Genetic Circuitry of Survival Motor Neuron, the Gene Underlying Spinal Muscular Atrophy

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Published Version</td>
<td>doi:10.1073/pnas.1301738110</td>
</tr>
<tr>
<td>Citable link</td>
<td><a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:12872186">http://nrs.harvard.edu/urn-3:HUL.InstRepos:12872186</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA">http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA</a></td>
</tr>
</tbody>
</table>
The genetic circuitry of Survival Motor Neuron, the gene underlying Spinal Muscular Atrophy

Anindya Sen1,*, Douglas N. Dimlich1,*, K. G. Guruharsha1,*, Mark W. Kankel1,*, Kazuya Hori1, Takakazu Yokokura1,2, Sophie Brachat2,*, Delwood Richardson2, Joseph Loureiro3, Rajeev Sivasankaran3, Daniel Curtis3, Lance S. Davidson4, Lee L. Rubin5, Anne C. Hart5, David Van Vactor1, and Spyros Artavanis-Tsakonas1. * Equal contribution

1. Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA. 2. Current affiliation: Okinawa Science and Technology Graduate University, 1919-1 Tancha, Onna-son, Okinawa 904-0495, Japan. 3. Developmental and Molecular Pathways, Novartis Institutes for Biomedical Research, 250 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA. 4. Current affiliation: Musculoskeletal Diseases, Novartis Institutes for Biomedical Research, Novartis Campus, CH-4002 Basel, Switzerland. 5. Department of Stem Cell and Regenerative Biology, Harvard Medical School, Boston, MA 02115, USA.

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

Submitted to Proceedings of the National Academy of Sciences of the United States of America

The clinical severity of the neurodegenerative disorder Spinal Muscular Atrophy (SMA) is dependent on the levels of functional Survival Motor Neuron (SMN) protein. Consequently, current strategies for developing treatments for SMA generally focus on augmenting SMN levels. To identify additional potential therapeutic avenues and achieve a greater understanding of SMN, we applied in vivo, in vitro, and in silico approaches to identify genetic and biochemical interactors of the Drosophila SMN homolog. We identified more than three hundred candidate genes that alter an SMN-dependent phenotype in vivo. Integrating the results from our genetic screens, large-scale protein interaction studies and bioinformatics analysis, we define a unique interactome for SMN which provides a knowledge base for a better understanding of SMA.

INTRODUCTION

Spinal Muscular Atrophy (SMA), the leading genetic cause of infant mortality, results from the partial loss of Survival Motor Neuron (SMN) gene activity (1). Numerous studies indicate that SMN functions as a central component of a complex which is responsible for the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs) [reviewed in (2)]. SMN is also reported to play additional roles, including mRNA trafficking in the axon (3). In humans, SMN is encoded by two nearly identical genes, SMN1 and SMN2, which are located on chromosome 5 (4). SMN2 differs from SMN1 in that only 10% of SMN2 transcripts produce functional SMN due to a single nucleotide polymorphism that results in inefficient splicing of exon 7 and translation of a truncated, unstable SMN protein (1, 5, 6). The clinical severity of SMA correlates with SMN2 copy number, which varies between individuals (7). As the small amount of functional SMN1 protein produced by each copy is capable of partially compensating for the loss of the SMN1 gene function, higher copy numbers of SMN2 typically result in milder forms of SMA. Therefore, genetic modifiers capable of increasing the abundance and/or specific activity of SMN hold promise as therapeutic targets.

The Drosophila genome harbors a single, highly conserved ortholog of SMN1/2, the Survival motor neuron (Snn) gene. SMN is essential for cell viability in vertebrates and Drosophila (8, 9). In Drosophila, zygotic loss of Snn function results in recessive larval lethality (not embryonic as might be expected) due to the rescue of early development by maternal contribution of Snn. The larval phenotype includes neuromuscular junction (NMJ) abnormalities that are reminiscent of those associated with the human disease, rendering this invertebrate organism an excellent system to model SMN biology (10-12). We previously described a genetic screen for modifiers of the lethal phenotype resulting from a complete loss of function Snn allele (13). This screen, though it probed half of the Drosophila genome, identified only a relatively small number of genes that affected the NMJ phenotype associated with Snn loss of function (13). In particular, it did not identify genes involved in snRNP biogenesis, the molecular functionality that is most clearly associated with SMN.

As the human disease state results from partial loss of SMN function, we reasoned that a screening paradigm using a hypomorphic Snn background (as opposed to a background that completely eliminates Snn function) would more closely resemble the genetic condition in SMA. Such a screen would therefore enhance our ability to detect novel elements of the Snn genetic network, and, consequently, add significantly to our efforts to both dissect the Snn genetic circuitry as well as identify potential clinically relevant targets with novel mode of action.

This complementary screen proved to be more sensitive than our previous screen and led to the identification of over 300 genetic interactors. Taking advantage of the recently established Drosophila Protein Interaction Map (DPiM) (14), we related the newly identified genetic interactors to the SMN protein interactome, producing an integrated Drosophila SMN biological network. Finally, the Drosophila Snn network was evaluated for its relevance to human biology by mapping Drosophila SMN network genes to their human homologs, and analyzing the human network using computational biology tools. The projection of the Drosophila Snn network derived from this study onto the human network derived from prior knowledge provides a rational basis for novel SMN functional hypotheses and network analysis.

Significance

Spinal Muscular Atrophy (SMA), the leading genetic cause of infant mortality, is a devastating neurodegenerative disease caused by reduced levels of Survival Motor Neuron (SMN) gene activity. Despite well-characterized aspects of the involvement of SMN in snRNP biogenesis, the gene circuitry affecting SMN activity remains obscure. Here, we use Drosophila as a model system to integrate results from large-scale genetic and proteomic studies, and bioinformatics analyses to define a unique SMN interactome to provide a basis for a better understanding of SMA. Such efforts not only help dissect the SMN biology but may also point to potential clinically relevant targets.

Reserved for Publication Footnotes

www.pnas.org — — PNAS | Issue Date | Volume | Issue Number | 1–77
intervention points that carry potential for so far unexplored clinical applications.

RESULTS

A genetic screen for modifiers of Smn-dependent lethality

We examined several Smn-RNAi strains to identify a hypomorphic Smn allele that could be used to model SMA in *Drosophila* more faithfully than alleles that completely abolish Smn function. We identified a transgenic strain, UAS-Smn-RNAi\_FL26B (FL26B) that displays a less severe phenotype than the allele used in our previous screen (13). Specifically, expression of FL26B under the control of tubulinGAL4 (tubGAL4::FL26B) results in late pupal lethality whereby approximately 50% of the pupae reach a more mature (pigmented) developmental stage prior to death (Figure 1A) than their less mature, unpigmented siblings.

We determined that this phenotype, measured by the ratio of pigmented to unpigmented pupae, is sensitive to Smn dosage, as reducing or increasing Smn copy number in the tubGAL4::FL26B genetic background resulted in enhancement or suppression, respectively (Figure 1A). In addition, the ability of wild type Smn (expressed by a UAS-Smn-GFP transgene) to rescue the lethality indicates that this phenotype does not result from off target RNAi effects. These results were corroborated using an independent Smn RNAi strain. Finally, we demonstrated that previously identified Smn modifiers altered the Smn RNAi phenotype in the expected fashion (Figure 1A). Together, these results demonstrate that the *tubGAL4::FL26B* phenotype is useful to detect changes in Smn functional activity and is thus suitable assay on which to base a modifier screen that will define and dissect the Smn genetic network.

Using this novel assay, we screened the Exelixis collection of genome-wide inser- 

![Fig. 1. Genetic modifiers of Smn using pupal lethality to screen the Exelixis collection of transposon insertions and their functional role to known Smn functions such as alternative splicing or SMA affected processes (neuronal and muscular). Enrichment significance is expressed as the $-\log_{10}$ (p-values).](image-url)
Fig. 2. The extended Drosophila genetic sub-network The sub-network of proteins connected to Smn and its genetic modifiers in the Drosophila Protein Interaction Map (DPiM). A total of 62 Smn genetic modifiers (diamonds with red border) are directly connected to 361 other proteins (circles), also known as first-degree neighbors through 3,800 interactions. The thickness of the gray lines connecting the proteins is proportional to the interaction score in the DPiM. Proteins belonging to individual clusters with GO term enrichment are shown with different colors. Proteins colored gray are part of clusters that are not enriched for any specific GO terms. Smn protein (indicated by an arrow head) itself is only connected to Nmdmc and shown as an interacting pair at the bottom.

several prediction algorithms show that out of the 340 Drosophila genes, at least 229 have human orthologs. Since a fraction of
these genes are represented by multiple paralogs, we identified a total of 322 human genes corresponding to the 229 Drosophila modifiers (see Materials and Methods, Dataset S1).

To assess the biological space covered by the novel Smn modifiers they were evaluated using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (17, 18) (http://david.abcc.ncifcrf.gov/), which identifies biological processes statistically overrepresented in the set of genetic modifiers. Analysis of the Drosophila SMN network with DAVID identified known SMN molecular activity (alternative splicing) and SMN-dependent processes ("neuron differentiation", "axon guidance", "axonogenesis", "muscle organ development" and "dendrite morphogenesis/development") (Figure 1C) (13, 19).

The predominant processes enriched in the modifier set reflect broad effects on morphogenesis and development reinforcing the notion that Smn depletion has pleiotropic consequences.

**Integration of Drosophila genetic and proteomic interactors**

To determine whether the genetic modifiers are intercon- nected through physical interactions, we placed them in the context of the recently generated Drosophila Protein Interaction Map (DPiM) (14). We first retrieved the set of proteins that co-purify with Drosophila Smn in DPiM, and asked whether any of the modifiers belong to this Smn sub-network. From all Drosophila proteins tested, 8 form protein complexes with Smn and interestingly one, NAD-dependent methylenetetrahydrofolate dehydrogenase (Nmdmc), is also a genetic modifier. Expansion of the Smn subnetwork to include proteins that form complexes with each of the eight members in turn identified 35 additional proteins. Some of these proteomic interactors have been previously associated with Smn function (e.g. Gemin2, Gemin3 and several snRNPs), and nearly half (20/43) are known to be involved in mRNA processing, a functionality closely linked to the documented biochemical role of SMN (Figure S1, Dataset S2).

To better understand the biological functions identified by the Smn modifiers, we extracted the Drosophila protein complexes, which include the Smn genetic modifiers in DPiM. We found that 62 of the proteins corresponding to genetic modifiers passed the stringent statistical criteria necessary for inclusion in the DPiM interactome (Materials and Methods) which includes only the top 5% of the total interactions scored in the coAP/MS analysis. These 62 proteins were associated with 50 separate Markov clusters, a statistical definition of significantly associated proteins (20), each of which may define a functional protein complex (see Figure 2 for the sub network of complexes identified by this analysis). Of these 50, we focused our attention on the 24 that are enriched for specific biological functions based on Gene Ontology (GO) terms – a system that provides a controlled vocabulary of terms for describing gene cellular and molecular functions (21).

The majority of these clusters harbor a single modifier, but 9 contained two or more (Table S3).

Inspecting the GO biological function terms of these 24 clusters (annotated in Figure 2), we find that many contain annotated functions previously linked to Smn activity including RNA metabolism ("RNA splicing", "mRNA binding") [reviewed in (3)], translation control ("eIF3 complex") (22-25), endocytosis ("Snap/SNARE complex") (26, 27), and protein transport ("fotillin complex") (28-30). Importantly, several genetic modifiers fall within protein complexes whose functions have not been previously associated with Smn function. These include complexes with phosphatase and kinase activities as well as those involved in intracellular signaling ("Toll signaling pathway" and "Hedgehog signaling") (Figure 3B). Two independent loss-of-function alleles of the Ecr4/abarm gene suppress Smn dependent
Fig. 4. Extended human Smn genetic sub-network Adding SMN1/2 first-degree neighbors to the network shown in Figure 3 generated an extended sub-network. In this interactome, 151 human orthologs of Drosophila Smn genetic modifiers (green circles) directly or indirectly connected to SMN1/SMN2. A total of 48 modifiers are connected to SMN1/SMN2 through 71 additional intermediate proteins (pink circles) from literature, seven among them (blue circles) were also identified in replicate SMN bait purifications in Drosophila. Different types of interactions are indicated with distinct colored lines.
encodes a Toll/interleukin-1 receptor homology (TIR) domain. Intriguingly, loss-of-function Ect4/dsarm mutations also suppress Wallerian degeneration phenotypes observed in Drosophila and mouse models (31). Together, these data suggest that the Wallerian degeneration pathway may also affect Smn pathobiology, an effect that may be mediated through Toll signaling. Hence this...
approach both confirmed and expanded the functional categories and pathways associated with SMN.

To further explore the relationships of the 62 proteins and their functional context within DFM, we carried out a first-degree neighbor analysis to identify other proteins directly connected in the network that may represent potential biochemical interactors. This retrieved 361 additional proteins that are linked to the 62 Smn modifier interactors (Figure 2, Dataset S3). These 361 proteins include 128 that are directly linked to at least two of the 62 modifiers (Table S3). A GO term (i.e. functionalities) analysis of these proteins reveals additional connections to the spliceosome, RNA binding and Snap/SNARE functions. Thus, considering genetic modifiers in the context of the DFM provides us with a novel perspective of the diverse molecular functions that can modulate SMN activity in vivo.

Overlapping the genetic modifiers on the human interactome

To study the Smn genetic circuitry in the human context, we generated a human view of the genetic Smn interactome taking advantage of the manually curated source of human molecular interactions from IPA (Ingenuity® Systems, www.ingenuity.com). This database integrates human gene relationships derived from a variety of allowed us analytical approaches, including proteomic studies. Using the human Smn proteins and the 322 human genes corresponding to the genetic modifiers identified in Drosophila (see above and Materials and Methods), we used the IPA knowledge base to derive a human SMN interaction network. Unlike DFM, IPA is not limited to physical interactions thus allowing consideration of other functional interactions including, for example, expression, localization, modification, and regulation. Such an approach allowed us to evaluate potential indirect relationships between the modifiers and SMN and uncover molecular functions beyond its canonical role in the SMN complex.

Based on the generated network, we found that orthologs of five modifiers HNRNPR, SNRPD1, SYNCRIPI, TRA2B and ZNF259 are directly related to SMN1/2 (Figure 3). HNRNPR, SNRPD1 and SYNCRIPI proteins physically interact with SMN1 and 2 and have a role in RNA splicing (32). Trab2 (also known as SF510 or Htra2-beta) was shown to regulate Smn2 protein levels by being a potent splicing enhancer (33). Finally, ZNF259 also known as ZPR1 was shown to be necessary for the localization of Smn1 to nuclear bodies (34) and more recently emerged as a key modifier of SMA pathology in patients (35). These findings support the relevance of the identified Drosophila modifiers in understanding the human pathways underlying SMA pathology.

Furthermore, 98 modifiers are indirectly related via these five interactors to human SMN. Together these 103 proteins representing one-third of the identified modifiers are interconnected in a human IPA database. In addition, we find another group of 19 proteins that make pair-wise functional interactions with other SMN genetic modifiers, but do not connect to the human Smn interactome. The remaining 177 proteins that are not connected in the human interactome (and the 19 that have pair-wise connections, 4 pairs of which are between two orthologs of the same Drosophila gene) potentially represent functions that have not been linked to SMN biology in human studies so far.

Expansion of the human SMN interactome beyond the 103 modifiers, by incorporating first-degree neighbor proteins of SMN in the database, connects an additional 48 modifiers (Figure 4). This expanded human SMN network contains intermediates that are known to associate physically with SMN (GEMINS, HNRNPs and LSM and SNRP family members) (3, 14) and signaling pathway elements known to affect SMN activity (FGF2, GSK3B, MAP3K5) (19, 36-39).

Validation of genetic modifiers at the larval NMJ

We chose to prioritize the modifiers for further functional characterization by using membership within both the Smn modifier network in DFM (Figure S1) and the expanded IPA Human SMN network (Figure 4) as the primary criterion. A total of 36 genes are shared between these interactomes (Figure 5, Dataset S4). The list includes 4 previously analyzed modifiers (Actn, Moes, Fim, cut up) (13, 27), 13 enhancers, (Sod, Hsp68, Hsf, step, CG17838, nsl1, shr, Vha5FDr, Rel, Hexo2, osa, CG13902, catlD) and 19 suppressors (CG30194, Neda4, Pka-R2, Rho1, Tango7, Arg1, 14-3-3-epsilon, Zpr1, CG9769, ceg11A, flw, cont, CG9062, 1(l3)72Ah, Karybeta3, HmgZ, Rhsn-5, sel, Paip2).

Our previous analyses (13) indicated a strong correlation between the strength of the lethal Smn phenotype with the severity of NMJ abnormalities. Therefore, examination of the effects of Drosophila modifiers on the Smn NMJ phenotype was used to validate their role in Drosophila and prioritize the corresponding orthologs for further investigation in vertebrate model systems. We used NMJ assays (13, 19) to sample the ability of a subset of these modifiers to alter the tubGAL4::FL26B NMJ phenotype.

Examination of third instar larvae carrying a combination of tubGAL4::FL26B and each of 20 modifier strains revealed that 11 out of the 20 (55 %) strains revealed a statistically significant change in the number of synaptic boutons and are modifiers of the Smn NMJ phenotype (Figure 6).

Effect of genetic modifiers on Smn protein levels and localization

Given that the severity of the disease phenotypes, in both patients and Drosophila models, correlates with SMN protein levels, we examined whether the prioritized genes affected SMN levels in Drosophila Smr2+ cells (40), the same cell line used to generate DFM. We used an image-based analysis (37) to quantify SMN protein levels in Smr2+ cells expressing inducible FLAG-HA tagged constructs corresponding to 21 Smn modifying genes available from the Universal Proteomics Resources (41). Untransfected cells within the same wells were used as controls. Surprisingly, we found that none of these ectopically expressed modifier genes significantly altered total Smn protein expression (Figure S2A). Since Smn is localized in both the cytoplasm and the nucleus, we also used this assay to evaluate whether any of these modifiers altered its distribution between these two compartments. We found seven modifiers significantly increased the nuclear Smn levels (Figure S2B and Dataset S5), consistent with the notion that some modifiers from the screen, which affect Smn lethality and NMJ phenotype, may directly affect Smn distribution between the nucleus and cytoplasm. It is worth noting that, a recent study (42) showed that mutant superoxide dismutase 1 (SOD1), known to cause familial ALS, alters the sub-cellular localization of the SMN protein and disrupts its recruitment to Cajal bodies thereby preventing the formation of nuclear `gels'. Sod was identified in our screen as an enhancer and was also shown to affect NMJ phenotype (Figure 6). A subset of modifiers does not alter either Smn levels or its localization. How these modifiers affect the functional Smn remains to be explored. Given these results, however, it is important to note that small changes in SMN function may have an important biological impact given that the severity of clinical manifestation in SMA patients correlates with small changes in SMN expression (1).

DISCUSSION

Different animal models for SMA-associated neuromuscular defects contributed significantly to a better understanding of the Spinal Muscular Atrophy etiology and genetics over the last few years. However, despite the well-characterized role for SMN in snRNP biogenesis, the links between its molecular function and the defects observed in SMA patients remain unclear. One of the key features of SMA is that the severity of the disease is dependent on SMN dosage, prompting the development of therapeutic strategies designed to increase SMN protein levels in patients. Still, it is essential to identify alternative approaches to modulate SMN activity. For this purpose, genetically tractable invertebrate...
systems may help to identify so far undiscovered elements of the SMN genetic circuitry. In particular, these organisms provide more flexible avenues to investigate the poorly understood role of SMN at the NMJ.

We have used Drosophila as our experimental system and previously described a genetic screen which uncovered a small number of SMN modifiers (13) of a strong loss of function mutant phenotype. In this screen, we identified functional links between SMn and the FGF pathway (13, 19), a relationship corroborated and extended by recent evidence in a severe mouse model of SMA, which demonstrated widespread alterations of the FGF-system in both muscle and spinal cord (38).

The relatively small number of modifiers recovered suggested a more sensitive genetic screen could provide extended information about the SMn genetic network. Our assessment of the lethal phase exhibited by a mild loss of function Smn RNAi allele, which more closely resembles the SMA hypomorphic condition, provided us with a more sensitive and quantifiable assay for genetic interaction. In comparison to our previous results, the RNAi-based screen provided us with a broader spectrum of modifiers including those related to the canonical role of Smn in snRNP biogenesis as well as additional elements of FGF and BMP signaling (13). Our careful mining of the screening modifier list based on functional enrichment and interactome analysis both in Drosophila and human, suggest that loss of Smn function may impact a range of developmental and maintenance-related programs of the whole neuromuscular system, including synaptic vesicle recycling, ion channels and signaling pathways that regulate intrinsic cellular functions. Finally, this analysis also uncovered biological processes not previously associated with Smn.

Among the newly recovered genes, many are associated with RNA metabolism; however, the majority is not involved with canonical SMN activity of snRNP biogenesis and includes factors involved in transcription, post-transcriptional modifications, RNA transport and translation regulation. Intriguingly, CG17838 is the Drosophila homolog of two closely related vertebrate RNA-binding proteins, hnRNP-R and SYNCRIP/hnRNP-Q, both of which bind to SMN in a yeast two-hybrid assay (32) and localize to mRNA containing granules that are transported in cultured neurons (28, 32, 43). Since both SMn and hnRNP-R affect localization of mRNA in axons (44, 45), this could have profound consequences on local translation in neurons (45).

Given the complexity of motor neuronal sub-cellular domains and their distance from the neuromuscular synapses, local regulation of the translation of synaptic proteins is likely to be important in synaptic plasticity and neurological diseases. In fact, many mRNA binding proteins (RBPs) that function as key regulators of local RNA translation are associated with neurological diseases, including FMRP in Fragile X Syndrome, ATXN-2 in Spinocerebellar Ataxia, and TDP-43 in Amyotrophic Lateral Sclerosis. Consistent with a possible role for Smn in affecting local translation, we recovered pumilio and elf-4E, which are thought to be a part of the local translational apparatus in neuromuscular synapses (46). Furthermore, we recovered another translation regulator, elf-4A, which negatively regulates BMP signaling components. Components of BMP signaling pathway have been shown to play a role in retrograde signaling in the NMJ (47, 48). Our results support the relationship between Smn and local translation and also provide an additional link to the retrograde signaling present in the neuromuscular system. Interestingly, perturbation of RNA translational control may result in deficits in endocytosis (49, 50), a process that has been suggested play a key role in neurodegenerative diseases, including Alzheimer’s (51) and Huntington’s diseases (52). Consistent with this notion, aberrant synaptic vesicle release at the NMJs in severe SMA mice may be evidence of impaired synaptic vesicle dynamics and/or abnormal active zone architecture (53). Further supporting a link between endocytosis and Smn (27), we identified Synaptotagmin 1, Synaptotagmin alpha, Synaptotagmin 2, and Dynamin positive RNAi knockdown, which demonstrated widespread alterations of the FGF-system in both muscle and spinal cord (38).

Since such genetic modifier screens are very sensitive and are able to recover a large number of modifiers that span a broad range of molecular functions, prioritization of candidates for further validation is essential. Here, bioinformatics mining allowed us to assemble a list of 36 Drosophila genes with human homologs for continued investigation. The majority of these tested genes showed a functional role in the structure and/or development of the NMJ in Drosophila, and some can alter the distribution of Smn in S2R+ cells, making them good candidates to pursue in vertebrate models of SMA. In addition, candidates may be drawn from a pool of modifiers that include members of signaling pathways such as GPCR, Kinas and Proteases, which are considered to be plausible small molecule targets, or secreted or membrane proteins, which may be targeted by antibodies. Our results thus provide an extensive list of novel genes and pathways that have now been functionally linked to an Smn-dependent phenotype and therefore represent potentially novel therapeutic targets.

**MATERIALS AND METHODS**

**Drosophila stocks and culture**

All Drosophila stocks were maintained on standard Drosophila medium at 25°C. The generation of the Smn alleles and controls used in this study (Smnnull, UAS-Smn-RNAiFL26B, UAS-Smn-GFP) were originally described in (13). The tubGAL4 and TM6B, Tb TubulinGAL8O chromosomes used to generate the screening stock were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN). The Exelixis Collection is housed in the Arthavanis-Tsakonas laboratory in the Department of Cell Biology at Harvard Medical School (Boston, MA).

**Genetic Modifier Screen**

Individual strains from the Exelixis Collection were tested for the ability to genetically modify the tubGAL4-induced IUS-Smn-RNAiFL26B pupal lethal phenotype by mating 3.5 males of the strain to 3 females of the wild-type. IUS-Smn-RNAiFL26B, tubGAL4TMT8M6B, Hb Tb tubulinGAL8O screening stock. After two days, adults were transferred to a fresh vial to create a duplicate cross and maintained with the integration of additional genetic markers for continued investigation. The majority of these tested genes showed a functional role in the structure and/or development of the NMJ in Drosophila, and some can alter the distribution of Smn in S2R+ cells, making them good candidates to pursue in vertebrate models of SMA. In addition, candidates may be drawn from a pool of modifiers that include members of signaling pathways such as GPCR, Kinas and Proteases, which are considered to be plausible small molecule targets, or secreted or membrane proteins, which may be targeted by antibodies. Our results thus provide an extensive list of novel genes and pathways that have now been functionally linked to an Smn-dependent phenotype and therefore represent potentially novel therapeutic targets.

**Gene Assignments for the Exelixis Collection of Transposon Insertions**
Data for Drosophila genes and Exelixis transposon insertion sites were obtained from the Discs, Droplets, and anti-HRP staining in the A3 segment muscle 4 as indicated. The muscle area for every animal was measured, and no significant difference was observed among different genotypes. At least 20-25 animals of each genotype were dissected for the bouton analysis. The ANOVA multiple comparison test was used for statistical analysis of the bouton number/muscle.

Microscopy

Immunofluorescent images were captured with a Nikon C1si spectral point scanning confocal connected to a Nikon TE200 inverted microscope equipped with DIC, phase, and epi-fluorescence optics, 40x Plan Fluor NA 1.4 objective lens and a Perkell EM-90 eyepiece. The Bouton Imaging System (BIS) was fixed at room temperature for 20 min in 4% paraformaldehyde (Electron microscopy sciences). The cells were washed in PBS-D T (0.3% sodium deoxycholate, 0.3% Triton X-100 in PBS) and incubated in 0.1% Triton X-100 for 30 min. Then the samples were re-incubated with mouse Alexa 488- and rabbit Alexa 568-conjugated secondary antibodies (1:500, Molecular Probes), followed by washing in PBS-T (0.1% Triton X-100 in PBS). The samples were then mounted in VECTASHIELD mounting medium with DAPI (Vector Laboratories). Adobe Photoshop CS5 was used to pseudocolor images.

Drosophila Smn modifiers

Drosophila S2R+ cells (40), a derivative of Schneider S2 cells, were cultured in Schneider Drosophila medium (Gibco) with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 25°C. Cells were transfected using TransIT-2020 (Mirus). One day after transfection, plasmid expression was induced with 0.35 mM CuSO₄ overnight. Harvested cells were plated on concanavalin A (0.5 mg/ml) and cultured in medium with DAPI at room temperature. Adobe Photoshop CS5 was used to pseudocolor images.

ACKNOWLEDGEMENTS

This work was initially supported by a grant from the SMA Foundation to S. Artavanis-Tasokas, A.C. Hart, and D. van Vactor. Additional support from Spinal Muscular Atrophy Program Project (P01NS050688) to S. Artavanis-Tasokas, L. Rubin, A.C. Hart, and D. van Vactor. Work on DPM project was supported by a grant from the National Institutes of Health (NIH S01HG003616) to S. Artavanis-Tasokas. A. Sen was supported by a postdoctoral fellowship from the Families of Spinal Muscular Atrophy. The authors thank Nina Makhortova for help with cell imaging and the Nikon Imaging Center at Harvard Medical School for help with light microscopy.
   ZPR1 is a potential modifier of spinal muscular atrophy. *Human molecular genetics*
   21(12):2742-2758.
38. Claus P, Bruns AF, & Grote C (2004) Fibroblast growth factor-2 (FGF2) binds directly to
   the survival of motoneuron protein and is associated with small nuclear RNAs. *Biochem J*
   384(Pt 1):559-565.
   line of Drosophila Schneider S2 cells that respond to wingless signaling. *J Biol Chem*
   273(48):32353-32359.
44. Karina N, et al. (2012) Mutant superoxide dismutase 1 (SOD1), a cause of amyotrophic lateral
   sclerosis, disrupts the recruitment of SMN, the spinal muscular atrophy protein to nuclear
   affecting cytoplasmic localization of beta-actin mRNA in fibroblasts and neurons. *The Journal
   axonal beta-actin mRNA translation in spinal motor neurons. *Human molecular genetics*
47. Fallini C, et al. (2011) The survival of motor neuron (SMN) protein interacts with the mRNA
   binding protein HuD and regulates localization of poly(A) mRNA in primary motor neurons.
   *J Neurosci* 31(10):3914-3925.
   ogy and controls posttranslational accumulation of translation factor eIF-4E. *Nature* 444(4166):
   663-676.
50. McCabe BD, et al. (2003) The BMP homolog Gbb provides a retrograde signal that regulates
   components of miRNA effector complexes and modulate miRNA activity. *Nature cell biology*
   11(9):1145-1149.
52. Lee YS, et al. (2009) Silencing by small RNAs is linked to endosomal trafficking. *Nature cell
   biology* 11(9):1150-1156.
   *Aging Res Rev* 8(3):147-149.
   and causes accumulation of cholesterol in vitro and in vivo. *Human molecular genetics*
55. Kogz L, et al. (2009) Impaired synaptic vesicle release and immaturity of neuromuscular
56. Gauthier J, et al. (2011) Truncating mutations in NNX2 and NNX1 in autism spectrum
   *Human molecular genetics* 18(5):988-996.
   cDNA and preliminary genomic organization of the DMD gene in normal and affected
59. Monaco AP, et al. (1986) Isolation of candidate cDNAs for portions of the Duchenne
60. Rosen DR, et al. (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with
   prehensive multi-species collection of ortholog groups. *Nucleic Acids Res* 34(Database issue):
   D136-D138.
66. Sayers EW, et al. (2012) Database resources of the National Center for Biotechnology
67. Li L, Stoeckert CJ, Jr., & Roos DS (2003) OrthoMCL: identification of ortholog groups for