Conserved Genes Act as Modifiers of Invertebrate SMN Loss of Function Defects

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Abstract

Spinal Muscular Atrophy (SMA) is caused by diminished function of the Survival of Motor Neuron (SMN) protein, but the molecular pathways critical for SMA pathology remain elusive. We have used genetic approaches in invertebrate models to identify conserved SMN loss of function modifier genes. Drosophila melanogaster and Caenorhabditis elegans each have a single gene encoding a protein orthologous to human SMN; diminished function of these invertebrate genes causes lethality and neuromuscular defects. To find genes that modulate SMN function defects across species, two approaches were used. First, a genome-wide RNAi screen for C. elegans SMN modifier genes was undertaken, yielding four genes. Second, we tested the conservation of modifier gene function across species; genes identified in one invertebrate model were tested for function in the other invertebrate model. Drosophila orthologs of two genes, which were identified originally in C. elegans, modified Drosophila SMN loss of function defects. C. elegans orthologs of twelve genes, which were originally identified in a previous Drosophila screen, modified C. elegans SMN loss of function defects. Bioinformatic analysis of the conserved, cross-species, modifier genes suggests that conserved cellular pathways, specifically endocytosis and mRNA regulation, act as critical genetic modifiers of SMN loss of function defects across species.

Introduction

Decreased Survival of Motor Neuron (SMN) protein function underlies most Spinal Muscular Atrophy (SMA) cases [1]. The SMN protein is ubiquitously expressed [2,3], yet SMA pathology is remarkably specific. Patients lose spinal α-motorneurons and experience muscular dysfunction with atrophy. Mild cases result in slowly progressing muscular weakness, while severe cases dramatically perturb proximal neuromuscular function resulting in childhood death [4]. There is no effective treatment for SMA and at least 1 in 40 people in the US population are carriers of SMN loss of function disease alleles [5–7].

The SMN protein is a component of the well-characterized Gem complex, which assembles splicing machinery in eukaryotes [8–10]. SMN also associates with β-actin mRNA during anterograde transport in neuronal processes suggesting a role for SMN in mRNA transport, sub-cellular localization and/or local translation [11–15]. In addition, SMN is found in post-synaptic densities and Z-discs of muscles along with other RNA processing proteins [11–19]. Roles for SMN in small nucleolar RNA (snoRNA) and microRNA (miRNA) pathways have also been suggested [20–22]. The relative contributions of SMN in these various compartments and the relative importance of SMN function in neurons and muscles for SMA pathology have been difficult to determine. Various tissue requirements for SMN function have been observed in different SMA model systems [23–27]. The diverse subcellular SMN localization and varied cellular requirements for SMN function suggest that this protein may act in multiple cellular compartments including the neuromuscular junction (NMJ) [28].

To determine in an unbiased fashion which cellular and molecular pathways are particularly relevant to SMA pathology, researchers have turned recently to genetic approaches in vertebrates and invertebrates. The identification of SMN loss of function modifier genes can reveal important biochemical pathways for SMA pathology. Studies in patients have already identified two genes that act as modifiers of SMA: SMN2 and Plastin 3 (PLS3).
Author Summary

Spinal Muscular Atrophy (SMA) is a common, untreatable, and often fatal neuromuscular disease predominately caused by reduced Survival Motor Neuron (SMN) protein function. Here, we use invertebrate models to identify and validate conserved genes that play a critical role in SMN loss of function neuromuscular defects. Decreased SMN function causes growth defects in the nematode Caenorhabditis elegans and in the fruit fly Drosophila melanogaster—as well as behavioral or synaptic connectivity defects between neurons and muscles, respectively. We found that a genetic modifier of SMA in patients, plastin, also affects SMN function in these invertebrate models. We undertook a genome-wide RNAi screen to identify genes whose perturbation alters the growth defects of C. elegans lacking SMN. These genes were validated in neuromuscular assays in nematode and fly models of SMA. Additionally, we used the C. elegans model to test SMN modifier genes previously identified in the Drosophila SMA model. Combined, these cross-species approaches identified fifteen genes that are important in both species when SMN function is decreased. Related mammalian proteins and the pathways in which they act (including endocytosis and RNA transport/translational control) are likely important players in SMA.

Two genes encode human SMN protein: SMN1 and SMN2. The SMN1 gene encodes only full-length SMN protein while the SMN2 gene encodes two different transcripts; 10% of SMN2 transcripts encode a full-length SMN protein identical to the SMN1 gene product. However, due to a change in the splice consensus sequence, 90% of SMN2 transcripts contain a stop codon at the beginning of exon 7 and, therefore, encode a truncated protein (called SMNdeltaEx7 or Δ7SMN) of diminished function and stability [1,29–31]. Humans have various numbers of SMN2 genes; patients with more copies of SMN2 generally have later onset/less severe symptoms than patients with fewer copies of SMN2. Decreased severity and delayed onset is usually attributed to increased full-length SMN levels from SMN2 in vivo [17,32–36].

PLS3 may modulate the severity of SMA. In several families, daughters who lack SMN1 and express PLS3 were remarkably unaffected [37]. PLS3 encodes a conserved calcium-binding, actin-bundling/stabilizing protein that is broadly expressed in various tissues including blood, muscles and neurons [38–40]. Loss of the yeast PLS3 ortholog, Sac6p, results in defective endocytosis [41,42]. Altering PLS3 levels modified SMN loss of function defects in zebrafish motorneurons consistent with results in human families and PLS3 co-precipitated with SMN from neuronal tissues [37]. However, increased PLS3 (due to profilin knockdown) did not decrease the defects in an SMA mouse model and it remains unclear how PLS3 might modify SMN neuromuscular defects [43].

Modifier genes identified in patient populations are clearly pertinent to SMA pathology. However, studies in humans are limited by kindred sizes and other considerations. As SMN orthologs are found in C. elegans and Drosophila melanogaster, it may be more efficient to identify SMA modifier genes in these powerful invertebrate models. SMN loss of function models have already been defined in C. elegans and Drosophila [18,25,44,45]. Loss of Drosophila Snnm (DmSnnm) causes larval lethality and NMJ defects; DmSnnm function is required in neurons and muscles in flies [26]. Loss of C. elegans SMN-1 (Cesmn-1) also causes neuromuscular function deficits followed by larval lethality [44]. Expression of Cesmn-1 in neurons dramatically restores neuromuscular function, whereas expression in muscles has little effect [44]. Given SMN conservation across species, genes that act as SMN loss of function modifiers in invertebrates could be important in SMA pathology in humans (e.g. PLS3) [37].

In a recent study, twenty-seven P-element transposon insertion lines were identified in Drosophila that modified SMN loss of function defects, and a role for the TGF-beta pathway in SMN loss of function pathology was delineated [26]. However, it remains unclear for several P-element lines which Drosophila gene near the transposon insertion site is responsible for modulating SMN phenotypic defects. The Drosophila P-element lines carried an inducible GAL4-UAS that could drive either over-expression or antisense RNAi expression of neighboring genes depending on transposon insertion site. Additionally, insertion of the P-element itself might perturb gene function. Eliminating ambiguity regarding modifier gene identity would increase the utility of the Drosophila study.

To explore the genetic circuitry affecting SMN activity in C. elegans, the Cesmn-1(·) growth defect phenotype was used as a metric in a rapid large-scale genetic screen. Growth may be affected by a variety of changes, such as body length and longevity. Subsequently, modifier genes were tested using a C. elegans behavioral assay, the pharyngeal pumping, which is likely more pertinent to SMN loss of function neuromuscular defects. In addition, to identify conserved invertebrate SMN modifier genes, we utilized previously described Drosophila assays to assess genetic interaction of DmSnnm with Drosophila orthologs of C. elegans modifier genes. In the study by Chang and co-workers, the DmSnnm lethal phenotype correlated with NMJ defects for virtually all DmSnnm modifier genes, suggesting that lethality and neuromuscular hibutton number are effective measures of genetic interaction with the Drosophila SMA ortholog [26].

Here, we define conserved genetic modifiers of SMN loss of function using C. elegans and Drosophila. We find that PLS3 orthologs act as SMN modifier genes in both invertebrate species. A genome-wide RNAi screen in C. elegans identified four new SMN modifier genes, including ncb-2 and flp-4, which also modify SMN loss of function defects in Drosophila. Candidate SMN modifier genes identified in a previous Drosophila screen were tested in C. elegans yielding twelve cross-species modifier genes. Examination of the literature for these genes suggested specific cellular pathways that are critical genetic modifiers of SMN function: endocytosis and RNA processing. These pathways may also be pertinent to SMN loss of function defects in patients with SMA.

Results

The previously described Cesmn-1(ok355) deletion allele causes a complete loss of Cesmn-1 function and is referred to herein as Cesmn-1(·) [44]. Cesmn-1(·) is recessive; heterozygous animals are overtly normal. To facilitate identification of heterozygous versus homozygous animals, we utilized the balanced strain hT2(bli-4(e937) let-2(q782) qIs48[myo-2p::GFP])/Cesmn-1(·) [44,46]. Heterozygous +/+ Cesmn-1(·) animals express pharyngeal GFP, homozygous Cesmn-1(·) progeny do not express GFP, and progeny homozygous for the hT2 balancer die as GFP-expressing embryos.

Although complete loss of SMN function causes lethality, C. elegans that are homozygous mutant for Cesmn-1(·) can survive for several days due to partial maternal rescue. It has been suggested that +/+ Cesmn-1(·) hermaphrodites load sufficient Cesmn-1 maternal protein and/or perhaps mRNA into oocytes to support development through embryogenesis and early larval stages [44]. Accordingly, homozygous Cesmn-1(·) larvae initially resemble wild type animals. Eventually maternally-loaded Cesmn-1 product is lost; Cesmn-1(·) animals grow more slowly than +/+ Cesmn-1(·) siblings,
are shorter, sterile, and most Cesmn-1(lf) animals die before reaching adulthood (Figure 1A). Combined, these defects decrease the average size of the Cesmn-1(lf) population versus control animals; decreased average population size will be referred to herein as a growth defect. This growth defect was harnessed in an automated assay to identify Cesmn-1(lf) modifier genes in a genome-wide screen.

**C. elegans** growth assay

To validate growth as an assay for SMN modifier gene identification, we first demonstrated that RNAi knockdown of Cesmn-1 or the invertebrate ortholog of Plastin 3 (PLS3) altered Cesmn-1(lf) growth. The C. elegans gene plst-1 (PlasSTin [actin bundling protein] homolog-1) encodes a predicted protein similar to PLS3.

To knockdown gene function, C. elegans were reared on bacteria producing double stranded RNA corresponding to the gene of interest, a strategy known as ‘feeding RNAi’ [47]. Feeding RNAi decreases gene transcripts in most C. elegans tissues although knockdown in neurons is generally less effective than knockdown in muscles, germline, and other tissues [48–50]. Here, animals were reared for two generations on solid media and RNAi feeding bacteria corresponding to Cesmn-1 or plst-1, allowing knockdown of maternal and zygotic transcripts (Figure 1B). Bacteria containing the empty RNAi feeding vector were used as a negative control (empty(RNAi)).

An automated system was used to simultaneously measure growth and determine genotype for the progeny of +/Cesmn-1(lf) animals (Figure 1C). The COPAS BioSorter (Union Biometrica, Holliston, MA) measures C. elegans length as ‘time-of-flight’, which is the time required for the animal to pass through the fluorescence-detection chamber [51]. Cesmn-1(lf) homozygous animals do not express GFP while +/Cesmn-1(lf) heterozygous animals express GFP and are longer than Cesmn-1 homozygous animals of the same late larval or adult stage. Animals smaller than L2 larval stage were excluded from this analysis to avoid bacterial debris. The percentage of large adult animals was determined for each genotype and RNAi treatment.

RNAi knockdown of Cesmn-1 decreased the percentage of large animals in both Cesmn-1(lf) homozygous and +/Cesmn-1(lf) heterozygous populations (Table 1, Rows 1 & 2). Initially, it seems counter-intuitive that the defects of Cesmn-1(lf) animals are exacerbated by Cesmn-1(RNAi). However, in this scenario, transcripts in both the somatic tissues and germline of +/Cesmn-1(lf) heterozygous animals are targeted and, consequently, maternally-loaded Cesmn-1 transcript and protein are depleted in homozygous Cesmn-1(lf) progeny, abrogating partially the observed maternal rescue. The ability of Cesmn-1(RNAi) to exacerbate Cesmn-1(lf) defects suggests that the effects of modifier genes can be assessed using RNAi feeding.

Knockdown of the C. elegans PLS3 ortholog, plst-1, increased the average length of the +/+Cesmn-1(lf) population, but did not significantly alter the average length of Cesmn-1(lf) animals (Table 1, Rows 1 & 3). Genetic interaction with plst-1 was further confirmed by using the plst-1 (tm4255) mutant allele (Table 2). The average length of +/+Cesmn-1(lf);plst-1(tm4255) adult animals was significantly increased in relation to +/+Cesmn-1(lf) animals. In contrast, the average length of homozygous Cesmn-1(lf);plst-1(tm4255) was not altered, recapitulating the results of plst-1(RNAi). Increased average adult length is an overall growth metric that may encompass a variety of changes; decreased plst-1 function, by RNAi or mutant allele, could increase length, cause sterility, and/or increase longevity in +/+Cesmn-1(lf) control animals. It appears that loss of Cesmn-1 function suppresses the effects of decreased plst-1 function (i.e. increased length was not observed in Cesmn-1(lf);plst-1(tm4255) homozygous mutant animals). The genetic and functional relationship between SMN and PLS3 bears further examination; as plst-1 and Cesmn-1 have opposing effects on the growth assay and since Cesmn-1(lf);plst-1(tm4255) animals resemble Cesmn-1(lf) single mutants, Cesmn-1 may act downstream of plst-1 in this growth assay [52].

**Growth modifier genes identified in C. elegans genome-wide screen**

To identify additional genes that modify SMN loss of function defects, a large-scale genome-wide screen for enhancers and suppressors of the Cesmn-1(lf) growth defect was undertaken. The growth assay was adapted to a higher-throughput 96-well, liquid

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**Figure 1. Survival and average length of Cesmn-1(lf) animals is decreased.** Homozygous loss of Cesmn-1(0f) results in slower growth and most animals die during larval stages. Animals that reach the adult stage are short-lived and sterile. A) Image of age-matched heterozygous [h72( leth/o)]/+myo-2::GFP[Cesmn-1(0f)/+]; Cesmn-1(lf) animals (described in text) and homozygous Cesmn-1(0f) individuals (below). Scale bar indicates 150 microns. B) To test the impact of candidate modifier genes on growth rates and size, a C. elegans growth assay was established. +/+Cesmn-1(lf) animals were collected as unhatched eggs, reared for 5 days/2 generations on standard C. elegans plates upon RNAi bacterial strains, as illustrated in the flow chart. Bacterial cultures expressed dsRNA corresponding to the gene of interest for RNAi knockdown; bacterial cultures containing vector with no insert were used as a control (‘empty’). C) Using an automated system, the length and fluorescence of animals was determined [51]; the smallest larvae in each culture were indistinguishable from debris and were excluded from the analysis as illustrated in the graph. Genotypes were discriminated by GFP fluorescence and length was determined as ‘time-of-flight’ through the laser chamber; each dot in the graph represents an individual animal. The disparate growth/survival rates of Cesmn-1(lf) and +/+Cesmn-1(lf) animals alters the percentage of ‘large’ animals in these mixed stage cultures. As illustrated graphically, +/+Cesmn-1(lf) and Cesmn-1(lf) animals can be sorted into blue and red boxes, respectively; animals designated as ‘large’ fall into shaded boxes. The fraction of large animals was calculated (# animals in shaded box/# animals in both shaded and unshaded box for each genotype) and is reported as % large with standard error of the mean (S.E.M.); significance was determined by Mann-Whitney U two-tailed test with p=0.05. At least three independent cultures of more than 200 animals were scored for each targeted gene.

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culture format and a previously described genome-wide C. elegans RNAi feeding library was used for gene knockdown (Figure 2A) [53]. Progeny of +/Cesmn-1(0f) animals were reared for two weeks (more than 2 generations) on RNAi feeding bacterial strains before assessment of growth using the COPAS Biosorter [51]. To identify RNAi clones that specifically altered the growth of Cesmn-1(0f) animals, a growth ratio of large to small animals was determined for each clone for Cesmn-1(0f) and for +/Cesmn-1(0f) genotypes. If the RNAi clone growth ratio was more than 2 standard deviations away from the mean for Cesmn-1(0f) animals and within 0.7 standard deviations of the mean for +/Cesmn-1(0f) animals in at least 40% of independent trials, then the corresponding gene was designated as an Cesmn-1(0f) modifier (Figure 2B). In the primary high-throughput screen, no suppressors were found, but four genes were identified as enhancers (Figure 2B). RNAi knockdown of these genes exacerbated homozygous Cesmn-1(0f) growth defects and did not significantly alter the growth of heterozygous +/Cesmn-1(0f) animals; ncbp-2, T02G5.3, grk-2, and flp-4. ncbp-2 encodes the C. elegans Cap Binding Protein 20 (CBP20 or Cbp20) ortholog [54]. T02G5.3 encodes a predicted protein of unknown function with no vertebrate orthologs based on BLAST analysis. grk-2 encodes one of two G-protein coupled receptor kinases. flp-4 encodes an FMRFamide family neuropeptide protein. The low number of modifiers identified in this screen versus the previous Drosophila screen may reflect the stringent criterion utilized here or the inefficiency of RNAi by feeding in neurons.

To determine if decreased adult body length accounts for the enhanced Cesmn-1(0f) growth defect upon knockdown of ncbp-2, T02G5.3, grk-2 and flp-4, the average body length of Cesmn-1(0f) young animals was determined (Text S1 and Table S4, top panel). Only ncbp-2(RNAi) significantly reduced the average body length of Cesmn-1(0f) animals suggesting that the enhanced growth defect caused by ncbp-2(RNAi) could be attributed to the Cesmn-1(0f) shorter body size. The other three enhancer genes may alter survival or growth as adult animals.

SMA is a neuromuscular disease and, therefore, our objective was the identification of modifier genes that impact SMN neuromuscular function. We then examined the impact of Cesmn-1 growth modifier genes on Cesmn-1 loss of function neuromuscular defects using RNAi and, when available, loss of function alleles of modifier genes.

**Table 1.** Conserved genes modify Cesmn-1(0f) defects in the growth assay.

<table>
<thead>
<tr>
<th>Ce gene</th>
<th>Dm gene</th>
<th>Cesmn-1(0f) % large ± SEM</th>
<th>+/Cesmn-1(0f) % large ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>none</td>
<td>18±2</td>
<td>46±1</td>
</tr>
<tr>
<td>Cesmn-1</td>
<td>Smn</td>
<td>6±4</td>
<td>31±1</td>
</tr>
<tr>
<td>plst-1</td>
<td>Flmbrin</td>
<td>15±3</td>
<td>55±3</td>
</tr>
<tr>
<td>uso-1</td>
<td>p115</td>
<td>7±4</td>
<td>44±1</td>
</tr>
<tr>
<td>hnr-85</td>
<td>Eip75B</td>
<td>8±5</td>
<td>44±1</td>
</tr>
<tr>
<td>egi-15</td>
<td>Breathless</td>
<td>9±5</td>
<td>45±1</td>
</tr>
<tr>
<td>att-6</td>
<td>Adf6</td>
<td>11±7</td>
<td>40±4</td>
</tr>
<tr>
<td>ape-1</td>
<td>CG18375</td>
<td>11±9</td>
<td>50±2</td>
</tr>
<tr>
<td>knc-2</td>
<td>SK</td>
<td>12±1</td>
<td>42±1</td>
</tr>
<tr>
<td>nck-3</td>
<td>Nek2</td>
<td>13±3</td>
<td>43±2</td>
</tr>
<tr>
<td>atn-1</td>
<td>Actin</td>
<td>23±4</td>
<td>43±1</td>
</tr>
<tr>
<td>cash-1</td>
<td>CG33172</td>
<td>26±5</td>
<td>64±11^*</td>
</tr>
<tr>
<td>dcl-1</td>
<td>cut up</td>
<td>15±8</td>
<td>66±3^*</td>
</tr>
</tbody>
</table>

C. elegans modifier genes are listed in column 1 and orthologous Drosophila genes are listed in column 2. Only genes that significantly altered the percentage of large Cesmn-1(0f) homozygous animals (column 3) or +/Cesmn-1(0f) heterozygous animals (column 4) are presented. Animals were reared for two generations on bacterial RNAi strains targeting the C. elegans gene prior to growth analysis as described in Figure 1. The percentage of large animals was determined for each genotype after RNAi knockdown of the indicated C. elegans gene and is reported with standard error of the mean (S.E.M.). Large animals include adults, L4 and L3 stage larvae. This assay does not distinguish between slow growth, changes in relative length, and altered viability. Three to six independent determinations were undertaken for each genotype/RNAi culture and the percentage of large animals in each trial was averaged. Significant changes from empty(RNAi) for each RNAi/gene type using the two-tailed Mann-Whitney U test (p<0.05) are indicated with an asterisk. See Table S1 for results for all genes tested and Materials and Methods for details.

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**Table 2.** C. elegans orthologs of PLS3, GRK, and FMRFamide modify Cesmn-1(0f) growth defects.

<table>
<thead>
<tr>
<th>Ce genotype</th>
<th>Ce gene</th>
<th>Cesmn-1(0f) % large ± SEM</th>
<th>+/Cesmn-1(0f) % large ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>Cesmn-1(0f)</td>
<td>27±2</td>
<td>44±2</td>
</tr>
<tr>
<td>plst-1(tm4255)</td>
<td>none</td>
<td>39±5</td>
<td>67±2^*</td>
</tr>
<tr>
<td>grk-2(n97)</td>
<td>none</td>
<td>10±1^*</td>
<td>41±1</td>
</tr>
<tr>
<td>flp-4(n353)</td>
<td>none</td>
<td>20±2</td>
<td>51±2</td>
</tr>
</tbody>
</table>

The growth defects of +/+Cesmn-1(0f) and Cesmn-1(0f) animals were examined in the presence of grk-2(n97), flp-4(n353) and plst-1(tm4255) loss of function alleles; alleles for each modifier gene are listed in column 1. The percentage of large animals was determined for Cesmn-1(0f) homozygous animals (column 3) or +/Cesmn-1(0f) heterozygous animals (column 4). At least three independent determinations were undertaken for each genotype. Significance versus control genotypes (+/+Cesmn-1(0f) and Cesmn-1(0f)) was determined using Chi-square analysis (p<0.05 indicated with an asterisk). S.E.M. is also reported.

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A recent study from the Sattelle laboratory demonstrated that loss of Cesmn-1 function causes progressive defects in C. elegans neuromuscular function in pharyngeal pumping [44]. C. elegans feeds on bacteria and other microorganisms using a small, discrete subset of neurons and muscles contained in the pharynx (Figure 3A) [55]. Pharyngeal cell specification, neuronal development, and myoblast fusion is completed within hours of hatching [56,57]. The pharynx pumps continuously and symmetrically at over 250 beats per minute in wild type animals when food is present and larval pumping is interrupted only by molting under standard culture conditions. We confirmed a previous report [44] that in early larval stages, the pumping rates of Cesmn-1(0f) animals are indistinguishable from control animals, but at larval later stages Cesmn-1(0f) pumping rates drop (Figure 3B). Cesmn-1(0f) animals have progressive defects in pharyngeal pumping, which occur earlier than reported locomotion defects. At day 2, 62% of Cesmn-1(0f) animals are moving spontaneously, but pumping rates have dropped dramatically (Figure 3B). Restoration of Cesmn-1 function in neurons almost completely restores pumping rates suggesting that Cesmn-1 is required in neurons for this behavior [44].

The efficacy of RNAi by feeding in this neuromuscular assay was assessed for Cesmn-1 and plst-1 using Cesmn-1(0f) and +/Cesmn-1(0f) animals. Animals were allowed to hatch on RNAi feeding plates and pumping rates were determined after three days. Either plst-1(RNAi) or Cesmn-1(RNAi) decreased Cesmn-1(0f) pumping rates, but not +/Cesmn-1(0f) pumping rates (Figure 3C). In addition, plst-1(0f) significantly decreased the pumping rates of...
Cesmn-1(lf) animals, validating the genetic interaction of plst-1 with Cesmn-1 in the neuromuscular pharyngeal pumping assay (Figure 3D). This exacerbation of Cesmn-1 loss of function defects by plst-1 manipulation is consistent with results in other organisms [37]. The ability of Cesmn-1(RNAi) and plst-1(RNAi) to alter pumping of homozygous mutant Cesmn-1(lf) animals suggests that candidate modifier genes can be assessed using RNAi knockdown in this neuromuscular assay.

The four modifier genes from the C. elegans growth screen were tested for function as Cesmn-1 neuromuscular modifier genes using the pharyngeal pumping assay. Results are summarized in Figure 4A. ncbp-2(RNAi) and T02G5.3(RNAi) enhanced and suppressed the pharyngeal pumping defects of Cesmn-1(lf) animals, respectively; flp-4(RNAi) and gk-2(RNAi) had no significant effect compared to controls. We suggest that ncbp-2 and T02G5.3 are likely modifiers of Cesmn-1(lf) neuromuscular defects based on RNAi results.

Loss of gk-2 enhances Cesmn-1(lf) growth and neuromuscular defects

RNAi knockdown of C. elegans genes by feeding is robust in virtually all cell types but can often be inefficient and can result in only partial loss of gene function, especially in the nervous system [58,59]. To address the specificity of the genetic modifiers, the RNAi results were confirmed by using mutant alleles when possible; alleles of ncbp-2 and T02G5.3 were not available.

A gk-2 loss of function allele has been previously described, gk-2(rt97) [60]. Loss of gk-2 significantly enhanced the growth defects of Cesmn-1(lf) animals (Table 2). Additionally, the pumping rates of gk-2(lf) animals derived from hT2 parents were not significantly lower than those of control animals, but the average pumping rates of Cesmn-1(lf) gk-2(lf) double mutant animals were significantly lower than the pumping rates of either single mutant (Figure 4B). This suggests that gk-2 loss enhances both Cesmn-1(lf) growth and pharyngeal pumping defects. A gk-2 gain of function allele is not available and transgenes are unstable in +/Cesmn-1(lf) animals (unpublished results and [44]).

To test the genetic interaction of flp-4 with Cesmn-1, we identified a flp-4 loss of function allele, flp-4(yn35), using PCR based screening techniques [61–65]. The flp-4(yn35) deletion removes all sequences encoding FLP-4 FMRFamide neuropeptides and likely causes a complete loss of flp-4 function ([66] and C. Li, in preparation). Although flp-4(yn35) reduced the percentage of Cesmn-1(lf) large animals in the growth assay, the difference was not statistical significant different (Table 2). Similar results were obtained using the pharyngeal pumping assay. The pumping rates of flp-4(lf) animals were slightly lower, but not significantly different than control animals. Loss of flp-4 function decreased pumping rates of Cesmn-1(lf) animals in five independent trials, but the difference was not statistically significant (p = 0.236, Figure 4B). Either flp-4 is not a bona fide modifier or flp-4(RNAi) may act off-target decreasing the function of more than one of the 32 other C. elegans FMRFamide genes [67].

Drosophila orthologs of plst-1, ncbp-2 and flp-4 modify SMN neuromuscular defects

SMN modifier genes that are conserved across species would be of considerable interest. Three of the candidate genes identified in the C. elegans screen encode conserved proteins with clear orthologs in other species: gk-2, flp-4, and ncbp-2. To determine if their orthologs modify SMN loss of function defects, we turned to the fruit fly Drosophila. Decreased function in the Drosophila SMN ortholog Smn(DmSmn) results in growth defects, early pupal arrest, and NMJ synaptic defects [26]. We utilized pre-existing Drosophila loss of function alleles and previously described Drosophila assays to assess genetic interaction of DmSmn with Drosophila orthologs of C. elegans modifier genes [18,25,26].

First, we determined if Fimbrin (Fim), the Drosophila ortholog of PLS3, modifies DmSmn loss of function defects in growth and NMJ assays. It has been shown that RNAi knockdown of DmSmn (DmSmn RNAi) results in 44% lethality in early pupal stages with 56% lethality at late pupal stages [26]. Loss of Fim alone does not cause larval or pupal lethality (data not shown). Three Fim loss of
Figure 3. Loss of C. elegans PLS3 ortholog enhances the pharyngeal pumping defects of Cesmn-1(lf) animals. During feeding the C. elegans pharynx contracts at over 200 times per minute to capture and grind bacteria. A) Pharyngeal pumping rates can be determined by videotaping feeding and counting contractions as movement of the grinder (indicated by arrow) at slower playback speeds; scale bar indicates 40 microns. B) The progeny of hT2[et8][myo-2p::GFP]/Cesmn-1(lf) (+/Cesmn-1(lf)) animals were allowed to hatch on bacterial strains on standard C. elegans plates. Pumping rates of +/Cesmn-1(lf) heterozygous and Cesmn-1(lf) homozygous animals were determined on different days (day 1–4) on a control ‘empty vector’ bacterial strain. Decreased locomotion (sinusoidal movement) was also scored as uncoordinated (Unc). At day 1, the pumping rates and locomotion of Cesmn-1(lf) animals are identical to +/Cesmn-1(lf) or wild type animals. By day 2, the average pumping rates dropped dramatically (as previously reported [44]) and roughly 20% of animals were uncoordinated (column on right). By day 3 and 4, the pumping rates and locomotion of the majority of surviving animals are defective. Expressing Cesmn-1 in neurons is sufficient to restore pumping rates to near normal levels [44]. The behavior of Cesmn-1(lf) animals is initially normal due to maternal loading of Cesmn-1 gene products, but as the maternal contribution is depleted, loss of Cesmn-1 causes progressive defects in neuromuscular function. C) Pumping rates of +/Cesmn-1(lf) and Cesmn-1(lf) animals were determined at 3 days post-hatching on bacterial feeding strains expressing dsRNA corresponding to Cesmn-1 and plst-1 genes; ‘empty vector’ bacterial cultures were used as a control (empty). plst-1(RNAi) or Cesmn-1(RNAi) significantly decreased Cesmn-1(lf) pumping rates, but not +/Cesmn-1(lf) pharyngeal pumps. D) Decreasing plst-1 function has no effect on pumping rates in heterozygous +/Cesmn-1(lf) animals, but pumping rates were significantly decreased in Cesmn-1(lf); plst-1(tm4255) double mutant animals. At least 20 animals were scored for each genotype in at least 2 independent trials. Pumping rates reported are the average of all animals in all trials. Because of the known impact of chromosomal pairing on Cesmn-1 function alleles were crossed into the DmA59Smn(RNAi) background and each accelerated death compared to DmA59Smn(RNAi) control animals (Figure 5A).

In Drosophila, loss of SMN function results in a dose-dependant decrease in process arborization at the NMJ and diminished numbers of synaptic specializations, termed synaptic boutons [26]. Boutons are visualized as coincident pre-synaptic synaptotagmin and post-synaptic Discs large protein immunoreactivity. The number of synaptic boutons found between Drosophila neurons and muscles provides a simple and readily quantifiable assessment of phenotypic severity. We determined if the Drosophila PLS3 ortholog Fim might also modify the NMJ defects of DmA59Smn. RNAi knockdown of DmA59Smn using the ubiquitous tubulin promoter (TubGAL4;SmnR-NAl) modestly decreased synaptic innervation in Drosophila larvae (reported as bouton numbers per muscle area, Figure 5B). Loss of Drosophila Fim function in Fim’/’ animals also modestly decreased bouton density. We found that effects of Fim’/’ animals were synergistic; bouton numbers were significantly decreased suggesting that loss of Fim function exacerbated DmA59Smn loss of function defects, being consistent with studies in vertebrate models of SMA [37]. These results suggest that PLS3 is a cross-species modifier of SMN function.

Next, Drosophila orthologs of candidate SMN modifier genes from C. elegans were examined. Chp20 and Fmrf were selected as Drosophila orthologs of ntcp-2 and flp-4, respectively, based on similarity and Drosophila loss of function alleles were obtained. (There are 32 genes in C. elegans encoding 32 FMRFamide-related neuropeptides, in contrast, three FMRFamide genes exist in Drosophila. There may be less redundancy in FMRFamide gene function in Drosophila [68,69,70]).

Heterozygous loss of DmA59Smn function in +/SmnT3AO or +/Smn10119 animals had no significant effect on bouton number as expected, SmnT3AO/Smn10119 animals had dramatically decreased bouton numbers (Figure 6). Loss of one copy of Cbc20 or Fmrf modestly decreased synaptic bouton number compared to control animals. However, simultaneous loss of one
copy of DmSmn and one copy of either modifier gene resulted in further synaptic bouton loss (Figure 6). The genetic interaction in trans-heterozygous animals is consistent with a strong genetic interaction between Smn and the two modifier genes. We were unable to obtain classical alleles of the grk-2 Drosophila ortholog. We conclude that Cbp20 and FMRFamide are conserved invertebrate enhancers of Smn loss of function defects and that this genetic interaction is conserved across species.

Testing Drosophila modifier genes in C. elegans assays

A previous Drosophila screen identified twenty-seven P-element insertion lines that altered Drosophila SMN (DmSmn) loss of function defects [26]. Cross-species validation of these genes might also help elucidate conserved pathways that are critical in SMN loss of function pathology. However, several genes flanked the P-element insertion site for many of these modifier lines and the precise DmSmn modifier gene could not be unambiguously identified. Therefore, 40 candidate modifier genes were reported [26]. We identified the likely C. elegans ortholog as 32 of these 40 genes using reciprocal BLAST similarity searching (Table S1). The ability of these genes to modify Cesmn-1(/f) growth defects was assessed by feeding Cesmn-1(/f) and +/+ Cesmn-1(/f) animals expressing the corresponding dsRNA; RNAi feeding clones were constructed for Cesmn-1(/f) and grk-2. RNAi knockdown of Cesmn-1(/f) animals. Knockdown of the C. elegans ortholog atn-1 significantly suppressed the growth defects of Cesmn-1(/f) animals without altering the percentage of large +/+ Cesmn-1(/f) siblings. Finally, C. elegans orthologs of two Drosophila genes were identified, whose genetic interaction with Cesmn-1 resembled the interaction of plst-1 with Cesmn-1, cash-1 and dlc-1. RNAi knockdown of these two genes increased the percentage of large animals in the +/+ Cesmn-1(/f) population without altering the Cesmn-1(/f) population. Growth assay results for these ten genes are found in Table 1 (Rows 4 through 13), results for all orthologs tested can be found in Table S1, and a discussion of modifier gene function is presented in Text S2.

For bona fide cross-species modifier genes, the impact of modifier genes on SMN loss of function defects should be conserved across species (i.e. enhancer genes should enhance in both species). For six cross-species genes, the impact of modifier gene loss on DmSmn and Cesmn-1 loss of function defects was conserved as expected. Specifically, the enhancement of Cesmn-1(/f) defects by RNAi knockdown of nhr-85, egl-15, and kcnl-2 was consistent with the effects of the corresponding Drosophila modifier genes on DmSmn [26]; the corresponding Drosophila insertion lines (d09801, f02864, and d03336) enhanced DmSmn defects and the transposon insertion in these lines are predicted to decrease function. The results for Drosophila orthologs of C. elegans uso-1 and nekl-3 were also consistent across species. The exacerbation of Cesmn-1(/f) growth defects observed after uso-1(RNAi) or nekl-3(RNAi) knockdown was consistent with the suppression of DmSmn defects observed after over-expression of the cognate Drosophila genes. There was also good concordance for the effect of actinin orthologs across invertebrate species. The d00712 Drosophila insertion line likely drives over-expression of the Drosophila gene Actinin (Actn) and enhances DmSmn defects [26,71,72], while

**Figure 4. C. elegans RNAi screen identifies conserved SMN modifier genes.**

A) Four genes were identified as modifiers of Cesmn-1(/f) growth defects in the C. elegans RNAi screen: ncbp-2, T02G5.3, grk-2 and flp-4. The pharyngeal pumping rates of homozygous mutant Cesmn-1(/f) and heterozygous control +/+ Cesmn-1(/f) animals were determined at day 3 as described in Figure 3. B) For two C. elegans candidate modifier genes, flp-4 and grk-2, loss of function alleles were available. grk-2(rt97) significantly decreased the pumping rates of Cesmn-1(/f) pumping rates, but did not reach statistical significance (p = 0.012 and p = 0.236 by two-tailed Mann-Whitney U test, respectively). Pumping rates were determined on day 3 post-hatching; at least 25 animals were scored for each genotype in at least 3 independent trials. Pumping rates reported are the average of all animals in all trials. Significance versus Cesmn-1(/f) is indicated with an asterisk. To control for genetic background, all animals tested were the progeny of animals carrying thebalancer chromosome hT2[myo-2p::GFP]; control animals are hT2/Cesmn-1(/f) siblings.

doi:10.1371/journal.pgen.1001172.g004
suppression of Cesmn-1(lf) growth defects by RNAi knockdown of Cesmn-1(lf) was observed here. For four genes, it is unclear if the results for Drosophila orthologs are concordant across species: atf-6, ape-1, dlc-1 and cash-1. For atf-6 and ape-1, the corresponding Drosophila transposons (d05057 and d05779) are inserted into the 1st intron of one of the two transcripts predicted for the orthologous Drosophila genes; accordingly, these transposons may perturb gene function or may drive over-expression of the predicted 2nd shorter transcript. For the genes with complex genetic interactions with Cesmn-1 (i.e. dlc-1 and cash-1), the function of Drosophila orthologs ctp and CKA are likely decreased by Drosophila insertion lines f02345 and f04448, which suppressed and enhanced DmSmn defects, respectively [26]. Overall, six of ten genes that modified DmSmn growth defects are clearly concordant with the C. elegans growth data, suggesting conserved roles as SMN loss of function modifiers.

Orthologs of three Drosophila genes modified Cesmn-1(lf) neuromuscular defects

C. elegans orthologs of DmSmn modifier genes identified in the previous Drosophila screen [26] were also rescreened using the pharyngeal pumping assay. We found that RNAi knockdown of daf-4 enhanced Cesmn-1(lf) pharyngeal pumping defects, while knockdown of kcnl-2 and nhr-25 suppressed Cesmn-1(lf) pumping defects (Table 3, rows 3 through 5).

Figure 5. Loss of PLS3 orthologs enhances SMN loss of function defects in invertebrates. Previous studies in vertebrates suggested that PLS3 orthologs in C. elegans and Drosophila might modify SMN loss of function defects [37]. A) A genetic interaction was found between the Drosophila PLS3 ortholog (Fimbrin or Fim) and DmSmn using previously described DmSmn RNAi knockdown lines [26] and Fim loss of function alleles. Percentage early or late larval lethality is reported. Ubiquitous RNAi knockdown of DmSmn using the tubGAL4 (TubGAL4) driver results in pupal death; modifier genes alter the percentage of animals that die at early versus late pupal stages (day 7 versus day 9 [26]). Loss of Fim function significantly increased the percentage of animals that died as early pupae (p≤0.05 by Chi-square analysis); Fim is an enhancer of DmSmn loss of function growth/survival defects. At least 100 animals of each genotype were scored in four replicates for the lethality assay. No significant variation was observed between control and experimental crosses with each independent trial. B) The Drosophila d02114 allele likely eliminates Fim function and in homozygous animals modestly perturbs NMJ morphology at larval muscle 4. Fimd02114 enhances DmSmn loss of function NMJ defects consistent with studies in vertebrates [37]. All strains carry the ubiquitous TubGAL4 driver. Significance of p≤0.05 versus single mutant strains was determined by ANOVA and is indicated with asterisk; S.E.M. is shown. In representative images, red corresponds to anti-Discs Large (DLG), green is anti-synaptotagmin and blue is DAPI; scale bar indicates 15 microns. doi:10.1371/journal.pgen.1001172.g005

suppression of Cesmn-1(lf) growth defects by RNAi knockdown of C. elegans atn-1 was observed here. For four genes, it is unclear if the results for Drosophila orthologs are concordant across species: atf-6, ape-1, dlc-1 and cash-1. For atf-6 and ape-1, the corresponding Drosophila transposons (d05057 and d05779) are inserted into the 1st intron of one of the two transcripts predicted for the orthologous Drosophila genes; accordingly, these transposons may perturb gene function or may drive over-expression of the predicted 2nd shorter transcript. For the genes with complex genetic interactions with Cesmn-1 (i.e. dlc-1 and cash-1), the function of Drosophila orthologs ctp and CKA are likely decreased by Drosophila insertion lines f02345 and f04448, which suppressed and enhanced DmSmn defects, respectively [26]. Overall, six of ten genes that modified DmSmn growth defects are clearly concordant with the C. elegans growth data, suggesting conserved roles as SMN loss of function modifiers.

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daf-4 encodes one of the C. elegans TGF-beta receptor subunits orthologous to Drosophila Wit (Witless). In C. elegans, daf-4 and TGF-beta/Dpp pathway function is required for cell specification at numerous stages and for transit through the stress-resistant, long-lived dauer stage [73]. RNAi knockdown of daf-4 exacerbated Cesmn-1(lf) pumping defects, consistent with the effect of TGF-beta pathway manipulation in Drosophila [26]. RNAi knockdown of two C. elegans genes diminished Cesmn-1(lf) neuromuscular defects: kcnl-2 and nhr-25. kcnl-2 encodes a likely C. elegans SK channel subunit and nhr-25 is one of the two C. elegans proteins most similar to Drosophila Usp (Ultraspiracle). No clear ortholog of Usp is found in the C. elegans genome. The corresponding Drosophila d00712 transposon insertion line likely drives over-expression of Usp resulting in enhancement of DmSmn defects [26]. This is consistent with C. elegans results. By contrast, the impact of SK/kcnl-2 loss in growth versus pumping assays is discordant. The d03336 transposon insertion is located in the SK gene, likely
perturbs SK function, and enhances DmSmn growth and Drosophila NMJ defects [26]. This is consistent with kcnl-2(RNAi) enhancement of C. elegans growth defects described above. The suppression of Cesmn-1(lf) pumping defects observed here after kcnl-2 knockdown may reflect differences in the requirement for kcnl-2 function in neuromuscular tissue and/or the relative inefficiency of RNAi knockdown in neurons.

Modifier gene interactions implicate specific pathways critical in neurodegenerative disease

To address the specificity of the invertebrate SMN modifier genes, the impact of their RNAi knockdown was examined on the number of synaptic boutons in the A2 segment of muscles 6 and 7, which were counted in third instar larvae (visualized using Discs large and synaptotagmin immunoreactivity, shown in red and green, respectively). Homozygous loss of DmSmn in DmSmn^{f1109}/DmSmn^{3A0} animals dramatically decreases bouton number but loss of one copy of DmSmn, Cbp20 or FMRF had little effect. Loss of one copy of either Cbp20 or FMRF in animals heterozygous for either DmSmn allele significantly decreased bouton numbers. This nonallelic noncomplementation suggests a strong genetic interaction between these two conserved modifier genes and DmSmn. Scale bar indicates 10 microns in representative images; S.E.M. is shown in graph; all transheterozygous combinations are significantly different from the corresponding single heterozygous controls with p<0.05 by ANOVA as indicated with asterisk.

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Figure 6. Loss of Drosophila Cbp20 or FMRF enhances DmSmn loss of function neuromuscular defects. For two C. elegans modifier genes, loss of function alleles were available for orthologous Drosophila genes: Cbp20 and FMRF [68–70]. Loss of either Drosophila gene enhanced the NMJ defects of DmSmn animals. The number of synaptic boutons in the A2 segment of muscles 6 and 7 was counted in third instar larvae (visualized using Discs large and synaptotagmin immunoreactivity, shown in red and green, respectively). Homozygous loss of DmSmn in DmSmn^{f1109}/DmSmn^{3A0} animals dramatically decreases bouton number but loss of one copy of DmSmn, Cbp20 or FMRF had little effect. Loss of one copy of either Cbp20 or FMRF in animals heterozygous for either DmSmn allele significantly decreased bouton numbers. This nonallelic noncomplementation suggests a strong genetic interaction between these two conserved modifier genes and DmSmn. Scale bar indicates 10 microns in representative images; S.E.M. is shown in graph; all transheterozygous combinations are significantly different from the corresponding single heterozygous controls with p<0.05 by ANOVA as indicated with asterisk.

doi:10.1371/journal.pgen.1001172.g006

A subset of these cross-species modifier genes interact, directly or indirectly, with previously described neurological or neuromuscular disease proteins suggesting common neurodegenerative pathways may be at work (i.e. ATF6 with VAPB/ALS8 or GPRK2 and SMN1 with FMRP) [75–77]. To determine if specific cellular mechanisms could be implicated in SMN loss of function pathology, the published literature and public databases were examined for physical and/or functional interactions between cross-species SMN modifier genes, SMN and neuromuscular disease genes. A protein/genetic interaction map was assembled and is presented in Figure 7 with references. We note that genes implicated in endocytosis and mRNA translational regulation unexpectedly predominate in this interaction map. These two cellular pathways may be pertinent to SMN loss of function pathology.

Discussion

Enormous effort over the last few decades has resulted in the successful identification of numerous neurodegenerative disease genes and the proteins they encode. However, in many cases there remains considerable controversy as to why perturbation of these genes results in neurodegeneration [78–81]. SMN plays a well-

Invertebrate Modifiers of SMN

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Invertebrate Modifiers of SMN

Table 3. RNAi knockdown of candidate genes alters Cesmn-1(lf) neuromuscular defects in the pharyngeal pumping assay.

<table>
<thead>
<tr>
<th>Ce gene</th>
<th>Dm gene</th>
<th>% change vs. control RNAi</th>
<th>Pumps/minute</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cesn-1</td>
<td>Smn</td>
<td>56±19*</td>
<td>101±2</td>
</tr>
<tr>
<td>pist-1</td>
<td>Fim</td>
<td>86±21*</td>
<td>106±1</td>
</tr>
<tr>
<td>daf-4</td>
<td>Wit</td>
<td>71±27*</td>
<td>93±2</td>
</tr>
<tr>
<td>kcnl-2</td>
<td>SK</td>
<td>147±50*</td>
<td>90±1</td>
</tr>
<tr>
<td>nhf-25</td>
<td>Usp</td>
<td>155±45*</td>
<td>91±5</td>
</tr>
</tbody>
</table>

C. elegans orthologs of DmSmn modifier genes whose RNAi knockdown altered Cesmn-1(lf) pharyngeal pumping defects are listed in column 1 and column 2, respectively. The percent (%) change in pumping rates on empty vector RNAi versus candidate gene RNAi was determined independently in four separate experiments and is reported with S.E.M. for both Cesmn-1(lf) homozygous mutant and +/Cesmn-1(lf) heterozygous control animals (columns 3 and 4). As pumping rates for each genotype/treatment varied day-to-day (due to food thickness, etc.), % change versus empty vector RNAi controls is reported and was used to determine significance.

The overall pharyngeal pumping rates in columns 5 and 6 were calculated by pooling all Cesmn-1(lf) animals reared with the RNAi bacteria indicated (n=40 animals). Animals were allowed to hatch on bacterial cultures carrying empty RNAi vector (empty) or RNAi constructs targeting C. elegans genes; pumping rates were determined after 3 days at the early adult or late L4 larval stage as illustrated in Figure 2. Significance (indicated with an asterisk) was determined by a two-sample t-test/Mann-Whitney U test according to sample-specific parameters (p≤0.05). Two additional genes that modified growth CG3172/cash-1 and CG18175/apc-1 (Table S2), reached significance here in pair-wise comparisons with controls. However, their knockdown enhanced Cesmn-1(lf) pumping defects in some trials and suppressed in other trials resulting in no significant change in overall pumping rates. See Table S2 for results for all genes tested and Materials and Methods for details.

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described and ubiquitous role in the Gemin complex and snRNP assembly [8–10], yet SMA specifically affects neuromuscular function, motorneuron survival, and leads to muscle atrophy. Given this neuromuscular specificity, it seems likely that loss of SMN function impacts cellular pathways outside of the Gemin complex. In addition, given the complexity of cellular signaling pathways, genetic pathways that are not directly involved in SMN activity may impact SMN loss of function pathology. To identify SMN modifier pathways, we have used a genetic approach. Unbiased genetic screens are powerful tools as they utilize functional criteria for the identification of genes critical for cellular function. In the case of SMN loss of function, genetic screens can reveal conserved genes and pathways that are important for neuromuscular dysfunction and pathology independent of initial assumptions about the roles of SMN in neurons and muscles. The identification of hitherto unsuspected molecular pathways that modulate SMN neuropathology, directly or indirectly, is expected to widen the range of targets for SMA therapy development.

Conserved genes that modify SMN loss of function defects in disparate species likely represent pathways that are important for SMN loss of function defects or pathology. C. elegans and Drosophila models have been used here to identify SMN loss of function modifier genes that ‘cross species’. It is difficult to estimate how many modifiers of SMN loss of function were missed in the models have been used here to identify SMN loss of function defects or pathology. Invertebrate models of SMN modifiers with SMN: endocytosis and RNA processing/translation control pathways. Regarding the former, it is of note that the yeast ortholog of PLS3, Sac6p, is a key player in endocytosis and Sac6p levels are critical when expanded polyglutamine neurodegenerative disease proteins are expressed in this system [42,89–91]. We suggest that 1) these two cellular mechanisms may be of particular importance in SMA pathology and 2) that unexpected and intimate connections exist between these two pathways. A pair of recently published studies found that the microRNA regulatory RISC complex and endocytosis are physically and functionally coupled in non-neuronal cells [92,93]. Interestingly, the RISC complex also contains Gemin complex proteins; the function of the Gemini and RISC complexes may be related, directly or indirectly [20]. We speculate that in normal animals, physically coupling the seemingly disparate pathways of endocytosis and local translational regulation may help coordinate synaptic activity and receptor signaling with protein translation during both synaptic development and neuron maintenance [94]. Defects in endocytosis have been suggested previously to play a pivotal role in neurodegenerative diseases in numerous scenarios. In such diseases, including SMA, perturbation of endocytosis may result in RNA translational control defects, or vice versa [92,93]. A recent study has demonstrated impaired synaptic vesicle release at the NMJs in severe SMA mice consistent with defects in synaptic vesicle endocytosis/recycling and/or defects in active zone organization [94]. Further
all animals were tested herein were the progeny of hT2(lethal)[myo-2p::GFP]/CeSmn-1[lf] parents. The use of RNAi sensitive C. elegans mutant strains was avoided as their behavior is not normal in many assays (Hart, unpublished observations) and because SMN complex/Sm proteins have been implicated in miRNA pathways [20,97,98]. We note that RNAi knockdown is not always effective. To control for genetic background effects, animals tested in these studies were either heterozygous for hT2 balancer chromosome or progeny of hT2 parents unless otherwise noted.

plst-1(tm4255) animals were obtained from the Japanese National Bioresource Project and were backcrossed four times before further study. The tm4255 allele is a 368 base pair deletion that removes one of the calponin-like, actin-binding homology (CH) domains; plst-1(tm4255) is likely a partial loss of function allele. To test the genetic interaction of plst-1 with Cesmn-1, the backcrossed plst-1(tm4255) allele was used to create a double mutant with Cesmn-1(lf). The self(4)(m) deletion allele was isolated by PCR-based screening of EMS-mutagenized animals. The yu33 allele is a 928 base pair deletion that removes exon 3 of flp-4 gene along with 5’ sequences (flanking sequences, ttcgaaaaacatatttaa and agegctgacgcaagctgct) [66]. The gk-2(rt07) loss of function allele was previously characterized [60].

Drosophila stocks were maintained on standard cornmeal/yeast/molasses/agar medium at 25°C. The mutations of Smn(T3D) and Smn01109 have been described previously [25]. Cbp20(f07077) is a Piggy-Bac insertion mutation from the Exelixis collection. The insertion location is 5’ upstream and adjacent to the start codon of the Cbp20 transcript. Fm7(KG1.300) and Fm1 alleles are loss of function alleles (Flybase). The line d03334 may have an unlinked lethal mutation on another chromosome. Fm7(right) and SmnRNAi; Fm7(right) have TubulinGal4 in the background; this Gal4 transgene does not alter Smn defects (data not shown).

Bioinformatics

C. elegans orthologs of Drosophila and human genes were identified by BLAST searching at NCBI. When a clear ortholog was not identified by reciprocal BLAST analysis, the most similar C. elegans genes were generally tested. plst-1 corresponds to exons of predicted adjacent genes Y104H12.2 and Y104H12.1 based on similarity searching. T02G5.3 corresponds to exons of T02G5.3, T02G5.2, and T02G5.1 based on high-throughput cDNA sequencing and gene prediction programs [99]. New gene predictions have been reported to Wormbase. To assemble the interaction map in Figure 7, literature pertaining to each modifier gene was examined at NCBI, ArceView, C. elegans and yeast on-line databases (Wormbase and SGD) to identify functional or direct interactions between modifier genes and neurodegenerative disease genes.

Construction of RNAi feeding clones for B0432.13, dhs-22 and ugt-49

The L4440 vector [47] was used to clone PCR products corresponding to B0432.13, dhs-22 and ugt-49 genes. Plasmids were transformed into the bacterial strain HT115(DE3) [47,49]. Primers used for cloning were: B0432.13 forward 5’-aagacttgtcagcattgct-3’, reverse 5’-taatccgatgtgctgtg-3’; dhs-22 forward 5’tactgctgtccagagggcag-3’, reverse 5’-tgctgtgctgtccagaggg-3’; ugt-49 forward 5’aagacttgtcagcattgct-3’, reverse 5’-aagacttgtcagcattgct-3’. C. elegans growth studies

For analysis of modifier genes, animals were reared for two generations/5 days on plates spread with bacterial RNAi strains from the Ahringer or Vidal RNAi libraries [53]. RNAi clones

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**Table 4. Summary: Invertebrate modifiers of SMN loss of function.**

<table>
<thead>
<tr>
<th>Ce gene</th>
<th>Dm gene</th>
<th>Hu gene</th>
<th>Change/Affect</th>
<th>Ce</th>
<th>Change/Affect</th>
<th>Dm</th>
</tr>
</thead>
<tbody>
<tr>
<td>plst-1</td>
<td>Fim</td>
<td>Plastin 3 (PLS3)</td>
<td>RNAi/Cmpx Gr, Enh Pump</td>
<td>Lof/Enh</td>
<td></td>
<td></td>
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<td>daf-4</td>
<td>Wit</td>
<td>TGFβ receptor (BMPRIII)</td>
<td>RNAi/Enh Pump</td>
<td>Lof/Enh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>knll-2</td>
<td>SK</td>
<td>SK channel (KCNN3)</td>
<td>RNAi/Enh Gr, Sup Pump</td>
<td>Lof/Enh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nhr-25</td>
<td>Usp</td>
<td>NHR LHR-1 (NRS2A)</td>
<td>RNAi/Sup Pump</td>
<td>OE/Enh</td>
<td></td>
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<tr>
<td>uso-1</td>
<td>p115</td>
<td>Vesicle docking (USO1)</td>
<td>RNAi/Enh Gr</td>
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<td>Eip75B</td>
<td>NHR RevErb (NR1D2)</td>
<td>RNAi/Enh Gr</td>
<td>Lof/Enh</td>
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<td>Atf6</td>
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<td>Btl</td>
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<td>RNAi/Enh Gr</td>
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<td>atr-1</td>
<td>Actn</td>
<td>a-actinin (ACTN)</td>
<td>RNAi/Cmpx Gr</td>
<td>Lof/Enh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cash-1</td>
<td>CG33172</td>
<td>Striatin (STRI9)</td>
<td>RNAi/Cmpx Gr</td>
<td>Lof/Enh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dlc-1</td>
<td>cut up</td>
<td>Dynnein light chain (DYNLL2)</td>
<td>RNAi/Cmpx Gr</td>
<td>Lof/Enh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ncbp-2</td>
<td>CBC20</td>
<td>Cap binding (CBP20)</td>
<td>RNAi/Enh Gr &amp; Pump</td>
<td>Lof/Enh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>grk-2</td>
<td>Gprk</td>
<td>GRK kinase (ABDRK1)</td>
<td>RNAi/Enh Gr &amp; Pump</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>flp-4</td>
<td>FMRF</td>
<td>Neuropeptide (NPFF)</td>
<td>RNAi/Enh Gr</td>
<td>Lof/Enh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T02G3.3</td>
<td>none</td>
<td>none</td>
<td>RNAi/Enh Gr, Sup Pump</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The summary table is divided into three sections corresponding to potential Cesmn-1(lf) modifier genes originally identified in humans, Drosophila, or C. elegans (1 in Hu, 40 in Dm, and 4 in Ce, respectively). Species and gene names are listed in the first three columns. The change in gene expression due to Drosophila GAL4-driven transposon insertion lines or C. elegans RNAi knockdown is listed in columns 4 and 5; effect on Drosophila gene function is predicted based solely on transposon insertion sites in some cases. RNAi: RNAi knockdown; Lof loss of function; decreased function or antisense; OE over-expression; ?: unclear; Enh: enhanced SMN loss of function defects; Sup: suppressed SMN loss of function defects, Cmpx: complex genetic interaction; not determined, n.d.). Column 4 includes the change in gene expression due to RNAi knockdown is not always effective. To control for genetic background effects, animals tested in these studies were the progeny of hT2 [lethal][myo-2p::GFP]/Cesmn-1[lf] parents unless otherwise noted. The progeny of hT2 animals were relatively resistant to Cesmn-1(RNAi) compared to wild type control strains in our assays (data not shown). Consequently, to keep the genetic background invariant, studies are warranted to ascertain the interdependence of endocytosis with transcontrolational control pathways and to explore the relevance of these pathways in neurodegenerative disease.

**Materials and Methods**

**Genetic analysis**

Cesmn-1(lf) homozygous strains cannot be maintained due to infertility; hT2(lethal)[myo-2p::GFP]/Cesmn-1(lf) hT2[blc-(e937, e937) let-37(e972) plas-48] (I;III) animals are fertile and were maintained using standard techniques [95]. The lack of homologous pairing for the rearranged chromosomes LGI and LGII in hT2 animals likely results in increased maternal/zygotic expression of Cesmn-1 and other balanced genes [96]. As expected, we found that the progeny of hT2 animals were relatively resistant to Cesmn-1(RNAi) compared to wild type control strains in our assays (data not shown). Consequently, to keep the genetic background invariant,
corresponding to modifier genes in Table 4 were sequenced to confirm accuracy. The hlh-4(RNAi) clone in the feeding library was incorrect. A Cesmn-1(ok355);hlh-4(tm604) double mutant strain was generated. hlh-4(tm604) did not affect the pharyngeal pumping rates of Cesmn-(lf) (data not shown) and hlh-4 was excluded from further analysis. Length and GFP fluorescence was determined using the COPAS Biosorter (Union Biometrica, Holliston, MA) and the percentage of large animals was determined for each genotype [51]. Three to six independent determinations were undertaken for each genotype/RNAi culture. Significant changes from empty(RNAi) were calculated for each RNAi/genotype using the two-tailed Mann-Whitney U test.

Figure 7. Interaction map of SMN modifier genes. A) Numerous published physical and/or functional interactions were found in the literature that connect many invertebrate SMN modifier genes. The names of vertebrate genes are used in the figure, but interactions are drawn from literature in any animal species. Each type of interaction is represented by different colors of connecting lines and references are provided in the table in part B. Not all interactions are indicated including interactions of FGF, TGF-beta and neuropeptide signaling pathways with endocytosis/cytoskeletal pathways, dynamin interactions with APP [101] and possible connection between VAPB, the ALS8 locus, and SK channels through riluzole [102–106]. The interaction analysis here is not exhaustive and additional interactions may exist. B) The type of interaction is indicated in the table [107–155]: P pull-down, Y yeast 2-hybrid, F functional/genetic, D direct physical interaction. Genes shaded in blue are cross-species invertebrate modifiers; genes shaded in yellow are mammalian genes whose perturbation can cause neurodegeneration; genes outlined in grey are SMA modifiers in drug studies or in patients. Genes pertinent to endocytosis include CIN85, EndoA, Alix, PLS3, CAV1, GPRK, Ataxin2, Profilin, EPS15, Phocein, DYNS1, and Striatin; genes pertinent to RNA processing include PABP, CBC20, Dcp5, SMN, and FMRP. Combined, our results and published studies support a model in which endocytosis and RNA translational control pathways are physically and functionally coupled [92,93]. In normal animals, coupling of these seemingly disparate pathways may help coordinate synaptic activity and protein synthesis in the neuromuscular system.

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C. elegans pharyngeal pumping

The average pharyngeal pumping rates of animals were determined after 3 days (at 25, 25 and 20°C) post-hatching on
empty vector (empty/RNAi) or candidate gene RNAi bacterial feeding strains. Animals were videotaped while feeding for 10 seconds with an AxioCam ICc1 camera on a Zeiss Stemi SV11 at 20 to 66 x magnification. Movies were slowed before counting pumping rates. Pharyngeal grinder movements in any axis were scored as a pumping event. Average pumping rates (± standard error of the mean, S.E.M) for each genotype/treatment were calculated independently in two to four separate experiments. The percent change in pumping rate on empty vector versus candidate gene RNAi was determined for each trial for both Cesmn-1(lf) homozygous and +/-Cesmn-1(lf) heterozygous animals and used to calculate the mean, S.E.M, and significance.

C. elegans genome-wide RNAi screen
kT2(kli-4(e937) let-7(g728) qk48(myo-2::GFP)) (III) animals were reared in liquid cultures in a 96-well plate format on RNAi feeding strains [53]. At least two independent cultures corresponding to each C. elegans RNAi feeding clone were established. Concentrated dsRNA expressing bacteria was added to cultures as necessary to prevent starvation. Cultures were maintained for 8 days at 25°C to generate sufficient animals for analysis. Length and fluorescence were determined using the COPAS BioSorter (Union Bionetrica, Holliston, MA). Data was exported to Excel (Microsoft Corp.) for analysis. Thirty-one clones were identified that modified the average length of Cesmn-1(lf) animals relative to +/-Cesmn-1(lf) siblings in both trials. Four of these genes altered Cesmn-1(lf) size relative to +/-Cesmn-1(lf) siblings in at least 40% of subsequent trials and these were selected as candidate modifier genes for neuromuscular analysis as described in the text.

Drosophila pupal lethality
Three males and three virgin females were placed on fresh food at 25°C on day 1. Eggs were collected for next 2 days (Set 1), and the parents transferred to fresh food. Eggs were collected for another 2 days (Set 2), and the parents discarded. The F1 animals were scored after 15 days, on the 16th day for the first set, and the 19th day for the second set, from day 1. White pupae were scored as early stage death and black pupae were scored as late stage death. Control crosses of tubGAL4:FL26B(Smn RNAi) outcrossed to the wild type strain were used as a control for every experimental set. Significance was determined by Chi-square analysis.

Drosophila NM analysis
Primary antibodies were used at the following dilutions: monoclonal anti-DLG (1:500) (Developmental Studies Hybridoma Bank), polyclonal anti-Synaptotagmin (1:1000) (a gift from Hugo Bellen). FITC- (1:40) and Cy5- (1:40) conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Jackson Immunoresearch Laboratories. Anti-Disc large used at 1:100 (Hybridoma) and anti-HRP used at 1:1000 (Cappell). 3th instar larvae were dissected and fixed for 5 minutes in Bouin’s fixative. Stained specimens were mounted in FluoroGuard Antifade Reagent (Bio-Rad), and images were obtained with a Zeiss LSM510 confocal microscope. Bouton numbers were counted based on the Discs large and Synaptotagmin staining in the A2 segment between muscles 6 and 7 or muscle 4 as indicated. The ratio of muscle area for the various genotypes was normalized to wild type. At least 10–12 animals of each genotype were dissected for the bouton analysis. The ANOVA multiple comparison test was used for statistical analysis.

Supporting Information
Table S1 Summary of C. elegans growth assays. Found at: doi:10.1371/journal.pgen.1001172.s001 (0.09 MB DOC)
Table S2 Summary of C. elegans pharyngeal pumping assays. Found at: doi:10.1371/journal.pgen.1001172.s002 (0.09 MB DOC)
Table S3 Invertebrate modifier genes specifically affect Cesmn-1(lf) pharyngeal pumping defects. Found at: doi:10.1371/journal.pgen.1001172.s003 (0.05 MB DOC)
Table S4 Body length determinations for Cesmn-1(lf) modifier genes. Found at: doi:10.1371/journal.pgen.1001172.s004 (0.05 MB DOC)

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Author Contributions
Conceived and designed the experiments: MD JNS AW HCC AS GK JH TB MBW JSS SAT ACH. Performed the experiments: MD JNS. Analyzed the data: MD TB MBW JSS CL DVV SAT ACH. Wrote the paper: MD JNS AW HCC AS GK JH TB MBW JSS CL DVV SAT ACH.

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