SNRP-27, the C. elegans homolog of the tri-snRNP 27K protein, has a role in 5′ splice site positioning in the spliceosome

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ABSTRACT

The tri-snRNP 27K protein is a component of the human U4/U6-U5 tri-snRNP and contains an N-terminal phosphorylated RS domain. In a forward genetic screen in C. elegans, we previously identified a dominant mutation, M141T, in the highly-conserved C-terminal region of this protein. The mutant allele promotes changes in cryptic 5′ splice site choice. To better understand the function of this poorly characterized splicing factor, we performed high-throughput mRNA sequencing analysis on worms containing this dominant mutation. Comparison of alternative splice site usage between the mutant and wild-type strains led to the identification of 26 native genes whose splicing changes in the presence of the snrp-27 mutation. The changes in splicing are specific to alternative 5′ splice sites. Analysis of new alleles suggests that snrp-27 is an essential gene for worm viability. We performed a novel directed-mutation experiment in which we used the CRISPR-cas9 system to randomly generate mutations specifically at M141 of SNRP-27. We identified eight amino acid substitutions at this position that are viable, and three that are homozygous lethal. All viable substitutions at M141 led to varying degrees of changes in alternative 5′ splicing of native targets. We hypothesize a role for this SR-related factor in maintaining the position of the 5′ splice site as U1snRNA trades interactions at the 5′ end of the intron with U6snRNA and PRP8 as the catalytic site is assembled.

Keywords: RS domain; cryptic splice sites; pre-mRNA splicing; spliceosome

INTRODUCTION

The two trans-esterification reaction steps of precursor messenger RNA (pre-mRNA) splicing are performed by the large ribonucleoprotein machine called the spliceosome. The spliceosome assembles onto the pre-mRNA at each intron through an ordered binding of its U6snRNP subunits. Early in this assembly, U1 snRNP recognizes the 5′ splice site through base-pairing interactions with the 5′ end of U1 snRNA (E complex). U2 snRNP is next recruited and identifies the branch point sequence via RNA–RNA interactions with U2snRNA (A complex). Next, the U4/U6-5′ tri-snRNP is recruited to the spliceosome (C complex), which carries out the first step of the splicing reaction (for a review, see Fica and Nagai 2017). In this first step of splicing, the 5′ splice site is catalytically committed by cleavage at the exon/intron junction and simultaneous formation of the intron lariat. As U1 leaves during B complex formation, U6 forms base-pairing interactions near the 5′ end of the intron prior to catalysis, and these interactions appear to be stabilized by PRP8 (Galej et al. 2016). The rearrangements in the spliceosome at the different steps are catalyzed and proof-read by a series of ATPase helicases (Koodathingal and Staley 2013). In the past three years, there has been an explosion of new information about the structures of the different spliceosomal complexes brought about by advances in cryo-electron microscopy (for a review, see Fica and Nagai 2017; Shi 2017). This has led to major advances in our understanding of the complex RNA–protein, protein–protein, and RNA–RNA interactions of the spliceosome. It is a very exciting time in the field, yet there

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remain challenges to understanding the splicing mechanism, including understanding the dynamic rearrangements of the spliceosome as it transitions from one complex to the next, which may be best served by continued genetic and biochemical studies (Mayerle and Guthrie 2017).

The formation of spliceosomal complexes and their rearrangements are guided by many proteins that interact with the pre-mRNA and/or associate with the spliceosomal subunits. These include the SR family of proteins, characterized by the presence of N-terminal RNA recognition motif(s), and a C-terminal RS domain, containing phosphoserine-arginine dipeptide repeats (Shepard and Hertel 2009). SR proteins assemble onto the pre-mRNA and guide spliceosomal components to the next, which may be best served by continued genetic and biochemical studies (Mayerle and Guthrie 2017). The RS domains are strikingly absent from proteins not homologs of classical SR proteins in S. cerevisiae, and an SNRNP27 homolog is also absent. For RS domain-containing proteins for which clear homologs are present in S. cerevisiae (i.e., SNRNP70 and PRP28), the RS domain is absent from the S. cerevisiae homolog.

We previously identified the C. elegans homolog of SNRPNP27, snrp-27, in a genetic screen for phenotypic suppressors of uncoordination for the unc-73(e936) allele, which contains a G→U substitution at the first nucleotide position of intron 15 (Dasah et al. 2009). Cryptic 5′ splice sites are defined as sites that are never used by the splicing machinery unless an authentic nearby 5′ splice site is altered by mutation. When that occurs, nearby cryptic sites, usually defined by GU dinucleotides, become substrates for splicing but with a 2/3 chance of being out-of-frame. A dominant mutation in SNRPNP27, M141T, in the highly conserved C-terminal region (a region with no detectable domain homology with other proteins) led to alteration of cryptic 5′ splice site usage in the unc-73(e936) allele, increasing the amount of in-frame splicing of unc-73(e936) with no apparent effects on worm viability. There is a very limited literature about this protein and its homologs. Human SNRNPNP27 was first identified as a gene of unknown function disrupted by HTLV-1 provirus insertion in a human T-cell line (then named HY-1) (Nakamura et al. 1994). The association of the human homolog with splicing was demonstrated when it was shown to be a component of the U4/U6-US tri-snRNP that is phosphorylated by a spliceosome-associated kinase (Fetzer et al. 1997). Even though it is detected in human tri-snRNPs prepared for cryo-EM structural analysis, its density has not been mapped in the proposed structure, likely due to the low complexity and potential unstructured nature of the protein (Agafonov et al. 2016). The Drosophila homolog, Yantar, was identified in a genetic deletion screen of lethal recessive mutants for factors that regulate hemocyte development; loss of Yantar is pupal lethal but leads to overproduction of hemocytes in larvae (Sinenko et al. 2004). In a reverse genetic screen for factors that affect splicing efficiency in Schizosaccharomyces pombe, deletion of the fission yeast homolog SPCC162.01c led to both the accumulation of unspliced forms of some messages and increased splicing efficiency for others (Larson et al. 2016). SNRNPNP27’s high enrichment in arginine and lysine residues leaves a very limited number of peptides that can be observed in mass spectrometry proteomic experiments after tryptic digest. This likely leads to underreporting of the presence of this protein because of low confidence due to the lack of multiple distinct peptide fragments that can be assigned to it.

In this paper, we further characterize the role of the dominant SNRPNP-27 mutation in splicing. We perform high-throughput mRNA sequencing analysis from the mutant strains and show that it activates alternative 5′ splice sites in 26 native genes; no other types of alternative splicing changes are detected. We confirmed these changes experimentally for 13 targets and characterize a likely role for this factor in 5′ splice site choice. Worms containing a CRISPRcas9-induced apparent null allele of this gene are inviable, with heterozygotes showing no apparent defects. We used a novel approach to randomize amino acid M141 and recovered viable worms with eight different substitutions at this position. We also identified three other substitutions that were homozygous lethal. All of the viable mutants led to changes in alternative 5′ splice site choice for the native targets tested, indicating that the highly conserved M141 is optimized for protein function. We propose that SNRPNP-27 functions during the handoff of the 5′ splice site from U1snRNA to U6snRNA and PRP8 to promote splicing fidelity.
RESULTS AND DISCUSSION

High-throughput mRNA-seq analysis of SNRP-27 M141T mutant strains

We have previously described the genetic screen that resulted in the isolation of snrp-27(az26) (Dassah et al. 2009). In that approach, we identified a suppressor allele of snrp-27 that altered the cryptic splicing of unc-73 (e936), resulting in a twofold increase in in-frame messages leading to improved coordination and movement. In order to determine whether the snrp-27(az26) allele also promotes changes in splicing of native genes, we compared high-throughput mRNA sequencing results of strains CB936 (unc-73(e936)i) versus SZ118 (unc-73(e936) snrp-27(az26)i). mRNA was isolated from young adult animals, cDNA prepared and high-throughput sequencing performed to obtain strand-specific 100-nucleotide (nt) paired-end reads. Two independent libraries from two independent RNA isolations were prepared from each strain and the resulting sequences were mapped to the C. elegans genome using STAR (Dobin et al. 2013).

We next set out to identify native genes whose alternative splicing is changed in the presence of the snrp-27 (az26) allele. We identified alternative splicing events and used MISO (Katz et al. 2010) to do four pairwise comparisons of the events in these libraries against each other (CB936 library 1 versus SZ118 library 1; CB936 library 1 versus SZ118 library 2; CB936 library 2 versus SZ118 library 1; CB936 library 2 versus SZ118 library 2) for all the de novo identified alternative splicing events in three categories (alternative 5′′ splice site, alternative 3′′ splice site, alternative cassette exon). Those events with a change in percent spliced in (delta PSI) value of ≥0.20 in all four library comparisons were further examined. There were 22 alternative 3′′ splice site events that met these criteria, but upon examination of individual reads supporting these events using Sashimi and display of library BAM tracks in the UCSC Genome Browser, only one was a potential library-specific alternative 3′′ splicing event. However, when tested directly by 32P RT-PCR analysis (data not shown), it showed no difference in alternative splicing between the strains. There were many alternative splicing events falsely flagged by our pipeline leading to a requirement that the flagged events be examined individually. False flagging was due to (i) pairwise comparison by MISO of two very minor isoforms when a third majority isoform was found sharing the same splice site, (ii) low coverage of reads for both isoforms of an alternatively spliced region such that a small number of changes led to large changes in percentages, (iii) complex splicing in a region that led to a false conclusion for a specific splicing event, or (iv) reads with alignment to multiple adjacent regions. Three alternative cassette exon events were flagged in this analysis as well, but upon examination none appeared to be true alternative splicing events dependent on snrp-27 mutation. Therefore, we could find no strong evidence for alternative 3′ splice site or cassette exons whose usage is misregulated in the presence of the SNRP-27 M141T mutation. In contrast, 61 alternative 5′′ splice site events showed changes in all four comparisons. We determined that 26 of the 61 alternative 5′′ splice site events have strong supportive evidence of alternative splicing differences between these strains. These are summarized in Figure 1. As snrp-27(az26) was identified as an allele that could alter cryptic 5′′ splice site usage, this connection to alternative 5′′ splice site usage of native C. elegans genes warrants further analysis to understand the nature of the SNRP-27 function.

We subjected 13 of the alternative 5′′ splicing events affected by the snrp-27(az26) mutation to experimental confirmation by doing reverse-transcription followed by PCR with 32P 5′′ end-labeled primers. With these alternative 5′′ splice sites in close proximity to each other (several are separated by only 3 nt), the resolution provided by separation of 32P-labeled PCR products on denaturing polyacrylamide gels is required to visualize the individual products. Six of the 13 alternative splicing events have 5′′ splice sites that are not separated by a multiple of 3 nt. This would imply that their use would result in alternative messages with different reading frames downstream from the alternative splicing event and may result in the formation of a premature termination codon (PTC). Alternative splicing was determined for five of these in a nonsense-mediated decay mutant background. Figure 2 shows representative 32P RT-PCR analyses of these 13 alternative splicing events. For simplicity, 32P RT-PCR results for only CB936 and SZ118 (strains with wild-type snrp-27 and M141T mutant snrp-27) are shown for all examples. For five of the alternative 5′′ splice sites not separated by a multiple of 3 nt, alternative splicing for the substrate in a nonsense-mediated decay-defective strain, CB4354 with a mutation in smg-3, is included.

The alternative splicing changes identified by high-throughput sequencing analysis are strongly supported by the individual RT-PCR reactions. The snrp-27(az26) allele promotes usage of the upstream 5′′ splice site for the three alternative 5′′ splice sites separated by 3 nt. These are interesting because the recognition sites for U1 snRNA and U6 snRNA are overlapping for these substrates, and the beginning intron sequence for the upstream snrp-27(az26)-promoted 5′′ splice site, GTTGTGA, is identical for all three substrates. The other alternative splicing events have 5′′ splice site sequences separated by 5 to 36 nt, and in these cases, either the upstream 5′′ splice site or the downstream 5′′ splice site can become preferentially used in the snrp-27(az26) mutant background. The 13 events tested had an average MISO delta PSI from the sequencing analysis of between 0.300 and 0.695, with stip-1 the weakest one tested. stip-1 had a confirmed change in alternative splicing in the snrp-27(az26)
mutations can be substrates for degradation by the nonsense-mediated decay (NMD) pathway (Maquat 2002). *C. elegans* is unique among model organism metazoans in that it is viable in the absence of NMD function (Pulak and Anderson 1993). In *C. elegans*, the smg genes (suppressors with morphogenetic effects on genitalia) are required for nonsense-mediated decay (Hodgkin et al. 1989). In order to confirm that we are seeing changes in 5′ splice site choice as opposed to changes in message stability in the snrp-27(az26) background, we isolated total RNA from smg-1(r869), smg-2(e2008) and smg-3(ma117) NMD-defective worms and tested 12 events for alternative 5′ splicing changes. In four of the five alternative splicing events tested that are not separated by a multiple of 3 nt, the alternative 5′ splice site usage was identical between wild-type and NMD mutant worms (Y71H2AM.2, mab-10, zim-1 and gon-14), indicating that neither alternative transcript is a substrate for the NMD pathways (only the smg-3 mutant is shown in Fig. 2 for simplicity). However, for the intron in C09E7.7, the NMD mutant backgrounds show higher usage of the upstream 5′ splice site relative to the CB936 strain. This indicates that usage of the upstream 5′ splice site in this case triggers NMD decay. It is notable that in the snrp-27(az26) mutant background (center lane—SZ118) there is also an increase in the steady-state level of transcripts that use the upstream 5′ splice site. Since those transcripts decay more rapidly via NMD than those that use the downstream 5′ splice site, it indicates snrp-27(az26) promotes an even stronger usage of the upstream 5′ splice site than is evidenced by just the relative steady state levels of the transcripts in that lane. Therefore, the changes observed in relative abundance of the different isoforms between CB936 and SZ118 are at the level of alternative splice site choice, not NMD-regulated message stability.

FIGURE 1. Alternative 5′ splice sites of native genes whose usage is changed as suggested by mRNA-seq analysis. For each of the 26 genes, the DNA sequence of the alternative 5′ splice site region is indicated. Lower case indicates intron sequences, while upper case indicates sequences that can be exons. The splicing regions are ordered by the increasing length of the distance between the two 5′ splice sites in two sets. The top set (whose names are in bold) was confirmed by 32P RT-PCR (Fig. 2). Also indicated is whether the alternative splicing has been previously annotated by Wormbase and the chromosomal location of the corresponding region in *C. elegans* genome release WS190/ce6. The average delta PSI of the four pairwise library comparisons and standard deviation are indicated. Black triangles indicate the 5′ splice site promoted by the snrp-27(az26) mutation. White triangles indicate the alternative 5′ splice site whose usage decreases in the snrp-27(az26) strains. Inverted triangles indicate 3′ splice site location for introns in which the 3′ splice site fits on the line. Lines ending in “…” indicate that the intron is longer than the sequence shown. Note that for ZIM-1 there are two alternative 3′ splice sites separated by 9 nt and both are indicated.
We focused on the first 7 nt of the 5′ end of the introns defined by the alternative 5′ splice sites as these sequences have overlapping interactions with U1snRNA and U6snRNA. SNRNP27 was isolated by its biochemical association with purified tri-snRNP complexes (Fetzer et al. 1997). As the tri-snRNP enters the spliceosome to form the B and then the B* complexes, the interaction of U1snRNA with the 5′ splice site is exchanged for U6snRNA interactions (Ares and Weiser 1995). Figure 3A,B shows the sequences of the splice sites and a Pictogram display (http://genes.mit.edu/pictogram.html) of the first 7 nt of these introns. A consensus Pictogram from 72,591 randomly chosen 5′ splice sites is shown at the top of Figure 3B and it matches the published Pictogram consensus for the C. elegans 5′ splice site (Lim and Burge 2001). Next is the Pictogram for the 26 alternative 5′ splice sites whose usage is reduced in the snrp-27(az26) background. They are a fairly good match to the consensus 5′ splice site sequence, with the exception that the +3 position of these 26 introns tend to exclude A residues (only 1/26 has an A at the +3 position), while the consensus 5′ splice site finds an A residue at 58% of total C. elegans introns (P-value from a hypergeometric test for nucleotide frequency of A at position +3 in the 5′ splice sites whose usage is reduced in the snrp-27(az26) background.}

5′ splice sites separated by 11 nt and alternative 3′ splice sites separated by 9 nt. The upstream 5′ splice site is strongly preferred in the snrp-27(+) background and shows splicing to both alternative 3′ splice sites at relatively equal levels. Both 3′ splice sites have relatively strong consensus sequences, so from previous work in our laboratory we might expect their alternative usage to not be regulated in a tissue-specific manner (Ragel et al. 2015). In contrast, the downstream 5′ splice site is strongly preferred in the snrp-27(az26) background, and it splices exclusively to the downstream 3′ splice site. This specificity in alternative 3′ ss usage correlated with the downstream 5′ splice site is likely due to the fact that the distance between the downstream 5′ splice site and the upstream 3′ splice site, 33 nt, is below the minimal threshold for intron size in this organism (Lim and Burge 2001), so that the downstream 5′ splice site can only be paired with the downstream 3′ splice site.

**Analysis of the sequence context of SNRP-27 M141T-promoted alternative splicing events**

We performed an analysis of the sequence context of the 5′ splice site intronic regions of the alternative isoforms.

- **FIGURE 2.** Confirmation of predicted az26-induced alternative splicing by 32P RT-PCR. Sample 32P RT-PCR reactions for each alternatively spliced region noted in bold in Figure 1. Genotypes of strains that were the source of RNA are indicated at the top of the figure. Alternative splicing events not separated by a multiple of 3 nt also show splicing for an NMD mutant strain. Black arrows indicate the 5′ splice site whose usage is promoted in the snrp-27(az26) strains, and the white arrows indicate its alternative splicing partner whose usage is reduced in the snrp-27(az26) strains.

- **TABLE:**

We performed an analysis of the sequence context of the 5′ splice site intronic regions of the alternative isoforms.
We looked at the nucleotide composition at positions +4, +5, and +6 for 110,339 annotated C. elegans introns beginning with GU. Of these 5’ splice sites, only 30,054 (27.2%) have less than two of three nucleotides at positions +4, +5, +6 match the AGU consensus. For the snrp-27(az26)-promoted 5’ splice sites, 21/26 (80.8%) have less than two of the three nucleotides in this region match the consensus. In contrast, only 1/26 of their alternative 5’ splice site partners have less than two of the three nucleotides match the consensus. The differences in nucleotide composition of the snrp-27 M141T-promoted alternative 5’ splice sites, especially at positions +4, +5, and +6 of the intron, suggest a model in which the mutation in snrp-27 relaxes the specificity by which U6 assembles with the pre-mRNA into an active site for splicing.

We tested whether the unusual abundance of G at the +6 position of the snrp-27(az26)-promoted 5’ splice sites was an important feature for this recognition. To do this, we generated two new alleles of the mab-10 gene using the CRISPRcas9 system. These new alleles result in a point mutation to the sixth position of the upstream 5’ splice site in mab-10 was chosen for analysis because it fit two criteria; mutation of this position resulted in no change in protein coding sequence as it is the wobble position of a proline codon, and this point mutation disrupt ed a HpyCH4III restriction site, leading to ease of screening and recovery of single-point mutants by PCR across this region followed by HpyCH4III digestion. In the snrp-27 (+) background, these two new alleles resulted in a point mutation to the sixth position of the upstream snrp-27(az26)-promoted intron; +6G→A in the az70 allele and +6G→T in the az71 allele (Fig. 3C). The +6 position of this 5’ splice site in mab-10 was chosen for analysis because it fit two criteria; mutation of this position resulted in no change in protein coding sequence as it is the wobble position of a proline codon, and this point mutation disrupted a HpyCH4III restriction site, leading to ease of screening and recovery of single-point mutants by PCR across this region followed by HpyCH4III digestion. In the snrp-27 (+) background, these two new alleles resulted in a point mutation to the sixth position of the upstream snrp-27(az26)-promoted intron; +6G→A in the az70 allele and +6G→T in the az71 allele (Fig. 3C).
For both alleles, the snrp-27 mutant was able to increase the usage of the upstream 5′ splice site even without a G at the +6 position of the intron. However, the relative increase in upstream 5′ splice site usage in the presence of the snrp-27 mutation was different for the two mab-10 mutant alleles relative to each other as well as relative to mab-10(+). The upstream mab-10 alternative 5′ splice site also has noncanonical C nucleotides at positions +4 and +5. These data indicate that the snrp-27 mutant increases the relative usage of this unusual upstream 5′ splice site, and that the presence of an intronic +6G is not essential for this to occur but may contribute to this recognition.

**Generation of new SNRP-27 M141 mutants and analysis of their role in splicing**

The SNRP-27 protein sequence is highly conserved in evolution. Figure 4A shows the alignment of the terminal 20aa of this protein from, C. elegans, human, Drosophila melanogaster, and Schizosaccharomyces pombe. There is striking conservation of the amino acid sequence of the protein in this 20-residue stretch at the C terminus. There is no homolog of this protein in Saccharomyces cerevisiae, and even a BLAST search (Altschul et al. 1990) with just this 20 amino acid conserved region yielded no potential homologs. The az26 M141T mutation is located within this region. In order to better understand the role of M141 in SNRP-27 function, we performed a novel genetic experiment in which we used the CRISPR-cas9 system coupled with oligonucleotide-directed recombination repair to specifically randomize the amino acid at position 141. Figure 4B outlines this repair strategy. We recovered 10 independent strains with nine different sequence alleles leading to eight different amino acid possibilities at position 141, including the original M141T substitution (Fig. 4C).
These strains all grow vigorously like wild-type, with the exception of the two M141A strains, which grow slightly slower and have fewer progeny.

We next set out to test whether these new snrp-27 M141 mutations promote changes in splicing. This is simplified by the fact that we have identified native genes whose alternative splicing changes in the presence of the snrp-27 (az26) mutation (Fig. 1). Figure 4D and E show the results of alternative 5′ splicing for T12C9.7 and mab-10 in the different strains listed in Figure 4C. For both alternative splicing events, the downstream 5′ splice site is strongly preferred in the wild-type snrp-27(+ ) backgrounds (CB936 (unc-73(e936) I) and N2) while any substitution to M141 leads to increased usage of the upstream 5′ splice site. This ranges from ~50% in the M141Y substitution strains to almost 100% in the M141A substitution strains, which also showed a mild growth defect. These results suggest that methionine at position 141 is optimized for SNRP-27 function in 5′ splice site choice, and that substitutions to this highly conserved position weaken this specificity. While this experiment did not saturate for every possible amino acid substitution at M141, we were able to generate and test a broad range of new alleles with different classes of amino acid side chains.

In addition to the viable substitutions at M141, we identified four different lines that were heterozygous for changes at M141, but for which the mutant allele could not be made homozygous (Fig. 4C). These alleles appeared completely normal as heterozygotes, indicating that these were recessive mutations, but were homozygous lethal. We sequenced PCR products across the mutant genomic region from heterozygous individuals, and by reading the electropherograms, we were able to deduce both the wild-type sequence as well as the sequence of the amino acid substitution in the mutant (Fig. 4C). M141G, M141F, and M141E were all homozygous lethal substitutions in this protein. This indicates that while several amino acid substitutions may lead to a functional protein with weakened ability to promote 5′ splice site specificity, other amino acid substitutions lead to a complete interference in protein function.

The homozygous lethal snrp-27 alleles raised the question as to whether snrp-27 is an essential gene. This two-exon gene is the sixth gene of an eight-gene operon, so we were concerned that creating a large deletion of snrp-27 to generate a null allele might affect the function of surrounding genes. Instead we generated an allele by CRISPR-cas9 in which we inserted a stop codon at amino acid 64 and asked whether these worms could be viable. This mutation truncated the protein partway through the RS domain and lacked the highly conserved C-terminal region. This allele was viable as a heterozygote and behaved normally, however this allele could not be made homozygous. Occasionally we would recover a sick late larval stage animal that was homozygous for the putative null mutant but could not maintain it because it was sterile. Given that the heterozygous worms with this mutation do not show any phenotypes, we do not believe the lethality of this allele to be the result of an RS domain poison product; we think of it as a putative null allele. The inability to make this putative null allele homozygous, along with the results that the M141G, M141F and M141E alleles appear normal as heterozygotes but are homozygous lethal, point to snrp-27 as an essential gene. This is consistent with the pupal lethal phenotype of the Drosophila Yantar gene deletion (Sinenko et al. 2004).

Conclusions

The tri-snRNP 27K protein is a poorly understood splicing factor. It was initially identified as a component of purified tri-snRNPs that could undergo phosphorylation in its RS domain (Fetzer et al. 1997). Our subsequent genetic study identified a dominant mutation, M141T, in the C. elegans homolog snrp-27 that promotes changes in cryptic 5′ splice site usage (Dassah et al. 2009). In this paper we present data that the M141T mutation promotes changes in alternative 5′ splice site usage for a number of native genes. Many amino acid substitutions at M141 in the highly conserved C terminus are viable and also promote the alternative 5′ splice site usage that we identified in the M141T allele. However, a subset of M141 substitutions lead to homozygous lethality (Fig. 4C), indicating that there is an essential role for this highly conserved region in protein function.

The ability of the SNRP-27 M141 alleles to promote alternative 5′ splice site usage on native genes may be due to a rare combination of conditions that allow for slippage of the spliceosome between two juxtaposed 5′ splice sites as the spliceosome assembles. In every case, the snrp-27(az26) promoted 5′ splice site has usage that can be detected at least at a low level in wild-type animals (Fig. 2). Perhaps the combination of a weaker 5′ splice site whose usage is reduced in the snrp-27(az26) background (note the lack of an A residue at the +3 position of these 5′ splice sites) and an unusual juxtaposition of bases in the M141T mutant-promoted 5′ splice site (Fig. 3A,B) combine to give a region of “slipperiness” that is enhanced in the snrp-27 mutant, resulting in activation of the neighboring 5′ splice site. This is consistent with its role in cryptic 5′ splice site suppression of unc-73(e936) (Dassah et al. 2009). We noted that 14 of the 26 M141T-promoted alternative 5′ splice sites have a G at the +6 position of the intron. There is evidence for noncanonical base pairing of U1snRNA with 5′ splice sites involving bulged nucleotides in order to accommodate a G at the +6 position of the intron (Roca and Krainer 2009; Roca et al. 2012, 2013; Tan et al. 2016). However, in the case of the alternative 5′ splice sites we noted here, the preferences change as the result of mutations in the SNRP-27 protein associated with the tri-snRNP, not
the initial U1snRNA interactions. We also note that the snrp-27(a26)-dependent change in alternative 5′ splice sites still occurs for the mab-10 event even when the +6G of the intron is changed to another nucleotide (Fig. 3C,D).

Based on our data, we propose a model for the role of the SNRP-27 M141 mutations in changes in alternative 5′ splice site usage. SNRP-27 is found associated with the spliceosome after U1snRNA has established a base-pairing interaction with the 5′ splice site. With the tri-snRNP (Fetzer et al. 1997), and interacts with the spliceosome until U1snRNA has established a base-pairing interaction with the 5′ splice site. As the tri-snRNP assembles into the spliceosome, U1 interactions with the 5′ end of the intron are replaced by interactions with U6 and PRP8 (Galej et al. 2016). Perhaps RS domain interactions between U1snRNP component U170K and SNRP-27 help to mediate the handoff of the 5′ splice site and ensure fidelity. For these unusual alternative 5′ splice sites whose relative usage changes in the presence of the SNRP-27 M141T mutant protein (Fig. 1), we hypothesize that this handoff becomes less efficient with the mutant SNRP-27, leading to a slippage event that allows U6 and PRP8 to assemble onto juxtaposed lower consensus alternative 5′ splice sites. While the new cryo-EM structural data have provided important new insights into the mechanism of splicing, it is clear that this is a dynamic process relying on multiple assembly steps. Our data strongly support the idea that there are additional factors not captured in these spliceosomal snapshots that play important roles in the fidelity of the assembly process.

MATERIALS AND METHODS

RNA isolation and high-throughput mRNA library production

RNA was isolated for library construction from the indicated strain taken from populations of worms enriched for young adults using TRIzol reagent (Invitrogen). mRNA libraries for high-throughput sequencing were prepared from 5 µg of total RNA after poly(A) selection using the NEXTflex Rapid Directional qRNA-Seq Kit from BioO Scientific. Paired directional 100 nt reads, eight libraries per lane were obtained from an Illumina HiSeq 4000 operated at UC Berkeley. FASTQ files of raw reads from the high-throughput RNA-seq libraries and processed .gtf files of alternative splicing events in each library have been submitted to the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) under accession number GSE113275. Libraries were prepared from two independent RNA isolations for each strain. These resulting sequences were trimmed to 80 bases on each end and resulting sequences were trimmed to 80 bases on each end and resulting sequences were trimmed to 80 bases on each end and resulting sequences were trimmed to 80 bases on each end.

Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) using STAR (Dobin 2013) and duplicate libraries were removed. The PCR mixtures were spiked with a32P 5′ end-labeled reverse primer at 2% of the concentration of unlabeled reverse primer. After 25 extension cycles, PCR products were recovered by phenol:CHCl3 extraction and ethanol precipitation. The ethanol precipitates were resuspended in formamide with dyes and 2 µL were separated on a 40 cm long 6% polyacrylamide urea denaturing gel in TBE buffer. Products were visualized with a Molecular Dynamics Typhoon PhosphorImager, and quantifications of band intensities were performed using ImageQuant software.

Calculation of significance of nucleotide frequency at specific positions in the snrp-27-affected alternative 5′ splice sites

We applied a hypergeometric test to determine the statistical significance of observed deviations in nucleotide frequencies at splice site positions. Briefly, we counted the number of times a nucleotide (e.g., “A”) occurred at a given position (e.g., “+3”) in a collection of 111,187 annotated 5′ splice sites, and in the subset of 26 affected pairs of alternative 5′ splice sites. We then applied a hypergeometric test in Python, using scipy.stats.hypergeom.pdf, to determine P-values and whether the observed nucleotide counts in the subset of affected sites diverged significantly from the genome-wide frequency.

32P RT-PCR

Starting with 4 µg of total mixed stage RNA, reverse transcription was performed with Avian Myeloblastosis Virus reverse transcriptase (Promega) with a primer specific for the downstream exon (see Tables 1 and 2) in 25 µL reaction mixtures. This was followed by polymerase chain reactions containing 1.3 µL of the reverse transcription product amplified with Taq polymerase using the same reverse primer as in the reverse transcription reaction along with a forward facing primer that anneals to the upstream exon. The PCR mixtures were spiked with a32P 5′ end-labeled reverse primer at 2% of the concentration of unlabeled reverse primer. After 25 extension cycles, PCR products were recovered by phenol:CHCl3 extraction and ethanol precipitation. The ethanol precipitates were resuspended in formamide with dyes and 2 µL were separated on a 40 cm long 6% polyacrylamide urea denaturing gel in TBE buffer. Products were visualized with a Molecular Dynamics Typhoon PhosphorImager, and quantifications of band intensities were performed using ImageQuant software.

CRISPR homologous recombination repair oligonucleotides

snrp-27 M141 mutagenesis

CGTGGATGCGGGTGGATGGCACTAAGAAACCTCGCGAGATACC GCCAAGTACNNNAACCGAAGAGGAGGATTCAATCGTCCACTT GATTITATGGGATAATTTA

Notes: The Met141 codon is replaced with three Ns (underlined) in which all nucleotides are randomly incorporated at these positions. The lower case “g” (underlined) is a silent mutation at the wobble position of Q139. It creates a GTAC RsaI restriction site which is used for detection of the altered snrp-27 coding...
region. The underlined lower-case “a” is a silent mutation at the wobble position of K144 that will disrupt the PAM sequence.

**snrp-27 null allele mutagenesis**

GTCGAAGCAGAAGTCCGCGCGATAAACGAGATCGGCGGGA
AAGAAGTCGA 
CTCACG 
TGAT CGAAAAGAACGTGATCGTGAA 
CGACAAAAAAAGGATCGTGA

Notes: The underlined C is a 1 nt insertion that changes reading frame at amino acid 64 resulting in a stop codon two codons downstream. It also creates a new SalI restriction site, GTCGAC that is useful for the detection and screening of altered alleles. The underlined T disrupts the PAM sequence and becomes the T of the out-of-frame TGA stop codon.

**mab-10 5’ss mutagenesis**

TCATCGTCTCCGTCACTGAATCTGAACACGACATCGTCGAATT 
CG GTACCHTTGG GTTAGTCTTTGTCGCTGGGCGTTTGGTAG 
CACACGCATGTCCATAA

Notes: The mutagenesis will disrupt an HpyCH4III site ACNGT (underlined) which is useful for detection of altered genes. The H with an underline is a substitution of a G nucleotide with A, C, or T randomly generated at this position during oligo synthesis.

**CRISPR crRNAs**

AltR crRNAs were synthesized by Integrated DNA Technology with the following crRNA guide sequences

| Gene       | Forward primer | Reverse primer |
|------------|----------------|----------------|
| snrp-27 M141 | GAGTCGTTACAAAGTGGAGC | TTCGCCATGGTCAAATTCCC |
| snrp-27 Null | CTCGGCATTCTGTTAGGAGC | TTCGCCATGGTCAAATTCCC |
| mab-10     | TTTCCGAAGCCTCTTCATGTC | CTACGCGCTGAATATAGTGC |

**snrp-27 M141 mutagenesis**

CCGCCAAUACAUGAACCGAA

**snrp-27 null allele**

GCGCGGAAGAAGAUGCAUCA

**mab-10**

AUCGUCGAAUUCGGUACCGU

**CRISPR Cas9 mutant production**

CRISPRCas9-directed mutagenesis was performed by micro-injecting Cas9 ribonucleoprotein complexes and homologous recombination repair oligonucleotides into the syncytial gonad of adult hermaphrodites (Paix et al. 2015). In addition to the crRNA and repair oligonucleotides for target genes snrp-27 and mab-10, we used co-CRISPR of dpy-10 II to the dominant roller cn64 allele for snrp-27 I mutagenesis or co-CRISPR of unc-58 X to the dominant paralysis e665 allele for mab-10 II mutagenesis using previously described crRNA and repair oligonucleotide sequences (Arribere et al. 2014). We prepared RNP complexes by incubating crRNAs and tracrRNA in 10 µl mixtures containing 500 pmols of tracrRNA, 450 pmols of target gene crRNA, and 50 pmols of co-CRISPR target crRNA (dpy-10 or unc-58). All RNAs in the mixture were synthesized by IDT using their proprietary Alt-R 5’ and 3’ end modifications to increase stability and had been resuspended at 100 µM concentration in IDT-supplied
Nuclease-Free Duplex buffer prior to mixing. The crRNA/ tracrRNA mixtures were heated to 95°C for 2 min followed by a 5 min incubation at room temperature. Five microliters of annealed RNA mixture were mixed with 5 µL of 40 µM S. pyogenes Cas9-NLS protein (in 20 mM HEPES-KOH pH 7.5, 150 mM KCl, 10% glycerol, 1 mM DTT), which was purchased from the QB3 MacroLab core facility at UC Berkeley. The cas9/cRNA/ tracrRNA mixture was incubated for 5min at room temperature to form RNP complexes. Then, 1 µL of 40 µM co-CRISPR repair oligo [either for dpy-10(m64) or unc-58(e665)] and 2 µL of 40 µM target gene repair oligo were added to make the injection mixture. The mixture was centrifuged at 13,000 g for 5min to clear any insoluble contaminants prior to filling an injection needle with the supernatant. In a typical experiment, 50 young adult C. elegans hermaphrodites were injected in their syncytial gonad with this mixture [Bristol N2 wild-type worms in all cases with the exception of the mab-10 mutagenesis experiment in which S211 snpr-27[aj256] worms were initially injected]. The P0 injected adults were allowed to recover for 4 h and each was placed on its own NGM agar plate. F1 progeny were screened for the dominant co-injection phenotype, and these were put onto individual plates and allowed to lay embryos. Then the adult was removed and DNA extracted from the single worm, and subject to PCR with primers spanning the targeted region of interest. Restriction digests were performed on the PCR products allowing us to identify F1s heterozygous for the mutation. F2 progeny of these heterozygous F1s that were wild-type in movement (had segregated away the unlinked dominant co-CRISPR allele) were followed by this same method in order to identify homozygous mutants in the target gene. The PCR products of the homozygous alleles were then subjected to Sanger sequencing methods to identify the new alleles of the target genes.

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