The RNA-binding protein SUP-12 controls muscle-specific splicing of the ADF/cofilin pre-mRNA in C. elegans

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Introduction

Pre-messenger RNA splicing is mediated by spliceosomes that contain small ribonucleoprotein particles and splicing factors (Jurica and Moore, 2003; Nilsen, 2003). Pre-mRNAs are often alternatively spliced to give rise to multiple mRNA species, which requires additional regulation of splice-site selection (Smith and Valcarcel, 2000; Maniatis and Tasic, 2002; Black, 2003). Tissue-specific alternative mRNA splicing is essential for expression of functionally different gene products from a single gene, and, thus, it is an important mechanism of cellular differentiation. Disruption of the splicing machinery is known to cause various human diseases (Nissim-Rafinia and Kerem, 2002; Stoilov et al., 2002; Faustino and Cooper, 2003). In particular, myotonic dystrophy has been linked to perturbation of the mechanism of alternative splicing in muscle (Kanadia et al., 2003; Nilsen, 2003). Pre-mRNAs are often rich, alanine- and glutamine-rich; EMSA, electrophoretic mobility shift assay; RRM, RNA recognition motif.

Actin depolymerizing factor (ADF)/cofilin proteins enhance actin filament dynamics by severing filaments and accelerating monomer dissociation from the pointed ends of the filaments (Bamburg, 1999; Bamburg et al., 1999; Maciver and Hussey, 2002; Ono, 2003). Essential functions of ADF/cofilin in vivo actin dynamics and cell viability have been demonstrated in several organisms (McKim et al., 1994; Gunsalus et al., 1995; Lappalainen and Drubin, 1997). In mammals, three ADF/cofilin isoforms are encoded by separate genes and expressed in different patterns of tissue distribution (Matsuzaki et al., 1988; Moriyama et al., 1990; Ono et al., 1994; Gillett et al., 1996; Thirion et al., 2001; Vartiainen et al., 2002). However, in the nematode Caenorhabditis elegans, the unc-60 gene undergoes alternative splicing and expresses two ADF/cofilin isoforms, UNC-60A and UNC-60B (McKim et al., 1994). Our previous studies have indicated that the two ADF/cofilin isoforms have different activities: UNC-60A strongly depolymerizes filaments, whereas UNC-60B binds to filaments with only weak depolymerizing activity (Ono and Benian, 1998; Ono, 1999; Mohri and Ono, 2003). More importantly, they are expressed in different tissues and required for specific actin-dependent processes: UNC-60A is expressed in nonmuscle cells and is required for embryonic cytokinesis (Ono et al., 1994), whereas UNC-60B is specifically expressed in the body wall muscle and regulates myofibril assembly (Ono et al., 2003).
The unc-60 gene has nine exons and only the first exon is shared by unc-60A and unc-60B (McKim et al., 1994). Therefore, the tissue-specific expression of unc-60A or unc-60B is proposed to be determined by selection of the first splice acceptor site at the 5'-end of either exon 2A or 2B (McKim et al., 1994). However, the regulatory mechanism of tissue-specific splicing of the unc-60 pre-mRNA is unknown.

In this work, we report identification and characterization of a putative splicing factor that regulates muscle-specific splicing of the unc-60 pre-mRNA in C. elegans. We cloned SUP-12, a conserved RNA-binding protein, as a suppressor of unc-60B.

Results

Identification of SUP-12 as a suppressor of unc-60B

In a previous effort to identify genes that functionally interact with unc-60, sup-12 mutant alleles st89 and st203 were isolated from a screen for extragenic suppressors of unc-60 mutants (G.R. Francis and R.H. Waterston, personal communication; Waterston, 1988). sup-12 mutations alone have only minor effects on motility of the worms (Fig. 1 A). However, in the unc-60B mutant backgrounds, sup-12 strongly suppressed the motility defects (Fig. 1 A). The suppression by sup-12 was also equally strong for a strong loss of function allele unc-60B(e677) and a null allele unc-60B(su158) (McKim et al., 1988), and the motility of the unc-60B;sup-12 double mutants was restored nearly to the level of wild-type worms (Fig. 1 A). The motility defects of the unc-60B mutants are caused by disorganization of actin filaments in the body wall muscle (Ono et al., 1999; Ono et al., 2003). sup-12(st203) exhibited slightly stronger suppressor effect than sup-12(st89) in worm motility (Fig. 1 A) and in actin organization in muscle (unpublished data). These results suggest that sup-12 genetically interacts with unc-60B and regulates actin organization in the body wall muscle.
By positional cloning and identification of mutation sites, we cloned the sup-12 gene and identified it as T22B2.4 (GenBank/EMBL/DDBJ accession no. NM_076273) that is mapped to the left arm of the X chromosome by the C. elegans Sequencing Consortium (1998). The SUP-12 protein (248 aa) is a putative RNA-binding protein that has a single RNA recognition motif (RRM) domain (Shamoo et al., 1995) in its NH2 terminus (residues 35–117) and alanine- and glutamine-rich (A/Q-rich) sequence in its COOH terminus (26 A and 24 Q in residues 121–246; Fig. 2 A). Missense mutations G59R, G77E, and G113E were found within the RRM domain in the sup-12 mutant alleles s2900, st89, and s2896, respectively (Fig. 2 A). s2901 has the same mutation as st89, although they were isolated in different laboratories. A mutation (G to A) was found in st203 at the splice donor site in the second intron. RT-PCR analysis showed that aberrantly spliced sup-12 mRNA was predominantly expressed in the sup-12(st203) mutants (unpublished data). RNA interference of sup-12 phenocopied the suppressor phenotype of sup-12 mutants and suppressed the Unc-60B phenotype of multiple unc-60B alleles (unpublished data). Homology searches revealed that SUP-12 is an orthologue of a human protein SEB-4 (Fig. 2 B). Two human SEB-4 isoforms (GenBank/EMBL/DDBJ accession no. AK095016 and NM_153020) are found in the database, but their function is unknown. In Xenopus, two SEB-4 isoforms, XSEB-4 and XSEB-4R, are reported: XSEB-4 is expressed in muscle precursor cells (Fetka et al., 2000), whereas XSEB-4R is strongly expressed in neuronal cells and involved in neural differentiation (Boy et al., 2004). Interestingly, Arabidopsis has at least six SEB-4 orthologues (Fig. 2 B), but functional studies on these proteins are not reported. However, no SEB-4 orthologues were found in yeasts and Drosophila. Sequence alignment of these proteins showed highly conserved sequences in the RRM domain in the NH2 terminus (unpublished data). The COOH-terminal halves are not highly conserved, yet the A/Q-rich sequences are present in all these proteins. The similarity in the sequences suggests that SUP-12 and SEB-4 homologues belong to a new family of functionally conserved RNA-binding proteins.

SUP-12 alters expression patterns of the unc-60 splice variants

To understand the mechanism of suppression of the Unc-60 mutant phenotype by sup-12, expression patterns of the splice variants of the unc-60 gene products were examined. Surprisingly, the protein level of UNC-60B, the muscle-specific isoform, was greatly reduced in the sup-12 mutants both in wild-type and unc-60B(e677) backgrounds (Fig. 3 B, lanes 1–6). In contrast, the total level of UNC-60A, the nonmuscle isoform, was not significantly altered by mutations in sup-12 or unc-60B (Fig. 3 B). Similar changes were detected at the mRNA levels: the unc-60B mRNA was reduced as compared with wild type, whereas the unc-60A mRNA was not significantly altered (Fig. 3 C). In wild-type background, the unc-60B mRNA was decreased in the sup-12 mutants (Fig. 3 C, lanes 1–3). unc-60B(e677) has a missense mutation (Ono et al., 1999) and had a greatly reduced level of the unc-60B mRNA (Fig. 3 C, lanes 4–6), which correlates with the reduced protein level (Fig. 3 B, lane 4). unc-60B(su158) has a 600-bp deletion in the unc-60B region without affecting unc-60A (Ono et al., 2003) and expressed a shorter unc-60B mRNA (Fig. 3 C, lane 7) that is not translated into a protein (Fig. 3 B, lane 7). Nonetheless, its mRNA level was still reduced in the sup-12 mutants (Fig. 3 C, lanes 8 and 9), strongly suggesting that SUP-12 affects the levels of the unc-60B mRNA, but not directly of the protein.
Altered expression of UNC-60A in the body wall muscle of the sup-12 mutants. Wild-type (A–C), unc-60B(e677) (D–F), sup-12(st203) (G–I), or unc-60B(e677); sup-12(st203) (J–L) worms were stained for UNC-60A (A, D, G, and J) and myoA, the muscle-specific myosin heavy chain (B, E, H, and K). Merged images are shown in C, F, I, and L. MyoA is a marker for the body wall muscle (arrows), but it is also expressed in the myoepithelial sheath of the ovary (asterisks). UNC-60A is widely expressed in nonmuscle tissues, but its expression in the body wall muscle is increased in the sup-12 mutants (arrows). Bar, 20 μm.

UNC-60A is expressed in a variety of nonmuscle cells (Ono et al., 2003). Therefore, changes in the level of UNC-60A in a subset of tissues may not be detected by the Northern blot analysis. Therefore, we examined the tissue distribution of UNC-60A by immunofluorescence microscopy and found that the level of UNC-60A in body wall muscle is altered by sup-12. In wild type and the unc-60B(e677) single mutant, UNC-60A was not detectable in body wall muscle (Fig. 4, A–F, arrows). However, in the unc-60B; sup-12 double mutant and the sup-12 single mutant, UNC-60A protein was detected in the diffuse cytoplasm (Fig. 4, G–L, arrows). These results indicate that the sup-12 mutations have opposite effects on the levels of the two splice variants, unc-60a and unc-60b, in muscle cells. Although UNC-60A and UNC-60B have quantitatively different biochemical activities, both isoforms can enhance actin filament dynamics by depolymerizing actin filaments (Ono and Benian, 1998; Ono, 1999; Ono et al., 1999). Thus, the suppression of the Unc-60 phenotype by sup-12 could be explained by the up-regulation of UNC-60A in muscle, which may compensate for the function of UNC-60B.

**SUP-12 is expressed in body wall muscle and localizes to the nuclei**

We next investigated expression and subcellular localization of SUP-12. An anti–SUP-12 antibody against its COOH terminus specifically recognized the SUP-12 protein by Western blot but did not detect the protein by immunofluorescence microscopy (unpublished data). The 3.1-kb promoter region of the sup-12 gene was able to drive expression of a reporter GFP in body wall muscle (Fig. 5 A, arrowheads) and pharynx (Fig. 5 A, asterisk). GFP-tagged SUP-12, which was expressed in the body wall muscle under the control of the myo-3 promoter (Okkema et al., 1993), predominantly localized to the nuclei (Fig. 5, B–D). Within the nuclei, GFP-SUP-12 was found in diffuse and often speckled patterns in the nucleoplasm but excluded from the globular region, possibly representing the nucleolus (Fig. 5 B). The speckled localization of SUP-12 in the nucleus is similar to patterns that are commonly observed for other splicing factors (Misteli, 2000; Dundr and Misteli, 2001; Lamond and Spector, 2003). Although the myo-3 promoter is active in the body wall muscle but not in the pharynx, myo-3–driven expression of GFP-SUP-12 was sufficient to rescue the Sup-12 mutant phenotype (Table I), indicating that GFP-SUP-12 is functional and expression of SUP-12 in the body wall muscle is functionally important for its interaction with unc-60. These results strongly suggest that SUP-12 is a muscle-specific regulator of pre-mRNA splicing in the nucleus.

Neither the NH2-terminal RRM domain nor the COOH-terminal A/Q-rich domain alone was able to rescue the sup-12 mutant phenotype (Table I). The RRM domain of SUP-12 (residues 1–117) localized to both nuclei and cytoplasm in a diffuse pattern (Fig. 5 E), whereas the A/Q-rich domain of SUP-12 (residues 118–248) strongly localized to the nuclei in a similar speckled pattern to the full-length protein and weakly to the cytoplasm (Fig. 5 F). These results suggest that either the RRM or the A/Q-rich domain is sufficient for nuclear localization, but both domains are required for the function of SUP-12.

**Direct binding of SUP-12 to the unc-60 pre-mRNA**

To determine whether the SUP-12 protein may be directly involved in pre-mRNA splicing of unc-60, we examined direct interaction between SUP-12 and unc-60 pre-mRNA in vitro by an electrophoretic mobility shift assay (EMSA). Recombinant GST-tagged full-length SUP-12 protein or the COOH-terminal portion (residues 118–248) of SUP-12 were poorly soluble (unpublished data) and therefore were not examined. However, the NH2-terminal portion (residues 1–117) of SUP-12 containing the RRM domain was stable and soluble as a GST-fusion protein. When purified GST-SUP-12 (RRM) was incubated with various portions of in vitro transcribed unc-60 pre-mRNA (Fig. 6 A), it caused a band shift of only the 978-nt RNA fragment that encompasses the sequence of exon 1 to a portion of exon 5A (Fig. 6, A and B). GST alone did not cause a band shift of the RNAs (Fig. 6 B). These results indicate that the RRM domain of SUP-12 is sufficient for direct RNA-binding and so could confer specificity for the 5′-region of the unc-60 pre-mRNA.

The SUP-12 binding region in the unc-60 pre-mRNA was further narrowed down by a pull-down assay with biotin-labeled RNA fragments (Fig. 6, C–G). We first tested interac-

### Table I. Dissection of the domains of SUP-12

<table>
<thead>
<tr>
<th>Domain</th>
<th>Residues</th>
<th>Rescue</th>
<th>Localization</th>
<th>RNA-binding</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-length</td>
<td>1–248</td>
<td>Yes</td>
<td>Nuclei</td>
<td>ND</td>
<td>Insoluble</td>
</tr>
<tr>
<td>RRM</td>
<td>1–117</td>
<td>No</td>
<td>Nuclei cytoplasm</td>
<td>Yes</td>
<td>Soluble</td>
</tr>
<tr>
<td>A/Q-rich</td>
<td>118–248</td>
<td>No</td>
<td>Nuclei cytoplasm</td>
<td>ND</td>
<td>Insoluble</td>
</tr>
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Figure 5. Dissection of the domains of SUP-12. The SUP-12 binding region in the unc-60 pre-mRNA was further narrowed down by a pull-down assay with biotin-labeled RNA fragments (Fig. 6, C–G). We first tested interac-
tions between GST-SUP-12 (RRM) and four RNA fragments, A1-1, A1-2, A1-3, and A1-4, within the unc-60A region (Fig. 6 C). GST-SUP-12 (RRM) bound to the magnetic beads in the presence of A1-1 or A1-2 (Fig. 6 E, lanes 2–5). However, in the presence of A1-3 or A1-4 (Fig. 6 E, lanes 6–9), the amounts of captured GST-SUP-12 (RRM) were not significantly different from that in the absence of RNA (Fig. 6 E, lane 1). GST alone did not bind to the beads in the presence or absence of RNA (Fig. 6 E), indicating that the RRM domain of SUP-12 mediated the interactions with RNAs.

We then used shorter RNA fragments of 10–152 bases in the pull-down assay with GST-SUP-12 (RRM) (Fig. 6, C and D) and characterized the interactions in a quantitative manner (Fig. 6, F and G). GST-SUP-12 (RRM) showed relatively strong binding with exon 1 (A1-1-1), the first intron (A1-1-2), and the second intron (A1-2-2) but did not significantly interact with exon 2A (A1-2-1; Fig. 6 F). Interestingly, an 18 nt truncation of A1-1-2 at the 3′-end (A1-1-2-ΔUG; Fig. 6, C and D) weakened the interaction with GST-SUP-12 (RRM; Fig. 6, F and G). The truncated region contains repeats of UG that have been reported to bind to several RNA-binding proteins (Mittag, 1996; Takahashi et al., 2000; Buratti et al., 2004). The RNA oligonucleotide UG (5′-UGUGUGCCUG-3′) strongly interacted with GST-SUP-12 (RRM; Fig. 6 E), whereas the oligonucleotide UC (5′-UCUCUCCUC-3′) showed nearly insignificant interaction (Fig. 6 E). Densitometric quantification of the results in Fig. 6 E indicates that only UG exhibited strong saturable binding with GST-SUP-12 (RRM; Fig. 6 F). Binding of GST-SUP-12 (RRM) to UG was saturated at a molar ratio of 1:1:1.0 with a dissociation constant of 0.31 μM, suggesting that they form a stoichiometric 1:1 complex with physiologically strong affinity. Removal of the UG repeats from A1-1-2 did not completely abolish the interaction of GST-SUP-12 (RRM) with A1-1-2-ΔUG (Fig. 6, F and G), suggesting that the UG repeats are sufficient but not necessary for this interaction. Binding of GST-SUP-12 (RRM) to A1-1-1, A1-1-2, or A1-2-2 did not reach saturation within the conditions used in this work (Fig. 6 G), so we were not able to determine stoichiometry and affinity. These results demonstrate that the RRM domain of SUP-12 directly interacts with the unc-60 pre-mRNA at multiple sites within exon 1 and the first and second introns. In particular, strong interaction of the SUP-12 RRM domain with the UG repeats near the 3′-splice site in the first intron supports that SUP-12 may function as a regulator of pre-mRNA splicing.

Discussion

In this work, we identified SUP-12 as a critical regulator of the muscle-specific pre-mRNA splicing of unc-60. sup-12 mutations suppressed muscle-specific defects in actin organization which was caused by unc-60B mutations. This suppression involved switching of the unc-60 isoforms in the muscle cells: sup-12 mutations enhanced muscle expression of UNC-60A, the nonmuscle ADF/cofilin isoforms, but reduced expression of UNC-60B, the muscle isoforms, thus allowing UNC-60A to compensate for the function of UNC-60B in the muscle cells. SUP-12 localized to the muscle nuclei and bound directly to exon 1 and the first and second introns of the unc-60 pre-mRNA in vitro, suggesting that SUP-12 is directly involved in muscle-specific splicing of the unc-60 pre-mRNA.

Our data indicate that, in muscle cells, SUP-12 normally inhibits production of the unc-60A mRNA, but it enhances expression of the unc-60B mRNA. Several possibilities for the
mechanism by which SUP-12 regulates expression of unc-60A and unc-60B could be considered. The most probable model is that the general splicing machinery may preferentially induce the splicing between exons 1 and 2A to produce unc-60A, whereas SUP-12 likely acts as an inhibitor of this splicing event. This model is strongly supported by the presence of UG repeats near the 3' end of the first intron, which strongly interacts with the RRM domain of SUP-12. In the human cystic fi-
brosis transmembrane conductance regulator pre-mRNA, the nuclear RRM protein TDP-43 binds to the UG repeats at the 3’-end of intron 8 and causes exon skipping (Niksic et al., 1999; Pagani et al., 2000; Buratti et al., 2004). Thus, SUP-12 and TDP-43 may negatively regulate splicing in a similar manner by binding to UG repeats and directly competing with the U2 auxiliary factor, which is an essential splicing factor that binds to 3’-splice sites (Merendino et al., 1999; Wu et al., 1999; Zorio and Blumenthal, 1999).

An alternative model is that SUP-12 may indirectly inhibit 3’-end processing of the unc-60A pre-mRNA. Because the SUP-12–binding sites on the unc-60 pre-mRNA are not close to the polyadenylation site of unc-60A, SUP-12 may have to interact with 3’-end processing factors to influence this process. The last exon (5A) for unc-60A that contains the 3’-untranslated region resides upstream of exon 2B (Fig. 3 A). In nonmuscle cells, the mRNA 3’-end processing factors may cleave the pre-mRNA and polyadenylate after exon 5A but before exon 2B is transcribed. Therefore, in muscle cells, SUP-12 may inhibit 3’-end processing at exon 5A and promote elongation of the pre-mRNA. Indeed, in case of polycistronic genes in C. elegans, an interaction between a 3’-end processing factor and a factor for trans-splicing is reported (Evans et al., 2001).

Also, it is possible that SUP-12 affects RNA stability or interacts with transcription factors and regulates transcription and pre-mRNA processing because splicing factors and transcription factors functionally interact and regulate pre-mRNA splicing in many instances (Bentley, 2002). In addition, we cannot exclude the possibility that SUP-12 may regulate relative stability of the two mRNAs.

The RRM domain of SUP-12 had activity to bind to the 5’-region of the unc-60 pre-mRNA but was unable to rescue the sup-12 mutant phenotype. This suggests that the COOH-terminal A/Q-rich sequence plays an important function. Although the function of the A/Q-rich sequence is unknown, it is intriguing that MEC-8, which regulates alternative splicing of the ADF/cofilin isoforms in muscle is necessary and sufficient for speckled localization in the nuclei (Spike et al., 2002), also contains an A/Q-rich region in addition to the RRM domain. The A/Q-rich sequence might have a regulatory function for a splicing factor or mediate interactions with other splicing factors, transcription factors, or 3’-end processing factors. We showed that the A/Q-rich region of SUP-12 is necessary and sufficient for speckled localization in the nuclei, suggesting that this region is important for SUP-12 to localize to speckles. Also, we noted that the bacterially expressed full-length SUP-12 protein was not only insoluble but also very susceptible for proteolysis (unpublished data), suggesting that the A/Q-rich sequence may regulate protein stability.

Furthermore, this work suggests that functional redundancy of the two ADF/cofilin isoforms in muscle is normally masked by tight regulation of tissue-specific splicing by sup-12. Thus, sup-12 mutations unmask the redundancy and allow UNC-60A to compensate for mutated UNC-60B in unc-60B mutants. From our previous work, it seemed logical to hypothesize that UNC-60B with weaker depolymerizing activity might be more suitable in muscle cells than UNC-60A, where less dynamic actin reorganization is needed than nonmuscle cells. Therefore, it is somewhat surprising that UNC-60A can substitute for UNC-60B in muscle. However, it is possible that, although the sup-12 mutants have apparently normal myofibrils, their muscle may exhibit different physiological properties from that of wild type under specific conditions. A number of human diseases are caused by alterations in the pre-mRNA splicing machinery (Nissim-Rafinia and Kerem, 2002; Stoilov et al., 2002; Faustino and Cooper, 2003). However, our results suggest that, the splicing machinery is a potential therapeutic target for certain genetic diseases in which manipulation of tissue-specific splicing machinery may reveal hidden functional redundancy among splice variants to compensate for a disease gene. We propose that SUP-12– and SEB-4–related proteins are a new family of tissue-specific splicing factors in multicellular organisms.

### Materials and methods

#### Nematode strains

Wild-type N2 and unc-60B(e677) [Waterston et al., 1980] were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN). sup-12(u89) and sup-12(mu203) were obtained from G.R. Francis (Exelixis, Inc., South San Francisco, CA) and R. Waterston [University of Washington, Seattle, WA]. sup-12(s2896), sup-12(s2900) and sup-12(s2901) were isolated by a screen for suppressors of unc-60B. In brief, unc-60B(e677) homozgyotes were mutagenized by ethyl methanesulfonate and 2F worms with improved motility were isolated. unc-60B(mu158) [Zengel and Epstein, 1980] was originally obtained from H.F. Epstein (University of Texas Medical Branch at Galveston, Galveston, TX) and characterized previously (Ono et al., 2003).

#### Cloning of sup-12

sup-12 was mapped to the left arm of the X chromosome that was included in the duplication mnDp33 [G.R. Francis and R.H. Waterston, personal communication]. We further narrowed down sup-12 by the snip-SNP mapping with polymorphisms in CB4856 (Wicks et al., 2001) to an interval between cosmid clones ZC64 and 106F4 that contained ~20 genes. We performed feeding RNA interference of 10 genes in wild type [Waterston et al., 1980] and examined for a suppressor phenotype for unc-60B. In brief, unc-60B(e677) homozygotes were mutagenized by ethyl methanesulfonate and 2F worms with improved motility were isolated. unc-60B(mu158). We found that T2282.4(DRNA) suppressed the motility defect of unc-60B(mu158) but did not affect motility of wild type. To confirm that T2282.4 is sup-12, we sequenced the T2282.4 gene in the sup-12 mutants and identified mutations in multiple sup-12 alleles (Results and Fig. 2 A).

#### Fluorescence microscopy

Actin filaments were visualized by staining adult worms with tetramethylrhodamine-phalloidin (Sigma-Aldrich) as described previously (Ozo, 2001). Immunofluorescent staining was performed on adult worms that were permeabilized with a freeze-crack method [Epstein et al., 1993] and fixed with methanol for 5 min at −20°C. Primary antibodies used were anti-UNC-60A [Ozo et al., 1999] and anti-myosin (mAb 5.6, obtained from H.F. Epstein; Miller et al., 1983). Secondary antibodies were Alexa488-labeled goat anti–rabbit IgG and Alexa647-labeled goat anti–mouse IgG [Molecular Probes]. To visualize nuclei, worms were fixed with 4% formaldehyde in PBS for 30 min at RT, permeabilized with acetone at −20°C, and stained with DAPI (Sigma-Aldrich) at 1 μg/ml in PBS containing 0.5% Triton X-100, 1 mM EDTA, and 0.05% sodium azide for 15 min.

Fluorescence samples were mounted with the Prolong antifading agent [Molecular Probes] and viewed by epifluorescence using an inverted microscope (model Eclipse TE2000; Nikon) with a 40× CFI Plan fluor objective (dry; NA 1.4). Images were captured by a SPOT RT Monochrome CCD camera (Diagnostic Instruments) and processed by the IPLab imaging software (Scanalytics, Inc.) and Adobe Photoshop 6.0.

#### Northern and Western blots

Total nematode RNA was isolated using a TRI reagent (Sigma-Aldrich). RNA samples (10 μg) were subjected to formaldehyde-agarose gel electrophoresis, transferred to positively charged nylon membranes (Millipore), and fixed by ultraviolet irradiation. cDNAs for unc-60A (503 bp), unc-60B (460 bp), and act (act-1) (1.1 kb) were amplified by PCR.
beled with digoxigenin with random priming, and used as probes for Northern blotting using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science). Western blot was performed as described previously [Ono and Ono, 2002] using the following primary antibodies: anti-UNC-60A [Ono et al., 1999], anti-UNC-60B [Ono et al., 1999], anti-actin monomeric C4 (ICN Biomedicals), and anti-a-tubulin (Amersham Biosciences).

Transgenic expression of GFP and GFP-SUP-12
To determine the promoter activity of sup-12, a 3,418-bp genomic fragment containing the 3,087-bp upstream region, exon 1, intron 1, and 58-bp of exon 2, was amplified by PCR using ExTaq DNA polymerase (Takara) and cloned into the gfp expression vector pPD9.6 (obtained from A. Fire, Stanford University, Stanford, CA) at the 5'-end of the gfp coding region. For expression of GFP-SUP-12 in body wall muscle, the sup-12 cDNA (yk1125e08, obtained from Y. Kohara, National Institute of Genetics, Mishima, Japan) was ligated in-frame with the 3’-end of gfp in pPD118.20 (obtained from A. Fire) that has the myo-3 promoter. For expression of fragments of SUP-12 as GFP-fusion proteins, fragments of the sup-12 cDNA encoding residues 1-117 and 118-248 were amplified by PCR and ligated in-frame with the 3’-end of gfp in pPD118.20. A synthetic stop codon was added for expression of residues 1-117. The plasmids were injected into hermaphrodite gonads of wild type or strains, A. Fire for expression vectors, H.F. Epstein for anti-myosin antibody and a strain, and K. Bhat and D. Kalman for critical comments.

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