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Spinal Muscular Atrophy: Why do low levels of SMN make motor neurons sick?

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Abstract

Many neurogenetic disorders are caused by the mutation of ubiquitously expressed genes. Spinal muscular atrophy is one such disorder and is caused by loss or mutation of the *survival motor neuron 1* gene (*SMN1*), leading to reduced SMN protein levels and a selective dysfunction of motor neurons. SMN, in collaboration with partner proteins, functions in the assembly of small nuclear ribonucleoproteins (snRNPs), which are important for pre-mRNA splicing. It has also been suggested that SMN might function in the assembly of other RNP complexes. Two hypotheses have been proposed to explain the molecular dysfunction that gives rise to SMA and its specificity to a particular group of neurons. The first hypothesis states that the loss of SMN's well-known function in snRNP assembly causes an alteration in the splicing of a specific gene (or genes). A second hypothesis proposes that SMN is critical for the transport of mRNA in neurons and disruption of this function results in SMA.

Introduction

Spinal muscular atrophies (SMA) are characterized by the loss of lower motor neurons and atrophy of muscle.[1] Proximal SMA (which we refer to as SMA) is a common genetic cause of infant death2 and the most frequent SMA.3–6. SMA is one of a number of neurological disorders associated with genes that play important roles in RNA metabolism (Supplementary Information S1 box). Although SMA is caused by reduced levels of a ubiquitously expressed protein, Survival Motor Neuron (SMN), it affects the lower motor neurons. A number of other neurogenetic disorders are caused by mutations in ubiquitously expressed genes, including amyotrophic lateral sclerosis (caused by mutations in *Super Oxide Dismutase 1*)[7], Huntington's disease (caused by an expanded CAG repeat in *Huntingtin*)[8] and several others. [9–12] Thus, understanding how SMN deficiency causes SMA could give us insight into the more general question of how a mutation in a ubiquitously expressed gene causes a neurological disease.

Two hypotheses have arisen to explain SMA. Reduction of SMN is proposed to affect pre-mRNA splicing of certain genes giving rise to SMA, or to disrupt its function in axons, resulting in reduced levels of certain transcripts in the distal axon. Papers have recently appeared providing evidence for both hypotheses. Here we discuss the function of SMN, SMA genetics and insights gained from animal models of the disease. We consider the evidence for each

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theory, how the proposed changes might result in SMA, and how strong evidence for the mechanism of SMA might be found. We aim in this article to provide a summary of the current debate in the field, as well as provoking critical discussion of each of the proposed disease mechanisms, so that experiments may be designed to resolve how deficiency of SMN causes SMA.

SMN Function

SMN is a 38kD protein found in the cytoplasm and nucleus of all cells.[13–17] An isoform of SMN comprising only the amino terminus (exons 1–3 and a part of intron 3) and found only in axons has also been reported.[18] However, this isoform is unlikely to play a role in SMA as some SMA patients carry missense mutations in exon 6 that would not alter its coding sequence. 19

SMN has been reported to bind to or associate with many proteins.[20–23] However, it is difficult to determine which of these interactors form functional complexes with SMN. Four criteria are useful in discerning the most likely functional SMN complexes. First, reciprocal immunoprecipitation of the endogenous forms of the interacting proteins should be shown. Second, isolation of a complex containing SMN as well as the protein in question from cells or tissues should be possible. Third, co-localization of SMN and the protein in question should be demonstrated. This co-localization may be only partial, that is, each protein may be found in separate locations (in other words only some of the immunofluorescent 'spots' may show overlap)however, where it occurs the overlap should be complete (that is, the spots for each protein on top of each other, rather than partially overlapping) to be considered as evidence for interaction.[24] Finally, the complex containing the SMN interacting protein should show a functional deficit on SMN reduction. At the present time only the SMN complex involved in snRNP assembly has met these criteria.

SMN in snRNP assembly

The best-characterized SMN complex is involved in snRNP assembly (Figure1).[20,25–31] Certain snRNPs are critical for the recognition of splice sites and the catalytic removal of introns from pre-mRNA. SnRNPs are composed of a U snRNA (U1, U2, U4, U5, U11 or U12) and for those snRNPs involved in splicing a heptameric ring of Sm proteins.[25,32] Although Sm proteins can self-associate onto snRNA *in vitro*,[32] the SMN complex is required for this process *in vivo*.[27,28] The SMN complex that performs this function in vertebrates consists of SMN, Gemins2–8, unr interacting protein (unrip) and ATP as the assembly reaction is ATP dependent (Figure 1).[25–27,33–35] Evidence to date indicates that SMN in this complex exists as an oligomer. The data include SMN's ability to self-oligomerize *in vitro*,[36,37] the interaction of the products of tagged SMN constructs in transfected cells[38,39] and the results of genetic complementation studies *in vivo*[40] (see below). Proteomic studies of a functional ATP dependent SMN complex that determine the precise stoichiometry of the components should be performed to further confirm the oligomeric nature of SMN as well as the number of SMN monomers in the active SMN complex.

In snRNP assembly, Sm proteins first form a complex with the chloride conductance regulatory protein (pICIn) which is thought to prevent inappropriate binding of Sm proteins to RNA.[22, 25–30,41] pICln exists in a complex known as the PRMT5 complex (PRMT=protein arginine methyltransferase) or methylsome which can methylate Sm proteins and is capable of transferring Sm proteins to the SMN complex.[41–44] (Figure1) The SMN complex then facilitates assembly of the Sm proteins onto the snRNA (Figure 1).[22,25–30,41,45–47] Although the basic mechanisms of SnRNP assembly are well characterized, there is a number of outstanding issues on which there is not full agreement (Supplementary Information S2 box). The Tudor domain of SMN has been shown to bind arginine and glycine rich domains (RG

and RGG domains) of various proteins such as the SmB, D1 and D3.[48,49] Once the Sm proteins are assembled onto the snRNA the m^7G-cap of the snRNA is hypermethylated to a m^3G-cap and the whole SMN complex with snRNA is transported into the nucleus.[50] In the nucleus snRNPs are further matured for pre-mRNA splicing but SMN's role in these steps is poorly defined.

Does SMN assemble other RNPs?

In addition to SMN's role in assembly of snRNPs, SMN has been called the master RNP assembler.[51] However, for which RNPs SMN is important remains poorly defined. Many RNA-binding protein targets of SMN have RG and RGG domains. Of particular relevance are the Sm-like proteins (Lsm proteins),[48,49,52–55] which share many features with the Sm proteins. Both Lsm and Sm proteins form heptameric ring structures and certain Lsm and Sm proteins contain RG domains which can bind the Tudor domain of SMN.[48,49,52–55]

Lsm10 and Lsm11 interact with Sm proteins to form a heptameric ring that binds the U7snRNAs.[55] SMN is important for assembly of this ring on U7 RNA *in vitro*.[55] Lsm2–8 proteins form a ring structure on the U6snRNA, a snRNA that is localized to the nucleus.[52–54] U6 snRNA levels do not appear to be altered in tissue from mice with SMA.[40,56] However specific assembly reactions to test SMN's importance in Lsm assembly on U6snRNPs have not been reported.

The Lsm1–7 protein ring is found in the cytoplasm.[52–54] The Lsm1–7 proteins have roles in mRNA decay[57] and stabilization.[58] Recently the Lsm1 and Lsm4 proteins have been shown to be present in dendrites and certain axons including spinal cord axons.[59] This axonal/dentritic RNP complex was associated with RNAs transported in axons and contained SMN.[59] Although SMN has yet to be shown to be important for Lsm ring assembly it is clear that both pICln[60] and SMN can bind Lsm4[48,49] making it possible that the SMN complex is important for assembly of these rings on mRNA.

The *Scd6* family is made up of conserved Lsm proteins that are associated with RNA granules. The *C. elegans* and Drosophila orthologues of *Scd6* – *CAR1* and *Trailer Hitch* respectively – also contain Sm like domains, form a 7-member ring that can bind RNA[61] and have a role in RNA localization.[61,62] The vertebrate orthologue of *trailer hitch*, Lsm14, assembles onto mRNAs and associates with decapping enzyme 1 (DCP1) and Me13B in distinct protein complexes that do not contain Lsm proteins 1–8 in non-neuronal cells .[63,64,65] Lsm1 and 4 have been reported in an RNP complex containing SMN in neuronal processes,59 but whether this complex or other vertebrate axonal RNP complexs contain Lsm14 is not known. No assay to examine the effect of reduced SMN on assembly of these Lsm RNPs has been reported. However as Lsm proteins are important in RNA localization and contain domains that can bind SMN we suggest that SMN reduction might affect the assembly of RNP complexes that are critical for mRNA transport. Alternatively SMN has been hypothesized[66–68] to function in a unique axonal complex with hnRNPQ/R[66,67] and ZBPs[69] to affect transport of β-actin message or other mRNAs yet to be identified.

Several other proteins have been reported to be able to interact with either SMN or the SMN complex.[20,21,70,71] For example the proteins FMRP,[72] hnRNPQ/R[66,73] and profilin[74] can bind SMN and thus could influence the function of SMN as an RNP assembler, although this has not yet been demonstrated.

SMN has also been suggested to be important for the assembly of snoRNPs 75,76, and knockdown of SMN alters the levels of U3snoRNA.[77] SnoRNPs are small nuclear RNA protein complexes were the snoRNA acts as a guide for the modification (methylation or pseudouridylation) of ribosomal RNA, transfer RNA and snRNA.[78] It is therefore necessary

to determine which RNP assembly reactions are dependent on SMN and which are critical to the development of SMA.

Genetics of SMA

In humans, two genes, *SMN1* and *SMN2*, encode SMN. SMA is an autosomal recessive disorder caused by loss or mutation of the *SMN1* gene and retention of SMN2[6,79] (Figure 2). Chromosomes that lack both *SMN1* and *SMN2* can occur in SMA carriers but homozygous loss of both genes has not been reported, presumably as a result of lethality.[79] Indeed, mice have a single *Smn* gene, the loss of which results in very early embryonic lethality.[80] Loss of just *SMN2* but not *SMN1* does occur in the human population and has no consequence.[79] The severity of SMA patients can be divided into five groups (0-IV) based on clinical presentation.[81] The copy number of *SMN2* is a major determinant of severity.[5,6,79,82,83] *SMN1* and *SMN2* are 99.9% identical,[84] have equivalent promoters,[85,86] and express RNA[6] and protein ubiquitously.[87,88] The functional difference between the two genes is a C-T change in exon 7, which alters the amount of exon 7 that is incorporated in the final SMN transcript; most transcripts from *SMN2* lack exon 7.[6,84,89–92] The absence of exon 7 disrupts SMN's ability to oligomerize, leading to an unstable protein that is rapidly degraded.[36,37,93,94] In essence, a single *SMN2* gene produces considerably less SMN protein than a *SMN1* gene[87,88] (Figure 2). Thus SMA is caused by low SMN protein levels rather than the complete loss of SMN.[87,88]

In 95% of SMA cases the *SMN1* gene is absent.[6,79] However, five percent of SMA cases have small mutations, such as small deletions, splice mutants and missense mutations, in *SMN1*.[95] These missense mutations can give us critical insight into the function of SMN that is disrupted in SMA. It is important to consider the context in which these missense mutations occur. *SMN2* is always present and thus interactions between the mutant protein and wild-type SMN protein produced by *SMN2* could occur. Table 1 summarizes the SMA missense mutations found in patients along with the activity of the resulting SMN molecules in various assays.

SMN can oligomerize with itself *in vitro*.[36,37] Furthermore, when SMN tagged with different epitopes is transfected into cells, subsequent immunopreciptation reveals that it self-interacts.[38,39] All mild mutations analyzed (those that occur in type II or III SMA when *SMN2* copy number is limited, i.e. 1 or 2 copies) produce proteins that are able to associate with wild-type SMN to form oligomers.[36,37] Other mutations, such as the loss of exon 7 and certain missense mutations in exon 6 (figure 2› table 1) severely disrupt SMN's ability to oligomerize *in vitro*.[36,37] All missense mutations that cause a major disruption of SMN's ability to form oligomers can be classified as severe alleles as when they occur in patients with limited *SMN2* copy number (1 or 2) they result in a severe phenotype.36›96 When SMN fails to oligomerize it is rapidly degraded and thus results in low SMN levels.94 Indeed it has been reported that SMN levels from a type I SMA patient with a mutation that disrupts oligomerization (Y272C), were similar to those of typical type I patients with complete loss of *SMN1*[87]. A second group of severe SMA missense mutations disrupt the binding of the Sm proteins to SMN but are able to oligomerize (Figure2› table1).[96–103] A number of reported mutations have been assayed for their ability to perform snRNP assembly in the presence of limiting amounts of wild-type SMN. All severe mutations assayed did not perform snRNP assembly whereas mild missense mutations did. 40›47›[104] Thus, mild SMN missense mutations retain the ability to oligomerize with wild-type SMN and bind Sm proteins. Our understanding of how these mutated proteins function has been further clarified by genetic complementation studies (Box 1), demonstrating that SMN functions as an oligomer and mild missense mutation are not functional by themselves. Thus *in vitro* assays for SMN function of the missense alleles should be performed in the presence of limiting amounts of wild-type SMN.

Models of SMA and the requirement for SMN

SMN deficiency has been modeled in several organisms (Box 2).[80,105–116] All species used to model SMA possess one *Smn* gene that is equivalent to *SMN1*. In all organisms, loss of SMN leads to lethality, however the exact time point of lethality is determined by the levels of maternal SMN (SMN derived from the mother's SMN gene as opposed to the fetus). In mice lacking *Smn* there is minimal maternal SMN and death occurs early.[80] However, in egg laying animals such as Zebrafish (unpublished observation) and Drosophila, death occurs later when maternal SMN levels drop below a critical point.[111] As would be expected, loss of SMN from a specific tissue in conditional *Smn* mutants results in the death of that tissue.[114,117,118]

In transgenic mice the genetic situation that occurs in SMA has modeled by the introduction of *SMN2* into mice lacking mouse *Smn*. When the *SMN2* copy number is low (2) SMA mice develop whereas high *SMN2* copy (8) number results in normal mice.[107,108] High neuronal expression of SMN driven by the Prion promoter rescues motor neuron loss of SMA mice and extends survival, even though high SMN levels are not restored in all tissues.119 This suggests that two copies of *SMN2* produce sufficient SMN for the normal function of most tissues, whereas motor neurons require higher levels of SMN, at least in mice.[119] Further experiments to determine the precise temporal and spatial requirement for high SMN levels are required. In conditional mouse mutants, or in species in which maternal SMN prevents embryonic lethality, it is likely that SMN levels could mimic levels that occur in SMA and might in turn model some aspects of the disease. However it is difficult to distinguish these features from those that are due to SMN levels that fall below what is required for most cells. It is therefore advisable to confirm observations made in these systems in animals where reduced levels of wild-type SMN are found everywhere.

There often appears to be inconsistencies between results obtained in different model systems, but we urge caution in this interpretation. For example, in Drosophila substantial rescue of SMN deficient animals can be obtained by expressing SMN in muscle.[111,120] In SMA mice, however, expression of SMN in muscle alone has little impact.[119] An examination of the drivers and mutants used in each case reveals two differences. In Drosophila the muscle driver used is expressed early in dividing mesodermal tissue, whereas in mice the actin promoter used drives SMN expression in postmitotic myofibers, but not in satellite cells or other premitotic mesodermal tissues.[111,119,120] Furthermore, in Drosophila functional SMN becomes depleted over time, whereas in SMA mice the human *SMN2* transgene will continue to produce SMN. Thus, in SMN deficient Drosophila, the expression of SMN in a wide range of premitotic mesoderm has a major impact, but the effect of SMN expression in postmitotic muscle fibers is unknown. In SMA mice, expression of SMN in postmitotic muscle fibers has no phenotypic effect and the impact of expression in mesoderm is unknown.

Invertebrates and non-mammalian vertebrates can be used to carry out experiments that cannot be done efficiently in mammals. We would advocate exploiting the strengths of each species. Genetic screens in *C. elegans* and Drosophila can be used to identify suppressors and genetic interactors120, which can then be tested in both zebrafish and SMA mice. To date, the genes identified by suppressor and synthetic lethal screens in Drosophila[120] (discussed below) have not been tested in vertebrate models. In zebrafish it is easy to test a large number of DNA constructs transiently, a select number of which can be followed up in transgenic mice or zebrafish. It is also advisable to confirm results obtained *in vitro* in cultured cells with *in vivo* systems.

In mice, a range of phenotypes can be obtained by titering the level of SMN or by introducing additional transgenes.[39,107,108,114,117–121] The introduction of 8 copies of *SMN2* completely rescues SMA mice, 2 copies of *SMN2* results in SMA mice that die at 5 days,[107,108] and one

copy of *SMN2* is embryonic lethal.[109] Furthermore, introduction of a transgene that expresses SMN lacking exon 7 (SMNΔ7) in SMA mice gives mice that survive 14 days[39] with specific motor defects122 and introduction of SMNA2G results in a mild SMA phenotype.98 It is clear that very small changes in SMN levels can have a major impact on phenotype.119

Understanding the cause of SMA

Two hypotheses have been put forward to explain the mechanism of SMA (Figure 3). The first suggests that the disrupted formation snRNPs affects the splicing of a select group of genes which are important for the motor neuron circuritry.[20,25,104] The second suggests that SMN has a function in axons that is disrupted in SMA.[20,25,67,104,113,123,124] It is also possible that the two hypothesis are linked, that is, reduced snRNP assembly could influence the splicing of a gene important for axons. Evidence for both hypotheses has been gathered, but there is still no clear indication of which is correct or whether both apply.

SMN in SnRNP assembly

According to the first hypothesis, SMN reduction causes an alteration in snRNP assembly, which in turn alters the amount or profile of snRNPs and thus causes an alteration in splicing. What are these alterations and how do they give rise to SMA? Assays to measure SMN's ability to assemble Sm proteins onto snRNA in both cells from SMA patients and tissues from SMA mice have been developed.[27,33,47,56,104,125–127] In mice, snRNP assembly levels change over development.[126] In addition, spinal cord extracts from postnatal day 3 severe SMA mice have low snRNP assembly activity. Mild SMA mice have reduced snRNP assembly activity, when compared to normal or carrier mice, but considerably more activity than severe SMA mice. [104] There is currently a complete correlation between the ability of an SMN protein complex to perform snRNP assembly and its ability to rescue SMA animals.[40,104] However, the introduction of constructs that correct assembly function could affect both the assembly of Sm proteins onto snRNA and the assembly of other RNP complexes. It is therefore important to assay the formation of various RNP complexes to determine which are dependent on SMN. Moreover, snRNP assembly has not been assayed directly in motor neurons of SMA mice. Regardless, the ability of SMN to perform assembly of RNP complexes appears to be critical in SMA.

When SMN is decreased in zebrafish, motor axon defects occur.113 A study has shown that reintroduction of assembled snRNPs can rescue these defects,[128] suggesting that snRNP assembly is the critical pathway affecting motor axons when SMN levels are reduced. However, as indicated by the authors, 50% of the zebrafish scored in the snRNP rescue assay were morphological defective (the study authors reported slightly delayed development and shortened and/or kinked body axis) and snRNPs also rescued these morphological defects (see supplemental table in reference 128). Thus, it is possible that the morphological defects give rise to abnormal motor axons due to the altered body muscles upon which these axons extend; thus, bringing into question the link between snRNPs and motor axon defects.[113,129] Knockdown of *Gemin2* and pICln were also reported to cause motor axon defects in zebrafish, again supporting a role snRNP assembly in the effects of SMN knock down.[128] However, studies that knocked down both *Smn* and *Gemin2* in individual motor neurons, thereby excluding morphological defects, demonstrated that knockdown of Gemin*2* did not cause motor axon defects whereas knockdown of Smn did.[113] To resolve if snRNPs are critical for motor axon defects it will be necessary to ask whether addition of snRNPs suppresses motor axon abnormalities when *Smn* is only knocked down in motor neurons as opposed to the whole organism. Then studies should be extended to genetic models of SMA in zebrafish and mice to ensure that rescue of other SMA phenotypes, including lethality, occurs.

The motor axonal phenotype observed in SMN-knockdown zebrafish can be corrected by reintroducing full-length SMN, but not mutant constructs or SMN lacking exon 7.[123] It was reported that the SMN mutant SMNA111G, which can perform snRNP assembly[40,47], was not capable of rescuing zebrafish axons, whereas a SMN which lacked exon 7 but had the additional amino acids VDQNQKE, and was predicted to be unable to perform snRNP assembly[123], was able to do so.[123] This was taken as evidence against this pathway being critical for axon function. However, we have subsequently obtained evidence that SMNA111G can correct SMA in mice and axon defects in zebrafish when expressed at high levels.[40] Furthermore it has not been possible to assay snRNP assembly in fish extracts and it is not clear whether the VDQNQKE allele does or does not perform snRNP assembly *in vivo*.

An important question for the snRNP hypothesis in SMA is what is the consequence of reduced snRNP assembly? One would predict a uniform reduction in the levels of all of the SMN-assembled snRNPs. However, there appears to be uneven changes in the snRNP profiles of tissues in SMA mice.[56,104] Most notable was a preferential reduction of minor snRNPs such as those containing U11 snRNA in the spinal cord of these mice.[40,56,104] This would suggest that some of the approximately 700 genes that use the minor U12/U11-dependent splicing pathway would be disrupted.[104,130,131] However, a clear demonstration of a SMN dependent downstream target that has a functional alteration in splicing has not been reported. Studies using both expression arrays and exon arrays have been undertaken to determine the effects of SMN reduction on downstream genes. Yet clear changes that can be convincingly related to SMA or even SMN levels directly as opposed to non-specific secondary changes due to the disease state of the mouse have not been obtained to date.[56,132–134] For example, a recent study used exon arrays to define changes in SMA mice (*SMN2*$^{+/+}$*; Smn*$^{-/-}$, SMNΔ7$^{+/+}$). These mice have an average life expectancy of 13 days and where examined at 11 days when the animals are rapidly declining in health. Many hundreds of genes were altered in 11 day old SMA mice in various tissues including spinal cord.[56] However it is difficult to know which of these changes are due to low SMN levels as opposed to secondary changes due to the state of the mouse. Indeed, kidney cytochrome p450 (Cy27b1), a proteins known to respond to stress stimuli, had the largest fold change in expression in the SMA sample[135] Furthermore, it is difficult to mechanistically relate these changes to an alteration in the minor splicing pathway. Although the list contains genes with minor introns, it also features numerous genes that lack them, in addition SMN itself is not listed in all tissues. It is thus hard to distinguish a splice or expression change due to stress, from changes due to SMN depletion. Given that there are a number of factors secondary to SMN reduction that might explain these splicing changes it is not possible to conclude that SMA is a pan-splicing disease rather than a disorder that affects splicing of specific targets important for motor neurons.

Overall, expression changes in SMA samples remain ill defined and the targets of reduced snRNPs have not been convincingly identified. Studies using arrays with exon junction probes[136], and the use of younger animals as well as suitable controls will aid this approach. Furthermore, analysis of gene expression in motor neurons will be useful[134] as well as the implementation of expression comparison using the new deep sequencing techniques. [137] Therefore we conclude that snRNP assembly is altered in SMA, correlates with phenotype severity, and leads to reduced levels of certain snRNPs. However what target genes are specifically affected remains unknown.

A role for SMN in axons

SMN has been found in neuronal axons and growth cones of cells in culture, where it is found in low abundance in a complex that does not contain Sm proteins.[38,66,68,74,124] Although it is relatively straight forward to detect SMN in the axons of cultured cells, it has not yet been convincingly detected in either axons or synapses *in vivo* by light microscopy in vertebrates.

[138] In part this is due to the difficulty in distinguishing whether granules or spots lie in an axon as opposed to an adjacent cell. This might be resolved by the use of specific neural preparations and electron microscopy. To confirm the localization of SMN in axons, the loss or reduction of punctuate SMN staining in axons and growth cones should be observed when SMN is knocked down or in SMA animals.

A role for SMN in axons was strengthened by the finding that knockdown of SMN in zebrafish leads to specific motor neuron axonal defects, including truncated and/or, branched axons. [113]. However, as described above, it is unclear whether these defects result from the loss of snRNP assembly or some other function of SMN. Currently no mutant form of SMN that clearly lacks snRNP assembly activity can correct SMA in animals or humans. There is a complete correlation between reduced ability to perform assembly of Sm proteins onto snRNA and the severity of SMA.[40,104] However, the reduced assembly of other RNP complexes, for example Lsm onto mRNA, could also correlate with severity of SMA and this has not been examined. It is therefore critical to both define the RNP complexes that SMN assembles and to determine whether they are disrupted in SMA.

Motor neurons cultured from SMA mice show a similar phenotype to zebrafish with reduced SMN, including short axons with small growth cones and reduced levels of β-actin mRNA transport to the growth cones.[67] This results in an altered distribution of the $Ca_v2.2$ channel and corresponding electrophysiological alterations.[139] An alteration of Ca channel distribution at the neuromuscular junction (NMJ) might influence neurotransmitter release and the development of active zones, as seen in mice lacking laminin β2.[140,141] Interestingly SMA mice do show developmental abnormalities of the NMJ with reduced arborization.[39,142–144] However, reduced folds at the NMJ do not necessarily indicate a severely impaired NMJ and do not account for the severe phenotype seen in SMA. As both $Ca_v2.1$ and $Ca_v2.2$ channels are important in the CNS synapses, it is currently not clear why motor neuron synapses and not CNS synapses are affected if alteration in Ca channels is critical to SMA. Patients with deficiency of laminin β2 do not demonstrate the classic fiber type grouping seen in type I SMA or the uniform atrophy seen in type 0 SMA and severe SMA mice.[145,146] Furthermore in patients lacking laminin β2 there is clear dysfunction of the CNS.[146] Thus it remains unclear whether SMA can be explained by an alteration of Ca channel distribution at the distal axon. Recently, it has been reported that in SMA mice with a relatively severe phenotype, there is reduced release of neurotransmiter at the NMJ, which could relate to altered Ca channel function at the NMJ or altered input to the motor neuron.[144] Further studies in SMA mouse models and humans[147] are required to resolve the specifics of what is altered at the NMJ.

In support of a critical role for β-actin in SMA, plastin3, a protein that stabilizes filamentous actin [148,149], has recently been reported to be a modifier of SMA.[150] However, there were some inconsistencies. In one discordant family T-plastin was expressed at similar levels in both the affected and unaffected sibs with the male sib being more severely affected. Second, in male patients with 2 copies of *SMN2* SMA was not modified by high plastin3 expression.[5, 151,152] The authors suggest that this discrepancy occurs because plastin3 is a sex-linked modifier with incomplete penetrance. However, it is difficult to eliminate the possibility that the expression of plastin3 is associated with, as opposed to the cause of, the modification. The possibility that a transcription factor activates or repress both *plastin3* and a modifying gene, such as *SMN2*, in spinal cord cannot be ruled out.

SMN and plastin3 are reported to form a complex,[150] however it is unclear whether SMN is associated with filamentous actin as the required assays have not been performed.[148,149] How could *plastin3* modify the SMA phenotype? The axonal defects that occur in cultured motor neurons from SMA mice or zerbrafish with knockdown of SMN, can be rescued by over-expression of plastin3.[150] However, whether this is because plastin3 encourages axon growth

or whether its effect is specific to SMA is unknown. Actin filaments are also important in the nucleus,[153] and it will be important to consider whether snRNP assembly or splicing could be altered by plastin3 expression. It will be important to express plastin3 in mouse models of SMA and determine whether the lethality due to SMA is corrected.

In severe SMA mice, motor neurons grow at a normal rate and do not display axonal branching or misdirection.[109] In severe SMA mice (*SMN2*$^{+/+}$, *Smn*$^{-/-}$) there are NMJs that are unoccupied by a motor neuron[109] indicating denervation. In intermediate SMA mice (*SMN2*$^{+/+}$; SMNΔ7$^{+/+}$; *Smn*$^{-/-}$ that live for 14 days) both loss of occupation and partial occupation of synapses occurs.[39,142,154] In addition, the motor neuron synapses that mature more rapidly during development are more vulnerable than those synapses that mature more slowly. [154] In addition the NMJ of SMA mice are generally immature with abnormal development and poor terminal arborization.[142–144] In mild SMA mice (*SMN2*$^{+/+}$; *Smn*$^{-/-}$; SMNA2G) electrophysiology reveals functional deficits at the NMJ, including intermittent neurotransmission failures.[142] In intermediate SMA mice (*SMN2*$^{+/+}$; *Smn*$^{-/-}$; SMNΔ7$^{+/+}$) electrophysiological studies show a reduced quantal content release of synaptic vesicles.[144] In contrast to mild SMA mice the intermediate SMA mice show facilitation during repetitive stimulation indicating a reduced probability of synaptic vesicle release[144] which could correlate with the alteration of Ca channels reported in motors from SMA mice in culture. In the future it will be important to study Ca Channel disruption *in vivo* in the NMJ of SMA mice as well as consider alteration of inputs to the motor neuron as responsible for defective output of the motor neuron in SMA. In all studies examining NMJs, the abnormal accumulation of neurofilament in motor neurons was observed resulting in varicosities in motor neurons and in certain cases neurofilament accumulation within the NMJ.[109,142,154] Interestingly, similar motor neuronal varicosities have been reported in SMA patients.[155] In Drosophila, reduction of SMN results in lethality and a decreased number of boutons. The latter phenotype can be modulated by changing the BMP signaling pathway.[120]

In conclusion, there is evidence for a role for SMN in the axon, but conclusive evidence that it is the axonal SMN complex that is critically disrupted in SMA is still lacking. Certain critical elements are not known. What is the function of SMN that is directly affected in SMA axons and can it be assayed? Or does disruption of snRNP assembly alter the splicing of a gene that is critical in these axon/NMJ functions? In the case of an axonal function does reduction of SMN directly affect the assembly of complexes onto mRNA leading to decreased mRNA transport to the distal axon? If so, which mRNAs besides β-actin are affected and can this be detected *in vivo*? Lastly, how might this disruption of RNA transport disrupt the lower motor neuron so specifically when SMN is found in the axons of all neurons? In this regards it is important to assay both *in vitro* and *in vivo* the affect of reduced SMN on proposed pathways.

Conclusion

It is currently unclear how reduced SMN levels cause SMA. This issue may be resolved by combining biochemical and genetic approaches. *In vitro* assembly assays of SMN missense mutations, both in homomeric and heteromeric complexes, combined with allelic complementation (Box 1) can be used to discern which SMN function is critically affected in SMA. Suppression or modification of the SMA phenotype can give invaluable insight into which process is critical in SMA. A directed suppression approach in which a specific RNP assembly reaction, such as snRNP assembly, is restored in SMA mice resulting in correction would give strong evidence for this pathway. However, it is difficult to identify such a suppressor that can be studied in a relatively straight forward manner in mice. In Drosophila and *C. elegans* non-directed suppressor and synthetic lethal screens can be performed. These screens ask what can suppress or enhance a specific phenotype without prior assumptions about a particular pathway. Such a screen in Drosophila has indicated the bone morphogenic protein

BMP signaling pathway as important in correction of the button phenotype but it is unclear whether this is a informational suppressor or relatively specific to suppression of SMA phenotypes.[120] Additional screens, in particular using knockdown as opposed to more severe mutants, should provide further insight into SMN dependent pathways. Desirable features of the suppressor include strong suppression, suppression of more than one SMN linked phenotype, elimination of informational suppressors, and more than one suppressor in a pathway. Furthermore, the ability to translate suppression into a vertebrate model will be important. However useful information can be obtained without fulfilling all these criteria. The identification of plastin3 as a potential modifier of SMA is of great interest and experiments to demonstrate that plastin3 does suppress the SMA phenotype in mice are critical. If plastin3 suppresses SMA in mice then it will be important to determine whether suppression is acting in the distal axon or via the nucleus.

SnRNP assembly is clearly altered in SMA, but are other SMN dependent assembly reactions affected? Importantly, what dictates the motor neuron specificity of this disease or how motor neuron specific is the disease? Detailed expression studies using arrays or sequencing technologies are clearly required, but it will be critical to distinguish a direct target from an indirect target. Presumably in the case of snRNPs, there would be different degrees of splicing dysfunction in mice of different severity. It is tempting to speculate that reduced SMN affects either the splicing or axonal transport of a limited number of target genes that are specific to motor neurons. Indeed altered splicing of a gene could in turn give rise to altered transport of mRNA. Restoration of expression of this defective gene(s) would be predicted to suppress the SMA phenotype, thus confirming a target gene. Answers to these questions may also provide insights into other disorders, such as ALS. We believe a careful use of genetics and biochemistry can dissect the immediate molecular targets of SMN deficiency and thus give information on the critical pathway in SMA.

TEXT BOX 1 Allelic complementation of SMN missense mutations

Allelic complementation occurs when the same protein, encoded by two different alleles, oligomerizes to form a functional unit with greater activity than homomers of proteins from either allele. [168,169] This is directly relevant to the analysis of SMA mild missense mutations.

Transgenic mice lacking *Smn*, but expressing 2 copies of human *SMN2* (SMA mice) 107, 108 exhibit very low snRNP assembly levels in spinal cord tissue and have severe SMA, dying at postnatal day 5 (figure, part 2).104,108 Expression of SMNA2G98 or SMNA111G40 missense alleles in this background (figure, part 4) increases their survival and increases snRNP assembly activity.[40,98,104] However, neither allele expressed alone (without *SMN2*) can rescue $Smn^{-/-}$ mice (figure, part 3).[40,98]

The expression of one copy of *SMN2* alone cannot rescue embryonic lethality in $Smn^{-/-}$ mice[109]; however the additional; expression of one SMNA111G allele can. Thus, the wild-type SMN produced by *SMN2* interacts with SMNA111G to give greater total function than the addition of a *SMN2* gene (SMA mice contain two copies of SMN2 and die at 5days). This indicates that two SMN proteins form a heteromer with substantial function.

Although unable to function *in vivo* by themselves, in the presence of limiting amounts of SMN, all mild SMA missense alleles assayed to date (figure, parts 2, 4 and 5) have substantial snRNP activity. This activity suggests that when small amounts of wild-type SMN from *SMN2* are added to mild mutant SMN, the resulting heteromers are functional. [40] High levels of expression of SMNA111G increases snRNP assembly in $Smn^{-/-}$ mice containing one copy of *SMN2* to a much greater extent than an additional *SMN2* copy, suggesting that the SMN complex is a heteromer of multiple subunits (an oligomer).

Severe alleles such as SMNI116F can interact with wild-type SMN but, when assayed in culture, SMNI116F does not complement low levels of wild-type SMN to result in increased snRNP assembly (figure, part 7).[47] Indeed, as they do not have a dominant negative affect in carrier patients these alleles would be predicted to interact with SMN from *SMN2* resulting in no net increase or decrease in total activity.

Could two missense mutations interact to produce a functional SMN oligomer? Although this situation would not arise in patients, this experiment can provide insights into the contribution of different protein domains to SMN function. Clearly, SMNA2G and SMNA111G do not complement each other.[40] However, it would be interesting to determine whether these N-terminal mutations can complement mild C-terminal mutations, which reduce, but do not eliminate, the ability to bind full-length SMN (this possibility is illustrated in part 5).[36,96]

The ability to assay SMN's snRNP assembly activity *in vitro*[44] will allow analysis of both homomer and heteromer activity of SMN missense mutations. This activity can then be correlated with complementation analysis *in vivo* to understand both the *in vitro* and *in vivo* properties affected by SMN missense mutations. In all cases knowledge of the stability and level of mutant proteins in patient derived cell lines is useful in directing future studies and interpretation of results. The figure below shows the outcome of experiments to date on allelic complementation of SMN missense alleles. The amount of SMN or activity in assembly is defined by none −, low +, moderate +++ and high ++++. A question mark (?) indicates allele has not been experimentally tested.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary Terms

Splicing	The removal of introns from pre-mRNA to obtain mRNA
Autosomal recessive disorder	The mutation to cause the disorder lies within a gene that is on an autosomal (not sex) chromosome and both copies of the gene need to be mutated for the disorder to occur
Missense mutations	A mutation that results in the substitution of an amino acid in a protein
UsnRNA	Small nuclear RNAs which are rich in uridine. The U1, U2, U4, U5 and U6 RNA in a complex with certain proteins form the major splicesome complex important for removal of major introns. The U11, U12, U4atac, U5, and U6atac RNA in a complex with certain proteins form the minor splicesome complex important for removal of minor introns
Sm/Lsm proteins	Sm proteins were first found as antigens in a patient with systemic lupus erythematosus and named after the patient. Lsm refers to like

	Sm proteins. Sm/Lsm are a group of RNA binding proteins that have a common three dimensional stucture and form a heptameric or hexameric ring strucutre
Complementation	Refers to a relationship between two different mutations that in the homozygous recessive state produce the same phenotype. The ability of two mutant alleles to interact with each other to produce a normal phenotype is complementation. In general this occurs when the mutations that give rise to the phenotype occur in two different genes. However allelic complementation can arise when different mutations in the same gene can interact with each other to produce a functional complex. This often occurs in oligomeric proteins
Tudor domain	A conserved 50 amino acid stretch originally found in the Tudor protein of Drosophila and often found in proteins with known roles in RNA metabolism
RNA granules	Cytoplasmic deposits that contain RNA. These can also be found in axons and dendrites. RNA in these granules may be transported to the distal axon for localized protein translation in the growth cone
P bodies	P-bodies are cytoplasmic domains that contain proteins involved in diverse post-transcriptional processes such as mRNA degradation, nonsense-mediated mRNA decay, translational repression, and RNA-mediated gene silencing
Active zones	A portion of the presynaptic membrane that faces the postsynaptic density across the synaptic cleft. It constitutes the site of synaptic vesicle clustering, docking and neurotransmitter release
Informational suppressor	A suppressor that will modify in a non-gene specific manner. In the case discussed here over expression of plastin3 could encourage axon growth thus rescuing the SMN deficient growth phenotype. If the growth defect was caused by a mutation in another gene in a different pathway over expression of plastin3 could also rescue that phenotype

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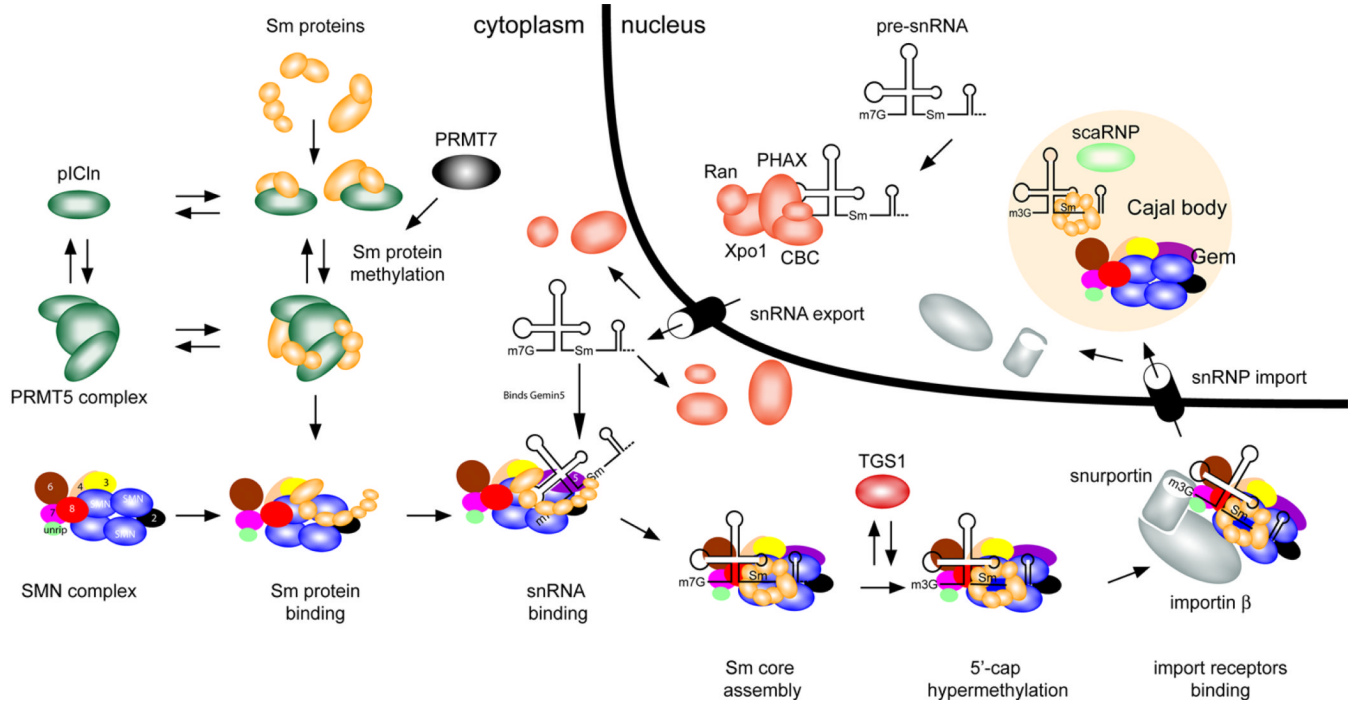

Figure 1. Function of SMN in snRNP assembly

Small nuclear ribonucleoproteins (snRNPs) are active in recognizing and removing introns from pre-mRNA in the nucleus. Each snRNP particle is composed of small nuclear RNA (snRNA) of approximately 150 nucleotides, several Sm proteins and a number of specific proteins that are unique for each snRNP. Survival motor neuron (SMN) functions in the cytoplasm to assemble Sm proteins onto the snRNAs to produce an active snRNP particle.[20, 25, 26, 32]

A) In the cytoplasm the 7 Sm proteins bind to the chloride conductance regulatory protein (pICln).[42, 43] *In vitro* studies reveal that pICln first binds the Sm proteins as two separate complexes: SmB, SmD3, and SmD1, SmD2.44 The latter subsequently binds SmE, SmF and SmG[44] The protein arginine methyltrasferase (PRMT5 complex) and PRMT7 methylate the Sm proteins SmB, SmD1 and SmD3.[42,43,156, 157] Sm proteins are released from pICln-PRMT5 complex and bind the SMN complex.

B1) The SMN complex is composed of SMN, Gemins2-8 and unrip. SMN is shown in the figure as an oligomer as it has been shown to self-associate and it has been suggested that oligomerization is critical for SMN function (see text). The exact numbers of SMN monomers in a SMN complex is unknown (it has been suggested to be an octomer).[158] The Gemins are shown as single units for simplicity as the exact stoichiometry of the SMN complex has not been determined.

B2) snRNA is transcribed in the nucleus and then binds the export proteins phosphorylated adaptor for RNA export (PHAX), Cap-binding complex (CBC), exportin (Xpo1) and ras-related nuclear protein GTP (Ran), which transport it to the cytoplasm.[25] In vertebrates, the snRNA is brought into the Sm protein-bound SMN complex by binding to Gemin5.46

C) The SMN complex places the Sm proteins onto the snRNA.[25, 44] The m^7G cap of the snRNA is hypermethylated by *trimethylguanosine sythetase 1 (TGS)*[25], allowing the SMN complex with the snRNA to bind snurportin and importin, which mediates transport of the SMN complex with an assembled snRNP into the nucleus.[25]

D) In the nucleus the SMN complex and snRNPs localize to the Cajal body and snRNPs undergo further maturation.[78] Depending on the cell type and developmental stage, SMN can localize as a separate body adjacent to the Cajal body.[13–15]

The figure is adapted from Pellizzoni.[25]

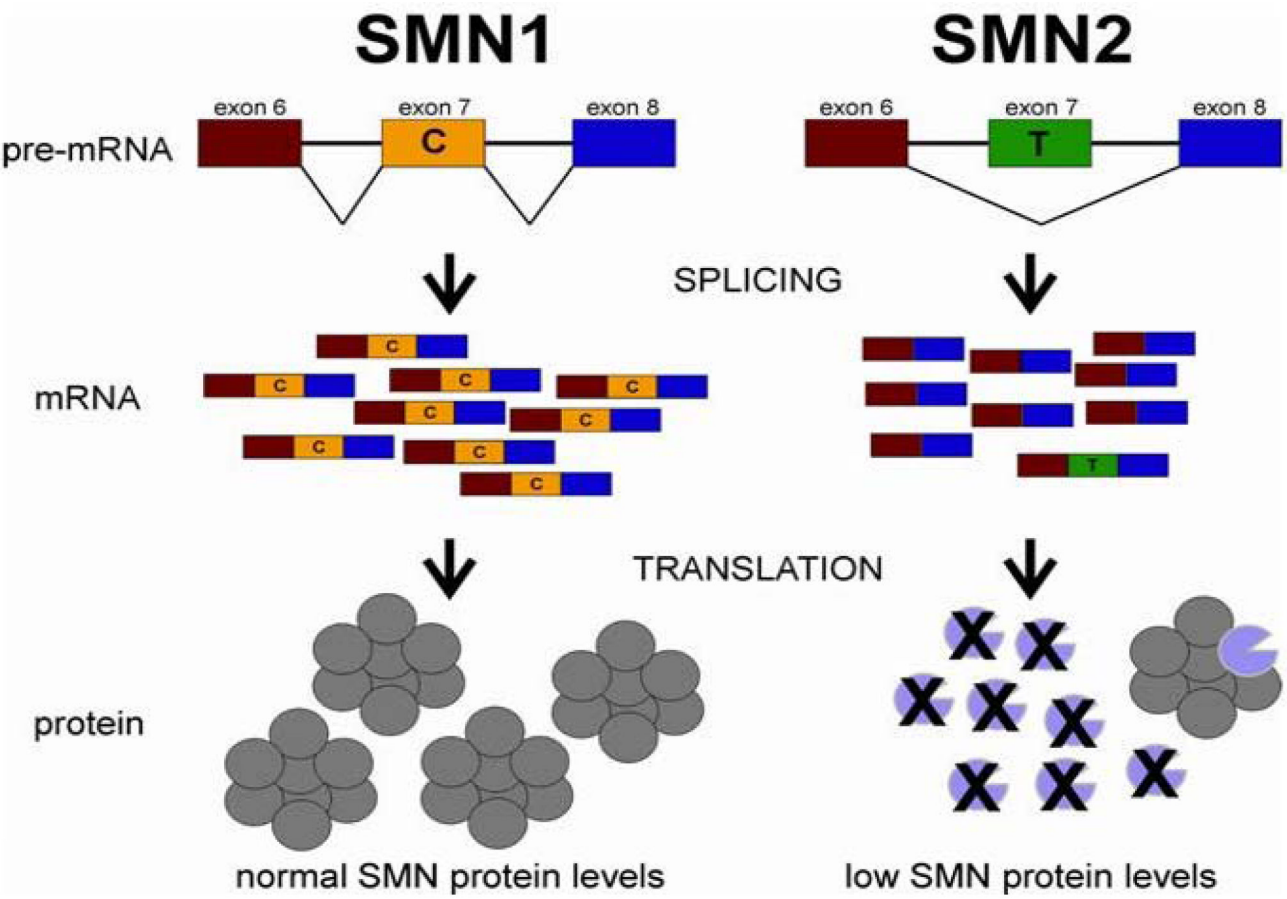

Figure 2A. *SMN1* and *SMN2* genes: structure and splicing

The *SMN1* and *SMN2* have identical gene structure and are 99.9% identical at the sequence level.[84] The essential difference between the two genes is a single nucleotide change in exon 7 (C or T as indicated). This single nucleotide change affects the splicing of the gene. Thus the majority of SMN transcripts from *SMN2* lack exon 7 whereas those from *SMN1* contain exon 7.[6, 84, 89–92, 121] However, because *SMN2* does produce some full-length SMN it can be viewed as a gene with reduced function but not loss of function. The loss of amino acids that are encoded by exon 7 results in the production of SMN protein with severely decreased oligomerization efficiency and stability.[36, 37, 93, 94] The SMN monomers are rapidly degraded.[94] Thus, loss of *SMN1* results in reduction of SMN levels in most tissues. The SMN oligomer is represented as an octomer based on gel filtration of SMN complexes formed *in vitro*[158] Figure is taken from Butchbach and Burghes.[159]

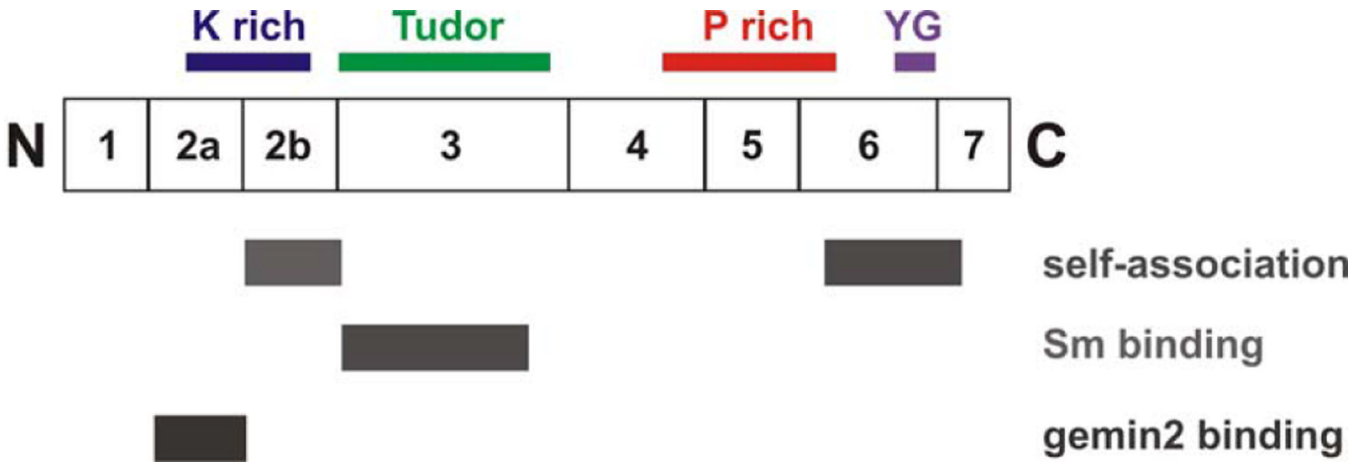

Figure 2B. Domains of SMN

A diagram of SMN showing the exons and domains. Exon 2B encodes a domain important in binding Gemin2 as well as self-association.[160] Both exon 2A and 2B are conserved. The K domain is rich in lysine, the Tudor domain is in exon 3 and has homology to other Tudor domains. The Tudor domain binds Sm proteins.[97] Exon 5 and part of exon 6 contain a proline rich domain that may influence profilin binding.[161] The C-terminal domain of exon 6 contains the conserved YG box and is important for self-association.[160, 162]

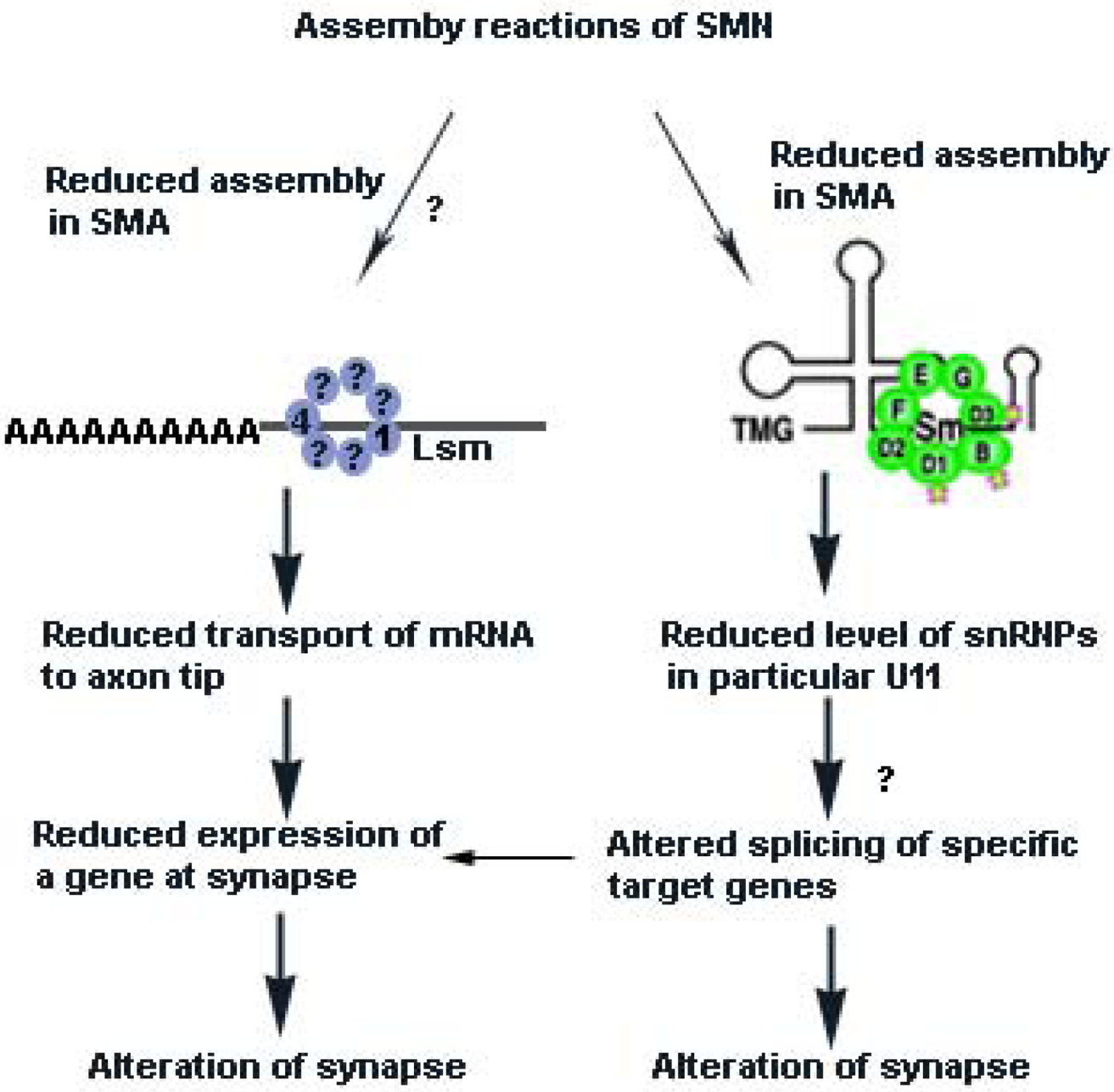

Figure 3. Mechanisms proposed to explain how reduced SMN levels cause SMA

According to one hypothesis, reduced SMN levels result in reduced assembly of Sm proteins onto snRNA. This unevenly alters the levels of specific endogenous snRNPs, such as those used to splice minor introns (particularly U11) from pre-mRNA.[40, 56, 104] It remains to be determined what the downstream target genes of the affected snRNPs are and how this specifically affects motor neuron function (indicated by a question mark (?)). One possibility is that the critical target gene is specific to motor neuron system. Alternatively, a function of critical importance to motor neurons could be disrupted.

In addition, it has been suggested that reduced levels of β-actin mRNA or other mRNA occur at the axon tip or synapse due to SMN having a function in axon RNA transport[66, 67, 68] at the

growth cones of motor neurons cultured from SMA mice. It has been proposed that hRNPQ/R66, 67 and ZBP69 participate with SMN in this complex and that the reduced β-actin transport leads to alteration of calcium channel distribution at the axon terminal139 which in turn could affect neurotransmitter release (see text). Lsm proteins 1 and 4 have been found in axons in an RNP complex.[59] We suggest that it is possible that reduced SMN levels affect the assembly of Lsm proteins required for axonal transport of mRNA, leading to reduced expression of specific genes at the synapse. However, a functional biochemical assay linking reduced SMN levels to an alteration in the formation of the required complex for transport of mRNA is lacking (indicated by ?). Whether other Lsm proteins, such as Lsm14, associate with this complex in neurons is not known.

We have not indicated other potential or known SMN dependent assembly pathways, such as assembly of U7 snRNA, as it is not clear how alteration of this pathway would give rise to SMA. However, we cannot eliminate the possibility that other RNP assembly reactions are affected by reduced SMN levels. Lastly, it is possible to unite the two hypotheses where reduced snRNP assembly causes reduced splicing of a target gene that is critical for transport of mRNA to the motor neuron synapse.

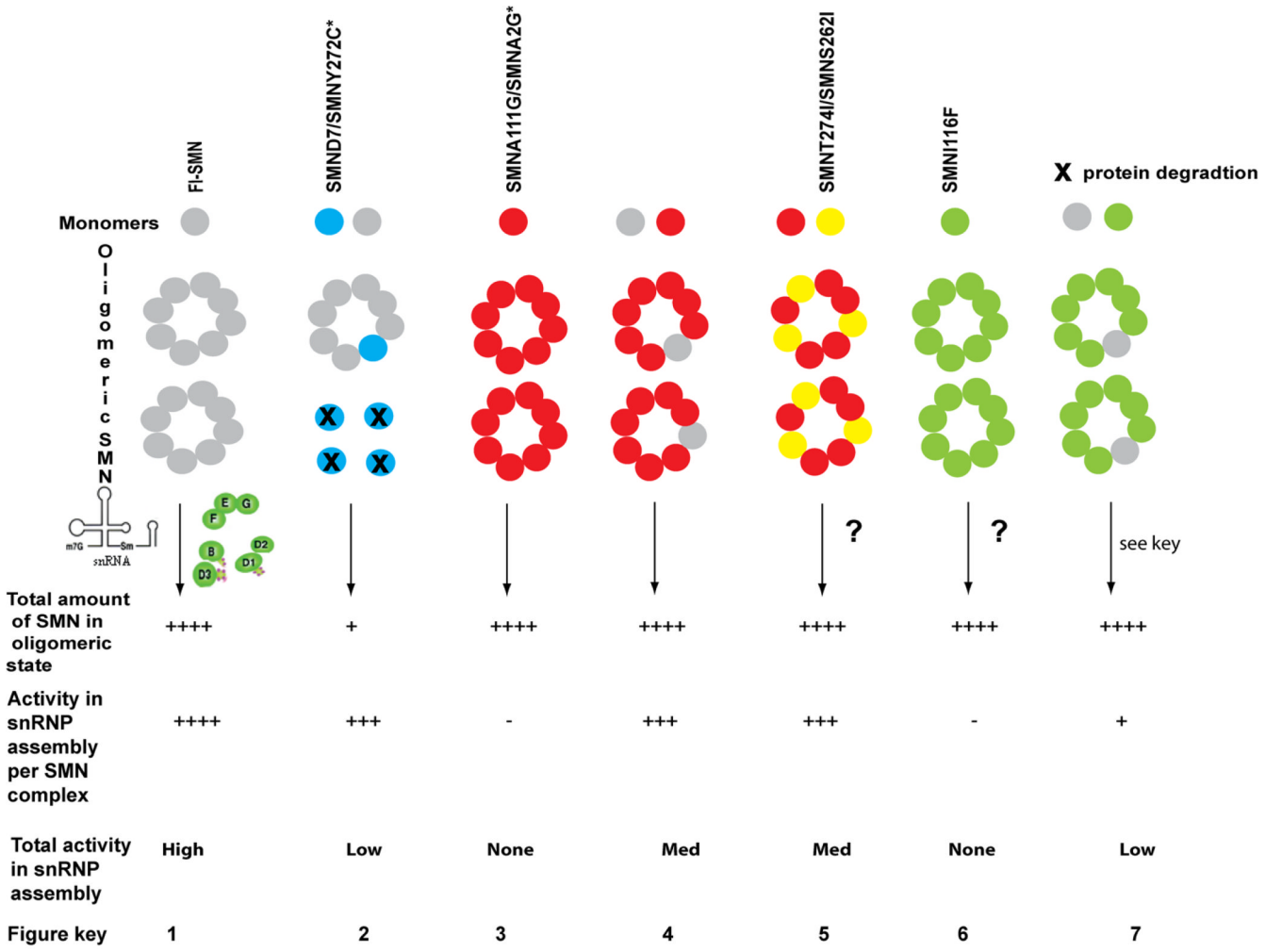

Figure for Text box1.

Table 1

Properties of missense mutations in SMA patients.

Mutation	Position of mutation		SMA type	*SMN2* copy number	Ability to Rescue mouse or fish SMA models	Ability to bind SMN	Ability to bind Sm proteins	Ability to Assemble snRNP when low amounts of non mutant SMN are present	References
	Exon	Domain							
A2G	1	ND	III	1	**High**	(++)	(++)	(++)	98, 103, 104
D30N	2a	Gemin2	II	2	ND	(+++)	(+++)	(+++)	47, 96
D44V	2a	Gemin2	III	1	ND	(+++)	(+++)	(++)	96,99
W92S	3	Tudor	I	3	ND	ND	(+)	ND	100
V94G	3	Tudor	II	3	ND	ND	ND	ND	101
G95R	3	Tudor	III	1	ND	(+++)	(+++)	ND	96
A111G	3	Tudor	Ib	2	**High**	(+++)	(+++)	(+++)40, 47	40, 4796, 123
I116F	3	Tudor	Ib	2	ND	ND	ND	(+)	47, 102
Y130C	3	Tudor	III	2	ND	ND	ND	ND	163
E134K	3	Tudor	I	2	ND	(+++)	(+)#	(+)	47, 96, 97
Q136E	3	Tudor	I	1	ND	ND	ND	(+)	47, 102
A188S	4	ND	I	ND	ND	ND	ND	ND	164
P245L	6	oligomer	III	ND	ND	ND	ND	ND	165
L260S	6	oligomer	II	2	ND	ND	ND	ND	101
S262G	6	oligomer	III	1	ND	ND	ND	ND	96
S262I	6	oligomer	III	1	ND	(++)	(++)	ND	36, 166
M263R	6	oligomer	I	2	ND	ND	ND	ND	101
M263T	6	oligomer	II	1	ND	ND	ND	ND	95
S266P	6	oligomer	II	2	ND	ND	ND	ND	163

Mutation	Position of mutation		SMA type	*SMN2* copy number	Ability to Rescue mouse or fish SMA models	Ability to bind SMN	Ability to bind Sm proteins	Ability to Assemble snRNP when low amounts of non mutant SMN are present	References
	Exon	Domain							
Y272C*	6	oligomer	II	2	**No**	(+)	(+)	(+)	36, 47, 123, 166
H273R	6	oligomer	II	ND	ND	ND	ND	ND	163
T274I	6	oligomer	III	1	ND	(++)	(++)	(+++)	36, 47, 166
G275S	6	oligomer	III	ND	ND	ND	ND	ND	101
G279C	7	oligomer	I	ND	ND	ND	ND	ND	167
G279V	7	oligomer	I	ND	**No**	(+)	(+)	(+)	36, 47, 123, 162

*The SMNY272C mutation has been reported in all three types of SMA with severity of the phenotype being dependent on *SMN2* copy number. This mutant SMN does not oligomerize efficiently and is rapidly turned over. SnRNP assembly has been assayed in mouse spinal cord tissue for SMNA2G and SMNA111G and in cell culture in all other cases indicated.[47]

+low binding or low activity;

++moderate binding or activity;

+++high binding or activity ND is not determined.

#Sm binding assay performed with just the Tudor domain of SMN and not full-length SMN.

Table 2

SMN mutant organisms

Organism	Mutation	Phenotype	References
S. pombe	Loss of *smn* by gene disruption	Lethal	105, 106, 115
C.elegans	RNAi knockdown and knockout of *smn*	Embryonic lethality and developmental defects, movement defects (with later knockdown of SMN)	110, 170
Drosophila	Smn^{73Ao} and Smn^{B} (G202S and S201F respectively).Mutations disrupt SMN self-association and can be considered equivalent to null alleles	Larval lethal or embryonic lethal if maternal SMN removed	111
	Smn^{E33} Loss of SMN in adult thorax	Lose ability to fly and jump	171
	siRNA knockdown of SMN	Lethality at different stages depending on knockdown.	120
Zebrafish	Antisense morpholino knockdown of *Smn* resulting in reduced Smn early but restoration of Smn levels later	Abnormal axon patterning and death.	113
Mouse	Loss of Smn by gene disruption of mouse *Smn* gene	Embryonic lethal	80
	Conditional (Cre-driven) loss of *Smn* in specific tissues at specific times	Death of cells in the tissue where *Smn* is removed	114, 117,118
	Reduced SMN levels through loss of mouse *Smn* and introduction of *SMN2* transgene.	Depends on *SMN2* copy number; 8 *SMN2* copies normal, 2 copies *SMN2* death at 5 days. Phenotype varies dependent on the deletion background or the presence of other SMN transgenes	107, 108
Human	Loss of *SMN1* and retention of *SMN2*	Phenotype is dependent on the copy number of *SMN2* and, more specifically, the amount of functional SMN complex	6