

SMN regulation in SMA and in response to stress: new paradigms and therapeutic possibilities

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Abstract Low levels of the survival of motor neuron (SMN) protein cause the neurodegenerative disease spinal muscular atrophy (SMA). SMA is a pediatric disease characterized by spinal motor neuron degeneration. SMA exhibits several levels of severity ranging from early antenatal fatality to only mild muscular weakness, and disease prognosis is related directly to the amount of functional SMN protein that a patient is able to express. Current therapies are being developed to increase the production of functional SMN protein; however, understanding the effect that natural stresses have on the production and function of SMN is of critical importance to ensuring that these therapies will have the greatest possible effect for patients. Research has shown that SMN, both on the mRNA and protein level, is highly affected by cellular stress. In this review we will summarize the research that highlights the roles of SMN in the disease process and the response of SMN to various environmental stresses.

Introduction

Protein coding genes account for an estimated 20,000 genes in the human genome; however, the sheer number of protein isoforms dramatically overshadows this number. A major source

of protein diversity is alternative splicing of mRNA. Splicing is the process by which immature pre-mRNA is processed to remove introns or non-protein-coding regions. Alternative splicing defines the system by which mature mRNA arising from a common gene can vary from transcript to transcript. This occurs by inclusion or exclusion of elements within the pre-mRNA, resulting in alternative protein-coding message. Therefore, alternative splicing generates protein diversity through interpreting a single genomic sequence in many different ways through alternative inclusion or exclusion of mRNA elements, known as exons. Ultimately, the inclusion and exclusion of exons lead to different protein isoforms arising from the same pre-mRNA transcript. Deep-sequencing of RNA has revealed that alternative splicing occurs in mRNAs from 95–100% of human protein coding genes that contain more than one exon, underscoring the importance of this process in development, cellular homeostasis and, when disrupted, in disease (Gerstein et al. 2014; Wang et al. 2008; Pan and Shai 2008; reviewed in Nilsen and Graveley 2010).

mRNA splicing is an essential step in normal gene expression that relies on a highly complex and dynamic mechanism comprising multiple *cis*- and *trans*-acting factors; thus, mutations in any one of these key elements may have profound effects on the health of the cell. The occurrence of mRNA splicing aberrations that contribute to a range of human disorders is well documented, with cancer, muscular dystrophy, retinitis pigmentosa, cardiomyopathies, amyotrophic lateral sclerosis, and spinal muscular atrophy among the most prominent examples (reviewed in Scotti and Swanson 2016).

Spinal muscular atrophy (SMA): the disease

Spinal muscular atrophy is an autosomal recessive pediatric neurodegenerative disease in which splicing alterations are

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associated with disease pathology. SMA is characterized by degeneration of alpha spinal motor neurons, which results in the denervation of target muscles, that consequently atrophy due to lack of stimulation. Interestingly, though all cells in an SMA patient exhibit decreased levels of the survival of motor neuron (SMN) protein, mainly motor neurons and certain populations of cortical neurons appear to be severely affected by this deficit (d'Errico et al. 2013). Due the pronounced effects of motor neuron degeneration in patients, much of the research on SMN deficiency has focused on these cells in SMA. However, with the development of numerous mouse models of SMA, there is growing evidence that other cell types and tissues are affected by the disease (reviewed in Shababi et al. 2014; Nash et al. 2016). While both males and females are affected by SMA, there is evidence supporting the existence of sex-specific manifestations of the disease that have not been extensively explored. Both humans and mouse models of SMA exhibit developmental defects in the male reproductive tract, with male sterility being observed in some mouse models (Howell et al. 2017; Riessland et al. 2010; Richert et al. 1986). While the genetic etiology of SMA is relatively simple, the reason that some cell types exhibit increased susceptibility to SMN deficiency is not completely understood. To approach this question, it will be important to consider the specific microenvironment of these cell types and to identify circumstances, such as cellular stress responses, that could exacerbate the phenotype in this context.

SMN is encoded by two genes in humans: *SMN1* and *SMN2*. The two genes are nearly identical, but a single base substitution in exon 7 accounts in large part for a deviation in the mRNA splicing patterns of the two genes (Lorson 1999; Cartegni and Krainer 2002; Kashima and Manley 2003; Lefebvre et al. 1995; Monani 1999) (Fig. 1). mRNA processing requires the mRNA message to be configured correctly by the spliceosome, which removes introns and determines which exons will be included in the final message. These decisions are determined by splice site strength, supportive or inhibitive sequences within the transcript, known as enhancers or repressors, respectively, and the availability and activity of protein accessory factors that bind these sequences. At the DNA level, the 6th nucleotide of exon 7 in *SMN1* is a cytosine that encodes an mRNA splicing enhancer sequence that functions in spliceosome recruitment and facilitates exon recognition. The respective residue in *SMN2* is a thymine, which alters the enhancer to become a repressor in the resultant mRNA, which blocks the spliceosome from including the exon (Lorson 1999; Cartegni and Krainer 2002; Kashima and Manley 2003; Lefebvre et al. 1995; Monani 1999). While the C to T substitution between *SMN1* and *SMN2* exon 7 is translationally silent, the result is an mRNA splicing pattern in which over 90% of *SMN1* transcripts include exon 7, while a substantial portion of

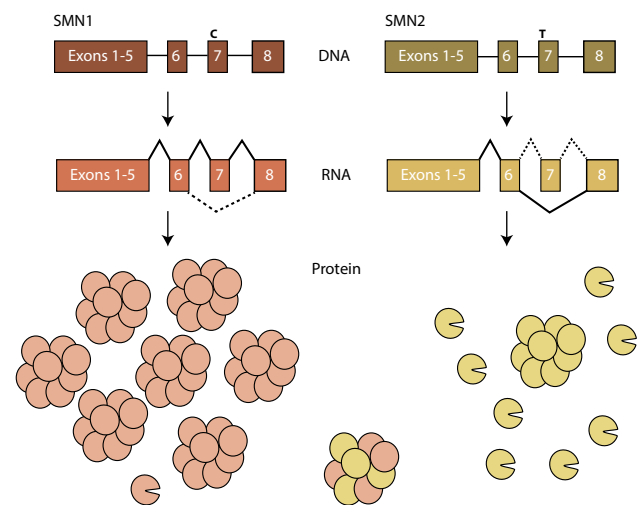


Fig. 1 *SMN1* and *SMN2* splicing. *SMN1* and *SMN2* genes contain a C to T nucleotide alteration in exon7. This alteration changes the splicing of the pre-mRNA, leading to exon inclusion in *SMN1* and predominant exon exclusion in *SMN2*. This alters the isoforms of protein produced, with *SMN1* producing mainly full-length protein, which oligomerizes and is stable, and *SMN2* producing mainly truncated proteins (depicted by missing wedge), which do not oligomerize and are rapidly degraded. The small amount of full-length protein that can be produced from *SMN2*, however, is identical to *SMN1* and the two populations can interact (figure adapted from Burghes and Beattie 2009)

SMN2 transcripts lack exon 7, depending on cell type and environmental context. This splicing alteration is functionally significant as transcripts lacking exon 7 produce truncated and unstable protein, which ultimately results in lower levels of functional SMN arising from the *SMN2* locus (Pelizzoni 1999; Burnett et al. 2009; Lorson 1998). While the role of the exon 7 C>T transition in causing exon exclusion is clear, there are many other sequences within *SMN1* and *SMN2* that also influence the final spliced products of the *SMN* transcripts. These regions of influence can extend a considerable distance from the exon itself and exert their effect through pre-mRNA secondary structure that modulates spliceosomal activity (Singh et al. 2015). These modes of splicing modulation are reviewed extensively by Singh et al. (2017b). Indeed, as the field advances, the spectrum of SMA-causing splicing mutations continues to broaden and we likely will continue to discover more sites of splicing modulation within *SMN1* and *SMN2*.

SMA is caused by mutation or deletion of *SMN1* and retention of *SMN2* (Lefebvre et al. 1995). The complete absence of SMN protein is embryonic lethal, but the presence of *SMN2* in the absence of *SMN1* in humans results in low levels of SMN protein, giving rise to SMA. A correlation between SMA severity and SMN protein levels has been observed both in SMA patients and in mouse models of SMA (Harada et al. 2002; Lefebvre et al. 1997; Hsieh-Li

et al. 2000; Monani et al. 2000; Coover et al. 1997). SMN levels can vary widely among SMA patients and consequently, SMA is a disease of variable severity ranging from Type I, the most severe, with paralysis and death as early as birth, to Type IV, which is mild, with muscular weakness but no effect on life span. The most significant correlative factor across the spectrum of disease severity is the copy number of *SMN2* genes (McAndrew 1997; Burghes 1997). The *SMN2* gene locus is amplified in some individuals, with improved disease outcomes in those with higher copy numbers (McAndrew 1997; Burghes 1997). Furthermore, mouse models with higher copy numbers of a human *SMN2* transgenes exhibited increased life span compared to littermates with fewer copy numbers (Hsieh-Li et al. 2000; Monani et al. 2000; Le et al. 2005). Therefore, SMN levels directly correlate to disease phenotypes (Lefebvre et al. 1997; Coover et al. 1997). For individuals with fewer copies of *SMN2*, it is critical to understand how splicing of this transcript responds to more subtle cues, both for understanding the disease and also for developing therapeutics. Splicing is a dynamic and malleable process that is influenced not only by development and tissue-specific cues, but also by environmental stresses. Environmental stresses are known to activate stress pathways, which in turn activate signaling cascades. These signaling cascades are mediated largely through kinase activation, with phosphorylation playing a key role. Many *trans*-acting splicing factors can be phosphorylated, which affects their cellular localization and activity. This can in turn alter the activity of the key splicing machinery (Stamm 2008). We review here the effect of environmental stresses on mRNA splicing and protein modification. We will focus on these stresses in the scope of SMA pathology, but these effects may be relevant to any number of other diseases and splicing products.

SMA: SMN function and the etiology of the disease

Years of extensive research into how SMN deficiency causes SMA have produced an expanding list of cellular processes that involve SMN function. In addition to a host of relatively well-defined roles in RNA metabolism, SMN has been implicated in signal transduction, intracellular trafficking, DNA recombination, endocytosis and autophagy (reviewed in Singh et al. 2017a, b). Though the role of SMN is widespread and diverse, its most well characterized function in the cell is that of snRNP assembly.

SMN in snRNP assembly

SMN is required for mRNA processing in all cells. Genetic depletion of SMN protein levels is pre-implantation

embryonic-lethal in mouse (Schrank 1997), while in zebrafish and *Drosophila*, translation-blocking morpholinos or genetic mutation to decrease functional *Smn* levels results in larval lethality (and a portion of zebrafish embryo lethality occurring between gastrulation and somitogenesis) (McWhorter et al. 2003; Boon et al. 2009; Winkler et al. 2005; Hao et al. 2013; Rajendra 2007; Chan et al. 2003). These studies from distantly related organisms illustrate the highly conserved and developmentally essential nature of SMN.

SMN functions in the biogenesis of small nuclear ribonucleic proteins (snRNPs) that are building blocks of the splicing machinery (Eggert et al. 2006; Pellizzoni 2007; Gubitz et al. 2004; Pellizzoni et al. 2002; Meister et al. 2001; Fischer et al. 1997; Liu et al. 1997; Ogawa et al. 2009). RNA components of snRNPs, snRNAs U1, U2, U4, and U5 are assembled with Sm protein pentameric rings and U6 assembling with an LSm ring (Pellizzoni et al. 2002; Wan et al. 2005). These snRNPs are critical to carry out the catalysis of splicing and are assisted by the Sm ring, which are brought together and assembled by the SMN complex (SMN protein and Gemin proteins 2–8) (Pellizzoni et al. 2002; Meister et al. 2000; Otter et al. 2007; Carissimi et al. 2006a, b). Reduced SMN protein levels decrease snRNP assembly in vitro and in vivo, and the cases in which snRNP assembly is most dramatically decreased corresponds to the most severe disease types (Workman et al. 2009; Shpargel et al. 2005; Sun et al. 2005; Bühler et al. 1999; Monani et al. 2003; Ogawa et al. 2007; Kotani et al. 2007; Clermont et al. 2004; Cuscó et al. 2004; Parsons 1998). Hence, reduced SMN complex levels and activity is a well-established cause of SMA (Shpargel et al. 2005; Sun et al. 2005; Ogawa et al. 2007; Kotani et al. 2007; Clermont et al. 2004; Workman et al. 2009; Alías et al. 2009; Cuscó et al. 2004; Prior 2007; Bühler et al. 1999; Rochette et al. 1997; Lorson 1998; Hahnen et al. 1997; Wang et al. 1998; Talbot et al. 1997; Seng et al. 2015).

It was recently demonstrated that, in addition to SMN deficiency per se as a cause of SMA, missense mutations that alter the ability of Sm proteins to bind or be released from the SMN complex can also cause the disease. Structural analysis of reconstituted SMN complexes in vitro, using either wild type SMN or SMN (E134K), a common SMA-causing mutant isoform that substitutes a lysine for glutamate at residue 134, found that the wild-type and mutant complexes were indistinguishable (Sun et al. 2005; Neuenkirchen et al. 2015). Furthermore, both complexes bound Sm protein similarly. However, upon addition of U1 snRNA, the transfer of Sm ring to snRNA was severely diminished in the SMN (E134K) samples. The lower transfer efficiency was found to be due to a much higher dissociation constant (K_d) of SMN (E134K) compared to wild-type SMN, indicating tighter binding or misarrangement (Neuenkirchen

et al. 2015). These experiments suggest that, in addition to Sm assembly onto snRNPs, Sm dissociation during the RNA remodeling of the splicing process may also play a role in the SMA disease phenotype. It should be noted that these experiments were performed in vitro in the absence of ATP. While the formation of snRNP assembly may be ATP-independent in vitro (Neuenkirchen et al. 2015; Raker 1996, 1999; Sumpter et al. 1992), the complex is ATP-dependent in vivo (Kleinschmidt 1989; Meister et al. 2001; Temsamani et al. 1991; Meister and Fischer 2002; Pellizzoni et al. 2002; Meister et al. 2000; Otter et al. 2007; Carissimi et al. 2006a, b). Therefore, the results should be verified in vivo to conclusively determine the role of Sm dissociation in addition to the formation of the SMN complex and association with Sm protein.

SMN and the minor spliceosome

The improper formation of the core spliceosomal subunits is clearly a manifestation of decreased SMN protein levels characteristic of the SMA disease state. Although it seems logical that the snRNP deficiency would lead to improper splicing, the role of downstream splicing events in SMA disease progression remains controversial. It has been shown that gemin levels and snRNP assembly are decreased in spinal cord from severe SMA mice (*SMN2^{+/+}; mSnn^{-/-}*) at postnatal day 3 (pnd3) and correlate with disease severity. Interestingly, the reduced SMN levels and snRNP assembly preferentially affects the levels of the U11 snRNP, an essential component of the minor spliceosome (Turunen et al. 2013). This finding lends credence to the hypothesis that the minor spliceosome is preferentially affected in SMA and affects the removal of U12-type introns that comprise less than 1% of all introns in human cells (Gabanella et al. 2007).

In one study, the decrease in minor spliceosomal subunits was indeed linked to aberrant splicing of transcripts containing the U12-type subtype of introns in *Drosophila* third instar larvae expressing the *Snn^{73A0}* loss-of-function allele compared to their wild-type counterparts (Lotti et al. 2012). Eighteen of the twenty-three U12 intron-containing genes in *Drosophila* were mis-spliced in the *Snn^{73A0}* mutant. Mis-splicing of one of the affected U12 intron-containing genes, *stasimon*, correlated with motor neuron pathology, where knock down of *stasimon* resulted in NMJ dysfunction that was, in turn, rescued by restoring *stasimon* expression. Overexpression of *stasimon* was also sufficient to rescue axonal pathfinding and outgrowth defects in *Snn* knockdown zebrafish (Lotti et al. 2012). It should be noted, however, that *stasimon* expression was not able to rescue the locomotion or viability defects.

In contrast, an earlier study that examined *Drosophila* third instar larvae from a cross between a mutant line carrying the null allele *Snn^D*, caused by an insertion between

the start codon and the Tudor domain (Rajendra 2007), and a mutant line carrying the *Snn^{X7}* allele that is deleted for most of the *Snn* region (Chang et al. 2008), showed decreased minor and major spliceosomal components but did not observe the same splicing defect specifically of minor intron-containing mRNA (Praveen et al. 2012). Of seven transcripts that were also identified by Lotti et al. in the study described above, only 2 exhibited similar levels of aberrant intron retention in response to the reduced *Snn* level in *Snn^DxSnn^{X7}* larvae. Moreover, low level expression of a wild type SMN transgene could rescue motility and viability defects without restoring normal snRNA levels or snRNP assembly, suggesting independent roles for SMN in motor function and snRNP assembly (Praveen et al. 2012). It was further shown that the changes in U12-intron containing transcripts could more likely stem from a developmental delay in the *Snn* mutant larvae since the splicing pattern of the transcripts varied more with developmental time than *Snn* mutant status (Garcia et al. 2013). Currently, the debate over the role of misspliced transcripts in the etiology of SMA remains unresolved.

More recently, genome-wide studies of splicing defects were performed in two different SMA mouse models: the severe Taiwanese model at PND1 and PND5, and an ASO-inducible SMA mouse model induced at 8 weeks and analyzed at 20 and 30 days post SMN depletion (Jangi et al. 2017; Doktor et al. 2017). Both studies report splicing defects in all tissues analyzed, specifically aberrant intron retention of both major and minor introns. Intron retention increased over time and the transcriptional profile was recapitulated with siRNA knock down of SMN in human HeLa cells, arguing against the influence of mouse development in inducing these changes (Doktor et al. 2017). Although minor introns are present in less than 1% of all genes, the sensitivity of U12 introns to low levels of SMN protein is illustrated by these studies, as evidenced by the large proportion of the minor intron containing genes that undergo mis-splicing in SMA mice. The most common changes observed in spinal cord mRNA after SMA disease induction is intron retention, with U2-type intron retention in 4628 transcripts (3.54% of major introns) and U12-type intron retention in 152 transcripts (31.54% of minor introns) (Jangi et al. 2017). However, the fact that these U12 intron-containing transcripts are prone to mis-splicing in the presence of decreased SMN levels may be an association and not causative of disease, since the number of mis-spliced U2 intron-containing mRNAs far exceeds the number of U12 intron-containing transcripts. Therefore, more detailed study of the most highly affected transcripts will be necessary to determine their function and the relationship of the splice variants to SMA pathology. It will be important to ask whether the changes in splicing cause SMA disease phenotype or are a consequence of disease progression.

Additional roles for SMN

In addition to SMN localization in the cell body where snRNP assembly occurs, in neurons SMN is also found within dendrites and axons. This raises the question of whether SMN protein has additional roles in axons independent of snRNP assembly that could contribute to the SMA disease phenotype. In a maternal-zygotic *Smn* mutant zebrafish line, transgenic expression of *Smn* specifically in motor neurons, driven by the *mx1* or *hb9* promoter, is sufficient to rescue axonal and dendritic morphological defects not only in motor neurons and dorsal root ganglia, but also Schwann cell myelination defects (Hao et al. 2015). Additionally, in a *Drosophila* model of SMA, restoration of basal levels of SMN in muscles or motor neurons did not improve muscle morphology or neuronal physiology. However, SMN expression in cholinergic interneurons and proprioceptive neurons rescues motor defects (Imlach et al. 2012). These findings from zebrafish and *Drosophila* SMA models suggest that SMA may be a disease affecting cells in a non-cell autonomous way, where normal motor neuron SMN levels are required for functional motor-sensory circuit establishment (Hao et al. 2015; Imlach et al. 2012).

SMN has also been implicated in mRNP trafficking (Fallini et al. 2012; Burghes and Beattie 2009; Pellizzoni 2007; Donlin-Asp et al. 2016). SMN and other members of the SMN complex (*gemin6*, *gemin7*, *gemin2*, and *gemin3*) colocalize in axons and dendrites but do not associate with splicing-specific proteins (Sm proteins) (Zhang et al. 2006; Sharma et al. 2005). However, co-staining of only two binding partners has been visualized at a time and it has not yet been shown that three or more of these endogenous proteins colocalize in axons together. Sm proteins are generally thought to function exclusively in splicing; however, SmB and SmD3 have also been shown to be involved in mRNA localization in *Drosophila*. Specifically, SmB and SmD3 colocalize with mRNPs that transport *oskar* mRNA in oocytes (Gonsalvez et al. 2010). SmD3 GFP-insertion mutants cause a delocalization of *oskar* mRNA deposition at the pole in oocytes. Similarly, SMN regulates the localization of several axonal mRNAs and mRNA-binding proteins (Sanchez et al. 2013; Rossoll et al. 2003; Fallini et al. 2012, 2014; Akten et al. 2011; Fallini et al. 2011). Colocalization of SMN and HuD in primary motor neuron axons was shown using immunohistochemistry, which was validated by SMN immunoprecipitation of rat spinal cord lysate and then probing for HuD (Fallini et al. 2011). Since HuD is a known mRNA-binding protein, immunoprecipitation was performed in the presence of RNase A, which indicated that mRNA is not required for SMN-HuD binding (Fallini et al. 2011). Furthermore, SMN knock down by shRNA prevents mRNA from being transported to the axons, demonstrating a critical role for SMN in the

distribution of mRNA (Fallini et al. 2011). However, it should be kept in mind that these conclusions are based on the overexpression of tagged proteins, and further in vivo work to analyze the role of the endogenous SMN in mRNA transport would be beneficial.

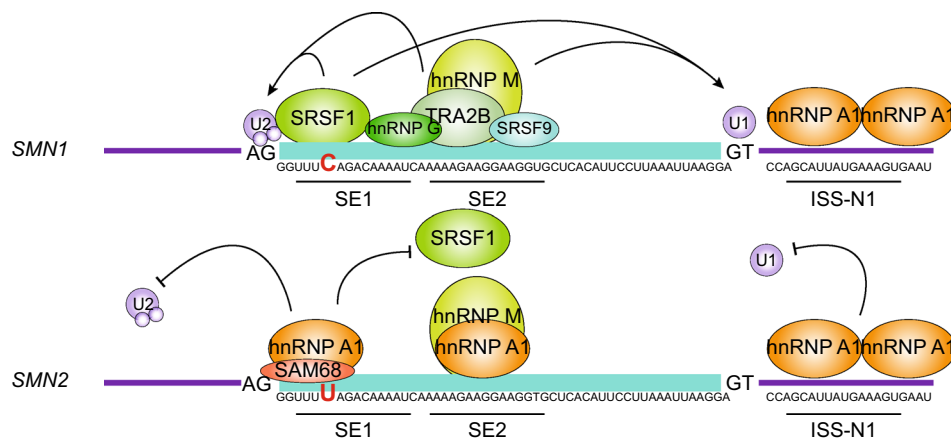
In addition to its a role in transporting mRNAs for local translation, SMN has been implicated in the regulation of translation itself. An in vitro transcription and translation assay utilizing rabbit reticulocyte lysates coupled with a luciferase assay show that SMN protein can act as a translation inhibitor, with this function being dependent on an intact Tudor domain (Sanchez et al. 2013). The effect of SMN on translation was found to affect the protein synthesis of the methyltransferase CARM1 (Sanchez et al. 2013). CARM1 stabilizes SMN mRNA through HuD interaction (Hubers et al. 2011). Therefore, CARM1 translational regulation by SMN represents a possible negative feedback loop. As this is not a global regulatory phenomenon, further experiments will be required to determine whether the translation of other mRNAs is affected by SMN activity and whether these phenomena are related to disease manifestation. In contrast, knockdown of SMN levels decreases local translation in axons, evidenced by nascent protein detection with the methionine analog, AHA (Fallini et al. 2016). Actin levels and growth-associated protein 43 (GAP43) that is associated with growth cones, are also decreased in axons both with SMN knock-down as well as in SMA versus control motor neurons. Hence, SMN may function both in repressing translation as well as transporting mRNA. These roles are not mutually exclusive, with translation silencing being required for transport of mRNA granules. It is important to note that the effect SMN has on translation is likely to differ between mRNAs from different genes and will require further analysis in the future. It is becoming increasingly evident that the multiple functions of SMN throughout a variety of cell types are likely to contribute in varying degrees to the SMA disease phenotype and that increasing SMN levels will likely ameliorate a broad range of defects.

Regulation of SMN splicing

Although a clear correlation exists between *SMN2* copy number and SMN protein levels, there are cellular processes that additionally affect SMN protein levels. One example is the splicing efficiency of the *SMN2* gene itself. Splicing of *SMN2* mRNA does not occur at a fixed rate, but is highly dynamic and context-dependent. One critical determinant of final the splicing outcome is the combinatorial binding of splicing factors to the region in and around exon 7 that determines whether the exon will be included in the transcript. *Trans*-acting factors bind pre-mRNA directly or as complexes with other proteins and

can influence splicing by either promoting or blocking interaction with the spliceosome (Kashima and Manley 2003; Cartegni et al. 2006; Vezain et al. 2010; Hua et al. 2008; Singh et al. 2006a, b; Hofmann 2000; Hofmann and Wirth 2002; Cléry et al. 2011; Chen et al. 2008; Pedrotti et al. 2010; Young et al. 2002; Passini et al. 2011) and function as positive and negative splicing factors, respectively. Sequences in the mRNA to which splicing regulators bind to facilitate inclusion are called splicing enhancers, while regions to which splicing regulators bind to prevent spliceosome recognition of target sequences are splicing silencers. The balance of positively and negatively acting protein–RNA interactions will largely influence the activity of the spliceosome (reviewed in Saltzman et al. 2011). Thus, while most *SMN2* mRNA lacks exon 7, the splicing efficiency can be skewed to favor exon 7 inclusion by increasing the activity of splicing-promoting factors, or to exacerbate exon exclusion by facilitating elements that promote exon 7 skipping.

Many of the identified splicing factors fall into two main classes of splicing regulators, serine arginine rich (SR) proteins and heterogeneous nuclear ribonuclear proteins (hnRNPs) (reviewed in Saltzman et al. 2011). The activity of these proteins is context dependent and many of them may regulate splicing either positively or negatively depending on the target sequence to which they are bound, post-translational modifications, and binding partners. The most well-characterized positively acting factors affecting *SMN2* exon 7 splicing are serine/arginine-rich splicing factor 1 (SRSF1) and transformer 2 protein homolog beta (Tra2B) (Hofmann 2000; Young et al. 2002). The most well-characterized negatively acting splicing factors are heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) and src-associated substrate in mitosis 68 (Sam68) (Fig. 2) (Hua et al. 2008; Singh et al. 2006a, b; Kashima and Manley 2003; Cartegni et al. 2006; Vezain et al. 2010; Pedrotti et al. 2010). Other factors that are also known to bind *SMN* mRNA and affect splicing of exon 7 are SRSF9, hnRNP G, and hnRNP Q1 (Table 1).



SAM68 binding site upstream of C>T (UUUUA) = Pedrotti et al 2010

SRSF9 = Young et al 2002

hnRNP G = Hofmann 2002

Tra2B = Hofmann 2000

exonic hnRNP A1 = Kashima 2003, Cartegni 2006

Tra2B orientation with hnRNP G and SRSF9 (all three bind RNA but dependent on TRA2) = Cléry 2011

ISSN1 hnRNP A1 = Hua 2008, Singh 2006

hnRNP Q = Chen 2008

Fig. 2 *SMN1* and *SMN2* exon 7 splicing regulators. *SMN1* is characterized by predominantly positive splicing regulation. SRSF1 and TRA2B bind directly to exon 7 splicing enhancer regions SE1 and SE2, respectively. TRA2B allows the cooperative binding of hnRNP G and SRSF9, which add further positive influence for exon recognition. These factors help to recruit U1 and U2 snRNPs to the exon boundaries. This snRNP recognition is critical for the inclusion of this exon. On the other hand, *SMN2* transcripts contain a nucleotide alteration from *SMN1*; C>U in SE1 region (marked in red). This nucleotide alteration changes the preferred protein-binding part-

ner from the positive SRSF1 to the negative hnRNP A1. Similarly, SAM68 is also capable of binding this region and exerting negative influence. Two more hnRNP A1 sites are known to induce exon 7 exclusion, located in SE2 region and the intronic silencing element, ISSN1. A GC-rich region overlapping the 5' end of ISSN1 also associates with long-distance interaction (*LD-I*); the negatively acting proteins decrease the recognition of the exon by U1 and U2 snRNPs, leading to exon 7 exclusion. hnRNP M binds to sequence in SE2 that overlaps the TRA2B binding site and promotes exon 7 inclusion in both *SMN1* and *SMN2* transcripts

Table 1 RNA binding proteins involved in SMN splicing

Protein	Effect on exon 7 splicing	Binding site	References
hnRNP A1 (exonic)	Inhibits exon 7 inclusion, <i>SMN2</i>	CAGGGU GAAGGAA	Kashima and Manley (2003) Cartegni et al. (2006) Prior et al. (2009) Vezain et al. (2010)
hnRNP A1 (intronic)	Inhibits exon 7 inclusion, <i>SMN2</i>	CCAGCAUUAUGAAAG	Hua et al. (2008) Singh et al. (2006)
hnRNP G	Promotes exon 7 inclusion, <i>SMN1</i>	CAAAAUCAAA	Hofmann and Wirth (2002) Cléry et al. (2011)
hnRNP M	Promotes exon 7 inclusion, <i>SMN1</i> and <i>SMN2</i>	GAAGGA	Cho et al. (2014)
hnRNP Q	Promotes exon 7 inclusion, <i>SMN1</i>	GGUUU	Chen et al. (2008)
SAM68	Inhibits exon 7 inclusion, <i>SMN2</i>	UUUUA	Pedrotti et al. (2010)
SRSF1	Promotes exon 7 inclusion, <i>SMN1</i>	CAGACAA	Cartegni et al. (2006)
SRSF9	Promotes exon 7 inclusion, <i>SMN1</i>	AGGAG, GAAGG	Young et al. (2002) Cléry et al. (2011)
TRA2B	Promotes exon 7 inclusion, <i>SMN1</i>	GGAGAA GAAGAA	Hofmann (2000) Cléry et al. (2011)

The splicing factors that are critical for *SMN* exon 7 splicing, SRSF1, Tra2B, hnRNP A1, and Sam68 (Hofmann 2000; Young et al. 2002; Hua et al. 2008; Singh et al. 2006a, b; Kashima and Manley 2003; Cartegni et al. 2006; Vezain et al. 2010; Pedrotti et al. 2010) are all RNA-binding proteins that recognize specific sequences in pre-mRNA to facilitate or repress recognition of exon 7 (Fig. 2). Transcripts from both *SMN1* and *SMN2* include the same *cis*-acting regulatory sites in their sequences that are bound by splicing factors to facilitate or block the recognition and inclusion of exon 7. These regulatory regions are known as splicing enhancer 1 (SE1), splicing enhancer 2 (SE2), and intronic splicing silencer 1 (ISSN1) (Lorson 1999; Hofmann 2000; Kashima and Manley 2003; Cartegni et al. 2006; Vezain et al. 2010; Hua et al. 2008; Singh et al. 2006a, b). The difference in splicing patterns of *SMN1* and *SMN2* transcripts is caused by a single genomic C-to-T transition in exon 7, resulting in a C-to-U transition in the mRNA (Lorson 1999; Kashima and Manley 2003; Cartegni et al. 2006; Vezain et al. 2010). In *SMN1*, the positive splicing factor, SRSF1 binds to SE1, with further positive exon recognition from TRA2B binding to SE2 (Cartegni and Krainer 2002; Hofmann 2000; Young et al. 2002). TRA2B binding to SE2 is required for the additional recruitment and binding of the positively acting splicing factors, hnRNP G and SRSF9. (Hofmann and Wirth 2002; Young et al. 2002; Passini et al. 2011; Cléry et al. 2011). The presence of these factors on the pre-mRNA promotes the recruitment of the initial splice site recognition snRNPs, U1 and U2, that bind to the 5' end of intron 6 and 3' end of the intron 7, respectively. The snRNP binding is essential for proper definition of the exon. In *SMN2* transcripts, the C-to-U substitution in SE1 results in decreased binding affinity for SRSF1 and increased affinity

for hnRNP A1, that in turn decreases exon definition by U1 and U2 (Lorson 1999; Cartegni and Krainer 2002). This strong influence by hnRNP A1 overpowers the ability of TRA2B to bind SE2. SAM68 is also found to bind in the region around SE1 and decrease exon definition (Pedrotti et al. 2010). Additionally, more negative splicing influence of exon 7 arises from hnRNP A1 binding to the intronic ISSN1 region (Singh et al. 2006a, b; Hua et al. 2008). In addition to these proximal RNA regulatory regions, variants in downstream sequences have been found to alter the efficiency of *SMN* exon 7 splicing by disrupting the normal secondary structure of the transcript, underscoring the potential for long-range effects in splicing regulation (Singh et al. 2015, 2017b).

Effects of stress on splicing

While several splicing factors are known to promote exon 7 inclusion in *SMN1* and exon 7 exclusion in *SMN2* splicing, the relative abundance of final spliced mRNA product is also affected by stresses and cell signaling processes, such as hypoxia, starvation, and temperature changes (Beebe et al. 2012; Sahashi et al. 2012). An example of this effect may be seen under the influence of oxidative stress induced by paraquat treatment, which has been shown to result in multiple aberrant *SMN2* splicing products in murine tissues (Seo et al. 2016). Protein modification affects the activity and localization of splicing factors and these changes are highly regulated. For instance, SRSF1 requires phosphorylation to promote spliceosome assembly, but must be dephosphorylated to catalyze splicing (Cao 1997). Not only is activity regulated by phosphorylation, but also physical availability.

distress due to muscle weakness. Concordantly, a hallmark of late-stage disease in a mouse model of severe SMA is increased levels of the hypoxic markers, Hif1alpha (Beebe et al. 2012) and Hif3alpha (Zhang et al. 2008). Additionally, hypoxic treatment of cultured SMA patient fibroblasts leads to increased levels of exon 7 skipping and subsequent reduced levels of stable, functional SMN protein. Importantly, this splicing alteration was found to be due to upregulation of the negative splicing factor hnRNP A1 (Beebe et al. 2012). While the mechanism of hnRNP A1 upregulation was not addressed in that study, hypoxia has also been shown to activate the AKT pathway, which phosphorylates hnRNP A1 (Jo et al. 2008). Further investigation of the possible involvement of the AKT pathway may identify additional mechanisms that link hypoxic stress and the regulation of *SMN2* splicing by hnRNP A1.

Notably, hyperbaric hyperoxia treatment of the SMA mice improved motor function and body weight (Beebe et al. 2012). The authors proposed that a feed-forward cycle contributes to the progression of the SMA disease state, in which motor neuron denervation of intercostal and diaphragm muscles leads to breathing impairment and hypoxia. This in turn decreases the proportion of full-length *SMN2* transcript, thereby decreasing the level of functional SMN protein, which in turn leads to further motor neuron damage. Consistent with this notion, vascular malformations in muscle and spinal cord have been described in three different SMA mouse models, Taiwanese, *SMNΔ7*, and Burghes' severe model (Somers et al. 2016; Nobutoki et al. 2015; Somers et al. 2012; Le et al. 2005; Monani et al. 2000; Hsieh-Li et al. 2000). Somers et al. analyzed muscle capillary beds in the severe Burghes mouse model at PND1, PND3, and PND5. The caudal band of the levator auris longus (LALc) that exhibits extensive NMJ pathology was stained for vascular beds. Samples from SMA mice exhibited much less densely organized capillary beds, with larger diameter capillaries and less intramuscular vascularization than wild-type samples at PND3 and PND5 but not at PND1. This decreased capillary bed density was similarly observed in the transverse abdominal muscle, that exhibits substantial muscular atrophy, and LAL rostral band (LALr), that shows mild muscular atrophy, indicating decreased capillary bed density did not correlate with only severely affected muscle types (Somers et al. 2012). Additionally, the Taiwanese and *SMNΔ7* SMA mouse models were also analyzed for capillary bed infiltration in muscle and spinal cord (Somers et al. 2016; Le et al. 2005; Monani et al. 2000). Spinal cord vasculature at pre-symptomatic stages matches that of non-disease littermates. However, over time, capillary beds in control animals grow denser while in SMA mice, they remain sparse, at approximately half the density of the controls (Somers et al. 2016). A similar vascular deficiency was observed in human SMA

patient muscle biopsies, with a capillary-to-filament ratio approaching tenfold lower in SMA patients than controls (Somers et al. 2016). However, other similarly denervating conditions were not examined in these studies, so whether this defect is due to denervation or results from reduced SMN in blood vessels remains unclear. Regardless of the primary cause, this condition creates a hypoxic microenvironment in the mouse spinal cord, as well as a disrupted blood–spinal cord barrier (Somers et al. 2016; Nobutoki et al. 2015).

Taken together, these findings identify hypoxia as a significant contributor to SMA disease onset and progression. SMA may lead to a hypoxic environment in patients through capillary bed deficiency as well as respiratory distress suffered by many SMA patients. These conditions may create a hypoxic environment that reduces the level of full-length *SMN2* transcripts as was observed in mice.

Oxidative stress

Neurons and other cells with high energy demands are subject to oxidative stress that results from a buildup of free radicals, including superoxide, hydrogen peroxide and other reactive oxygen species (ROS) as a byproduct of energy production from the mitochondrial electron transport chain. The well-established role of oxidative stress as a factor in the pathology of several neurodegenerative diseases (reviewed in Niedzielska et al. 2016; Kim et al. 2015) has prompted investigation into whether it contributes to SMA pathology as well.

One of the first studies to indicate that SMN deficiency results in oxidative stress used siRNA-mediated knock-down of SMN in NSC-34 cells, a motor neuron-like hybrid cell line, to demonstrate that 72 h after transfection, with a 66% reduction in SMN protein, cells showed increased levels of ATP, cytochrome C oxidase and hydrogen peroxide, indicating a defect in mitochondrial energy production (Acasadi et al. 2009). The knock-down cells also displayed increased mitochondrial membrane potential and a reduced number of cells with neurite outgrowth. Similar results were seen in long-term cultures of motor neurons derived from in vitro differentiation of WT human embryonic stem cells (hESCs) (Wang et al. 2013). In lines where SMN expression was knocked-down with stably transfected shRNA, mitochondrial superoxide production was significantly increased, as measured by MitoSOX staining. These cultures also showed increased caspase 3/7 activity relative to control cells, and long-term cultures (6 weeks) showed a greater loss of motor neurons, as measured by staining for HB9, a motor neuron marker. In cultures treated with the antioxidant *N*-acetylcysteine (NAC), caspase 3/7 levels and motor neuron survival returned to control levels. While the SMN knock-down cultures showed a dramatic reduction in axon length, it is

unclear whether this effect was due to oxidative stress since the effect of NAC treatment was not analyzed.

To better understand how SMN deficiency could induce oxidative stress, two recent studies examined mitochondrial dysfunction in vitro and in vivo. Using live cell imaging of motor neurons derived from SMA type 1 iPSCs, Xu et al (2016) found a reduction in size, number, and axonal transport of mitochondria in the SMA cells compared to WT cells. NAC treatment returned mitochondrial numbers and transport to WT levels. The mitochondrial defects were not observed in telencephalic glutamatergic (forebrain) neurons derived from the same iPSCs, suggesting that the impaired mitochondrial transport was specific to motor neurons. After long-term (6 weeks) culture, the SMA motor neurons displayed an increased number of axonal swellings and increased caspase 3/7 activity relative to WT cells, reflecting increased degeneration and apoptosis. NAC treatment ameliorated these defects as well, suggesting that oxidative stress that accompanies mitochondrial dysfunction in SMA motor neurons contributes to motor neuron degeneration. Similar results were obtained by Miller et al. (2016) using motor neurons isolated by cell sorting from spinal cords of SMA mice ($\Delta 7$ SMA or *Smn*^{-/-}; *SMN2*^{tg/-}) at P9. The mutant motor neurons showed reduced mitochondrial transport and oxygen consumption, as well as increased oxidative stress, as measured by live-imaging of signal from transfected mito-roGFP, a redox-sensitive GFP targeted to mitochondria. No differences in mitochondrial transport or oxygen consumption were observed between SMA and WT midbrain neurons, suggesting a higher susceptibility of motor neurons to mitochondrial dysfunction due to SMN deficiency. Ultrastructural analysis by electron microscopy of mitochondria in SMA mouse spinal cords at P3 (presymptomatic) and P9 (symptomatic) found reduced size and increased mitochondrial edema at P3, and additional morphological abnormalities at P9, indicating that mitochondrial dysfunction may be present before disease symptoms are manifest in vivo.

Additional in vivo evidence of compromised mitochondrial function due to SMA deficiency recently came from analysis of a zebrafish model of SMA (Boyd et al. 2017). Morpholino-induced knock-down of SMN expression in embryos resulted in reduced ATP-linked respiration and lower levels of ATP synthase subunit alpha (APT5A), a subunit of mitochondrial ATP synthase. The SMA morphants display a well-characterized defect in axonal outgrowth of motor neurons (McWhorter et al. 2003) and this phenotype could be partially rescued by overexpression of the glycolytic enzyme Pfkfb3, suggesting that ATP deficiency due to mitochondrial dysfunction contributes to the impaired axonal outgrowth.

Although these studies, taken together, implicate mitochondria dysfunction, bioenergetic deficiency and oxidative stress in SMA motor neuron pathology, very little is

currently known about the specific mechanisms by which SMN deficiency affects mitochondria. It should be noted that the occurrence of oxidative stress in SMN-deficient cells is still unresolved. For example, Patitucci et al. (2016), analyzing motor neurons and astrocytes derived from SMA patient iPSCs, found no evidence of increased oxidative stress in the SMA cells. Surprisingly, staining for ROS using DHE indicated lower ROS in SMA motor neurons than WT cells. This may be accounted for by the dramatically elevated levels of catalase, that decomposes hydrogen peroxide, detected in the SMA cells. siRNA-mediated knockdown of catalase expression resulted in increased ROS levels in SMA motor neurons, but other markers of oxidative stress were not induced. What triggers the dramatic induction of catalase in the SMA motor neurons in vitro is not known.

In addition to SMA deficiency potentially giving rise to oxidative stress, conversely, SMN function may be impaired by oxidative stress. A study using an in vitro snRNP assembly high-throughput screen found that the ROS-generating compounds beta-lapachone, menadione, and peroxide inhibit the assembly of Sm rings onto snRNAs (Wan et al. 2008). Closer examination showed that under oxidative stress, SMN forms disulfide bonds between multiple SMN proteins, which was postulated to render them unable to function in snRNP assembly. These data suggest that SMN function is sensitive to oxidizing environments. However, these experiments were performed either in vitro in the absence of reducing agents, or in lysed cells, where conditions almost certainly do not maintain the in vivo redox state of SMN upon cellular lysis. Thus, more experiments will be required to determine whether in vivo, where glutathione levels are maintained in the millimolar range, a sufficient imbalance of oxidizing and reducing agents exists to cause SMN crosslinking.

Finally, a novel way in which SMN deficiency may render cells vulnerable to oxidative stress was proposed with the recent discovery that the SMN complex binds SECIS-binding protein 2 (SBP2), which is required for specialized translation of mRNA encoding selenoproteins (Gribbling-Burrer et al. 2017). Incorporation of the amino acid selenocysteine into nascent protein requires SBP2 binding to a Selenocysteine Insertion Sequence (SECIS) in the 3' UTR of mRNA. Selenoproteins are known to be involved in protection from oxidative stress through their oxidoreductase activity (reviewed in Labunskyy et al. 2014; Papp et al. 2010). mRNA levels for several selenoproteins were significantly lower in spinal cords from SMN-deficient versus WT mice, while in the brain, there was no difference (Gribbling-Burrer et al. 2017). If the lower transcript levels are found to result in lower selenoprotein levels in the spinal cord, it would represent a possible mechanism by which SMA motor neurons are made more susceptible to damage from oxidative stress. Thus, SMN deficiency could promote oxidative damage both

through mitochondrial dysfunction, as discussed above, as well as by impairing mechanisms that are protective against oxidative stress. If the elevated oxidative stress were sufficient to cause SMN inactivation through cross-linking, it would represent another feed-forward loop, as discussed above with regard to hypoxia, in which SMN deficiency causes a stress condition that, in turn, further reduces levels of functional SMN.

Adaptation to temperature changes

The heat shock response (HSR) is a highly conserved, global stress response that a cell undergoes when faced with thermal stress. Thermal stress causes aberrant protein folding and leads to cellular dysfunction and is, therefore, counteracted by the induction of precise protein folding quality control mechanisms (reviewed in Balchin et al. 2016). At the other end of the temperature spectrum, hypothermia and cold-shock are additional sources of cellular stress. Cold-shock suppresses transcription and may also be associated with oxidative stress due to associated changes in reactive oxygen species (Al-Fageeh et al. 2006). A large collaborative study analyzed the differences in care for SMA patients in North America, Australasia, and Europe (Bladen et al. 2014). Within the data from this study, Tsai et al. identified a correlation between climate and SMA type III ambulation, in which regions with cooler climates (Germany/Austria, Switzerland, and the UK) have patients with a more extended period of ambulation than those in countries with warmer climates (Argentina, Hungary, Ukraine, and Serbia) (Tsai et al. 2016; Bladen et al. 2014). The interpretation of these data by Tsai et al. did not take into consideration differences in health care systems of these countries or dietary or cultural considerations that may also contribute to the phenotypic severity (Tsai et al. 2016). Additionally, it is not clear that the classification of cooler vs. warmer climates is well delimited, since the “warmer” regions experience the following average temperatures for hottest and coldest months, respectively: Argentina (76–53 °F at most equatorial location and 56–35 °F at its most southern regions), Hungary (70–30 °F), Ukraine (66–24 °F), Serbia (80 °F–39 °C). Likewise, the “cooler” countries experience the following yearly temperatures: Germany/Austria (72 °C–38 °F), Switzerland (65–32 °F), UK (65–42 °F).

These concerns notwithstanding, Tsai et al. (2016) did find that repeated transient hypothermia treatment of a severe SMA mouse model (Taiwanese) ameliorated disease manifestations in early postnatal SMA pups. Specifically, hypothermia treatment consisted of exposing neonatal pups to crushed ice for 50 s either daily or every 3 days during which their core body temperature dropped from 33 to 20 °C. The pups were then warmed to restore normal body temperature within 5 min. Following this treatment,

SMA pups displayed improved muscle morphology in the quadriceps, intercostal, and diaphragm muscles, as well as improved motor end plate occupancy in the quadriceps. While clearly beneficial to muscle biology, the hypothermia treatment led to only a modest increase in average lifespan and no SMA animals survived beyond 14 days of age. The authors found a correlation between activated p38MAPK and increased SMN protein levels in the brain and spinal cord of hypothermia-treated SMA mice. Consistent with this result, p38 MAPK was previously shown to increase the stability of SMN transcripts, leading to increased SMN protein (Farooq et al. 2009). However, there appeared to be an uncoupling of the protein and mRNA upregulation. For instance, in the spinal cord, SMN transcript levels were significantly upregulated with daily hypothermia treatment while transcript levels were not upregulated with hypothermia treatments given every three days. On the other hand, spinal cord SMN protein levels were not increased significantly with daily treatments, but were significantly upregulated with the every third day treatment (Tsai et al. 2016). Therefore, while the mechanism needs clarification, there is a significant increase in the SMN protein levels of brain and spinal cord upon daily hypothermia treatment, which may indicate interplay between a potential cold shock mechanism and other stress pathways known to upregulate p38 MAPK. These data suggest that the cold-shock pathway may be harnessed to increase SMN protein levels and as a potential therapeutic intervention for SMA.

Metabolic stress

Recently, it was shown that intracellular bacterial infection by *Shigella*, *Salmonella*, and *Listeria* leads to aggregation of snRNA and the SMN complex (Tsalikis et al. 2015). The aggregation of these factors rendered them temporarily inactive. This response was caused by metabolic stress (amino acid starvation and ER stress) triggered by membrane damage. The formation of these aggregate bodies limits the availability of snRNA and greatly affects their functional association with the Sm protein ring. Hence, bacterial infection may reduce splicing capacity due to decreased snRNP availability. Because decreased snRNP assembly and splicing are known to be hallmarks of SMA, it may also contribute to the increased mortality due to infections in the SMA patient population (Cobben 2008; Finkel and Richard 2013). However, most infections experienced by SMA patients are respiratory, which are most often caused by *Streptococcus pneumoniae*, *Haemophilus* species, *Staphylococcus aureus*, and *Mycobacterium tuberculosis* (Speert 2006). These bacteria include both intracellular and extracellular pathogens and therefore, the effect of infection by both intracellular and extracellular bacteria should be similarly analyzed with

regard to snRNA and SMN complex aggregation and relevance to SMA.

Another metabolic stress recently found to be linked to SMN deficiency is oxidative phosphorylation. A transcriptional profiling study revealed that mRNA involved in ribosomal processes and oxidative phosphorylation were preferentially decreased in an intermediate-severity SMA mouse model (Murray et al. 2015). Oxidative phosphorylation is critical for production of energy from the mitochondria, and importantly, this study compared vulnerable and non-vulnerable motor neuron populations (as previously determined by NMJ loss), all at pre-symptomatic time points. Interestingly, the p53 pathway, which is known to trigger cell death, was activated preceding NMJ loss, indicating that metabolic deficiencies may program cell death before NMJ denervation.

Starvation stress

Another environmental stress that affects SMN mRNA processing is starvation, which is also a significant morbidity associated with SMA. Prolonged weight loss begins a starvation-signaling cascade, in which AKT plays a role. It has been shown in a mild SMA mouse model that starvation exacerbates *SMN2* mis-splicing (Sahashi et al. 2012). Furthermore, when starvation occurs during early postnatal development, the mis-splicing is greater than in adults undergoing starvation (Sahashi et al. 2012). Similar results were observed in all tissues analyzed (spinal cord, heart, liver). Though no regulatory splicing factors were implicated in this study, it is reasonable to hypothesize that due to signaling similarities between starvation and hypoxia, the key modulator in this context is hnRNP A1. Further research will elucidate common mechanisms by which these stress pathways affect *SMN2* splicing.

SMN in cytoplasmic stress granules

Stress granules (SG) are dense aggregates of protein and RNA that appear transiently in the nucleus and cytoplasm in response to a variety of stress conditions. Different types of SGs have distinct compositions depending on their cellular sublocalization, cell type, and the type of stress under which they appear (Aulas et al. 2017; Mahboubi and Stochaj 2017). Nuclear SGs, also called nuclear stress bodies, are known to include the heat-shock response transcription factors HSF1/2, as well as pre-mRNA processing factors including HAP (hnRNP A1 interacting protein), hnRNP M, Sam68, and SR splicing factors (Denegri 2001). Cytoplasmic SGs commonly include mRNA bound to stalled translation initiation complexes with phosphorylated eIF2a initiation factor, along with the RNA-binding proteins TIA-1 and TIAR that are associated with repression of translation, in addition to perhaps dozens of other RNA-binding and

non-RNA-binding proteins in various combinations under different conditions (Damgaard and Lykke-Anderson 2011; reviewed in Panas et al. 2016; Mahboubi and Stochaj 2017). Broadly, cytoplasmic stress granules are thought to provide a mechanism to very rapidly alter or reprioritize gene expression by transiently sequestering a subset of mRNAs from translation and allowing specific stress response genes to be expressed (reviewed in Anderson and Kedersha 2007).

SMN immunofluorescence signal co-localizes with TIA-1 and TIAR in cytoplasmic SGs that appear in HELA cells in response to heat shock, arsenite or UV irradiation (Hua and Zhou 2004). Further, this study found that overexpressed SMN co-immunoprecipitates with TIA-1/R proteins in extracts from heat shocked HeLa cells. P19 embryonal carcinoma cells in which SMN expression is knocked down by a stably transfected shRNA directed against SMN display a reduced number of SG-positive cells in response to arsenite, and a reduced level of phosphorylated eIF2a and lower cell viability in response to arsenite or hydrogen peroxide treatment (Zou et al. 2011). These studies demonstrate that SMN functions in SGs that promote cell survival under stress conditions. Although numerous studies have investigated the subcellular localization of SMN in neurons and other cell types, mainly in vitro, questions remain regarding the presence and/or role of SMN specifically in stress granules in relevant cell types in vivo. The many SMA mouse models currently available provide a resource to address the question of how SMN deficiency affects stress granule formation and function in relevant cell types during disease progression.

In vivo evidence that SMN participates in SG formation in motor neurons recently came from a study of a mouse model of amyotrophic lateral sclerosis (ALS), a neurodegenerative disease that, like SMA, is marked by loss of motor neurons and progressive muscle weakness. SMN was found to be upregulated and associated with HuR-positive SGs that appear in motor neurons of the lumbar spinal cord and brain in male TDP-43^{A315T} transgenic mice, a model of ALS (Perera et al. 2016). TAR DNA binding protein 43 (TDP-43) is a multifunctional DNA- and RNA-binding protein involved in regulation of transcription, translation, and alternative splicing (reviewed in Ratti and Buratti 2016). Moreover, TDP-43 binds to SMN in co-IP experiments, colocalizes with SMN-containing nuclear gems, and is recruited into SGs in response to arsenite stress in vitro (Liu-Yesucevitz et al. 2010). Aberrant cytoplasmic aggregates of TDP-43, that may result from persistent SGs (Parker et al. 2012), are present in motor neurons in a large majority of ALS and frontotemporal lobar degeneration (FTLD) patients (Neumann et al. 2006). Transgenic overexpression of SMN, specifically in neurons of the TDP-43^{A315T} mice, rescued motor neuron loss and delayed onset of hindlimb paralysis in females, but not males (Perera et al. 2016). The reason for the sex-specific effects on SG formation and response

to SMN overexpression is not clear, but TDP-43^{A315T} males showed a significantly earlier onset of weight loss and hindlimb paralysis than females and androgen receptor levels were reduced by nearly 50% in spinal cords of TDP-43^{A315T} versus WT males, while they were not different between TDP-43^{A315T} and WT females.

These results add to a growing body of evidence indicating that despite important differences between SMA and ALS, including genetic etiology, age of onset, and specific neurons affected, the two disorders share common underlying disease mechanisms in motor neurons (reviewed in Achsel et al. 2013). Lowering SMN levels in a mouse model of ALS by crossing *Smn*^{+/-} animals to the SOD1^{G93A} transgenic model of ALS resulted in poorer rotorod performance and reduced lifespan in the SOD1^{G93A}; *Smn*^{+/-} animals compared to SOD1^{G93A} alone (Turner et al. 2009). Likewise, genetic analysis of ALS patients previously indicated that reduced levels of SMN increased susceptibility to and severity of ALS (Veldink et al. 2005). By contrast, a more recent study that found a significant association between *SMN1* gene duplications (but not *SMN1* deletions or *SMN2* copy number) and sporadic ALS leaves the question of how SMN levels contribute to ALS pathogenesis in humans unresolved (Blauw et al. 2012).

Fused in sarcoma (FUS), a DNA and RNA-binding protein that shuttles between the nucleus and cytoplasm, and functions in DNA repair, transcription, splicing, and mRNA transport (Qiu et al. 2014; Reber et al. 2016; Yasuda et al. 2017), is yet another link between the neurodegenerative diseases SMA and ALS. Mutations in FUS are associated with cases of familial ALS and aberrant FUS-positive aggregates have been detected in motor and spinal neurons (Kwiatkowski et al. 2009; Vance et al. 2009). Exogenously expressed FUS carrying ALS-associated mutations localizes to SGs in vitro in response to oxidative, thermal, mitochondrial, and ER stress (reviewed in Aulas and Vande Velde 2015). Physical interaction between WT FUS and SMN has been demonstrated by co-IP in lysates from HeLa cells, neural-derived cell lines N2a and NSC-34 cells, as well as mouse brain (Yamazaki et al. 2012; Groen et al. 2013; Sun et al. 2015). Groen et al. found that expression of several different ALS-associated mutant forms of FUS in mouse primary cortical neurons causes mislocalization of SMN into FUS-positive cytoplasmic aggregates, with reduced SMN detected in axons. Neurons transfected with the R521C FUS mutant showed reduced axon branching and growth cone area compared to WT FUS, and this defect could be rescued by overexpression of SMN. The authors speculate that mutant FUS traps SMN in cytoplasmic aggregates, thereby preventing SMN from performing its normal function in mRNA transport along axons. An abnormally enhanced interaction between SMN and mutant FUS was confirmed by Sun et al. (2015) and they further observed a reduced

number of SMN-containing nuclear gems in ALS patient fibroblasts with FUS mutations and in spinal motor neurons of transgenic mice expressing ALS-associated R521C FUS.

The physical interaction between SMN, TDP-43 and FUS, their association with SGs or aberrant aggregates in motor neuron disease, along with their role in RNA metabolism, specifically splicing, all point toward dysregulated splicing as a factor in motor neuron loss in SMA and ALS. The protective function of SGs in regulating RNA metabolism under stress conditions may be compromised either by SMN deficiency in SMA or by their abnormal persistence and sequestration of SMN in ALS.

Concluding remarks

SMN transcripts and protein are sensitive to external and internal stimuli and responsive to a variety of stresses. We have reviewed here the responses of *SMN* mRNA and protein to hypoxic stress, oxidative stress, changes in temperature, metabolic stress, and starvation. The mechanisms of cellular response to these stress states share regulatory steps or key factors. The fact that these pathways either affect SMN directly or factors that are critical for the proper production of SMN protein is important for cell maintenance. This is an especially critical piece of information as the splicing of *SMN2* and protein status relates directly to the severity of SMA, and the disease state can in turn lead directly back to splicing and protein mishandling aberrations in SMN. We propose that these stresses that decrease the amount of SMN levels can act in a feed-forward cycle, in which an accumulation of stresses resulting from disease progression further exacerbates the disease pathology.

The understanding of stress manifestations in disease will help us to understand disease progression in SMA as well as related neurodegenerative diseases. This understanding can extend even beyond the realm of neurodegeneration and find applications in diseases such as cancers that also revolve around these stress pathways.

It has been shown in mouse models of SMA that SMN restoration as therapy for the disease loses efficacy as the disease progresses and that earlier time points are more efficacious (Robbins et al. 2014; Lutz et al. 2011; Le et al. 2011). Even complete restoration of SMN in severe SMA mice is unable to rescue them if the addition of SMN is after this therapeutic window, indicating that after a certain point, the secondary symptoms of SMA and not just the original cause of the disease must be treated. Therefore, if we are aware of the stress pathways that predispose SMA individuals to the highest level of morbidity, we can customize treatment options for these secondary ailments in addition to restoring SMN protein levels in these individuals.

As clinical trials advance and treatment options improve for these patients, we are entering a new era of improved prognosis for SMA. Understanding and mitigating the cellular stresses left in the wake of lowered SMN levels will be the next step for patients and families.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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