

The role of plastin in motor neuron diseases and identifying genetic
modifiers of spinal muscular atrophy

Melissa B. Walsh

B.S. Neuroscience, Lafayette College, 2004

M.S. Biology, Montclair State University, 2006

Thesis

Submitted in partial fulfillment of the requirements for the Degree of Doctorate of Philosophy in
the Department of Molecular Biology, Cellular Biology and Biochemistry at Brown University.

Providence, Rhode Island

May, 2015

© 2014 by Melissa B. Walsh

This dissertation, by Melissa B. Walsh, is accepted in its present form by the
Department of Molecular Biology, Cell Biology and Biochemistry as satisfying the
dissertation requirement for the degree of Doctor of Philosophy.

Date_____

Advisor (Signature)_____
Anne C. Hart, PhD, Advisor
Brown University, Neuroscience Department

Recommended to the Graduate Council

Date_____

Reader (Signature)_____
Robert A. Reenan, PhD, Committee Chair
Brown University, MCB Department

Date_____

Reader (Signature)_____
Eric M. Morrow, MD, PhD, Committee Member
Brown University, MCB Department

Date_____

Reader (Signature)_____
Kimberly L. Mowry, PhD, Committee Member
Brown University, MCB Department

Date_____

Reader (Signature)_____
Mustafa Sahin, MD, PhD, Outside Reader
Boston Children's Hospital

Approved by the Graduate Council

Date_____

Dean (Signature)_____
Peter M. Webber, PhD, Dean of Graduate School
Brown University

Melissa B. Walsh

Providence, Rhode Island
(610) 248-9932 • melissa_hoh@brown.edu

Education

Brown University, Providence, RI 2009-present
Ph.D. candidate, Molecular Biology, Cellular Biology & Biochemistry Graduate Program

Montclair State University, Montclair, NJ 2004-2006
M.S. Biology

Lafayette College, Easton, PA 2000-2004
B.S. Neuroscience, Minor Environmental Science

Research Experience

Graduate Thesis Research 2010 - present
MCB PhD Program, Brown University, Providence RI
Advisor: Dr. Anne C. Hart

- Characterizing the role of plastin in motor neuron diseases in *C. elegans* using behavioral, biochemical and molecular techniques.
- Identifying genetic modifiers of PLS3 and *smn-1* in *C. elegans*, an invertebrate model of Spinal Muscular Atrophy (SMA).

Senior Research Assistant 04/2009 – 09/2009
Brown University, Providence RI
Advisor: Dr. Stephen L. Helfand

- Worked independently to set up and carry out projects to develop a model for Parkinson's disease in the fruit fly. Responsible for continuing studies in the genetics of aging and helping train lab members in various molecular techniques.

Research Assistant, Senior Research Assistant 09/2006 – 09/2008
Brown University, Providence RI
Advisor: Dr. Yih-Woei Fridell

- Carried out research in the genetics of aging using the *Drosophila* model. Utilized techniques for immunohistochemistry, *in situ* hybridization, insect tissue culture, nucleic acid isolation and purification, and real-time PCR. Also, ran and analyzed physiological and biochemical assays to assess aging in *Drosophila*. Responsible for maintaining fly stocks, and laboratory supplies and for implementing protocols and data analysis on all research projects.

Master's Research 2005-2006
Montclair University, Montclair NJ
Advisor: Dr. Reginald Halaby

- Studied the effects of lysosomal-mediated apoptosis in triptolide- (a Chinese herb) treated MCF-7 breast cancer cells. Responsibilities included cell culturing techniques, MTT cell viability assays, and fluorescence microscopy

Teaching & Mentoring Experience

- Brown University, Providence RI 2013 - present
Project ARISE: Advancing Rhode Island Science Education
• Providing high school teachers with molecular biology equipment and training
- Brown University, Providence RI 2012 - present
College undergraduate student mentor and trainer
- Okinawa Institute of Science & Technology, Okinawa JA summer 2012
Tutor for the OIST Graduate Neurodevelopmental course and lab
- Brown University, Providence, RI 2011
Graduate Teaching Assistant for Undergraduate Course Developmental Biology
• Assisted in Lab course
- Permanent Substitute Teacher 09/2008 – 03/2009
Greater New Bedford Vocational High School, New Bedford MA
Super Intendent Michael Shea
• Permanent substitute for a 9th grade Biology class for the first semester. For the rest of the year, helped set-up the laboratory program for the Science department.

Publications

Deep conservation of genes required for both *D. melanogaster* and *C. elegans* sleep includes a role for dopaminergic signaling. Komudi Singh, Jennifer Y. Ju, **Melissa B. Walsh**, Michael A. DiIorio, Anne C. Hart. Sleep, 2014

C. elegans Notch signaling regulates adult chemosensory response and larval molting quiescence. Singh K, Chao MY, Somers GA, Komatsu H, Corkins ME, larkins-Ford J, Tacy T, Dionne HM, **Walsh MB**, Beaumont EK, Hart DP, Lockery SR, Hart AC. Current Biology. 2011

Conserved genes act as modifiers of invertebrate SMN loss of function defects. Dimitriadi M, Sleight JN, Walker A, Chang Hc, Sen A, Kalloo G, Harris J, Barsby T, **Walsh MB**, Satterlee JS, Li C, Van Vactor D, Artavanis-Tsakonas S, Hart AC. PLoS Genetics. 2010

Yih-Woei C. Fridell, **Melissa Hoh**, Orsolya Kréneisz, Suzanne Hosier, Chengyi Chang, Dane Scantling, Daniel K. Mulkey and Stephen L. Helfand. 2009. Increased uncoupling protein (UCP) activity in *Drosophila* insulin producing neurons attenuates insulin signaling and extends lifespan. Aging, 2009

Conferences

- Society for Neuroscience 2013
Poster Presentation
Walsh, M.B., Wingrove, E., Hart, A.C. “The role of plastin in Spinal Muscular Atrophy”
- Families of Spinal Muscular Atrophy 2013

Selected for Platform Presentation

Walsh, M.B., Wingrove, E., Hart, A.C. “The role of plastin in SMA and other neurodegenerative diseases”

Families of Spinal Muscular Atrophy

2012

Poster Presentation

Walsh, M.B. & Hart, A.C. “The role of plastin in SMA”

C. elegans International Worm Meeting

2011

Poster Presentation

Walsh, M.B. & Hart, A.C. “The role of plastin in SMA”

Professional Associations

Society for Neuroscience

2013

Genetics Society of America

2011

American Association for the Advancement of Science

2011

Acknowledgements

The work presented in this dissertation was supported by Brown University's Neuroscience Program, Molecular Biology, Cellular Biology and Biochemistry Program and the National Institute of Neurological Disorders and Stroke. First and foremost, I would like to thank my advisor, Dr. Anne C. Hart, for the opportunity to perform my thesis research under her guidance. Her limitless knowledge and enthusiasm for the field of *C. elegans* and neurodegenerative disease, provided an invaluable resource throughout my time at Brown. She fully supported my research and gave me the intellectual freedom to expand my research interests and expertise, which in return fueled my passion for science. Not only was she an excellent mentor, she is also one of the most talented and thought provoking scientists. Under her guidance, I have received exceptional training that has prepared me well for future work in the scientific community. I would also like to thank the collaborators in our program project grant, Dr. David VanVactor, Dr. Spyros Artavanis-Tsakonas and Dr. Lee Rubin for their insight into the field and availability of resources. Specifically, Dr. Rubin for allowing me to extend my research into a vertebrate model, in his lab at Harvard.

Next, I would like to thank my Committee members, Dr. Robert Reenan, Dr. Kim Mowry and Dr. Eric Morrow. Their insight into everything from invertebrate genetics to human disease has provided fundamental knowledge and guidance throughout my thesis. I would also like to thank the faculty and support staff of the MCB and Neuroscience Departments.

Additionally, I would like to thank the members of the Hart lab, both past and present. They are colleagues, advisors and friends and it was a pleasure to work along side of them. I would like to give a special thanks to Emily Wingrove, who trained under me as an undergraduate; her unwillingness to give up was inspirational. I would also like to thank fellow graduate student Altar Sorkaç. Not only was he a sounding board for all things scientific, he is a great person and a friendship I will cherish the rest of my life. In particular, from the Hart lab I would like to thank former post-doc Dr. Komudi Singh, without her I wouldn't be the scientist I

am today. Her endless knowledge in *C. elegans*, molecular biology and biochemistry was an inspiration and drove me to become a better scientist.

Importantly, I would like to thank all my friends along the way. Their support and laughter made this process much more enjoyable. I am blessed to have such amazing people in my life, who inspire me every day. Lastly, but most importantly, I would like to thank my family, who have encouraged and supported my decisions from the very beginning. A special thanks to my husband, who has been my rock since day one and can always make me laugh after a long day in the lab. I would like to dedicate this thesis to him and my parents, for their endless love and support.

Table of Contents

Title page.....	i
Copyright page.....	ii
Signature page.....	iii
Curriculum vitae.....	iv
Acknowledgements	vii
Table of Contents.....	ix
 <u>Chapter 1: Introduction</u>	 1
Neurodegenerative diseases.....	2
Overview of Spinal Muscular Atrophy.....	2
Clinical Knowledge.....	3
Genetics of SMA.....	4
SMN protein function.....	5
PLS3 as a genetic modifier of SMA.....	7
Overview of Amyotrophic Lateral Sclerosis.....	8
Genetics of ALS.....	9
hnRNPs.....	10
Overview of Polyglutamine toxicity diseases.....	12
Genetics of Polyglutamine diseases.....	12
<i>Caenorhabditis elegans</i> as a model system.....	13
Modeling SMA in <i>C. elegans</i>	14
Modeling ALS in <i>C. elegans</i>	15
Modeling polyQ disease in <i>C. elegans</i>	15
Overview of thesis.....	16
References.....	18

<u>Chapter 2: PLS3 and hnRNP F/H act as genetic modifiers in neurodegenerative disease..</u>	30
Introduction.....	31
Methods.....	33
Results.....	38
Slow recovery after exhaustion by <i>Cesmn-1(lf)</i> animals.....	38
Increased plastin suppresses <i>Cesmn-1</i> defects and acts in neurons.....	40
Plastin suppresses defects in <i>C. elegans</i> ALS and polyglutamine toxicity models..	42
Identification of <i>sym-2</i> as a genetic modifier of PLS3oe and <i>Cesmn-1</i> mutants.....	43
<i>sym-2</i> acts as a genetic modifier in the <i>C. elegans</i> ALS model.....	44
PLS3, hnRNP F & hnRNP H colocalize with SMN in vertebrate neurons.....	45
Discussion.....	46
References.....	51
Figures.....	57

Chapter 3: Identification of genetic modifiers in *Caenorhabditis elegans*

<u><i>smn-1</i> mutant animals.....</u>	71
Introduction.....	72
Methods.....	73
Results.....	76
The identification of genetic modifiers of PLS3 oe mutant animals.....	76
The identification of genetic modifiers of <i>Cesmn-1</i> mutant animals.....	77
Do genetic modifiers directly interact with PLS3 or SMN?.....	77
Discussion.....	79
Figures.....	82
References.....	86

<u>Chapter 4: Summary, discussion and conclusions</u>	90
Genetic modifiers of disease.....	91
Common pathways are perturbed in neurodegenerative diseases.....	92
Possible model for how PLS3 ameliorates SMA defects.....	95
Current and future studies.....	96
Implications.....	97
References.....	99
<u>Appendix</u>	118
Conserved Genes Act as Modifiers of Invertebrate SMN Loss of Function Defects	
<i>C. elegans</i> Notch Signaling Regulates Adult Chemosensory Response and Larval Molting Quiescence	
Deep conservation of genes required for both <i>D. melanogaster</i> and <i>C. elegans</i> sleep includes a role for dopaminergic signaling	

List of Tables and Figures

Chapter 1:

Figure 1: SMA patients are unable to produce the required amount of full-length SMN protein...4

Table 1: Common genes known to carry ALS-causing mutations.....9

Chapter 2:

Table 1: Strains used in study.....57

Figure 1: *Cesmn-1(cb131)* mutant animals have decreased locomotion after stimulation and slower recovery, that is rescued with *Cesmn-1* insertion.....59

Figure 2: Increased plastin suppresses *Cesmn-1* defects and acts in neurons.....61

Figure 3: PLS3 suppresses the locomotion and pharyngeal pumping defects in *C. elegans* ALS and polyQ toxicity models.....63

Figure 4: *Cesym-2* is a genetic modifier of PLS3 oe, *Cesmn-1* mutants and SODG85R mutants when assessed by locomotion.....65

Figure 5: PLS3, hnRNP F & hnRNP H pull-down and colocalize with SMN in vertebrate neurons.....67

Supplementary Figure 1: PLS3 suppressed the resistance to the cholinesterase inhibitor aldicarb in *Cesmn-1(cb131)*;PLS3 double mutants.....69

Supplementary Figure 2: PLS3 does not rescue the locomotion defects in GABA *unc-25* mutants.....70

Chapter 3:

Table 1: Candidate genetic modifiers screened in *C. elegans* behavioral assays.....82

Figure 1: Genetic modifiers of the PLS3 oe increased locomotion in liquid.....83

Figure 2: Genetic modifiers of the *Cesmn-1(cb131)* locomotion in liquid defect.....84

Figure 3: DDX5 binds directly to SMN and PLS3 in a yeast two-hybrid system.....85

Chapter 4:

Figure 1: Possible model for how PLS3 ameliorates SMA defect in motor neuron processes...95

Chapter 1

Overview of Neurodegenerative Diseases and Genetic Modifiers

1.1 Neurodegenerative diseases

Neurodegenerative diseases are increasingly prevalent due to the rise in average lifespan. This growth in incidence correlates with a high demand for treatment, which is not readily available for most of these diseases, fueling the investigation to better understand the mechanisms behind them. The identification of genes that enhance the likelihood of having or directly causing neurodegeneration has led to improvements in diagnosis; but there is no consensus on the molecular and cellular mechanisms underlying these diseases. To further understand these mechanisms many vertebrate models were created. However, due to the complexity of the vertebrate brain and other limitations, such as gene manipulation and time, researchers have looked to invertebrate model systems such as *Caenorhabditis elegans* (*C. elegans*) or *Drosophila melanogaster* (*Drosophila*) to delineate the molecular and cellular pathways underlying neurodegeneration.

1.2 Overview of Spinal Muscular Atrophy

Spinal Muscular Atrophy (SMA) is an autosomal recessive neurodegenerative disease characterized by loss of alpha motor neurons and muscle atrophy that leads to paralysis and ultimately death (Crawford and Pardo, 1996). It is the number one genetic cause of death for children under two years of age in the United States, affects approximately 1 in 10,000 live births and has a high carrier frequency in the Caucasian population, roughly 1 in 40 (Pearn, 1978, Crawford and Pardo, 1996). SMA is caused by diminished levels of the Survival of Motor Neuron (SMN) protein. SMN is ubiquitously expressed and known to play a fundamental role in snRNP assembly (Fischer et al., 1997, Liu et al., 1997). While the role of SMN in spliceosome assembly has been well studied, it is still unclear how depletion of a ubiquitously expressed protein can specifically cause motor neuron death. Insights into new pathways involving SMN have

occurred over the last several years (Hua and Zhou, 2004, Rossoll and Bassell, 2009, Coady and Lorson, 2011, Fallini et al., 2011, Zou et al., 2011); however, despite a plethora of studies, we still do not know what drives SMA pathogenesis. Understanding disease mechanism could provide insight into the molecular and cellular pathway(s) behind SMA and offer new therapeutic approaches to a disease for which no treatment is currently effective.

Clinical Knowledge

SMA affects the lower motor neurons that are found in the spinal cord and relay information to the muscle. Degeneration of lower motor neurons in individuals with SMA leads to muscle weakening and atrophy throughout the body, usually starting from muscles located closest to the trunk (i.e., shoulders, hips and back). The disease does not impact the brain; hence, individuals maintain intellectual ability and all senses. Due to the wide range of clinical severity in patients, SMA is divided into four categories depending on the age of onset and disease severity (Russman, 2007).

Type I: Also known as Werdnig-Hoffman disease, this is the most severe form of the disorder. Children are diagnosed at birth or within the first few months of life. Affected patients have severe muscle atrophy and are unable to support their head or sit unassisted. Severe muscle atrophy leads to difficulty in breathing and swallowing causing complications that can lead to death by approximately two years of age (Byers and Banker, 1961, Werdnig, 1971).

Type II: Diagnosis is made within the first year of life due to characteristic muscle weakness. Patients with type II SMA are mostly able to sit without support, but are wheel chair bound due to their inability to stand or walk (Dubowitz, 1964).

Type III: Also known as Kugelberg-Welander disease or juvenile type SMA. It has much milder characteristics with symptoms developing as late as adolescence. Patients

generally reach adulthood and can walk without assistance; although they may have mobility trouble later on in life (Kugelberg, 1967).

Type IV: This is the newest category of SMA, known as adult onset, where symptoms often occur after age 30. Affected patients experience mild to moderate muscle weakness, typically in proximal muscles (Russman et al., 1992, Russman et al., 1996).

Genetics of SMA

The gene causing SMA, *SMN1*, was first identified in 1990, to be located on chromosome 5q (Brzustowicz et al., 1990). Unaffected humans contain two intact copies of genes encoding for *SMN* due to a duplication event: a telomeric and a centromeric copy. The duplicated centromeric copy (*survival motor neuron 2 gene (SMN2)*), only present in humans, is very similar to the telomeric copy; but it contains a single nucleotide change (C>T) in exon 7. This single nucleotide change affects *SMN2* pre-mRNA splicing and typically results in a truncated protein of diminished function and stability (*SMN Δ 7*). The protein product of *SMN2* is full-length only 10% of the time, whereas the rest is truncated and quickly degraded (Lefebvre et al., 1995, Coover et al., 1997) (Figure 1).

Figure 1

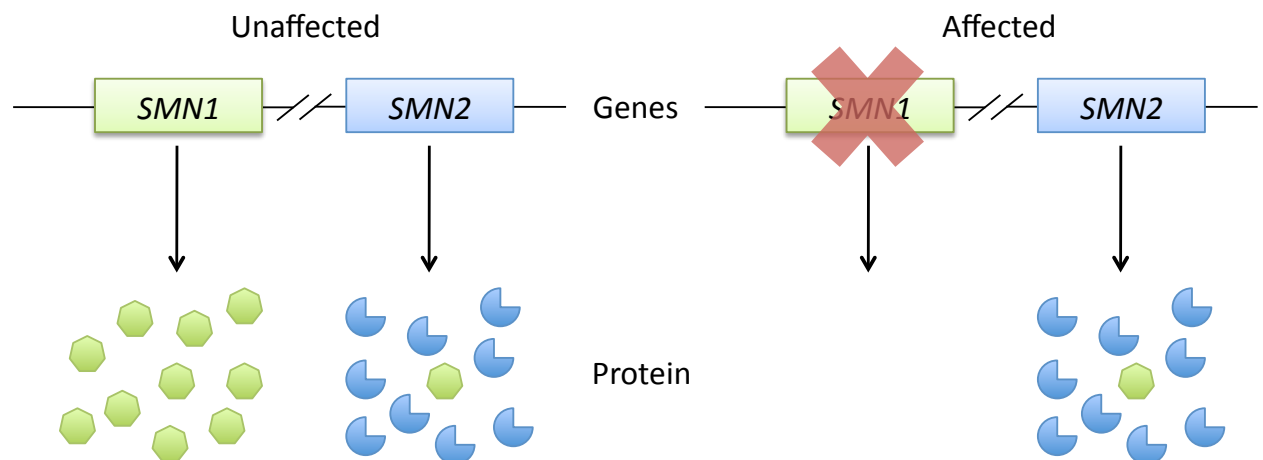


Figure 1: SMA patients are unable to produce the required amount of full-length SMN protein.

SMA (affected) patients contain homozygous mutations in *SMN1*. *SMN2* is only able to produce 10% full protein and the rest is quickly degraded.

SMA patients are homozygous for either a deletion or loss-of-function mutation in the telomeric copy of the *survival motor neuron 1 gene (SMN1)* (Crawford and Pardo, 1996). *SMN2* acts as a genetic modifier of the disease with complete loss of *SMN1* expression. This is because *SMN2* is located in a highly unstable region of chromosome 5q13 and is often present in multiple copies. The highly variant expression of *SMN2* is able to modulate SMA severity in a copy-dependent manner. Studies show that patients with mild symptoms of SMA (SMA Type III) can have as many as four or five *SMN2* copies compared to Type I patients that may only have one (Lefebvre et al., 1995, Prior et al., 2004).

SMN protein function

The 38kDa SMN protein is found in the nucleus and cytoplasm of all cells. Its various domains give a better understanding of the proteins function. Exon 2b encodes a K domain that is rich in lysine (K-rich) and is responsible for GEMIN-2 binding (Young et al., 2000). Directly following is a Tudor domain, encoded by exon 3, that is present in proteins that have a role in RNA metabolism (Buhler et al., 1999). Both domains are essential for SMN's canonical role in snRNP biogenesis (Buhler et al., 1999, Zhang et al., 2011). Exon 5 and part of exon 6 encode a proline-rich (P-rich) region that may be important for the interaction with profilin, an actin-binding protein (Giesemann et al.,

1999). The C-terminal domain is encoded by exon 6 and contains the self-oligomerization domain of SMN allowing it to dimerize into a functional complex with itself (Lorson et al., 1998, Burnett et al., 2009).

SMN has been reported to bind or associate with many proteins (Paushkin et al., 2002, Eggert et al., 2006, Simic, 2008). However, it is unclear which of these interactions are critical in SMA pathogenesis. SMA is thought to be predominantly caused by disruption of SMN's canonical role in snRNP biogenesis and splicing (Lefebvre et al., 1995, Battle et al., 2006). In this paradigm, SMN binds to Sm proteins which function in the assembly of the snRNP complex. This complex is crucial for the recognition of splice sites within pre-mRNAs, followed by the removal of introns. It contains SMN, GEMINS 2-8 and the UNR-interacting protein (UNRIP) (Pellizzoni et al., 2002, Gubitz et al., 2004, Pellizzoni, 2007). Mutations in SMN that disrupt formation of this complex formation cause alterations in splicing (Gabanella et al., 2007, Zhang et al., 2008). It is speculated that specific genes that are mis-spliced when SMN is depleted are crucial for motor neuron circuitry; thus making these cells particularly susceptible to degeneration (Lotti et al., 2012).

Recent studies have shown SMN in a complex without Sm proteins, but instead found in granules along axons and in growth cones of various neuronal types, including motor neurons (Pagliardini et al., 2000, Rossoll et al., 2003, Zhang et al., 2006, Akten et al., 2011, Fallini et al., 2011). To further support a role for SMN in axon granules, SMN was shown to either directly interact or associate with proteins that have known roles in axon function, such as FMRP, HuD and TDP-43 (Wang et al., 2002, Piazzon et al., 2008, Akten et al., 2011). Most SMN interacting proteins in axons are either involved in cytoskeletal dynamics or mRNA-binding regulating transport, stability and local translation in neurons (Giesemann et al., 1999, Rossoll et al., 2002, Oprea et al., 2008, Fallini et al., 2011). Animal models of SMA exhibit decreased axon outgrowth and

smaller growth cones, underlining the importance of SMN's role in motor neurons (Rossoll et al., 2003). Data from these studies and others point to a neuron specific role of SMN, where it is hypothesized that SMN is involved in the trafficking of RNAs for local translation. A clear understanding of SMN's role in axon granules and the RNAs that are locally translated are yet to be identified. Without further knowledge it is unclear whether SMA is a result of the loss of SMN's canonical role in pre-mRNA splicing, or its putative role in axon granule formation and local mRNA translation.

PLS3 as a genetic modifier of SMA

Previously, *SMN2* was the only identified genetic modifier of SMA. In 2008, a study identified Plastin 3 (PLS3), which is found in a complex with SMN and actin, as a gender-specific protective modifier in female SMA patients (Oprea *et al.*, 2008). PLS3 is broadly expressed and contains a conserved calcium-binding domain and is an actin-bundling/stabilizing protein (Delanote *et al.*, 2005). PLS3 is known to play a role in cytoskeleton dynamics, which is crucial for axon growth, pathfinding and branching (Dent and Gertler, 2003). Not much is known about the role of PLS3 outside of its canonical function as an actin-bundling protein. This function or another role yet to be identified could affect SMA pathogenesis in motor neurons.

Oprea and co-workers identified an increase in the PLS3 protein level in asymptomatic individuals with a homozygous mutation of *SMN1* compared to their symptomatic siblings (Oprea *et al.*, 2008). The same study showed that increased PLS3 levels improved axon length and growth defects observed in motor neurons of SMA mice and in zebrafish (Oprea *et al.*, 2008). More recently, the same group developed a PLS3 over-expression (oe) model in mice and found that presynaptic F-actin levels increased in the PLS3 oe mice in an SMA background (Ackermann et al., 2013). The authors conclude that PLS3 can restore defective processes in SMA mutant mice that are

dependent on actin dynamics. By contrast, data from another group suggested that PLS3 acts through an actin-independent component to rescue SMA defects (Lyon et al., 2014). They found that other actin cytoskeleton organizing proteins were not able to modify SMN mutant zebrafish, suggesting a specific role for PLS3. To follow up this theory, the same authors found that the calcium binding residues within the EF hands were essential for PLS3 rescue in motor neurons (Lyon et al., 2014), implying an actin-independent role. Together, these contradictory findings suggest that it is unclear whether plastin acts as an actin bundling protein to suppress the symptoms caused by diminished SMN, or whether plastin plays another role in the SMA paradigm.

1.3 Overview of Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's disease, is the most common motor neuron disease with adult onset. It is characterized by both upper and lower motor neuron degeneration, leading to rapid muscle wasting. Average lifespan of patients after diagnosis is less than five years and death is usually caused by respiratory failure (Rowland and Shneider, 2001a). ALS, in general, affects people between 40 and 60 years of age, with a slightly higher incidence in males (Alonso et al., 2009). It is estimated that approximately 5,000 people in the United States are diagnosed with the disease each year. There is no effective cure for ALS. The only FDA approved drug to treat ALS is Rilutek, however it increases lifespan of patients by only approximately three months (Rowland and Shneider, 2001a). The mechanism of Rilutek or riluzole is unclear. It has diverse targets including potassium channels (Grunnet et al., 2001, Cao et al., 2002), calcium channels (Huang et al., 1997, Duprat et al., 2000) and sodium channels (Benoit and Escande, 1991, Song et al., 1997). Similar to some other neurodegenerative diseases, there are familial and sporadic cases of ALS. It is estimated that 10% of ALS cases are classified as familial, for the majority of which the

genetic etiology is understood. The other 90% of cases occur randomly throughout the population, arising from *de novo* mutations in genes associated with ALS or due to unknown explanations (Rowland and Shneider, 2001b).

Genetics of ALS

The ability to obtain a more complete familial history and genetic information through sequencing, has led the majority of ALS researchers to focus on familial cases. The first gene identified as the cause of some familial ALS cases was discovered over 20 years ago in 1993. Researchers found that dominant mutations in the gene, superoxide dismutase 1 (*SOD1*) lead to the disease phenotype (Rosen, 1993). Since then, over a hundred *SOD1* mutations have been reported and mutations in this gene account for approximately 12% of familial cases and 1% of sporadic cases (Chio et al., 2008). With mutations spread throughout the entire gene, it is still unclear how the disrupted function of this protein leads to motor neuron degeneration.

Since the discovery of *SOD1* over a dozen other genes in varying genetic pathways were identified to cause ALS (Table 1). The functional understanding of these genes in both normal and mutated form provides insight into possible mechanisms of ALS. They include genes involved in RNA processing, protein recycling and possibly motor neuron development and maintenance, among others (Sreedharan et al., 2008, Johnson et al., 2010, Fecto et al., 2011, Wu et al., 2012).

Table 1: Common genes known to carry ALS-causing mutations

Gene	Percentage explained		Putative protein function
	Familial ALS	Sporadic ALS	
<i>TARDBP</i>	4	1	RNA metabolism

Gene	Percentage explained		Putative protein function
	Familial ALS	Sporadic ALS	
<i>SQSTM1</i>	1	<1	Ubiquitination; autophagy
<i>C9ORF72</i>	40	7	DENN protein
<i>VCP</i>	1	1	Proteasome; vesicle trafficking
<i>OPTN</i>	<1	<1	Vesicle trafficking
<i>FUS</i>	4	1	RNA metabolism
<i>PFN1</i>	<1	<1	Cytoskeletal dynamics
<i>SOD1</i>	12	1–2	Superoxide metabolism
<i>UBQLN2</i>	<1	<1	Proteasome

*Table modified from Renton *et al.*, Nature Neuroscience, 2014

Values represent the percentage of ALS by each gene in populations of European ancestry. References for mutations identified are (Rosen, 1993, Sreedharan *et al.*, 2008, Kwiatkowski and Vanderburg, 2009, Johnson *et al.*, 2010, Deng *et al.*, 2011, Fecto *et al.*, 2011, Chio *et al.*, 2012, Majounie *et al.*, 2012, Wu *et al.*, 2012), respectively.

It is likely that there are many unidentified genes that cause ALS. Also, there are additional genes not listed in Table 1 that are implicated in the pathogenesis of ALS and reported as rare causes of ALS or ALS-like syndromes (Hadano *et al.*, 2001, Chen *et al.*, 2004).

hnRNPs

Some of the genes associated with ALS are important for RNA processing and a subset of genes from this pathway that is of particular interest is the ribonucleoproteins (RNPs). These proteins form complexes with other proteins and RNAs that make up

heterogeneous nuclear ribonucleoprotein (hnRNP) complexes. hnRNP complexes are most commonly found within the nucleus and act as post-transcriptional modifiers of newly synthesized pre-mRNAs. While they are best known for their functions in the nucleus, studies have also implicated a cytoplasmic role (Dreyfuss et al., 2002). hnRNPs are categorized based on their affinity and sequence-specific RNA interactions (Dreyfuss et al., 1993). The most abundant of these are designated hnRNP A1 through U, all containing one or more RNA-binding motifs and at least one other domain that either regulates protein-protein interactions or subcellular localization (Dreyfuss et al., 1993). Their interaction with RNAs and proteins suggests multiple roles for hnRNPs, perturbation of which would have detrimental consequences.

Redundancy in this protein family is present, but not exclusive. A recent study has connected a particular hnRNP, hnRNP A, to ALS and other neurodegenerative diseases (Kim et al., 2013). In this report two families demonstrated a mutation in either hnRNPA1 or hnRNPA2B1, causing multisystem proteinopathy (MSP), a syndrome reflecting all aspects of protein disruption that leads to disease pathology. The same study also found in one family the mutations also caused ALS. Identification of disease causing mutations in these hnRNPs is not surprising. Both are known to directly interact with TDP-43, another known ALS causing gene (Buratti et al., 2005) and their fly homologs have been shown to suppress degeneration in an MSP model by RNAi knockdown of hnRNP A (Ritson et al., 2010).

Identification of ALS causing mutations in hnRNPs, along with other genes such as FUS and TARDBP, suggest the importance of RNA processing in disease pathogenesis, a pathway that has been implicated in other neurodegenerative disorders such as SMA. Further studies looking at the role of hnRNPs in neurodegeneration is necessary to determine if the RNA processing pathway is a possible therapeutic target in neurodegeneration.

1.4 Overview of Polyglutamine toxicity diseases

Polyglutamine (PolyQ) disease is a result of expanded repeats of glutamine within a protein (He et al., 2010). This expansion causes late-onset progressive neurodegeneration in many identified proteins within which it resides. The most common form of polyQ disease is Huntington's disease, but this category of neurodegenerative diseases also includes most of spinocerebellar ataxias (SCAs) (Lee et al., 2003, Jacobi et al., 2012). Symptoms typically appear mid-life and progress for 10-25 years. Since the disease is a result of an abnormal gene expansion, it is most commonly genetically inherited with equal penetrance in males and females (Zoghbi and Orr, 2000). Unlike the previously discussed neurodegenerative diseases that mainly affect motor neurons, polyQ disease impacts a much broader range of neurons. For example, patients can exhibit cognitive defects and emotional instability throughout all stages of the disease. It is hypothesized that the cause of this wide range of defects is the formation of abnormal mutant protein inclusions (Nalavade et al., 2013). Most therapeutic strategies target the expanded polyglutamine protein itself with the aim to prevent the formation of inclusions.

Genetics of Polyglutamine disease

The abnormal expansion of the trinucleotide CAG (glutamine) repeat within a gene causes polyQ diseases. Disease severity and onset is correlated to the size of the repeats. For example, Huntington's disease occurs when there are more than 35 CAG repeats encoded within the huntingtin gene, and the age of onset decreases with more repeats (Bates GP, 2002). The large amount of repeats renders the gene unstable, with a propensity to further expand in successive generations. Aggregation of the mutant protein could disrupt cellular function and is implicated in the etiology of neurodegeneration in polyQ disease. These polyQ expansions are described as gain-of-

function mutations due to protein accumulation and aberrant interactions. Normally, misfolded wild-type proteins are degraded by the proteasome. In the case of diseased individuals the proteasome is not able to keep up with the formation of new misfolded proteins, leading to hallmark inclusion formation. Inclusions can then recruit other proteins forming large complexes that can be toxic to the cell (Macdonald et al., 1993). As the repeat expansion in a variety of proteins leads to similar neurodegeneration, polyQ disease may stem from a common mechanism. Understanding this pathway would not only give us insight into polyQ disease but possibly other neurodegenerative diseases that also exhibit protein inclusions.

Taken together, studying the pathology of SMA, ALS and polyQ disease could identify overlapping pathways that are perturbed in all three diseases. Previous studies already implicate perturbation in RNA processing and protein degradation across diseases. Manipulation of a single gene or genetic pathway in order to ameliorate disease pathology has not been done, but for the first time seems promising. Identification of this gene or genetic pathway would provide therapeutic targets that could be beneficial for multiple diseases.

1.5 *Caenorhabditis elegans* as a model system

Caenorhabditis elegans (*C. elegans*) are microscopic nematodes that provide a powerful model organism to understand gene function and genetic pathways. The animal's simple genetics along with transparent body and ease of cultivation make *C. elegans* an ideal system for studying neuronal function and the molecular basis for human disease. The ability to propagate a large quantity of animals from egg to adulthood within several days at minimal cost, allows high throughput screening behaviorally and genetically (Brenner, 1974). With a completely sequenced genome and identification of every neuron, genetic analysis is much faster and easier. In particular *C.*

C. elegans is a powerful model organism in which to study neurodegenerative disease, such as SMA and ALS, because the genome contains orthologs of all disease-associated genes of interest, along with conserved neurotransmitter systems (Culetto and Sattelle, 2000). For these reasons, *C. elegans* have emerged as a powerful *in vivo* model system to study the biological pathways disrupted in major neurodegenerative diseases. The rest of this chapter will outline the disease models used within this dissertation.

Modeling SMA in C. elegans

The *C. elegans* *SMN1* ortholog (*smn-1*) is located on Chromosome I and is highly conserved (Miguel-Aliaga et al., 1999). There are two established models to study SMA with diminished levels of SMN-1 similar to those seen in human patients. In the complete loss-of-function allele (*ok355*) a deletion removes the majority of the gene; and the hypomorphic allele (*cb131*) contains a single point mutation in a region that is conserved in humans (Sleigh et al., 2011). *smn-1 (ok355)* animals have severe behavioral defects and rarely survive to adulthood (Briese et al., 2009). Strains are maintained by balancing the deletion with a GFP marker that is homozygous inviable. Most animals homozygous for the deletion survive to the last larval stage (L4) due to maternal loading of *smn-1* mRNA and SMN-1 protein. Behavioral defects include locomotion, pharyngeal pumping and growth defects (Dimitriadi et al., 2010). Recent unpublished data from our lab shows mislocalization of synaptic proteins at the neuromuscular junction (NMJ) via fluorescently tagged proteins, along with NMJ structural changes at the EM level (paper in submission). On the other hand *cb131* is a much less severe allele, more similar to Type IIIb SMA. These animals look superficially wild-type, but have growth defects, subtle locomotion defects and are sensitive to pharmacological assays that disrupt the NMJ (Sleigh et al., 2011). Most defects in both

alleles implicate the importance for functional *smn-1* at the NMJ, similar to the importance of *SMN1* in human patients. Therefore, *C. elegans smn-1* mutant animals provide a beneficial model to study the pathogenesis of SMA along with biological pathways pertinent to the disease.

Modeling ALS in C. elegans

There are various published models for ALS in *C. elegans*. For the purpose of this study we focus on one model in particular established by the Horwich lab in 2009. In this model, pan-neuronal expression of either human WT SOD1 or mutant G85R SOD1 protein, resulted in a range of behavioral defects (Wang et al., 2009a). For example, the mutant animals displayed locomotion defects, sensitivity to pharmacological assays and show protein aggregation. Looking more closely at the NMJ, fluorescent labeling of synaptic proteins showed mislocalization of proteins and EM data revealed abnormal processes in the nerve cord. These animals still contain the endogenous *SOD1* gene and a multi-copy integration of either WT SOD1 or mutant G85R SOD1, designating it as an over-expression model. However, its use is beneficial since it displays neuronal and behavioral defects similar to other models that are also observed in current SMA models.

Modeling polyQ disease in C. elegans

Polyglutamine toxicity in *C. elegans* is demonstrated as both age and polyQ tract length dependent, recapitulating critical aspects of human polyQ repeat disorders (Brignull et al., 2007). Conversely, expression polyQ tracts in different cells shows varying threshold requirements for toxicity. In this thesis a neuronal polyQ model obtained from the Morimoto lab was utilized (Brignull et al., 2006). Animals expressed

varying lengths of polyQ repeats under the control of a pan-neuronal promoter. Similar to previous disease models discussed, behavior analysis and imaging of the fluorescently tagged repeats was carried out. Animals with expanded repeats, particularly 67, show locomotion and pharyngeal pumping defects compared to animals with only 19 repeats (Brignull et al., 2006). Also, aggregation positively correlated with the behavioral results from these animals. This *C. elegans* model provides a unique tool for investigating the basis of neuron-specific toxicity associated with polyQ proteins. By employing the behavioral NMJ defects demonstrated in this model along with the other disease models, one could test for potential cross disease genetic modifiers.

1.6 Overview of thesis

The work described in this thesis demonstrates genetic modifiers in the context of several neurodegenerative diseases. The second chapter examines a previously identified SMA genetic modifier, PLS3 and its role across SMA, ALS, and polyQ diseases. It is known that PLS3 can ameliorate SMA symptoms in patients and model systems of SMA, but it has not been studied in the context of ALS or polyQ disease. We addressed this context and also identified a novel genetic modifier of SMA and ALS, *sym-2*. The genetic and biochemical methods used in this chapter implicate a new complex that contains SMN, PLS3 and HNRNP F/H.

The third chapter of this thesis identifies several other genetic modifiers for both *smn-1* mutant animals and PLS3 oe animals. Using the model system *C. elegans* we screened through candidate genes and identified new modifiers that may give insight into alternative pathways involved in SMA pathogenesis. Further investigation of these genes will be necessary to better understand if they modify model organisms across species.

The final discussion chapter summarizes the results and presents a possible mechanism by which PLS3 ameliorates SMA pathogenesis. It also emphasizes the importance of *C. elegans* as a model organism in identifying new genetic modifiers in a high throughput method. The data presented in this thesis provide a new cross disease modifier, PLS3 in *C. elegans* disease models of SMA, ALS and polyQ disease. It also identifies novel genetic modifiers of SMA and ALS that may play a role in the mechanism of PLS3 suppression in SMA.

References

- Ackermann B, Krober S, Torres-Benito L, Borgmann A, Peters M, Barkooie SMH, Tejero R, Jakubik M, Schreml J, Milbradt J, Wunderlich TF, Riessland M, Tabares L, Wirth B (Plastin 3 ameliorates spinal muscular atrophy via delayed axon pruning and improves neuromuscular junction functionality. *Hum Mol Genet* 22:1328-1347.2013).
- Akten B, Kye MJ, Hao LT, Wertz MH, Singh S, Nie DY, Huang J, Merianda TT, Twiss JL, Beattie CE, Steen JAJ, Sahin M (Interaction of survival of motor neuron (SMN) and HuD proteins with mRNA cpg15 rescues motor neuron axonal deficits. *P Natl Acad Sci USA* 108:10337-10342.2011).
- Alonso A, Logroscino G, Jick SS, Hernan MA (Incidence and lifetime risk of motor neuron disease in the United Kingdom: a population-based study. *Eur J Neurol* 16:745-751.2009).
- Bates GP HP, Jones AL (eds) (Huntington's Disease. Oxford University Press Oxford, UK.2002).
- Battle DJ, Lau CK, Wan LL, Deng HY, Lotti F, Dreyfuss G (The gemin5 protein of the SMN complex identifies snRNAs. *Mol Cell* 23:273-279.2006).
- Benoit E, Escande D (Riluzole specifically blocks inactivated Na channels in myelinated nerve fibre. *Pflugers Arch* 419:603-609.1991).
- Brenner S (Genetics of *Caenorhabditis-Elegans*. *Genetics* 77:71-94.1974).
- Briese M, Esmaeili B, Fraboulet S, Burt EC, Christodoulou S, Towers PR, Davies KE, Sattelle DB (Deletion of *smn-1*, the *Caenorhabditis elegans* ortholog of the spinal muscular atrophy gene, results in locomotor dysfunction and reduced lifespan. *Hum Mol Genet* 18:97-104.2009).

- Brignull HR, Moore FE, Tang SJ, Morimoto RI (Polyglutamine proteins at the pathogenic threshold display neuron-specific aggregation in a pan-neuronal *Caenorhabditis elegans* model. *J Neurosci* 26:7597-7606.2006).
- Brignull HR, Morley JF, Morimoto RI (The stress of misfolded proteins: *C. elegans* models for neurodegenerative disease and aging. *Adv Exp Med Biol* 594:167-189.2007).
- Brzustowicz LM, Lehner T, Castilla LH, Penchaszadeh GK, Wilhelmsen KC, Daniels R, Davies KE, Leppert M, Ziter F, Wood D, Dubowitz V, Zerres K, Hausmanowapetrusewicz I, Ott J, Munsat TL, Gilliam TC (Genetic-Mapping of Chronic Childhood-Onset Spinal Muscular-Atrophy to Chromosome-5q11.2-13.3. *Nature* 344:540-541.1990).
- Buhler D, Raker V, Luhrmann R, Fischer U (Essential role for the tudor domain of SMN in spliceosomal U snRNP assembly: implications for spinal muscular atrophy. *Hum Mol Genet* 8:2351-2357.1999).
- Buratti E, Brindisi A, Giombi M, Tisminetzky S, Ayala YM, Baralle FE (TDP-43 binds heterogeneous nuclear ribonucleoprotein A/B through its C-terminal tail - An important region for the inhibition of cystic fibrosis transmembrane conductance regulator exon 9 splicing. *Journal of Biological Chemistry* 280:37572-37584.2005).
- Burnett BG, Munoz E, Tandon A, Kwon DY, Sumner CJ, Fischbeck KH (Regulation of SMN Protein Stability. *Mol Cell Biol* 29:1107-1115.2009).
- Byers RK, Banker BQ (Infantile muscular atrophy. *Arch Neurol* 5:140-164.1961).
- Cao YJ, Dreixler JC, Couey JJ, Houamed KM (Modulation of recombinant and native neuronal SK channels by the neuroprotective drug riluzole. *Eur J Pharmacol* 449:47-54.2002).

- Chen YZ, Bennett CL, Huynh HM, Blair IP, Puls I, Irobi J, Dierick I, Abel A, Kennerson ML, Rabin BA, Nicholson GA, Auer-Grumbach M, Wagner K, De Jonghe P, Griffin JW, Fischbeck KH, Timmerman V, Cornblath DR, Chance PF (DNA/RNA helicase gene mutations in a form of juvenile amyotrophic lateral sclerosis (ALS4). *Am J Hum Genet* 74:1128-1135.2004).
- Chio A, Traynor BJ, Lombardo F, Fimognari M, Calvo A, Ghiglione P, Mutani R, Restagno G (Prevalence of SOD1 mutations in the Italian ALS population. *Neurology* 70:533-537.2008).
- Coady TH, Lorson CL (SMN in spinal muscular atrophy and snRNP biogenesis. *Wires Rna* 2:546-564.2011).
- Coover DD, Le T, McAndrew PE, Strasswimmer J, Crawford TO, Mendell JR, Androphy E, Prior TW, Burghes AHM (The survival motor neuron protein (SMN) in spinal muscular atrophy (SMA). *Am J Hum Genet* 61:A329-A329.1997).
- Crawford TO, Pardo CA (The neurobiology of childhood spinal muscular atrophy. *Neurobiol Dis* 3:97-110.1996).
- Culetto E, Sattelle DB (A role for *Caenorhabditis elegans* in understanding the function and interactions of human disease genes. *Hum Mol Genet* 9:869-877.2000).
- Delanote V, Vandekerckhove J, Gettemans J (Plastins: versatile modulators of actin organization in (patho)physiological cellular processes. *Acta Pharmacol Sin* 26:769-779.2005).
- Dent EW, Gertler FB (Cytoskeletal dynamics and transport in growth cone motility and axon guidance. *Neuron* 40:209-227.2003).
- Dimitriadis M, Sleight JN, Walker A, Chang HC, Sen A, Kalloo G, Harris J, Barsby T, Walsh MB, Satterlee JS, Li C, Van Vactor D, Artavanis-Tsakonas S, Hart AC (Conserved Genes Act as Modifiers of Invertebrate SMN Loss of Function Defects. *PLOS Genetics* 6:-.2010).

- Dreyfuss G, Kim VN, Kataoka N (Messenger-RNA-binding proteins and the messages they carry. *Nat Rev Mol Cell Bio* 3:195-205.2002).
- Dreyfuss G, Matunis MJ, Pinolroma S, Burd CG (Hnnp Proteins and the Biogenesis of Messenger-Rna. *Annu Rev Biochem* 62:289-321.1993).
- Dubowitz V (Infantile Muscular Atrophy. A Prospective Study with Particular Reference to a Slowly Progressive Variety. *Brain* 87:707-718.1964).
- Duprat F, Lesage F, Patel AJ, Fink M, Romey G, Lazdunski M (The neuroprotective agent riluzole activates the two P domain K(+) channels TREK-1 and TRAAK. *Mol Pharmacol* 57:906-912.2000).
- Eggert C, Chari A, Lagerbauer B, Fischer U (Spinal muscular atrophy: the RNP connection. *Trends Mol Med* 12:113-121.2006).
- Fallini C, Zhang HL, Su YH, Silani V, Singer RH, Rossoll W, Bassell GJ (The Survival of Motor Neuron (SMN) Protein Interacts with the mRNA-Binding Protein HuD and Regulates Localization of Poly(A) mRNA in Primary Motor Neuron Axons. *Journal of Neuroscience* 31:3914-3925.2011).
- Fecto F, Yan JH, Vemula SP, Liu ED, Yang Y, Chen WJ, Zheng JG, Shi Y, Siddique N, Arrat H, Donkervoort S, Ajroud-Driss S, Sufit RL, Heller SL, Deng HX, Siddique T (SQSTM1 Mutations in Familial and Sporadic Amyotrophic Lateral Sclerosis. *Arch Neurol-Chicago* 68:1440-1446.2011).
- Fischer U, Liu Q, Dreyfuss G (The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. *Cell* 90:1023-1029.1997).
- Gabanella F, Butchbach MER, Saieva L, Carissimi C, Burghes AHM, Pellizzoni L (Ribonucleoprotein Assembly Defects Correlate with Spinal Muscular Atrophy Severity and Preferentially Affect a Subset of Spliceosomal snRNPs. *PLoS One* 2.2007).

- Gall JG (Small granules in the amphibian oocyte nucleus and their relationship to RNA. *J Biophys Biochem Cytol* 2:393-396.1956).
- Giesemann T, Rathke-Hartlieb S, Rothkegel M, Bartsch JW, Buchmeier S, Jockusch BM, Jockusch H (A role for polyproline motifs in the spinal muscular atrophy protein SMN - Profilins bind to and colocalize with SMN in nuclear gems. *Journal of Biological Chemistry* 274:37908-37914.1999).
- Grunnet M, Jespersen T, Angelo K, Frokjaer-Jensen C, Klaerke DA, Olesen SP, Jensen BS (Pharmacological modulation of SK3 channels. *Neuropharmacology* 40:879-887.2001).
- Gubitz AK, Feng WQ, Dreyfuss G (The SMN complex. *Exp Cell Res* 296:51-56.2004).
- Hadano S, Hand CK, Osuga H, Yanagisawa Y, Otomo A, Devon RS, Miyamoto N, Showguchi-Miyata J, Okada Y, Singaraja R, Figlewicz DA, Kwiatkowski T, Hosler BA, Sagie T, Skaug J, Nasir J, Brown RH, Scherer SW, Rouleau GA, Hayden MR, Ikeda JE (A gene encoding a putative GTPase regulator is mutated in familial amyotrophic lateral sclerosis 2 (vol 29, pg 166, 2001). *Nature Genetics* 29:352-352.2001).
- He XH, Lin F, Qin ZH (Current understanding on the pathogenesis of polyglutamine diseases. *Neurosci Bull* 26:247-256.2010).
- Hua Y, Zhou J (Rpp20 interacts with SMN and is re-distributed into SMN granules in response to stress. *Biochem Biophys Res Commun* 314:268-276.2004).
- Huang CS, Song JH, Nagata K, Yeh JZ, Narahashi T (Effects of the neuroprotective agent riluzole on the high voltage-activated calcium channels of rat dorsal root ganglion neurons. *J Pharmacol Exp Ther* 282:1280-1290.1997).
- Jacobi H, Hauser TK, Giunti P, Globas C, Bauer P, Schmitz-Hubsch T, Baliko L, Filla A, Mariotti C, Rakowicz M, Charles P, Ribai P, Szymanski S, Infante J, van de Warrenburg BP, Durr A, Timmann D, Boesch S, Fancellu R, Rola R, Depondt C,

- Schols L, Zdzienicka E, Kang JS, Ratzka S, Kremer B, Stephenson DA, Melegh B, Pandolfo M, Tezenas du Montcel S, Borkert J, Schulz JB, Klockgether T (Spinocerebellar ataxia types 1, 2, 3 and 6: the clinical spectrum of ataxia and morphometric brainstem and cerebellar findings. *Cerebellum* 11:155-166.2012).
- Johnson JO, Mandrioli J, Benatar M, Abramzon Y, Van Deerlin VM, Trojanowski JQ, Gibbs JR, Brunetti M, Gronka S, Wu J, Ding JH, McCluskey L, Martinez-Lage M, Falcone D, Hernandez DG, Arepalli S, Chong S, Schymick JC, Rothstein J, Landi F, Wang YD, Calvo A, Mora G, Sabatelli M, Monsurro MR, Battistini S, Salvi F, Spataro R, Sola P, Borghero G, Galassi G, Scholz SW, Taylor JP, Restagno G, Chio A, Traynor BJ, Consortium I (Exome Sequencing Reveals VCP Mutations as a Cause of Familial ALS. *Neuron* 68:857-864.2010).
- Kim HJ, Kim NC, Wang YD, Scarborough EA, Moore J, Diaz Z, MacLea KS, Freibaum B, Li SQ, Molliex A, Kanagaraj AP, Carter R, Boylan KB, Wojtas AM, Rademakers R, Pinkus JL, Greenberg SA, Trojanowski JQ, Traynor BJ, Smith BN, Topp S, Gkazi AS, Miller J, Shaw CE, Kottlors M, Kirschner J, Pestronk A, Li YR, Ford AF, Gitler AD, Benatar M, King OD, Kimonis VE, Ross ED, Weihl CC, Shorter J, Taylor JP (Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS. *Nature* 495:467-+.2013).
- Kugelberg E (Functions of the spinal cord. Experimental and clinical aspects. Conclusion. *Electroencephalogr Clin Neurophysiol Suppl* 25:95-27.1967).
- Lee WY, Jin DK, Oh MR, Lee JE, Song SM, Lee EA, Kim GM, Chung JS, Lee KH (Frequency analysis and clinical characterization of spinocerebellar ataxia types 1, 2, 3, 6, and 7 in Korean patients. *Arch Neurol* 60:858-863.2003).
- Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, Viollet L, Benichou B, Cruaud C, Millasseau P, Zeviani M, Lepaslier D, Frezal J, Cohen D, Weissenbach J,

- Munnich A, Melki J (Identification and Characterization of a Spinal Muscular Atrophy-Determining Gene. *Cell* 80:155-165.1995).
- Liu Q, Fischer U, Wang F, Dreyfuss G (The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins. *Cell* 90:1013-1021.1997).
- Lorson CL, Strasswimmer J, Yao JM, Baleja JD, Hahnen E, Wirth B, Le T, Burghes AHM, Androphy EJ (SMN oligomerization defect correlates with spinal muscular atrophy severity. *Nature Genetics* 19:63-66.1998).
- Lotti F, Imlach WL, Saieva L, Beck ES, Hao le T, Li DK, Jiao W, Mentis GZ, Beattie CE, McCabe BD, Pellizzoni L (An SMN-dependent U12 splicing event essential for motor circuit function. *Cell* 151:440-454.2012).
- Lyon AN, Pineda RH, Hao LT, Kudryashova E, Kudryashov DS, Beattie CE (Calcium binding is essential for plastin 3 function in Smn-deficient motoneurons. *Hum Mol Genet* 23:1990-2004.2014).
- Macdonald ME, Ambrose CM, Duyao MP, Myers RH, Lin C, Srinidhi L, Barnes G, Taylor SA, James M, Groot N, Macfarlane H, Jenkins B, Anderson MA, Wexler NS, Gusella JF, Bates GP, Baxendale S, Hummerich H, Kirby S, North M, Youngman S, Mott R, Zehetner G, Sedlacek Z, Poustka A, Frischauf AM, Lehrach H, Buckler AJ, Church D, Doucettstamm L, Odonovan MC, Ribaramirez L, Shah M, Stanton VP, Strobel SA, Draths KM, Wales JL, Dervan P, Housman DE, Altherr M, Shiang R, Thompson L, Fielder T, Wasmuth JJ, Tagle D, Valdes J, Elmer L, Allard M, Castilla L, Swaroop M, Blanchard K, Collins FS, Snell R, Holloway T, Gillespie K, Datson N, Shaw D, Harper PS (A Novel Gene Containing a Trinucleotide Repeat That Is Expanded and Unstable on Huntingtons-Disease Chromosomes. *Cell* 72:971-983.1993).

- Miguel-Aliaga I, Culetto E, Walker DS, Baylis HA, Sattelle DB, Davies KE (The *Caenorhabditis elegans* orthologue of the human gene responsible for spinal muscular atrophy is a maternal product critical for germline maturation and embryonic viability. *Hum Mol Genet* 8:2133-2143.1999).
- Nalavade R, Griesche N, Ryan DP, Hildebrand S, Krauss S (Mechanisms of RNA-induced toxicity in CAG repeat disorders. *Cell Death Dis* 4:e752.2013).
- Oprea GE, Krober S, McWhorter ML, Rossoll W, Muller S, Krawczak M, Bassell GJ, Beattie CE, Wirth B (Plastin 3 is a protective modifier of autosomal recessive spinal muscular atrophy. *Science* 320:524-527.2008).
- Pagliardini S, Giavazzi A, Setola V, Lizier C, Di Luca M, DeBiasi S, Battaglia G (Subcellular localization and axonal transport of the survival motor neuron (SMN) protein in the developing rat spinal cord. *Hum Mol Genet* 9:47-56.2000).
- Paushkin S, Gubitz AK, Massenet S, Dreyfuss G (The SMN complex, an assemblysome of ribonucleoproteins. *Curr Opin Cell Biol* 14:305-312.2002).
- Pearn J (Incidence, Prevalence, and Gene Frequency Studies of Chronic Childhood Spinal Muscular-Atrophy. *J Med Genet* 15:409-413.1978).
- Pellizzoni L (Chaperoning ribonucleoprotein biogenesis in health and disease. *Embo Rep* 8:340-345.2007).
- Pellizzoni L, Yong J, Dreyfuss G (Essential role for the SMN complex in the specificity of snRNP assembly. *Science* 298:1775-1779.2002).
- Piazzon N, Rage F, Schlotter F, Moine H, Branlant C, Massenet S (In vitro and in cellulo evidences for association of the survival of motor neuron complex with the fragile x mental retardation protein. *Journal of Biological Chemistry* 283:5598-5610.2008).

- Prior TW, Swoboda KJ, Scott HD, Hejmanowski AQ (Homozygous SMN1 deletions in unaffected family members and modification of the phenotype by SMN2. *Am J Med Genet A* 130A:307-310.2004).
- Ritson GP, Custer SK, Freibaum BD, Guinto JB, Geffel D, Moore J, Tang WX, Winton MJ, Neumann M, Trojanowski JQ, Lee VMY, Forman MS, Taylor JP (TDP-43 Mediates Degeneration in a Novel *Drosophila* Model of Disease Caused by Mutations in VCP/p97. *Journal of Neuroscience* 30:7729-7739.2010).
- Rosen DR (Mutations in Cu/Zn Superoxide-Dismutase Gene Are Associated with Familial Amyotrophic-Lateral-Sclerosis (Vol 362, Pg 59, 1993). *Nature* 364:362-362.1993).
- Rossoll W, Bassell GJ (Spinal muscular atrophy and a model for survival of motor neuron protein function in axonal ribonucleoprotein complexes. *Results Probl Cell Differ* 48:289-326.2009).
- Rossoll W, Jablonka S, Andreassi C, Kroning AK, Karle K, Monani UR, Sendtner M (Smn, the spinal muscular atrophy-determining gene product, modulates axon growth and localization of beta-actin mRNA in growth cones of motoneurons. *Journal of Cell Biology* 163:801-812.2003).
- Rossoll W, Kroning AK, Ohndorf UM, Steegborn C, Jablonka S, Sendtner M (Specific interaction of Smn, the spinal muscular atrophy determining gene product, with hnRNP-R and gry-rbp/hnRNP-Q: a role for Smn in RNA processing in motor axons? *Hum Mol Genet* 11:93-105.2002).
- Rowland LP, Shneider NA (Amyotrophic lateral sclerosis - Reply. *New Engl J Med* 345:1132-1132.2001).
- Russman BS (Spinal muscular atrophy: clinical classification and disease heterogeneity. *J Child Neurol* 22:946-951.2007).

- Russman BS, Buncher CR, White M, Samaha FJ, Iannaccone ST (Function changes in spinal muscular atrophy II and III. The DCN/SMA Group. *Neurology* 47:973-976.1996).
- Russman BS, Iannaccone ST, Buncher CR, Samaha FJ, White M, Perkins B, Zimmerman L, Smith C, Burhans K, Barker L (Spinal muscular atrophy: new thoughts on the pathogenesis and classification schema. *J Child Neurol* 7:347-353.1992).
- Simic G (Pathogenesis of proximal autosomal recessive spinal muscular atrophy. *Acta Neuropathol* 116:223-234.2008).
- Sleigh JN, Buckingham SD, Esmaeili B, Viswanathan M, Cuppen E, Westlund BM, Sattelle DB (A novel *Caenorhabditis elegans* allele, *smn-1(cb131)*, mimicking a mild form of spinal muscular atrophy, provides a convenient drug screening platform highlighting new and pre-approved compounds. *Hum Mol Genet* 20:245-260.2011).
- Song JH, Huang CS, Nagata K, Yeh JZ, Narahashi T (Differential action of riluzole on tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels. *J Pharmacol Exp Ther* 282:707-714.1997).
- Sreedharan J, Blair IP, Tripathi VB, Hu X, Vance C, Rogelj B, Ackerley S, Durnall JC, Williams KL, Buratti E, Baralle F, de Belleruche J, Mitchell JD, Leigh PN, Al-Chalabi A, Miller CC, Nicholson G, Shaw CE (TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science* 319:1668-1672.2008).
- Wang IF, Reddy NM, Shen CKJ (Higher order arrangement of the eukaryotic nuclear bodies. *P Natl Acad Sci USA* 99:13583-13588.2002).
- Wang J, Farr GW, Hall DH, Li F, Furtak K, Dreier L, Horwich AL (An ALS-Linked Mutant SOD1 Produces a Locomotor Defect Associated with Aggregation and Synaptic Dysfunction When Expressed in Neurons of *Caenorhabditis elegans*. *PLOS Genetics* 5:-.2009).

- Werdnig G (Two early infantile hereditary cases of progressive muscular atrophy simulating dystrophy, but on a neural basis. 1891. Arch Neurol 25:276-278.1971).
- Wu CH, Fallini C, Ticozzi N, Keagle PJ, Sapp PC, Piotrowska K, Lowe P, Koppers M, McKenna-Yasek D, Baron DM, Kost JE, Gonzalez-Perez P, Fox AD, Adams J, Taroni F, Tiloca C, Leclerc AL, Chafe SC, Mangroo D, Moore MJ, Zitzewitz JA, Xu ZS, van den Berg LH, Glass JD, Siciliano G, Cirulli ET, Goldstein DB, Salachas F, Meininger V, Rossoll W, Ratti A, Gellera C, Bosco DA, Bassell GJ, Silani V, Drory VE, Brown RH, Landers JE (Mutations in the profilin 1 gene cause familial amyotrophic lateral sclerosis. Nature 488:499-+.2012).
- Young PJ, Man NT, Lorson CL, Le TT, Androphy EJ, Burghes AHM, Morris GE (The exon 2b region of the spinal muscular atrophy protein, SMN, is involved in self-association and SIP1 binding. Hum Mol Genet 9:2869-2877.2000).
- Zhang HL, Xing L, Rossoll W, Wichterle H, Singer RH, Bassell GJ (Multiprotein complexes of the survival of motor neuron protein SMN with gemins traffic to neuronal processes and growth cones of motor neurons (vol 26, pg 8622, 2006). Journal of Neuroscience 26.2006).
- Zhang Z, Lotti F, Dittmar K, Younis I, Wan L, Kasim M, Dreyfuss G (SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. Cell 133:585-600.2008).
- Zoghbi HY, Orr HT (Glutamine repeats and neurodegeneration. Annu Rev Neurosci 23:217-247.2000).
- Zou T, Yang X, Pan D, Huang J, Sahin M, Zhou J (SMN deficiency reduces cellular ability to form stress granules, sensitizing cells to stress. Cell Mol Neurobiol 31:541-550.2011).

Chapter 2

PLS3 and hnRNP F/H act as genetic modifiers in neurodegenerative disease

Melissa B. Walsh¹, Emily Wingrove², Lee L. Rubin³, Anne C. Hart²

¹Department of Molecular Biology, Cellular Biology & Biochemistry, Brown University, Providence, RI

²Department of Neuroscience, Brown University, Providence, RI

³Department of Stem Cell & Regenerative Biology, Harvard University, Cambridge, MA

Emily Wingrove helped with the swimming behavioral assay for the ALS and polyQ models used. Maria Dimitriadi and Bryn Bliska created the *Cesmn-1* rescue construct and associated strains. Natalia Rodriguez Muela undertook mouse motor neuron differentiation and Lance Davidow helped with image analysis of colocalization; both are in the Rubin lab.

Introduction

Neurodegenerative diseases are becoming increasingly prevalent due to increased longevity, and these diseases have no cure. In many cases genes that cause disease have been identified, but little is known about the mechanisms underlying their pathology. Several neurodegenerative diseases affect motor neurons with varying degrees of specificity, including Spinal Muscular Atrophy (SMA), Amyotrophic Lateral Sclerosis (ALS), and some polyglutamine expansion diseases (polyQ) (i.e., Huntington's, Spinocerebellar Ataxia (SCA) 2 and SCA6). Most therapy development aims to correct the expression of the mutated gene in these diseases. In the case of SMA, either the use of histone deacetylase (HDAC) inhibitors to modify chromatin structure or proteasome inhibitors to decrease degradation leads to increased protein levels of SMN (Miller and Cleveland, 2005, Narver et al., 2008, Kwon et al., 2011). In ALS and Huntington's, both regarded as gain-of-function diseases, gene silencing through RNAi and antisense technologies is utilized as therapeutic targets (Miller et al., 2005, Lu and Yang, 2012). Alternatively, instead of targeting specific genes, recent studies suggest that common mechanisms/pathways, such as RNA metabolism and axon outgrowth and maintenance, may be disrupted in multiple diseases (Lin et al., 1998, Ackermann et al., 2013, Bertini and Houlden, 2014). Correction of these disrupted pathways through genetic modification could act as therapeutic targets across multiple diseases. Therefore, it is possible that common genetic modifiers exist for these diseases.

Genes that cause or modify multiple neurodegenerative diseases have been identified. Mutation of the vesicle-trafficking protein VAPB (vesicle-associated membrane protein-associated protein B) can lead to late-onset SMA and/or ALS (Nishimura et al., 2004). Expanded repeats in Ataxin-2 causes the polyQ disease SCA2 and increases the risk of ALS (Farg et al., 2013, Tazen et al., 2013). Mutations in either heterogeneous ribonucleoprotein A1 or A2B1 (hnRNP A1 or hnRNP A2B1) can cause either ALS or

multisystem proteinopathy syndrome (MSP), another degenerative disorder (Kim et al., 2013). These data suggest common links across diseases that can be genetically manipulated to alter disease onset and severity. Genetic modifiers have been reported for SMA, ALS and PolyQ diseases (Oprea et al., 2008, Gusella and MacDonald, 2009, Riboldi et al., 2011); but it remains unclear if any of these genes have an impact in other diseases. This study focuses on Plastin 3 (PLS3); increased PLS3 ameliorates symptoms in Type III SMA patients and PLS3 is found in a complex with SMN (Oprea et al., 2008). SMA is caused by homozygous loss of function mutations in Survival Motor Neuron 1 (*SMN1*) that leads to decreased Survival Motor Neuron (SMN) protein levels (Lefebvre et al., 1995). The mechanism through which increased levels of PLS3, an actin bundling protein, help patients with decreased levels of SMN, a protein essential for small nuclear ribonucleic particle (snRNP) assembly, is unknown. If PLS3 and SMN act in a protein complex or pathway that is disrupted in multiple diseases, PLS3 might be a potential genetic modifier of these other diseases (Ackermann et al., 2013). Determining if PLS3 is a cross disease modifier and identifying other genes within this genetic pathway could lead to the identification of a mechanism of action.

In this study we demonstrate that PLS3 is a genetic modifier in the model system *Caenorhabditis elegans* when the SMN1 ortholog, *Cesmn-1* levels are diminished. We further demonstrate that PLS3 is a genetic modifier across several disease models using previously established ALS (Wang et al., 2009a) and polyQ (Brignull et al., 2006) *C. elegans* models. Using candidate proteins that independently pull-down with *DmSMN* (SMN ortholog) and *Dmfimbrin* (PLS3 ortholog), allowed us to identify the *C. elegans* ortholog of hnRNP F/H, *sym-2*, as a genetic modifier across SMA and ALS disease models in *C. elegans*. The genetic analysis suggests that *sym-2* acts in the same pathway as PLS3 and *Cesmn-1*. Combined with the biochemical and molecular analyses

in vertebrate models done in this study, the results presented here suggest that hnRNP F/H and PLS3 are likely in a complex with SMN that is pertinent to SMA pathology.

Methods

Caenorhabditis elegans strains

Strains listed in Table 1 were maintained at 20°C under standard conditions (Brenner, 1974). Experiments using *Cesmn-1(ok355)*, were performed using the first generation progeny of animals heterozygous for the *hT2* balancer to preserve a common maternal genetic background. RNAi studies were undertaken in a sensitized background containing the transgene, *uls72* that expresses the SID-1 double stranded RNA channel in neurons (Calixto et al., 2010). All integrated transgenes were backcrossed to wild type at least three times before final strain construction. The transgenic strains *rtSi27* and *rtSi28* were generated by Mos-mediated single-copy insertion (Frokjaer-Jensen et al., 2008). The *dpy-30* promoter was subcloned into pPD49.26 from the *C.elegans* expression vector pS235, containing the *dpy-30* promoter via SphI and NheI sites. Human PLS3 CDS (ATG to TAA) was amplified from pcDNA3.1_PLS3.V5-His6 TOPO vector using primers 5'-GAACGCTAGCATGGATGAGATGGCTACCAC and 5'-CAGGGGAATGAAGAGAGTGTAACCCGGGGTTC and subcloned behind the *dpy-30* promoter creating *dpy-30p::PLS3*. Either the promoter alone or the promoter with the PLS3 coding sequence was cloned into a modified pCFJ66 via SphI and NheI or SphI and XmaI sites, respectively. The final constructs pHA0606 *dpy-30p::unc-54UTR*, *unc-119(+)* and pHA0607 *dpy-30p::PLS3::unc-54UTR*, *unc-119(+)* were injected at 50 ng/μl with the standard MosSCI cocktail and injected into EG4322 (*unc-119(ed3)III;cxTi10816 IV*) animals. Single-copy insertion events were identified based on rescue of *unc-119* in non-fluorescent animals and confirmed by PCR genotyping. Transgenic strains for neuronal and muscle specific rescue were generated by cloning the *unc-119* or *myo-3*

promoter using SphI and NheI sites into pHA0606 and pHA0607, in place of the *dpy-30* promoter. Both single-copy and multi-copy transgenes were generated by injection of 50 ng/μl of targeting plasmid into EG4322 (*unc-119(ed3)III;cxTi10816 IV*) animals. Multi-copy insertion events were identified based on the rescue of *unc-119* in the presence of fluorescent animals. The *CepIst-1* over-expression animals were created by amplifying *pIst-1* from the yk1465f08 clone using primers 5'-GGTACCGGTTTAATTACCCAAGTTTG and 5'-GAGCTCCAGAAAAACCGAAAAAATCC. The cDNA was placed behind the *dpy-30p* in pPD49.26 using KpnI and SacI restriction sites. Either promoter alone or promoter with *pIst-1* coding sequence was injected in *Cesmn-1(cb131);ChR2* animals at 30 ng/μl along with the co-injection marker *elt-2p::GFP*.

C. elegans assays

Retinal Feeding

Retinal feeding was done as previously described (Liu et al., 2009) with some modifications. Briefly, transgenic animals were cultivated in the dark at 20°C on NGM plates with OP50 bacteria with or without all-trans retinal (Sigma), approximately 18 hours before experiments. Plates containing all-trans retinal were prepared by spreading 100μl of OP50 culture mixed with 1.6μl of 100mM all-trans retinal stock (dissolved in ethanol) onto 2.5cm plates containing 5ml of NGM. Animals were illuminated with a mercury bulb and a Zeiss bandpass filter set was used to excite channelrhodopsin at 450-490 nm for 20 seconds before analyzing swimming behavior.

Swimming analysis

For the swimming assay, young adult animals were picked singly into wells of a PDMS chip containing 2μl of PBS. Animals were given 5 minutes to settle and assayed at a room temperature, which was maintained between 22°C and 24°C. 10-second

videos were taken with an AxioCam R3.0 camera at 12 frames/second, on a Zeiss Discovery V20 at 12.5 x 11 magnification. Individual animal movies were analyzed using the NABAS software, which was modified for image formats yielding body bends per minute (Krajacic et al., 2012). Average body bends per minute ($\pm$ SEM) were combined from at least three independent trials ($n \geq 30$ animals in total).

Pharyngeal pumping analysis

The pharyngeal pumping assay was performed in the last larval stage or young adults as previously described (Dimitriadi et al., 2010). A pharyngeal grinder movement in any axis was scored as a pumping event. Average pumping rates ($\pm$ SEM) were combined from at least three independent trials ($n \geq 25$ animals in total).

RNAi by feeding

For the RNAi-mediated knockdown, second generation (F2) animals reared on either control vector *pL4440* or *sym-2* (ZK1067.6) dsRNA feeding vector in HT115 bacteria were assayed. The *sym-2* RNAi clone contains genomic DNA, corresponding to the last 3 exons and the 3'UTR of the gene, amplified by primers 5'-CTCGATCTATTCCATCAAAATCG and 5'-TGAGAGGATTGCCTTATGATTGT inserted into the *pL4440* vector (Kamath et al., 2003a). The clones were verified by sequencing.

Aldicarb

Aldicarb sensitivity was conducted as described (Mahoney et al., 2006 Nature Protocols) with some modifications. Aldicarb (Sigma Aldrich) was added to NGM at a final concentration of 1 mM. The day of the assay 30 μ l of OP50 bacteria was seeded in the center of the plates. The plates were allowed to dry without lids for at least 20 minutes. Approximately 15 young adult animals were transferred with a platinum wire onto an aldicarb assay plate. The animals were examined every hour and scored as paralyzed if they did not pump or move after being prodded with a platinum wire. The experimenter was blinded to the genotypes of the tested animals. Average fractional of

paralyzed animals ($\pm$ SEM) was obtained combining at least 3 independent trials ($n \geq 30$ animals in total).

Protein extraction

Whole adult mouse brains of Gad2-EGFP mice were prepared by homogenization, using a Polytron homogenizer in ~20mL of cold 1X RIPA buffer and then incubated on ice for 30 minutes. Supernatant was collected after a 20-minute centrifuge step at 13,500 RPM, in 4°C and stored at -80°C. The protein concentrations were determined using the BSA Protein Assay kit (Bradford), according to the manufacturer's protocol.

Co-immunoprecipitation (Co-IP)

750 μ g of lysate was incubated with 2 μ g of antibody for 1 h on a rotator at 4°C. During incubation protein G beads were washed 5 times in PBS and final bead suspension was added to lysate mixture and incubated for 3 hours on a rotator at 4°C. The bead mixture was centrifuged down and washed three times, removing all supernatant. The beads were then boiled in 2x SDS loading dye at 95°C for 5 minutes to elute the proteins. Eluted proteins were run on an SDS-polyacrylamide gel for Western blotting.

Western analysis

Equal amounts of protein preparations or Co-IP were run and separated on a 10% SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. Membranes were blocked with 5% milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) overnight at 4°C. They were then probed with the appropriate antibody for 1 hour at RT. Antibodies were used at the following dilutions: mouse anti-SMN antibody (BD Transduction Laboratories, diluted 1:2,500), mouse anti-PLS3 antibody (Santa Cruz, diluted 1:1,000), rabbit anti-hnRNP-H antibody (One World Lab, diluted 1:10,000), and

rabbit anti-hnRNP F antibody (Abcam, diluted 1:2,500). After washing with TBST, membranes were incubated with HRP-conjugated secondary antibodies (anti-mouse or -rabbit immunoglobulin-horseradish peroxidase, both at 1:2,500 dilution) at RT for 30 minutes, and the blots were developed using ECL and visualized on autoradiography films. The presence of protein was determined based on the size of the band compared to a ladder

Mouse Motor Neuron Differentiation

Mouse wild-type hb9::GFP ES colonies were differentiated into motor neurons using an embryoid body method as described previously (Wichterle et al., 2002). Briefly, after 2 days, EBs were treated with retinoic acid (RAc; 100nM, Sigma-Aldrich) and a hedgehog agonist (100nM, Curis Inc.). On day 3, EBs were treated with RAc (100nM) and the hedgehog agonist (1 μ M) and incubated for 96h. Papain (Worthington Biochemical) and DNase I were used to dissociate EBs. Motor neurons were then dissociated, plated into poly-ornithine coated 96-well plates (PerkinElmer) at a density of 20,000 cells per well and cultured in DMEM-F12 medium (Invitrogen) containing 2% FBS (Invitrogen), B-27 supplement (Invitrogen), 20ng/ml GDNF and BDNF, CNTF (R&D Systems), insulin, progesterone, BSA, selenite and apotransferrin (Sigma-Aldrich).

Immunocytochemistry

Day 13 motor neuron cultures were fixed using 8% PFA for 15 minutes, followed by permeabilization and blocking using 0.25% Triton X-100 and 10% normal goat serum in PBS for 30 minutes. Detection of endogenous proteins was performed using the following antibodies: mouse anti-SMN antibody (BD Transduction Laboratories, diluted 1:200), rabbit anti-SMN antibody (Santa Cruz, diluted 1:200), mouse anti-PLS3 antibody (Santa Cruz, diluted 1:500), rabbit anti-hnRNP H antibody (One World Lab, diluted 1:1,000), and rabbit anti-hnRNP F antibody (Abcam, diluted 1:500). Secondary

antibodies for detection included AlexaFluor 546 (Gt anti-Rb, 1:300, or Gt anti-Ms, 1:300) and AlexaFluor 647 (Gt anti-Rb, 1:300).

Colocalization analysis

Images were taken using the Opera model QEHS from Perkin Elmer with laser excitation at 405, 488, 561 and 635 nm and a 40x water immersion lens. Confocal stacks were taken using a Nipkow spinning disk at 1µm slice intervals. Images were then stacked together and analyzed for colocalization of antibody staining using Perkin Elmer's Columbus program. Columbus has a graphical user interface using Acapella (<http://www.perkinelmer.com/pages/020/cellularimaging/products/columbus.xhtml>).

Results

Slow recovery after exhaustion of *Cesmn-1(lf)* animals

The *C. elegans* genome contains a single ortholog of SMN1, *smn-1*, referred to here as *Cesmn-1* for clarity. Severe loss of function in *Cesmn-1(ok355)* animals causes neuromuscular junction defects (Briese et al., 2009, Dimitriadi et al., 2010). The majority of the studies in *C. elegans* have used the *ok355* allele. A less severe allele, *Cesmn-1(cb131)*, was recently reported with a mutation that encodes an amino acid substitution of aspartic acid to asparagine that mimics an aspartic acid to a valine mutation found in a Type III SMA patient (Sun et al., 2005, Sleight et al., 2010). To determine if the *Cesmn-1(cb131)* animals have defects reminiscent of *Cesmn-1(ok355)* animals, we assessed locomotion in liquid using an automated scoring program (Krajacic et al., 2012). No locomotion defects during swimming were observed in *Cesmn-1(cb131)* adults after 5 minutes (assessed as body bends per minute, Figure 1A, $p=0.746$). However, it is known that these animals are egg laying defective and have decreased sensitivity to the

acetylcholinesterase inhibitor, pyridostigmine bromide; both behaviors controlled by the NMJ (Sleigh et al., 2011). Thus, we hypothesized that prolonged activation of cholinergic motor neurons using channelrhodopsin (ChR2) might reveal latent defects (Nagel et al., 2005).

To test this hypothesis, we assayed *Cesmn-1(cb131)* animals with ChR2 expressed in cholinergic neurons for swimming defects after ChR2 activation by blue light stimulus. ChR2 fused to mCherry is expressed in cholinergic neurons using the promoter of the *unc-17* gene (Liu et al., 2009). Upon blue light stimulation in the presence of all-trans retinal, the animals displayed a rigid curling behavior similar to what has been previously described upon activation of ChR2 in the cholinergic neurons (Liu et al., 2009). Neither mutant nor control ChR2 animals showed any difference in locomotion before blue light exposure, but both showed a decrease in locomotion immediately following 20 seconds of blue light exposure. However, the *Cesmn-1(cb131);ChR2* animals displayed an even further reduction in locomotion by 42%, immediately after blue light exposure compared to the control *ChR2* animals (Figure 1A, $p=0.001$). To assess if the introduction of ChR2 into the *Cesmn-1(cb131)* mutant animals depressed neuronal function permanently, recovery was evaluated over a course of time. Locomotion of *Cesmn-1(cb131);ChR2* animals recovered to control levels approximately 20 minutes after blue light stimulation (Figure 1A, $p=0.869$). We conclude that *Cesmn-1(cb131)* animals exhibit locomotive defects immediately following stimulation and slower recovery after exhaustion of cholinergic neurons.

To confirm that this defect was due to diminished levels of SMN, we assessed rescue of the defect with the introduction of a single extra copy of *Cesmn-1* (Dimitriadi et al., 2014, under review). A single extra copy of the *C. elegans smn-1* gene is able to significantly increase locomotion rates in the *Cesmn-1(cb131);ChR2* animals post blue-light exposure (Figure 1B, $p=0.043$); thus verifying that the behavioral defect observed is

due to the diminished levels of SMN in the *Cesmn-1(cb131)* animals. Using this assay and other identified behavioral defects in the *Cesmn-1* mutant animals, we are able to assess genetic modifiers of SMA in *C. elegans*

Increased plastin suppresses *Cesmn-1* defects and acts in neurons

PLS3 is a protective modifier only in patients with Type III SMA (Oprea et al., 2008, Hao et al., 2012). Type III SMA patients have functional SMN present, but not in sufficient amounts to completely ameliorate defects. This could be due to the fact that some SMN-1 must be present for PLS3 to have a beneficial effect (Hao et al., 2012). We therefore used the less severe *Cesmn-1(cb131)* animals to determine if PLS3 is able to rescue two neuromuscular defects, locomotion after exhaustion and aldicarb (an acetylcholinesterase inhibitor) sensitivity. Using the exhaustion paradigm, we tested whether the over-expression of PLS3 in all tissues was able to restore locomotion to wild type levels in *Cesmn-1(cb131)* animals. Multiple copies of PLS3 driven by a ubiquitous promoter (*dpy-30*) increased locomotion by 38% when swimming in a wild type background (Figure 2A, $p=0.003$); whereas single copy insertion of the same transgene has no effect in the wild type background (data not shown). Using the single copy PLS3 transgene, we created a triple mutant with the *Cesmn-1(cb131);ChR2* animals. *Cesmn-1(cb131);PLS3oe;ChR2* animals have increased locomotion rates, compared to control *Cesmn-1(cb131);ChR2* animals (Figure 2B, $p=0.002$). *Cesmn-1(cb131)* animals also show resistance to aldicarb, an acetylcholinesterase inhibitor (response measured as fraction of paralyzed animals over time). Double mutant *Cesmn-1(cb131);PLS3oe* showed a wild type paralysis curve and rescued the synaptic defects of *Cesmn-1(cb131)* (Supplementary Figure 1, $p=0.009$). Together, these results indicate the PLS3 oe is able to rescue neuromuscular defects of *Cesmn-1(cb131)* in two separate paradigms.

To determine if plastin is a cross species modifier, we examined whether the *C. elegans* ortholog of PLS3, *plst-1* is also able to rescue *Cesmn-1(cb131)* defects. For

this, animals carrying an extrachromosomal array containing the ubiquitously expressed promoter, *dpy-30* driving *plst-1* were assayed. Introduction of the transgene ameliorated the locomotion defect of the *Cesmn-1(cb131);ChR2* animals (Figure 2B, $p=0.006$). Overall, using the exhaustion defects of *Cesmn-1* mutant animals we showed that plastin is a conserved cross species modifier.

To determine where PLS3 functions to suppress the locomotion defect of *Cesmn-1(cb131)* animals, tissue specific promoters were used driving expression in either neurons or muscle. Expression of PLS3 in the neurons (*unc-119p*) only, but not in muscle (*myo-3p*,) increased locomotion in *Cesmn-1(cb131);ChR2* animals (Figure 2C, $p=0.05$ $p=0.237$, respectively). These results are consistent with a recent study in vertebrates (Ackermann et al., 2013), which corroborates PLS3 oe is sufficient in neurons, arguing a neuron specific role for PLS3 in the suppression of defects in SMN depleted animals.

To demonstrate that the PLS3 oe model suppresses multiple *Cesmn-1* neuromuscular defects, we assessed pharyngeal pumping rates of *Cesmn-1(ok355)* animals. *C. elegans* feed on bacteria using a subset of muscles and neurons in the pharynx when food is present. This behavior is commonly used to assess the neuromuscular function of an animal. Wild type animals pump roughly 250 times per minute in the presence of food. The pumping rates of PLS3 oe animals were reduced by 11% compared to wild type (Figure 2D, $p=0.002$). It is already established that *Cesmn-1(ok355)* animals have a significant reduction in pharyngeal pumping rates (Briese et al., 2009, Dimitriadi et al., 2010). We can therefore test this second *Cesmn-1* mutant allele using the pharyngeal pumping behavior to assess if PLS3 oe is able to rescue. Our data shows that the single copy insertion of the *dpy-30p::PLS3* transgene is able to significantly increase pharyngeal pumping rates by 50% in the *Cesmn-1(ok355)* animals, but not restore it back to wild type levels (Figure 2D, $p=0.011$). Taken together with the

Cesmn-1(cb131);ChR2 locomotion data, this further supports that PLS3 oe is able to rescue defects in multiple CeSMN-1 mutants.

Plastin suppresses defects in *C. elegans* ALS and polyglutamine toxicity models

PLS3 is suggested to be a genetic modifier of other neurodegenerative diseases, besides SMA (Ackermann et al., 2013). To test this hypothesis we assayed locomotion and pharyngeal pumping in previously established *C. elegans* models of ALS and polyQ disease (Brignull et al., 2006, Wang et al., 2009a). In the ALS model, human cDNA for wild type SOD1 or mutant SODG85R is expressed at multiple copies using a pan-neuronal *C. elegans* promoter (*snb-1*). The polyQ toxicity model uses the *rgef-1* pan-neuronal promoter to express YFP containing either 19, or 67 glutamine repeats, serving as control and disease/toxic models respectively. Both ALS and polyQ models have decreased locomotion and pharyngeal pumping rates at the young adult stage (Figure 3; $p=6 \times 10^{-17}$, $p=1.1 \times 10^{-8}$, $p=6.6 \times 10^{-15}$, $p=3.7 \times 10^{-10}$, respectively). To determine if PLS3 suppresses the defects of the disease models, both disease/toxic mutants and their respective controls were crossed into the single copy PLS3 oe animals.

Over-expression of PLS3 in the G85R mutant background increased locomotion in liquid by 61% compared to the control animals (Figure 3A, $p=3 \times 10^{-6}$). Unexpectedly, SOD wild type animals with PLS3 showed a decrease in locomotion by 50% compared to SOD wild type animals without PLS3 (Figure 3A, $p=1.7 \times 10^{-5}$). Results of pharyngeal pumping are similar to locomotion in that the PLS3 over expression animals had increased pumping rates by 35%, back to control levels in the G85R mutant animals, compared to G85R alone (Figure 3B, $p=4.1 \times 10^{-8}$). These data allowed us to conclude that PLS3 is able to suppress the behavioral defects assessed in the *C. elegans* ALS model.

We next tested rescue of these two behaviors in the polyQ model described previously. We observed no significant change in locomotion between the polyQ67 with or without PLS3 (Figure 3C, $p=0.135$). However, PLS3 is able to increase pharyngeal pumping rates in the polyQ67 animals by 13% compared to polyQ67 animals without PLS3 (Figure 3D, $p=5.2 \times 10^{-3}$). Together these results indicate that PLS3 modifies behavioral defects across *C. elegans* neurodegenerative disease models.

As a control to ensure that the effects of PLS3 oe on behavior are disease-specific, we assessed the locomotion of *unc-25* mutant animals in the presence of PLS3 oe. The *unc-25* gene encodes for the *C. elegans* ortholog of mammalian GAD1 and is required for γ -aminobutyric acid (GABA) synthesis. Among other behavioral defects, these animals have decreased locomotion (Jin et al., 1999). We recapitulated the 65% decrease in locomotion in liquid and see no change in locomotion rates in the presence of PLS3 (Supplementary Figure 2, $p=5 \times 10^{-25}$, $p=0.576$, respectively). Thus, PLS3 is not able to rescue locomotive defects in GABA mutants and suggesting that the rescue is specific to the disease models tested.

Identification of *sym-2* as a genetic modifier of PLS3oe and *Cesmn-1* mutants

PLS3 and SMN co-immunoprecipitate in a complex together with actin in HEK293 cells and murine spinal cord (Oprea et al., 2008), but the identities of any other proteins in this complex are unknown. Identifying candidate proteins in this complex would lead to a better understanding of how PLS3 ameliorates symptoms in SMA patients. Having validated *C. elegans* SMA models through rescue by PLS3 oe, we looked to identify more *Cesmn-1* genetic modifiers by a targeted screen. Proteomic pull-down analysis done in S2 cells with *Drosophila melanogaster* (*Dm*) SMN and the ortholog of PLS3, fimbrin, provided a list of candidates that pulled down with both proteins of interest independently (Sen et al., 2013). Prioritizing these candidate proteins

by frequency of pull-down, orthology to both *H. sapiens* and *C. elegans* and availability of *C. elegans* loss-of-function(*lf*) alleles, we tested 11 candidate modifier genes. Each of these *lf* alleles was first screened using the PLS3 oe mutant in a wild type background to assess suppression of increased locomotion. Genes whose loss suppressed PLS3 oe increased locomotion were sequentially crossed into *Cesmn-1* mutants and these animals were assayed for locomotion and pharyngeal pumping assays.

One gene of particular interest that came out of the screen was *sym-2*, the ortholog to hnRNP F/H, a heterogeneous nuclear ribonucleoprotein involved in processing pre-mRNAs. *sym-2(lf)* mutants suppressed the increased locomotion of PLS3 oe animals (Figure 4A, $p=0.007$). Further analysis in *Cesmn-1(cb131)* mutant animals showed that *sym-2(lf)* also suppressed the locomotion after exhaustion defect (Figure 4B, $p=0.006$). Pharyngeal pumping analysis of the double mutant with *Cesmn-1(ok355)* did not show complete rescue, but showed a subset of the population rescued, suggesting only a subtle effect on the more severe *Cesmn-1* mutant allele. Taken together, these data suggest that *sym-2* is a genetic modifier of both PLS3 oe, *Cesmn-1(cb131)* mutant animals and to a lesser extent *Cesmn-1(ok355)* animals. The discrepancy between *Cesmn-1* alleles suggests that in order for *sym-2* to act as a genetic modifier, some SMN-1 is necessary. The identification of *sym-2* as a genetic modifier in these behavioral paradigms, suggests a genetic interaction with SMN and PLS3 and therefore SYM-2 is likely to act through the same mechanism as PLS3.

***sym-2* acts as a genetic modifier in the *C. elegans* ALS model**

Previously we observed that PLS3 modifies behavior across *C. elegans* disease models, suggesting perturbation of a common genetic pathway. To determine whether *sym-2* is able to also modify behavioral defects in these disease models, we assessed locomotion in the ALS and polyQ mutant animals. Results show that *sym-2* knockdown

by RNA interference (RNAi) significantly ameliorated G85R locomotion (Figure 4C, $p=3.7 \times 10^{-8}$), suggesting that *sym-2* is a cross disease modifier. However, knockdown of *sym-2* by RNAi in the polyQ disease model does not affect the locomotion of the polyQ mutant animals (data not shown). Combined, these results suggest that while *sym-2* knockdown is able to modify the SMN and ALS mutant animals, it may be specific to those two disease models. However, the penetrance of RNAi by feeding is variable and incomplete in some cases, which could account for the lack of effect on the polyQ animals (Kamath et al., 2003b, Simmer et al., 2003).

PLS3, hnRNP F & hnRNP H colocalize with SMN in vertebrate neurons

To determine if both PLS3 and hnRNP F & H are found in the same complex as SMN in vertebrate neuronal tissue, Co-IP was performed with the proteins of interest. Initially, we confirmed that all proteins are present in mouse brain lysate independently of each other. Subsequently, we used immunoprecipitation of endogenous SMN protein to isolate any complexes containing SMN and blotted for SMN, PLS3, hnRNP F and hnRNP H. Western blots show that all 4 proteins of interest coimmunoprecipitate with SMN (Figure 5A). To rule out any background effect, all 4 proteins of interest were blotted for, in a control IP that did not contain any antibody. No bands were present in these blots (data not shown), arguing SMN does associate with PLS3, hnRNP F and hnRNP H, although we cannot conclude whether all these proteins are found in the same complex.

Further analysis was performed in mouse primary motor neuron cultures to assess for colocalization of SMN with the proteins of interest. Studies show that SMN is found in granules along neuronal processes (Pagliardini et al., 2000). New evidence also supports the presence of several hnRNPs to be located in granules in the neuronal processes as well (Rossoll et al., 2002, Liu et al., 2008b). However, both hnRNP F and H have only been previously discussed in the context of their nuclear role in splicing

(Wang et al., 2012, Gupta et al., 2013). Labeling of endogenous proteins in the mouse primary motor neuron cultures, shows the presence of SMN, PLS3, hnRNP F and hnRNP H in a punctate pattern along neuronal processes at day 13 neuronal cultures (Figure 5B). Automated analysis using Columbus from Perkin Elmer also shows colocalization of SMN with all 3 proteins of interest in the neurites (Figure 5B). This shows for the first time that hnRNP F and H are found in neuronal processes of motor neurons and that they colocalize with SMN. Collectively, these data suggest an interaction between SMN, PLS3 and hnRNP F/H that could be important in disease pathology or the mechanism behind PLS3 suppression of SMA.

Discussion

Here we report for the first time that PLS3, a gene not previously implicated in ALS or polyQ pathogenesis is a cross disease modifier. We also identify *sym-2* as a new genetic modifier of our PLS3 oe model and the *C. elegans* models of SMA and ALS. The majority of the work presented here is done in previously defined *C. elegans* models of SMA, ALS and polyQ disease. All of these models have neuromuscular defects that can be assessed by locomotion or pharyngeal pumping. PLS3 is able to ameliorate these defects, but it is still unclear why. Previously identified proteomic interactions of SMN and fimbrin (the *Drosophila* ortholog of PLS3), independently in S2 cells (Sen et al., 2013) allowed us to compile a list of possible genes that PLS3 could be acting through to modify SMA. These genes implicate several pathways, including splicing and axonal transport. Through genetic analysis, *sym-2*, the ortholog to hnRNP F & H1,2,3 was found to modify *Cesmn-1* and PLS3 oe mutant animals. We also observed *sym-2* as a modifier of the ALS mutant animals, but not PolyQ. Finally, SMN pulled down with PLS3, hnRNP F and hnRNP H in mouse brain tissue and colocalized with these proteins in primary motor neurons of wild type mice. The results here suggest that PLS3 may ameliorate

SMA defects through a novel complex containing hnRNP F and/or H, that acts in the processes of motor neurons. These data reveal a possible complex containing SMN and PLS3 that could give us insight into the mechanism behind PLS3 suppression of SMA.

Plastin is a cross species and cross disease modifier of *C. elegans* SMA, ALS & Polyglutamine models

Consistent with vertebrate data (Ackermann et al., 2013), our data showed that PLS3 expression is sufficient in neurons to rescue the behavioral defects of *Cesmn-1* mutant animals. Over expression of the *C. elegans* ortholog to PLS3, *p1st-1*, also shows rescue of the locomotion defect proving plastin to be a cross species modifier. These data suggest the high conservation of both *Cesmn-1* and *p1st-1*, validating the nematode *C. elegans* model to study these genes. The neuron specific rescue of the PLS3 model similar to previous studies in vertebrate models (Oprea et al., 2008, Hao et al., 2012, Ackermann et al., 2013) also validates this system as a way to screen for genetic modifiers of disease. It is known that genes causing ALS and Polyglutamine disease affect motor neurons, similarly to SMA. The idea that a common mechanism is disrupted across these diseases to cause neuromuscular defects has been widely speculated. For the first time we show that manipulation of a single gene, PLS3 can ameliorate the neuromuscular defects of seen in three disease models. Overall, our results suggest that the mechanism through which PLS3 is acting could be a common mechanism that is disrupted across neurodegenerative diseases. These data also validate the model used for further analysis of genetic modifiers of SMA.

hnRNPs are a cross disease modifier of *C. elegans* SMA and ALS models

The only identified human genetic modifiers of SMA are SMN2 and PLS3 (Lefebvre et al., 1995, Prior et al., 2004, Oprea et al., 2008). The model system *C. elegans* provides an efficient tool to screen for behavioral modifiers of SMN mutant

animals. Identification of new genetic modifiers paves the way to further analysis in vertebrate models and these genes can possibly be used as therapeutic targets of SMA. In this study we have identified the ortholog of hnRNP F & H1,2,3, *sym-2*, as a genetic modifier in the *C. elegans* models for SMA and ALS. Loss of *sym-2* is able to increase locomotion in both models while also suppressing the PLS3 oe increased locomotion. These data demonstrate a genetic interaction between *Cesmn-1*, PLS3 and *sym-2* and suggest a similar interaction is occurring in vertebrates. Other hnRNPs are implicated in neurodegenerative diseases, hnRNPA1 & A2B1 loss-of-function mutations are found in both ALS and MSP patients (Kim et al., 2013). Our data show an opposite effect of hnRNP perturbation in disease, reinforcing the fact that these proteins can be extremely different from one another. Little is known about the function of RNPs outside their role in transcription and RNA and protein binding. The possibility that one class of RNPs binds a different subset of RNAs and proteins compared to another is highly likely, and thus could explain the difference in effects when you alter their expression levels. We suggest that hnRNP F/H perturbation may confer protection against SMN-1 depletion, but further studies are necessary to address this conclusively.

SMN, PLS3 and hnRNPs could be acting in a complex together in motor neuron processes

With the *C. elegans* behavioral data implicating the genetic interaction between SMN, PLS3 and hnRNPs we turned to a vertebrate system to determine if they are acting in the same complex together in higher organisms. Here, we report using biochemical analysis that in mouse brain SMN pulls-down with PLS3, hnRNP F and hnRNP H, independently. This analysis is done using endogenous proteins to ensure that manipulation of protein expression is not the cause of interaction. To support these findings, we also assessed colocalization of proteins of interest in mouse primary motor

neuron cultures. Consistent with pull-down data we see colocalization of SMN with PLS3, hnRNP F and hnRNP H. Interestingly, the colocalization is found in motor neuron processes, implicating an axon granule interaction between SMN and PLS3. The localization of SMN in processes is consistent with previous studies identifying SMN within axon granules (Rossoll et al., 2002). However, it has not been previously shown that these granules also contain PLS3 and hnRNP F/H. Our results suggest a non-nuclear role for these proteins possibly in the same complex, which has not been previously established.

How do these proteins act together to modify disease?

It is still unknown how PLS3 is able to ameliorate symptoms in SMA patients without altering SMN levels. One possible model is that SMN is found in axon granules and is part of an RNA/protein complex that also contains PLS3 and various hnRNP's. SMN loss impairs axonal trafficking of the RNP granules that synthesize proteins essential for axon growth and maintenance, including F-actin, CPG15 and synaptophysin (Taylor et al., 2009, Akten et al., 2011, Ackermann et al., 2013). Furthermore loss of SMN could cause decreased local translation through mis-splicing of local RNAs at the growth cones of motor neurons, critical for neuron survival. Over-expression of PLS3 may be able to stabilize these granules via actin in order to increase transport and local translation of essential proteins. hnRNPs may compete for binding in these complexes and with the loss of the particular subset hnRNP F & H, recruitment of other hnRNP's such as A1 & A2B1 may be upregulated. This upregulation along with increased stabilization of the granules through PLS3 increases transport of these granules and increases local translation of RNAs; two essential processes in axon growth and maintenance. Additional studies are needed to further understand the interaction between SMN, PLS3 and hnRNP F/H in the context of SMA. Also,

understanding the interaction between PLS3 and hnRNPs in axon granules could lead to novel therapeutic interventions for related neurodegenerative disorders that also have motor neuron and axon outgrowth defects.

References:

- Ackermann B, Krober S, Torres-Benito L, Borgmann A, Peters M, Barkooie SMH, Tejero R, Jakubik M, Schreml J, Milbradt J, Wunderlich TF, Riessland M, Tabares L, Wirth B (Plastin 3 ameliorates spinal muscular atrophy via delayed axon pruning and improves neuromuscular junction functionality. *Hum Mol Genet* 22:1328-1347.2013).
- Akten B, Kye MJ, Hao LT, Wertz MH, Singh S, Nie DY, Huang J, Merianda TT, Twiss JL, Beattie CE, Steen JAJ, Sahin M (Interaction of survival of motor neuron (SMN) and HuD proteins with mRNA cpg15 rescues motor neuron axonal deficits. *P Natl Acad Sci USA* 108:10337-10342.2011).
- Bertini E, Houlden H (Defects of RNA metabolism in the pathogenesis of spinal muscular atrophy. *Neurology* 82:1298-1299.2014).
- Brenner S (Genetics of *Caenorhabditis-Elegans*. *Genetics* 77:71-94.1974).
- Briese M, Esmaeili B, Fraboulet S, Burt EC, Christodoulou S, Towers PR, Davies KE, Sattelle DB (Deletion of *smn-1*, the *Caenorhabditis elegans* ortholog of the spinal muscular atrophy gene, results in locomotor dysfunction and reduced lifespan. *Hum Mol Genet* 18:97-104.2009).
- Brignull HR, Moore FE, Tang SJ, Morimoto RI (Polyglutamine proteins at the pathogenic threshold display neuron-specific aggregation in a pan-neuronal *Caenorhabditis elegans* model. *J Neurosci* 26:7597-7606.2006).
- Calixto A, Chelur D, Topalidou I, Chen XY, Chalfie M (Enhanced neuronal RNAi in *C. elegans* using SID-1. *Nature Methods* 7:554-U102.2010).
- Dimitriadi M, Sleight JN, Walker A, Chang HC, Sen A, Kalloo G, Harris J, Barsby T, Walsh MB, Satterlee JS, Li C, Van Vactor D, Artavanis-Tsakonas S, Hart AC

- (Conserved Genes Act as Modifiers of Invertebrate SMN Loss of Function Defects. *PLOS Genetics* 6:-.2010).
- Farg MA, Soo KY, Warraich ST, Sundaramoorthy V, Blair IP, Atkin JD (Ataxin-2 interacts with FUS and intermediate-length polyglutamine expansions enhance FUS-related pathology in amyotrophic lateral sclerosis. *Hum Mol Genet* 22:717-728.2013).
- Frokjaer-Jensen C, Davis MW, Hopkins CE, Newman BJ, Thummel JM, Olesen SP, Grunnet M, Jorgensen EM (Single-copy insertion of transgenes in *Caenorhabditis elegans*. *Nat Genet* 40:1375-1383.2008).
- Gupta SK, Kostı I, Plaut G, Pivko A, Tkacz ID, Cohen-Chalamish S, Biswas DK, Wachtel C, Ben-Asher HW, Carmi S, Glaser F, Mandel-Gutfreund Y, Michaeli S (The hnRNP F/H homologue of *Trypanosoma brucei* is differentially expressed in the two life cycle stages of the parasite and regulates splicing and mRNA stability. *Nucleic Acids Research* 41:6577-6594.2013).
- Gusella JF, MacDonald ME (Huntington's disease: the case for genetic modifiers. *Genome Med* 1.2009).
- Hao LT, Wolman M, Granato M, Beattie CE (Survival Motor Neuron Affects Plastin 3 Protein Levels Leading to Motor Defects. *Journal of Neuroscience* 32:5074-5084.2012).
- Jin YS, Jorgensen E, Hartweg E, Horvitz HR (The *Caenorhabditis elegans* gene *unc-25* encodes glutamic acid decarboxylase and is required for synaptic transmission but not synaptic development. *Journal of Neuroscience* 19:539-548.1999).
- Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, Kanapin A, Le Bot N, Moreno S, Sohrmann M, Welchman DP, Zipperlen P, Ahringer J (Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* 421:231-237.2003a).

- Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, Kanapin A, Le Bot N, Moreno S, Sohrmann M, Welchman DP, Zipperlen P, Ahringer J (Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* 421:231-237.2003b).
- Kim HJ, Kim NC, Wang YD, Scarborough EA, Moore J, Diaz Z, MacLea KS, Freibaum B, Li SQ, Molliex A, Kanagaraj AP, Carter R, Boylan KB, Wojtas AM, Rademakers R, Pinkus JL, Greenberg SA, Trojanowski JQ, Traynor BJ, Smith BN, Topp S, Gkazi AS, Miller J, Shaw CE, Kottlors M, Kirschner J, Pestronk A, Li YR, Ford AF, Gitler AD, Benatar M, King OD, Kimonis VE, Ross ED, Weihl CC, Shorter J, Taylor JP (Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS. *Nature* 495:467-+.2013).
- Krajacic P, Shen XN, Purohit PK, Arratia P, Lamitina T (Biomechanical Profiling of *Caenorhabditis elegans* Motility. *Genetics* 191:1015-U1613.2012).
- Kwon DY, Motley WW, Fischbeck KH, Burnett BG (Increasing expression and decreasing degradation of SMN ameliorate the spinal muscular atrophy phenotype in mice. *Hum Mol Genet* 20:3667-3677.2011).
- Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, Viollet L, Benichou B, Cruaud C, Millasseau P, Zeviani M, Lepaslier D, Frezal J, Cohen D, Weissenbach J, Munnich A, Melki J (Identification and Characterization of a Spinal Muscular Atrophy-Determining Gene. *Cell* 80:155-165.1995).
- Lin CL, Bristol LA, Jin L, Dykes-Hoberg M, Crawford T, Clawson L, Rothstein JD (Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis. *Neuron* 20:589-602.1998).
- Liu Q, Hollopeter G, Jorgensen EM (Graded synaptic transmission at the *Caenorhabditis elegans* neuromuscular junction. *P Natl Acad Sci USA* 106:10823-10828.2009).

- Liu YY, Gervasi C, Szaro BG (A crucial role for hnRNP K in axon development in *Xenopus laevis*. *Development* 135:3125-3135.2008).
- Lu XH, Yang XW ("Huntingtin holiday": progress toward an antisense therapy for Huntington's disease. *Neuron* 74:964-966.2012).
- Miller T, Cleveland D (Merits of a new drug trial for ALS? Response. *Science* 308:632-633.2005).
- Miller TM, Kaspar BK, Kops GJ, Yamanaka K, Christian LJ, Gage FH, Cleveland DW (Virus-delivered small RNA silencing sustains strength in amyotrophic lateral sclerosis. *Ann Neurol* 57:773-776.2005).
- Nagel G, Brauner M, Liewald JF, Adeishvili N, Bamberg E, Gottschalk A (Light activation of channelrhodopsin-2 in excitable cells of *Caenorhabditis elegans* triggers rapid Behavioral responses. *Current Biology* 15:2279-2284.2005).
- Narver HL, Kong LL, Burnett BG, Choe DW, Bosch-Mare M, Taye AA, Eckhaus MA, Sumner CJ (Sustained Improvement of Spinal Muscular Atrophy Mice Treated with Trichostatin A Plus Nutrition. *Ann Neurol* 64:465-470.2008).
- Nishimura AL, Mitne-Neto M, Silva HCA, Richieri-Costa A, Middleton S, Cascio D, Kok F, Oliveira JRM, Gillingwater T, Webb J, Skehel P, Zatz M (A mutation in the vesicle-trafficking protein VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis. *Am J Hum Genet* 75:822-831.2004).
- Oprea GE, Krober S, McWhorter ML, Rossoll W, Muller S, Krawczak M, Bassell GJ, Beattie CE, Wirth B (Plastin 3 is a protective modifier of autosomal recessive spinal muscular atrophy. *Science* 320:524-527.2008a).
- Oprea GE, Kroeber S, McWhorter ML, Rossoll W, Mueller S, Krawczak M, Bassell GJ, Beattie CE, Wirth B (Plastin 3 is a protective modifier of autosomal recessive spinal muscular atrophy. *Science* 320:524-527.2008b).

- Pagliardini S, Giavazzi A, Setola V, Lizier C, Di Luca M, DeBiasi S, Battaglia G (Subcellular localization and axonal transport of the survival motor neuron (SMN) protein in the developing rat spinal cord. *Hum Mol Genet* 9:47-56.2000).
- Prior TW, Swoboda KJ, Scott HD, Hejmanowski AQ (Homozygous SMN1 deletions in unaffected family members and modification of the phenotype by SMN2. *Am J Med Genet A* 130A:307-310.2004).
- Riboldi G, Nizzardo M, Simone C, Falcone M, Bresolin N, Comi GP, Corti S (ALS genetic modifiers that increase survival of SOD1 mice and are suitable for therapeutic development. *Prog Neurobiol* 95:133-148.2011).
- Rossoll W, Kroning AK, Ohndorf UM, Steegborn C, Jablonka S, Sendtner M (Specific interaction of Smn, the spinal muscular atrophy determining gene product, with hnRNP-R and gry-rbp/hnRNP-Q: a role for Smn in RNA processing in motor axons? *Hum Mol Genet* 11:93-105.2002).
- Sen A, Dimlich DN, Guruharsha KG, Kankel MW, Hori K, Yokokura T, Brachat S, Richardson D, Loureiro J, Sivasankaran R, Curtis D, Davidow LS, Rubin LL, Hart AC, Van Vactor D, Artavanis-Tsakonas S (Genetic circuitry of Survival motor neuron, the gene underlying spinal muscular atrophy. *P Natl Acad Sci USA* 110:E2371-E2380.2013).
- Simmer F, Moorman C, van der Linden AM, Kuijk E, van den Berghe PV, Kamath RS, Fraser AG, Ahringer J, Plasterk RH (Genome-wide RNAi of *C. elegans* using the hypersensitive rrf-3 strain reveals novel gene functions. *PLoS Biol* 1:E12.2003).
- Sleigh JN, Buckingham SD, Cuppen E, Viswanathan M, Westlund BM, Sattelle DB (A novel point mutation in the *Caenorhabditis elegans* smn-1 gene provides a useful model for investigating Spinal Muscular Atrophy. *Neuromuscular Disord* 20:S22-S23.2010).

- Sleigh JN, Buckingham SD, Esmaeili B, Viswanathan M, Cuppen E, Westlund BM, Sattelle DB (A novel *Caenorhabditis elegans* allele, *smn-1(cb131)*, mimicking a mild form of spinal muscular atrophy, provides a convenient drug screening platform highlighting new and pre-approved compounds. *Hum Mol Genet* 20:245-260.2011).
- Sun Y, Grimm M, Schwarzer V, Schoenen F, Fischer U, Wirth B (Molecular and functional analysis of intragenic SMN1 mutations in patients with spinal muscular atrophy. *Hum Mutat* 25:64-71.2005).
- Taylor AM, Berchtold NC, Perreau VM, Tu CH, Jeon NL, Cotman CW (Axonal mRNA in Uninjured and Regenerating Cortical Mammalian Axons. *Journal of Neuroscience* 29:4697-4707.2009).
- Tazen S, Figueroa K, Kwan JY, Goldman J, Hunt A, Sampson J, Gutmann L, Pulst SM, Mitumoto H, Kuo SH (Amyotrophic lateral sclerosis and spinocerebellar ataxia type 2 in a family with full CAG repeat expansions of ATXN2. *JAMA Neurol* 70:1302-1304.2013).
- Wang EM, Aslanzadeh V, Papa F, Zhu HY, de la Grange P, Cambi F (Global Profiling of Alternative Splicing Events and Gene Expression Regulated by hnRNPH/F. *PLoS One* 7.2012).
- Wang J, Farr GW, Hall DH, Li F, Furtak K, Dreier L, Horwich AL (An ALS-Linked Mutant SOD1 Produces a Locomotor Defect Associated with Aggregation and Synaptic Dysfunction When Expressed in Neurons of *Caenorhabditis elegans*. *PLOS Genetics* 5:-.2009).
- Wichterle H, Lieberam I, Porter JA, Jessell TM (Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110:385-397.2002).

Figures:

Table 1: Strains used in this study

Strain name	Genotype
EG5425	<i>oxIs364</i>
HA2163	<i>smn-1(cb131);oxIs364</i>
LM99	<i>smn-1(ok355)/hT2(I;III)</i>
HA1981	<i>+hT2(I;III)</i>
SP2230	<i>sym-2(mn617)II</i>
HA2686	<i>sym-2(mn617)II;oxIs364X</i>
HA2687	<i>smn-1(cb131)I;sym-2(mn617)II;oxIs364X</i>
HA2682	<i>smn-1(cb131)I;myo-3p::PLS3::unc-54UTR, unc-119(+);oxIs364X</i>
HA2683	<i>smn-1(cb131);myo-3p::unc-54UTR;oxIs364X</i>
HA2684	<i>smn-1(cb131)I ;unc119p::PLS3::unc-54UTR, unc-119(+);oxIs364X</i>
HA2685	<i>smn-1(cb131)I ;unc119p::unc-54UTR;oxIs364X</i>
HA2627	<i>+hT2(I;III);dpy-30p::PLS3::unc-54UTR, unc-119(+)(IV)</i>
HA2629	<i>+hT2(I;III);dpy-30p::unc-54UTR(IV)</i>
HA2628	<i>smn-1(ok355)/hT2(I;III);dpy-30p::unc-54UTR(IV)</i>
HA2626	<i>smn-1(ok355)/hT2(I;III);dpy-30p::PLS3::unc-54UTR, unc-119(+)(IV)</i>
HA2688	<i>sym-2(mn617)II; dpy-30p::PLS3::unc-54UTR</i>
HA2689	<i>sym-2(mn617)II; dpy-30p::unc-54UTR</i>
AM82	<i>rmEx95[pF25B3.3::Q19::CFP]</i>
AM81	<i>rmEx164 [pF25B3.3p::Q67::CFP]</i>
CB156	<i>unc-25(e156)III.</i>
LC108	<i>uls69[pCFJ90(myo-2p::mCherry) +unc 119p::sid-1]</i>

SOD1-WT	<i>Psnb1::human SOD1-YFP</i>
SOD1-G85R	<i>Psnb1::humanSOD1-G85R-YFP</i>
HA2690	<i>Psnb1::human SOD1-YFP</i> ; dpy-30p::PLS3::unc-54UTR, unc-119(+)(IV)
HA2691	<i>Psnb1::human SOD1-YFP</i> ; dpy-30p::unc-54UTR(IV)
HA2692	<i>Psnb1::humanSOD1-G85R-YFP</i> ; dpy-30p::PLS3::unc-54UTR, unc-119(+)(IV)
HA2693	<i>Psnb1::humanSOD1-G85R-YFP</i> ; dpy-30p::unc-54UTR(IV)
HA2694	<i>rmEx95[pF25B3.3::Q19::CFP]</i> ; dpy-30p::PLS3::unc-54UTR, unc-119(+)(IV)
HA2695	<i>rmEx95[pF25B3.3::Q19::CFP]</i> ; dpy-30p::unc-54UTR(IV)
HA2696	<i>rmEx164 [pF25B3.3p::Q67::CFP]</i> ; dpy-30p::PLS3::unc-54UTR, unc-119(+)(IV)
HA2697	<i>rmEx164 [pF25B3.3p::Q67::CFP]</i> ; dpy-30p::unc-54UTR(IV)

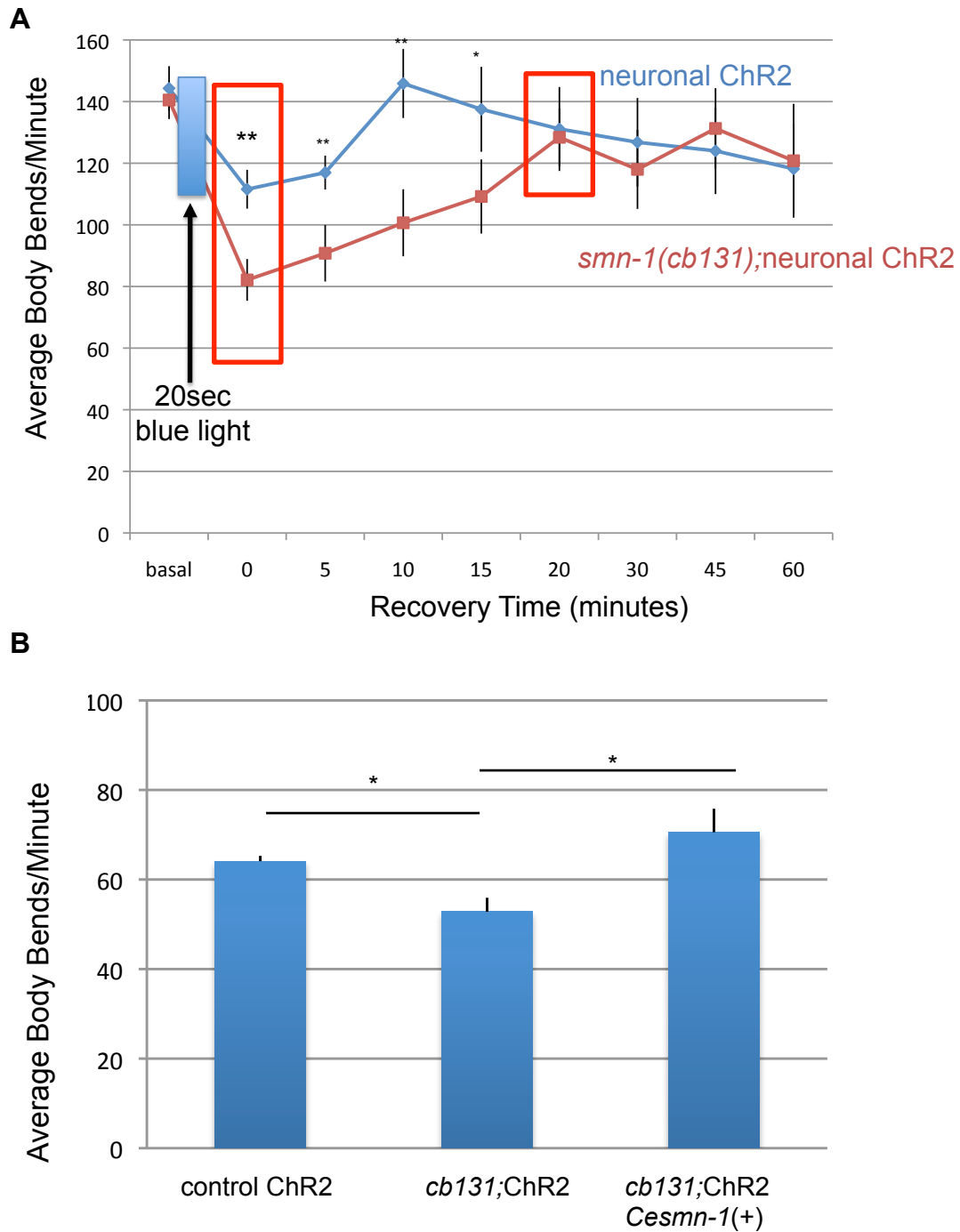


Figure 1: *Cesmn-1(cb131)* mutant animals have decreased locomotion after stimulation and slower recovery, that is rescued with *Cesmn-1* insertion.

Identification of a novel behavioral defect in *Cesmn-1(cb131)* partial loss-of-function mutants using channelrhodopsin that is specific to *Cesmn-1* loss. A) *Cesmn-1(cb131)* partial loss-of-function animals expressing channelrhodopsin (ChR2) in cholinergic neurons, show a locomotion defect and longer recovery period after a 20 second blue light stimulation in the presence of retinol compared to non- *Cesmn-1* mutant animals. As a control, animals not exposed to retinol showed no changes in locomotion after blue light stimulation for 20 seconds (data not shown) B) Post blue light exposure a single copy insertion of *Cesmn-1* driven by it's promoter is able to rescue the locomotion defects of *Cesmn-1(cb131);ChR2* animals. Data are presented as the average body bends per minute over time ($\pm$ SEM). Data represent 3 independent trials, n>30. *p<0.05, **p<0.01

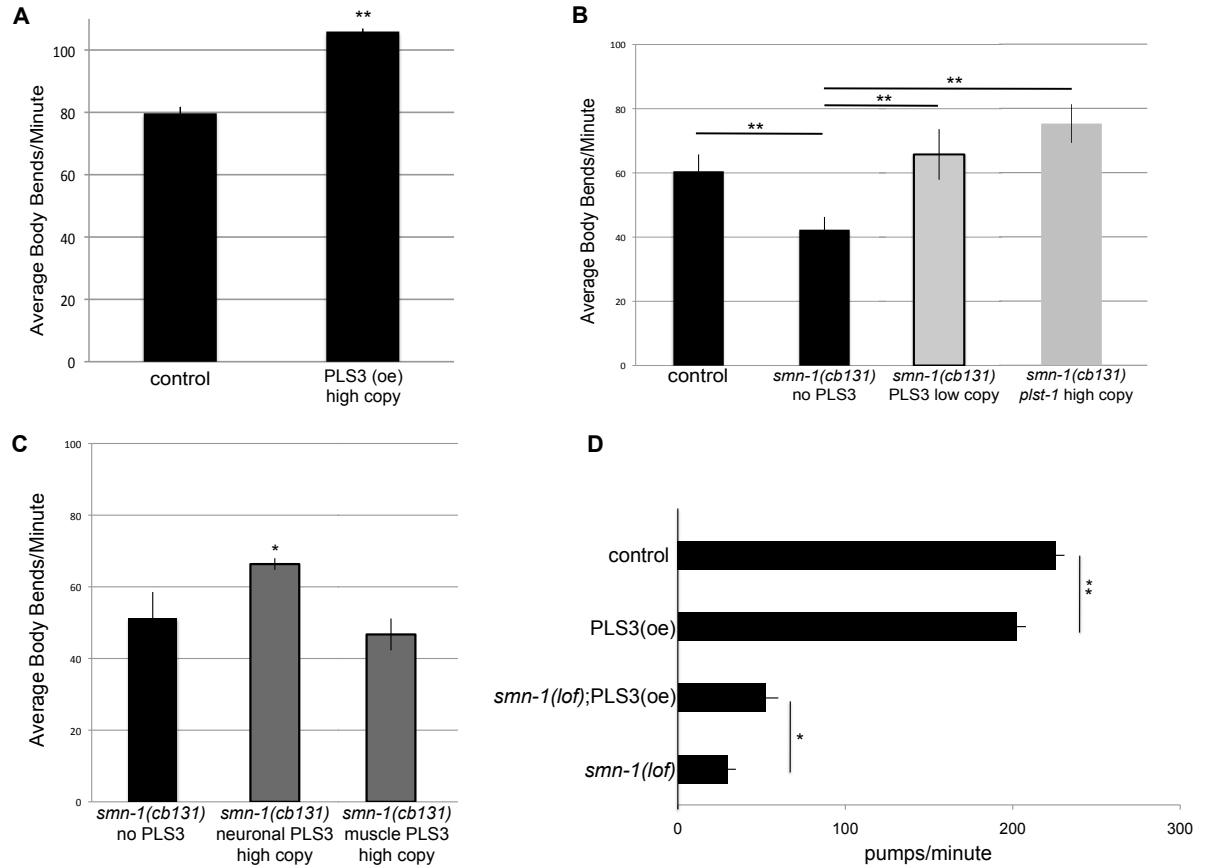


Figure 2: Increased plastin suppresses *Cesmn-1* defects and acts in neurons.

Locomotion in liquid and pharyngeal pumping behaviors were assessed using with the *Cesmn-1(cb131)* partial loss-of-function mutant or the *Cesmn-1(ok355)* complete loss-of-function mutant in the presence of plastin over-expression to assess for rescue of the behavioral defects found in *Cesmn-1* mutants. A) A multi-copy integration of PLS3, ubiquitously expressed in wild type background causes increased locomotion in liquid. B) Ubiquitous expression of PLS3 at a single copy rescues the locomotion defect after blue light stimulation in the *Cesmn-1(cb131)*;ChR2 animals. Multi-copy expression of the *C. elegans* ortholog *plst-1*, ubiquitously expressed is also able to rescue the locomotion defect. C) Only neuronal expression of PLS3 at a multi copy levels is able to

rescue the locomotion defect in *Cesmn-1(cb131)*;ChR2 mutants. Multi copy muscle and single copy neuronal or muscle, are not sufficient for rescue. D) Assessment of pharyngeal pumping in *Cesmn-1(ok355)* animals with ubiquitous somatic expression of human PLS3 improved pumping rates compared to *Cesmn-1(ok355)*;PLS3 animals versus [no cDNA] transgenic control animals. SEM is shown; Mann-Whitney U-test: *p<0.05, **p<0.01.

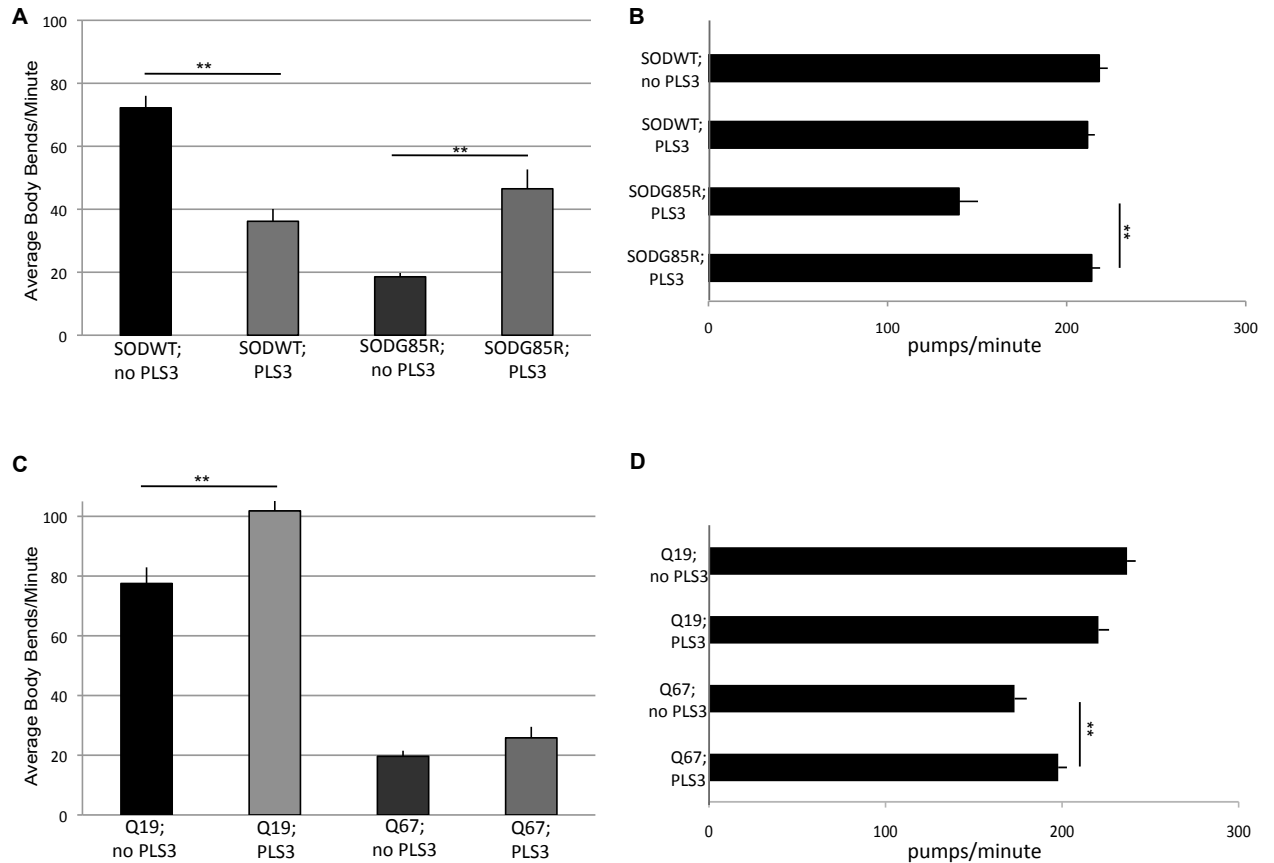


Figure 3: PLS3 suppresses the locomotion and pharyngeal pumping defects in *C. elegans* ALS and polyQ toxicity models.

The ALS and polyQ toxicity models in *C. elegans* used have decreased locomotion in liquid and decreased pharyngeal pumping rates at the young adult stage. PLS3 was crossed into these mutants and their respective controls to assess for rescue in either locomotion or pharyngeal pumping. A) Ubiquitous somatic expression of human PLS3 improved locomotion in SODG85R;PLS3 double mutant animals versus no PLS3 cDNA controls and decreased locomotion in SODWT control animals, assessed as body bends per minute in liquid. B) Ubiquitous PLS3 expression increased pharyngeal pumping rates in SODG85R;PLS3 double mutant animals compared to SODG85R mutants alone. C) PLS3 oe does not increase locomotion in the polyQ toxicity model with 67 repeats but

increases locomotion in the control polyQ animals with 19 repeats. D) The polyQ toxicity model with 67 repeats has increased pharyngeal pumping rates in double mutants with PLS3 compared to animals with 67 polyQ repeats and no PLS3 cDNA. SEM is shown; Mann-Whitney U-test: * $p < 0.05$, ** $p < 0.01$.

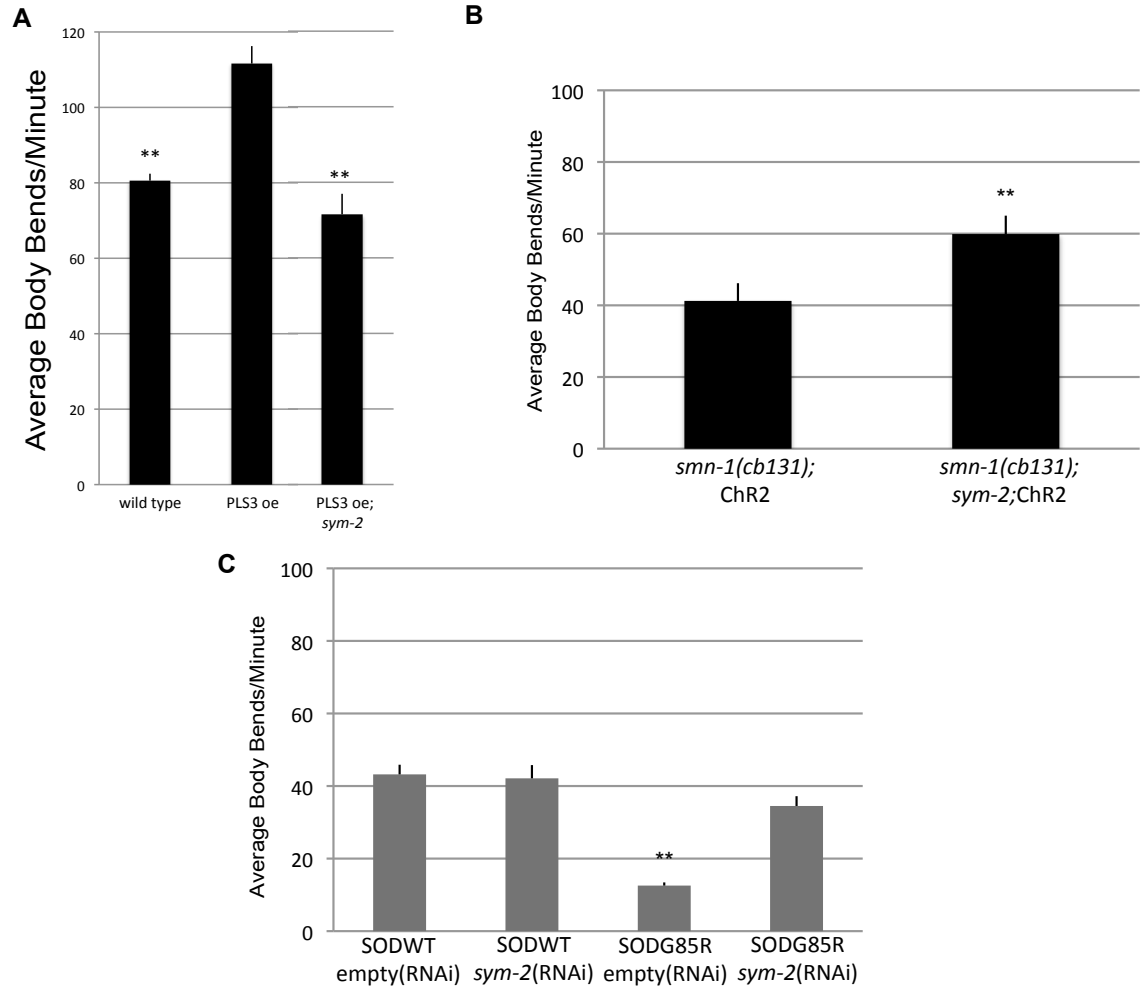


Figure 4: *Cesym-2* is a genetic modifier of PLS3 oe, *Cesmn-1* mutants and SODG85R mutants when assessed by locomotion.

A) Loss of *Cesym-2* suppressed the increased locomotion of PLS3 oe animals in a wild type background, assessed as body bends per minute in liquid. Both wild type and double mutant PLS3oe;*sym-2* had significantly less locomotion compared to the PLS3 oe single mutant. B) *Cesmn-1(cb131);*Chr2 animals have increased locomotion when *Cesym-2* levels are decreased using a loss-of-function allele in *Cesmn-1;sym-2;Chr2*

triple mutants compared to controls with normal levels of *Cesym-2*. C) Ubiquitous RNAi knockdown of *Cesym-2* in the *sid-1*(neuronal sensitive) background, increased locomotion of SODG85R mutant animals compared to SODG85R mutants without decreased *Cesym-2*. SEM shown, data represent 3 independent trials, $n>30$, $**p<0.01$.

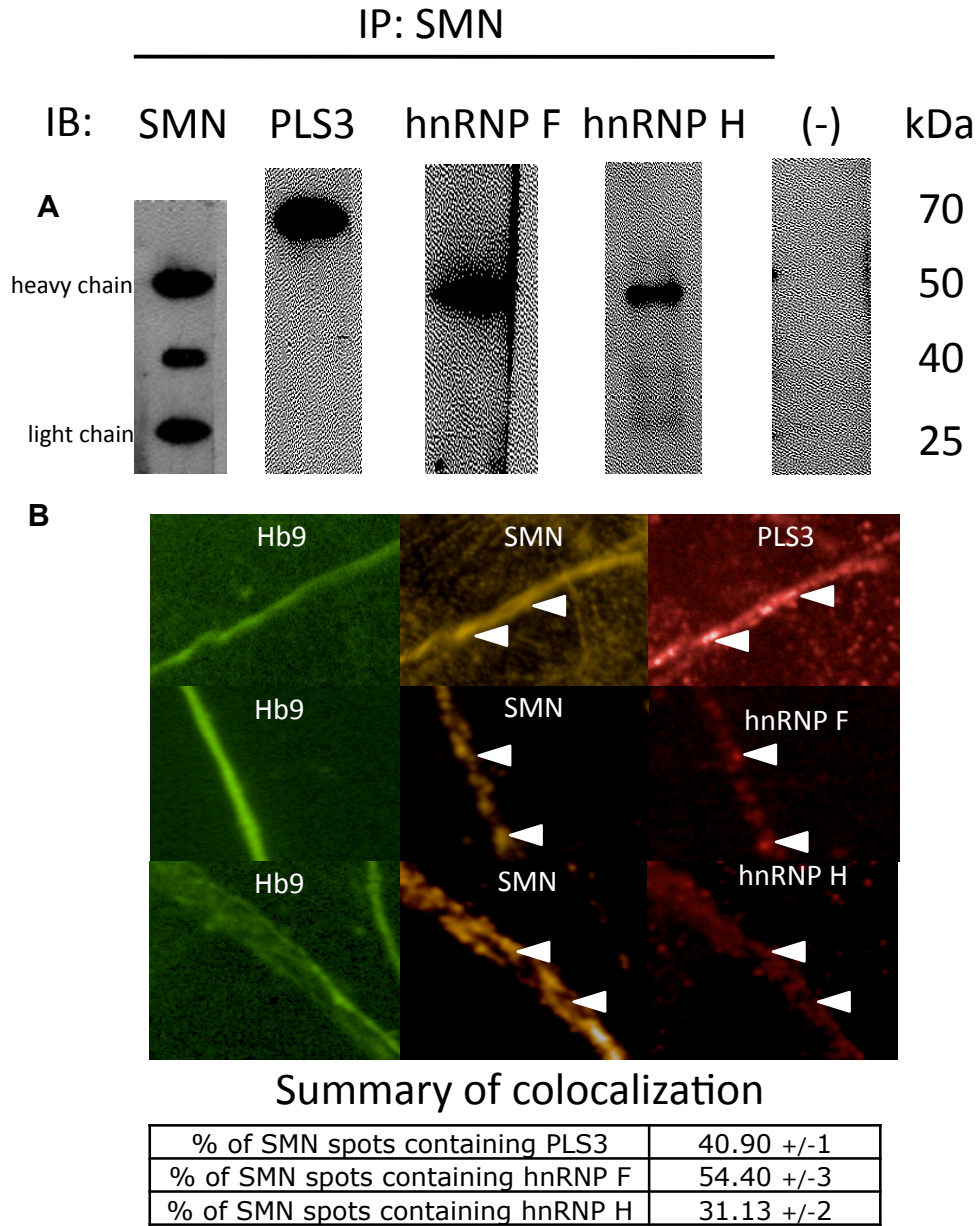
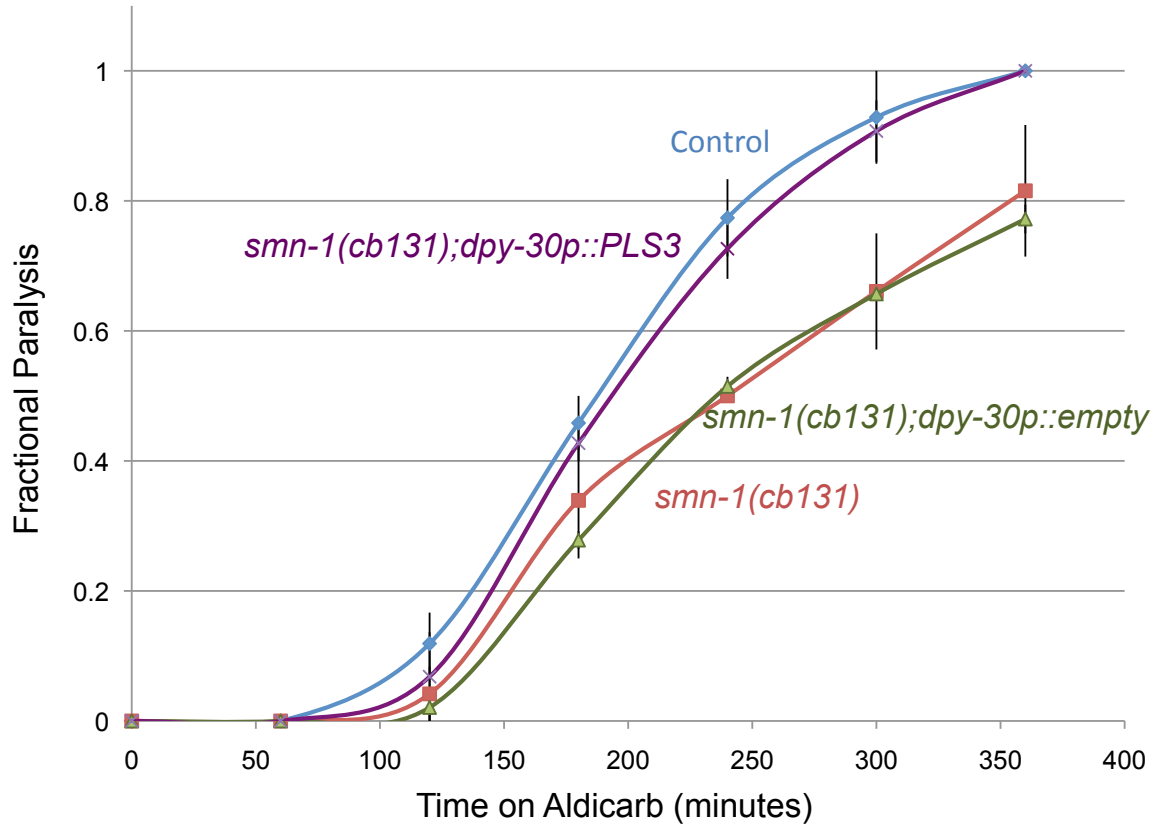


Figure 5: PLS3, hnRNP F & hnRNP H pull-down and colocalize with SMN in vertebrate neurons.

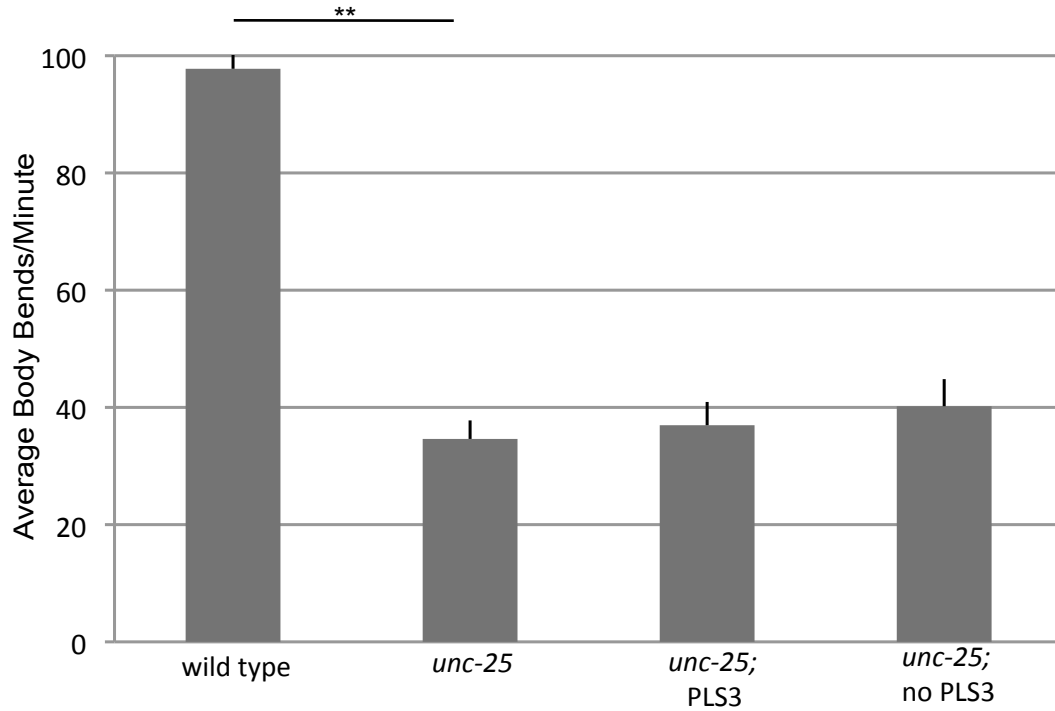
A) Western blot analysis showed that immunoprecipitation with monoclonal SMN antibody to mouse whole brain extract also co-immunoprecipitates SMN (lane 1), PLS3 (lane 2), hnRNP F (lane 3) and hnRNP H (lane 4) in mouse brain lysate. As a control, immunoprecipitation without any antibody does not show any band. B)

Immunocytochemistry of wild type mouse primary motor neuron cultures at day 13 are labelled with Hb9 (motor neuron label), SMN, PLS3, hnRNP F and hnRNPH. Images represent an example of antibody staining and show labeling in the motor neuron processes along with antibody staining. Arrowheads point to punctate spots of protein along the motor neuron processes. Quantitative analysis using Columbus software identifies the amount of colocalization of each protein with SMN compared to the total amount of SMN spots in motor neuron processes by percentage $\pm$ SD.



Supplementary Figure 1: PLS3 suppressed the resistance to the cholinesterase inhibitor aldicarb in *Cesmn-1(cb131);PLS3* double mutants.

Aldicarb induced paralysis of animals treated with 1mM of aldicarb on NGM plates seeded with 30μl of bacteria. *Cesmn-1(cb131)* mutant animals show a resistance to paralysis over time in the presence of aldicarb, an acetylcholinesterase inhibitor when compared to wild-type control animals. PLS3 oe suppressed the resistance to paralysis in double mutant *Cesmn-1(cb131);PLS3* animals compared to double mutants with *Cesmn-1(cb131)* and no PLS3 cDNA . Statistics were done using a log-rank test with an $n>30$, over 3 independent trials.



Supplementary Figure 2: PLS3 does not rescue the locomotion defects in GABA *unc-25* mutants.

unc-25 (GABA synthesis mutants) have decreased locomotion in liquid compared to wild type control animals (Jin et al., 1999). Ubiquitous over-expression of PLS3 is not able to rescue the locomotion defects in *unc-25* mutant animals assessed in the double mutants *unc-25*;PLS3 oe and *unc-25*;no PLS3 cDNA experimental and control animals, respectively. Locomotion is assessed as body bends per minutes in liquid, $n>30$ over three independent trials. SEM is shown; Mann-Whitney U-test: ** $p<0.01$

Chapter 3

Identification of genetic modifiers in *Caenorhabditis elegans smn-1* mutant animals

Melissa B. Walsh¹, Anne C. Hart²

¹Department of Molecular Biology, Cellular Biology & Biochemistry, Brown University, Providence, RI

²Department of Neuroscience, Brown University, Providence, RI

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disorder caused by the degeneration of alpha motor neurons. SMA is the leading genetic cause of death in children under the age of two and has no cure (Crawford and Pardo, 1996). Disease severity varies and can be correlated to the presence of genetic modifiers. Irrespective of severity, all SMA patients have a homozygous deletion or mutation in the *Survival of Motor Neuron (SMN1)* gene, and retain at least one copy of the almost identical *SMN2* gene. *SMN2* varies in a single C>T nucleotide change in exon 7 that perturbs pre-mRNA splicing and results in a truncated protein of diminished function (*SMN Δ 7*) (Monani et al., 1999). *SMN2* produces low levels of full-length SMN protein and while it cannot compensate for the loss of *SMN1* in SMA, it does act as a genetic modifier of SMA in a copy-dependent manner (Feldkotter et al., 2002, Mailman et al., 2002). Currently, there is one other known genetic modifier in patients, plastin 3 (PLS3) an actin bundling protein. PLS3 was identified as a protective modifier in a small number of female SMA patients and was seen to pull-down in a complex with SMN and actin (Oprea et al., 2008).

SMN is ubiquitously expressed and was identified to play a role in numerous protein complexes. Perhaps its most common function is in the Gemin complex, where SMN is required for biogenesis of small ribonucleoprotein (snRNP) particles essential for pre-mRNA splicing (Pellizzoni et al., 2002). SMN is also found in RNP granules along axons, which is thought to have involvement in mRNA transport and local translation (Akten et al., 2011, Fallini et al., 2011). Additionally, SMN is required in stress granule formation (Hua and Zhou, 2004, Zou et al., 2011) and recent studies showed it is required for the maintenance of embryonic stem cells and neuronal differentiation in mice (Chang et al., 2014). The molecular defect responsible for motor neuron degeneration in SMA is still unknown. It is clear that SMN is involved in various cellular

processes; therefore, identifying the role in which SMN is pertinent for SMA pathogenesis is essential in understanding the disease.

One method to identify essential pathways disrupted in SMA pathogenesis is to screen for genetic modifiers and identify their role in pathogenesis or define their interaction with SMN. As previously mentioned, one known genetic modifier of SMA is PLS3 and these two proteins are found in a complex together with actin. In this study we use the nematode, *Caenorhabditis elegans* (*C. elegans*) to screen candidate modifiers that affect both PLS3 and SMN mutated animals in functional analyses. The behavioral data collected, suggests a genetic interaction of SMN and PLS3 with several genes involved in RNA processing and transport. Further analysis assessed if any of the genetic modifiers also directly bound to PLS3 or SMN in a yeast two-hybrid system. Combined, these results uncovered a number of new *smn-1(lf)* genetic modifiers in *C. elegans* including a single dead box helicase (DDX5) that has a weak direct interaction with both PLS3 and SMN. Utilizing behavioral analyses in a high throughput organism, allowed the identification of new genes that can then be selectively screened in SMA vertebrate models.

Materials and Methods

Strains

C. elegans were grown on nematode growth media (NGM) agar plates seeded with OP50 bacteria and maintained at 20C as previously described (Brenner, 1974). For experiments involving balanced strains, all animals were first generation progeny of animals heterozygous for the *hT2* or *nT1* balancer to maintain a common genetic background. Loss-of-function genetic modifiers were obtained from the *Caenorhabditis* Genetic Center and the *C. elegans* Knockout Consortium. The following strains were

used: N2, SP2230 *sym-2(mn617)*II, VC659 *hrp-1(ok963)* IV/*nT1(qIs51)* IV;V, WM53 *alg-2(ok304)*II, HR1184 *+/szT1[lon-2(e678)]* I; *nmy-1(sb115)* *dpy-8(e130)/szT1* X., VC1784 *hsp-1(gk1230)*IV, VC1191 *pab-1(ok1656)* I/*hT2[bli-4(e937)* *let-?(q782)* *qIs48](I;III)*, VC1260 F58E10.3&*aip-1(ok277)* V/*nT1[qIs51]* (IV;V), VC1635 *psf-1(gk1206)* III/*hT2[bli-4(e937)* *let-?(q782)* *qIs48](I;III)*, VC1310 *cey-1(ok1805)* II, VC1057 *tbb-4(ok1461)* X, HS143 *msi-1(os1)* III, EG5425 (*oxIs364*)X, HA2163 (*smn-1(cb131);oxIs364*) X.

Retinal Feeding

Retinal feeding was done as previously described (Liu et al., 2009) with some modifications. Briefly, transgenic animals were cultivated in the dark at 20C on NGM plates with OP50 bacteria with or without all-trans retinal (Sigma), approximately 18 hours before experiments. Plates containing all-trans retinal were prepared by spreading 100ul of OP50 culture mixed with 1.6ul of 100mM all-trans retinal stock (dissolved in ethanol) onto 2.5cm plates containing 5ml of NGM. Animals were illuminated with a mercury bulb and a Zeiss bandpass filter set was used to excite channelrhodopsin at 450-490 nm for 20 seconds before analyzing swimming behavior.

Swimming analysis

For the swimming assay, young adult animals were picked singly into wells of a PDMS chip containing 2ul of PBS. Animals were given 5 minutes to settle and assayed at a room temperature that was maintained between 22C and 24C. 10-second videos were taken with an AxioCam R3.0 camera at 12 frames/second, on a Zeiss Discovery V20 at 12.5 x 11 magnification. Individual animal movies were analyzed using the NABAS software, which was modified for image formats yielding body bends per minute (Krajacic et al., 2012). Average body bends per minute ($\pm$ SEM) were combined from at least three independent trials ($n \geq 30$ animals in total).

Yeast two-hybrid screen

The AH109 yeast strain was used to assess interactions of human cDNAs that were cloned into pDBLeu and pEXP-AD502 binding domain and activation domain vectors, respectively. Human PLS3 CDS (ATG to TAA), was provided from the Wirth lab and amplified from pcDNA3.1_PLS3.V5-His6 TOPO vector. Human SMN cDNA was provided by the Rubin lab and amplified from pEF-DEST51 vector. All other cDNAs were purchased from Thermo Scientific. Clones and primers used for amplification are listed in the table below.

Protein Name	Clone/Seq ID	Primers used for amplification
SMN	20996	5'- CCCGGGtgcatgagcagcggcggcagtg 5'- CCATGGattaagnaatgtgagcac
PLS3	005032.6	5'- CCCGGGtgatgagatggctaccactcag 5'- CCATGGttacactctcttcattcccctg
DDX5	3528578	5'- GTCGACttattgggaatatcctg 5'- ACTAGTttattgggaatatcctg
DDX17	3345982	5'- GTCGACccactgcccggtttgagcgtag 5'- ACTAGTtcatttacgtgaaggaggaggagggagg
DDX20	4651697	5'- GTCGACcgcggcggcatttgaagc 5'- ACTAGTtcactggttactatgcac
EIF2C2	3356425	5'- GTCGACctggattttaaaagtattgaag 5'- ACTAGTtcaagcaaagtacatggtgcgc
HNRNP F	4104978	5'- GTCGACcatgctgggcccctgagggagg 5'-ACTAGTctagtcatagccacccatgc
NONO	5108266	5'-GTCGACccagagtaataaaacttttaacttg 5'-ACTAGTtagtatcggcgacg
PABPC1	3940309	5'-GTCGACcaaccccagtgccccag 5'-GCGGCCGCTtaaacagttggaacaccgg
SFPQ	5262885	5'-GTCGACctctcgggatcggttcgg 5'-ACTAGTctaaaatcggggtttttgtttggcc

The SMN and PLS3 baits were used to screen the prey using triple drop out plates (–His/–Leu/–Trp) according to the manufacturers instructions (Invitrogen ProQuest system).

Results

The identification of genetic modifiers of PLS3 oe mutant animals

Studies have confirmed PLS3 as a genetic modifier of SMA models from zebrafish to mice (Oprea et al., 2008, Ackermann et al., 2013, Lyon et al., 2014). It is known that SMN and PLS3 co-immunoprecipitate in a complex together with actin, but the other proteins in the complex are unknown. Identification of these proteins may give insight into possible mechanisms through which PLS3 suppresses SMA defects. Previous proteomic data collected from *Drosophila* S2 cells, discovered proteins that associate with *DmSMN* and *Dmfimbrin* (PLS3 ortholog), independently (Sen et al., 2013). Drawing on this data a list of candidate genetic modifiers was compiled based on the following requirements: the frequency in which the proteins interacted with one another, presence of both *H. sapiens* and *C. elegans* orthologs, and whether a loss-of-function (*lf*) *C. elegans* allele was available to assess behavior. From these criteria, a list of 11 candidate genes was created to test genetic modifiers of PLS3 and *Cesmn-1* mutant animals in a behavioral screen (Table 1).

As described earlier, a *C. elegans* model where PLS3 is over expressed (oe) with multiple copies in a wild type background, increases locomotion in liquid by 38% (Chapter 2 and Figure 1, $p = 0.003$). Potential interactions between PLS3 and candidate genetic modifiers were examined by constructing double mutant strains. If a gene is required for PLS3 function in locomotion, loss of this gene should suppress the increased locomotion phenotype. However, if a gene were not involved in the PLS3 function in locomotion, loss of it would be inconsequential in this neuromuscular

phenotype. First, double mutant animals were constructed with PLS3 (oe) and loss of function alleles for genes listed in Table 1. Loss of *hrp-1*, *nmy-1*, *hsp-1*, *msi-1*, *cey-1* and *tbb-4* did not suppress the increased locomotion of PLS3 animals (data not shown). In contrast, loss of *alg-2*, *ddx-17*, *pab-1*, *psf-1* and *sym-2* did suppress the increased locomotion of PLS3 animals (Figure 1; $p=0.007$, $p=0.008$, $p=0.039$, $p=0.005$ and $p=0.007$, respectively). These results indicate a possible genetic interaction between PLS3 and the identified five genes that affected locomotion. Next, further analysis was carried out to determine if they were also genetic modifiers in *Cesmn-1* mutant animals.

The identification of genetic modifiers in *Cesmn-1* mutant animals

After the discovery of PLS3 genetic modifiers, those genes were sequentially assessed for behavioral modification in *Cesmn-1* mutant animals. It is already established that *Cesmn-1(cb131);ChR2* animals have decreased locomotion in liquid after stimulation of cholinergic neurons by ChR2 compared to controls (Chapter 2 and Figure 2). Thus, we utilized this quantitative neuromuscular phenotype to screen for genetic modifiers. Double mutants were constructed with *Cesmn-1(cb131);ChR2* and loss of function alleles for *alg-2*, *ddx-17*, *pab-1*, *psf-1*, and *sym-2*. In the case of *sym-2(lf)* and *psf-1(lf)*, double mutants suppressed the *Cesmn-1(cb131);ChR2* decreased locomotion (Figure 2, $p=0.006$, $p=0.009$, respectively). In contrast, *ddx-17(lf)* enhanced the *Cesmn-1(cb131);ChR2* defect, while *alg-2(lf)* and *pab-1(lf)* had no affect (Figure 2, $p=0.02$). It should be noted that double mutants with *pab-1(lf)* and *Cesmn-1* were assayed as trans-heterozygotes, due to their close proximity on the genome. Together, these results identify three genetic modifiers of *Cesmn-1(cb131);ChR2* mutant animals that were proven to function behaviorally with PLS3 mutant animals.

Do genetic modifiers directly interact with PLS3 or SMN?

To test for an *in vivo* protein-protein interaction between SMN or PLS3 and the genetic modifiers identified, the yeast two-hybrid system was employed. For this

analysis, human cDNA clones were used (see methods for the list of clones screened). Fusion constructs were generated containing the full-length cDNA sequences of *smn-1* or PLS3 fused to the GAL4 DNA-binding domain or modifier sequences fused to the activation domain. The competent yeast strain AH109 was transformed with binding and activation domain constructs. Upon interaction of proteins, active GAL4 transcription factors are formed, driving transcription of several reporter genes (*Ade*, *His*, *Leu*). Induction of these reporter genes should result in colony growth on selective media lacking the three amino acids (Figure 3). Positive and negative controls were included on each selective plate and screening was carried out in triplicate. SMN is known to bind itself and was therefore used as a positive control (Lorson et al., 1998, Burnett et al., 2009). We found a robust, consistent interaction with SMN and SMN as bait and prey, indicating a relatively high reliability of our screening process (Figure 3). However, DDX20 (Gemin 3) a known binding partner of SMN was also applied and did not show any interaction, suggesting a higher false negative rate and that some potential interactions could be missed in our screen (Figure 3) (Charroux et al., 1999). Co-transformation of DDX5-AD with SMN-BD and PLS3-BD showed induction of all three-reporter genes by this assay (Figure 3). However, growth was limited compared to positive controls, suggesting a weak interaction between proteins. No other genetic modifier screened showed a consistent interaction with either SMN or PLS3. Results from the screen suggest that DDX5 could interact in a complex with SMN, PLS3 and actin, but further analysis is necessary. Proteins that failed to induce transcription could still be part of a functional complex with SMN and PLS3, but require intermediate binding partners.

Conclusions

Here we identify several novel genetic modifiers of PLS3 and *Cesmn-1* mutant animals that are involved in mRNA processing. The work presented is based on previously defined *C. elegans* models of PLS3 oe and SMA where over-expression of PLS3 or decreased function of the *C. elegans* SMN ortholog, *Cesmn-1*, results in neuromuscular function defects (Chapter 2). We found that loss of *alg-2*, *sym-2*, *psf-1*, *ddx-17* and *pab-1* suppressed the behavioral phenotype of PLS3 animals, suggesting that these genes are required for increased locomotion in PLS3 animals. Additionally, genetic interactions with *Cesmn-1* mutants were identified with *sym-2*, *psf-1* and *ddx-17*. Further biochemical analysis using a yeast two-hybrid screen identified the *C. elegans* ortholog of *ddx-17*, DDX5 to directly bind to SMN and PLS3 *in vivo*. Taken together these results suggest DDX5 as a putative component of the SMN, PLS3 and actin complex. This novel interaction between the three proteins has potential pertinence to SMA pathogenesis.

***psf-1* acts as a genetic suppressors of *Cesmn-1* mutants, through PLS3**

Identification of genetic suppressors of SMA is important in finding new therapeutic targets. Using invertebrate model systems to quickly screen for suppressors is a valuable tool that could give insight into disease mechanism and lead to more specific screens in vertebrate model systems in the future. In this study we identify two genes that suppress the *Cesmn-1* mutant defects and also suppress PLS3 mutant defects, *sym-2* (previously discussed in Chapter 2) and *psf-1*. The human orthologs of *psf-1* are NONO and SFPQ, binding partners that are known to have involvement in several nuclear processes. They are DNA and RNA binding proteins that are implicated in pre-mRNA splicing and spliceosome formation (Rosonina et al., 2005). These proteins are also shown to interact with SMN (Fuller et al., 2010). While our yeast two-hybrid screen did not show a direct interaction of either of these proteins with SMN or PLS3, intermediate-binding partners could be required. One could hypothesize that

these four proteins form a nuclear complex critical for splicing and that disruption of this complex due to SMN loss perturbs splicing function. However, further behavioral and biochemical analyses are necessary to understand the role of all four proteins in this paradigm.

DDX5 binds SMN and PLS3 and the invertebrate ortholog acts through PLS3 to enhance *Cesmn-1* defects

The *ddx-17* gene is orthologous to the human genes DDX5 and DDX17. These genes encode DEAD box proteins that are involved in pre-mRNA splicing and alteration of RNA secondary structure (Lorsch, 2002). The two proteins are 90% identical at the core and differ only in N- and C- termini (Lamm et al., 1996). They work synergistically and are known to associate with hnRNPs (Hegele et al., 2012), including orthologs to *sym-2*, another identified genetic modifier in this screen. Loss of *ddx-17* exacerbated the *Cesmn-1* mutant defects, acting as an enhancer to SMN diminished protein levels. Interestingly, of the genetic modifiers identified, DDX5 was the only protein to show a direct interaction with SMN and PLS3. It is not surprising to see an interaction with SMN, given the role of DEAD box helicases in splicing; however the observation that DDX5 also interacts with PLS3 is unexpected. Together, the genetic and biochemistry analyses suggest that SMN, PLS3 and DDX5 form a complex together. Combining the genetic data discussed here and the literature, we found that hnRNPs, in particular H1,2,3, are also implicated to associate with all these proteins. SMN, DDX5 and hnRNP H play a critical role in pre-mRNA splicing (Lorsch, 2002, Pellizzoni et al., 2002, Gupta et al., 2013). Several studies identify PLS3 as a component of large protein complexes involved in RNA processing/metabolism (Ho et al., 2002, Blagoev et al., 2003). These along with our data suggest that PLS3 may also be functioning in splicing and that disruption of this large complex could contribute to the pathogenesis of SMA.

Summary

The genetic and biochemical studies presented here demonstrate that invertebrate model organisms such as *C. elegans* provide a fast and effective tool for identifying genetic pathways pertinent to SMN mutants. Previous studies suggest that the role of SMN in splicing is the primary perturbation causing SMA pathogenesis. For example, analysis of SMN target genes identified Stasimon as a protein required for motor circuit function. When Stasimon expression was restored, defects of *Drosophila* and *Danio rerio* (zebrafish) SMN mutants at the neuromuscular junction were corrected (Lotti et al., 2012). Additionally, SMN from SMA patients showed a decreased ability to interact with Gemin3, a functional complex that is critical in splicing (Charroux et al., 1999). These studies along with others show that SMN function in splicing is critical in SMA pathology. Identifying genetic modifiers that target SMN splicing complexes could lead towards identification of novel therapeutic interventions for SMA.

Figures

<u><i>C.elegans</i></u> <u>gene</u>	<u><i>D.melanogaster</i></u> <u>gene</u>	<u><i>H.sapiens</i></u> <u>protein</u>	<u>Description/Function</u>	
<i>sym-2</i>	glo	hnRNP F/H	heterogeneous ribonucleoprotein	nuclear
<i>hrp-1</i>	Hrb98DE	hnRNP A	heterogeneous ribonucleoprotein	nuclear
<i>alg-2</i>	AGO2	EIF2C2	Argonaute 2, part of RISC	
<i>nmy-1</i>	zip	MYH10	myosin heavy chain	
<i>hsp-1</i>	Hsc70-4	HSPA8	heat-shock protein	
<i>pab-1</i>	pAbp	PABPC1	poly(A) binding protein	
<i>ddx-17</i>	Rm62	DDX5/17	ATP-dependent RNA helicase	
<i>msi-1</i>	sqd	MSI2	RNA-binding protein	
<i>psf-1</i>	nonA	NONO/SFPQ	splicing factor, proline- and glutamine-rich	
<i>cey-1</i>	yps	YBX1	Y-box protein	
<i>tbb-4</i>	betaTub56D	TUBB4B	tubulin beta-2C chain	

Table 1: Candidate genetic modifiers screened in *C. elegans* behavioral assays.

A list of candidate genetic modifiers of PLS3 and *Cesmn-1*, compiled from proteomic data in S2 cells (Sen et al., 2013). *C. elegans*, *Drosophila* and *H. sapiens* orthologs are listed, along with a brief description of their function.

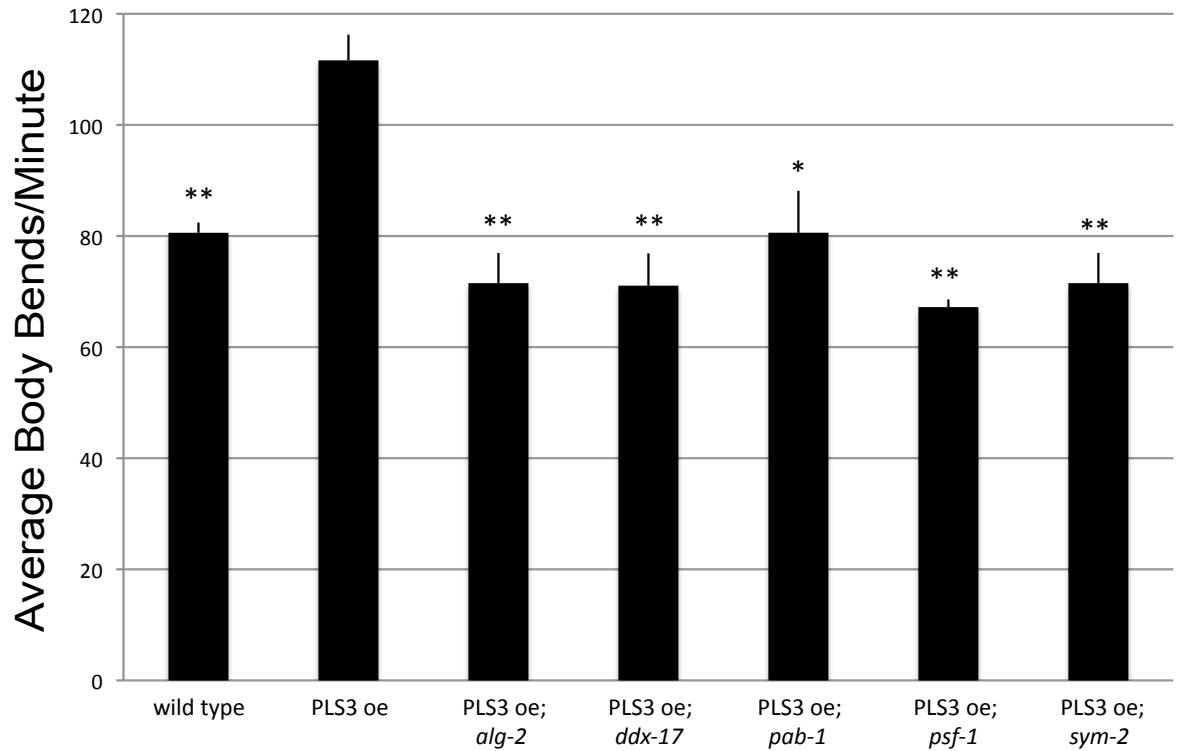


Figure 1: Genetic modifiers of the PLS3 oe increased locomotion in liquid.

A multi-copy integration of PLS3 ubiquitously expressed in wild type background increased locomotion in liquid (measured as average body bends per minute) compared to wild type animals (first two bars). All loss-of-function alleles of candidate modifiers were crossed into PLS3 oe animals and double mutants were assessed for a change in the increased locomotion of PLS3 oe on its own. This genetic screening identified *alg-2*, *ddx-17*, *pab-1*, *psf-1* and *sym-2* deficient animals to suppress the increased locomotion of PLS3 oe animals. Locomotion assessment of candidate gene single mutant loss-of-function animals showed no significant changes. Data shown is from three independent trials with SEM shown. All samples were compared to the PLS3 oe animals using a student t-test; * $p < 0.05$, ** $p < 0.01$.

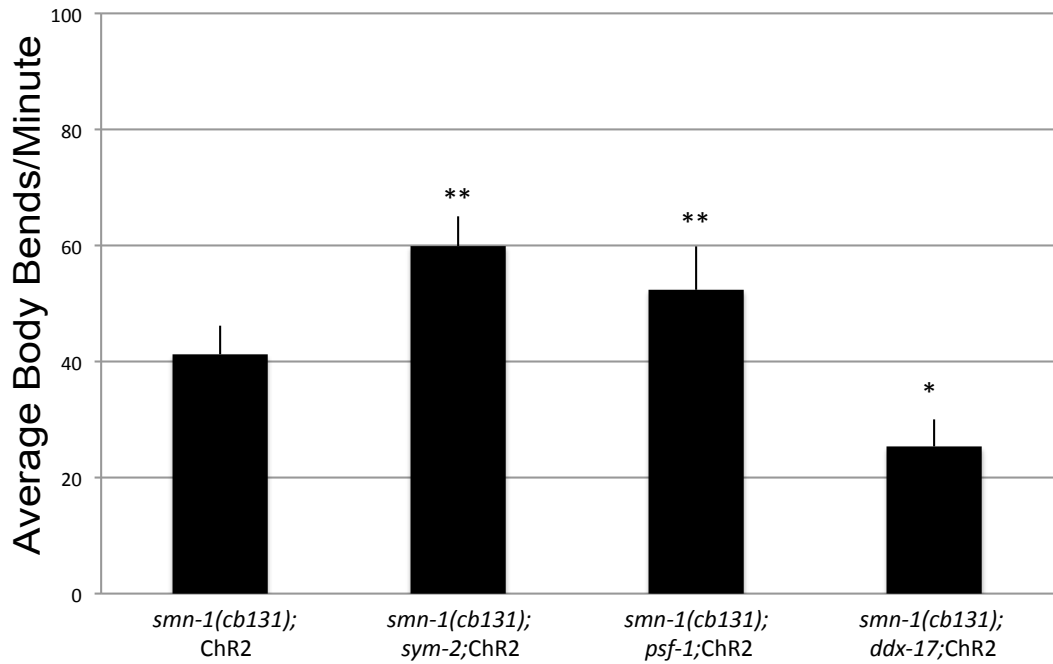


Figure 2: Genetic modifiers of the *Cesmn-1(cb131)* locomotion in liquid defect.

From the candidate genes that suppressed PLS3 or increased locomotion rates, loss of function alleles for *sym-2*, *psf-1* and *ddx-17* were the only mutants that altered locomotion in liquid of the *Cesmn-1(cb131);ChR2* mutant animals. Triple mutants were assessed and *Cesmn-1(cb131);sym-2;ChR2* and *Cesmn-1(cb131);psf-1;ChR2* suppressed the locomotion defect of *Cesmn-1(cb131);ChR2* animals. The triple mutant *Cesmn-1(cb131);ddx-17;ChR2* enhanced the *Cesmn-1(cb131);ChR2* defect. Data shown is from three independent trials with SEM shown. All samples are compared to *Cesmn-1(cb131);ChR2* mutants for significance using a student t-test; * $p < 0.05$, ** $p < 0.01$.

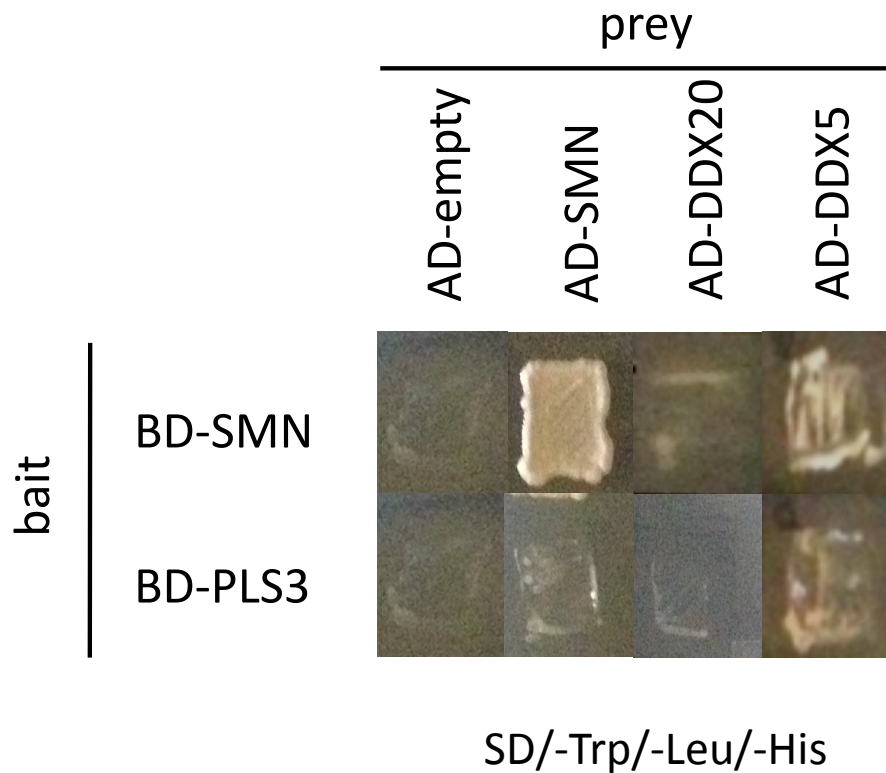


Figure 3: DDX5 binds directly to SMN and PLS3 in a yeast two-hybrid system.

Yeast AH109 cells were co-transformed with the expression plasmids for fusion proteins of both BD (bait) and AD (prey) and selected on a double dropout (SD/-Trp/-Leu) medium plate. Each colony was streaked on selection media plates of double dropout and triple dropout (SD/-Trp/-Leu/-His) and incubated for 4 days at 30°C. Images shown are of growth on triple dropout plates. SMN showed a strong interaction with itself, while DDX5 showed some growth when transformed with both SMN and PLS3 as the bait. An empty prey showed no interaction with either SMN or PLS3 as a negative control. DDX20 or Gemin 3 also did not show any direct interaction with SMN or PLS3 in these experiments. Experiments were done in triplicate; a single colony patch is shown as a representative of the experiments.

References

- Ackermann B, Krober S, Torres-Benito L, Borgmann A, Peters M, Barkooie SMH, Tejero R, Jakubik M, Schreml J, Milbradt J, Wunderlich TF, Riessland M, Tabares L, Wirth B (Plastin 3 ameliorates spinal muscular atrophy via delayed axon pruning and improves neuromuscular junction functionality. *Hum Mol Genet* 22:1328-1347.2013).
- Akten B, Kye MJ, Hao LT, Wertz MH, Singh S, Nie DY, Huang J, Merianda TT, Twiss JL, Beattie CE, Steen JAJ, Sahin M (Interaction of survival of motor neuron (SMN) and HuD proteins with mRNA cpg15 rescues motor neuron axonal deficits. *P Natl Acad Sci USA* 108:10337-10342.2011).
- Blagoev B, Kratchmarova I, Ong SE, Nielsen M, Foster LJ, Mann M (A proteomics strategy to elucidate functional protein-protein interactions applied to EGF signaling. *Nat Biotechnol* 21:315-318.2003).
- Brenner S (Genetics of *Caenorhabditis-Elegans*. *Genetics* 77:71-94.1974).
- Burnett BG, Munoz E, Tandon A, Kwon DY, Sumner CJ, Fischbeck KH (Regulation of SMN Protein Stability. *Mol Cell Biol* 29:1107-1115.2009).
- Chang WF, Xu J, Chang CC, Yang SH, Li HY, Hsieh-Li HM, Tsai MH, Wu SC, Cheng WT, Liu JL, Sung LY (SMN is required for the maintenance of embryonic stem cells and neuronal differentiation in mice. *Brain Struct Funct*.2014).
- Charroux B, Pellizzoni L, Parkinson RA, Shevchenko A, Mann M, Dreyfuss G (Gemin3: A novel DEAD box protein that interacts with SMN, the spinal muscular atrophy gene product, and is a component of gems. *J Cell Biol* 147:1181-1194.1999).
- Crawford TO, Pardo CA (The neurobiology of childhood spinal muscular atrophy. *Neurobiol Dis* 3:97-110.1996).

- Fallini C, Zhang HL, Su YH, Silani V, Singer RH, Rossoll W, Bassell GJ (The Survival of Motor Neuron (SMN) Protein Interacts with the mRNA-Binding Protein HuD and Regulates Localization of Poly(A) mRNA in Primary Motor Neuron Axons. *Journal of Neuroscience* 31:3914-3925.2011).
- Feldkotter M, Schwarzer V, Wirth R, Wienker TF, Wirth B (Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. *Am J Hum Genet* 70:358-368.2002).
- Fuller HR, Man NT, Lam le T, Thanh le T, Keough RA, Asperger A, Gonda TJ, Morris GE (The SMN interactome includes Myb-binding protein 1a. *J Proteome Res* 9:556-563.2010).
- Gupta SK, Kosti I, Plaut G, Pivko A, Tkacz ID, Cohen-Chalamish S, Biswas DK, Wachtel C, Ben-Asher HW, Carmi S, Glaser F, Mandel-Gutfreund Y, Michaeli S (The hnRNP F/H homologue of *Trypanosoma brucei* is differentially expressed in the two life cycle stages of the parasite and regulates splicing and mRNA stability. *Nucleic Acids Research* 41:6577-6594.2013).
- Hegele A, Kamburov A, Grossmann A, Sourlis C, Wowro S, Weimann M, Will CL, Pena V, Luhrmann R, Stelzl U (Dynamic protein-protein interaction wiring of the human spliceosome. *Mol Cell* 45:567-580.2012).
- Ho Y, Gruhler A, Heilbut A, Bader GD, Moore L, Adams SL, Millar A, Taylor P, Bennett K, Boutilier K, Yang L, Wolting C, Donaldson I, Schandorff S, Shewnarane J, Vo M, Taggart J, Goudreau M, Muskut B, Alfarano C, Dewar D, Lin Z, Michalickova K, Willems AR, Sassi H, Nielsen PA, Rasmussen KJ, Andersen JR, Johansen LE, Hansen LH, Jespersen H, Podtelejnikov A, Nielsen E, Crawford J, Poulsen V, Sorensen BD, Matthiesen J, Hendrickson RC, Gleeson F, Pawson T, Moran MF, Durocher D, Mann M, Hogue CW, Figgeys D, Tyers M (Systematic

- identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature* 415:180-183.2002).
- Hua Y, Zhou J (Rpp20 interacts with SMN and is re-distributed into SMN granules in response to stress. *Biochem Biophys Res Commun* 314:268-276.2004).
- Krajacic P, Shen XN, Purohit PK, Arratia P, Lamitina T (Biomechanical Profiling of *Caenorhabditis elegans* Motility. *Genetics* 191:1015-U1613.2012).
- Lamm GM, Nicol SM, Fuller-Pace FV, Lamond AI (p72: a human nuclear DEAD box protein highly related to p68. *Nucleic Acids Res* 24:3739-3747.1996).
- Liu Q, Hollopeter G, Jorgensen EM (Graded synaptic transmission at the *Caenorhabditis elegans* neuromuscular junction. *P Natl Acad Sci USA* 106:10823-10828.2009).
- Lorsch JR (RNA chaperones exist and DEAD box proteins get a life. *Cell* 109:797-800.2002).
- Lorson CL, Strasswimmer J, Yao JM, Baleja JD, Hahnen E, Wirth B, Le T, Burghes AHM, Androphy EJ (SMN oligomerization defect correlates with spinal muscular atrophy severity. *Nature Genetics* 19:63-66.1998).
- Lotti F, Imlach WL, Saieva L, Beck ES, Hao le T, Li DK, Jiao W, Mentis GZ, Beattie CE, McCabe BD, Pellizzoni L (An SMN-dependent U12 splicing event essential for motor circuit function. *Cell* 151:440-454.2012).
- Lyon AN, Pineda RH, Hao LT, Kudryashova E, Kudryashov DS, Beattie CE (Calcium binding is essential for plastin 3 function in Smn-deficient motoneurons. *Hum Mol Genet* 23:1990-2004.2014).
- Mailman MD, Heinz JW, Papp AC, Snyder PJ, Sedra MS, Wirth B, Burghes AH, Prior TW (Molecular analysis of spinal muscular atrophy and modification of the phenotype by SMN2. *Genet Med* 4:20-26.2002).
- Monani UR, Lorson CL, Parsons DW, Prior TW, Androphy EJ, Burghes AH, McPherson JD (A single nucleotide difference that alters splicing patterns distinguishes the

- SMA gene SMN1 from the copy gene SMN2. *Hum Mol Genet* 8:1177-1183.1999).
- Oprea GE, Krober S, McWhorter ML, Rossoll W, Muller S, Krawczak M, Bassell GJ, Beattie CE, Wirth B (Plastin 3 is a protective modifier of autosomal recessive spinal muscular atrophy. *Science* 320:524-527.2008).
- Pellizzoni L, Yong J, Dreyfuss G (Essential role for the SMN complex in the specificity of snRNP assembly. *Science* 298:1775-1779.2002).
- Rosonina E, Ip JY, Calarco JA, Bakowski MA, Emili A, McCracken S, Tucker P, Ingles CJ, Blencowe BJ (Role for PSF in mediating transcriptional activator-dependent stimulation of pre-mRNA processing in vivo. *Mol Cell Biol* 25:6734-6746.2005).
- Sen A, Dimlich DN, Guruharsha KG, Kankel MW, Hori K, Yokokura T, Brachat S, Richardson D, Loureiro J, Sivasankaran R, Curtis D, Davidow LS, Rubin LL, Hart AC, Van Vactor D, Artavanis-Tsakonas S (Genetic circuitry of Survival motor neuron, the gene underlying spinal muscular atrophy. *P Natl Acad Sci USA* 110:E2371-E2380.2013).
- Zou T, Yang X, Pan D, Huang J, Sahin M, Zhou J (SMN deficiency reduces cellular ability to form stress granules, sensitizing cells to stress. *Cell Mol Neurobiol* 31:541-550.2011).

Chapter 4

Summary, Discussion & Conclusions

Deficiency of SMN causes detrimental effects at the neuromuscular junction that lead to the motor neuron disease Spinal Muscular Atrophy (SMA). A promising avenue for understanding how depleted levels of SMN lead to motor neuron specific degeneration is the identification of genetic modifiers. Genetic modifiers of SMA and other neurodegenerative diseases could lead to a better understanding of targeted pathways that are disrupted in these diseases and to possible therapeutic targets. One of the goals of this thesis was to characterize a previously identified genetic modifier in SMA patients, PLS3 and determine if it is a cross disease modifier. Using PLS3 as a model for genetic modifiers of disease in *C. elegans*, we also identified new genetic modifiers that alter the *Cesmn-1* mutant phenotypes and tested them in ALS and polyQ toxicity models. The results obtained led us to the conclusions discussed below.

Genetic modifiers of disease

The actin bundling protein PLS3 is found to be upregulated in individuals that are asymptomatic for SMA even though they carry homozygous mutations in the SMN-1 gene. PLS3 over-expression (oe) restored axon outgrowth and suppressed behavioral defects in SMA zebrafish and mouse models (Oprea et al., 2008, Ackermann et al., 2013). PLS3 was also found in a protein complex along with SMN and actin, suggesting a genetic interaction between the proteins. While a previous study suggests that the role PLS3 plays in actin bundling is crucial for suppression of SMN defects, another study identifies the calcium-binding domain of PLS3 to be essential for suppression (Ackermann et al., 2013, Lyon et al., 2014). The discrepancy in the role of PLS3 in SMN mutants of these studies show that more needs to be understood about the mechanism of PLS3 suppression in SMA. These studies also highlight the importance of PLS3 in axon outgrowth and neuronal function in general, suggesting that PLS3 could be acting in a pathway that when manipulated would be beneficial across neurodegenerative

diseases. While these experiments suggest PLS3 as a future therapeutic target for SMA, they also underline the beneficial effects of identifying common modifiers across diseases.

Chapter two discussed evidence of common genes perturbed across the neurodegenerative diseases SMA, ALS and certain polyQ diseases, highlighting the importance of identification of genetic modifiers (Nishimura et al., 2004, Oprea et al., 2008, Farg et al., 2013, Kim et al., 2013, Tazen et al., 2013). However, perturbation of these genes (VAPB, Ataxin-2 and hnRNPA) is detrimental and causes disease. There has yet to be identified a single gene manipulation that is beneficial in multiple diseases, which is why understanding the role PLS3 plays in SMA and if it is a genetic modifier of other diseases is crucial in understanding disease pathology.

Common pathways are perturbed in neurodegenerative diseases

The data presented in Chapter two has confirmed that PLS3 is a genetic modifier in *Cesmn-1* mutants and that its expression in neurons is sufficient for rescue. An improvement of behavioral defects in ALS and polyQ models was also seen. This improvement across disease models could be due to a common pathway that is perturbed in multiple diseases. Protein misfolding and aggregation occur in most neurodegenerative diseases (Macdonald et al., 1993, Cudkowicz and Brown, 1997, Bates GP, 2002, Wang et al., 2009b). Because of this it is widely accepted that protein quality control plays a crucial role in neuronal function and survival. Studies have shown that manipulation of these pathways is able to alter pathogenesis of neurodegenerative diseases (Cummings et al., 2001, Bonini, 2002). Specifically, the ubiquitin-proteasome pathway (UPP) may contribute to neurodegenerative disease progression in various ways including: recruitment or sequestration to protein aggregates, overloading of the pathway by misfolded protein, or in some cases having the opposite effect where

proteins are degraded too quickly (Hasselgren et al., 2002, Bricceno et al., 2012). The cross disease effects of the proteosome pathway highlight the importance of identifying new pathways that are misregulated across diseases. Identification of new pathways would lead to a better understanding of disease mechanism along with possible new therapeutic targets for those diseases.

The identification of hnRNP F/H as a genetic modifier of SMA and ALS models through PLS3, implicates another pathway that could be perturbed across neurodegenerative diseases. It is known that SMN loss impairs axonal trafficking of the RNP granules that synthesize proteins essential for axon growth and maintenance, including F-actin, CPG15 and synaptophysin (Taylor et al., 2009, Akten et al., 2011, Ackermann et al., 2013). Furthermore loss of SMN could cause decreased local translation through mis-regulation of RNA at the growth cones of motor neurons. Actin also plays an essential role in axon growth and development. The actin cytoskeleton of growth cones allows for the process of elongation and studies show that decreased levels of actin perturb neuron outgrowth (Rossoll et al., 2002). Since PLS3 is an actin-bundling protein and localizes to axons, PLS3 may play a crucial role in neuronal processes (Ackermann et al., 2013). While the role of hnRNPs is still being studied outside of the nucleus, studies have identified several hnRNPs to be localized in granules along the axon and identified as crucial in axon development (Rossoll et al., 2002, Liu et al., 2008a). hnRNP R was identified to modulate actin content in growth cones and when perturbed caused a decrease in actin localization. This suggests that functional hnRNP R is required for localization of actin in growth cones (Rossoll et al., 2002). With the building evidence that both PLS3 and hnRNPs play a role in axons that is pertinent to SMA, the question remains if these proteins and this pathway are also important in other neurodegenerative diseases. Reduced axon growth and disruption of axonal transport is observed in other motoneuron disorders (Yonekawa et al., 1998,

Hadano et al., 2001, Yang et al., 2001). The identification of PLS3 and hnRNP F/H as modifiers of the *C.elegans* ALS model, suggests that their role in axons may be a pertinent to pathogenesis in ALS as well.

While our data suggests a common role for SMN, PLS3, hnRNP F & hnRNP H in motor neuron axons, we cannot exclude the possibility that all four proteins could also play a role in other places, such as the nucleus. SMN and hNRNPs play a critical role in pre-mRNA splicing (Pellizzoni et al., 2002, Gupta et al., 2013). Several studies have also identified PLS3 as a component of complexes involved in RNA processing and metabolism (Ho et al., 2002, Blagoev et al., 2003). The genetic screening in Chapter three also identified the protein DDX5 as a genetic modifier in *C. elegans smn-1* mutants. Using a yeast two-hybrid system DDX5 was also found to bind to both SMN and PLS3. DDX5 is a dead box protein that is a putative RNA helicase. It is known to play various cellular roles that involve translation initiation, splicing and spliceosome assembly (Lorsch, 2002). Perturbation of splicing would affect many genes and is one of the most common therapeutic targets for SMA. A decrease in proteins that are required for splicing such as SMN and DDX5 could lead to alterations in splicing of essential genes that are required for motor neuron survival and thus lead to motor neuron disease. While the splicing pathway is known to be perturbed in SMA, the connection to other neurodegenerative diseases is not as clear. However, specific RNA processing proteins such as FUS and TDP43, when mutated cause ALS and motor neuron disease (Gitcho et al., 2008, Liscic et al., 2008, Vance et al., 2009). The connection between RNA processing proteins and disease suggests a pathway that is perturbed across disease. However, future work connecting these proteins and identifying which of the pathways discussed is pertinent to disease pathogenesis could provide additional therapeutic targets to neurodegenerative diseases such as SMA and ALS.

Possible model for how PLS3 ameliorates SMA defects

A possible model for how PLS3 is able to ameliorate SMA defects is reflected in the vertebrate co-localization data described in Chapter two. This data showed that SMN co-localized in motor neuron processes with PLS3, hnRNP F and hnRNP H in a punctate manner. The punctate pattern of co-localization along with previous studies that identify the independent proteins in granules along axons, suggests a specific complex where all 4 proteins interact. This complex would presumably contain other proteins along with mRNAs that are being transported down the processes for local translation (figure 1). Loss of SMN would disrupt the formation of this complex leading to less mRNA transport and less local translation at growth cones during critical time periods such as motor neuron development or maintenance. The increased expression of PLS3 may either stabilize these complexes or recruit more of them to the processes, therefore increasing the amount of mRNA transport and local translation, restoring axon outgrowth. While it is also shown that actin is found in a complex with SMN and PLS3 (Oprea et al., 2008), it is unclear if actin mRNA is one of the crucial mRNAs being misregulated with loss of SMN and if less local translation of actin leads to weaker or more unstable processes.

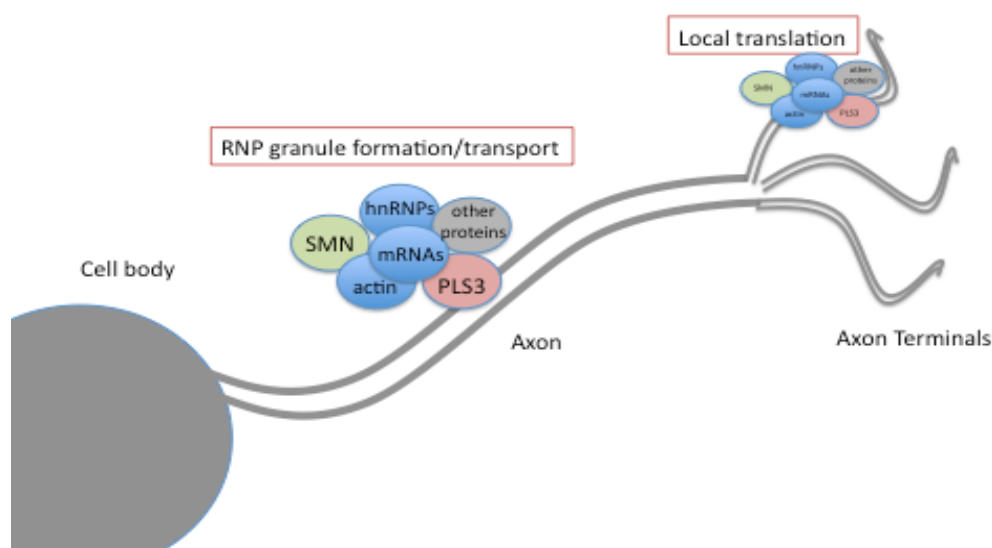


Figure 1: Possible model for how PLS3 ameliorates SMA defects in motor neuron processes.

SMN, PLS3, hnRNPs and other proteins along with mRNAs are found in a complex along motor neuron processes. This complex could be crucial for transport and local translation of mRNAs at critical time periods such as axon outgrowth and maintenance. Disruption of this complex formation would lead to less local translation of mRNAs crucial to motor neuron survival.

Current and future studies

Identification of genetic modifiers can be limited in vertebrate models of disease due to time and genetics. Therefore, it can be advantageous to screen for new modifiers using invertebrate systems such as *C. elegans*. This model organism provides an efficient way to identify genes and pathways pertinent to disease pathogenesis. We have identified *sym-2*, *psf-1* and *ddx-17* to modify *Cesmn-1* mutant phenotypes and *sym-2* to modify an ALS model in *C. elegans*. This data provides enough evidence to possibly test if these genes are modifiers in other model organisms and other disease models. The results could further elucidate a genetic pathway that is pertinent across neurodegenerative diseases.

Ongoing studies plan to validate the interaction between SMN, PLS3 and hnRNP F/H further, in vertebrate models. A more extensive co-IP could show that all the proteins of interest are found in a complex together in vertebrate neuronal tissue. We can do this with sequential IPs, first with SMN and then with PLS3, followed by immunoblotting for hnRNP F and hnRNP H. This data would show all 4 proteins of interest in a complex together for the first time and would suggest a new role for SMN possibly significant to SMA. Continuing co-localization studies in mouse primary motor neurons and triple staining for SMN, PLS3 and hnRNP F or H would allow us to identify

if all of the proteins co-localize on the processes and would complement the immunoprecipitation data planned. Further analysis in mouse primary motor neuron cultures will allow us to determine if loss of SMN alters hnRNP F/H levels via western blot analysis. It is known that diminished levels of SMN decrease hnRNP R levels in axons, but it is unclear if we will see an opposite effect on a different family of hnRNPs (Rossoll et al., 2002).

Finally, as discussed in Chapter 3, is there an unidentified genetic interaction between SMN, PLS3, DDX5 and hnRNP H? Combined genetic analysis in this thesis and protein interaction studies done previously suggest that all these proteins could be components of a large complex involved in RNA processing (Ho et al., 2002, Lorsch, 2002, Pellizzoni et al., 2002, Gupta et al., 2013). Future genetic epistasis studies in *C. elegans* looking at combination of mutants in the locomotion paradigm would allow us to better determine if all of the genes act in the same pathway or if they act in different pathways. For example, additive behavioral defects would suggest genes acting in parallel pathways, whereas non-additivity of phenotypes might suggest genes acting in the same pathway. This genetic testing along with co-immunoprecipitation to determine if all four proteins are found in a complex together in vertebrate neuronal tissue would further characterize their interactions and determine if they are in a complex together.

Implications

There is currently no treatment for SMA and treatments for other neurodegenerative diseases remains limited. Most treatments currently target symptoms of the diseases and are not able to get at the mechanistic cause underlying the disease itself. Understanding common pathways that are perturbed across these diseases, as a strategy for disease therapy requires an understanding of shared genetic modifiers. Work in this thesis identified PLS3 and hnRNP F/H as genetic modifiers across disease.

Targeting either of these proteins improved the behavioral phenotypes in *C. elegans* models, pointing to their role in neurodegeneration. This data provides incentive for future work identifying if these proteins modify across disease in vertebrate models.

References:

- Ackermann B, Krober S, Torres-Benito L, Borgmann A, Peters M, Barkooie SMH, Tejero R, Jakubik M, Schreml J, Milbradt J, Wunderlich TF, Riessland M, Tabares L, Wirth B (Plastin 3 ameliorates spinal muscular atrophy via delayed axon pruning and improves neuromuscular junction functionality. *Hum Mol Genet* 22:1328-1347.2013).
- Akten B, Kye MJ, Hao LT, Wertz MH, Singh S, Nie DY, Huang J, Merianda TT, Twiss JL, Beattie CE, Steen JAJ, Sahin M (Interaction of survival of motor neuron (SMN) and HuD proteins with mRNA cpg15 rescues motor neuron axonal deficits. *P Natl Acad Sci USA* 108:10337-10342.2011).
- Alonso A, Logroscino G, Jick SS, Hernan MA (Incidence and lifetime risk of motor neuron disease in the United Kingdom: a population-based study. *Eur J Neurol* 16:745-751.2009).
- Bates GP HP, Jones AL (eds) (Huntington's Disease. Oxford University Press Oxford, UK.2002).
- Battle DJ, Lau CK, Wan LL, Deng HY, Lotti F, Dreyfuss G (The gemin5 protein of the SMN complex identifies snRNAs. *Mol Cell* 23:273-279.2006).
- Benoit E, Escande D (Riluzole specifically blocks inactivated Na channels in myelinated nerve fibre. *Pflugers Arch* 419:603-609.1991).
- Bertini E, Houlden H (Defects of RNA metabolism in the pathogenesis of spinal muscular atrophy. *Neurology* 82:1298-1299.2014).
- Blagoev B, Kratchmarova I, Ong SE, Nielsen M, Foster LJ, Mann M (A proteomics strategy to elucidate functional protein-protein interactions applied to EGF signaling. *Nat Biotechnol* 21:315-318.2003).
- Bonini NM (Chaperoning brain degeneration. *Proc Natl Acad Sci U S A* 99 Suppl 4:16407-16411.2002).

- Brenner S (Genetics of *Caenorhabditis-Elegans*. Genetics 77:71-94.1974).
- Bricceno KV, Sampognaro PJ, Van Meerbeke JP, Sumner CJ, Fischbeck KH, Burnett BG (Histone deacetylase inhibition suppresses myogenin-dependent atrogene activation in spinal muscular atrophy mice. Hum Mol Genet 21:4448-4459.2012).
- Briese M, Esmaeili B, Fraboulet S, Burt EC, Christodoulou S, Towers PR, Davies KE, Sattelle DB (Deletion of *smn-1*, the *Caenorhabditis elegans* ortholog of the spinal muscular atrophy gene, results in locomotor dysfunction and reduced lifespan. Hum Mol Genet 18:97-104.2009).
- Brignull HR, Moore FE, Tang SJ, Morimoto RI (Polyglutamine proteins at the pathogenic threshold display neuron-specific aggregation in a pan-neuronal *Caenorhabditis elegans* model. J Neurosci 26:7597-7606.2006).
- Brignull HR, Morley JF, Morimoto RI (The stress of misfolded proteins: *C. elegans* models for neurodegenerative disease and aging. Adv Exp Med Biol 594:167-189.2007).
- Brzustowicz LM, Lehner T, Castilla LH, Penchaszadeh GK, Wilhelmsen KC, Daniels R, Davies KE, Leppert M, Ziter F, Wood D, Dubowitz V, Zerres K, Hausmanowapetrusewicz I, Ott J, Munsat TL, Gilliam TC (Genetic-Mapping of Chronic Childhood-Onset Spinal Muscular-Atrophy to Chromosome-5q11.2-13.3. Nature 344:540-541.1990).
- Buhler D, Raker V, Luhrmann R, Fischer U (Essential role for the tudor domain of SMN in spliceosomal U snRNP assembly: implications for spinal muscular atrophy. Hum Mol Genet 8:2351-2357.1999).
- Buratti E, Brindisi A, Giombi M, Tisminetzky S, Ayala YM, Baralle FE (TDP-43 binds heterogeneous nuclear ribonucleoprotein A/B through its C-terminal tail - An important region for the inhibition of cystic fibrosis transmembrane conductance

- regulator exon 9 splicing. *Journal of Biological Chemistry* 280:37572-37584.2005).
- Burnett BG, Munoz E, Tandon A, Kwon DY, Sumner CJ, Fischbeck KH (Regulation of SMN Protein Stability. *Mol Cell Biol* 29:1107-1115.2009).
- Byers RK, Banker BQ (Infantile muscular atrophy. *Arch Neurol* 5:140-164.1961).
- Calixto A, Chelur D, Topalidou I, Chen XY, Chalfie M (Enhanced neuronal RNAi in *C. elegans* using SID-1. *Nature Methods* 7:554-U102.2010).
- Cao YJ, Dreixler JC, Couey JJ, Houamed KM (Modulation of recombinant and native neuronal SK channels by the neuroprotective drug riluzole. *Eur J Pharmacol* 449:47-54.2002).
- Chang WF, Xu J, Chang CC, Yang SH, Li HY, Hsieh-Li HM, Tsai MH, Wu SC, Cheng WT, Liu JL, Sung LY (SMN is required for the maintenance of embryonic stem cells and neuronal differentiation in mice. *Brain Struct Funct*.2014).
- Charroux B, Pellizzoni L, Parkinson RA, Shevchenko A, Mann M, Dreyfuss G (Gemin3: A novel DEAD box protein that interacts with SMN, the spinal muscular atrophy gene product, and is a component of gems. *J Cell Biol* 147:1181-1194.1999).
- Chen YZ, Bennett CL, Huynh HM, Blair IP, Puls I, Irobi J, Dierick I, Abel A, Kennerson ML, Rabin BA, Nicholson GA, Auer-Grumbach M, Wagner K, De Jonghe P, Griffin JW, Fischbeck KH, Timmerman V, Cornblath DR, Chance PF (DNA/RNA helicase gene mutations in a form of juvenile amyotrophic lateral sclerosis (ALS4). *Am J Hum Genet* 74:1128-1135.2004).
- Chio A, Calvo A, Mazzini L, Cantello R, Mora G, Moglia C, Corrado L, D'Alfonso S, Majounie E, Renton A, Pisano F, Ossola I, Brunetti M, Traynor BJ, Restagno G (Extensive genetics of ALS: a population-based study in Italy. *Neurology* 79:1983-1989.2012).

- Chio A, Traynor BJ, Lombardo F, Fimognari M, Calvo A, Ghiglione P, Mutani R, Restagno G (Prevalence of SOD1 mutations in the Italian ALS population. *Neurology* 70:533-537.2008).
- Coady TH, Lorson CL (SMN in spinal muscular atrophy and snRNP biogenesis. *Wires Rna* 2:546-564.2011).
- Coover DD, Le T, McAndrew PE, Strasswimmer J, Crawford TO, Mendell JR, Androphy E, Prior TW, Burghes AHM (The survival motor neuron protein (SMN) in spinal muscular atrophy (SMA). *Am J Hum Genet* 61:A329-A329.1997).
- Crawford TO, Pardo CA (The neurobiology of childhood spinal muscular atrophy. *Neurobiol Dis* 3:97-110.1996).
- Cudkovic ME, Brown RH (CuZnSOD-associated amyotrophic lateral sclerosis - Reply. *Ann Neurol* 42:274-274.1997).
- Culetto E, Sattelle DB (A role for *Caenorhabditis elegans* in understanding the function and interactions of human disease genes. *Hum Mol Genet* 9:869-877.2000).
- Cummings CJ, Sun Y, Opal P, Antalffy B, Mestral R, Orr HT, Dillmann WH, Zoghbi HY (Over-expression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in SCA1 mice. *Hum Mol Genet* 10:1511-1518.2001).
- Delanote V, Vandekerckhove J, Gettemans J (Plastins: versatile modulators of actin organization in (patho)physiological cellular processes. *Acta Pharmacol Sin* 26:769-779.2005).
- Deng HX, Chen W, Hong ST, Boycott KM, Gorrie GH, Siddique N, Yang Y, Fecto F, Shi Y, Zhai H, Jiang H, Hirano M, Rampersaud E, Jansen GH, Donkervoort S, Bigio EH, Brooks BR, Ajroud K, Sufit RL, Haines JL, Mugnaini E, Pericak-Vance MA, Siddique T (Mutations in UBQLN2 cause dominant X-linked juvenile and adult-onset ALS and ALS/dementia. *Nature* 477:211-215.2011).

Dent EW, Gertler FB (Cytoskeletal dynamics and transport in growth cone motility and axon guidance. *Neuron* 40:209-227.2003).

Dimitriadi M, Sleight JN, Walker A, Chang HC, Sen A, Kalloo G, Harris J, Barsby T, Walsh MB, Satterlee JS, Li C, Van Vactor D, Artavanis-Tsakonas S, Hart AC (Conserved Genes Act as Modifiers of Invertebrate SMN Loss of Function Defects. *PLOS Genetics* 6:-.2010).

Dreyfuss G, Kim VN, Kataoka N (Messenger-RNA-binding proteins and the messages they carry. *Nat Rev Mol Cell Bio* 3:195-205.2002).

Dreyfuss G, Matunis MJ, Pinolroma S, Burd CG (Hnnp Proteins and the Biogenesis of Messenger-Rna. *Annu Rev Biochem* 62:289-321.1993).

Dubowitz V (Infantile Muscular Atrophy. A Prospective Study with Particular Reference to a Slowly Progressive Variety. *Brain* 87:707-718.1964).

Duprat F, Lesage F, Patel AJ, Fink M, Romey G, Lazdunski M (The neuroprotective agent riluzole activates the two P domain K(+) channels TREK-1 and TRAAK. *Mol Pharmacol* 57:906-912.2000).

Eggert C, Chari A, Lagerbauer B, Fischer U (Spinal muscular atrophy: the RNP connection. *Trends Mol Med* 12:113-121.2006).

Fallini C, Zhang HL, Su YH, Silani V, Singer RH, Rossoll W, Bassell GJ (The Survival of Motor Neuron (SMN) Protein Interacts with the mRNA-Binding Protein HuD and Regulates Localization of Poly(A) mRNA in Primary Motor Neuron Axons. *Journal of Neuroscience* 31:3914-3925.2011).

Farg MA, Soo KY, Warraich ST, Sundaramoorthy V, Blair IP, Atkin JD (Ataxin-2 interacts with FUS and intermediate-length polyglutamine expansions enhance FUS-related pathology in amyotrophic lateral sclerosis. *Hum Mol Genet* 22:717-728.2013).

- Fecto F, Yan JH, Vemula SP, Liu ED, Yang Y, Chen WJ, Zheng JG, Shi Y, Siddique N, Arrat H, Donkervoort S, Ajroud-Driss S, Sufit RL, Heller SL, Deng HX, Siddique T (SQSTM1 Mutations in Familial and Sporadic Amyotrophic Lateral Sclerosis. Arch Neurol-Chicago 68:1440-1446.2011).
- Feldkotter M, Schwarzer V, Wirth R, Wienker TF, Wirth B (Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. Am J Hum Genet 70:358-368.2002).
- Fischer U, Liu Q, Dreyfuss G (The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. Cell 90:1023-1029.1997).
- Frokjaer-Jensen C, Davis MW, Hopkins CE, Newman BJ, Thummel JM, Olesen SP, Grunnet M, Jorgensen EM (Single-copy insertion of transgenes in Caenorhabditis elegans. Nat Genet 40:1375-1383.2008).
- Fuller HR, Man NT, Lam le T, Thanh le T, Keough RA, Asperger A, Gonda TJ, Morris GE (The SMN interactome includes Myb-binding protein 1a. J Proteome Res 9:556-563.2010).
- Gabanella F, Butchbach MER, Saieva L, Carissimi C, Burghes AHM, Pellizzoni L (Ribonucleoprotein Assembly Defects Correlate with Spinal Muscular Atrophy Severity and Preferentially Affect a Subset of Spliceosomal snRNPs. PLoS One 2.2007).
- Giesemann T, Rathke-Hartlieb S, Rothkegel M, Bartsch JW, Buchmeier S, Jockusch BM, Jockusch H (A role for polyproline motifs in the spinal muscular atrophy protein SMN - Profilins bind to and colocalize with SMN in nuclear gems. Journal of Biological Chemistry 274:37908-37914.1999).
- Gitcho MA, Baloh RH, Chakraverty S, Mayo K, Norton JB, Levitch D, Hatanpaa KJ, White CL, 3rd, Bigio EH, Caselli R, Baker M, Al-Lozi MT, Morris JC, Pestronk A,

- Rademakers R, Goate AM, Cairns NJ (TDP-43 A315T mutation in familial motor neuron disease. *Ann Neurol* 63:535-538.2008).
- Grunnet M, Jespersen T, Angelo K, Frokjaer-Jensen C, Klaerke DA, Olesen SP, Jensen BS (Pharmacological modulation of SK3 channels. *Neuropharmacology* 40:879-887.2001).
- Gubitz AK, Feng WQ, Dreyfuss G (The SMN complex. *Exp Cell Res* 296:51-56.2004).
- Gupta SK, Kosti I, Plaut G, Pivko A, Tkacz ID, Cohen-Chalamish S, Biswas DK, Wachtel C, Ben-Asher HW, Carmi S, Glaser F, Mandel-Gutfreund Y, Michaeli S (The hnRNP F/H homologue of *Trypanosoma brucei* is differentially expressed in the two life cycle stages of the parasite and regulates splicing and mRNA stability. *Nucleic Acids Research* 41:6577-6594.2013).
- Gusella JF, MacDonald ME (Huntington's disease: the case for genetic modifiers. *Genome Med* 1.2009).
- Hadano S, Hand CK, Osuga H, Yanagisawa Y, Otomo A, Devon RS, Miyamoto N, Showguchi-Miyata J, Okada Y, Singaraja R, Figlewicz DA, Kwiatkowski T, Hosler BA, Sagie T, Skaug J, Nasir J, Brown RH, Scherer SW, Rouleau GA, Hayden MR, Ikeda JE (A gene encoding a putative GTPase regulator is mutated in familial amyotrophic lateral sclerosis 2 (vol 29, pg 166, 2001). *Nature Genetics* 29:352-352.2001).
- Hao LT, Wolman M, Granato M, Beattie CE (Survival Motor Neuron Affects Plastin 3 Protein Levels Leading to Motor Defects. *Journal of Neuroscience* 32:5074-5084.2012).
- Hasselgren PO, Wray C, Mammen J (Molecular regulation of muscle cachexia: it may be more than the proteasome. *Biochem Biophys Res Commun* 290:1-10.2002).
- He XH, Lin F, Qin ZH (Current understanding on the pathogenesis of polyglutamine diseases. *Neurosci Bull* 26:247-256.2010).

- Hegele A, Kamburov A, Grossmann A, Sourlis C, Wowro S, Weimann M, Will CL, Pena V, Luhrmann R, Stelzl U (Dynamic protein-protein interaction wiring of the human spliceosome. *Mol Cell* 45:567-580.2012).
- Ho Y, Gruhler A, Heilbut A, Bader GD, Moore L, Adams SL, Millar A, Taylor P, Bennett K, Boutilier K, Yang L, Wolting C, Donaldson I, Schandorff S, Shewnarane J, Vo M, Taggart J, Goudreault M, Muskat B, Alfarano C, Dewar D, Lin Z, Michalickova K, Willems AR, Sassi H, Nielsen PA, Rasmussen KJ, Andersen JR, Johansen LE, Hansen LH, Jespersen H, Podtelejnikov A, Nielsen E, Crawford J, Poulsen V, Sorensen BD, Matthiesen J, Hendrickson RC, Gleeson F, Pawson T, Moran MF, Durocher D, Mann M, Hogue CW, Figeys D, Tyers M (Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature* 415:180-183.2002).
- Hua Y, Zhou J (Rpp20 interacts with SMN and is re-distributed into SMN granules in response to stress. *Biochem Biophys Res Commun* 314:268-276.2004).
- Huang CS, Song JH, Nagata K, Yeh JZ, Narahashi T (Effects of the neuroprotective agent riluzole on the high voltage-activated calcium channels of rat dorsal root ganglion neurons. *J Pharmacol Exp Ther* 282:1280-1290.1997).
- Jacobi H, Hauser TK, Giunti P, Globas C, Bauer P, Schmitz-Hubsch T, Baliko L, Filla A, Mariotti C, Rakowicz M, Charles P, Ribai P, Szymanski S, Infante J, van de Warrenburg BP, Durr A, Timmann D, Boesch S, Fancellu R, Rola R, Depondt C, Schols L, Zdzienicka E, Kang JS, Ratzka S, Kremer B, Stephenson DA, Melegh B, Pandolfo M, Tezenas du Montcel S, Borkert J, Schulz JB, Klockgether T (Spinocerebellar ataxia types 1, 2, 3 and 6: the clinical spectrum of ataxia and morphometric brainstem and cerebellar findings. *Cerebellum* 11:155-166.2012).

- Jin YS, Jorgensen E, Hartweg E, Horvitz HR (The *Caenorhabditis elegans* gene *unc-25* encodes glutamic acid decarboxylase and is required for synaptic transmission but not synaptic development. *Journal of Neuroscience* 19:539-548.1999).
- Johnson JO, Mandrioli J, Benatar M, Abramzon Y, Van Deerlin VM, Trojanowski JQ, Gibbs JR, Brunetti M, Gronka S, Wu J, Ding JH, McCluskey L, Martinez-Lage M, Falcone D, Hernandez DG, Arepalli S, Chong S, Schymick JC, Rothstein J, Landi F, Wang YD, Calvo A, Mora G, Sabatelli M, Monsurro MR, Battistini S, Salvi F, Spataro R, Sola P, Borghero G, Galassi G, Scholz SW, Taylor JP, Restagno G, Chio A, Traynor BJ, Consortium I (Exome Sequencing Reveals VCP Mutations as a Cause of Familial ALS. *Neuron* 68:857-864.2010).
- Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, Kanapin A, Le Bot N, Moreno S, Sohrmann M, Welchman DP, Zipperlen P, Ahringer J (Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* 421:231-237.2003a).
- Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, Kanapin A, Le Bot N, Moreno S, Sohrmann M, Welchman DP, Zipperlen P, Ahringer J (Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* 421:231-237.2003b).
- Kim HJ, Kim NC, Wang YD, Scarborough EA, Moore J, Diaz Z, MacLea KS, Freibaum B, Li SQ, Molliex A, Kanagaraj AP, Carter R, Boylan KB, Wojtas AM, Rademakers R, Pinkus JL, Greenberg SA, Trojanowski JQ, Traynor BJ, Smith BN, Topp S, Gkazi AS, Miller J, Shaw CE, Kottlors M, Kirschner J, Pestronk A, Li YR, Ford AF, Gitler AD, Benatar M, King OD, Kimonis VE, Ross ED, Weihl CC, Shorter J, Taylor JP (Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS. *Nature* 495:467-+.2013).

- Krajacic P, Shen XN, Purohit PK, Arratia P, Lamitina T (Biomechanical Profiling of *Caenorhabditis elegans* Motility. *Genetics* 191:1015-U1613.2012).
- Kugelberg E (Functions of the spinal cord. Experimental and clinical aspects. Conclusion. *Electroencephalogr Clin Neurophysiol Suppl* 25:95-27.1967).
- Kwiatkowski TJ, Vanderburg CR (Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis (vol 323, pg 1205, 2009). *Science* 324:465-465.2009).
- Kwon DY, Motley WW, Fischbeck KH, Burnett BG (Increasing expression and decreasing degradation of SMN ameliorate the spinal muscular atrophy phenotype in mice. *Hum Mol Genet* 20:3667-3677.2011).
- Lamm GM, Nicol SM, Fuller-Pace FV, Lamond AI (p72: a human nuclear DEAD box protein highly related to p68. *Nucleic Acids Res* 24:3739-3747.1996).
- Lee WY, Jin DK, Oh MR, Lee JE, Song SM, Lee EA, Kim GM, Chung JS, Lee KH (Frequency analysis and clinical characterization of spinocerebellar ataxia types 1, 2, 3, 6, and 7 in Korean patients. *Arch Neurol* 60:858-863.2003).
- Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, Viollet L, Benichou B, Cruaud C, Millasseau P, Zeviani M, Lepaslier D, Frezal J, Cohen D, Weissenbach J, Munnich A, Melki J (Identification and Characterization of a Spinal Muscular Atrophy-Determining Gene. *Cell* 80:155-165.1995).
- Lin CL, Bristol LA, Jin L, Dykes-Hoberg M, Crawford T, Clawson L, Rothstein JD (Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis. *Neuron* 20:589-602.1998).
- Liscic RM, Grinberg LT, Zidar J, Gitcho MA, Cairns NJ (ALS and FTLD: two faces of TDP-43 proteinopathy. *Eur J Neurol* 15:772-780.2008).

- Liu Q, Fischer U, Wang F, Dreyfuss G (The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins. *Cell* 90:1013-1021.1997).
- Liu Q, Hollopeter G, Jorgensen EM (Graded synaptic transmission at the *Caenorhabditis elegans* neuromuscular junction. *P Natl Acad Sci USA* 106:10823-10828.2009).
- Liu Y, Gervasi C, Szaro BG (A crucial role for hnRNP K in axon development in *Xenopus laevis*. *Development* 135:3125-3135.2008a).
- Liu YY, Gervasi C, Szaro BG (A crucial role for hnRNP K in axon development in *Xenopus laevis*. *Development* 135:3125-3135.2008b).
- Lorsch JR (RNA chaperones exist and DEAD box proteins get a life. *Cell* 109:797-800.2002).
- Lorson CL, Strasswimmer J, Yao JM, Baleja JD, Hahnen E, Wirth B, Le T, Burghes AHM, Androphy EJ (SMN oligomerization defect correlates with spinal muscular atrophy severity. *Nature Genetics* 19:63-66.1998).
- Lotti F, Imlach WL, Saieva L, Beck ES, Hao le T, Li DK, Jiao W, Mentis GZ, Beattie CE, McCabe BD, Pellizzoni L (An SMN-dependent U12 splicing event essential for motor circuit function. *Cell* 151:440-454.2012).
- Lu XH, Yang XW ("Huntingtin holiday": progress toward an antisense therapy for Huntington's disease. *Neuron* 74:964-966.2012).
- Lyon AN, Pineda RH, Hao LT, Kudryashova E, Kudryashov DS, Beattie CE (Calcium binding is essential for plastin 3 function in Smn-deficient motoneurons. *Hum Mol Genet* 23:1990-2004.2014).
- Macdonald ME, Ambrose CM, Duyao MP, Myers RH, Lin C, Srinidhi L, Barnes G, Taylor SA, James M, Groot N, Macfarlane H, Jenkins B, Anderson MA, Wexler NS, Gusella JF, Bates GP, Baxendale S, Hummerich H, Kirby S, North M, Youngman S, Mott R, Zehetner G, Sedlacek Z, Poustka A, Frischauf AM, Lehrach H, Buckler

AJ, Church D, Doucettstamm L, Odonovan MC, Ribaramirez L, Shah M, Stanton VP, Strobel SA, Draths KM, Wales JL, Dervan P, Housman DE, Altherr M, Shiang R, Thompson L, Fielder T, Wasmuth JJ, Tagle D, Valdes J, Elmer L, Allard M, Castilla L, Swaroop M, Blanchard K, Collins FS, Snell R, Holloway T, Gillespie K, Datson N, Shaw D, Harper PS (A Novel Gene Containing a Trinucleotide Repeat That Is Expanded and Unstable on Huntingtons-Disease Chromosomes. *Cell* 72:971-983.1993).

Mailman MD, Heinz JW, Papp AC, Snyder PJ, Sedra MS, Wirth B, Burghes AH, Prior TW (Molecular analysis of spinal muscular atrophy and modification of the phenotype by SMN2. *Genet Med* 4:20-26.2002).

Majounie E, Renton AE, Mok K, Dopper EG, Waite A, Rollinson S, Chio A, Restagno G, Nicolaou N, Simon-Sanchez J, van Swieten JC, Abramzon Y, Johnson JO, Sendtner M, Pamphlett R, Orrell RW, Mead S, Sidle KC, Houlden H, Rohrer JD, Morrison KE, Pall H, Talbot K, Ansorge O, Hernandez DG, Arepalli S, Sabatelli M, Mora G, Corbo M, Giannini F, Calvo A, Englund E, Borghero G, Floris GL, Remes AM, Laaksovirta H, McCluskey L, Trojanowski JQ, Van Deerlin VM, Schellenberg GD, Nalls MA, Drory VE, Lu CS, Yeh TH, Ishiura H, Takahashi Y, Tsuji S, Le Ber I, Brice A, Drepper C, Williams N, Kirby J, Shaw P, Hardy J, Tienari PJ, Heutink P, Morris HR, Pickering-Brown S, Traynor BJ (Frequency of the C9orf72 hexanucleotide repeat expansion in patients with amyotrophic lateral sclerosis and frontotemporal dementia: a cross-sectional study. *Lancet Neurol* 11:323-330.2012).

Miguel-Aliaga I, Culetto E, Walker DS, Baylis HA, Sattelle DB, Davies KE (The *Caenorhabditis elegans* orthologue of the human gene responsible for spinal muscular atrophy is a maternal product critical for germline maturation and embryonic viability. *Hum Mol Genet* 8:2133-2143.1999).

- Miller T, Cleveland D (Merits of a new drug trial for ALS? Response. Science 308:632-633.2005).
- Miller TM, Kaspar BK, Kops GJ, Yamanaka K, Christian LJ, Gage FH, Cleveland DW (Virus-delivered small RNA silencing sustains strength in amyotrophic lateral sclerosis. Ann Neurol 57:773-776.2005).
- Monani UR, Lorson CL, Parsons DW, Prior TW, Androphy EJ, Burghes AH, McPherson JD (A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. Hum Mol Genet 8:1177-1183.1999).
- Nagel G, Brauner M, Liewald JF, Adeishvili N, Bamberg E, Gottschalk A (Light activation of channelrhodopsin-2 in excitable cells of *Caenorhabditis elegans* triggers rapid Behavioral responses. Current Biology 15:2279-2284.2005).
- Nalavade R, Griesche N, Ryan DP, Hildebrand S, Krauss S (Mechanisms of RNA-induced toxicity in CAG repeat disorders. Cell Death Dis 4:e752.2013).
- Narver HL, Kong LL, Burnett BG, Choe DW, Bosch-Mare M, Taye AA, Eckhaus MA, Sumner CJ (Sustained Improvement of Spinal Muscular Atrophy Mice Treated with Trichostatin A Plus Nutrition. Ann Neurol 64:465-470.2008).
- Nishimura AL, Mitne-Neto M, Silva HCA, Richieri-Costa A, Middleton S, Cascio D, Kok F, Oliveira JRM, Gillingwater T, Webb J, Skehel P, Zatz M (A mutation in the vesicle-trafficking protein VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis. Am J Hum Genet 75:822-831.2004).
- Oprea GE, Krober S, McWhorter ML, Rossoll W, Muller S, Krawczak M, Bassell GJ, Beattie CE, Wirth B (Plastin 3 is a protective modifier of autosomal recessive spinal muscular atrophy. Science 320:524-527.2008).

- Pagliardini S, Giavazzi A, Setola V, Lizier C, Di Luca M, DeBiasi S, Battaglia G (Subcellular localization and axonal transport of the survival motor neuron (SMN) protein in the developing rat spinal cord. *Hum Mol Genet* 9:47-56.2000).
- Paushkin S, Gubitz AK, Massenet S, Dreyfuss G (The SMN complex, an assemblyosome of ribonucleoproteins. *Curr Opin Cell Biol* 14:305-312.2002).
- Pearn J (Incidence, Prevalence, and Gene Frequency Studies of Chronic Childhood Spinal Muscular-Atrophy. *J Med Genet* 15:409-413.1978).
- Pellizzoni L (Chaperoning ribonucleoprotein biogenesis in health and disease. *Embo Rep* 8:340-345.2007).
- Pellizzoni L, Yong J, Dreyfuss G (Essential role for the SMN complex in the specificity of snRNP assembly. *Science* 298:1775-1779.2002).
- Piazzon N, Rage F, Schlotter F, Moine H, Branlant C, Massenet S (In vitro and in cellulo evidences for association of the survival of motor neuron complex with the fragile x mental retardation protein. *Journal of Biological Chemistry* 283:5598-5610.2008).
- Prior TW, Swoboda KJ, Scott HD, Hejmanowski AQ (Homozygous SMN1 deletions in unaffected family members and modification of the phenotype by SMN2. *Am J Med Genet A* 130A:307-310.2004).
- Riboldi G, Nizzardo M, Simone C, Falcone M, Bresolin N, Comi GP, Corti S (ALS genetic modifiers that increase survival of SOD1 mice and are suitable for therapeutic development. *Prog Neurobiol* 95:133-148.2011).
- Ritson GP, Custer SK, Freibaum BD, Guinto JB, Geffel D, Moore J, Tang WX, Winton MJ, Neumann M, Trojanowski JQ, Lee VMY, Forman MS, Taylor JP (TDP-43 Mediates Degeneration in a Novel Drosophila Model of Disease Caused by Mutations in VCP/p97. *Journal of Neuroscience* 30:7729-7739.2010).

- Rosen DR (Mutations in Cu/Zn Superoxide-Dismutase Gene Are Associated with Familial Amyotrophic-Lateral-Sclerosis (Vol 362, Pg 59, 1993). *Nature* 364:362-362.1993).
- Rosonina E, Ip JY, Calarco JA, Bakowski MA, Emili A, McCracken S, Tucker P, Ingles CJ, Blencowe BJ (Role for PSF in mediating transcriptional activator-dependent stimulation of pre-mRNA processing in vivo. *Mol Cell Biol* 25:6734-6746.2005).
- Rossoll W, Bassell GJ (Spinal muscular atrophy and a model for survival of motor neuron protein function in axonal ribonucleoprotein complexes. *Results Probl Cell Differ* 48:289-326.2009).
- Rossoll W, Jablonka S, Andreassi C, Kroning AK, Karle K, Monani UR, Sendtner M (Smn, the spinal muscular atrophy-determining gene product, modulates axon growth and localization of beta-actin mRNA in growth cones of motoneurons. *Journal of Cell Biology* 163:801-812.2003).
- Rossoll W, Kroning AK, Ohndorf UM, Steegborn C, Jablonka S, Sendtner M (Specific interaction of Smn, the spinal muscular atrophy determining gene product, with hnRNP-R and gry-rbp/hnRNP-Q: a role for Smn in RNA processing in motor axons? *Hum Mol Genet* 11:93-105.2002).
- Rowland LP, Shneider NA (Amyotrophic lateral sclerosis - Reply. *New Engl J Med* 345:1132-1132.2001a).
- Rowland LP, Shneider NA (Medical progress: Amyotrophic lateral sclerosis. *New Engl J Med* 344:1688-1700.2001b).
- Russman BS (Spinal muscular atrophy: clinical classification and disease heterogeneity. *J Child Neurol* 22:946-951.2007).
- Russman BS, Buncher CR, White M, Samaha FJ, Iannaccone ST (Function changes in spinal muscular atrophy II and III. The DCN/SMA Group. *Neurology* 47:973-976.1996).

- Russman BS, Iannacone ST, Buncher CR, Samaha FJ, White M, Perkins B, Zimmerman L, Smith C, Burhans K, Barker L (Spinal muscular atrophy: new thoughts on the pathogenesis and classification schema. *J Child Neurol* 7:347-353.1992).
- Sen A, Dimlich DN, Guruharsha KG, Kankel MW, Hori K, Yokokura T, Brachat S, Richardson D, Loureiro J, Sivasankaran R, Curtis D, Davidow LS, Rubin LL, Hart AC, Van Vactor D, Artavanis-Tsakonas S (Genetic circuitry of Survival motor neuron, the gene underlying spinal muscular atrophy. *P Natl Acad Sci USA* 110:E2371-E2380.2013).
- Simic G (Pathogenesis of proximal autosomal recessive spinal muscular atrophy. *Acta Neuropathol* 116:223-234.2008).
- Simmer F, Moorman C, van der Linden AM, Kuijk E, van den Berghe PV, Kamath RS, Fraser AG, Ahringer J, Plasterk RH (Genome-wide RNAi of *C. elegans* using the hypersensitive *rff-3* strain reveals novel gene functions. *PLoS Biol* 1:E12.2003).
- Sleigh JN, Buckingham SD, Cuppen E, Viswanathan M, Westlund BM, Sattelle DB (A novel point mutation in the *Caenorhabditis elegans* *smn-1* gene provides a useful model for investigating Spinal Muscular Atrophy. *Neuromuscular Disord* 20:S22-S23.2010).
- Sleigh JN, Buckingham SD, Esmaeili B, Viswanathan M, Cuppen E, Westlund BM, Sattelle DB (A novel *Caenorhabditis elegans* allele, *smn-1(cb131)*, mimicking a mild form of spinal muscular atrophy, provides a convenient drug screening platform highlighting new and pre-approved compounds. *Hum Mol Genet* 20:245-260.2011).
- Song JH, Huang CS, Nagata K, Yeh JZ, Narahashi T (Differential action of riluzole on tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels. *J Pharmacol Exp Ther* 282:707-714.1997).

- Sreedharan J, Blair IP, Tripathi VB, Hu X, Vance C, Rogelj B, Ackerley S, Durnall JC, Williams KL, Buratti E, Baralle F, de Belleruche J, Mitchell JD, Leigh PN, Al-Chalabi A, Miller CC, Nicholson G, Shaw CE (TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science* 319:1668-1672.2008).
- Sun Y, Grimm M, Schwarzer V, Schoenen F, Fischer U, Wirth B (Molecular and functional analysis of intragenic SMN1 mutations in patients with spinal muscular atrophy. *Hum Mutat* 25:64-71.2005).
- Taylor AM, Berchtold NC, Perreau VM, Tu CH, Jeon NL, Cotman CW (Axonal mRNA in Uninjured and Regenerating Cortical Mammalian Axons. *Journal of Neuroscience* 29:4697-4707.2009).
- Tazen S, Figueroa K, Kwan JY, Goldman J, Hunt A, Sampson J, Gutmann L, Pulst SM, Mitumoto H, Kuo SH (Amyotrophic lateral sclerosis and spinocerebellar ataxia type 2 in a family with full CAG repeat expansions of ATXN2. *JAMA Neurol* 70:1302-1304.2013).
- Vance C, Rogelj B, Hortobagyi T, De Vos KJ, Nishimura AL, Sreedharan J, Hu X, Smith B, Ruddy D, Wright P, Ganesalingam J, Williams KL, Tripathi V, Al-Saraj S, Al-Chalabi A, Leigh PN, Blair IP, Nicholson G, de Belleruche J, Gallo JM, Miller CC, Shaw CE (Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science* 323:1208-1211.2009).
- Wang EM, Aslanzadeh V, Papa F, Zhu HY, de la Grange P, Cambi F (Global Profiling of Alternative Splicing Events and Gene Expression Regulated by hnRNPH/F. *PLoS One* 7.2012).
- Wang IF, Reddy NM, Shen CKJ (Higher order arrangement of the eukaryotic nuclear bodies. *P Natl Acad Sci USA* 99:13583-13588.2002).
- Wang J, Farr GW, Hall DH, Li F, Furtak K, Dreier L, Horwich AL (An ALS-Linked Mutant SOD1 Produces a Locomotor Defect Associated with Aggregation and Synaptic

- Dysfunction When Expressed in Neurons of *Caenorhabditis elegans*. *PLOS Genetics* 5:-.2009a).
- Wang J, Farr GW, Zeiss CJ, Rodriguez-Gil DJ, Wilson JH, Furtak K, Rutkowski DT, Kaufman RJ, Ruse CI, Yates JR, Perrin S, Feany MB, Horwich AL (Progressive aggregation despite chaperone associations of a mutant SOD1-YFP in transgenic mice that develop ALS. *P Natl Acad Sci USA* 106:1392-1397.2009b).
- Werdnig G (Two early infantile hereditary cases of progressive muscular atrophy simulating dystrophy, but on a neural basis. 1891. *Arch Neurol* 25:276-278.1971).
- Wichterle H, Lieberam I, Porter JA, Jessell TM (Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110:385-397.2002).
- Wu CH, Fallini C, Ticozzi N, Keagle PJ, Sapp PC, Piotrowska K, Lowe P, Koppers M, McKenna-Yasek D, Baron DM, Kost JE, Gonzalez-Perez P, Fox AD, Adams J, Taroni F, Tiloca C, Leclerc AL, Chafe SC, Mangroo D, Moore MJ, Zitzewitz JA, Xu ZS, van den Berg LH, Glass JD, Siciliano G, Cirulli ET, Goldstein DB, Salachas F, Meininger V, Rossoll W, Ratti A, Gellera C, Bosco DA, Bassell GJ, Silani V, Drory VE, Brown RH, Landers JE (Mutations in the profilin 1 gene cause familial amyotrophic lateral sclerosis. *Nature* 488:499-+.2012).
- Yang Y, Hentati A, Deng HX, Dabbagh O, Sasaki T, Hirano M, Hung WY, Ouahchi K, Yan J, Azim AC, Cole N, Gascon G, Yagmour A, Ben-Hamida M, Pericak-Vance M, Hentati F, Siddique T (The gene encoding alsin, a protein with three guanine-nucleotide exchange factor domains, is mutated in a form of recessive amyotrophic lateral sclerosis. *Nat Genet* 29:160-165.2001).
- Yonekawa Y, Harada A, Okada Y, Funakoshi T, Kanai Y, Takei Y, Terada S, Noda T, Hirokawa N (Defect in synaptic vesicle precursor transport and neuronal cell death in KIF1A motor protein-deficient mice. *J Cell Biol* 141:431-441.1998).

- Young PJ, Man NT, Lorson CL, Le TT, Androphy EJ, Burghes AHM, Morris GE (The exon 2b region of the spinal muscular atrophy protein, SMN, is involved in self-association and SIP1 binding. *Hum Mol Genet* 9:2869-2877.2000).
- Zhang HL, Xing L, Rossoll W, Wichterle H, Singer RH, Bassell GJ (Multiprotein complexes of the survival of motor neuron protein SMN with gemins traffic to neuronal processes and growth cones of motor neurons (vol 26, pg 8622, 2006). *Journal of Neuroscience* 26.2006).
- Zhang R, So BR, Li P, Yong J, Glisovic T, Wan L, Dreyfuss G (Structure of a key intermediate of the SMN complex reveals Gemin2's crucial function in snRNP assembly. *Cell* 146:384-395.2011).
- Zhang Z, Lotti F, Dittmar K, Younis I, Wan L, Kasim M, Dreyfuss G (SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. *Cell* 133:585-600.2008).
- Zoghbi HY, Orr HT (Glutamine repeats and neurodegeneration. *Annu Rev Neurosci* 23:217-247.2000).
- Zou T, Yang X, Pan D, Huang J, Sahin M, Zhou J (SMN deficiency reduces cellular ability to form stress granules, sensitizing cells to stress. *Cell Mol Neurobiol* 31:541-550.2011).

Appendix

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

In this paper, I contributed data to the pharyngeal pumping assays and growth assays done in *C. elegans* mutant strains.

C. elegans Notch Signaling Regulates Adult Chemosensory Response and Larval Molting Quiescence

In this paper I did the genetic cross to make *osm-7;osm-11* double mutants. I also contributed behavioral analysis of *osm-7;osm-11* double mutants in the octanol response behavioral analysis and undertook locomotion behavioral assessment of *osm-7*, *osm-11* and *osm-7;osm-11* mutants (Supplementary figure).

Deep conservation of genes required for both *D. melanogaster* and *C. elegans* sleep includes a role for dopaminergic signaling

In this paper I did the genetic cross to create *dop-1;acy-1* double mutants. I assessed quiescence behavior on *dop-1*, *dop-1;acy-1* and a second allele for *goa-1* mutant animals. I also reviewed and contributed edits to the final manuscript.

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

Maria Dimitriadi^{1,9}, James N. Sleight^{2,3,9}, Amy Walker^{2,9}, Howard C. Chang⁴, Anindya Sen⁴, Geetika Kalloo¹, Jevede Harris², Tom Barsby^{2,3}, Melissa B. Walsh⁵, John S. Satterlee², Chris Li⁶, David Van Vactor⁴, Spyros Artavanis-Tsakonas^{4,7}, Anne C. Hart^{1*}

1 Department of Neuroscience, Brown University, Providence, Rhode Island, United States of America, **2** Center for Cancer Research, Massachusetts General Hospital, Department of Pathology, Harvard Medical School, Boston, Massachusetts, United States of America, **3** Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom, **4** Department of Cell Biology, Harvard Medical School, Boston, Massachusetts, United States of America, **5** Department of Molecular and Cellular Biology, Brown University, Providence, Rhode Island, United States of America, **6** Department of Biology, City College – City University of New York, New York, New York, United States of America, **7** Collège de France, Paris, France

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.

Citation: Dimitriadi M, Sleight JN, Walker A, Chang HC, Sen A, et al. (2010) Conserved Genes Act as Modifiers of Invertebrate SMN Loss of Function Defects. PLoS Genet 6(10): e1001172. doi:10.1371/journal.pgen.1001172

Editor: Gregory S. Barsh, Stanford University, United States of America

Received: February 4, 2010; **Accepted:** September 21, 2010; **Published:** October 28, 2010

This is an open-access article distributed under the terms of the Creative Commons Public Domain declaration which stipulates that, once placed in the public domain, this work may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose.

Funding: This work was supported by the Spinal Muscular Atrophy Foundation (DVV, SA-T, ACH), by National Institutes of Health (NIH R01 NS42459) and NIH/NICRR/RCMI Grant G12-RR03060 (CL), and by the City College of New York, respectively. Also by NIH R01 NS26084 (SA-T) and a Families of SMA Postdoctoral Fellowship (AS). Some nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: anne_hart@brown.edu

9 These authors contributed equally to this work.

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 Gemin 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 over-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 motoneurons 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* Smn (*DmSmn*) causes larval lethality and NMJ defects; *DmSmn* 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(lf)* 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 *DmSmn* with *Drosophila* orthologs of *C. elegans* modifier genes. In the study by Chang and co-workers, the *DmSmn* lethal phenotype correlated with NMJ defects for virtually all *DmSmn* modifier genes, suggesting that lethality and neuromuscular bouton number are effective measures of genetic interaction with the *Drosophila* SMN 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 *ncbp-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(lf)* [44]. *Cesmn-1(lf)* 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(lf)* (abbreviated *+ / Cesmn-1(lf)*) [44,46]. Heterozygous *+ / Cesmn-1(lf)* animals express pharyngeal GFP, homozygous *Cesmn-1(lf)* 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(lf)* can survive for several days due to partial maternal rescue. It has been suggested that *+ / Cesmn-1(lf)* 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(lf)* larvae initially resemble wild type animals. Eventually maternally-loaded *Cesmn-1* product is lost; *Cesmn-1(lf)* animals grow more slowly than *+ / Cesmn-1(lf)* 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* (*PLaSTin* (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 the 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

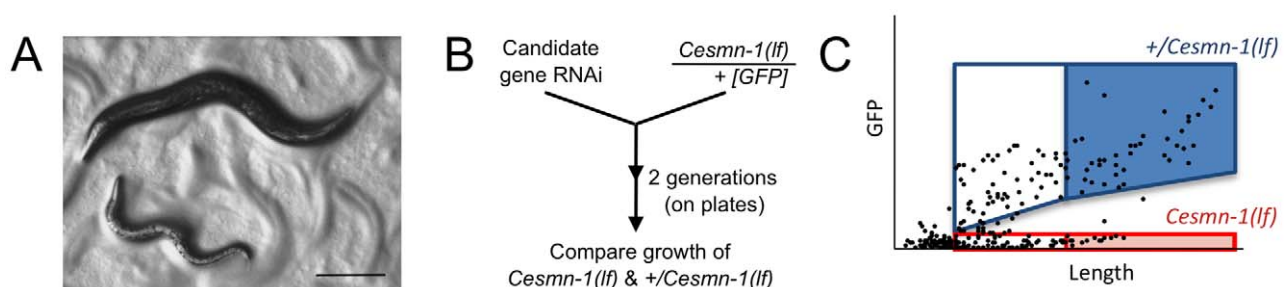


Figure 1. Survival and average length of *Cesmn-1(lf)* animals is decreased. Homozygous loss of *Cesmn-1(lf)* results in slower growth and most animals die during larval stages. Animals that reach the adult stage are short-lived and sterile. A) Image of age-matched heterozygous *hT2(lethal)[myo-2p::GFP]/Cesmn-1(lf)* (*+ / Cesmn-1(lf)*, described in text) and homozygous *Cesmn-1(lf)* 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 $p \leq 0.05$. At least three independent cultures of more than 200 animals were scored for each targeted gene. doi:10.1371/journal.pgen.1001172.g001

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

Ce gene	Dm gene	<i>Cesmn-1(lf)</i>	<i>+Cesmn-1(lf)</i>
		% large $\pm$ SEM	% large $\pm$ SEM
none	none	18 $\pm$ 2	46 $\pm$ 1
<i>Cesmn-1</i>	<i>Smn</i>	6 $\pm$ 4*	31 $\pm$ 1*
<i>plst-1</i>	<i>Fimbrin</i>	15 $\pm$ 3	55 $\pm$ 3*
<i>uso-1</i>	<i>p115</i>	7 $\pm$ 4*	44 $\pm$ 1
<i>nhr-85</i>	<i>Eip75B</i>	8 $\pm$ 5*	44 $\pm$ 1
<i>egl-15</i>	<i>Breathless</i>	9 $\pm$ 5*	45 $\pm$ 1
<i>atf-6</i>	<i>Atf6</i>	11 $\pm$ 7*	40 $\pm$ 4
<i>ape-1</i>	<i>CG18375</i>	11 $\pm$ 9*	50 $\pm$ 2
<i>kcnl-2</i>	<i>SK</i>	12 $\pm$ 1*	42 $\pm$ 1
<i>nekl-3</i>	<i>Nek2</i>	13 $\pm$ 3*	43 $\pm$ 2
<i>atn-1</i>	<i>Actinin</i>	23 $\pm$ 4*	43 $\pm$ 1
<i>cash-1</i>	<i>CG33172</i>	26 $\pm$ 5	64 $\pm$ 11*
<i>dlc-1</i>	<i>cut up</i>	15 $\pm$ 8	66 $\pm$ 3*

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(lf)* homozygous animals (column 3) or *+Cesmn-1(lf)* 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/genotype 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. doi:10.1371/journal.pgen.1001172.t001

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(lf)* 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(lf)*

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

Ce genotype	Ce gene	<i>Cesmn-1(lf)</i>	<i>+Cesmn-1(lf)</i>
	RNAi target	% large $\pm$ SEM	% large $\pm$ SEM
	none	27 $\pm$ 2	44 $\pm$ 2
<i>plst-1(tm4255)</i>	none	39 $\pm$ 5	67 $\pm$ 2*
<i>grk-2(rt97)</i>	none	10 $\pm$ 1*	41 $\pm$ 1
<i>flp-4(yn35)</i>	none	20 $\pm$ 2	51 $\pm$ 2

The growth defects of *+Cesmn-1(lf)* and *Cesmn-1(lf)* animals were examined in the presence of *grk-2(rt97)*, *flp-4(yn35)* 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(lf)* homozygous animals (column 3) or *+Cesmn-1(lf)* heterozygous animals (column 4). At least three independent determinations were undertaken for each genotype. Significance versus control genotypes (*+Cesmn-1(lf)* and *Cesmn-1(lf)*) was determined using Chi-square analysis ($p < 0.05$ indicated with an asterisk). S.E.M. is also reported. doi:10.1371/journal.pgen.1001172.t002

animals, a growth ratio of large to small animals was determined for each clone for *Cesmn-1(lf)* and for *+Cesmn-1(lf)* genotypes. If the RNAi clone growth ratio was more than 2 standard deviations away from the mean for *Cesmn-1(lf)* animals and within 0.7 standard deviations of the mean for *+Cesmn-1(lf)* animals in at least 40% of independent trials, then the corresponding gene was designated as an *Cesmn-1(lf)* 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(lf)* growth defects and did not significantly alter the growth of heterozygous *+Cesmn-1(lf)* 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(lf)* growth defect upon knockdown of *ncbp-2*, *T02G5.3*, *grk-2* and *flp-4*, the average body length of *Cesmn-1(lf)* young animals was determined (Text S1 and Table S4, top panel). Only *ncbp-2(RNAi)* significantly reduced the average body length of *Cesmn-1(lf)* animals suggesting that the enhanced growth defect caused by *ncbp-2(RNAi)* could be attributed to the *Cesmn-1(lf)* 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.

RNAi knockdown of *ncbp-2*, *grk-2*, and *T02G5.3* modified *Cesmn-1(lf)* neuromuscular defects

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(lf)* animals are indistinguishable from control animals, but at later larval stages *Cesmn-1(lf)* pumping rates drop (Figure 3B). *Cesmn-1(lf)* animals have progressive defects in pharyngeal pumping, which occur earlier than reported locomotion defects. At day 2, 62% of *Cesmn-1(lf)* 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(lf)* and *+Cesmn-1(lf)* 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(lf)* pumping rates, but not *+Cesmn-1(lf)* pumping rates (Figure 3C). In addition, *plst-1(lf)* significantly decreased the pumping rates of

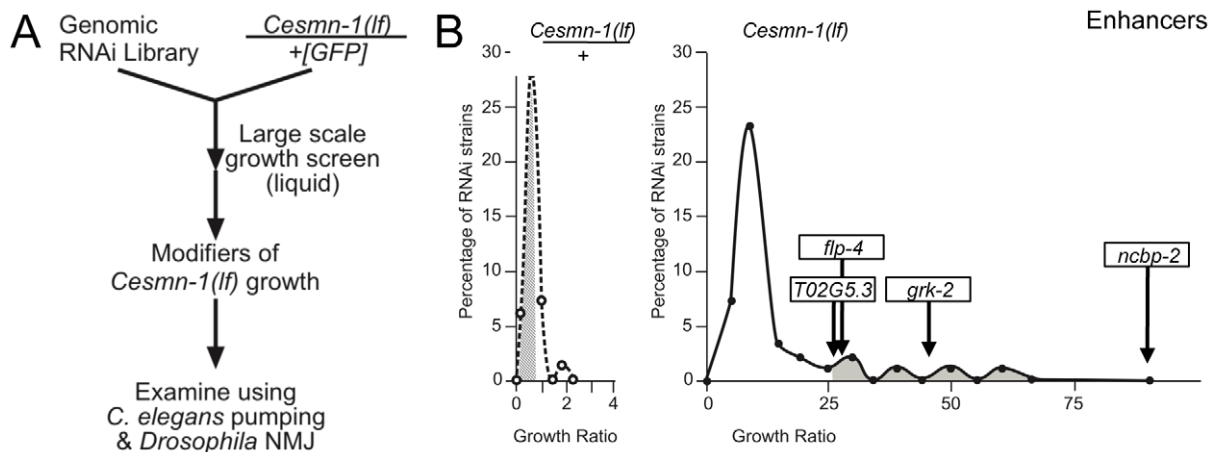


Figure 2. Genome-wide RNAi screen for *Cesmn-1(lf)* modifier genes. A) To identify genes whose knockdown modifies the growth of *Cesmn-1(lf)* animals, the progeny of *hT2(lethal)[myo-2p::GFP]/Cesmn-1(lf) (+/Cesmn-1(lf))*, described in text) were reared for more than 2 generations in 96-well, liquid culture format on bacteria expressing dsRNA corresponding to over 16,500 *C. elegans* genes. Modifier genes identified in this assay were tested subsequently in neuromuscular assays in *C. elegans* and *Drosophila*. B) *Cesmn-1(lf)* and *+/Cesmn-1(lf)* length was measured for each RNAi clone and a 'growth' ratio of large:small animals was determined for each genotype. Representative graphs illustrate the distribution of RNAi clone growth ratios. Candidate enhancer genes were those with a growth ratio more than 2 standard deviations above the mean for *Cesmn-1(lf)* (shaded in right graph) and within 0.7 standard deviations of the mean for *+/Cesmn-1(lf)* (shaded in left graph) for each 96-well plate. No suppressors were identified using similar criteria. Two independent determinations were made for each clone in the original screen. Candidate genes were retested in at least quadruplicate and enhanced growth in at least 40% of trials before designation as growth modifier genes; average growth ratios of enhancers from the *C. elegans* screen are indicated. doi:10.1371/journal.pgen.1001172.g002

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 *grk-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 *grk-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 *grk-2* loss of function allele has been previously described, *grk-2(rt97)* [60]. Loss of *grk-2* significantly enhanced the growth defects of *Cesmn-1(lf)* animals (Table 2). Additionally, the pumping rates of *grk-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) grk-2(lf)* double mutant animals were significantly lower than the pumping rates of either single mutant (Figure 4B). This suggests that *grk-2* loss enhances both *Cesmn-1(lf)* growth and pharyngeal pumping defects. A *grk-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 statistically significant (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: *grk-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

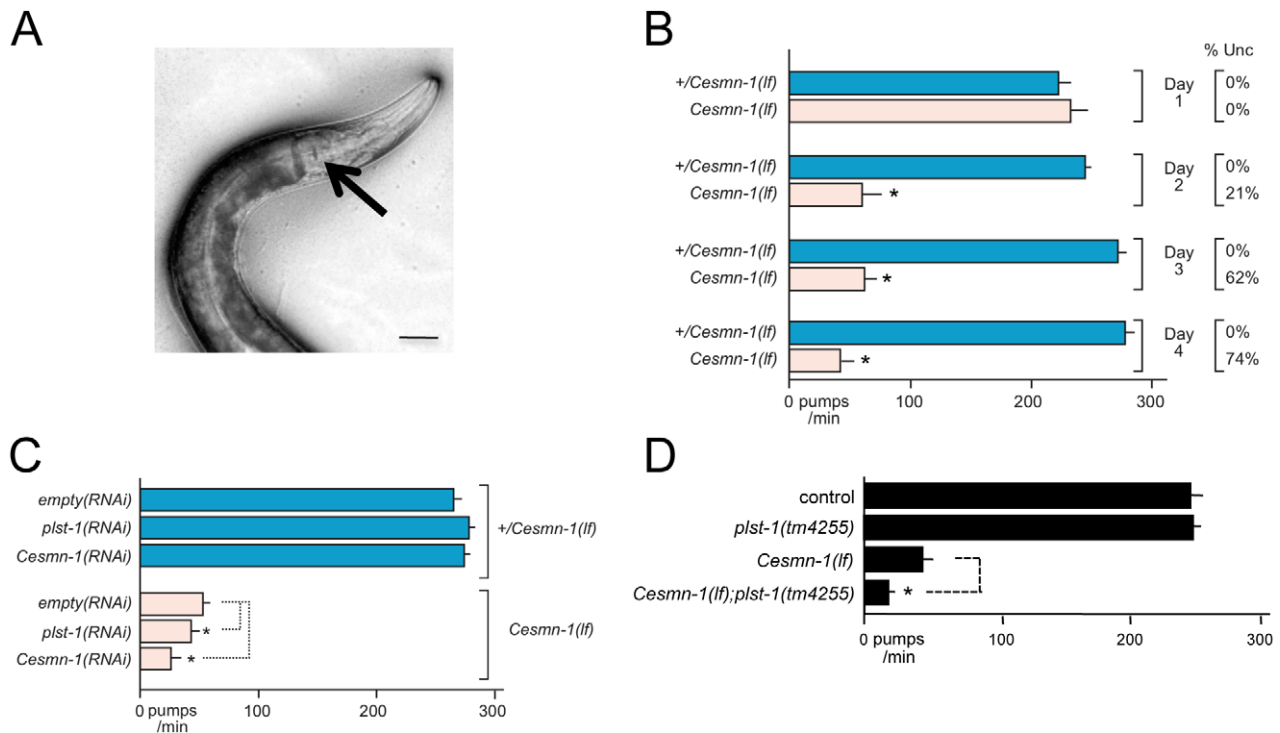


Figure 3. Loss of *C. elegans* PLS3 ortholog enhances the pharyngeal pumping defects of *Cesmn-1(lf)* animals. During feeding the *C. elegans* pharynx contracts at over 200 times per minute to capture and grind bacteria. A) Pharyngeal pumping rates can be determined by videotaping feeding and counting contractions as movement of the grinder (indicated by arrow) at slower playback speeds; scale bar indicates 40 microns. B) The progeny of *hT2(1ethal)[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 histone methylation and gene expression [96], all animals tested herein were the progeny of animals carrying the *hT2* balancer chromosome that prevents recombination. S.E.M. is reported. Significance of $p \leq 0.05$ is indicated with an asterisk and was determined using either an unpaired two-sample *t*-test or a Mann-Whitney *U* two-tailed test according to sample-specific parameters [100]. doi:10.1371/journal.pgen.1001172.g003

function alleles were crossed into the *DmSmn(RNAi)* background and each accelerated death compared to *DmSmn(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 *DmSmn*. RNAi knockdown of *DmSmn* using the ubiquitous tubulin promoter (TubGAL4;*SmnR*-*RNAi*) modestly decreased synaptic innervation in *Drosophila* larvae (reported as bouton numbers per muscle area, Figure 5B). Loss of *Drosophila* Fim function in *Fim^{d02114}* animals also modestly decreased bouton density. We found that effects of *Fim^{d02114}* and *DmSmn* knockdown were synergistic; bouton numbers were significantly

decreased suggesting that loss of Fim function exacerbated *DmSmn* 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. Cbp20 and Fmr were selected as *Drosophila* orthologs of *ncbp-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 *DmSmn* function in +/*Smn^{73Ao}* or +/*Smn⁸⁰¹¹⁰⁹* animals had no significant effect on bouton number as expected, *Smn^{73Ao}/Smn⁸⁰¹¹⁰⁹* animals had dramatically decreased bouton numbers (Figure 6). Loss of one copy of Cbp20 or Fmr modestly decreased synaptic bouton number compared to control animals. However, simultaneous loss of one

A

Ce gene	<i>Cesmn-1(lf)</i> % change	<i>+Cesmn-1(lf)</i> % change	<i>Cesmn-1(lf)</i> <i>empty(RNAi)</i> Pumps/min	<i>Cesmn-1(lf)</i> <i>gene(RNAi)</i> Pumps/min
<i>ncbp-2</i>	55 ± 14*	94 ± 7	30 ± 5	16 ± 4
<i>T02G5.3</i>	156 ± 32*	98 ± 3	30 ± 5	45 ± 8
<i>grk-2</i>	107 ± 29	109 ± 10*	32 ± 8	34 ± 11
<i>flp-4</i>	103 ± 10	110 ± 6*	32 ± 8	32 ± 9

B

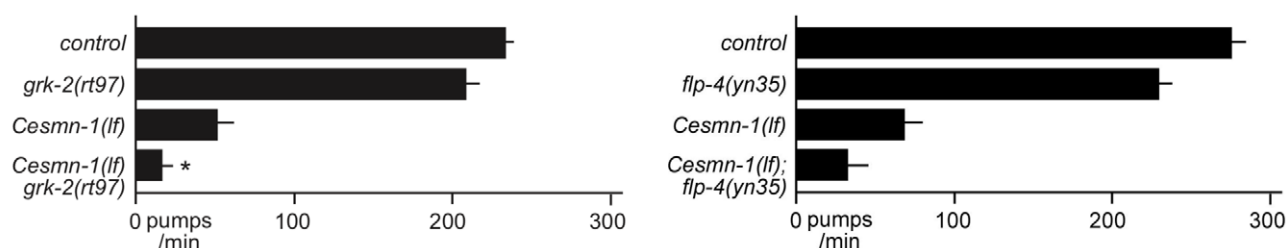


Figure 4. *C. elegans* RNAi screen identifies conserved SMN modifier genes. A) Four genes were identified as modifiers of *Cesmn-1(lf)* growth defects in the *C. elegans* RNAi screen: *ncbp-2*, *T02G5.3*, *grk-2* and *flp-4*. The pharyngeal pumping rates of homozygous mutant *Cesmn-1(lf)* and heterozygous control *+Cesmn-1(lf)* 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(lf)* animals, while *flp-4(yn35)* lowered *Cesmn-1(lf)* 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(lf)* is indicated with an asterisk. To control for genetic background, all animals tested were the progeny of animals carrying the balancer chromosome *hT2[myo-2p::GFP]*; control animals are *hT2/Cesmn-1(lf)* siblings.

doi:10.1371/journal.pgen.1001172.g004

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* orthologs for 32 of these 40 genes using reciprocal BLAST similarity searching (Table S1). The ability of these genes to modify *Cesmn-1(lf)* growth defects was assessed by feeding *Cesmn-1(lf)* and *+Cesmn-1(lf)* animals bacteria expressing the corresponding dsRNA; RNAi feeding clones were constructed for *B0432.13*, *dhs-22* and *ugt-49* [53]. Twelve genes crossed species and modified *Cesmn-1(lf)* defects in one or both *C. elegans* assays.

Orthologs of ten *Drosophila* genes modified *Cesmn-1(lf)* growth defects

Knockdown of seven *C. elegans* genes (*uso-1*, *nhr-85*, *egl-15*, *atf-6*, *ape-1*, *kcnl-2* and *nekl-3*) orthologous to *DmSmn* modifier genes specifically enhanced *Cesmn-1(lf)* growth defects, but did not

significantly alter the percentage of large heterozygous *+Cesmn-1(lf)* animals. Knockdown of the *C. elegans* ortholog *atn-1* significantly suppressed the growth defects of *Cesmn-1(lf)* animals without altering the percentage of large *+Cesmn-1(lf)* 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(lf)* population without altering the *Cesmn-1(lf)* 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(lf)* 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(lf)* 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

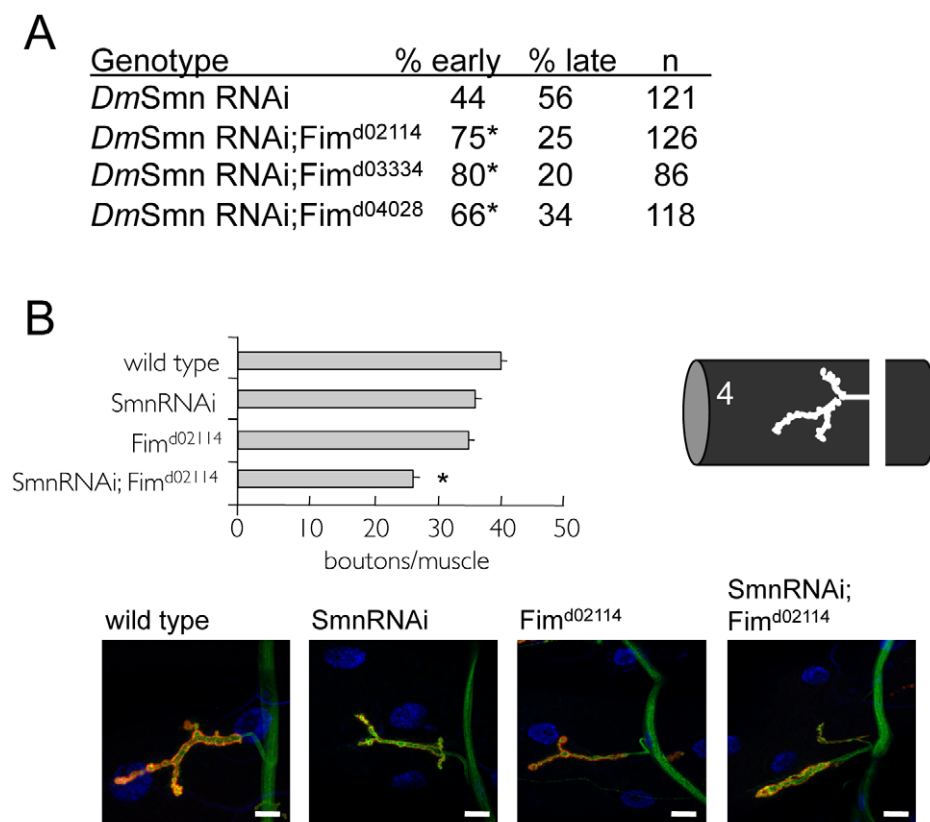


Figure 5. Loss of PLS3 orthologs enhances SMN loss of function defects in invertebrates. Previous studies in vertebrates suggested that PLS3 orthologs in *C. elegans* and *Drosophila* might modify SMN loss of function defects [37]. A) A genetic interaction was found between the *Drosophila* PLS3 ortholog (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 *tubulinGAL4* (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 at early pupae ($p \leq 0.05$ 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. *Fim*^{d02114} enhances *DmSmn* loss of function NMJ defects consistent with studies in vertebrates [37]. All strains carry the ubiquitous TubGAL4 driver. Significance of $p \leq 0.05$ 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.

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 *daf-4* enhanced *Cesmn-1(lf)* pharyngeal pumping defects, while knockdown of *kcnl-2* or *nhr-25* suppressed *Cesmn-1(lf)* pumping defects (Table 3, rows 3 through 5).

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)* pumping 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 (Ultraspacule). 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

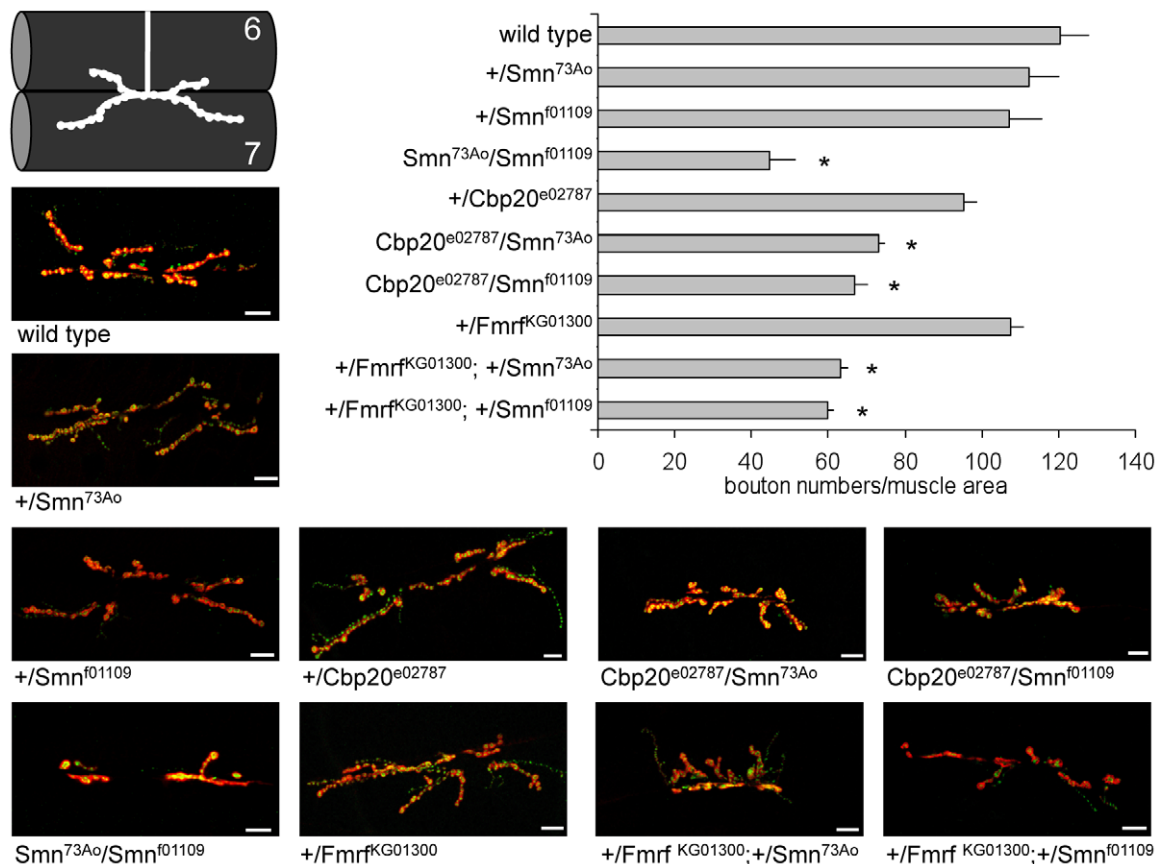


Figure 6. Loss of *Drosophila* Cbp20 or FMRF enhances *DmSmn* loss of function neuromuscular defects. For two *C. elegans* modifier genes, loss of function alleles were available for orthologous *Drosophila* genes: Cbp20 and FMRF [68–70]. Loss of either *Drosophila* gene enhanced the NMJ defects of *DmSmn* 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*^{73Ao} 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 $p \leq 0.05$ by ANOVA as indicated with asterisk. doi:10.1371/journal.pgen.1001172.g006

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 an unrelated pharyngeal pumping defective strain. Loss of *egl-30(ad805)*, which perturbs $G_q\alpha$ function in *C. elegans*, decreases their pharyngeal pumping rates [74]. RNAi knockdown did not significantly alter *egl-30* pharyngeal pumping rates for any modifier gene (Table S3), suggesting that these genes are likely specific modifiers of SMN loss of function defects.

Combined the results described here define eleven conserved genes that modify invertebrate SMN ortholog function in at least one assay in both *C. elegans* and *Drosophila* (summarized in Table 4).

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-

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

		<i>Cesmn-1(lf)</i>	<i>+/Cesmn-1(lf)</i>	<i>Cesmn-1(lf)</i>	<i>Cesmn-1(lf)</i>
		% change vs. control RNAi		<i>empty(RNAi)</i>	<i>candidate(RNAi)</i>
<i>Ce</i> gene	<i>Dm</i> gene	Pumps/min			
<i>Cesmn-1</i>	Smn	56±19*	101±2	53±9	27±6
<i>plst-1</i>	Fim	86±21*	106±1	53±9	43±8
<i>daf-4</i>	Wit	71±27*	93±2	38±7	26±6
<i>kcnl-2</i>	SK	147±50*	90±1	42±7	48±8
<i>nhr-25</i>	Usp	155±45*	91±5	48±8	67±11

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 ($p \leq 0.05$). Two additional genes that modified growth CG33172/*cash-1* and CG18375/*ape-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.

doi:10.1371/journal.pgen.1001172.t003

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 genome-wide *C. elegans* RNAi screen. ‘Growth’ encompasses a variety of factors; slow progression through the larvae stages, reduced growth in the adult stage, longevity, body size, different culture format (liquid versus plates), or a combination of these. Additionally, identification of genetic modifiers for a null allele can be more challenging as compared to identification of genetic modifiers for partial loss of function alleles [82]. No genetic screen can identify all modifier genes pertinent to a pathway and important players can be missed (e.g. miRNAs). Despite this, there is excellent concordance of modifier gene action in *C. elegans* and *Drosophila*. In most cases, genes that enhanced SMN loss of function defects in *Drosophila* also enhanced SMN loss of function defects in *C. elegans* and vice versa. This concordance suggests that the genetic relationships between SMN and these modifier genes are conserved across species. Orthologous genes are likely also important in SMN loss of function pathology in vertebrate species,

as suggested by other invertebrate modifier screens that have identified conserved human disease-related genes and/or functional pathways [83–88].

Thus far, there are only two published human SMA modifier genes: Plastin 3 (PLS3) and SMN2. The role of SMN2 is clear as it provides a modicum of functional SMN protein. However, the role of PLS3 in SMA is controversial and it is unclear how PLS3 levels might modulate severity in SMA patients [37,43]. We find that invertebrate PLS3 orthologs act as modifiers in *C. elegans* and *Drosophila* models. This cross-species interaction of PLS3 and SMN both increases confidence in the invertebrate models and suggests that plastin-associated pathways are important for SMN function at a fundamental level in multiple contexts.

In the bioinformatic analysis presented in Figure 7, we independently identified two cellular pathways that connect multiple modifier genes with SMN: endocytosis and RNA processing/translational 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 Gemin 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

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

Ce gene	Dm gene	Hs gene	Change/Affect Ce	Change/Affect Dm
<i>plst-1</i>	Fim	Plastin 3 (PLS3)	RNAi/Cmpx Gr, Enh Pump	Lof/Enh
<i>daf-4</i>	Wit	TGFβ receptor (BMPRII)	RNAi/Enh Pump	Lof/Enh
<i>kcnl-2</i>	SK	SK channel (KCNN3)	RNAi/Enh Gr, Sup Pump	Lof/Enh
<i>nhr-25</i>	Usp	NHR LRH-1 (NR5A2)	RNAi/Sup Pump	OE/Enh
<i>uso-1</i>	p115	Vesicle docking (USO1)	RNAi/Enh Gr	OE/Sup
<i>nhr-85</i>	Eip75B	NHR RevErb (NR1D2)	RNAi/Enh Gr	Lof/Enh
<i>atf-6</i>	Atf6	Atf6 trans. factor (ATF6)	RNAi/Enh Gr	?/Sup
<i>egl-15</i>	Btl	FGF receptor (FGFR3)	RNAi/Enh Gr	Lof/Enh
<i>ape-1</i>	CG18375	p53 inhibition (PPP1R13)	RNAi/Enh Gr	?/Enh
<i>nekl-3</i>	Nek2	NIMA family kinase (NEK7)	RNAi/Enh Gr	OE/Sup
<i>atn-1</i>	Actinin	a-actinin (ACTN)	RNAi/Sup Gr	OE/Enh
<i>cash-1</i>	CG33172	Striatin (STRN)	RNAi/Cmpx Gr	Lof/Enh
<i>dlc-1</i>	cut up	Dynein light chain (DYNLL2)	RNAi/Cmpx Gr	Lof/Sup
<i>ncbp-2</i>	CBC20	Cap binding (CBP20)	RNAi/Enh Gr & Pump	Lof/Enh
<i>grk-2</i>	Gprk	GRK kinase (ADRBK1)	RNAi/Enh Gr & Pump	n.d.
<i>flp-4</i>	FMRF	Neuropeptide (NPFF)	RNAi/Enh Gr	Lof/Enh
<i>T02G5.3</i>	none	none	RNAi/Enh Gr, Sup Pump	n.d.

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 *Hs*, 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 *C. elegans* assay revealing modifier activity (Gr: growth; Pump: pharyngeal pumping.) RNAi knockdown was sufficient to modify pumping rates for *T02G5.3* and *ncbp-2*; mutant alleles of *grk-2* exacerbated *Cesmn-1(lf)* pumping defects. *flp-4* loss decreased *Cesmn-1(lf)* pumping rates in 4 out of 5 trials, but did not reach significance overall. Discordance between *Drosophila* and *C. elegans* results is indicated with ≠. doi:10.1371/journal.pgen.1001172.t004

studies are warranted to ascertain the interdependence of endocytosis with translational 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[bli-4(e937) let-? (q782)qIs48] (I;III)*) animals are fertile and were maintained using standard techniques [95]. The lack of homologous pairing for the rearranged chromosomes LGI and LGIII 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,

all animals were tested herein were the progeny of *hT2(lethal)[myo-2p::GFP]* 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 *flp-4(yn35)* deletion allele was isolated by PCR-based screening of EMS-mutagenized animals. The *yn35* allele is a 928 base pair deletion that removes exon 3 of *flp-4* gene along with 5' sequences (flanking sequences, ttctgaaaaactttaataa and agctgcgccgagccgagctt) [66]. The *grk-2(rt97)* 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*^{73Ao} and *Smn*¹⁰¹⁰⁹ have been described previously [25]. *Cbp20*^{e02787} 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. *Fmf*^{KG1300} and *Fim* alleles are loss of function alleles (Flybase). The line d03334 may have an unlinked lethal mutation on another chromosome. *Fim*^{d02114} and *Smn*RNAi; *Fim*^{d02114} have Tubulin:Gal4 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 Y104H12BR.1 and Y104H12BL.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, AceView, *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'-acaagctctgcacatcgctg-3', reverse 5'-ttaatgccgcatcctcttg-3'; *dhs-22* forward 5'-tatgtctgtg-cagaagcgaag-3', reverse 5'-ctgcttgatcctgtgtattc-3'; *ugt-49* forward 5'-acgtggatgtagctgaatgg-3', reverse 5'-acgtgaagaacagcaacgaac-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

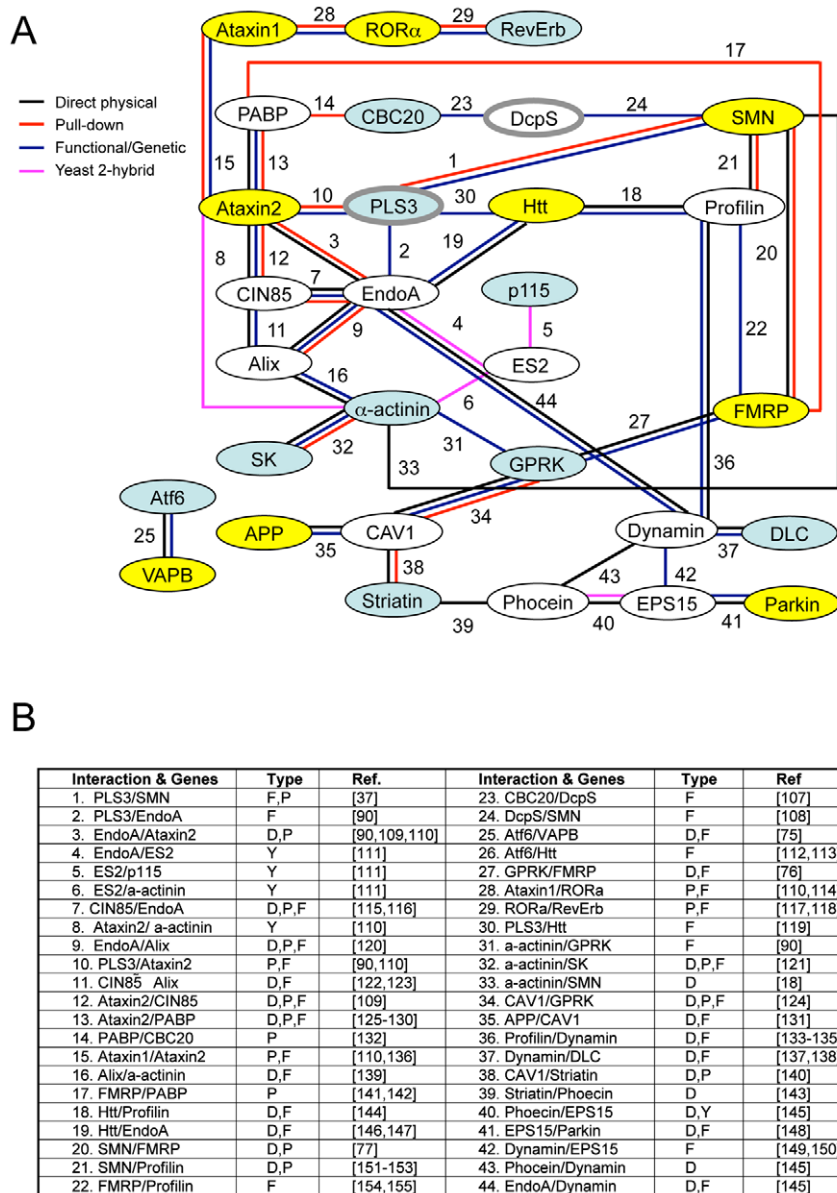


Figure 7. Interaction map of SMN modifier genes. A) Numerous published physical and/or functional interactions were found in the literature that connect many invertebrate SMN modifier genes. The names of vertebrate genes are used in the figure, but interactions are drawn from literature in any animal 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, dynein 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, Dynamin, and Striatin; genes pertinent to RNA processing include PABP, CBC20, DcpS, 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.
doi:10.1371/journal.pgen.1001172.g007

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-1(f)* (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.

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× magnification. Movies were slowed before counting pumping rates. Pharyngeal grinder movements in any axis were scored as a pumping event. Average pumping rates ($\pm$ 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

hT2(bli-4(e937) let-2(q782) qIs48[myo-2p::GFP]) (I;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 Biometrica, 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 NMJ 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 (Cappel). 3rd 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)

Text S1 Methods for supplementary files.

Found at: doi:10.1371/journal.pgen.1001172.s005 (0.02 MB DOC)

Text S2 Supplementary discussion of invertebrate modifier genes.

Found at: doi:10.1371/journal.pgen.1001172.s006 (0.33 MB DOC)

Acknowledgments

We are grateful for advice and/or reagents from the *C. elegans* knockout consortia and numerous members of the *C. elegans* community including Prof. D. B. Sattelle for sharing information on *Cesmn-1(lf)* pumping defects prior to publication. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center.

Author Contributions

Conceived and designed the experiments: MD JNS AW HCC AS GK JH TB MBW JSS CL DVV SAT ACH. Performed the experiments: MD JNS AW HCC AS GK JH TB MBW JSS SAT ACH. Analyzed the data: MD JNS AW HCC AS GK JH TB MBW JSS DVV SAT ACH. Contributed reagents/materials/analysis tools: CL DVV. Wrote the paper: MD JNS AW HCC AS ACH.

References

- Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, et al. (1995) Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* 80: 155–165.
- Boda B, Mas C, Giudicelli C, Nepote V, Guimiot F, et al. (2004) Survival motor neuron *SMN1* and *SMN2* gene promoters: identical sequences and differential expression in neurons and non-neuronal cells. *Eur J Hum Genet* 12: 729–737.
- Monani UR, McPherson JD, Burghes AH (1999) Promoter analysis of the human centromeric and telomeric survival motor neuron genes (*SMNC* and *SMNT*). *Biochim Biophys Acta* 1445: 330–336.
- Pearn J (1980) Classification of spinal muscular atrophies. *Lancet* 1: 919–922.
- Cusin V, Clermont O, Gerard B, Chanterreau D, Elion J (2003) Prevalence of *SMN1* deletion and duplication in carrier and normal populations: implication for genetic counselling. *J Med Genet* 40: e39.
- McAndrew PE, Parsons DW, Simard LR, Rochette C, Ray PN, et al. (1997) Identification of proximal spinal muscular atrophy carriers and patients by analysis of *SMNT* and *SMNC* gene copy number. *Am J Hum Genet* 60: 1411–1422.
- Ogino S, Leonard DG, Rennert H, Ewens WJ, Wilson RB (2002) Genetic risk assessment in carrier testing for spinal muscular atrophy. *Am J Med Genet* 110: 301–307.
- Gubitz AK, Feng W, Dreyfuss G (2004) The SMN complex. *Exp Cell Res* 296: 51–56.
- Ohn T, Kedersha N, Hickman T, Tisdale S, Anderson P (2008) A functional RNAi screen links O-GlcNAc modification of ribosomal proteins to stress granule and processing body assembly. *Nat Cell Biol* 10: 1224–1231.
- Hua Y, Zhou J (2004) Survival motor neuron protein facilitates assembly of stress granules. *FEBS Lett* 572: 69–74.

11. Fan L, Simard LR (2002) Survival motor neuron (SMN) protein: role in neurite outgrowth and neuromuscular maturation during neuronal differentiation and development. *Hum Mol Genet* 11: 1605–1614.
12. Rossoll W, Jablonka S, Andreassi C, Kroning AK, Karle K, et al. (2003) Smn, the spinal muscular atrophy-determining gene product, modulates axon growth and localization of beta-actin mRNA in growth cones of motoneurons. *J Cell Biol* 163: 801–812.
13. Francis JW, Sandrock AW, Bhide PG, Vonsattel JP, Brown RH, Jr. (1998) Heterogeneity of subcellular localization and electrophoretic mobility of survival motor neuron (SMN) protein in mammalian neural cells and tissues. *Proc Natl Acad Sci U S A* 95: 6492–6497.
14. Giavazzi A, Setola V, Simonati A, Battaglia G (2006) Neuronal-specific roles of the survival motor neuron protein: evidence from survival motor neuron expression patterns in the developing human central nervous system. *J Neuropathol Exp Neurol* 65: 267–277.
15. Pagliardini S, Giavazzi A, Setola V, Lizier C, Di Luca M, et al. (2000) Subcellular localization and axonal transport of the survival motor neuron (SMN) protein in the developing rat spinal cord. *Hum Mol Genet* 9: 47–56.
16. Gennarelli M, Lucarelli M, Capon F, Pizzuti A, Merlini L, et al. (1995) Survival motor neuron gene transcript analysis in muscles from spinal muscular atrophy patients. *Biochem Biophys Res Commun* 213: 342–348.
17. Coovert DD, Le TT, McAndrew PE, Strasswimmer J, Crawford TO, et al. (1997) The survival motor neuron protein in spinal muscular atrophy. *Hum Mol Genet* 6: 1205–1214.
18. Rajendra TK, Gonsalvez GB, Walker MP, Shpargel KB, Salz HK, et al. (2007) A *Drosophila melanogaster* model of spinal muscular atrophy reveals a function for SMN in striated muscle. *J Cell Biol* 176: 831–841.
19. Walker MP, Rajendra TK, Saieva L, Fuentes JL, Pellizzoni L, et al. (2008) The SMN complex localizes to the sarcomeric Z-disc and is a proteolytic target of calpain. *Hum Mol Genet*.
20. Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, et al. (2002) miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. *Genes Dev* 16: 720–728.
21. Pellizzoni L, Baccon J, Charroux B, Dreyfuss G (2001) The survival of motor neurons (SMN) protein interacts with the snoRNP proteins fibrillarin and GAR1. *Curr Biol* 11: 1079–1088.
22. Whitehead SE, Jones KW, Zhang X, Cheng X, Terns RM, et al. (2002) Determinants of the interaction of the spinal muscular atrophy disease protein SMN with the dimethylarginine-modified box H/ACA small nucleolar ribonucleoprotein GAR1. *J Biol Chem* 277: 48087–48093.
23. Cifuentes-Diaz C, Frugier T, Tiziano FD, Lacene E, Roblot N, et al. (2001) Deletion of murine SMN exon 7 directed to skeletal muscle leads to severe muscular dystrophy. *J Cell Biol* 152: 1107–1114.
24. Shafey D, Cote PD, Kothary R (2005) Hypomorphic Smn knockdown C2C12 myoblasts reveal intrinsic defects in myoblast fusion and myotube morphology. *Exp Cell Res* 311: 49–61.
25. Chan YB, Miguel-Alia I, Franks C, Thomas N, Trulzsch B, et al. (2003) Neuromuscular defects in a *Drosophila* survival motor neuron gene mutant. *Hum Mol Genet* 12: 1367–1376.
26. Chang HC, Dimlich DN, Yokokura T, Mukherjee A, Kankel MW, et al. (2008) Modeling spinal muscular atrophy in *Drosophila*. *PLoS ONE* 3: e3209. doi:10.1371/journal.pone.0003209.
27. McWhorter ML, Monani UR, Burghes AH, Beattie CE (2003) Knockdown of the survival motor neuron (Smn) protein in zebrafish causes defects in motor axon outgrowth and pathfinding. *J Cell Biol* 162: 919–931.
28. Kariya S, Park GH, Maeno-Hikichi Y, Leykekhman O, Lutz C, et al. (2008) Reduced SMN protein impairs maturation of the neuromuscular junctions in mouse models of spinal muscular atrophy. *Hum Mol Genet* 17: 2552–2569.
29. Lorson CL, Androphy EJ (2000) An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene SMN. *Hum Mol Genet* 9: 259–265.
30. Monani UR, Coovert DD, Burghes AH (2000) Animal models of spinal muscular atrophy. *Hum Mol Genet* 9: 2451–2457.
31. Monani UR, Lorson CL, Parsons DW, Prior TW, Androphy EJ, et al. (1999) A single nucleotide difference that alters splicing patterns distinguishes the SMA gene *SMN1* from the copy gene *SMN2*. *Hum Mol Genet* 8: 1177–1183.
32. Lefebvre S, Burlet P, Liu Q, Bertrand S, Clermont O, et al. (1997) Correlation between severity and SMN protein level in spinal muscular atrophy. *Nat Genet* 16: 265–269.
33. Feldkotter M, Schwarzer V, Wirth R, Wienker TF, Wirth B (2002) Quantitative analyses of *SMN1* and *SMN2* based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. *Am J Hum Genet* 70: 358–368.
34. Wirth B, Herz M, Wetter A, Moskau S, Hahnen E, et al. (1999) Quantitative analysis of survival motor neuron copies: identification of subtle *SMN1* mutations in patients with spinal muscular atrophy, genotype-phenotype correlation, and implications for genetic counseling. *Am J Hum Genet* 64: 1340–1356.
35. Lefebvre S, Burglen L, Frezal J, Munnich A, Melki J (1998) The role of the SMN gene in proximal spinal muscular atrophy. *Hum Mol Genet* 7: 1531–1536.
36. Burghes AH (1997) When is a deletion not a deletion? When it is converted. *Am J Hum Genet* 61: 9–15.
37. Oprea GE, Krober S, McWhorter ML, Rossoll W, Muller S, et al. (2008) Plastin 3 is a protective modifier of autosomal recessive spinal muscular atrophy. *Science* 320: 524–527.
38. Bretscher A (1981) Fimbrin is a cytoskeletal protein that crosslinks F-actin in vitro. *Proc Natl Acad Sci U S A* 78: 6849–6853.
39. Bretscher A, Weber K (1980) Fimbrin, a new microfilament-associated protein present in microvilli and other cell surface structures. *J Cell Biol* 86: 335–340.
40. Glenney JR, Jr., Kaulfus P, Matsudaira P, Weber K (1981) F-actin binding and bundling properties of fimbrin, a major cytoskeletal protein of microvillus core filaments. *J Biol Chem* 256: 9283–9288.
41. Adams AE, Botstein D, Drubin DG (1989) A yeast actin-binding protein is encoded by SAC6, a gene found by suppression of an actin mutation. *Science* 243: 231–233.
42. Kubler E, Riezman H (1993) Actin and fimbrin are required for the internalization step of endocytosis in yeast. *EMBO J* 12: 2855–2862.
43. Bowerman M, Anderson CL, Beauvais A, Boyl PP, Witke W, et al. (2009) SMN, profilin 1a and plastin 3: a link between the deregulation of actin dynamics and SMA pathogenesis. *Mol Cell Neurosci* 42: 66–74.
44. Briese M, Esmacili B, Fraboulet S, Burt EC, Christodoulou S, et al. (2009) Deletion of *smn-1*, the *Caenorhabditis elegans* ortholog of the spinal muscular atrophy gene, results in locomotor dysfunction and reduced lifespan. *Hum Mol Genet* 18: 97–104.
45. Miguel-Alia I, Culetto E, Walker DS, Baylis HA, Sattelle DB, et al. (1999) The *Caenorhabditis elegans* orthologue of the human gene responsible for spinal muscular atrophy is a maternal product critical for germline maturation and embryonic viability. *Hum Mol Genet* 8: 2133–2143.
46. McKim KS, Peters K, Rose AM (1993) Two types of sites required for meiotic chromosome pairing in *Caenorhabditis elegans*. *Genetics* 134: 749–768.
47. Timmons L, Fire A (1998) Specific interference by ingested dsRNA. *Nature* 395: 854.
48. Kamath RS, Martinez-Campos M, Zipperlen P, Fraser AG, Ahringer J (2001) Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biol* 2: RESEARCH0002.
49. Timmons L, Court DL, Fire A (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* 263: 103–112.
50. Rual JF, Ceron J, Koreth J, Hao T, Nicot AS, et al. (2004) Toward improving *Caenorhabditis elegans* phenome mapping with an ORFeome-based RNAi library. *Genome Res* 14: 2162–2168.
51. Pulak R (2006) Techniques for analysis, sorting, and dispensing of *C. elegans* on the COPAS flow-sorting system. *Methods Mol Biol* 351: 275–286.
52. Huang LS, Sternberg PW (2006) Genetic dissection of developmental pathways. *WormBook*. pp 1–19.
53. Kamath RS, Ahringer J (2003) Genome-wide RNAi screening in *Caenorhabditis elegans*. *Methods* 30: 313–321.
54. Lall S, Piano F, Davis RE (2005) *Caenorhabditis elegans* decapping proteins: localization and functional analysis of Dcp1, Dcp2, and DcpS during embryogenesis. *Mol Biol Cell* 16: 5880–5890.
55. Avery L (1993) The genetics of feeding in *Caenorhabditis elegans*. *Genetics* 133: 897–917.
56. Sulston JE, Horvitz HR (1977) Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev Biol* 56: 110–156.
57. Sulston JE, Schierenberg E, White JG, Thomson JN (1983) The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev Biol* 100: 64–119.
58. Simmer F, Moorman C, van der Linden AM, Kuijk E, van den Bergh PV, et al. (2003) Genome-wide RNAi of *C. elegans* using the hypersensitive *rif-3* strain reveals novel gene functions. *PLoS Biol* 1: e12. doi:10.1371/journal.pbio.0000012.
59. Kennedy S, Wang D, Ruvkun G (2004) A conserved siRNA-degrading RNase negatively regulates RNA interference in *C. elegans*. *Nature* 427: 645–649.
60. Fukuto HS, Ferkey DM, Apicella AJ, Lans H, Sharmeen T, et al. (2004) G protein-coupled receptor kinase function is essential for chemosensation in *C. elegans*. *Neuron* 42: 581–593.
61. Barrett PL, Fleming JT, Gobel V (2004) Targeted gene alteration in *Caenorhabditis elegans* by gene conversion. *Nat Genet* 36: 1231–1237.
62. Gengyo-Ando K, Mitani S (2000) Characterization of mutations induced by ethyl methanesulfonate, UV, and trimethylpsoralen in the nematode *Caenorhabditis elegans*. *Biochem Biophys Res Commun* 269: 64–69.
63. Gilchrist EJ, O'Neil NJ, Rose AM, Zetka MC, Haughn GW (2006) TILLING is an effective reverse genetics technique for *Caenorhabditis elegans*. *BMC Genomics* 7: 262.
64. Jansen G, Hazendonk E, Thijssen KL, Plasterk RH (1997) Reverse genetics by chemical mutagenesis in *Caenorhabditis elegans*. *Nat Genet* 17: 119–121.
65. Lesa GM (2006) Isolation of *Caenorhabditis elegans* gene knockouts by PCR screening of chemically mutagenized libraries. *Nat Protoc* 1: 2231–2240.
66. Liu T, Kim K, Li C, Barr MM (2007) FMRFamide-like neuropeptides and mechanosensory touch receptor neurons regulate male sexual turning behavior in *Caenorhabditis elegans*. *J Neurosci* 27: 7174–7182.
67. Li C, Kim K (2008) Neuropeptides. *WormBook*. pp 1–36.
68. Mount SM, Salz HK (2000) Pre-messenger RNA processing factors in the *Drosophila* genome. *J Cell Biol* 150: F37–44.
69. Lasko P (2000) The *Drosophila melanogaster* genome: translation factors and RNA binding proteins. *J Cell Biol* 150: F51–56.

70. Nambu JR, Murphy-Erdosh C, Andrews PC, Feistner GJ, Scheller RH (1988) Isolation and characterization of a *Drosophila* neuropeptide gene. *Neuron* 1: 55–61.
71. Roulier EM, Fyrberg C, Fyrberg E (1992) Perturbations of *Drosophila* alpha-actinin cause muscle paralysis, weakness, and atrophy but do not confer obvious nonmuscle phenotypes. *J Cell Biol* 116: 911–922.
72. Fyrberg E, Kelly M, Ball E, Fyrberg C, Reedy MC (1990) Molecular genetics of *Drosophila* alpha-actinin: mutant alleles disrupt Z disc integrity and muscle insertions. *J Cell Biol* 110: 1999–2011.
73. Inoue T, Thomas JH (2000) Targets of TGF-beta signaling in *Caenorhabditis elegans* dauer formation. *Dev Biol* 217: 192–204.
74. Brundage L, Avery L, Katz A, Kim UJ, Mendel JE, et al. (1996) Mutations in a *C. elegans* Gqalpha gene disrupt movement, egg laying, and viability. *Neuron* 16: 999–1009.
75. Gkogkas C, Middleton S, Kremer AM, Wardrope C, Hannah M, et al. (2008) VAPB interacts with and modulates the activity of ATF6. *Hum Mol Genet* 17: 1517–1526.
76. Wang H, Wu LJ, Kim SS, Lee FJ, Gong B, et al. (2008) FMRP acts as a key messenger for dopamine modulation in the forebrain. *Neuron* 59: 634–647.
77. Piazzon N, Rage F, Schlotter F, Moine H, Brantant C, et al. (2008) *In vitro* and *in cellulo* evidences for association of the survival of motor neuron complex with the fragile X mental retardation protein. *J Biol Chem* 283: 5598–5610.
78. Bauer PO, Nukina N (2009) The pathogenic mechanisms of polyglutamine diseases and current therapeutic strategies. *J Neurochem* 110: 1737–1765.
79. Crawford TO, Pardo CA (1996) The neurobiology of childhood spinal muscular atrophy. *Neurobiol Dis* 3: 97–110.
80. Goedert M, Spillantini MG (2006) A century of Alzheimer's disease. *Science* 314: 777–781.
81. Lang AE, Lozano AM (1998) Parkinson's disease. First of two parts. *N Engl J Med* 339: 1044–1053.
82. Jorgensen EM, Mango SE (2002) The art and design of genetic screens: *Caenorhabditis elegans*. *Nat Rev Genet* 3: 356–369.
83. Dimitriadis M, Hart AC (2010) Neurodegenerative disorders: Insights from the nematode *Caenorhabditis elegans*. *Neurobiol Dis*.
84. Fiuza UM, Arias AM (2007) Cell and molecular biology of Notch. *J Endocrinol* 194: 459–474.
85. Kim SK (2007) Common aging pathways in worms, flies, mice and humans. *J Exp Biol* 120: 1607–1612.
86. Leicht DT, Balan V, Kaplun A, Singh-Gupta V, Kaplun L, et al. (2007) Raf kinases: function, regulation and role in human cancer. *Biochim Biophys Acta* 1773: 1196–1212.
87. Schlegel A, Stainier DY (2007) Lessons from “lower” organisms: what worms, flies, and zebrafish can teach us about human energy metabolism. *PLoS Genet* 3: e199. doi:10.1371/journal.pgen.0030199.
88. Silverman GA, Luke CJ, Bhatia SR, Long OS, Vetica AC, et al. (2009) Modeling molecular and cellular aspects of human disease using the nematode *Caenorhabditis elegans*. *Pediatr Res* 65: 10–18.
89. Kaksonen M, Toret CP, Drubin DG (2005) A modular design for the clathrin- and actin-mediated endocytosis machinery. *Cell* 123: 305–320.
90. Ralser M, Nonhoff U, Albrecht M, Lengauer T, Wanker EE, et al. (2005) Ataxin-2 and huntingtin interact with endophilin-A complexes to function in plastin-associated pathways. *Hum Mol Genet* 14: 2893–2909.
91. Singer-Kruger B, Nemoto Y, Daniell L, Ferro-Novick S, De Camilli P (1998) Synaptotagmin family members are implicated in endocytic membrane traffic in yeast. *J Cell Sci* 111 (Pt 22): 3347–3356.
92. Gibbins DJ, Ciaudo C, Erhardt M, Voinnet O (2009) Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. *Nat Cell Biol* 11: 1143–1149.
93. Lee YS, Pressman S, Andress AP, Kim K, White JL, et al. (2009) Silencing by small RNAs is linked to endosomal trafficking. *Nat Cell Biol* 11: 1150–1156.
94. Kong L, Wang X, Choe DW, Polley M, Burnett BG, et al. (2009) Impaired synaptic vesicle release and immaturity of neuromuscular junctions in spinal muscular atrophy mice. *J Neurosci* 29: 842–851.
95. Brenner S (1974) The genetics of *Caenorhabditis elegans*. *Genetics* 77: 71–94.
96. Bean CJ, Schaner CE, Kelly WG (2004) Meiotic pairing and imprinted X chromatin assembly in *Caenorhabditis elegans*. *Nat Genet* 36: 100–105.
97. Bilinski SM, Jaglarz MK, Szymanska B, Etkin LD, Kloc M (2004) Sm proteins, the constituents of the spliceosome, are components of nuage and mitochondrial cement in *Xenopus* oocytes. *Exp Cell Res* 299: 171–178.
98. Dostie J, Mourtelas Z, Yang M, Sharma A, Dreyfuss G (2003) Numerous microRNPs in neuronal cells containing novel microRNAs. *RNA* 9: 180–186.
99. Hillier LW, Reinke V, Green P, Hirst M, Marra MA, et al. (2009) Massively parallel sequencing of the polyadenylated transcriptome of *C. elegans*. *Genome Res* 19: 657–666.
100. Townend J (2002) Practical statistics for environmental and biological scientists. Chichester, UK: John Wiley and Sons Ltd.
101. Gunawardena S, Goldstein LS (2001) Disruption of axonal transport and neuronal viability by amyloid precursor protein mutations in *Drosophila*. *Neuron* 32: 389–401.
102. Cao YJ, Dreixler JC, Couey JJ, Houamed KM (2002) Modulation of recombinant and native neuronal SK channels by the neuroprotective drug riluzole. *Eur J Pharmacol* 449: 47–54.
103. Bensimon G, Lacomblez L, Meininger V (1994) A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. *N Engl J Med* 330: 585–591.
104. Couratier P, Sindou P, Esclaire F, Louvel E, Hugon J (1994) Neuroprotective effects of riluzole in ALS CSF toxicity. *Neuroreport* 5: 1012–1014.
105. Haddad H, Cifuentes-Diaz C, Miroglia A, Roblot N, Joshi V, et al. (2003) Riluzole attenuates spinal muscular atrophy disease progression in a mouse model. *Muscle Nerve* 28: 432–437.
106. Nishimura AL, Mitne-Neto M, Silva HC, Richieri-Costa A, Middleton S, et al. (2004) A mutation in the vesicle-trafficking protein VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis. *Am J Hum Genet* 75: 822–831.
107. Shen V, Liu H, Liu SW, Jiao X, Kiledjian M (2008) DcpS scavenger decapping enzyme can modulate pre-mRNA splicing. *RNA* 14: 1132–1142.
108. Singh J, Salcius M, Liu SW, Staker BL, Mishra R, et al. (2008) DcpS as a therapeutic target for spinal muscular atrophy. *ACS Chem Biol* 3: 711–722.
109. Nonis D, Schmidt MH, van de Loo S, Eich F, Dikic I, et al. (2008) Ataxin-2 associates with the endocytosis complex and affects EGF receptor trafficking. *Cell Signal* 20: 1725–1739.
110. Lim J, Hao T, Shaw C, Patel AJ, Szabo G, et al. (2006) A protein-protein interaction network for human inherited ataxias and disorders of Purkinje cell degeneration. *Cell* 125: 801–814.
111. Li S, Armstrong CM, Bertin N, Ge H, Milstein S, et al. (2004) A map of the interactome network of the metazoan *C. elegans*. *Science* 303: 540–543.
112. Jeong H, Then F, Melia TJ, Jr., Mazzulli JR, Cui L, et al. (2009) Acetylation targets mutant huntingtin to autophagosomes for degradation. *Cell* 137: 60–72.
113. Jia K, Hart AC, Levine B (2007) Autophagy genes protect against disease caused by polyglutamine expansion proteins in *Caenorhabditis elegans*. *Autophagy* 3: 21–25.
114. Serra HG, Duvick L, Zu T, Carlson K, Stevens S, et al. (2006) RORalpha-mediated Purkinje cell development determines disease severity in adult SCA1 mice. *Cell* 127: 697–708.
115. Petrelli A, Gilestro GF, Lanzardo S, Comoglio PM, Migone N, et al. (2002) The endophilin-CIN85-Cbl complex mediates ligand-dependent downregulation of c-Met. *Nature* 416: 187–190.
116. Kaneko T, Maeda A, Takefuji M, Aoyama H, Nakayama M, et al. (2005) Rho mediates endocytosis of epidermal growth factor receptor through phosphorylation of endophilin A1 by Rho-kinase. *Genes Cells* 10: 973–987.
117. Guillaumond F, Dardente H, Giguere V, Cermakian N (2005) Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. *J Biol Rhythms* 20: 391–403.
118. Forman BM, Chen J, Blumberg B, Klierer SA, Henshaw R, et al. (1994) Cross-talk among ROR alpha 1 and the Rev-erb family of orphan nuclear receptors. *Mol Endocrinol* 8: 1253–1261.
119. Freeman JL, Pitcher JA, Li X, Bennett V, Lefkowitz RJ (2000) alpha-Actinin is a potent regulator of G protein-coupled receptor kinase activity and substrate specificity *in vitro*. *FEBS Lett* 473: 280–284.
120. Chatellard-Cause C, Blot B, Cristina N, Torch S, Missotten M, et al. (2002) Alix (ALG-2-interacting protein X), a protein involved in apoptosis, binds to endophilins and induces cytoplasmic vacuolization. *J Biol Chem* 277: 29108–29115.
121. Lu L, Zhang Q, Timofeyev V, Zhang Z, Young JN, et al. (2007) Molecular coupling of a Ca2+-activated K+ channel to L-type Ca2+ channels via alpha-actinin2. *Circ Res* 100: 112–120.
122. Chen B, Borinstein SC, Gillis J, Sykes VW, Bogler O (2000) The glioma-associated protein SETA interacts with AIP1/Alix and ALG-2 and modulates apoptosis in astrocytes. *J Biol Chem* 275: 19275–19281.
123. Schmidt MH, Hoeller D, Yu J, Furnari FB, Cavenee WK, et al. (2004) Alix/AIP1 antagonizes epidermal growth factor receptor downregulation by the Cbl-SETA/CIN85 complex. *Mol Cell Biol* 24: 8981–8993.
124. Carman CV, Lisanti MP, Benovic JL (1999) Regulation of G protein-coupled receptor kinases by caveolin. *J Biol Chem* 274: 8858–8864.
125. Ralser M, Albrecht M, Nonhoff U, Lengauer T, Lechtrach H, et al. (2005) An integrative approach to gain insights into the cellular function of human ataxin-2. *J Mol Biol* 346: 203–214.
126. Lessing D, Bonini NM (2008) Polyglutamine genes interact to modulate the severity and progression of neurodegeneration in *Drosophila*. *PLoS Biol* 6: e29. doi:10.1371/journal.pbio.0060029.
127. Ciosk R, DePalma M, Priess JR (2004) ATX-2, the *C. elegans* ortholog of ataxin 2, functions in translational regulation in the germline. *Development* 131: 4831–4841.
128. Satterfield TF, Pallanck LJ (2006) Ataxin-2 and its *Drosophila* homolog, ATX2, physically assemble with polyribosomes. *Hum Mol Genet* 15: 2523–2532.
129. Nonhoff U, Ralser M, Welzel F, Piccini I, Balzeret D, et al. (2007) Ataxin-2 interacts with the DEAD/H-box RNA helicase DDX6 and interferes with P-bodies and stress granules. *Mol Biol Cell* 18: 1385–1396.
130. Kozlov G, Trempe JF, Khaleghpour K, Kahvejian A, Ekiel I, et al. (2001) Structure and function of the C-terminal PABC domain of human poly(A)-binding protein. *Proc Natl Acad Sci U S A* 98: 4409–4413.
131. Ikezu T, Trapp BD, Song KS, Schlegel A, Lisanti MP, et al. (1998) Caveolae, plasma membrane microdomains for alpha-secretase-mediated processing of the amyloid precursor protein. *J Biol Chem* 273: 10485–10495.

132. Massenet S, Pellizzoni L, Paushkin S, Mattaj JW, Dreyfuss G (2002) The SMN complex is associated with snRNPs throughout their cytoplasmic assembly pathway. *Mol Cell Biol* 22: 6533–6541.
133. Gareus R, Di Nardo A, Rybin V, Witke W (2006) Mouse profilin 2 regulates endocytosis and competes with SH3 ligand binding to dynamin 1. *J Biol Chem* 281: 2803–2811.
134. Schafer DA, Weed SA, Binns D, Karginov AV, Parsons JT, et al. (2002) Dynamin2 and cortactin regulate actin assembly and filament organization. *Curr Biol* 12: 1852–1857.
135. Witke W, Podtelejnikov AV, Di Nardo A, Sutherland JD, Gurniak CB, et al. (1998) In mouse brain profilin I and profilin II associate with regulators of the endocytic pathway and actin assembly. *EMBO J* 17: 967–976.
136. Al-Ramahi I, Perez AM, Lim J, Zhang M, Sorensen R, et al. (2007) dAtaxin-2 mediates expanded Ataxin-1-induced neurodegeneration in a *Drosophila* model of SCA1. *PLoS Genet* 3: e234. doi:10.1371/journal.pgen.0030234.
137. Ghosh-Roy A, Desai BS, Ray K (2005) Dynein light chain 1 regulates dynamin-mediated F-actin assembly during sperm individualization in *Drosophila*. *Mol Biol Cell* 16: 3107–3116.
138. Navarro-Lerida I, Martinez Moreno M, Roncal F, Gavilanes F, Albar JP, et al. (2004) Proteomic identification of brain proteins that interact with dynein light chain LC8. *Proteomics* 4: 339–346.
139. Pan S, Wang R, Zhou X, He G, Koomen J, et al. (2006) Involvement of the conserved adaptor protein Alix in actin cytoskeleton assembly. *J Biol Chem* 281: 34640–34650.
140. Gaillard S, Bartoli M, Castets F, Monneron A (2001) Striatin, a calmodulin-dependent scaffolding protein, directly binds caveolin-1. *FEBS Lett* 508: 49–52.
141. Khandjian EW, Huot ME, Tremblay S, Davidovic L, Mazroui R, et al. (2004) Biochemical evidence for the association of fragile X mental retardation protein with brain polyribosomal ribonucleoproteins. *Proc Natl Acad Sci U S A* 101: 13357–13362.
142. Gagne JP, Bonicalzi ME, Gagne P, Ouellet ME, Hendzel MJ, et al. (2005) Poly(ADP-ribose) glycohydrolase is a component of the FMRP-associated messenger ribonucleoproteins. *Biochem J* 392: 499–509.
143. Castets F, Bartoli M, Barnier JV, Baillat G, Salin P, et al. (1996) A novel calmodulin-binding protein, belonging to the WD-repeat family, is localized in dendrites of a subset of CNS neurons. *J Cell Biol* 134: 1051–1062.
144. Shao J, Welch WJ, Diprospero NA, Diamond MI (2008) Phosphorylation of profilin by ROCK1 regulates polyglutamine aggregation. *Mol Cell Biol* 28: 5196–5208.
145. Baillat G, Gaillard S, Castets F, Monneron A (2002) Interactions of phocoin with nucleoside-diphosphate kinase, Eps15, and Dynamin I. *J Biol Chem* 277: 18961–18966.
146. Sittler A, Walter S, Wedemeyer N, Hasenbank R, Scherzinger E, et al. (1998) SH3GL3 associates with the Huntingtin exon 1 protein and promotes the formation of polyglutamine-containing protein aggregates. *Mol Cell* 2: 427–436.
147. Qin ZH, Wang Y, Sapp E, Cuiffo B, Wanker E, et al. (2004) Huntingtin bodies sequester vesicle-associated proteins by a polyproline-dependent interaction. *J Neurosci* 24: 269–281.
148. Fallon L, Belanger CM, Corera AT, Kontogiannina M, Regan-Klapisz E, et al. (2006) A regulated interaction with the UIM protein Eps15 implicates parkin in EGF receptor trafficking and PI(3)K-Akt signalling. *Nat Cell Biol* 8: 834–842.
149. Salcini AE, Hilliard MA, Croce A, Arbucci S, Luzzi P, et al. (2001) The Eps15 *C. elegans* homologue EHS-1 is implicated in synaptic vesicle recycling. *Nat Cell Biol* 3: 755–760.
150. Krishnan KS, Rikhy R, Rao S, Shivalkar M, Mosko M, et al. (2001) Nucleoside diphosphate kinase, a source of GTP, is required for dynamin-dependent synaptic vesicle recycling. *Neuron* 30: 197–210.
151. Sharma A, Lambrechts A, Hao le T, Le TT, Sewry CA, et al. (2005) A role for complexes of survival of motor neurons (SMN) protein with gemins and profilin in neurite-like cytoplasmic extensions of cultured nerve cells. *Exp Cell Res* 309: 185–197.
152. Giesemann T, Rathke-Hartlieb S, Rothkegel M, Bartsch JW, Buchmeier S, et al. (1999) A role for polyproline motifs in the spinal muscular atrophy protein SMN. Profilins bind to and colocalize with smn in nuclear gems. *J Biol Chem* 274: 37908–37914.
153. Bowerman M, Shafey D, Kothary R (2007) Smn depletion alters profilin II expression and leads to upregulation of the RhoA/ROCK pathway and defects in neuronal integrity. *J Mol Neurosci* 32: 120–131.
154. Reeve SP, Bassetto L, Genova GK, Kleyner Y, Leyssen M, et al. (2005) The *Drosophila* fragile X mental retardation protein controls actin dynamics by directly regulating profilin in the brain. *Curr Biol* 15: 1156–1163.
155. Tessier CR, Broadie K (2008) *Drosophila* fragile X mental retardation protein developmentally regulates activity-dependent axon pruning. *Development* 135: 1547–1557.

C. elegans Notch Signaling Regulates Adult Chemosensory Response and Larval Molting Quiescence

Komudi Singh,^{1,8} Michael Y. Chao,^{2,8} Gerard A. Somers,³ Hidetoshi Komatsu,³ Mark E. Corkins,¹ Jonah Larkins-Ford,³ Tim Tucey,³ Heather M. Dionne,³ Melissa B. Walsh,⁴ Emma K. Beaumont,^{1,5} Douglas P. Hart,⁶ Shawn R. Lockery,⁷ and Anne C. Hart^{1,*}

¹Department of Neuroscience, Brown University, 185 Meeting Street Box GL-N, Providence, RI 02912, USA

²Department of Biology, California State University, San Bernardino, 5500 University Parkway, San Bernardino, CA 92407, USA

³Center for Cancer Research, Massachusetts General Hospital and Department of Pathology, Harvard Medical School, Charlestown, MA 02129, USA

⁴Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, 185 Meeting Street Box G-L, Providence, RI 02912, USA

⁵Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK

⁶Department of Mechanical Engineering, Massachusetts Institute of Technology, Building 3-246, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

⁷Institute of Neuroscience, University of Oregon, 1254 University of Oregon, Eugene, OR 97403, USA

Summary

Background: The conserved DOS-motif proteins OSM-7 and OSM-11 function as coligands with canonical DSL (Delta, Serrate, and LAG-2) ligands to activate *C. elegans* Notch receptors during development. We report here that Notch ligands, coligands, and the receptors LIN-12 and GLP-1 regulate two *C. elegans* behaviors: chemosensory avoidance of octanol and quiescence during molting lethargus.

Results: *C. elegans* lacking *osm-7* or *osm-11* are defective in their response to octanol. We find that OSM-11 is secreted from hypodermal seam cells into the pseudocoelomic body cavity and acts non-cell autonomously as a diffusible factor. OSM-11 acts with the DSL ligand LAG-2 to activate LIN-12 and GLP-1 Notch receptors in the neurons of adult animals, thereby regulating octanol avoidance response. In adult animals, overexpression of *osm-11* and consequent Notch receptor activation induces anachronistic sleep-like quiescence. Perturbation of Notch signaling alters basal activity in adults as well as arousal thresholds and quiescence during molting lethargus. Genetic epistasis studies reveal that Notch signaling regulates quiescence via previously identified circuits and genetic pathways including the *egl-4* cGMP-dependent kinase.

Conclusions: Our findings indicate that the conserved Notch pathway modulates behavior in adult *C. elegans* in response to environmental stress. Additionally, Notch signaling regulates sleep-like quiescence in *C. elegans*, suggesting that Notch may regulate sleep in other species.

Introduction

Notch signaling is a highly conserved signaling pathway with well-characterized roles in development and cell fate specification. In the canonical Notch pathway, transmembrane Notch receptors are activated by binding to transmembrane ligands containing a highly conserved DSL (Delta, Serrate, and LAG-2) domain. Activated Notch receptors are cleaved by the gamma secretase/presenilin complex and the intracellular (IC) domain of the receptor translocates to the nucleus. Notch IC then acts in conjunction with a transcription factor called Suppressor of Hairless in *Drosophila* or LAG-1 in *C. elegans*, and transcription of target genes is activated [1]. The two *C. elegans* Notch receptors, LIN-12 and GLP-1, play well-characterized roles during *C. elegans* development. For example, LIN-12 plays critical roles at multiple steps of vulval cell fate specification and differentiation [2], whereas GLP-1 signaling negatively regulates mitotic exit and meiotic entry in the germline [3].

The *C. elegans* proteins encoded by *osm-7*, *osm-11*, *dos-1*, *dos-2*, and *dos-3* are predicted to encode secreted or transmembrane proteins containing an EGF repeat conforming to the DOS-motif consensus (Delta and OSM-11) [4]. DOS motifs are also found in canonical DSL ligands of other metazoans [4]. In *C. elegans*, OSM-11 acts with DSL ligands in vulval cell fate specification to help activate LIN-12 Notch [4]. Because *C. elegans* DSL-domain ligands lack the DOS motif, they may function as bipartite ligands with DOS-motif proteins to activate *C. elegans* Notch receptors. DOS-motif proteins also play poorly understood nondevelopmental roles. Loss of OSM-11 or OSM-7 causes defects in osmotic avoidance behavior, decreases defecation rates, increases internal osmolyte levels, increases sensitivity to anoxia [5], and alters expression of specific innate immunity proteins [6–8].

Several lines of evidence suggest that Notch signaling plays a nondevelopmental role in adult nervous systems across species. Notch receptors are expressed in the adult nervous systems of mammals [9], *Drosophila* [10], and *C. elegans* [11, 12] (this study). Altering Notch signaling changes neuronal activity [13, 14], and this affects various processes including spatial learning, memory, long-term potentiation, and neuromuscular junction synaptic plasticity [10, 15–18]. In *C. elegans*, LIN-12 Notch receptor function is required in the RIG interneurons of adult *C. elegans* to regulate the rate at which animals spontaneously initiate backward locomotion (i.e., reversals) during forward locomotion [11].

Here we describe roles for the Notch pathway in two *C. elegans* behaviors. First, Notch ligands function in adult animals to activate GLP-1 and LIN-12 Notch receptors expressed in neurons to modulate chemosensory avoidance of the odorant 1-octanol. Second, Notch ligands and receptors regulate quiescence during *C. elegans* molting lethargus, a sleep-like behavior. Decreased Notch signaling reduces arousal thresholds during the L4-to-adult molt, and ectopic expression of OSM-11 in adult animals is sufficient to induce anachronistic quiescence, which is dependent on the EGL-4 cGMP-dependent kinase (PKG) and on the LIN-3/LET-23 EGF pathway. Combined, these and other results presented

⁸These authors contributed equally to this work

*Correspondence: anne_hart@brown.edu

herein reveal hitherto unsuspected roles for Notch signaling regulating *C. elegans* behavior.

Results

The Notch Coligand OSM-11 and Related DOS-Motif Proteins Are Required for Normal Octanol Response

Wild-type *C. elegans* avoid the noxious chemical odorant 1-octanol by rapidly initiating backward locomotion [19, 20]. We found that *osm-11(rt142)* complete loss-of-function (null) animals were defective in octanol response (Figure 1A). Another DOS gene was required for octanol response; *osm-7(tm2256)* null animals were as defective in octanol response as animals lacking *osm-11*. By contrast, *dos-1(ok2398)* null and *dos-2(tm4515)* loss-of-function animals were only modestly defective (Figure 1A). We confirmed these results by RNA interference (RNAi) knockdown (see Figure S1A available online). Octanol response defects were not further exacerbated in *osm-7;osm-11* double mutants (data not shown). Combined, these results suggest that *osm-11* and *osm-7* Notch coligand genes play essential and nonredundant roles in chemosensory avoidance of octanol; we focused here on the role of *osm-11*.

We found that *osm-11* acts in adult animals to modulate behavior. No morphological defects were observed in the octanol-sensing chemosensory amphid neurons in *osm-11(null)* animals (data not shown), consistent with a nondevelopmental role. To confirm that *osm-11* function in adult animals was sufficient to regulate octanol response, we manipulated *osm-11* levels in adult animals. First, wild-type animals were reared on *E. coli* expressing double-stranded RNA corresponding to *osm-11* [*osm-11(RNAi)*]; these animals were severely defective in octanol response. However, when moved as adults to standard, non-RNAi *E. coli*, octanol response was fully restored ("Recovered from RNAi," Figure 1B). In a reciprocal experiment, wild-type animals were transferred to plates containing *E. coli* expressing *osm-11(RNAi)* as L4 larvae; as adults, these animals were defective in octanol response (Figure 1B). Finally, an inducible heat-shock promoter was utilized to express *osm-11* cDNA (*hsp::osm-11*) after cell fate specification was complete. Induction of *osm-11* expression fully restored normal octanol response within hours (Figure 1B). Combined, these results indicate that the neural circuitry required for response to octanol is intact in animals lacking *osm-11* and that *osm-11* function is required only in adult animals for response to octanol.

Previous work has shown that *osm-11* animals have increased internal glycerol levels reminiscent of animals adapted to high external osmolarity [7]. To determine whether *osm-11* regulation of octanol response is dependent on glycerol accumulation, we examined the octanol response in *osm-11* animals lacking the glycerol biosynthesis genes *gpdh-1* and *gpdh-2*. The loss of both *gpdh-1* and *gpdh-2* did not suppress the octanol response defect of *osm-11* animals (Figure S1B), suggesting that this behavioral defect may be independent of glycerol accumulation.

osm-11 Acts Non-Cell Autonomously to Regulate Octanol Avoidance in Adults

osm-11 is expressed in the hypodermal seam cells and spermathecae of adult animals but is not expressed in neurons [4]. The seam cells lie along the lateral sides of the body contacting both the cuticle and the body cavity (pseudocoelom); they help secrete cuticle at each molt, but their roles in adult

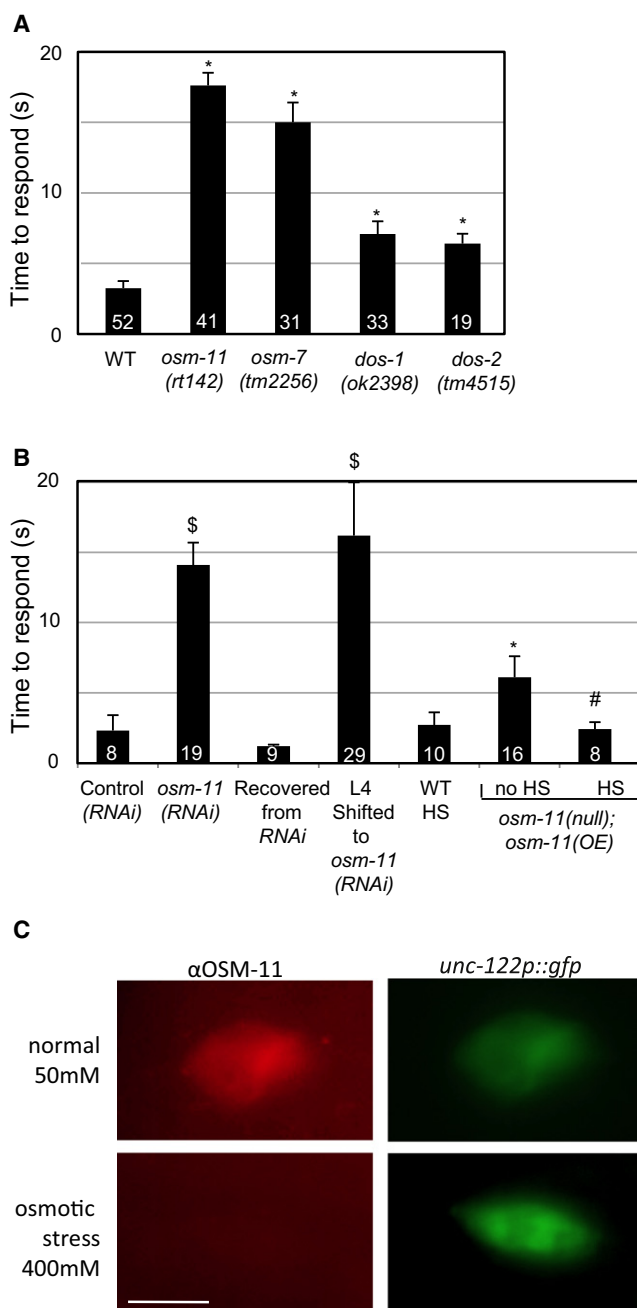


Figure 1. *C. elegans* DOS-Motif Proteins Are Required for Response to Octanol

(A) Octanol responses of animals carrying the likely null alleles *osm-11(rt142)*, *osm-7(tm2256)*, *dos-1(ok2398)*, and partial loss-of-function allele *dos-2(tm4515)*. * $p < 0.0005$ versus wild-type (WT).

(B) *osm-11* function is not required during development for octanol response in adults. \$ $p < 10^{-4}$ versus control (RNAi); * $p > 0.05$ versus WT; # $p > 0.05$ versus *osm-11(null); osm-11(OE)* no heat shock (HS).

(C) OSM-11 protein accumulates in coelomocytes. *osm-11p::gfp* reporter constructs do not drive GFP expression in coelomocytes [4]. Top left: representative image of OSM-11 accumulation in a coelomocyte using α OSM-11 antiserum [4]. Bottom left: osmotic stress likely results in diminished OSM-11 secretion by seam cells into pseudocoelom. Top and bottom right: GFP expression (*unc-122p::gfp*) in coelomocytes [53]. Scale bar represents 6 μ m.

Time to respond to octanol in (A) and (B) is reported as mean time to initiate backward locomotion (s) $\pm$ standard error of the mean (SEM). See Supplemental Experimental Procedures for details.

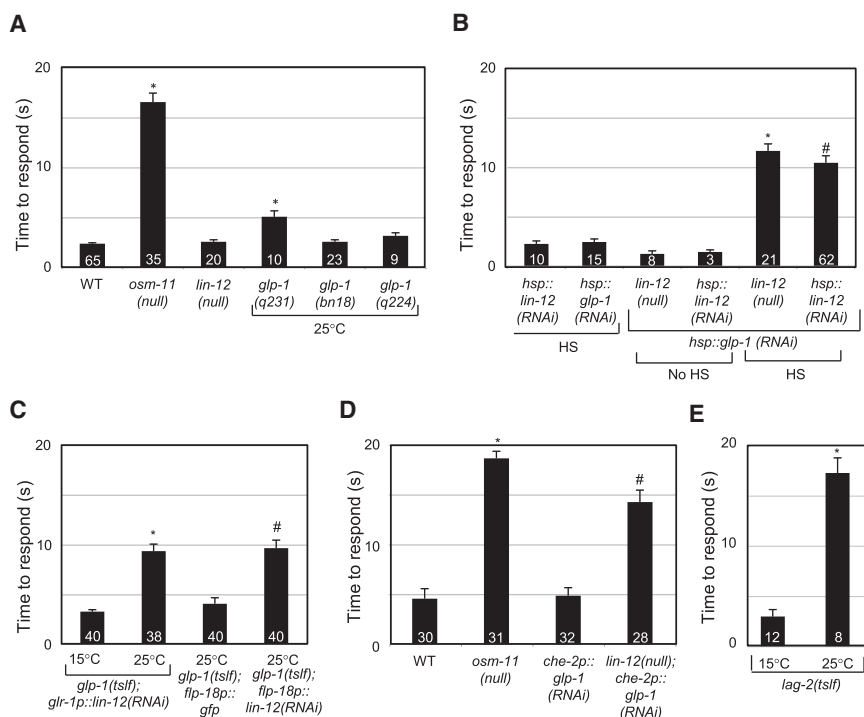


Figure 2. *C. elegans* Notch Receptors and DSL Ligand Are Required for Normal Octanol Response

(A) Decreasing function of either of the Notch receptor genes *lin-12* or *glp-1* alone does not dramatically impair octanol response. * $p < 10^{-11}$ versus wild-type (WT).

(B) Knockdown of both Notch receptors impairs octanol response in adult animals. # $p < 0.05$ versus *hsp::lin-12*(RNAi) HS; * $p < 0.05$ versus *lin-12*(null).

(C) Notch receptors are required in neurons. *lin-12* function is required in a subset of interneurons for normal octanol response. *lin-12*(RNAi) in *glp-1*- and *flp-18*-expressing neurons impairs octanol response. * $p < 10^{-11}$ versus animals at 15°C; # $p < 10^{-4}$ versus *glp-1*(tslf);*flp-18p::glp-1*.

(D) *glp-1* functions in ciliated sensory neurons to regulate octanol response. *glp-1*(RNAi) in *che-2*-expressing ciliated sensory neurons impairs octanol response in *lin-12*(null) animals. * $p < 10^{-12}$ versus WT; # $p < 10^{-8}$ versus *che-2p::glp-1*(RNAi).

(E) *lag-2* function is required in adult animals for octanol response. * $p < 10^{-7}$. Time to respond to octanol is reported as mean time to initiate backward locomotion (s) $\pm$ SEM. See Supplemental Experimental Procedures for details.

animals remain unexplored. Expression of *osm-11* cDNA in seam cells and hypodermal tissues [21] restored octanol response in *osm-11*(null) animals (*wrt-6p::osm-11*, Figure S1C). In tissue culture studies, OSM-11 is a secreted protein. Furthermore, in vivo OSM-11 can diffuse within the body cavity to activate Notch signaling at distant sites in developmental contexts [4]. We detected OSM-11 immunoreactivity in coelomocytes (Figure 1C), which are endocytic cells that clear secreted proteins from the pseudocoelom. Coelomocyte clearance of secreted proteins requires the function of the *cup-4* and *cup-5* genes. Loss of *cup-4* function dramatically decreases coelomocyte endocytosis, whereas loss of *cup-5* function results in aberrant accumulation of internalized proteins in enlarged endocytic vacuoles [22, 23]. Based on the OSM-11 immunoreactivity, the OSM-11 protein did not accumulate in the coelomocytes of *cup-4* animals but accumulated to excessive levels in *cup-5* animals. However, OSM-11 immunoreactivity in seam cells was unchanged (data not shown). Because the *osm-11* promoter does not drive expression in coelomocytes [4], OSM-11 is likely secreted into the pseudocoelom, diffuses, and accumulates in coelomocytes.

Prolonged exposure to high external osmolarity, loss of *osm-11*, or loss of *osm-7* results in increased levels of the osmolyte glycerol and behavioral changes [5]. We found that when *C. elegans* were reared on standard plates containing 51 mM NaCl, all coelomocytes contained OSM-11 immunoreactivity (Figure 1C), but rearing *C. elegans* on 400 mM NaCl plates decreased or eliminated OSM-11 immunoreactivity, whereas immunoreactivity in the seam cells was not altered (data not shown). This suggests that osmotic stress may diminish OSM-11 secretion by the seam cells into the body cavity. OSM-11 likely modulates octanol response, and perhaps physiological changes, in a non-cell-autonomous manner. Additional rescue experiments support this

conclusion (Figure S1C). Taken together, these results suggest that OSM-11 is a secreted, diffusible protein released into the body cavity that modulates octanol response in a non-cell-autonomous manner.

Notch Receptors Are Required for Octanol Avoidance

Because OSM-11 is required for octanol response, the canonical Notch signaling pathway might play a hitherto unsuspected role in *C. elegans* chemosensory behavior. Therefore, we examined the role of the *C. elegans* Notch receptors LIN-12 and GLP-1 in octanol avoidance using animals harboring the *lin-12*(n941) null allele or any of three *glp-1* temperature-sensitive, partial loss-of-function (*tslf*) alleles. *lin-12*(null) animals responded robustly to octanol. Two strains of animals carrying *glp-1*(tslf) alleles that were shifted to the restrictive temperature as adults had normal responses, and animals carrying the third allele had only slightly impaired response (Figure 2A). *lin-12* and *glp-1* are tightly linked (<22 kb apart), and loss of both Notch receptors results in embryonic lethality [24]. Therefore, a combination of transgene-based RNAi and/or mutant alleles was used to simultaneously decrease activity of both *C. elegans* Notch receptors.

Induction of either *glp-1* or *lin-12* RNAi knockdown using a heat-shock promoter in otherwise normal adult animals [*hsp::glp-1*(RNAi) and *hsp::lin-12*(RNAi), respectively] alone did not alter octanol response (Figure 2B), consistent with the relatively normal response of *lin-12* and *glp-1* loss-of-function animals. However, RNAi knockdown of *glp-1* in *lin-12*(null) animals dramatically impaired octanol response after heat-shock induction. Similarly, RNAi knockdown of *lin-12* in *glp-1*(tslf) animals impaired octanol response when animals were shifted to the restrictive temperature (Figure 2C; data not shown). Additionally, transgenic animals in which *lin-12* and *glp-1* could be simultaneously knocked down in adults [*hsp::glp-1*(RNAi); *hsp::lin-12*(RNAi)] were defective in octanol

response several hours after heat-shock induction (Figure 2B). These results suggest that both LIN-12 and GLP-1 Notch receptors play a role in adult animals in modulating response to octanol but that the two receptors function redundantly.

Notch Receptors Act in Nonoverlapping Subsets of Neurons

Where might Notch receptors function to regulate octanol response? LIN-12 is required for proper development of the somatic gonad, and GLP-1 is required for germ cell proliferation, but laser ablation of the gonad did not result in octanol response defects (Figure S1D). We considered whether *C. elegans* Notch receptors might function in the nervous system to regulate behavior. *lin-12p::gfp* is expressed in RIG interneurons, and *glr-1p::lin-12(RNAi)* knockdown of *lin-12* in *glr-1*-expressing interneurons (which include RIG) is sufficient to replicate *lin-12* loss-of-function defects in reversal rates during locomotion [11]. *glr-1p::lin-12(RNAi)* in *glp-1(tslf)* animals at the permissive temperature (15°C) did not perturb octanol avoidance. However, *glr-1p::lin-12(RNAi)* significantly impaired octanol response in *glp-1(tslf)* animals raised at the restrictive temperature (25°C), consistent with *lin-12* acting in *glr-1*-expressing neurons (Figure 2C) [25, 26]. To further narrow down the site of *lin-12* action, we used the *flp-18* promoter to drive *lin-12(RNAi)* in AVA, RIM, AIY, and RIG interneurons along with M2 and M3 pharyngeal neurons. Whereas the control *flp-18p::gfp* transgene had no effect in *glp-1(tslf)* animals, the *flp-18p::lin-12(RNAi)* transgene perturbed octanol response in *glp-1(tslf)* animals raised at the restrictive temperature (25°C). Combined, these results indicate that *lin-12* is likely required for octanol response in AVA, RIM, and/or RIG interneurons, which express both *glr-1* and *flp-18* (Figure 2C).

Redundant function of the two Notch receptor genes could be explained by their expression in the same cells or by action in different parts of the response circuit. Our results suggest that the GLP-1 Notch receptor is required in a different set of neurons from LIN-12 for octanol response. We generated a *glp-1p::gfp* reporter construct that was expressed in many neurons, including the ciliated ASH, AWB, and ADL sensory neurons that detect octanol (Figure S2A). To determine the site of *glp-1* function, we knocked down *glp-1* by RNAi using the *osm-10* promoter (for ASH, ASI, PHA, and PHB), the *gpa-11* promoter (for ASH and ADL [27]), and the *che-2* promoter (for all ciliated sensory neurons including ASH, ADL, and AWB [28]). None of these promoters drive expression in AVA, RIM, or RIG interneurons where *lin-12* likely acts (see Figure S6). RNAi knockdown of *glp-1* in *osm-10*- and *gpa-11*-expressing neurons only modestly affected octanol response in *lin-12(null)* animals (Figure S2B). However, RNAi knockdown in the larger set of *che-2*-expressing neurons dramatically impaired octanol response in *lin-12(null)* animals (Figure 2D). These results suggest that *glp-1* normally acts in *che-2*-expressing ciliated neurons to modulate octanol response and that *lin-12* is primarily required in AVA, RIM, and/or RIG interneurons. The redundant function of Notch receptors is likely due to properties of the neural circuit and not due to coexpression of the two genes in the same neurons.

Notch Ligands act via Notch Receptors to Regulate Octanol Avoidance

If LIN-12 and GLP-1 Notch receptors are activated by OSM-11 to regulate octanol response, then increasing Notch signaling should ameliorate *osm-11* loss-of-function defects. We

utilized two Notch gain-of-function alleles: *lin-12(n137n460)*, a cold-sensitive gain-of-function (*csgf*) allele, and *glp-1(ar202)*, a temperature-sensitive gain-of-function (*tsgf*) allele, to test this hypothesis. The gain-of-function alleles had little or no effect on octanol response in otherwise normal animals regardless of temperature (Figure S2C). Both of the receptor gain-of-function alleles partially suppressed the octanol response defect of *osm-11(null)* animals at the restrictive temperature (Figure S2C), suggesting that OSM-11 acts via the Notch receptors.

Canonical DSL-domain Notch ligands, such as LAG-2, activate Notch receptors in concert with DOS-motif proteins in developmental contexts [4]. The cellular expression pattern of a *lag-2p::gfp* reporter [29] suggested that *lag-2* is expressed in many neurons of adult *C. elegans*, including the AVA command interneurons that regulate locomotion and help initiate reversals in response to aversive stimuli (Figure S2D). We found that octanol response requires *lag-2* function. *lag-2(q420tslf)* animals were defective in their response to octanol at the restrictive temperature but responded normally when reared at the permissive temperature (Figure 2E). Taken together, these results suggest that the DSL-domain ligand LAG-2 and the DOS-motif coligand OSM-11 both contribute to activation of neuronal Notch receptors in adult animals to modulate octanol response.

Identification of Neuronal Notch Targets

Direct transcriptional targets of the Notch pathway that are expressed in neurons would be excellent candidates for downstream targets of Notch signaling in octanol avoidance pathways, but few functional targets of Notch receptors beyond helix-loop-helix transcription factors have been identified. A previous study identified a list of 163 likely Notch target genes with four or more LAG-1 consensus binding sites in upstream or intronic regulatory sequences [30]. Of these, only 22 were likely or definitely expressed in neurons based on known expression patterns or predicted protein function. Two of these genes, *egl-4* and *lst-1*, acted downstream of Notch receptors based on genetic criteria.

egl-4 encodes a cGMP-dependent kinase (PKG) that is expressed in many head neurons and has been previously implicated in several *C. elegans* behaviors [31–34]. *egl-4(n479)* loss-of-function animals respond normally to octanol (data not shown). However, the gain-of-function *egl-4(ad450)* allele partially suppressed the octanol response defect of *osm-11(null)* animals in double-mutant animals (Figure S2E). This result is consistent with *egl-4* acting, directly or indirectly, as one of several downstream targets of Notch receptor activation.

lst-1 is a direct transcriptional target of *lin-12* Notch signaling in vulval cell fate specification and encodes a protein lacking vertebrate orthologs [30]. *lst-1p::gfp* is expressed in many *C. elegans* head neurons, including the octanol-sensing AWB neurons [35]. Animals lacking *lst-1* were as defective in their octanol response as *osm-11(null)* animals (Figure S2E). Because the *lin-12(csgf)* allele suppressed the octanol response defect of *lst-1(null)* animals, we conclude that *lin-12* likely acts via additional target genes to regulate octanol response.

OSM-11 Overexpression Induces Quiescence in Adults via Notch Receptors

Adult *C. elegans* overexpressing *osm-11* [*osm-11(OE)*] via the *hsp::osm-11* transgene exhibited uncharacteristic

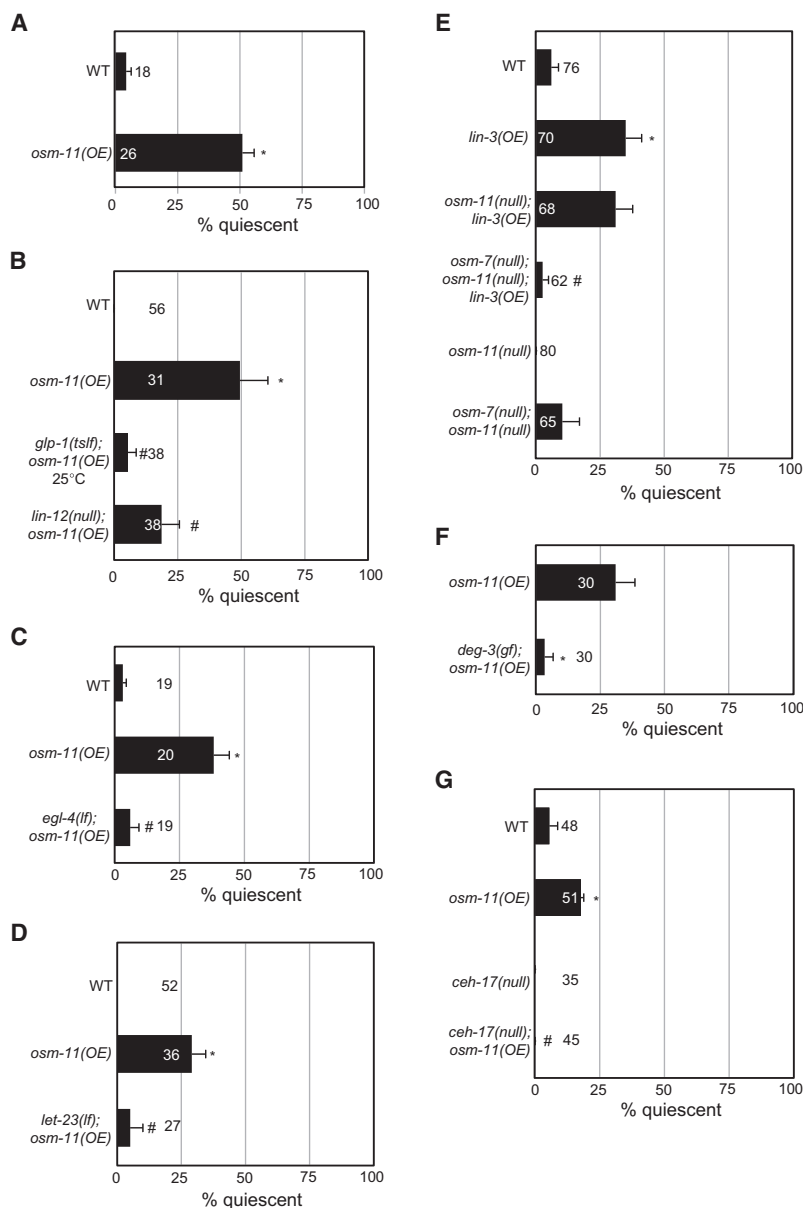


Figure 3. OSM-11 Overexpression Induces Quiescence in Adults

In all panels, *osm-11* was expressed under the heat-shock promoter (*hsp::osm-11*).

(A) OSM-11 overexpression (OE) induces spontaneous, transient, and anachronistic quiescence in adult animals. * $p < 10^{-5}$ versus wild-type (WT).

(B) *osm-11(OE)*-induced adult quiescence requires Notch receptor function. *glp-1(tslf)* or *lin-12(null)* suppresses *osm-11(OE)*-induced quiescence. * $p < 0.05$ versus WT; # $p < 0.05$ versus *osm-11(OE)*.

(C) OSM-11 overexpression-induced quiescence is suppressed by *egl-4(lf)*. * $p < 0.0001$ versus WT; # $p < 0.05$ versus *osm-11(OE)*.

(D) Loss of LET-23 EGF receptor function [*let-23(lf)*] suppresses *osm-11(OE)*-induced quiescence. * $p < 0.001$ versus WT; # $p < 0.05$ versus *osm-11(OE)*.

(E) Simultaneous loss of *osm-7* and *osm-11* suppresses LIN-3 EGF overexpression [*lin-3(OE)*]-induced adult quiescence. * $p < 0.01$ versus WT; # $p < 0.05$ versus *lin-3(OE)*.

(F) *deg-3*-induced neurodegeneration suppresses *osm-11(OE)*-induced quiescence. * $p < 0.05$. Because the locomotion of *deg-3(u662)* animals is severely uncoordinated, pharyngeal pumping was used to assess quiescence.

(G) Defective axon outgrowth in *ceh-17*-expressing neurons suppresses *osm-11(OE)*-induced quiescence. *ceh-17(null)* affects processes of ALA and a subset of other neurons. * $p < 0.05$ versus WT; # $p < 0.001$ versus *osm-11(OE)*.

Quiescence behavior is reported as percent of quiescent adult animals after OSM-11 overexpression $\pm$ SEM. See Supplemental Experimental Procedures for details.

during quiescent bouts, but they responded normally between quiescent bouts. During ectopic quiescence, *osm-11(OE)* animals failed in $47\% \pm 9\%$ of trials to respond to body touch. By contrast, only $10\% \pm 0\%$ of heat-shocked wild-type or $8\% \pm 4\%$ of non-heat-shocked wild-type (*osm-11(OE)*) animals failed to respond [$n = 30$, $p < 0.01$ for *osm-11(OE)* heat shock versus WT or no heat shock]. Similar results were observed using dilute octanol as a stimulus (data not shown). The diminished sensory responses during quiescence suggest that overexpression of OSM-11 might anachronistically activate the quiescence observed during *C. elegans* molting lethargus.

bouts of spontaneous and transient quiescence after heat-shock induction, during which locomotion and pharyngeal pumping ceased for several minutes regardless of the presence of food (Figure 3A). Control animals did not display noticeable changes in behavior after a 1 hr recovery from heat shock. Between quiescent bouts, *osm-11(OE)* animals moved with coordinated sinusoidal locomotion and had normal pharyngeal pumping rates.

The quiescence of *osm-11(OE)* animals was similar to the sleep-like quiescence observed in *C. elegans* during larval molting lethargus [36, 37] in four ways. First, the quiescent state was transient. Second, animals entered and exited the quiescent state spontaneously. Third, quiescent *osm-11(OE)* animals could be “woken” by prodding with a metal wire. This perturbation elicited short bursts of coordinated sinusoidal locomotion. Fourth, *osm-11(OE)* animals exhibited increased arousal thresholds to mechanosensory stimulation

We did not observe inappropriate quiescence in transgenic adult animals overexpressing Notch receptors. To confirm that *osm-11* overexpression induced inappropriate quiescence by activating Notch receptors, we examined *osm-11(OE)* quiescence in animals lacking Notch receptor function. We found that either loss of the *lin-12* Notch receptor or diminished function of the *glp-1* Notch receptor suppressed *osm-11(OE)*-induced locomotion and pumping quiescence (Figure 3B). These results suggest that increasing *osm-11* levels inappropriately activates Notch signaling, resulting in behavioral quiescence.

OSM-11-Induced Quiescence Requires Previously Defined Quiescence Pathways

Does *osm-11* overexpression induce quiescence through signaling pathways previously implicated in *C. elegans* molting lethargus quiescence? *C. elegans* quiescence requires the

function of EGL-4 PKG [36, 37]. We found that loss of *egl-4* suppressed *osm-11(OE)*-induced quiescence (Figure 3C); this is consistent with OSM-11 overexpression leading to inappropriately increased *egl-4* activity with consequent quiescence in adult animals.

Endogenous *C. elegans* molting quiescence also requires LIN-3 EGF and LET-23 EGF receptor function [37]. LET-23 receptor function is required in the ALA neuron. Loss of *let-23*, the ALA neuron, or ALA processes is sufficient to decrease quiescence induced by LIN-3 EGF ligand overexpression [37]. Loss of the LET-23 EGF receptor suppressed *osm-11(OE)*-induced locomotion and pumping quiescence in adult animals (Figure 3D), suggesting that EGF receptor signaling is required for Notch-induced quiescence. We also examined the relationship between *osm-11* and *lin-3*. Overexpression of *lin-3* [*lin-3(OE)*] in transgenic adult animals using the heat-shock promoter (*hsp::lin-3*) causes anachronistic quiescence [37] reminiscent of quiescence induced in *osm-11(OE)* animals. We found that loss of *osm-11* was not sufficient to suppress *lin-3OE*-induced quiescence, but simultaneous loss of both *osm-11* and *osm-7* dramatically suppressed *lin-3OE*-induced quiescence (Figure 3E). Thus, *osm-7* and *osm-11* may play redundant roles in *lin-3(OE)*-induced anachronistic quiescence.

The role of the ALA neuron in *osm-11* overexpression quiescence was examined using two strategies. First, the ALA neuron (among others) was genetically ablated in *deg-3(u662)* animals [38]. Inappropriate *osm-11*-induced quiescence was suppressed in *deg-3(u662);osm-11(OE)* animals (Figure 3F). Second, ALA (and SIA) processes were perturbed in the *ceh-17(np1)* background [39], which also suppressed *osm-11(OE)*-induced quiescence (Figure 3G). Combined, these results suggest that Notch-induced quiescence in adult *C. elegans* requires the ALA neuron and EGF signaling that normally regulate quiescence during molting lethargus.

Notch Signaling Alters Quiescence during Molting Lethargus

To determine whether altering Notch signaling affects quiescence during L4-to-adult (L4/A) molting lethargus, we developed a simple microfluidics-based approach to assess quiescence in multiple animals simultaneously during L4/A lethargus (Figure S3). Total quiescence was determined for L4/A lethargus by loading mid-L4-stage larvae into microfluidic chambers and recording activity into adulthood (Figure 4A). Both *lin-12(null)* and *glp-1(tslf)* animals had increased total quiescence versus control animals (Figure 4B). Also, Notch receptor gain-of-function alleles resulted in increased total quiescence [*glp-1(ar202tsf)* and *lin-12(n137n460csgf)* animals, Figure 4C]. Similar results were observed in animals overexpressing LIN-12 [*lin-12p::lin-12*, Figure S4A]. Increasing GLP-1 receptor activity in ciliated sensory neurons was sufficient to increase total quiescence in wild-type animals [*che-2p::glp-1(IC)*, Figure 4D], and expression of GLP-1(IC) in ciliated neurons partially restored quiescence in *glp-1(tslf)* animals (Figure 4D).

Next, we examined the impact of Notch ligands. Decreasing *lag-2* function increased total quiescence [*lag-2(q420tsf)*, Figure 4E]. Similarly, loss of either *osm-7* or *osm-11* increased total quiescence (Figure 4E). However, simultaneous loss of function of both *osm-7* and *osm-11* resulted in decreased total quiescence (Figure 4E), which was unexpected given the increased quiescence of single-mutant animals. Combined, these results suggest that Notch receptors and ligands regulate L4/A quiescence in a complex fashion.

Notch Signaling Impacts the Duration of L4/A Lethargus

The decreased total quiescence of *osm-7(null);osm-11(null)* animals could be caused by a shorter L4/A lethargus. Therefore, the impact of Notch signaling on the duration of lethargus was determined. The L4/A lethargus was significantly shorter for *osm-7(null);osm-11(null)* animals (Table S1A). The decreased lethargus period of *osm-7(null);osm-11(null)* animals suggested that they were defective in lethargus entry or maintenance. There was no difference in the time at which *osm-7(null);osm-11(null)* animals entered lethargus (Figure S4D). However, *osm-7(null);osm-11(null)* animals exited lethargus early (Figure 4F; Figure S4E), suggesting that premature exit from L4/A lethargus causes decreased total quiescence.

To reconcile the complex effects of Notch pathway manipulation on L4/A quiescence, we delineated the impact of Notch signaling on two other components of quiescence: basal locomotion activity (hereafter referred as basal activity) and arousal threshold.

Notch Signaling Regulates Basal Activity and Arousal Thresholds

One factor that could influence quiescence maintenance is basal activity, which was assessed as body bends per minute [40]. Animals lacking both *osm-7* and *osm-11* had twice as many body bends per minute compared to control animals either at the adult stage or between L4/A quiescent bouts (Table S1B). The dramatically increased basal activity likely contributes to the decreased total quiescence of *osm-7;osm-11* animals.

During quiescence, animals have high arousal thresholds because they respond slowly or less robustly to sensory stimulation [36]. To assess arousal threshold, we used the mechanosensory body touch assay [41]. Wild-type adult animals responded to light touch in 100% of trials, but quiescent L4/A animals responded in roughly 50% of trials (Table 1), reflecting an increased arousal threshold. Decreasing Notch signaling lowered arousal thresholds during L4/A quiescence; quiescent *lin-12(null)* or *glp-1(tslf)* animals responded more frequently than control quiescent animals (Table 1). Conversely, increasing Notch receptor function increased arousal thresholds; *glp-1(tsgf)*, *lin-12(csgf)*, *che-2p::glp-1(IC)*, and *lin-12(OE)* animals had increased arousal thresholds compared to their respective controls (Table 1). Also, decreasing *lag-2* function or loss of *osm-7* and/or *osm-11* decreased arousal thresholds. Overall, Notch signaling correlated with arousal thresholds during L4/A quiescence (Table 1; Table S1C). Low arousal thresholds may reflect poor-quality quiescence. We speculate that poor-quality quiescence leads to compensatory increases in quiescence, as observed in both total L4/A quiescence and duration of lethargus (Figure 4; Table S1A). Overall, our results indicate that the Notch signaling pathway regulates basal activity, duration of lethargus, arousal thresholds, and quiescence in *C. elegans*.

Discussion

Like sleep, quiescence is a complex behavior dependent on multiple processes including arousal threshold and cessation of activity. We propose a model (Figure 5) wherein Notch signaling levels directly correlate with arousal levels during the L4/A molt. Increased arousal thresholds resulted in either inappropriate or increased quiescence. The consequences of decreased Notch activity are more complicated. Increased

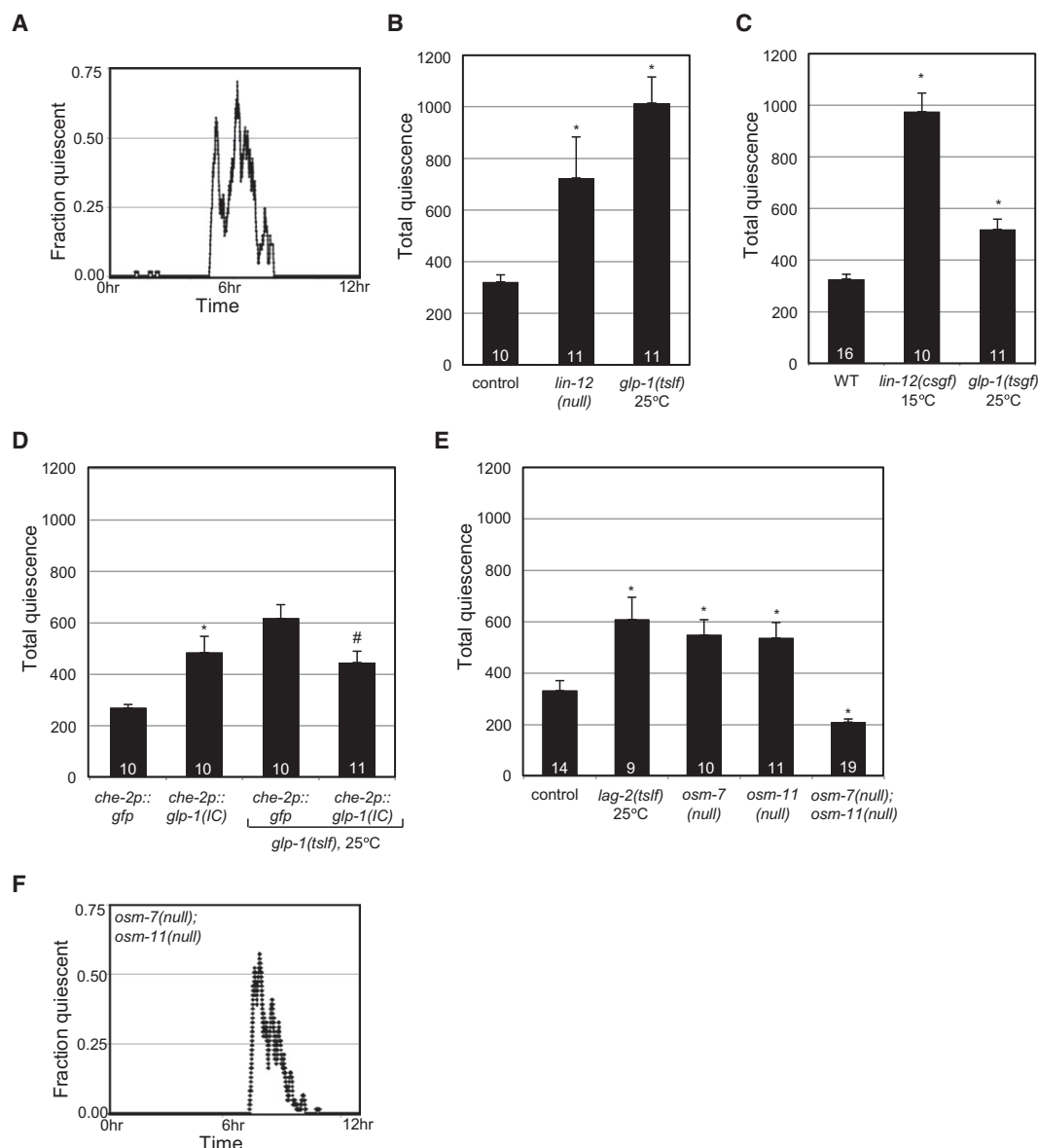


Figure 4. Notch Signaling Regulates L4-to-Adult Molting Quiescence

(A) Representative fractional quiescence graph. Animals exhibit L4/A molting lethargus quiescence for a few hours before adulthood.
(B) Loss of LIN-12 receptor or decreased GLP-1 receptor function increases total L4/A quiescence. * $p < 0.02$ versus control (*mgIs49*).
(C) Increased LIN-12 or GLP-1 function increases total L4/A molting quiescence. * $p < 10^{-4}$ versus wild type (WT).
(D) Increasing GLP-1 receptor activity in ciliated sensory neurons increases total L4/A quiescence in wild-type animals [*che-2p::glp-1(IC)*]. Expression of *che-2p::glp-1(IC)* transgene restores total quiescence in *glp-1*(tslf) animals to normal levels. * $p < 0.003$ versus *che-2p::gfp*; # $p < 0.02$ versus *glp-1*(tslf);*che-2p::gfp*.
(E) Notch ligands and coligands regulate total L4/A quiescence. * $p < 0.002$ versus control (*mgIs49*).
(F) Representative fractional quiescence graph of *osm-7*(null);*osm-11*(null) for comparison with (A).
Total L4/A quiescence in (B)–(E) is calculated as area under the curve $\pm$ SEM. See Supplemental Experimental Procedures for details.

basal activity and/or decreased arousal thresholds resulted in homeostatic compensation in which animals increased quiescence to compensate for poor-quality quiescence. Further decreases in Notch signaling left *osm-7*(null);*osm-11*(null) animals unable to maintain quiescence as a result of dramatically increased basal activity and decreased arousal thresholds. The results presented here are consistent with Notch signaling playing a major, albeit complex, role in the regulation of behavioral quiescence. Notch pathway regulation of L4/A quiescence is specific, because the Notch signaling pathway

genes have no impact on satiety quiescence (Y.-j. You and L. Avery, personal communication).

The presence of sleep-like states in *Drosophila* and *C. elegans* permits the dissection of conserved pertinent pathways [42]. In *Drosophila*, CREB has been implicated in sleep homeostasis [43] and a novel GPI-anchored protein (with a possible homolog in *C. elegans*) encoded by *sleepless* regulates sleep via an interaction with the Shaker K^+ channel [44]. cAMP and EGFR signaling have been implicated in sleep regulation in both *Drosophila* and *C. elegans* [36, 37, 43, 45].

Table 1. Notch Activity Regulates Arousal during L4/A Molting Quiescence

	% Failed to Respond	n
Wild-type	50 ± 2	221
<i>osm-11(null)</i>	29 ± 5 ^a	71
<i>osm-7(null)</i>	25 ± 5 ^a	71
<i>osm-7(null);osm-11(null)</i>	44 ± 4 ^a	76
<i>lag-2(tslf)</i> , 25°C	31 ± 1 ^b	35
<i>glp-1(tslf)</i> , 25°C	27 ± 3 ^a	49
<i>glp-1(tsgf)</i> , 25°C	70 ± 5 ^a	31
<i>lin-12(null)</i>	40 ± 1 ^a	51
<i>lin-12(csgf)</i> , 15°C	65 ± 1 ^a	37
<i>lin-12p::gfp</i>	45 ± 2	40
<i>lin-12p::lin-12</i>	62 ± 3 ^c	43
<i>che-2p::gfp</i>	46 ± 2	31
<i>che-2p::glp-1(lc)</i>	61 ± 3 ^d	35

Notch activity regulates arousal thresholds during L4/A molting quiescence. % of animals failing to respond to body touch with a hair during quiescent bouts is reported. Notch activity correlates with arousal thresholds. High Notch activity results in animals with high arousal thresholds, and vice versa.

^a $p < 10^{-3}$ by analysis of variance.

^b $p < 0.002$ versus wild type.

^c $p < 0.003$ for *lin-12p::lin-12* versus *lin-12p::gfp* in *pha-1* rescue.

^d $p < 0.008$ for *che-2p::glp-1(lc)* compared to transgenic control animals expressing *che-2p::gfp* in wild type background. See supplemental methods for Table 1 for details. *osm-11(OE)*-induced quiescence in adult animals also results in increased arousal thresholds. (See text.)

Interestingly, the mammalian OSM-11 ortholog DLK1 is expressed in the ventral tegmental area, substantia nigra pars compacta, and Raphe nuclei of adult rat and human brains [46], regions of the brain that have been implicated in sleep-wake cycle regulation [47].

Previous studies identified two signaling pathways that regulate quiescence in *C. elegans*: EGF and PKG [36, 37]. *egl-4* PKG functions downstream of EGF signaling, because loss of *egl-4* prevents inappropriate quiescence caused by *lin-3* EGF overexpression [37]. We found that loss of *egl-4* PKG function also prevents OSM-11-induced anachronistic quiescence, suggesting that *egl-4* also acts downstream of Notch signaling. However, loss of *osm-7* and *osm-11* suppressed LIN-3 EGF-induced quiescence, suggesting that there may be crosstalk and/or feedback regulation between the Notch and EGF signaling pathways.

Multiple lines of evidence suggest that OSM-11 is expressed in hypodermal seam cells [4] but is secreted and acts non-cell autonomously. OSM-11 likely acts on sensory neurons in the head, including those that coexpress GLP-1 and EGL-4 PKG, and on the RIG interneurons that express LIN-12. However, other neurons are also likely involved. The ALA neuron is required for quiescence [37]; interestingly, ALA is one of three *C. elegans* neurons with processes directly underlying seam cells. OSM-11 secreted from the seam cells may act directly on the ALA neuron. Or, because Notch receptor expression has not been observed in ALA, the ALA neuron may regulate OSM-11 secretion from seam cells. There are two other classes of neurons with processes underneath the seam cells: CAN and PVD. The function of the CAN neurons remains unclear, although they associate with the excretory canal cell that regulates *C. elegans* osmotic balance [48]. The PVD neurons are involved in mechanical touch response [49], which might occur during osmotic swelling and/or shrinking. A functional interaction of PVD and/or CAN with the seam cells would be consistent with a role for OSM-11 in osmotic stress [7].

Our results support an ethological model in which OSM-11 and Notch signaling modulate neuronal function, behavior, and adaptation to environmental stress. Adaptation to high external osmolarity or loss of DOS coligands *osm-7* or *osm-11* causes both physiological and behavioral changes in *C. elegans*. Increased external osmolarity decreases OSM-11 levels in the pseudocoelom, consistent with osmotic regulation of OSM-11 secretion from the seam cells. Either osmotic adaptation or loss of DOS coligands causes physiological adaptation to high environmental osmolarity via an unknown pathway that induces *gpdh-1* expression and consequent glycerol osmolyte synthesis [50]. Additionally, either osmotic adaptation or DOS gene loss causes behavioral changes including decreased response to high osmolarity and octanol. These behavioral changes are dependent on Notch receptor signaling in the neurons of adult animals and are not dependent on *gpdh-1* upregulation. It is tempting to speculate that these physiological and behavioral changes are adaptive and are coordinated by global regulation of Notch receptor signaling. Humoral OSM-11 may play a key role in regulating Notch signaling in various tissues to coordinate physiological and behavioral adaptation to osmotic stress. Because Notch receptor signaling has been recently been implicated in regulation of *C. elegans* heterochronic genes [51, 52], humoral OSM-11 acting on Notch receptors may also play a pivotal role in the temporal regulation of physiological and behavioral events at the larval molt, including quiescence.

Supplemental Information

Supplemental Information includes six figures, one table, Supplemental Discussion, and Supplemental Experimental Procedures and can be found with this article online at [doi:10.1016/j.cub.2011.04.010](https://doi.org/10.1016/j.cub.2011.04.010).

Acknowledgments

This work was supported by National Institutes of Health (NIH) grants R01 GM078171 and NS055813 (A.C.H.), NIH MH051383 and RC1AI087059 (S.R.L.), and the Massachusetts General Hospital Medical Discovery Fund and NIH R15 DA021667-01 (M.Y.C.). We are grateful for advice and/or reagents from the *C. elegans* knockout consortia and numerous members of the *C. elegans* community. We thank Leon Avery and Young-jai You for communicating unpublished results. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources.

Received: February 18, 2010

Revised: December 29, 2010

Accepted: April 6, 2011

Published online: May 5, 2011

References

- Fortini, M.E. (2009). Notch signaling: The core pathway and its post-translational regulation. *Dev. Cell* 16, 633–647.
- Sternberg, P.W. (2005). Vulval development. In *WormBook, The C. elegans Research Community*, ed. 10.1895/wormbook.1.6.1, <http://www.wormbook.org>.
- Kimble, J., and Crittenden, S.L. (2005). Germline proliferation and its control. In *WormBook, The C. elegans Research Community*, ed. 10.1895/wormbook.1.13.1, <http://www.wormbook.org>.
- Komatsu, H., Chao, M.Y., Larkins-Ford, J., Corkins, M.E., Somers, G.A., Tucey, T., Dionne, H.M., White, J.Q., Wani, K., Boxem, M., and Hart, A.C. (2008). OSM-11 facilitates LIN-12 Notch signaling during *Caenorhabditis elegans* vulval development. *PLoS Biol.* 6, e196.
- Frazier, H.N., 3rd, and Roth, M.B. (2009). Adaptive sugar provisioning controls survival of *C. elegans* embryos in adverse environments. *Curr. Biol.* 19, 859–863.

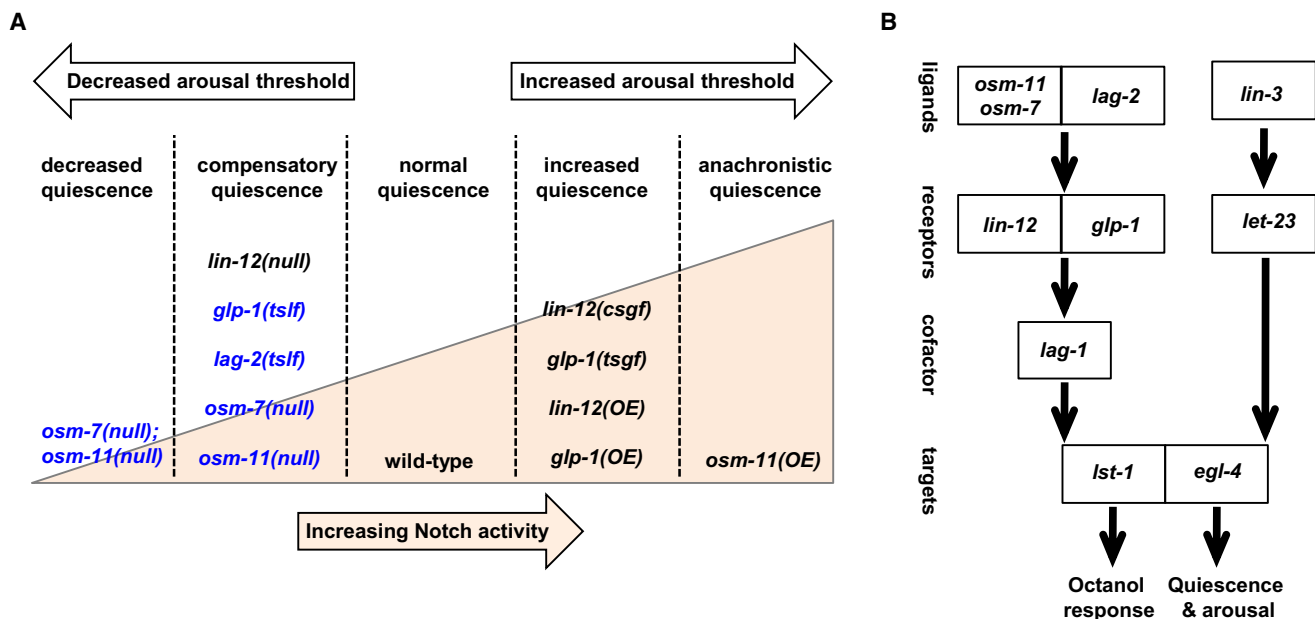


Figure 5. Model for Notch Signaling Regulating Multiple Aspects of L4-to-Adult Molting Quiescence

(A) Schematic model summarizing the impact of various Notch pathway genes. Gene name and type of allele is listed; Notch activity increases left to right (illustrated by peach wedge). For each genotype, the impact of altered Notch signaling on total L4/A quiescence is listed above the wedge; OSM-11 coligand overexpression also induced anachronistic quiescence in adult animals. Decreased Notch signaling decreased arousal threshold during L4/A molting lethargus, whereas increased Notch signaling increased arousal thresholds (white arrows). Decreased *glp-1* Notch receptor function, decreased DSL ligand function, or loss of DOS coligands increased *C. elegans* basal locomotion activity (blue text), whereas loss of *lin-12* Notch receptor decreased basal activity. (B) Genetic pathway illustrating relationships between *C. elegans* genes regulating octanol response and quiescence. *egl-4* PKG also contributes to, but is not required for, octanol response; the role of the EGF pathway in octanol response has not been addressed. Neuronal Notch receptors act redundantly and in different populations of neurons in this behavior: *lin-12* in a subset of interneurons, and *glp-1* in ciliated sensory neurons. Notch receptors may also act elsewhere to regulate quiescence. Activation of either EGF or Notch pathways by ligand overexpression induces anachronistic quiescence, and these pathways are both required for normal L4/A molting quiescence. EGF and Notch pathways may act in parallel, converging on *egl-4* to regulate molting quiescence (illustrated), and/or there may be functional interrelationships between these signaling pathways.

- Lamitina, T., Huang, C.G., and Strange, K. (2006). Genome-wide RNAi screening identifies protein damage as a regulator of osmoprotective gene expression. *Proc. Natl. Acad. Sci. USA* 103, 12173–12178.
- Wheeler, J.M., and Thomas, J.H. (2006). Identification of a novel gene family involved in osmotic stress response in *Caenorhabditis elegans*. *Genetics* 174, 1327–1336.
- Pujol, N., Zugasti, O., Wong, D., Couillault, C., Kurz, C.L., Schulenburg, H., and Ewbank, J.J. (2008). Anti-fungal innate immunity in *C. elegans* is enhanced by evolutionary diversification of antimicrobial peptides. *PLoS Pathog.* 4, e1000105.
- Berezovska, O., McLean, P., Knowles, R., Frosh, M., Lu, F.M., Lux, S.E., and Hyman, B.T. (1999). Notch1 inhibits neurite outgrowth in postmitotic primary neurons. *Neuroscience* 93, 433–439.
- Ge, X., Hannan, F., Xie, Z., Feng, C., Tully, T., Zhou, H., Xie, Z., and Zhong, Y. (2004). Notch signaling in *Drosophila* long-term memory formation. *Proc. Natl. Acad. Sci. USA* 101, 10172–10176.
- Chao, M.Y., Larkins-Ford, J., Tukey, T.M., and Hart, A.C. (2005). *lin-12* Notch functions in the adult nervous system of *C. elegans*. *BMC Neurosci.* 6, 45.
- Ouellet, J., Li, S., and Roy, R. (2008). Notch signalling is required for both dauer maintenance and recovery in *C. elegans*. *Development* 135, 2583–2592.
- Costa, R.M., Drew, C., and Silva, A.J. (2005). Notch to remember. *Trends Neurosci.* 28, 429–435.
- Yoon, K., and Gaiano, N. (2005). Notch signaling in the mammalian central nervous system: Insights from mouse mutants. *Nat. Neurosci.* 8, 709–715.
- Costa, R.M., Honjo, T., and Silva, A.J. (2003). Learning and memory deficits in Notch mutant mice. *Curr. Biol.* 13, 1348–1354.
- Wang, Y., Chan, S.L., Miele, L., Yao, P.J., Mackes, J., Ingram, D.K., Mattson, M.P., and Furukawa, K. (2004). Involvement of Notch signaling in hippocampal synaptic plasticity. *Proc. Natl. Acad. Sci. USA* 101, 9458–9462.
- Song, Q., Sun, K., Shuai, Y., Lin, R., You, W., Wang, L., and Zhong, Y. (2009). Suppressor of Hairless is required for long-term memory formation in *Drosophila*. *J. Neurogenet.* 23, 405–411.
- de Bivort, B.L., Guo, H.F., and Zhong, Y. (2009). Notch signaling is required for activity-dependent synaptic plasticity at the *Drosophila* neuromuscular junction. *J. Neurogenet.* 23, 395–404.
- Chao, M.Y., Komatsu, H., Fukuto, H.S., Dionne, H.M., and Hart, A.C. (2004). Feeding status and serotonin rapidly and reversibly modulate a *Caenorhabditis elegans* chemosensory circuit. *Proc. Natl. Acad. Sci. USA* 101, 15512–15517.
- Troemel, E.R., Chou, J.H., Dwyer, N.D., Colbert, H.A., and Bargmann, C.I. (1995). Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans*. *Cell* 83, 207–218.
- Hao, L., Aspöck, G., and Bürglin, T.R. (2006). The hedgehog-related gene *wrt-5* is essential for hypodermal development in *Caenorhabditis elegans*. *Dev. Biol.* 290, 323–336.
- Fares, H., and Greenwald, I. (2001). Regulation of endocytosis by CUP-5, the *Caenorhabditis elegans* mucolipin-1 homolog. *Nat. Genet.* 28, 64–68.
- Patton, A., Knuth, S., Schaheen, B., Dang, H., Greenwald, I., and Fares, H. (2005). Endocytosis function of a ligand-gated ion channel homolog in *Caenorhabditis elegans*. *Curr. Biol.* 15, 1045–1050.
- Lambie, E.J., and Kimble, J. (1991). Two homologous regulatory genes, *lin-12* and *glp-1*, have overlapping functions. *Development* 112, 231–240.
- Hart, A.C., Sims, S., and Kaplan, J.M. (1995). Synaptic code for sensory modalities revealed by *C. elegans* GLR-1 glutamate receptor. *Nature* 378, 82–85.
- Maricq, A.V., Peckol, E., Driscoll, M., and Bargmann, C.I. (1995). Mechanosensory signalling in *C. elegans* mediated by the GLR-1 glutamate receptor. *Nature* 378, 78–81.

27. Jansen, G., Thijssen, K.L., Werner, P., van der Horst, M., Hazendonk, E., and Plasterk, R.H. (1999). The complete family of genes encoding G proteins of *Caenorhabditis elegans*. *Nat. Genet.* 21, 414–419.
28. Fujiwara, M., Ishihara, T., and Katsura, I. (1999). A novel WD40 protein, CHE-2, acts cell-autonomously in the formation of *C. elegans* sensory cilia. *Development* 126, 4839–4848.
29. Bellefleur, R., Anna-Arriola, S.S., Gao, D., Li, Y., Hodgkin, J., and Kimble, J. (1999). The gon-1 gene is required for gonadal morphogenesis in *Caenorhabditis elegans*. *Dev. Biol.* 216, 382–393.
30. Yoo, A.S., Bais, C., and Greenwald, I. (2004). Crosstalk between the EGFR and LIN-12/Notch pathways in *C. elegans* vulval development. *Science* 303, 663–666.
31. Fujiwara, M., Sengupta, P., and McIntire, S.L. (2002). Regulation of body size and behavioral state of *C. elegans* by sensory perception and the EGL-4 cGMP-dependent protein kinase. *Neuron* 36, 1091–1102.
32. L'Etoile, N.D., Coburn, C.M., Eastham, J., Kistler, A., Gallegos, G., and Bargmann, C.I. (2002). The cyclic GMP-dependent protein kinase EGL-4 regulates olfactory adaptation in *C. elegans*. *Neuron* 36, 1079–1089.
33. Raizen, D.M., Cullison, K.M., Pack, A.I., and Sundaram, M.V. (2006). A novel gain-of-function mutant of the cyclic GMP-dependent protein kinase egl-4 affects multiple physiological processes in *Caenorhabditis elegans*. *Genetics* 173, 177–187.
34. Hukema, R.K., Rademakers, S., Dekkers, M.P., Burghoorn, J., and Jansen, G. (2006). Antagonistic sensory cues generate gustatory plasticity in *Caenorhabditis elegans*. *EMBO J.* 25, 312–322.
35. Cuppen, E., van der Linden, A.M., Jansen, G., and Plasterk, R.H. (2003). Proteins interacting with *Caenorhabditis elegans* Galpha subunits. *Comp. Funct. Genomics* 4, 479–491.
36. Raizen, D.M., Zimmerman, J.E., Maycock, M.H., Ta, U.D., You, Y.J., Sundaram, M.V., and Pack, A.I. (2008). Lethargus is a *Caenorhabditis elegans* sleep-like state. *Nature* 451, 569–572.
37. Van Buskirk, C., and Sternberg, P.W. (2007). Epidermal growth factor signaling induces behavioral quiescence in *Caenorhabditis elegans*. *Nat. Neurosci.* 10, 1300–1307.
38. Treinin, M., and Chalfie, M. (1995). A mutated acetylcholine receptor subunit causes neuronal degeneration in *C. elegans*. *Neuron* 14, 871–877.
39. Pujol, N., Torregrossa, P., Ewbank, J.J., and Brunet, J.F. (2000). The homeodomain protein CePHOX2/CEH-17 controls antero-posterior axonal growth in *C. elegans*. *Development* 127, 3361–3371.
40. Hart, A.C. (2006). Behavior. In *WormBook, The C. elegans Research Community*, ed. 10.1895/wormbook.1.87.1, <http://www.wormbook.org>.
41. Chalfie, M., Sulston, J.E., White, J.G., Southgate, E., Thomson, J.N., and Brenner, S. (1985). The neural circuit for touch sensitivity in *Caenorhabditis elegans*. *J. Neurosci.* 5, 956–964.
42. Crocker, A., and Sehgal, A. (2010). Genetic analysis of sleep. *Genes Dev.* 24, 1220–1235.
43. Hendricks, J.C., Williams, J.A., Panckeri, K., Kirk, D., Tello, M., Yin, J.C., and Sehgal, A. (2001). A non-circadian role for cAMP signaling and CREB activity in *Drosophila* rest homeostasis. *Nat. Neurosci.* 4, 1108–1115.
44. Koh, K., Joiner, W.J., Wu, M.N., Yue, Z., Smith, C.J., and Sehgal, A. (2008). Identification of SLEEPLESS, a sleep-promoting factor. *Science* 321, 372–376.
45. Foltenyi, K., Greenspan, R.J., and Newport, J.W. (2007). Activation of EGFR and ERK by rhomboid signaling regulates the consolidation and maintenance of sleep in *Drosophila*. *Nat. Neurosci.* 10, 1160–1167.
46. Jensen, C.H., Meyer, M., Schroder, H.D., Kliem, A., Zimmer, J., and Teisner, B. (2001). Neurons in the monoaminergic nuclei of the rat and human central nervous system express FA1/dlk. *Neuroreport* 12, 3959–3963.
47. Monti, J.M., and Monti, D. (2007). The involvement of dopamine in the modulation of sleep and waking. *Sleep Med. Rev.* 11, 113–133.
48. White, J., Southgate, E., Thomson, J.N., and Brenner, S. (1986). The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 314, 1–340.
49. Way, J.C., and Chalfie, M. (1989). The mec-3 gene of *Caenorhabditis elegans* requires its own product for maintained expression and is expressed in three neuronal cell types. *Genes Dev.* 3 (12A), 1823–1833.
50. Rohlfing, A.K., Miteva, Y., Hannehalli, S., and Lamitina, T. (2010). Genetic and physiological activation of osmosensitive gene expression mimics transcriptional signatures of pathogen infection in *C. elegans*. *PLoS ONE* 5, e9010.
51. Li, J., and Greenwald, I. (2010). LIN-14 inhibition of LIN-12 contributes to precision and timing of *C. elegans* vulval fate patterning. *Curr. Biol.* 20, 1875–1879.
52. Solomon, A., Mian, Y., Ortega-Cava, C., Liu, V.W., Gurumurthy, C.B., Naramura, M., Band, V., and Band, H. (2008). Upregulation of the let-7 microRNA with precocious development in lin-12/Notch hypermorphic *Caenorhabditis elegans* mutants. *Dev. Biol.* 316, 191–199.
53. Loria, P.M., Hodgkin, J., and Hobert, O. (2004). A conserved postsynaptic transmembrane protein affecting neuromuscular signaling in *Caenorhabditis elegans*. *J. Neurosci.* 24, 2191–2201.

Deep conservation of genes required for both *D. melanogaster* and *C. elegans* sleep includes a role for dopaminergic signaling

Komudi Singh¹, Jennifer Y. Ju¹, Melissa B. Walsh², Michael A. Dilorio¹, Anne C. Hart¹

¹Department of Neuroscience, Brown University, 185 Meeting Street, Box GL-N, Providence, RI 02912

²Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, 185 Meeting Street, Providence, RI 02912

Corresponding author: Anne C. Hart

Office: 401-863-2822, Departmental Fax 401-863-1074, E-mail address: anne_hart@brown.edu

Mailing address and site of performance: Department of Neuroscience, Brown University, 185 Meeting Street, Box GL-N, Providence, RI 02912

No off-label or investigational use. No conflict of interest for any author.

Supported by National Institutes of Health NIH R01 GM078171 and NS055813

Objectives

Cross-species conservation of sleep-like behaviors predicts the presence of conserved molecular mechanisms underlying sleep. However, limited experimental evidence of conservation exists. Here, this prediction is tested directly.

Measurements and results

During lethargus, *Caenorhabditis elegans* spontaneously sleep in short bouts that are interspersed with bouts of spontaneous locomotion. We identified twenty six genes required for *Drosophila melanogaster* sleep. Twenty orthologous *C. elegans* genes were selected based on similarity. Their impact on *C. elegans* sleep and arousal during the last larval lethargus was assessed. The twenty most similar genes altered both the quantity of sleep and arousal thresholds. In eighteen cases, the direction of change was concordant with *Drosophila* studies published previously. Additionally, we delineated a conserved genetic pathway by which dopamine regulates sleep and arousal. In *C. elegans* neurons, G-alpha S, adenylyl cyclase, and PKA act downstream of D1 dopamine receptors to regulate these behaviors. Finally, a quantitative analysis of genes examined herein revealed that *C. elegans* arousal thresholds were directly correlated with amount of sleep during lethargus. But, bout duration varies little and was not correlated with arousal thresholds.

Conclusions

The comprehensive analysis presented here suggests that conserved genes and pathways are required for sleep in invertebrates and, likely, across the entire animal kingdom. The genetic pathway delineated in this study implicates G-alpha S and previously known genes downstream of dopamine signaling in sleep. Quantitative analysis of various components of quiescence suggests that interdependent or identical cellular and molecular mechanisms are likely to regulate both arousal and sleep entry.

Introduction

Sleep is observed in all animal species, but the genetic underpinnings of mammalian sleep remain largely unknown.¹ However, the recent identification of several genes and DNA polymorphisms affecting human sleep strongly suggests that this behavior is genetically regulated.²⁻⁴ Sleep in model organisms, including zebrafish, fruit flies, and nematodes, has been defined based on behavioral changes shared with human sleep. These changes include sleep-specific posture, spontaneous cessation of movement/feeding, increased arousal thresholds, rapid reversibility, and homeostatic response to sleep deprivation.⁴⁻⁶ While genetic studies have identified many genes required for sleep in these species,^{5, 7-11} generally the cross-species relevance of these genes has not been examined. Given the shared characteristics of sleep and the conservation of this behavior across animal kingdom, it seemed likely that the genes and pathways required for sleep are conserved. Studies in mice, fruit flies and nematodes have established a role for the epidermal growth factor (EGF) signaling pathway, protein kinase G (PKG), cyclic AMP (cAMP)/ protein kinase A (PKA)/ cAMP response element binding (CREB), dopamine, neuropeptides, and the Notch signaling pathway in sleep in two or more of these model systems.^{6, 12-26} Here, we more comprehensively address the conservation of genes required for sleep using two invertebrate model organisms.

Caenorhabditis elegans (*C. elegans*) sleep is called quiescence⁶ while *Drosophila melanogaster* sleep is called rest.²⁷ In *Drosophila*, rest is temporally coordinated with the solar light-dark cycle via circadian proteins such as Period, whose abundance oscillates rhythmically on a 24-hour cycle.²⁸ Under standard culture conditions, *C. elegans* quiescence occurs during a period called lethargus and is temporally coordinated with molting of the larval cuticle, which occurs at the end of each larval stage.⁶ Interestingly, the *C. elegans* Period ortholog, *lin-42*, regulates both cuticle molting and quiescence.²⁹ While the roles of circadian genes in sleep regulation does vary between these invertebrate species, the

role of other genes required for entry, maintenance, and exit from sleep may be more directly conserved. However, little experimental evidence exists to support this hypothesis.

In order to test this putative conservation, we focused on two well-known invertebrate model systems, *Drosophila* and *C. elegans*. Published *Drosophila* literature was reviewed to shortlist genes required for rest. The impact of orthologous *C. elegans* genes on quiescence and arousal was assessed. We found that *C. elegans* orthologs of *Drosophila* genes required for rest also altered both the amount of *C. elegans* quiescence and arousal during quiescence. Correlation analysis for the *C. elegans* genes tested herein revealed a simple and direct correlation between arousal thresholds and quiescence bout entry. Finally, the comprehensive analysis presented here allowed us to delineate a conserved genetic pathway acting downstream of the DOP-1 D1 receptors that regulates *C. elegans* quiescence.

Materials and Methods

***C. elegans* strains:** Strains and alleles examined are listed in Supplemental Table S1. Animals were reared on standard NGM media seeded with OP50 *E. coli* at 25°C and assayed at 24°C. Animals from the RNAi sensitized strain, HA2518, were transferred to RNAi feeding plates as L4 larvae and their progeny were assayed for quiescence and arousal defects. In some cases, the impact of RNAi was not seen until the F2 generation. To control for potential off-target impact of the RNAi knockdown, RNAi clones for non-overlapping regions of the gene of interest were tested. The only potential *inso-1(RNAi)* off-target gene is *csn-1*, encoding a component of a COP9 signalosome complex. RNAi studies used NGM plates with 1mM ampicillin and 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG).³⁰

Plasmids and transgenic strains: Transgenic animals were generated by microinjection of plasmids using standard methods.³¹ Co-injection markers were transcriptional fusions pGH#8³² *rab-3p::mCherry* (25ng/μl) or pPD48.33³³ *myo-2::gfp* (5ng/μl). Primers are listed in Supplemental Table S2. Results for at least two independent transgenic lines were determined and combined for each transgenic genotype reported.

dop-1p::gfp pHA#651, 4.3kb of sequence upstream the *dop-1* start codon was PCR amplified and cloned into the pPD49.26³⁴ *Xma*I site. GFP was cloned behind the *dop-1* promoter using KpnI and EcoRI sites.

dop-1p::kin-2(RNAi) pHA#652, 2.5 kb of *kin-2* genomic sequence containing exons was PCR amplified and cloned in reverse orientation behind the *dop-1* promoter of pHA#581, using KpnI and SacI sites.

dop-1p::crh-1 pHA#653, the *crh-1* cDNA sequence of 945 bp was PCR amplified and cloned behind the *dop-1* promoter of pHA#581, using NheI and KpnI sites.

Total quiescence: RNAi feeding clones described below:

crh-1(RNAi) feeding clone pHA#665 469 bp of the *crh-1* cDNA sequence was cloned into pL4440³⁰ vector using NheI and NcoI sites.

egl-4(RNAi) feeding clone pHA#666 2.1 kbp of the *egl-4* genomic sequence containing exons was PCR-amplified and cloned into pL4440 vector using the SphI site.

cul-3(RNAi) feeding clones pHA#657 and pHA#661 each contain a non-overlapping, PCR-amplified *cul-3* cDNA sequence cloned into XmaI site of pL4440.

lgc-38(RNAi) feeding clones pHA#654 and pHA#664 contain a non-overlapping PCR-amplified *lgc-38* genomic and cDNA sequence, respectively cloned into NheI and SacI sites of pL4440.

cya-1(RNAi) feeding clones pHA#662 and pHA#663 contain a non-overlapping, PCR-amplified *cya-1* cDNA sequence cloned into NheI and XmaI sites of pL4440 vector.

inso-1(RNAi) feeding clones pHA#659 and pHA#660 each contain a non-overlapping, PCR-amplified *inso-1* cDNA sequence cloned into the SpeI site of pL4440 vector.

jnk-1(RNAi) feeding clone pHA#667 1.8 kbp of the *jnk-1* genomic sequence containing exons was PCR-amplified and cloned into pL4440 vector using NheI and SacI sites.

Arousal threshold: RNAi feeding clones described below. The impact on quiescence of these clones was virtually identical to the impact of clones above that altered quiescence (Fig. 2A).

inso-1(RNAi) feeding clone pHA#656, 624 bp of *inso-1* genomic sequence containing exons was PCR-amplified and cloned into pL4440 vector using the SpeI site.

cul-3(RNAi) feeding clone pHA#657 (described above).

cya-1(RNAi) feeding clone pHA#655, 1.47 kbp of *cya-1* genomic sequence containing exons was PCR-amplified and cloned into pL4440 vector using NheI and XmaI sites.

lgc-38(RNAi) feeding clone pHA#654 (described above).

crh-1(RNAi) feeding clone pHA#665 (described above).

jnk-1(RNAi) feeding clone pHA#667 (described above).

Quiescence and arousal threshold assays: Quiescence during the L4-to-adult molting lethargus was determined using the previously published microfluidic chamber-based assays.¹⁹ Image subtraction was performed using the previously published Matlab analysis program.¹⁹ A single pixel change above camera noise was the metric for movement detection (lack of quiescence). Cameras used for image acquisition were AxioCam ICc1 (Zeiss) (pixel size: 4.65µm x 4.65 µm), AxioCam MRc Rev3 (Zeiss) (pixel size: 6.45µm x 6.45 µm) and Stingray F201c (Allied Vision Technologies) (pixel size: 4.4µm x 4.4 µm). Unless indicated otherwise, early-L4 stage larvae were loaded into 1mmx4mm microfluidic chambers 4-6 hrs prior to lethargus and the activity was monitored for 12hrs. HA2518 animals were loaded in the microfluidic chambers as black-dot/late-L4 stage larvae, just as lethargus began and activity was

recorded for 8hrs. Images were acquired at 10 seconds interval. Unlike the previous analysis presented in Singh *et al*, in the present study quiescence onset time was defined as a time point after which the fractional quiescence remains >0.1 for at least 20 minutes. The quiescence exit time was defined as the time point after which the fractional quiescence had remained <0.1 for at least 20 minutes. The total quiescence was calculated by counting the number of images where no pixel change was detected (compared to the previous image, above camera noise) between quiescence onset and exit time points. Each image subtraction that did not detect motion represents 10 seconds of quiescence. Quiescence onset time was subtracted from quiescence exit time to determine the duration of lethargus quiescence and was reported in hours. The number of quiescent bouts that were 10s or longer was determined and total number of bouts occurring between the quiescence onset and exit time was reported. Bout frequency was calculated by dividing total number of bouts by lethargus duration for each animal. The duration of all the quiescent bouts occurring between the quiescence onset and exit time was measured and the average bout duration was calculated and reported in seconds. We calculated bout durations for each animal and then averaged the results of all animals in a genotype/treatment. Other bout metrics were calculated similarly. *goa-1(lf)* and *gsa-1(gf)* animals had profoundly decreased total quiescence. As a result, the fractional quiescence cutoff of >0.1 for 20 minutes was never exceeded. Therefore, these animals have total quiescence=0 mins, bout number =0, and lethargus duration=0 hrs (see Supplemental Table S3). Since lethargus duration for *goa-1(lf)* and *gsa-1(gf)* animals was equal to zero, bout number and bout duration for these genotypes could not be determined and were excluded from the correlation analysis presented in Fig. 4. Quiescence and arousal thresholds of *pde-4(ok1439)* animals were presented as *pde-4(ce268)* animals exhibited normal quiescence (total quiescence of *pde-4(ce268)* = 59±6 mins, p value = 0.27). *pde-4(ce268)* changes a single amino acid while *pde-4(ok1439)* deletes 1100 bp and likely results in a complete loss of function. For genotypes with most and least total quiescence, bout duration was re-examined at one frame per second interval. In this re-analysis bout

duration >1 second was measured and total quiescence determined. This analysis is described in Supplemental file S4.

For arousal threshold determination, response to body touch³⁶ was used; exceptions noted below. A hair was placed on the agar plate evenly seeded with OP50 bacteria and flexed to contact the animal behind the pharynx; locomotion within one second was scored as a response and percentage of animals failing to respond is reported. Arousal thresholds of HA2518 RNAi sensitized animals and *cnb-1(lf)* animals were performed using 1-octanol diluted to 60% concentration with ethanol. Average response time to 60% octanol of quiescent animals reared on experimental and control RNAi strains is reported in seconds. During quiescence, animals responded to touch or dilute 1-octanol by initiating backward locomotion. For touch, any immediate locomotion was scored as response. For dilute octanol, time to initiate locomotion was recorded. Octanol assays stopped at 20 seconds. For all genotypes, at least half of the trials were done by an observer blinded as to the genotypes/treatment. Control genotypes were always run in parallel (also blinded). Non-quiescent animals in lethargus were used as controls to determine the basal response to mechanical or chemosensory stimuli. Any immobile, non-feeding animal was considered quiescent for arousal threshold determination. For *gsa-1* and *goa-1*, rare quiescent animals identified during the appropriate developmental stage were scored for arousal. Arousal threshold results are presented for at least 3 independent trials with $N \geq 6$ per trial for each genotype tested. Following exceptions should be noted: *goa-1*: $N=1$ and *kin-2*: $N=5$ animals (due to scarcity of quiescent animals).

Statistics: Genes examined here are *C. elegans* orthologs of *Drosophila* genes that alter the quantity of *Drosophila* rest. Genes that impact circadian behavior or genes that only alter homeostasis were not included in the analysis. Orthologs were identified and similarity was assessed based on E values from BLAST at NCBI.³⁷ The Expect (E) values for all the *C. elegans* genes compared to *Drosophila* genes are listed in Supplemental Table S5. The least similar was RCN-1 ($E=3e-27$). At least 8 or 20 animals were

tested for each quiescence or arousal threshold determination, respectively. For each of the twenty genes examined, independent sets of wild type or control animals were tested in parallel and these were used to determine significance by student's t test (Supplemental Table S3 and figure legends). Because multiple comparisons were made, we calculated the false discovery rate (FDR).^{38, 39} The FDR q-value was set at the standard 0.05 to ascribe significance (Supplemental Table S5). A similar statistical analysis was performed for arousal thresholds (Supplemental Table S5).

Results

Lethargus quiescence requires the function of conserved genes

The *Drosophila* rest and *C. elegans* quiescence share behavioral characteristics of sleep including cessation of movement, altered arousal and homeostatic regulation.^{4-6, 40} The presence of these common hallmarks suggests that conserved processes regulate sleep in these invertebrate species. For clarity herein, we will refer to sleep in *Drosophila* as rest and sleep in *C. elegans* as quiescence.

To determine if orthologs of *Drosophila* genes required for rest also play a conserved role in *C. elegans* quiescence, we first identified genes that altered the quantity of *Drosophila* rest. Genes involved in circadian regulation or solely in homeostasis were excluded from this analysis. *C. elegans* orthologs of *Drosophila* genes were identified based on protein sequence similarity using BLAST (Supplemental Table S5) and the closest *C. elegans* ortholog was examined in this study. No unambiguous orthologs could be found for six *Drosophila* genes: *Hyperkinetic*,⁴¹ *Fragile X mental retardation*,⁴² *homer*,⁴³ *Relish*,⁴⁴ *sleepless*,⁴⁵ and *Regulator of cyclin A*.⁴⁶ Publicly available deletion, missense, or nonsense alleles were obtained for the remaining *C. elegans* genes, whenever possible (Fig. 1, Supplementary Table S1). If no alleles were available, then RNA interference (RNAi) by feeding was employed to test the impact of genes on *C. elegans* quiescence (see Fig. 2). *C. elegans* L4-to-adult lethargus quiescence was assessed using a microfluidic chamber-based assay. Total time spent in quiescence in minutes, which will be

referred to as total quiescence, was reported (Fig. 1, see Methods and ¹⁹). All genes tested altered *C. elegans* quiescence. For eighteen genes, the change in *C. elegans* total quiescence was as would be predicted from *D. melanogaster* literature. When disruption of *Drosophila* gene function increased rest, disruption of the *C. elegans* ortholog increased total quiescence, - and *vice versa*. Exceptions were the *C. elegans* ortholog of *Drosophila basket* and *cyclin A* (described below). Results are summarized in Supplemental Table S3, S6, presented graphically in Figures 1 and 2, and detailed in the following paragraphs.

Shaker and the calcineurin pathway (*shk-1*, *tax-6*, *cnb-1*, *rcn-1*): *Drosophila Shaker* encodes a voltage-gated potassium channel, which regulates membrane repolarization and synaptic signaling.⁴⁷ Disruption of *Drosophila Shaker* function reduces rest.⁴⁸ Consistent with this, decreased total quiescence was observed in animals carrying a deletion allele of the *C. elegans Shaker* ortholog, *shk-1* (Fig. 1A). The calcineurin signaling pathway has also been implicated in *Drosophila* rest.^{49, 50} Calcineurin is a Ca²⁺/calmodulin dependent protein phosphatase complex containing both a catalytic subunit and a regulatory subunit. Disruption of either catalytic subunit, *Pp2B-14D* or *CanA-14F*, or either regulatory subunit, *CanB* or *CanB2*, results in reduced *Drosophila* rest. Additionally, loss of the calcineurin regulator *sarah* reduces *Drosophila* rest.⁴⁹ In *C. elegans*, the catalytic and regulatory subunits of calcineurin are encoded by *tax-6* and *cnb-1*, respectively^{51, 52}; the calcineurin regulator *sarah* is encoded by *rcn-1*.⁵³ We found that loss of *rcn-1*, *cnb-1*, or *tax-6* function decreased *C. elegans* quiescence. Combined, these results suggest that *Shaker* potassium channels and calcineurin play conserved roles in sleep across species (Fig. 1A).

Neurotransmitter systems (*dop-1*, *dat-1*, *ser-4*, *unc-25*, *unc-49*, *lgc-38*, *tbh-1*, *tdc-1*): In *Drosophila*, loss of dopamine signaling increases rest; therefore dopamine is said to have a wake-promoting effect.^{54, 55} For example, increasing synaptic dopamine by disrupting the dopamine transporter *fumin* significantly

decreases *Drosophila* rest.¹² Conversely, loss of the D1 dopamine receptor *DopR* increases *Drosophila* rest.⁵⁵ The *C. elegans* orthologs of *fumin* and *DopR* genes are *dat-1* and *dop-1*, respectively.^{56, 57} Consistent with the *Drosophila* results, *dat-1* loss of function decreased total quiescence, and as expected, the *dop-1* loss of function increased total quiescence (Fig. 1B).

In *Drosophila*, loss of serotonin signaling decreases rest; serotonin is said to have a sleep-promoting effect.⁵⁸ Loss of the *Drosophila* serotonin receptor 5-HT1A decreases rest.⁵⁸ In *C. elegans*, *ser-4*⁵⁹ encodes the protein most similar to *Drosophila* 5-HT1A. Consistent with *Drosophila* results, *ser-4* loss of function decreased total quiescence in *C. elegans* (Fig. 1B).

GABA signaling also impacts *Drosophila* rest. Overexpression of the only *Drosophila* GABA_A receptor, *Resistant to dieldrin* (*Rdl*), in LNvs (large ventral lateral neurons) results in increased rest.²⁴ In addition, inhibiting GABAergic neurons via ectopic *Shaw* channel expression decreases *Drosophila* rest.⁶⁰ To test the impact of GABA signaling on *C. elegans* quiescence, the role of GABA receptor function in quiescence was determined. Similarity searching suggested that in *C. elegans* both *unc-49* and *lgc-38* are orthologs of *Drosophila* *Rdl*.⁶¹ Neither *unc-49(lf)* nor *lgc-38(RNAi)* alone impacted quiescence (total quiescence = 60±3 mins, p value = 0.66 or total quiescence = 66±5 mins, p value = 0.26, respectively and Fig. 2D) possibly due to redundant function of the *unc-49* and *lgc-38*. Since no alleles were available for *lgc-38*, the gene was knocked down using two non-overlapping RNAi clones in *unc-49(lf)* animals and quiescence determined. *unc-49(lf)* animals reared on *lgc-38(RNAi)* feeding clones had significantly decreased total quiescence compared to those reared on empty vector control (Fig. 2C). Complete loss of GABA biosynthesis does not cause lethality in *C. elegans*. Therefore, we tested animals completely lacking the function of *unc-25*, which encodes glutamic acid decarboxylase.⁶² These animals have decreased total quiescence, which is consistent with the impact of GABA loss on *Drosophila* rest (Fig. 1C).

Octopamine, a biogenic amine, also regulates *Drosophila* sleep.⁶³ Loss of octopamine biosynthesis pathway genes, tyrosine decarboxylase (*Tdc*) and tyramine beta hydroxylase (*TβH*), increases rest.⁶³ *tdc-1* and *tbh-1* are the *C. elegans* orthologs of *Drosophila* *Tdc* and *TβH* genes.⁶⁴ Consistent with the *Drosophila* sleep defects observed in *Tdc* and *TβH* flies, *tdc-1(lf)* and *tbh-1(lf)* *C. elegans* had increased total quiescence (Fig. 1C).

cAMP signaling (*acy-1*, *pde-4*, *kin-2*, *crh-1*): The duration of *Drosophila* sleep is inversely proportional to cAMP signaling, PKA activity, and CREB activity.⁶⁵ The *Drosophila* *rutabaga* and *dunce* genes encode adenylyl cyclase and phosphodiesterase, respectively, and they antagonistically regulate cAMP levels. Loss of *rutabaga* increases rest, while loss of *dunce* decreases rest.⁶⁵ *C. elegans* cAMP levels are regulated by two orthologous genes: *acy-1* and *pde-4*.⁶⁶ Complete loss of *acy-1* causes lethality. Therefore, a previously described *acy-1* gain of function allele was tested.⁶⁷ Either *acy-1* gain of function or *pde-4* loss of function likely increases cAMP levels.⁶⁸ We found that *acy-1(gf)* or *pde-4(lf)* animals exhibited decreased total quiescence, consistent with results in *Drosophila* (Fig. 1D).

Two conserved downstream components of cAMP signaling pathway are protein kinase A (PKA) and cAMP response element binding protein (CREB).⁶⁹ In *Drosophila*, over-expression of PKA pan-neuronally or with a heat shock promoter decreases rest.^{65, 70} Conversely, blocking *Drosophila* CREB activity increases rest.⁶⁵ *C. elegans* *kin-2* encodes the regulatory subunit of PKA and *kin-2* loss of function increases PKA catalytic activity.⁶⁷ As predicted from the *Drosophila* study,⁶⁵ *kin-2* loss of function decreased *C. elegans* total quiescence (Fig. 1D). *crh-1* encodes the *C. elegans* CREB ortholog.⁷¹ *crh-1(lf)* animals had increased total quiescence, which is consistent with CREB function in *Drosophila* rest (Fig. 1D). Combined, these results suggest that the cAMP signaling cascade plays a conserved role in *Drosophila* and *C. elegans* sleep.

Other signaling (*goa-1*, *jnk-1*): The *Drosophila* G_o protein is encoded by the *G_oα47a* gene and has been implicated in rest. Pan-neuronal expression of G_o increases rest, while RNAi knockdown of G_o decreases rest.⁷² In *C. elegans*, G_o is encoded by *goa-1*.⁷³ Complete loss of *goa-1* function decreased *C. elegans* quiescence supporting a conserved role for G_o protein in invertebrate sleep (Fig. 1E).

The *Drosophila* gene *basket* encodes a C-Jun N-terminal kinase that is required for rest.⁷⁴ RNAi knockdown of *basket* decreases rest.⁷⁴ In *C. elegans*, the closest ortholog of *Drosophila basket* is encoded by *jnk-1*.⁷⁵ *C. elegans jnk-1* loss of function caused increased total quiescence (Fig. 1E). This gene is one of only two *C. elegans* orthologs that had discordant results between species, which is discussed below.

BTB, cullin, and other signaling (*inso-1*, *cul-3*, *cya-1*): RNAi knockdown of either *Drosophila insomniac* or *Cullin-3* decreases rest. Consistent with this result, *insomniac* loss of function alleles also decreases rest.⁷⁶ In *C. elegans*, *cul-3* is the predicted ortholog of *Cullin-3*.⁷⁷ The *C. elegans* gene most similar to *insomniac* is C52B11.2, which we here name *inso-1*. These *C. elegans* genes have not been previously characterized. A *cul-3* mutant allele is not available. A previously uncharacterized *inso-1(gk344)* deletion allele exists, which removes the predicted first exon of *inso-1*, but this mutation did not alter quiescence (total quiescence = 50±3 mins, p value = 0.09). However, examination of *inso-1* cDNA clones suggested that translation in *inso-1(gk344)* animals might initiate at methionine (22) in exon 2 and might not dramatically alter gene function. Therefore, we employed RNAi knockdown to test the impact of these two genes on *C. elegans* quiescence. Expression of double stranded RNA channels in neurons increases *C. elegans* sensitivity to RNAi by feeding.⁷⁸ We confirmed that *egl-4(RNAi)* in this sensitized background resulted in decreased total quiescence (total quiescence = 42±6 mins, p value = 0.047), which is consistent with previous work using *egl-4(lf)* mutant animals.⁶ The impact of *cul-3*, the *C. elegans* ortholog of *Cullin-3*, on quiescence was tested using two independent RNAi clones. Knockdown of *cul-3*

by RNAi decreased total quiescence (Fig. 2A). Two independent *inso-1* RNAi clones were tested. One decreased quiescence compared to the control RNAi animals (Fig. 2A).⁷⁶ While decreased quiescence is consistent with the impact of the *Drosophila insomniac* on rest, potential off-target effects of *inso-1* RNAi cannot be ruled out. See methods.

Neuronal depletion of *Drosophila CycA*, which encodes cyclin A, decreases rest.⁴⁶ The *C. elegans* ortholog of cyclin A is *cya-1*.⁷⁹ As no deletion alleles were available for *cya-1*, RNAi knockdown in the sensitized background was employed to test impact on quiescence. RNAi knockdown of *cya-1* using two independent clones increased *C. elegans* total quiescence compared to animals raised on control bacteria containing the empty vector (Fig. 2A). C-Jun N-terminal kinase and cyclin A are the only two genes for which the change in sleep was inconsistent between the two species.

In summary, all twenty *C. elegans* genes tested here impacted quiescence. For eighteen genes, the impact on quiescence was consistent with predictions based on the *Drosophila* literature. Taken together, these results suggest deep conservation of genes required for invertebrate sleep.

Conserved genes also regulate arousal during *C. elegans* quiescence

Altered arousal is an essential component of sleep. *C. elegans* and *Drosophila* also exhibit increased arousal thresholds during sleep as their responsiveness to sensory stimuli is reduced.^{4, 5, 77} In *Drosophila*, disruption of *fumin*, *G α 47A* or *basket* gene function perturbs arousal thresholds during rest.^{12, 72} To independently assess the impact of these genes on arousal during *C. elegans* quiescence, we examined response using chemosensory and/or mechanosensory stimuli.^{19, 36} Failure to respond to touch with a hair during quiescence is presented for all *C. elegans* alleles and is reported as % failed to respond to touch (Fig. 3). Response to 1-octanol during quiescence is reported for RNAi studies (Fig. 2B, 2C, and 2E) as the requisite genetic background and/or bacterial strain causes hypersensitivity to

mechanosensory stimuli. Response to 1-octanol during quiescence is reported for *cnb-1(lf)* animals (Fig. 2F) as these animals exhibited defective response to mechanosensory stimuli. In RNAi studies, non-quiescent animals responded to dilute 1-octanol as quickly as control animals with one exception. Non-quiescent *inso-1(RNAi)* animals were defective in their response to dilute 1-octanol (8 ± 1 sec) compared to non-quiescent wild type animals (4 ± 0.1 sec), complicating analysis. Quiescent wild type animals increased their response time by $167 \pm 11\%$ but *inso-1(RNAi)* animals only increased their response time by $63 \pm 13\%$ suggesting that *inso-1* loss decreases arousal thresholds during quiescence (Fig. 2C).

All twenty genes that impacted *C. elegans* quiescence also altered arousal thresholds (Fig. 2B, 2C, 2E, 2F and 3, Supplemental Table S3, S6). We found decreased arousal thresholds in *dat-1* and *goa-1* loss of function animals, which is consistent with the decreased arousal thresholds of *Drosophila fumin* and *G α 47A* animals.^{12, 72} The only other comparison possible across species was for C-Jun N-terminal kinase orthologs. *C. elegans jnk-1* loss of function decreased arousal thresholds, while *Drosophila basket* RNAi is reported to have no impact on arousal during rest.⁷⁴ For the seventeen other orthologous genes, additional studies in *Drosophila* will be required to test if their function in arousal across species is conserved.

Assessing correlations between arousal and quiescence

Arousal thresholds likely reflect depth and/or quality of sleep. We considered the possibility that arousal might correlate with other metrics of *C. elegans* sleep, which include total time spent in quiescence, lethargus duration and/or quiescence bout characteristics (number and duration). Total quiescence, lethargus duration, and bout number are intrinsically correlated based on the methods used to detect and calculate *C. elegans* quiescence. Therefore, we only considered possible correlations of arousal with bout duration and bout number. The impact of *C. elegans* genes tested above on these parameters is reported in Supplemental Table S3. For *lgc-38(RNAi)*, *cul-3(RNAi)*, *inso-1(RNAi)*, *cya-*

1(RNAi), and *cnb-1(lf)* animals, arousal thresholds were determined by chemosensory response (see Fig. 2B, 2C, 2E & 2F), which complicated comparison to the other fifteen genes, whose arousal thresholds were determined by mechanosensory response. Therefore, we excluded these five genes from the correlation analysis described below.

We noted that genotypes with increased arousal thresholds usually had both increased total quiescence, and *vice versa* (Supplemental Table S3). Total quiescence is a function of quiescent bout duration and bout number. One possibility is that arousal threshold changes might impact bout duration. We tested this by looking for correlation between these two parameters and found that bout duration did not directly correlate with arousal thresholds (Fig. 4A). This is not surprising because there is remarkably little variation in bout duration across genotype. Another possibility was that arousal threshold changes might impact bout number, as altered arousal might influence entry into quiescence. We found that bout number directly correlated with arousal thresholds (Fig. 4B). In summary, we conclude that mechanisms governing bout duration are likely independent from the mechanisms regulating arousal thresholds. Increased bout number reflects increased propensity to enter a quiescent state. Together, these results make it likely that common genetic pathways likely regulate arousal thresholds and quiescence bout entry.

Genetic pathway for dopamine regulation of *C. elegans* lethargus quiescence

Combined with the previously published *Drosophila* and murine studies, our cross-species comparison of genes required for sleep (Fig. 1B) confirms that decreased dopamine signaling increases sleep (Fig. 1B and ^{12, 13, 80}). Past studies have determined the site of action of D1 receptor as well as the neuronal circuitry of dopamine signaling in *Drosophila* sleep.^{81, 82} However, little is known about the signaling cascade acting downstream of the D1 receptor in sleep. We undertook genetic and phenotypic

rescue experiments to assemble a signaling pathway downstream of dopamine regulating quiescence in *C. elegans*.

In humans, D1 dopamine receptors couple with various G α proteins, including G_s and G_o.⁸³ Loss of the DOP-1 D1 receptor increased quiescence (Fig. 1B, *dop-1(lf)*). The *C. elegans* G_o ortholog *goa-1* is unlikely to act downstream of the DOP-1 D1 receptor as loss of *goa-1* decreased total quiescence (Fig. 1E, *goa-1(lf)*). Therefore, the role of *C. elegans* G_s ortholog GSA-1 was assessed. Loss of *gsa-1* function causes lethality; therefore, a previously characterized *gsa-1(gf)* gain of function allele was tested.⁶⁶ *gsa-1* gain of function decreased total quiescence (Fig. 5A, *gsa-1(gf)*), which was consistent with increased signaling downstream of the DOP-1 D1 receptor. If *gsa-1* functions downstream of *dop-1*, then *gsa-1(gf)* should suppress the increased quiescence of *dop-1(lf)* animals. We found that *gsa-1(gf);dop-1(lf)* double mutant animals had decreased total quiescence compared to *dop-1(lf)* animals (Fig. 5A). Combined, these results suggest that *gsa-1* functions downstream of dopamine and D1 dopamine receptors in *C. elegans* quiescence.

The *C. elegans* adenylyl cyclase *acy-1* is known to act downstream of *gsa-1* in synaptic signaling and locomotion.⁶⁷ Similar to the *gsa-1(gf)* impact on quiescence, *acy-1(gf)* decreased total quiescence (Fig. 1D, *acy-1(gf)*). If *acy-1* functions downstream of *dop-1* and *gsa-1*, then *acy-1* gain of function should suppress the increased quiescence of *dop-1(lf)* animals. Indeed, *acy-1(gf); dop-1(lf)* double mutant animals have decreased quiescence compared to the *dop-1(lf)* animals (Fig. 5A). Together, these results suggest that both *gsa-1* and *acy-1* function downstream of or in parallel to *dop-1* D1 receptor in quiescence.

Next, we determined if the components of the cAMP signaling pathway, adenylyl cyclase, PKA, and CREB, function in the DOP-1 D1 receptor expressing neurons. The impact of adenylyl cyclase activity in DOP-1 D1 receptor expressing neurons was assessed first. Overexpression of adenylyl cyclase in DOP-

1 D1 receptor expressing neurons of wild type animals was sufficient to decrease quiescence (Fig. 5B). Additionally, over-expressing adenylyl cyclase in the D1 dopamine receptor expressing neurons of *dop-1(lf)* animals suppressed their quiescence defects (Fig. 5B). Increased PKA activity in *kin-2(lf)* animals resulted in decreased total quiescence (Fig. 1D, *kin-2(lf)*), which was concordant with the impact of *acy-1(gf)* on quiescence (Fig. 1D, *acy-1(gf)*). To determine if increased PKA activity in DOP-1 D1 receptor expressing neurons was sufficient to regulate quiescence, we knocked down *kin-2* in these neurons. As predicted, RNAi knockdown of *kin-2* in DOP-1 D1 receptor expressing neurons partially recapitulated the quiescence defects of *kin-2(lf)* animals (Fig. 5C). Additionally, RNAi knockdown of *kin-2* in DOP-1 D1 receptor expressing neurons of *dop-1(lf)* animals suppressed their quiescence defects (Fig. 5C). These results suggest that manipulating adenylyl cyclase and PKA activity in these neurons was sufficient to regulate quiescence.

We found that global loss of CREB function in *crh-1(lf)* animals increased quiescence (Fig. 1D, *crh-1(lf)*). We confirmed this by RNAi knockdown of *crh-1* (Supplemental Table S3). However, it is not clear if CRH-1 CREB functions in *C. elegans* DOP-1 D1 neurons. Simultaneous over-expression of two *crh-1* splice isoforms in DOP-1 D1 expressing neurons did not rescue the quiescence defects of *dop-1(lf)* animals (total quiescence of *dop-1(lf);dop-1p::crh-1(cDNA)* = 82 ± 7 mins, p value = 0.88). This might be due to insufficient phosphorylation of CRH-1 in *dop-1(lf)* animals.⁶⁹ Expression of these two *crh-1* splice isoforms did not ameliorate the total quiescence defects of *crh-1(lf)* animals (total quiescence of *crh-1(lf);dop-1p::crh-1(cDNA)* = 85 ± 7 mins, p value = 0.79). As described in other systems, CRH-1 may function downstream of other receptors or signaling cascade that may increase or decrease quiescence.^{71, 84} Combined, these results suggest that cAMP/adenylyl cyclase, and PKA function downstream of or parallel to dopamine and the D1 dopamine receptor in *C. elegans* neurons to regulate quiescence.

Discussion

Sleep has many shared characteristics and is ubiquitous across the animal kingdom suggesting that the genes and pathways required for this behavior are conserved. However, experimental evidence to support this hypothesis is limited. Previous work has independently identified roles in sleep for protein kinase G (PKG), neuropeptides, EGF and Notch signaling in both flies and nematodes.^{6, 14, 15, 17-19, 22, 23} To test molecular conservation of sleep more broadly, we took advantage of two well-defined invertebrate model systems.^{4, 5} A *Drosophila* literature survey allowed us to shortlist twenty-six genes required for rest. *C. elegans* orthologs of twenty of these genes were systematically tested for roles in quiescence and arousal. Disruption of nearly all of the orthologous *C. elegans* genes increased or decreased quiescence as would be predicted from *Drosophila* studies. We found few exceptions to this, which are discussed below. Although only one allele was tested for most genes in the cross-species comparison, the results presented here demonstrate that conserved genes play critical roles in sleep in two well-defined invertebrate species.

Diminished response to sensory stimuli is an essential feature of sleep. Twenty genes altered *C. elegans* total quiescence, allowing a comprehensive analysis of their impact on arousal. In every genotype, arousal thresholds during quiescence were significantly different from wild type *C. elegans*. As few *Drosophila* studies report changes in arousal during rest,^{12, 48, 74, 85} we can only speculate regarding cross species roles for these genes in arousal. For genes encoding the dopamine transporter or G_o, the impact on arousal thresholds was clearly conserved between these two species. It seems likely that many of the other genes described here will play a conserved role in arousal in *Drosophila* and other animals.

Only two of the genes tested here, *jnk-1* and *cya-1*, had a discordant impact on *C. elegans* and *Drosophila* sleep. RNAi knockdown of *Drosophila basket*, which encodes C-Jun N-terminal kinase, results

in decreased rest with no change in arousal thresholds.⁷⁴ This is not concordant with either RNAi knockdown or loss of function of the *C. elegans* *basket* ortholog *jnk-1*; both manipulations increased total *C. elegans* quiescence and decreased arousal thresholds (⁷⁴ and this study). Also, RNAi knockdown of *Drosophila* *cycA*, which encodes cyclin A, decreases rest, but knockdown of *C. elegans* *cya-1* increased quiescence (⁴⁶ and this study). It is unclear why these two genes had discordant effects on sleep in flies and nematodes. Possibly, their downstream targets differ in these species or these genes regulate different aspects of sleep in *C. elegans* and *Drosophila*. Previous work established that PKG and EGF play concordant roles in invertebrate sleep,^{6, 17, 18} but the Notch pathway impacts different aspects of *C. elegans* and *Drosophila* sleep.^{15, 19} We conclude that most genes play conserved roles in sleep across species, but a subset of genes play species-specific roles.

Given the large number of genes characterized here, we were able to examine for the first time possible correlations between quiescence metrics, such as bout number, bout duration, arousal thresholds, and others. Examination of *C. elegans* quiescence suggested that genotypes with increased total quiescence also had an increased number of quiescent bouts and had an increased lethargus duration, - and *vice versa* (Supplementary Table S3). There was little change in bout duration or frequency despite the large number of genotypes tested. In other words, increased quiescence generally occurs when mutant animals have more quiescent bouts and spend more time in lethargus. Therefore, common genetic mechanisms likely impact arousal thresholds and bout entry. However, bout duration may be independently regulated from arousal thresholds and may require other genes that will be identified in future studies.

We did discover a simple and relatively direct correlation between arousal thresholds and total time spent in quiescence for fifteen genes. In other words, most of the genetic perturbations that decreased total quiescence also resulted in lowered arousal thresholds. And, genetic perturbations that

increased quiescence were almost always associated with higher arousal thresholds. Based on these observations, we considered two possibilities. Low arousal thresholds might result in an inability either to establish or to maintain quiescence during a bout. Counter intuitively, genotypes with aberrantly high arousal thresholds did not have increased bout duration, but they did have increased number of quiescent bouts late in lethargus and had increased lethargus duration. High arousal thresholds may indicate an inappropriate propensity to establish the quiescent state, resulting in more quiescent bouts. Low arousal thresholds may reflect an inability to enter the quiescent state. Alternatively, genes altering arousal thresholds may regulate lethargus duration. However, it is unclear if quiescence changes are the cause or the result of altered arousal thresholds. Also, it is unclear if quiescence and arousals are independently regulated by these fifteen genes. This may be clarified in future studies that directly examine molecular and cellular mechanisms underlying arousal and sleep.

Dopamine plays a conserved role in sleep in flies and mice.^{12, 13, 54} Here, we extend this role to *C. elegans*. However, the signaling pathways downstream of dopamine and dopamine receptors relevant to sleep were largely unknown. To establish a coherent model of dopamine signaling in sleep, we assembled a genetic pathway based on phenotype and confirmed action in a subset of *C. elegans* neurons using a combination of genetic epistasis and rescue experiments. Double mutant analysis suggested that G_s and adenylyl cyclase function in the dopamine signaling pathway, downstream of the DOP-1 D1 receptors. Additionally, activation of PKA or overexpression of adenylyl cyclase (ACY), only in neurons expressing DOP-1 D1 receptors- was sufficient to rescue the *dop-1* quiescence defects (Fig. 5) suggesting that ACY and PKA act in parallel or downstream of D1 receptors. DOP-1 D1 receptors are widely expressed in the *C. elegans* nervous system;⁸⁶ further studies will be needed to determine precisely which *dop-1* expressing neurons are critical for sleep. It is likely that dopamine signaling *via* G_s , adenylyl cyclase, PKA, and perhaps CREB are required for sleep in all animals.

Results presented here confirm that the genetic underpinnings of sleep are broadly conserved in invertebrates. Some vertebrate orthologs of genes required for invertebrate sleep have been implicated previously in sleep.^{1, 4} However, the connections between these genes and signaling pathways in vertebrate sleep remain mysterious. Invertebrate model systems can be used to rapidly identify and delineate these conserved pathways.

Figure legends

Fig. 1. Lethargus quiescence requires the function of conserved genes. L4-to-adult lethargus quiescence was determined. Total quiescence was reported in minutes. Error bars represent standard error of the mean (s.e.m.). Numbers inside or above the bar indicate sample size. For each gene examined, independent sets of wild type control animals were tested in parallel and these were used to determine significance by student's t test. p value < 0.05* <0.01** and < 0.001*** versus wild type. Results were grouped by pathway/function and controls were pooled for concise presentation. Control is *dop-1* promoter driving *gfp* (*dop-1p::gfp*). (A) Loss of calcineurin signaling components (*rcn-1*, *cnb-1*, *tax-6*) or Shaker potassium channel (*shk-1*) function decreased total quiescence. (B) Loss of dopamine transporter (*dat-1*) or serotonin receptor (*ser-4*) function decreased total quiescence. Loss of D1 dopamine receptor (*dop-1*) function increased total quiescence. (C) Loss of GABA synthesis (*unc-25*) decreased total quiescence. Loss of genes required for octopamine biosynthesis increased total quiescence: tyrosine decarboxylase (*tdc-1*) and dopamine beta hydroxylase (*tbh-1*). (D) Adenylyl cyclase (*acy-1*) gain of function or loss of phosphodiesterase (*pde-4*) decreased total quiescence. Loss of CREB (*crh-1*) function increased total quiescence. Loss of protein kinase A regulatory subunit (*kin-2*) function decreased total quiescence. (E) Loss of G_o (*goa-1*) decreased total quiescence. Loss of C-Jun N-terminal kinase (*jnk-1*) function increased total quiescence.

Fig. 2. Lethargus quiescence and altered arousal during sleep requires the function of conserved

genes. For RNAi feeding experiments, HA2158 animals expressing the SID-1 double stranded RNA channel in neurons were used.⁷⁸ Total quiescence during L4-to-adult lethargus reported in minutes. Arousal thresholds were determined by measuring the time to respond to 60% dilute 1-octanol. Response time is reported in seconds. Error bars represent standard error of the mean (s.e.m.). Grey and black bars represent independent clones in 2A and 2D. Numbers inside the bar indicate sample size. For each gene examined, independent sets of control animals were tested in parallel and these were used to determine significance by student's t test. p value < 0.05* <0.01** and < 0.001*** versus control(RNAi), *unc-49(lf)*, or wild type. Results were grouped and controls were pooled for concise presentation. (A) RNAi knockdown using two independent clones of insomniac (*inso-1*) and cullin-3 (*cul-3*) decreased total quiescence with one exception. See Methods for possible off-target gene for *inso-1(RNAi)*. RNAi knockdown of cyclin A (*cya-1*) increased total quiescence. (B) RNAi knockdown of *egl-4* and *cul-3* in sensitized HA2158 animals lowered arousal thresholds during quiescence compared to their respective controls. RNAi knockdown of *cya-1* increased arousal thresholds. (C) Non-quiescent *inso-1(RNAi)* animals were mildly 1-octanol sensing defective. Quiescent wild type animals' response time to dilute 1-octanol increased by 167±11%. However, *inso-1(RNAi)* animals increased their response time by 63±13% suggesting that these animals maintain inappropriately low arousal thresholds during quiescence. (D) RNAi knockdown of the *lgc-38* GABA A receptor decreased quiescence in animals lacking the *unc-49* GABA A receptor. (E) RNAi knockdown of the *lgc-38* in *unc-49(lf)* animals also decreased arousal thresholds. (F) Non-quiescent *cnb-1(lf)* animals were defective in their response to mechanosensory stimuli, but responded normally to dilute 1-octanol. Therefore, dilute 1-octanol response was used to demonstrate heightened arousal thresholds during quiescence.

Fig. 3. Conserved genes regulate arousal. Arousal thresholds were determined by counting the number of quiescent animals responding to body touch. Percent of animals failing to respond to body touch is

reported in all the figure panels. Error bars represent standard error of the mean (s.e.m.). Non-quiescent animals rarely failed to respond to body touch with a failure rate of 0-10% for all genotypes. Due to their profound quiescence defects, very few quiescent *goa-1(lf)* (n=2) and *kin-2(lf)* (n=5) animals were found, even when >100 animals were sampled. For each gene examined, independent sets of wild type control animals were tested in parallel and these were used to determine significance by student's t test. p value < 0.05* <0.01** and <0.001*** versus wild type. Results were grouped by pathway/function and controls were pooled for concise presentation. (A) Loss of either *C. elegans* calcineurin signaling genes (*rcn-1* and *tax-6*) or loss of *C. elegans* Shaker (*shk-1*) decreased arousal thresholds. (B) Loss of dopamine transporter (*dat-1*) or serotonin receptor (*ser-4*) function decreased arousal thresholds. Loss of D1 dopamine receptor (*dop-1*) function increased arousal thresholds. (C) Loss of GABA synthesis gene (*unc-25*) decreased arousal thresholds. Loss of octopamine biosynthesis genes tyrosine decarboxylase (*tdc-1*) and dopamine beta hydroxylase (*tbbh-1*) increased arousal thresholds. (D) Loss of phosphodiesterase (*pde-4*) or protein kinase A regulatory subunit (*kin-2*) function decreased arousal thresholds. Gain of adenylyl cyclase (*acy-1*) function decreased arousal thresholds. Loss of CREB increased arousal thresholds (*crh-1(lf)*=63±0.6 versus parallel wild type control=51±0.4, p value=0.005). (E) Loss of G_o (*goa-1*) or C-Jun N-terminal kinase (*jnk-1*) function decreased arousal thresholds.

Fig. 4. Assessing correlations between arousal and quiescence. Quiescence bout number and bout duration during lethargus were examined versus arousal thresholds for genotypes tested in Figures 1 and 3. Wild type is represented as an empty circle. Genes implicated in the dopamine signaling pathway are represented as green and orange rhombi, which represent decreased and increased quiescence, respectively. (A) No correlation was observed between quiescent bout duration and arousal thresholds ($r=-0.051$, p value=0.580). (B) A direct correlation was detected between quiescent bout numbers and arousal thresholds. Correlation coefficient $r=0.887$, p value=1e-08. See methods for statistical details.

Fig. 5. Genetic pathway for dopamine regulation of *C. elegans* lethargus quiescence. Genetic epistasis and rescue experiments delineate a dopamine signaling pathway functioning in the regulation of *C. elegans* quiescence. Total quiescence was reported in minutes. Error bars represent standard error of the mean (s.e.m.). Numbers inside or above the bar indicate sample size. Results were grouped and controls were pooled for concise presentation. Control is *dop-1* promoter driving *gfp* (*dop-1p::gfp*). (A) Gain of Gs (*gsa-1*) function or *dop-1;gsa-1* double mutant animals had decreased total quiescence. *dop-1;acy-1* double mutant animals had decreased total quiescence. (B) Expression of *acy-1* cDNA in DOP-1 D1 receptor expressing neurons decreased total quiescence in wild type animals. Expression of *acy-1* cDNA in DOP-1 D1 receptor expressing neurons of *dop-1(lf)* animals suppressed the quiescence defect. (C) RNAi knockdown of *kin-2* in DOP-1 D1 receptor expressing neurons decreased total quiescence in wild type animals. In *dop-1(lf)* animals, RNAi knockdown of *kin-2* in DOP-1 D1 receptor expressing neurons suppressed the quiescence defect. *p value< 0.05 **p value< 0.01 ***p value<0.005 versus wild type in A; versus transgenic control for each genotype in B and C. (D) A model for dopamine signaling mediated regulation of quiescence. Dopamine binding to D1 dopamine receptor DOP-1 activates downstream signaling *via* Gs (*gsa-1*). This results in the activation of adenylyl cyclase (*acy-1*) that produces cAMP. cAMP activates protein kinase A (PKA) to promote wakefulness. Consistently, knockdown of the PKA regulatory subunit (*kin-2*) in D1 receptor expressing neurons decreased quiescence. It is possible that phosphodiesterase 4 (*pde-4* in *C. elegans*) regulates cAMP levels in this pathway.

Abbreviations

ACY, adenylyl cyclase

AMP, adenosine monophosphate

BLAST, basic local alignment search tool

BTB, BR-C, ttk and bab

cAMP, cyclic AMP

cnb-1, calcineurin B

CREB, cyclic AMP response element binding

crh-1, CREB homolog

cul-3, cullin

cya-1, cyclin A

dat-1, dopamine transporter

DNA, deoxyribonucleic acid

dop-1, dopamine receptor

EGF, epidermal growth factor

egl-4, egg laying defective

FDR, false discovery rate

GABA, gamma-aminobutyric acid

gf, gain of function

GFP, green fluorescent protein

goa-1, G protein, O, alpha subunit

gsa-1, G protein, subunit alpha or G-alpha S

inso-1, insomniac-1

IPTG, isopropyl β -D-1-thiogalactopyranoside

jnk-1, Jun N-terminal kinase

kin-2, protein kinase

L4, larvae stage 4

lf, loss of function

lgc-38, ligand gated ion channel

myo-2, myosin heavy chain structural genes

NGM, nematode growth medium

pde-4, phosphodiesterase

PKA, protein kinase A

PKG, protein kinase G or cGMP dependent kinase

rab-3, RAB family

rcn-1, regulator of calcineurin

RNA, ribonucleic acid

RNAi, RNA interference

ser-4, serotonin/octopamine receptor family

shk-1, shaker family of potassium channels

tax-6, abnormal chemotaxis

tbh-1, tyramine beta hydroxylase

tdc-1, tyrosine decarboxylase

unc-25, uncoordinated

unc-49, uncoordinated

Acknowledgments

This work was supported by the National Institutes of Health NIH R01 GM078171 and NS055813 (A.C.H.). We thank Dr. Huiyan Huang, Altar Sorkac and other members of the laboratory for helpful discussion and feed-back. We are grateful for advice and/or reagents from the *C. elegans* knockout consortia and numerous members of the *C. elegans* community, including Dr. Mark Alkema. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

References

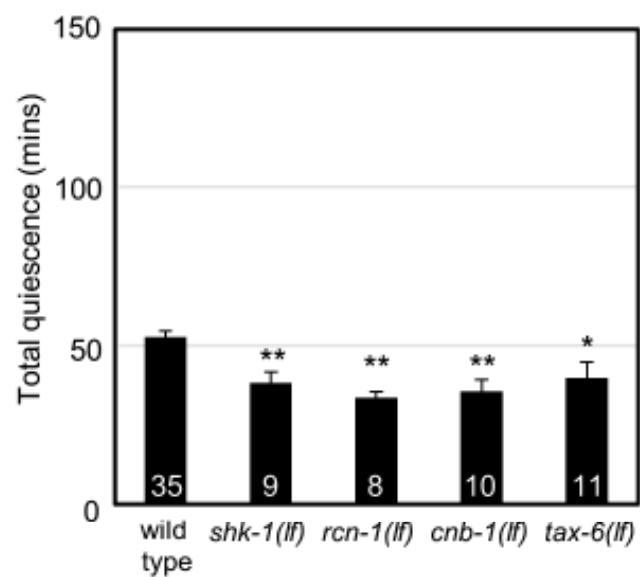
1. Caylak E. The genetics of sleep disorders in humans: narcolepsy, restless legs syndrome, and obstructive sleep apnea syndrome. *Am J Med Genet A* 2009;149A:2612-26.
2. Retey JV, Adam M, Honegger E, et al. A functional genetic variation of adenosine deaminase affects the duration and intensity of deep sleep in humans. *Proc Natl Acad Sci U S A* 2005;102:15676-81.
3. Kotronoulas G, Stamatakis A, Stylianopoulou F. Hormones, hormonal agents, and neuropeptides involved in the neuroendocrine regulation of sleep in humans. *Hormones (Athens)* 2009;8:232-48.
4. Sehgal A, Mignot E. Genetics of sleep and sleep disorders. *Cell* 2011;146:194-207.
5. Nelson MD, Raizen DM. A sleep state during *C. elegans* development. *Curr Opin Neurobiol* 2013.
6. Raizen DM, Zimmerman JE, Maycock MH, et al. Lethargus is a *Caenorhabditis elegans* sleep-like state. *Nature* 2008;451:569-72.
7. Yokogawa T, Marin W, Faraco J, et al. Characterization of sleep in zebrafish and insomnia in hypocretin receptor mutants. *PLoS Biol* 2007;5:e277.
8. Allada R, Siegel JM. Unearthing the phylogenetic roots of sleep. *Curr Biol* 2008;18:R670-R9.
9. Shaw P, Ocorr K, Bodmer R, Oldham S. *Drosophila* aging 2006/2007. *Exp Gerontol* 2008;43:5-10.
10. Crocker A, Sehgal A. Genetic analysis of sleep. *Genes Dev* 2010;24:1220-35.
11. Bushey D, Cirelli C. From genetics to structure to function: exploring sleep in *Drosophila*. *Int Rev Neurobiol* 2011;99:213-44.
12. Kume K, Kume S, Park SK, Hirsh J, Jackson FR. Dopamine is a regulator of arousal in the fruit fly. *J Neurosci* 2005;25:7377-84.
13. Dzirasa K, Ribeiro S, Costa R, et al. Dopaminergic control of sleep-wake states. *J Neurosci* 2006;26:10577-89.
14. Langmesser S, Franken P, Feil S, Emmenegger Y, Albrecht U, Feil R. cGMP-dependent protein kinase type I is implicated in the regulation of the timing and quality of sleep and wakefulness. *PLoS One* 2009;4:e4238.
15. Seugnet L, Suzuki Y, Merlin G, Gottschalk L, Duntley SP, Shaw PJ. Notch signaling modulates sleep homeostasis and learning after sleep deprivation in *Drosophila*. *Curr Biol* 2011;21:835-40.
16. Donlea J, Leahy A, Thimman MS, et al. Foraging alters resilience/vulnerability to sleep disruption and starvation in *Drosophila*. *Proc Natl Acad Sci U S A* 2012;109:2613-8.
17. Van Buskirk C, Sternberg PW. Epidermal growth factor signaling induces behavioral quiescence in *Caenorhabditis elegans*. *Nat Neurosci* 2007;10:1300-7.
18. Foltenyi K, Greenspan RJ, Newport JW. Activation of EGFR and ERK by rhomboid signaling regulates the consolidation and maintenance of sleep in *Drosophila*. *Nat Neurosci* 2007;10:1160-7.
19. Singh K, Chao MY, Somers GA, et al. *C. elegans* Notch signaling regulates adult chemosensory response and larval molting quiescence. *Curr Biol* 2011;21:825-34.
20. Belfer SJ, Chuang HS, Freedman BL, et al. *Caenorhabditis*-in-drop array for monitoring *C. elegans* quiescent behavior. *Sleep* 2013;36:689-98G.
21. Graves LA, Hellman K, Veasey S, Blendy JA, Pack AI, Abel T. Genetic evidence for a role of CREB in sustained cortical arousal. *J Neurophysiol* 2003;90:1152-9.
22. Turek M, Lewandrowski I, Bringmann H. An AP2 Transcription Factor Is Required for a Sleep-Active Neuron to Induce Sleep-like Quiescence in *C. elegans*. *Curr Biol* 2013;23:2215-23.
23. Choi S, Chatzigeorgiou M, Taylor KP, Schafer WR, Kaplan JM. Analysis of NPR-1 reveals a circuit mechanism for behavioral quiescence in *C. elegans*. *Neuron* 2013;78:869-80.
24. Parisky KM, Agosto J, Pulver SR, et al. PDF cells are a GABA-responsive wake-promoting component of the *Drosophila* sleep circuit. *Neuron* 2008;60:672-82.
25. Iwanir S, Tramm N, Nagy S, Wright C, Ish D, Biron D. The microarchitecture of *C. elegans* behavior during lethargus: homeostatic bout dynamics, a typical body posture, and regulation by a central neuron. *Sleep* 2013;36:385-95.

26. Nagy S, Wright C, Tramm N, Labello N, Burov S, Biron D. A longitudinal study of *Caenorhabditis elegans* larvae reveals a novel locomotion switch, regulated by Galphas signaling. *Elife* 2013;2:e00782.
27. Hendricks JC, Finn SM, Panckeri KA, et al. Rest in *Drosophila* is a sleep-like state. *Neuron* 2000;25:129-38.
28. Sehgal A, Joiner W, Crocker A, et al. Molecular analysis of sleep: wake cycles in *Drosophila*. *Cold Spring Harb Symp Quant Biol* 2007;72:557-64.
29. Monsalve GC, Van Buskirk C, Frand AR. LIN-42/PERIOD controls cyclical and developmental progression of *C. elegans* molts. *Curr Biol* 2011;21:2033-45.
30. Timmons L, Fire A. Specific interference by ingested dsRNA. *Nature* 1998;395:854.
31. Mello CC, Kramer JM, Stinchcomb D, Ambros V. Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J* 1991;10:3959-70.
32. Frokjaer-Jensen C, Davis MW, Ailion M, Jorgensen EM. Improved Mos1-mediated transgenesis in *C. elegans*. *Nat Methods* 2012;9:117-8.
33. Fire A, Harrison SW, Dixon D. A modular set of lacZ fusion vectors for studying gene expression in *Caenorhabditis elegans*. *Gene* 1990;93:189-98.
34. Fire A, Kondo K, Waterston R. Vectors for low copy transformation of *C. elegans*. *Nucleic Acids Res* 1990;18:4269-70.
35. Bringmann H. Agarose hydrogel microcompartments for imaging sleep- and wake-like behavior and nervous system development in *Caenorhabditis elegans* larvae. *J Neurosci Methods* 2011;201:78-88.
36. Chalfie M, Sulston JE, White JG, Southgate E, Thomson JN, Brenner S. The neural circuit for touch sensitivity in *Caenorhabditis elegans*. *J Neurosci* 1985;5:956-64.
37. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990;215:403-10.
38. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. *Behav Brain Res* 2001;125:279-84.
39. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)* 1995;57:289-300.
40. Shaw PJ, Cirelli C, Greenspan RJ, Tononi G. Correlates of sleep and waking in *Drosophila melanogaster*. *Science* 2000;287:1834-7.
41. Bushey D, Huber R, Tononi G, Cirelli C. *Drosophila* Hyperkinetic mutants have reduced sleep and impaired memory. *J Neurosci* 2007;27:5384-93.
42. Bushey D, Tononi G, Cirelli C. The *Drosophila* fragile X mental retardation gene regulates sleep need. *J Neurosci* 2009;29:1948-61.
43. Naidoo N, Ferber M, Galante RJ, et al. Role of Homer proteins in the maintenance of sleep-wake states. *PLoS One* 2012;7:e35174.
44. Williams JA, Sathyanarayanan S, Hendricks JC, Sehgal A. Interaction between sleep and the immune response in *Drosophila*: a role for the NFkappaB relish. *Sleep* 2007;30:389-400.
45. Koh K, Joiner WJ, Wu MN, Yue Z, Smith CJ, Sehgal A. Identification of SLEEPLESS, a sleep-promoting factor. *Science* 2008;321:372-6.
46. Rogulja D, Young MW. Control of sleep by cyclin A and its regulator. *Science* 2012;335:1617-21.
47. Papazian DM, Schwarz TL, Tempel BL, Jan YN, Jan LY. Cloning of genomic and complementary DNA from Shaker, a putative potassium channel gene from *Drosophila*. *Science* 1987;237:749-53.
48. Cirelli C, Bushey D, Hill S, et al. Reduced sleep in *Drosophila* Shaker mutants. *Nature* 2005;434:1087-92.
49. Nakai Y, Horiuchi J, Tsuda M, et al. Calcineurin and its regulator sra/DSCR1 are essential for sleep in *Drosophila*. *J Neurosci* 2011;31:12759-66.
50. Tomita J, Mitsuyoshi M, Ueno T, et al. Pan-neuronal knockdown of calcineurin reduces sleep in the fruit fly, *Drosophila melanogaster*. *J Neurosci* 2011;31:13137-46.

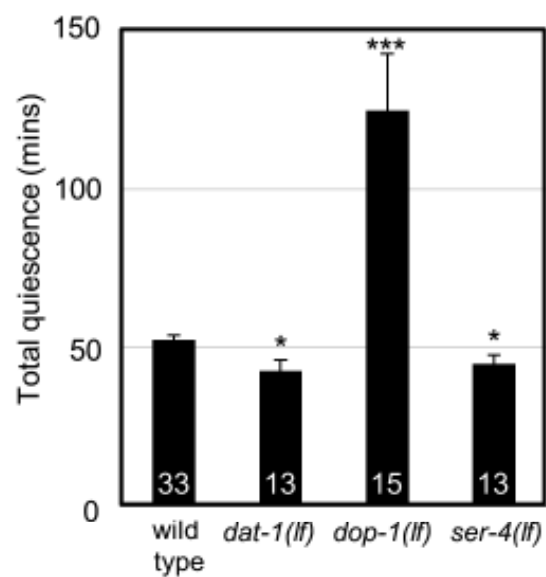
51. Bandyopadhyay J, Lee J, Lee J, et al. Calcineurin, a calcium/calmodulin-dependent protein phosphatase, is involved in movement, fertility, egg laying, and growth in *Caenorhabditis elegans*. *Mol Biol Cell* 2002;13:3281-93.
52. Song HO, Ahnn J. Calcineurin may regulate multiple endocytic processes in *C. elegans*. *BMB Rep* 2011;44:96-101.
53. Lee JI, Dhakal BK, Lee J, et al. The *Caenorhabditis elegans* homologue of Down syndrome critical region 1, RCN-1, inhibits multiple functions of the phosphatase calcineurin. *J Mol Biol* 2003;328:147-56.
54. Andretic R, van Swinderen B, Greenspan RJ. Dopaminergic modulation of arousal in *Drosophila*. *Curr Biol* 2005;15:1165-75.
55. Lebestky T, Chang JS, Dankert H, et al. Two different forms of arousal in *Drosophila* are oppositely regulated by the dopamine D1 receptor ortholog DopR via distinct neural circuits. *Neuron* 2009;64:522-36.
56. Jayanthi LD, Apparsundaram S, Malone MD, et al. The *Caenorhabditis elegans* gene T23G5.5 encodes an antidepressant- and cocaine-sensitive dopamine transporter. *Mol Pharmacol* 1998;54:601-9.
57. Suo S, Sasagawa N, Ishiura S. Identification of a dopamine receptor from *Caenorhabditis elegans*. *Neurosci Lett* 2002;319:13-6.
58. Yuan Q, Joiner WJ, Sehgal A. A sleep-promoting role for the *Drosophila* serotonin receptor 1A. *Curr Biol* 2006;16:1051-62.
59. Carre-Pierrat M, Baillie D, Johnsen R, et al. Characterization of the *Caenorhabditis elegans* G protein-coupled serotonin receptors. *Invert Neurosci* 2006;6:189-205.
60. Agosto J, Choi JC, Parisky KM, Stilwell G, Rosbash M, Griffith LC. Modulation of GABAA receptor desensitization uncouples sleep onset and maintenance in *Drosophila*. *Nat Neurosci* 2008;11:354-9.
61. Bamber BA, Beg AA, Twyman RE, Jorgensen EM. The *Caenorhabditis elegans* unc-49 locus encodes multiple subunits of a heteromultimeric GABA receptor. *J Neurosci* 1999;19:5348-59.
62. Jin Y, Jorgensen E, Hartweg E, Horvitz HR. The *Caenorhabditis elegans* gene unc-25 encodes glutamic acid decarboxylase and is required for synaptic transmission but not synaptic development. *J Neurosci* 1999;19:539-48.
63. Crocker A, Sehgal A. Octopamine regulates sleep in *Drosophila* through protein kinase A-dependent mechanisms. *J Neurosci* 2008;28:9377-85.
64. Alkema MJ, Hunter-Ensor M, Ringstad N, Horvitz HR. Tyramine Functions independently of octopamine in the *Caenorhabditis elegans* nervous system. *Neuron* 2005;46:247-60.
65. Hendricks JC, Williams JA, Panckeri K, et al. A non-circadian role for cAMP signaling and CREB activity in *Drosophila* rest homeostasis. *Nat Neurosci* 2001;4:1108-15.
66. Reynolds NK, Schade MA, Miller KG. Convergent, RIC-8-dependent G α signaling pathways in the *Caenorhabditis elegans* synaptic signaling network. *Genetics* 2005;169:651-70.
67. Schade MA, Reynolds NK, Dollins CM, Miller KG. Mutations that rescue the paralysis of *Caenorhabditis elegans* ric-8 (synembryn) mutants activate the G α (s) pathway and define a third major branch of the synaptic signaling network. *Genetics* 2005;169:631-49.
68. Charlie NK, Thomure AM, Schade MA, Miller KG. The Dunce cAMP phosphodiesterase PDE-4 negatively regulates G α (s)-dependent and G α (s)-independent cAMP pools in the *Caenorhabditis elegans* synaptic signaling network. *Genetics* 2006;173:111-30.
69. Delghandi MP, Johannessen M, Moens U. The cAMP signalling pathway activates CREB through PKA, p38 and MSK1 in NIH 3T3 cells. *Cell Signal* 2005;17:1343-51.
70. Joiner WJ, Crocker A, White BH, Sehgal A. Sleep in *Drosophila* is regulated by adult mushroom bodies. *Nature* 2006;441:757-60.
71. Kimura Y, Corcoran EE, Eto K, et al. A CaMK cascade activates CRE-mediated transcription in neurons of *Caenorhabditis elegans*. *EMBO Rep* 2002;3:962-6.

72. Guo F, Yi W, Zhou M, Guo A. Go signaling in mushroom bodies regulates sleep in *Drosophila*. *Sleep* 2011;34:273-81.
73. Lochrie MA, Mendel JE, Sternberg PW, Simon MI. Homologous and unique G protein alpha subunits in the nematode *Caenorhabditis elegans*. *Cell Regul* 1991;2:135-54.
74. Takahama K, Tomita J, Ueno T, Yamazaki M, Kume S, Kume K. Pan-neuronal knockdown of the c-Jun N-terminal Kinase (JNK) results in a reduction in sleep and longevity in *Drosophila*. *Biochem Biophys Res Commun* 2012;417:807-11.
75. Kawasaki M, Hisamoto N, Iino Y, Yamamoto M, Ninomiya-Tsuji J, Matsumoto K. A *Caenorhabditis elegans* JNK signal transduction pathway regulates coordinated movement via type-D GABAergic motor neurons. *EMBO J* 1999;18:3604-15.
76. Stavropoulos N, Young MW. Insomniac and Cullin-3 regulate sleep and wakefulness in *Drosophila*. *Neuron* 2011;72:964-76.
77. Kipreos ET, Lander LE, Wing JP, He WW, Hedgecock EM. *cul-1* is required for cell cycle exit in *C. elegans* and identifies a novel gene family. *Cell* 1996;85:829-39.
78. Calixto A, Chelur D, Topalidou I, Chen X, Chalfie M. Enhanced neuronal RNAi in *C. elegans* using SID-1. *Nat Methods* 2010;7:554-9.
79. Kreutzer MA, Richards JP, De Silva-Udawatta MN, et al. *Caenorhabditis elegans* cyclin A- and B-type genes: a cyclin A multigene family, an ancestral cyclin B3 and differential germline expression. *J Cell Sci* 1995;108 (Pt 6):2415-24.
80. Liu Q, Liu S, Kodama L, Driscoll MR, Wu MN. Two dopaminergic neurons signal to the dorsal fan-shaped body to promote wakefulness in *Drosophila*. *Curr Biol* 2012;22:2114-23.
81. Ueno T, Tomita J, Tanimoto H, et al. Identification of a dopamine pathway that regulates sleep and arousal in *Drosophila*. *Nat Neurosci* 2012;15:1516-23.
82. Seugnet L, Suzuki Y, Vine L, Gottschalk L, Shaw PJ. D1 receptor activation in the mushroom bodies rescues sleep-loss-induced learning impairments in *Drosophila*. *Curr Biol* 2008;18:1110-7.
83. Kimura K, White BH, Sidhu A. Coupling of human D-1 dopamine receptors to different guanine nucleotide binding proteins. Evidence that D-1 dopamine receptors can couple to both Gs and G(o). *J Biol Chem* 1995;270:14672-8.
84. Johannessen M, Delghandi MP, Moens U. What turns CREB on? *Cell Signal* 2004;16:1211-27.
85. Wu MN, Koh K, Yue Z, Joiner WJ, Sehgal A. A genetic screen for sleep and circadian mutants reveals mechanisms underlying regulation of sleep in *Drosophila*. *Sleep* 2008;31:465-72.
86. Wani KA, Catanese M, Normantowicz R, Herd M, Maher KN, Chase DL. D1 dopamine receptor signaling is modulated by the R7 RGS protein EAT-16 and the R7 binding protein RSBP-1 in *Caenorhabditis elegans* motor neurons. *PLoS One* 2012;7:e37831.

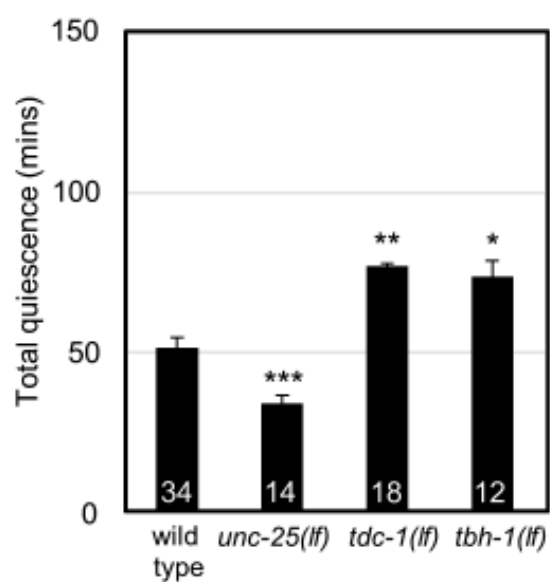
A



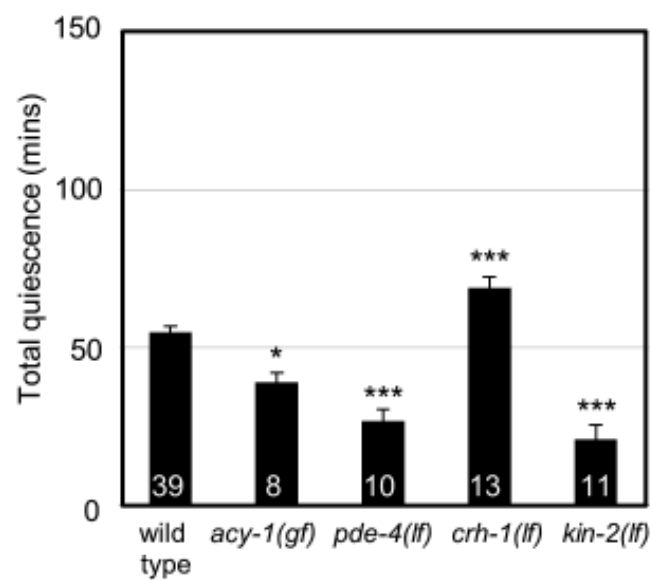
B



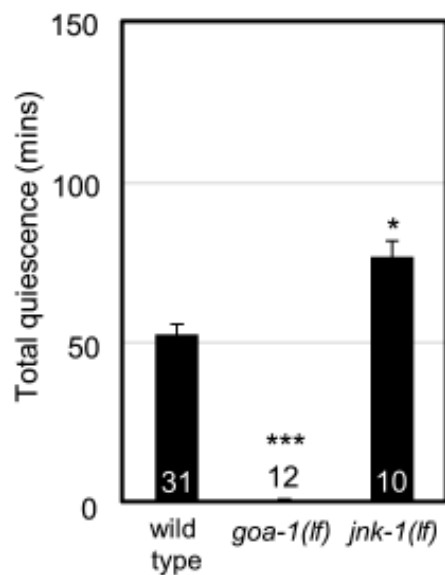
C



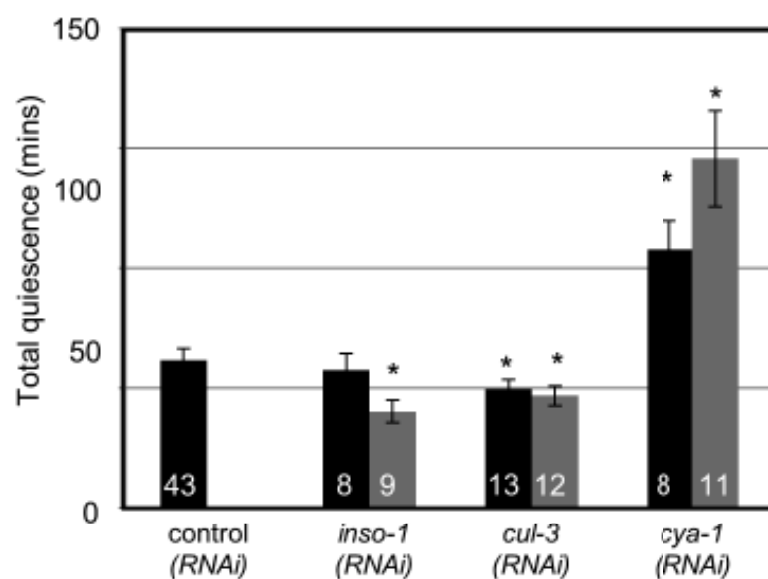
D



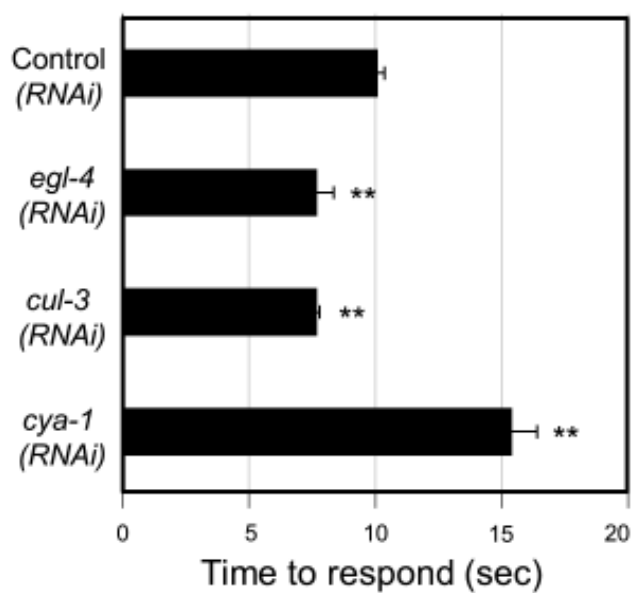
E



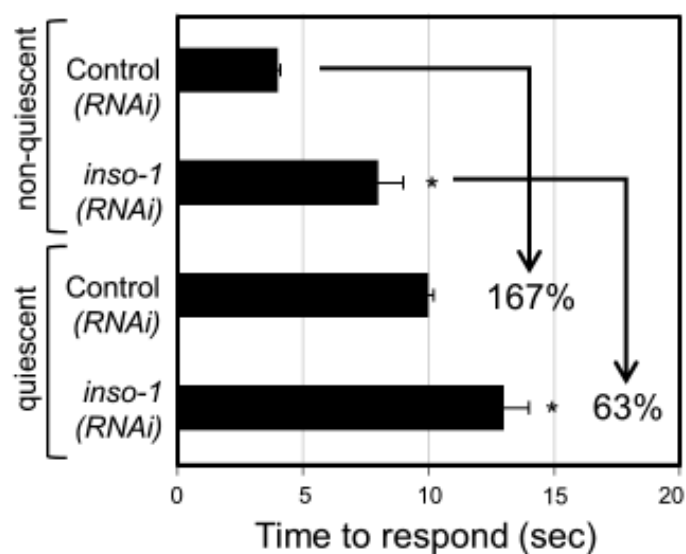
A



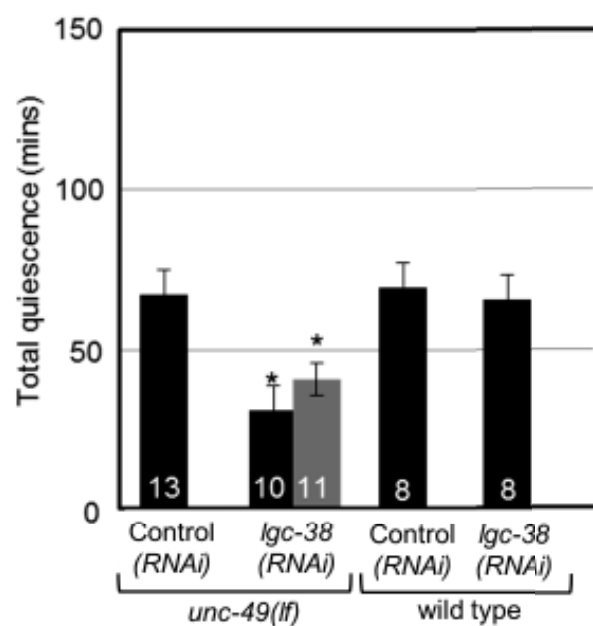
B



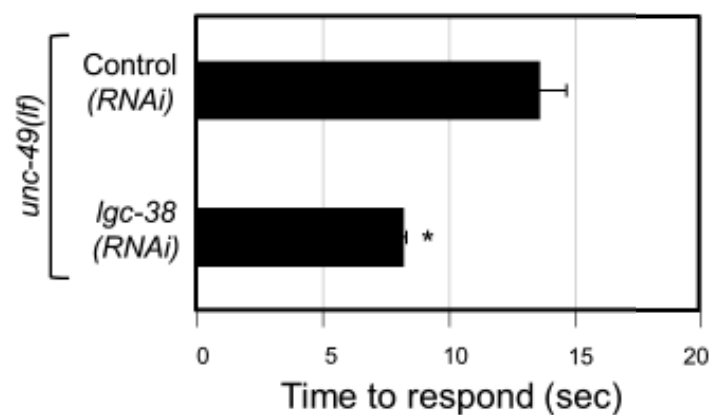
C



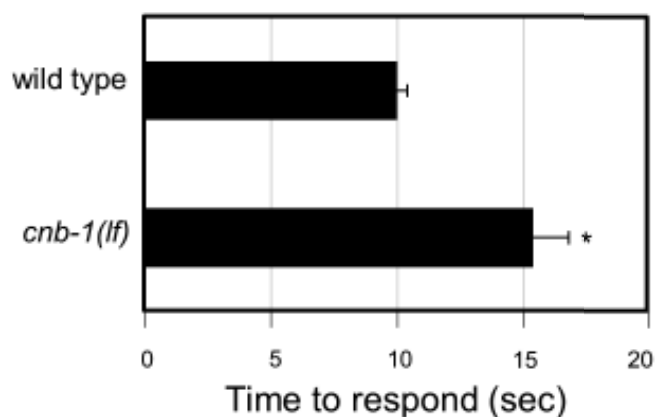
D



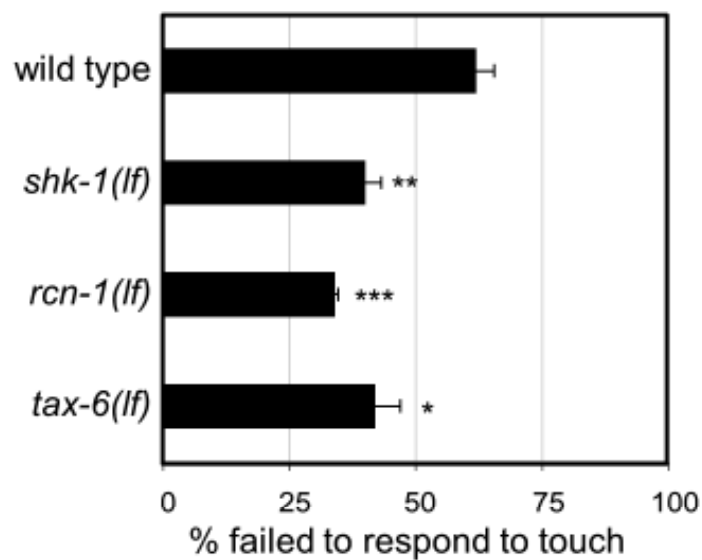
E



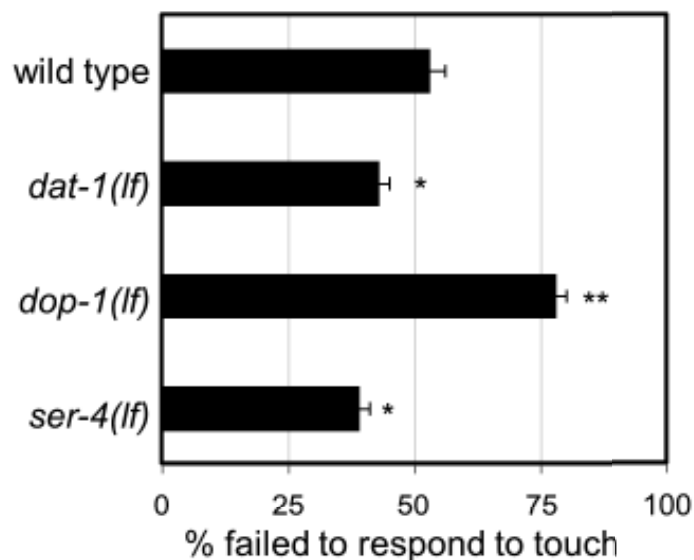
F



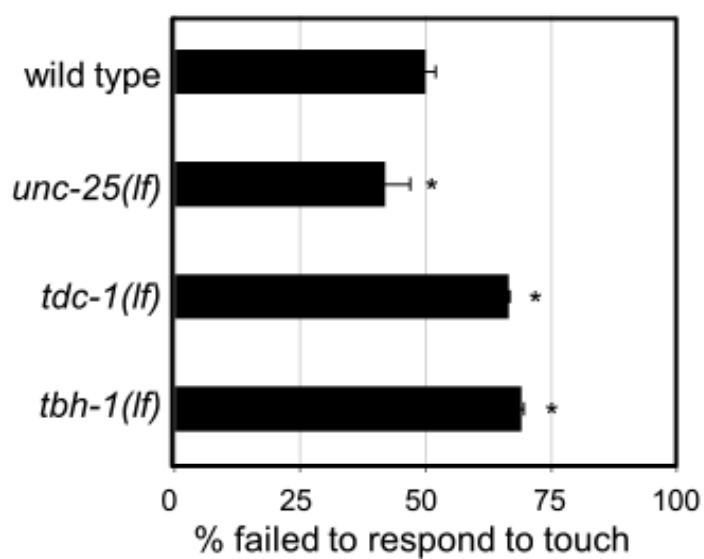
A



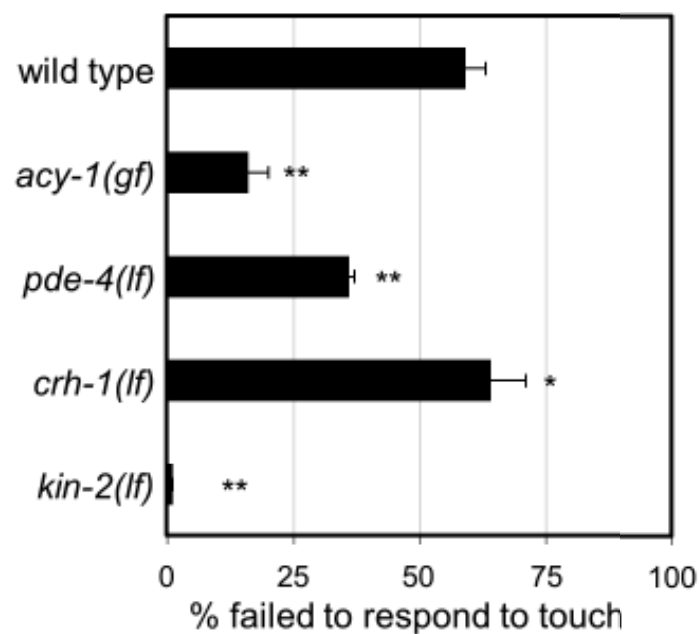
B



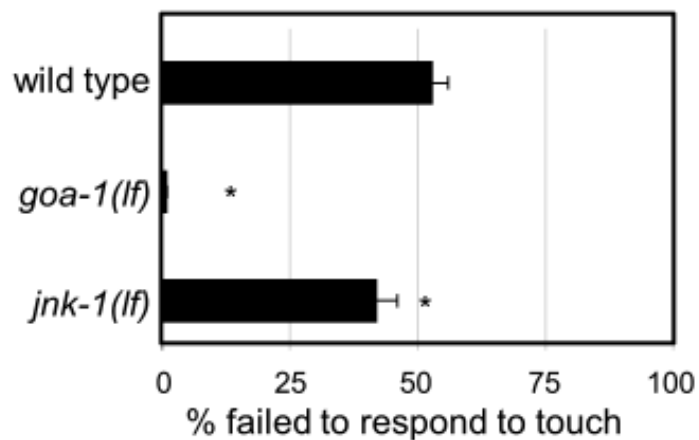
C

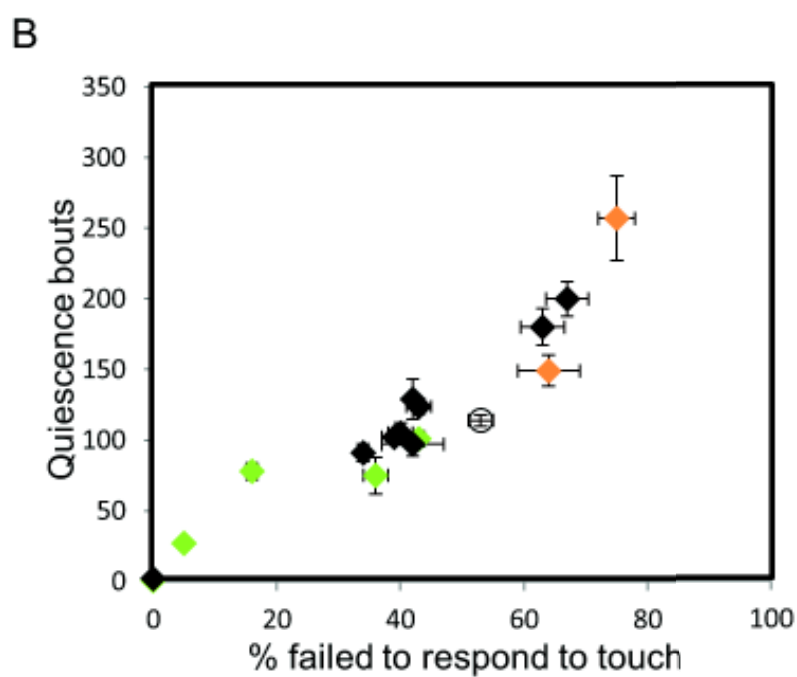
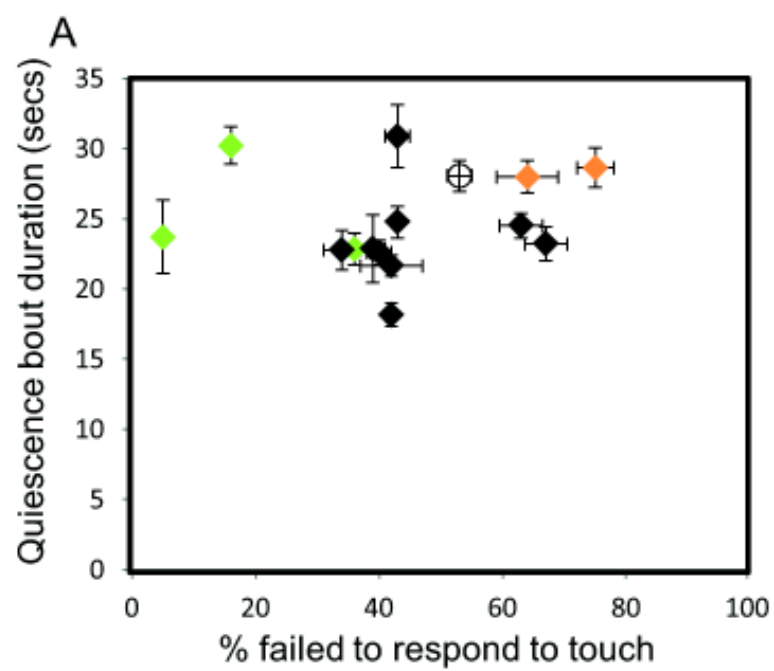


D

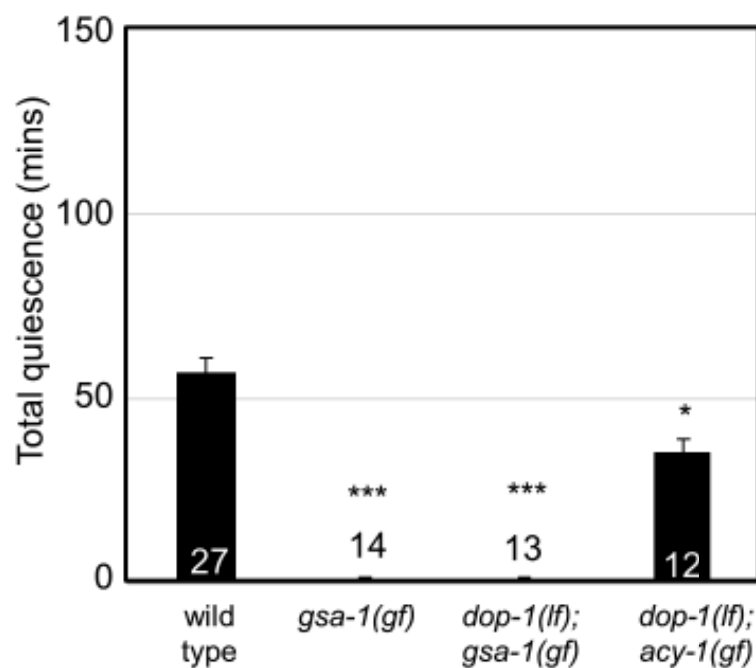


E

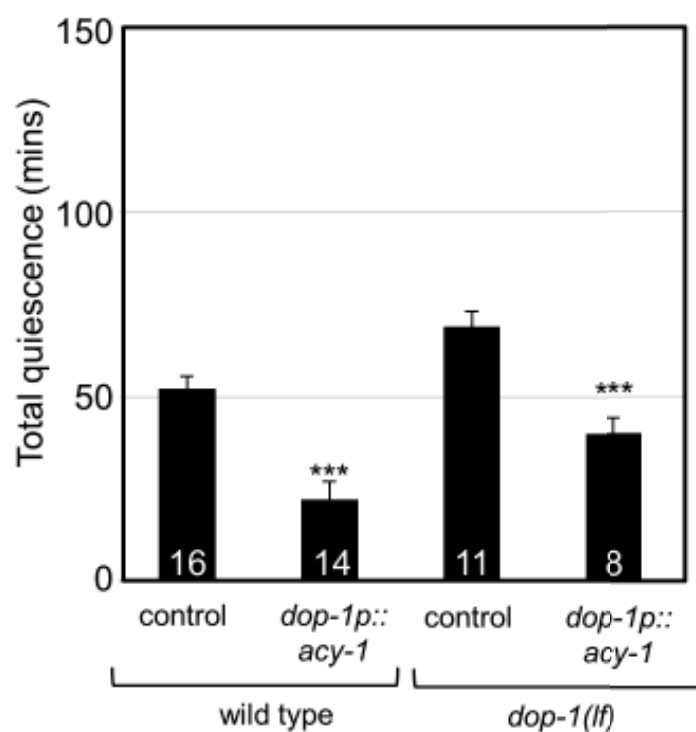




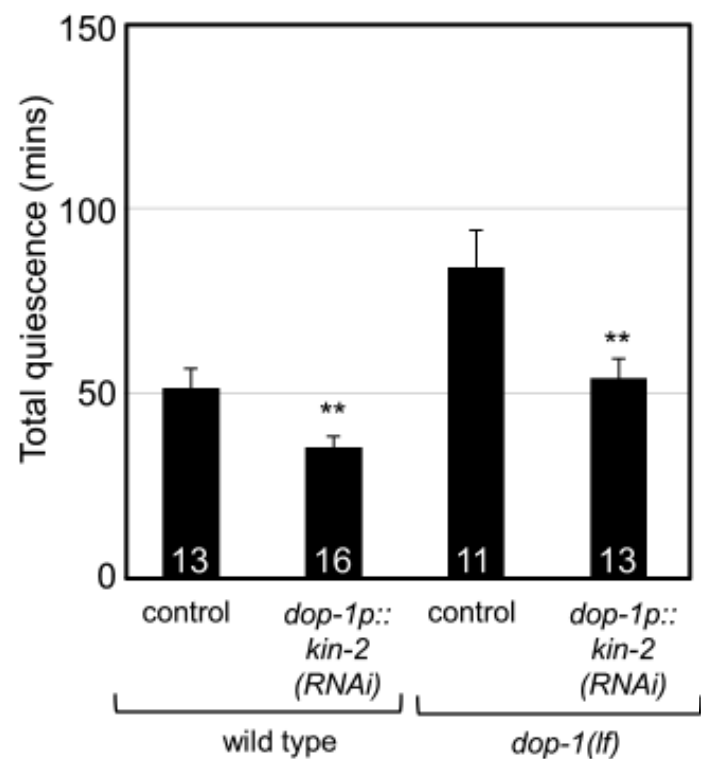
A



B



C



D

