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A *Drosophila* model of Spinal Muscular Atrophy uncouples the snRNP biogenesis functions of *survival motor neuron* from locomotion and viability defects

Kavita Praveen[a], **Ying Wen**[b], and **A. Gregory Matera**[a,b]

[a]Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, NC 27599

[b]Departments of Biology and Genetics, Program in Molecular Biology and Biotechnology, University of North Carolina, Chapel Hill, NC 27599

Summary

The Spinal Muscular Atrophy (SMA) protein, survival motor neuron (SMN), functions in the biogenesis of small nuclear ribonucleoproteins (snRNPs). SMN has also been implicated in tissue-specific functions, however, it remains unclear which of these is important for the etiology of SMA. *Smn* null mutants are larval lethals and show significant locomotion defects as well as reductions in minor-class spliceosomal snRNAs. Despite these reductions, we found no appreciable defects in splicing of mRNAs containing minor-class introns. Transgenic expression of low levels of either wild-type or an SMA patient-derived form of SMN rescued the larval lethality and locomotor defects, however, snRNA levels were not restored. Thus, the snRNP biogenesis function of SMN is not a major contributor to the phenotype of *Smn* null mutants. These findings have major implications for SMA etiology because they show that SMN's role in snRNP biogenesis can be uncoupled from the organismal viability and locomotor defects.

Introduction

Spinal muscular atrophy (SMA) is caused by recessive loss-of-function mutations in the *survival motor neuron 1 (SMN1)* gene (Lefebvre et al., 1995). The disease is characterized by motor neuron loss, atrophy of the proximal muscles, and progressive symmetrical paralysis. SMA symptoms vary in severity, and are classified by types I to IV, ranked in decreasing order (Ogino and Wilson, 2004). In the most severe form (SMA type I), which is also the most common, death usually occurs by 2 years of age.

SMN is the central component of a multimeric protein assemblage known as the SMN complex (Burghes and Beattie, 2009). The best characterized function of this complex is in the biogenesis of small nuclear ribonucleoproteins (snRNPs), primary components of the spliceosome. The SMN complex chaperones assembly of Sm proteins onto spliceosomal snRNAs (Meister et al., 2001; Pellizzoni et al., 2002). Complete loss of SMN is lethal in all organisms investigated to date. SMA, therefore, is a disease that arises due to a reduction in SMN levels, not a complete loss. Humans have two paralogs of SMN, named *SMN1* and *SMN2*, both of which contribute to total cellular levels of SMN protein. *SMN2*, however,

contains a base change that primarily leads to production of a truncated, unstable protein product. Current estimates suggest that *SMN2* produces only 10-15% of the level of full-length protein produced by *SMN1* (Lorson et al., 2010). Whilst this amount is not enough to completely compensate for loss of *SMN1, SMN2* is sufficient to rescue embryonic lethality.

A causative link between *SMN1* and SMA was established over 15 years ago, however, the mechanism of disease pathology remains unclear. In addition to roles in snRNP biogenesis, SMN has been implicated in a number of tissue-specific processes, including: axonal pathfinding (McWhorter et al., 2003; Sharma et al., 2005), axonal transport of β-actin mRNP (Rossoll et al., 2003), neuromuscular junction formation and function (Chan et al., 2003; Kariya et al., 2008), myoblast fusion (Shafey et al., 2005) and maintenance of muscle architecture (Rajendra et al., 2007; Walker et al., 2008; Bowerman et al., 2009). Thus it is not surprising that therapeutic approaches targeting SMA are primarily focused on increasing production of full-length SMN protein from the *SMN2* gene in patients (Lorson et al., 2010). While these approaches are extremely important, it is clear that a deeper understanding of the molecular etiology of SMA will be essential to develop effective treatments and minimize side-effects.

Here, we present a new *Drosophila* model system to study SMA patient-derived loss-of-function mutations in the background of an *Smn* null allele. *Smn* null mutants display a reduction in the levels of a subset of snRNAs and considerable defects in larval locomotion. Transgenic expression of FLAG-tagged wild-type (WT) dSMN rescues larval locomotion and organismal viability but, surprisingly, fails to rescue snRNA levels. Expression of an Smn^{T205I} construct (which mimics SMN^{T274I} in humans) also rescues the larval motility and viability defects, but the majority of these animals die as pupae with an snRNA profile similar to that of the wild-type transgenics. These data show that the observed decreases in snRNA levels in *Smn* null animals are not major contributors to organismal phenotype, and indicate that non-snRNP biogenesis functions of SMN play important roles in SMA pathology.

Results and Discussion

Characterization of *Smn* null flies

In order to minimize genetic background effects arising from recessive second-site mutations, we used flies bearing two different *Smn* null alleles for phenotypic characterization. $Smn^{D/+}$ flies (Rajendra et al., 2007) were crossed with $Smn^{X7/+}$ (Chang et al., 2008) to produce $Smn^{D/X7}$ trans-heterozygous mutants, herein referred to as $Smn^{-/-}$. $Smn^{-/-}$ larvae begin to die in early third instar, ~80 hrs (day 4) post egg laying (Fig. 1A). The primary lethal phase continues through day 5, after which time the number of larvae reduces gradually. Consistent with previous observations (Shpargel et al., 2009), 20-30% of *Smn* null larvae are ‘long lived’ and can survive for many days without undergoing metamorphosis or exhibiting the wandering behavior typical of the late third instar. Larval dSMN levels are highest at 1 day post egg laying (DPE), then decrease and remain constant. In $Smn^{-/-}$ mutants, dSMN levels are already reduced on day 1 and are almost undetectable by 2 DPE (Fig. 1B). Thus, a significant fraction of animals are able to survive for long periods with very low levels of dSMN.

To determine whether a correlation exists between dSMN and snRNA levels, developmentally staged $Smn^{-/-}$ and Oregon-R (OR) larvae were harvested and split into two groups of ~20-50 animals. One group was used to extract protein for western analysis and the other for RNA extraction and northern blotting (Fig. 1C). The snRNA levels in both OR and *Smn* null flies were greatest on day 1, after which time they decreased and remained constant throughout larval development. This was a surprising finding in the mutant larvae,

since dSMN levels are severely reduced by 2 DPE and yet, no dramatic or progressive reduction in snRNA levels was observed. We did note a slight decrease in U1, U5 and U11 snRNAs in the mutants and a greater decrease in levels of U12 and U4atac snRNAs (Fig. 1C). These findings are consistent with observations in the mammalian system that minor-class snRNAs (particularly U4atac and U12) are more sensitive to SMN depletion (Gabanella et al., 2007; Workman et al., 2009). We also note that levels of U6atac appear to be upregulated in the *Smn*$^{-/-}$ larvae, although the molecular basis for this is currently unknown. We did not detect an increase in unassembled snRNAs in mutant larvae, as shown by co-immunoprecipitation with anti-Sm antibody, Y12 (Fig. S1). Thus, as observed in mammalian cell culture systems (Sauterer et al., 1988), total *Drosophila* snRNA levels reflect total snRNP levels.

The observed decrease in U4atac and U12 snRNAs in *Smn* null larvae was neither exacerbated by prolonged reduction of dSMN, nor did it result in appreciable defects in the splicing of minor-class (U12-type) introns. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was performed using RNA from *Smn*$^{-/-}$ and wild-type larvae. Relative levels of spliced mRNAs from genes that contain U12-type introns were measured (Fig. 2). *U6atac*$^{-/-}$ larvae, which are known to be defective in U12-dependent splicing (Otake et al., 2002; Pessa et al., 2010), were used as positive controls. As shown in Fig. 2, mRNAs from these genes were significantly reduced in *U6atac* mutants, whereas they were largely unaffected in *Smn* mutants.

Transgenic expression of an SMA patient-derived mutation in dSMN rescues *Smn*$^{-/-}$ larval lethality

Although analysis of *Smn* null mutants is informative, the many putative functions of SMN make it difficult to parse out the precise cause of lethality. We thus wanted to develop *Drosophila* SMA models that perturb only a subset of SMN functions rather than affecting all of them. As a proof of principle, we created flies expressing an SMA patient-derived point mutation, T205I. This mutation corresponds to an SMA type II/III mutation, T274I, located in the YG box domain of SMN (Sun et al., 2005). We generated transgenic flies carrying either a wild-type or T205I mutant transgene, inserted at site 86Fb on chromosome 3 (Bischof et al., 2007). To preserve endogenous expression patterns, constructs are driven by the native *Smn* promoter and contain native 3' and 5' flanking sequences. Transgenic proteins can be distinguished from the endogenous by the presence of an N-terminal 3X FLAG tag.

Expression of the wild-type construct (*Smn*^{WT}) in the *Smn*$^{-/-}$ background results in >70% rescue of lethality (Fig. 3A). The surviving adults are fertile, with no apparent defects in flight or motility. Similarly, expression of the *Smn*^{T205I} construct fully rescues larval lethality of the null mutant. And while ~25% of *Smn*^{T205I} mutants eclose as adults, the majority of these animals die as pupae (Fig. 3A). This partial rescue of the null phenotypeis consistent with the human *SMN*^{T274I} mutation, which causes a relatively mild form of SMA (Sun et al., 2005). Note that both the WT and T205I rescue animals show some developmental delay, as they pupate and eclose a day later than OR controls.

Smn^{T205I} is moderately defective in self-oligmerization

A number of human SMA-derived mutations in the YG box region of SMN, including *SMN*^{T274I}, have been shown to be defective in oligomerization (Lorson et al., 1998). We tested a panel of dSMN proteins, including T205I, for similar defects. FLAG- and Myc-tagged versions of each construct were expressed in *Drosophila* S2 cells and co-immunoprecipitation profiles compared. Immunoprecipitation was performed with anti-FLAG antibody and the amount of Myc-tagged protein that co-precipitated was measured.

As shown in Fig. 3B, the amount of Myc-dSMN(T205I) precipitated by FLAG-dSMN(T205I) was reduced compared to the amount of Myc-dSMN(WT) precipitated by FLAG-dSMN(WT), indicating a deficiency in its ability to self-oligomerize. This conservation between the human and fly SMN mutants also extends to other SMA-derived point mutations. Both Smn^{G206S} (corresponding to human SMN^{G275S}) and Smn^{Y203C} (SMN^{Y272C}) mutants were defective in oligomerization, similar to reports of their human counterparts (Lorson et al., 1998). Furthermore, the severity of these defects is also conserved, as Y203C was less effective at binding to itself than either T205I or G206S (Fig. 3D). Likewise, Y272C has a more severe oligomerization defect than T274I or G275S (Lorson et al., 1998). Thus, T205I displays a relatively mild defect in self-oligomerization.

SMN molecules that are unable to oligomerize are unstable (Burnett et al., 2009). Thus we expected that T205I transgenic flies might display reduced levels of FLAG-dSMN compared to transgenic flies expressing WT FLAG-dSMN. However, levels of FLAG-dSMN in both the WT and T205I transgenic lines were equivalent, as analyzed by western blotting (Fig. 3C). This finding indicates that the mild defect in T205I oligomerization does not significantly affect dSMN levels. In addition, this suggests that the lethality of the T205I mutant is not due to insufficient protein levels, but rather due to a molecular defect in dSMN.

As shown in Fig. 3C, expression of both transgenic FLAG-dSMN constructs (WT and T205I) was significantly below the level of endogenous dSMN. The considerable degree of rescue achieved with such low expression levels shows that dSMN is made in greater quantities than required by the fly. Similar observations have been made in mammalian cell lines wherein the phenotypic effects of SMN reduction are observed only when SMN levels are reduced by 85-95% (Zhang et al., 2008; Bowerman et al., 2011). Interestingly, we found that expression of the WT transgene with an Actin5C promoter failed to rescue the null mutant beyond pupal stages, despite the fact that this construct expressed much higher levels of dSMN protein than the native promoter driven construct (Fig. 3D). These results suggest that the Actin5C promoter fails to express dSMN in some critical tissue or developmental window.

Transgenic expression of Smn^{T205I} rescues larval locomotion

Consistent with previous reports (Chan et al., 2003; Shpargel et al., 2009), we found that $Smn^{-/-}$ animals display significant defects in larval locomotion. To further characterize these locomotor defects, we employed two previously described assays (Wu et al., 2003; Ubhi et al., 2007). In the ‘righting’ assay, each larva was placed on its dorsal surface and the time taken to return to its ventral surface (the crawling position) was measured (Fig. 3E). The $Smn^{-/-}$ larvae took a significantly longer time to return to the crawling position compared to OR. Larval motility was rescued in animals carrying the WT transgene, and to a large extent in the T205I transgenic animals, which showed a relatively mild defect. In the ‘burrowing’ assay, ~30 larvae were placed atop a dish containing 1.5% agar/molasses media and incubated at room temperature in the dark. After 2 hours, the number of larvae remaining on top of the medium was recorded. The assay was repeated 3 times per genotype. We found that the $Smn^{-/-}$ mutants do not burrow into the medium at all (Fig. 3F). However, we also noticed that if there were surface defects (cuts) present in the media, some of these larvae worked themselves into the crevices. Importantly, the burrowing behavior was restored in both the WT and T205I transgenic animals (Fig. 3F). We conclude that Smn^{T205I} rescues the larval viability and locomotor defects observed in the null mutants.

Smn^{T205I} and *Smn*^{WT} transgenic larvae are functional in snRNP biogenesis

Phenotypically, the *Smn*^{T205I} flies more closely resemble *Smn*^{WT} animals than they do the *Smn* null mutants. To determine the degree to which this resemblance extends to the molecular level, we quantified the snRNAs in *Smn*$^{-/-}$, *Smn*^{WT} and *Smn*^{T205I} animals. Larvae from each of the three aforementioned genotypes, along with OR, were harvested just prior to the beginning of the lethal phase (i.e. at ~76 hours) and snRNA levels were analyzed by northern blotting (Fig. 4A). As noted earlier, U1, U4, U5, U11, U12 and U4atac snRNAs showed a reduction in the null animals (Fig. 4B). Again, U12 and U4atac levels were the most severely affected (~60% reduction). The *Smn*^{WT} and *Smn*^{T205I} animals showed very similar snRNA profiles, despite differences in adult viability. Both transgenes failed to rescue U1, U4 and U4atac snRNA levels and only partially rescued U12 and U5 snRNAs (Fig. 4B). Thus, we conclude that the *Smn*^{T205I} mutant is functional in snRNP biogenesis. In support of this interpretation, the human counterpart to this mutation, *SMN*^{T274I}, was shown to be active in a HeLa cell Sm-core snRNP assembly assay (Shpargel and Matera, 2005).

We also analyzed minor-class intron splicing in the two transgenic rescue lines. Among the four mRNAs that showed slight but significant reductions in the *Smn* null mutant background (Fig. 2), there was no correlation among the *Smn*^{WT} and *Smn*^{T205I} samples (Fig. S2). Two of the mRNAs (CG18177 and CG15081) showed no significant differences upon expression of either transgene. CG11839 mRNA was restored to OR levels only by expression of *Smn*^{WT}, whereas CG33108 levels were restored only by expression of *Smn*^{T205I} (Fig. S2).

Smn^{T205I} is a good model for intermediate SMA

The human *SMN*^{T274I} mutation was identified in patients with milder forms of SMA, types II/III (Sun et al., 2005). These patients show a later age of onset, decreased severity of symptoms and longer life expectancy than type I patients (Ogino and Wilson, 2004). The *Drosophila* equivalent of this mutation, *Smn*^{T205I}, recapitulates several key features of intermediate SMA, including a slight deficiency in motor function and a much longer lifespan than the null mutant. We also observed a mild defect in oligomerization of *Smn*^{T205I}, suggesting that on a molecular level, this *Drosophila* protein behaves similarly to the human mutation. We conclude that the *Smn*^{T205I} flies represent a good model for intermediate SMA.

SMN, snRNA levels and SMA

As shown above, transgenic expression of *Smn*^{WT} was able to rescue the locomotion and viability defects observed in *Smn* null mutants, however, a majority of the snRNAs were largely unrestored to wild-type levels. Given the considerable degree of phenotypic rescue, this was an unexpected finding, suggesting that the observed reduction in snRNAs in the null animals is not a major contributor to larval locomotion and viability.

Consistent with our findings, previous studies using mouse models of SMA have also reported decreases in snRNA levels, particularly for U12 and U4atac (Gabanella et al., 2007; Workman et al., 2009). Other investigators reported widespread pre-mRNA splicing changes in both minor- and major-class introns in late-symptomatic SMA mice (Zhang et al., 2008). However, subsequent studies revealed that these splicing defects are likely a secondary consequence of severe SMN loss, as pre- and early-symptomatic SMA mice did not show an enrichment of unspliced introns (Baumer et al., 2009).

In our *Drosophila* system, we note that U12 and (to a lesser extent) U5 levels were partially rescued by transgenic expression of *Smn*^{WT} and *Smn*^{T205I}, although U4atac levels remained low (Fig. 4B). Thus, one explanation for our results could be that splicing of minor-class

(U12-specific) introns is sensitive to small changes in snRNA concentrations and that U12 and U5 are limiting factors, whereas U4atac is not. We tested this hypothesis by using qRT-PCR to measure mRNA levels in 18 of the 20 predicted minor-class introns in the *Drosophila* transcriptome (Lin et al., 2010). As shown in Fig. 2, a mutation in the *U6atac* gene resulted in pronounced defects in splicing of minor-class introns, however, splicing of these same mRNAs was largely unaffected in *Smn* null mutants. Among the minor-intron transcripts we analyzed, the steady-state levels of two mRNAs, CG15081 (*Phb2/Rea* in mice) and CG33108 (ortholog unknown), were reduced by roughly 50% in $Smn^{-/-}$ larvae. It is possible that reduction in levels of one or more of these mRNAs could contribute to the phenotype of *Smn* null mutants, however, CG15081 heterozygotes are completely viable (see Flybase). A similar decrease in levels of the *Phb2* mRNA in mice was shown to affect neither organismal viability nor motor function (Park et al., 2005) strongly suggesting that haploinsufficiency for CG15081 is not the cause of the *Smn* phenotype.

These observations question the significance of snRNA levels in human SMA etiology. In this regard, it is important to note that recessive point mutations in the gene encoding human U4atac snRNA do not phenocopy SMA. Instead, loss of U4atac function causes a disease known as microcephalic osteodysplastic primordial dwarfism type I (MOPD I), which is characterized by severe intrauterine growth retardation and multiple organ abnormalities (Edery et al., 2011; He et al., 2011). Similar to the fruitfly *U6atac* mutants (Pessa et al., 2010; this work), cells derived from MOPD I patients display marked defects in splicing of minor-class introns, whereas splicing of major-class (U2-type) introns is unaffected (Edery et al., 2011; He et al., 2011). Although we cannot entirely exclude the possibility that tissue-specific defects in minor-intron splicing may be causative for SMA, our finding that transgenic expression of Smn^{WT} can rescue viability and fertility without restoring U4atac levels effectively uncouples the observed global snRNA deficits from the organismal phenotype. Moreover, it is important to note that the *Smn* and *U6atac* mutants were analyzed just prior to onset of the lethal phase. At this timepoint, *Smn* null animals already display significant motor function defects. We therefore conclude that perturbations in minor spliceosome levels are not likely to be causative for the larval locomotion and viability defects observed in *Smn* null mutants.

With the possible exception of U5, Smn^{T205I} animals have nearly identical snRNA profiles to those of the Smn^{WT} animals, yet most of the T205I animals die as pupae. Given the intermediate phenotype of both humans and fruit flies expressing the T274I/T205I mutation and the fact that human SMN^{T274I} is active in Sm-core assembly (Shpargel and Matera, 2005), these findings strongly suggest that mutation of this residue disrupts a second, essential function of SMN protein. This does not mean that splicing plays no role in downstream SMA pathology; it clearly does. There is strong evidence for a negative feedback loop wherein low levels of SMN protein exacerbate exon skipping of human *SMN2*, leading to a further reduction in SMN expression (Jodelka et al., 2010; Ruggiu et al., 2011). However, because *Smn* is a single-exon gene in *Drosophila*, our system also uncouples protein-based defects in SMN from autologous feedback regulation via splicing. In conclusion, our results demonstrate that the reduction in snRNA levels observed in *Smn* mutants is not a major contributor to organismal lethality, and indicate that non-snRNP biogenesis functions of SMN play critical roles in the etiology of SMA. Molecular identification of this second SMN function will be an important subject of future investigation.

Experimental procedures

Fly stocks and genetics

All stocks were cultured on molasses and agar at room temperature (24 ± 1°C) in half-pint bottles. Oregon-R was used as the wild-type allele. The Smn^{X7} microdeletion allele (Chang et al., 2008) was a gift from S.Artavanis-Tsakonis (Harvard University, Cambridge, USA). The Smn^{D} (f01109) transposon insertion allele was obtained from the Exelixis collection at Harvard Medical School and isogenized to remove non-*Smn* flies contaminating this stock (Rajendra et al., 2007). WT and T205I transgene constructs were made using a ~3kb fragment encompassing the entire *Smn* coding region, cloned into the pAttB vector (Bischof et al. 2007). A 3X FLAG tag was inserted downstream of the start codon. The constructs were injected into embryos by BestGene Inc. (Chino Hills, CA). To rescue the *Smn* null phenotype, the WT and T205I transgenic lines were recombined with the Smn^{D} line. Recombinants were identified by genotyping, verified by western blotting for FLAG-dSMN expression and subsequently crossed with Smn^{X7} for analysis. $U6atac^{k01105}/CyO\ Dfm\text{-}Yfp$ flies were a gift from B. McCabe (Columbia University, USA).

Antibodies and western blotting

Larval, pupal 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 protease inhibitor cocktail (Invitrogen) and clearing the lysate by centrifugation. Western analysis 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 anti-α tubulin (Sigma) and 1 in 10,000 for polyclonal anti-Myc (Santa Cruz) were used. Anti-FLAG antibody crosslinked to agarose beads (EZview Red Anti-FLAG M2 affinity gel, Sigma) was used to immunoprecipitate FLAG tagged proteins from cells.

Northern blotting

Larvae, pupae and adult *Drosophila* were homogenized in TRIZOL (Invitrogen) and total RNA was extracted following manufacturer's instructions. RNA was run on a 10% polyacrylamide-urea gel (Invitrogen), transferred to a nylon membrane, and probed with ^{32}P-labeled PCR products corresponding to the *D. melanogaster* U1, U2, U4, U5, U6, U11, U12, U4atac, U6atac, and 7SK cDNAs. The blots were visualized using a Typhoon phosphorimager (Molecular Dynamics) and quantification was performed by densitometry using ImageQuant software.

Larval motility assays

Larvae used for the motility assays were ~76 hours old. For the righting assay, larvae were placed on a 3% agarose plate for 120 sec to allow them to equilibrate to the new environment. They were then placed on their ventral surfaces and the time taken to return to a crawling position was noted. The assay was terminated at 120 sec. For the burrowing assay, thirty larvae were placed on a molasses plate with 1.5% agarose. The plates were kept in the dark and the number of larvae remaining on the surface was counted after 2 hours. Statistical significance for righting and burrowing assays was determined using Student's T-test and chi-squared test, respectively.

Real-time PCR

One microgram of total RNA from ~76 hour larvae was used in a 20μl reverse transcriptase reaction with random hexamer primers (Superscript III first strand synthesis system,

Invitrogen), and ~50-100ng of the cDNA was used for qRT-PCR. Real-time PCR reactions were carried out on an Applied Biosystems 7900HT Fast Real-time PCR machine using Maxima® SYBR Green/Rox qPCR master mix (Fermentas). Three technical replicates were run for each reaction and three biological replicates were tested for each genotype. A 2-step PCR reaction was used and the data were analyzed using the ΔΔCt method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Drosophila SMN mutants display locomotor defects and reductions in snRNA levels

dSMN null mutants larval lethal but have no appreciable defects in pre-mRNA splicing

Larval lethality rescued by SMA patient mutation that is functional in snRNP assembly

New SMA model system highlights importance of non-snRNP assembly functions of SMN

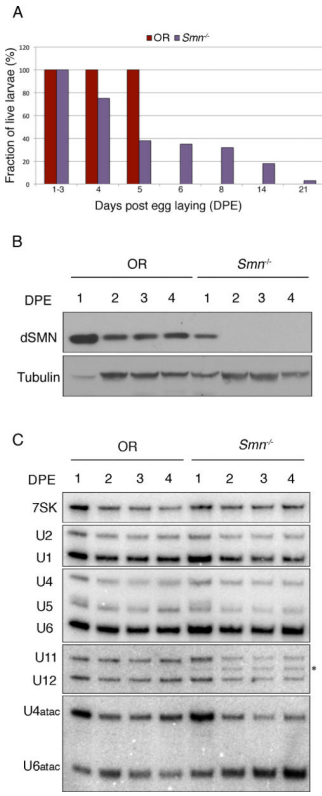

Figure 1.

Characterization of *Smn* null flies. ***A***. *Smn*$^{-/-}$ mutants begin dying 4 days post egg laying (DPE), with a number of larvae surviving for extended periods without pupariation. Note that by 6 DPE the OR control larvae had all pupated. For each genotype n ≥ 100 larvae, collected on day 1 post egg laying. ***B***. Developmental western blot of dSMN levels in control (OR) and *Smn* null animals. ***C***. Developmental northern blot showing major and minor class spliceosomal snRNA levels in OR and *Smn* null larvae. 7SK and U6 snRNA are shown as loading controls. These snRNA levels are reflective of the total snRNP levels, as shown in Fig. S1. An asterisk marks a heteroallelic variant U11 snRNA present in the *Smn* null background (see Fig. S1).

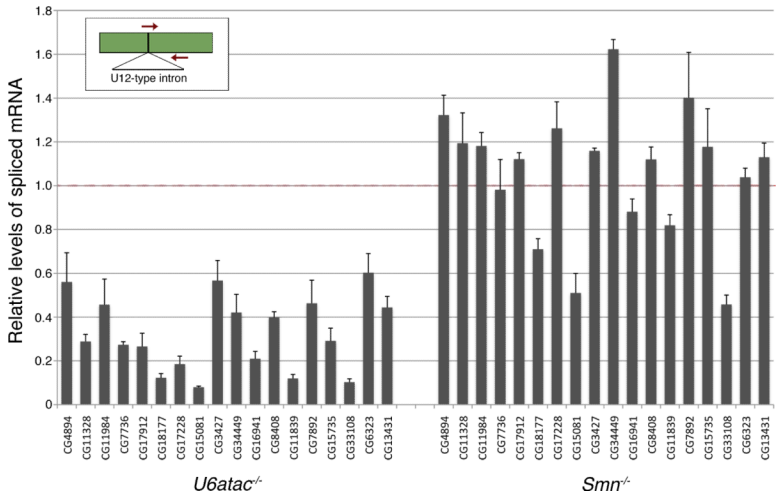

Figure 2.

Analysis of minor-class intron splicing defects in *Smn* and *U6atac* mutants. mRNA levels for eighteen genes containing putative U12-dependent introns in ~76 hour OR, $Smn^{-/-}$ and $U6atac^{+/-}$ larvae were measured by qRT-PCR, and normalized to OR using *Rpl32* mRNA. The data represent three biological and technical replicates. Primers were designed to cross the exon junction flanking the U12-type intron, such that only spliced mRNAs would be detected (inset). The $U6atac^{+/-}$ larvae showed robust defects in splicing of these introns (avg. = 0.32). Nine of the eighteen mRNAs were reduced in $U6atac^{+/-}$ animals with p-values < 0.01, eight had $p < 0.05$ and the levels of one mRNA approached, but did not reach, significance (CG7892, p ~0.08). In contrast, splicing of these same introns was relatively unaffected in $Smn^{-/-}$ animals (avg. = 1.06). Although a majority of the mRNAs were not affected, we note that the four mRNAs showing the greatest decrease in the $Smn^{-/-}$ mutants were also the most severely affected mRNAs in $U6atac^{+/-}$ mutants. Each of these (CG18177, CG33108, CG15081 and CG11839) had p-values < 0.05, although none were below 0.01.

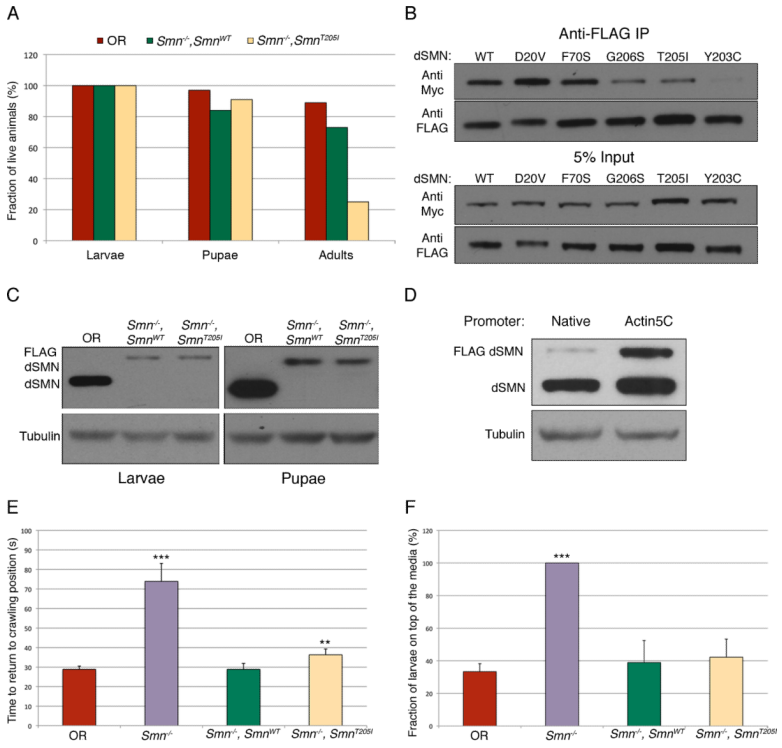

Figure 3.

Transgenic rescue of *Smn* null animals with an SMA patient-derived point mutation, T205I. ***A***. Analysis of *Smn* null flies expressing Smn^{WT} and Smn^{T205I} transgenic constructs. ~100 first instar larvae were collected and the fraction of live animals at subsequent developmental stages was measured. ***B***. dSMN(T205I) shows mild oligomerization defects. Lysates from cells co-expressing FLAG and Myc tagged versions of each dSMN point mutant and WT were immunoprecipitated with anti-FLAG antibody followed by western with anti-Myc antibody. ***C***. Western blot of lysates from Smn^{WT} and Smn^{T205I} larvae and pupae probed with anti-SMN. Tubulin was used as loading control. ***D***. Western analysis of lysates from flies heterozygous for the native and Actin5C promoter-driven *FLAG-Smn* constructs. Blots probed with anti-SMN; anti-tubulin as control. ***E***. Smn^{WT} and Smn^{T205I} animals rescued locomotion defects present in $Smn^{-/-}$ larvae. Graph shows the performance of $Smn^{-/-}$, Smn^{WT} and Smn^{T205I} larvae compared to OR in the righting assay. At least 18 larvae were measured for each genotype and Student's T-test was used to calculate p-values. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$. ***F***. Graph illustrating results of the burrowing assay for OR, $Smn^{-/-}$, Smn^{WT} and Smn^{T205I} larvae. A chi-squared test was performed to determine significance.

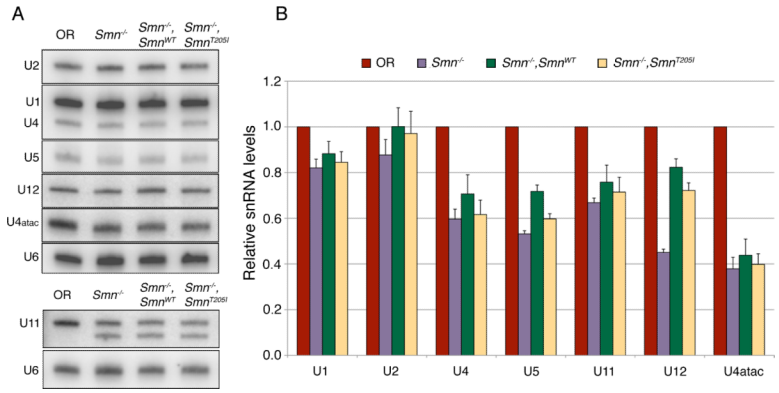

Figure 4.

Smn^{WT} and *Smn*^{T205I} animals have similar snRNA profiles. ***A***. Northern blot showing levels of major and minor class snRNAs in ~76 hour larvae from OR, *Smn*$^{-/-}$, *Smn*^{WT} and *Smn*^{T205I} lines. U6 snRNA was used as a control for loading. ***B.*** Graph illustrating the relative levels of snRNAs in *Smn*$^{-/-}$, *Smn*^{WT} and *Smn*^{T205I} larvae compared to OR. Northern blots from at least four biological replicates were used to quantify snRNA levels, using U6 for normalization. Student's T-test was used for statistical analysis. See Fig. S2 for additional information.