Published online 14 February 2013 *Nucleic Acids Research, 2013, Vol. 41, No. 7* ***4015–4025***
doi:10.1093/nar/gkt097

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

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Received December 30, 2012; Revised January 24, 2013; Accepted January 25, 2013

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),

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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 heterologous 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 *a* subunit of V_0 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 Primescript 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 χ^2 test: $\chi^2 = (n_w + n_m)\ (e_w - n_w p)^2 / n_w n_m\ p(1-p)$, 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). $\Delta\Psi$ was calculated by using the following equation: $\Delta\Psi = \{c_{1m}/(c_{1m} + c_{2m}) - c_{1w}/(c_{1w} + c_{2w})\} \times 100$, 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 χ^2 test: $\chi^2 = (c_{1w} + c_{2w} + c_{1m} + c_{2m})\ (c_{1w}\ c_{2m} - c_{1m}\ c_{2w})^2 / (c_{1w} + c_{2w})\ (c_{1m} + c_{2m})\ (c_{1w} + c_{1m})\ (c_{2w} + c_{2m})$, 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 GGS_6, 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.

Electophoretic mobility shift assay

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

RESULTS

mRNAseq 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 mRNAseq 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 introns 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)T GTTGTG 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 UUGUUGUGUUGU 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/R) 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[a]	Exon	Chr	Str	Exon start	Exon end	Exon size	ΔPSI	*P*-value
WBGene00010796	*M01A8.2*	Uncharacterized	CE	4	III	–	9267323	9267526	3n	−56.0	4.36E−34
WBGene00006594	*tom-1*	Tomosyn	ME	13A	I	+	5552883	5553372	3n+1	35.9	1.11E−16
				13B			5554744	5554867	3n+1	−35.9	
WBGene00004336	*ret-1*	Reticulon	CE	5	V	–	14836315	14836395	3n	61.7	1.42E−16
WBGene00006768	*unc-32*	V-ATPase a subunit	ME	4a	III	+	8906432	8906586	3n+2	9.2	1.16E−01
				4b			8906875	8906981	3n+2	−37.0	1.81E−14
				4c			8907219	8907340	3n+2	27.8	7.78E−07
WBGene00015561	*C07A12.7*	Uncharacterized	CE	4	X	–	4519324	4519425	3n	28.6	2.21E−15
WBGene00016384	*C33H5.18*	Phosphatidate cytidylyltransferase	CE	2	IV	–	7815454	7815483	3n	−27.1	1.94E−12
WBGene00007799	*nrx-1*	Neurexin	CE[b]	24	V	+	10842967	10843494	3n	−26.3	1.09E−11
WBGene00006807	*unc-75*	CELF3-6	CE	8	I	+	11600245	11600394	3n	41.0	1.08E−09
WBGene00000228	*atn-1*	Alpha-actinin	ME	4a	V	+	12480705	12480865	3n+2	25.1	2.93E−08
				4b			12481091	12481173	3n+2	−25.1	
WBGene00006755	*unc-16*	JIP3	CE	16	III	+	9553998	9554081	3n	−30.5	9.56E−08
WBGene00001130	*dyn-1*	Dynamin	CE	8	X	+	15572436	15572517	3n+1[c]	−24.2	1.59E−07
WBGene00006768	*unc-32*	V-ATPase a subunit	ME	7a	III	+	8909141	8909263	3n	−25.7	2.12E−06
				7b			8909989	8910129	3n	25.7	
WBGene00000899	*daf-3*	co-SMAD	CE	3	X	–	824068	824169	3n	−34.8	4.98E−06
WBGene00002251	*lat-1*	Latrophilin receptor family	ME	3A	II	+	8903309	8903415	3n+2	−25.6	1.86E−05
				3B			8903528	8903640	3n+2	25.6	
WBGene00006876	*vab-10*	Microtubule–actin crosslinking factor 1	CE	17	I	–	11770982	11771323	3n	−15.6	1.74E−04
WBGene00020097	*larp-1*	La-related protein	CE	11	III	–	5011104	5011280	3n	15.4	1.64E−03
WBGene00004739	*scd-1*	Uncharacterized	CE	8	X	–	12505931	12505951	3n	−6.4	3.67E−03
WBGene00006438	*tag-60*	Uncharacterized	CE	9	IV	+	9128466	9128669	3n	11.5	4.46E−03
WBGene00021534	*mvk-1*	Mevalonate kinase	CE	4	III	–	6130157	6130435	3n	12.3	8.61E−03
WBGene00016415	*C34F11.3*	AMP deaminase	CE	7	II	+	5204650	5204685	3n	−6.2	1.40E−02
WBGene00008549	*din-1*	DAF-12-interacting protein	CE	15	II	–	11623852	11624439	3n	8.7	2.55E−02
WBGene00004053	*pme-5*	Poly(ADP-ribose) polymerase	CE	5	V	–	1281508	1281621	3n	−10.4	2.91E−02
WBGene00007010	*alx-1*	Programmed cell death 6-interacting protein	CE	8	III	+	9961984	9962091	3n	−7.6	3.29E−02
WBGene00000102	*akt-1*	Akt/PKB	ME	6a	V	+	10250812	10251003	3n	10.8	5.53E−02
				6b			10251298	10251504	3n	−10.8	

[a]CE, cassette exon; ME, mutually exclusive exons.
[b]CE, cassette exon with alternative 3′-splice sites.
[c]Carrying a termination codon.

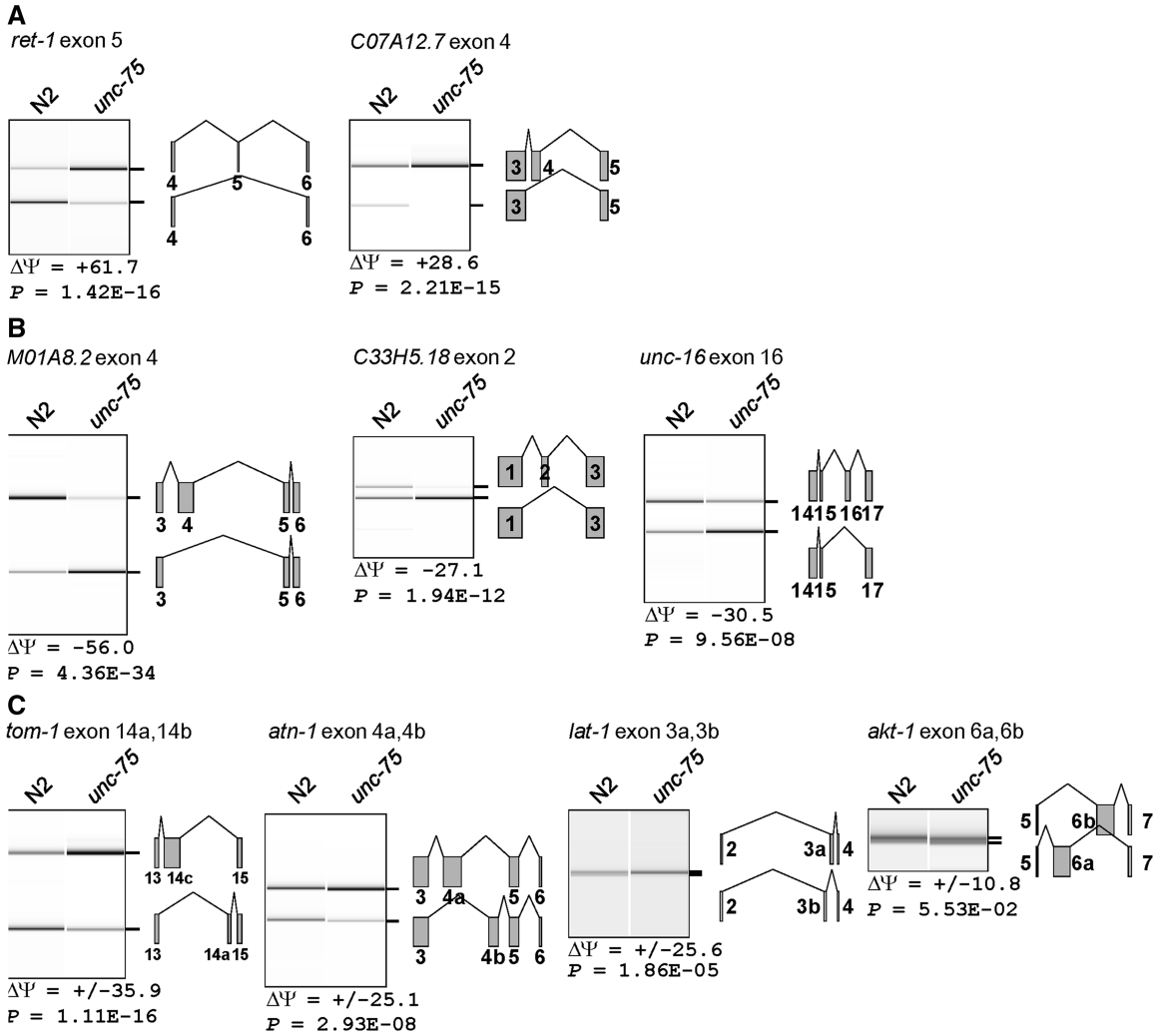

Figure 1. RT–PCR validation of UNC-75-regulated alternative splicing events. (**A**) UNC-75-repressed cassette exons. (**B**) UNC-75-activated cassette exons. (**C**) UNC-75-regulated mutually exclusive exons. Total RNAs were extracted from synchronized L1 worms of N2 (wild type) and the *unc-75 (yb1701)*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

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 ΔE4-EGFP in the nervous system (Figure 2J and Supplementary Figure S3D). Disruption of UUGUU GUG 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.

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

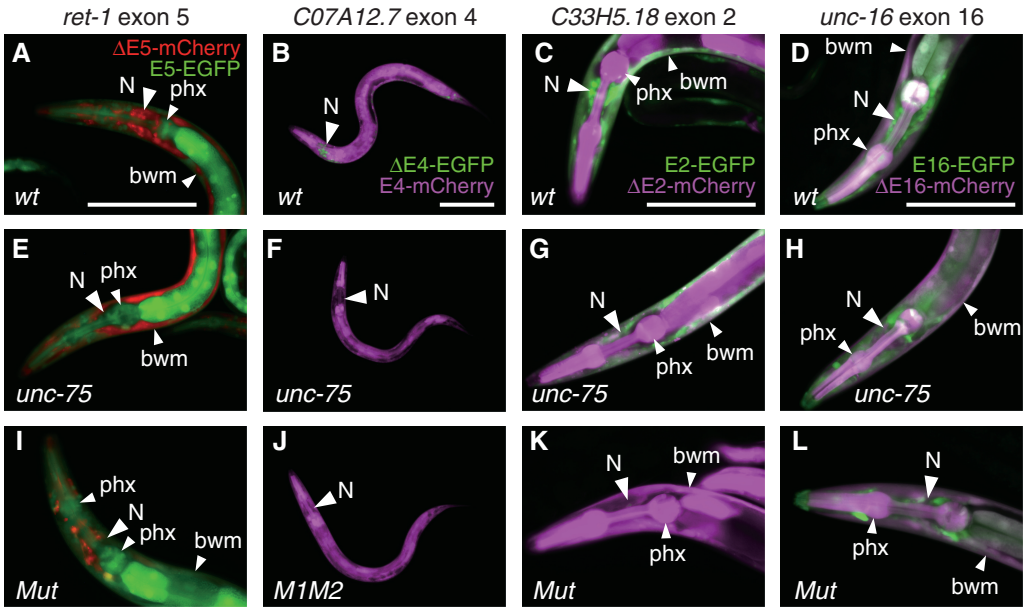

Figure 2. UNC-75 regulates its target exons in a neuron-specific manner. (**A–L**) Fluorescence images of reporter worms for *ret-1* exon 5 (A, E, I), *C07A12.7* exon 4 (B, F, J), *C33H5.18* exon 2 (C, G, K) and *unc-16* exon 16 (D, H, L) in the wild-type (A–D) and *unc-75 (yb1701)* (E–H) backgrounds and with mutations (Mut or M1M2) in the (G/U)UGUUGUG elements (I–L). Scale bars, 100 μm. bwm, body wall muscles; N, nervous system and phx, pharynx. Color images with a dual band-pass filter for the *ret-1* exon 5 reporters (A, E, I) and pseudo-colored merged images of mCherry and EGFP in magenta and green, respectively, for the others.

Table 2. GO analysis of the genes with UNC-75-regulated exons

Ontology type	GO term ID	Term	Count in expressed genes (N = 15 946)	Count in genes with affected exons (n = 23)	Fold enrichment	P-value (Fischer's exact test)
Biological process	GO:0040010	Positive regulation of growth rate	1714	9	3.64	3.76E−04
	GO:0040039	Inductive cell migration	131	3	15.88	8.51E−04
	GO:0030421	Defecation	44	2	31.51	1.81E−03

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 mRNAseq 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

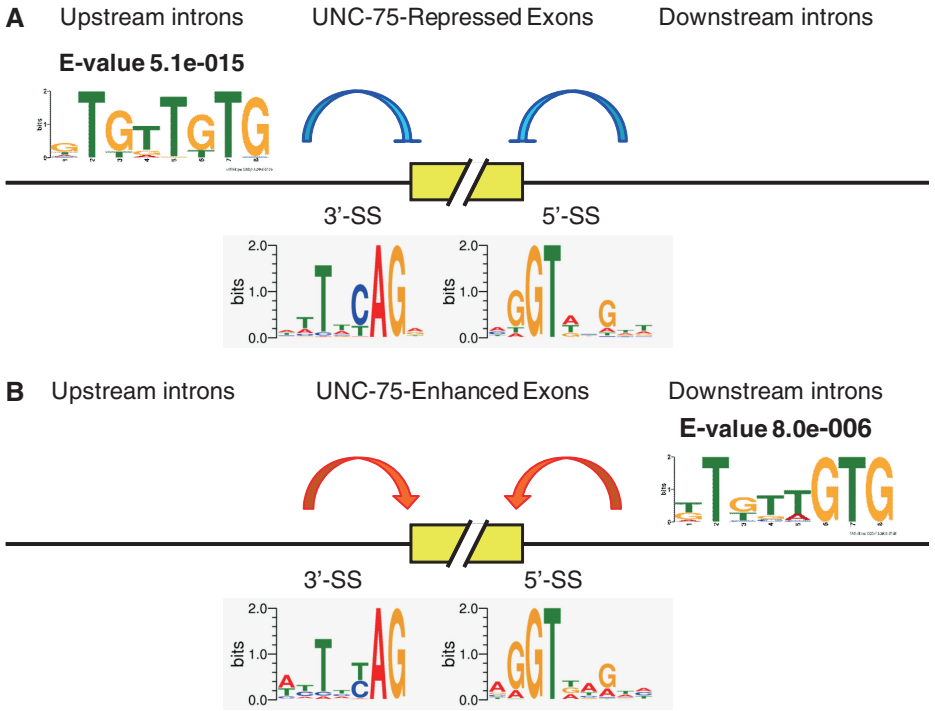

Figure 3. Summary of the motif analyses. Enriched motifs with *E*-values < 1 in the MEME analysis are indicated for upstream and downstream flanking introns of UNC-75-repressed (**A**) and -activated (**B**) cassette and mutually exclusive exons. Sequence logos of the 5′- and 3′-splice sites (SS) of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

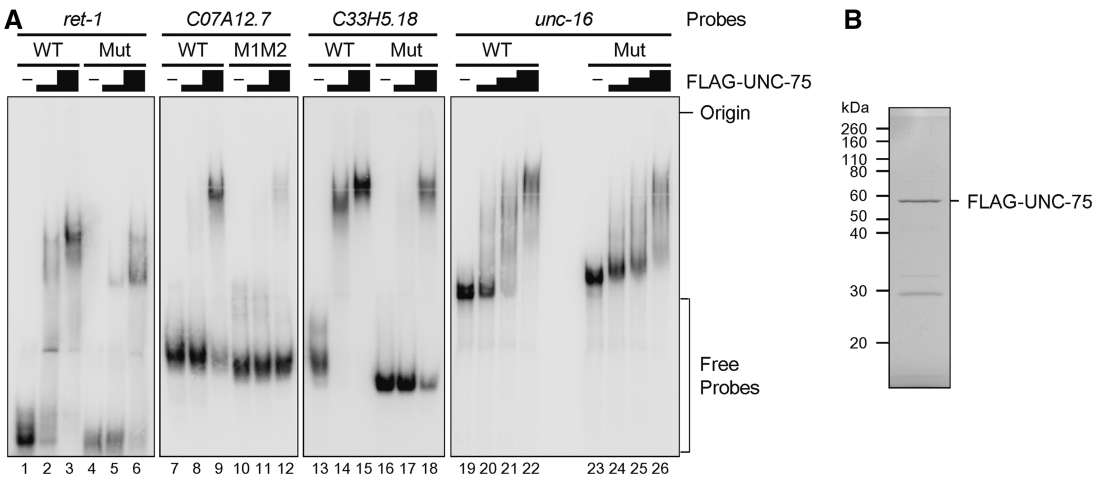

Figure 4. UNC-75 directly and specifically binds to the (G/U)UGUUGUG element(s) in the upstream or downstream of its target exons. (**A**) EMSA using radiolabeled wild-type (WT) and mutant (Mut or M1M2) probes without (−) or with 4-fold (*ret-1*, *C07A12.7* and *C33H5.18*) or 2-fold (*unc-16*) dilution 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).

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 *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.

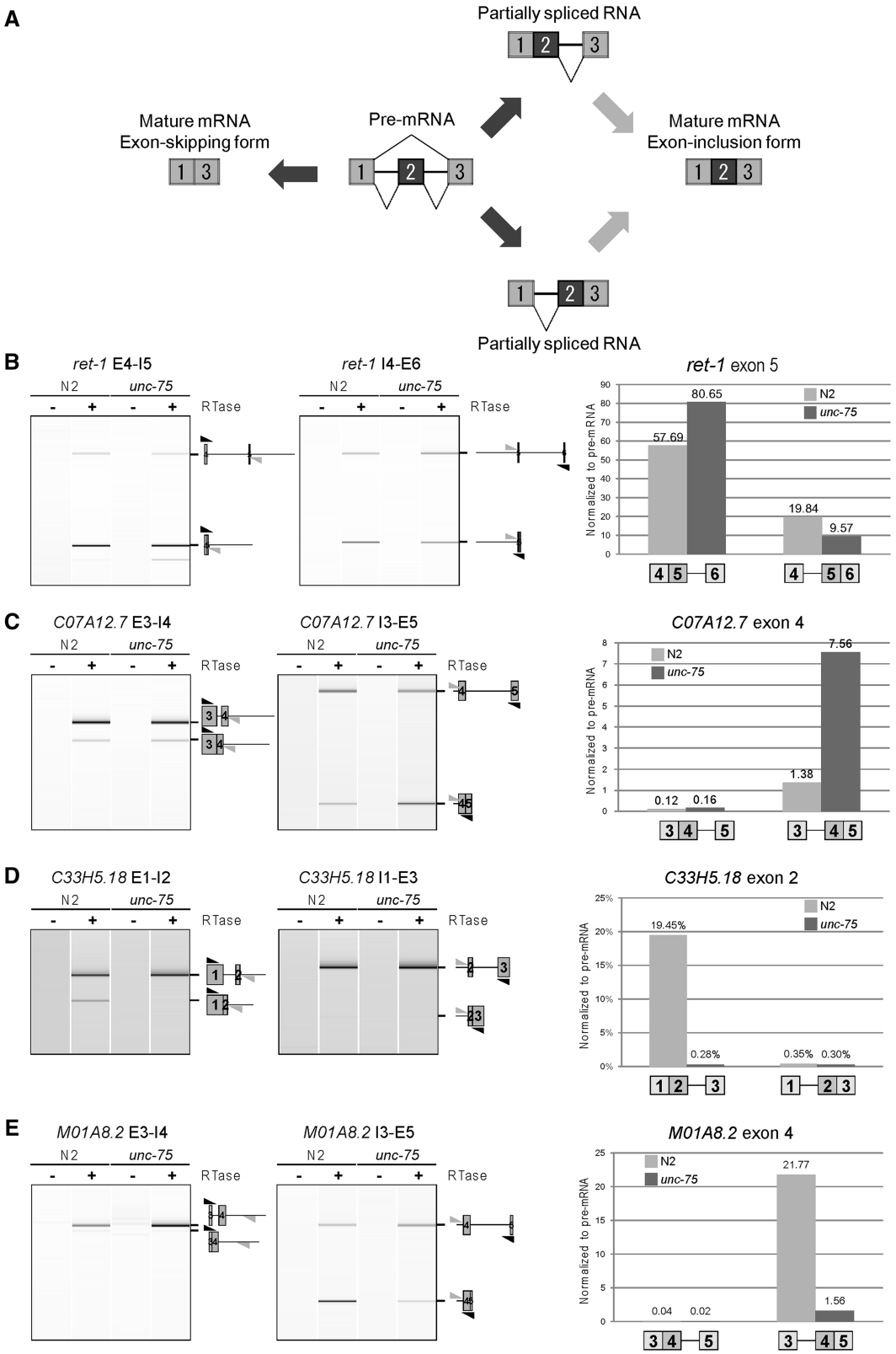

Figure 5. UNC-75 can regulate excision of upstream and downstream flanking introns of its target exons. (**A**) Schematic illustration of the order of intron excision from a pre-mRNA for an exon-skipping isoform and an exon-inclusion isoform of mature mRNAs. Note that there are two forms of partially 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)UGUU GUG 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)UGUU GUG 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.

ACKNOWLEDGEMENTS

We thank Arun K. Ramani and Andrew G. Fraser of University of Toronto for detailed information about alternative splicing events in *C. elegans*. We thank Marina Togo and Hiroshi Kurokawa for technical assistance. We thank *Caenorhabditis* Genetics Center for strains.

FUNDING

Grant-in-Aid for Scientific Research on Innovative Areas 'RNA regulation' [No. 20112004] from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to H.K.); a Grant-in-Aid for Scientific Research 'Challenging Exploratory Research' [No. 24657116] from the Japan Society for the Promotion of Science (JSPS) (to H.K.) and a Grant from Japan Science and Technology Agency (JST), PRESTO (to H.K.). Funding for open access charge: a Grant-in-Aid for Scientific Research on Innovative Areas 'RNA regulation' [No. 20112004] from

the Ministry of Education, Culture, Sports, Science and Technology of Japan (to H.K.).

Conflict of interest statement. None declared.

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