TITLE:

A genetic screen for suppressors of a mutated 5' splice site identifies factors associated with later steps of spliceosome assembly

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RUNNING TITLE

Genes that alter cryptic splicing

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ABSTRACT

Many alleles of human disease genes have mutations within splicing consensus sequences that activate cryptic splice sites. In *C. elegans*, the *unc-73(e936)* allele has a G-to-U mutation at the first base of the intron downstream of exon 15, which results in an uncoordinated-phenotype. This mutation triggers cryptic splicing at the -1 and +23 positions, and retains some residual splicing at the mutated wild-type (wt) position. We previously demonstrated that a mutation in *sup-39*, a U1 snRNA gene, suppresses *e936* by increasing splicing at the wt splice site. We report here the results of a suppressor screen in which we identify three proteins that function in cryptic splice site choice. Loss-of-function mutations in the non-essential splicing factor *smu-2* suppress *e936* uncoordination through changes in splicing. SMU-2 binds SMU-1, and *smu-1(RNAi)* also leads to suppression of *e936*. A dominant mutation in the conserved C-terminal domain of the *C. elegans* homolog of the human tri-snRNP 27K protein, which we have named SNRP-27, suppresses *e936* uncoordination through changes in splicing. We propose that SMU-2, SMU-1 and SNRP-27 contribute to the fidelity of splice site choice after the initial identification of 5' splice sites by U1 snRNP.
INTRODUCTION

Pre-mRNA splicing takes place in a large ribonucleoprotein complex called the spliceosome (BURGE et al. 1999). Components of this splicing machinery assemble at conserved signal sequences within the pre-mRNA. The 5' splice site consensus sequence $M_{-3}A_{-2}G_{-1}|G_{+1}U_{+2}R_{+3}A_{+4}G_{+5}U_{+6}$ and the 3' splice site consensus sequence $Y_{-3}A_{-2}G_{-1}|R_{+1}$ (M is either A or C; R is a purine, and Y is a pyrimidine) define the limits of the intron. Base-pairing interactions between the 5' end of the U1 snRNA and the 5' splice site consensus sequence occur early in spliceosome assembly. It is the nearly invariable GU dinucleotide at the first two positions of the 5' end of the intron that defines the beginning of the intron. The 5' consensus sequence is essential but insufficient for splice site selection, as 5' splice sites with weaker consensus matches may require additional determinants for proper activation (SANFORD et al. 2005).

Mutations that disrupt the 5' consensus splice signal can lead to genetic disease in humans (COHEN et al. 1994; NELSON and GREEN 1990). Approximately 15% of point mutations that cause genetic diseases affect pre-mRNA splicing consensus sequences (KRAWCZAK et al. 1992). For some specific disease genes, as many as 50% of the known heritable alleles alter splicing (ARS et al. 2000; PAGENSTECHER et al. 2006; ROCA et al. 2003; TERAOKA et al. 1999). Among all the positions of the 5' splice site consensus sequence, the highest proportion of human disease mutations occur at the +1G position (BURATTI et al. 2007). The fidelity of pre-mRNA splice site choice is largely disrupted by this defect, since this mutation causes splicing at this site to be either abolished or
out-competed by the activation of nearby cryptic 5’ splice sites (COHEN et al. 1994; NELSON and GREEN 1990). Cryptic splice sites are used only when the wild-type splice donor is disrupted by mutation, as they tend to have very weak splice donor consensus sequences outside of a 5’-GU dinucleotide that defines the beginning of the intron (ROCA et al. 2003). Suppression of mutations to the 5' splice site consensus sequence in vivo has been achieved through the expression of U1 snRNAs containing compensatory base substitutions (ZHUANG and WEINER 1986); however suppression of mutations to the +1 position of the intron using reverse genetic approaches has not been successful (COHEN et al. 1994; NELSON and GREEN 1990; NEWMAN et al. 1985).

We have used a specific allele of the C. elegans unc-73 gene, e936, which contains a G to U mutation at the first nucleotide of intron 16 (STEVEN et al. 1998), as a model for studying cryptic splice site choice (ROLLER et al. 2000; ZAHLER et al. 2004). unc-73 encodes a RAC guanine nucleotide exchange factor that is expressed in neurons and is important for axon guidance (STEVEN et al. 1998). The e936 allele induces the use of three different cryptic 5' splice sites (Fig 1A). Two of these 5' splice sites, located at the -1 and +23 positions, and define introns beginning with GU. The third 5' splice site used is at the mutated wild-type (wt) position, and is referred to as “wt” since splicing at this site still produces wild-type unc-73 mRNA and protein, even though the intron begins with UU (ROLLER et al. 2000). Use of either the -1 or +23 cryptic site causes a shift in the reading frame and loss of gene function. In e936 animals, 90% of the stable messages of unc-73 are out-of-frame, yet the phenotype is not as severe as for
other alleles in this gene. This indicates that the 10% of steady-state messages that are in-frame have some functional role.

In a previous genetic screen for extragenic suppressors of e936 movement defects, Way and colleagues identified sup-39 (RUN et al. 1996). It was subsequently shown that mutations in sup-39 alter cryptic splice site choice of e936 (ROLLER et al. 2000). sup-39 encodes a U1 snRNA gene with a compensatory mutation at the position that normally base pairs with the +1G. This allows sup-39 to base pair with an intron with a +1U (ZAHLER et al. 2004). This dominant suppressor increases usage of the mutated splice site, and improves the fraction of in-frame messages from e936 from 10% to 33%, with a dramatic improvement in coordination. A similar mutant U1 snRNA suppressor with a different compensatory substitution, sup-6(st19), was found to suppress the intronic +1G to A transition of unc-13(e309) to allow for splicing at the mutated wild-type site, even though the intron begins with AU instead of GU (ZAHLER et al. 2004).

We are interested in identifying additional factors that play a role in cryptic 5’ splice site choice. To do this, we took advantage of unc-73(e936), in which modest increases in the use of the wt splice site lead to dramatic increases in coordination, as a sensitive screen for changes in cryptic splice site choice. In this paper we report that the proteins SMU-1 and SMU-2, which are non-essential factors previously shown to have a role in alternative splicing (SPARTZ et al. 2004), have a role in selection of cryptic 5’ splice sites. We also report the
MATERIALS AND METHODS

Growth and Maintenance of Worms: Worms were grown at 20° on (NGM) hardened agar in Petri dishes (9-cm and 5-cm) with limited supply of *E. coli* for food (LEWIS and FLEMING 1995). The CB936 (*unc-73(e936)*), JW101 (*unc-73(e936); sup-39(je5)*), Hawaiian CB4856, and Bristol N2 strains were provided by the Caenorhabditis Genetics Center (CGC) at the University of Minnesota.

N-Ethyl-N-nitrosurea (ENU) Mutagenesis: The protocol previously developed for identifying e936 suppressors with EMS mutagenesis (RUN et al. 1996) was adapted for mutagenesis with N-ethyl-N-nitrosourea (ENU). Synchronized L4 worms were washed with M9 solution and placed into a 15 ml conical tube. Worms were concentrated by centrifugation at 3,000 x *g* and resuspended in 2 ml of M9. A 50 mM ENU stock was diluted to 0.33 mM in M9 solution and 20 µl of this were added to the vial with the worm suspension. The vial was rotated for 4 hrs at room temperature. After rotation, worms were collected by centrifugation and the ENU supernatant was removed. The worm pellet was washed two times with 4 ml of M9, rotating between washes. Then worms were plated onto NGM plates with *E. coli* as a food source and allowed to recover overnight.

Mutant Screen: The screen for suppressed animals was performed essentially as described by Run *et al.* (RUN et al. 1996). The day following the mutagenesis with ENU described above, four healthy mutagenized worms (P₀) were placed on the edge of an *E. coli*-seeded 10-cm NGM agar plates, and 250 such plates were
made. Plates were incubated for one week at 20°, allowing sufficient time for the 
F2 generation to reach adulthood. Putative suppressed animals were identified by 
their ability to migrate to the far side of the plate containing food. The screening 
process also involved tapping the head of the migrated worms and looking for 
coordinated, sinusoidal forward and backward movement. We screened 200,000 
mutagenized haploid genomes:

$$250 \text{ plates } \times 4 \text{ } P_0 \text{ adults per plate } \times 100 \text{ } F_1 \text{ progeny/ } P_0 \times 2 \text{ genomes/ } F_1$$

**Thrashing Assay:** To measure how well the suppressed worms move 
compared to the uncoordinated mutant *unc-73(e936)* worms, a thrash test was 
performed (TSALIK and HOBERT 2003). Live L4 worms were transferred to a plate 
filled with liquid M9 solution at 20°. For a period of 60 sec, worms were observed 
and the number of times that they bent across their body axis in a rhythmic or 
rapid manner was recorded.

**RNA Extraction:** RNA extractions were performed as described previously 
(ROLLER et al. 2000).

**Reverse Transcription:** To generate complementary DNAs from extracted 
RNA, an oligonucleotide primer complementary to a portion of *unc-73* exon 17 
was used in reverse transcriptase (RT) reactions: 5'-'ACT TGT CCA TCA AAA 
TCT GC-3'. Reverse transcription reactions were performed as previously 
described (ROLLER et al. 2000). For the experiment in Fig 4 only, the RT primer
used was the same as the exon 16 reverse primer used in the PCR reactions (see below).

**Polymerase Chain Reactions (PCRs) and determination of relative cryptic splice site usage:** Primers corresponding to unc-73 exon 15 (5’-AGA AGT TGT ACG GAT AAG AC-3’) and exon 16 (5’-GAA ACT TCA ATG CGT TTA GC-3’) were used in PCR reactions with Taq DNA Polymerase and unc-73 cDNAs as previously described (ROLLER et al. 2000). PCR cycles were as follows: 94° for 5 min; then 30 cycles of 94° for 1 min; 59° for 1 min for annealing, and 72° for 1 min for extension. γ<sup>32</sup>P labeling of oligonucleotides for PCR and separation of labeled unc-73 RT-PCR products on 6% polyacrylamide urea gels were performed as previously described (ROLLER et al. 2000). Gels were dried onto Whatman paper and visualized with an Amersham Biosciences Typhoon. Quantitation of comparative splice site usage was done using ImageQuant software (Molecular Dynamics) as previously described (ROLLER et al. 2000). For each strain, the average quantitation of at least three independent RNA extractions and RT-PCRs was reported.

**Dominant/Recessive Assay:** Genetic crosses were used to determine whether each individual extragenic suppressor is dominant or recessive. Tests were performed as described by Run et al. (RUN et al. 1996). Some of the extragenic suppressors showed partial suppression of the e936 uncoordinated phenotype when heterozygous, indicating semi-dominant suppression.
**Mapping Suppressors:** To map *unc-73(e936)* suppressors to a chromosome, the snip-SNP approach was taken (Wicks et al. 2001). Each suppressor was mated with *unc-73(+) ; sup-39(+) * males of the Hawaiian strain CB4856, for which thousands of single nucleotide polymorphisms (SNPs) relative to the N2 strain are known. The F₁ progeny were then segregated and allowed to self-fertilize. Uncoordinated F₂ animals were picked onto individual plates and the F₃ animals were scored. The Unc F₂ animals that had 100% uncoordinated progeny must be homozygous *unc-73(e936)/unc-73(e936) ; sup(CB+)/sup(CB+).* DNA was extracted from these worms and PCRs were done across known SNP regions, and digested with *DraI* restriction enzyme, to indicate whether the locus is derived from CB4856 or N2. The location of the suppressor gene in every case is a location that always corresponds to the CB4856 genome.

**Bacterial Mediated RNAi:** The RNAi bacterial feeding method was used to introduce dsRNA into the worms (Timmons et al. 2001). The following RNAi feeding vectors from the Ahringer RNAi screening set were used: *smu-1 I-6J07* and empty vector L4440 (Kamath and Ahringer 2003; Kamath et al. 2003). *E. coli* bacterial strain HT115 (DE3) transformed with these plasmids was grown in 10 ml of liquid culture in LB with 50µg/ml Ampicillin. NGM plates containing IPTG and 50µg/ml carbenicillin were seeded with one drop of overnight culture. After overnight room temperature incubation, *unc-73(e936)* worms at the L4 stage were transferred to the seeded plates. Worms were allowed to grow at 15° for 72
hrs or 22° for 36-40 hrs. Adult worms were moved to a new, freshly seeded plate. Adults continued to be moved each day to freshly seeded plates, and unhatched embryos were counted to account for embryonic lethality. The progeny were scored for uncoordination in a thrash assay.

**Construction of snrp-27 trans-genes.** The *snrp-27* genomic region was amplified from the genome of N2 and az26 worms with PCR primers 5’ GTA CCG GTT CGC GAG CAA TGG GAC G 3’ and 5’ GTA TGC TAG CTT CCA ATG AGT TTG TCA TCC 3’, which contained sites for the restriction enzymes *Age* I and *Nhe* I respectively. PCR products were ligated into the TOPO TA vector pCR2.1 (Invitrogen) according to the manufacturer’s instructions. Clones containing inserts were sequenced to confirm a 100% match to the worm genome, with the exception of the point mutation in the *az26*-derived clones. Confirmed inserts were removed from the vector by digestion with *Age* I and *Nhe* I and cloned into these same sites found in the polylinker pPD103.05 (a gift from the Andrew Fire laboratory). This Fire Vector contains the constitutive *let-858* promoter followed by a polylinker containing the *Age* I and *Nhe* I sites followed by the rest of the *let-858* gene. These plasmids were mixed with a *sur-5*::GFP co-expression marker plasmid (pTG96_2) (Gu et al. 1998) and total N2 DNA digested with *Pvu* II restriction enzyme as carrier (final concentrations of injection mix are 30 ng/µl *Plet-858::snrp-27* plasmid, 20 ng/µl pTG96_2 plasmid and 80 ng/µl *Pvu* II-digested N2 total DNA). DNA was injected into the gonads of young adult N2 worms and GFP-expressing lines carrying extrachromosomal arrays
were established (Jin 1999). To establish strains homozygous for *unc-73(e936)* and containing these extrachromosomal arrays, N2 males were crossed to the array-containing hermaphrodites. GFP-expressing males from the first generation were then crossed to *unc-73(e936)* hermaphrodites. F2 animals from this cross that expressed GFP and had somewhat uncoordinated movement were picked and confirmed to be homozygous for e936.
RESULTS

A screen for suppressors of \textit{unc-73(e936)} uncoordination yields genes that alter cryptic splicing. Having previously established that suppression of \textit{unc-73(e936)} uncoordination provides a very sensitive assay for changes in cryptic splice site selection (ROLLER et al. 2000), we decided to repeat the previous screen for \textit{unc-73(e936)} suppressors (RUN et al. 1996) in order to potentially identify additional classes of cryptic splicing factors. The only difference to our approach this time was that we used N-ethyl-N-nitrosourea (ENU) as the mutagen instead of ethyl methane sulfonate (EMS).

We screened the F2 progeny of 200,000 ENU mutagenized CB936 (\textit{unc-73(e936)}) genomes for animals that showed suppression of the uncoordination defect. When the suppressed worms were isolated and tapped on the head, they were able to move backward, unlike the CB936 parent strain, but sometimes their heads would flop from side to side, unable to make complete sinusoidal movements. The suppressed worms were able to respond to mechanical stimulus, but appeared slightly lethargic relative to wild-type (N2) worms. Overall, their movement was much more responsive and energetic than \textit{unc-73(e936)} animals, but not as responsive as the \textit{sup-39} U1 snRNA suppressors of \textit{e936} that were previously identified.

We isolated seven independent suppressed animals from the screen, allowed them to propagate and grow, and collected their RNA for RT-PCR analysis to determine whether the suppressors were acting at the level of splicing. All but two of the isolated suppressor strains showed detectable
changes in the ratio of cryptic splice site usage compared to that of unc-73(e936) (Fig 1B). The two suppressors that did not show any detectable changes in splicing ratios, az22 and az28, were not studied further. These may represent suppressors of the loss of unc-73 RAC-GEF function, but their suppression does not appear to be at the level of splicing (Fig 1B, lanes 4 and 10).

One of the suppressors appeared to be intragenic to unc-73. Suppressor az23 did not segregate uncoordinated animals in the F2 generation after crossing to N2 males, and it displayed an extra band in the gel showing the various unc-73(e936) spliced transcripts (Fig 1B, lane 9). This extra band was cloned and sequenced in order to identify the new splice junction. This new splice site was found nine nucleotides upstream of the original splice donor, generating a message that is in frame. The genomic sequence of unc-73 for this potential intragenic suppressor was obtained and we determined that az23 was a second site A to T mutation in exon 15, eight nucleotides upstream of the original splice site (and e936 mutation). This created a new 5’ GU dinucleotide that acted as a splice donor from the -9 position and was used in 31% of the steady-state unc-73 transcripts (Fig 1C). Use of this new splice donor removes only three amino acids from the in-frame message, and led to phenotypic suppression of the e936 mutant animals. Run et al. reported isolating 8 intragenic suppressors of e936 (Run et al. 1996), but as that report came out before the molecular identification of unc-73 and the e936 allele (Steven et al. 1998), those suppressors were not characterized as leading to a change in splicing, although that is a likely assumption.
**Extragenic suppressors of *unc-73(e936)* alter cryptic splice site usage.** We further investigated how well the four extragenic splicing suppressors identified from our suppressor screen suppress the *e936* mutation. We assayed the improved movement of the suppressed animals using a thrashing assay (TSALIK and HOBERT 2003). These four extragenic suppressors showed significant improvement in coordinated movement in comparison to the *unc-73(e936)* mutant (Fig 2A). There was a range of improvement, with *az27* being the weakest of the suppressors. None appeared to move as well as wild-type or the previously described *sup-39(je5)* suppressor.

We quantitated the relative levels of steady-state messages that were spliced at the -1, mutated wild-type (wt), and +23 splice sites for each of these extragenic suppressors (Fig 2B). ImageQuant software (Molecular Dynamics) was used to determine the radioactivity in each band from the PhosphorImager image, and the fraction of RT-PCR product representing each mRNA from each RNA sample was calculated as described previously (ROLLER et al. 2000). Averages of a minimum of three different RT-PCR reactions for each are presented, and a representative RT-PCR reaction for each of the suppressors except *az23* is shown at the bottom of the Fig 2B. We previously demonstrated that the relative stabilities of the three isoforms are not regulated by nonsense-mediated decay, so these ratios appear to correspond to the relative usage of the different splice sites by the splicing machinery (ROLLER et al. 2000). Consistent with the data from the thrashing assay, these four suppressors showed increases
in levels of messages that were spliced at the wt position relative to the *unc-73(e936)* mutant strain. All four suppressors showed approximately a two-fold increase in the steady-state level of messages that were spliced at the wt position. The -1 and wt positions appear to be present in a 1:1 ratio, indicating that, like *sup-39*, these suppressors work to increase usage of the overlapping -1/wt splice donors relative to the +23 site, while the choice between -1 and wt is likely made at a later step in spliceosome assembly (ROLLER *et al.* 2000). None of these four suppressor alleles had as strong a suppressor activity at the level of splicing as the U1 snRNA suppressor *sup-39(je5)*. However, *je5* also led to 50% embryonic lethality, and we observed no embryonic lethality with any of these new extragenic suppressors (data not shown).

A series of genetic crosses was carried out on each of the four new alleles to determine whether they are dominant or recessive suppressors of the *e936* mutation (RUN *et al.* 1996). While doing these crosses, we observed that for three of the suppressors, *az24*, *az25* and *az26*, there was an easy-to-score intermediate suppressed phenotype for animals that were heterozygous for the suppressor and homozygous for *unc-73(e936)*; the heterozygous suppressor animals move better than the *e936* animals, but not as well as the animals that are homozygous for the suppressor. We refer to the heterozygous suppressor animals as having an "unc-ish" phenotype. The 1(wt):2(unc-ish):1(unc) ratio observed from scoring progeny of *e936;az24/*+, *e936;az25/*+ and *e936;az26/*+ animals is consistent with these suppressors being semi-dominant. In contrast,
the e936 suppressor az27 behaved as would be expected for a true recessive allele.

**Mapping the isolated extragenic suppressors of *unc-73(e936).*** We used snip-SNP mapping between N2 and CB4856 (Wicks et al. 2001) to narrow down the location of the extragenic suppressor mutations on the chromosomes of *C. elegans*. Suppressors az25 and az26 both mapped to chromosome I, and were linked to *unc-73*. The SNP mapping placed both az25 and az26 between base positions 8,416,835 and 9,910,535 on chromosome I. az24 mapped between base positions 7,763,699 and 9,149,301 on chromosome III. az27 mapped between base positions 3,117,765 and 4,305,268 on chromosome II. We were immediately able to rule out the possibility that any of these four suppressors represent one of the twelve *C. elegans* U1 snRNA genes based on these genomic positions. To narrow down the possible identity of potential suppressor genes, we compared the SNP mapping results of the extragenic suppressors to a table of chromosomal locations of *C. elegans* homologs of mammalian splicing factors, spliceosome-associated proteins and spliceosomal core components identified from proteomic experiments (mammalian spliceosomal protein table courtesy of Melissa Jurica). Using this approach combined with sequencing of candidate genes, we found that the az27 suppressor strain had a premature termination codon in smu-2, the worm homolog of the human RED protein. For both az25 and az26 we found an identical missense mutation in the gene R05D11.7, the worm homolog of the human tri-snRNP 27K protein. We were
unable to identify a potential gene for az24 with this approach. We next set out to confirm whether these mutations in smu-2 and R05D11.7 are indeed suppressors of e936.

**Loss-of-function mutations in smu-2 and smu-1 are extragenic suppressors of the e936 mutation.** We sequenced the smu-2 gene from the az27 animals, and found a lesion in the third exon, a T to G transversion that results in a nonsense mutation at codon 111 (there are 547 amino acids in the protein). This mutation is predicted to be a null mutation, and fits with the genetic evidence that strain az27 is a recessive suppressor of unc-73(e936). SMU stands for suppressor of mechanosensory and uncoordination defects genes. smu-1 and smu-2 were isolated in a screen for suppressors of the synthetic lethality of mec-8 and unc-52 mutations (LUNDQUIST and HERMAN 1994). unc-52 encodes a homolog of the mammalian perlecan extracellular matrix protein and mec-8 encodes an RNA recognition motif-containing (RRM) protein whose loss leads to a variety of mechanosensory and chemosensory defects (LUNDQUIST *et al.* 1996). unc-52 is an essential gene, but a subset of alleles containing mutations in a region of three consecutive alternative exons have a less severe late larval-onset paralysis phenotype. These viable unc-52 mutations combined with loss of mec-8 lead to synthetic lethality through changes in the ability to skip the mutant exon by alternative splicing. smu-1 and smu-2 mutants were identified as suppressors of this mec-8;unc-52 synthetic lethality. They cause changes in unc-52 splicing and can suppress some of the alleles found in the alternatively
spliced region by changing the relative use of the exons (LUNDQUIST and HERMAN 1994; SPARTZ et al. 2004). The smu-2 gene encodes a ubiquitously expressed nuclear protein with 40% homology to human RED protein, a component of purified spliceosomes (DECKERT et al. 2006; JURICA and MOORE 2003; SPARTZ et al. 2004). SMU-2 interacts with SMU-1, a protein 62% identical to human spliceosome-associated protein fSAP57 (DECKERT et al. 2006; JURICA and MOORE 2003). SMU-2 has been shown to be required for the stability of the SMU-1 protein (SPARTZ et al. 2004).

We confirmed that az27 has a mutation in smu-2 by testing a known recessive allele of smu-2 for the ability to suppress unc-73(e936). A double-mutant strain was made containing both unc-73(e936) and smu-2(mn416), which carries a recessive mutation in the first intron of the gene at the 3’ splice site (SPARTZ et al. 2004). We compared spliced e936 transcripts from mn416 suppressed animals to that of az27 and found that the ratios of splice site usage are the same between the two (Fig 3A). Consistent with the RT-PCR results, we measured the same amount of coordinated movement between unc-73(e936);smu-2(mn416) double mutant animals and e936;az27 animals. These results confirm that mutant alleles of the smu-2 gene are suppressors of e936 uncoordination and that they function at the level of splicing (Fig 3B).

Previously, SMU-2 was shown to bind to and stabilize another protein called SMU-1, and loss of either protein had similar ability to suppress unc-52;mec-8 synthetic lethality (SPARTZ et al. 2004). Since the SMU-2 and SMU-1 proteins interact, we asked whether smu-1(RNAi) could also suppress the unc-
73(e936) defect. The smu-1 gene was knocked-down in unc-73(e936) animals by RNAi feeding (KAMATH et al. 2001; TIMMONS et al. 2001). After RNAi, the animals were measured for coordinated movement by a thrashing assay. The unc-73(e936); smu-1(RNAi) animals suppressed the e936 phenotype and moved in a more coordinated manner (77 ± 43 thrashes/min for unc-73(e936); smu-1(RNAi) animals vs. 2 thrashes/min for unc-73(e936) animals). We also fed bacteria harboring the empty L4440 RNAi plasmid vector to unc-73(e936) as a negative feeding control, and these showed no change in uncoordinated movement relative to untreated unc-73(e936). The γ-32P labeled RT-PCR products of e936 showed a slight increase in the usage of the -1 and mutated wild-type wt splice sites in smu-1(RNAi) animals, compared to unc-73(e936) (data not shown). However, this change in splice site usage was fairly subtle and the standard deviation was quite high, perhaps consistent with the high standard deviation found in the thrash assay for the RNAi suppressed worms. Still, the clear ability to find wild-type moving e936 worms with RNAi of smu-1 is consistent with a loss of SMU-1/SMU-2 leading to suppression of e936 through alteration of cryptic splicing.

A dominant suppressor of e936 is the C. elegans homolog of human tri-snRNP 27K. The semi-dominant suppressor strains carrying az25 and az26 were found to have identical lesions in the C. elegans gene R05D11.7, leading to a missense mutation in the protein coding region. R05D11.7 is the C. elegans homolog of the human tri-snRNP 27K (SNRNP27), a phosphoprotein component
of the U4/U6.U5 spliceosomal tri-snRNP complex (FETZER et al. 1997). We have
named this C. elegans gene snrp-27. Figure 4A shows an alignment between the
human and worm proteins; these two proteins share 54% amino acid identity.
The position of the Met to Thr amino acid substitution found in az25 and az26 at
codon 141 is indicated. This amino acid substitution is contained within a 17
amino acid stretch of 100% sequence identity between worms and humans for
this protein; there is no known function for this C-terminal domain.

Confirming the molecular identity of snrp-27(az26) as a suppressor of unc-73(e936)
presents two challenges. One challenge is due to the semi-dominant
nature of the mutation in az26. A transgene experiment to confirm that this allele
is an e936 suppressor will require expressing the dominant mutant version of the
gene in an e936 background. The second challenge is that snrp-27 is the sixth
gene in an eight gene operon, so it is not possible to clone it in its native
promoter context, as the entire operon is too big to fit on a plasmid. We reasoned
that a core component of the spliceosome is likely to be ubiquitously expressed,
so we made two constructs in which we placed the wild-type or az26 forms of
snrp-27 under the control of the promoter for the ubiquitously expressed gene let-
858 (KELLY et al. 1997). Extrachromosomal arrays carrying either snrp-27(+) or
snrp-27(az26) under the control of the let-858 promoter were successfully
obtained, including three independent lines for snrp-27(az26) (Fig 4B). Genetic
crosses were done so that these extrachromosomal arrays were moved into an
unc-73(e936) homozygous background, and in this context they were tested for
phenotypic and molecular suppression of e936.
We performed the thrash test assay on e936 worms expressing the various snrp-27 extrachromosomal arrays (Fig 4C). For the thrash assay, we picked L4 worms that express the GFP co-expression marker contained in the arrays, without regard to their movement capability on the plate, and counted thrashes/min. For the snrp-27(az26)-expressing arrays we found an improvement in the thrashes/min relative to the snrp-27(+)expressing array, which showed no significant difference from e936 strain lacking the array. Consistent with the high standard deviations in thrashes/min for the snrp-27(az26)-expressing worms, some animals moved as well as wild type (up to 126 thrashes/min) and others behaved more like e936 (less than 10 thrashes/min), suggesting a broad range of movement capabilities for the strains carrying the snrp-27(az26) arrays. We decided on another movement assay to measure whether these arrays could rescue the e936 movement defect. When wild-type animals are tapped on the head, 100% of them complete two full sinusoidal cycles of backwards movement, while only 8% of e936 animals tested were capable of this movement. 100% of suppressed unc-73(e936) snrp-27(az26) animals were capable of this movement. For e936 worms expressing the snrp-27(+) extrachromosomal array, only 5% were capable of this backwards movement, while between 65% and 75% of e936 worms carrying snrp-27(az26) extrachromosomal arrays were capable of sinusoidal backwards movement. This is consistent with expression of the mutant form of snrp-27 serving as a dominant suppressor of the e936 uncoordination phenotype.
We next decided to test whether the snrp-27(az26) transgene suppression of e936 occurs at the level of splicing. Because only about 50% of the progeny of the snrp-27 array-carrying worms inherit the extrachromosomal array, and because only about 2/3 of snrp-27(az26) array-carrying worms show improved movement, we needed to isolate RNA from array-carrying worms showing improved movement in order to have a reasonable chance of observing changes in the ratio of unc-73(e936) cryptic splicing products. For each of the e936; Ex snrp-27(az26) lines, 200 L4 worms expressing GFP and capable of backwards movement were picked and RNA was extracted. RNA was also extracted from 200 e936; Ex snrp-27(+) worms, although none showed wild-type movement. RT-PCR assays on unc-73 were performed as in previous experiments (Fig 4D) along with e936 and e936 az26 controls. The quantitation of the relative splice site usage indicates that the snrp-27(az26) arrays improve splicing at the -1/wt sites relative to e936, but the snrp-27(+) array does not. This is consistent with suppression at the level of splice site choice. The dominant suppressor transgene arrays do not change splicing to the same extent as found in the unc-73(e936);snrp-27(az26) suppressor strain; this may be due to the semi-dominance of az26 and the fact that the strains that express the dominant extrachromosomal arrays for snrp-27(az26) also carry, and presumably still express, two chromosomal copies of snrp-27(+).
DISCUSSION

We have taken advantage of an extremely sensitive assay, in which small changes in cryptic splice site choice lead to dramatic phenotypic changes in movement, to identify extragenic and intragenic suppressors with a role in cryptic splice site determination. We identified extragenic suppressors of e936 that led to ~2-fold increases in the usage of the mutated wild-type splice site, as verified by RT-PCR analysis. These splicing suppressors led to dramatic increases in coordinated movement, ranging from 8-fold to 20-fold in a thrash assay. The intragenic suppressor that we identified, az23, helped confirm that changes in splicing lead to movement defect suppression.

We identified an apparent null mutation in the smu-2 gene as an extragenic suppressor of e936 that functions at the level of splicing. smu-2(az27) acts as an extragenic suppressor of the e936 splicing defect, as does a previously described allele, smu-2(mn416). SMU-2 binds and stabilizes SMU-1 (SPARTZ et al. 2004), and we provide evidence that smu-1(RNAi) can also suppress the e936 unc-phenotype. Our study uncovers potential new roles for the SMU-1/SMU-2 complex as fidelity factors for splicing. It was shown previously that recessive mutations in smu-2 and smu-1 affect the accumulation of alternatively spliced transcripts of unc-52 (SPARTZ et al. 2004). In addition to promoting the skipping of unc-52 exon 17, it was also demonstrated that smu-1(mn415) can enhance the phenotype of unc-52(e1421) (SPIKE et al. 2001). e1421 contains a splice donor mutation, from g|gttaag to g|gttaaa, in the intron downstream of alternative exon 16 (ROGALSKI et al. 1995). This previously
reported result is also consistent with a role for the smu genes in altering usage of weak 5' splice sites. smu-1 and smu-2 are not essential for splicing or viability, but when the splicing machinery is challenged with suboptimal cryptic splice sites, these proteins appear to have a role in the 5' splice site selection process. RED protein, the mammalian homolog of SMU-2, was previously reported to have been found in the spliceosomal C-complex by mass spectrometry (JURICA and MOORE 2003). It was also reported to be found in all the active B-complexes of the spliceosome, along with fSAP57, the mammalian homolog of SMU-1 (DECKERT et al. 2006). These findings suggest that SMU-2 interacts with the spliceosome after the assembly of the tri-snRNP.

We identified a dominant mutation in the highly conserved C-terminal region of snrp-27, the C. elegans homolog of the human tri-snRNP 27K protein. The identical alleles az25 and az26 were isolated independently as suppressors of e936, suggesting that this is a very specific gain of function substitution in the protein. Tri-snRNP 27K was identified as a component of the U4/U6.U5 tri-snRNP that was capable of undergoing multiple phosphorylations (FETZER et al. 1997). The N-terminal half of the protein is a serine- and arginine-rich domain typical of SR proteins and the U1-70K protein, but unlike those proteins snrp-27 contains no RNA recognition motif or any other recognizable domains. It has been shown that stable binding of the U1 snRNP to the 5' splice site (formation of the spliceosomal E-complex) is mediated by interactions between the SR domains of ASF/SF2, an SR protein, and U1-70K, a protein component of U1 snRNP (KOHTZ et al. 1994). Additional studies indicated the requirement for SR
proteins in the subsequent recruitment of the U4/U6.U5 tri-snRNP to the assembling spliceosome (ROSCIGNO and GARCIA-BLANCO 1995). Since phosphorylated SR domains serve as protein-protein intermolecular bridges, it has been proposed that tri-snRNP 27K may be the component of the tri-snRNP that mediates tri-snRNP recruitment to the pre-mRNA through bridging with SR proteins and U1 snRNP (FETZER et al. 1997). We speculate that this potential interaction may be directly related to a role for SNRP-27 in cryptic splice site choice through mediating the assembling tri-snRNP’s interactions with different potential 5’ splice donors.

When the best 5’ splice site is eliminated by mutation, the splicing machinery is left with multiple weaker cryptic 5’ splice sites from which it may choose. The pressure to make a choice is likely the result of SR proteins binding to the exon and recruiting U1 snRNP, which then must choose its best base-pairing partner. Perhaps multiple U1 snRNAs land on the pre-mRNA at these multiple weak sites, and a decision must be made at a subsequent step of splicing to choose which will become a site of catalysis. In alternative splicing of the SV40 T antigen pre-mRNA, there are two alternative 5’ splice sites, and U1 can be directed by a subset of SR proteins to simultaneously assemble at both of them. When this happens, the site closest to the 3’ splice site is chosen (ZAHLER and ROTH 1995). It is very interesting that the mammalian homologs of SMU-1, SMU-2 and SNRP-27 are all factors found to be associated with later steps of spliceosome assembly, as opposed to being splicing factors associated with the initial recognition step of 5’ splice sites (JURICA and MOORE 2003). Perhaps the
role of SMU-1/SMU-2 is to ensure that when multiple U1 snRNAs bind a
message, the one most proximal to the 3' splice site or that has the best base-
pairing interaction with the U1 snRNA is preferred. In the absence of either of
these SMU factors, there will be less preference for recruitment of the tri-snRNP
components to one specific U1 snRNA. In the case of \textit{e936}, the loss of SMU
proteins might lead to less use of the stronger, more proximal +23 splice site and
more use of the other cryptic sites. For the dominant missense suppressor in the
SNRP-27 protein, the ability to choose which of the multiple 5' splice sites to
interact with may be altered. Further understanding of the binding partners for
SNRP-27, especially in the C-terminal region that contains the dominant
mutation, will be helpful in understanding how the spliceosome distinguishes
among different weak cryptic splice sites during its assembly transitions.
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FIGURE LEGENDS

FIGURE 1. (A) Diagram of the *unc-73* gene between exons 15 and 16. The position of the -1 and +23 cryptic 5' splice sites are indicated by arrows. The intronic *e936* (+1G→U) point mutation is highlighted. (B) γ-32P labeled RT-PCR results across the cryptic splicing region of *unc-73(e936)* for different strains. Lanes 1, 2, and 3 are loaded with RT-PCR reactions from wild-type (N2), *unc-73(e936);sup-39(je5)*, and *unc-73(e936)* RNA respectively. The lines carrying the suppressor alleles and *e936* follow in lanes 4 to 10 as indicated. (C) The *unc-73* genomic sequence from exon 15 (upper case), and intron 15 (lower case). The locations of the *az23* and *e936* mutational substitutions are indicated below. The position of the -9 cryptic splice donor activated in *e936az23* is indicated by an arrow above.

FIGURE 2. Quantitation of *unc-73(e936)* movement defect suppression and cryptic splice site usage in the presence of the isolated extragenic suppressor mutations. (A) Results from ten sets of worm thrashing experiments performed on the indicated strains. (B) Representation of splice site usage as determined by γ-32P labeled RT-PCR and quantitated from a minimum of three different sets of reactions for each. Representative PhosphorImager images of the RT-PCR reactions for the different strains are shown directly below the column of quantitative data for each strain.
FIGURE 3. A comparison of az27 and a known allele of smu-2, smu-2(mn416) in suppression of unc-73(e936). (A) Measurements of unc-73(e936) splice site usage in the presence of smu-2(mn416) and az27 as determined by γ^{32}P labeled RT-PCR. (B) Results from eleven sets of worm thrashing experiments each performed on L4 larvae from wild-type (N2), unc-73(e936), unc-73(e936);smu-2(mn416), and unc-73(e936);smu-2(az27) strains.

FIGURE 4. The az26 allele of snrp-27 is a dominant suppressor of e936 cryptic splicing. (A) Alignment of human tri-snRNP 27K protein and C. elegans SNRP-27. The Met at position 141 that is changed to Thr in az25 and az26, and the corresponding base substitution, are indicated. (B) The snrp-27 transgene contents of the four extrachromosomal array lines to be tested. (C) Thrash test and backward movement assay results for various lines. For array-containing strains, GFP+ L4 animals were picked and assayed. (D) RT-PCR results and quantitation of the relative level of the bands for the various lines. For the array-containing lines, 200 individual L4 worms were picked and RNA extracted and tested by RT-PCR.


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A  Representation of Splice Site Usage

Percent Splicing at Cryptic 5' Splice Site

N2  unc-73(e936)  smu-2(mn416); unc-73(e936)  smu-2(a27)
+ 23  0  80  61  64
wt  100  10  18  17
-1  0  10  21  19

B  Representation of Coordinated Movement

Average Number of Thrashes per Minute

N2  unc-73(e936)  smu-2(mn416); unc-73(e936)  smu-2(a27)
N=11  128  2  32  35
A. Human Tri-snRNP 27K and C. elegans SNRP-27

Human MGRSRSRSPRRERRRSRSRRRERSRRRRERRRRER-DRRRSHRSRSRPRHR 58
Worm MGRDRRSR-DRKRRSRRSRSRVER---RRERSRRERDARKNEKRRSRRSRSRPDRK 56
** ** ** .......................... ** .......................... .......................... ..........................

Human STSP-SPSR-LKERRDKEKETKETKSRQ---ITEEDLEGKTEEIEEMMKLMGFAKFSDS 114
Worm DRRRERSRDRKRRDREKRRKDRPKKREKQEEISLQ8SNoticeDA-MMAAMGFPPGFTD 115
** ** ** .......................... ** .......................... .......................... ..........................

Human TKGKVDGSVNAAYINVSQKRKYRQYNKRGNGFNPRLDIA 155
Worm TKNQVNDVNDG-CVNIKPRRYQYNKGGFNPRLDFMG 155
** ** ** .......................... ** .......................... .......................... ..........................

141
M
N2 ATG az26 ACG T

B. snrp-27 expression arrays with let-858 promoter

azEx64 = Plet-858::snrp-27 (+) line 1
azEx65 = Plet-858::snrp-27 (az26) line 1
azEx66 = Plet-858::snrp-27 (az26) line 2
azEx67 = Plet-858::snrp-27 (az26) line 3

C. Movement assays for e936 worms carrying snrp-27 arrays

<table>
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<th>thrashes/min (N=10)</th>
<th>% crawl backward (N=40)</th>
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<tr>
<td>N2</td>
<td>138 ± 27</td>
<td>100%</td>
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<tr>
<td>e936</td>
<td>4 ± 6</td>
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</tr>
<tr>
<td>e936; az26</td>
<td>93 ± 55</td>
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<tr>
<td>e936; azEx64</td>
<td>7 ± 4</td>
<td>5%</td>
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<tr>
<td>e936; azEx65</td>
<td>34 ± 30</td>
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<tr>
<td>e936; azEx66</td>
<td>54 ± 52</td>
<td>75%</td>
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<tr>
<td>e936; azEx67</td>
<td>19 ± 13</td>
<td>68%</td>
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D. RT-PCR analysis of unc-73 splicing in snrp-27 array strains

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<tr>
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<tr>
<td>% +23</td>
<td>85</td>
<td>65</td>
<td>87</td>
<td>73</td>
<td>76</td>
<td>76</td>
</tr>
<tr>
<td>% -1 + wt</td>
<td>15</td>
<td>35</td>
<td>13</td>
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