Plastin 3 Expression Does Not Modify Spinal Muscular Atrophy Severity in the Δ7 SMA Mouse

Vicki L. McGovern

Aurelie Massoni-Laporte

Xueyong Wang
Wright State University - Main Campus, xueyong.wang@wright.edu

Thanh T. Le

Hao T. Le

See next page for additional authors

Follow this and additional works at: https://corescholar.libraries.wright.edu/ncbp

Part of the Medical Cell Biology Commons, Medical Neurobiology Commons, Medical Physiology Commons, Neurosciences Commons, and the Physiological Processes Commons

Repository Citation
McGovern, V. L., Massoni-Laporte, A., Wang, X., Le, T. T., Le, H. T., Rich, M. M., & Burghes, A. H. (2015). Plastin 3 Expression Does Not Modify Spinal Muscular Atrophy Severity in the Δ7 SMA Mouse. PLOS ONE. https://corescholar.libraries.wright.edu/ncbp/1081

This Article is brought to you for free and open access by the Neuroscience, Cell Biology & Physiology at CORE Scholar. It has been accepted for inclusion in Neuroscience, Cell Biology & Physiology Faculty Publications by an authorized administrator of CORE Scholar. For more information, please contact library-corescholar@wright.edu.
Authors
Vicki L. McGovern, Aurelie Massoni-Laporte, Xueyong Wang, Thanh T. Le, Hao T. Le, Mark M. Rich, and Arthur H. M. Burghes

This article is available at CORE Scholar: https://corescholar.libraries.wright.edu/ncbp/1081
Plastin 3 Expression Does Not Modify Spinal Muscular Atrophy Severity in the Δ7 SMA Mouse

Vicki L. McGovern, Aurélie Massoni-Laporte, Xueyong Wang, Thanh T. Le, Hao T. Le, Christine E. Beattie, Mark M. Rich, Arthur H. M. Burghes

1 Department of Molecular and Cellular Biochemistry, The Ohio State University Wexner Medical Center, Columbus, Ohio, United States of America, 2 Department of Neuroscience, Cell Biology, and Physiology, Wright State University, Dayton, Ohio, United States of America, 3 Department of Neuroscience, The Ohio State University Wexner Medical Center, Columbus, Ohio, United States of America, 4 Department of Neurology, The Ohio State University Wexner Medical Center, Columbus, Ohio, United States of America

* These authors contributed equally to this work.
* burghes.1@osu.edu

Abstract

Spinal muscular atrophy is caused by loss of the SMN1 gene and retention of SMN2. The SMN2 copy number inversely correlates with phenotypic severity and is a modifier of disease outcome. The SMN2 gene essentially differs from SMN1 by a single nucleotide in exon 7 that modulates the incorporation of exon 7 into the final SMN transcript. The majority of the SMN2 transcripts lack exon 7 and this leads to a SMN protein that does not effectively oligomerize and is rapidly degraded. However the SMN2 gene does produce some full-length SMN and the SMN2 copy number along with how much full-length SMN the SMN2 gene makes correlates with severity of the SMA phenotype. However there are a number of discordant SMA siblings that have identical haplotypes and SMN2 copy number yet one has a milder form of SMA. It has been suggested that Plastin3 (PL3) acts as a sex specific phenotypic modifier where increased expression of PL3 modifies the SMA phenotype in females. To test the effect of PL3 overexpression we have over expressed full-length PL3 in SMA mice. To ensure no disruption of functional or post-translational processing of PL3 we did not place a tag on the protein. PL3 protein was expressed under the Prion promoter as we have shown previously that SMN expression under this promoter can rescue SMA mice. High levels of PL3 mRNA were expressed in motor neurons along with an increased level of PL3 protein in total spinal cord, yet there was no significant beneficial effect on the phenotype of SMA mice. Specifically, neither survival nor the fundamental electrophysiological aspects of the neuromuscular junction were improved upon overexpression of PL3 in neurons.
Introduction

Proximal Spinal Muscular Atrophy (SMA) is an autosomal recessive disorder and the leading genetic cause of infant mortality [1, 2]. The disorder is characterized by loss of alpha motor neurons in anterior spinal cord and atrophy of muscle [3]. SMA is caused by loss of the Survival Motor Neuron 1 gene (SMN1) and retention of SMN2 [4, 5]. The SMN1 and SMN2 genes essentially differ at a single nucleotide within exon 7 that results in disruption of a splice modulator and the majority of the SMN2 transcript lacking exon 7 [6–10]. The SMN lacking exon 7 encoded amino acids does not efficiently oligomerize and thus is rapidly degraded [11, 12]. This leads to low SMN levels in SMA [13, 14]. The major modulator of the SMA phenotype is the SMN2 gene itself as it does produce some full-length SMN protein. Specifically, there is an inverse correlation of SMN2 copy number to phenotypic severity where mild type III SMA cases have more copies of SMN2 than severe type 1 cases [15, 16]. Furthermore, certain SMN2 alleles, namely the variant 859G>C, alters the incorporation of SMN exon 7 and thus results in greater SMN production [17, 18]. Indeed, a study of Spanish SMA patients showed that type 1 patients do not have this variant, type 2 patients (with two copies of SMN2) are heterozygous for this variant, and type 3b patients (with two copies of SMN2) are homozygous for this variant [19]. Thus there is a clear relationship between the amount of full-length SMN that can be produced by a particular genotype and the severity of SMA. However there are some discrepancies to the SMN2 copy number rule. In particular, there are reports on a series of families where siblings with identical haplotypes, including SMN2 copy number, have markedly different phenotypes [15, 20–25]. While this may be more common in type 2 and 3 cases [20, 22, 23] siblings with discordant phenotypes also occur with type 1 SMA [24, 26–28]. Furthermore, there appears to be a gradation of phenotypic severity where type 1 and type 2 SMA occurs in the same family, or type 2 and type 3 SMA, or type 3 SMA and unaffected siblings. This strongly implies that the modifier of SMA phenotype can alter all SMA types in much the same way that SMN2 copy number alters SMN levels and SMA.

There are two major paths that can be considered for modification of SMA phenotype in discordant siblings. First, there could be alteration in a factor that acts on the SMN2 loci to influence the amount of full-length SMN produced by SMN2. In this regard, many proteins have been reported to bind SMN exon 7 and the surrounding introns to regulate the incorporation of exon 7 [29, 30]. The second possibility involves modifiers of the SMA phenotype that do not alter SMN levels. SMN has been shown to function in the assembly of Sm proteins onto snRNA to form SnRNPs and has been suggested to play a role in a series of other assembly reactions [31]. SMN has also been proposed to have other functional roles particularly in the axon. Indeed knockdown of Smn in zebrafish results in axonal abnormalities and motor neurons cultured from severe mice have been reported to have reduced transport of β-actin to the growth cone [32, 33]. However there is no defect of axon out growth in vivo in the SMA mouse embryo [34]. Genes that could influence these SMN functions or their outcome might act as modifiers of the SMA phenotype.

Studying discordant families has produced reports of potential modifiers of SMA. Originally it was reported that SMN levels were elevated in fibroblast cultures of milder cases in discordant families [35]. Subsequently the levels were reported to be the same [36] at least in lymphoblasts. Analysis of the expression changes that occur in lymphoblasts revealed that PLS3 (PLS3, T-Plastin, or T-fimbrin; MIM 300131, Xq23) had elevated levels in some siblings with discordant phenotypes. The authors concluded that PLS3 acts as a female specific modifier of SMA. All the sib pairs except one contained a male and female where the female was asymptomatic. Thus, even if expression of PLS3 were found in the male patients it would not improve the phenotype. As such it is difficult to interpret the significance of this observation based on a single
female sib pair. In a second study no association of PLS3 expression was found in discordant female sib pairs [37]. In fact, PLS3 expression was slightly increased in the affected female sibling and not the asymptomatic individual. Recently, a tagged form of PLS3 protein has been investigated in SMA mice [37]. The authors report some mild benefits to the SMA phenotype under certain conditions. However, PLS3 expression is highly modulated at the protein level and the placement of a tag can affect both function and protein turnover [37]. We thus have investigated whether the overexpression of PLS3 without a tag can modify the SMA phenotype in mice.

In order to determine if PLS3 acts as a modifier of the SMA phenotype we generated transgenic mice expressing human PLS3 under control of the Prion (PrP) promoter. We have shown previously that the PrP:SMN transgene resulted in high expression of SMN in all neurons completely rescued the SMA phenotype in the mouse [38]. We proposed that if PLS3 is a SMA modifier then high expression of human PLS3 under control of this same promoter should alter survival and phenotype of Δ7 SMA mice. We found no increase in weight or survival of PrP:PLS3, Δ7 SMA mice in three different transgenic lines. Furthermore, we found no improvement in neuromuscular junction physiology in these PrP:PLS3 Δ7 SMA mice. Our results indicate that PLS3 is not a viable therapeutic target to modify the SMA phenotype in humans.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the University Laboratory Animal Resources at The Ohio State University and Wright State University. Our protocol was approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC), Office of Responsible Research Practices, under Permit Number 2008A0089. Anesthesia was administered with Isoflurane according to our animal protocol. Carbon Dioxide followed by cervical dislocation for secondary means of confirmation was used for euthanasia according to our approved protocol.

Generation of PLS3 expressing transgenes

Human PLS3 cDNA (Clone ID 6064540, Open Biosystems) was end filled and cloned into the Prion (PrP) vector that contains the mouse prion promoter and exon 1, intron 1, part of exon 2 [34]. Human PLS3 cDNA was directionally cloned between the end filled KpnI and XhoI/SalI sites in PrP vector exon 2. The resulting construct was sequenced, linearized by digestion with PvuI, gel purified and dialyzed. The PrP:PLS3 plasmid was transfected into MN1 cells using Lipofectamine 2000 Transfection Reagent according to the manufacturer’s instructions (Invitrogen). After expression PLS3 was confirmed by Western blot the construct was injected into fertilized FVB/N Δ7 (JAX 5025) mouse oocytes to generate transgenic mice. The PrP:PLS3 transgene was detected with PrP exon 2 FP 5′ GGACTCGTGAGTATATTCCAG and PLS3 RP 5′ GAAGGCTTGCAAATCCTACT. Three founder mice, named PLS-14, PLS-39 and PLS-46, were bred to Δ7 mice (SMN2+/−, SMN1−/−:Δ7SMN1−/−). PLS-14 female founder would not breed therefore an ovary transfer was performed into a FVB/N female and then progeny were bred to Δ7 mice. The SMN2 transgene and mouse knockout allele were detected as previously described [38, 39]. PLS-39 and PLS-46 lines were bred to homozygosity, PLS-14 is not homozygous viable. Each transgenic line conformed to Mendelian autosomal patterns of inheritance thus multiple transgenic insertion sites were not detected. Homozygosity of the transgene was determined by qPCR on genomic tail DNA.
Weight and survival measurements

Mice were housed and fed at no more than 5 per cage according to our IACUC approved animal protocol and the Standard Operating Procedure for SMA mice SMA_M.2.2.003 (Treat-NMD.eu). Weight and survival analysis was performed as previously described for the Δ7 line [40, 41]. A similar number of male and female mice were observed and weighed at minimum once per day from the date of birth (P0) until death or day 21 (P21). Any change in behavior, appearance or survival was noted. Mice where humanely euthanized when they achieved exclusion criteria including the inability of neonatal SMA mice to go to the mother (homing) to suckle and loss of greater than 20% of maximum weight that particular animal achieved according to our IACUC approved animal protocol. Required steps were taken to minimize suffering of the mice including administration of systemic analgesia (Motrin) in the water bottle at 100mg/5ml providing a dose of approximately 30 mg/kg when needed. All animals were grouped according to genotype. Kaplan-Meier survival curves and mean weights were graphed with SigmaPlot.

Expression of PLS3 in Brain and Spinal cord of PrP:PLS3 SMA mice

RNA was isolated from brain and total spinal cord at P12 using TRIzol reagent (Invitrogen), purified with the RNeasy kit (Qiagen) and converted to cDNA as previously described [38]. Primers used to detect cDNA include: Prion:PLS3 transgene, FP: 5’ CCGATCACGAGACCCGATTCT, RP: 5’ GCACCTCGGAAATCTTGTCA, probe: FAM–ATCGGTGGCGAGACT–MGB; mouse Pfs3, FP: 5’ CCGCAGTCCCTATGAGTCTT (mouse specific), RP: 5’ GAGTTCTCAAGCTCTATCCTTTTGGA, Probe:FAM–ACATGGATGAGAAGGGC–MGB; Mouse cyclophilin, FP: 5’ GTCAACCCCACC GTGGATCTT, RP: 5’ TTGGAACCTTGTGCTGCAACA, Probe: VIC–CTTGCGCCGCTCT–MGB.

Reactions were run on the ABI 7300 Real-Time PCR System. Relative human and mouse PLS3 levels were determined by normalizing to mouse cyclophilin (PIPB) expression. Three technical replicates and five to seven biological replicates were performed for each sample. All three transgenic lines as well as a non-transgenic control were tested.

Expression of PLS3 in LCM isolated motor neurons of PrP:PLS3 SMA mice

Motor neurons were collected from fresh frozen spinal cord sections on a Zeiss Palm Robo 3 Laser Capture Microdissection System. Motor neurons were located based on size and location in the anterior horn after Nissl staining for contrast. RNA was isolated with the Ambion RNA- aqueous Micro kit (AM1931) and aRNA was generated with the Arcturus PicoPure RNA Isolation Kit (ABI KIT0204). Droplet generation and reader analysis were performed on the QX200 (Bio-Rad). 15,000 to 18,000 droplets containing cDNA, primers, probe, 2x ddPCR SuperMix for Probes, and droplet generation oil were generated and amplified. Primers used to detect cDNA include: Prion:PLS3 transgene, FP: 5’ CCGATCACGAGACCCGATTCT, RP: 5’ GCACCTCGGAAATCTTGTCA, probe: FAM–ATCGGTGGCGAGACT–MGB; mouse Pfs3, FP: 5’ CCGCAGTCCCTATGAGTCTT (mouse specific), RP: 5’ GAGTTCTCAAGCTCTATCCTTTTGGA, Probe:FAM–ACATGGATGAGAAGGGC–MGB; Mouse cyclophilin, FP: 5’ GTCAACCCCACC GTGGATCTT, RP: 5’ TTGGAACCTTGTGCTGCAACA, Probe: VIC–CTTGCGCCGCTCT–MGB. A sufficient number of positive and negative droplets were read by the QX200 reader and quantified using the QuantaSoft software (Bio-Rad). The concentration of transcripts was determined using Poisson statistical distributions and relative human or mouse plastin levels were determined by normalizing to mouse cyclophilin expression. Two technical replicates (for a total of >20,000 droplet PCR reactions) and three biological replicates were performed for each sample. All three transgenic lines as well as a non-transgenic control were tested.
Protein expression of PLS3 spinal cord

Brain and spinal cord tissues were harvested from 3 PrP:PLS3 male mice at P10 for each transgenic line and non-transgenic controls. Protein isolation and western blots were performed as previously described [42]. The antibody used to detect PLS3 (1:250, GenTEX, 103323) is not specific for human PLS3 thus the total amount of mouse and human PLS3 protein was detected. Accurate size detection of the PLS3 protein was confirmed by detecting PrP:PLS3 protein isolated from transfected MN-1 cells. Blots were incubated with anti-rabbit Fab fragment HRP (1:10,000, Jackson Immunoresearch, 111-035-006). Three concentrations of the same protein sample were loaded on the gel (90µg, 45µg and 25µg). Blots were probed with mouse anti beta-tubulin mAb (1:10,000, Abcam Ab7291) to measure protein loading and developed using the ECL system as described by the manufacturer (GE Healthcare Life Sciences). Blots where scanned and quantified as described (http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/) and the area under each peak determined with ImageJ software. Statistical analysis was performed with SigmaPlot. All samples collected for RNA and protein analysis were from male mice.

Zebrashed axon correction

Zebrashed embryos were maintained at ~28.5°C and staged by hours post fertilization (hpf) [43]. Transgenic Tg(mnx1:GFP)os26 [44] embryos expressing GFP in their motor axons (referred to as Tg(mnx1:GFP) embryos) were used for smn morpholino (MO) and human PLS3 mRNA injections. The antisense smn MO was described previously by McWhorter et al. [33]. One cell-stage embryos were injected with 9ng of smn MO with or without 250 pg of synthetic human PLS3 mRNA.

To generate mRNA, human PLS3 in pCMV.spot 6 vector was subcloned into pCS2+ vector and linearized with NotI. Capped RNA was generated using the Sp6 mMESSAGE mMACHINE kit (Ambion, Austin, TX) following the manufacturer’s instructions.

To visualize motor axons, 28 hpf Tg(mnx1:GFP) embryos were anesthetized with tricaine (160 µg/ml) and fixed overnight at 4°C in 4% formaldehyde/PBS. After removing from fix, embryos were mounted on glass cover slips for observation under a Zeiss Axioplan microscope, scored [45] and imaged on a Leica confocal microscope. Ten motor axons were scored per animal and animals were designated as containing severe, moderate, mild, or no defects based on criteria in Carrel et al. [45]. Three separate experiments were performed and for each condition (control, smn MO and smn MO + PLS3 RNA), n was between 19–24 embryos. Data was plotted as mean ± SEM for the three experiments and Mann-Whitney non-parametric rank test was used to test significance.

Electrophysiological recording from neuromuscular junctions (NMJs)

Physiology was performed on P10–P11 mouse NMJs from the tibialis anterior muscle as previously described [46]. Briefly, muscle was perfused with Ringer solution containing (in millimoles per liter): NaCl, 118; KCl, 3.5; CaCl2, 2; MgSO4, 0.7; NaHCO3, 26.2; NaH2PO4, 1.7; glucose, 5.5 (pH 7.3–7.4, 20–22°C) equilibrated with 95%O2 and 5% CO2. All NMJs were imaged by staining with 4-Di-2-ASP and impaled within 100 um of the endplate. Muscle fibers were crushed away from the endplate band and voltage clamped to -45 mV. Quantal content was determined directly by dividing evoked endplate current (EPC) amplitude by the average miniature endplate current (MEPC) amplitude for a given NMJ. Repetitive stimulation was given by applying a 50 Hz train of 10 pulses. Statistics. All data are expressed as mean ± SEM.
Statistical analysis

Quantitative data are expressed as mean ± SEM. Values for number of animals are given in Results and the figure legends. Kaplan-Meier survival curves were generated with SigmaPlot and statistical significance was determined using the log-rank test. The Holm-Sidak method was used for all pairwise multiple comparisons. Significance of weight data were determined with one-way ANOVA and by the Compare Growth Curve function found in the R-Package (Statmod). Specific tests for qPCR, ddPCR and western blot analysis are as described in the figure legends. Values of p<0.05 were considered significant.

Results

Generation of PLS3 expressing transgenes

We have previously expressed SMA mice using SMN driven by the prion promoter (PrP) [38]. Thus this promoter expresses in the required spatial and temporal pattern in the nervous system. The construct contains human PLS3, without a tag, expressed under the mouse prion promoter. While tagging a protein at the amino terminus is useful in detection of the protein, the tag can alter protein function and/or the decay of the protein [47, 48]. The construct used for generating the transgenes is diagrammed in Fig 1. The construct was injected into the pronucleus of fertilized FVB/N Δ7 (JAX 5025) mouse oocytes. A total of 3 expressing lines named PLS-14, PLS-39 and PLS-46 were obtained. The PrP-PLS3 lines were crossed to the Δ7 SMA mouse model to obtain mice that contained the PrP:PLS3 transgene, two copies of SMN2, and the SMNΔ7 transgene (PrP:PLS3 Δ7/Δ7; SMN2 Δ7/Δ7; SMNΔ7 Δ7/Δ7; Smn Δ7/Δ7). The progeny for each line were interbred to obtain SMA mice containing the PrP:PLS3 transgene (PLS-14 Δ7/Δ7; SMN2 Δ7/Δ7; SMNΔ7 Δ7/Δ7; Smn Δ7/Δ7), (PLS-39 Δ7/Δ7; SMN2 Δ7/Δ7; SMNΔ7 Δ7/Δ7; Smn Δ7/Δ7), (PLS-46 Δ7/Δ7; SMN2 Δ7/Δ7; SMNΔ7 Δ7/Δ7; Smn Δ7/Δ7). Mice that were homozygous for the PLS-14 transgene were not viable.

Functionality of PLS3 coding sequence used to generate transgenic mice

To test whether the PLS3 cDNA used in generating our transgenic lines encoded functional PLS3 protein, we tested PLS3 mRNA in zebrafish. Decreasing smn transiently in zebrafish embryos using an smn morpholino has been shown to result in motor axon defects [33]. Furthermore, injecting human PLS3 RNA into these smn morphants rescued the axonal defects [36, 49]. Therefore, we tested the PLS3 sequence used to construct our transgene in this same assay and found that it was able to significantly rescue the smn morphant motor axon defects (Fig 2). This finding demonstrates that the PLS3 coding sequence used to generate our transgenic mice produced functional PLS3 protein.

![Diagram of the PrP:PLS3 construct](https://example.com/diagram.png)

**Fig 1. Diagram of the PrP:PLS3 construct.** A construct containing the mouse Prion (PrP) promoter, exon 1, intron 1 and part of exon 2 was fused to the human PLS3 cDNA. This same promoter was used previously to express SMN in neurons [49]. Arrows indicate the location of PrP exon 1 forward primer and PLS3 reverse primer used to specifically amplify PLS3 transcripts produced by this transgene.

doi:10.1371/journal.pone.0132364.g001
**Fig 2. Coding sequence of the plastin 3 transgene is functional.** (A) Lateral view of 28 hpf Tg(mnx1:GFP) showing ventrally extending motor axons of uninjected, smn morpholino (MO) injected, or smn MO + plastin 3 RNA (PLS3) injected embryos. (B) Embryos from three separate experiments (n = 19–24 embryos/experiment) were scored as having severe, moderate, mild, or no defects based on criteria in Carrel et al [45]. Mean ± SEM was plotted and significance was determined by two-tailed Mann-Whitney non-parametric rank test. *p = 0.0001, **p<0.0001.

doi:10.1371/journal.pone.0132364.g002

**Expression of PLS3 in brain and spinal cord tissue**

To determine expression of PrP:PLS3 in the brain and spinal cord we used quantitative qRT-PCR. Endogenous mouse Pls expression ([Fig 3A and 3B]) and human PLS3 expression ([Fig 3C and 3D]) was measured by quantitative RT-qPCR in the brain ([Fig 3A and 3C]) and in total spinal cord ([Fig 3B and 3D]) tissue at P10 for each transgenic line (PLS-14, PLS-39 and PLS-46). We found that the expression of the PrP:PLS3 transgene was nearly 100 fold increased over endogenous mouse Pls3 levels in both brain and spinal cord in all three transgenic lines. (n = 5–7 for each transgenic line and tissue). We used primers located in PrP exon 2 and PLS3 exon 1 to specifically detect the transgenic expression of human PLS3 from PrP:PLS3. Mouse Pls was specifically amplified using a forward primer that was unique to mouse Pls. The highest
level of PLS3 expression was found in line PLS-14 which showed a 300-fold increase in total spinal cord samples.

Expression of PLS3 in motor neurons

To ensure that PrP:PLS3 was indeed expressed in neurons we sectioned P10 lumbar spinal cord (L3-L5) from each transgenic line. The motor neurons were isolated by laser capture microdissection (LCM) and RNA was extracted. One round of aRNA amplification (Arcturus) was followed by quantitative PCR using droplet digital PCR (ddPCR, Bio-Rad). The amount of PLS3 expression detected in the motor neuron is more than 100x greater than the endogenous
Fig 4. Expression of PLS3 and endogenous mouse Pls3 in LCM isolated motor neurons. Plasmin expression was measured by quantitative RT-ddPCR in the motor neurons isolated from lumbar spinal cord tissue at P10 for each transgenic line (A) PLS3 expression is greatest in transgenic line PLS-14, (PLS-14: 170.5±45.3 p<0.05 vs. control, t-test, PLS-39: 151.0±57.7, PLS-46: 96.2±27.8, control: 0±0.0 RFU). Only PLS-14 expression was statistically different from control thus we pursued this line for protein analysis. PLS3 expression was not detected in the non-transgenic control motor neurons indicating the specificity of our primers. (B) Expression of mouse Pls3 is unchanged in the transgenic PrP:PLS3 lines when compared to a non-transgenic control, (PLS-14: 0.9±0.3, PLS-39: 1.4±0.2, PLS-46: 1.2±0.3, control: 1.1±0.1 RFU, not statistically significant from control.) Note that overexpression of human PLS3 is more than 100 fold greater than the amount of mouse Pls3 expression in the motor neuron. These results are similar to the expression assayed by qPCR in total spinal cord samples. (n = 3 mice for each transgenic line and control). RFU is defined as Relative Fluorescent Units.

doi:10.1371/journal.pone.0132364.g004

mouse Pls3 expression for each transgenic line examined (Fig 4A). The level of mouse Pls3 expression for each transgenic line was no different from that of a non-transgenic age matched control (Fig 4B).

Total Plasmin protein expression in spinal cord tissue

We next examined the expression of total PLS3 protein. The Plasmin antibody used was first tested to ensure that it reacted with PLS3 protein at the correct size (~70kD). MN-1 cells were transiently transfected with the PrP:PLS3 construct and the approximately 70kd PLS3 protein was detected by Western blot on transfected cells. In the case of transfected MN-1 cells a marked increase in total PLS3 expression was observed at the protein level. Western blot analysis of total spinal cord from P10 mice expressing PLS3 is shown in Fig 5. Despite a 100-fold increase in PLS3 mRNA expression, the increase in total PLS3 protein levels was only 2 fold when compared to non-transgenic animals. There was a significant increase in PLS3 protein in the PLS-14 line (p<0.005) (Fig 5A). This result is similar to the findings of Ackerman et al in which the PLS3 transgene was tagged and therefore more easily detected [50]. However the levels indicated for total PLS3 and mouse Pls3 combined are similar to Figure 7 in Ackerman et al. [50]. Thus PLS3 mRNA is dramatically increased in the spinal cord but post-translational regulation mechanisms present in the mouse limit the level of PLS3 protein that can be obtained. Any regulation of the PLS3 protein is important to consider in determining if PLS3 expression alters SMA.

Effect of PLS3 expression on SMA phenotype

We measured the weight of PrP:PLS3 mice to determine if overexpression of PLS3 in neurons increased the weight of Δ7 SMA the mouse. We found that the weight is not increased in three PrP:PLS3 transgenic lines in the Δ7 SMA mouse (Fig 6). The weight of each PrP:PLS3 transgenic line in the presence and absence of mouse Snn was measured daily until weaning at 21 days of age. The three PrP:PLS3 transgenic lines (PLS-14+/−, SMN2+/−, Smn−/−; Δ7SMN−/−),
n = 22, average max. weight 3.9 g (PLS3+/+, SMN2+/+, Smn−/−, Δ7SMN+/+, n = 20, average max. weight 3.5 g), (PLS-46+/+, SMN2+/+, Smn−/−, Δ7SMN+/+, n = 32 average max. weight 3.7 g) weigh slightly less than Δ7 SMA mice (SMN2+/+, Smn−/−, Δ7SMN+/+, n = 11, average max. weight 4.0 g). There is no statistical difference in weight between Δ7 SMA mice or any of the transgenic PrP:PLS3 expressing lines as determined by the CompareGrowthCurve function found in the R-Package (Statmod).

To determine if survival of the Δ7 SMA mouse is improved upon overexpression of PLS3 in neurons we monitored survival. Survival is not increased in three PrP:PLS3 transgenic lines in the Δ7 SMA mouse (Fig 7). The Kaplan–Meier survival curve for PLS3 transgenic lines: (PLS-14+/+, SMN2+/+, Smn−/−, Δ7SMN+/+, n = 20), (PLS-39+/+, SMN2+/+, Smn−/−, Δ7SMN+/+, n = 20), (PLS-46+/+, SMN2+/+, Smn−/−, Δ7SMN+/+, n = 30), and SMA (SMN2+/+, Smn−/−, Δ7SMN+/+, n = 58). The median survival of PLS-14; Smn−/− (14.8 ± 0.9 days), PLS-46; Smn−/− (13.6 ± 0.7 days) and Δ7 SMA mice (Smn−/−) (15.7 ± 0.4 days) were statistically different from controls (log-rank p < 0.001). Survival of and Δ7 SMA mice (Smn−/−) (15.7 ± 0.4 days) was not statistically different from PLS-14; Smn−/− (14.8 ± 0.9 days), or PLS-46; Smn−/− (13.6 ± 0.7 days) (p < 0.001, Holm-Sidak pairwise comparison). PLS-39; Smn−/− (13.4 ± 0.3 days) mice died on average 2 days before SMA controls.

Electrophysiology of PLS3 SMA mice

We and others have previously shown that early stages of SMA disease pathogenesis are characterized by functional abnormalities of the neuromuscular junction (NMJ) [46, 51–53]. It has recently been reported that PLS3 expression rescues function of the NMJ in mice with SMA [50]. In order to determine whether expression of PLS3 rescues functional NMJ abnormalities we examined the physiology of NMJs in the tibialis anterior muscle of mice on P10 to P13 as previously described [46, 51]. Each PrP:PLS3; Δ7SMA mouse was compared to an age-matched littermate that was studied on the same day.
Fig 6. Weight of each PrP:PLS3 transgenic line in the presence and absence of mouse Smn. Mice were weighted every day until weaning at 21 days of age. Each of the three PrP:PLS3 transgenic lines (PLS-14++, SMN2++; Smn++; Δ7SMN++/+, n = 22, average max. weight 3.9g), (PLS-39++, SMN2++; Smn++; Δ7SMN++/+, n = 20, average max. weight 3.5g), (PLS-46++, SMN2++; Smn++; Δ7SMN++/+, n = 32 average max. weight 3.7g) weigh slightly less than Δ7 SMA mice (SMN2++; Smn++; Δ7SMN++/+, n = 11, average max. weight 4.0g). There is no statistical difference in weight between SMA mice (Smn++) with or without the transgene.

doi:10.1371/journal.pone.0132364.g006

The most dramatic abnormality in SMA is a 60% reduction in endplate current (EPC) amplitude, which is determined by both the number of synaptic vesicles released following nerve stimulation (quantal content) and the amplitude of the muscle response to the transmitter released from a single vesicle (quantal amplitude) [46, 51]. When plasin SMA mice were compared to their unaffected littermates they had a 60% reduction in EPC amplitude (p < 0.05, Fig 7) that was very similar to the reduction we found previously in the same line of SMA mice at P10-P14 [46, 51]. The reduction in endplate current amplitude was due to both reduction in quantal content and quantal amplitude (Fig 8) with the magnitude of reduction of both parameters similar to what we found previously in SMA mice at P10-P14 [51].

Previously, we found an increase in MEPC and EPC time constants that was likely due to prolonged expression of embryonic acetylcholine receptors (AChRs) [46, 51]. A similar increase in EPC time constant was present in PrP:PLS3 SMA mice (p < 0.05, Fig 8). Finally, we and others previously found that a reduction in the probability of synaptic vesicle release as shown by increased facilitation during repetitive stimulation was a likely contributor to reduced quantal content in SMA NMJs [46, 51, 53]. A similar increase in facilitation was present in PrP:PLS3 SMA mice relative to control littermates (p < 0.05, Fig 8).
**Discussion**

The overexpression of PLS3 has been suggested to modify the SMA phenotype [36]. In particular, it has been suggested to act as a female specific modifier thus overexpression of PLS3 would only alter female SMA patients. This finding was reported after the identification of higher PLS3 expression in lymphoblasts of the less severely affected SMA individual of siblings with identical haplotypes but variant phenotype. However, an increase in PLS3 expression does not occur in all haploidentical cases. Thus it was reported that PLS3 is a female specific modifier of SMA phenotype that is not always penetrant [36]. A close examination of the 6 pedigrees studied reveals that in all but one example (family #34), the severely affected case was male [35, 36].

doi:10.1371/journal.pone.0132364.g007
Thus low PLS3 expression would not be predicted to have any impact on these individuals anyway. In essence, the initial evidence for PLS3 modifying the SMA phenotype comes down to one family where the two mildly affected female patients showed a one-fold (BW279) and 1.6 fold (BW283) increase in PLS3 transcript compared to their more severe sister (BW280) [36]. In a separate study female patients with the more severe phenotype showed high PLS3 expression compared to their less severe female siblings [54]. Thus it does not seem PLS3 expression can always modify SMA in females and it is unclear why this modification would be partially penetrant and female specific. Lastly no clear insight into how increased PLS3 expression occurs has been presented. Does it occur due to an alteration of regulatory sequence at the PLS3 locus, alteration of methylation at the PLS3 locus alteration of a transregulator of PLS3 expression or altered escape from X inactivation at that loci. In the latter case it can be noted that PLS3 has been reported to undergo X inactivation [55] and it is hard to see how this would specifically give rise to high PLS3 expression in certain individuals. Furthermore males do show high PLS3 expression but modification is not reported to occur in this case (family #800, individual LN421 found in Oprea, et al.) [36]. Regardless it is important to address the issue of how PLS3 is activated as well as why PLS3 expression is only believed to operate in certain individuals.

In a subsequent study, Stratigopoulos et al. [56] found no difference in PLS3 expression in 47 female SMA patients when all ages, SMA types or SMN2 copy number were compared. An inverse correlation between PLS3 expression and SMA severity was only identified when females were grouped by age (pre and post pubescence). No change in PLS3 expression was identified in males grouped by SMA severity or SMN2 copy number. PLS3 levels were found to be 50% lower in older males. Finally, expression of PLS3 did not correlate with the functional measures of CMAP or MUNE in males or females [56].

Recently, Yanyan et al. found higher levels of PLS3 in type 3 female SMA children (over the age of 3) compared to type 2 female children [57]. Yet the level of PLS3 expression was always higher in females than in males and correlated positively with SMN2 copy number. The level of
PLS3 was higher in SMA patients 3 to 12 years of age compared to healthy controls. Thus it is suggested that PLS3 may be playing some compensatory role in SMA, however levels of PLS3 were highest in healthy controls under age of 3. PLS3 is unlikely to be useful as a biomarker due to the alteration of expression in blood with patient age and sex [37].

Although PLS3 mRNA is elevated in lymphoblasts at the mRNA level there are differences in the reports of protein expression. Opera et al. [36] reported altered PLS3 protein levels whereas Bernal et al. [54] found that PLS3 protein levels where not detectable in lymphoblasts and not significantly altered in fibroblasts of patients that had different mRNA levels of PLS3. Similarly, in our study we observed only a 2 fold increase in PLS3 protein in transgenic mice heavily overexpressing (up to 300 fold higher) PLS3 mRNA indicating the likely occurrence of posttranslational regulation of PLS3 expression. Hao et al. [58] have reported in zebrafish that reduction of SMN resulted in reduced PLS3 protein levels whereas in the mouse Ackerman et al. [59] found that SMN levels did not alter PLS3 levels. Other studies have shown that PLS3 levels are increased under various conditions. For instance cisplatin-resistant human bladder, prostatic, and head and neck cancer cell lines express high levels of PLS3 when compared to cisplatin-sensitive cells [59]. High PLS3 levels have also been found in Sezary Syndrome patients and this was associated with loss of CD26. In addition, PLS3 positive cells showed hypomethylation of the PLS3 CpG island at sites 95–99 [60,61]. Interestingly, the polymorphism SNP PLS3 rs871773 T allele is associated with a higher protein expression of the PLS3 gene in colon cancer and an increased risk of recurrence of colon cancer [62]. If PLS3 does alter severity of SMA, defining the role of both PLS3 rs871773 and the hypomethylation of sites 95–99 is important as it gives a mechanism of PLS3 activation and may even result in a DNA marker that could be followed in patient material. However, this does not explain why increased PLS3 expression only modifies female SMA patients and is often non-penetrant. Indeed our results show no marked alteration of SMA phenotype in mice with a 100-fold increase in mRNA expression of PLS3. The studies we present here do not support a role for PLS3 in SMA. Moreover, the lack of penetrance in modifying the phenotype in males, as well as certain female cases, is difficult to reconcile.

Previously, overexpression of PLS3-V5, which contains an amino terminal tag, was reported to improve the Taiwanese model of SMA [50,63] but only very slightly and under specific conditions [50]. No improvement of survival of the Taiwanese 2 copy SMN2 mice was seen on a C57BL/6 background with overexpression of PLS3-V5 and only marginal improvement of muscle fiber size and connectivity of the NMJ. In a F1 mixed background of FVB/N and C57BL/6 the mean survival rate was increased by 2 days and the maximum survival was not increased [50]. In our experience with the Δ7 SMA mice this kind of survival increase is not significant and can vary between tests. In essence, the differences in survival between the current study and that of Ackerman et al. are minimal and we suggest there is no significant improvement in survival of Δ7 SMA mice with overexpression of PLS3. Alternatively, the modest increase in survival of 2 days in the mixed background Taiwanese SMA mouse model could be due to a neuroprotective effect of PLS3. A slight increase in survival was also observed in the Taiwanese SMA mice upon administration of neuroprotective factors IGF-1 [64], cardiotrophin-1 [65], and Bcl-xL [66].

In our study as well as that of Ackerman et al., electrophysiology studies of the function of the NMJ were performed. Ackerman et al. reported a small improvement in the time constant of the endplate potential and quantal content [50]. However this was only on a mixed background and is unlikely to have a major impact on NMJ function. We did not find evidence to suggest significant improvement in either parameter. There is no evidence to suggest that expression of PLS3 improved any of the pre- and postsynaptic physiologic deficits at the neuromuscular junction in our study of Δ7 SMA mice. One difference between our study and the
previous study is that we used voltage clamp of muscle fibers to directly measure synaptic currents whereas the study by Ackermann et al. measured endplate potentials. Endplate potentials can be affected by changes in muscle fiber properties (fiber size and specific membrane resistance) that are unrelated to synaptic function. These differences might account for the difference in findings relating to time constant, however, it seems unlikely that a difference in muscle fiber property could account for the difference between the two studies on quantal content. We cannot rule out that overexpression of PLS3 has a very modest effect on synaptic function that would be picked up with study of more mice. Driving expression of human PLS3 in motor neurons rescued the NMJ defects and motor function in zygotic zebrafish smn1 mutants suggesting that under low conditions of SMN, PLS3 can indeed benefit vertebrate motor neurons [58].

In conclusion, we have shown in the Δ7 mouse model of SMA no beneficial effects of PLS3 overexpression in the neuron. This is also consistent with the study of Bowerman et al. [67] using a milder model of SMA where loss of Profilin results in increased PLS3 expression but no modification of the SMA phenotype [67]. A puzzling feature of all the reports of PLS3 modification is that the effect is proposed to be sex-specific and partially-penetrant. To date, this hypothesis has not been replicated in any animal studies and is not explained by PLS3’s location on the X chromosome because a transgene on an autosome will not be subjected to inactivation. It is clear that there are males in the population that express PLS3 but this does not modify the SMA phenotype in humans. Why would this be the case? We suggest that modifiers of SMA need to be revisited in the human population in discordant sibling pairs. A genetic modifier that has a DNA change on a solid mechanistic base as to why altered plastin expression occurs can be studied in these individuals. In this case it would be preferable to study haploidentical pairs of discordant type 1 and type 2, or type 2 and type 3 siblings. The genetic modifier will not be present in any severe SMA type 1 patient therefore type 1 patient DNA can be used to exclude false modifiers. Indeed there are SNPs and methylation changes associated with altered PLS3 expression that could be investigated in SMA. Currently PLS3 can be viewed as a candidate modifier where an understanding of mechanism of activation, and DNA changes associated with increased expression is not understood, nor why modification only occurs under certain circumstances. However an alternative explanation is that PLS3 is in fact not the critical modifier of SMA phenotype. Thus studies that remain open to the possibility of defining alternate modifiers in SMA are of critical importance.

Acknowledgments
We thank Xiaohui Li and Kaitlyn Corlett for genotyping assistance.

Author Contributions
Conceived and designed the experiments: VLM AML XW TTL CEB MMR AHMB. Performed the experiments: VLM AML XW TTL. Analyzed the data: VLM AML TTL CEB MMR AHMB. Contributed reagents/materials/analysis tools: CEB MMR AHMB. Wrote the paper: VLM CEB MMR AHMB.

References
1. Roberts DF, Chavez J, Court SD. The genetic component in child mortality. Arch Dis Child. 1970; 45(239):33–8. PMID: 4245389.
2. Pearn J. Incidence, prevalence, and gene frequency studies of chronic childhood spinal muscular atrophy. J Med Genet. 1978; 15(6):409–13. PMID: 745211.
3. Crawford TO, Pardo CA. The neurobiology of childhood spinal muscular atrophy. Neurobiol Dis. 1996; 3(2):97–110. PMID: 9173917.
4. Lefebvre S, Burglen L, Rebouillot S, Clermont O, Burlet P, Viollet L, et al. Identification and characterization of a spinal muscular atrophy-determining gene. Cell. 1995; 80(1):155–65. PMID: 7813012.

5. Burghes AH, Beattie CE. Spinal muscular atrophy: why do low levels of survival motor neuron protein make motor neurons sick? Nat Rev Neurosci. 2009; 10(8):597–609. PMID: 19584893. doi: 10.1038/nnm2670

6. Monani UR, Lorson CL, Parsons DW, Prior TW, Androphy EJ. Burghes AH, et al. A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. Hum Mol Genet. 1999; 8(7):1177–83. PMID: 10369862.

7. Lorson CL, Hahnen E, Androphy EJ, Wirth B. A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. Proc Natl Acad Sci U S A. 1999; 96(11):6307–11. PMID: 10339583.

8. Kashima T, Manley JL. A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy. Nat Genet. 2003; 34(4):460–3. PMID: 12833158.

9. Cartegni L, Krainer AR. Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1, Nat Genet, 2002; 30(4):377–84. PMID: 11925564.

10. Gennarelli M, Lucarelli M, Capon F, Pizzuti A, Merlino L, Angelini C, et al. Survival motor neuron gene transcript analysis in muscles from spinal muscular atrophy patients. Biochem Biophys Res Commun. 1995; 213(3):342–8. PMID: 7639755.

11. Lorson CL, Strasswimmer J, Yao JM, Baleja JD, Hahnen E, Wirth B, et al. SMN oligomerization defect correlates with spinal muscular atrophy severity. Nat Genet. 1998; 19(1):63–6. PMID: 959291.

12. Burnett BG, Munoz E, Tandon A, Kwon DY, Sumner CJ, Fischbeck KH. Regulation of SMN protein stability. Mol Cell Biol. 2009; 29(5):1107–15. PMID: 19103745. doi: 10.1128/MCB.01262-08

13. Coovett DD, Le TT, McAndrew PE, Strasswimmer J, Crawford TO, Mendell JR, et al. The survival motor neuron protein in spinal muscular atrophy, Hum Mol Genet. 1997; 6(8):1205–14. PMID: 9259265.

14. Lefebvre S, Burlet P, Liu Q, Bertrand S, Clermont O, Munnoch A, et al. Correlation between severity and SMN protein level in spinal muscular atrophy, Nat Genet. 1997; 16(3):265–9. PMID: 9207792.

15. McAndrew PE, Parsons DW, Simard LR, Rochette C, Ray PN, Mendell JR, et al. Identification of proximal spinal muscular atrophy carriers and patients by analysis of SMNT and SMN2 gene copy number. Am J Hum Genet. 1997; 60(6):1411–22. PMID: 9186562.

16. Burghes AH. When is a deletion not a deletion? When it is converted. Am J Hum Genet. 1997; 61(1):9–15. PMID: 9245977.

17. Prior TW, Krainer AR, Hua Y, Swoboda KJ, Snyder PC, Bridgeman SJ, et al. A positive modifier of spinal muscular atrophy in the SMN2 gene, Am J Hum Genet. 2008; 85(3):408–13. Epub 2009/08/01. S0002-9297(08)00345-0 [pii] doi: 10.1016/j.ajhg.2008.08.002 PMID: 19716110; PubMed Central PMCID: PMC2771537.

18. Vezain M, Saugier-Weber P, Goina E, Touraine R, Manel V, Toutain A, et al. A rare SMN2 variant in a previously unrecognized composite splicing regulatory element induces exon 7 inclusion and reduces the clinical severity of spinal muscular atrophy. Hum Mutat. 2010; 31(1):E1110–25. Epub 2009/12/03. doi: 10.1002/humu.21173 PMID: 19953846.

19. Bernal S, Alias L, Barcelo MJ, Aluo-Rallo E, Martinez-Hernandez R, Gamez J, et al. The c.859G>C variant in the SMN2 gene is associated with types II and III SMA and originates from a common ancestor. J Med Genet. 2010; 47(9):640–2. doi: 10.1136/jmg.2010.079004 PMID: 20577007.

20. Capon F, Levato C, Merlino L, Angelini C, Mostacciolo ML, Polliano L, et al. Discordant clinical outcome in type III spinal muscular atrophy sibships showing the same deletion pattern. Neuromuscular Disorders. 1996; 6(4):261–4. PMID: 8887955.

21. Burghes AH, Inghram SE, Kote-Jarai Z, Rosenfeld S, Herta N, Nadkami N, et al. Linkage mapping of the spinal muscular atrophy gene. Hum Genet. 1994; 93(3):305–12. PMID: 8125483.

22. Cobben JM, van der Stege G, Grootscholten P, de Visser M, Scheffer H, Buys CH. Deletions of the survival motor neuron gene in unaffected siblings of patients with spinal muscular atrophy, Am J Hum Genet. 1995; 57(4):805–8. PMID: 7573039.

23. Hahnen E, Forkert R, Marke C, Rudnik-Schoneborn S, Schonling J, Zerres K, et al. Molecular analysis of candidate genes on chromosome 5q13 in autosomal recessive spinal muscular atrophy: evidence of homoygous deletions of the SMN gene in unaffected individuals. Hum Mol Genet. 1995; 4(10):1927–33. PMID: 8595417.

24. Dubowitz V. Infantile muscular atrophy: a prospective study with particular reference to a slowly progressive variety. Brain. 1964; 87:707–18. PMID: 14236013
25. SCHMID PC. [Study of the clinical aspects of infantile spinal progressive muscular atrophy of the Werdnig-Hoffman type]. Z Kinderheilkd. 1958; 81(1):13–25. PMID: 13604538.

26. Parano E, Pavone L, Falsaperla R, Trifletti R, Wang C. Molecular basis of phenotypic heterogeneity in siblings with spinal muscular atrophy. Ann Neurol. 1996; 40(2):247–51. Epub 1996/08/01. doi: 10.1002/ana.410400218 PMID: 8773609.

27. DiDonato CJ, Ingraham SE, Mendell JR, Prior TW, Lenard S, Moxley RT 3rd, et al. Deletion and conversion in spinal muscular atrophy patients: is there a relationship to severity? Ann Neurol. 1997; 41(2):230–7. PMID: 9029072.

28. Rochette CF, Surih LC, Ray PN, McAndrew PE, Prior TW, Burghes AH, et al. Molecular diagnosis of non-deletion SMA patients using quantitative PCR of SMN exon 7. Neurogenetics. 1997; 1(2):141–7. PMID: 10732817.

29. Bebee TW, Gladman JT, Chandler DS. Splicing regulation of the survival motor neuron genes and implications for treatment of spinal muscular atrophy. Front Biosci. 2010; 15:1191–204. Epub 2010/06/03. 3670 [pii]. PMID: 20515750.

30. Singh NK, Singh NN, Androphy EJ, Singh RN. Splicing of a critical exon of human Survival Motor Neu- ron is regulated by a unique silencer element located in the last intron. Mol Cell Biol. 2006; 26(4): 1333–46. Epub 2006/02/02. 26/4/1333 [pii] doi: 10.1128/MCB.26.4.1333-1346.2006 PMID: 16449646; PubMed Central PMCID: PMC1687187.

31. Li DK, Tisdale S, Lotti F, Pellizzoni L. SMN control of RNP assembly: from post-transcriptional gene regulation to motor neuron disease. Semin Cell Dev Biol. 2014; 32:22–9. doi: 10.1016/j.semcdb.2014.04.026 PMID: 24769255; PubMed Central PMCID: PMC4110182.

32. Rossoll W, Jablonka S, Andreassi C, Kroning AK, Karle K, Monani UR, et al. Smn, the spinal muscular atrophy-determining gene product, regulates axon growth and localization of beta-actin mRNA in growth cones of motoneurons. J Cell Biol. 2003; 163(4):801–12. PMID: 14623865.

33. McWhorter ML, Monani UR, Burghes AH, Beattie CE. Knockdown of the survival motor neuron (Smn) protein in zebrafish causes defects in motor axon outgrowth and pathfinding. J Cell Biol. 2003; 162(5): 919–32. PMID: 12952942.

34. McGovern VL, Gavrillina TO, Beattie CE, Burghes AH. Embryonic motor axon development in the severe SMA mouse. Hum Mol Genet. 2008; 17(18):2900–9. Epub 2008/07/08. doi: 10.1093/hmg/ddn189 PMID: 18603534; PubMed Central PMCID: PMC2722893.

35. Helmken C, Hofmann Y, Schoenen F, Oprea G, Raschke H, Rudnik-Schoneborn S, et al. Evidence for a modifying pathway in SMA discordant families: reduced SMN levels decrease the amount of its interacting partners and Htra2-betas1. Hum Genet; 2003; 114(1):11–21. PMID: 14520560.

36. Oprea GE, Krober S, McWhorter ML, Rossoll W, Muller S, Krawczak M, et al. Plastin 3 is a protective modifier of autosomal recessive spinal muscular atrophy, Science, 2008; 320(5875):524–7; PMID: 18440926. doi: 10.1126/science.1155085

37. Hasanzad M, Azad M, Kahrizi K, Safar BS, Nafisi S, Keyhanioudiz Z, et al. Carrier frequency of SMA by quantitative analysis of the SMN1 deletion in the Iranian population. European journal of neurology: the official journal of the European Federation of Neurological Societies. 2010; 17(1):160–2. doi: 10.1111/j.1468-1331.2009.02693.x PMID: 19538222.

38. Gavrillina TO, McGovern VL, Workeman E, Crawford TO, Gogliotti RG, DiDonato CJ, et al. Neuronal SMN expression corrects spinal muscular atrophy in severe SMA mice while muscle-specific SMN expression has no phenotypic effect. Hum Mol Genet. 2008; 17(8):1063–75. Epub 2008/01/08. doi: 10.1093/hmg/ddm379 PMID: 18178576; PubMed Central PMCID: PMC2835941.

39. Le TT, Pham LT, Butchbach ME, Zhang HL, Monani UR, Coover DD, et al. SMN Delta7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN. Hum Mol Genet. 2005; 14(6):845–57. PMID: 15703163.

40. Arnold WD, Porensky PN, McGovern VL, Iyer CC, Duque S, Li X, et al. Electrophysiological Biomarkers in Spinal Muscular Atrophy: Preliminary Proof of Concept. Ann Clin Transl Neurol. 2014; 1(1):34–44. PMID: 24511555; PubMed Central PMCID: PMC3914317.

41. Iyer CC, McGovern VL, Wise DO, Glass DJ, Burghes AH. Deletion of atrophy enhancing genes fails to ameliorate the phenotype in a mouse model of spinal muscular atrophy, Neuromuscul Disord. 2014; 24(5):436–44. doi: 10.1016/j.nmd.2014.02.007 PMID: 24658734; PubMed Central PMCID: PMC4005840.

42. Le TT, McGovern VL, Alvino IE, Wang X, Massoni-Laporte A, Rich MM, et al. Temporal requirement for high SMN expression in SMA mice. Hum Mol Genet. 2011; 20(18):3578–81. Epub 2011/06/16. doi: 10.1093/hmg/ddr275 PMID: 21672918; PubMed Central PMCID: PMC3159555.

43. Westerfield. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio). Eugene, OR: University of Oregon Press; 1995.
44. Dalgin G, Ward AB, Hao IT, Beattie CE, Nechiporuk A, Prince VE. Zebrafish mnx1 controls cell fate choice in the developing endocrine pancreas. Development. 2011; 138(21):4597–608. doi: 10.1242/dev.067736 PMID: 21989008; PubMed Central PMCID: PMCPMC3190380.

45. Carrel TL, McWhorter ML, Workman E, Zhang H, Wolstencroft EC, Lorson C, et al. Survival motor neuron function in motor axons is independent of functions required for small nuclear ribonucleoprotein biogenesis. J Neurosci. 2006; 26(43):11014–22. doi: 10.1523/JNEUROSCI.1637-06.2006 PMID: 17065443.

46. Kong L, Wang X, Choe DW, Polley M, Burnett BG, Bosch-Marce M, et al. Impaired synaptic vesicle release and immaturity of neuromuscular junctions in spinal muscular atrophy mice. J Neurosci. 2009; 29(3):842–51. PMID: 19158308; doi: 10.1523/JNEUROSCI.4434-08.2009

47. Leduc P, Duez C, Vanhove M, Lejeune A, Fonze E, Charlier P, et al. Unexpected influence of a C-terminal-fused His-tag on the processing of an enzyme and on the kinetic and folding parameters. FEBS Lett. 1997; 413(2):194–6. PMID: 9280280.

48. Alvarez-Castelao B, Munoz C, Sanchez I, Goethals M, Vandekerckhove J, Castano JG. Reduced protein stability of human DJ-1/PARK7 L166P, linked to autosomal recessive Parkinson disease, is due to direct endoproteolytic cleavage by the proteasome. Biochim Biophys Acta. 2012; 1823(2):524–33. doi: 10.1016/j.bbapap.2011.11.010 PMID: 22173095.

49. Lyon AN, Pineda RH, Hao IT, Kudryashova E, Kudryashov DS, Beattie CE. Calcium binding is essential for plastin 3 function in Smn-deficient motoneurons. Hum Mol Genet. 2014; 23(8):1990–2004. doi: 10.1093/hmg/ddt456 PMID: 24271012; PubMed Central PMCID: PMCPMC3959813.

50. Ackermann B, Krober S, Torres-Benito L, Borgmann A, Peters M, Hosseini Barkooie SM, et al. Plastin 3 ameliorates spinal muscular atrophy via delayed axon pruning and improves neuromuscular junction functionality. Hum Mol Genet. 2013; 22(7):1328–47. Epub 2012/12/25. PMID: 23253681.

51. Martinez TL, Kong L, Wang X, Osborne MA, Crowder ME, Van Meeteren JP, et al. Survival motor neuron protein in motor neurons determines synaptic integrity in spinal muscular atrophy. J Neurosci. 2012; 32(25):8703–15. Epub 2012/06/23. 32(25):8703 [pii] doi: 10.1523/JNEUROSCI.0294-12.2012 PMID: 22723710; PubMed Central PMCID: PMC3462658.

52. Ruiz R, Casanas JJ, Torres-Benito L, Cano R, Tabares L. Altered intracellular Ca2+ homeostasis in nerve terminals of severe spinal muscular atrophy mice. J Neurosci. 2010; 30(3):849–57. Epub 2010/01/22. 30(3):849 [pii] doi: 10.1523/JNEUROSCI.4486-09.2010 PMID: 20088893.

53. Ling KK, Lin MY, Zingg B, Feng Z, Ko CP. Synaptic defects in the spinal and neuromuscular circuitry in a mouse model of spinal muscular atrophy. PLoS One. 2011; 6(11):e25457. Epub 2011/11/19. doi: 10.1371/journal.pone.0015457 PMID: 21085654; PubMed Central PMCID: PMC2978709.

54. Bernal S, Alcaro-Rallo E, Martinez-Hernandez R, Alias L, Rodriguez-Alvarez FJ, Millan JM, et al. Plastin 3 expression in discordant spinal muscular atrophy (SMA) siblings. Neuromuscul Disord. 2011; 21(6): 413–9. doi: 10.1016/j.nmd.2011.03.009 PMID: 21546251.

55. Tinker AV, Brown CJ. Induction of XIST expression from the human active X chromosome in mouse/human somatic cell hybrids by DNA demethylation. Nucleic Acids Res. 1988; 16(12):2935–40. PMID: 9611238; PubMed Central PMCID: PMC147638.

56. Stratiopoulos G, Lanzano P, Deng L, Guo J, Kaufmann P, Darras B, et al. Association of plastin 3 expression with disease severity in spinal muscular atrophy only in postpubertal females. Arch Neurol. 2010; 67(10):1252–6. doi: 10.1001/archneurol.2010.238 PMID: 20937953.

57. Yanyan C, Yujin Q, Jinti B, Yuwei J, Hong W, Fang S. Correlation of PLS3 expression with disease severity in children with spinal muscular atrophy. Journal of human genetics. 2014; 59(1):24–7. doi: 10.1038/jhg.2013.111 PMID: 24172247.

58. Hao T, Wolman M, Granato M, Beattie CE. Survival motor neuron affects plastin 3 protein levels leading to motor defects. J Neurosci. 2012; 32(15):5074–84. doi: 10.1523/neurosci.5808-11.2012 PMID: 22496553; PubMed Central PMCID: PMC3355766.

59. Hisano T, Ono M, Nakayama M, Naito S, Kuwano M, Wada M. Increased expression of T-plastin gene in cisplatin-resistant human cancer cells: identification by mRNA differential display. FEBS Lett. 1996; 39(1):101–7. PMID: 8941723.

60. Jones CL, Ferreira S, McKenzie RC, Tosi I, Caesar JA, Bagan M, et al. Regulation of T-plastin expression by promoter hypomethylation in primary cutaneous T-cell lymphoma. J Invest Dermatol. 2012; 132(2):2042–9. doi: 10.1038/jid.2012.106 PMID: 22495182.

61. Begue E, Michel L, Jean-Louis F, Bagot M, Bensussan A. Promoter Hypomethylation and Expression of PLS3 in Human Sezary Lymphoma Cells. SOJ Immunology. 2013; 1(1):4.

62. Szkandera J, Winder T, Stolz M, Weissmueller M, Langsenlehner T, Pichler M, et al. A common gene variant in PL53 predicts colon cancer recurrence in women. Tumour Biol. 2013; 34(4):2183–8. doi: 10.1007/s13277-013-0754-7 PMID: 23549653.
63. Hsieh-Li HM, Chang JG, Jong YJ, Wu MH, Wang NM, Tsai CH, et al. A mouse model for spinal muscular atrophy. Nat Genet. 2000; 24(1):66–70. PMID: 10615130.
64. Bosch-Marce M, Wee CD, Martinez TL, Lipkes CE, Choe DW, Kong L, et al. Increased IGF-1 in muscle modulates the phenotype of severe SMA mice. Hum Mol Genet. 2011; 20(9):1844–53. doi: 10.1093/hmg/ddr067 PMID: 21325354; PubMed Central PMCID: PMC3071675.
65. Lesbordes JC, Cifuentes-Diaz C, Miroglio A, Joshi V, Bordet T, Kahn A, et al. Therapeutic benefits of cardiotrophin-1 gene transfer in a mouse model of spinal muscular atrophy. Hum Mol Genet. 2003; 12(11):1233–9. PMID: 12761038.
66. Tsai LK, Tsai MS, Ting CH, Wang SH, Li H. Restoring Bcl-x(L) levels benefits a mouse model of spinal muscular atrophy. Neurobiol Dis. 2008; 31(3):381–7. doi: 10.1016/j.nbd.2008.05.014 PMID: 18590823.
67. Bowerman M, Anderson CL, Beauvais A, Boyl PP, Witke W, Kothary R. SMN, profilin Ila and plastin 3: a link between the deregulation of actin dynamics and SMA pathogenesis. Mol Cell Neurosci. 2009; 42(1):66–74. doi: 10.1016/j.mcn.2009.05.009 PMID: 19497369.