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Intron 7 conserved sequence elements regulate the splicing of the SMN genes

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Abstract

Proximal spinal muscular atrophy (SMA) is a neuromuscular disease caused by low levels of the survival motor neuron (SMN) protein. In humans there are two nearly identical *SMN* genes, *SMN1* and *SMN2*. The *SMN2* gene generates a truncated protein, due to a C to T nucleotide alteration in exon 7, which leads to inefficient RNA splicing of exon 7. This exclusion of *SMN* exon 7 is central to the onset of the SMA disease. Exon 7 splicing is regulated by a number of exonic and intronic splicing regulatory sequences and the trans-factors that bind them. Here we identify conserved intronic sequences in the *SMN* genes. Five regions were examined due to conservation and their proximity to exons 6 through 8. Using mutagenesis two conserved elements located in intron 7 of the *SMN* genes that affect exon 7 splicing have been identified. Additional analysis of one of these regions showed decreased inclusion of exon 7 in *SMN* transcripts when deletions or mutations were introduced. Furthermore, multimerization of this conserved region was capable of restoring correct *SMN* splicing. Together these results describe a novel intronic splicing enhancer sequence located in the final intron of the *SMN* genes. This discovery provides insight into the splicing of the *SMN* genes using conserved intronic sequence as a tool to uncover regions of importance in pre-messenger RNA splicing. A better understanding of the way *SMN* pre-mRNA is spliced can lead to the development of new therapies.

Keywords

Spinal Muscular Atrophy (SMA); Survival Motor Neuron (SMN); pre-mRNA Splicing; Intronic Splicing Enhancer

Introduction

Proximal Spinal Muscular Atrophy (SMA) is a common neuro-degenerative disease, occurring in approximately 1 in 6,000 live births. The disease is characterized by the loss of alpha-motor neurons resulting in progressive muscle atrophy and eventually leading to paralysis and death. Homozygous loss of the *Survival Motor Neuron-1 (SMN1)* gene is responsible for greater than 98% of SMA cases (Lefebvre et al. 1995). *SMN1* is located on chromosome 5q13, and produces a ubiquitously expressed protein (Lefebvre et al. 1995; Novelli et al. 1997).

There is, however, a nearly identical gene located on chromosomal segment 5q13, *SMN2*, which can produce a protein with identical function of *SMN1* (Lefebvre et al. 1995). There are only a small number of nucleotide differences between *SMN1* and *SMN2*, most of which have been shown to have no effect on *SMN* levels or protein function. However, a C to T translationally silent nucleotide difference within *SMN* exon 7 causes this gene to be improperly spliced thereby excluding exon 7 in the majority of *SMN2* derived transcripts. Although the majority of the *SMN2* pre-mRNA results in transcripts lacking exon 7, *SMN2* does produce a small amount of full-length transcript and thus protein (Lorson et al. 1999; Monani et al. 1999). Disease symptoms can show varied intensities with lower levels of *SMN* protein resulting in a more severe disease phenotype, and complete absence of the *Smn* gene is embryonic lethal in mice. (Coovert et al. 1997; Lefebvre et al. 1997; Schrank et al. 1997). While *SMN2* is capable of generating full-length *SMN2* protein, the levels it produces are insufficient to prevent the onset of disease. Since *SMN2* is capable of producing full-length functional *SMN*, and increased levels of *SMN2* would be predicted to lessen the severity of the disease, therapeutics aimed at increasing the levels of full length *SMN* mRNA and protein are actively being investigated. Understanding the regulation of the events in *SMN* splicing can therefore lead to the generation of new therapeutic options in the treatment of SMA.

Various cis-elements contained within exons and introns are essential for the proper regulation of pre-mRNA splicing. Splicing elements that enhance exon inclusion are called the exonic or intronic splicing enhancer (ESE or ISE) depending upon their location within the gene. Additionally, elements can also interfere with inclusion of the exon and are likewise called exonic or intronic splicing silencers (ESS and ISS) (Black 2003). *SMN* exon 7 pre-messenger RNA (pre-mRNA) splicing is extensively regulated through a complex interaction between positive and negative RNA splicing elements. Splicing enhancer and silencer proteins serve as direct or indirect binding partners in the *SMN* genes. The splicing regulatory proteins SF2/ASF, hnRNP A1, Tra2B1, SRp30c, hnRNP-G, and hnRNP-Q have all been shown to bind some of these exonic regions to affect *SMN* exon 7 splicing (Hofmann et al. 2000; Cartegni et al. 2002; Hofmann et al. 2002; Young et al. 2002; Kashima et al. 2003; Chen et al. 2008). Additionally, RNA binding proteins FUBP, PTB, and hnRNP A1 have also been shown to bind regions of intronic RNA that influence *SMN* splicing (Kashima et al. 2007; Hua et al. 2008; Baughan et al. 2009).

While only the human genome harbors the duplication and mutation event that created the *SMN2* gene, the *Survival Motor Neuron* gene is evolutionally conserved. DiDonato et al. showed that the exon of the mouse and human *SMN* genes are highly conserved and this conservation leads to a similar conservation in the amino acid sequence with nucleotide sequence of exon 7 being 81% conserved between mouse and human and the amino acid sequence being 75% conserved between mouse and human (DiDonato et al. 1997). While the C>T alteration is not normally found in the mouse genome, it has been shown that introduction of a C>T mutation into a mouse minigene model of *Smn* cause a similar disruption of *Smn* splicing and leads to the increased exclusion of the mouse *Smn* exon 7 (DiDonato et al. 2001, Gladman and Chandler unpublished data). Knowing that the *SMN2* gene splicing can be recapitulated in the *Smn* genes of other organisms suggests that other splicing elements located in the *SMN* genes might also be conserved. To date, no search of the conserved intronic regions of the *SMN* gene has been undertaken. As organisms evolve their genomes change. Sequence homology between species is, to a large degree, most common in exonic sequences that code for amino acids. Intronic regions often show less homology since they play only a minor role in the actual properties of the resulting protein. Conserved regions in introns and non-coding exons often indicate a functional significance for gene regulation. By comparing the *SMN* gene in seventeen different species, conserved intronic sequences were identified in intron 6 and intron 7 of the *SMN* genes. Here we use

the human *SMN* sequence to assess the role of these conserved, yet previously unexamined, regions in the regulation of pre-mRNA splicing of the *SMN* genes. In this manuscript we report the identification of five conserved intronic elements in the *SMN* gene, two of which we show to have a regulatory role in the splicing of *SMN*.

Materials and methods

Generation of splicing competent minigenes

The pSMN-1 minigenes were constructed to contain the wildtype human genomic *SMN1* fragment containing exon 6, exon 7, and exon 8 and their corresponding intronic sequences. This was achieved by PCR amplification using high fidelity taq polymerase (Roche, Indianapolis, IN, USA) and hSMN X6 FW primer (5'-ATAATTCCCCCACCACCTC-3') and hSMN X8 RV primer (5'-CACATACGCCTCACATACA-3') cloning into Stratagene's (La Jolla, CA, USA) CMV-2B vector. Site directed mutagenesis was then performed to convert the exon 7 C>T using QuikChange XL Site Directed Mutagenesis Kit (Stratagene) to create the SMN2-like minigene designated pSMN-2. The CMV-2B vector was chosen so the minigene would contain the vector specific 5' FLAG tag sequence. This unique sequence can be used during PCR experiments to avoid the detection of endogenous mouse or human *SMN* transcripts. Dr. Ravindra Singh provided the Casp3Avr minigene and Casp3Avr I7-1 was generated using QuikChange XL Site Directed Mutagenesis Kit (Stratagene). Deletion and mutations in the SMN vectors were generated used QuikChange XL Site Directed Mutagenesis Kit (Stratagene). Mutagenesis primers used are listed in Supplemental Table 1. All minigenes were sequenced to confirm desired mutations.

Sequence alignment for identification of evolutionarily conserved sequences

The May 2004 genomic assembly of the human *SMN* gene was accessed on the UCSG Genome Browser (genome.ucsc.edu). The species conservation tracks showing the alignments were obtained through the conservation link comparing the *SMN* genes of seventeen different species. While the sequence elements were not equally conserved in every organism examined, genomic blocks that were present in both human and mouse and located in intron 6 and intron 7 were selected for analysis as the C>T alteration in mouse and human leads to altered exon 7 splicing. We also produced an alignment of these conserved elements discussed in this manuscript using the UCSG Genome Browser March 2006 comparing the human genomic assembly to 9 other species to display the levels of conservation.

Cell Culture

Unless otherwise stated, all tissue culture media and supplements were purchased from Invitrogen (Carlsbad, CA, USA). Human embryonic kidney 293 (HEK-293) cells (American Type Culture Collection, Manassas, VA, USA), mouse motor neuron-like (NSC-34) cells, non-small cell lung carcinoma (H1299) cells (American Type Culture Collection), and mouse myoblast (C2C12) cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Dr. Brian Kaspar generously provided the NSC-34 cells.

Transfection and *in vivo* splicing assays

All reagents were used according to the manufacturer's recommendations. Transient transfections of cells with plasmid DNA were performed with Fugene 6 (Roche). Cells were plated 24 h prior to transfection so that their density on the day of transfection was ~60%. Unless indicated otherwise, total RNA was isolated 24 h after transfection with RNeasy

Mini Kit (Qiagen, Valencia, CA, USA). To generate cDNA, reverse transcription was carried out with Transcriptor Reverse Transcriptase (Roche) using a random primer p(dN)₆. Generally, 5.0 µg of total RNA was used per 20 µl of reaction mixture. Minigene specific spliced products were subsequently amplified with Taq polymerase (Sigma, St. Louis, MO, USA) and the following primer combinations: FLAG (5'-GATTACAAGGATGACGACGATAAG-3') and hSMN X8as1 (5'-CCCTTCTCACAGCTCATAAAATTAC-3') for SMN minigenes. Casp3 splicing products were amplified using Modified P55 primer (5'-GCCGCATCCTTTGAATTTGCGCAAG-3') and P1 primer (5'-CGACTCACTATAGGCTAGCC-3'). Analysis and quantifications of spliced products were performed using ImageQuant 5.2 (GE Healthcare Live Sciences, Piscataway, NJ, USA). Results were confirmed by a minimum of three independent experiments.

Results

Generation of an *SMN* minigene and Identification of Conserved Elements

SMN splicing competent minigenes were generated for use in transfection experiments (Fig. 1a). These minigenes, when transfected into cultured cells, demonstrated the characteristic splicing observed in the *SMN* genes with the pSMN-1 minigene producing all full-length transcript and the pSMN-2 minigene generating up to 70% exon 7 skipped isoform (Fig. 1b). Regions of *SMN* intronic sequence homology were identified using the University of California Santa Cruz Genome Browser. A multispecies alignment of the conserved elements identified revealed varying levels of conservation (Supplemental Fig 1 and Supplemental Tables 2–6). Since the C>T alteration in mouse and human can lead to exon 7 splicing defects, conserved sequence blocks located in intron 6 and intron 7 in both human and mouse were selected for further analysis. Using this technique five regions of homology were selected (Fig. 1c). Three were identified in intron 6. I6-1, a 29 nucleotide long sequence, is located in close proximity to exon 6. In the middle of intron 6 the second conserved region, I6-2, was identified containing 150 nucleotides. Finally I6-3, also located in intron 6, is 20 nucleotides long and located near the polypyrimidine tract near the intronic boundary of exon 7. In intron 7 two previously unexamined regions were identified, I7-1 which is 31 nucleotides long and I7-2 which was 60 nucleotides long. Both were found within 150 nucleotides of exon 7.

Deletion Analysis of Conserved Regions

To determine the effect of these conserved regions on the splicing of *SMN*, site directed mutagenesis was used to individually delete the conserved regions in the minigene constructs. The mutated constructs were then transfected into HEK 293 cells, which have been used previously to study splicing of the *SMN* genes, and the splicing assessed by RT-PCR. Deletions of the intron 6 regions (I6-1, I6-2 or I6-3) displayed no significant change in their splicing ratios as compared to wildtype pSMN-1 or pSMN-2 control minigenes (Fig. 2a–c). When I7-1 or I7-2 was deleted in the minigene system an alteration in *SMN* splicing ratios was observed (Fig. 2d–e). Deletion of I7-1 leads to a 42% increase in exon 7 skipping of the pSMN-1 minigene and complete skipping in the pSMN-2 minigenes. The I7-2 region, when deleted also showed an increase in the exon 7 skipped isoform but only in the pSMN-2 minigene. This would suggest that both I7-1 and I7-2 act as intronic splicing enhancers (ISEs). Due to its robust alteration of splicing seen in the deletion of I7-1 further analysis was conducted to better elucidate its role as a cis-element in RNA splicing. Similar to the HEK 293 cell line, all cell types investigated supported exon 7 exclusion in our I7-1 deletion mutations. Both a neuronal (NSC-34) and muscle (C2C12) cell line were utilized as these tissue types most effected in the SMA disease. These results suggest that I7-1 mediated splicing of exon 7 is not cell type specific (Fig. 2g).

I7-1 Acts as an Intronic Splicing Enhancer

Intron 7 of the *SMN* gene has previously been characterized for intronic splicing elements. Two important regulatory elements, the intronic splicing silencer, ISS-N1, and the intronic splicing enhancer element 2, which has been shown to form a stem loop structure, have been identified (Miyaso et al. 2003; Singh et al. 2006). The I7-1 region is located between these two previously identified splicing regulatory regions. Located 5' of I7-1 is the intronic splicing silencer ISS-N1 and located 3' of I7-1 is the intronic splicing enhancer element 2. The deletion of I7-1 in our minigene would have the consequence of bringing ISS-N1 and element 2 in closer proximity to each other, which could potentially lead to the changes in splicing we observed. To determine if the observed changes in splicing was the result of the I7-1 sequence itself, the native sequence was replaced with a neutral sequence containing no putative SR proteins as predicted using the ESEFinder 3.0 program (Fig. 3a) (Cartegni et al. 2003). Minigenes containing this neutral filler sequence continued to show a pronounced increase in exon 7 skipping in both *SMN* minigenes, with the pSMN-1 minigene skipping exon 7 in 45% of the transcripts and pSMN-2 producing only exon 7 skipped transcripts. These results indicate that the observed changes in splicing were not the result of changing the distance between the two previously identified splicing elements, but rather determined by the specific sequence of I7-1 (Fig. 3b). This further suggests that the I7-1 sequence contains a cis-element essential to the proper splicing of the *SMN* gene.

In an attempt to determine the relevant region of the I7-1 regulatory element small deletions were made and tested using the *in vivo* splicing assay (Fig. 3a). The region of I7-1 was divided into 3 portions and each was deleted from the pSMN minigenes and used in our *in vivo* transfection assay. Further investigation found that deleting these smaller portions of the I7-1 did not result in as severe of a splicing phenotype as seen with the complete deletion of I7-1 or the replacement of I7-1 with the neutral filler sequence. Partial deletion 1 (pΔ1 I7-1) and partial deletion 2 (pΔ2 I7-1) of I7-1 caused no change in splicing as compared to our wildtype minigenes. However partial deletion 3 (pΔ3 I7-1) resulted in a 22% increase in exon 7 skipping (Fig. 3c) with 87% of the transcripts generated lacking exon 7. This increase is only observed in the pSMN-2 minigene, but to a lesser extent than generated when the whole I7-1 sequence was deleted. None of the smaller deletions were capable of causing the changes in the pSMN-1 minigene, which continued to produce only full-length transcript.

While the changes present were less severe in the pΔ3 I7-1 minigene as compared to the complete deletion of I7-1, the increase in skipping observed still indicates that this region of I7-1 was necessary for the correct regulation of the *SMN* transcript. To better characterize this ten nucleotide region at the end of I7-1, four nucleotide alterations were made in the minigene (Fig. 3a) and used in the *in vivo* splicing assay. Disruption of these regions showed that the last seven nucleotides were essential in regulating the splicing of the *SMN* gene as their disruption resulted in an increase of exon 7 skipped transcript (Fig. 3d).

To test whether the last 7 nucleotides of I7-1 could activate the splicing of the *SMN* exon 7 containing the C to T transition three tandem repeats of these nucleotides were inserted into the minigene (Fig. 4a). The insertion of the sequence in multiple repeats increased the inclusion of *SMN* exon 7 to full-length levels (Fig. 4b). Altogether, these data demonstrate that I7-1 is a new cis-element that acts as an intronic splicing enhancer for *SMN* and that the last seven nucleotides of I7-1 are capable of correcting *SMN* splicing levels when repeated in tandem.

To examine the role of I7-1 outside of the *SMN* genes we inserted the I7-1 sequence in a heterologous context. We used a Casp3Avr minigene that recapitulates the partial skipping of the endogenous caspase 3 gene that has been used previously to examine the splicing of

intronic splicing regulators of the SMN gene (Singh et al. 2006). When I7-1 was inserted downstream of the alternatively spliced exon we saw a significant increase in exon included transcript further defining the sequence of I7-1 as an intronic splicing enhancer (Figure 5a). An *in silico* search of predicted binding proteins was done using multiple online programs. Using ESEFinder 3.0, RegRNA, and ESRsearch on the I7-1 sequence revealed multiple RNA binding proteins (Figure 5b) (Cartegni et al. 2003; Zhang et al. 2004; Huang et al. 2006). In addition to these predicted binding proteins, the rich A/T content of this region could suggest a role of any of the KH domain containing RNA binding proteins. A genome wide search of I7-1 revealed that while the whole 31 nucleotide sequence is not found elsewhere in the human genome, fragments of I7-1 could be found in multiple protein coding RNA transcripts (Figure 5c). These sequences were found in both exonic and intronic sequence. While many of the genes examined encode multiply spliced isoforms, the I7-1 fragments were not associated with any exon known to be alternatively spliced. If the role of I7-1 is necessary for constitutive splicing in the genes examined, its function may only be elucidated if mutations were found in these sites to affect splicing. It is interesting to note that three of the genes identified (*ACTR3*, *SKAP1*, and *NEK11*) contain I7-1 sequence elements downstream of a 5' splice-site, as seen in the *SMN* genes.

Discussion

The human genome harbors two copies of the essential gene, *survival motor neuron*. *SMN1* mRNA expresses full-length transcript, while *SMN2* produces only low levels of full-length transcript. The critical difference between *SMN1* and *SMN2* is a silent nucleotide transition in *SMN* exon 7 (Lefebvre et al. 1995; Lefebvre et al. 1998). We have examined conserved regions present the *SMN* genes in an attempt to identify additional cis-acting elements responsible for the correct splicing of the *SMN* exon 7 containing the C to T transition. In this manuscript five regions were identified that appear to be important regulatory elements due to their level of conservation and their proximity to exons 6 through 8. Mutagenesis of our *SMN* minigenes has identified two conserved elements that effect exon 7 splicing, I7-1 and I7-2. Additional analysis of the sequence of I7-1 demonstrated that this element acts as an intronic splicing enhancer because of the dramatic decrease in *SMN* transcripts that include exon 7 is observed when it is deleted or mutated in the minigene.

Splicing enhancers have an important function in pre-mRNA splicing. Spliced RNAs are regulated by these important cis-elements and the trans-factors that bind them leading to greater transcript diversity and consequently more protein diversity. We hypothesize that I7-1 acts as a cis-element in which a yet unidentified protein binds and facilitates the correct splicing of the *SMN* gene, perhaps by helping define exon 7 to the splicing machinery through direct interaction with the exonic splicing machinery or through interaction with other known intron 7 splicing regulators (Fig 6). In this way ISE I7-1 could facilitate the inclusion of exon 7 into the *SMN* transcript. Since the last nucleotides of I7-1 were capable of correcting splicing of the *SMN* minigene when multiple copies of the element are included in tandem in the pre-mRNA, it is likely that at least one important trans-factor binds this region to facilitate more efficient splicing. Its nearness to element 2, a previously identified structural ISE, could suggest that the two may function together to regulate *SMN* splicing. This region of the intron could represent a “hot spot” in RNA exon 7 splicing regulation, as three splicing elements ISS-N1, ISE I7-1, and Element 2 have all been found in close proximity to each other and exon 7.

This close proximity and the competing functions of known elements located in intron 7 (ISS-N1 acts as a silencer while ISE I7-1 and element 2 functions as enhancers) suggests that intron 7 plays a powerful role in regulating *SMN* exon 7 splicing. Additionally, these elements or the proteins that bind them may be interacting in a cooperative or antagonistic

fashion to regulate *SMN* splicing. Any factors that bind ISE 17-1 may be involved in the steric hindrance of proteins that bind ISS-N1. In this proposed model ISE 17-1 acts as an enhancer through the inhibition of the strong intronic silencer ISS-N1 (Fig 6, model 2). Another possibility is that ISE 17-1 is involved in the regulation of the RNA folding of the *SMN* intron and could be important in the correct formation of element 2, the known structural enhancer located 3' of ISE 17-1. In this proposed model ISE 17-1 would act as an enhancer by stabilizing the structure intronic splicing enhancer element 2 (Fig 6, model 3). Finally ISE 17-1 could be regulating splicing in a fashion that doesn't directly involve either ISS-N1 or element 2 but instead facilitates splicing through the binding of a protein that directly alters the pre-mRNA splicing architecture of the *SMN* genes (Fig 6, model 1). Additionally since none of these models is mutually exclusive, ISE 17-1 could be involved in all of these functions through the interaction of one or multiple proteins. Further characterization of the interaction between splicing regulators located in intron 7 will help elucidate the actual role that ISE 17-1 plays in pre-mRNA splicing.

While only two of the conserved regions we identified affected the splicing of the *SMN* genes in the cell-lines analyzed, we cannot exclude that the three remaining conserved regions have an important function in regulating the *SMN* pre-mRNA. Additionally these regions may contain sequence elements that facilitate *SMN* splicing that are not detectable by simple deletion from our minigenes system due to redundant elements located elsewhere in the gene. These regions could play a role in RNA stability, localization, or splicing under specific conditions that are unknown and not tested here. Stability and localization have been shown to be important for regulation of other RNA transcripts. The RNA-binding protein HuR has been shown to play a major role in regulating p21 expression by enhancing p21 mRNA stability after stress (Wang et al. 2000). It is possible, then, that the *SMN* genes could be similarly regulated after stress, during development, or in a tissue specific manner by the conserved regions that are present in the gene.

The existence of the *SMN2* gene as a potential therapeutic target in SMA makes understanding the regulation of *SMN* pre-mRNA splicing vital in developing new tools to treat this disease. Currently research is being done using small oligos to improve the outcome of SMA through correction of splicing. This research has shown that introns 6 and 7 can be targeted by small oligos to improve exon 7 splicing in *in vitro* and in an *in vivo* mouse model of SMA (Hua et al. 2008). Additionally, the use of trans-splicing RNAs has been shown to increase *SMN* full-length transcripts (Coady et al. 2007). Development of more effective small oligo therapies can be achieved by gaining a better understanding of the RNA splicing architecture of the *SMN* genes. Understanding the dynamic interactions involved in the regulation of the *SMN* RNA will assist in finding a treatment for this disease.

In summary, we found two elements located in intron 7 of the *SMN* genes that enhance the splicing of *SMN* exon 7 containing the C to T alteration. These findings bring new insight into our understanding of human *SMN* splicing and provide a deeper understanding for the correction of genes associated with disease. However, in diseases like SMA a better understanding of the way pre-mRNA is spliced can lead to the development of new therapies designed at increasing full length transcript by exploiting the already present cis-elements and the proteins that bind them.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 2. Deletion of conserved regions located in intron 7 of *SMN* lead to decreased full length *SMN* transcript. (a–e) pSMN minigenes were generated lacking the specific deletions of the conserved regions located in intron 6 and intron 7 and transfected into HEK 293 cells. After 24 hr the RNA was harvested and splice products visualized by RT-PCR. Deletion of I7-1 and I7-2 altered the splicing ratio as compared to wildtype controls. (f) Graphic representation of the observed splicing changes displayed as percent change of full-length transcript in both minigenes. Analysis and quantifications of spliced products was performed using ImageQuant 5.2. Results were confirmed by a minimum of three independent experiments. (g) *In vivo* splicing pattern of I7-1 deletions in the mouse motor neuron-like (NSC-34) cells, non-small cell lung carcinoma (H1299) cells, and mouse myoblast (C2C12) cells

Fig. 3. I7-1 acts as an intronic splicing enhancer. (a) Schematic of the deletions or mutations made in the pSMN minigene vectors with dashed lines representing deletions and the presence of a grey boxes showing a change from the wildtype sequence. (b) Splice products generated by deletions or replacement of I7-1 with a neutral filler sequence. (c) Splice products generated by smaller stepwise deletions of I7-1. (d) Splice products generated by mutations in the last ten nucleotides of I7-1. (e) Graphic representation of the observed splicing changes displayed as a percent change from full length transcript in both minigenes. Analysis and quantifications of spliced products was performed using ImageQuant 5.2. Results were confirmed by a minimum of three independent experiments

Fig. 4. Tandem repeat of the 3' portion of I7-1 enhances the splicing of *SMN* exon 7. (a) Schematic of the deletions or mutation made in the pSMN minigene. The black box represents the 3' portion of I7-1. (b) Splice products generated by the tandem repeats. Analysis and quantifications of spliced products was performed using ImageQuant 5.2. Results were confirmed by a minimum of three independent experiments

Fig. 5.

Heterologous context of I7-1. (a) The I7-1 sequence was inserted downstream of exon 6 in the Casp3Avr splicing minigene with the black box represents the I7-1. The splice products generated in both minigenes were analyzed and quantifications of spliced products were performed using ImageQuant 5.2. The insertion of I7-1 increased full length transcript. Results were confirmed by a minimum of three independent experiments. (b) Potential I7-1 interacting proteins as predicted by multiple predictive programs. (c) A BLAST search of I7-1 sequence with a schematic of the location of I7-1 sequence shown as a black box and the actual sequence depicted to the right of each graphic. The genes which contain potential I7-1 elements are *epidermal growth factor receptor (EGFR)*, *armadillo repeat-containing protein 2 (ARMC2)*, *actin-related protein 3 (ACTR3)*, *src kinase-associated phosphoprotein 1 (SKAP1)*, and *serine/threonine-protein kinase Nek11 (NEK11)*

Fig. 6.

Hypothesized models of I7-1 as an ISE. Model of I7-1's effect as an ISE with a protein, represented by an ellipse (+), likely binding to the 3' end of I7-1 which is represented by the hatched box. This protein would bind I7-1 and be required for correct splicing of the *SMN* pre-RNA. In model 1 ISE I7-1 works independently on the 5' splice site to enhance exonic inclusion. In model 2 ISE I7-1 inhibits the effect of the known intronic splicing suppressor ISS-N1 and thus enhances exon 7 inclusion. In model 3 ISE I7-1 enhances the known structural intronic splicing enhancer Element 2 and thus enhances exon 7 inclusion