Position-dependent and neuron-specific splicing regulation by the CELF family RNA-binding protein UNC-75 in Caenorhabditis elegans

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ABSTRACT

A large fraction of protein-coding genes in metazoans undergo alternative pre-mRNA splicing in tissue- or cell-type-specific manners. Recent genome-wide approaches have identified many putative-binding sites for some of tissue-specific trans-acting splicing regulators. However, the mechanisms of splicing regulation in vivo remain largely unknown. To elucidate the modes of splicing regulation by the neuron-specific CELF family RNA-binding protein UNC-75 in Caenorhabditis elegans, we performed deep sequencing of poly(A)+ RNAs from the unc-75(+)- and unc-75-mutant worms and identified more than 20 cassette and mutually exclusive exons repressed or activated by UNC-75. Motif searches revealed that (G/U)UGUUGUG stretches are enriched in the upstream and downstream introns of the UNC-75-repressed and -activated exons, respectively. Recombinant UNC-75 protein specifically binds to RNA fragments carrying the (G/U)UGUUGUG stretches in vitro. Bi-chromatic fluorescence alternative splicing reporters revealed that the UNC-75-target exons are regulated in tissue-specific and (G/U)UGUUGUG element-dependent manners in vivo. The unc-75 mutation affected the splicing reporter expression specifically in the nervous system. These results indicate that UNC-75 regulates alternative splicing of its target exons in neuron-specific and position-dependent manners through the (G/U)UGUUGUG elements in C. elegans. This study thus reveals the repertoire of target events for the CELF family in the living organism.

INTRODUCTION

Alternative splicing of pre-mRNAs greatly contributes to proteome diversity in metazoans. Recent transcriptome analyses of diverse human tissues and cell lines revealed that a large fraction of protein-coding genes undergo alternative pre-mRNA splicing in tissue- or cell-type-specific manners (1). Alternative splicing patterns are coordinately determined by a limited number of trans-acting regulatory factors and corresponding cis-elements (2–5). Recent genome-wide analyses of protein–RNA interactions by crosslinking and immunoprecipitation experiments combined with deep sequencing identified many binding sites and target alternative splicing events for some of such splicing regulators in living cells or tissues (6,7). However, the molecular mechanisms underlying the tissue-specific alternative splicing regulation in vivo remain largely unknown.

The CELF family of RNA-binding proteins is evolutionarily conserved and shares overall domain structures; three RNA recognition motif (RRM) domains and a divergent domain between RRM2 and RRM3 (8,9). The CELF family proteins are subdivided into two subfamilies according to the sequence similarities (8). CELF1-2 subfamily members CELF1 (also known as CUG-BP1) and CELF2 (also known as CUG-BP2) are broadly expressed with high expression in skeletal muscle, heart and brain in vertebrates (10–12). There are many reports on the molecular functions of the CELF1-2 subfamily; they preferentially bind to GU- or UG-rich elements in vitro (13–18),...
in cultured cells (19–21) and in brain (22), and are shown to regulate various aspects of mRNA metabolism including splicing (17,23–27), translation (28–31) and decay (16,19,20). On the other hand, CELF3-6 subfamily members CELF3 to CELF6 are predominantly expressed in the nervous system (12,32–35). Although some reports revealed that they function as splicing regulators in heterogeneous minigene systems (32,36–39), their endogenous target genes are almost unknown presumably due to redundancy in their functions.

In the nematode Caenorhabditis elegans, there are two members of the CELF family proteins; ETR-1 and UNC-75 are the sole members of the CELF1-2 and CELF3-6 subfamilies, respectively (11,40). ETR-1 is specifically expressed in body wall muscles and vulval muscles and is essential for embryonic development (41), but its molecular function is to be elucidated. UNC-75 was characterized as a pan-neuronal protein and is localized to dynamic nuclear speckles, eventually affecting synaptic transmission (40). We have recently identified UNC-75 as a neuron-specific alternative splicing regulator; UNC-75 is required for neuron-specific selection of two sets of mutually exclusive exons of the unc-32 gene, encoding the α subunit of V0 complex of vacuolar-type H + -ATPases (42). In this study, we identify UNC-75-regulated alternative splicing events by deep sequencing of mRNAs from the wild-type and the unc-75 mutant and demonstrate that UNC-75 regulates the target exons via (G/U)UGUUGUG elements in position-dependent and neuron-specific manners.

MATERIALS AND METHODS

Worm culture and microscopy

Worms were cultured following standard methods. The strains used are N2, KH1668: smg-2 (yb979) I, KH1752: unc-75 (yb1701) I and KH1857: smg-2(yb979) unc-75(yb1701) I. Transgenic worms were generated as described previously (43). Images of fluorescence reporter worms were captured using a fluorescence compound microscope (DM6000B, Leica) equipped with a color, cooled CCD camera (DFC310FX, Leica) and processed with Photoshop (Adobe).

Deep sequencing and data analysis

Total RNAs were extracted from synchronized L1 worms by using RNeasy Midi kit (Qiagen). Poly (A)+ RNAs were repeatedly purified with FastTrack MAG (Invitrogen), fragmented in 1× Fragmentation buffer (Ambion) and reverse transcribed using random hexamer and Primerscript II (Takara). Second strand cDNAs were synthesized by using RNase H (Invitrogen) and DNA polymerase I (Invitrogen). Deep sequencing was performed by utilizing Genome Analyzer II (Illumina) following standard protocols. We obtained 25-nt sequence tags and mapped them to the genome sequence and RefSeq models (WS190/ce6, UCSC Genome Browser) as well as to a custom exon-exon junction library. The numbers of uniquely mapped sequence tags for smg-2 and smg-2 unc-75 are 10 259 115 and 6 333 712, respectively. Significance of difference in the exon inclusion level was estimated by using the following equation after Pearson’s χ² test: \[ \chi^2 = \frac{(n_w + n_m) - (e_w - n_w)p)^2}{n_w n_m} \], where \( n \) and \( e \) indicate actual number of sequence tags associated with a given RefSeq model and a given exon plus its left and right junctions, respectively, and \( w \) and \( m \) indicate wild-type and mutant, respectively, \( p = (e_w + e_m)/(n_w + n_m) \) and degree of freedom (df) = 1.

Gene ontology (GO) analysis was performed with GO data (WS220, Wormbase) using Ekuseru-Toukei 2010 (Social Survey Research Information, Tokyo, Japan). Genomic sequences of the genes in C. elegans, Caenorhabditis brenneri, Caenorhabditis briggsae, Caenorhabditis japonica and Caenorhabditis remanei were extracted from Wormbase (http://www.wormbase.org/). Multiple EM for Motif Elicitation (MEME) analysis was performed by using tools available at http://tools.genouest.org/tools/meme/. Sequence logos were generated by using WebLogo (44) at http://weblogo.threeplusone.com/create.cgi.

Reverse transcriptase–polymerase chain reaction

Reverse transcriptase–polymerase chain reaction (RT–PCR) was performed essentially as described previously for amplifying mature mRNAs (43) and partially spliced RNAs (45). RT–PCR products were analyzed by using BioAnalyzer (Agilent). ΔΨ was calculated by using the following equation: \[ \Delta \Psi = \frac{c_{1w}}{c_{2w}} - \frac{c_{1m}}{c_{2m}} \], where \( c_i \) indicates molar concentration of isoform \( i \) (1 or 2) and \( m \) and \( w \) indicate mutant and wild-type, respectively. Significance of difference in the ratio of mRNA isoforms was analyzed by using the following equation modified from Pearson’s χ² test: \[ \chi^2 = \frac{(c_{1w} + c_{2w} - c_{1m} - c_{2m})^2}{(c_{1w} + c_{2w}) (c_{1m} + c_{2m})} \], where \( c_i \) indicates concentration in 0.1 ng/μl of isoform \( i \) and degree of freedom (df) = 1. The sequences of the RT–PCR products were confirmed by direct sequencing or by cloning and sequencing. Sequences of the primers used in the RT–PCR assays are available in Supplementary Table S1.

Construction of reporter minigenes

Symmetric fluorescence reporter minigenes were constructed as described previously (43). In this study, we utilized EGFP and mCherry cassettes in pENTR-L5/L2 vector that has a linker sequence GGS6, six repeats of Gly-Gly-Ser and lacks initiation codon. The detailed sequence information is available upon request to H.K. Sequences of the primers used in the plasmid construction are available in Supplementary Table S2.

Electrophoretic mobility shift assay

Recombinant FLAG-tagged full-length UNC-75 protein was prepared as described previously (42). 32P-labeled RNA probes were prepared as described previously by using template PCR products amplified with primers in Supplementary Table S3. Electrophoretic mobility shift assays (EMSAs) were performed as described previously (45).
RESULTS

mRNAsseq revealed UNC-75-regulated alternative splicing events

In order to systematically identify alternative splicing events regulated by UNC-75, we performed transcriptome analysis by deep sequencing of poly(A)+ RNAs. To effectively detect alternative mRNA isoforms that may be rapidly degraded by nonsense-mediated mRNA decay (NMD), we sequenced poly(A)+ RNAs from NMD-deficient smg-2 mutant and smg-2 unc-75 double-mutant worms. Since neuron-specific mRNA isoforms become relatively less abundant at later stages (42), we analyzed poly(A)+ RNAs from synchronized L1 worms. By comparing actual numbers of sequence tags associated with a given RefSeq models and a given exon and its junctions (see ‘Materials and Methods’ section for detail), together with genome-wide information about alternative splicing events from the modENCODE project (46), we predicted alternative splicing events affected in the unc-75 mutant and validated by RT–PCR analysis of total RNAs from synchronized L1 larvae.

Table 1 summarizes the 24 experimentally validated alternative splicing events including unc-32 exons 4 and 7 and unc-75 exon 8 (42). These events include 7 cassette exons derepressed in the unc-75 mutant (Figure 1A and Supplementary Figure S1A), 11 cassette exons repressed in the unc-75 mutant (Figure 1B and Supplementary Figure S1B) and 6 cases of mutually exclusive exons (Figure 1C). The sizes of all the 18 cassette exons are multiple of three bases except for dyn-1 exon 8 that is the second last exon containing an alternative termination codon. The sizes of the mutually exclusive exons are not necessarily multiple of three bases but maintain the frame whichever of the exons are selected. Thus, none of these UNC-75-regulated alternative isoforms appear to be NMD substrates, despite the use of the NMD-deficient worms in the mRNAsseq analysis.

UNC-75 regulates its target exons in a neuron-specific manner

To analyze spatio-temporal selection patterns of the UNC-75-regulated exons, we constructed symmetric pairs of bi-chromatic fluorescence splicing reporter minigenes under the control of a ubiquitous promoter for two of the UNC-75-repressed cassette exons ret-1 exon 5 and C07A12.7 exon 4 and two of the UNC-75-activated cassette exons C33H5.18 exon 2 and unc-16 exon 16 (Figure 2A-D and Supplementary Figures S2–S5). All these exons exhibited tissue-specific selection patterns, which varied from gene to gene. ret-1 exon 5 was skipped in hypodermis and body wall muscles as well as in the nervous system (Figure 2A and Supplementary Figure S2A and B). C07A12.7 exon 4 was skipped exclusively in the nervous system (Figure 2B and Supplementary Figure S3A and B). C33H5.18 exon 2 was included in body wall muscles and the nervous system (Figure 2C and Supplementary Figure S4A and B). unc-16 exon 16 was included in hypodermis and the nervous system (Figure 2D and Supplementary Figure S5A and B). In the unc-75-mutant background, the nervous system turned the selection patterns while the other tissues were not affected in all cases (Figure 2E–H and Supplementary Figures S2C, S3C, S4C and S5C). The direction of the color change of the nervous system was consistent with the change in the splicing patterns of the endogenous genes in the unc-75 mutant (Figure 1A and B). These results indicated that the fluorescence splicing reporters represented the selection patterns of the endogenous genes and that UNC-75 regulates its target exons in a neuron-specific manner.

GO analysis of the 23 genes with the UNC-75-regulated exons revealed that GO terms ‘positive regulation of growth rate’, ‘inductive cell migration’ and ‘defecation’ are enriched in the UNC-75-regulated genes (Table 2). GO terms related to neuron-specific functions or structures were not enriched in the UNC-75-regulated genes presumably because UNC-75 regulates neuron-specific alternative splicing of genes that would be expressed not only in the nervous system but also in some other tissues.

(G/U)UGUUGUG stretches are enriched in the upstream and downstream introns of the UNC-75-repressed and -activated exons, respectively

To elucidate regulatory elements for UNC-75, we searched for enriched motifs in upstream and downstream flanking exons of the UNC-75-regulated exons in C. elegans and of orthologous exons in other species of the genus Caenorhabditis by MEME analysis (47). (G/T)TGTGTG motif was the most enriched motif in the upstream introns of 55 putative UNC-75-repressed exons as well as in the downstream introns of 53 putative UNC-75-activated exons (Figure 3). The motif overlaps with UUGUUGUGUGUG element, the binding site for UNC-75 experimentally identified in unc-32 intron 7a (42), suggesting that UNC-75 regulates the target exons via the (G/U)UGUUGUG motifs. In contrast, no motif was found to be enriched in the downstream introns of the putative UNC-75-repressed exons or in the upstream introns of the putative UNC-75-activated exons (Figure 3).

Collection of the splice site sequences from the UNC-75-regulated exons and orthologous exons (Figure 3) revealed that these splice sites are less related to the consensus of the 3′- (TTTTCAG/C1) and 5′- (AG/GTAAGTT) splice sites in C. elegans (48,49) and are therefore considered to be weaker.

The (G/U)UGUUGUG elements are involved in the regulation of the UNC-75-target exons in a position-dependent manner

To test whether the (G/U)UGUUGUG elements are required for the regulation of the target exons for UNC-75, we mutagenized the (G/U)UGUUGUG elements in the fluorescence reporter minigenes. Disruption of GUGAUGUG stretch in the upstream intron of the ret-1 exon 5 reporter (Supplementary Figure S2A) affected the expression of ΔE5-mCherry in
Table 1. UNC-75-regulated alternative splicing events confirmed by RT–PCR

| Gene WB ID         | Gene Public Name | Gene product         | AS type | Exon | Chr | Str | Exon start | Exon end | Exon size | ΔPSI | P-value  |
|--------------------|------------------|----------------------|---------|------|-----|-----|-------------|----------|-----------|------|----------|
| WBGene00010796     | M01A8.2          | Uncharacterized      | CE      | 4    | III | –   | 926732     | 9267526  | 3n       | –56.0| 4.36E–34 |
| WBGene00016384     | C33H5.18         | Phosphatidylinositol  | CE      | 2    | IV  | –   | 78154     | 781548   | 3n       | –27.1| 1.94E–12 |
| WBGene00006799     | unc-1            | Reticulon V-ATPase a subunit | ME   | 4a   | III | +   | 8906432   | 8906891  | 3n+2    | –37.0| 1.81E–14 |
| WBGene00006768     | unc-75           | Alpha-actinin        | ME      | 4b   | –   | –   | 8906785   | 8907219  | 3n+2    | 27.8 | 7.78E–07 |
| WBGene00006807     | unc-32           | Alpha-actinin        | ME      | 4b   | –   | –   | 8909141   | 8909989  | 3n+2    | –25.1| 2.93E–08 |
| WBGene00006755     | unc-16           | JIP1                 | ME      | 4b   | –   | –   | 8909989   | 8910749  | 3n+2    | 25.7 | 2.72E–10 |
| WBGene00016384     | C33H5.18         | Phosphatidylinositol transferase | CE   | 2    | IV  | –   | 78154     | 781548   | 3n       | –27.1| 1.94E–12 |
| WBGene00006799     | unc-1            | Reticulon V-ATPase a subunit | ME   | 4a   | III | +   | 8906432   | 8906891  | 3n+2    | –37.0| 1.81E–14 |
| WBGene00015561     | C07A12.7         | Uncharacterized      | CE      | 4    | X   | –   | 4519324   | 4519412  | 3n       | –28.6| 2.21E–15 |

aCE, cassette exon; ME, mutually exclusive exons.
bCE, cassette exon with alternative 3′-splice sites.
cCarrying a termination codon.
the nervous system and body wall muscles and not in hypodermis (Figure 2I and Supplementary Figure S2D). Disruption of UUGUUGUG and GUGUUGUG stretches in the upstream intron of the C07A12.7 exon 4 reporter (Supplementary Figure S3A) abolished the expression of E4-EGFP in the nervous system (Figure 2J and Supplementary Figure S3D). Disruption of UUGUU stretch in the downstream intron of the C33H5.18 exon 2 reporter (Supplementary Figure S4A) affected the expression of E2-EGFP in the nervous system and body wall muscles (Figure 2K and Supplementary Figure S4D). Disruption of GGUGUUGUG stretch in the downstream intron of the unc-16 exon 16 reporter (Supplementary Figure S5A) affected the expression of E16-EGFP in the nervous system and not in hypodermis (Figure 2L and Supplementary Figure S5D). These results confirmed that the (G/U)UGUUGUG elements actually play roles in the regulation of the UNC-75-target exons in a position-dependent manner.

**Figure 1.** RT–PCR validation of UNC-75-regulated alternative splicing events. (A) UNC-75-repressed cassette exons. (B) UNC-75-activated cassette exons. (C) UNC-75-regulated mutually exclusive exons. Total RNAs were extracted from synchronized L1 worms of N2 (wild type) and the unc-75 (yb1701) mutant. Splicing patterns of the mRNAs are schematically shown on the right. \( \Delta \Psi \), change in percentage of an exon-inclusion isoform (PSI or \( \Psi \), percent-spliced-in) to the total in molar concentration. \( P \)-values were calculated as described in 'Materials and Methods' section.

**UNC-75 specifically recognizes the target RNAs via the (G/U)UGUUGUG elements in vitro**

We tested direct and specific recognition of the target RNAs carrying the (G/U)UGUUGUG elements by UNC-75 in vitro. In EMSAs, recombinant full-length UNC-75 protein efficiently shifted the mobility of intact ret-1, C07A12.7, C33H5.18 and unc-16 probes and less efficiently of mutant probes with the same substitutions as in the mutant reporters (Figure 4), confirming that UNC-75 specifically binds to these RNAs in a (G/U)UG UUGUG element-dependent fashion.

**UNC-75 regulates excision of the upstream and/or downstream flanking introns of its target exons**

To obtain mechanistic insights into the role of UNC-75 in the regulation of its target exons, we determined the major order of excision of the upstream and downstream flanking introns by comparing the relative amounts of
two partially spliced RNAs to the pre-mRNA for the UNC-75-regulated cassette exons between the wild-type and unc-75 mutant worms, assuming that the relative amounts of the partially spliced RNAs are directly correlated with the relative amount of the exon-inclusion isoform of mature mRNAs (Figure 5A).

Among the UNC-75-repressed exons, excision of the upstream intron was derepressed for ret-1 exon 5 (Figure 5B), tag-60 exon 9 (Supplementary Figure S6A), mvk-1 exon 4 (Supplementary Figure S6B) and din-1 exon 15 (Supplementary Figure S6C), while excision of the downstream intron was derepressed for C07A12.7 exon 4 (Figure 5C) in the unc-75 mutant. Excision of both upstream and downstream introns was derepressed in the unc-75 mutant for larp-1 exon 11 (Supplementary Figure S6D). These results suggested that UNC-75 promotes skipping of cassette exons by repressing excision of the upstream and/or downstream introns in vivo.

Among the UNC-75-activated exons, excision of the upstream intron was diminished for C33H5.18 exon 2 (Figure 5D), unc-16 exon 16 (Supplementary Figure S7A) and dyn-1 exon 8 (Supplementary Figure S7B), while excision of the downstream intron was diminished for M01A8.2 exon 4 (Figure 5E) and nrx-1 exon 24 (Supplementary Figure S7C) in the unc-75 mutant. These results suggested that UNC-75 promotes inclusion of cassette exons by enhancing excision of either the upstream or downstream intron in vivo.

**DISCUSSION**

In this study, we identified more than 20 validated endogenous target events for UNC-75 by comparing the genome-wide mRNA-seq data between the wild-type and mutant strains (Table 1). UNC-75 regulates the target splicing events only in the nervous system (Figure 2). As the contribution of the nervous system to the total mRNAs in the whole animals is relatively small (42), the splicing changes of the target genes in the unc-75 mutant are often small (Figure 1 and Supplementary Figure S1). Indeed, a previous study utilizing a splicing-sensitive microarray for 352 cassette exons did not identify an alternative splicing event affected in the unc-75 mutant (50).

In this study, we evaluated inclusion level changes of all the exons in the RefSeq models and the method does not
rely on the information about alternative isoforms. Yet the deep sequencing of the mRNAs from the whole animals in combination with the information from the modENCODE project enabled identification of the alternative splicing events affected in the \textit{unc-75} mutant, emphasizing the feasibility of this approach in identifying target events. This study did not necessarily identify all or most of the UNC-75 target events considering the following situations: (i) only the L1 worms were analyzed, (ii) some target genes may be expressed only in a small subset of neurons in the nervous system and the expected splicing changes in the whole animal are too subtle and (iii) the RefSeq models miss some genes, novel exons or untranslated regions. Future sequencing of the mRNAs in more depth and evaluation of all the exons based on precise gene models will improve the accuracy of exon inclusion level estimations and increase the specificity of target event predictions.

**Figure 3.** Summary of the motif analyses. Enriched motifs with \( E \)-values < 1 in the MEME analysis are indicated for upstream and downstream flanking introns of UNC-75-repressed (A) and -activated (B) cassette and mutually exclusive exons. Sequence logos of the 5’- and 3’-splice sites (SS) of these exons are also indicated.

**Figure 4.** UNC-75 directly and specifically binds to the (G/U)UGUUGUG element(s) in the upstream or downstream of its target exons. (A) EMSA using radiolabeled wild-type (WT) and mutant (Mut or M1M2) probes without (−) or with 4-fold (\textit{ret-1}, \textit{C07A12.7} and \textit{C33H5.18}) or 2-fold (\textit{unc-16}) dilution series of full-length FLAG-tagged UNC-75 protein. The mutant probes have the same substitutions as in the mutant reporter minigenes in Figure 2I–L and Supplementary Figures S2–S5. (B) SDS–PAGE and CBB staining of recombinant FLAG-tagged full-length UNC-75 protein used in (A).
Figure 5. UNC-75 can regulate excision of upstream and downstream flanking introns of its target exons. (A) Schematic illustration of the order of intron excision from a pre-mRNA for an exon-skipping isoform and an exon-inclusion isoform of mature mRNAs. Note that there are two forms of partially spliced RNAs, both of which are intermediates for the exon-inclusion isoform. (B–E) Comparison of the amounts of partially spliced RNAs in N2 (wild-type) and unc-75 (yb1701) worms for ret-1 exon 5 (B), C07A12.7 exon 4 (C), C33H5.18 exon 2 (D) and M01A8.2 exon 4 (E). (Left) RT–PCR analyses with upstream exonic and downstream intronic primers. (Middle) RT–PCR with upstream intronic and downstream exonic primers. Schematic structures of the PCR products are indicated on the right. Black and gray arrowheads indicate positions and directions of the exonic and intronic primers, respectively. RTase, reverse transcriptase. (Right) Relative amounts of the partially spliced RNAs normalized to the pre-mRNA.
Collection of the genomic sequences of the validated target genes for UNC-75 in *C. elegans* and of the orthologous genes in the same genus led to identification of the (G/U)UGUUGUG stretch as the only motif enriched in the flanking introns of the UNC-75-regulated exons (Figure 3) and full-length UNC-75 specifically bound to the RNA fragments carrying the (G/U)UGUUGUG elements in *vitro* (Figure 4). As all three RRMs of UNC-75 are required for recognition of the target sequence and RRM3 by itself can specifically recognize the UUGUUGUGUUGU stretch in the cis-element in the case of *unc-32* (42), it is likely that the (G/U)UGUUGUG elements in the other target genes are also recognized by RRM3 and that stretches recognized by RRM1 or RRM2 are too diversified to be identified in the MEME analysis. Consistent with the amino acid sequence similarities between the two subfamilies in the CELF family (8), the CELF1-2 subfamily proteins in vertebrates have also been shown to bind to a variety of U/G-rich sequences (13–21). The recognition sequences for the CELF family may vary in a context-dependent manner.

The fluorescence alternative splicing reporters revealed that the UNC-75-regulated exons exhibited tissue-specific and not necessarily neuron-specific selection patterns (Figure 2 and Supplementary Figures S2–S5). Consistent with its specific expression in the nervous system (40), UNC-75 regulates its target exons only in the nervous system (Figure 2 and Supplementary Figures S2–S5). The (G/U)UGUUGUG elements, on the other hand, regulate splicing not only in the nervous system but also in body wall muscles for *ret-1* exon 5 and *C33H5.18* exon 2, suggesting that other factor(s) may recognize the same elements in body wall muscles and regulate the exons in the same way as UNC-75. The most likely regulator for these exons in muscles is the sole member of the CELF1-2 subfamily proteins, ETR-1, which has been shown to be muscle specific (41). The reporters also revealed position-dependent regulation of the UNC-75-target exons by the (G/U)UGUUGUG elements (Figure 2 and Supplementary Figures S2–S5), similar to tissue-specific splicing factors Nova (51,52), RBFOX family (53,54), PTBP1 (55), TIA proteins (56) and TDP-43 (57) in mammals. Although it is still unclear how these tissue-specific splicing factors differentially affect splicing of the target exons in a position-dependent manner (6), there may be a general assumption that the splicing factors bound to introns repress or activate splicing of the introns that they directly bind to. Unexpectedly, we found from the analyses of the partially spliced RNAs or the processing intermediates that UNC-75 may regulate excision of the other flanking intron of the target exons in some cases (Figure 5 and Supplementary Figures S6 and S7). Our data do not preclude the possibility that UNC-75 binds to both the upstream and downstream introns in these cases, although the (G/U)UGUUGUG elements in the upstream and downstream introns are essential for repressing *C07A12.7* exon 4 and activating *C33H5.18* exon 2, respectively, in the nervous system (Figure 2 and Supplementary Figures S3 and S4). It is also unclear whether UNC-75 alone is sufficient for the neuron-specific splicing regulation of the target genes. Other factors bound to the same intron, the regulated exon or the other intron may cooperate with UNC-75 to repress or activate the cassette exons. Similar cross-exon regulation of intron excision has been reported for alternative splicing regulation of exon 9 of the human *ATP5C1* gene encoding mitochondrial ATP synthase γ subunit, where RBFOX1 represses excision of intron 9 by binding to GCAUG stretches in intron 8 to interfere with E complex formation on intron 9 (58). Interestingly, *ATP5C1* intron 9 has been shown to be excised by U1-independent U2-type splicing which contributes to alternative splicing regulation although the molecular mechanism is to be elucidated (59,60).

Genome-wide analysis of PTBP1–RNA interactions suggested that binding of PTBP1 near the constitutive 3′-splice site of the downstream introns interferes with the recognition of the constitutive 3′-splice site to promote the use of the alternative exons (61). This mechanism is unlikely to explain the activation of *C33H5.18* exon 2 by UNC-75 because the UUGUUGUG element resides very close to the 5′-splice site of intron 2 (Supplementary Figure S4A). Further biochemical and genetic approaches will identify factors and sequences mediating such cross-exon regulation of alternative splicing.

**ACCESSION NUMBERS**

The sequence data for the *smg-2* and *smg-2 unc-75* mutants are deposited to Sequence Read Archive (SRA) under the Accession number DRA000864.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online: Supplementary Tables 1–3 and Supplementary Figures 1–7.

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REFERENCES

1. Wang, E.T., Sandberg, R., Luo, S., Khrebtukova, I., Zhang, L., Mayr, C., Kingsmore, S.F., Schroth, G.P. and Burge, C.B. (2008) Alternative isoform regulation in human tissue transcriptomes. Nature, 456, 470–476.

2. Black, D.L. (2003) Mechanisms of alternative pre-messenger RNA splicing. Annu. Rev. Biochem., 72, 391–436.

3. Nilsen, T.W. and Graveley, B.R. (2010) Expansion of the eukaryotic proteome by alternative splicing. Nature, 463, 457–463.

4. Chen, M. and Manley, J.L. (2009) Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. Nat. Rev. Mol. Cell Biol., 10, 741–754.

5. Kasotra, A. and Cooper, T.A. (2011) Functional consequences of developmentally regulated alternative splicing. Nat. Rev. Genet., 12, 715–729.

6. Witten, J.T. and Ule, J. (2011) Understanding splicing regulation through RNA splicing maps. Trends Genet., 27, 89–97.

7. Darnell, R.B. (2010) HITS-CLIP: panoramic views of protein–RNA interaction within living cells. Wiley Interdiscip Rev RNA, 1, 266–286.

8. Dasgupta, T. and Ladd, A.N. (2012) The importance of CELF proteins and polyypyrimidine tract binding protein. RNA, 18, 845–866.

9. Gallo, J.M. and Spickett, C. (2010) The role of CELF proteins in neurological disorders. RNA Biol., 7, 474–479.

10. Roberts, R., Timchenko, N.A., Miller, J.W., Reddy, S., Caskey, C.T., Swanson, M.S. and Timchenko, L.T. (1997) Altered phosphorylation and intracellular distribution of a CUG(n) triplet repeat RNA-binding protein in patients with myotonic dystrophy type 1. Proc. Natl Acad. Sci. USA, 94, 13221–13226.

11. Good, P.J., Chen, Q., Warner, S.J. and Herring, D.C. (2000) A family of human RNA-binding proteins related to the Drosophila Bruno translational regulator. J. Biol. Chem., 275, 28583–28592.

12. Ladd, A.N., Charlet, N. and Cooper, T.A. (2001) The CELF family of RNA-binding proteins is implicated in cell-specific and developmentally regulated alternative splicing. Mol. Cell Biol., 21, 1285–1296.

13. Mori, D., Sasagawa, N., Kino, Y. and Ishiura, S. (2008) Quantitative analysis of CUG-BP1 binding to RNA repeats. J. Biochem., 143, 377–383.

14. Teplova, M., Song, J., Gav, H.Y., Teplova, A. and Patel, D.J. (2010) Structural insights into RNA recognition by the alternate-splicing regulator CUG-binding protein 1. Structure, 18, 1364–1377.

15. Marquis, J., Paillard, L., Audic, Y., Cosson, B., Danos, O., Le Bec, C. and Osborne, H.B. (2006) CUG-BP/CELF1 requires UGU-rich sequences for high-affinity binding. Biochem. J., 400, 291–301.

16. Vlasova, I.A., Tahoe, N.M., Fan, D., Larsson, O., Rattenbacher, B., Sternjohn, J.R., Vasdewani, J., Karypis, G., Reilly, C.S., Bitterman, P.B. et al. (2008) Conserved GU-rich elements mediate mRNA decay by binding to CUG-binding protein 1. Mol. Cell, 29, 263–270.

17. Dembowski, J.A. and Grabowski, P.J. (2009) The CUGBP2 splicing factor regulates an ensemble of branchpoints from perimeter binding sites with implications for autoregulation. PLoS Genet., 5, e1000595.

18. Tsuda, K., Kuwasako, K., Takahashi, M., Someya, T., Inoue, M., Terada, T., Kobayashi, N., Shirouzu, M., Kigawa, T., Tanaka, A. et al. (2009) Structural basis for the sequence-specific RNA-recognition mechanism of human CUG-BP1 RRM3. Nucleic Acids Res., 37, 5151–5166.

19. Rattenbacher, B., Beisang, D., Wiesner, D.L., Jeschke, J.C., von Hohenberg, M., St. Louis-Vlasova, I.A. and Bohjanen, P.R. (2010) Analysis of CUGBP1 targets identifies GU-repeat sequences that mediate rapid mRNA decay. Mol. Cell Biol., 30, 3970–3980.
39. Han,J. and Cooper,T.A. (2005) Identification of CELF splicing activation and repression domains in vivo. Nucleic Acids Res., 33, 2769–2780.
40. Loria,P.M., Duke,A., Rand,J.B. and Hobert,O. (2003) Two neuronal, nuclear-localized RNA binding proteins involved in synaptic transmission. Curr. Biol., 13, 1317–1323.
41. Milne,C.A. and Hodgkin,J. (1999) ETR-1, a homologue of a protein linked to myotonic dystrophy, is essential for muscle development in Caenorhabditis elegans. Curr. Biol., 9, 1243–1246.
42. Kuroyanagi,H., Watanabe,Y. and Hagiwara,M. (2013) CELF family RNA-binding protein UNC-75 regulates two sets of mutually exclusive exons of the unc-32 gene in neuron-specific manners in Caenorhabditis elegans. PLoS Genet., 9, e1003377.
43. Kuroyanagi,H., Ohno,G., Sakane,H., Maruoka,H. and Hagiwara,M. (2010) Visualization and genetic analysis of alternative splicing regulation in vivo using fluorescence reporters in transgenic Caenorhabditis elegans. Nat. Protoc., 5, 1495–1517.
44. Crooks,G.E., Hon,G., Chandonia,J.M. and Brenner,S.E. (2004) WebLogo: a sequence logo generator. Genome Res., 14, 1188–1190.
45. Ohno,G., Ono,K., Togo,M., Watanabe,Y., Ono,S., Hagiwara,M. and Kuroyanagi,H. (2012) Muscle-specific splicing factors ASD-2 and SUP-12 cooperatively switch alternative pre-mRNA processing patterns of the ADF/cofilin gene in Caenorhabditis elegans. PLoS Genet., 8, e1002991.
46. Ramani,A.K., Calarco,J.A., Pan,Q., Mavandadi,S., Wang,Y., Nelson,A.C., Lee,L.I., Morris,Q., Blencowe,B.J., Zhen,M. et al. (2011) Genome-wide analysis of alternative splicing in Caenorhabditis elegans. Genome Res., 21, 342–348.
47. Bailey,T.L., Boden,M., Buske,F.A., Frith,M., Grant,C.E., Clementi,L., Ren,J., Li,W.W. and Noble,W.S. (2009) MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res., 37, W202–W208.
48. Hollins,C., Zorio,D.A., MacMorris,M. and Blumenthal,T. (2005) U2AF binding selects for the high conservation of the C. elegans 3’ splice site. RNA, 11, 248–253.
49. Sheth,N., Rocca,X., Hastings,M.L., Roeder,T., Krainer,A.R. and Sachidanandam,R. (2006) Comprehensive splice-site analysis using comparative genomics. Nucleic Acids Res., 34, 3955–3967.
50. Barberan-Soler,S., Medina,P., Estrella,J., Williams,J. and Zahler,A.M. (2011) Co-regulation of alternative splicing by diverse splicing factors in Caenorhabditis elegans. Nucleic Acids Res., 39, 666–674.
51. Ule,J., Stefani,G., Mele,A., Ruggiu,M., Wang,X., Taneri,B., Gaasterland,T., Blencow,B.J. and Darnell,R.B. (2006) An RNA map predicting Nova-dependent splicing regulation. Nature, 444, 580–586.
52. Licatalosi,D.D., Mele,A., Fak,J.J., Ule,J., Kayikci,M., Chi,S.W., Clark,T.A., Schweitzer,A.C., Blume,J.E., Wang,X. et al. (2008) HTIS-CLIP yields genome-wide insights into brain alternative RNA processing. Nature, 456, 464–469.
53. Zhang,C., Zhang,Z., Castle,J., Sun,S., Johnson,K., Krainer,A.R. and Zhang,M.Q. (2008) Defining the regulatory network of the tissue-specific splicing factors Fox-1 and Fox-2. Genes Dev., 22, 2550–2563.
54. Yeo,G.W., Coufal,N.G., Liang,T.Y., Peng,G.E., Fu,X.D. and Gage,F.H. (2009) An RNA code for the FOX2 splicing regulator revealed by mapping RNA–protein interactions in stem cells. Nat. Struct. Mol. Biol., 16, 130–137.
55. Llorian,M., Schwartz,S., Clark,T.A., Hollandier,D., Tan,L.Y., Spellman,R., Gordon,A., Schweitzer,A.C., de la Grange,P., Ast,G. et al. (2010) Position-dependent alternative splicing activity revealed by global profiling of alternative splicing events regulated by PTB. Nat. Struct. Mol. Biol., 17, 1114–1123.
56. Wang,Z., Kayikci,M., Briese,M., Zarnack,K., Luscombe,N.M., Rot,G., Zupan,B., Curt,T. and Ule,J. (2010) iCLIP predicts the dual splicing effects of TIA–RNA interactions. PLoS Biol., 8, e1000530.
57. Tollervey,J.R., Curt,T., Rogelj,B., Briese,M., Cereda,M., Kayikci,M., Konig,J., Hortobagyi,T., Nishimura,A.L., Zupunski,V. et al. (2011) Characterizing the RNA targets and position-dependent splicing regulation by TDP-43. Nat. Neurosci., 14, 452–458.
58. Fukumura,K., Kato,A., Jin,Y., Ideue,T., Hirose,T., Kataoka,N., Fujisawa,T., Sakamoto,H. and Inoue,K. (2007) Tissue-specific splicing regulator Fox-1 induces exon skipping by interfering E complex formation on the downstream intron of human F1gamma gene. Nucleic Acids Res., e1000530.
59. Fukumura,K. and Inoue,K. (2009) Role and mechanism of U1-independent pre-mRNA splicing in the regulation of alternative splicing. RNA Biol., 6, 395–398.
60. Fukumura,K., Taniguchi,I., Sakamoto,H., Ohno,M. and Inoue,K. (2009) U1-independent pre-mRNA splicing contributes to the regulation of alternative splicing. Nucleic Acids Res., 37, 1907–1914.
61. Xue,Y., Zhou,Y., Wu,T., Zhu,T., Ji,X., Kwon,Y.S., Zhang,C., Yeo,G., Black,D.L., Sun,H. et al. (2009) Genome-wide analysis of PTB–RNA interactions reveals a strategy used by the general splicing repressor to modulate exon inclusion or skipping. Mol. Cell, 36, 996–1006.