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-deendent 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.

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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 SMN2 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 (Snm) gene. SMN is essential for cell viability in vertebrates and Drosophila (8, 9). In Drosophila, zygotic loss of Smn function results in recessive larval lethality (not embryonic as might be expected) due to the rescue of early development by maternal contribution of Smn. 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 Smn 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 Smn 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 Smn background (as opposed to a background that completely eliminates Smn function) would more closely resemble the genetic condition in SMA. Such a screen would therefore enhance our ability to detect novel elements of the Smn genetic network, and, consequently, add significantly to our efforts to both dissect the Smn genetic circuitry as well as identify potential clinically relevant targets with novel mode of action.

This comprehensive 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 genetic network. Finally, the Drosophila Smn network was evaluated for its relevance to human biology by mapping Drosophila Smn genetic network genes to their human homologs, and analyzing the human network using computational biology tools. The projection of the Drosophila Smn network derived from this study onto the human network derived from prior knowledge provides a rational basis for novel Smn functional hypotheses and network

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.

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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::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 insertional mutations (https://drosophila.med.harvard.edu/) for dominant modification of the lethality associated with the tubGAL4::FL26B strain (see Figure 1B for scheme) and identified nearly 1600 candidate strains. To eliminate false positives, all interacting insertions were restested. Only those that were not lethal in trans with tubGAL4 (13) and for which results could be clearly repeated were finally designated as modifiers. From this analysis, we identified 303 modifying strains (129 enhancers and 174 suppressors), which represents nearly 2% of the collection and indicates that this phenotype is useful to detect changes in the fractions of pigmented vs. unpigmented pupae.

(B) The screening strategy to identify genetic modifiers of the Smn pupal lethality phenotype using the Exelixis collection (illustrated for insertions on the 3rd chromosome). The lethal phase for all Smn & TE (16) pupae in individual test crosses are scored and compared to those observed in control crosses (more survival = enhancers, more lethality = suppressors).

(C) Drosophila functional categories over-represented in the genetic modifier list. GO biological functions with the highest significant relation to known Smn functions such as alternative splicing or SMA affected processes (neuronal and muscular). Enrichment significance is expressed as the –log₁₀ (p-values).

Fig. 1. Genetic modifiers of Smn using pupal lethality to screen the Exelixis collection of transposon insertions and their functional role in Smn-modified survival. (A) The lethality associated with the tubGAL4::FL26B strain (see Figure 1B for scheme) and identified nearly 1600 candidate strains. To eliminate false positives, all interacting insertions were restested. Only those that were not lethal in trans with tubGAL4 (13) and for which results could be clearly repeated were finally designated as modifiers. From this analysis, we identified 303 modifying strains (129 enhancers and 174 suppressors), which represents nearly 2% of the collection and indicates that this phenotype is useful to detect changes in the fractions of pigmented vs. unpigmented pupae.

No gene could be assigned for 36 of the 303 insertions. Careful consideration of the Exelixis collection may be near multiple coding regions, unambiguous gene assignments are not always possible. Given this consideration, we determined that these 303 insertions potentially affected 340 Drosophila genes. In most cases, single genes were affected by single insertions, though 14 genes scored in the screen were represented by 2 or more alleles. Careful human to Drosophila homology mapping using a combination of
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 interconnected 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 methylentetrahydrofolate 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).

Inspecting the GO biological function terms of these 24 clusters harboring a single modifier, but 9 contained two or more (Table S3).}

Fig. 3. Human Smn genetic modifiers

networkingguinity Pathway Analysis indicates that about one-third of the human orthologs of Drosophila Smn genetic modifiers (green circles) are connected in a network involving 282 interactions with other modifiers and SMN1/SMN2 (yellow). Different types of interactions are indicated with distinct colored lines. A small number of modifiers (19 genes) have only interactions with other modifiers but not SMN1/SMN2 (4 pairs are two human orthologs of the same Drosophila gene). Nearly two-thirds of the modifiers (177 genes, not shown) have no interactions that would connect them to SMN1/SMN2 or other modifiers.
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.

lethality. The recovery of *Ect4/dsarm* may provide additional evidence linking the Toll signaling pathway to Smn activity as it...
Fig. 5. Network of 36 high-priority genetic interactors of Smn. The network shows 36 human orthologs of Drosophila Smn genetic modifiers (green circles) connected to SMN1/SMN2 in human. These 36 modifiers are present in Drosophila DPM as well as human IPA based network and were selected for functional validation in NMJs. The intermediate proteins (pink circles) shown provide the shortest path to connect the modifiers to SMN1/SMN2. Different types of interactions are indicated with distinct colored lines.

Fig. 6. Genetic modifiers of Smn regulate NMJ morphology. (A) An NMJ derived from muscle 6/7 of a tubulinGAL4::UAS-Smn-RNAiFL26 (tubGAL4::FL26B) 3rd instar larva. (B, C) A reduction in NMJ size is observed upon introduction of enhancers c024569 (B) and d02738 (C) into the tubGAL4::FL26B background. (D, E, F) Introduction of a suppressors c05501, c06705 into this screening background leads to an increase in NMJ size, whereas suppressor d05711 (F) does not result in significant modification of the NMJ. (G) Quantitation of bouton numbers/muscle in individuals of indicated genotypes, which include enhancers (red) and suppressors (green), normalized per muscle surface area (MSA) and expressed as percentage change compared to Tub Gal4:Smn RNAi alone. The ANOVA multiple comparison test was used for statistical analysis of the bouton numbers/muscle. Significance P<0.05. Scale Bar = 50 mm. n = 20. All preparations were stained with anti-HRP (red) and anti-Dlg (green). The muscle nucleus was labeled using DAPI.

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
To further explore the relationships of the 62 proteins and their functional context within DPiM, 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 DPiM provides us with a novel perspective of the diverse molecular functions that can modulate SMN activity in vivo.

Overlaying 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 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 DPiM, 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, SRNPD1, SYNCRIP, TRA2B and ZNF259 are directly related to SMN1/2 (Figure 3). HNRNR, SRNPD1 and SYNCRIP proteins physically interact with SMN1 and 2 and have a role in RNA splicing (32). Tra2b (also known as SFRS10 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 DPiM (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, Hsp, step, CG17838, ns1, shb, VhaSFD, Rel, Hex2o, rsa, CG13902, cathD) and 19 suppressors (CG30194, Neda4, Pka-R2, Rh1, Tango7, Argk, 14-3-3-epsilon, Zpr1, CG9769, cenG1A, flw, cont, CG9062, l(3)72Ab, Karybeta3, HmgZ, Rhbn-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 reveal 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 S2R+ cells (40), the same cell line used to generate DPiM. We used an image-based analysis (37) to quantify SMN protein levels in S2R+ 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, www.ingenuity.com).
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 described here 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 interaction 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 FMRF in Fragile X Syndrome, ATXN-2 in Spinocerebellar Ataxia, and TDP-43, FUS (fused in sarcoma), ANG and ATXN-2 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 defects 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 Smn 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 Synaptotagmin1, Synaptotagmin-alpha, and synaptotagmin-beta, the Drosophila homologs of N-ethylmaleimide sensitive factor (NSF), which are core components of synaptic vesicle recycling. We also recovered genes that are directly required for synaptic transmitter release, such as methuselah, or indirectly, such as bruchpilot, which plays a role in constructing the active zone.

Though many of the recovered genes broadly impact the neuromuscular system, a subset includes the Drosophila homologs for several disease related genes, including Nrx-1 (schizophrenia and Autism Spectrum Disorders) (54, 55), Dystrophin (Duchenne’s Muscular Dystrophy) (56, 57), Superoxide dismutase (42, 58), RhoA, (Amyotrophic Lateral Sclerosis) (59) and Ect4/dSarm (Wallarian degeneration) (31). Our recovery of these genes suggests the genetic network identified by our screen may overlap, perhaps significantly, the genetic networks impacted by other human neurological disorders. If true, the use of Drosophila to explore other neurological disease networks via genetic screens, and the use density. Accordingly, adults were discarded for approaches, could identify common therapeutic targets which could potentially be tested in other disease models.

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, Kinases and Proteases, which are considered to be plausible small molecule targets, or secreted or membrane proteins, which may be targeted by antibiotics. Our results thus provide an extensive list of novel genes and pathways that have now been functionally linked to a 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 constructs used in this study (Smn<sup>d2</sup>, UAS-Smn-RNAi<sup>UAS</sup>, UAS-Smn-GFP) were originally described in (13). The tubulinGAL4 and TM6B, Tb Hu tubulinGAL80 chromosomes used to generate the screening stock were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN). The Exelixis Collection is housed in the Artavanis-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 UAS-Smn-RNAi<sup>UAS</sup> pupal lethal phenotype by mating 3-5 males of the strain to 3 females of the w, UAS-Smn-RNAi<sup>UAS</sup>, tubGAL4TMTM6B, Hu Tb tubGAL80 screening stock. After two days, adults were transferred to a fresh vial to create a duplicate cross and maintained with the integration of additional genetically encoded approaches, could identify common therapeutic targets which could potentially be tested in other disease models.

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, Kinases and Proteases, which are considered to be plausible small molecule targets, or secreted or membrane proteins, which may be targeted by antibiotics. Our results thus provide an extensive list of novel genes and pathways that have now been functionally linked to a 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 Database for Annotation, Visualization, and Integrated Discovery (DAVID) and the Genome Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 6.7. The transposon insertion sites were used to create gene assignments according to the following criteria: a transposon was considered to map to insertion sites of the transposons were then used to create gene assignments, according to the following criteria: a transposon was considered to map to a particular gene if its insertion site is located in the transcription unit of the gene itself or within 1 kb upstream of the transcription start site or 100 bp downstream of the transcription termination site.

**Mapping Drosophila genes to human orthologs**

FlyBase version 5.39 identifies 15,233 Drosophila genes, which were iteratively mapped to human orthologs using prediction algorithms, several prediction algorithms. Multiple predictions were combined into a single prediction by ordering the algorithms based upon lowest false positive and highest false negative rates (see 61), and choosing the first prediction. The methods used (in order) were inParanoid version 7 (62), orthoMCL version 5 (63), Homologene build 65 (64) and, orthoMCL version 2 (65). The inParanoid predictions were selected using a probability score of 0.4. As a result, of the 15,233 Drosophila genes considered, 6,821 could be mapped to 6,703 human genes. This dataset was used to assign the human orthologs of Drosophila Smn modifiers as shown in Dataset 51.

**Functional enrichment**

The functional enrichment Gene Ontology terms of the Drosophila genetic modifiers was assessed using EASE statistics available through the Database for Annotating, Visualization, and Integrated Discovery (DAVID). Bioinformatic analyses were carried out using the DAVID collection as a reference set (67, 68). Human and Drosophila protein-protein and genetic interactions were visualized and analyzed (first neighbors) using Cytoscape (66). Cytoscape Biological networks were created using the functional categories of the retrieved clusters in the Drosophila sub-networks. The human network was generated through the use of IPA (Ingenuity Systems, www.ingenuity.com) and further visualized and mined in Cytoscape.

**Neuromuscular junction analyses**

Third instar larvae were dissected in cold 1X phosphate buffered saline (PBS) and fixed at room temperature (RT) for 20 min, in 4% Parafomaldehyde (PFA). The preparations were washed overnight at 4°C with primary antibody. The primary antibody was washed off with PTA at RT. The samples were incubated at RT with secondary antibody for 90 min. This was followed by PTX wash, and the tissues were washed off with PTX at RT. The samples were incubated at RT with secondary antibodies (1:500, Molecular Probes), followed by washing in PBS-T (0.1% Tween 20). The samples were incubated in Vectashield Mounting Media with DAPI (Vector Laboratories) at room temperature. Adobe Photoshop CS5 was used to pseudocolor images.

**Drosophila S2R+ cells**

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. HR cells (S. Guo, et al.) were cultured in Drosophila media supplemented with 1% (v/v) Glucose, 1% (v/v) L-glutamine, and 0.3 mM sodium acetate. HR cells were fixed at room temperature for 20 min in 4% paraformaldehyde (Electron microscopy sciences). The cells were washed in PBS-D (0.1% sodium deoxycholate, 0.3% Triton X-100 in PBS) and incubated in 0.1% Triton X-100 overnight at 4°C. The viable cell surface was then blocked with 3% (w/v) goat serum, 1% (w/v) bovine serum albumin, and 0.1% (w/v) sodium azide in PBS. The samples were incubated in 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 imaged in VECTASHIELD mounting medium with DAPI (Vector Laboratories).

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