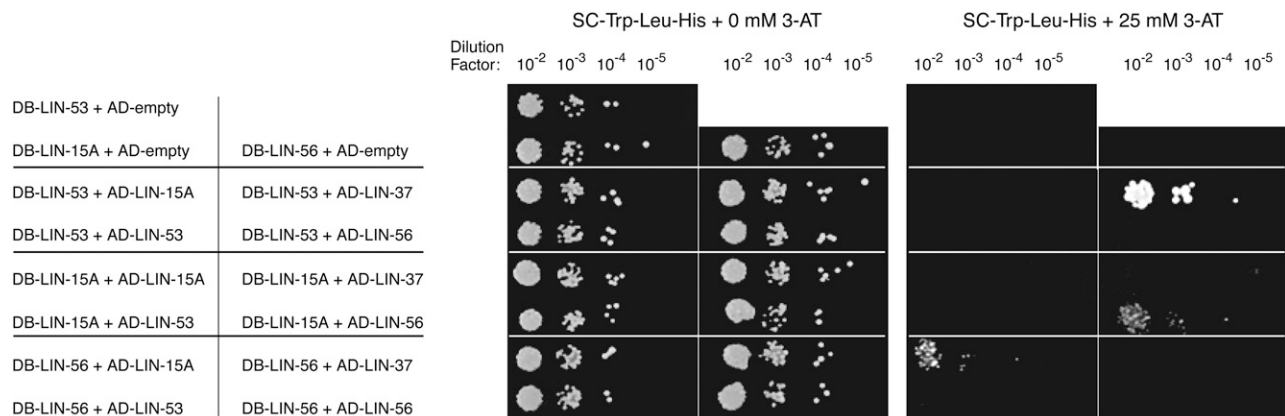


FIGURE 5.—LIN-15A and LIN-56 interact with each other in the yeast two-hybrid system. Growth of cotransformant Y190 colonies on SC-Trp-Leu-His media in either the absence or the presence of 25 mM 3-AT. Interaction of the DB- and AD-fusion proteins results in increased expression of a *GAL1::HIS3* reporter gene, permitting colony formation in the absence of histidine and presence of 3-AT, a competitive inhibitor of the enzyme encoded by *HIS3*. Plasmids cotransformed into each strain are indicated at left. Interactions are evident between DB-LIN-15A and AD-LIN-56, DB-LIN-56 and AD-LIN-15A, and DB-LIN-53 and AD-LIN-37, as previously reported (WALHOUT *et al.* 2000a). DB, Gal4 DNA-binding domain. AD, GAL4 activation domain.

both the wild-type and *lin-56(lf)* RNA samples but not from the *lin-15A(lf)* RNA samples (Figure 4B). The decreased amount or mislocalization of LIN-15A protein in *lin-56(lf)* as compared to wild-type animals thus likely results from a post-transcriptional event, consistent with the hypothesis that LIN-56 is required for the translation, stability, or nuclear localization of LIN-15A protein.

Overexpression of *lin-56* does not rescue the *lin-15AB(lf)* synMuv phenotype, nor does overexpression of *lin-15A* rescue the *lin-56(lf)* synMuv phenotype: If *lin-15A* acts only to control the translation or stability of LIN-56 protein, then restoring the level of LIN-56 protein should rescue the *lin-15A(lf); synMuvB(lf)* synMuv phenotype. To test this possibility, we induced a *lin-56* cDNA under the control of heat-shock promoters shortly after L1 lethargus in *lin-56(n2728); lin-15B(n744)* animals and rescued the synMuv phenotype (Table 1). The same treatment failed to rescue the synMuv phenotype of *lin-15AB(e1763)* animals (Table 1). As assayed by immunoblot, LIN-56 protein was produced under these conditions at levels at least 10-fold greater than those of endogenous LIN-56 in the wild type (supporting information, Figure S1). The mechanism that normally prevents LIN-56 protein accumulation in *lin-15AB(e1763)* animals is likely overwhelmed by the level of LIN-56 produced by heat-shock overexpression. Also, LIN-56 produced from the heat-shock promoters in *lin-15AB(e1763)* animals did not obviously differ in electrophoretic mobility from that in *lin-56(n2728); lin-15B(n744)* animals by immunoblot (Figure S1). Since overexpression of LIN-56 failed to rescue the synMuv phenotype of a *lin-15A(lf); synMuvB(lf)* mutant, *lin-15A* cannot function only to positively regulate the translation or stability of LIN-56 protein.

Likewise, if *lin-56* acts only to control the translation or stability of LIN-15A protein, then restoring the level of LIN-15A protein should rescue the *lin-56(lf); synMuvB(lf)* synMuv phenotype. To test this possibility, we induced a *lin-15A* cDNA under the control of heat-shock promoters shortly after L1 lethargus in *lin-36(n766); lin-15A(n767)* animals and rescued the synMuv phenotype (Table 2). The same treatment failed to rescue the synMuv phenotype of *lin-56(n2728); lin-36(n766)* animals (Table 2). Since overexpression of *lin-15A* failed to rescue the synMuv phenotype of a *lin-56(lf); synMuvB(lf)* mutant, *lin-56* is unlikely to act only by positively regulating the translation or stability of LIN-15A protein.

LIN-56 and LIN-15A physically interact: Since LIN-56 protein levels are reduced in a *lin-15A* mutant background and LIN-15A nuclear accumulation is reduced in a *lin-56* mutant background, we hypothesized that LIN-56 and LIN-15A might normally be stabilized by association with each other in a functional complex. To investigate if LIN-56 and LIN-15A can interact, we used the GAL4-based yeast two-hybrid system (FIELDS and SONG 1989). The class B synMuv proteins LIN-37 and

LIN-53 were previously shown to interact using this approach (WALHOUT *et al.* 2000a; see also Figure 5). The class A synMuv proteins LIN-56 and LIN-15A interacted specifically with each other, as detected by using yeast cotransformed with either constructs DB-LIN-15A and AD-LIN-56 or DB-LIN-56 and AD-LIN-15A (Figure 5). By contrast, neither LIN-56 nor LIN-15A appeared to interact specifically with either LIN-37 or LIN-53 (Figure 5).

DISCUSSION

LIN-56 and LIN-15A might function as a complex *in vivo*: *lin-56* and *lin-15A* encode putative transcriptional regulators that are present in most or all cells in *C. elegans*. The LIN-56 and LIN-15A proteins share a THAP-like C₂CH motif and are dependent on each other for wild-type levels and/or localization. *lin-15A* is required post-transcriptionally for LIN-56 protein expression or stability, and *lin-56* is required for the expression, stability, or nuclear localization of LIN-15A. As overexpression of LIN-56 did not rescue the *lin-15A(lf)* synMuv phenotype, it seems unlikely that *lin-15A* functions only to regulate LIN-56 protein levels. Likewise, expression of *lin-15A* was unable to rescue the *lin-56(lf)* synMuv phenotype, suggesting that *lin-56* does not function only to regulate LIN-15A levels. Rather, given the ability of LIN-56 and LIN-15A to interact in the yeast two-hybrid system, we favor a model in which LIN-56 and LIN-15A associate in a functional complex required for the inhibition of vulval cell fates, with the absence of one of the complex subunits resulting in the instability or loss of nuclear localization of the other(s). This model is supported by genetic evidence showing that, unlike all other pairs of class A synMuv genes, *lin-15A* and *lin-56* are unable to function independently of each other in vulval development (ANDERSEN *et al.* 2008).

Class A synMuv genes likely directly regulate gene expression: The THAP C₂CH domain is conserved from *C. elegans* to humans and was initially proposed to bind DNA on the basis of its similarity to a region of the *Drosophila* P-element transposase (ROUSSIGNE *et al.* 2003). The THAP domain of the human protein THAP1 has since been shown to possess zinc-dependent sequence-specific DNA-binding activity *in vitro* (CLOUAIRE *et al.* 2005). Furthermore, coimmunoprecipitation studies revealed that THAP1 associates *in vivo* with the promoter of a pRB/E2F cell-cycle target gene (CAYROL *et al.* 2007).

Several *C. elegans* proteins contain THAP domains, THAP-like domains, or both (REDDY and VILLENEUVE 2004; CLOUAIRE *et al.* 2005). Analysis of a subset of the THAP domain-containing proteins in *C. elegans* suggests that they might mediate access to chromatin remodeling and chromatin-modifying complexes (REDDY and VILLENEUVE 2004). Specifically, HIM-17

and the class B synMuv proteins LIN-15B and LIN-36 contain one or more THAP domains. HIM-17 is required both for accumulation of histone H3 methyl-lysine 9 in the germline and for meiotic recombination, suggesting a link between these two processes (REDDY and VILLENEUVE 2004). The class B synMuv proteins are thought to silence genes required for vulval cell-fate specification through chromatin remodeling, most likely including histone H3 lysine-9 methylation (LU and HORVITZ 1998; SOLARI and AHRINGER 2000; VON ZELEWSKY *et al.* 2000; CEOL and HORVITZ 2001; COUTEAU *et al.* 2002; ANDERSEN and HORVITZ 2007). Furthermore, the class B synMuv protein LIN-35 Rb acts with HIM-17 in meiotic recombination (REDDY and VILLENEUVE 2004).

The region of similarity between the class A synMuv proteins LIN-56 and LIN-15A shares the C₂CH signature of the THAP domain but lacks additional conserved residues, such as an invariant tryptophan and a C-terminal AVPTIF motif (CLOUAIRE *et al.* 2005). This variant THAP-like domain is found also in HIM-17 and LIN-15B (REDDY and VILLENEUVE 2004). Given the nuclear localization of LIN-56 and LIN-15A, we suggest that this variant C₂CH motif is also likely to mediate interaction with DNA. Together with the nuclear localization of LIN-8 and its physical interaction with LIN-35 Rb (DAVISON *et al.* 2005), these results strongly suggest that class A synMuv proteins inhibit vulval cell-fate specification through the regulation of transcription.

The class A synMuv genes, including both *lin-15A* and *lin-56*, repress expression of *lin-3* EGF redundantly with the class B synMuv genes (CUI *et al.* 2006; ANDERSEN *et al.* 2008). It was previously shown that RNAi of *lin-3* can suppress the *lin-15A(lf); synMuvB(lf)* synMuv phenotype (CUI *et al.* 2006), and we have shown that RNAi of *lin-3* can also suppress the *lin-56(lf); synMuvB(lf)* synMuv phenotype, consistent with the hypothesis that both *lin-15A* and *lin-56* act upstream of *lin-3*. Given their molecular identities, the class B synMuv genes likely repress gene expression by chromatin remodeling. On the basis of their potential DNA-binding THAP-like domains and nuclear localization, we propose that the class A synMuv genes also regulate transcription. Both class A and class B synMuv proteins might directly repress expression of *lin-3*. Alternatively, either one or both classes of synMuv proteins could directly regulate the expression of another protein, which subsequently represses *lin-3*.

Class A synMuv genes might have functions beyond those in vulval development: Mutation of the class A synMuv genes, unlike mutation of the class B synMuv genes, has not been reported to result in defects other than those associated with vulval development. Specifically, mutation of the class A synMuv genes does not appear to cause cell-cycle defects (BOXEM and VAN DEN HEUVEL 2002; FAY *et al.* 2002), reduced gene expression from repetitive transgene arrays (HSIEH *et al.* 1999), or sterility or lethality (LU and HORVITZ 1998; BEITEL *et al.*

2000; MELENDEZ and GREENWALD 2000; VON ZELEWSKY *et al.* 2000; CEOL and HORVITZ 2001; BELFIORE *et al.* 2002; COUTEAU *et al.* 2002; DUFOURCQ *et al.* 2002; UNHAVAITHAYA *et al.* 2002). Nonetheless, the broad expression patterns of the LIN-8 (DAVISON *et al.* 2005), LIN-15A, and LIN-56 proteins suggest that additional roles might exist for the class A synMuv genes. Perhaps the class A synMuv genes function redundantly with loci other than the class B synMuv genes to regulate biological processes other than the vulval cell-fate decision. This possibility seems particularly likely for *lin-8*, which is a member of a novel *C. elegans* gene family with 16 other genes (DAVISON *et al.* 2005).

Implications for mammalian tumorigenesis: The inhibition of vulval development in *C. elegans* involves multiple redundant pathways: both a class A and a class B synMuv gene must be inactivated for ectopic vulval development to occur (FERGUSON and HORVITZ 1989). The class B synMuv genes include counterparts of the mammalian tumor-suppressor gene Rb and genes that interact with Rb (LU and HORVITZ 1998; CEOL and HORVITZ 2001; HARRISON *et al.* 2006). pRb and the related proteins p107 and p130 play critical roles in mammalian cell-cycle regulation, apoptosis, development, and differentiation (CLASSON and HARLOW 2002), and Rb is often mutated in human cancers (NEVINS 2001). Inappropriate activation of EGF/Ras signaling is also a common event in cancers (NORMANNO *et al.* 2006). Because the class A synMuv genes function redundantly with an Rb pathway to repress transcription of an EGF ligand and inhibit Ras-mediated vulval cell-fate specification, we propose that analogous THAP domain proteins in mammals might act as tumor-suppressor genes by repressing the expression of EGF-like ligands.

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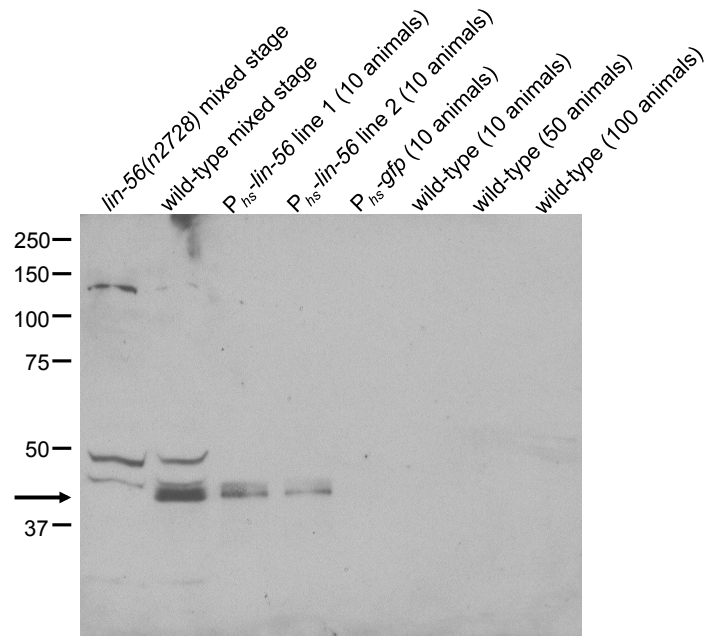


FIGURE S1.—*Phs-lin-56* transgenic animals express LIN-56 at least 10-fold higher than wild-type animals. Immunoblot of wild-type, *lin-56(n2728)*, or transgenic protein extracts probed with anti-LIN-56 antibodies. The arrow indicates the band that corresponds to LIN-56. Molecular weights of marker proteins are indicated at left in kilodaltons.

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