Spinal Muscular Atrophy in *Drosophila* and Mouse

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ABSTRACT

Spinal Muscular Atrophy (SMA) is a recessive genetic disorder that is characterized by selective loss of lower motor neurons and atrophy of muscle. It is the most common genetic cause of infant mortality and is caused by the loss of the *SMN1* (Survival Motor Neuron) gene and retention of *SMN2,* which results in low levels of SMN protein. SMN functions in assembly of Sm proteins onto snRNAs and these RNPs function in splicing. Several mouse models have been developed to study the different types of SMA. Intermediate SMA mice (*SMN2 +/+; Smn -/-; SMNΔ7 +/+*) live an average of 14 days and express low levels of SMN protein. To investigate if expressing SMN in neurons would increase survival past 14 days, intermediate SMA mice were crossed to mice containing the ChAT-SMN transgene. ChAT-SMN expresses SMN in mature neurons. The results indicate increased expression of SMN in mature neurons does not increase survival of intermediate SMA mice past their normal lifespan.

Additionally, we are creating a Drosophila model of SMA to study the effect of Smn missense mutations on survival. Drosophila has one *Smn* gene and loss of Smn protein is lethal at the larval stage. To investigate the effect of missense mutations on survival, we are introducing them into a Smn null background to see if survival is increased past the larval lethal stage. Patient mutations SMND44V and G279V (D20V and G210V in Drosophila) were chosen due to 100% identity of amino acids between human and fly and the type of SMA they cause in humans. D44V is a mild form of SMA (Type III) while G279V causes a severe form of SMA (Type I). Site-directed mutagenesis was performed to introduce the missense mutations into Drosophila *Smn* and the gene was subcloned into the pUAST vector. The plasmid will be injected in Drosophila embryos and the resulting flies crossed into the *Smn* null background. Three additional mutations, V94G, G95R, and Y130C (V72G, G73R, Y107C in Drosophila), were selected to study their effect on survival as well. Site-directed mutagenesis was performed on Drosophila *Smn* and the constructs remain to be subcloned and sequenced. Based on the results of these experiments, biochemical assays will be performed to determine the level of snRNP assembly in surviving flies to see if there is a relationship between levels of snRNP assembly and the type of SMA. Additionally, mutations that extend the flies past the larval stage will be chosen to create transgenic mice to see if they have a similar effect in mice.

INTRODUCTION

Background

Spinal Muscular Atrophy (SMA) is an autosomal recessive disorder that is characterized by selective loss of lower motor neurons and atrophy of muscle (1). It is the most common genetic cause of infant mortality in humans (2). SMA is caused by loss of the telomeric *survival motor neuron* (*SMN1)* gene and retention of the centromeric *SMN2* gene (3,4). Loss of both SMN genes is embryonic lethal (4,7,8). *SMN1* and *SMN2* are 99.9% identical and the critical difference between the two genes is a C-T change in exon 7 of *SMN2*, which does not alter the encoded amino acids but does alter the amount of exon 7 incorporated in the final SMN transcript (3,9,10,11,12,13). The *SMN1* gene produces full-length transcripts whereas the majority of the *SMN2* transcripts lack exon 7(3, 13). The lack of exon 7 leads to an unstable protein that is unable to oligomerize efficiently and is rapidly degraded (14,15). As a result, a single *SMN2* gene produces considerably less SMN protein than a *SMN1* gene (5,6). Thus the disease is caused, not by lack of SMN protein, but by low levels of SMN protein (5,6).

The severity of SMA can be divided into three groups based on clinical presentation. Type I SMA is the most severe form of SMA with onset before 6 months and death occurring by the age of 2 years (1). Type II SMA is intermediate in severity with onset before 18 months of age and patients never gaining the ability to walk (1). Type III SMA is the mildest form of the disease with onset after 18 months and retains the ability to walk (1).

The most well characterized function of SMN is in snRNP (small nuclear ribonucleic protein) assembly (16,17,18,19). SnRNPs contain small nuclear RNA in a specific protein complex (16,20). The SMN protein assembles Sm protein onto snRNA (16,17,18). SnRNPs in the nucleus are important for the splicing of genes (16). SnRNP assembly is essential for cell

survival (16), however, it is unclear if disruption of snRNP assembly is the cause of SMA. In addition to its role in assembly of snRNPs, SMN also associates with numerous other proteins (16,21,22). SMN has been found in the axons and growth cones of neurons (23, 24, 25, 26). In contrast to the motor neuron cell body, antibody staining in motor axons appears to indicate that the vast majority of SMN is not complexed to a known snRNP component, Gemin2 (27). This has led to competing hypotheses of whether the function of SMN that is disrupted in SMA is involved in splicing or if there is another unknown SMN complex in axons that leads to the motor neuron disorder.

The *SMN* gene is highly conserved among different species, including Drosophila and mouse (28, 38). In both species there is one *survival motor neuron* (*Smn*) gene that is equivalent to *SMN1* (28,36,38). Complete loss of this gene results in early embryonic lethality in mice (39) and larval lethality in Drosophila (40), as would be expected given SMN's critical role in snRNP assembly (18).

Drosophila Models of SMA

Five percent of SMA cases have small mutations including missense mutations in *SMN1*. Severity of the mutation corresponds to the type of SMA the patient has while also taking into account the *SMN2* copy number, which has been shown to modulate the phenotype with increasing copies of *SMN2* (30). Several SMA patient mutations occur in amino acids that are conserved between fly and human. Figure 1 shows the alignment of human, mouse, Zebrafish, and Drosophila *SMN* along with reported SMA patient missense mutations. Two Drosophila mutant *Smn* alleles, Smn^{73Ao} and Smn^{B} (G202S and S201F) have been reported that disrupt oligomerization and are equivalent to the severe Y272C mutation in humans (28,32). These mutations are effectively null alleles for Smn function. Homozygous Smn^{73Ao} mutants are larval

lethal and embryonic lethal in the absence of maternal Smn (40). The restoration of *Smn* expression in all tissues is required to rescue larval lethality and ubiquitous expression of UAS-*Smn* using the GAL4 system does rescue the larval lethality (40). **Table 1** shows five of the conserved mutations that were used in this experiment.

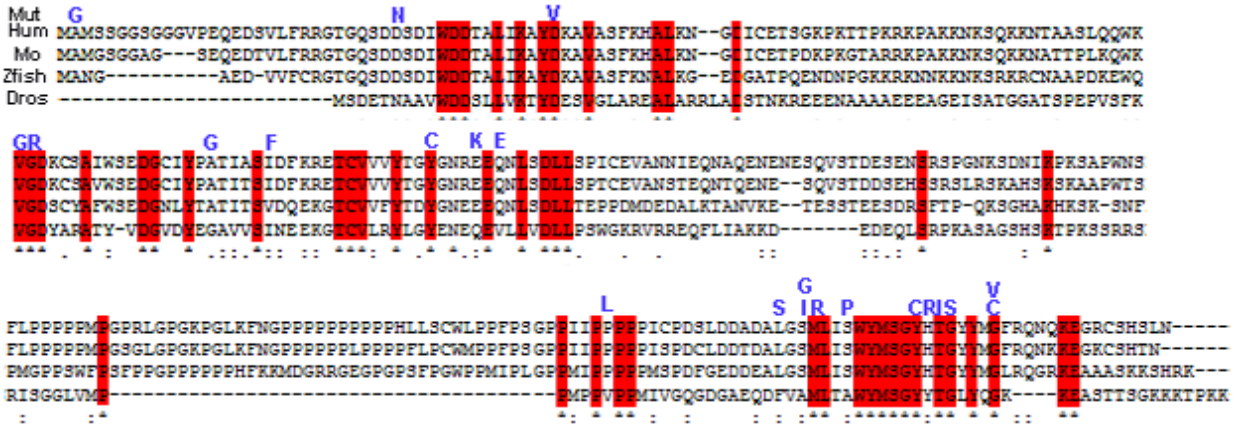

Figure 1. Alignment of SMN. The blue letters indicate patient missense mutations, red highlights the identical amino acids. (Mut) mutation, (Hum) Human, (Mo) Mouse, (Zfish) Zebrafish, (Dros) Drosophila, (*****) identical, (**:**) conserved, (**.**) similar amino acids (29).

Human Mutation in *SMN1*	Exon	SMA type	SMN2 Copy #	Fly Mutation in *Smn*
D44V	2a	3	1	D20
V94G	3	2	3	V72
G95R	3	3	1	G73
Y130C	3	3	2	Y107
G279V	7	1	ND	G210

Table 1. Missense Mutations in *SMN1*

Mouse Models of SMA

Several mouse models have been developed to investigate SMN function in SMA. The embryonic lethal phenotype caused by complete loss of the mouse *Smn* gene is rescued by the introduction of two copies of *SMN2*, resulting in mice with severe SMA that live an average of 5

days, whereas eight copies of *SMN2* allows the mice to live normal lifespans (30). *SMN2* produces the required functional SMN protein for cells to survive. An intermediate SMA mouse model has also been developed that is representative of type II SMA. While *SMN2* produces some full-length SMN, the majority of the transcripts generated from *SMN2* lack exon 7 (3, 13). In the intermediate SMA model, a transgene was constructed that placed an SMN cDNA lacking exon 7 (SMNΔ7) under the control of the SMN promoter. The SMNΔ7 mice were then crossed onto the severe SMA background. Expression of SMN Δ7 modulates the severe SMA phenotype and allows the mice to live an average of 14 days (43).

RESULTS

Aim 1

Site-Directed Mutagenesis. Five SMA patient missense mutations that occur in amino acids of 100% identity between human *SMN1* and Drosophila *Smn* were chosen for site-directed mutagenesis. The mutations chosen were D44V, V94G, G95R, Y130C, and G279V (D20, V72, G73, Y107, and G210 in fly). These mutations, shown in **Table 1**, are of varying severities due to the type of SMA they cause and the number of copies of *SMN2*. D44V and G95R are mild mutations (33, 34), V94G and Y130C (35, 37) are intermediate mutations, and G279V (41) corresponds to a severe mutation. To investigate the effect of missense mutations on survival, we are introducing them into the Smn^{73Ao} null background to see if survival is increased past the larval lethal stage. All five missense mutations have been introduced to Drosophila *Smn* in the pOT2 vector using site-directed mutagenesis.

To screen for clones containing the desired mutations, a series of diagnostic digests were utilized. Potential clones were digested with EcoRI and XhoI to confirm the presence of the

841bp *Smn* insert. Digests specific for each mutation were set up to detect the presence of the mutation in the different clones. For D20, a new PshAI site was created by the mutation. Potential clones were double digested with PshAI and XhoI and the presence of an extra band compared to wild type *Smn*/pOT2 indicated the mutation was present. Because the mutation was localized close to the 5′ end of *Smn*, the PshAI and XhoI digest essentially excised the insert whereas wild type *Smn* was only linearized. Potential G210 clones were digested with EcoRI and BsaJI. The G210 mutation causes *Smn* to lose a BsaJI site and positive clones were identified by the absence of a band as compared to wild type fly *Smn*. Five D20 mutations and one G210 mutation were identified and confirmed through sequencing.

Mutations V72 and G73 also utilized digests to identify potential positive clones but used a different screening approach due to the complicated banding pattern that resulted from digesting the entire plasmid. To identify clones containing these mutations, the *Smn* insert only was amplified using a primer in the pOT2 vector just outside the 5′ end of the gene and an internal primer at the 3′ end. After confirming the presence of Drosophila *Smn* by a positive band after running out an aliquot of PCR reaction on a 1.2% agarose gel, diagnostic digests were set up on the remaining PCR product to identify positive clones. V72 gains a new MnlI site and G73 gains a new RsaI site. As many small fragments resulted from this digest, the product was run out on a MetaPhor Agarose high resolution gel and compared to the wild type *Smn* pattern to identify clones. The Y107 mutation did not result in any changes in the restriction enzyme sites and will have to be identified through sequencing. All three mutations remain to be sequenced to confirm positive clones.

Subcloning into pUAST vector. The 9056bp pUAST vector contains 3′ and 5′ P-elements, UAS sequences, and a white^{+} marker for eye color. These features allow a method of introducing

our clone to the Drosophila genome through P-element transformation, a method to express the construct using the GAL4 system, and a marker to identify transformants. The 841bp *Smn* gene containing either the D20 or G210 mutation as well as wild type *Smn* was excised from the pOT2 vector using EcoRI and XhoI. The pUAST vector was digested with same enzymes and then dephosphorylated to ensure that in future ligation steps the vector would not close on itself without the insertion of the *Smn* gene. Both sets of digests were run out on a 0.7% gel to separate the fragments. The bands corresponding to approximately 850 kb in the fly *Smn*/pOT2 digest and 9 kb in the pUAST digest were removed from the gel and gel purified to obtain the DNA. The presence of different sticky ends from EcoRI and XhoI in the linearized pUAST vector and *Smn* allowed for ligation in the correct orientation of the two fragments and recircularization of the plasmid.

Positive clones were identified using the same method as in the pOT2 vector. Potential positive clones were screened by first digesting with EcoRI and XhoI to confirm the presence of the insert and then digesting with the mutations' respective diagnostic restriction enzymes. Positive clones were identified by comparing the banding pattern of the mutated fly *Smn*/pUAST with wild type and detecting differences. D20/pUAST gains a new PshAI site and the existence of a band at approximately 850 kb after a XhoI and PshAI double digest indicated the presence of the mutation. G210/pUAST potential clones were digested with EcoRI and BsaJI and resulted in many different fragment lengths. The banding pattern was compared with wild type and the presence of a new band at approximately 750 bp identified the mutants. The positive clones were sequenced to confirm the mutation was present and the gene remained intact. V72, G73, and Y107 still remain to be subcloned in the pUAST vector.

Germline transformation in *Drosophila.* P-element transformation was used to introduce the mutated and wild type Drosophila *Smn*/pUAST construct into Drosophila embryos. The pUAST vector contains a 5′ and 3′ P-element flanking the *Smn* gene and several other necessary sequences such as a marker for eye color and UAS binding sequences. P-elements are transposable elements that are able to randomly insert themselves into genomic DNA. The pUAST vector contains P-elements engineered without a transposase gene regulating DNA insertion. Defective P-elements require *trans*-acting transposase of the intact element for transposition. This transposase is provided by a helper plasmid that is co-injected with the construct of interest. The *Smn*/pUAST constructs were co-injected with Δ2-3 transposase, a P-element that is active in supplying transposase but due to a deletion of exons 2 and 3 is unable to transpose itself. This allows only the P-elements within the pUAST vector to be randomly inserted into the Drosophila germline. The construct was injected into embryos before the formation of the pole cells so that a stable, transmissible germline was created.

The P-element containing the *Smn* gene contained a white^{+} marker that allowed identification of transformants by the presence of orange or red eyes in the w^{-} fly stocks. All crossing and maintenance of fly stocks was done by Dr. McGovern.

Expression of the mutated *Smn* genes will occur through the GAL4 system. The GAL4 system allows for directed gene expression in Drosophila. The *Smn* gene was subcloned behind GAL4 binding sites and is silent in the absence of GAL4. Flies carrying *UAS-Smn* will be crossed to flies expressing GAL4 and the *trans*-acting protein will activate the gene to produce mutant protein. Our transformants will be crossed to flies expressing GAL4 under the tubulin promoter to allow for ubiquitous expression of UAS-Smn in the flies. The flies will then be

assessed for survival to determine if the mutations extend the lifespan of the flies past larval lethality.

Aim 2

Mouse Breeding. An intermediate SMA mouse model (*SMN2 +/+; Smn -/-; SMNΔ7 +/+*) was previously developed by Le, et al (2005). These Δ7 mice live an average of 14 days and express SMNΔ7 protein and low levels of full length SMN protein. To investigate if increased expression of SMN in mature neurons or muscle would extend survival past 14 days, intermediate SMA mice (*SMN2 +/+; Smn +/-; SMNΔ7 +/+*) were crossed to mice containing the ChAT-SMN transgene (*SMN2 +/+; Smn +/-;* ChAT-SMN) or HSA-SMN transgene (*SMN2 +/+; Smn +/-;* HSA-SMN). ChAT-SMN expresses high levels of SMN in mature motor neurons under the choline acetyltransferase promoter beginning at postnatal day 5 (PND05) (44). HSA-SMN expresses high levels of SMN under the human skeletal actin promoter in muscle tissue only (42). **Table 2** summarizes the results. A total of 10 intermediate SMA animals positive for ChAT-SMN were produced and had a mean survival of 13.9 days. One intermediate SMA mouse with HSA-SMN resulted from the crosses and lived for 12.5 days. Intermediate SMA mice crossed to both ChAT-SMN and HSA-SMN resulted in three progeny that survived an average of 16.8 days. Overall, the 14 intermediate SMA mice that resulted from these crosses lived an average of 14.4 days.

Genotype	Number of Animals	Average Days Survived
Intermediate SMA mice	**Ref. 1**	**14**
Intermediate SMA mice + ChAT-SMN	**10**	**13.9**
Intermediate SMA mice + HSA-SMN	**1**	**12.5**
Intermediate SMA mice + ChAT-SMN+ HSA-SMN	**3**	**16.8**
Total Animals	**14**	**14.4**

Table 2. Summary of mouse genotypes and survival

Increased expression of SMN in mature neurons under the ChAT promoter or in muscle only under the human skeletal actin promoter does not appear to rescue intermediate SMA mice. Neither transgene was able to significantly extend survival of the SMNΔ7 mice past the normal 14 days by itself or in combination with each other.

DISCUSSION

Aim 1. The five *SMN* missense mutations introduced in to Drosophila *Smn* were chosen because they occur in amino acids of 100% identity between the two species and for the severity of the mutation in humans. The flies will be crossed onto a *Smn* null background and survival will be assessed to see if the mutant protein allows the flies to live past the larval lethal stage.

I would predict the milder *SMN* mutations allow for survival. While it is unknown what role of SMN is affected in SMA, it could be either snRNP assembly or another unknown function specific to motor neurons. The milder mutations like D44V and G95R are predicted to result in the least disruption of SMN's function in SMA.

Flies that survive past the larval stage will be used for snRNP assays to measure the level of snRNP assembly that can help determine if there is a correlation between disruption of snRNP assembly and the severity of SMA. If snRNP assembly is the SMN complex implicated in SMA, it would be expected that severe *SMN* mutations would have the greatest reduction in snRNP levels and milder *SMN* mutations would have a mildly reduced disruption of snRNP assembly. The results from snRNP assays will help researchers to move closer to identifying the role of SMN that is disrupted in motor neurons and leads to SMA. If a mutation rescues flies then the mutation will be introduced into mice to generate a mammalian model of SMA. While Drosophila is a powerful genetic model that allows a fast and easy way to screen the mutations, a mouse model may more closely mimic the human phenotype.

Aim 2. Expressing increased levels of SMN in mature neurons or muscle did not increase survival of intermediate SMA mice past the normal 14 day lifespan. Furthermore, increased expression of SMN in muscle tissue in addition to increased expression in mature neurons did not result in rescue of the animals either.

In a recently published study, severe SMA mice were rescued by the increased expression of SMN in neurons during embryonic development whereas expression of SMN in muscle only was insufficient to rescue these mice. Severe SMA mice live an average of 5 days (30). Full-length SMN was expressed under the Prion promoter (PrP) and reached high levels in neurons at embryonic day 15 (50). These mice survived an average of 297 days, with expression of PrP-SMN resulting in complete rescue (42). Expression of HSA-SMN in muscle resulted in no extension of survival (42). The authors concluded that high expression of full-length SMN in neurons can correct the severe SMA phenotype in mice, while high SMN levels in muscle alone has no impact.

ChAT-SMN was also previously shown not to rescue severe SMA mice. While PrP-SMN expression is high embryonically, high expression of ChAT-SMN occurs later at PND05. This coincides with the time of death of the SMA animals. In this model it is likely that the increased expression of SMN was occurring too late to rescue the severe SMA mice. Since intermediate SMA mice live an average of 14 days, it was plausible that ChAT-SMN expression could rescue these mice. The results indicate that ChAT-SMN either does not express SMN early enough or not at a high enough level to rescue intermediate SMA mice or it needs to be expressed in more than just motor neurons.

These results taken in context with the study expressing PrP-SMN and HSA-SMN in severe SMA mice indicate timing and levels of expression is important for rescue and high SMN expression needs to take place at least before PND05 in severe and intermediate SMA mice. The results also indicate that high expression of SMN in muscle alone is not sufficient to rescue severe or intermediate SMA mice. SMN expression in muscle in addition to SMN expression in mature neurons in intermediate SMA mice also is not sufficient to rescue. This suggests that earlier expression of SMN in motor neurons or additional neural subtypes is required for rescue of intermediate SMA mice.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Drosophila *Smn* cDNA (clone LD36939) was obtained from the Drosophila Genomic Resource Center. The 841bp *Smn* fragment was previously cloned into the pOT2 vector between EcoRI and XhoI. SMA missense mutations D44V, V94G, G95R, Y130C, and G279V (D20V, V72G, V73G, Y107C, and G210V in Drosophila) were selected for site-directed mutagenesis. Stratagene QuikChange Site-Directed Mutagenesis Kit protocol was followed. The PCR was conducted in a 50 μl reaction with 5 μl 10x *PfuTurbo* reaction buffer, 50 ng dsDNA template, 125 ng forward and reverse oligonucleotide primers, 1 μl 10 mM dNTPs, and 1 μl PfuTurbo DNA polymerase (2.5 U/μl, Stratagene). The PCR primers for D20V were 5′-GGTGAAGACGTACG**T**CGAGTCGGTGGGAC-3′ and 5′-GTCCCACCGACTCG**A**CGTACGTCTTCACC-3′, for V72G were 5′-CAGTATCCTTCAAAG**G**AGGCGGCGACTACGCCAG-3′ and 5′-CTGGCGTAGTCGCCT**C**CTTTGAAGGATACTG-3′, for G73R were 5′-GTATCCTTCAAAGTA**C**GCGACTACGCCAGGG-3′ and 5′-

CCCTGGCGTAGTCGC**G**TACTTTGAAGGATAC-3′, for Y107C were 5′-CTCCGCTATTTGGGCT**G**TGAGAACGAGCAGGAG-3′ and 5′-CTCCTGCTCGTTCTCA**C**AGCCCAAATAGCGGAG-3′, and for G210V were 5′-CGGGCCTCTACCAGG**T**AAAGAAAGAAGCCAG-3′ and 5′-CTGGCTTCTTTCTTT**A**CCTGGTAGAGGCCCG-3′. PCR reaction conditions are as follows: 95℃ for 2 minutes; 12 cycles of 95℃ for 30 seconds and 68℃ for 11 minutes. The methylated, non-mutated parental strand was digested with 2 μl *DpnI* (Invitrogen) for one hour at 37℃. The *DpnI* digested product was transformed into XL1 Blue supercompetent cells (Stratagene) according to the manufacturer's protocol and grown on LB and chloramphenicol plates to screen for potential mutants.

Subcloning into pUAST vector. Drosophila *Smn* in the pOT2 vector and a separate sample of the pUAST vector were digested in a 40 μl reaction with EcoRI and XhoI and run out on a 0.7% agarose gel to separate the fragments. The 841 bp fragment corresponding to *Smn* and an approximately 9 kb fragment corresponding to the pUAST vector were excised and gel purified using the QIAquick Gel Extraction kit (Qiagen #28706). The *Smn* fragment and linearized vector were then ligated using DNA ligase. The ligation product was transformed into DH5α cells and plated on LB and ampicillin plates. DNA was purified using a phenol/chloroform extraction. Possible positive clones were screened by digestion with (1) EcoRI and XhoI to determine if they contained the insert and (2) PshAI and XhoI for D20, BsaJI and EcoRI for G210, MnlI for V72 and RsaI for G73. Clones were also screened through whole colony PCR using the primers T7 5′- TAATACGACTCACTATAGGG -3′ and flySmn1R 5′-GATGGAATTACTTCTTGGGTGTC-3′ to amplify the *Smn* insert in the pOT2 vecor and pUAST 1F 5'-CAAAGTGAACACGTCGCTAAGC-3' and flySmn1R 5′-

GATGGAATTACTTCTTGGGTGTC-3′ to determine the presence of the *Smn* insert in the pUAST vector. The reaction conditions were as follows: 95°C for 5 min; 30 cycles of 95°C for 1 min, 59°C for 1 min, 72°C for 1 min; 72°C for 5 min.

The PCR product of potential clones containing the insert was then digested with the restriction enzyme indicated above as a diagnostic for the mutation. DNA was purified using the QIAprep Spin Miniprep kit (Qiagen #27106) and positive clones were confirmed through sequencing at the Plant-Microbe Genomics Facility at The Ohio State University.

Germline transformation in *Drosophila.* Injection DNA of sequenced clones was obtained by using the QIAGEN Plasmid Midi Kit (Qiagen #12143). Collection chambers with w^- fruitflies were set up several days in advance by Dr. McGovern. Embryo collections were taken the day of injections on an hourly basis. Embryos were dechorionated with 50% bleach for 2 minutes and rinsed with water. Embryos were aligned on an apple juice plate slice horizontally with the micropyle facing the left and transferred to a strip of tape. The embryos were desiccated for 3 minutes and 30 seconds on drying pellets in a large petri dish and then covered with a mixture of 90% 700 Halocarbon oil, 10% 95 Halocarbon oil (Sigma). DNA containing *Smn* in the pUAST vector was co-injected with Δ2-3 transposase and buffer by Dr. McGovern. Transformants were identified by crossing to w^-flies and detecting the presence of orange to red eyes in the next generation.

Mouse genotyping. PCR reactions were set up to determine the presence or absence of mouse *Smn*, mouse knockout, *SMN2*, HSA-SMN, ChAT-SMN, Δ7, and PrP-SMN depending on the genotypes of the parents. The primers used to detect mouse *Smn* were mSMNex2AF 5′-TTTTCTCCCTCTTCAGAGTGAT-3′ and mSMNex2BR 5′-CTGTTTCAAGGGAGTTGTGGC-3′. The mouse knockout allele was genotyped with primers

mSMNex2AF 5′-TTTTCTCCCTCTTCAGAGTGAT-3′ and Bgal3R 5′-GAGTAACAACCCGTCGGATTC-3′. *SMN2* copy number was confirmed using primers that flank the insertion site of the *SMN2* transgene. The primers are as follows: Tg89F 5′-CTGACCTACAGGGATGAGG-3′, Tg89Grm7negativeR 5′-CCCAGGTGGTTTATAGACTCAGA-3′, and Tg89SMNpositiveR 5′-GGTCTGTTCTACAGCCACAGC-3′. HSA-SMN was detected with the primer HSA 5F 5′-GAGCCGAGAGTAGCAGTTGT-3′ and a SMN exon 1-2 junction primer 1.2R 5′-AGAATCATCGCTCTGGCCTGTGCC-3′. ChAT-SMN was genotyped using the primer Chat5F 5′-CTGGATTAAGAATCGCTAGG-3′ and an exon 3 primer 2.3R 5′-AGTAGATCGGACACAGATTTTGCT-3′. The primers used to genotype Δ7 were SMNproF 5′-TGGAGTTCGAGACGAGGCCTAAGC-3′ and 2.3R 5′-AGTAGATCGGACACAGATTTTGCT-3′. Prp-SMN was detected using the primers Prion ex2 F 5′-GGACTCGTGAGTATATTTCAG-3′ and the SMN exon 1-2 junction primer 1.2R 5′-AGAATCATCGCTCTGGCCTGTGCC-3′. The thermal cycling conditions for mouse *Smn* and mouse knockout allele PCR reactions are 95℃ for 3 min; 35 cycles of 95℃ for 1 min, 57℃ for 1 min, 72℃ for 1 min; 72℃ for 5 min. The thermal cycling conditions for *SMN2* PCR reaction are 94℃ for 3 min; 30 cycles of 94℃ for 1 min, 63℃ for 1 min, 72℃ for 1 min; 72℃ for 10 min. The thermal cycling conditions for HSA-SMN, ChAT-SMN, Δ7, and Prp-SMN PCR reactions are 95℃ for 5 min; 30 cycles of 95℃ for 1 min, 59℃ for 1 min, 72℃ for 1 min; 72℃ for 5 min.

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