Supplementary Table legends

Table S1: List of genes and alternative exons surveyed using our two color splicing reporter. Related to Figure 1.
Expression patterns reported on Wormbase are included in the table, in addition to whether or not differential splicing in distinct neurons was observed.

Table S2: List of differential junction usage between wildtype and unc-75(e950), exc-7(rh252), and double mutant animals. Related to Figure 4.
Spreadsheets containing information about chromosome, strand, junction start and stop coordinates (based on UCSC ce6), number of times each junction was counted in wild type and each mutant, the calculated relative junction usage difference, p-value determined by Fisher's exact test, and CDS identifier for the given gene. Each workbook contains a different mutant comparison (wild type vs. *unc-75*, wild type vs. *exc-7*, and wild type vs. double mutants).

Table S3: GO enrichment analysis of the network of genes containing junctions regulated by UNC-75 or EXC-7. Related to Figure 4.
Top GO categories identified and their FDR-corrected p-values are shown.

Table S4: List of genes with transcript junctions regulated by UNC-75, analyzed for whether loss of function analysis reported locomotion or aldicarb sensitivity defects. Related to Figure 6.
Spreadsheet contains data used to generate Figure 6A, B, and C. Orange boxes indicate the presence of a phenotype, grey boxes indicate the absence of a phenotype, and white boxes indicate that the phenotype was not assessed in the literature.

Supplementary Movie Legends

Movie S1: A movie showing wild type and mutant animals thrashing in liquid M9 media. Related to Figure 6.

Norris *et al*. Table S1

Table S1: List of genes and alternative exons surveyed using our two color splicing reporter

Gene and alternative exon	Annotated Expression Pattern (Wormbase)	neuron-specific splicing observed with bichromatic reporter?
unc-16 exon 16	expressed in neurons of the ventral cord, retrovesicular and preanal ganglia, the nerve ring, intestinal cells, seam and hypodermal cells, body wall and head muscle, and pharynx.	YES
gar-2 exon 11	expressed from mid-embryogenesis through adulthood with expression seen in ciliated head neurons, ventral cord neurons, and the HSN (hermaphrodite-specific neuron)	YES
clr-1 exon 7	expressed in some neurons and mesodermal cells	NO
slo-1 exons 9 and 10	expressed in neurons and in body wall and vulval muscle	NO
unc-36 exon 7	expressed in most neurons and virtually all muscle tissue	NO
slo-2 exon 18	expressed in body wall, vulval, and pharyngeal/ intestinal valve muscles, nerve ring processes, and some motor neurons in the ventral nerve cord	NO
cle-1 exon 18	expressed predominantly in neurons	YES
itr-1 exon 22	expressed in the adult intestine, pharynx, excretory cell, germ line, and spermatheca, with limited neuronal expression.	NO
mod-1 exon 9	expressed in the cell bodies and axons (the latter presumably due to overexpression) of neurons in the head, tail, and ventral nerve cord.	YES
madd-2 exon 8	expressed in vulva muscles; head mesodermal cell; head neurons; tail neurons	NO
dyn-1 exon 8	expressed in motor neurons, intestinal cells, and pharyngeal muscle.	YES
sad-1 exon 15	expressed in the nervous system, where it localizes asymmetrically to the synapse-rich regions of axons.	YES
vps-45 exon 4	expressed in pharynx; body wall muscle; tail neurons; unidentified cells in head	NO
nhx-5 exon 17	expressed in the anterior, lateral and ventral, and retrovesicular ganglion near the pharynx, in the ventral nerve cord, and in the pre-anal, dorso-rectal, and lumbar ganglion near the tail. Animals demonstrated labeling of the lumbar ganglion in an adult animal. HSN (hermaphrodite-specific neuron) was also labeled as were the vulC cells of the vulva and the excretory cell.	YES

Supplementary Table 2: GO enrichment analysis of the network of genes containing junctions regulated by UNC-75 or EXC-7

splicing network	Top GO categories	p-value
UNC-75	I band	0.00136
	Substrate-specific transporter activity	0.00547
	Gated channel activity	0.00547
	Calcium channel activity	0.00547
EXC-7	Locomotory behaior	1.16E-13
	Behavior	3.86E-13
	Response to stimulus	6.29E-11
	Gamete generation	9.81E-11

Detailed Experimental Procedures

Strain Maintenance and Microscopy

C. elegans were maintained by standard techniques (Brenner, 1974) on NGM agar plates seeded with OP50 *E. coli*. All images of two color reporter transgene-expressing animals were captured with a Zeiss Axioskop 2 fluorescence compound microscope and processed in ImageJ. The alleles used in this study were: *unc-75(e950) I, kcnl-2(ok2818) I, Y95B8A.12(ok1820) I, vab-10(e698) I, acs-13(ok2815) I, acs-13(ok2861) I, exc-7(rh252) II, shk-1(ok1581) II, pqn-80(tm4167) III, M01A8.2(gk470) III, sms-3(ok3540) III, lst-4(tm2423) IV, pkg-2(ok966) IV, Y61A9LA.1(tm3303) V, Y61A9LA.1(tm3444) V, cca-1(gk30) X, cca-1(ad1650) X*, and *zxIs6 [Punc-17::chop-2(H134R)-yfp; lin-15(+)]*.

Plasmid, fosmid, and transgenic animal creation

Isoform-specific fosmids were created by fosmid recombineering according to standard protocols (Tursun et al., 2009) by introducing cDNA sequences that were pre-spliced for a given *unc-64* isoform into an otherwise endogenous genomic locus. *C. elegans* alternative splicing reporters were created using the plasmid RG6 (Orengo et al., 2006) as a starting template. We modified this plasmid by replacing dsRed with mRFP, and also by placing the EGFP open reading frame upstream of the mRFP reading frame by PCR stitching. We subsequently introduced XhoI and NotI cloning sites into the plasmid to allow for further cloning of alternative exons and flanking genomic regions of interest. Next, we amplified the entire cassette spanning from the start methionine codon to the end of mRFP reading frame by PCR with primers containing gateway compatible recombination sites. This PCR product was then inserted into pDONR221 (Invitrogen) by a Gateway BP cloning reaction (Invitrogen) as recommended by the manufacturer, creating an entry vector. We also constructed several destination vectors that drove expression of each reporter pan-neuronally, or with endogenous promoter sequences. Reporters were placed in these destination vectors by a Gateway LR reaction. Our codon-optimized eBFP plasmid was synthesized *de novo* (GenScript) and also cloned into pDONR221 by a Gateway BP cloning reaction. Through an LR cloning reaction, we then placed the eBFP coding sequence into a destination vector containing the *unc-25* GABA-specific promoter. A plasmid containing GFP tagged UNC-75 expressed from its native

promoter was kindly provided from Dr. Oliver Hobert. Details of fosmid, plasmid, and oligonucleotide sequences are available upon request.

Transgenic animals were created by a standard microinjection protocol (Kadandale et al., 2009), and when required, transgenes were integrated into the genome by UV irradiation.

Mutagenesis and Screening

P_0 worms expressing the *unc-16* exon 16 alternative splicing reporter were mutagenized with ethyl methanesulfonate (EMS) at 47 mM for four hours. Single F_1s were sorted into individual wells of a 96-well plate by a Union Biometrica COPAS worm sorter. After 4-7 days of growth in NGM liquid culture media at room temperature, an aliquot of each well was transferred to an optical imaging 96 well plate (Nunc). The *unc-16* alternative splicing reporter pattern in the pool of F_2-F_3 mutant worms was analyzed fluorescently on a Zeiss Axioskop 2 microscope. Wells containing mutant animals with altered reporter patterns were isolated. To identify causative mutations, we back-crossed identified mutants four times to our ancestral strain carrying the *unc-16* splicing reporter, and prepared cDNA libraries from mutant genomic DNA using the Illumina TruSeq kit as recommended by the manufacturer. We then performed whole-genome sequencing, generating 150 base paired-end reads, and aligned the reads back to the *C. elegans* reference genome using the software MAQgene according to previously described protocols (Bigelow et al., 2009; Sarin et al., 2008). Polymorphisms that were common to all mutant genomes were considered to be present in the ancestral reporter strain, and were ruled out for further analysis. We focused on missense and nonsense mutations that were unique in each strain, and eventually confirmed causal mutations in *unc*-75 and *exc*-7 using a combination of Sanger sequencing, the use of other existing alleles to replicate observed phenotypes, and transgene rescue experiments.

Consensus Binding Motif Determination

Consensus binding motifs were determined by RNAcompete, in which recombinant GST-tagged UNC-75 or EXC-7 were incubated with 75-fold molar excess of a complex pool of 30-41 nucleotide RNAs consisting of all possible 9-mers repeated at least 16

times. The protein was recovered by affinity selection and the associated RNAs were assayed by microarray and computational analysis as previously described (Ray et al., 2009; Ray et al., 2013).

Electrophoretic Mobility Shift Assay

^{32}P-labeled RNA probes were transcribed with the MEGAshortscript T7 kit (Ambion) and incubated with varying concentrations of recombinant GST-tagged UNC-75 and EXC-7 full-length protein for 30 minutes at room temperature in binding buffer (10 mM HEPES, 100 mM KCl, 20 mM NaCl, 0.025% NP-40, 2 mM DTT, 10 % glycerol, 0.2 ng/µl BSA, 2 mM $MgCl_2$), then run on a 6% polyacrylamide gel. The sequences for each of the RNA probes were the following: WT UNC-75 sequence: TGTTGTGCACTGTTGTGCACTGTTGTGCAC. WT EXC-7 sequence: TAAGTTCACTAAGTTCACTAAGTTCAC. Mutant UNC-75 sequence: TGCTATGCACTGCTATGCACTGCTATGCAC. Mutant EXC-7 sequence: CAAACCCAC-CAAACCCACCAAACCCAC.

mRNA-Seq analysis to identify differential junction usage

Total RNA was isolated from synchronized L4 worms using Tri reagent (Sigma Aldrich), and cDNA libraries from Poly A+ enriched RNA was prepared using the TruSeq RNA kit (Illumina) according to manufacturer's instructions. Deep sequencing was performed on an Illumina HiSeq 2000 according to standard protocols, generating 150 base paired-end reads. Reads were mapped to the *C. elegans* genome (version WS190) using the RNA-Seq Unified Mapper (RUM) under default settings (Grant et al., 2011). Alternative junctions were identified by mining the set of high quality junction reads mapped by RUM, searching for reads spanning exons connected by alternative splice sites. The relative usage of an alternative junction for each strain was calculated as the ratio of the alternative junctions utilized (j_1/j_2). To measure differences in relative junction usage, we calculated the differential relative junction usage percentage between wild type and mutant animals ($100\% \times ([j_1/(j_1 + j_2)]_{wildtype} - [j_1/(j_1 + j_2)]_{mutant})$). Alternative splicing in mutants was defined according to experimentally-validated cutoffs of both >20% change in relative junction usage, and significant ($p < 0.01$) difference in junction ratios as

determined by Fisher's exact test. Clustering of RNA-Seq data for heat map construction was generated by using unweighted Pearson correlations performed by Cluster and visualized with TreeView (Eisen Lab, UC Berkeley) (Eisen et al., 1998).

RT-PCR assays

Alternative splicing events detected by our mRNA-Seq analysis were validated by semi-quantitative RT-PCR assays with the Qiagen OneStep RT-PCR kit. 30 nanograms of total RNA was used as input, and reactions were carried out according to the manufacturer's recommendations. Amplified products were resolved on 2% agarose gels and stained with ethidium bromide. Images were captured using an Alpha Imager HP (Alpha Innotech), and then densitometry was performed using ImageJ. All primers used for RT-PCR assays are available upon request.

Bioinformatic analysis

Cassette exons with altered exon junction usage between WT and *unc-75; exc-7* were grouped into two categories: increased skipping in mutant (51 exons) and increased inclusion in mutant (19 exons). Sequences were obtained for the cassette exons, as well as their neighboring introns and exons. The top three RNAcompete motifs for each factor were queried against each full exon and intron sequence. Equal numbers of introns and exons not regulated by *unc-75* or *exc-7* were similarly analyzed and plotted as a negative control. P-values were obtained via Chi-squared analysis between the values of the experiment and negative control.

Phenotypic Analyses

Aldicarb assays were performed as previously described (Loria et al., 2003) on plates of young adult worms at 15°C and 1 mM aldicarb. A total of 20-30 worms were counted in each assay for each strain, and each assay was replicated a total of three times. Worms were considered paralyzed if they did not move in response to a light nose touch. Coordination assays were performed by placing young adult worms in a drop of M9 buffer, and after one minute of equilibration, counting the thrashing rate (frequency of

number of body bends per minute) of animals recorded under a stereomicroscope. A total of 15-20 worms were analyzed from each strain tested.

Network Analysis

Gene Ontology analysis was performed using the software GOstat (Beissbarth and Speed, 2004), using as a background the entire set of genes in the *C. elegans* genome with curated GO annotations. Functional categories were scored as significantly over-represented if they had an FDR-corrected significance score less than 0.1. Gene interaction networks were generated with GeneMANIA (Warde-Farley et al., 2010), using the set of UNC-75 regulated targets as a starting query list. The resulting network was visualized and edited using Cytoscape.

Electrophysiology

The dissection of the *C. elegans* was described previously (Richmond and Jorgensen, 1999). Briefly, one-two days old hermaphrodite adults were glued to a sylgard-coated cover glass covered with bath solution. Two-three days old hermaphrodite adults of *unc-75* and *unc-75; exc-7* mutants were used sometimes in this study due to developmental delay. The integrity of the anterior ventral body muscle and the ventral nerve cord were visually examined via DIC microscopy, and anterior muscle cells were patched using fire-polished 4−6 MΩ resistant borosilicate pipettes (World Precision Instruments, USA). Membrane currents were recorded in the whole-cell configuration by a Digidata 1440A and a MultiClamp 700A amplifier, using the Clampex 10 software and processed with Clampfit 10 (Axon Instruments, Molecular Devices, USA). Data were digitized at 10−20 kHz and filtered at 2.6 kHz.

The recording solutions were as described in our previous studies (Richmond and Jorgensen, 1999). Specifically, the pipette solution contains (in mM): K-gluconate 115; KCl 25; $CaCl_2$ 0.1; $MgCl_2$ 5; BAPTA 1; HEPES 10; Na_2ATP 5; Na_2GTP 0.5; cAMP 0.5; cGMP 0.5, pH7.2 with KOH, ~320 mOsm. The bath solution consists of (in mM): NaCl 150; KCl 5; $CaCl_2$ 5; $MgCl_2$ 1; glucose 10; sucrose 5; HEPES 15, pH7.3 with NaOH, ~330 mOsm. Under these conditions, the reversal potentials are ~+20 mV for acetylcholine receptors, respectively (Gao and Zhen, 2011). Cell resistance and

capacitance were determined with Clampex (Molecular Devices) by applying a 10-mV depolarizing pulse with a holding potential of -60 mV, which was used to calculate the current density of ESPCs (pA/pF). Leak currents were subtracted. Light stimulation of *zxIs6* was performed with an LED lamp (KSL-70; RAPP OptoElectronic) at a wavelength of 470 nm (8 mW/mm^2), controlled by the Axon amplifier software. zxIs6 was cultured in the dark at 22 °C on OP50-seeded NGM plates supplemented with all-trans retinal as described previously (Gao and Zhen, 2011). All chemicals were from Sigma. Experiments were performed at room temperatures (20−22℃).

Two-tailed Mann-Whitney U test was used to compare the difference of the electrophysiological datasets. Subsequent analysis and graphing were performed using Excel (Microsoft), Igor Pro (Wavemetrics) and Clampfit (Molecular Devices). In this study, N refers to the number of recordings (one cell per animal). All data are presented as mean ± SEM.

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