

## The Fox-1 Family and SUP-12 Coordinately Regulate Tissue-Specific Alternative Splicing In Vivo<sup>∇†</sup>

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Many pre-mRNAs are alternatively spliced in a tissue-specific manner in multicellular organisms. The Fox-1 family of RNA-binding proteins regulate alternative splicing by either activating or repressing exon inclusion through specific binding to UGCAUG stretches. However, the precise cellular contexts that determine the action of the Fox-1 family in vivo remain to be elucidated. We have recently demonstrated that ASD-1 and FOX-1, members of the Fox-1 family in *Caenorhabditis elegans*, regulate tissue-specific alternative splicing of the fibroblast growth factor receptor gene, *egl-15*, which eventually determines the ligand specificity of the receptor in vivo. Here we report that another RNA-binding protein, SUP-12, coregulates the *egl-15* alternative splicing. By screening for mutants defective in the muscle-specific expression of our alternative splicing reporter, we identified the muscle-specific RNA-binding protein SUP-12. We identified juxtaposed conserved stretches as the *cis* elements responsible for the regulation. The Fox-1 family and the SUP-12 proteins form a stable complex with *egl-15* RNA, depending on the *cis* elements. Furthermore, the *asd-1; sup-12* double mutant is defective in sex myoblast migration, phenocopying the isoform-specific *egl-15(5A)* mutant. These results establish an in vivo model that coordination of the two families of RNA-binding proteins regulates tissue-specific alternative splicing of a specific target gene.

Tissue-specific alternative splicing of pre-mRNAs is considered to be a major source of proteomic complexity in metazoans (7, 21). Consequences of the alternative splicing for protein structure and function as well as for cellular processes have been discussed (45). Recent global studies demonstrated that as many as two-thirds of human genes have multiple isoforms of mature mRNAs (28, 39). Consistent with the functional significance of the fine regulation of the pre-mRNA processing, it is estimated that at least 15% of human hereditary diseases are caused by defects in posttranscriptional processing (15, 19). Utilization of alternative splicing microarrays revealed that many alternative splicing events are controlled in a tissue- and cell-type- and/or developmental stage-dependent manner, raising the novel question of how so many genes are regulated by a limited number of regulatory factors (8, 34).

Studies on multiple model genes established a view that a complex interplay among both positive and negative functions of various *trans*-acting factors and multiple exonic and intronic *cis*-acting elements affects tissue-specific alternative splicing. SR protein family members and heterogeneous nuclear ribonucleoproteins (hnRNPs) that bind to the exonic splicing enhancers and silencers, respectively, have been shown to regulate constitutive and alternative splicing of several model genes (6, 33). Conditional knockout and transgenic models demon-

strated that balanced expression of these ubiquitous factors regulates a specific subset of target genes in a tissue-specific manner in vivo (44, 53). In other cases, tissue-specific RNA-binding proteins regulate tissue-specific alternative splicing by binding to their specific target sequences (6). The function of the Nova family, consisting of brain-specific KH-type RNA-binding proteins (11, 54), has been the most thoroughly studied. They bind to YCAY clusters (11, 47) and regulate a specific subset of brain-specific alternative splicing in vivo to modulate synaptic functions (48). Recent computational comparison of the intronic sequences among alternatively spliced genes predicted many other uncharacterized candidate *cis* elements (46, 55). Further experimental identification of the *trans* factors and elucidation of rules for their functional coordination would contribute to deciphering the “cellular codes” underlying tissue-specific and developmentally regulated alternative splicing in living organisms (8, 33, 34).

Members of the Fox-1 family of RNA-binding proteins are tissue-specific alternative splicing regulators that specifically bind to (U)GCAUG. Experimental studies had demonstrated that the (U)GCAUG stretches are involved in cell-type-dependent regulation of well-studied model exons from alternatively spliced genes (16, 22, 26, 29, 32, 38). Computational analysis of the brain-specific alternative cassette exons demonstrated that GCAUG pentanucleotide and UGCAUG hexanucleotide elements are the most overrepresented elements in the downstream introns (10). The UGCAUG stretch is phylogenetically and spatially conserved in the flanking introns of brain-enriched exons among multiple orthologous vertebrate genes (37). Jin et al. first demonstrated that zebrafish (*zFox-1*) and murine (*mFox-1*) homologs of *Caenorhabditis elegans* FOX-1 specifically bind to the (U)GCAUG stretch in vitro, and they

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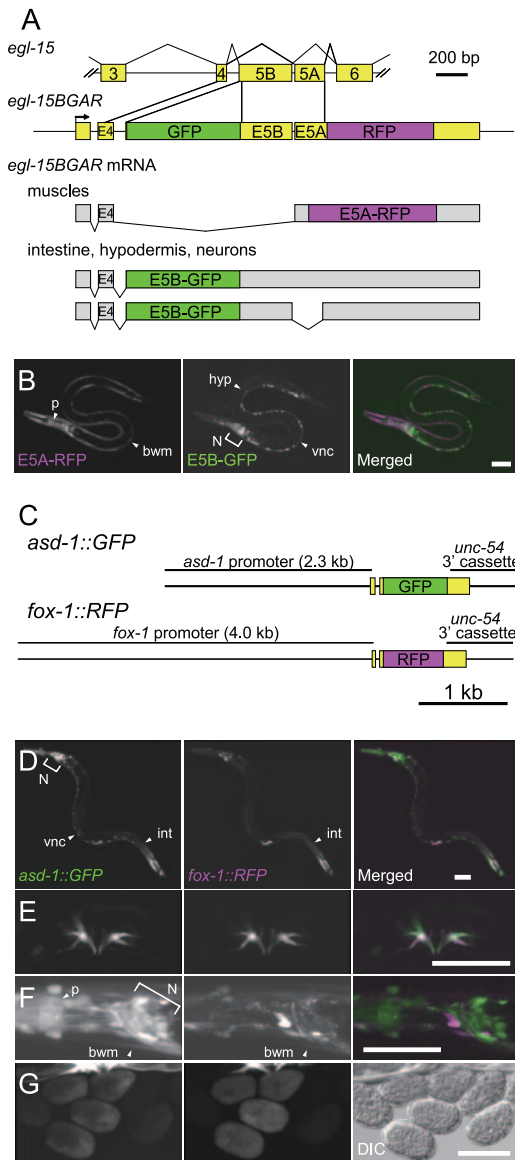


FIG. 1. Expression patterns of the *egl-15* alternative splicing reporter and the Fox-1 family. (A) The *egl-15BGAR* reporter minigene. Top, schematic drawing of the genomic fragment spanning exons 3 through 6 of the *egl-15* gene. Boxes indicate exons, and horizontal lines indicate introns. Middle, schematic drawing of the *egl-15BGAR* minigene. The GFP cDNA was inserted into exon 5B (E5B), and the RFP cDNA was connected in frame to exon 5A (E5A). Bottom, expression and structures of the mRNAs derived from the *egl-15BGAR* minigene. Putative open reading frames are colored in magenta for E5A-RFP fusion proteins and in green for E5B-GFP fusion proteins. Note that repression of E5B results in splicing between exon 4 (E4) and E5A to produce E5A-RFP, while inclusion of E5B results in production of either of the two isoforms producing the GFP protein (E5B-GFP). (B) Tissue-dependent expression patterns of the *egl-15BGAR* reporter. Expression patterns of E5A-RFP (left) and E5B-GFP (middle) and a merged image (right) are shown. (C) Schematic drawing of the *asd-1* and *fox-1* transcriptional fusion constructs. Genomic fragments 5' flanking the initiation codons of the *asd-1* and *fox-1* genes were used to drive expression of the GFP and RFP cDNAs, respectively. (D to G) Expression patterns of *asd-1::GFP* (left column) and *fox-1::RFP* (middle column) and merged views (right column). (D) Lateral view of an adult worm. (E) Enlarged lateral view of an adult vulva. (F) Enlarged lateral view of an adult head. (G) Embryos. A differ-

provided evidence that expression of the vertebrate Fox-1 proteins in cultured cells promotes the inclusion of the fibronectin EIIIB exon, mimics the muscle-specific skipping of the mitochondrial ATP synthase F1 $\gamma$ , and affects the choice of mutually exclusive exons of the  $\alpha$ -actinin pre-mRNA (27). Since this discovery, other laboratories have also reported that closely related members of the Fox-1 family of RNA-binding proteins regulate cell-type-specific alternative splicing of several genes through UGCAUG stretches in mammalian cells (3, 41, 43, 49, 56). It is yet to be elucidated, however, what precisely determines whether the Fox-1 family proteins enhance or inhibit inclusion of the alternatively spliced exons.

*C. elegans* is an excellent model organism to study mechanisms of regulation of alternative splicing in vivo. At least 5% of the total genes in *C. elegans* are alternatively spliced in manners similar to those for vertebrates (30, 40). We have recently developed a transgenic reporter worm system that can monitor the expression profiles of alternatively spliced exons at single-cell resolution in vivo by utilizing the *egl-15* gene as a model (31). The *egl-15* gene encodes the sole homolog of the fibroblast growth factor receptors in *C. elegans* (17), and selection of its mutually exclusive alternative exon 5A and exon 5B confers a functional difference (5). Stern and colleagues provided genetic evidence that the *egl-15(5A)* isoform is required for the migration of the sex myoblast towards the center of the body, where its specific ligand, EGL-17, is expressed and secreted, while the *egl-15(5B)* isoform exerts functions essential in embryonic and larval development (13, 14, 20). A functional difference between the *egl-15* isoforms has also been reported in axon outgrowth and maintenance in the nervous system (12). In a previous study, we constructed a reporter minigene, *egl-15BGAR*, so that we could monitor the selection of the *egl-15* exons 5B and 5A through expression of green fluorescent protein (GFP) and red fluorescent protein (RFP), respectively (Fig. 1A) (31). We have demonstrated that the pre-mRNA from the *egl-15BGAR* minigene is alternatively spliced into three isoforms, yielding the two fluorescent proteins in a tissue-specific manner (Fig. 1A and B) (31). In muscle tissues, such as egg-laying vulval muscle, body wall muscles, pharyngeal muscles, and an anal muscle, *egl-15BGAR* pre-mRNA is predominantly spliced to form the E5A-RFP isoform (Fig. 1A and B, left panel), while in epidermal tissues such as intestine and hypodermis and in neurons, the E5B-GFP isoform is exclusively or predominantly expressed (Fig. 1A and B, middle panel).

We have also provided evidence that the Fox-1 family of RNA-binding proteins regulates the tissue-specific alternative splicing of the *egl-15* gene through the UGCAUG stretch in intron 4 (31). Through screening for mutants defective in E5A-RFP expression from the *egl-15BGAR* reporter in the body wall muscles, we isolated mutants with a characteristic color phenotype, "chimera," and identified the *alternative-splicing-defective-1 (asd-1)* gene, a member of the Fox-1 family (31). Our genetic evidence led to a model that ASD-1 and FOX-1 redundantly regulate the tissue-specific selection of the alter-

ential interference contrast (DIC) image is shown instead of a merged image in the right panel of panel G. Bars, 50  $\mu$ m. bwm, body wall muscles; hyp, hypodermis; int, intestine; N, neurons in the head ganglia; p, pharynx; vnc, ventral nerve cord.

native exons of the *egl-15* reporter and the endogenous *egl-15* gene by binding to the UGCAUG stretch in intron 4 to repress inclusion of the upstream exon 5B and to allow inclusion of the downstream exon 5A. This work was the first demonstration that Fox-1- and UGCAUG-mediated tissue-specific alternative splicing is evolutionarily conserved in nematodes as in vertebrates, reinforcing the feasibility of genetic studies with *C. elegans* to decipher complex codes of alternative splicing in vivo.

In the present study, we demonstrate that the expression of the *asd-1* and *fox-1* is observed beyond muscular tissues and is insufficient to explain the tissue-dependent profiles of the *egl-15* reporter expression. We therefore screened for further mutants defective in muscle-specific alternative splicing of the *egl-15BGAR* reporter. We provide genetic evidence that another, muscle-specific RNA-binding protein, SUP-12, regulates the muscle-specific alternative splicing of the endogenous *egl-15* gene by cooperatively binding with the Fox-1 family proteins to the juxtaposed *cis* elements in intron 4. This study provides the first genetic evidence that two families of tissue-specific factors coregulate tissue-specific alternative splicing of a target gene in vivo through cooperative binding to their juxtaposed *cis* elements in a specific subset of tissues where expression of the two regulator families overlaps.

#### MATERIALS AND METHODS

**Worm culture, mutant screening, and mapping.** We cultured worms by standard methods. We prepared transgenic lines as described previously (31). We mutagenized the transgenic reporter allele *ybIs733 [myo-3::EGL-15BGAR lin-15 (+)]* with ethyl methanesulfonate as described previously (23, 31). Since the completely “green” mutants were sterile, we randomly isolated F<sub>1</sub> worms with a fluorescence-assisted worm sorter (Cepas Biosort; Union Biometrica) and screened the F<sub>2</sub> worms for siblings with color phenotypes. We eventually found that all the green *sup-12* mutants are sterile when they are homozygous for the transgenic reporter allele, while they are fertile when heterozygous for the reporter allele. We can therefore maintain the homozygous *sup-12* alleles as reporter heterozygotes. We performed single-nucleotide polymorphism-based mapping as described previously (31, 51).

**Minigene construction.** We constructed the promoter vectors as Gateway vectors (Invitrogen). We amplified the *asd-1* promoter fragment with PCR primers GCAGGTACCGCACTGACTGGGATGATGAGC and TATGGTACCCG CCGTTGTGATTGTAGAGA (underlining indicates KpnI digestion sites), digested the fragment with KpnI, and subcloned the fragment into pDEST-PL (31) to construct pDEST-*asd-1*-p. We amplified the *fox-1* promoter fragment with TGATTACGCCAAGCTTCGACACGTGCATTAGGCACA and CCTTTGGC CAATCCCTACAGGGCTTGGATGGGCAGA and subcloned the fragment into pDEST-PL (31) to construct pDEST-*fox-1*-p. The nucleotide sequences of these vectors are available in the *C. elegans* promoter database (<http://www.shigen.nig.ac.jp/c.elegans/promoter/index.jsp>). We mutagenized the *myo-3::egl-15BGAR* minigene (31) to construct the *myo-3::egl-15BGAR<sup>ctc</sup>* minigene by utilizing QuikChange (Stratagene) and mutagenizing oligonucleotide DNAs TTCCATGCATGGTCTCCT TTGTTTTCAGA and TCTGAAAACAAAGGAGACCATGCATGGAA.

**Microscopy.** We used confocal microscopes (Fluoview FV500 and FV1000; Olympus), for image scanning and processed the acquired images with MetaMorph (Molecular Devices).

**Sequence alignment.** We aligned the amino acid sequences of the RNA recognition motif (RRM) domains of the SUP-12 family of RNA-binding proteins by the Clustal W method using MegAlign (DNASTAR). The accession numbers of the sequences used are NP\_508674 (SUP-12), NP\_059965 (*Homo sapiens* Rnpc1), CAG31969 (*Gallus gallus* SEB4), AAP42281 (*Xenopus laevis* SEB4), CAB96420 (*Xenopus laevis* MTG-1a), BAD12194 (*Danio rerio* seb-4), AAH81649 (*Danio rerio* Rnpc1), AAM61541 (*Arabidopsis thaliana* SEB-4), and NP\_665898 (*Homo sapiens* A2BP1/Fox-1).

**In vitro binding assay.** We prepared His-tagged and FLAG-tagged recombinant proteins by using Ni-nitrilotriacetic acid Sepharose beads (QIAGEN) and M2 agarose beads (Sigma), respectively, according to standard protocols. We prepared the bacterial expression constructs as Gateway expression vectors (In-

vitrogen) by modifying a cold-induction vector (pCold II; Takara). We synthesized RNA probes by in vitro transcription with [ $\alpha$ -<sup>32</sup>P]UTP and T7 RNA polymerase. We performed all the in vitro binding experiments with RNA binding buffer consisting of 100 mM KCl, 5% glycerol, and 1% Triton X-100 in HEPES-KOH (pH 7.9) supplemented with 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride). We performed UV cross-linking essentially as described previously (31) in the presence of 130 ng/ $\mu$ l *Escherichia coli* tRNA. We performed electrophoretic mobility shift assay (EMSA) essentially as described previously (31) in the presence of 130 ng/ $\mu$ l *E. coli* tRNA and 50 ng/ $\mu$ l bovine serum albumin. We performed pull-down experiments with FLAG-tagged recombinant proteins bound to M2 agarose beads (Sigma) and purified His-tagged recombinant proteins in a total volume of 1 ml in the presence of 100 ng/ $\mu$ l yeast tRNA and 50 ng/ $\mu$ l bovine serum albumin. The *egl-15* RNA, AUUUUUCCA UGCAUGGUGUCUUUG, was chemically synthesized (Hokkaido System Science).

**Reverse transcription-PCR (RT-PCR).** We prepared total RNAs and amplified the endogenous *egl-15* and *inf-1* cDNA fragments as previously described (31).

#### RESULTS

**Expression of *asd-1* and *fox-1* is not restricted to muscle.** In order to investigate whether expression of either *asd-1* or *fox-1* is correlated with the tissue-dependent expression of the E5A-RFP isoform from the *egl-15BGAR* reporter, we analyzed the expression profiles of the *asd-1* and *fox-1* genes. We prepared promoter fusion constructs (Fig. 1C) and generated transgenic worms by coinjecting the two minigenes. We found that both *asd-1* and *fox-1* are expressed in a relatively broad range of tissues (Fig. 1D to G). Consistent with our previous observation that *asd-1* and *fox-1* are redundantly required for the expression of the E5A isoform in muscle (Fig. 1B) (31), they are robustly expressed in vulval muscles (Fig. 1E) and modestly expressed in body wall muscles and in the pharynx (Fig. 1D and F). However, we also observed expression of both *asd-1* and *fox-1* in the intestine (Fig. 1D) and expression of *asd-1* in the nervous system (Fig. 1D and F), where the E5B isoform predominated (Fig. 1B) (31). Since *fox-1* was originally identified as a regulator of sex determination in *C. elegans* (24, 42), we investigated the expression of these genes in embryogenesis and found that they become widely expressed in the early embryos before morphogenesis (Fig. 1G). These expression profiles raised a possibility that mere expression of the Fox-1 family proteins is insufficient to regulate the tissue-specific alternative splicing of the *egl-15* reporter in vivo.

***sup-12* mutants are also defective in *egl-15* reporter expression.** In order to search for other factors involved in the regulation of tissue-specific expression of the *egl-15* reporter, we screened for further color mutants. The parental transgenic reporter allele, *ybIs733*, predominantly expressed E5A-RFP in body wall muscles (Fig. 2A). In the screening, we randomly isolated many F<sub>1</sub> animals and searched the next generation for color mutants so that we could obtain sterile mutants (see Materials and Methods for details). We isolated several novel green (Fig. 2B and C) and chimera (Fig. 2D) mutants as well as chimera, “orange,” and other mutants similar to those we have reported previously (Fig. 2E and F) (31). Although all the green mutants were sterile in the reporter homozygous background, they were fertile in the reporter heterozygous background, which allowed us to maintain the homozygous mutant alleles. Most of the chimera mutants were indistinguishable from the *asd-1* (*yb978*) mutants (31) and turned out to carry new alleles of the *asd-1* gene, including both nonsense and



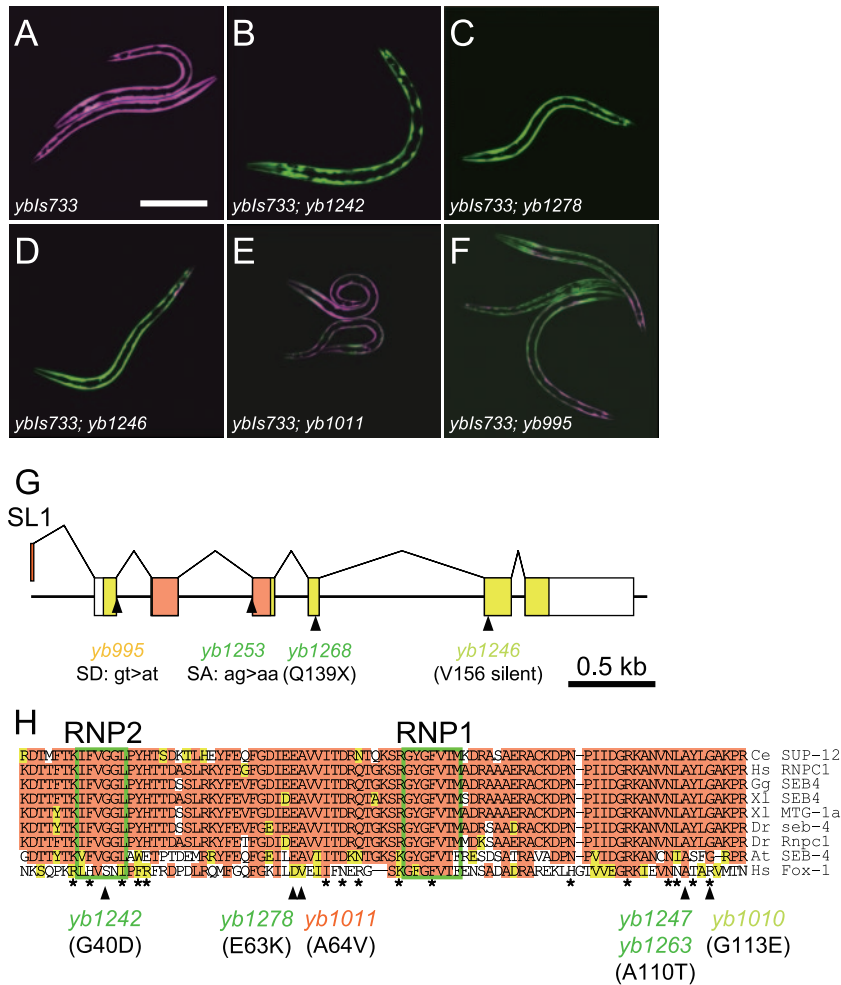


FIG. 2. *sup-12* mutants defective in tissue-specific expression of the *egl-15* alternative splicing reporter. (A) The parent allele, *ybls733*, expressing the *egl-15BGAR* minigene in the body wall muscles, used for the mutant screening. (B to F) *sup-12* mutant alleles identified in the screening. (B and C) Green mutants *yb1242* (B) and *yb1278* (C). (D to F) chimera mutants, *yb1246* (D), *yb1011* (E), and *yb995* (F). Bar, 100  $\mu$ m. (G) Schematic representation of the *sup-12* gene and the mutations identified in the screening. Open reading frames are colored. The RRM is orange. (H) Alignment of the RRM amino acid sequences among the SUP-12 family and comparison to that of the human Fox-1. Identical residues are shaded in orange, and residues with similar properties are shaded in yellow. Gaps were introduced to maximize the alignment. Conserved RNP1 and RNP2 motifs are boxed in green. Positions and allele names of the missense mutations identified within the RRM are indicated. Allele names are colored to reflect the severity of the color phenotype of the *egl-15* reporter expression. Asterisks indicate residues of human Fox-1 involved in recognition of the UGCAUG RNA (2). Ce, *C. elegans*; Hs, *Homo sapiens* (human); Gg, *Gallus gallus* (chick); Xl, *Xenopus laevis* (frog); Dr, *Danio rerio* (zebrafish); At, *Arabidopsis thaliana* (plant). Panel F is reproduced from reference 31.

missense mutations (see Wormbase at <http://www.wormbase.org/> for further allele information), demonstrating reproducibility of the color mutant screening and the *asd-1* color phenotype.

Single-nucleotide polymorphism-based chromosome mapping and sequencing of the mutant genome revealed that the green mutants (Fig. 2B and C) and unidentified chimera mutants (Fig. 2D to F) carry alleles of the *sup-12* gene (Fig. 2G and H). The *sup-12* gene was originally cloned as a gene responsible for the genetic suppression of *unc-60B* mutants (1). *sup-12* encodes an evolutionarily conserved RNA-binding protein specifically expressed in body wall muscles, pharynx, vulval muscles, and an anal depressor muscle (1, 18, 35), consistent with our previous observation that the E5A-RFP form of the *egl-15* reporter was predominantly expressed in the muscle

(Fig. 1B) (31). SUP-12 protein has a highly conserved N-terminal RRM (Fig. 2H) and a C-terminal AQ-rich domain (1). The *yb1253* allele is likely to be a null allele, since it has a mutation in the splice acceptor of exon 3 corresponding to the C-terminal half of the RRM (Fig. 2G). This leads to cryptic splicing causing a one-base deletion in the mature *sup-12* mRNA (data not shown). Although other green mutants with missense mutations within the RRM are indistinguishable from *yb1253* mutants in the color phenotype, they may be reduction-of-function (*rf*) alleles, since the *yb1278*(E63K) allele (Fig. 2C) has a less severe growth phenotype than the *yb1253* allele in the *asd-1* or *fox-1* mutant background (see below) and the recombinant SUP-12(E63K) protein has a trace of RNA-binding activity (see below). All the missense mutations affected residues within the RRM, which is well

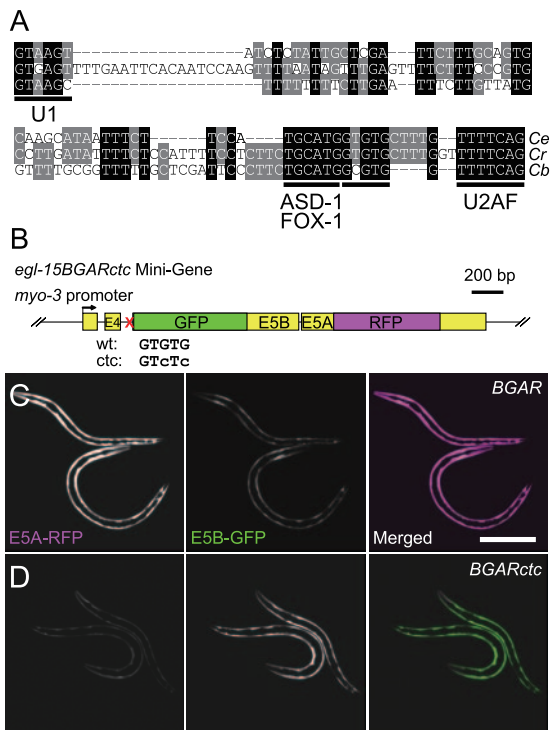


FIG. 3. *cis* elements for the tissue-specific alternative splicing of the *egl-15* gene. (A) Alignment of the nucleotide sequence of *egl-15* intron 4 among three related nematodes. Nucleotides conserved among all three species are shaded in black, and those conserved in two of the three species are in gray. Conserved stretches are underlined, and the known binding factors are indicated. *Ce*, *C. elegans*; *Cr*, *C. remanei*; *Cb*, *C. briggsae*. (B) Schematic representation of the modified reporter minigene, *egl-15BGARctc*. wt, wild type. (C and D) Expression of E5A-RFP (left panels) and E5B-GFP (middle panels) and merged views (right panels) of the *egl-15BGAR* reporter (C) and the *egl-15BGARctc* reporter (D) in the body wall muscles. Bar, 100  $\mu$ m.

conserved among the family members, and caused various color phenotypes ranging from green to almost red chimera (Fig. 2H), suggesting that they are *rf* alleles with variable RNA-binding properties.

We also mapped the genes responsible for the orange phenotype (31) and found that the phenotype is caused by mutation in the *smg-1* (see Fig. S1 in the supplemental material) and *smg-2* (see Fig. S2 in the supplemental material) genes. We conclude that the E5B-GFP isoforms of the mRNAs derived from the *egl-15BGAR* reporter (Fig. 1A) are substrates for nonsense-mediated mRNA decay (see the supplemental material).

**A GUGUG stretch is an essential *cis* element for *egl-15* reporter regulation.** As the *sup-12* gene encodes an RNA-binding protein, we searched for a *cis* element involved in regulation by the SUP-12 protein. We have previously reported that the conserved *cis* element for the ASD-1 and FOX-1 proteins, UGCAUG, resides in intron 4 of the *egl-15* pre-mRNA (Fig. 3A) (31). Further comparison of the nucleotide sequences of *egl-15* intron 4 among three related nematodes revealed that another short stretch, GYGUG, is conserved just downstream of the UGCAUG stretch (Fig. 3A). Anyanful et al. have reported that SUP-12 preferentially binds to a (UG)<sub>4</sub>

repeat in vitro (1), consistent with the sequence of the short stretch. In order to investigate whether the GYGUG stretch is involved in the tissue-specific expression of the *egl-15BGAR* reporter, we constructed a mutant minigene in which the GUGUG stretch is mutagenized to GUCUC (Fig. 3B). We found that the disruption of the GUGUG stretch resulted in the green phenotype (Fig. 3C and D), as did the disruption of the UGCAUG stretch (31). This result indicates that the GUGUG stretch is also required for the muscle-specific expression of the *egl-15* reporter in *C. elegans*.

**SUP-12 specifically binds to the GUGUG stretch in *egl-15* intron 4 in vitro.** In order to characterize the RNA-binding property of the SUP-12 protein, we performed UV cross-linking and EMSA. We prepared a radioisotope-labeled RNA probe from the wild-type *egl-15* intron 4 and two mutant probes, UcgAUG and cUc, in which the UGCAUG and GUGUG stretches were mutagenized, respectively (see Fig. S3A and B in the supplemental material). We also prepared recombinant full-length proteins (see Fig. S3C in the supplemental material). UV cross-linking experiments (Fig. 4A) revealed that the SUP-12 protein binds to the wild-type (lane 1) and the UcgAUG (lane 2) probes but not to the cUc probe (lane 3). The mutant SUP-12(E63K) protein binds only weakly to these probes (lanes 4 to 6). On the other hand, ASD-1 and FOX-1 bind to the wild-type and the cUc probes but not to the UcgAUG probe (lanes 7 to 12), while the mutant ASD-1(G140R) protein did not bind to any of these probes (lanes 13 to 15), confirming the sequence specificity of the Fox-1 family RNA-binding proteins (31). EMSA revealed the same RNA-binding properties (Fig. 4B). SUP-12 shifted the mobility of the wild-type (lanes 2 to 5) and the UcgAUG (lanes 10 to 13) probes in a dose-dependent manner but not that of the cUc probe (lane 17). SUP-12(E63K) has little effect on the mobility of the wild-type probe (lane 6). These results indicate that SUP-12 specifically binds to the GUGUG stretch of *egl-15* intron 4.

**ASD-1/FOX-1 and SUP-12 cooperatively bind to *egl-15* intron 4.** We next investigated whether interaction between the Fox-1 family and SUP-12 affects the RNA-binding properties of each other (Fig. 5A). SUP-12 enhanced the UV cross-linking of the FOX-1 and ASD-1 proteins to the wild-type RNA probe by 1.7- and 1.9-fold, respectively (lanes 2, 3, 4, and 5). In the same way, FOX-1 and ASD-1 enhanced the UV cross-linking of the SUP-12 protein to the wild-type probe by 3.0- and 3.2-fold, respectively (lanes 1, 2, and 4), but the ASD-1(G140R) mutant protein had little effect (1.2-fold) (lanes 1, 6, and 7). EMSA also revealed cooperative binding (Fig. 5B). Coincubation of the wild-type RNA probe with both the Fox-1 family and the SUP-12 proteins promoted formation of more stable complexes (lanes 3 and 5) than did incubation with any of the single proteins (lanes 2, 4, and 6). The stable complex was not formed when the ASD-1(G140R) protein (lane 9) or either of the mutant probes (lanes 10 to 13 and 14 to 18) was used, indicating that formation of the stable complex requires the specific binding of both Fox-1 family members and SUP-12 to the *egl-15* RNA. Unexpectedly, SUP-12(E63K), which binds only weakly to *egl-15* RNA, can also form the stable complex with the Fox-1 family proteins and the *egl-15* RNA (lanes 7 and 8), suggesting a direct interaction between SUP-12 and the Fox-1 family proteins. We therefore investigated the physical interaction between the two families of RNA-binding proteins

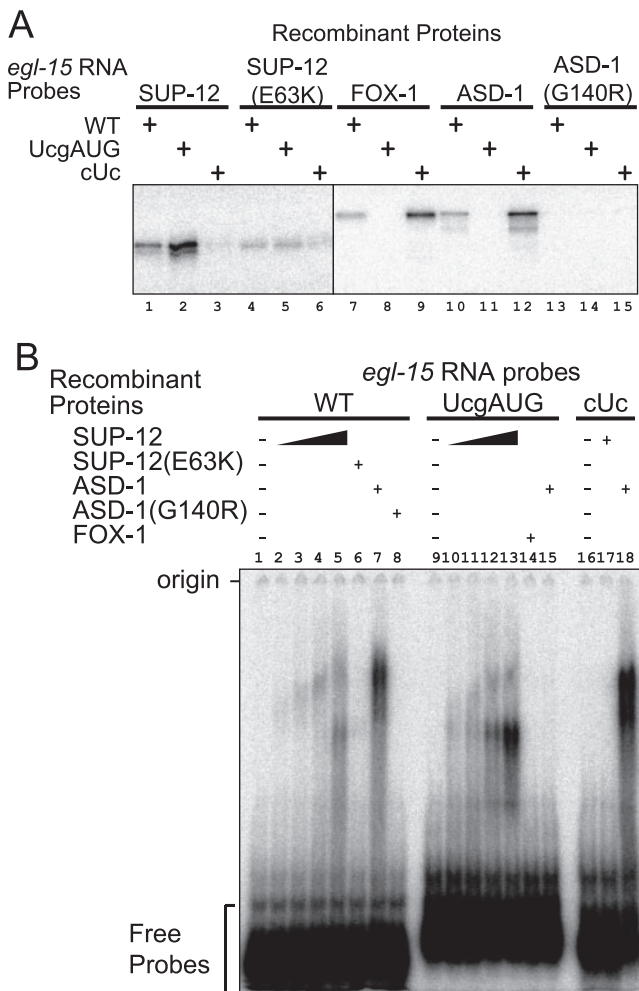


FIG. 4. Specific binding of SUP-12 to the GUGUG stretch in *egl-15* intron 4. (A) UV cross-linking assays with single recombinant proteins. Recombinant proteins and RNA probes used in the assay are indicated. WT, wild type. (B) EMSA with single recombinant proteins. Recombinant proteins and RNA probes used in the assay are indicated. Twofold dilution series of SUP-12 proteins were used in lanes 2 to 5 and 10 to 13.

in vitro by pull-down experiments using purified recombinant proteins (Fig. 5C). SUP-12 (lane 3) but not GFP (lanes 1 and 2) bound to ASD-1 and FOX-1 proteins even in the absence of the *egl-15* RNA, and *egl-15* RNA enhanced the binding of SUP-12 to ASD-1 and FOX-1 by 2.9- and 1.9-fold, respectively (lane 4).

**sup-12 and the Fox-1 family cooperatively regulate the endogenous *egl-15* gene.** We investigated whether *sup-12* is involved in the alternative splicing regulation of the endogenous *egl-15* in vivo. Since the putative null allele of the *sup-12* mutant, *yb1253*, is normal in egg-laying behavior (data not shown), we analyzed the genetic interaction between *sup-12* and the *fox-1* family. The *asd-1; sup-12 (yb1253)* double mutant grew to sterile adults in which the vulval muscles differentiated in the posterior of the body (Fig. 6A and B), phenocopying the aberrant migration of the sex myoblasts in the *egl-15(5A)* isoform-specific mutant (20) and the *asd-1; fox-1* double mutant (31). The *fox-1 sup-12 (yb1253)* double mutant arrested as

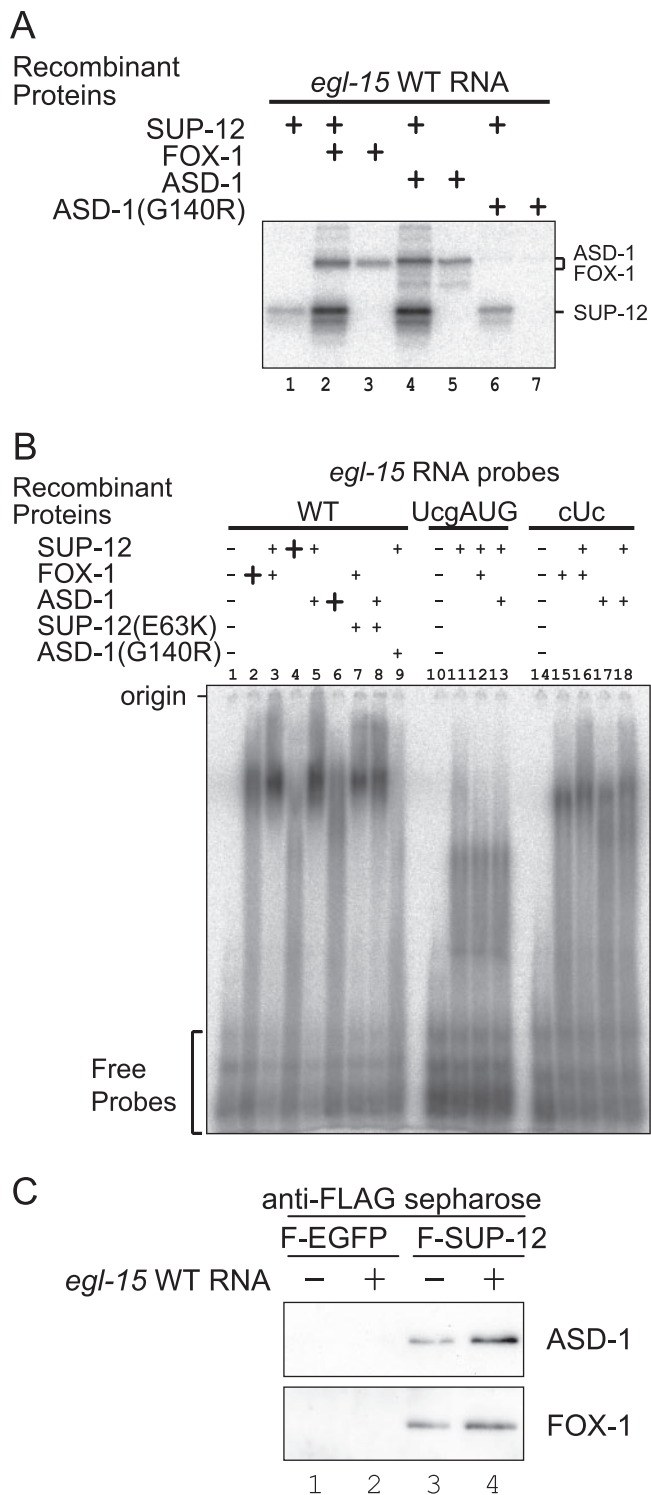


FIG. 5. Cooperative binding of the Fox-1 family and SUP-12 to the juxtaposed *cis* elements in *egl-15* intron 4. (A) UV cross-linking with combinations of recombinant proteins. WT, wild type. (B) EMSA with combinations of recombinant proteins. Large plus signs indicate twice the amount of the recombinant proteins compared to small plus signs. (C) Pull-down assay. Purified FLAG-tagged proteins and a synthesized RNA used in the pull-down assay are indicated.

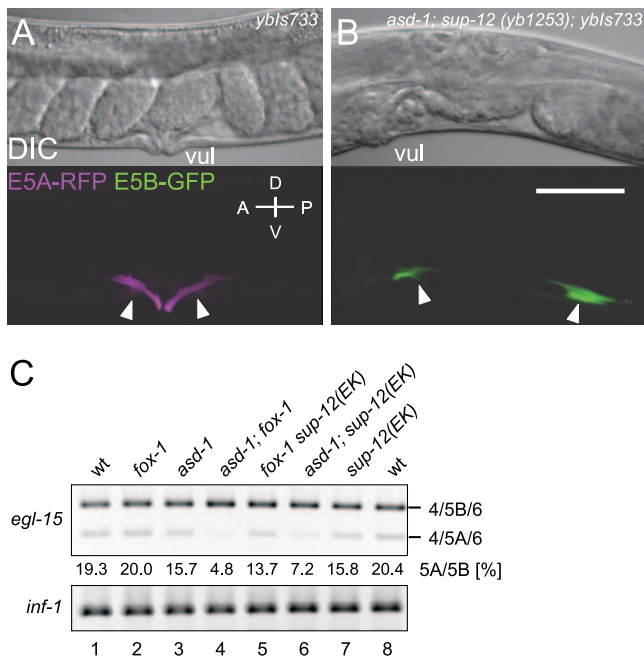


FIG. 6. Regulation of the endogenous *egl-15* gene by *sup-12*. (A and B) Lateral views of adult bodies expressing the *egl-15*BGAR reporter in the wild-type background (A) and in the *asd-1; sup-12* (*yb1253*) background (B). Top, differential interference contrast (DIC) images. Bottom, merged confocal views. vul, vulva. Arrowheads indicate vulval muscles. Anterior is to the left and dorsal is to the top. Bar, 50  $\mu$ m. (C) RT-PCR analysis of the endogenous *egl-15* gene. The amount of the *egl-15*(5A) isoform relative to that of the *egl-15*(5B) isoform in each strain is indicated. *inf-1* cDNA was also amplified as a loading control. The genotypes of the worms used are indicated. wt, wild type.

larvae, and we could not investigate the vulval muscle phenotype. On the other hand, *asd-1; sup-12* (E63K) and *fox-1 sup-12* (E63K) double mutants were fertile and their vulval muscles were normally formed (data not shown), indicating that *sup-12* function is more seriously affected by the *yb1253* allele. We then compared the amounts of the *egl-15* mRNA isoforms in the single mutants and the fertile double mutants by RT-PCR (Fig. 6C). Although the amounts of the *egl-15*(5A) isoform in the single mutants (lanes 2, 3, and 7) were comparable to that in the wild type (lanes 1 and 8), we found a  $\sim$ 2.8-fold reduction in the *asd-1; sup-12* (E63K) double mutant (lane 6) as in the *asd-1; fox-1* double mutant ( $>$ 4-fold) (lane 4). These results indicate that SUP-12 functions as a coregulator of the muscle-specific alternative splicing of the endogenous *egl-15* gene in vivo.

DISCUSSION

We have provided genetic and biochemical evidence that the muscle-specific and evolutionarily conserved RNA-binding protein SUP-12 cooperatively regulates, with ASD-1 and FOX-1, the tissue-specific alternative splicing of the fibroblast growth factor receptor gene *egl-15*. Figure 7 summarizes the coordinated binding of the Fox-1 family and SUP-12 to the *egl-15* RNA. The Fox-1 family and SUP-12 can specifically bind alone to the *egl-15* RNA through the UGCAUG and the GUGUG stretches, respectively (left and right panels). The Fox-1 family and SUP-12 can directly bind to each other even in the absence of the target RNA (middle panel). When the three components meet together, they form a more stable ternary complex through their sequence-specific binding prop-

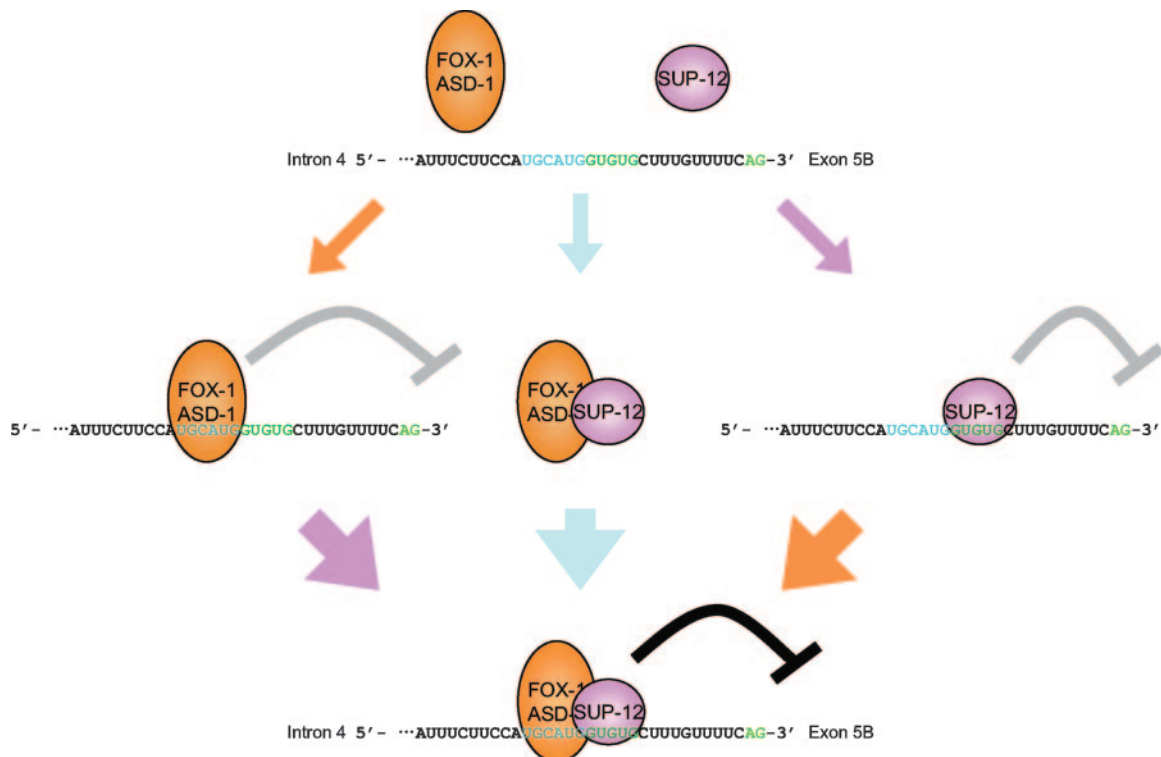


FIG. 7. Schematic representation of the in vitro binding of ASD-1, FOX-1, and SUP-12 to the *egl-15* pre-mRNA. See text for details.



erties (bottom panel). This scheme also explains the mode of the *egl-15* alternative splicing regulation. The two families of RNA-binding proteins form a stable complex on intron 4 to repress selection of E5B (bottom panel), which eventually leads to the selection of the downstream E5A in muscle. Disruption of either of the UGCAUG or the GUGUG stretches or mutation in the *asd-1*, *fox-1*, or *sup-12* gene (left and right panels) also leads to derepression of E5B. The molecular mechanisms of E5B repression by the Fox-1 family/SUP-12/*egl-15* intron 4 ternary complex formation remain to be elucidated. Although *C. elegans* does not have a recognizable branch point consensus or a polypyrimidine tract (25), the putative branch point is the A at position -20 in the UGCAUG element, which is the first A upstream of the 3' splice site for E5B (Fig. 3A) and is close to the positions where the putative branch point A is frequently found (9). It is reasonable to suggest that the SUP-12/Fox-1 complex directly competes with the branch point binding proteins or that the complex interferes with the recruitment of U2 snRNP by U2AF. Mutation in any one of the *asd-1*, *fox-1*, and *sup-12* genes did not cause an egg-laying (Egl) phenotype, suggesting that the Egl phenotype is the binary readout of the amount of the endogenous *egl-15(5A)* isoform in the sex myoblasts. On the other hand, *asd-1* and *sup-12* single mutants showed reproducible color phenotypes in the body wall muscles (Fig. 2) (31), suggesting that the color phenotype is quantitative and directly reflects the net amounts of the mature reporter mRNA isoforms in that tissue. Our approach with the alternative splicing reporter worm system thus allowed us to identify the *trans*-acting factors involved in the tissue-specific regulation and led us to the conclusion that the endogenous *egl-15* gene is coordinately regulated by these three RNA-binding proteins *in vivo*.

Previous work suggested that SUP-12 is a muscle-specific splicing regulator of the *unc-60* gene (1). The *unc-60* gene, encoding two related isoforms of actin-depolymerizing factor (also known as cofilin), has a common first exon and two separate series of downstream exons (36). Exons 2A through 5A of the *unc-60A* isoform are selected in nonmuscle tissues, while exons 2B through 5B of the *unc-60B* isoform reside downstream of the polyadenylation signal of *unc-60A* and are selected only in muscle tissues. Genetic evidence indicated that SUP-12 represses expression of *unc-60A* in muscle, allowing selection of downstream *unc-60B* (1). SUP-12 has been shown to bind to UG-rich stretches in intron 1 and intron 2A of the *unc-60* pre-mRNA *in vitro* (1). Therefore, the situations of the *sup-12*-dependent muscle specific exon repression are quite similar for *unc-60* and *egl-15*, although the Fox-1 family RNA-binding consensus, (U)GCAUG, is absent from the *unc-60* pre-mRNA. It is not yet clear whether other *trans* factors and *cis* elements are involved in regulation of the *unc-60* gene or whether SUP-12 functions as the sole repressor. As the SUP-12 family of RNA-binding proteins are evolutionarily conserved among metazoans and plants (Fig. 3F) (1), it is likely that the family is involved in the regulation of tissue-specific alternative splicing in other multicellular organisms.

A remarkable feature of the Fox-1 family RNA-binding proteins is that their single evolutionarily conserved RRM binds to a highly specific hexameric nucleotide stretch (2, 27). In contrast, other tissue-specific alternative splicing regulator families usually have multiple copies of RNA-binding domains or

form dimers. For instance, the Nova family has three KH motifs (54), the CELF/Bruno-like family has three RRMs (4), PTB and its paralogue nPTB have four RRMs (50), and the GSG/STAR family forms homodimers (52). Although the target sequence of the Fox-1 family, UGCAUG, is the most highly overrepresented hexanucleotide in the downstream introns of tissue-specific alternative exons (10, 37), it is unlikely that all the UGCAUG-containing pre-mRNAs are spliced in a common way depending merely on the expression of the Fox-1 family proteins, because such binary regulation mechanisms would reduce diversity in gene expression. In fact, mammalian pre-mRNAs regulated by the Fox-1 family usually have multiple copies of other *cis* elements involved in alternative splicing regulation. Furthermore, the UGCAUG stretch may function as both an enhancer and a silencer of the cassette exons (27, 49). These characteristic features of the Fox-1 family proteins raise a question about what other tissue-specific regulators or components of the general splicing apparatus mediate tissue-specific and context-dependent alternative splicing regulation by the Fox-1 family. Our present study provides the first example that two families of RNA-binding proteins, each of which has only one RNA-binding domain, cooperatively form a stable complex on a target pre-mRNA that has a tandem cluster of the *cis* elements for each of the families. The two Fox-1 family proteins, ASD-1 and FOX-1, are coexpressed in a broad range of tissues, redundantly function through high sequence specificity, and may contribute to confer robustness to *egl-15* regulation, while their muscle-specific partner protein, SUP-12, may contribute to confer tissue specificity and also to specify the targets to a smaller subset. This kind of coordinated regulation of alternative splicing by the Fox-1 family may be evolutionarily conserved in higher eukaryotes, since (i) the mammalian Fox-1 family members are coexpressed in neurons and muscles (41, 49) and the target pre-mRNAs usually have multiple copies of the UGCAUG stretches (3, 27, 41, 43, 49, 56), allowing robust regulation, and (ii) the domain composition of the Fox-1 family is well conserved (27), suggesting conservation of the physical interaction with other regulators.

Multiple *cis* elements are usually involved in cell-type-specific regulation of alternative splicing. Functional antagonism between juxtaposed enhancer and silencer elements via positive and negative regulatory factors have been reported in both exonic and intronic regions from multiple model genes (34). The functional coordination of the Fox-1 family and SUP-12 presented in this study suggest the presence of more orchestrated modes of splicing regulation. Recent global analyses of tissue-specific splicing patterns and comparison of the genomic sequences predicted many uncharacterized candidate *cis* elements (10, 46, 55). Further identification of *trans*-acting factors and characterization of their RNA-binding properties, as in the present study, would lead to understanding of the tissue-specific splicing regulation and to prediction of the effect of nucleotide polymorphisms on gene expression.

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