Self-oligomerization regulates stability of Survival Motor Neuron (SMN) protein isoforms by sequestering an SCFSImb degron

Gray, Kelsey M.; Kaifer, Kevin A.; Baillat, David; Wen, Ying; Bonacci, Thomas R.; Ebert, Allison D.; Raimer, Amanda C.; Spring, Ashlyn M.; Have, Sara ten; Glascock, Jacqueline J.; Gupta, Kushol; Van Duyne, Gregory D.; Emanuele, Michael J.; Lamond, Angus I.; Wagner, Eric J.; Lorson, Christian L.; Matera, A. Gregory

Published in:
Molecular Biology of the Cell

DOI:
10.1091/mbc.E17-11-0627

Publication date:
2018

Publisher Version
Published in the public portal

Link to publication in Discovery Research Portal

Citation for published version (APA):
Self-oligomerization regulates stability of survival motor neuron protein isoforms by sequestering an SCF$^{\text{Slmb}}$ degron

Kelsey M. Gray$^{a,b}$, Kevin A. Kaifer$^c$, David Baillat$^d$, Ying Wen$^b$, Thomas R. Bonacci$^{a,e}$, Allison D. Ebert$^f$, Amanda C. Raimer$^{a,b}$, Ashlyn M. Spring$^b$, Sara ten Have$^g$, Jacqueline J. Glascock$^c$, Kushol Gupta$^h$, Gregory D. Van Duyne$^h$, Michael J. Emanuele$^{a,e}$, Angus I. Lamond$^b$, Eric J. Wagner$^d$, Christian L. Lorson$^c$,* and A. Gregory Matera$^{a,b,*}$

$^a$Curriculum in Genetics and Molecular Biology and Lineberger Comprehensive Cancer Center, $^b$Integrative Program in Biological and Genome Sciences, Department of Biology and Department of Genetics, and $^c$Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27599; $^d$Molecular Pathogenesis and Therapeutics Program, Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri, Columbia, MO 65211; $^e$Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX 77550; $^f$Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, WI 53226; $^g$Centre for Gene Regulation and Expression, School of Life Sciences, University of Dundee, Dundee DD15EH, UK; $^h$Department of Biochemistry and Biophysics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104

ABSTRACT Spinal muscular atrophy (SMA) is caused by homozygous mutations in human SMN1. Expression of a duplicate gene (SMN2) primarily results in skipping of exon 7 and production of an unstable protein isoform, SMNΔ7. Although SMN2 exon skipping is the principal contributor to SMA severity, mechanisms governing stability of survival motor neuron (SMN) isoforms are poorly understood. We used a Drosophila model system and label-free proteomics to identify the SCF$^{\text{Slmb}}$ ubiquitin E3 ligase complex as a novel SMN binding partner. SCF$^{\text{Slmb}}$ interacts with a phosphor degron embedded within the human and fruitfly SMN YG-box oligomerization domains. Substitution of a conserved serine (S270A) interferes with SCF$^{\text{Slmb}}$ binding and stabilizes SMNΔ7. SMA-causing missense mutations that block multimerization of full-length SMN are also stabilized in the degron mutant background. Overexpression of SMNΔ7$^{S270A}$, but not wild-type (WT) SMNΔ7, provides a protective effect in SMA model mice and human motor neuron cell culture systems. Our findings support a model wherein the degron is exposed when SMN is monomeric and sequestered when SMN forms higher-order multimers.

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 6 mo of life and rarely live past 2 yr (Prior, 2010; Wee et al., 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). Humans have two SMN paralogues, named SMN1 and SMN2, both of which contribute to total cellular levels of survival motor neuron (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 (Coover et al., 1997; Lefebvre et al., 1997).

Whereas a causative link between SMN1 and SMA was established in the early 1990s, the molecular role of SMN in disease etiology remains unclear. SMN is the central component of a multifactorial protein assembly known as the SMN complex (Li et al., 2014; Matera and Wang, 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 mRNA (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 neuro-muscular 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 (Ramsen 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 fly 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 SCFSnb E3 ubiquitin ligase complex as a novel SMN interactor. Importantly, this interaction is conserved from flies to humans. We show that SCFSnb binding requires a phosphodegron 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 (Hsu et al., 2010; Kwon et al., 2013; Han et al., 2016). Given our findings in fruit-fly embryos, SMN is likely targeted by multiple E3 ubiquitin ligases.

RESULTS

Flag-SMN interacts with 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 under Materials and Methods, we intercrossed hemizygous Flag-SmnWT, Smn1/2/Smn2 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-SmnWT 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 (0–12 h) embryos from Flag-SmnWT/WT, Smn1X/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 seven of the canonical Sm-core snRNP proteins (Figure 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 copurify with SMN in S2 cells (Kroiss et al., 2008; Cauchi et al., 2010; Gunarathna 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 Figure 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 copurified numerous factors that are part of the ubiquitin proteasome system (UPS; Figure 1C). Among these UPS proteins, we identified Cullin 1 (Cul1), Skp1-related A (SkpA), and supernumerary limbs (Slmb), as being highly enriched
Slmb mediates degradation of SMN

<table>
<thead>
<tr>
<th>Known SMN Interactors</th>
<th>Protein</th>
<th>Gene #</th>
<th>Category</th>
<th>Ctrl (pS/P)</th>
<th>SMN (pS/P)</th>
<th>Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMN</td>
<td>CG16725</td>
<td>UPS, SCF E3</td>
<td>3.8</td>
<td>1455.2</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Gem3</td>
<td>CG66339</td>
<td>UPS, SCF E3</td>
<td>3.1</td>
<td>1052.2</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Gem5</td>
<td>CG10419</td>
<td>UPS, SCF E3</td>
<td>0.0</td>
<td>372.6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>SmD3</td>
<td>CG30149</td>
<td>UPS, SCF E3</td>
<td>0.0</td>
<td>96.5</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>SmI</td>
<td>CG18591</td>
<td>Sm protein</td>
<td>0.9</td>
<td>439.8</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>SmD2</td>
<td>CG9762</td>
<td>Sm protein</td>
<td>0.4</td>
<td>364.5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>SmD3</td>
<td>CG1249</td>
<td>Sm protein</td>
<td>0.0</td>
<td>303.4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>SmI</td>
<td>CG16792</td>
<td>Sm protein</td>
<td>0.0</td>
<td>88.9</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>EmI</td>
<td>CG1244</td>
<td>Sm protein</td>
<td>0.0</td>
<td>47.3</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>EmD3</td>
<td>CG8427</td>
<td>Sm protein</td>
<td>0.3</td>
<td>31.1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>SmI</td>
<td>CG13073</td>
<td>Sm protein</td>
<td>0.0</td>
<td>20.0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>SmI</td>
<td>CG0392</td>
<td>Sm protein</td>
<td>0.0</td>
<td>13.3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>SmD3</td>
<td>CG12938</td>
<td>Sm protein</td>
<td>0.0</td>
<td>11.2</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 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 with 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. (>

To test the functional consequences of this conserved interaction between SMN and SCFSlmb/B-TrCP, a cell-based ubiquitylation assay
FIGURE 2: Conserved interaction between SMN and the SCF^{Slmb/B-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-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. Copurification of each of the SCF components with endogenous SMN was detected. (C) An in vitro binding assay tested direct interaction between human SMN\Delta5-Gemin2 (SMN\Delta Gem2) (Martin et al., 2012; Gupta et al., 2015) and purified GST-tagged proteins. SMN\Delta 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 homologues 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.
was performed (Figure 2F). Protein lysate from HEK 293T cells transfected with 6xHis-Flag-ubiquitin and GFP-SMN was purified using a Ni²⁺ 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 (Figure 2F). Ubiquitylation levels were further increased following addition of both proteins together.

These experiments demonstrate that SCF<sub>Slmb/B-TrCP</sub> can ubiquitylate SMN in vivo.

**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 [AQ 3] levels (Figure 3A). Following Slmb RNAi, endogenous SMN levels were modestly increased as compared with 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 Figure 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), an inhibitor of protein synthesis, following Slmb depletion following a 3-d 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 specifically 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 h post–CHX treatment. At 6 h post–CHX treatment, there is a modest increase in full-length SMN levels following Slmb RNAi as compared with the initial time point (0 h) and as compared with 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 (Fuchs et al., 2004; Jin et al., 2005; Frescas and Pagano, 2008). There are also several known variants of this motif, for example, DDGFVD, SSGYFS, and TSGCSS (Kim et al., 2015). As shown in Figure 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 (264MSGYHT-GYYMEMLA282) that was thought to be created in SMNΔ7 by SMN2 alternative splicing (Cho and Dreyfuss, 2010). As shown in Figure 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 (264MSGYHT-GYYMEMLA282) that was thought to be created in SMNΔ7 by SMN2 alternative splicing (Cho and Dreyfuss, 2010).
serine-to-alanine mutation were generated using the vSmn backbone, including both the full-length (e.g., vSmnG206S) and truncated (e.g., vSmnA7A) versions of the protein (Figure 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 Figure 4A. The S201A mutation was created in this construct as well (MGLRQ*G206S). To mimic a constitutively phosphorylated state, we also introduced serine to aspartate mutations, vSmnG206D and vSmnΔ7D. We transfected each of these constructs, Flag-tagged and driven by the native vSmn promoter, into S2 cells and measured protein levels by Western blotting (Figure 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 (Supplemental Figure S2). As shown, the vSmnG206S and vSmnΔ7 constructs exhibited increased protein levels compared with their serine containing counterparts, whereas levels of the S201D mutants were reduced, suggesting that the phosphodegron 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 Flag-vSmn, vSmnΔ7A, and Flag-vSmnS201A were created. Here again, we observed that the S201A mutation increased protein levels of both full-length SMN and SMNΔ7 (Supplemental Figure S3).

serine-to-alanine mutation was transfected in Drosophila S2 cells. The constructs containing the serine to alanine mutation are as follows: SmnG206C→SmnG206A, SmnG206S→SmnG206A, SmnG206D→SmnG206A, SmnG206E→SmnG206A, and SmnG206V→SmnG206A. The serine to alanine mutation has a stabilizing effect on SMN mutants with poor self-oligomerization capability. *p < 0.05, n = 3.

**Normalized fold change as compared with vSmn levels is indicated at the bottom. *p < 0.01, ns = not significant.**
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 (Figure 5, A–C). As described previously (Praveen et al., 2014), SmnG206S 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 (SmnG206S/S201A) express markedly increased levels of SMN protein (Figure 5C), and a good fraction of these animals complete development (Figure 5A). Moreover, SmnG206S/S201A larvae display significantly improved locomotor activity as compared with SmnG206S or SmnG206S/S201A null mutants (Figure 5B).

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 (unpublished data), 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 cotransfected the appropriate Flag-Smn constructs with Myc-Slmb in S2 cells. Protein lysates were then Flag-immunoprecipitated and probed with anti-Myc antibody (Figure 4C). The S201A mutation decreased binding of Slmb to both the full-length and the truncated SMN isoforms (Figure 4C). However, the vSmnΔ7S construct coprecipitated the greatest amount of Slmb protein, despite the fact that it is present at much lower levels in the input lysate (Figure 4C). Because SMNA7 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 (Figure 4D and Supplemental Figure S2). The S201A degron mutation has a clear stabilizing effect on the G206S and Y203C constructs, as compared with 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.
These results strongly suggest that both the structure of the G206S mutant protein as well as its instability contribute to the organisomal phenotype.

**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 in the early 2000s (Hsieh-Li et al., 2000; Monani 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 (postnatal 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 ubiquitination machinery and stabilizing endogenous SMN. In such a scenario, the prediction would be that SMNΔ7 is less protective than SMNΔ7S because it is not a very good substrate for SCFSIMb.

We therefore compared the stabilizing effects of overexpressing GFP-tagged SMNΔ7S/Δ7A (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 10 h. As shown in Figure 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 (Figure 6A, table). We then calculated a “stabilization factor” by taking a ratio of these two ratios. As shown (Figure 6A, graph), the protective benefit of overexpressing Δ7S versus Δ7A at t = 0 h 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 SMNΔ7A is a relatively good SCFSIMb 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 control-induced pluripotent stem cell (iPSC) motor neuron cultures were transduced with lentiviral vectors expressing an mCherry control protein or SMNΔ7A (Figure 6B). At 4 wk postdifferentiation, no statistical difference was observed between control and SMA motor neurons; however, by 6 wk, SMA motor neuron numbers had decreased significantly to ~7% of the total cell population (Figure 6B). In contrast, expression of SMNΔ7A maintained motor neuron numbers to approximately the same level as the controls and nearly twofold greater than untreated cells (Figure 6B). Thus expression of SMNΔ7A improves survival of human iPSCs when differentiated into motor neuron lineages.

**SMNΔ7A is a protective modifier of intermediate SMA mouse phenotypes**

To examine the importance of the Smb degron in a mammalian organisomal system, we previously developed SMA mouse models were utilized. As mentioned above, the “Delta7” mouse (SmnΔ7/Δ7;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 (SmnΔ2B/-) is a model of intermediate SMA (Bowerman et al., 2012; Rindt et al., 2015) and these animals survive much longer, 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 d, similar to the results obtained with the full-length AAV9-SMN construct (Figure 7A). In contrast, untreated 2B/- animals lived, on average, only 30 d. Mice treated with AAV9-SMNΔ7S survived an average of 45 d (Figure 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 (Figure 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 (Supplemental Figure 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 (Supplemental Figure 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 life-span 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 (Figure 7B). Treatment with full-length SMN cDNA resulted in animals that were clearly stronger and more mobile, consistent with the weight data (Figure 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 (Figure 7C). Animals treated with AAV9-SMNΔ7D and -SMNΔ7S did not survive long enough for testing.

**SCFSIMb primarily targets unstable SMN monomers**

As indicated in Figure 8, our findings suggest a model whereby SMN and SMNΔ7 degradation is in part mediated by SCFSIMb, a multicomponent E3 ubiquitin ligase composed of Slmb, SkpA, Cul1, and Roc1 (Jiang and Struhl, 1998, Patton et al., 1998a,b; Zheng et al., 2002). Our work demonstrates that B-TRCP/Slmb binds directly to SMN (Figure 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.

**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 copurify 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$^\text{Slmb}$ 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 SMN$\Delta^7$S$^{S270A}$, but not SMN$\Delta^7$ or SMN$\Delta^7^T^D^O$, a phosphomimetic, is a protective modifier of SMA phenotypes in animal models and human iPSC cultures.

The SCF$^\text{Slmb}$ degron is exposed by SMN$^2$ exon skipping

A previous study posited that a phosphodegron 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 SMN$\Delta^7$. Notably, the degron mutation has a greater effect on SMN levels (both full-length and $\Delta^7$) 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 SMN$^2$ 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 (Hsu et al., 2010; Kwon et al., 2013; Han et al., 2016). 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 time frames. 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<sub>Smn</sub> 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). Lyssine 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., 2002; Kirkin et al., 2009; Lin and Lin, 2010). Interestingly, recent work has implicated defects in endocytosis in SMA (Custer and Androphy, 2014; Dimitriadi et al., 2016; Hosseinibar-kooie 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 pull down, we identified three E2 proteins as potential SMN interacting partners (Figure 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-κB 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, 2010). Interestingly, recent work has implicated defects in endocytosis in SMA signaling in *Drosophila* (Zhou et al., 2005; Paquette et al., 2010). Previously, we and others have shown that JNK signaling is dysregulated in animal models of SMA (Garcia et al., 2013, 2016; Genabai et al., 2015; Ahmad et al., 2016). Moreover, mutations in all three components of SCF<sub>Smn</sub> 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 GSK3β (Figure 8), as this kinase recognizes a motif (Sxx(S/T); Liu et al., 2007; Lee et al., 2013) that includes the degron and extends N-terminally to the MGLR motif, numbering as per human SMN. In support of this hypothesis, we identified the Drosophila GSK3β orthologue, Shaggy (Sgg), in our SMN pull downs (Figure 1C). Moreover, GSK3β inhibitors as well as siRNA-mediated knockdown of GSK3β were shown to increase SMN levels, primarily by stabilizing the protein (Makhortova et al., 2011; Chen et al., 2012). Finally, GSK3β is also responsible for phosphorylation of a degron in β-catenin, a well-characterized SCF<sup>E3</sup><sub>Smnb</sub> substrate (Liu et al., 2002).

SMA mice have low levels of UBA1 (E1), ultimately leading to accumulation of β-catenin (Wishart et al., 2014). Pharmacological inhibition of β-catenin improved neuromuscular pathology in Drosophila, zebrafish, and mouse SMA models. β-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). β-Catenin also regulates motor neuron differentiation by retrograde signaling from skeletal muscle (Li et al., 2008). The connections of UBA1 and multiple SCF<sup>E3</sup><sub>Smnb</sub> 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<sup>E3</sup><sub>Smnb</sub> 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 (Figure 8) whereby accessibility of the SMN phosphodegron is regulated by self-multimerization, providing an elegant mechanism for balancing functional activity with degradation.

MATERIALS AND METHODS

Fly stocks and transgenes

Oregon-R was used as the wild-type control. The Smn<sup>−/−</sup> microdeletion allele (Chang et al., 2008) was a gift from S. Artavanis-Tsakonis (Harvard University, Cambridge, MA). 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 (Chino Hills, CA) as described in Praveen et al., 2014. In short, a ~3 kb 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 QS (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 fruit fly 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

Drosophila embryos (0–12 h) were collected from Oregon-R control and Flag-SMN flies, dechorionated, flash frozen, and stored at –80°C. Embryos (approximately 150) were then homogenized on ice (AQ 9) with a Potter tissue grinder in 5 ml of lysis buffer containing 100 mM potassium acetate, 30 mM HEPES–KOH at pH 7.4, 2 mM magnesium acetate, 5 mM dithiothreitol (DTT), and protease inhibitor cocktail. Lysates were centrifuged twice at 20,000 rpm for 20 min at 4°C and dialyzed for 5 h at 4°C in Buffer D (HEPES 20 mM, pH 7.9, 100 mM KCl, 2.5 mM MgCl<sub>2</sub>, 20% glycerol, 0.5 mM DTT, PMSF [AQ 10] 0.2 mM). Lysates were clarified again by centrifugation at 20,000 rpm for 20 min at 4°C. Lysates were flash frozen using liquid nitrogen and stored at –80°C before use. Lysates were then thawed on ice, centrifuged at 20,000 rpm for 20 min at 4°C and incubated with rotation with 100 μl of EZview Red Anti-FLAG M2 affinity gel (Sigma) for 2 h at 4°C. Beads were washed a total of six times using buffer with KCl concentrations ranging from 100 to 250 mM with rotation for 1 min at 4°C in between each wash. Finally, Flag proteins were eluted 3 consecutive times with one bed volume of elution buffer (Tris 20 mM, pH 8, 100 mM KCl, 10% glycerol, 0.5 mM DTT, PMSF 0.2 mM) containing 250 μg/ml 3XFLAG peptide (sigma). The entire eluate was used for mass spectrometry analysis on an Orbitrap Velos instrument, fitted with a Thermo Easy-spray 50-cm 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 (Life Technologies) supplemented with 1% penicillin/streptomycin and filter sterilized. Cells were removed from the flask using a cell scraper and passedag to maintain a density of ~10<sup>5</sup>–10<sup>6</sup> cells/ml. S2 cells were transfected to filter sterilized SF900 SFM (Life Technologies) without antibiotics prior to transfection with Cellflectin II (Invitrogen). Transfections were performed according to Cellflectin II protocol in a final volume of 4 ml in a T-25 flask containing 10<sup>5</sup> cells that were plated 1 h before transfection. The total amount of DNA used in transfections was 2.5 μg. Human embryonic kidney HEK-293T and HeLa cells were maintained at 37°C with 5% CO<sub>2</sub> in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies). Cells (1 × 10<sup>5</sup>–2 × 10<sup>6</sup>) were plated in T-25 flasks and transiently transfected with 1–2 μg 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 transfections, HeLa cells were plated subconfluently in T-25 flasks and transfected with 10 nm of siRNA (gift from Mike Emanuele lab) and 17 μl Lipofectamine RNAi MAX (Invitrogen) in 5 ml total media according to manufacturer’s instructions. After 48 h of transfection, cells were harvested. For RNAi in S2 cells using dsRNA, 10<sup>5</sup> cells were plated in each well of a six-well plate in 1 ml of media. Cells were treated approximately every 24 h with 10 μg/ml dsRNA targeted against Slmb, Oskar, or Gaussia Luciferase (as controls) as described in Rogers and Rogers, 2008.

In vitro binding assay

GST and GST-SMN were purified from Escherichia 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 [AQ 12] and purified using Glutathione sepharose 4B beads. GST-B-TrCP1
was purchased from Novus Biologicals (cat# H00008945). SMN•Gem2 complexes were coexpressed in E. coli using SMNΔS and Gemin2(12–280) constructs, as described in Gupta et al. (2015). Glutathione sepharose 4B beads were washed 3x with phosphate-buffered saline (PBS). GST alone, GST-SMN, or GST-B-TrCP1 were attached to beads during 4-h-overnight incubation at 4°C in PBS with rotation. Beads were then washed 3x with modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 1 mM EDTA, 1% NP-40). Beads (20 μl) with ~2 μg attached GST-tagged protein (as determined by Coomassie stain with BSA standard) were added to 200 μl modified RIPA buffer with 100 μg/ml BSA block. SMN•Gem2 (2 μg) was added, and the mixture was rotated end over end at 4°C overnight. Beads were then washed 3x with modified RIPA buffer, and 10 μl SDS loading buffer was added followed by boiling for 5 min.

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 h and then harvested in PBS. Of the cell suspension, 80% was lysed in 6 M 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. One-third of these cells were frozen and taken as the 0 h time point. The remaining of the cells were replated in six-well plates. Cycloheximide (CHX; 100 μg/ml) was added to each sample, and cells were harvested at 2 and 6 h following treatment.

Immunoprecipitation
Clariﬁed cell lysates were precleared with immunoglobulin G agarose beads for 1 h at 4°C and again precleared overnight at 4°C. The precleared lysates were then incubated with anti-FLAG antibody cross-linked to agarose beads (EZview Red Anti-FLAG M2 afﬁnity gel, Sigma) for 2 h at 4°C 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 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM 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 4°C. S2 cell lysates were prepared by suspending cells in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM 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 4°C. Human cells (293T and HeLa) were ﬁrst gently washed in ice-cold 1x PBS and 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 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM 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 13,000 at 4°C. Western blotting [AQ 16] 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 2500 for the afﬁnity puriﬁed anti-dSMN, 1 in 20,000 (fly) or 1 in 5000 (human) for anti–α-tubulin (Sigma), 1 in 10,000 for monoclonal anti-Flag (Sigma), 1 in 1000 for anti-Slimb (gift from Greg Rogers), 1 in [AQ 17] 2500 for anti-human SMN (BD Biosciences), 1 in 1000 for anti–B-TrCP (gift from MB Major lab), 1 in 10,000 for polyclonal anti-Myc (Santa Cruz), and 1 in 2000 for anti-GST (Abcam) were used.

Larval locomotion
Smm control and mutant larvae (73–77 h 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 s. 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 analysis of variance (ANOVA).

SMA mouse models
Two previously developed SMA mouse models were utilized. The “Delta7” mouse (Smm< sup>-/-;SMN2;SMNΔS) is a model of severe SMA (Le et al., 2005). The “2B/-” mouse (Smm<sup>85X</sup>) 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; Glasscock 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 maintained as floating spheres of neural progenitor cells in the neural progenitor growth medium Stemline (Sigma) supplemented with N2 supplement [Life Technologies], 5 μg/ml Heparin [Sigma], 1x Non-Essential Amino Acids [Life Technologies], and 1x Antibiotic-Antimycotic [Life Technologies] plus 0.1 μM all-trans retinoic acid (RA) for 2 wk; 1 μM Purmorphamine (PMN; Stemgent) was added during the second week. Spheres were then dissociated with TrypLE Express (Life Technologies) and plated onto Matrigel-coated 12-mm coverslips in neuro 1x N2 Supplement [Life Technologies], 5 μg/ml Heparin [Sigma], 1x Non-Essential Amino Acids [Life Technologies], and 1x Antibiotic-Antimycotic [Life Technologies] plus 0.1 μM all-trans retinoic acid (RA) for 2 wk; 1 μM Purmorphamine (PMN; Stemgent) was added during the second week. Spheres were then dissociated with TrypLE Express (Life Technologies) and plated onto Matrigel-coated 12-mm coverslips in neuro 1x N2 Supplement [Life Technologies], 5 μg/ml Heparin [Sigma], 1x Non-Essential Amino Acids [Life Technologies], and 1x Antibiotic-Antimycotic [Life Technologies] plus 0.1 μM all-trans retinoic acid (RA) for 2 wk; 1 μM Purmorphamine (PMN; Stemgent) was added during the second week. Spheres were then dissociated with TrypLE Express (Life Technologies) and plated onto Matrigel-coated 12-mm coverslips in neuro 1x N2 Supplement [Life Technologies], 5 μg/ml Heparin [Sigma], 1x Non-Essential Amino Acids [Life Technologies], and 1x Antibiotic-Antimycotic [Life Technologies] plus 0.1 μM all-trans retinoic acid (RA).
EF1α promoter. Uninfected cells served as controls. Cells were analyzed at 1 and 3 wk postinfection, which was 4 and 6 wk of total differentiation (Ebert et al., 2009; Sareen et al., 2013).

Immunocytochemistry

Coverslips were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 20 min at room temperature and rinsed with PBS. Cells were blocked with 5% normal donkey serum (Millipore) and permeabilized in 0.2% Triton X-100 (Sigma) for 30 min at room temperature. Cells were then incubated in primary antibody solution for 1 h, rinsed with PBS and incubated in secondary antibody solution for 1 h at room temperature. Finally, nuclei were labeled with Hoechst nuclear stain (Sigma) to label DNA and mounted onto glass slides using FluoroMount medium (SouthernBiotec). Primary antibodies used were mouse anti-SMI-32 (Covance SMI-32R; 1:1000) and rabbit anti-mCherry (ThermoFisher; 1:1000). Secondary antibodies 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.

ACKNOWLEDGMENTS

This work was supported by the National Institute of General Medical Sciences R01-GM118636 (to A.G.M.). K.M.G. was supported by graduate research fellowship DGE-1144081 from the National Science Foundation. Work in the Wagner lab (D.B. and E.J.W.) was supported by the Welch Foundation (H1889). We also thank the Peifer, Major, and Rogers laboratories for reagents, advice, and expertise.

REFERENCES


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.

ACKNOWLEDGMENTS

This work was supported by the National Institute of General Medical Sciences R01-GM118636 (to A.G.M.). K.M.G. was supported by graduate research fellowship DGE-1144081 from the National Science Foundation. Work in the Wagner lab (D.B. and E.J.W.) was supported by the Welch Foundation (H1889). We also thank the Peifer, Major, and Rogers laboratories for reagents, advice, and expertise.

REFERENCES


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.

ACKNOWLEDGMENTS

This work was supported by the National Institute of General Medical Sciences R01-GM118636 (to A.G.M.). K.M.G. was supported by graduate research fellowship DGE-1144081 from the National Science Foundation. Work in the Wagner lab (D.B. and E.J.W.) was supported by the Welch Foundation (H1889). We also thank the Peifer, Major, and Rogers laboratories for reagents, advice, and expertise.

REFERENCES


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.

ACKNOWLEDGMENTS

This work was supported by the National Institute of General Medical Sciences R01-GM118636 (to A.G.M.). K.M.G. was supported by graduate research fellowship DGE-1144081 from the National Science Foundation. Work in the Wagner lab (D.B. and E.J.W.) was supported by the Welch Foundation (H1889). We also thank the Peifer, Major, and Rogers laboratories for reagents, advice, and expertise.

REFERENCES


14  |  K. M. Gray et al.  | Molecular Biology of the Cell


Pelizzonzi L, Yong J, Dreyfuss G (2002). Essential role for the SMN complex in the assembly of the Sm core particles and in processing of histone RNA. Genes Dev 16, 703–709.

Pellizzoni L, Yong J, Dreyfuss G (2002). Essential role for the SMN complex in the assembly of the Sm core particles and in processing of histone RNA. Genes Dev 16, 703–709.


SMN protein levels inversely correlate with the severity of spinal muscular atrophy. The SCF<sup>Smn</sup> E3 ligase complex interacts with a degron embedded within the C-terminal self-oligomerization domain of SMN. The findings elucidate a model whereby accessibility of the SMN degron is regulated by self-multimerization.
Author Queries

[AQ 1]: Please provide abbreviations list with definitions.  
[AQ 2]: Please define GST at first occurrence.  
[AQ 3]: Please define dsRNA at first occurrence.  
[AQ 4]: Matera and Van Duyne: if applicable please update and add to ref list. Otherwise unpublished per journal style.  
[AQ 5]: Please spell out DAPI.  
[AQ 6]: There is a Tan et al., 2008 in list; change year here?  
[AQ 7]: Please define JNK at first occurrence.  
[AQ 8]: Please spell out UTR.  
[AQ 9]: Does gr mean grem (g)?  
[AQ 10]: Please define PMSF at first occurrence.  
[AQ 11]: Please define SFM at first occurrence.  
[AQ 12]: Please spell out IPTG.  
[AQ 13]: Please define RIPA at first occurrence.  
[AQ 14]: Please define BSA at first occurrence.  
[AQ 15]: Use of “immunoglobulin” instead of “immune-globulin” OK?  
[AQ 16]: rpm or × g?  
[AQ 17]: Give Rogers and major lab affiliations.  
[AQ 18]: Please spell out NIM.  
[AQ 19]: Please spell out BDNF and GDNF.  
[AQ 20]: Please spell out MOI.  
[AQ 21]: Bocca et al. not cited in text; delete from list?  
[AQ 22]: Garcia et al., 2016: Please provide volume number.  
[AQ 23]: Li et al., 2014: please provide volume number and page range.  
[AQ 24]: Lim and Lim not cited in the text; delete from list?  
[AQ 25]: Monani 2005 not cited in text; delete from list?  
[AQ 26]: Noureddine et al., 2002 not cited in text; delete from list?  
[AQ 27]: Ojeda et al., 2011: Please provide page range.  
[AQ 28]: added “a” and “b” labels Please check citations (a,b always cited together, OK?)  
[AQ 29]: Pearn 1982: Please provide volume number.  
[AQ 30]: Schrank B et al., 1997 not cited in text; delete from list?  
[AQ 31]: Swoboda et al., 2015 not cited in text; delete from list?  
[AQ 32]: please note that Tan et al., 2007 was cited in text; add to list?  
[AQ 33]: Wirth 2000 not cited in text; delete from list?