SURVIVAL MOTOR NEURON PROTEIN INTERACTION PARTNERS IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

Kelsey Marie Gray: Survival Motor Neuron protein interaction partners
in *Drosophila melanogaster*
(Under the direction of A. Gregory Matera)

Spinal Muscular Atrophy (SMA) is a neuromuscular disorder that results from biallelic loss-of-function mutations in the human *survival motor neuron 1* (*SMN1*) gene. Tissue-specific and housekeeping functions have been ascribed to SMN; however, their relevance to SMA pathology is not well understood. We generated transgenic *Drosophila melanogaster* that express only flag-tagged wild-type SMN. Our objective is to characterize novel protein-protein interactions of SMN. We collected embryos and analyzed Flag-purified lysates by mass spectrometry. We identified Flag-SMN along with other known interactors such as the Sm proteins and the Gemins.

We also identified Slmb, SkpA, and Cullin 1 as being highly enriched in Flag-SMN samples as compared to the control sample. Together, these proteins comprise the SCF^{Slmb} E3 ubiquitin ligase. These interactions were verified in *Drosophila* S2 cells and human cells. *In vitro* experiments revealed Slmb and SMN can directly interact. Identification of a putative Slmb degron in the self-oligomerization domain of SMN led us to generate a serine to alanine mutation that stabilizes full length and truncated SMN, with strongest effects on SMN with poor self-oligomerization capability. This same point mutation decreases SMN's interaction with Slmb, demonstrating the putative Slmb

degron is indeed mediating degradation of SMN. Finally, expression of truncated SMN stabilized by the mutation modifies viability of a mild SMA mouse model.

We identified additional protein interactions of SMN with CG2941, nucleosome assembly protein 1 (Nap1), and Bendless (Ben). Each of these interactions was verified in cell culture or using antibodies generated specifically for the protein of interest. Preliminary investigation of CG2941 has revealed it is an essential gene that produces protein that localizes to both the nucleus and the cytoplasm.

We have examined SMN protein interactions in the context of developing *Drosophila melanogaster* embryos, with follow-up studies in mouse, and human systems. When SMN is unable to self-oligomerize, the Slmb degron is highly accessible, and thus SMN is degraded. SMN also interacts with previously unknown partners that may be relevant to SMA pathology. This work elucidates a disease-relevant mechanism where SMN levels are regulated by self-multimerization and identifies candidate proteins for further study of the molecular mechanisms underlying SMA.

To my parents,

my brother, Dorian,

and my cat, Emma

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my parents. I was raised in an incredibly fortunate home where I knew I would be supported through earning a graduate degree from the time I was born. They have always been there to motivate me and to support my love of science. They taught me to persevere, and I am incredibly thankful for that today. I appreciate my brother for always being there to ask me questions to which I don't know the answer, as a reminder that I always have more to learn.

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LIST OF ABBREVIATIONS

AAV9	Adeno-associated virus serotype 9
ATP	Adenosine triphosphate
Ben	Bendless
CHX	Cycloheximide
Cul1	Cullin 1
dsRNA	Double-stranded RNA
DNA	Deoxyribonucleis acid
dSMN	*Drosophila* SMN
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methane sulphonate
FLAG	Polypeptide epitope
GF	Giant fiber neuron
GFP	Green fluorescent protein
GST	Glutathione S-transferase
HEK	Human embryonic kidney cells
Impβ	Importinβ
IP	Immunoprecipitation
iPSC	Induced pluripotent stem cells
m^7G	7-methylguanosine
m_3G	Trimethylguanosine
Myc	c-Myc epitope

Nap1	Nucleosome assembly protein
NP-40	Nonident P-40
mRNA	messenger RNA
mRNP	messenger RNP
NMJ	Neuromuscular junction
OR	Oregon-R
PBS	Phosphate buffered saline
PHAX	Phosphorylated adaptor for snRNA export
PLS3	Plastin 3
PRMT5	Protein arginine methyltransferase
RIG	Rigor Mortis
RNA	Ribonucleic acid
RNAi	RNA interference
RNP	Ribonucleoprotein
S2	Schneider 2
scaRNP	small cajal body specific RNP
SETX	Senataxin
siRNA	Short interfering RNA
SkpA	Skp1-related A
Slmb	Supernumerary limbs
SMA	Spinal muscular atrophy
SMN	Survival motor neuron
snoRNP	small nucleolar RNP

snRNA	small nuclear RNA
snRNP	small nuclear RNP
SPN1	Snurportin1
Tgs1	Trimethylguanosine synthase
TMG	2,2,7-trimethylguanosine
UAS	Upstream activating sequence
Unrip	unr-interacting protein
UPS	Ubiquitin proteasome system
U-snRNA	Uridine rich snRNA
UTR	Untranslated region
WT	Wild-type

CHAPTER I: Introduction

Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is a common neuromuscular disorder that is the most prevalent genetic cause of infant mortality (Pearn 1980). SMA has a carrier frequency of 1 in 50 and an incidence rate of 1 in 6,000-10,000 (Ogino et al. 2002). The disease presents clinically as the degeneration of motor neurons in the anterior horn of the spinal cord (Crawford and Pardo 1996). Without stimulation from the neuron, the proximal muscles atrophy, leading to subsequent loss of motor function, and ultimately symmetrical paralysis. Since the timing of the onset of symptoms and their severity can vary, SMA has historically been classified into subtypes (Ogino S 2004). More recently, clinicians have recognized that SMA is better characterized as a continuous spectrum disorder, ranging from severe (prenatal onset) to nearly asymptomatic (Tiziano et al. 2013)

While there are several different ways to classify SMA, one of the most commonly used systems separates SMA cases into three types based on the severity of the phenotype, which is determined by the age of onset and the level of maximum motor function achieved by the patient (Ogino S 2004). Type I SMA is the most common form of the disease, affecting ~60% of SMA patients (Nicole et al. 2002; Ogino S 2004). Type I SMA, alternatively known as Werdnig-Hoffman disease, is also the most severe form.

Symptoms begin to appear as early as *in utero* and are clearly detectable during the first 6 months of life (Wee et al. 2010; Prior 2010). Affected infants experience progressive muscle weakness, which correlates with a reduced ability to swallow. These infants most frequently die from complications such as progressive and restrictive respiratory failure by 2 years of age (Kolb and Kissell 2015). Type II SMA is less severe with symptom onset occurring between 6 and 18 months of age. These children experience developmental motor delays and are unable to stand or walk, although they can sit unsupported. The lifespan of type II patients can vary from 2 to 30 years, with death usually occurring as a result of respiratory infections. SMA type III, also known as Kugelberg-Welander disease, is the mildest form with an age of onset after 2 years. Most type III patients are able to stand and walk, but often need to use a wheelchair in adulthood due to muscle weakness. Many type III patients have a normal life expectancy since the disease progress is slow.

SMA Genetic Etiology

In 1990, linkage analysis was used to map the SMA-causing gene to chromosome 5q11.2-13.3 in several affected families (Brzustowicz et al. 1990; Melki et al. 1990). Lefebvre et al. (1995) discovered a gene in that chromosomal region, *survival of motor neuron 1* (*SMN1*), that was identified to be the causative gene in SMA (Bussaglia et al. 1995; Rodrigues et al. 1995; van der Steege et al. 1995; Chang et al. 1997). At this time, it was determined that over 95% of SMA patients have deletions of *SMN1* (Lefebvre et al. 1995; Campbell et al. 1997).

Further investigation of the 5q chromosome region revealed the presence of at least two *SMN* paralogs in most people (Fig. 1.1). There is a 500 kb inverted duplication

located at 5q11-13 that resulted in a telomere-proximal copy (*SMN1)* and a centromere-proximal copy (*SMN2*) of the *SMN* gene (Lefebvre et al. 1995). Five base pair differences distinguish the two *SMN* copies at the nucleotide level, but each of these nucleotide changes leave the amino acid sequence of the protein unchanged. The nucleotide difference that affects the functionality of the proteins produced by each of the genes is a change from C to T in exon 7 of the *SMN2* gene (Lorson et al. 2010). This change affects splicing and results in the exclusion of exon 7 (*SMNΔ7*) in ~90% of the *SMN2* transcripts (Fig. 1.1). This *SMNΔ7* transcript encodes a truncated and unstable protein. The last 16 amino acids of SMN are replaced in SMNΔ7 by four amino acids, EMLA, encoded by exon 8.

Current estimates suggest the remaining ~10-15% of transcripts are full length and encode protein that is fully functional and indistinguishable from that produced by *SMN1* (Lorson et al. 1999; Monani et al. 1999; Lorson et al. 2000). Thus, both *SMN1* and *SMN2* contribute to total cellular levels of SMN protein.

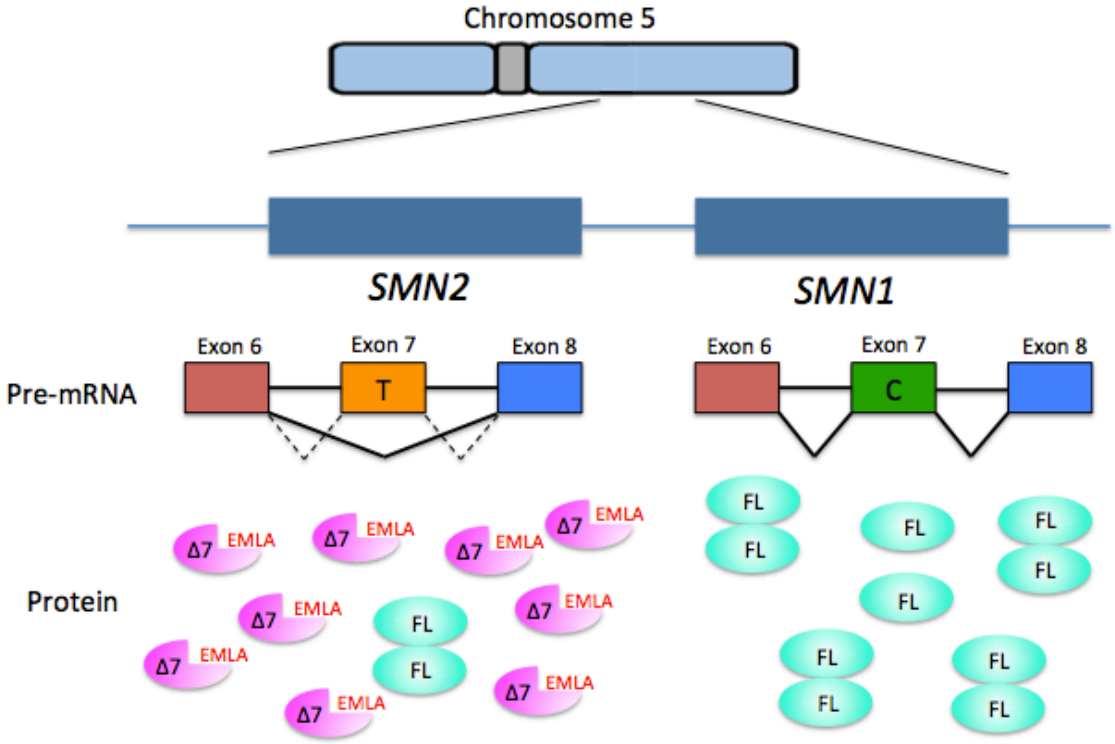


Figure 1.1. SMN1 and SMN2 are found on human chromosome five. SMN2 cannot fully compensate for loss of SMN1, but is the most significant modifier of the disease phenotype. A base change from C to T in the SMN2 gene results in the exclusion of exon 7 and production of a truncated and unstable protein with the addition of four amino acids (EMLA) (Δ7) in ~90% of the transcripts and full-length (FL) protein in ~10% of the transcripts.

Complete loss of SMN expression is lethal in all organisms investigated to date (Schrank et al. 1997; O'Hearn et al. 2016); therefore, SMA is a disease that arises due to hypomorphic reduction in SMN levels (Lefebvre et al. 1995). Although the amount of full-length protein produced by *SMN2* is not enough to compensate for loss of *SMN1*, *SMN2* is sufficient to rescue embryonic lethality (Monani et al. 2000). Studies in SMA patients have revealed that decreased levels of functional, full length SMN protein correlate with the phenotypic severity of SMA (Lefebvre et al. 1997; Coovert et al. 1997). These findings contributed to the formulation of a connection between these genes and SMA.

SMA is a gene dosage disorder with *SMN2* being the primary genetic modifier of the phenotype. There is an inverse correlation between the number of *SMN2* copies in the genome and disease severity (Vitali et al. 1999). Mildly affected patients generally have more copies of *SMN2* than those with more severe phenotypes. Consistent with this observation, levels of SMN protein in cells from SMA type I patients are reduced to 5-20% of levels in controls (Lefebvre et al. 1997; Vitali et al. 1999). In contrast, type III SMA patient cells have SMN levels that are comparable to controls. One potential explanation for this finding is that type I SMA is caused by deletions and/or mutations in the *SMN1* gene, whereas type III SMA results from gene conversion events that convert *SMN1* to *SMN2* (Campbell et al. 1997). In the latter case, there would be more copies of *SMN2*, since *SMN1* was converted, and thus more functional SMN protein. This would result in a milder SMA phenotype. While SMA typically results from homozygous deletion of *SMN1* gene (Lefebvre et al. 1995), a small fraction of SMA patients in all three categories of severity have lost one copy of *SMN1* and the remaining copy contains a missense mutation (Burghes and Beattie 2009). While the genetic etiology of the disease is well-established, the molecular role of SMN in the disease is largely unknown. Several animal models of SMA, including *Drosophila melanogaster*, are used to address this open question.

SMN Protein and the SMN Complex

The *SMN1* gene and protein are highly conserved in evolution, with homologs found in all major eukaryotic model organisms investigated except *S. cerevisiae*. The human SMN protein is 294 amino acids long and has three well described domains: the Gemin2 binding domain, the Tudor domain, which is found in many RNA-binding

proteins, and the C-terminal region called the YG box (Fig. 1.2). The Tudor domain is thought to be involved in binding of SMN to Sm proteins (Buhler et al. 1999). The YG box is the most well conserved region in the protein and is involved in SMN self-oligomerization (Lorson et al. 1998). Interestingly, primates are the only species that have more than one copy of the *SMN* gene, and only humans have the C to T base change that defines the *SMN2* gene. Researchers have failed to detect any *SMNΔ7* mRNA in our closest relative, the chimpanzee (DiDonato et al. 1997; Rochette et al. 2001).

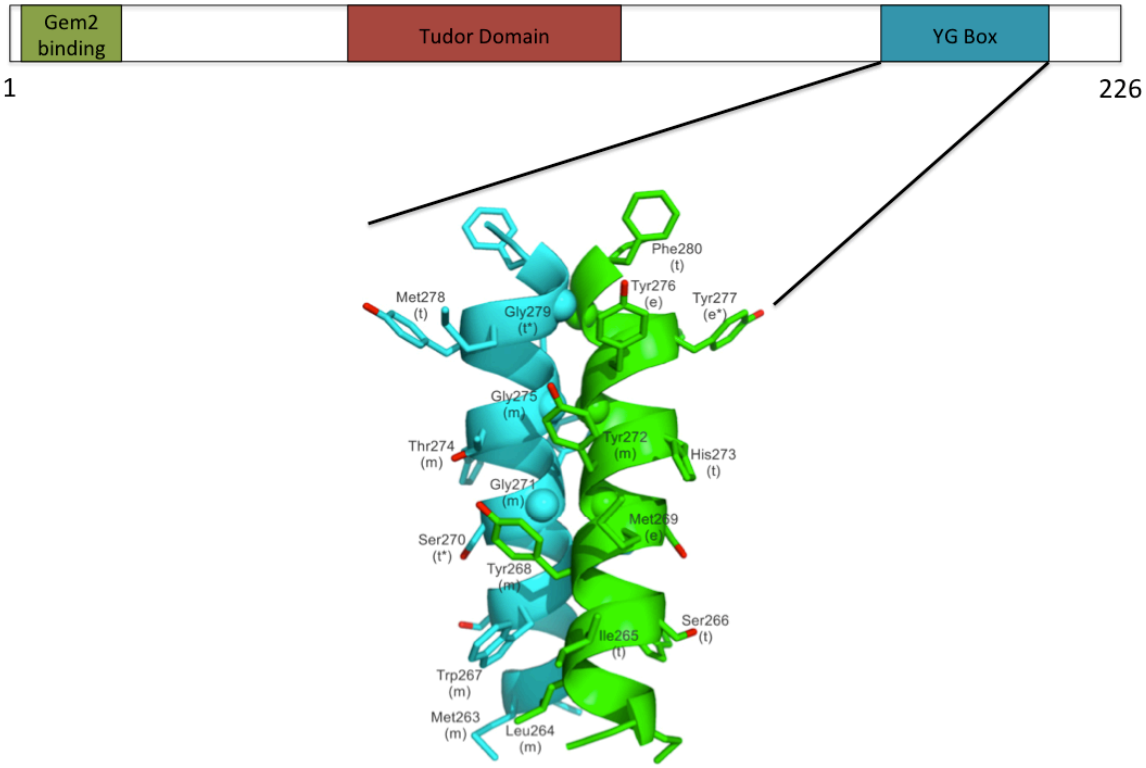


Figure 1.2. Major domains in the SMN protein. Survival of motor neuron protein is conserved from yeast to humans and contains three functional domains. The SMN protein has an N-terminal domain that is important for binding to Gemin2, a member of the canonical SMN complex. The tudor domain, which is present in many RNA binding proteins, interacts with Sm proteins. The YG box mediates SMN self-oligomerization. This domain forms a glycine zipper structure, similar to that seen in transmembrane proteins. Figure modified from the thesis of Kavita Praveen (Praveen, 2012) using YG Box structure from Martin et al. 2012.

Consistent with its function in the essential process of snRNP biogenesis, SMN is ubiquitously expressed (Coovert et al. 1997; Burlet et al. 1998) and localizes to the cytoplasm as well as the nucleus. In the cytoplasm, SMN is diffuse, whereas SMN is found in nuclear foci called Cajal bodies in the nuclei of most tissues (Carvalho et al. 1999). Cajal bodies contain high levels of snRNPs, small nucleolar ribonucleoproteins (snoRNPs), small Cajal body specific ribonucleoproteins (scaRNPs) as well as other proteins involved in RNP metabolism (reviewed in Matera et al. 2006).

SMN is found as part of a large multimeric complex consisting of SMN, the Gemins, and Unrip in the cytoplasm (Charroux et al. 1999; Charroux et al. 2000; Baccon et al. 2002; Gubitz et al. 2002; Pellizzoni et al. 2002; Grimmler et al. 2005; Carissimi et al. 2006) (Fig. 1.3). The entire SMN complex is required for proper snRNP assembly *in vivo*. SMN and Gemin2 alone are not sufficient to restore RNP assembly activity in *Xenopus* egg extracts immunodepleted for these proteins (Meister et al. 2001). A number of additional studies have confirmed this requirement for the other members of the SMN complex (Meister et al. 2001; Feng et al. 2005; Shpargel et al. 2005; Battle et al. 2006; Ogawa et al. 2007). It is possible that the other SMN complex members may be involved in snRNP-independent functions of SMN (Zhang et al. 2006; Walker et al. 2008; Todd et al. 2010).

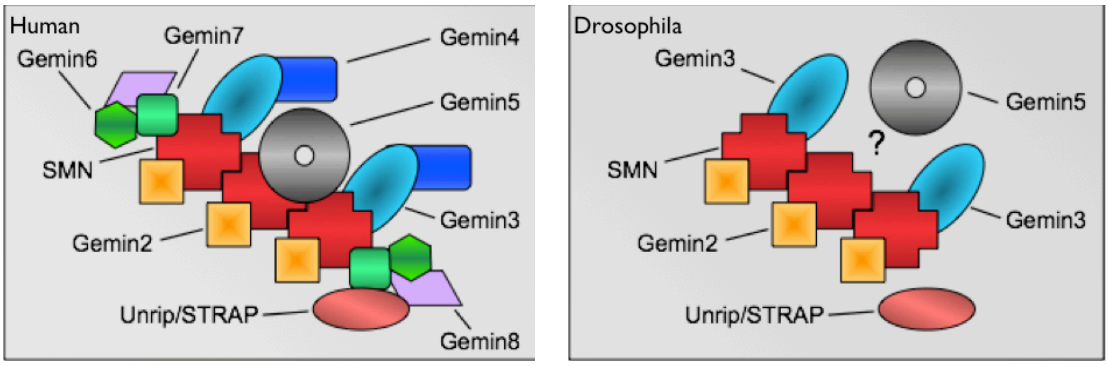


Figure 1.3. Comparison of core SMN complex members in human (mammal) and *Drosophila*. Only verified members are shown in each representation and the stoichiometry is not intended to reflect true relative amounts of protein. The human SMN complex includes Gemin2/3/4/5/6/7/8 and Unrip/STRAP. Drosophila has a lesser verified SMN complex, consisting only of Gemin2/3 and Unrip. The *Drosophila* homolog of Gemin5, *rig*, is likely a member of the complex. However, *rig* has not been definitively shown to play a role in snRNP biogenesis. Figure from Greg Matera, unpublished.

While orthologs of Gemin2 can be identified in all species, the other Gemins are not as well conserved (Fig. 1.3). A bioinformatic investigation to identify Gemin homologs suggested that Gemins 3 and 5 are the most ancestral Gemins in the complex (Kroiss et al. 2008). Putative homologs of Gemins 4, 6, 7, and 8 were only found in animals at the time, suggesting they are newer additions to the complex. Both Dipterans that were analysed, *D.melanogaster* and *A.gambiae*, seemed to have only Gemins 2, 3 and 5 (Fig. 1.3).

HeLa cells require SMN and Gemins 2, 3, 4 and 5 for snRNP assembly (Feng et al. 2005; Shpargel et al. 2005). A minimal SMN complex, consisting of SMN and Gemin2 only, is sufficient for assembly of Sm proteins onto snRNAs *in vitro* (Kroiss et al. 2008). Therefore, while other SMN complex members may not be essential for the assembly reaction, they enhance snRNP assembly *in vivo*. These enhancement functions include improving specificity of assembly of the Sm ring on the snRNA and speeding up the reaction. As previously mentioned, the SMN complex is associated with the Sm-

snRNA complex throughout the cytoplasmic phase of assembly (Massenet et al. 2002) and is also important for re-import of the immature snRNP into the nucleus (Narayanan et al. 2002; Narayanan et al. 2004). This presents the possibility that Gemins have functions in steps following the assembly of Sm proteins onto snRNAs.

The Gemins are very structurally different, and the precise function of most of them in the SMN complex is not clear. However, there are suggestions for the functions of several of them. For example, Gemin2 has been reported to stabilize SMN by enhancing SMN self-association through the N-terminal Gemin2 binding domain (Ogawa et al. 2007). Additionally, Gemin2 binds a pentamer of the Sm proteins D1, D2, E, F and G directly, as visualized in a crystal structure of Gemin2 with the Gemin2-binding domain of SMN (Zhang et al. 2011). Gemin3 contains a DEAD box domain with potential helicase activity; therefore, Gemin3 may perform the ATP dependent step of the assembly reaction (Charroux et al. 1999). Gemin5 has been shown to bind snRNAs directly, which could contribute to distinguishing them from other RNAs (Battle et al. 2006). This mechanism provides specificity to the assembly reaction (Battle et al. 2006). Gemins 6 and 7 form a heterodimer that is similar to the structure created by heterodimers of SmB/SmD3 and SmD1/SmD2 (Ma et al. 2005). Gemins 6 and 7 also interact with a subset of Sm proteins. It has been suggested that Gemins 6 and 7 act as "place holders" for SmB/SmD3 in the Sm protein pentamer that binds Gemin2 (Zhang et al. 2011) before assembly onto the snRNA. Functions have not been assigned to the remaining members of the SMN complex.

SMN Function

A role of SMN in RNA metabolism was the first suggested cellular function (Liu et al. 1996). This was determined due to the observation that SMN protein can associate with the RNA binding domain of hnRNP U, an RNA binding protein. The role of SMN in RNA metabolism was bolstered when, in 1997, Liu et al. showed that SMN, along with Gemin2, co-purified with a set of proteins that bind to uridine-rich small nuclear ribonucleoproteins (U-snRNPs). These proteins are called Sm proteins. Fischer et al. (1997) determined the functional significance of the interaction by using *Xenopus* oocytes to show that SMN and Gemin2 were involved in an early step in spliceosomal U-snRNP biogenesis. These data were corroborated in 2001 by Meister et al. who showed that the SMN complex was required for *in vivo* assembly of Sm proteins onto U-snRNAs.

snRNP Biogenesis

SMN is expressed in all tissues of animals (Matera and Wang 2014; Tripsianes et al. 2011; Li et al. 2014). The best-characterized function for the ubiquitous SMN protein is in the assembly of Sm-class snRNPs. Sm-class snRNPs are made up of uridine-rich snRNAs, non-coding RNAs that perform diverse roles in RNA metabolism (Mattaj et al. 1993; Tern and Steitz, 1997). Sm-class snRNPs also contain several specific proteins that are unique to each snRNA and a set of 7 common Sm proteins.

The Sm-class snRNPs form the core components of the spliceosome. Two distinct classes of spliceosomes exist: the ‘major’ spliceosome and the ‘minor’ spliceosome (Levine and Durbin 2001). The ‘major’ spliceosome is responsible for over 99% of intron splicing in the human genome while the ‘minor’ spliceosome removes the remaining <1% of introns. Based on this information, it is reasonable to predict that the cell would

require higher levels of the components of the major-class than there are of the minor-class. Indeed, the major-class snRNAs are ~100 fold more abundant than the snRNAs that make up the minor-class spliceosome (Zieve and Sauterer 1990). U1, U2, U4 and U6 snRNAs make up the major-class and U11, U12, U4atac and U6atac snRNAs comprise the minor-class (Levine and Durbin 2001). The U5 snRNA is shared by both spliceosomes (Patel and Steitz 2003).

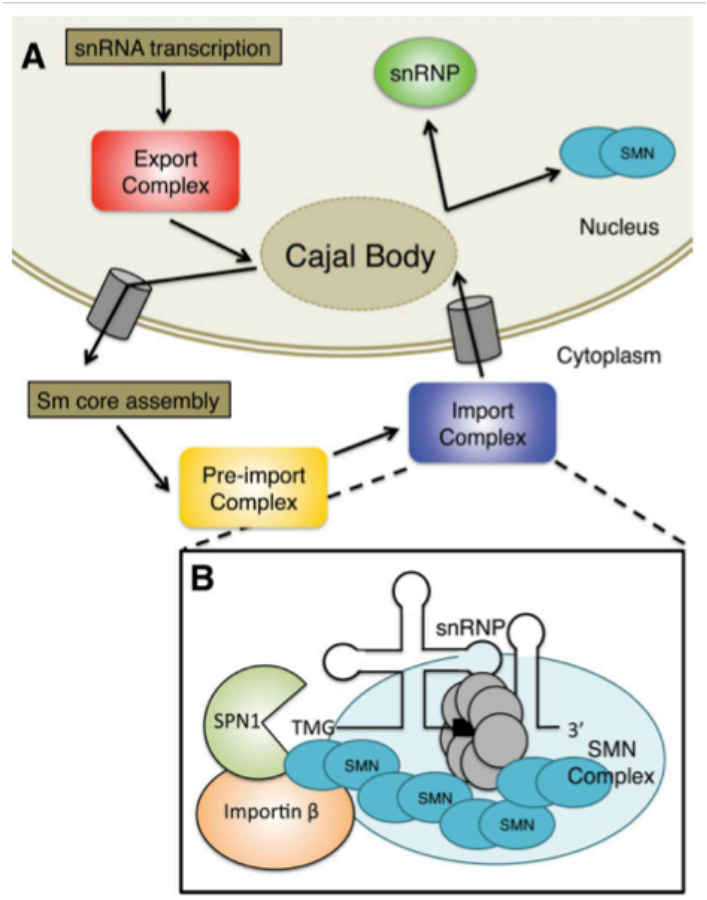


Figure 1.4. Overview of the mammalian snRNP biogenesis pathway. **A.** After snRNA transcription by RNA polymerase II, the pre-snRNA is bound by PHAX to create the export complex and this complex often enters the Cajal body. PHAX then recruits CRM1 and Ran to export the pre-snRNA out to the cytoplasm. In the cytoplasm, the export complex disassembles. Sm core assembly occurs when the snRNA is loaded with a seven membered ring of Sm proteins by the SMN complex. The pre-snRNA is further modified by methylation of the 7-methyl guanosine (m^7G) cap to a trimethylguanosine (m_3G) cap and trimming of the 3' end of the snRNA by an exonuclease (EXO). **B.** The snRNA is imported into the nucleus, along with the SMN complex, by Snurportin (SPN1) and

Importin β, where it localizes to the Cajal body. There, the snRNA binds other proteins and acquires further modifications. Figure from Raimer, Gray, and Matera 2016.

The life cycle of the Sm-class U-snRNAs takes place in both the cytoplasm and the nucleus (Fig. 1.4). Sm-class snRNAs are transcribed by RNA polymerase II and contain additional nucleotides at the 3' end and a monomethylated m^7GpppG (m^7G) cap structure at the 5' end (Cougot et al. 2004) (Fig 1.4A). After 3' end processing to remove the extraneous nucleotides, pre-snRNA transcripts are exported from the nucleus by a set of factors that includes the cap-binding complex (CBP80 and CBP20), the snRNA-specific export adaptor phosphorylated adaptor RNA export (PHAX), and arsenite resistance 2 (ARS2) (Hallais et al. 2013; Frey et al. 1995; Frey et al. 1999; Ohno et al. 2000; Frey et al. 2001; Suzuki et al. 2010). These proteins link the 5' cap of the snRNA to the nuclear export receptor chromosome region maintenance 1 (CRM1/Exportin1). This entire complex interacts with nuclear pore proteins to promote export to the cytoplasm (Fornerod et al. 1997). The snRNA nuclear export complex dissociates upon phosphorylation of PHAX in the cytoplasm (Kitao et al. 2008; Ohno et al. 2000). The SMN protein complex regulates the entire cytoplasmic phase of the snRNP cycle (Fig. 1.4B). Specific phases of snRNP biogenesis in the cytoplasm regulated by SMN include Sm core assembly, trimethylguanosine (TMG) cap formation, and Snurportin1 binding to the TMG cap structure.

The SMN complex serves as a scaffold for Sm core assembly upon which Sm proteins and snRNA are assembled (Fig. 1.4B). The seven Sm proteins are called SmB/B', SmD1, SmD2, SmD3, SmE, SmF, and SmG. In an adenosine triphosphate (ATP) dependent reaction, these proteins are assembled onto a conserved motif of the

pre-snRNA called the 'Sm-site' to form a ring (Kambach et al. 1999; Will et al. 2001; Meister et al. 2002; Pellizzoni et al. 2002; Yong et al. 2004; Golembe et al. 2005; Paushkin et al. 2002). Assembly of the Sm core not only stabilizes the snRNA by protecting it from nucleases, but also is required for downstream RNA-processing steps.

The Sm proteins are delivered to the SMN complex due to the activity of the protein arginine methyltransferase 5 (PRMT5) complex, consisting of PRMT5, pICln, and WD45 (Mep50) (Brahms et al. 2000; Brahams et al. 2001; Friesen et al. 2001; Meister et al. 2001; Friesen et al. 2002). The PRMT5 complex symmetrically dimethylates C-terminal arginine residues within SmB, SmD1, and SmD3 (Meister et al. 2001; Friesen et al. 2001). These methylation marks enhance the interaction between the Sm proteins and SMN. In *Drosophila*, Sm protein methylation is not necessary for snRNP assembly (Gonsalvez et al. 2008). Thus, while many of the biochemical properties of snRNP biogenesis are conserved between flies and mammals, this is an important caveat to consider.

Gemin5 is thought to be the component of the SMN complex responsible for recognition of Sm-class snRNAs (Yong et al. 2010). Although the assembly of the Sm core onto the snRNA can occur spontaneously and non-specifically *in vitro*, the SMN complex is thought to provide specificity, to avoid assembly of Sm cores onto non-target RNAs (Pellizzoni et al. 2002; Yong et al. 2010), and to accelerate formation of the final product from kinetically trapped intermediates (Chari et al. 2008). The role SMN plays in snRNP assembly is crucial because without Sm core assembly snRNPs are incapable of nuclear re-import. This would prevent their participation in active splicing within the nucleus.

Following Sm-core assembly, an RNA methyltransferase called trimethylguanosine synthase (TGS1) is recruited to the m7G cap (Mouaikel et al. 2002; Verheggen et al. 2002). The SMN complex does not immediately dissociate from the RNA after Sm-core assembly, suggesting that SMN may play a role in the recruitment of TGS1 to the complex (Mouaikel et al. 2003). Additionally, it has been shown that TGS1 directly interacts with SMN both *in vivo* and *in vitro* supporting the role of SMN in recruitment. TGS1 hypermethylates the cap to form a 2,2,7-trimethylguanosine (TMG) cap structure. A properly assembled Sm core is required for this process as well as for 3'-end maturation (Mouaikel et al. 2002; Mattaj 1986; Neuman de Vegvar and Dahlberg 1990).

Once the cytoplasmic phase of snRNP biogenesis is complete, Importinβ (Impβ) binds the import adaptor Snurportin 1 (SPN1) that attaches to the TMG cap and imports the partially assembled pre-snRNP, along with the SMN complex, back into the nucleus (Fig. 1.4B) (Palacios et al. 1997; Huber et al. 1998; Narayanan et al. 2004). Interaction between SMN and Impβ, and observations that snRNP import is defective in the presence of some SMN mutations, indicate that SMN may also function in facilitating snRNA nuclear import (Narayanan et al. 2002; Narayanan et al. 2004). This means it is possible that SMN plays a role in every stage of snRNP development, in both the cytoplasm and the nucleus.

The process of snRNP assembly and import is rapid, taking place in approximately one hour, as shown by pulse chase (Gonsalvez et al. 2007). Once in the nucleus, pre-snRNPs localize to Cajal bodies, are released from the SMN complex, further modified, and bound by other snRNP-specific proteins. Mature snRNPs can be

stored in nuclear domains called speckles, where snRNPs are thought to be kept while not participating in splicing (Sleeman and Lamond 1999; Fakan 1994). snRNPs may also localize to active transcription sites in perichromatin fibrils where they actively participate in splicing.

Two spliceosomal snRNAs, U6 and U6atac, have not been discussed thus far and do not follow the same assembly pathway as the others. U6 and U6atac are transcribed by RNA polymerase III and acquire a γ-monomethyl cap after transcription. They bound by seven Sm-like (Lsm) proteins (Lsm2-Lsm8) (Achsel et al. 1999), and are referred to as "Lsm-class" snRNAs. These Lsm proteins are used in place of the Sm proteins that assemble around the other snRNAs. The assembly of U6 and U6atac is thought to occur entirely within the nucleus and appears to be independent of SMN.

snRNP independent functions of SMN

Although snRNP biogenesis remains by far the most well established function of SMN, the protein has also been implicated in other global and tissue-specific roles. For example, SMN has been reported to influence the activity of a viral transcription activator (Strasswimmer et al. 1999). SMN has also been suggested to interact with RNA helicase A and RNA polymerase II (Pellizzoni et al. 2001), invoking the possibility that it may function in transcriptional regulation. Further evidence supporting this function of SMN was provided when Zhao et al. (2015) demonstrated that SMN binds the RNA polymerase II C-terminal domain after it is symmetrically dimethylated on arginine residue R1810 (Fig. 1.5). SMN also interacts with Senataxin (SETX), a helicase that unwinds R-loops around transcription termination sites (Suraweera et al. 2009), allowing

XRN2 exonuclease to be recruited, thereby contributing to transcription termination (Skourti-Stathaki et al. 2011).

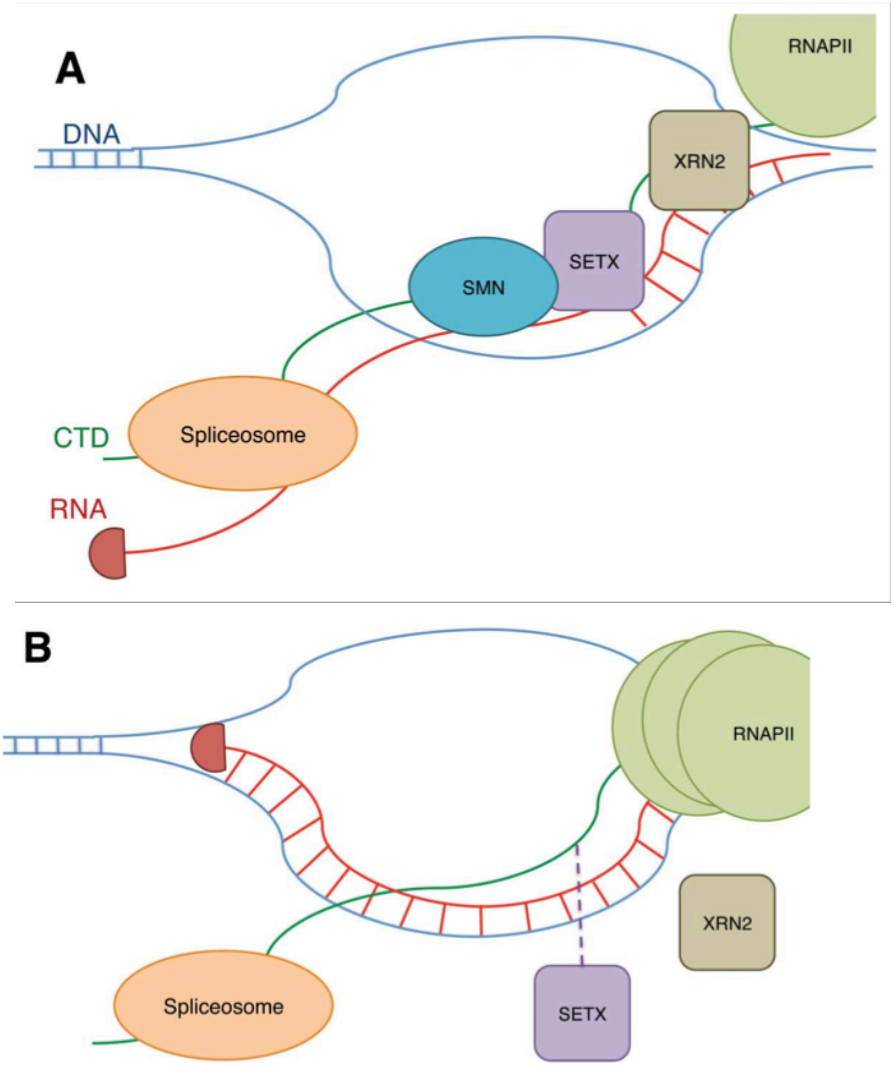


Figure 1.5. The potential role of SMN in transcriptional regulation. **A.** SMN interaction with R1810me2s on the C-terminal domain (CTD) of RNA polymerase II (RNAPII) stabilizes SETX, an RNA/DNA helicase, interaction with the CTD. SETX allows the spliceosome to access and splice the RNA by preventing R-loop formation. **B.** Following reduction of SMN or loss of R1810 methylation, SETX is not recruited as efficiently resulting in an increase in R-loop formation. RNAPII accumulates at the transcription termination site. Figure from Raimer, Gray, and Matera 2016.

A number of observations have raised the possibility of neuron and muscle specific roles for SMN. One such function is in regulating actin dynamics via SMN's

connections to Profilin and Plastin3, which are actin bundling proteins (Giesemann et al. 1999; Sharma et al. 2005; Oprea et al. 2008; Ackermann et al. 2013). This function of SMN may be the primary cause for defects observed in SMA, such as problems with endocytosis at the synapse (Custer and Androphy 2014; Dimitriadi et al. 2016; Hosseinibarkooie et al. 2016). The localization of SMN to growth cones in differentiating neurons (Fan and Simard 2002; Sharma et al. 2005) along with defects in axonal growth of motor neurons in mouse and zebrafish models of SMA (McWhorter et al. 2003; Rossoll et al. 2003) indicate a possible function for SMN in neurite outgrowth and axonal pathfinding.

One of the most well-supported snRNP independent roles for SMN is in mRNP assembly and transport along motor axons (Rossoll et al. 2003). Evidence for this role includes defects in localization of β-actin mRNA in developing motor neurons of SMA mice. SMN may regulate levels of other proteins through a connection with phosphatase and tensin homolog-mediated (PTEN-mediated) protein synthesis pathways (Ning et al. 2010) and other modes of translational regulation (Sanchez et al. 2013). SMN has also been implicated in neuromuscular junction (NMJ) formation and function (Fan et al. 2002; Chan et al. 2003; Kariya et al. 2008; Kong et al. 2009; Voigt et al. 2010).

It is challenging to distinguish between primary and secondary effects in neurons and muscles in SMA models since cell autonomy is difficult to establish when the functions of motor neurons and muscles are highly interconnected. Nonetheless, SMN has been suggested to play a role in some muscle-specific functions. Mouse cells differentiating into muscle fibers were used to show that reduced levels of SMN resulted in myoblast fusion defects and morphologically abnormal myotubes (Shafey et al. 2005).

The severity of these defects was proportional to the level of SMN expression. Rajendra et al. (2007) observed localization of SMN to sarcomeres, the contractile units of muscle fibers, in *Drosophila* and in mice. The final piece of evidence for a muscle specific function of SMN is that flight muscles in an adult *Drosophila* model of SMA were severely disorganized, suggesting a role for SMN in maintenance of muscle architecture (Walker et al. 2008; Bowerman et al. 2009).

The central question facing researchers in the SMA field is how the loss of a protein with an essential and ubiquitous function can cause a primarily neuromuscular disease. Current hypotheses suggest that certain tissues, such as neurons or muscles, may have a greater requirement for snRNPs thus making them more sensitive to low levels of SMN and more susceptible to defects in snRNP biogenesis. It is also possible that disruptions to the tissue-specific functions of SMN lead to SMA. These hypotheses need not be mutually exclusive.

Modeling SMA

Drosophila SMA models

Integral aspects of cell and developmental biology in humans are conserved in *Drosophila*. For example, approximately 75% of disease-causing loci in humans have homologs in the fly (Reiter et al. 2001). This conservation means that by modeling human disease causing mutations in the fly, we are able to study human disorders in a system that is likely to respond with similar pathology as that observed in humans. The fruitfly is an especially attractive model organism for numerous reasons including the availability of a sequenced genome, the ease of implementing several different genetic tools, and a short generation time. Additionally, neuromuscular development in adult flies

resembles that of vertebrates in many ways, thus making *Drosophila* well-suited for study of disorders such as SMA (Fernandes et al. 1999).

The *Drosophila Smn* ortholog is an intron-less gene that codes for a 226 amino acid protein. Due to the absence of introns, the mechanism of splicing of *SMN2* that occurs in humans is not optimally studied in this model system. The human and fly homologs share 23.5% identity and 36.7% similarity; therefore, several other elements of SMN biology can be effectively modeled. The regions of the protein showing greatest conservation correspond to the Gemin2 binding site near the N-terminus, the Tudor domain, and the YG box self-oligomerization domain at the C-terminus (Fig. 1.6) (Miguel-Aliaga et al. 2000). The *Drosophila* SMN complex participates in the assembly of Sm proteins onto snRNAs, indicating that the function of human SMN in snRNP biogenesis is conserved in the fly (Rajendra et al. 2007).

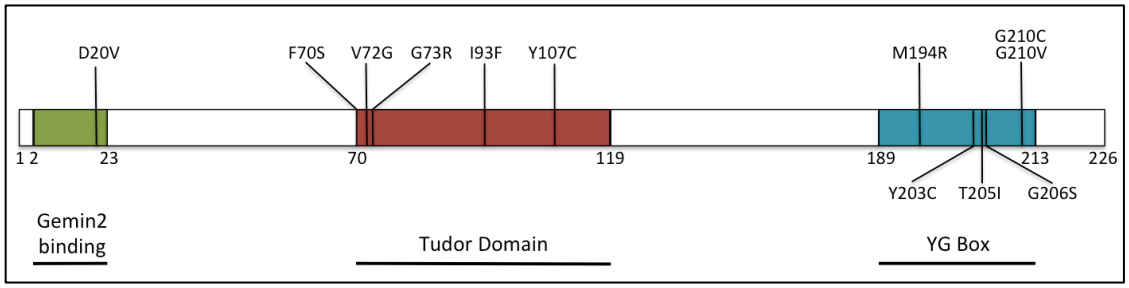


Figure 1.6. SMA patient mutations in the Drosophila Smn gene. Many point mutations in SMN1 that cause SMA are at amino acid residues that are conserved in Drosophila. These point mutations span all three known functional domains of SMN, the Gemin2 binding domain, the tudor domain, and the YG Box self-oligomerization domain. This point mutations lead to mild, intermediate, and severe defects in the fly, thus representing the varying severity of the disease observed in humans. Figure modified from the thesis of Kavita Praveen (Praveen, 2012).

Chan et al. (2003) isolated two point mutations in the YG box self-oligomerization domain of SMN through a small-scale ethyl methane sulphonate (EMS) mutagenesis screen. Mutant animals died during late larval stages and showed loss of

mobility and coordination. Several additional *Smn* mutants have also been generated via transposon-mediated mutagenesis (Rajendra et al. 2007; Shpargel et al. 2009). These animals vary in the severity of their phenotype based on the location of the insertion. Using one of these lines that contained a transposon insertion upstream of *Smn*, an *Smn* null allele was used in an imprecise excision screen to generate a deletion that removes the promoter, open reading frame, and part of the 3` UTR of SMN (Chang et al. 2008). This fly model of SMA, called Smn^{X7}, is useful for the study of larval phenotypes but not adult phenotypes since animals die as larvae without pupating. In addition, these models do not recapitulate human SMA, which is caused by a reduction, but not complete loss, of functional protein.

The number of *Drosophila melanogaster* SMA models expanded when a series of flies were developed where the endogenous *Smn* gene is replaced with a *Flag-Smn* transgene expressing either wild-type SMN or SMN containing patient-derived point mutations at conserved amino acids (Fig. 1.6) (Praveen et al. 2012; Praveen et al. 2014). Although it is highly similar to human *SMN1* and *SMN2*, the entire open reading frame of fruitfly *Smn* is contained within a single exon, and so only full-length SMN protein is expressed in *Drosophila* (Rajendra et al. 2007). Without complications introduced by splicing, the fruitfly is an excellent model for investigating the functions of the protein in isolation. When modeled in the fly, SMA-causing point mutations recapitulate the range of phenotypic severity seen in humans (Praveen et al. 2014; Garcia et al. 2016).

Other animal models of SMA

SMA has been modeled in numerous additional organisms including mice, fish, worms and fission yeast (Miguel-Aliaga et al. 1999; Hsieh-Li et al. 2000; Monani et al.

2000; Owen et al. 2000; McWhorter et al. 2003). Severe reduction or complete knockout of *Smn* in all of these organisms is lethal. Moderate reductions in SMN levels in *C. elegans* resulted in an uncoordinated phenotype and lack of muscle tone. This ultimately resulted in paralysis in this model system. Moderate loss of SMN in zebrafish led to defects in motor axon pathfinding. This occurred without defects in muscles or overt movement problems.

To achieve a milder reduction in SMN levels and obtain a mouse model that more closely resembled the human disorder, two groups generated mice that expressed the human *SMN2* gene in the background of a homozygous mouse *Smn* mutation (*Smn-/-; SMN2)* (Hsieh-Li et al. 2000; Monani et al. 2000). The inclusion of the human *SMN2* gene in the mouse genome meant that the splicing pattern of the gene in humans was also a factor in this model. This rescued the embryonic lethality of *Smn-/-* mice and they presented with many of the pathological features observed in SMA patients. These include a shorter lifespan, motor neuron degeneration, and developing muscle weakness over time (Monani et al. 2000). As observed in the human disease, varying the number of copies of *SMN2* varied the severity of the phenotype from that resembling type I (1 or 2 copies) patients to complete rescue (8-10 copies). In this way, the full spectrum of the human disease can be represented in this model.

As SMA research progressed, additional mouse models were generated to use in studying different aspects of the disease. The 'Delta7' mouse (*$Smn^{-/-}$;SMN2;SMNΔ7*), is a model of severe SMA (Le et al. 2005), and affected mice usually die between postnatal day 10 (P10) and P18. This model differs from the previous mouse model of the disease because it includes a version of SMN in the genome that exclusively encodes for the

SMNΔ7 transcript, meaning that its splicing cannot be modulated, in addition to having a copy of human *SMN2*. The 2B/– mouse ($Smn^{2B/-}$) is a model of intermediate SMA (Bowerman et al. 2012; Rindt et al. 2015) and these animals survive much longer before dying, typically between P25 and P45. This provides a longer window for conducting experiments, which can provide insights into disease pathology that cannot be gained when animals die very early.

Oligomeric properties of SMN complexes

The C-terminal YG box self-oligomerization domain of SMN has been examined using X-ray crystallographic studies (Martin et al. 2012; Gupta et al. 2015). Hydrophobic interactions, similar to those found in glycine zipper domains of certain transmembrane channel proteins, are responsible for dimerization of the protein. (Fig. 1.2). The core of this YG box helical domain contains a highly conserved YxxxYxxxY motif.

More than half of the known SMA patient missense mutations are found within the YG box (Burghes et al. 2009; Li et al. 2014; Wirth 2000). Speaking to the importance of oligomerization in SMN function, mutations that completely disrupt SMN's ability to self-interact display severe phenotypes in human SMA patients and in animal models (Lefebvre et al. 1997; Lorson et al. 1998; Praveen et al. 2014; Clermont et al. 2004; Wirth et al. 1999; Pellizzoni et al. 1999; Workman et al. 2009).

The exact composition and stoichiometry of the various complexes formed by SMN are not well understood. *In vitro*, SMN-Gem2 exists as a stable heterodimer that, for purposes of discussing higher order oligomerization, can be considered as a single structural unit (Fischer et al. 1997; Sarachan et al. 2012). As the concentration of SMN-Gem2 increases in solution, this unit exists in an equilibrium mixture containing dimers,

tetramers and octamers (Gupta et al. 2015). There are two possibilities for the way in which SMN tetramers are formed: by a dimer of dimers or by forming symmetric bundles. Further analysis of this question has revealed they are composed of a dimer of dimers.

Only dimers and tetramers are detected in fission yeast, in contrast to human SMN-Gem2, which forms dimers to octamers and possibly even larger complexes. Octamers appear to form due to self-association of tetramers, although a hexameric SMN complex cannot be ruled out. Human SMN-Gem2 co-sediments with Gemins3–8 *in vivo* (Paushkin et al. 2000), however, the relative stoichiometries of these proteins are unknown.

SMN Protein Stability

The stability of full-length SMN and SMNΔ7 protein has been the subject of many studies, given that SMA is caused by low levels of SMN protein. SMNΔ7 protein produced from the *SMN2* gene is known to be unstable. The SMNΔ7 protein has a twofold shorter half-life than full-length SMN in cells (Burnett et al. 2009). SMNΔ7 is also not thought to be fully functional compared with full-length SMN protein (Lorson et al. 1998; Pellizzoni et al. 1999). Biochemical studies have shown that SMNΔ7 protein does not oligomerize well and interactions with established binding partners, such as the snRNP Sm proteins, are decreased. Mutations in full-length SMN that inhibit oligomerization and prevent complex formation also reduce half-life, suggesting that SMN protein stability is modulated by complex formation (Burnett et al. 2009).

The cause of SMNΔ7 instability was investigated by Cho and Dreyfuss (2010) using a luciferase reporter system of protein stability. They identified a degron motif at

the C-terminus of SMNΔ7 (^{268}YMSGYHTGYYMEMLA^{282}). It was thought to be created in SMNΔ7 by *SMN2* alternative splicing since it includes the EMLA amino acids that are specific to SMNΔ7. Identified through scanning alanine mutagenesis of the C-terminus, mutation of serine 270 to alanine was shown to stabilize SMNΔ7 constructs in human cells. Overexpression of SMNΔ7^{S270A} in SMN-deficient chicken DT40 cells rescued their viability, indicating some functionality of stabilized SMNΔ7 protein. Factors responsible for specifically mediating SMNΔ7 degradation have not been identified.

Intracellular proteins can be degraded or cleaved by a variety of different proteolytic systems including the calcium-activated neutral protease (calpain) system, lysosomal proteases, autophagy, and the ubiquitin proteasome system (UPS). SMN has been shown to be a proteolytic target of Calpain (Walker et al. 2008; Fuentes et al. 2010). Inhibition of the lysosome, autophagy, and the proteasome revealed that only proteasome inhibition significantly increased SMN protein levels. This and other experiments show ubiquitylation pathways regulate the stability and degradation of SMN (Chang et al. 2004; Burnett et al. 2009; Hsu et al. 2010).

Ubiquitin Proteasome System (UPS) and SMN

In the UPS, proteins are tagged by linkage to ubiquitin through the action of three factors (Fig. 1.7) (Petroski 2008). E1 proteins activate ubiquitin and transfer it to the E2 enzyme. E2 proteins conjugate ubiquitin to their substrates. E3 proteins recognize the substrate and assist in the transfer of ubiquitin from the E2. Since E3 ligases confer substrate specificity, they are often considered as candidates for targeted inhibition of protein degradation.

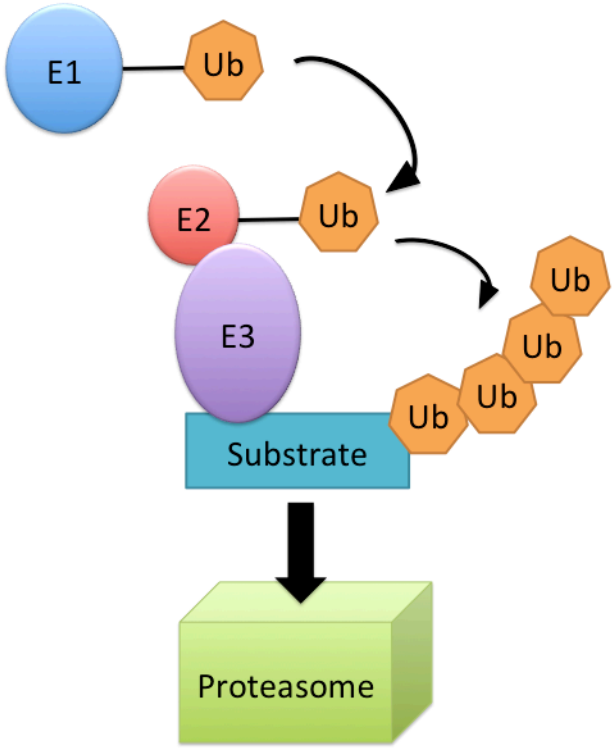


Figure 1.7. The ubiquitin proteasome system (UPS) involves three types of proteins: E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes, and E3 ubiquitin ligase enzymes. E3 ubiquitin ligases recognize target substrates and ubiquitylate them, leading to their degradation by the proteasome. In other cases, ubiquitylation occurs without leading directly to proteasome targeting and degradation. Figure adapted from Elledge lab, Harvard.

Ubiquitin homeostasis is thought to be especially important for neuromuscular pathology in SMA (Groen and Gillingwater 2015). X-linked infantile SMA is caused by mutations in ubiquitin-like modifier activating enzyme 1 (UBA1) (Ramser et al. 2008; Schmutzler et al. 2008). Furthermore, mouse models of SMA have disrupted levels of the E1 protein, UBA1 (Wishart et al. 2014). Finally, ubiquitylation pathways have been shown to specifically affect axonal and synaptic stability (Korhonen and Lindholm 2004).

SMN protein degradation via the UPS has been well-established (Chang et al. 2004; Burnett et al. 2009; Kwon et al. 2011). Since the E3 ligases are the most specific

part of the UPS for their ubiquitylation targets, investigators have studied E3 ligases of SMN using candidate approaches (Han et al. 2016; Hsu et al. 2010; Kwon et al. 2013). As outlined below, researchers have discovered E3 ligases that target SMN for degradation in cultured human cells through these studies. It is therefore likely that SMN is targeted by multiple E3 ubiquitin ligases, as this mechanism of regulation has been demonstrated for a number of proteins (e.g. p53; Jain and Barton 2010). Ubiquitylation does not always result in immediate destruction of the target (Mukhopadhyay and Riezman 2007; Ikeda and Dikic 2008; Liu and Walters 2010). Different functions arise based on the type of lysine linkage between ubiquitin molecules and the length of the ubiquitin chain that is created.

Mindbomb1 (Mib1)

Kwon et al. (2013) identified the E3 ubiquitin ligase, mind bomb 1 (Mib1), as interacting with and ubiquitylating SMN, leading to subsequent degradation. It was originally chosen as a candidate E3 ubiquitin ligase due to its role in inhibition of the outgrowth of neurites in cultured neurons (Choe et al. 2007). Additionally, loss of Mib1 in *Drosophila melanogaster* had been shown to increase the number of synaptic boutons at neuromuscular junctions, producing synaptic overgrowth. Evidence for Mib1 targeting SMN for degradation included the experiment that revealed Mib1 knockdown in cultured cells increases SMN protein levels. Additionally, in SMN deficient *C. elegans*, neuromuscular function improved following knockdown of the Mib1 ortholog as measured in a pharyngeal pumping assay, indicating a physiological role for Mib1 in modulating SMN. Kwon et al. (2013) also mapped the interacting domains of Mib1 and

SMN, demonstrating the interaction is mediated by the N-terminal domain of Mib1 and the part of SMN protein encoded for by exon 6.

Itch

In some cases, protein ubiquitylation does not result in destruction of the target. Different types of ubiquitin lysine linkages or different chain lengths can affect other aspects of protein function within the cell, including cellular localization. SMN is found in both the cytoplasm and nucleus. In the nucleus, SMN is concentrated in nuclear bodies – Cajal bodies, gems, and the nucleolus of neurons (Stanek and Neugebauer, 2006; Liu and Dreyfuss 1996). Posttranslational modifications are thought to affect this cellular localization. Han et al. (2016) reported that the E3 ubiquitin ligase called Itch directly interacts with and monoubiquitylates SMN. This had a modest effect on protein degradation and a more pronounced effect on cellular localization of SMN. This mislocalization of SMN was shown to impair Cajal body integrity and findings suggested impairment of snRNP maturation.

SCF^{Slmb}/$SCF^{B\text{-}TrCP}$

SCF complexes are one type of E3 ligase that operates as part of the UPS. SCF complexes are different from many other E3 ubiquitin ligases because multiple proteins function together to ubiquitylate substrates, in contrast to other E3 ligases that are single proteins (Willems et al. 2004). *SCF^{Slmb}/$SCF^{B\text{-}TrCP}$* consists of Cullin1 (Cul1), Slmb/B-TrCP, and SkpA. Cul1 forms the major structural scaffold of this horseshoe-shaped, multi-subunit complex (Zheng et al. 2002). Slmb/B-TrCP is an F-box protein and is the substrate recognition component (Jiang and Struhl 1998). SkpA is a bridging protein

essential for interaction of Cul1 with the F-box protein (Patton et al. 1998a; Patton et al. 1998b).

The SCF^{Slmb}/$SCF^{B\text{-}TrCP}$ complex is one of the best-characterized SCF E3 ligases in animals (Willems et al. 2004). SCF^{Slmb} is the *Drosophila melanogaster* homolog of $SCF^{B\text{-}TrCP}$ in mammals (Bocca et al. 2001). Initial analysis of this SCF pathway revealed that SCF^{Slmb}/$SCF^{B\text{-}TrCP}$ catalyzes the phosphorylation-dependent ubiquitylation of the NFKB inhibitor IKB and the transcription factor B-catenin (Yaron et al. 1998; Winston et al. 1999). Most known substrates contain the amino acid sequence DGSXXS degron motif or a variant thereof. Both serine, or in some cases threonine, residues need to be phosphorylated for efficient recognition of the degron in the substrate by the E3 ligase. Since the discovery and characterization of these two substrates of SCF^{Slmb}/$SCF^{B\text{-}TrCP}$ numerous additional substrates have been identified using substrate trapping proteomics analysis and other approaches (Kim et al. 2015; Skwarek et al. 2014). The work described here describes the identification of a novel SCF^{Slmb}/$SCF^{B\text{-}TrCP}$ substrate, SMN protein.

Research Objectives

Spinal muscular atrophy is a neuromuscular disorder caused by loss of, or mutation in, the *SMN1* gene. The best-characterized function of SMN is in the biogenesis of snRNPs, core components of the spliceosome. The mechanism of SMA disease pathology has remained unclear despite over 20 years of research. One of the most pressing questions in the field relates to the fact that SMA is primarily a neuromuscular disorder, but SMN is known to have an ubiquitous and essential function. Our approach

to elucidate critical functions of SMN in SMA is to examine SMN protein interaction partners in a whole organism during early stages of development. To do this, we used previously created transgenic flies expressing only Flag-SMN to characterize SMN complexes using Flag immunoprecipitation followed by mass spectrometry. These interactions have provided insight into the mechanisms of degradation of SMN. Additionally, identification of novel binding partners provides the foundation for exploration of molecular mechanisms related to alternative functions of SMN.

CHAPTER II: Self-oligomeriation regulates stability of Survival Motor Neuron (SMN) protein isoforms by sequestering an SCF^{Slmb} degron[1]

Introduction

Spinal muscular atrophy (SMA) is a common neuromuscular disorder, recognized as the most prevalent genetic cause of early childhood mortality (Pearn 1980). Patients with the most severe form of the disease, which is also the most common, become symptomatic in the first six months of life and rarely live past two years (Wee et al. 2010; Prior 2010). Because the onset of symptoms and their severity can vary, SMA has historically been classified into three subtypes (Ogino and Wilson 2004). More recently, clinicians have recognized that SMA is better characterized as a continuous spectrum disorder, ranging from acute (prenatal onset) to nearly asymptomatic (Tiziano et al. 2013). Clinically, SMA patients experience degeneration of motor neurons in the anterior horn of the lower spinal cord (Crawford and Pardo 1996). This leads to progressive atrophy of proximal muscle groups, ultimately resulting in loss of motor function and symmetrical paralysis. The cause of death is often restrictive respiratory failure (Kolb and Kissell 2015).

SMA typically results from homozygous deletion of the *survival motor neuron 1* (*SMN1*) gene (Lefebvre et al. 1995). A small fraction of SMA patients have lost one copy of *SMN1* and the remaining copy contains a point mutation (Burghes and Beattie 2009).

[1] This chapter previously appeared as an article in Molecular Biology of the Cell. The original citation is as follows: Gray, K. M., Kaifer, K. A., Baillat, D., et al. (2018). Self-oligomerization regulates stability of survival motor neuron protein isoforms by sequestering an SCF^{Slmb} degron. *Molecular Biology of the Cell*, 29(2), 96-110.

Humans have two *SMN* paralogs, named *SMN1* and *SMN2*, both of which contribute to total cellular levels of SMN protein. *SMN2* exon 7 contains a silent base change that alters splicing to primarily produce a truncated, unstable protein product called SMNΔ7 (Lorson et al. 1999; Monani et al. 1999; Lorson and Androphy 2000). The last 16 amino acids of SMN are replaced in SMNΔ7 by four amino acids, EMLA, encoded by exon 8. Current estimates suggest that *SMN2* produces 10-15% of the level of full-length protein produced by *SMN1* (Lorson et al. 2010). Complete loss of SMN is lethal in all organisms investigated to date (O'Hearn et al. 2016). Although the amount of full-length protein produced by *SMN2* is not enough to compensate for loss of *SMN1*, *SMN2* is sufficient to rescue embryonic lethality (Monani et al. 2000). SMA is therefore a disease that arises due to a hypomorphic reduction in SMN levels (Lefebvre et al. 1995). Furthermore, relative levels of the SMN protein correlate with the phenotypic severity of SMA (Coovert et al. 1997; Lefebvre et al. 1997).

Whereas a causative link between *SMN1* and SMA was established over 20 years ago, the molecular role of SMN in disease etiology remains unclear. SMN is the central component of a multimeric protein assemblage known as the SMN complex (Matera and Wang 2014; Li et al. 2014). The best-characterized function of this complex, which is found in all tissues of metazoan organisms, is in the cytoplasmic assembly of small nuclear ribonucleoproteins (snRNPs), core components of the spliceosome (Fischer et al. 1997; Meister et al. 2001; Pellizzoni et al. 2002).

Although it is ubiquitously expressed, SMN has also been implicated in a number of tissue-specific processes related to neurons and muscles. These functions include actin dynamics (Oprea et al. 2008; Ackermann et al. 2013), axonal pathfinding (Fan and

Simard 2002; McWhorter et al. 2003; Sharma et al. 2005), axonal transport of β-actin mRNP (Rossoll et al. 2003), phosphatase and tensin homolog-mediated (PTEN-mediated) protein synthesis pathways (Ning et al. 2010), translational regulation (Sanchez et al. 2013), neuromuscular junction formation and function (Chan et al. 2003; Kariya et al. 2008; Kong et al. 2009; Voigt et al. 2010), myoblast fusion (Shafey et al. 2005) and maintenance of muscle architecture (Rajendra et al. 2007; Walker et al. 2008; Bowerman et al. 2009).

Ubiquitylation pathways have been shown to regulate the stability and degradation of SMN (Chang et al. 2004; Burnett et al. 2009; Hsu et al. 2010) as well as axonal and synaptic stability (Korhonen and Lindholm 2004). In the ubiquitin proteasome system (UPS), proteins destined for degradation are tagged by linkage to ubiquitin through the action of three factors (Petroski 2008). E1 proteins activate ubiquitin and transfer it to the E2 enzyme. E2 proteins conjugate ubiquitin to their substrates. E3 proteins recognize the substrate and assist in the transfer of ubiquitin from the E2. Because E3 ligases confer substrate specificity, they are typically considered as candidates for targeted inhibition of protein degradation. Ubiquitin homeostasis is thought to be particularly important for neuromuscular pathology in SMA (Groen and Gillingwater 2015). Indeed, mouse models of SMA display widespread perturbations in UBA1 (ubiquitin-like modifier activating enzyme 1) levels (Wishart et al. 2014). Furthermore, mutations in UBA1 are known to cause X-linked infantile SMA (Ramser et al. 2008; Schmutzler et al. 2008).

Given the importance of these processes to normal development as well as neurodegenerative disease, we set out to identify and characterize novel SMN binding

partners. Previously, we developed *Drosophila melanogaster* as a model system wherein the endogenous *Smn* gene is replaced with a *Flag-Smn* transgene (Praveen et al. 2012). Although it is highly similar to human *SMN1* and *SMN2*, the entire open reading frame of fruitfly *Smn* is contained within a single exon, and so only full-length SMN protein is expressed in *Drosophila* (Rajendra et al. 2007). When modeled in the fly, SMA-causing point mutations recapitulate the full range of phenotypic severity seen in humans (Praveen et al. 2014; Garcia et al. 2016). Using this system, we carried out proteomic profiling of Flag-purified embryonic lysates and identified the SCF^{Slmb} E3 ubiquitin ligase complex as a novel SMN interactor. Importantly, this interaction is conserved from flies to humans. We show that SCF^{Slmb} binding requires a phospho-degron motif located within the SMN self-oligomerization domain, mutation of which stabilizes SMNΔ7 and, to a lesser extent, full-length SMN. Additional studies in flies, mice and human cells elucidate a disease-relevant mechanism whereby SMN protein stability is regulated by self-oligomerization. Other E3 ligases have been reported to target SMN for degradation in cultured human cells (Han et al. 2016; Hsu et al. 2010; Kwon et al. 2013). Given our findings in fruit fly embryos, SMN is likely targeted by multiple E3 ubiquitin ligases.

Experimental procedures

Fly stocks and transgenes

Oregon-R was used as the wild-type control. The Smn^{X7} microdeletion allele (Chang et al. 2008) was a gift from S. Artavanis-Tsakonis (Harvard University, Cambridge, USA). This deficiency removes the promoter and the entire SMN coding region, leaving only the final 44bp of the 3' UTR. All stocks were cultured on molasses and agar at room temperature (24 ± 1°C) in half-pint bottles. The Smn transgenic

constructs were injected into embryos by BestGene Inc. (Chino Hills, CA) as described in Praveen et al. 2014. In short, a ~3kb fragment containing the entire *Smn* coding region was cloned from the *Drosophila* genome into the pAttB vector (Bischof et al. 2007). A 3X FLAG tag was inserted immediately downstream of the start codon of dSMN. Point mutations were introduced into this construct using Q5 (NEB) site-directed mutagenesis according to manufacturer's instructions. The basal Smn construct used, vSmn, contained three single amino acid changes and the addition of the MGLR motif to make fruitfly Smn more similar to the evolutionarily conserved vertebrate Smn. Subsequently generated constructs used vSmn as a template and consist of the amino acid changes detailed in Figure 4. Y203C, G206S, and G210V were previously published in Praveen et al. 2014.

Drosophila embryo protein lysate and mass spectrometry

0-12h Drosophila embryos were collected from Oregon-R control and Flag-SMN flies, dechorionated, flash frozen, and stored at -80C. Embryos (approx. 1gr) were then homogenized on ice with a Potter tissue grinder in 5 mL of lysis buffer containing 100mM potassium acetate, 30mM HEPES-KOH at pH 7.4, 2mM magnesium acetate, 5mM dithiothreitol (DTT) and protease inhibitor cocktail. Lysates were centrifuged twice at 20000 rpm for 20min at 4C and dialyzed for 5h at 4C in Buffer D (HEPES 20mM pH 7.9, 100mM KCl, 2.5 mM $MgCl_2$, 20% glycerol, 0.5 mM DTT, PMSF 0.2 mM). Lysates were clarified again by centrifugation at 20000 rpm for 20 min at 4C. Lysates were flash frozen using liquid nitrogen and stored at -80C before use. Lysates were then thawed on ice, centrifuged at 20000 rpm for 20 min at 4C and incubated with rotation with 100 mL of EZview Red Anti-FLAG M2 affinity gel (Sigma) for 2h at 4C. Beads were washed a

total of six times using buffer with KCl concentrations ranging from 100mM to 250mM with rotation for 1 min at 4C in between each wash. Finally, Flag proteins were eluted 3 consecutive times with one bed volume of elution buffer (Tris 20mM pH 8, 100 mM KCl, 10% glycerol, 0.5 mM DTT, PMSF 0.2 mM) containing 250ug/mL 3XFLAG peptide (sigma). The entire eluate was used for mass spectrometry analysis on an Orbitrap Velos instrument, fitted with a Thermo Easy-spray 50cm column.

Tissue culture and transfections

S2 cell lines were obtained from the Drosophila Genome Resource Center (Bloomington, IL). S2 cells were maintained in SF900 SFM (Gibco) supplemented with 1% penicillin/streptomycin and filter sterilized. Cells were removed from the flask using a cell scraper and passaged to maintain a density of approximately 10^6-10^7 cells/mL. S2 cells were transferred to filter sterilized SF900 SFM (Gibco) without antibiotics prior to transfection with Cellfectin II (Invitrogen). Transfections were performed according to Cellfectin II protocol in a final volume of 4 mL in a T-25 flask containing 10^7 cells that were plated one hour before transfection. The total amount of DNA used in transfections was 2.5ug. Human embryonic kidney HEK-293T and HeLa cells were maintained at 37C with 5% CO_2 in DMEM (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco). $1x10^6$ - $2x10^6$ cells were plated in T-25 flasks and transiently transfected with 1-2ug of plasmid DNA per flask using Lipofectamine (Invitrogen) or FuGENE HD transfection reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Cells were harvested 24-72 h posttransfection.

For siRNA transections, HeLa cells were plated subconfluently in T-25 flasks and transfected with 10nm of siRNA (Gift from Mike Emanuele lab) and 17uL Lipofectamine RNAi MAX (Invitrogen) in 5mL total media according to manufacturers instructions. After 48h of transfection cells were harvested. For RNAi in S2 cells using dsRNA, 10^7 cells were plated in each well of a 6-well plate in 1 mL of media. Cells were treated ~ every 24h with 10ug/mL dsRNA targeted against Slmb, Oskar, or Gaussia Luciferaese (as controls) as described in Rogers and Rogers 2008.

In vitro binding assay

GST and GST-SMN were purified from *E. coli*. In brief, cells transformed with BL21*GST-SMN were grown at 37˚C overnight and then induced using 1 mM IPTG. Recombinant protein was extracted and purified using Glutathione sepharose 4B beads. GST-B-TrCP1 was purchased from Novus Biologicals (cat# H00008945). SMN•Gem2 complexes were co-expressed in *E. coli* using SMNΔ5 and Gemin2(12-280) constructs, as described in Gupta et al. (2015). Glutathione sepharose 4B beads were washed 3x with PBS. GST alone, GST-SMN, or GST-B-TrCP1 were attached to beads during 4h-overnight incubation at 4˚C in PBS with rotation. Beads were then washed 3x with modified RIPA buffer (50mM Tris-HCl, pH 7.5, 250 mM NaCl, 1mM EDTA, 1% NP-40). 20uL of beads with ~2ug attached GST-tagged protein (as determined by Coomassie stain with BSA standard) were added to 200uL modified RIPA buffer with 100ug/mL BSA block. 2ug of SMN•Gem2 was added and the mixture was rotated end over end at 4˚C overnight. Beads were then washed 3x with modified RIPA buffer and 10uL SDS loading buffer was added followed by boiling for 5 minutes.

In vivo ubiquitylation assay

The *in vivo* ubiquitylation assay was performed as described previously (Choudhury et al. 2016). Briefly, HEK-293T cells were transfected as indicated in 10 cm dishes using Lipofectamine2000 (Thermo Fisher Scientific). The day after, cells were treated with 20 μM of MG132 for 4 hours and then harvested in PBS. 80% of the cell suspension was lysed in 6M Guanidine-HCl containing buffer and used to pull down His-Ubiquitinated proteins on Ni2+-NTA beads, while the remaining 20% was used to prepare inputs. Ni2+ pull down eluates and inputs were separated through SDS-PAGE and analyzed by western blot.

Cycloheximide treatment

Following RNAi treatment, S2 cells were pooled, centrifuged and resuspended in fresh media. 1/3 of these cells were frozen and taken as the 0h timepoint. The remainders of the cells were replated in 6 well plates. 100ug/mL cycloheximide (CHX) was added to each sample, and cells were harvested at 2 and 6 hours following treatment.

Immunoprecipitation

Clarified cell lysates were precleared with immune-globulin G (IgG) agarose beads for 1h at 4C and again precleared overnight at 4C. The precleared lysates were then incubated with Anti-FLAG antibody crosslinked to agarose beads (EZview Red Anti-FLAG M2 affinity gel, Sigma) for 2h at 4C with rotation. The beads were washed with lysis buffer or modified lysis buffer six times and boiled in SDS gel-loading buffer. Eluted proteins were run on an SDS-PAGE for western blotting.

Antibodies and Western blotting

Larval and adult lysates were prepared by crushing the animals in lysis buffer (50mM Tris-HCl, pH 7.5, 150 mM NaCl, 1mM EDTA, 1% NP-40) with 1X (adults) or 10x (larvae) protease inhibitor cocktail (Invitrogen) and clearing the lysate by centrifugation at 13,000 RPM for 10 min at 4ºC. S2 cell lysates were prepared by suspending cells in lysis buffer (50mM Tris-HCl, pH 7.5, 150 mM NaCl, 1mM EDTA, 1% NP-40) with 10% glycerol and 1x protease inhibitor cocktail (Invitrogen) and disrupting cell membranes by pulling the suspension through a 25 gauge needle (Becton Dickinson). The lysate was then cleared by centrifugation at 13,000 RPM for 10 min at 4ºC. Human cells (293Ts and HeLas) were first gently washed in ice-cold 1X PBS, then collected in ice-cold 1X PBS by scraping. Cells were pelleted by spinning at 1000 rpm for 5 min. The supernatant was removed and cells were resuspended in ice cold lysis buffer (50mM Tris-HCl, pH 7.5, 150 mM NaCl, 1mM EDTA, 1% NP-40) and allowed to lyse on ice for 30 min. After lysing, the lysate was cleared by centrifuging the cells for 10 min at 13000 at 4C. Western blotting on lysates was performed using standard protocols. Rabbit anti-dSMN serum was generated by injecting rabbits with purified full-length dSMN protein (Pacific Immunology Corp, CA), and was subsequently affinity purified. For Western blotting, dilutions of 1 in 2,500 for the affinity purified anti-dSMN, 1 in 20,000 (fly) or 1 in 5,000 (human) for anti-α tubulin (Sigma), 1 in 10,000 for monoclonal anti-Flag (Sigma), 1 in 1,000 for anti-Slmb (gift from Greg Rogers), 1 in 2,500 for anti-human SMN (BD Biosciences), 1 in 1,000 for anti-B-TrCP (gift from MB Major lab), 1 in 10,000 for polyclonal anti-Myc (Santa Cruz), and 1 in 2,000 for anti-GST (abcam) were used.

Larval locomotion

Smn control and mutant larvae (73-77 hours post egg-laying) were placed on a 1.5% agarose molasses tray five at a time. The tray was then placed in a box with a camera and the larvae were recorded moving freely for 60 seconds. Each set of larvae was recorded three times, and one video was chosen for analysis based on video quality. The videos were then converted to AVI files and analyzed using the wrMTrck plug-in of the Fiji software. The "Body Lengths per Second" was calculated in wrMTrck by dividing the track length by half the perimeter and time (seconds). P-values were generated using a multiple comparison ANOVA.

SMA Mouse Models

Two previously developed SMA mouse models were utilized. The 'Delta7' mouse (*Smn*$^{-/-}$*;SMN2;SMNΔ7*), is a model of severe SMA (Le et al. 2005). The '2B/–' mouse (*Smn*$^{2B/-}$) is a model of intermediate SMA (Bowerman et al. 2012; Rindt et al. 2015). Adeno-associated virus serotype 9 (AAV9) delivered SMN cDNA isoforms to these SMA mice, as previously described (Foust et al. 2010; Passini et al. 2010; Valori et al. 2010; Dominguez et al. 2011; Glascock et al. 2012). Gross motor function was measured using a modified tube-test which tests the ability of mice to splay their legs and maintain a hanging position.

Human iPSC Cell culture

Human iPSCs from two independent unaffected control and two SMA patient lines were grown as pluripotent colonies on Matrigel substrate (Corning) in Nutristem medium (Stemgent). Colonies were then lifted using 1mg/ml Dispase (Gibco) and maintained as floating spheres of neural progenitor cells in the neural progenitor growth

medium Stemline (Sigma) supplemented with 100ng/ml human basic fibroblast growth factor (FGF-2, Miltenyi), 100ng/ml epidermal growth factor (EGF, Miltenyi), and 5μg/ml heparin (Sigma-Aldrich) in ultra-low attachment flasks. Aggregates were passaged using a manual chopping technique as previously described (Svendsen et al. 1998; Ebert et al. 2013). To induce motor neuron differentiation, neural progenitor cells were cultured in neural induction medium (1:1 DMEM/F12 (Gibco), 1x N2 Supplement (Gibco), 5μg/mL Heparin (Sigma), 1x Non-Essential Amino Acids (Gibco), and 1x Antibiotic-Antimycotic (Gibco)) plus 0.1μM all-trans retinoic acid (RA) for two weeks; 1μM Purmorphamine (PMN, Stemgent) was added during the second week. Spheres were then dissociated with TrypLE Express (Gibco) and plated onto Matrigel-coated 12mm coverslips in NIM plus 1μM RA, 1μM PMN, 1x B27 Supplement (Gibco), 200ng/mL Ascorbic Acid (Sigma), 1μM cAMP (Sigma), 10ng/mL BDNF (Peprotech), 10ng/mL GDNF (Peprotech)). One week post-plating, cells were infected with lentiviral vectors (MOI = 5) expressing mCherry alone or SMN S270A-IRES-mCherry. Transgenes in both viruses were under the control of the EF1α promoter. Uninfected cells served as controls. Cells were analyzed at 1 and 3 weeks post-infections, which was 4 and 6 weeks of total differentiation (Ebert et al. 2009; Sareen et al. 2013).

Immunocytochemistry

Coverslips were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 20 minutes at room temperature and rinsed with PBS. Cells were blocked with 5% Normal Donkey Serum (Millipore) and permeabilized in 0.2% TritonX-100 (Sigma) for 30 minutes at room temperature. Cells were then incubated in primary antibody solution for 1 hour, rinsed with PBS, and incubated in secondary antibody solution for 1 hour at

room temperature. Finally, nuclei were labeled with Hoechst nuclear stain (Sigma) to label DNA and mounted onto glass slides using FluoroMount medium (SouthernBiotech). Primary antibodies used were mouse anti-SMI-32 (Covance SMI-32R, 1:1000) and rabbit anti-mCherry (ThermoFisher, 1:1000). Secondary antibodies used were donkey anti-rabbit Cy3 (Jackson Immunoresearch 711-165-152) and donkey anti-mouse AF488 (Invitrogen A21202).

Immunocytochemical Analysis

Images were acquired from five random fields per coverslip using an inverted fluorescent microscope (Nikon) and NIS Elements software. Images were blinded and manually analyzed for antigen specificity with NIS Elements software.

Results

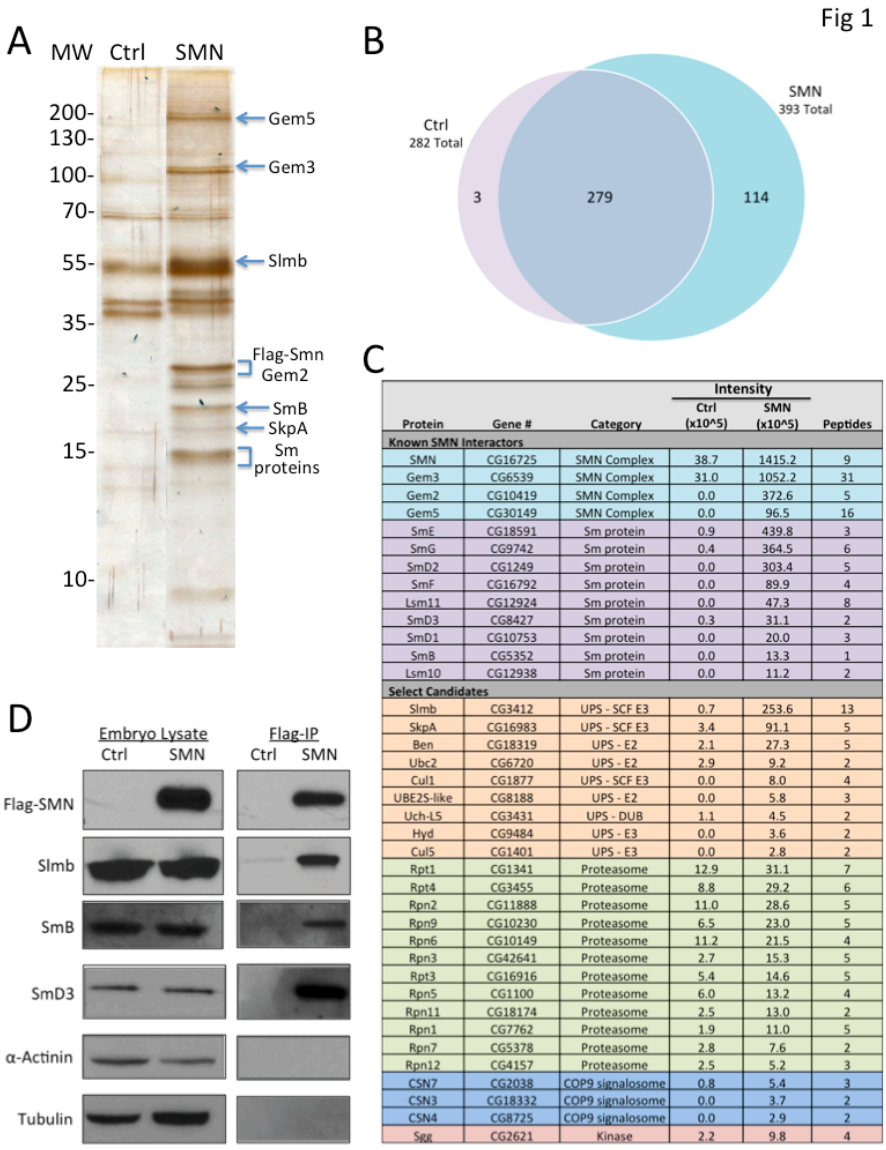
Flag-SMN interacts with UPS (ubiquitin proteasome system) proteins

We previously generated transgenic flies that express Flag-tagged SMN proteins in an otherwise null *Smn* background (Praveen et al. 2012). To preserve endogenous expression patterns, the constructs are driven by the native promoter and flanking sequences. As described in the Methods, we intercrossed hemizygous *Flag-*Smn^{WT},Smn^{X7}/Smn^{D} animals to establish a stock wherein all of the SMN protein, including the maternal contribution, is epitope-tagged. After breeding them for >100 generations, essentially all of the animals are homozygous for the *Flag-*Smn^{WT} transgene, but second site recessive mutations are minimized due to the use of two different *Smn* null alleles. Adults from this stock display no apparent defects and have an eclosion frequency (~90%) similar to that of wild-type (Oregon-R) animals.

We collected (0-12h) embryos from *Flag-Smn*$^{WT/WT}$,*Smn*$^{X7/D}$ (SMN) and Oregon-R (Ctrl) animals and analyzed Flag-purified lysates by 'label-free' mass spectrometry. In addition to Flag-SMN, we identified SMN complex components Gemin2 and Gemin3, along with all seven of the canonical Sm-core snRNP proteins (Fig. 1A). We also identified the U7-specific Sm-like heterodimer Lsm10/11 (Pillai et al. 2003) and the Gemin5 orthologue, Rigor mortis (Gates et al. 2004). Previous studies of Schneider2 (S2) cells transfected with epitope-tagged *Smn* had identified most of the proteins listed above as SMN binding partners in *Drosophila* (Kroiss et al. 2008). However, despite bioinformatic and cell biological data indicating that Rigor mortis is part of the fruit fly SMN complex, this protein failed to co-purify with SMN in S2 cells (Kroiss et al. 2008; Cauchi et al. 2010; Guruharsha et al. 2011). On the basis of our purification data, we conclude that the conditions are effective and that Rigor mortis/Gemin5 is an integral member of the SMN complex in flies.

A detailed proteomic analysis of these flies will be presented elsewhere. As shown in Fig. 1B, our preliminary analysis identified 396 proteins, 114 of which were detected only in the Flag-SMN sample and not in the control. An additional 279 proteins were detected in both the Flag purification and control samples. In addition to SMN complex members, we co-purified numerous factors that are part of the ubiquitin proteasome system (UPS; Fig. 1C). Among these UPS proteins, we identified Cullin 1 (Cul1), Skp1-related A (SkpA), and supernumerary limbs (Slmb), as being highly enriched (>10 fold) in Flag-SMN samples as compared to the control. Together, these proteins comprise the SCF^{Slmb} E3 ubiquitin ligase. Cul1 forms the major structural scaffold of this horseshoe-shaped, multi-subunit complex (Zheng et al. 2002). Slmb is an

F-box protein and is the substrate recognition component (Jiang and Struhl 1998). SkpA is a bridging protein essential for interaction of Cul1 with the F-box protein (Patton et al. 1998a; Patton et al. 1998b). Because of its role in substrate recognition, Slmb is likely to be the direct interacting partner of SMN within the SCF^{Slmb} complex. For this reason, we focused on Slmb for the initial validation. As shown, Slmb was easily detectable in Flag-purified eluates from embryos expressing Flag-SMN and nearly undetectable in those from control embryos (Fig. 1D). SmB and SmD3 were also easily detectable by western blot in Flag-purified embryonic lysates and were used as positive controls for known protein interaction partners of SMN. Tubulin and α-Actinin were not detected as interacting with SMN in our purification and demonstrate the specificity of the detected SMN interactions.



Protein	Gene #	Category	Intensity Ctrl (x10^5)	Intensity SMN (x10^5)	Peptides
Known SMN Interactors					
SMN	CG16725	SMN Complex	38.7	1415.2	9
Gem3	CG6539	SMN Complex	31.0	1052.2	31
Gem2	CG10419	SMN Complex	0.0	372.6	5
Gem5	CG30149	SMN Complex	0.0	96.5	16
SmE	CG18591	Sm protein	0.9	439.8	3
SmG	CG9742	Sm protein	0.4	364.5	6
SmD2	CG1249	Sm protein	0.0	303.4	5
SmF	CG16792	Sm protein	0.0	89.9	4
Lsm11	CG12924	Sm protein	0.0	47.3	8
SmD3	CG8427	Sm protein	0.3	31.1	2
SmD1	CG10753	Sm protein	0.0	20.0	3
SmB	CG5352	Sm protein	0.0	13.3	1
Lsm10	CG12938	Sm protein	0.0	11.2	2
Select Candidates					
Slmb	CG3412	UPS - SCF E3	0.7	253.6	13
SkpA	CG16983	UPS - SCF E3	3.4	91.1	5
Ben	CG18319	UPS - E2	2.1	27.3	5
Ubc2	CG6720	UPS - E2	2.9	9.2	2
Cul1	CG1877	UPS - SCF E3	0.0	8.0	4
UBE2S-like	CG8188	UPS - E2	0.0	5.8	3
Uch-L5	CG3431	UPS - DUB	1.1	4.5	2
Hyd	CG9484	UPS - E3	0.0	3.6	2
Cul5	CG1401	UPS - E3	0.0	2.8	2
Rpt1	CG1341	Proteasome	12.9	31.1	7
Rpt4	CG3455	Proteasome	8.8	29.2	6
Rpn2	CG11888	Proteasome	11.0	28.6	5
Rpn9	CG10230	Proteasome	6.5	23.0	5
Rpn6	CG10149	Proteasome	11.2	21.5	4
Rpn3	CG42641	Proteasome	2.7	15.3	5
Rpt3	CG16916	Proteasome	5.4	14.6	5
Rpn5	CG1100	Proteasome	6.0	13.2	4
Rpn11	CG18174	Proteasome	2.5	13.0	2
Rpn1	CG7762	Proteasome	1.9	11.0	5
Rpn7	CG5378	Proteasome	2.8	7.6	2
Rpn12	CG4157	Proteasome	2.5	5.2	3
CSN7	CG2038	COP9 signalosome	0.8	5.4	3
CSN3	CG18332	COP9 signalosome	0.0	3.7	2
CSN4	CG8725	COP9 signalosome	0.0	2.9	2
Sgg	CG2621	Kinase	2.2	9.8	4

Figure 2.1. Flag-SMN immunopurified lysates contain known protein interaction partners and ubiquitin proteasome system (UPS) proteins. ***A***. Lysates from Oregon-R control (Ctrl) *Drosophila* embryos and embryos expressing only transgenic Flag-SMN (SMN) were Flag-immunopurified and protein eluates were separated by gel electrophoresis and silver stained. Band identities predicted by size using information from panels C and D. ***B***. Direct mass spectrometric analysis of the eluates (which were not gel purified) identified a total of 396 proteins, 114 of which were detected only in SMN sample and 279 of which were detected in both SMN and Ctrl samples. ***C***. Flag-purified eluates were analyzed by 'label-free' mass spectrometry. Numerous proteins that copurify with Flag-SMN are part of the ubiquitin proteasome system (UPS). Of these UPS proteins, Cullin 1 (Cul 1), SkpA, and supernumerary limbs (Slmb), were highly enriched (at least 10 fold) in the SMN sample as compared to Ctrl. ***D***. Western blot analysis of Flag-purified eluates was used to further validate the presence or absence of

SMN interaction partners. Flag-SMN was successfully purified from SMN embryos, but was undetectable in the control. As positive controls for known protein interaction partners of SMN, SmB and SmD3 were also easily detectable by western blotting using anti-Sm antibodies. The presence of Slmb was verified using anti-Slmb. α-Actinin and Tubulin were not enriched in our purification and are used as negative controls to demonstrate specificity.

SCF^{Slmb} is a bona fide SMN interaction partner that ubiquitylates SMN

As an E3 ubiquitin ligase, the SCF^{Slmb} complex is a substrate recognition component of the ubiquitin proteasome system. As outlined in Fig. 2A, E3 ligases work with E1 and E2 proteins to ubiquitylate their targets. The interaction of SCF^{Slmb} with SMN was verified in a reciprocal co-immunoprecipitation, demonstrating that Flag-tagged SCF components form complexes with endogenous SMN (Fig. 2B) in S2 cells. SCF complexes are highly conserved from flies to humans: SkpA is 77% identical to human Skp1, Cul1 is 63% identical, and Slmb is 80% identical to its human homologs, B-TrCP1 and B-TrCP2. Slmb/B-TrCP is the SCF component that directly contacts substrates of the E3 ligase. We therefore tested the interaction of recombinant human SMN in complex with Gemin2 (SMN•Gem2) (Gupta et al. 2015) with GST-tagged B-TrCP1 and -SMN proteins in an *in vitro* binding assay. As shown in Fig. 2C, SMN•Gem2 did not interact with GST alone, but was detected at high levels following pulldown with either GST-SMN (positive control) or GST-B-TrCP1. We also tested the interaction of Flag-tagged *Drosophila* SCF components with endogenous human SMN in HEK 293T cells (Fig. 2D). Accordingly, human SMN was co-precipitated with Flag-Cul1 and Flag-Slmb and at lower levels following Flag-SkpA immunoprecipitation. Flag-B-TrCP1 and Flag-B-TrCP2, the two human homologs of Slmb, also copurified with endogenous human SMN in HEK 293T cells (Fig. 2E). Altogether, these data

demonstrate a conserved interaction between SMN and the $SCF^{Slmb/B\text{-}TrCP}$ E3 ubiquitin ligase complex.

In order to test the functional consequences of this conserved interaction between SMN and $SCF^{Slmb/B\text{-}TrCP}$, a cell based ubiquitylation assay was performed (Fig. 2F). Protein lysate from HEK 293T cells transfected with 6xHis-Flag-ubiquitin and GFP-SMN was purified using a Ni^{2+} pull down for the tagged ubiquitin. Baseline levels of ubiquitylated GFP-SMN were detected using anti-GFP antibody. Following transfection of Flag-B-TrCP1 or Flag-B-TrCP2, the levels of ubiquitylated SMN markedly increased (Fig. 2F). Ubiquitylation levels were further increased following addition of both proteins together. These experiments demonstrate that $SCF^{Slmb/B\text{-}TrCP}$ can ubiquitylate SMN *in vivo*.

Fig 2

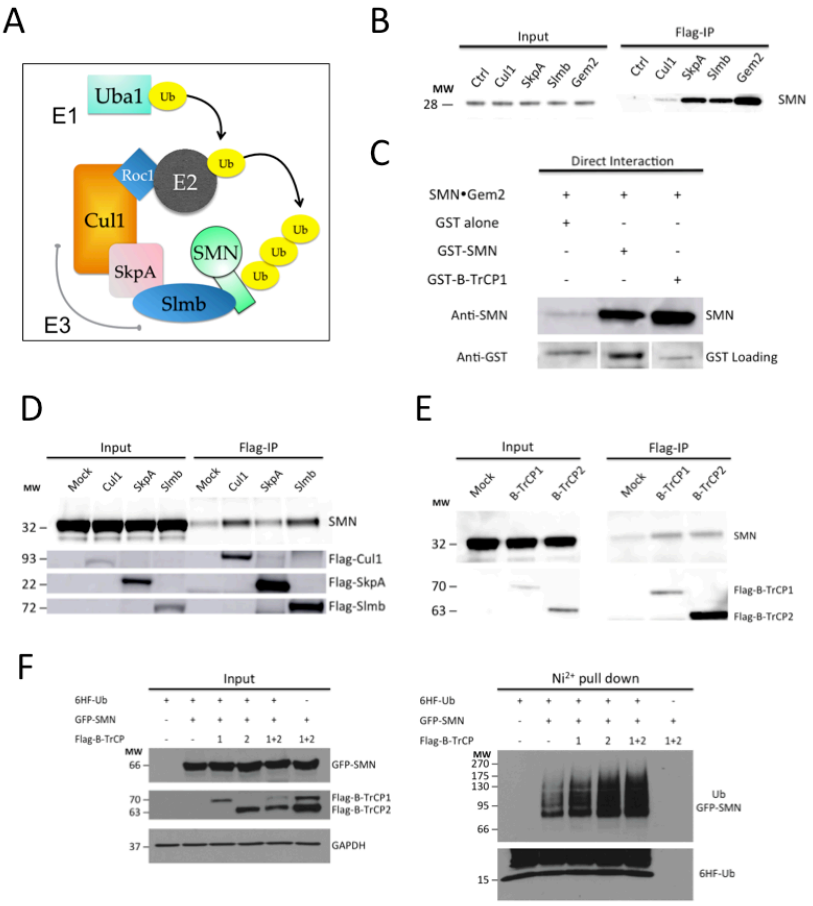


Figure 2.2. Conserved interaction between SMN and the SCF$^{Slmb/B\text{-}TrCP}$ E3 ubiquitin ligase results in ubiquitylation of SMN. ***A***. E3 ligases work with E1 and E2 proteins to ubiquitylate their targets. The SCF$^{Slmb/B\text{-}TrCP}$ E3 ubiquitin ligase is made up of three proteins: Cul1, SkpA, and Slmb. The E3 ubiquitin ligase is the substrate recognition component of the ubiquitin proteasome system. ***B***. Following Cul1-Flag, SkpA-Flag, Flag-Slmb and Flag-Gem2 immunoprecipitation from Drosophila S2 cell lysates, western analysis using anti-SMN antibody for endogenous SMN was carried out. Co-purification of each of the SCF components with endogenous SMN was detected. ***C***. An *in vitro* binding assay tested direct interaction between human SMNΔ5-Gemin2 (SMN•Gem2) (Martin et al. 2012; Gupta et al. 2015) and purified GST-tagged proteins. SMN•Gem2 did not interact with GST protein alone, but bound to GST tagged *Drosophila* SMN (GST-SMN) and GST tagged human B-TrCP1 (GST-B-TrCP1). Levels of GST alone, GST-SMN, and GST-B-TrCP1 were detected using anti-GST antibody. ***D***. The interaction of Flag-tagged Drosophila SCF components with endogenous human SMN was tested in in HEK 293T cells. Human SMN was detected at high levels following immunoprecipitation of Drosophila Flag-Cul1 and Flag-Slmb and detected at a lower level following Drosophila Flag-SkpA immunoprecipitation. ***E***. Flag-tagged versions of the human homologs of Slmb, Flag-B-TrCP1 and Flag-B-TrCP2, interact with endogenous human SMN in HEK 293T cells demonstrated by Flag-immunopurification

followed by immunodetection of SMN. ***F***. Protein lysate from HEK 293T cells transfected with 6xHis-Flag-ubiquitin (6HF-Ub) and GFP-SMN was purified using a Ni^{2+} pull down for the tagged ubiquitin. Baseline levels of ubiquitylated GFP-SMN were detected using anti-GFP antibody. Following transfection of Flag-B-TrCP1 or Flag-B-TrCP2, the levels of ubiquitylated SMN markedly increased. Ubiquitylation levels were further increased following addition of both proteins together. In the input, GFP-SMN was detected using anti-GFP antibody, Flag-B-TrCP1 and Flag-B-TrCP2 were detected using anti-Flag antibody, and GAPDH was detected by anti-GAPDH antibody. In the Ni^{2+} pull down, ubiquitylated GFP-SMN was detected using anti-GFP antibody and 6HF-Ub was detected using anti-Flag antibody to verify successful pull down of tagged ubiquitin.

Depletion of Slmb/B-TrCP results in a modest increase in SMN levels

Given that one of the primary functions of protein ubiquitylation is to target proteins to the proteasome, we examined whether depletion of Slmb by RNA interference (RNAi) using dsRNA in S2 cells would increase SMN levels (Fig. 3A). Following Slmb RNAi, endogenous SMN levels were modestly increased as compared to cells treated with control dsRNA. We obtained similar results using an siRNA that targets both B-TrCP1 and B-TrCP2 in HeLa cells. As shown in Fig. 3B, we detected a modest increase in levels of full-length SMN following B-TrCP RNAi, but not control RNAi. Next, we treated S2 cells with cycloheximide (CHX), in the presence or absence of dsRNA targeting Slmb, to determine whether differences in SMN levels would be exacerbated when production of new proteins was prevented (Fig. 3C). SMN protein levels were also specifically targeted using dsRNA against *Smn* as a positive control for the RNAi treatment. At 6 hours post-CHX treatment there was a modest increase in full-length SMN levels following Slmb RNAi as compared to the initial timepoint (0h) or the negative control (Ctrl) RNAi (Fig. 3C). Together, these data indicate that Slmb/B-TrCP participates in the regulation of SMN protein levels.

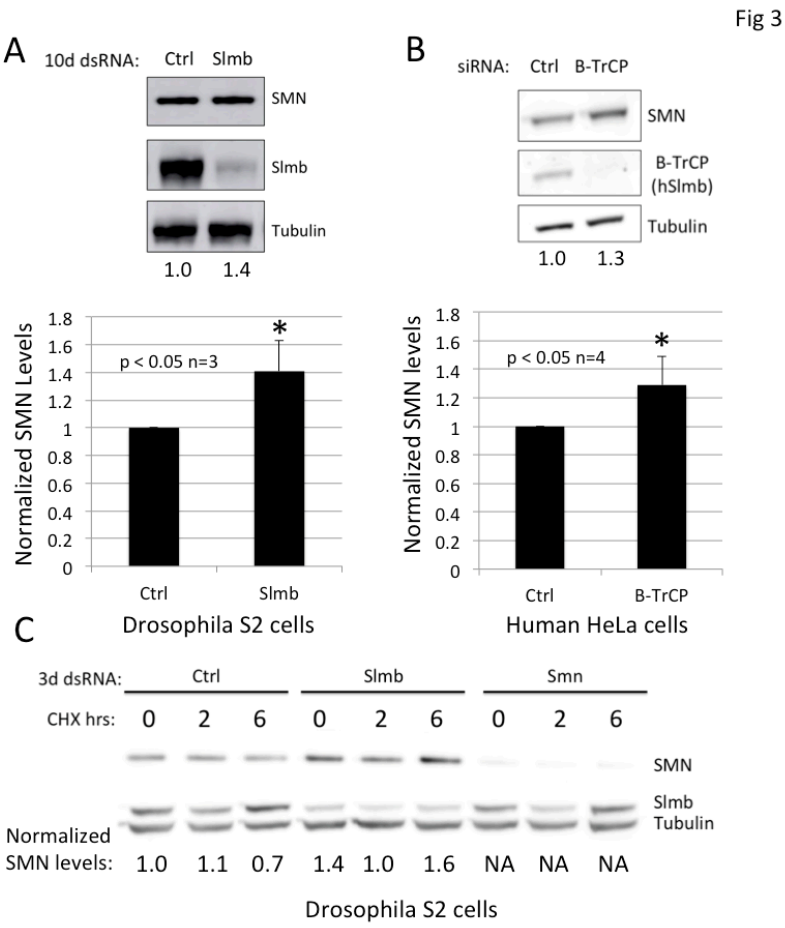


Figure 2.3. Depletion of Slmb/B-TrCP results in an increase of SMN levels. ***A***. Depletion of Slmb using 10 day (10d) treatment with dsRNA in *Drosophila* S2 cells resulted in modestly increased SMN levels. Following Slmb RNAi, full-length SMN levels were increased as compared to cells treated with control dsRNA against Gaussia Luciferase, which is not expressed in S2 cells. ***B***. The effect of B-TrCP depletion on SMN levels in human cells was tested using siRNA that targets both B-TrCP1 and B-TrCP2 in HeLa cells. We detected a modest increase in levels of full-length endogenous SMN after B-TrCP RNAi but not control (scramble) RNAi. ***C***. *Drosophila* S2 cells were treated with cycloheximide (CHX), an inhibitor of protein synthesis, following Slmb depletion following a 3d dsRNA treatment to test whether differences in protein levels would be exacerbated when the production of new protein was prevented. SMN protein levels were also directly targeted using dsRNA against *Smn* as a positive control for the RNAi treatment. As a negative control (Ctrl), dsRNA against *oskar*, which is not expressed in S2 cells, was used. Protein was collected at 0, 2, and 6 hours post CHX treatment. At 6 hours post-CHX treatment there is a modest increase in full-length SMN levels following *Slmb* RNAi as compared to the initial timepoint (0h) and as compared to control RNAi treatment.

Identification and characterization of a Slmb/B-TrCP degradation signal in SMN

Studies of numerous UPS substrates in a variety of species have revealed the presence of degradation signals (called degrons) that are required for proper E3 target recognition and binding. Slmb/B-TrCP canonically recognizes a consensus DpSGXXpS/T degron, where p indicates a phosphoryl group (Jin et al. 2005; Frescas and Pagano 2008; Fuchs et al. 2004). There are also several known variants of this motif, for example: DDGFVD, SSGYFS, TSGCSS (Kim et al. 2015). As shown in Fig. 4A, we identified a putative Slmb/B-TrCP degron (^{269}MSGYHT^{274}) in the highly conserved self-oligomerization domain (YG Box) of human SMN. Interestingly, this sequence was previously identified as part of a larger degron motif (^{268}YMSGYHTGYYMEMLA^{282}) that was thought to be created in SMNΔ7 by *SMN2* alternative splicing (Cho and Dreyfuss 2010). In particular, mutation of S270 (S201 in flies) to alanine was shown to dramatically stabilize SMNΔ7 constructs in human cells, and overexpression of SMNΔ7^{S270A} in SMN-deficient chicken DT40 cells rescued their viability (Cho and Dreyfuss 2010). However, factors responsible for specifically mediating SMNΔ7 degradation have not been identified.

In order to develop a more disease-relevant *Drosophila* system to investigate SMN YG box function, we generated a 'vertebrate-like' SMN construct, called vSmn (Fig. 4A). Transgenic flies expressing Flag-vSmn and Flag-vSmn^{S201A} in the background of an *Smn^{X7}* null mutation are fully viable (Fig. S1). In fact, the eclosion frequencies of these animals are consistently higher than those that express Flag-Smn^{WT} (Fig. S1). Additional *Smn* mutant constructs were generated using the vSmn backbone, including both the full-length (e.g. vSmn^{S201A}) and truncated (e.g. vSmnΔ7A) versions of the

protein (Fig. 4A). To test the effects of overall protein length and distance of the putative degron from the C-terminus, we also generated vSmn constructs that are the same length as SMNΔ7, replacing the MEMLA* motif (the amino acids introduced by human *SMN2* splicing) with MGLRQ*, see Fig. 4A. The S201A mutation was created in this construct as well (MGLRQ*^{S201A}). To mimic a constitutively phosphorylated state, we also introduced serine to aspartate mutations, vSmn^{S201D} and vSmnΔ7D. We transfected each of these constructs, Flag-tagged and driven by the native *Smn* promoter, into S2 cells and measured protein levels by western blotting (Fig. 4B). We note that these constructs are expressed at levels far below those of endogenous SMN protein in S2 cells; moreover, they do not affect levels of endogenous SMN (Fig. S2). As shown, the vSmn^{S201A} and vSMNΔ7A constructs exhibited increased protein levels compared to their serine containing counterparts, whereas levels of the S201D mutants were reduced, suggesting that the phospho-degron motif identified in human SMNΔ7 (Cho and Dreyfuss 2010) is also conserved in the fly protein. In addition to examining protein levels of each of these constructs in cell culture, transgenic flies expressing vSmn, vSmn^{S201A}, vSmnΔ7S, and vSmnΔ7A were created. Here again, we observed that the S201A mutation increased protein levels of both full-length SMN and SMNΔ7 (Fig. S3).

The MGLRQ* construct is present at levels that are similar to wild-type (vSmn) and much higher than vSmnΔ7S. Based on the crystal structures of the SMN YG box (Martin et al. 2012; Gupta et al. 2015), the presence of the MGLR insertion in *Drosophila* SMN is predicted to promote self-oligomerization (A.G. Matera and G.D. Van Duyne, unpublished), thus stabilizing the protein within the SMN complex (Burnett et al. 2009). By the same logic, the relative inability of vSmnΔ7S to self-interact would be predicted

to lead to its destruction. To determine whether the observed increase in SMN protein levels correlated with its ability to interact with Slmb, we co-transfected the appropriate Flag-Smn constructs with Myc-Slmb in S2 cells. Protein lysates were then Flag-immunoprecipitated and probed with anti-Myc antibody (Fig. 4C). The S201A mutation decreased binding of Slmb to both the full-length and the truncated SMN isoforms (Fig. 4C). However, the vSmnΔ7S construct co-precipitated the greatest amount of Slmb protein, despite the fact that it is present at much lower levels in the input lysate (Fig. 4C). Because SMNΔ7 is defective in self-interaction, this result suggests that the degron is more accessible to Slmb when SMN is monomeric and cannot efficiently oligomerize.

SMN self-oligomerization regulates access to the Slmb degron

To examine the connection between SMN self-oligomerization and degron accessibility more closely, we took advantage of two SMA patient-derived point mutations (Y203C and G206S) that are known to destabilize the full-length protein and to decrease its self-oligomerization capacity (Praveen et al. 2014). As a control, we also employed an SMA-causing mutation (G210V) that does not disrupt SMN self-oligomerization (Praveen et al. 2014; Gupta et al. 2015). Next, we introduced the S201A degron mutation into all three of these full-length SMN constructs, transfected them into S2 cells and carried out western blotting (Figs. 4D and S2). The S201A degron mutation has a clear stabilizing effect on the G206S and Y203C constructs, as compared to the effect of S201A paired with G210V. Hence, we conclude that the Slmb degron is exposed when SMN is present predominantly as a monomer, whereas it is less accessible when the protein is able to form higher order multimers.

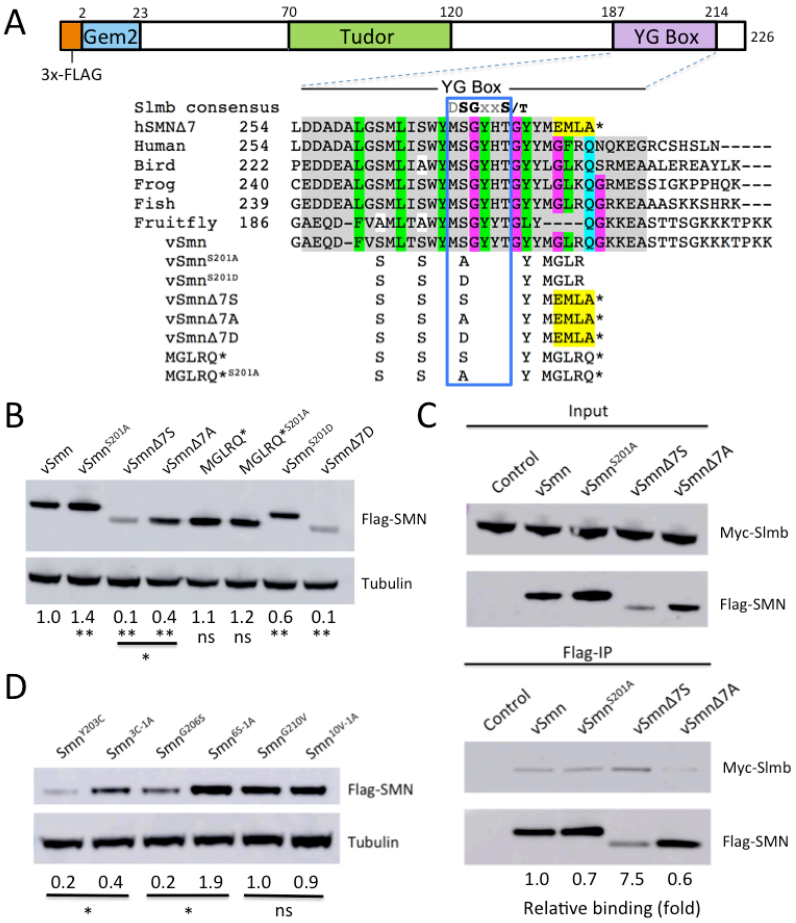


Figure 2.4. Identification and mutation of a putative Slmb/B-TrCP phospho-degron ***A***. Identification of a conserved putative Slmb phospho-degron (DpSGXXpS/T motif variant) in the C-terminal self-oligomerization domain (YG Box) of SMN. The amino acid sequence of Smn from a variety of vertebrates is shown to illustrate conservation of this motif and rationale for the amino acid changes. Full-length human SMN is labeled as "Human" and the truncated isoform is labeled "hSMNΔ7". Endogenous *Drosophila melanogaster* Smn is labeled "Fruitfly". To generate a more vertebrate-like SMN, key amino acids in Drosophila SMN were changed to amino acids conserved in vertebrates. Using this SMN backbone, a serine to alanine mutation was made in the putative degron in both full-length (vSMN^{S201A}) and truncated SMNΔ7 (vSMNΔ7A). An additional SMN construct that is the same length as SMNΔ7, but has the amino acid sequence GLRQ (the next amino acids in the sequence) rather than EMLA (the amino acids introduced by mis-splicing of *SMN2*) was made. The same serine to alanine mutation was made in this construct as well (MGLRQ* and MGLRQ*^{S201A}). Finally, to mimic a phosphorylated serine the full-length SMN construct (vSmn^{S201D}) and truncated SMN (vSmnΔ7D) were made. ***B***. Western blotting was used to determine protein levels of each of these SMN constructs, with expression driven by the endogenous promoter, in Drosophila S2 cells. Both the vSMN and vSMNΔ7S proteins show increased levels when the serine is mutated

to an alanine, indicating disruption of the normal degradation of SMN. Additionally, MGLRQ* protein is present at higher levels than is vSMNΔ7S and protein levels do not change when the serine is mutated to an alanine. Normalized fold change as compared to vSmn levels is indicated at the bottom. *p<0.05, **p<0.01 n=3. ***C***. Flag-tagged SMN constructs were co-transfected with Myc-Slmb in Drosophila S2 cells. Protein lysates were Flag-immunoprecipitated and probed with anti-Myc antibody to detect SMN-Slmb interaction. In both full-length SMN (vSMN) and truncated SMN (vSMNΔ7), serine to alanine mutation decreased interaction of Slmb with SMN. Truncated SMN (vSMNΔ7) showed a dramatically increased interaction with Slmb as compared to full-length SMN (vSMN), despite the fact it is present at lower levels. ***D***. Full-length SMN constructs containing point mutations known to decrease self-oligomerization (Smn^{Y203C} and Smn^{G206S}) and a mutation that does not disrupt self-oligomerization in the fly (Smn^{G210V}) with and without the serine to alanine mutation were transfected in Drosophila S2 cells. The constructs containing the serine to alanine mutation are as follows: Smn^{Y203C}->Smn^{3C-1A}, Smn^{G206S}->Smn^{6S-1A}, Smn^{G210V}->Smn^{10V-1A}. The serine to alanine mutation has a stabilizing effect on SMN mutants with poor self-oligomerization capability. *p<0.05, n=3.

Mutation of the Slmb degron rescues viability and locomotion defects in SMA model flies

Next, we examined the effect of mutating the Slmb degron in the context of the full-length protein *in vivo*. We characterized adult viability, larval locomotion and SMN protein expression phenotypes of the G206S mutants in the presence or absence of the degron mutation, S201A (Fig. 5A-C). As described previously (Praveen et al. 2014), Smn^{G206S} animals express very low levels of SMN and fail to develop beyond larval stages. In contrast, flies bearing the S201A degron mutation in addition to G206S (Smn^{6S-1A}) express markedly increased levels of SMN protein (Fig. 5C) and a good fraction of these animals complete development (Fig. 5A). Moreover, Smn^{6S-1A} larvae display significantly improved locomotor activity as compared to Smn^{G206S} or Smn^{X7} null mutants (Fig. 5B). These results strongly suggest that both the structure of the G206S mutant protein as well as its instability contribute to the organismal phenotype.

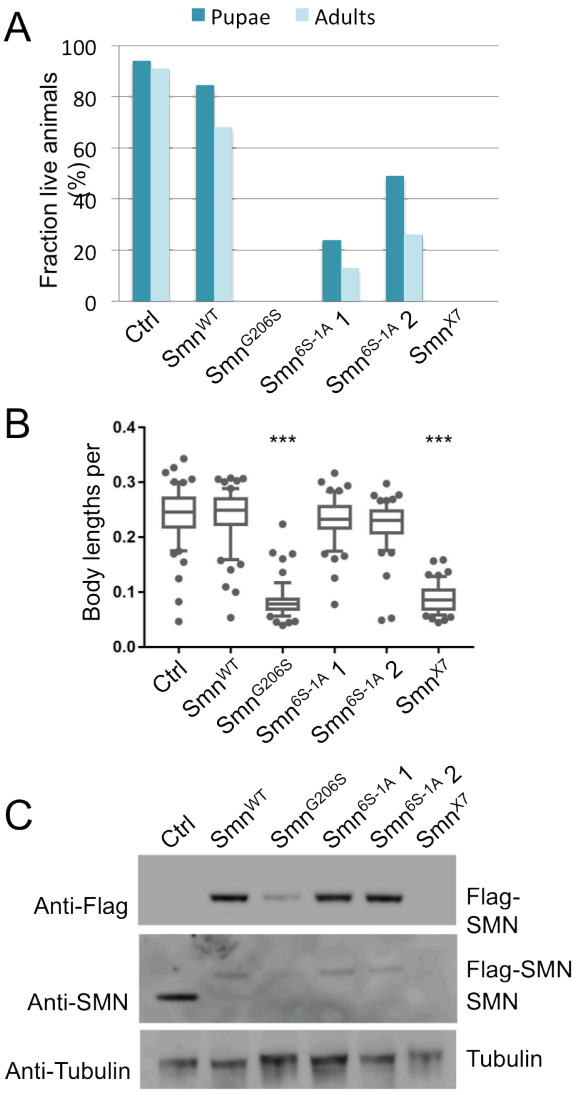


Figure 2.5. Mutation of the Slmb degron rescues defects in SMA model flies. ***A***. Viability analysis of an SMA point mutation (G206S) in the presence and absence of the degron mutation, S201A. Flies with the following genotypes were analyzed in this experiment: Oregon-R (Ctrl), *Flag-Smn*^{WT}*,Smn*^{X7}*/Smn*^{X7} (Smn^{WT}), *Flag-Smn*^{G206S}*,Smn*^{X7}*/Smn*^{X7} (Smn^{G206S}), *Flag-Smn*^{G206,S201A}*,Smn*^{X7}*/Smn*^{X7} (Smn$^{6S\text{-}1A}$) or *Smn*^{X7}*/Smn*^{X7} (Smn^{X7}). The data for each genotype are expressed as a fraction of pupae or adults over the total number of starting larvae, n=200. Expression of the WT transgene (*Smn*^{WT}) shows robust rescue of the null (*Smn*^{X7}) phenotype (~68% adults). *Smn*^{G206S} is a larval lethal mutation. In two independent recombinant lines of *Smn*$^{6S\text{-}1A}$ (*Smn*$^{6S\text{-}1A}$*1* and *Smn*$^{6S\text{-}1A}$*2*) a fraction of the larvae complete development to become adults. ***B***. Locomotor ability of early 3rd instar larvae was determined by tracking their movement for one minute and then calculating the velocity. To account for potential differences in larval size, speed is expressed as average body lengths per second moved. Genotypes are as in panel A. *Smn*^{G206S} larvae move similarly to null animals. The motility of *Smn*$^{6S\text{-}1A}$*1* and *Smn*$^{6S\text{-}1A}$*2* larvae is not different from Ctrl or *Smn*^{WT} larvae. ***p<0.001, n=50 to 60

larvae. ***C***. Larval protein levels were examined by western blotting; genotypes as in panel A. Lysates from hemizygous mutant lines were probed with anti-Flag or anti-SMN antibodies as indicated. The slower migrating bands represent the Flag-tagged transgenic proteins and the faster migrating band corresponds to endogenous SMN, which is present only in the Ctrl (note Oregon-R has two copies *Smn* whereas the transgenics have only one). Smn^{G206S} has very low levels of SMN protein. Flies bearing the S201A degron mutation in addition to G206S ($Smn^{6S\text{-}1A}$) express markedly increased levels of SMN protein.

GFP-SMNΔ7 overexpression stabilizes endogenous SMN and SMNΔ7 in cultured human cells

Increased *SMN2* copy number correlates with a milder clinical phenotype in SMA patients (Oskoui et al. 2016). This phenomenon was successfully modeled in mice over a decade ago (Monani et al. 2000; Hsieh-Li et al. 2000), showing that high copy number *SMN2* transgenes fully rescue the null phenotype, whereas low copy transgenes do not. Moreover, transgenic expression of a human *SMNΔ7* cDNA construct in a low-copy *SMN2* background improves survival of this severe SMA mouse model from P5 (post-natal day 5) to P13 (Le et al. 2005). Although the truncated SMN likely retains partial functionality, the protective effect of *SMNΔ7* overexpression may not entirely be intrinsic to the protein. That is, SMNΔ7 could also act as a 'soak-off' factor, titrating the ubiquitylation machinery and stabilizing endogenous SMN. In such a scenario, the prediction would be that SMNΔ7A is less protective than SMNΔ7S because it is not a very good substrate for SCF^{Slmb}.

We therefore compared the stabilizing effects of overexpressing GFP-tagged $SMN\Delta7^{S270A}$ (SMNΔ7A) and SMNΔ7 (SMNΔ7S) on endogenous human SMN and SMNΔ7. HEK 293T cells were transfected with equivalent amounts of GFP-SMNΔ7A or -SMNΔ7S. The following day, cells were harvested after treatment with cycloheximide (CHX) for zero to ten hours. As shown in Fig. 6A, western blotting with anti-SMN

showed that the SMNΔ7S construct exhibits a clear advantage over SMNΔ7A in its ability to stabilize endogenous SMN and SMNΔ7. By comparing band intensities within a given lane, we generated average intensity ratios for each time point using replicate blots (Fig. 6A, table). We then calculated a 'stabilization factor' by taking a ratio of these two ratios. As shown (Fig. 6A, graph), the protective benefit of overexpressing Δ7S vs. Δ7A at t=0 hr was roughly 3.0x for endogenous SMNΔ7 and 1.75x for full-length SMN. Thus, as predicted above, the GFP-SMNΔ7A construct was much less effective at stabilizing endogenous SMN isoforms. Because SMNΔ7 is a relatively good SCF^{Slmb} substrate, overexpression of this isoform protects full-length SMN from degradation.

As mentioned above, experiments in an SMN-deficient chicken DT40 cell line showed that expression of SMNΔ7A, but not SMNΔ7S, rescued cellular proliferation (Cho and Dreyfuss 2010). These results suggest that, when stable, SMNΔ7 is intrinsically functional. To examine SMNΔ7A functionality in a more disease-relevant cell type, control and SMA induced pluripotent stem cell (iPSC) motor neuron cultures were transduced with lentiviral vectors expressing an mCherry control protein or SMNΔ7A (Fig. 6B). At 4 weeks post-differentiation, no statistical difference was observed between control and SMA motor neurons, however by 6 weeks, SMA motor neuron numbers had decreased significantly to approximately 7% of the total cell population (Fig. 6B). In contrast, expression of SMNΔ7A maintained motor neuron numbers to approximately the same level as the controls, and nearly two-fold greater than untreated cells (Fig. 6B). Thus expression of SMNΔ7A improves survival of human iPSCs when differentiated into motor neuron lineages.

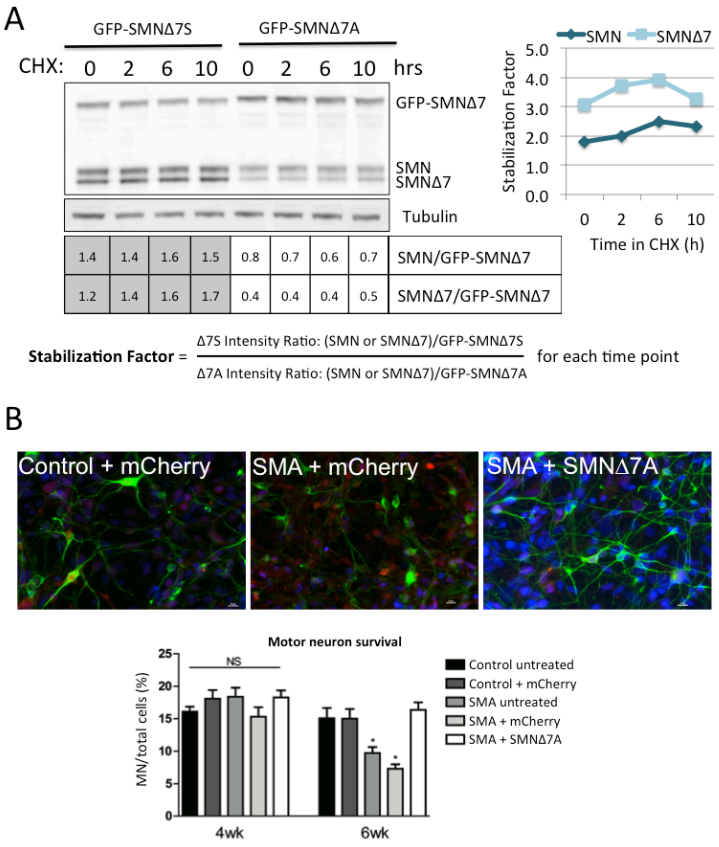


Figure 2.6. Stabilization of endogenous SMN and SMNΔ7 in cultured human cells. ***A***. HEK 293T cells were transfected with equivalent amounts of GFP-SMNΔ7A or -SMNΔ7S. The following day, cells were harvested after treatment with cycloheximide (CHX) for zero to ten hours. Western blotting with anti-SMN showed that SMNΔ7S stabilizes endogenous SMN and SMNΔ7 to a greater extent than SMNΔ7A. By comparing band intensities within a given lane, we generated average intensity ratios for each time point using replicate blots. We then calculated a 'stabilization factor' by taking a ratio of these two ratios. The protective benefit of overexpressing Δ7S vs. Δ7A at t=0 hr was roughly 3.0x for endogenous SMNΔ7 and 1.75x for full-length SMN. ***B***. SMNΔ7A (S270A) expression protects SMA iPSC-derived motor neurons. Control motor neurons were left untreated or transduced with a lentiviral vector expressing an mCherry control. SMA motor neurons were left untreated, transduced with a lentiviral vector expressing an mCherry control, or a lentiviral vector expressing SMNΔ7A (S270A). At 4 weeks of differentiation, there was no difference in motor neuron survival between control and SMA iPSC motor neuron cultures in any of the treatment groups. However, at 6 weeks, SMI-32 positive motor neurons showed selective loss in SMA iPSC motor neuron cultures in the untreated and lenti-mCherry groups compared to control iPSC motor neuron cultures. In contrast, lenti-SMNΔ7A expression fully protects SMA iPSC-derived motor neurons. Representative images of control and SMA iPSC-derived motor neurons labeled with SMI-32 (green) and mCherry (red). Nuclei are stained with DAPI and shown in blue. *p<0.05 by ANOVA. NS = not significant. n=3

SMNΔ7A is a protective modifier of intermediate SMA mouse phenotypes

To examine the importance of the Slmb degron in a mammalian organismal system, two previously developed SMA mouse models were utilized. As mentioned above, the 'Delta7' mouse (*Smn*$^{-/-}$*;SMN2;SMNΔ7*), is a model of severe SMA (Le et al. 2005), and affected pups usually die between P10 and P18 (Avg. P15). The '2B/–' mouse (*Smn*$^{2B/-}$) is a model of intermediate SMA (Bowerman et al. 2012; Rindt et al. 2015) and these animals survive much longer before dying, typically between P25 and P45 (Avg. P32). Adeno-associated virus serotype 9 (AAV9) was selected to deliver the SMN cDNA isoforms to these SMA mice, as this vector has previously been shown to enter and express in SMA-relevant tissues and can dramatically rescue the SMA phenotype when expressing the wild-type SMN cDNA (Foust et al. 2010; Passini et al. 2010; Valori et al. 2010; Dominguez et al. 2011; Glascock et al. 2012).

Delivery of AAV9-SMNΔ7A at P1 significantly extended survival in the intermediate 2B/– animals, resulting in 100% of the treated pups living beyond 100 days, similar to the results obtained with the full-length AAV9-SMN construct (Fig. 7A). In contrast, untreated 2B/– animals lived, on average, only 30 days. Mice treated with AAV9-SMNΔ7S survived an average of 45 days (Fig. 7A). Mice treated with AAV9-SMNΔ7D, a phosphomimetic of the wild-type serine 270 residue, have an average life span that is equivalent or slightly shorter than that of untreated 2B/– mice (Fig. 7A). These results not only highlight the specificity of the S270A mutation in conferring efficacy to SMNΔ7, but also illustrate that AAV9-mediated delivery of protein alone does not improve the phenotype.

We also analyzed the effects of SMNΔ7A expression in the severe Delta7 mouse model (Le et al. 2005). Treatment with AAV9-SMNΔ7A had only a very modest effect on Delta7 mice, as none of the animals (treated or untreated) survived weaning (Fig. S4). These findings are similar to the results in *Drosophila*. Transgenic expression of SMNΔ7A in the *Smn* null background is not sufficient to rescue larval lethality (Fig. S3). Thus expression of SMNΔ7A provides a clear protective benefit to the viability of intermediate mice, but not to severe SMA models.

Consistent with the lifespan data, AAV9-SMNΔ7A treated 2B/– mice gained significantly more weight than either untreated or AAV-SMNΔ7S treated animals, nearly achieving the same weight as pups treated with full-length AAV-SMN (Fig. 7B). Treatment with full-length SMN cDNA resulted in animals that were clearly stronger and more mobile, consistent with the weight data (Fig. 7C). Although they did not perform as well as mice treated with full-length SMN cDNA, the SMNΔ7A treated animals retained strength and gross motor function at late time points (e.g. P100), as measured by their ability to splay their legs and maintain a hanging position using a modified tube-test, (Fig. 7C). Animals treated with AAV9-SMNΔ7D and -SMNΔ7S did not survive long enough for testing.

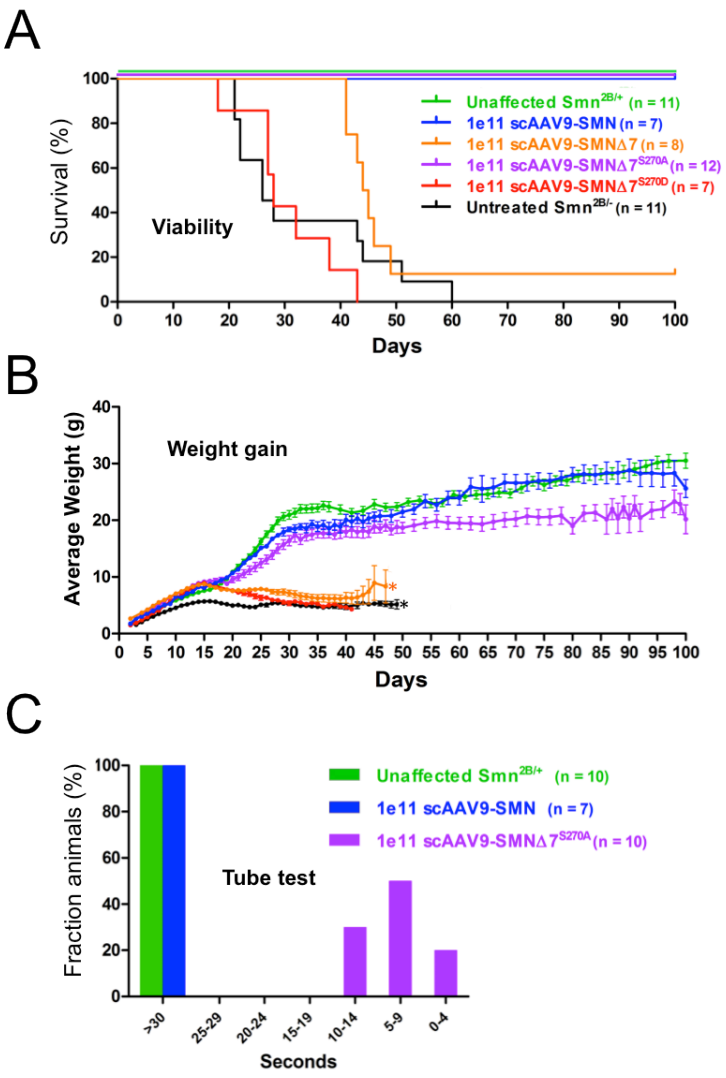


Figure 2.7. SMNΔ7A is a protective modifier of intermediate SMA phenotypes in mice. ***A***. Mouse genotypes include control unaffected *Smn*$^{2B/+}$ mice, which have a wild-type *Smn* allele, *Smn*$^{2B/–}$ (2B/–) mice treated with scAAV9 expressing different versions of SMN, and untreated 2B/– mice, which are an intermediate mouse model of SMA. 1e11 denotes the viral dose. scAAV9-SMN expresses full-length SMN, scAAV9-SMNΔ7 expresses truncated SMN, scAAV9-SMNΔ7^{S270A} expresses truncated SMN with the S to A change in the degron, and scAAV9-SMNΔ7^{S270D} expresses truncated SMN with a phosphomimic in the degron. Delivery of AAV9-SMNΔ7A at P1 significantly extended survival in the intermediate 2B/– animals, resulting in 100% of the treated pups living beyond 100 days, similar to the results obtained with the full-length AAV9-SMN construct. Untreated 2B/– animals lived, on average, only 30 days. Mice treated with AAV9-SMNΔ7S survived an average of 45 days. Mice treated with AAV9 expressing SMNΔ7D had an average life span equivalent or slightly worse than that of untreated 2B/– mice. ***B***. Average weight (measured over time) of the animals used in panel A. AAV9-SMNΔ7A treated mice also gained significantly more weight than either untreated

or AAV-SMNΔ7S treated animals, nearly achieving the same weight as 2B/– pups treated with full-length SMN cDNA. ***C***. Mouse genotypes include control unaffected $Smn^{2B/+}$ mice, which carry a wild-type *Smn* allele, and 2B/– mice treated with scAAV9 expressing different versions of SMN. scAAV9-SMN expresses full-length SMN and scAAV9-SMNΔ7^{S270A} expresses truncated SMN with the S to A change in the degron. AAV-SMNΔ7A treated animals retained their improved strength and gross motor functions at late time points (P100), as measured by their ability to splay their legs and maintain a hanging position using a modified tube-test.

SCF^{Slmb} primarily targets unstable SMN monomers

As indicated in Fig. 8, our findings suggest a model whereby SMN and SMNΔ7 degradation is in part mediated by SCF^{Slmb}, a multi-component E3 ubiquitin ligase composed of Slmb, SkpA, Cul1, and Roc1 (Zheng et al. 2002, Jiang and Struhl 1998, Patton et al. 1998a; Patton et al. 1998b). Our work demonstrates that B-TrCP/Slmb binds directly to SMN (Fig. 2) and is one of a growing number of E3 ligases in the cell that can target SMN protein (Kwon et al. 2013; Han et al. 2016). SMN monomers, such as those created in SMNΔ7, are the primary targets for degradation. As shown in the model, partially-active SMN•SMNΔ7 dimers and active SMN oligomers are also substrates, but to a lesser extent.

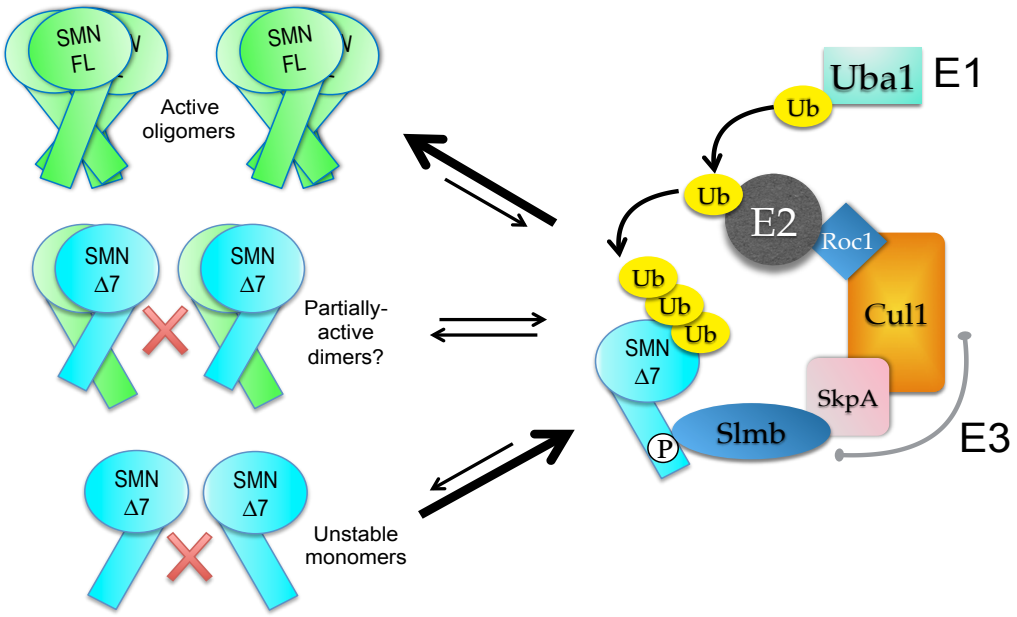


Figure 2.8. Proposed model of SMN as a substrate of SCF^{Slmb} E3 ubiquitin ligase. Unstable SMN monomers, such as those created in SMNΔ7, are the primary substrates for degradation. Active oligomers of full-length SMN (SMN-FL) and partially active SMN-FL•SMNΔ7 dimers (Praveen et al. 2014; Gupta et al. 2015) would be targeted to a lesser extent. SCF^{Slmb} is a multi-component E3 ubiquitin ligase composed of Slmb, SkpA, Cul1, and Roc1 (see text for details). This E3 ligase complex functions together with E1 and E2 proteins in the ubiquitin proteasome system (UPS) to tag proteins for degradation by linkage to ubiquitin (Ub). Phosphorylation (P) by GSK3β and/or another kinase (see text) is predicted to trigger ubiquitylation.

Discussion

Factors that recognize the putative SMNΔ7-specific degron have not been identified and the molecular mechanisms governing proteasomal access to SMN and SMNΔ7 remain unclear. In this study, we isolated factors that co-purifiy with SMN from *Drosophila* embryos that exclusively express Flag-SMN. This approach reduces potential bias towards SMN partner proteins that may be more abundant in a given tissue or cell line (Charroux et al. 1999; Meister et al. 2001; Pellizzoni et al. 2002; Kroiss et al. 2008; Trinkle-Mulcahy et al. 2008; Guruharsha et al. 2011). Here, we identify the SCF^{Slmb} E3 ubiquitin ligase complex as a novel SMN binding partner whose interaction is conserved

in human. Depletion of Slmb or B-TrCP by RNAi resulted in an increase in steady-state SMN levels in *Drosophila* and human cells, respectively. We also showed that ectopic expression of SMNΔ7^{S270A}, but not SMNΔ7 or SMNΔ7^{S270D}, a phosphomimetic, is a protective modifier of SMA phenotypes in animal models and human iPSC cultures.

The SCF^{Slmb} degron is exposed by SMN2 exon skipping

A previous study posited that a phospho-degron was specifically created by exon 7 skipping and that this event represented a key aspect of the SMA disease mechanism (Cho and Dreyfuss 2010). Our identification of a putative Slmb binding site located in the C-terminal self-oligomerization domain of *Drosophila* and human SMN has allowed us to explore the molecular details of this hypothesis. The mutation of a conserved serine within the Slmb degron not only disrupted the interaction between SMN and Slmb, but also stabilized full-length SMN and SMNΔ7. Notably, the degron mutation has a greater effect on SMN levels (both full-length and Δ7) when made in the context of a protein that does not efficiently self-oligomerize. These and other findings strongly suggest that the Slmb degron is uncovered when SMN is monomeric, whereas it is less accessible when SMN forms higher-order multimers. On the basis of these results, we conclude that *SMN2* exon skipping does not *create* a potent protein degradation signal; rather, it *exposes* an existing one.

SMN targeting by multiple E2 and E3 systems

SMN degradation via the UPS is well-established (Chang et al. 2004; Burnett et al. 2009; Kwon et al. 2011). Using candidate approaches, investigators have studied other E3 ligases that have been reported to target SMN for degradation in cultured human cells (Han et al. 2016; Hsu et al. 2010; Kwon et al. 2013). Given our findings, it is therefore

likely that SMN is targeted by multiple E3 ubiquitin ligases, as this regulatory paradigm has been demonstrated for a number of proteins (e.g. p53; Jain and Barton 2010). Targeting of a single protein by multiple E3 ligases is thought to provide regulatory specificity by expressing the appropriate degradation complexes only within certain tissues, subcellular compartments or developmental timeframes. Moreover, ubiquitylation does not always result in immediate destruction of the target; differential use of ubiquitin lysine linkages or chain length can alter a protein's fate (Mukhopadhyay and Riezman 2007; Ikeda and Dikic 2008; Liu and Walters 2010).

Avenues of future exploration include determination of the E2 proteins that partner with SCF^{Slmb} as well as the types of ubiquitin lysine chain linkages they add to SMN. These two questions are interconnected, as ubiquitin linkage specificity is determined by the E2 (Ye and Rape 2009). Lysine 48 (K48) linked chains typically result in degradation of the targeted protein by the 26S proteasome, whereas lysine 63 (K63) linkage is more commonly associated with lysosomal degradation and nonproteolytic functions such as endocytosis (Tan et al. 2007; Kirkin et al. 2009; Lim and Lim 2010). Interestingly, recent work has implicated defects in endocytosis in SMA (Custer and Androphy 2014; Dimitriadi et al. 2016; Hosseinibarkooie et al. 2016). It remains to be determined how the ubiquitylation status of SMN might intersect with endocytic functions.

Does SMN function as a signaling hub?

In the Flag-SMN pulldown, we identified three E2 proteins as potential SMN interacting partners (Fig. 1C). Among them, Bendless (Ben) is particularly interesting. Ben physically interacts with TRAF6, an E3 ligase that functions together with

Ube2N/Ubc13/Ben in human cells (Kim and Choi 2017). TRAF6 is an activator of NF-kB signaling, and its interaction with SMN is thought to inhibit this activity (Kim and Choi 2017). Notably, Ube2N/Ben heterodimerizes with Uev1a to form K63 ubiquitin linkages on target proteins (Ye and Rape 2009; van Wijk and Timmers 2010; Komander and Rape 2012; Marblestone et al. 2013; Zhang et al. 2013). Furthermore, Ben-Uev1a is involved in upstream activation of both JNK and IMD signaling in *Drosophila* (Paquette et al. 2010; Zhou et al. 2005). Previously, we and others have shown that JNK signaling is dysregulated in animal models of SMA (Garcia et al. 2013; Genabai et al. 2015; Garcia et al. 2016; Ahmad et al. 2016). Moreover, mutations in all three components of SCF^{Slmb} lead to constitutive expression of antimicrobial peptides, which are also downstream of the IMD pathway (Khush et al. 2002). Together, these findings suggest the interesting possibility of SMN functioning as a signaling hub that links the UPS to the JNK and IMD pathways, all of which have been shown to be disrupted in SMA.

Phosphorylation of the Slmb degron within SMN

As Slmb is known to recognize phospho-degrons, one of the first questions raised by our study concerns the identity of the kinase(s) responsible for phosphorylating the degron in SMN. A prime candidate is GSK3b (Fig. 8), as this kinase recognizes a motif (SxxxS/T; Liu et al. 2007; Lee et al. 2013) that includes the degron and extends N-terminally (^{262}SxxxSxxxSxxxT^{274}, numbering as per human SMN). In support of this hypothesis, we identified the *Drosophila* GSK3b orthologue, Shaggy (Sgg), in our SMN pulldowns (Fig. 1C). Moreover, GSK3b inhibitors as well as siRNA mediated knockdown of GSK3b were shown to increase SMN levels, primarily by stabilizing the protein (Makhortova et al. 2011; Chen et al. 2012). Finally, GSK3b is also responsible for

phosphorylation of a degron in b-catenin, a well-characterized SCF^{Slmb} substrate (Liu et al. 2002). SMA mice have low levels of UBA1 (E1) ultimately leading to accumulation of b-catenin (Wishart et al. 2014). Pharmacological inhibition of b-catenin improved neuromuscular pathology in *Drosophila*, zebrafish, and mouse SMA models. b-catenin had previously been shown to regulate motor neuron differentiation and stability by affecting synaptic structure and function (Murase et al. 2002; Li et al. 2008; Ojeda et al. 2011). b-catenin also regulates motor neuron differentiation by retrograde signaling from skeletal muscle (Li et al. 2008). The connections of UBA1 and multiple SCF^{Slmb} substrates to motor neuron health thus places the UPS at the center of SMA research interest.

Concluding remarks

In summary, this study identifies conserved factors that regulate SMN stability. To our knowledge, this work represents the first time that SMN complexes have been purified in the context of an intact developing organism. Using this approach, we have demonstrated that the SCF^{Slmb} E3 ligase complex interacts with a degron embedded within the self-oligomerization domain of SMN. Our findings establish plausible connections to disease-relevant cellular processes and signaling pathways. Further, they elucidate a model (Fig. 8) whereby accessibility of the SMN phospho-degron is regulated by self-multimerization, providing an elegant mechanism for balancing functional activity with degradation.

Supplementary Data

Genotype	Pupation (%)	Eclosion (%)
Flag-Smn^{WT},Smn^{X7}/Smn^{X7}	99	73
Flag-vSmn,Smn^{X7}/Smn^{X7}	86	83
Flag-vSmn^{S201A},Smn^{X7}/Smn^{X7}	100	97.5

Figure S2.1. Transgenic flies expressing Flag-vSmn and Flag-vSmn^{S201A} in the background of an *Smn^{X7}* null mutation are fully viable. The eclosion frequencies of these animals are consistently higher than those that express Flag-Smn^{WT} in the background of an *Smn^{X7}* null mutation. The data for each genotype are expressed as a fraction of pupae or adults over the total number of starting larvae, n=200.

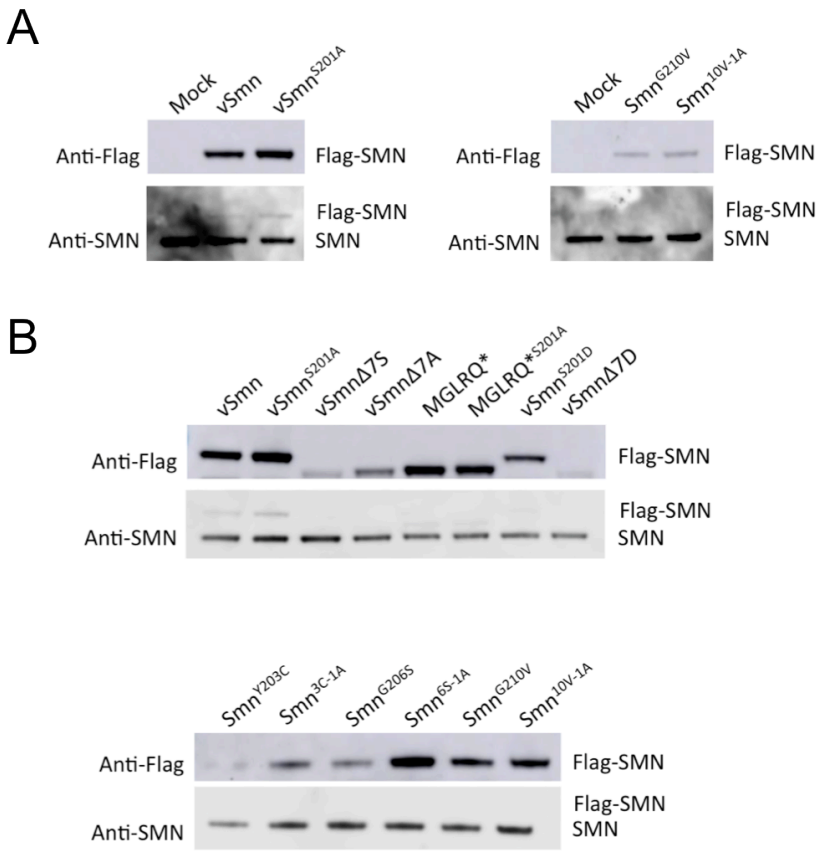


Figure S2.2. A. The expression of endogenous SMN in S2 cells following transient transfection of either modified 'vertebrate' SMN constructs (vSMN and vSMN^{S201A}) or Drosophila SMN constructs (Smn^{G210V} and Smn^{10V-1A}) is unaffected, as compared to mock transfection. Protein is detected by anti-Flag antibody or anti-SMN antibody as indicated to the left of the blots. **B.** Transient transfections in S2 cells express Flag-SMN from the endogenous promoter. Protein levels of all transfected constructs are lower than endogenous SMN protein levels. Protein is detected by anti-Flag antibody or anti-SMN antibody as indicated to the left of the blots.

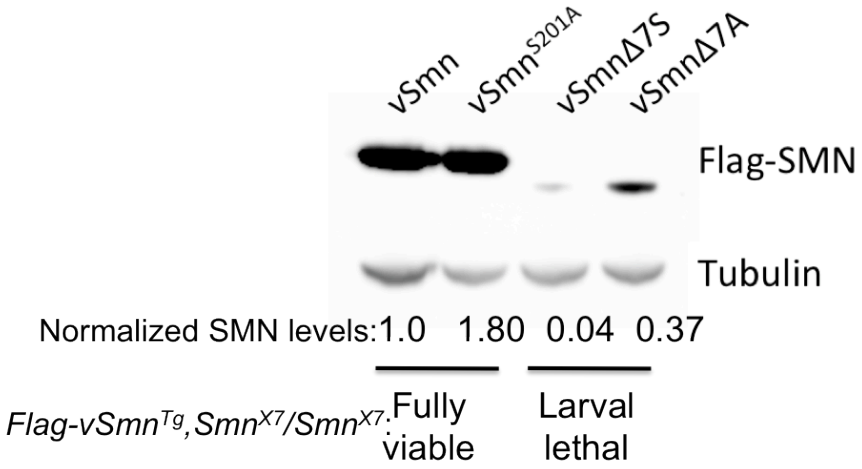


Figure S2.3. Western blotting was used to determine protein levels of each of the SMN constructs, with expression driven by the endogenous promoter, in transgenic adult flies. Protein lysates were made by pooling 40-50 adult flies. Flies with the following genotypes were analyzed in this experiment: *Flag-vSmn,Smn^{X7}/Tm6b* (vSmn), *Flag-vSmn^{S201A},Smn^{X7}/Tm6b* (vSmn^{S201A}), *Flag-vSmnΔ7S,Smn^{X7}/Tm6b* (vSmnΔ7S) or *Flag-vSmnΔ7A,Smn^{X7}/Tm6b* (vSmnΔ7A). Both the vSMN and vSMNΔ7S proteins show increased levels when the serine is mutated to an alanine, indicating disruption of the normal degradation of SMN. Flag-SMN was detected using anti-Flag antibody. Normalized fold change as compared to vSmn levels is indicated at the bottom.

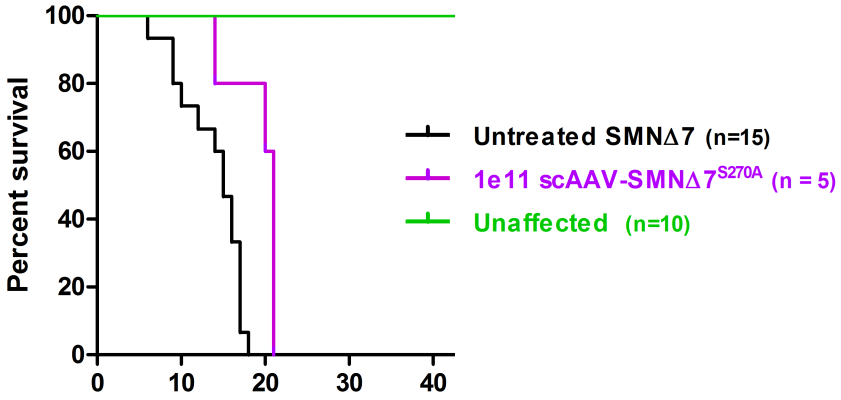


Figure S2.4 Survival analysis of the effects of SMNΔ7A expression in the severe Delta7 mouse model. Genotypes include untreated SMNΔ7 mice, which are a severe mouse model of SMA, SMNΔ7 mice treated with scAAV9 expressing SMNΔ7^{S270A}, truncated SMN with the S to A change in the degron, and control unaffected mice, which have a wild-type Smn allele. Treatment with AAV9-SMNΔ7A had only a very modest effect on viability and none of the animals survived weaning. 1e11 denotes the viral dose.

CHAPTER III: Novel SMN interaction partners related to a variety of cellular functions

Introduction

Spinal muscular atrophy (SMA) is a pediatric neuromuscular disorder caused by mutations in or loss of *survival motor neuron 1* (*SMN1*). Approximately 95% of SMA patients have deletions in *SMN1*, and the remaining ~5% have point mutations in *SMN1* (Burghes and Beattie, 2009). The etiology of SMA remains poorly understood despite the progress that has been made in this field, especially therapeutically. SMN's most well understood function is in the biogenesis of spliceosomal uridine-rich small nuclear ribonucleoproteins (snRNPs). Several putative neuron and muscle-specific functions for SMN have also been purported (Fan et al., 2002; McWhorter et al., 2003; Sharma et al., 2005). Thus far, no definitive link between any function of SMN and SMA etiology has been shown. Moving forward, the key question in SMA research is to understand which function or functions of SMN are critical in causing the disease. This will be important for detailing the basic biology as well as refining the therapies that are currently in use.

In Chapter II, we showed evidence suggesting that SMN interacts with the multi-protein E3 ubiquitin ligase, SCF^{Slmb}. This protein complex is responsible for contributing to ubiquitylating SMN leading to degradation via the ubiquitin proteasome system (UPS). Here, we extend the analysis to examine additional SMN protein interaction partners. Flag-SMN was immunopurified from fly embryos, and this lysate was analysed using

LC-MS/MS. Here, we validate SMN interaction with three of the proteins detected at high levels in SMN immunopurified lysate: CG2941, Nucleosome assembly protein 1 (Nap1), and bendless (ben). Further, we present preliminary characterization of CG2941 including determination of cellular localization and fruit fly viability following knockdown of CG2941 in the whole fly as well as the other two genes present in the gene triplication. Therefore detection and investigation of proteins interacting with SMN in the developing *Drosophila melanogaster* embryo provide insight into the functions of SMN that are relevant in whole organisms early in development and therefore possibly in the onset of SMA.

The protein produced from the CG2941 gene was detected in abundance from the Flag-immunopurified embryonic lysates. This gene is present in *Drosophila melanogaster* as a gene triplication on the X chromosome, with CG32786 and CG32783 having a highly similar DNA sequence. At the amino acid level, this protein is not conserved. The protein is found only in *Drosophila*, and a protein with some sequence similarity is predicted in mosquitos. The possibility remains for conservation at the structural or functional levels. FlyAtlas Anatomical Expression data shows it to be most highly expressed in the larval central nervous system and in the adult ovary. Previously, CG2941 had been shown to co-purify with two other members of the SMN complex: Gemin2 and Gemin3 (Guruharsha et al. 2011). In humans, SMN operates as part of the multiprotein complex consisting of Gemins2-8 and Unrip. The fruit fly complex has been thought to operate as a smaller complex including SMN, Gemin2/3/5, and potentially Unrip. Given the available information, CG2941 is an strong candidate for acting as one of the Gemins not previously identified in the fruit fly SMN complex.

Nucleosome assembly protein (Nap1) has been associated with multiple aspects of chromatin remodelling and associated changes in gene expression. Nap1 works with SWI/SNF complexes to remodel chromatin and facilitate transcription factor binding to DNA (Chen et al. 1994; Cote et al. 1994; Walter et al. 1995). Nap1 preferentially removes and replaces the H2A-H2B histone heterodimer and its variants, thereby facilitating nucleosome sliding (Park et al. 2004). The expression of genes involved in neurulation has been shown to specifically be affected by Nap1 interaction with chromatin (Rogner et al. 2000). In addition to affecting gene expression through chromatin remodelling, Nap1 has also been suggested to directly interact with transcriptional activators (Asahara et al. 2002; Shikama et al. 2000; Rehtanz et al. 2004). Finally, it has been suggested that Nap1 may play a role in chromatin regulation, upstream of direct remodelling, by shuttling histones into the nucleus (Park and Luger 2006; Mosammaprast et al. 2002). It is yet to be determined whether SMN might be involved in the roles of Nap1 that have previously been established, or whether they have a novel function separate from their individual roles.

Bendless (ben) was originally identified in a Drosophila melanogaster screen for mutations affecting the giant fiber (GF) synapse using jumping behavior as a read-out for such mutations (Thomas and Wyman 1984). Bendless mutants were named as such because they were found to have a GF neuron that failed to bend and reach it's connection with the target motorneuron. Ben is highly expressed in the fruit fly nervous system during development and regulates synaptic connectivity by contributing to degradation of target proteins on the presynaptic side (Muralidhar and Thomas 1993). Bendless is an E2 ubiquitin conjugating enzyme that heterodimerizes with Uev1a to form

ubiquitin linkages on target proteins (Ye and Rape 2009; van Wijk and Timmers 2010; Komander and Rape 2012; Marblestone et al. 2013; Zhang et al. 2013). As SMN and Ben have both been suggested to affect neuronal development, this is an interesting avenue of further investigation.

Experimental Procedures

Fly stocks

RNAi lines were obtained from the Bloomington TRIP collection. The identifying numbers listed on the chart are stock numbers. Each of the RNAi constructs are expressed from one of five VALIUM vectors and require Gal4 for expression. All stocks were cultured on molasses and agar at room temperature (24 ± 1°C).

Antibodies and Western blotting

Embryonic lysates were prepared by crushing the animals in lysis buffer (50mM Tris-HCl, pH 7.5, 150 mM NaCl, 1mM EDTA, 1% NP-40) with 1X protease inhibitor cocktail (Invitrogen) and clearing the lysate by centrifugation at 13,000 RPM for 10 min at 4ºC. S2 cell lysates were prepared by suspending cells in lysis buffer (50mM Tris-HCl, pH 7.5, 150 mM NaCl, 1mM EDTA, 1% NP-40) with 10% glycerol and 1x protease inhibitor cocktail (Invitrogen) and disrupting cell membranes by pulling the suspension through a 25 gauge needle (Becton Dickinson). The lysate was then cleared by centrifugation at 13,000 RPM for 10 min at 4ºC. Cell fractionation was performed using a standard protocol (West et al. 2008). In brief, following centrifugation, cytoplasmic extracts were taken from the top 0.2mL and the nuclear pellet was resuspended in 0.2mL RIPA buffer. Western blotting on lysates was performed using standard protocols. Rabbit

anti-dSMN serum was generated by injecting rabbits with purified, full-length dSMN protein (Pacific Immunology Corp, CA), and was subsequently affinity purified. For Western blotting, dilutions of 1 in 2,500 for the affinity purified anti-dSMN, 1 in 10,000 for monoclonal anti-FLAG (Sigma), and 1 in 500 for the Nap1 antibody (gift from M. Kiledijan) were used.

Immunoprecipitation

Lysates were incubated with Anti-FLAG antibody crosslinked to agarose beads (EZview Red Anti-FLAG M2 affinity gel, Sigma) for 2h-ON at 4C with rotation. The beads were washed with RIPA lysis buffer or three times and boiled in SDS gel-loading buffer. Eluted proteins were run on an SDS-PAGE for western blotting.

Drosophila embryo protein lysate and mass spectrometry

0-12h Drosophila embryos were collected from Oregon-R control and Flag-SMN flies, dechorionated, flash frozen, and stored at -80C. Embryos (approx. 1gr) were then homogenized on ice with a Potter tissue grinder in 5 mL of lysis buffer containing 100mM potassium acetate, 30mM HEPES-KOH at pH 7.4, 2mM magnesium acetate, 5mM dithiothreitol (DTT) and protease inhibitor cocktail. Lysates were centrifuged twice at 20000 rpm for 20min at 4C and dialyzed for 5h at 4C in Buffer D (HEPES 20mM pH 7.9, 100mM KCl, 2.5 mM $MgCl_2$, 20% glycerol, 0.5 mM DTT, PMSF 0.2 mM). Lysates were clarified again by centrifugation at 20000 rpm for 20 min at 4C. Lysates were flash frozen using liquid nitrogen and stored at -80C before use. Lysates were then thawed on ice, centrifuged at 20000 rpm for 20 min at 4C and incubated with rotation with 100 mL of EZview Red Anti-FLAG M2 affinity gel (Sigma) for 2h at 4C. Beads were washed a total of six times using buffer with KCl concentrations ranging from 100mM to 250mM

with rotation for 1 min at 4C in between each wash. Finally, Flag proteins were eluted 3 consecutive times with one bed volume of elution buffer (Tris 20mM pH 8, 100 mM KCl, 10% glycerol, 0.5 mM DTT, PMSF 0.2 mM) containing 250ug/mL 3XFLAG peptide (sigma). The entire eluate was used for mass spectrometry analysis on an Orbitrap Velos instrument, fitted with a Thermo Easy-spray 50cm column.

Tissue culture and transfections

S2 cell lines were obtained from the Drosophila Genome Resource Center (Bloomington, IL). S2 cells were maintained in SF900 SFM (Gibco) supplemented with 1% penicillin/streptomycin and filter sterilized. Cells were removed from the flask using a cell scraper and passaged to maintain a density of approximately 10^6-10^7 cells/mL. S2 cells were transferred to filter sterilized SF900 SFM (Gibco) without antibiotics prior to transfection with Cellfectin II (Invitrogen). Transfections were performed according to Cellfectin II protocol in a final volume of 4 mL in a T-25 flask containing 10^7 cells that were plated one hour before transfection. The total amount of DNA used in transfections was 2.5ug.

Immunofluorescence

Immunofluoresce in S2 cells was performed according to the previously published protocol (Rogers and Rogers 2008). S2 cells were seeded onto coverslips with ConA. Cells were fixed in 10% formaldehyde and washed three times in PBST. Cells were incubated with blocking solution for 10 min at RT, then transferred to 4C with primary antibody (1:250 dilution) for ON incubation. Cells were washed three times in PBST and the secondary antibody was incubated for 1h at 4C. Following the final three washes in PBST, cells were mounted on microscope slides and imaged using confocal microscopy.

Results

Flag-SMN interacts with proteins involved in a variety of cellular processes

We previously generated transgenic flies that express Flag-tagged SMN proteins in a null *Smn* background, carrying a deletion of endogenous *Drosophila Smn* (Praveen et al. 2012). To preserve endogenous expression patterns, the constructs are driven by the native promoter and flanking sequences. We crossed hemizygous *Flag-*Smn^{WT},Smn^{X7}/Smn^{D} animals to establish a stock where all of the SMN protein, including the maternal contribution, is Flag-tagged. Adults from this stock display no apparent defects and have an eclosion frequency (~90%) similar to that of wild-type (Oregon-R) animals. We collected (0-12h) embryos from Flag-SMN and Oregon-R (Ctrl) animals and analyzed Flag-purified lysates by LC-MS/MS. In addition to the proteins described in Chapter II, we conducted preliminary verification experiments for three of the 396 detected proteins (Fig 3.1 A).

I verified the interaction of CG2941 with SMN via reciprocal co-immunoprecipitation, demonstrating that Flag-tagged CG2941 co-purifies with endogenous SMN in S2 cells (Fig. 3.1 B). Nap1 was easily detectable in Flag-purified eluates from embryos expressing Flag-SMN and nearly undetectable in those from control embryos (Fig. 3.1 C). The interaction of Ben with SMN was also verified in a reciprocal co-immunoprecipitation, demonstrating that overexpressed Flag-tagged Ben co-purifies with endogenous SMN in S2 cells (Fig. 3.1 D). Cul1-Flag IP represents a low level of protein-protein interaction with SMN. Ben interaction with SMN is detected at higher levels than Cul1 interaction with SMN. Slmb interaction with SMN is a positive control for protein interaction with SMN, as related to the findings presented in Chapter

II. In S2 cells, endogenous Ben is not detected in immunoprecipitation of Flag-SMN or flag-tagged members of the SCF complex (data not shown).

A

Protein	Gene #	Category	Ctrl (x10^5)	SMN (x10^5)	Peptides
Gaulos	CG2941	SMN Complex?	1.82	1297	34
Nap1	CG5330	chromatin remodeling	4.86	1289	12
Ben	CG18319	E2	2.07	27.33	5

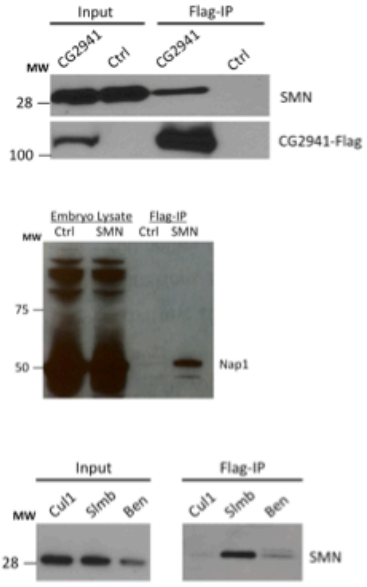


Figure 3.1. Flag-SMN immunopurified lysates contain novel interaction partners with a variety of cellular functions. ***A.*** Flag-purified eluates were analyzed by 'label-free' mass spectrometry. Numerous proteins that copurify with Flag-SMN are previously unknown interaction partners. Each of the three proteins in this table: CG2941, Nap1, and Ben are highly enriched in the Flag-SMN sample (SMN) as compared to the control (Ctrl). Numbers (x10^5) indicate intensities of peptides from each protein as detected by LC-MS/MS. ***B.*** CG2941 interaction with SMN was verified by expressing CG2941-Flag in S2 cells and immunoprecipitating using Flag beads. SMN is detected in a CG2941 but not in the control (Ctrl) sample. SMN was detected using *Drosophila* SMN antibody and CG2941-Flag was detected using anti-Flag antibody. ***C.*** Flag purified embryonic lysates were probed with anti-Nap1 antibody to verify the identity of Nap1 as a protein interacting with SMN. No signal is detected in the Ctrl Flag-IP, but there is a clear Nap1 band in Flag-SMN embryonic lysate. ***D.*** Ben interaction with SMN was verified by expressing Ben-Flag in S2 cells and immunoprecipitating lysates using Flag beads. SMN is detected above background (Cul1 in this blot) in Ben-Flag purified lysate. Flag-Slmb is a positive control for protein interaction with SMN. SMN was detected using *Drosophila* SMN antibody.

CG2941-Flag localizes to both the cytoplasm and nucleus

We generated an S2 cell line that stably expresses CG2941-Flag. The S2 cells were cultured in media containing puromycin to maintain selection for the plasmid expressing CG2941-Flag. Cell fractionation was used to make protein lysates from both the cytoplasm and nucleus of these S2 cells. These fractions were run on a 12% gel and visualized using silver staining (Fig. 3.2 A). There are proteins associated with CG2941 in both the cytoplasm and nucleus. Some bands may be common interacting proteins in both the cytoplasm and nucleus, whereas others are specific for each compartment. These lysates were also probed with anti-Flag antibody to test presence of CG2941-Flag in each of the eluted fractions from each cellular compartment. This served as a positive control for the immunoprecipitation and provides insight into CG2941 by way of knowing its cellular localization.

This cell line was also used for immunofluorescence experiments (Fig. 3.2 B). S2 cells were stained with DAPI to visualize nuclei. Anti-SMN antibody stains SMN predominantly in the cytoplasm as expected. SMN is present in nuclear bodies in the nucleus, but is not detected in all cell types. CG2941-Flag was detected using anti-Flag antibody. While not all cells appear to express CG2941-Flag from the plasmid, CG2941 was present in both the nucleus and cytoplasm when it was expressed. Using a combination of cell fractionation and immunofluorescence, we have determined CG2941 to be present in both the nucleus and cytoplasm of S2 cells.

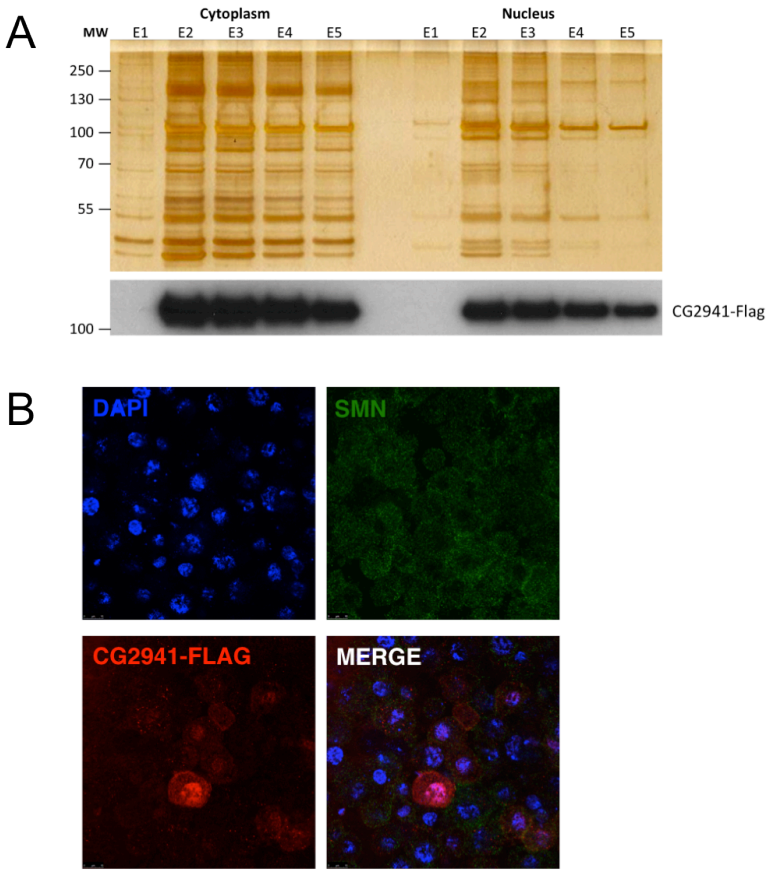


Figure 3.2. CG2941 is in both the nucleus and the cytoplasm of S2 cells. ***A.*** S2 cells stably expressing CG2941-Flag under Puromycin selection contain CG2941 in both the cytoplasmic and nuclear cellular compartments. Cellular fractionation was used to obtain lysates to silver stain (top) and probe with anti-Flag antibody (bottom). Experiment completed by David Baillat. ***B.*** S2 cells stably expressing CG2941-Flag under Puromycin selection demonstrate CG2941 protein localization to both the cytoplasm and nucleus. S2 cells were stained with DAPI to show the nucleus, anti-SMN antibody shows protein localization mainly to the cytoplasm, and CG2941 is in both the cytoplasm and the nucleus as detected by anti-Flag antibody.

CG2941 RNAi reduces fly viability

As the CG2941 gene and both of the genes in the gene triplication code for proteins of unknown function, we wanted to determine whether loss of this protein affected viability. There are four Bloomington TRiP lines that target one or multiple of the genes in the triplication (Fig. 3.3 A). Depending on the vector from which they are expressed, the RNAi will be present more in the soma or the germline when combined

with a Gal4 driver. The vectors used in the experiment include VALIUM20, which is effective in both the soma and the germline, and the vectors VALIUM21 and VALIUM22, which express well in the germline but not in the soma.

We first expressed CG2941 RNAi from each of these fly lines using a ubiquitously expressed Actin5C-Gal4 driver (Fig. 3.3 B). Overall, those that expressed only in the germline did not affect viability. In contrast, the RNAi line that targets only CG2941 and not the other genes in the triplication but is expressed in the soma, decreased viability and the RNAi construct targeting all three genes resulted in an even greater decrease is fly viability. Since the RNAi lines expressed in the germline did not affect viability, we tested fertility in 35667 RNAi flies and found they were able to produce offspring. For each of the RNAi lines that were found to affect viability, we made observations regarding the developmental stage as which most of the flies experienced developmental arrest. The 53989 RNAi was found to be pharate pupal lethal and 60490 was early pupal lethal. These differences in developmental arrest could be due to the differences of targeting one of the genes as opposed to targeting all three. Alternatively, these two RNAi lines may reduce protein levels to differing extents.

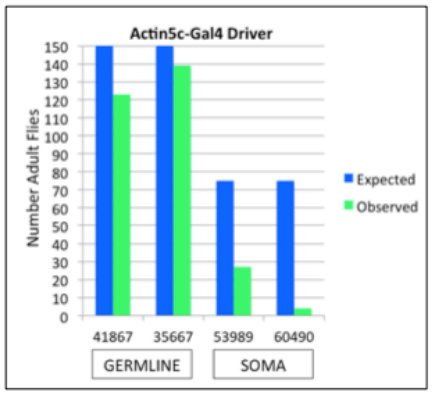
We also expressed each of the RNAi constructs in flies using a different ubiquitous Gal4 driver, Tubulin-Gal4, to test whether similar effects of viability would be detected (Fig. 3.3 C). Again, expression of CG2941 RNAi only in the germline did not affect viability, but expression in the soma resulted in decreased viability. Since CG2941 had previously been shown to be highly expressed in the larval central nervous system we also expressed CG2941 RNAi tissue-specifically in neurons using the Elav-Gal4 driver.

We did not detect any effect on fly viability in this RNAi (data not shown). This result suggests that CG2941 has an essential function that is not specific to neurons.

A

	CDS	3' UTR	Soma	Germline
41867		X		++
53989	X		++	+
35667	X			++
60490	X		++	+

B



C

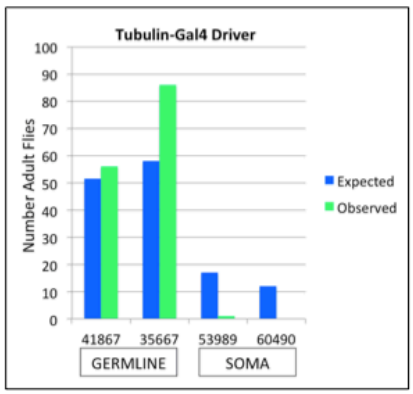


Figure 3.3. CG2941 is likely an essential gene in *Drosophila*. ***A.*** Four different RNAi lines were tested for their effects on *Drosophila* viability. Three of the dsRNAs expressed in these lines were designed to specifically target CG2941 (41867, 53989, and 35667), whereas the fourth line (60490) is intended to target all three members of the gene triplication in *Drosophila*. The other primary distinction between these lines is that two are optimized for expression in the germline, while the other two are mostly expressed in the soma. The RNAi may be targeted to the coding sequence (CDS) or the 3' UTR. ***B.*** Knockdown of CG2941 in the germline using the ubiquitous Actin5C-Gal4 driver does not affect *Drosophila* viability. In contrast, knockdown in the soma reduces adult viability. Blue bars indicate the expected number of adult flies based on mendelian ratios and green bars demonstrate the actual number of adult flies observed. ***C.*** Knockdown of CG2941 in the germline using the ubiquitous Tubulin-Gal4 driver does not affect *Drosophila* viability. In contrast, knockdown in the soma reduces adult viability. Blue and green bars represented as in panel B.

Discussion

In this work, we have described new protein interaction partners of SMN in *Drosophila melanogaster* embryos. All previous SMN interacting partners were originally identified using cell culture and *in vitro* techniques, meaning ours is the first

investigation of proteins in complex with SMN in an intact developing organism. This approach provided insight into biologically relevant interactions that occur early in development, the key SMA time window. The three novel interactions highlighted here are between SMN and CG2941, nucleosome assembly protein 1 (Nap1), and bendless (Ben). Each of these proteins relate to different aspects of established or suggested functions of SMN. By considering the cellular roles of the proteins that interact with SMN in early organismal development, we can generate new hypotheses about SMA relevant functions of SMN.

CG2941 as a member of the core SMN complex?

Detection of CG2941 co-purification with Gemin2 and Gemin3 (Guruharsha et al. 2011) provided the first hint that this protein may be a member of the canonical SMN complex in *Drosophila melanogaster*. Our detection of high levels of CG2941 in Flag-SMN purification from fly embryonic lysates, the presence of CG2941 in both the nucleus and cytoplasm, and suggestion of CG2941 as an essential gene all provided credence to this hypothesis. Lanfranco et al. (2017) recently published findings suggesting CG2941 as a *Drosophila* Gemin4 homolog in addition to identifying proteins with similarities to Gemin6/7/8. This suggests that *Drosophila* have all of the proteins that are present in the mammalian SMN complex, as opposed to having a smaller complex that carried out the same functions, as previously thought.

The identified homologs are divergent at the amino acid level, but display secondary structure conservation. Additionally, they affect neuromuscular survival and function. CG2941 genetically interacts with Gemin3 as shown by defects in third instar larvae mobility, adult viability, and adult lifespan following CG2941 RNAi in a

hypomorphic Gemin3 mutant background (Lanfranco et al. 2017). In accordance with our findings, CG2941 (Gaulos) was found in complex with SMN following immunoprecipitation of tagged proteins from S2 cells. This study added to our findings of adult viability defects in flies with ubiquitous knockdown of CG2941 through investigation of tissue-specific effects of CG2941 RNAi in mesoderm, muscle, the central nervous system, and motor neurons. Each of these was found to have an effect on adult viability, adult lifespan, and motor function. Motor function defects were assessed using a climbing assay and examination of flight.

An important next step of the investigation of CG2941 as a *bona fide* Gemin4 homolog will be determination of the role of CG2941 in snRNP biogenesis; the most well established function of the SMN complex. This may be tested by measuring snRNA levels in the whole organism following CG2941 RNAi and conducting biochemical assays to determine the ability of CG2941 to work with the remainder of the SMN complex to assembly Sm proteins onto snRNAs. Since CG2941 also has similarity to other human proteins, as determined by bioinformatics (Lanfranco et al. 2017), investigation of additional functions of this protein could provide insight into alternative functions of the SMN complex that may have relevance in humans. Finally, since CG2941 is present in flies as part of a gene triplication, determination of the significance of the other two genes in the triplication will be important for future studies of the SMN complex in flies when applying that information to the SMN complex in humans.

Chromatin remodelling and transcriptional regulation in SMA

Nucleosome assembly protein (Nap1) has well-established roles in chromatin remodelling and the associated changes in gene expression. Nap1 facilitates nucleosome

sliding by removing and replacing the H2A-H2B histone heterodimer and its variants (Park et al. 2004). Nap1 may also play a role in chromatin regulation, upstream of direct remodelling, by shuttling histones into the nucleus (Park and Luger 2005; Mosammaprast et al. 2002). Especially relevant in the context of SMA, Nap1 interaction with chromatin affects the expression of genes involved in neurulation (Rogner et al. 2000). While SMN protein had not previously been known to interact with Nap1, SMN has been implicated in nuclear-cytoplasmic shuttling as well as neuronal development and these processes have been considered in relation to SMA.

Although the primary role of SMN in cytoplasmic snRNP biogenesis is well studied, the connections of SMN to chromatin remodelling and regulating transcription involve the less understood functions of nuclear SMN. As SMN has not been suggested to directly affect active chromatin remodelling, SMN function with Nap1 in this cellular activity would be novel. SMN has been suggested to facilitate nuclear import of snRNPs, as part of their lifecycle involving both nuclear and cytoplasmic compartments (Narayanan et al. 2002; Narayanan et al. 2004). Potentially using the same or different nuclear import adapters, SMN works with Nap1 to shuttle histones into the nucleus. Several lines of evidence suggest the involvement of SMN in transcriptional regulation, which has the potential to relate to the protein-protein interaction of SMN with Nap1. Specific and general transcription factors have been shown to concentrate in nuclear Cajal bodies, which include SMN (Carvalho et al. 1999). Additionally, this concentration has been shown to be physiologically regulated, indicating the potential for functional significance. Transient associations of Cajal bodies with chromatin may be dependent on active transcription at that chromosomal locus (Platani et al. 2002). Further evidence

supporting the role of SMN in transcriptional regulation was provided when Zhao et al. (2015) demonstrated that SMN binds symmetric dimethylation of an arginine residue (R1810) of RNA polymerase II C-terminal domain. SMN also interacts with Senataxin (SETX), a helicase that unwinds R-loops around transcription termination sites (Fig. 1.5) (Suraweera et al. 2009), contributing to transcription termination via XRN2 exonuclease recruitment (Skourti-Stathaki et al. 2011). It remains an open question as to whether SMN and Nap1 function together in any of these contexts or whether there is simply overlap in their functions. The verification of their interaction provides impetus to further investigate this open area of research.

SMN and cellular signalling pathways

Previously, we and others have shown that JNK signaling is dysregulated in animal models of SMA (Garcia et al. 2013; Genabai et al. 2015; Garcia et al. 2016; Ahmad et al. 2016). Bendless (Ben) is involved in upstream activation of JNK signaling in *Drosophila* (Paquette et al. 2010; Zhou et al. 2005). This activation is accomplished due to Ben forming a heterodimer with Uev1a. (Ye and Rape 2009; van Wijk and Timmers 2010; Komander and Rape 2012; Marblestone et al. 2013; Zhang et al. 2013). Uev1a maintains a structural conformation that permits only K63 ubiquitin linkages on target proteins. K63 ubiquitin linkages are associated with formation of signaling complexes, rather than leading to protein degradation via the ubiquitin proteasome system (Ye and Rape 2009). Detecting SMN and Ben purifying together from S2 cell lysates suggests that Ben may be working with an E3 ubiquitin ligase to ubiquitylate SMN as a substrate. Alternatively, and not mutually exclusively, SMN may be regulating Ben activity leading to downstream effects on JNK signaling.

Through other protein interactions, Ben is involved in additional signaling pathways, such as those where NF-kB is an effector. For example, Ben has been shown to physically interact with TRAF6, an E3 ligase (Kim and Choi 2017). TRAF6 is an activator of NF-kB signaling, and its interaction with SMN is thought to inhibit this activity (Kim and Choi 2017). Since SMN has also been shown to interact with Ben, there are multiple mechanisms for regulation that may be hypothesized based on this interaction network. Future studies elucidating these details will be important to fully understanding the role of SMN in cellular signaling pathways. Together, these findings suggest the interesting possibility of SMN functioning as a signaling hub that is a part of JNK and NF-kB signaling, both of which have been shown to be disrupted in SMA.

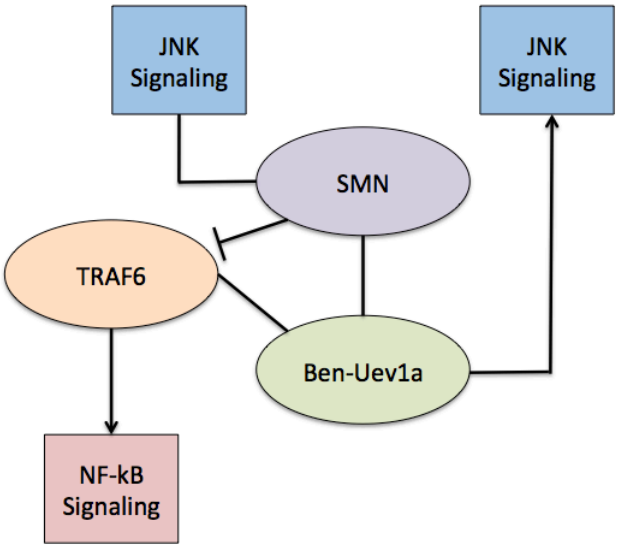


Figure 3.4. Diagram of the connections between SMN, TRAF6, and Ben-Uev1a proteins (Ovals) and JNK and NF-kB signaling pathways (Squares). Lines between proteins indicate they have been found in complex with one another. Lines connecting to signaling pathways show that the indicated proteins affect the activity of the cellular signaling pathway. In the cases where activating (arrows) or inhibitory (block line) effects are known, these are indicated.

CHAPTER IV: Conclusions and Future Directions

Molecular characterization of SMN function using SMA patient mutations

Several groups have established *Drosophila* as a model organism for studying SMA (Chan et al, 2003; Rajendra et al, 2007; Chang et al, 2008). *Smn* is an essential gene in flies and SMN's function in snRNP biogenesis is conserved between humans and flies. *Smn* null animals have locomotor defects suggestive of neuromuscular problems (Praveen et al. 2012). Characterization of SMA-causing point mutations in SMN (Praveen et al. 2014) has revealed they are similar to their human mutant counterparts on a molecular level. Flies carrying these mutations recapitulate the full range of phenotypic severity observed in SMA patients. Therefore, it is likely that pathological mechanisms that result in SMA are similar in flies and humans.

As a continuation of the preliminary molecular characterization conducted in Praveen et al. (2014), changes in the entire proteomes of the various SMA-causing point mutants of SMN could be analysed. Changes in protein levels that are common to all or most of the point mutations are most likely to be involved in SMA etiology, considering all of the point mutation result in the development of SMA in patients. Changes in protein levels that are specific to certain point mutations or certain categories of point mutations (ie. protein domain or disease severity) will also be interesting. These findings will be related to the separation-of-function discoveries that this allelic series was intended to uncover. Finally, changes in protein level may be analysed in relation to other phenotypes

of these flies such as locomotor ability. As is a caveat with all SMA-related studies, there may be tissue-specific changes in protein level that are not detected using this approach. Additionally, changes in protein localization within cells, particularly in polarized neurons, will also not be detected by analysing the whole proteome from whole animals.

In addition to examination of the entire proteome it is interesting to identify protein interactions with SMN. In this work, we purified wild-type SMN from Flag-Smn transgenic flies. This protein purification was then analysed using LC-MS/MS to identify the proteins that co-purified with SMN. These studies may be extended by purifying mutant complexes from transgenic flies and comparing and contrasting co-purifying proteins. We hypothesize that each of the point mutations may abrogate or reduce key interactions between SMN and unknown proteins. Similar to the whole proteome studies, SMN protein-protein interactions may be considered in terms of severity of the phenotype (severe, intermediate and mild) or location of the point mutation within the protein. As all mutations cause SMA in humans, changes in binding partners common to all the mutants, and that are also restored in the WT rescue flies, would be of interest in the context of SMA pathology. Changes in binding partners that are specific for certain point mutations or sets of point mutations would provide insight into the functions of SMN within the cell.

The previously described experiments examining the entire proteome or SMN binding partners in transgenic flies should be carried out at a timepoint in fly development near the onset of SMA-like symptoms rather than just before death. This approach is most likely to provide information about the molecular happenings that cause SMA rather than a readout of all the problems present in a dying organism. Another

interesting avenue would be to examine SMN protein interaction partners over the course of development in a whole organism. This may be accomplished by doing Flag immunopurifications from lysates of transgenic flies expressing wild-type Flag-SMN at multiple developmental stages. A catalog of SMN protein interaction partners throughout development would provide insight into the functions of SMN over time.

The most well-known function of the canonical SMN complex is its role in the assembly of snRNPs. The Flag-immunopurified protein lysates containing SMN complexes with mutant SMN could be used in snRNP assembly assays to determine the impact of each of the point mutations on the ability of the SMN complex to carry out snRNP assembly, as determined by the addition of Sm proteins to an snRNA. Since SMN is known to perform this snRNP assembly function in the cell, SMN may also be involved in biogenesis of RNPs other than splicing/spliceosomal snRNPs. Research in our lab has revealed that Sm proteins co-purify with specific mRNAs from *Drosophila* ovaries and S2 cells, presenting these mRNAs as potential candidates for assembly by SMN (Lu et al. 2014). The first step in this line of experimentation would be to determine whether wild-type SMN complexes are able to assemble each of these candidate RNPs. Following these experiments, it would be interesting to determine whether any or all of these RNP assembly functions were disrupted by point mutations in SMN.

The SMN complex and protein stability

In our preliminary isolations of the SMN complex from transgenic flies with wild-type Flag-SMN, we identified core members of the SMN complex as well as many other interacting partners. Previously, *Drosophila* were thought to have a minimal SMN complex consisting of only of SMN, Gemin2, Gemin3, and rig, which is thought to likely

be the Gemin5 homolog (Kroiss et al. 2008). As there is now evidence that Gemin4/6/7/8 are also present in *Drosophila*, any studies conducted using the fruit fly model system will be more directly relevant to the functions of the mammalian SMN complex than previously expected. We and others (Guruharsha et al. 2011; Lanfranco et al. 2017) have identified CG2941 as interacting with SMN. Further characterization has provided evidence pointing to its identity as a Gemin4 homolog in *Drosophila*. This additional characterization included demonstration of CG2941 genetic interaction with Gemin3 using CG2941 RNAi and a hypomorphic allele of Gemin3. Gemin3 has been suggested to play a central role in formation of the SMN complex and SMN protein levels in the cell.

Shpargel et al. (2009) reported a decrease in SMN levels in the absence of Gemin3 in *Drosophila*. Two SMA mutations in SMN (Y203C and G206S) that disrupt interaction with Gemin3 are known to destabilize the protein (Praveen et al. 2014). One possibility is that SMN degrades more quickly when it is unable to bind Gemin3. The Y203C and G206S mutations also display defects in self-oligomerization. This may mean that the instability of SMN is due to a lack of self-oligomerization. It is possible that proteins with these mutations fail to bind Gemin3 because of an inability to oligomerize. However, another SMN mutation (T205I) displays oligomerization defects, yet retains the ability to bind Gemin3. Thus, it is unlikely that interaction between Gemin3 and SMN is dependent on SMN oligomerization. In addition, experiments with the human counterpart of the Y203C mutation (Y272C) show that its failure to interact with Gemin3 is independent of its ability to oligomerize (Charroux et al, 1999).

Although Gemin3 binds SMN independently of SMN oligomerization status, Gemin3 may influence higher order oligomerization of the SMN complex. If this is the case, there must also be other determinants, such as the activity of E3 ubiquitin ligases, since overexpression of YFP-SMN in a Gemin3 mutant background in *Drosophila* restores endogenous SMN levels (Shpargel et al, 2009). This suggests that the presence of additional exogenous SMN has a protective effect against the degradation of endogenous SMN, similar to the finding in Chapter II where over expression of GFP-SMN in human cells protected endogenous SMN.

An investigation by Burnett et al. (2009) into the stability of SMN found a strong correlation between SMN stability and its incorporation into complexes. SMN that is unable to oligomerize has a shorter half-life than SMN that enters into a complex (Burnett et al., 2009). In agreement with the *Drosophila* studies described above, these investigators demonstrated that the oligomerization deficient human SMN mutations, Y272C, G279V and SMNΔ7, had significantly shorter half-lives than wild-type SMN. The experiment was conducted using human cell-based pulse-chase assays. Both Y272C and SMNΔ7 are known to have defects in binding Gemin3 (Charroux et al, 1999). These findings continue to support the idea that the defect in binding Gemin3 could be contributing to the instability of the Y272C and SMNΔ7 mutants in human cells. These studies together suggest that Gemin3 binds to SMN independent of SMN oligomerization status, but may still play a role in SMN stability. These findings leave open the possibility that defects in self-oligomerization of SMN contribute to protein stability as well.

Further characterization of the oligomerization defects using *in vitro* assays, and determination of the stability of the Y203C, G206S and T205I mutant proteins in a quantitative manner will help provide information about the two mechanisms proposed above. We can test whether Gemin3 plays a role in SMN oligomerization by determining whether SMN forms higher order complexes in the absence of Gemin3. Gemin3 can be knocked down in S2 cells and analytical ultracentrifugation can be used to obtain the profile of SMN complexes, and thus provide a measure of oligomerization state. A shift of the sedimentation profile to a lower fraction when compared to the control would indicate that SMN from Gemin3 knockdown cells is not incorporated into oligomeric complexes as well. This would suggest that Gemin3 impacts the oligomerization of SMN. A potential problem with this approach is that SMN is co-depleted when Gemin3 is knocked down. Since SMN is ubiquitylated and degraded via the proteasome (Burnett et al, 2009; Chapter II), the analytical ultracentrifugation assay can be performed in the presence of a proteasome inhibitor, such as MG132, to stabilize SMN levels. Potential mechanisms for Gemin3 influencing the stability of SMN are by stabilizing the association of Gemin2 and SMN or by contributing to masking of the Slmb degron (Chapter II) or another unknown degron in SMN.

We have shown in Chapter II that SMN self-oligomerization affects the stability of SMN by hiding the Slmb degron that is located in the YG-box self-oligomerization domain of SMN. Further investigations of this topic may include a more detailed analysis of the precise oligomeric states of SMN where the Slmb degron is accessible and those where it is not. From our findings it is unknown whether SMN dimers are sufficient to bury the degron and prevent ubiquitylation or whether higher order oligomers of the

SMN complex are required to fully protect the protein from SCF^{Slmb} degradation. Additionally, studies regarding the role of other members of the SMN complex, such as Gemin3, in SMN ubiquitylation and degradation by SCF^{Slmb} have not been conducted. The previous findings about the role of Gemin3 related to SMN stability present the possibility that Gemin3 is involved in regulating the accessibility of an SMN degron. This may be the Slmb degron as discussed in Chapter II, or another degron with an unidentified E3 ubiquitin ligase.

One of the likely regulators of SCF^{Slmb} interaction with the Slmb degron is the phosphorylation status of the degron. As Slmb is known to recognize phospho-degrons, identifying the kinase(s) responsible for phosphorylating the degron in SMN is an important and logical next step (Fig 4.1A). A top candidate is GSK3b (Liu et al. 2007; Lee et al. 2013). This kinase recognizes a motif (SxxxS/T) that includes the degron and extends N-terminally (^{262}SxxxSxxxSxxxT^{274}, numbering as per human SMN) (Fig. 4.1B). We identified the *Drosophila* GSK3b ortholog, Shaggy (Sgg), in our SMN pulldowns from fly embryonic lysates lending further support to the role of GSK3β in SMN phosphorylation (Chapter II). GSK3b-specific inhibitors and siRNA-mediated knockdown of GSK3b have been shown to increase SMN levels (Makhortova et al. 2011; Chen et al. 2012). These findings plausibly connect SMN phosphorylation to downstream protein degradation pathways. Finally, GSK3b is responsible for phosphorylation of a degron in beta-catenin, one of the most well-characterized SCF^{Slmb} substrates (Liu et al. 2002). This indicates the presence of a functional GSK3β kinase motif in established SCF^{Slmb} substrates.

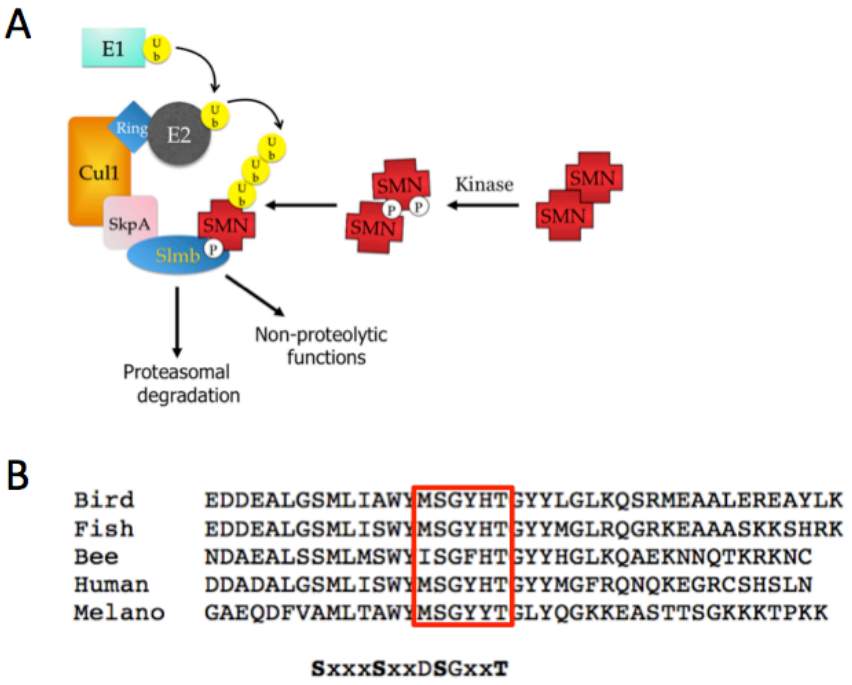


Figure 4.1. GSK3β is a candidate kinase for phosphorylation of the Slmb degron. **A.** SMN is degraded via the ubiquitin proteasome system. Slmb is the substrate recognition component of the SCF^{Slmb} E3 ubiquitin ligase. The Slmb degron in all other known substrates is a phospho-degron. Preliminary evidence suggests that GSK3β is the kinase targeting SMN. **B.** GSK3β has a known target sequence in the proteins it phosphorylates. This amino acid sequence is found in the C-terminal YG Box of SMN and includes the Slmb degron (red box) and amino acids N-terminally of that sequence.

The interplay between SMN oligomerization status and the presence of phosphorylation on the serine and threonine residues in the Slmb degron motif is not currently understood. One possibility is that if SMN is unable to interact with itself, the degron is open for kinases to phosphorylate the critical residues leading to interaction with SCF^{Slmb}. Alternatively, and not mutually exclusively, the kinase may be able to phosphorylate the residues even when SMN is in a complex. In this case, the phosphorylation of the residues may lead to breakdown of the complex and subsequent degradation of SMN. Definitive identification of the kinase and phosphorylation of the predicted amino acid residues are the obvious next steps of the line of experimentation.

One approach would be to knockdown GSK3β in S2 cells using RNAi and harvest protein lysate using buffer that includes phosphatase inhibitors. This protein could be analyzed using Western blots to generally detect SMN phosphorylation and mass spectrometry to detect specific amino acids that are phosphorylated in controls and no longer phosphorylated following GSK3β knockdown. A potential pitfall of these experiments is that the C-terminus of SMN is not typically detected well using mass spectrometry; however, it is likely that procedures could be optimized for detection of these residues. Detection of the C-terminus of SMN using mass spectrometry would also contribute to knowledge of which lysines are ubiquitylated based on identification of ubiquitin conjugation sites.

Ubiquitylation of SMN and endocytosis

The process of protein ubiquitylation involves three main steps (Petroski 2008). First, an E1 protein activates ubiquitin and transfers it to the E2 enzyme. E2 proteins conjugate ubiquitin to their substrates. E3 proteins recognize the substrate and assist in the transfer of ubiquitin from the E2. In chapter II, we identified SMN as a novel substrate of the E3 ubiquitin ligase SCF^{Slmb}. Future exploration of the entire process that results in SMN ubiquitylation includes determination of the E2 proteins that partner with SCF^{Slmb}. One of the known E2 proteins to work with SCF E3 ligases is UbcD1 (Bocca et al. 2001). Adding to the list of known functional E2 partners of SCF^{Slmb} would contribute both to knowledge of SMN biology, as well as the more general biology of the ubiquitin proteasome system.

Bendless (Ben) is a candidate E2 for being involved in SMN function, as suggested in Chapter III. We detected Ben interaction with SMN in embryonic lysates

and verified this interaction using Ben-Flag overexpression and immunoprecipitation in S2 cells. Ben has been shown to physically interact with TRAF6, an E3 ligase, in human cells (Kim and Choi 2017). The possibility remains that Ben works with other E3 ubiquitin ligases as well. We did not detect an interaction of Ben with any of the components of SCF^{Slmb} in S2 cells (data not shown), but detection of physical interaction between E2 and E3 proteins is not always possible, even when they are known to work together. One way to determine the ability of Ben to work as an E2 with SCF^{Slmb} would be to use *in vitro* ubiquitylation assays. These assays could compare the ubiquitylation ability of the E2 known to work with SCF^{Slmb} (UbcD1) with that of Ben. This experiment is complicated due to the complex nature of SCF^{Slmb} as a multi-protein E3 ligase, especially since Cul1 is activated by neddylation. Nevertheless, such an experiment would provide clear evidence for the ability of SCF^{Slmb} to work with Ben (or other candidates) to ubiquitylate substrates.

While ubiquitylation is most well-known in terms of protein degradation via the ubiquitin proteasome system, ubiquitylation does not always result in immediate destruction of the target. Linking ubiquitin molecules using different lysine residues and the presence of different chain lengths can alter a protein's fate (Mukhopadhyay and Riezman 2007; Ikeda and Dikic 2008; Liu and Walters 2010). Ubiquitin linkage specificity is determined by the E2 (Ye and Rape 2009). Lysine 48 (K48) linked chains typically result in degradation of the targeted protein by the 26S proteasome, whereas lysine 63 (K63) linkage is generally associated with lysosomal degradation and nonproteolytic functions such as endocytosis (Tan et al. 2008; Kirkin et al. 2009; Lim and Lim 2011). Ben heterodimerizes with Uev1a to form K63 ubiquitin linkages on target

proteins (Ye and Rape 2009; van Wijk and Timmers 2010; Komander and Rape 2012; Marblestone et al. 2013; Zhang et al. 2013). Uev1a alters protein conformations to ensure ubiquitin linkage occurs only at K63. We also detected Uev1a in our Flag-SMN purifications from embryonic lysates, providing further evidence that SMN truly interacts with the Ben-Uev1a heterodimer in the whole organism. Considering K63 ubiquitin linkages are associated with functions separate from the proteasome, such as endocytosis, it is interesting that recent work has implicated defects in endocytosis in SMA (Custer and Androphy 2014; Dimitriadi et al. 2016; Hosseinibarkooie et al. 2016).

These defects in endocytosis may be downstream effects of disruptions of SMN related to the ubiquitin proteasome system and SMN interactions with actin binding proteins. Additionally, these same disruptions directly connected to the loss of SMN may lead to defects in autophagy. The interconnection between autophagy and endocytosis is well-established (Barth and Kohler 2014). Autophagy and endocytosis share the same effector molecules and affect one another. Abnormal autophagy is a common feature of neurodegenerative diseases (Custer and Androphy 2014). Specifically in spinal muscular atrophy, there appears to be an accumulation of autophagosomes and their associated cargo in multiple different cell types. Dimitriadi et al. (2016) determined that low levels of SMN lead to defects in endosomal trafficking. This is believed to have the downstream consequence of impairing synaptic function, potentially making endocytic trafficking a main determinant of SMA disease pathology. Hosseinibarkooie et al. (2016) has conducted preliminary investigations of the molecular mechanisms underlying this endocytic defect in SMA (Fig. 4.2). Reduced endocytosis caused by low levels of SMN protein was rescued by overexpression of PLS3, a known protective modifier of SMA

(Oprea et al. 2008). PLS3 is an F-actin binding protein (Delanote et al. 2005) and using proteomic and biochemical techniques Hosseinibarkooie et al. (2016) identified another F-actin binding protein, CORO1C that also modifies the endocytic defects. It remains to be determined how the ubiquitylation status of SMN might intersect with endocytic functions.

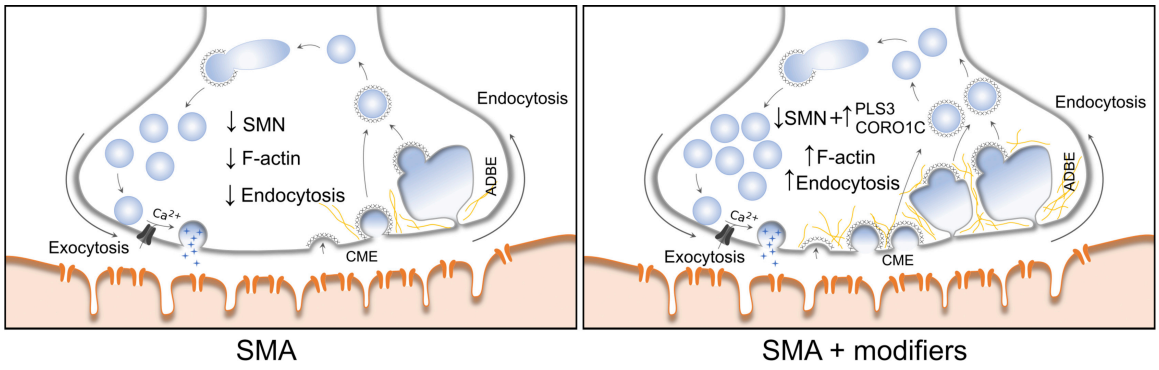


Figure 4.2. Increasing levels of PLS3 and CORO1C result in increased levels of F-actin and improved endocytic function. Endocytic function includes clathrin-mediated endocytosis (CME) and activity-dependent bulk endocytosis (ADBE). Figure is taken from Hosseinibarkooie et al, 2016.

Additional functions of SMN

A number of alternative functions for SMN in addition to its role in endocytosis have been put forward. Several lines of evidence have revealed connections between SMN and proteins involved in actin homeostasis and function (Rossoll et al, 2003; Shafey et al, 2005; Rajendra et al, 2007; Walker et al, 2008; Bowerman et al, 2009; Oprea et al, 2008). This potential role for SMN in actin cytoskeletal dynamics is closely related to its role in endocytosis. Numerous studies have suggested particular functions for SMN at the neuromuscular junction (NMJ) in mice (Kariya et al., 2008; Murray et al., 2008; Kong et al., 2009; Michaud et al., 2010; Voigt et al., 2010; Dachs et al., 2011), however the extent of NMJ defects in *Drosophila Smn* mutants is unclear. Chan et al

(2003) studied *Smn* null mutants and observed an enlargement of boutons at the larval NMJ with no difference in bouton number. In contrast, Chang et al (2008) reported a decrease in bouton number in *Smn* null flies as well as high levels of SMN at the wild-type NMJs. In other studies in the fly, nothing more than a faint accumulation of SMN at the NMJ has been detected (Matera lab, unpublished). One explanation for these discrepancies is that the NMJ defects observed by Chang et al (2008) are the result of a second-site mutation, since other *Smn* null alleles in the fly do not show NMJ defects (B.McCabe, personal communication). Several groups have confirmed the presence of NMJ defects in SMA mouse models, making the role of SMN at the NMJ in humans uncertain.

Information regarding SMN protein-protein interactions may be used to inform previously unknown functions of SMN or to provide further evidence for suggested roles of SMN. Our detection of SMN interaction with nucleosome assembly protein 1 (Nap1) brings forth the consideration of the interplay between SMN and chromatin, as Nap1 has its most well-established role in chromatin remodelling. SMN has been suggested to have the ability to bind chromatin, specifically interacting with methylated histone H3K79 (Sabra et al. 2013). SMN interaction with this chromatin mark was shown to affect SMN localization to damaged centromeres. Multiple methods were used to demonstrate interaction of SMN with mono- and di-methylated H3K79, although our lab was unable to recapitulate these results (unpublished). This may be due to a variety of reasons, including the use of different histone peptides in the *in vitro* pulldown assays.

H3K79 methylation is known to promote transcription by enhancing transcription elongation (Veloso et al. 2014). Several lines of evidence suggest the involvement of

SMN in transcriptional regulation. Specific and general transcription factors have been shown to concentrate in nuclear Cajal bodies, which include SMN (Carvalho et al. 1999). Additionally, this concentration has been shown to be physiologically regulated, indicating the potential for functional significance. Transient associations of Cajal bodies with chromatin may be dependent on active transcription at that chromosomal locus (Platani et al. 2002). Further evidence supporting the role of SMN in transcriptional regulation was provided when Zhao et al. (2015) demonstrated that SMN binds symmetric dimethylation of an arginine residue (R1810) of RNA polymerase II C-terminal domain. SMN also interacts with Senataxin (SETX), a helicase that unwinds R-loops around transcription termination sites (Suraweera et al. 2009), contributing to transcription termination via XRN2 exonuclease recruitment (Skourti-Stathaki et al. 2011). It remains an open question as to whether SMN and Nap1 function together in any of these contexts involving chromatin and transcriptional regulation or whether there is simply overlap in their functions. The verification of their interaction provides impetus to further investigate this open area of research.

Role of SMN as a signaling hub

A possible explanation for the numerous purported functions of SMN is that SMN works to connect different aspects of cellular signaling, both within and between distinct cellular processes. For example, SMN has been shown in complex with E1 (UBA1, Wishart et al. 2014), E2 (Ben, Chapter III), and E3 (SCF^{Slmb}, Chapter II; TRAF6, Kim and Choi 2017) proteins in the UPS. Perhaps in addition to being degraded via the UPS, SMN is in-turn regulating protein degradation within the cell.

SMN may not only be interacting with different proteins in the UPS, but may also be a key component in connecting the UPS with other cellular signaling pathways. The E2, Ben, is involved in the activation of both JNK and IMD signaling in *Drosophila* (Paquette et al. 2010; Zhou et al. 2005). Additionally, SMN interaction with TRAF6 is thought to inhibit the ability of TRAF6 to activate NF-kB signaling (Kim and Choi 2017). Mutations in SCF^{Slmb} proteins lead to constitutive expression of antimicrobial peptides, which are also downstream of the IMD pathway (Khush et al. 2002). The UPS, JNK signaling, and the IMD pathway have all been shown to be disrupted in SMA. This places SMN in a central role, potentially coordinating the activity of numerous processes within the cell.

Summary

The ultimate goal of SMA research is to discover which functions of SMN are most relevant to the disease in order to develop the most effective treatments. This discovery is important not only for SMA therapy, but also for understanding fundamental concepts in RNA biology and neuromuscular development. Previous research conducted in our lab has strongly suggested that the etiology of SMA lies in functions of SMN that are independent of snRNP biogenesis. To explore alternative functions and mechanisms of regulation of SMN we purified SMN complexes from intact, developing *Drosophila melanogaster* embryos.

Using this approach, we have identified conserved factors that regulate SMN stability. This study demonstrated that the SCF^{Slmb} E3 ligase complex interacts with a degron embedded within the self-oligomerization domain of SMN. Our findings suggest

a molecular model wherein accessibility of the SMN degron is regulated by self-multimerization, providing an elegant mechanism for balancing functional activity with degradation. Additionally, we verified three novel protein interaction partners of SMN: CG2941, nucleosome assembly protein 1 (Nap1), and Bendless (Ben). Further biochemical and genetic studies related to these and other candidate proteins will provide insight into the physiological pathways that give rise to SMA.

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