Genetic modifiers of ALS-associated defects in a *C. elegans sod-1* model

By
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THESIS

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PUBLICATIONS

1. **Yanagi KS,** Osborne J, Tsai J, Lins JJ, Stinson LA, Walsh MB, Mahapatra A, Aderogba R, Kruskop J, and Hart AC. Genetic suppressors of stress-induced glutamatergic neuron degeneration in *C. elegans* SOD-1G85R model. *In prep.*

2. **Osborne J, Yanagi KS, and Hart AC.** Genetic interactions in a *C. elegans sod-1* ALS model: glutamatergic neuron degeneration***. Micropublication. January 15, 2021.*** doi: 10.17912/micropub.biology.000338

3. **Yanagi KS,** Wu Z, Amaya J, Chapkis N, Duffy A, Hajdarovic KH, Held A, Mathur A, Russo K, Ryan VH, Steinert B, Whitt JP, Brown RH, Fallon JR, Fawzi NL, Lipscombe D, Reenan RA, Wharton KA, and Hart AC. Meta-analysis of genetic modifiers of reveals candidate dysregulated pathways of ALS. *Neuroscience.* Jan 1, 2019.
   doi: 10.1016/j.neuroscience.2018.10.033

4. Baskoylu SN, Yersak J, O'Hern P, Grosser S, Simon J, Kim S, Schuch K, Dimitriadi M, **Yanagi KS**, Lins J, and Hart AC. Single copy/knock-in models of ALS SOD1 in C. elegans suggest loss and gain of function have different contributions to cholinergic and glutamatergic neurodegeneration. *PLoS Genetics*. October 8, 2018.
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TEACHING EXPERIENCE

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Neurogenetics Teaching Assistant, Brown University Spring 2017

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2. **Yanagi KS**, Osborne J, Tsai J, Lins JJ, Stinson LA, Walsh MB, Mahapatra A, Hart AC. Identifying suppressors of stress-induced neurodegeneration in a knock-in SOD-1 Amyotrophic lateral sclerosis model. International *C. elegans* Meeting 2019, Los Angeles, CA, June 2019.

3. **Yanagi KS**, Lins JJ, Stinson LA, Walsh MB, Mahapatra A, Hart AC. Genetic modifiers of stress-induced neurodegeneration in a *C. elegans sod-1* knock-in model. Society for Neuroscience Annual Meeting, San Diego CA, November 2018.

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5. **Yanagi KS**, Lins JJ, Stinson LA, Walsh MB, Mahapatra A, Hart AC. Genetic modifiers of stress-induced neurodegeneration in a *C. elegans sod-1* knock-in model. RNA metabolism in Neurological Disease, San Diego CA, November 2018.

6. **Yanagi KS**, Lins JJ, Stinson LA, Walsh MB, Mahapatra A, Char S, Hart AC. Identification of suppressors of stress-induced neurodegeneration in a knock-in SOD-1 model. Mind Brain Research Day, Providence RI, March 2018.

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8. **Yanagi KS**, Lins JJ, Stinson LA, Walsh MB, Mahapatra A, Char S, Hart AC. Identifying genes that suppress stress-induced neurodegeneration in a knock-in SOD1 Amyotrophic Lateral Sclerosis model. International Worm Meeting, Los Angeles CA.

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Honors thesis: Genetic Pathway Analysis of Amyotrophic Lateral Sclerosis in C. elegans

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Genetic suppressors of ALS

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Honors thesis: Genetic Suppressors of Defects in a SOD1 Model of Amyotrophic Lateral Sclerosis in C. elegans

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PROFESSIONAL SOCIETIES

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Abstract

Amyotrophic lateral sclerosis (ALS) is a progressive degenerative disease that results from the loss of glutamatergic and cholinergic motor neurons. Variations in many genes have been genetically linked with ALS, including the superoxide dismutase 1 (SOD1) gene that encodes an enzyme that catalyzes the breakdown of superoxide radicals. Further, multiple cellular processes including oxidative stress response and RNA homeostasis are implicated in ALS pathogenesis. There is a wide range of clinical variability observed in the ALS patient population which poses major complications for the development of treatments. Genetic modifiers, genes that enhance or suppress disease-associated defects, can contribute to the heterogeneity in the patient population and provide valuable insight into pathogenic mechanisms. Here we sought to identify genetic modifiers of ALS using two approaches: (1) a meta-analysis of previously published genetic modifiers of ALS-associated defects in model organisms or genetic modifiers identified through genome-wide association studies and (2) a novel forward genetic screen for suppressors of stress-induced glutamatergic neuron degeneration in a *C. elegans sod-1G85R* model. From our suppressor screen, we identified loss of an RNA binding protein is a suppressor of stress-induced glutamatergic and cholinergic neuron degeneration. Interestingly, many other ALS-linked genes have RNA binding capabilities, and the disruption of RNA granules are thought to be key mediators of neurodegeneration. These results suggests that RNA-based regulatory mechanisms may underly common pathways leading to degeneration.

CHAPTER ONE

Introduction

1.1 Introduction to Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS), also known as Lou Gherig's disease, is an adult-onset neuromuscular disease resulting from the progressive and selective degeneration of motor neurons. Both spinal 'lower' motor neurons which innervate muscles and cortical 'upper' motor neurons are vulnerable to degeneration in ALS patients (Brown and Al-Chalabi, 2017). Oculomotor and sensory neurons are largely spared (Gizzi et al., 1992; Nijssen et al., 2017; Nimchinsky et al., 2000). While degeneration of lower motor neurons leads to symptoms including muscle weakness, atrophy, fasciculations, degeneration of upper motor neurons causes hyperflexia, clonus, and cognitive impairment. Although most cases of ALS are sporadic (sALS) and do not have an associated family history, approximately 10% of cases have a family history of disease (fALS). Despite cases being classified as either sALS or fALS, these ALS cases are clinically and genetically indistinguishable.

ALS patients are typically diagnosed between 50-60 years of age and many exhibit a rapid disease progression after clinical diagnosis. Though most ALS cases are diagnosed during mid-late adulthood, there are cases of juvenile ALS (Rabin et al., 1999). Patients die due to respiratory failure three to five years post diagnosis (Swinnen and Robberecht, 2014; Zarei et al., 2015). Further, in the United States, ALS affects approximately 2 per 100,000 people every year and males have a higher risk for ALS (Longinetti and Fang, 2019). Why motor neurons selectively and progressively degenerate in patients with ALS is unclear, but both environment and genetics likely play a role in disease pathogenesis.

Currently, there is no cure for ALS. The only treatments available to patients are Riluzole/Rilutek and Edaravone/Radicava. Riluzole was the first treatment approved for ALS patients by the United States Food and Drug Association (Miller et al., 2002). One of early hypothesis of how motor neurons are selectively vulnerable in ALS patients is that excess glutamate causes degeneration. Riluzole is thought to act on the excessive glutamate-excitation hypothesis by blocking glutamate release (Doble, 1996). An alternative hypothesis is that cells prone to degeneration release excess free radicals, which in turn, damage neighboring cells. Edaravone is a free radical scavenger that helps to prevent the secondary cell damage, thus protecting additional cells (Sawada, 2017; Takei et al., 2017). These treatments only show modest improvements in patient outcome, only delaying the progression of associated ALS symptoms by approximately 3 months. The lack of effective treatments highlights the need for the discovery of additional therapeutic targets for drug development.

1.2 Genetics of Amyotrophic Lateral Sclerosis

Advances in whole genome sequencing technology have led to a flurry of discoveries identifying ALS-causal variants in over 20 functionally distinct genes. Mutations in hundreds of additional are identified as risk or modifier genes (Andersen and Al-Chalabi, 2011). Adding to the genetic heterogeneity and complexity of ALS, mutations in ALS-linked genes may also present as frontotemporal dementia (FTD). FTD is primarily a cognitive disorder resulting from the atrophy in the frontal and temporal lobes (Convery et al., 2019; Olney et al., 2017). Although much work has gone into identifying ALS-linked genes and understanding the pathology of the mutations, it is still unclear why mutations in genes encoding functionally diverse proteins cause the same disease.

Mutations in Superoxide dismutase 1 (SOD1) linked with ALS

The Cu/Zn superoxide dismutase, SOD1, is a ubiquitously expressed, evolutionarily conserved protein (Fukai and Ushio-Fukai, 2011) that catalyzes the breakdown of superoxide radicals (McCord and Fridovich, 1969) through the following reaction:

$$2O_2\bullet + H_2 \rightarrow O_2 + H_2O_2$$

SOD1 is primarily a cytosolic enzyme, but high concentrations can localize in the mitochondrial intermembrane space and on the outer membrane. Interestingly, in the cytosol of motor neurons, SOD1 is particularly abundant (Popović-Bijelić et al., 2016). In addition to SOD1, there are two other superoxide dismutases in mammals: SOD2 and SOD3. SOD2 is primarily localized in the mitochondrial matrix and depends on manganese (Mn) for enzymatic activity. SOD3 primarily found in extracellular spaces, such as the extra cellular matrix and uses Cu/Zn for enzymatic activity. Though SOD2 and SOD3 have similar functions as SOD1 in different cellular compartments, there is very little evidence to suggest involvement in ALS pathogenesis (Tomkins et al., 2001).

Mutations in superoxide dismutase 1 (SOD1) were first described and genetically linked with ALS in 1993 (Deng et al., 1993; Rosen, 1993). There are now over a hundred ALS-linked mutations in the SOD1 gene and are observed throughout the SOD1 gene (Saccon et al., 2013). Mutations in SOD1 cause approximately 20% of fALS cases and 4% sALS cases. Most mutations in SOD1 have an autosomal dominant inheritance pattern where there is a family history of the disease. However, a asparagine to alanine (D90A) mutation is inherited in an autosomal recessive manner in a Scandinavian population (Al-Chalabi et al., 1998). In the United States, the alanine to valine mutation at position 4

(A4V) is one of the most common mutations found in the SOD1 patient population and has a rapid disease progression (Rosen et al., 1994)

A glycine to arginine mutation at position 85 (G85R) was one of the first mutations identified in patients to cause ALS (Rosen, 1993). The G85R mutation occurs within the zinc-binding site of SOD1 and is thought to cause the inactivity of SOD1 (Borchelt et al., 1994; Cao et al., 2008).

Models of SOD1 ALS

Animal models of SOD1 ALS are pivotal tools that aid in elucidating mechanisms behind disease associated defects. Mouse models where mutant SOD1 is overexpressed have been extensively studied and exhibit motor neuron degeneration (Joyce et al., 2011). SOD1 null mice did not develop motor neuron disease and only showed increases in motor neuron degeneration after axotomy (Reaume et al., 1996; Shefner et al., 1999). Further, some SOD1 mutations do not show a change in superoxide dismutase activity. However, additional studies have showed that loss of SOD1 function has a major impact on the a range of phenotypes including reduced locomotion and decreased grip strength (Fischer and Glass, 2010; Shefner et al., 1999). Mice overexpressing wildtype SOD1, normally used as controls, exhibit ALS-like phenotypes (Graffmo et al., 2013).

One of the widely studied models of SOD1 ALS is the human SOD1G93A overexpression in mice (Gurney et al., 1994). In mammalian models, both loss and gain of function mechanisms seem to contribute to motor neuron degeneration. Transgenic mice overexpressing SOD1G85R display an ALS-like fast-progressing motor neuron disease with SOD1 positive aggregates (Bruijn et al., 1997).

Models of SOD1 ALS in *C. elegans*

Caenorhabditis elegans is a non-parasitic nematode. Established as a model organism in 1963, *C. elegans* has become a versatile model to study both basic biological processes and human disease (Brenner, 1974). The ease of rearing in the laboratory due to its short generation time and the optically transparent features makes *C. elegans* an ideal organism to study basic biological functions. In addition to having a fully sequenced genome with a majority of the major cellular pathways and genes conserved, the cell fate lineage of all 959 somatic cells and 302 neurons are fully mapped in *C. elegans*. Further, the *C. elegans* nervous system is well characterized with the major neurotransmitter class neurons conserved including glutamatergic and cholinergic neurons (White et al., 1976). Although *C. elegans* models do not always capture the complete human disease, these models are particularly powerful for unbiased, discovery-based analysis such as forward genetic screens.

Multiple *C. elegans* ALS models that recapitulate various aspects of ALS have been published **(Table 1.1)**. Overexpression of mutant human SOD1 caused sensitivity to oxidative stress (Oeda et al., 2001). Further, overexpression models also recapitulated ALS-like symptomology including locomotion defects and increased aggregation (Wang et al., 2009). Recently, advances in genetic engineering have given rise to single-copy models through editing endogenous genes to express mutant patient alleles of a specific disease. Novel single-copy/knock-in models of SOD-1 ALS have glutamatergic and cholinergic neuron degeneration, lifespan defects, locomotion defects, and increased aggregation after exposure to oxidative stress (Baskoylu et al., 2018). The single-copy/knock-in models generated will complement the work done in the overexpression

models and provide insight into molecular mechanisms dysregulated in disease. Invertebrate animal models help to bridge the gap between *in vitro* and mammalian models and provide insight from physiologically relevant data.

Other known ALS genes

In addition to SOD1, mutations in over 20 genes have been found in patients with ALS **(Table 1.2)**. Some ALS-linked mutations occur in genes that encode RNA binding proteins such as FUS (Kwiatkowski et al., 2009; Vance et al., 2009) and TDP43 (Arai et al., 2006). Many of the ALS-causal mutations in these RNA binding proteins are in the low-complexity domain. More recently, a hexanucleotide expansion in C9orf72 and found in approximately 60% of patients with ALS has garnered much of the attention (DeJesus-Hernandez et al., 2011; Renton et al., 2011). As more ALS-linked mutations are identified in additional genes, the complexity of the disease grows. The diversity of ALS-linked genes raises the questions if these are distinct motor neuron diseases and if common pathways facilitate the degeneration of motor neurons in patients with ALS.

1.3 Potential pathogenic mechanisms of SOD1 ALS

Although much research has been done, it is still unclear why mutations in SOD1 cause ALS. Early findings suggested that mutations in SOD1 caused a reduction of SOD1 activity (Deng et al., 1993; Rosen, 1993). Loss of SOD1 function could lead to the buildup of superoxide radicals and cell death. However, there is not a definitive correlation between SOD1 activity and disease progression. The G85R mutation causes a decrease in superoxide dismutase activity, yet, some mutations, such as G37R, do not cause a decrease in the enzymatic activity of SOD1 *in vitro* (Borchelt et al., 1994). Alternatively,

SOD1 could act through gain of function mechanisms and cause the selective neurodegeneration of motor neurons. Mutated SOD1 protein can misfold and forms aggregates in patients and in ALS models. Cytosolic aggregates are a common factor of pathological hallmarks in ALS patients. Many patients exhibit aggregation of the ubiquitinated TDP43 (Neumann et al., 2006). Although patients with mutations in the SOD1 gene largely do not have TDP43 aggregates, mutated SOD1 can aggregate in ALS patients (Gill et al., 2019; Kato et al., 2000; Shibata et al., 1996). Further, mutant SOD1 aggregation is recapitulated in transgenic mice models overexpressing the human G85R patient allele (Kato et al., 2000; Watanabe et al., 2001). More recent studies have suggested that both loss and gain of function mechanisms contribute to degeneration (Baskoylu et al., 2018; Şahin et al., 2017).

Regulation of the oxidative stress response

Reactive oxygen species (ROS) and free radicals are known signaling components regulating integral cellular processes including cell survival. Low doses of ROS tend to activate cell survival signaling pathways, while high doses contribute to the activation of apoptotic, necrosis, and other cell death pathways (Redza-Dutordoir and Averill-Bates, 2016). SOD1 is a key facilitator of maintaining the balance of ROS in cells as one of its main functions is catalyzing the breakdown of superoxide radicals. Thus, a favorable hypothesis is cellular damage and neurodegeneration is caused by buildup of ROS and excess oxidative stress (Barber et al., 2006).

RNA binding proteins and RNA homeostasis as key mediators of degeneration

Cytoplasmic RNA granules are important spatial and temporal translational modulators. These RNA granules contain RNA-binding proteins that stabilize mRNAs for

location-specific translation. Two prominent classes of RNA granules implicated in neurodegenerative disease are stress granules (SGs) and RNA transport granules. SGs form in response to various stressors and are composed of stalled mRNAs that rapidly respond to changes in the cellular environment (Aulas et al., 2017). On the other hand, neuronal RNA granules are motile units that transport specific RNAs to regulate local translation (Kiebler and Bassell, 2006). Disruption of RNA granules is a consistent reoccurring theme that occurs in ALS (Li et al., 2013b). Several ALS-linked genes are components of RNA granules including TDP43, FUS, and hnRNPA1. Additionally, axonal transport defects are observed in models of ALS-linked mutations (Alami et al., 2014). Further, in many patients with ALS, the RNA binding protein TDP43 is known to form aggregates (Neumann et al., 2006). Whether or not these ubiquitinated TDP43 aggregates are what ultimately causes the degeneration of motor neurons is unclear.

Non-canonical roles for SOD1: regulation of transcription and translation?

Unlike many other ALS-causal genes, SOD1 does not contain RNA-binding motifs. However, recent reports suggest that mutant SOD1 can interact with mRNAs and may act as a nuclear transcription factor regulating the oxidative stress response (Reddi and Culotta, 2013; Tsang et al., 2014). Mutant SOD1 can localize to RNA-rich structures and has the potential to associate with certain mRNAs (Butti and Patten, 2018; Li et al., 2009). In particular SOD1 has been shown to interact with and bind to vascular endothelial growth factor (VGEF) mRNA regulating its expression (Lu et al., 2007). SOD1 has also been observed to associate with an anti-apoptotic agent Bcl-2 (Pasinelli et al., 2004), which suggests a role in regulating some cell survival pathways. Mutant SOD1 may also regulate RNA processes by interacting with RNA granules. Recent reports suggest that mutant SOD1 interacts with RNA granule proteins G3BP and TIA1 (Gal et al., 2016; Huai and Zhang, 2019; Lu et al., 2009). These pieces of evidence suggest that in addition to its

canonical role as a superoxide dismutase, SOD1 has the capacity to function as an RNA binding protein.

1.4 Clinical variability in ALS patients

ALS is clinically heterogeneous. Clinical presentation varies between patients: location of onset, time of onset, type of motor neuron affected, and progression of disease (Swinnen and Robberecht, 2014; Takeda et al., 2020). One family with a S76T (asparagine to tyrosine mutation at the position 76) had different times of onset of ALS symptoms and progression of the disease (Andersen et al., 1997). The biology behind the phenotypic variation in patients is unclear. However, several factors such as additional genetic and environmental factors may contribute to the phenotypic variability.

1.5 Genetic modifiers

Genetic interactions give rise to complex traits. Causal variants, mutant alleles of genes, are well-studied in the context of disease. Yet, challenges remain in fully understanding the complexities of clinical variability within a patient population. The wide range of clinical presentations observed in many neurodegenerative diseases can partly be attributed to genetic modifiers or modifier genes. These genetic modifiers influence the penetrance, dominance, and expressivity of specific traits (Matsui et al., 2017; Prelich, 1999). The concept of genetic modifiers was first introduced in (Haldane, 1941) and has since become an important field of study.

Genetic suppressors and suppressor mechanisms

Genetic suppressors are genes that can counteract the deleterious effects of a mutation and have been extensively studied to provide insight into penetrance of

mutations in disease and natural genetic variability. Understanding gene-suppressor interactions will help elucidate the genotype-phenotype correlation and the penetrance of a trait. In the context of disease, genetic interactions between disease-causing genes and genetic suppressors can impact clinical variability within human patients and be used to guide the development of treatments. *C. elegans* have been a particularly powerful model organism to identify genetic suppressors (Hodgkin et al., 1987). There are four main mechanisms of action that suppressors act through: 1) Informational suppressors, 2) Activity suppressors, 3) Amount suppressors, and 4) Intragenic suppressors (Prelich, 1999). Understanding how genetic variation contribute to phenotypic penetrance provides unique insight into cellular processes that influence specific phenotypes (Figure 1.2).

Identifying modifiers of neurodegenerative diseases

Genetic screens are powerful tools to identify genes involved in biological processes and, in the context of disease, can provide novel targets for the development of therapies. In humans, GWAS are particularly useful for the discovery of genetic modifiers that influence disease development, susceptibility, and progression. However, there are still many challenges with GWAS: sample size and diversity of patient population and the identification of causal mutations (Donnelly, 2008).

Genetic screens in models of disease provide complement GWAS. Modifiers of disease-associated phenotypes in these models were also identified through GWAS studies to influence patient symptoms or progression. There are two major classes of genetic screens: forward and reverse genetics. Forward genetic approaches examine how alterations in genes influence a phenotype of interest (investigations starting at a phenotype of interest and identifying the gene(s) involved). Forward genetics can uncover previously unassociated genes that may be involved in a specific phenotype. Conversely,

in reverse genetics the phenotype of a genetic alteration is explored (investigations starting from a gene of interest and exploring the phenotypic consequences of altering the gene). Both approaches provide unique insights into the genetics behind phenotypic traits.

In *C. elegans*, screens for modifiers of ALS primarily employed reverse genetic techniques and have examined locomotion or aggregation phenotypes associated with ALS **(Table 3.2)**. These RNAi based approaches have identified numerous genes that suppressed and enhanced models of *SOD1ALS*. Analysis of these genetic modifiers showed that components of pathways, such as proteastasis, are thought to be dysregulated in ALS pathology (Wang et al., 2009). Additionally, forward genetic screens have also identified modifiers of G4C2-associated locomotion defects (Wang et al., 2016). However, no group has completed a forward genetic screen for modifiers of neurodegeneration in a *C. elegans* ALS model.

In **Chapter 3**, we screened for suppressors of stress-induced glutamatergic neuron degeneration in a single-copy *sod-1G85R* model. We identified that loss of *imph-1* suppresses stress-induced glutamatergic neuron degeneration. In the following section, I will introduce *imph-1/IGF2BPs* and relevant literature.

1.6 Insulin growth factor 2 binding protein (IGF2BPs)

Insulin-like growth factor 2 mRNA binding proteins (IGF2BPs) modulate important aspects of cellular function including spatial and temporal control of translation of target mRNAs. IGF2BPs were first purified in 1999 and the family consists of IGF2BP1, IGF2BP2, and IGF2BP3 (Nielsen et al., 1999). IGF2BPs have two RNA recognition motifs and four hnRNPK homology (KH) domains and have high degree of amino acid similarity (Bell et al., 2013). Of the three IGF2BP family members, IGF2BP2 is the most highly expressed

in the developed nervous system. However, this does not omit IGF2BP1 and IGF2BP3 from being involved in the maintenance of neuronal function. In *C. elegans*, RNA binding proteins with KH domains, including *imph-1*, can associate with miRNAs (Haskell and Zinovyeva, 2021). Recent single cell RNA sequencing has supported that *C. elegans imph-1* is expressed in the PHA/PHB glutamatergic sensory neurons (Taylor et al., 2020).

IGF2BPs has strong association with beta-actin mRNAs (Hüttelmaier et al., 2005; Zhang et al., 2001). In *Drosophila*, the homolog of IGF2BPs, *Imp*, promotes synaptic terminal growth (Boylan et al., 2008) and can regulate neural stem cell growth through the stabilization of *myc* (Samuels et al., 2020). Further, IGF2BPs are associated with SGs and other RNA granules. IGF2BP1 colocalizes with the canonical stress granule protein G3BP (Atlas et al., 2004). Recent studies have suggested a larger role of IGF2BPs in the formation of stress granules. After osmotic stress, IGF2BPs has been shown to rapidly form liquid-like droplets and may recruit G3BP and TIA1 (Zeng et al., 2020). Together these data suggest that IGF2BPs have the potential to play critical roles in the maintenance and development of the nervous system.

Accumulating evidence link variants of IGF2BPs with cancer and type II diabetes (Diabetes Genetics Initiative of Broad Institute of Harvard and MIT, Lund University, and Novartis Institutes of BioMedical Research et al., 2007) and may promote these diseases through destabilizing critical mRNAs. No variants in IGF2BPs are currently linked directly to cause human disease.

IGF2BPs are linked with ALS and other related neurodegenerative diseases, such as spinal muscular atrophy (SMA), a neuromuscular disease caused by loss of function of the survival for motor neuron protein. Overexpression of IGF2BP1 ameliorates neurite

growth in *in vitro* models of SMA (Fallini et al., 2014). Further, IGF2BP1 was identified as a modifier of C9orf72 dipeptide toxicity through a CRISPR-based knock down screen (Kramer et al., 2018). Interestingly, IGF2 is a candidate biomarker for ALS and may act as a protective factor providing resilience to motor neurons (Allodi et al., 2016; Osborn et al., 2018). Finally, IGF2BP2 has recently been identified as a component of a complex containing an ALS-linked gene *ANXA11* and aids in facilitating trafficking of RNA granules by acting as a part of a tether to lysosomes (Liao et al., 2019). These pieces of evidence suggest that there is a potential common role for IGF2BPs in neuromuscular disorders.

1.7 Commonalities between related neurodegenerative disorders

Neurodegenerative diseases are influenced by a combination of genetic and environmental factors. However, many neurodegenerative diseases share similar pathological hallmarks such as protein aggregation. Aggregates of TDP43 are often found in patients with ALS (Kabashi et al., 2008; Sreedharan et al., 2008). These aggregates are also observed in patients with a disease often comorbid with ALS, Frontotemporal dementia (FTD) (Cairns et al., 2007; Liscic et al., 2008). Clinically, some ALS patients present with FTD-like cognitive decline and FTD patients present with ALS-like motor neuron dysfunction (Murphy et al., 2007). Genetically, mutations in the same gene can cause both ALS and FTD. The clinical and genetic similarities suggest that ALS and FTD may be on the same spectrum of disease.

A predominantly childhood neuromuscular disease, spinal muscular atrophy (SMA), also shares many pathological hallmarks with ALS (Bowerman et al., 2018). SMA is caused by loss of the survival of motor neuron 1 (SMN1) gene and results from the loss of motor neurons. Interestingly, mutations in the vesicle-associated membrane protein/synaptobrevin-associated membrane protein B (VAPB) cause both a rapidly

progressing form of ALS and juvenile ALS (Nishimura et al., 2004). Additionally, recent reports suggest that the Activating Signal Cointegrator 1 (ASC-1) complex is a common factor affected in both ALS and SMA (Chi et al., 2018). In both ALS and SMA, the common underlying factor is the selective vulnerability of motor neurons. The identification of common factors that influence the development of both ALS and SMA suggest common pathways may lead to the degeneration in motor neuron diseases.

The pathological, clinical, and genetic similarities between many neurogenerative diseases suggest that common factors lead to disease. In Chapter 3, I examine if loss of the ALS suppressor, *imph-1*, can modify SMA-associated survival defects in a *C. elegans* model.

1.8 Significance

Major advances have propelled our understanding of the genetics of ALS and underlying pathology, yet there is still no cure for ALS patients. The inherent clinical and genetic heterogeneity of the ALS patient population may cause potentially helpful experimental drugs to fail in clinical trials (Ludolph and Jesse, 2009). Understanding additional factors may aid in the development of more targeted therapies for patients with ALS.

Powerful forward genetic tools aid in the identification of novel genes that are integral to the degeneration and death of motor neurons in ALS. I completed the first forward genetic screen on glutamatergic neuron degeneration in a *C. elegans* single-copy/knock-in models of SOD-1ALS. The genetic suppressors found through unbiased methods should complement the RNAi based screens done in *C. elegans* overexpression models of ALS. I established that *imph-1(lf)* suppresses stress-induced glutamatergic neuron degeneration and cholinergic neuron degeneration. Additionally, *imph-1(lf)*

extends survival on oxidative stress. Further, I have started to investigate additional candidate suppressor genes of glutamatergic neuron degeneration.

In **Chapter Two**, I focus on established genetic modifiers of ALS. This analysis shows that many pathways are implicated in ALS pathogenesis and that the recent advances of whole genome sequencing has produced a flurry of papers that describe genetic modifiers. Many of the modifiers that were used in the analysis in Chapter two were identified through screens in models where overexpression of a disease allele was used. Thus, in **Chapter Three**, I hope to complement previous studies by discussing the identification of genetic modifiers of glutamatergic neuron degeneration in single-copy models of ALS. Here, we have established *imph-1/IGF2BP* as a suppressor of glutamatergic and cholinergic neuron degeneration as well as discuss many other candidates generated in suppressor lines. Finally, in **Chapter Four**, I will summarize the results presented in this thesis as well as discuss future directions. Together, the results presented in this thesis will contribute to common mechanisms that may drive selective and progressive neurodegeneration in ALS patients.

1.9 Figures and Tables

Table 1.1 Models of SOD1 ALS in *C. elegans*. Early models generated overexpressed ALS patient alleles such as A4V, G37R, G85R, and G93A. Of these, the G93A patient allele is the most well studied. Overexpression of human SOD1 containing these patient alleles all exhibit varying ALS-associated phenotypes including sensitivity to oxidative stress, aggregation, locomotion defects, and selective neurodegeneration. To complement overexpression models, single-copy models have also been generated which also exhibit sensitivity to oxidative stress, aggregation, locomotor defects, and neurodegeneration that are allele dependent.

Model Generation	ALS-associated phenotypes	Reference
Overexpression of human SOD1 with A4V, G37R, or G93A ALS patient mutations under *hsp16-2* or *myo-3* promoter	Oxidative stress sensitivity	(Oeda et al., 2001)
Overexpression of human SOD1 with G85R ALS patient mutation under *snb-1* promoter (panneuronal)	Locomotion defects Aggregation in neurons	(Wang et al., 2009)
Overexpression of human SOD1 with G85R, G93A, or 127X patient mutations under *unc-54* promoter (muscle)	Aggregation in body wall muscle	(Gidalevitz et al., 2009)
Overexpression of human SOD1 with G93A patient allele under the *unc-25* promoter (GABAergic motor neurons)	Locomotion defects Aggregates in neurons	(Li et al., 2013a, 2014)
Single-copy models of A4V, H71Y, L84V, G85R, or G93A patient alleles	Glutamatergic and cholinergic neuron degeneration (oxidative stress dependent) Locomotor defects Aggregation in neurons	(Baskoylu et al., 2018)

Table 1.2 Summary of previous screens for modifiers of ALS in *C. elegans*. Almost all screens previously completed in *C. elegans* have used reverse genetic approaches to identify modifiers of ALS-associated defects. Further, in the published literature, there are no genetic screens for modifiers of neurodegeneration. In other model organisms such as *D. melanogaster* and *S. cerevisiae*, forward and reverse genetics have been used to identify modifiers of ALS-associated defects. Through the identification of genetic modifiers, pathological mechanisms underlying the selective degeneration can be uncovered.

Gene	Description of screen	Reference
SOD1	Genome wide RNAi screen for modifiers of protein aggregation in *Psnb-1::hSOD1G85R::YFP*	(Wang et al., 2009)
SOD1	Shelf screen (candidate gene list generated from modifiers of polyQ aggregation) for modifiers of *Punc-54::hSOD1G93A* aggregation	(Silva et al., 2011)
C9orf72	Forward genetic screen for suppressors of locomotion defects in *Phsp-16::(G4C2)$_{29}$::GFP* animals	(Wang et al., 2016)
SOD1	Forward genetic screen for suppressors of locomotion of *Psnb-1::hSOD1G85R*	(Lu et al., 2019)
TDP43	Genome wide RNAi screen for suppression of motor dysfunction in *Psnb-1::hTDP-43(M337V)*	(Liachko et al., 2019)

Table 1.3 A list of select genes with known mutations that cause ALS or are known risk factors for ALS. Also listed are the corresponding *C. elegans* orthologs.

Homo sapiens	***C. elegans***
SOD1	*sod-1*
C9orf72	*alfa-1*
TDP43	*tdp-1*
FUS	*fust-1*
HNRNPA1	*hrp-1*
HNRNPA2/B1	*hrp-1*
ATXN2	*atx-2*
GRN	*grn-1*
VCP	*cdc-48.1*
VAPB	*vpr-1*
DAO	*daao-1*
SQSTM1	*sqst-1*
PFN1	*pfn-1*
UBQLN2	*ubql-1*
FIG4	*figo-1*

Figure 1.1 Mutations in SOD1 may cause degeneration through a variety of mechanisms. Mutant SOD1 has been shown to aggregate in patients as well as animal models of ALS. Further, mutated SOD1 colocalize with RNA binding proteins (RBPs) and may undertake a new function. Mutations in SOD1 may also alter the activity of the enzyme and lead to increases in oxidative stress. This may cause damage to other cellular organelles and lead to dysfunction and degeneration of neurons.

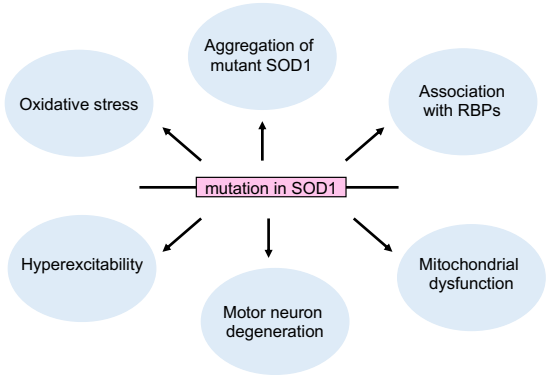
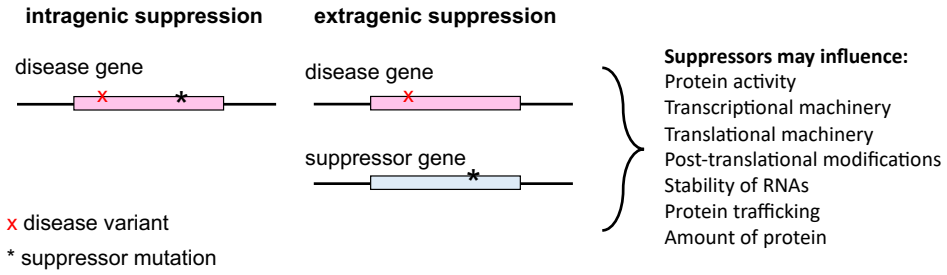


Figure 1.2 Mechanisms of suppression. (A) Intragenic suppression occurs through a second site mutation in the gene of interest. These mutations can be true revertants: suppressor mutation restores DNA sequence to that of the wild type or restores the wildtype or less pathogenic codon. Intragenic suppression could also cause second site mutations in the same gene, causing a codon change, that rescues the function of the protein. (B) Extragenic suppressors are those identified in second genes that ameliorate a phenotype of interest. Genetic suppressors may alter transcription (informational suppressors), translation (amount suppressors), or specific activity of proteins (activity suppressors).



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CHAPTER TWO

Meta-analysis of genetic modifiers reveals candidate dysregulated pathways in Amyotrophic Lateral Sclerosis

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2.1 Abstract

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease that has significant overlap with frontotemporal dementia (FTD). Mutations in specific genes have been identified that can cause and/or predispose patients to ALS. However, the clinical variability seen in ALS patients suggests that additional genes impact pathology, susceptibility, severity, and/or progression of the disease. To identify molecular pathways involved in ALS, we undertook a meta-analysis of published genetic modifiers both in patients and in model organisms and undertook bioinformatic pathway analysis. From 72 published studies, we generated a list of 946 genes whose perturbation (1) impacted ALS in patient populations, (2) altered defects in laboratory models, or (3) modified defects caused by ALS gene ortholog loss of function. Herein, these are all called modifier genes. We found 727 modifier genes that encode proteins with human orthologs. Of these, 43 modifier genes were identified as modifiers of more than one ALS gene/model, consistent with the hypothesis that shared genes and pathways may underlie ALS. Further, we used a gene ontology-based bioinformatic analysis to identify pathways and associated genes that may be important in ALS. To our knowledge this is the first comprehensive survey of ALS modifier genes. This work suggests that shared molecular mechanisms may underlie pathology caused by different ALS disease genes. Surprisingly, few ALS modifier genes have been tested in more than one disease model. Understanding genes that modify ALS-associated defects will help to elucidate the molecular pathways that underlie ALS and provide additional targets for therapeutic intervention.

Key words ALS, FTD, genetic modifiers, pathway analysis

2.2 Introduction

Amyotrophic Lateral Sclerosis (ALS) is a progressive disease that results in selective degeneration and death of upper (cortical) and lower (spinal) motor neurons. First described by Jean-Martin Charcot in 1869 (Rowland, 2001), ALS is characterized by muscle weakness, paralysis, respiratory failure, and death typically within 3-5 years of symptom onset. Within the past two decades, over 20 genes have been identified and/or implicated in ALS (Baker et al. 2006; D. Brenner et al. 2016; Chaussenot et al. 2014; Y. Z. Chen et al. 2004; Chesi et al. 2013; Chow et al. 2009; Cirulli et al. 2015; Couthouis et al. 2012; Cruts et al. 2006; Daoud et al. 2012; DeJesus-Hernandez et al. 2011; Deng et al. 1993; Elden et al. 2010; Figlewicz et al. 1994; Freischmidt et al. 2015; Greenway et al. 2006; Gros-Louis et al. 2004; Hutton et al. 1998; Johnson et al. 2014, 2010; Kabashi et al. 2008; Kenna et al. 2016; H. J. Kim et al. 2013; Kwiatkowski et al. 2009; Leblond et al. 2014; Leung et al. 2004; Maruyama and Kawakami 2013; Millecamps et al. 2014; Mitchell et al. 2010; Munch et al. 2005, 2004; Nishimura et al. 2004; Parkinson et al. 2006; Pensato et al. 2015; Rademakers and van Blitterswijk 2014; Renton et al. 2011; Rosen et al. 1993; Skibinski et al. 2005; Skvortsova et al. 2004; Smith et al. 2014; Sreedharan et al. 2008; Takahashi et al. 2013; Teyssou et al. 2013, 2014; Ticozzi et al. 2011; Van Deerlin et al. 2008; Vance et al. 2009; Wu et al. 2012; Y. Yang et al. 2001; Sreedharan and Brown 2013)

Together, mutations in superoxide dismutase 1 (SOD1), TAR DNA binding protein (TARDBP), fused in sarcoma (FUS), and chromosome 9 open reading frame 72 (C9orf72) account for approximately 60-70% of ALS cases with a family history. Other genes, including VAMP-associated protein B (VAPB), valosin-containing protein (VCP), and optineurin (OPTN), account for 30-40% of familial cases. The proteins encoded by these

genes are involved in a variety of pathways, including oxidative stress (Barber, Mead, and Shaw 2006), protein aggregation (Bruijn *et al.*, 1998), and neuroinflammation (Hooten *et al.*, 2015). However, despite the varied roles of these proteins in healthy cell function, disease alleles of the aforementioned genes can lead to ALS. Even though we have made progress on understanding some aspects of ALS, we do not understand how or why mutations in functionally diverse proteins can cause what appears to be a single disease.

Insights into ALS pathological mechanisms came from the discovery that mutations in a subset of these genes can also cause frontotemporal dementia (FTD), with characteristic degeneration of frontal and temporal lobe neurons (Ratnavalli *et al.*, 2002). ALS and FTD share many pathological hallmarks, including ubiquitinated inclusions, which have been observed in lower motor neurons and cortical neurons of patients with ALS. Furthermore, approximately 50% of ALS patients develop FTD-like symptoms and around 40% of FTD patients develop ALS-like symptoms (Ferrari et al., 2011; Ji et al., 2017; Lomen-Hoerth et al., 2002, 2003; Strong, 2008). These observations suggest that ALS and FTD are related and may share pathways leading to neurodegeneration (Arai et al. 2006; Leigh et al. 1991; Liscic et al. 2008; Mackenzie and Feldman 2005). One strategy that can be used to delineate shared pathways, is to find “genetic modifiers” or “modifier genes” of ALS and FTD genes, which can reveal pathological mechanisms.

Broadly defined, “modifier genes” are genes with alleles that ameliorate or exacerbate defects caused by an allele of another gene. Modifier genes, in patients, may influence clinical presentation of disease including disease onset, severity, penetrance, or progression. Classical genetic studies in model organisms have extensively used modifier gene analysis to dissect function and dysfunction, contributing to our understanding of neurodegenerative diseases (Alexander, Marfil, and Li 2014; M. Dimitriadi and Hart 2010;

Gama Sosa, De Gasperi, and Elder 2012; Plantie et al. 2015; Therrien and Parker 2014; Verbandt, Cammue, and Thevissen 2016). Large scale forward genetic screens for modifiers are possible in small, genetically tractable organisms, such as *S. cerevisiae*, *C. elegans*, and *D. melanogaster* (Chen and Burgoyne, 2012; Sin *et al.*, 2014). These can yield unexpected insights into mechanisms and complement hypothesis-driven studies. Most animal models of ALS compare the consequences of expressing a human protein containing the disease mutation versus the wild type form of the protein. These models have been used to identify genetic modifiers of ALS-associated defects and we surveyed their results. Also, ALS alleles may cause loss of function, which may contribute to disease pathology. Therefore, we surveyed the results of previous studies focused on identifying modifiers of either disease models or ALS-gene ortholog loss of function.

Further, ALS modifier genes have also been identified in human populations and may help explain variation in clinical presentation or disease progression. The site of onset (bulbar or spinal), age of onset, progression rate, and level of cognitive impairment can differ between patients even within the same family (Swinnen and Robberecht 2014). The variability observed in ALS patients may be, in part, due to a result of different alleles of modifier genes that affect progression, penetrance or onset—even if these modifier alleles do not cause disease *per se*. Risk genes are also of interest, as they may reveal pathways critical for disease, even if risk genes are neither necessary nor sufficient to cause disease. Genome wide association studies (GWAS) and linkage analysis in humans with ALS have been used to identify genetic modifiers (Giess *et al.*, 2002; Gros-Louis *et al.*, 2004; Lee *et al.*, 2013).

Here, we undertook a comprehensive literature search and identified 946 genes that act as modifiers of ALS-associated defects in *S. cerevisiae, C. elegans, D.*

melanogaster, M. musculus, or human patients. As shared mechanisms may underlie ALS, we used a gene ontology bioinformatics approach to identify pathways pertinent to disease. This bioinformatic analysis focused on 727 modifier genes that are orthologous to human genes, some of which have been identified in human studies. The results suggest that shared pathways may underlie ALS, regardless of the disease gene involved.

2.3 Methods

Literature Search We searched the literature in PubMed from September 7, 2016 - December 31, 2016 and identified studies that reported modifier genes in ALS models or modifiers of ALS ortholog loss of function (Figure 2.1). Specifically, we examined papers in PubMed reporting genetic modifiers of SOD1, TDP43, C9orf72, FUS, or VAPB. For SOD1, TDP43, C9orf72, and FUS; two independent co-authors searched the literature. The literature review included, but was not limited to, genome-wide screens and candidate genes reported to modify phenotypes in *S. cerevisiae*, *C. elegans*, *D. melanogaster*, *M. musculus,* and cell culture models. Additionally, we searched for genetic modifiers of ALS in patients, which was reviewed by Ghasemi and Brown (Ghasemi and Brown, 2017). A database was assembled in Microsoft Excel with the NCBI GeneID, modifier gene name, human ortholog name, ALS model used, type of screen (RNAi knockdown, genome-wide screen) and impact of modifier on phenotype. The total number of modifier genes (946) corresponds to all modifier genes identified in the literature survey; we did not reexamine this list to identify and eliminate orthologous genes independently identified in different model organisms, which would modestly reduce this number. Additionally, using OMIM (Online Mendelian Inheritance of Man) a list of genes known to cause ALS and/or FTD was compiled. At least 2 independent coauthors checked each ALS gene after the

database was assembled to ensure the accuracy. All data used in the analysis are included in the manuscript and supplemental files.

Human Ortholog Identification Human orthologs of modifier genes found in model organisms were identified using best match similarity with BLAST (NCBI) at blast.ncbi.nlm.nih.gov/Blast.cgi based on protein sequences. If there was more than one best match, then up to three were reported in the "other orthologs" column in Supplemental File 1. When genes with identical statistical scores were called as best match, both genes were included in the bioinformatics analysis. For example, *Hbr98DE* gene is an ortholog of both hnRNPA1 and hnRNPA2B1. Human ortholog identification was verified with DIOPT (http://www.flyrnai.org/diopt) (Hu et al., 2011). If no human ortholog was found, the modifier gene was not included in bioinformatics analysis presented herein.

Gene Ontology Bioinformatic Analysis

Gene ontology (GO) bioinformatic analysis was performed independently for lists of human genes and/or orthologs of modifier genes identified in other species. GO terms that describe Biological Processes are tested to determine if these were over-represented in the curated gene lists, compared to the rest of the transcriptome, using a hypergeometric test implemented in the GOstat package (Falcon and Gentleman, 2007). GO terms with a p-value less than 0.05 after Bonferroni correction were considered overrepresented. In addition, a list of modifier genes associated with more than one ALS genes was assembled and independently subjected to GO analysis. The supplemental files containing lists of modifier genes and other data use in the bioinformatic analysis are available at https://doi.org/10.26300/7asw-k867.

2.4 Results

To identify modifier genes associated with ALS, we searched the PubMed literature database at the National Center for Biotechnology Information (NCBI) for modifiers of SOD1, TDP43, FUS, C9orf72, VAPB, and other ALS genes. In total, 72 studies were found reporting modifier genes 1) in ALS models, 2) in human patients, or 3) for loss of function alleles of ALS gene orthologs. The resulting list of genes is available in Supplemental File 1. Here, we provide an abbreviated background for ALS genes that served as the basis for our search, including a brief description of ALS models used in modifier gene studies. For each ALS gene, gene ontology bioinformatic pathway analysis was undertaken and pathways that were enriched in gene ontology analysis are discussed.

SOD1

In 1993, the discovery that point mutations in superoxide dismutase 1 (SOD1) cause ALS revolutionized the field (Deng et al. 1993; Rosen et al. 1993). SOD1 is an evolutionarily conserved, ubiquitously expressed protein that catalyzes breakdown of superoxide radicals into hydrogen peroxide and water. As the second most common gene whose mutation causes familial ALS (fALS), mutations in SOD1 account for approximately 20% of fALS cases and 5% of sporadic ALS (sALS) cases (Kaur et al., 2016). Over 100 mutations have been identified in SOD1, and almost all disease alleles are dominant in patients. From many studies, it seems likely that disease alleles cause a toxic gain of function, but loss of function may contribute to disease pathology (Bruijn et al. 1998; Saccon et al. 2013).

Two non-exclusive hypotheses for SOD1-associated ALS motor neuron degeneration dominate the field: the aggregation hypothesis and the oxidative stress hypothesis. Mutant SOD1 protein aggregates in the cytosol of SOD1 ALS patient cells are thought to confer toxicity or reduce SOD1 enzymatic activity (Stieber, Gonatas, and Gonatas 2000; M. Watanabe et al. 2001). Early studies in mice supported a toxic gain of function hypothesis, as SOD1 null mice do not exhibit ALS-like pathology and overexpression of mutant human SOD1 resulted in reduced enzymatic activity (Gurney et al. 1994; Reaume et al. 1996). However, how SOD1 mutations cause ALS is still debated. More recent studies suggest that SOD1 loss of function also contributes to ALS dysfunction and degeneration. One possible mechanism is that mutations in SOD1 cause loss of function by aggregation, causing abnormal buildup of superoxide radicals or hydrogen peroxide, the substrate and byproducts of SOD1 action, respectively (Beckman et al., 1993). SOD1 activity is decreased in patients with ALS, suggesting that SOD1 loss of function may contribute to pathology (Rosen et al. 1993; Y. Watanabe et al. 1997). SOD1-mediated motor neuron death may be caused by a combination of the loss and gain of function consequences of patient alleles of SOD1 (Şahin et al. 2017).

ALS models have been created by overexpressing mutant human SOD1 protein in numerous model organisms and comparing the deleterious consequences of the mutant protein to the consequences of overexpressed wild type human SOD1 protein. Two of the most frequently used patient alleles in SOD1 ALS models are missense mutations that result in a glycine to arginine substitution at position 85 (G85R) or a glycine to alanine substitution at position 93 (G93A). As SOD1 loss of function may also contribute to ALS-associated defects, modifier genes that suppress defects associated with SOD1 loss of function alleles are also of interest. In the literature, we found 33 articles that, in combination, yielded 164 modifier genes in either SOD1 ALS model animals or animals

lacking SOD1 ortholog function. These are listed in Supplemental File 1 in the SOD1 tab (Allodi et al. 2016; Bahadorani et al. 2013; Boccitto, Lamitina, and Kalb 2012; Chloupkova, LeBard, and Koeller 2003; Couillard-Despres et al. 1998; Dadon-Nachum et al. 2015; Dobrowolny et al. 2008; Giess et al. 2002; Hetz et al. 2009; Jablonski et al. 2015; Kieran et al. 2007; Kumimoto, Fore, and Zhang 2013; Lambrechts et al. 2003; Lapinskas et al. 1995; Liu et al. 2002; Lobsiger et al. 2005; Lorenzl et al. 2006; L. Lu et al. 2009; Lunn et al. 2009; Marden et al. 2007; Ohta et al. 2016; Pitzer et al. 2008; Reyes et al. 2010; Riddoch-Contreras et al. 2009; Sharp et al. 2008; Silva et al. 2011; Strain et al. 1998; Teuling et al. 2008; Kenna et al. 2016; J. Wang et al. 2009; Y. S. Yang, Harel, and Strittmatter 2009; Zhai et al. 2005).

We hypothesized that shared pathways might link SOD1 modifier genes. To identify these connections, we undertook gene ontology enrichment analysis of the assembled SOD1 modifier gene lists and identified enriched Gene Ontology (GO) pathways. Initially, this analysis was complicated by the diversity of model organisms used for modifier gene identification. To facilitate cross-species comparisons and bioinformatic analysis, the closest human ortholog of each modifier gene was identified based on amino acid similarity using reciprocal BLAST analysis. Proteins that lacked a human ortholog were excluded from bioinformatic analysis. This bioinformatic analysis revealed enrichment of pathways integral for endogenous SOD1 function: “response to reactive oxygen species” (GO:0000302) and “regulation of oxidative stress-induced intrinsic apoptotic signaling” (GO:1902175). The complete SOD1 pathway analysis is presented in Supplemental File 2, SOD1 tab, and top hits are illustrated in Figure 2.2A (pathways with odds ratio > 5). The most enriched pathway for SOD1 modifier genes was “neurotransmitter reuptake” (GO:0098810). The SOD1 modifier genes from the literature

survey that led to bioinformatic analysis identification of “neurotransmitter reuptake” are shown in Figure 2.6.

TDP43

Ubiquitinated inclusions in affected patient neurons are a frequent pathological hallmark of ALS (Arai et al. 2006; Leigh et al. 1991; Ling, Polymenidou, and Cleveland 2013; Liscic et al. 2008; Mackenzie and Feldman 2005; Maekawa et al. 2009; Neumann et al. 2006). In 2006, TAR DNA binding protein 43 (TDP43), was identified as the ubiquitinated protein in intracellular aggregates in both ALS and FTD (Neumann et al. 2006). TDP43, encoded by the TARDBP gene, is a ubiquitously expressed nucleic acid binding protein that play critical roles in RNA splicing and microRNA biogenesis (Buratti and Baralle, 2008). Over 40 missense mutations in TARDBP have been identified in ALS cases (Sreedharan and Brown, 2013). These missense mutations are almost always located in the glycine-rich C-terminal domain of the protein, which has important roles in protein-protein interactions and liquid-liquid phase separation (Sreedharan et al., 2008; Van Deerlin et al., 2008; Wang et al., 2018; Yokoseki et al., 2008).

TDP43 is predominantly found in the nucleus, with a minor fraction of the protein cycling through the cytosol. However, cytosolic TDP43 dramatically increases in patients carrying TARDBP fALS alleles, in many sALS patients, and in a large fraction of fALS patients carrying mutations in other causal genes. TDP43 mislocalization may contribute to the degeneration of motor neurons in ALS/FTD. One hypothesis is that mutant TDP43 acts through a gain of toxic function mechanism by aggregating and inhibiting the endogenous function of normal TDP43. In this model, TDP43 loss of function defects would contribute to neurodegeneration. Alternatively, mutations in TDP43 could alter

endogenous RNA splicing and microRNA biogenesis via disruption of functional interactions (Boeynaems et al. 2016; Freibaum et al. 2015; Jovicic et al. 2015; Kramer et al. 2016; K. H. Lee et al. 2016; Mori et al. 2016; Xu et al. 2013; K. Zhang et al. 2015) or mutant TDP43 protein may act in an abnormal cellular compartment, resulting in neurodegeneration and indicative of a gain of toxic function mechanism.

We found eleven published studies that, in combination, reported 93 modifier genes of TDP43 ALS/FTD phenotypes (Supplemental File 1, TDP43 Tab), for either mutant TDP43 overexpression, wildtype TDP43 overexpression, or TDP43 ortholog loss of function (Armakola et al., 2012; Chou et al., 2015; Elden et al., 2010; Figley and Gitler, 2013; Jablonski et al., 2015; Kim et al., 2014, 2012; Liachko et al., 2013; Sreedharan et al., 2015; Zhan et al., 2013, 2015). We undertook bioinformatics analysis, as described above, with these TDP43 modifier genes and found only 4 enriched GO pathways with odds ratio above 5 (Figure 2.2B). Pathways are listed in Supplemental File 2, TDP43 tab, and include “G/M2 cell cycle regulation” (GO:0000086, GO:0044839) and “regulation of viral transcription” (GO:0019083, GO:0032897), for which TDP43 roles have already been described (Ignatius et al., 1995; Yamashita et al., 2014). Modifier genes that led to bioinformatic analysis identification of “G/M2 cell cycle regulation” are shown in Figure 2.6.

FUS

Originally characterized as a liposarcoma oncogene, mutations in the Fused in Sarcoma gene (FUS) were found in a cohort of 197 British ALS patients in 2009. The FUS protein is a ubiquitously expressed RNA-binding protein involved in splicing and stress granule formation (K. Zhang et al. 2015). Mutations in FUS cause approximately 4-5% of all familial ALS cases. Patient mutations can be found throughout the FUS protein, but

mutation of the C-terminal nuclear localization signal (NLS) is most frequently observed (Ju et al. 2011; Ling, Polymenidou, and Cleveland 2013). In some cases, FUS mutations result in FTD, and patients with FUS-linked FTD usually show ALS symptoms (Nolan et al., 2016). These FTD patients present with FUS-immunoreactive inclusions; these inclusions are also present in the motor neurons of FUS ALS patients who lack FTD symptoms (Deng et al., 2010; Hewitt et al., 2010; Rademakers et al., 2010).

In most FUS ALS patients examined, mutant FUS is mislocalized from the nucleus and protein aggregates form in the cytoplasm (Dormann et al. 2010; Vance et al. 2009). Furthermore, cytoplasmic FUS incorporates into membraneless organelles - phase separated liquid structures (*e.g.* stress granules), which may drive mutant FUS aggregation (Bosco et al., 2010; Burke et al., 2015; Patel et al., 2015). Cytoplasmic aggregation of FUS may inhibit the maturation of RNAs integral for the survival of motor neurons, as nuclear FUS is important for mRNA splicing (Colombrita et al., 2015; Sun et al., 2015). Alternatively, mutant FUS may act via a gain of function mechanism where patient mutations may subvert DNA repair mechanisms, leading to cumulative increases in DNA damage (Hill et al., 2016).

We found five articles that identified 72 modifiers of FUS (Armakola et al., 2012; Chen et al., 2016; Farg et al., 2013; Ju et al., 2011; Sun et al., 2011) (Supplemental File 1, FUS tab). Many of these suppressor and enhancer genes were identified in genome-wide modifier screens in yeast expressing mutant human FUS at high levels (Sun et al., 2011). Our bioinformatic analysis identified 34 GO terms/pathways that were enriched (Figure 2.2C, Supplemental File 2, FUS tab). Many of these are related to cellular pathways associated with normal FUS protein function, including “RNA processing” (GO:0006396) and “translation” (GO:0006412). The FUS modifier genes associated with

the most enriched GO term “nuclear-transcribed mRNA poly(A) tail shortening” (GO:0000289) are shown in Figure 2.6.

C9orf72

In 2011, expansion of GGGGCC (G_4C_2) repeats in the non-coding region of chromosome 9 open reading frame 72 (C9orf72) was identified in ALS patients. C9orf72 expansion is one of the most common causes of ALS and FTD and accounts for approximately 40% of fALS cases (Rademakers et al., 2012). The number of G_4C_2 repeats varies dramatically between patients; Southern blot analysis from one family revealed pathogenic repeats ranging from 700-1,600 (DeJesus-Hernandez et al. 2011; Haeusler, Donnelly, and Rothstein 2016). In addition to the typical ALS motor neuron functional defects, *C9orf72* ALS patients may have earlier disease onset, cognitive and behavioral impairment, and decreased survival compared to other patients (Rademakers et al., 2012).

Why G_4C_2 repeats cause disease is still unclear; studies have suggested the C9orf72 protein has roles in the endolysosomal pathway and vesicle trafficking (Aoki et al., 2017; Corrionero and Horvitz, 2018). Three non-exclusive mechanisms have been proposed: decreased C9orf72 protein expression, toxic expanded G_4C_2 repeat RNAs, and/or toxic dipeptide repeat (DPR) proteins generated by repeat-associated non-AUG (RAN) translation of G_4C_2 repeat RNAs (Haeusler et al., 2016).

Considerable evidence suggests that high level expression of either G_4C_2 repeat-derived RNAs or DPR proteins can be toxic. G_4C_2 repeat RNA may sequester RNA binding proteins and splicing factors, thus disrupting their normal functions and causing neurodegeneration (Lee et al., 2013; Mori et al., 2016; Xu et al., 2013). This model is

supported by the observation that overexpression of Pur-α, an RNA-binding protein that physically interacts with repeat RNAs, suppresses G_4C_2-mediated neurodegeneration in mouse neuronal cells and *D. melanogaster* (Xu et al., 2013). However, DPR proteins are also toxic. These are produced through RAN translation of G_4C_2 repeat RNA, which occurs in the absence of an AUG initiation codon and from both sense and antisense G_4C_2 repeat strands (Zu et al., 2013). Different DPR proteins have varying levels of toxicity: the arginine-rich DPRs, poly(GR) and poly(PR) are most toxic (Jovicic et al., 2015; Kwon et al., 2014; Wen et al., 2014), poly(GA) is moderately toxic, and poly(GP) and poly(PA) are the least toxic (Freibaum et al., 2015; Wen et al., 2014).

We found eight articles that identified modifier genes for G_4C_2 RNA and/or DPR toxicity (Supplemental file 1, C9orf72 tab) (Boeynaems et al. 2016; Freibaum et al. 2015; Jovicic et al. 2015; N. J. Kramer et al. 2016; K. H. Lee et al. 2016; Mori et al. 2016; Xu et al. 2013; K. Zhang et al. 2015). Multiple unbiased genetic screens were undertaken in *D. melanogaster* and *S. cerevisiae* for modifiers of poly(PR) toxicity (Boeynaems et al., 2016; Jovicic et al., 2015). Screens in a *D. melanogaster* eye poly(PR) model yielded modifiers encoding proteins that directly interact with poly(GR) and poly (PR) peptides (Lee et al., 2016). In a candidate-based screen using *D. melanogaster* expressing $(G_4C_2)_{30}$ repeats, RanGAP was identified as a suppressor of neurodegeneration (Zhang et al., 2015). No modifiers of *C9orf72* loss of function have been reported.

From these eight studies, we assembled a list of 285 genetic modifiers with human orthologs of G_4C_2 toxicity (Supplemental File 1, C9orf72 tab). Gene ontology bioinformatic analysis revealed 98 enriched GO pathways (Supplemental File 2, C9orf72 tab). These include “nuclear pore assembly”, “protein import”, and “protein export” (Figure 2.3, Figure 2.4). Additionally, “gene silencing by miRNA” (GO:0035195) and metabolism-associated

pathways were enriched in this dataset. Genes associated with the most enriched pathway in our bioinformatics analysis, “nuclear pore complex assembly” (GO:0051292), are presented in Figure 2.6.

VAPB and other ALS genes

Most studies that report ALS modifier genes focus on SOD1, TDP43, C9orf72, or FUS. Mutations in other genes also lead to ALS and insights into disease pathogenesis may arise from analysis of these other disease genes. In 2004, a P56S mutation in the Vesicle-Associated Membrane Protein-Associated Protein B/C (VAPB) gene was identified in seven different Brazilian families with afflicted individuals showing ALS and/or late-onset spinal muscular atrophy (Nishimura et al., 2004). VAPB protein interacts with SNARE proteins and regulates vesicular transport. Although the severity, presentation, and progression of disease varies between families, the VAPB P56S mutation was dominant (Nishimura et al., 2004). The P56S mutation lies in the VAPB protein Major Sperm Protein (MSP) domain, which likely mediates protein dimerization and other protein-protein interactions.

The functional consequences of VAPB P56S that lead to ALS are poorly understood, but both gain of function (Kuijpers et al., 2013; Ratnaparkhi et al., 2008) and loss of function mechanisms (Kabashi et al., 2013) have been proposed. In normal cells, VAPB mediates membrane interactions between mitochondria and the endoplasmic reticulum, which are critical for mitochondrial calcium regulation and ATP production (Stoica et al., 2014, 2016). Mutations in either TDP43 or FUS can disrupt VAPB function, ultimately leading to disrupted mitochondrial calcium uptake and decreasing ATP production (Stoica et al., 2014, 2016). We found two articles describing genetic modifiers

of VAPB (Deivasigamani et al., 2014; Sanhueza et al., 2015), as well as one article describing genetic modifiers of OPTN (Akizuki et al., 2013) and one article describing VCP modifier genes (Ritson et al., 2010) (Supplemental File 1, Other tab). We searched for modifier genes of other fALS-linked genes, but did not uncover additional modifier studies in the published literature. VAPB modifiers were identified in two different *D. melanogaster* screens. Deivasigamani et al. upregulated or downregulated *D. melanogaster VAPB* (*dVAP*) ortholog levels, which results in altered bristles (Deivasigamani et al., 2014). Sanhueza et al., found that high level expression of dVAP[P58S] in the *D. melanogaster* eye leads to reduced eye size and used this observation to identify 85 modifier genes (Sanhueza et al., 2015). Only one pathway, "single-organism cellular localization" (GO:1902580), was significantly enriched in our gene ontology bioinformatics.

Modifier genes associated with more than one ALS gene

If patient alleles in the genes listed above lead to a single disease, which we call ALS, then one would expect commonalities in disease mechanism and pathological processes. Accordingly, one might expect common pathways to arise from modifier gene analyses. This hypothesis is supported by previous work demonstrating that some modifier genes impact ALS-associated defects in more than one ALS model. In total, 946 modifier genes were identified from the literature with 727 corresponding human orthologs (Supplemental File 1). To look for commonalities between ALS modifier genes, we compiled a list of modifier genes with impact on more than one ALS causal gene (Table 2.1). For example, if a gene modified defects in both an SOD1 ALS model and a TDP43 ALS model, it was included in Table 2.1 (and in Supplemental File 1, multiple ALS genes). In addition, Table 1 includes modifier genes identified in ALS patient GWAS or genetic studies that have been validated in ALS models, as these are likely relevant to disease.

In total, 43 modifier genes have functional impact on more than one ALS gene and are listed in Table 2.1.

Two genes, KPNB1 and TARDBP, were identified as genetic modifiers in more than two ALS models. KPNB1 was reported as a modifier of C9orf72, VAPB, and TDP43 ALS models. In an RNAi screen conducted in *D. melanogaster* C9orf72 model of ALS, decreased KPNB1 function enhanced PR25-mediated eye degeneration (Boeynaems et al., 2016). In HeLa cells, KNPB1 knockdown enhanced the cytosolic localization of TDP43 (Kim et al., 2012). Additionally, overexpression of the *D. melanogaster* ortholog of KPNB1, *Fs(2)Ket*, resulted in suppression of the rough eye phenotype in a VAPB model of ALS. TARDBP was reported as a modifier of C9orf72, VAPB, and VCP. RNAi knockdown of TARDBP in a C9orf72 model suppressed a viability defect and the rough-eye phenotype in a *D. melanogaster* model (Lee et al., 2016). TARDBP has also been reported as a suppressor of VCP-related degeneration (Ritson et al., 2010) and acts as a suppressor in an overexpression model of VAPB (Deivasigamani et al., 2014). While TARDBP was intentionally selected for assessment in these studies, the nuclear pore complex protein, KPNB1, was independently identified in less biased screens.

To identify common pathways associated with modifier genes, we undertook bioinformatic analysis with the genes listed in Table 2.1. Forty-two GO terms were enriched in this analysis. Pathways enriched in in this modifier gene list included GO terms associated with protein transport or metabolic processes. The most enriched GO term was “protein import into nucleus, translocation” (GO:0000060).

2.5 Discussion

Modifier gene studies have the potential to dramatically increase our understanding of ALS pathogenesis and to provide insight into variation in patient symptoms, penetrance of disease, and disease progression. Modifier gene studies can provide insights into pathways associated with neuronal dysfunction and neurodegeneration in ALS. Furthermore, common genetic modifiers may link ALS caused by mutations in different genes, suggesting a common mechanism of motor neuron degeneration. Additionally, modifier genes can be used to identify pathways and targets for therapeutic intervention. Many genetic modifiers of ALS have been discovered through hypothesis-driven experiments, forward genetic screens, or genetic studies in human populations. But, to our knowledge, a comprehensive listing and analysis of modifier genes pertinent to ALS has not been undertaken previously.

It is likely that both loss and gain of function mechanisms contribute to ALS pathology. Therefore, we included modifiers of both loss- and gain of function in our survey, as well as overexpression of wildtype or mutant protein. In total, we compiled a list of 727 modifier genes with human orthologs. Many modifier genes were found in *S. cerevisiae*, *C. elegans*, *D. melanogaster*, *M. musculus*, and cell culture models of ALS. Additionally, genetic modifiers were identified in ALS patients through linkage analysis and genome wide association studies. In total, we identified 72 articles in the published literature that reported 727 modifier genes with human orthologs for SOD1, TDP43, FUS, C9orf72, VAPB, VCP, or OPTN. We searched for modifiers of other ALS-linked genes, but did not find any in the published literature. Interestingly, the 727 genes identified as modifiers of ALS corresponds to roughly 5% of human genes, consistent with the complexity of this disease. We appreciate the enormous effort these original studies represent and we hope

to highlight the importance of these studies and leverage their results to identify common pathways pertinent to ALS. We note that additional modifier genes have been reported in subsequent studies, including those by (Nicholas J. Kramer et al. 2018).

Many of the pathways that were enriched in our bioinformatics analysis are associated with the endogenous functions of genes implicated in ALS and are established as dysregulated in ALS patients, including “response to reactive oxygen species” (GO:0000302) in SOD1 and “RNA processing “(GO:0006396) in FUS. Interestingly, metabolic processes were identified as enriched pathways in all of our modifier gene lists. This commonality highlights the importance of previous studies demonstrating that metabolism is affected in ALS patients (Mattiazzi et al., 2002).

FUS and TARDBP encode RNA-associated proteins and it has been suggested that they act in the same pathways in ALS pathogenesis (Honda et al., 2013). The analysis of FUS and TARDBP modifier genes could be interpreted to support this hypothesis. When GO pathways are examined, 2 of the 12 enriched GO terms found from the list of TDP43 modifier were also included in the list of 60 FUS-modifier enriched GO terms: “cellular macromolecule metabolic process” and “viral transcription”. There were over 2000 GO pathway terms available in our bioinformatic analysis; the small overlap we observed between FUS and TDP43 is significant; but may reflect the importance of RNA-binding proteins in these processes.

The comprehensive literature search reveals that relatively few ALS modifier genes have been tested in other models of ALS or have been identified in more than one independent modifier screen. Overall, only 43 modifier genes are reported to modify more than one ALS gene. Of these 43 genes, KNBP1 and TARDBP were reported to be

modifiers in more than two ALS models. An inherent bias against the publication of “failure to suppress cross-species” may partially account for this, as well as a bias against reporting negative results, or a lack of motivation to re-test modifier genes identified in other species/models. To fully understand why mutations in specific genes cause ALS and to identify therapeutic targets, we suggest that modifier genes should be tested in multiple ALS models. This should expose commonalities and differences between ALS caused by mutations in different genes and inform the selection of therapeutic targets.

ALS modifier gene studies have already increased our understanding of pathways that may be dysregulated in this devastating disease. We provide the first comprehensive review of published ALS modifier genes and undertook bioinformatic analysis. These data suggest that common pathways may underlie ALS caused by mutations in different genes. We expect that as additional modifier genes are identified and tested in additional models of ALS, more commonalities between the different ALS genes will be found and additional therapeutic targets will be developed for the treatment of this disease.

2.6 Figures and Tables

Table 2.1. Genetic modifiers that may modify multiple ALS genes. This list includes the human orthologs that were identified as modifiers of more than one ALS-causal genes (*e.g.* SRRT orthologs were reported as modifiers in both SOD1 and TDP43 models). Additionally, human genes reported as modifiers through GWAS or linkage analysis studies are also included in this list.

Human Gene	Organism	Modifier for	Reference
ATXN2	Yeast, Human	TDP43, Human	Elden et al. (2010), Figley and Gitler (2013), Lee et al. (2011)
BMP2	Nematode, Fly	SOD1, C9orf72	Wang et al. (2009), Zhang et al. (2015)
CCNB1	Yeast, Fly	FUS, VAPB	Sun et al. (2011), Sanhueza et al. (2015)
CCT4	Nematode, Fly	SOD1, C9orf72	Wang et al. (2009), Lee et al. (2016)
CDC6	Yeast, Fly	C9orf72, VAPB	Deivasigamani et al. (2014), Jovicic et al. (2015)
CHGB	Mouse, Human	SOD1, Human	Gros-Louis et al. (2009), Ohta et al. (2016)
COA4	Nematode, Yeast	SOD1, FUS	Wang et al. (2009), Sun et al. (2011)
DAZAP1	Fly	C9orf72, VCP	Ritson et al. (2010), Zhang et al. (2015)
DBR1	Yeast	TDP43, FUS	Armakola et al. (2012), Figley and Gitler (2013)
DNAJA1	Yeast, Fly	SOD1, C9orf72	Strain et al. (1998), Lee et al. (2016)
FBXW7	Nematode, Yeast	SOD1, FUS	Wang et al. (2009), Sun et al. (2011)
GNAQ	Fly	C9orf72, VAPB	Deivasigamani et al. (2014), Zhang et al. (2015)
HNRNPC	Yeast, Fly	C9orf72, FUS	Sun et al. (2011), Lee et al. (2016)
IMPA1	Yeast, Fly	FUS, VAPB	Sun et al. (2011), Deivasigamani et al. (2014)
IPO5	Fly	C9orf72, VAPB	Deivasigamani et al. (2014), Lee et al. (2016)
KPNB1	HeLa cells, Fly	TDP43, C9orf72, VAPB	Kim et al. (2012), Sanhueza et al. (2015), Boeynaems et al. (2016)
MMP9	Mouse, Human	SOD1, Human	Lorenzl et al. (2006), Kaplan et al. (2014)
MRPL33	Yeast	TDP43, FUS	Sun et al. (2011), Armakola et al. (2012)
NAA20	Fly	C9orf72, VAPB	Deivasigamani et al. (2014), Zhang et al. (2015)
NCL	Yeast, Fly	C9orf72, FUS	Sun et al. (2011), Lee et al. (2016)
NOB1	Nematode, Yeast	SOD1, C9orf72	Wang et al. (2009), Jovicic et al. (2015)
NUP50	Fly	TDP43, C9orf72	Zhan et al. (2013), Freibaum et al. (2015), Boeynaems et al. (2016)
NUP107	Fly	FUS, C9orf72	Sun et al. (2011), Boeynaems et al. (2016)
PDE6D	Nematode, Fly	SOD1, VAPB	Silva et al. (2011), Deivasigamani et al. (2014)
PELO	Yeast, Fly	TDP43, C9orf72	Armakola et al. (2012), Jovicic et al. (2015)
PGM1	Yeast, Fly	TDP43, C9orf72	Kim et al. (2014), Zhang et al. (2015)
PIAS1	Nematode, Fly	SOD1, VAPB	Wang et al. (2009), Sanhueza et al. (2015)
POLD3	Yeast, Fly	C9orf72, VAPB	Deivasigamani et al. (2014), Jovicic et al. (2015)
PPP1R3C	Fly, Yeast	C9orf72, FUS	Sun et al. (2011), Zhang et al. (2015)
RAN	Human cells, Fly	TDP43, C9orf72	Kim et al. (2012), Freibaum et al. (2015)
RBM10	Fly	C9orf72, VAPB	Sanhueza et al. (2015), Lee et al. (2016)
SAMD4A	Yeast	TDP43, C9orf72	Kim et al. (2014), Jovicic et al. (2015)
SCARB1	Fly	C9orf72, VAPB	Sanhueza et al. (2015), Zhang et al. (2015)
SIRT1	Fly	C9orf72, VAPB	Sanhueza et al. (2015), Zhang et al. (2015)
SLC17A5	Nematode, Fly	SOD1, C9orf72	Wang et al. (2009), Zhang et al. (2015)
SOD1	Fly	SOD1, VAPB	Kumimoto et al. (2013), Deivasigamani et al. (2014)
SRRT	Fly	C9orf72, VAPB	Deivasigamani et al. (2014), Freibaum et al. (2015)
TARDBP	Fly	TDP43, C9orf72, VAPB, VCP	Deivasigamani et al. (2014), Lee et al. (2016), Ritson et al. (2010), Sreedharan et al., (2015), Wang et al. (2011)
TNPO1	Fly	C9orf72, VAPB	Boeynaems et al. (2016), Deivasigamani et al. (2014)
TOP1	Nematode, Fly	SOD1, C9orf72	Lee et al. (2016), Wang et al. (2009)
UBR5	Yeast, Fly	TDP43, C9orf72	Kim et al. (2014), Lee et al. (2016)
UQCRC2	Nematode, Fly	SOD1, VAPB	Deivasigamani et al. (2014), Silva et al. (2011)
XPO1	Nematode, Fly	SOD1, C9orf72	Silva et al. (2011), Zhang et al. (2015)

Figure 2.1. Schematic representation of the workflow used to compile and analyze the list of genetic modifiers of ALS.

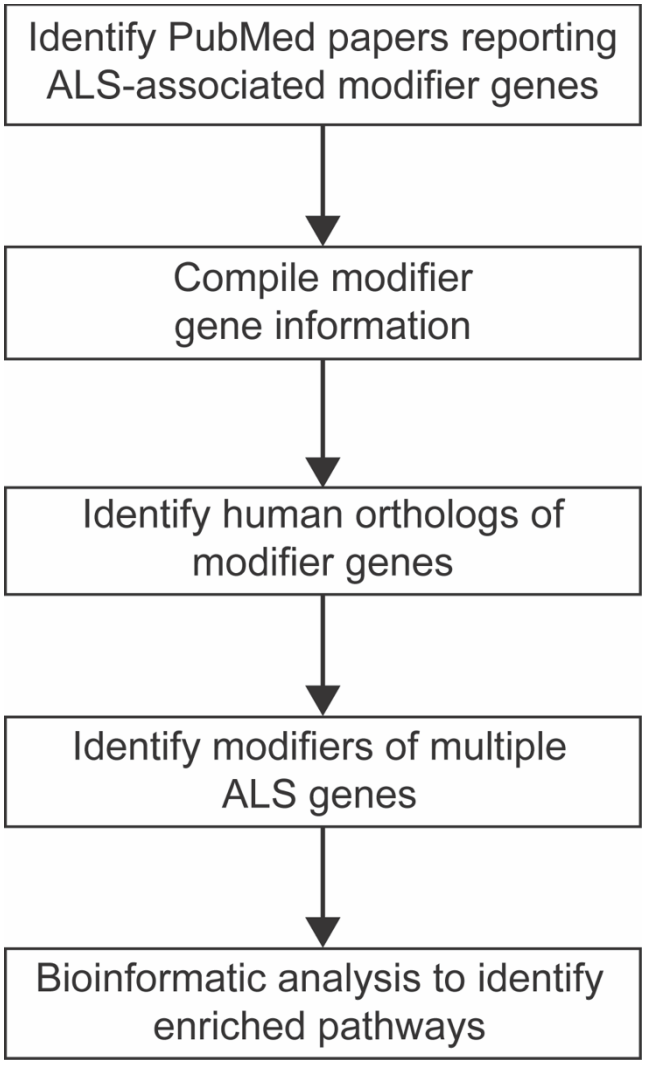
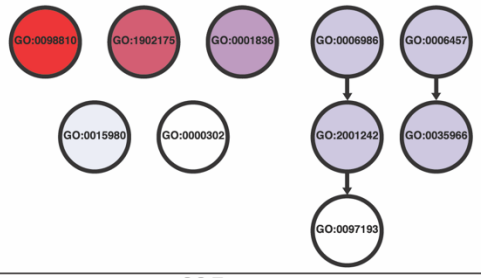


Figure 2.2. GO terms enriched for SOD1, TDP43 and FUS. A) Diagram (top) shows relationship between GO terms enriched in gene ontology analysis of SOD1 genetic modifiers. Arrows indicate related terms that are “nested” inside a broader category. GO terms above odd ratios of 5 or greater are listed (below); the most highly enriched genes are at the top of the list. Darker red hues indicate a higher odds ratio (the magnitude of enrichment). For example, “neurotransmitter reuptake (GO:0098810)” has an odds ratio of 29.86 and is shown in red. This indicates that we observe more genes associated with “neurotransmitter reuptake” in the list of SOD1 genetic modifiers than expected. Though it is enriched in our dataset, “intrinsic apoptotic signaling pathway (GO:0097193)” has an odds ratio of 5.35 and is shown in white. In this case, we still observe more genes than expected with “intrinsic apoptotic signaling pathway” in our dataset, but not to the same extent as “neurotransmitter reuptake” genes. B) GO terms enriched in TDP43 modifiers, presented as in panel A. C) GO terms enriched in FUS modifiers, presented as in panel A.

A. SOD

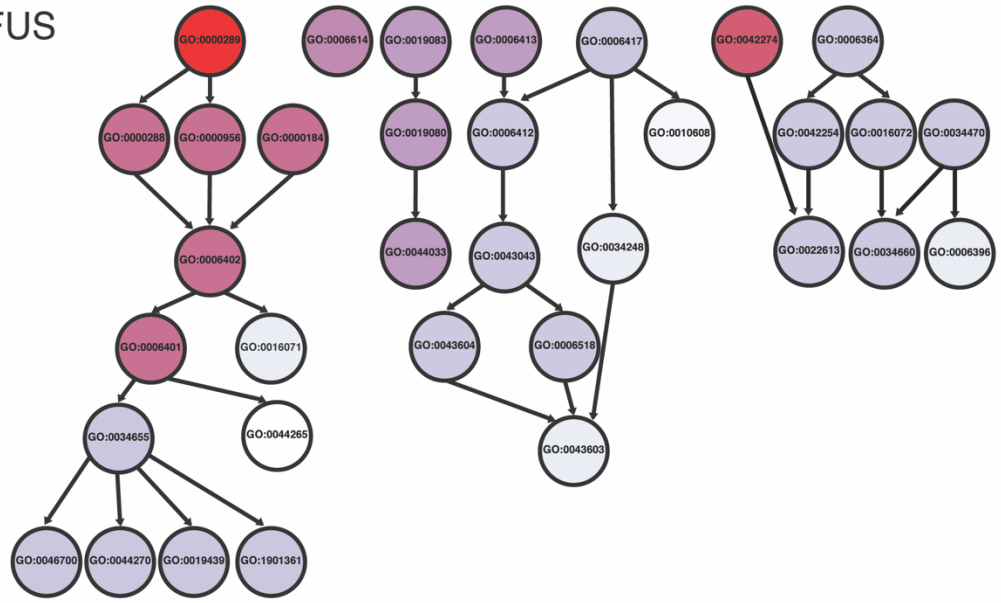


GOBPID	GO Term
GO:0098810	neurotransmitter reuptake
GO:1902175	regulation of oxidative stress-induced intrinsic apoptotic signaling pathway
GO:0001836	release of cytochrome c from mitochondria
GO:2001242	regulation of intrinsic apoptotic signaling pathway
GO:0006986	response to unfolded protein
GO:0006457	protein folding
GO:0035966	response to topologically incorrect protein
GO:0015980	energy derivation by oxidation of organic compounds
GO:0000302	response to reactive oxygen species
GO:0097193	intrinsic apoptotic signaling pathway

B. TDP43

GOBPID	GO Term
GO:0032897	negative regulation of viral transcription
GO:0019083	viral transcription
GO:0000086	G2/M transition of mitotic cell cycle
GO:0044839	cell cycle G2/M phase transition

C. FUS



GOBPID	GO Term	GOBPID	GO Term
GO:0000289	nuclear-transcribed mRNA poly(A) tail shortening	GO:0006412	translation
GO:0042274	ribosomal small subunit biogenesis	GO:0046700	heterocycle catabolic process
GO:0000288	nuclear-transcribed mRNA catabolic process, deadenylation-dependent decay	GO:0043043	peptide biosynthetic process
GO:0000956	nuclear-transcribed mRNA catabolic process	GO:0044270	cellular nitrogen compound catabolic process
GO:0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	GO:0034660	ncRNA metabolic process
GO:0006402	mRNA catabolic process	GO:0019439	aromatic compound catabolic process
GO:0006401	RNA catabolic process	GO:0022613	ribonucleoprotein complex biogenesis
GO:0006614	SRP-dependent cotranslational protein targeting to membrane	GO:1901361	organic cyclic compound catabolic process
GO:0019083	viral transcription	GO:0043604	amide biosynthetic process
GO:0019080	viral gene expression	GO:0006518	peptide metabolic process
GO:0006413	translational initiation	GO:0006417	regulation of translation
GO:0034470	ncRNA processing	GO:0006396	RNA processing
GO:0044033	multi-organism metabolic process	GO:0016071	mRNA metabolic process
GO:0042254	ribosome biogenesis	GO:0034248	regulation of cellular amide metabolic process
GO:0034655	nucleobase-containing compound catabolic process	GO:0043603	cellular amide metabolic process
GO:0006364	rRNA processing	GO:0010608	posttranscriptional regulation of gene expression
GO:0016072	rRNA metabolic process	GO:0044265	cellular macromolecule catabolic process

Figure 2.3 Diagram of GO terms enriched for C9orf72. Illustration of the relationship between GO terms enriched in gene ontology analysis of C9orf72 genetic modifiers. Arrows indicate related terms that are "nested" inside a broader category. Darker red hues are GO terms that were more enriched in the modifier list.

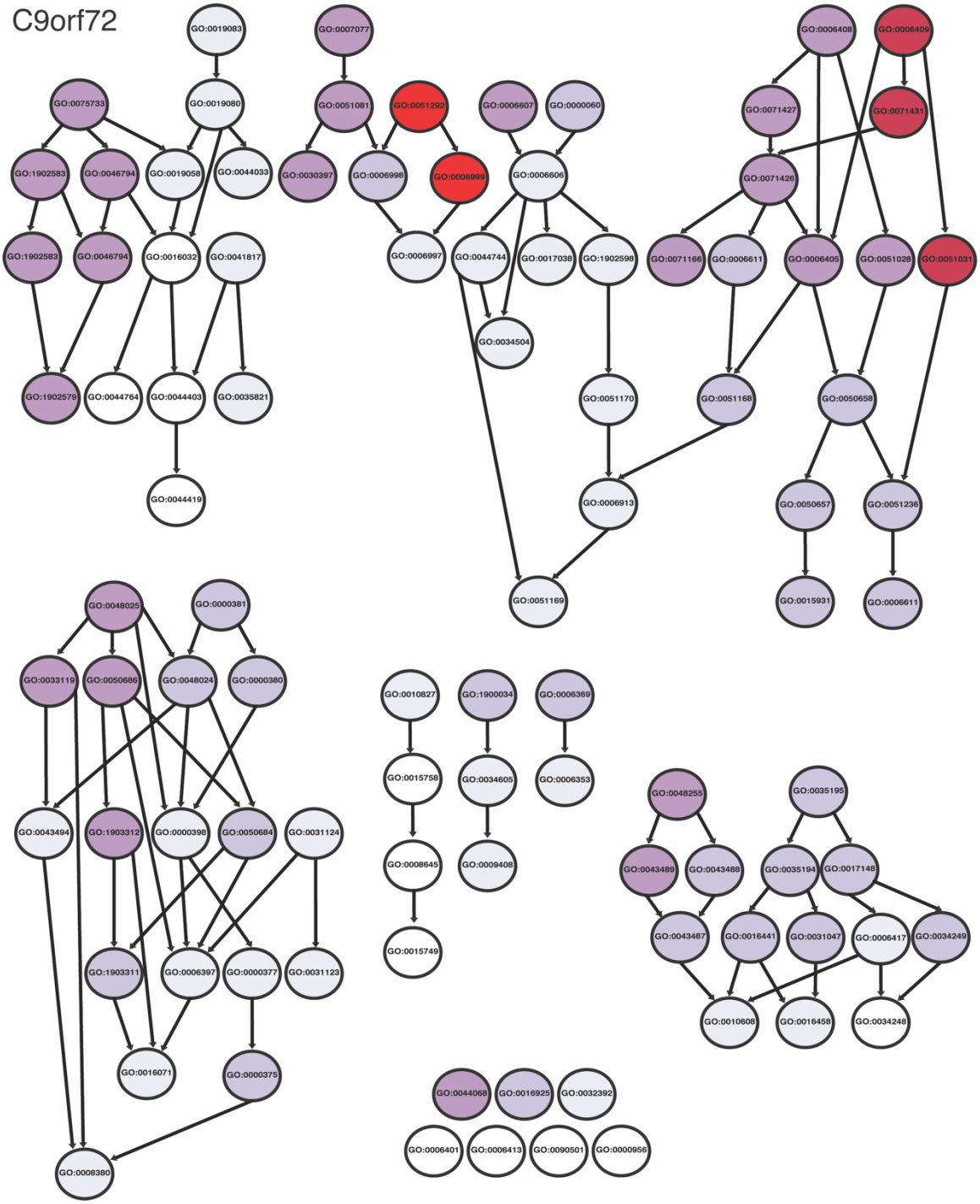


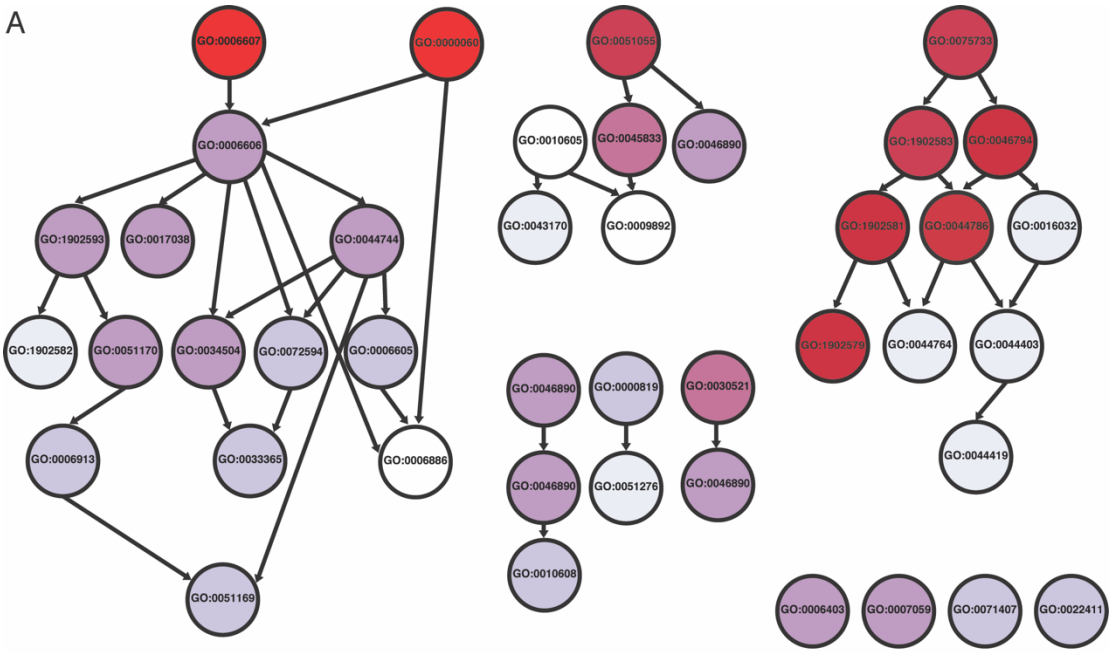
Figure 2.4. List of GO terms enriched for C9orf72. GO terms with odd ratios of 5 or more are listed; the most highly enriched genes are at the top of the list.

C9orf72

GOBPID	Term
GO:0051292	nuclear pore complex assembly
GO:0006999	nuclear pore organization
GO:0006409	tRNA export from nucleus
GO:0071431	tRNA-containing ribonucleoprotein complex export from nucleus
GO:0051031	tRNA transport
GO:0046931	pore complex assembly
GO:0006607	NLS-bearing protein import into nucleus
GO:0075733	intracellular transport of virus
GO:1902581	multi-organism cellular localization
GO:1902583	multi-organism intracellular transport
GO:0048025	negative regulation of mRNA splicing, via spliceosome
GO:0046794	transport of virus
GO:0007077	mitotic nuclear envelope disassembly
GO:0044766	multi-organism transport
GO:1902579	multi-organism localization
GO:0030397	membrane disassembly
GO:0051081	nuclear envelope disassembly
GO:0048255	mRNA stabilization
GO:0033119	negative regulation of RNA splicing
GO:0050686	negative regulation of mRNA processing
GO:0043489	RNA stabilization
GO:0006406	mRNA export from nucleus
GO:0071427	mRNA-containing ribonucleoprotein complex export from nucleus
GO:0071426	ribonucleoprotein complex export from nucleus
GO:0044068	modulation by symbiont of host cellular process
GO:0006405	RNA export from nucleus
GO:0071166	ribonucleoprotein complex localization
GO:0051028	mRNA transport
GO:1903312	negative regulation of mRNA metabolic process
GO:0050657	nucleic acid transport
GO:0050658	RNA transport
GO:0051236	establishment of RNA localization
GO:0000381	regulation of alternative mRNA splicing, via spliceosome
GO:0006403	RNA localization
GO:0006611	protein export from nucleus
GO:0048024	regulation of mRNA splicing, via spliceosome
GO:0000060	protein import into nucleus, translocation
GO:0016925	protein sumoylation
GO:0051168	nuclear export
GO:1900034	regulation of cellular response to heat
GO:0050684	regulation of mRNA processing
GO:0000380	alternative mRNA splicing, via spliceosome
GO:0015931	nucleobase-containing compound transport
GO:0006998	nuclear envelope organization
GO:0031047	gene silencing by RNA
GO:0017148	negative regulation of translation
GO:0043488	regulation of mRNA stability
GO:1903311	regulation of mRNA metabolic process
GO:0035195	gene silencing by miRNA
GO:0043487	regulation of RNA stability
GO:0034249	negative regulation of cellular amide metabolic process
GO:0006369	termination of RNA polymerase II transcription
GO:0010827	regulation of glucose transport
GO:0006913	nucleocytoplasmic transport
GO:0051169	nuclear transport
GO:0043484	regulation of RNA splicing
GO:0035194	posttranscriptional gene silencing by RNA
GO:0031124	mRNA 3'-end processing
GO:0019080	viral gene expression
GO:0016441	posttranscriptional gene silencing
GO:0034605	cellular response to heat
GO:0000377	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile
GO:0000398	mRNA splicing, via spliceosome
GO:0044033	multi-organism metabolic process
GO:0000375	RNA splicing, via transesterification reactions
GO:0031123	RNA 3'-end processing
GO:0019083	viral transcription
GO:0006997	nucleus organization
GO:0016458	gene silencing
GO:0051817	modification of morphology or physiology of other organism involved in symbiotic interaction
GO:0032392	DNA geometric change
GO:0051170	nuclear import
GO:0006606	protein import into nucleus
GO:0044744	protein targeting to nucleus
GO:0010608	posttranscriptional regulation of gene expression
GO:0019058	viral life cycle
GO:1902593	single-organism nuclear import
GO:0008380	RNA splicing
GO:0006417	regulation of translation
GO:0006397	mRNA processing
GO:0035821	modification of morphology or physiology of other organism
GO:0016071	mRNA metabolic process
GO:0009408	response to heat
GO:0034248	regulation of cellular amide metabolic process
GO:0006353	DNA-templated transcription, termination
GO:0034504	protein localization to nucleus
GO:0017038	protein import
GO:0090501	RNA phosphodiester bond hydrolysis
GO:0006413	translational initiation
GO:0044403	symbiosis, encompassing mutualism through parasitism
GO:0000956	nuclear-transcribed mRNA catabolic process
GO:0015758	glucose transport
GO:0044419	interspecies interaction between organisms
GO:0008645	hexose transport
GO:0015749	monosaccharide transport
GO:0016032	viral process
GO:0044764	multi-organism cellular process
GO:0006401	RNA catabolic process

Figure 2.5. GO terms enriched for genes reported to be modifiers of multiple ALS genes. Diagram (top) shows relationship between GO terms enriched in gene ontology analysis. Arrows indicate related terms that are "nested" inside a broader category. Darker red hues are GO terms that were more enriched in the modifier list. GO terms above odd ratios of 5 or greater are listed (below); the most highly enriched genes are at the top of the list.

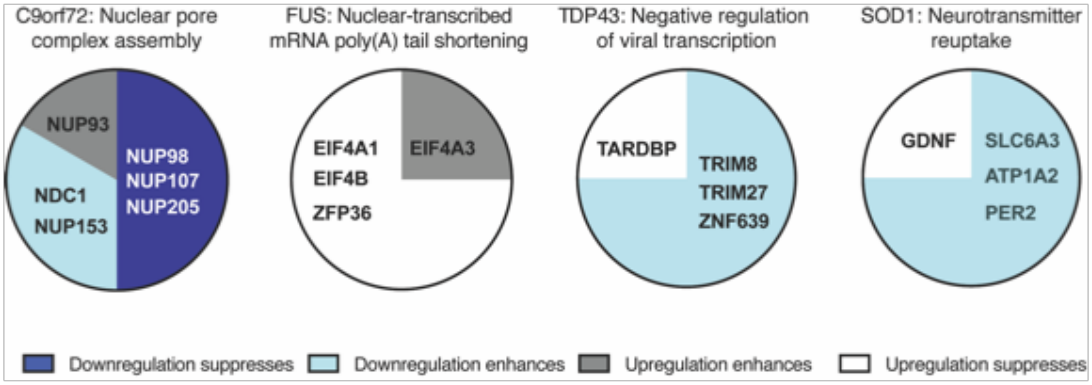
A



B

GOBPID	GO Terms	GOBPID	GO Terms
GO:0000060	protein import into nucleus, translocation	GO:0000819	sister chromatid segregation
GO:0006607	NLS-bearing protein import into nucleus	GO:0006403	RNA localization
GO:0051055	negative regulation of lipid biosynthetic process	GO:0006913	nucleocytoplasmic transport
GO:0075733	intracellular transport of virus	GO:0072594	establishment of protein localization to organelle
GO:1902581	multi-organism cellular localization	GO:0051169	nuclear transport
GO:1902583	multi-organism intracellular transport	GO:0007059	chromosome segregation
GO:0046794	transport of virus	GO:0010608	posttranscriptional regulation of gene expression
GO:0044766	multi-organism transport	GO:0033365	protein localization to organelle
GO:1902579	multi-organism localization	GO:0006605	protein targeting
GO:0030521	androgen receptor signaling pathway	GO:0071407	cellular response to organic cyclic compound
GO:0045833	negative regulation of lipid metabolic process	GO:0022411	cellular component disassembly
GO:0030518	intracellular steroid hormone receptor signaling pathway	GO:1902582	single-organism intracellular transport
GO:0006606	protein import into nucleus	GO:0016032	viral process
GO:0044744	protein targeting to nucleus	GO:0044764	multi-organism cellular process
GO:1902593	single-organism nuclear import	GO:0044403	symbiosis, encompassing mutualism through parasitism
GO:0046890	regulation of lipid biosynthetic process	GO:0044419	interspecies interaction between organisms
GO:0034504	protein localization to nucleus	GO:0043170	macromolecule metabolic process
GO:0043488	regulation of mRNA stability	GO:0051276	chromosome organization
GO:0051170	nuclear import	GO:0006886	intracellular protein transport
GO:0043487	regulation of RNA stability	GO:0010605	negative regulation of macromolecule metabolic process
GO:0017038	protein import	GO:0009892	negative regulation of metabolic process

Figure 2.6. Modifier genes in the most enriched GO term for ALS genes. The most enriched GO term for each ALS genes is listed. Each pie chart contains the names of all human genes (or orthologs) that were associated with the top GO term. Genes are grouped and color coded based on originally reported perturbation of the modifier gene and their impact on ALS-associated defects. For example, knockdown of the NUP98 *D. melanogaster* ortholog ameliorated ALS-associated defects in a *D. melanogaster* C9orf72; this gene was classified as “downregulation suppressed”.



2.7 Supplemental Files and Information

Supplemental File 1. List of genes reported to modify ALS-associated defects. The page labeled “Modifiers of multiple” contains the genes that were found to modify two or more ALS genes. Pages are labeled with the name of the ALS gene that the genetic modifier was observed to modify. Each tab contains the following information: the originally reported gene, the human ortholog, and the reference.

Supplemental File 2. Gene ontology analysis results listing enriched GO terms for each of the analyses reported in the main text and the Figures. Each page lists the gene ontology term, the odds ratio, p-value, expected counts (the number of times a gene should be in this category given the number of genes on our list), the count (the number of times a gene is associated with the specific GO term here), and size (the number of genes associated with the called GO term).

Supplemental File 3. Genes associated with enriched GO terms. The modifier genes associated with each enriched GO term are reported from the original analysis output.

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CHAPTER THREE

Suppressors of stress-induced glutamatergic neuron degeneration in a *C. elegans sod-1G85R* model

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KSY and ACH conceptualized experiments. RA, JJL, AM, LA, and MBW screened for suppressors of glutamatergic neuron degeneration. KSY, JK, JO, and JT performed behavioral, survival, and neuronal degeneration assays. ACH acquired funding.

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3.1 Abstract

Genetic variants in superoxide dismutase 1 (SOD1) cause Amyotrophic lateral sclerosis (ALS), a progressive neurodegenerative disease that results from the selective of motor neurons. Although decades of research have advanced our understanding of ALS, there is no cure and current treatments extend the lifespan of patients by a couple of months. Genetic modifiers, genes that modulate disease severity, may provide insight into the progressive and selective degeneration of motor neurons in ALS patients as well as identify novel targets for treatments. Here, we undertook the first forward genetic screen for suppressors of stress-induced glutamatergic neuron degeneration in a single-copy model of *sod-1G85R*. After mutagenesis, we recovered 45 lines that suppressed stress-induced glutamatergic neuron degeneration in *sod-1G85R* mutant animals. In three independently isolated lines, we observed mutations in exonic regions of *imph-1*/IGF2BP. IGF2BPs are RNA binding proteins and are components of RNA granules in cells, including stress granules. Thus, we examined if loss of classical stress granule genes suppressed glutamatergic neuron degeneration. These results coupled with evidence that RNA binding proteins, such as FUS and TDP43 cause ALS, suggest that RNA-based regulatory mechanisms may be a common factor that underlies ALS pathogenesis.

3.2 Introduction

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease that results in motor neuron degeneration. Both glutamatergic and cholinergic neurons degenerate and can cause progressive muscle weakness, difficulty swallowing, and death due to respiratory failure typically within 3-5 years after disease onset. Mutations in over 30 genes have been linked with ALS, including over 100 mutations in superoxide dismutase 1 (SOD1) and RNA binding proteins FUS and TDP43, are known to cause ALS (Deng et al., 1993; Kabashi et al., 2008; Kwiatkowski et al., 2009; Rosen, 1993; Saccon et al., 2013; Vance et al., 2009). SOD1 catalyzes the breakdown of superoxide radicals into hydrogen peroxide and water (Wang et al., 2018). However, why mutations in SOD1 cause ALS is unknown.

The clinical and genetic heterogeneity of the ALS patient population suggests many mechanisms may be disrupted and underlie ALS pathology. Previous screens for modifiers of ALS were predominantly completed in overexpression models and on other pathological defects, such as aggregation (Silva et al., 2011; Wang et al., 2009). Although these screens have yielded genes that influence aggregation, they still do not target the crux of ALS: the degeneration of neurons. Here, we took advantage of genetic tools in *C. elegans* models of ALS to identify additional genes that may influence neurodegeneration.

We have completed the first forward genetic screen for suppressors of neuron degeneration in a single-copy model of SOD1 ALS. We identified multiple mutagenized lines that suppressed glutamatergic neuron degeneration and have identified *imph-1* as a suppressor of glutamatergic and cholinergic neuron degeneration. Coupled with the identification of an RNA binding protein from our forward genetic screen, studies of mutant

SOD1 and other ALS-linked genes implicate for a large role of RNA dysregulation in ALS pathogenesis. mRNA transport, localization, and transport are all critical functions to maintain cellular homeostasis. These functions are critical in neurons as they constantly need to adapt to changes in the cellular environment. This suggests a critical role for RNA binding proteins, particularly those that are involved in stress and neuronal transport granules. However, the link between SOD1 and the RNA binding proteins that cause ALS is poorly understood.

Additionally, in this chapter I will discuss some of the other candidate genes that we are currently investigating, including *phy-3*, and genes whose loss of function modified in a *C. elegans* SOD1G93A overexpression model.

3.3 Materials and Methods

C. elegans culture and strain information

C. elegans strains in supplementary tables were maintained under standard conditions at 20˚C or 25˚C (Brenner, 1974).

Dye-filling assay for glutamatergic neuron degeneration

Almost all PHA/PHB glutamatergic neurons degenerate in *sod-1G85R*^{M} animals after exposure to 2.5mM paraquat (Baskoylu et al., 2018). We assessed neurodegeneration using a dye filling assay and determined the percentage of animals with intact PHA/PHB neurons (protocol adapted from (Perkins et al., 1986). L4 animals were placed on 2.5mM paraquat (Sigma, 75365-73-0) overnight. Day 1 adults were incubated in DiI (Fisher, DiIC18(5) D307) for 90 minutes and then allowed to recover

before scoring. Animals were scored for intact phasmids under a fluorescent dissecting microscope (Ziess steroscope). Animals with two or fewer phasmid neurons were considered to have degeneration. Twenty animals were counted and the number of animals with phasmids intact were calculated.

To assess glutamatergic neuron degeneration of PHA/PHB neurons under a higher power, animals were immobilized with 30 mg/mL BDM in M9 buffer and mounted on 2% (vol/vol) agar pads. Fluorescent PHA/PHB cell bodies were visualized under 63x or 100x objective (Zeiss Axioplan2) and scored for the presence or absence of dye.

Screen for suppressors of glutamatergic neuron degeneration

HA2633 (rtSi006 [sod-1p::sod-1G85R^{M}::sod-1 3'UTR + Cbr-unc-119(+)] IV; vsIs48 [unc-17p::GFP]) L4 larvae were mutagenized with 47 mM ethyl methyl sulfonate for 4 hrs (Brenner, 1974). After 1 hr recovery, late L4 worms were picked onto new dishes seeded with OP50 and incubated overnight. Three adults P0 were picked onto new plates for a 4hr egg lay at 25˚C resulting in 20-30 eggs. From here, we used two different strategies to screen for suppressors of glutamatergic neuron degeneration (Figure 3.1).

Strategy 1: Pooled F1 screen

Approximately 3 days post-egg lay, the F1 generation was removed. When a majority of the F2 worms developed to L4s, the plate was screened for suppression of glutamatergic neuron degeneration as described below. Lines with 25% or more animals with their phasmids intact were retained and three animals with their phasmids intact were singled for rescreening in subsequent generations.

Strategy 2: Clonal F1 screen

L4 F1 larvae were singled onto new plates seeded with OP50 and allowed to lay eggs for 6 hrs. The F1 generation was removed. When a majority of the F2 generation was at the L4 larval stage, the plate was screened for suppression of glutamatergic neuron degeneration as described below.

Rescreen

We next rescreened the F3 or F4 generation for lines that retained suppression of glutamatergic neuron degeneration. Strains were serially passaged multiple times before stocks were frozen. After approximately 1 month, lines were thawed and rescreened a third time for retainment of suppression of the dye filling defect. Select lines were chosen for 2x backcrossed through HA2720 (*sod-1(tm776); hjSi20; vsIs48*).

Strategy for Whole genome sequencing

For our non-backcrossed strategy, 0x lines were sent for whole genome sequencing using Illumina (HiSeq 2500/4000) at Beijing Genome Institute. For our backcrossed lines strategy, two independently isolated backcrossed lines were used for whole genome sequencing. Whole genome sequencing data was analyzed using CloudMap on Galaxy (Minevich et al., 2012). Using the unmapped mutant workflow and variant subtraction workflow from CloudMap, we subtracted mutations from the serially passaged original *sod-1G85R* strain from suppressor lines to identify EMS-induced mutations. We focused our analysis on EMS-induced mutations causing stop codons,

splice junction changes, frameshift mutations, and missense amino acid changes. Candidate missense and nonsense mutations were confirmed with Sanger sequencing or restriction mapping.

Cholinergic neuron degeneration

The cholinergic marker, vsIs48 [*unc-17p::GFP*], was used to assess cholinergic neuron degeneration. L4 animals were treated with 2.5 mM paraquat overnight and day 1 adults were scored. Animals were mounted on 2% (vol/vol) agar pads. 30 mg/mL BDM was used to immobilize animals. Cholinergic neurons posterior to the vulva were counted and animals missing two or more neurons were scored as animals with degeneration.

Survival assay

Animals were reared at 20˚C or 25˚C under standard conditions. To avoid counting progeny and overcrowding, animals were transferred to new plates at least every other day until all animals stopped laying eggs. Animals were scored as dead if they no longer responded to light taps on the head or tail. For survival on oxidative stress, animals were picked onto 2.5 mM paraquat plates instead of NGM plates. In the presence or absence of oxidative stress, animals were censored if they bagged or left the plate. Floxuridine (FUDR) was not used in any survival experiments.

Data Availability

All whole genome sequence results are archived in their raw form as well as putative exonic SNPs identified through CloudMap at a Brown designated DOI. This

includes sequences from the 34 0x backcrossed lines and 11 2x backcrossed lines with two independent sibling isolates. These data files include position of mutation, the putative amino acid changes, and identification of the genes SNPs occur in, as well as other metrics relating to the quality and coverage of the sequence reads.

3.4 Results

A forward genetic screen for suppression of oxidative stress-induced glutamatergic neuron degeneration

We undertook a classical forward genetic screen to identify suppressors of stress-induced glutamatergic neuron degeneration caused by *sod-1G85R*. We mutagenized *sod-1G85R*^{M} animals and screened the F_2 generation. We retained lines that had 25% or more animals with dye-filled neurons. In the candidate lines identified, suppression of the dye-filling defect varied between lines (Figure 3.2). Intriguingly some lines almost fully rescued the stress-induced glutamatergic neuron defect in *sod-1G85R* animals.

Whole genome sequencing identifies *imph-1* as a suppressor of glutamatergic neuron degeneration

To facilitate the identification of candidate suppressors, we focused our analysis on homozygous non-synonymous de novo mutations. After whole genome sequencing, we observed that *imph-1* had candidate alleles in three independently isolated lines (Supplementary Figure 3.1). G28R is located in a predicted low-complexity domain while R341C and Q653* are located in KH1 domains.

imph-1(lf) modifies ALS-associated defects in *sod-1G85R* animals

To test if loss of *imph-1* suppresses stress-induced glutamatergic neuron degeneration in *sod-1G85R*, we used a preexisting loss of function allele, *tm1623*. *imph-1(lf)* did not cause degeneration of PHA/PHB neurons after exposure to oxidative stress. However, *sod-1G85R; imph-1(lf)* double mutants showed partial suppression of glutamatergic neuron degeneration. In addition, we examined if *imph-1(lf)* suppressed glutamatergic neuron degeneration in *sod-1(tm776)* animals and observed that *imph-1(lf)* partially suppressed the loss of function defects of *sod-1(tm776)*. As cholinergic motor neuron degeneration is a hallmark of ALS (Huynh et al., 2016), we examined cholinergic neurons in the ventral nerve cord. After exposure to oxidative stress, cholinergic neurons in *sod-1G85R* animals degenerate (Baskoylu et al., 2018). We observed that *imph-1(lf)* completely rescued cholinergic neuron degeneration in *sod-1G85R* animals (Figure 3.3). From these results, we conclude that *imph-1(lf)* suppresses both stress-induced glutamatergic and cholinergic neuron degeneration.

Does loss of stress granule proteins modify glutamatergic neuron degeneration?

Exogenous stressors may influence the development and progression of ALS. The human orthologs of IMPH-1 are IGF2BP1, IGF2BP2, and IGF2BP3. IGF2BPs are involved in promoting cell growth and survival (Boylan et al., 2008) and in the formation of granules after stressful events (Zeng et al., 2020). Thus, we decided to examine whether loss of stress granules modified stress-induced glutamatergic neuron degeneration in *sod-1G85R* mutant animals. We focused our analysis on two RNA binding proteins Ras GTPase-activating protein-binding protein 1 (G3BP1) and Nucleolysin TIA-1 isoform p40 (TIA1). The *C. elegans* G3BP1 ortholog is *gtbp-1* and the TIA1 orthologs are *tiar-1* and

tiar-2. Loss of a single gene did not significantly modify glutamatergic neuron degeneration (Figure 3.4). This suggest that loss of a single gene encoding granule proteins may be insufficient to modify glutamatergic neuron degeneration.

imph-1(lf) suppresses stress-associated survival defects of *sod-1G85R* animals

Oxidative stress is thought to be a major factor that influences the development and progression of ALS (Barber et al., 2006). Lifespan of *sod-1G85R* animals is decreased on oxidative stress (Baskoylu et al., 2018). To determine if *imph-1(lf)* modified oxidative stress-induced survival defects, we examined lifespan of animals exposed to 2.5 mM paraquat (Figure 3.5). *imph-1(lf)* extended the median survival time of *sod-1G85R* animals by two days (Figure 3.5). Interestingly, we found that the *imph-1(lf)* mutant animals survived, on average, one day longer than the respective wildtype controls (Figure 3.5). This suggest that *imph-1(lf)* may be resistant to oxidative stress and confer resistance to *sod-1G85R* animals.

imph-1(lf) modifies defects associated with SMA

There are many clinical and pathological hallmarks that are shared between ALS and a predominately childhood neuromuscular disease spinal muscular atrophy (SMA). SMA is the leading genetic cause of infant death and is caused by loss of function of the survival of motor neuron (SMN) protein (Mercuri et al., 2020). IGF2BP1 has been observed to modify neuronal growth defects observed in *in vitro* models of SMA (Fallini et al., 2014). To test the hypothesis that there are common mechanisms underlying the cellular and molecular mechanisms of neurodegeneration in both these neurodegenerative disorders, we crossed *imph-1(lf)* on to established models of SMA

which have major neuromuscular junction and survival defects (Briese et al., 2009; Dimitriadi et al., 2016; O'Hern et al., 2017). To assess if *imph-1(lf)* modified defects observed in *C. elegans* SMA model, we examined if *imph-1(lf)* modified survival. (Figure 3.6). *smn-1(rt248)* animals had a median survival of 8 days, while *smn-1(rt248); imph-1(lf)* animals had a median survival time of 6 days.

3.5 Discussion

Here we report the first classical forward genetic screen for suppressors of glutamatergic neuron degeneration in single-copy *sod-1G85R* mutant animals. The identification of *imph-1/IGF2BP* as a genetic suppressor of glutamatergic neuron degeneration suggests that common pathways, such as RNA dysregulation, may underly ALS pathologies.

Screening strategies used to identify suppressors of stress-induced glutamatergic neuron degeneration

To identify suppressors of stress-induced glutamatergic neuron degeneration, we used two methods: pooled F1 strategy and clonal F1 strategy. Both strategies yielded suppressors of glutamatergic neuron degeneration. However, from our pooled F1 strategies, many putative suppressor lines isolated in the initial screening stages, did not pass subsequent rounds of rescreening. Thus, we switched to singling F1s following mutagenesis of *sod-1G85R*^{M} animals. The clonal F1 screening strategy had two main advantages. First, putative suppressor lines identified in the initial screen passed subsequent rescreens at a higher rate. Second, the lineage of the lines identified from the clonal F1 screen was known and putative suppressor lines could not originate from the same F1 parent. While suppressor lines can be isolated from both pooled and clonal

F1 strategies, the clonal F1 strategy yielded more suppressor lines that were not false positives.

imph-1 as a modifier of *SOD1ALS:* potential for cross-species modification?

imph-1 is homologous to the IGF2BP family of RNA binding proteins (Supplemental Figure 3.1). We identified *imph-1*, an RNA binding protein, as a suppressor of stress-induced glutamatergic neuron degeneration (Figure 3.3). Further examination revealed that loss of *imph-1* also suppressed cholinergic neuron degeneration and extended survival of *sod-1G85R* animals on oxidative stress. Recently, the fly ortholog of *imph-1*, *Imp*, was shown to modify the degenerative eye defect in models of FUS and TDP43 (Kankel et al., 2020). Additional studies have shown that IGF2BPs can modify in models of C9orf72 (Kramer et al., 2018) and are components of granules with other ALS-linked genes (Liao et al., 2019). This suggests that there may be a conserved role for IGF2BPs in ALS.

Role of RNA binding proteins in ALS

Recent studies show an increasing importance of RNA binding protein and RNA homeostasis mechanisms in the development, progression, and prognosis of ALS. Although, *gtbp-1(lf)*, *tiar-1(lf)*, and *tiar-2(lf)* did not independently modify glutamatergic neuron degeneration (Figure 3.4), there may be compensatory mechanisms at play. For example, *tiar-1(lf)* may be compensated for by *tiar-2* and vice versa. Future experiments could address these compensatory mechanisms by examining double and triple mutants. It is also likely that stress granlues are not the critical RNA granules dysregulated in SOD1 ALS. Other granule genes such as STAU1/*stau-1* could be examined as candidate

neuronal transport granules. Mutations in numerous RNA binding proteins cause ALS. Further disruption of spatial and temporal localization of RNAs is known to be important in ALS pathongenesis. IGF2BPs play important roles in these processes as they localize and interact with to RNA granules and associate with other RNA binding proteins. Further, there may be a more important role for IGF2BPs. IGF2BP2 forms a part of a molecular tether complex, that aids in the movement of RNA granules attached to lysosomes (Liao et al., 2019). In this complex there are other proteins that are genetically linked with ALS such as ANEX11.

Resistance to oxidative stress

An alternative hypothesis is that loss of *imph-1* may confer resistance to oxidative stress and protect against neurodegeneration. In order for cell survival, cells must maintain a steady level of reactive oxygen species needed for cell signaling. Superoxide dismutases help maintain this balance by breaking down excess superoxide radicals to less toxic hydrogen peroxide and water. Further, these superoxide radicals may build up and cause cellular damage to motor neurons in ALS patients. We observed that while *imph-1(lf)* suppresses oxidative stress-associated survival defects in *sod-1G85R* mutant animals*, imph-1(lf)* animals have an extended survival on oxidative stress (Figure 3.5). This suggests that *imph-1(lf)* confers oxidative stress resistance and may protect against neurodegeneration through the activation of compensatory stress regulation pathways. There are many mRNA targets of IGF2BPs, and recent reports have found that IGF2BPs do bind to oxidative stress response genes (Samuels et al., 2020).

imph-1 may act as a modifier of multiple neuromuscular diseases

ALS shares many genetic and pathologic factors with spinal SMA. Recent studies have suggested that similar mechanisms cause motor neuron degeneration in both of these diseases (Chi et al., 2018; Mirra et al., 2017). Further, SMN1 gene duplications are associated with influencing the development of ALS (Blauw et al., 2012; Corcia et al., 2006). Thus, it seems likely that common mechanisms underly both ALS and SMA. Previous reports IGF2BPs have been shown to modify defects in SMA models (Fallini et al., 2014). When lifespan of animals was evaluated, we observed that *imph-1(lf)* enhanced survival defects of *smn-1(rt248)* while having no significant effect on *smn-1(ok355)* (Figure 3.6).

3.6 Other candidate genes

In this section, I will discuss a subset of additional candidate suppressor genes and the mechanisms that may be involved.

Backcrossing and putative identification of *phy-3/P4HA* as a suppressor for neurodegeneration

We used a modified version of the sibling subtraction method (Joseph et al., 2018) and identified many mutations on chromosome V after backcrossing. To identify the causal mutation in the suppressor strain, we used recombination mapping and RFLP analysis, which led us to the identification of *phy-3* (Figure 3.6A). To test if *phy-3(lf)* caused suppression of glutamatergic neuron degeneration, we used a preexisting loss of function allele, *ok199.* We observed partial suppression of glutamatergic neuron degeneration (Figure 3.6B). PHY-3 is homologous to P4HA, a prolyl hydroxylase that is involved in metabolism. It commonly complexes with protein disulfide isomerases (PDI). PDIs are

known to enhance defects in ALS (Perri et al., 2017; Woehlbier et al., 2016). Thus, we explored if loss of the PDI orthologs *pdi-1* enhance glutamatergic neuron degeneration and observed *pdi-1(lf)* does not enhance defects in *sod-1G85R* or *sod-1(-)* animals (Figure 3.6C-D). However, there are numerous orthologs of PDIs in *C. elegans,* including *pdi-2*, which is essential for *C. elegans* development (Winter et al., 2007). These genes may act redundantly to compensate for the loss of *pdi-1*. Additional double mutant analysis should be done to examine the effects of loss of both *pdi-1* and *pdi-2* on modification of glutamatergic neuron degeneration. Additionally, there is a possibility that *pdi-1(lf)* may enhance cholinergic neuron degeneration but not glutamatergic neuron degeneration. As different mechanisms may underly glutamatergic (loss of function) and cholinergic (gain of function) neuron degeneration, modifier genes may only impact a subset of disease-associated defects.

Overlap of candidate modifiers with a previous RNAi screen

In our attempt to identify causative suppressor gene, we compared exonic SNPs generated in our random mutagenesis to established modifiers of aggregation a *C. elegans* overexpression hSOD1G93A model (Silva et al., 2011). From this analysis we identified seven candidate genes (Figure 3.7). In the next section, I will briefly summarize the candidate genes.

MIG-6/PAPLN are components of the extracellular matrix and in *C. elegans* is necessary for distal tip cell migration (Kawano et al., 2009). Recently, a role in axonal guidance, remodeling, and dendritic patterning has been proposed for *mig-6* (Ramirez-Suarez et al., 2019). OGDH-1/OGDH is involved in metabolism of glucose, glutamate, and α-ketoglutaric acid (Sen et al., 2012). Many patients with ALS exhibit metabolic changes

and this may implicate metabolic mechanisms that are dysregulated in ALS. METR-1/MTR is a methionine synthase involved in processing amino acids. TAG-335/GMPPB is a subunit of GDP mannose pyrophosorylase (Ning and Elbein, 2000). Knockdown of *tag-335* has been observed to induce ER stress and the unfolded protein response (Higuchi-Sanabria et al., 2020). CLEC-19/MRC1 encodes a mannose receptor that binds carbohydrates and may play a role in endocytosis (Moseman et al., 2013). RNR-2/*RRM2B* is a ribonucleotide reductase involved in regulating mitochondrial activity and may play a role in the oxidative stress response (Cho et al., 2015). F43G9.12/GCFC2 an understudied DNA binding factor. However, recent GWAS reports suggest that the GCFC2 loci may influence hippocampal neurodegeneration (Melville et al., 2012).The putative functions of these modifier genes suggest a multitude of pathways have the potential to modify ALS-associated defects in *C. elegans*. The ability to regulate the levels of cellular stress either through the unfolded protein response and regulation oxidative stress response seem to be important factors.

3.7 Overall conclusions

Many pathways likely contribute to neurodegeneration in ALS. Due to the unbiased nature of classical forward genetic screens, previously unknown mechanisms that lead to neurodegeneration may be revealed. Here, we suggest that RNA binding proteins may play a larger role in SOD1 ALS than previously expected. Further efforts must be put into identifying the remainder of the suppressors isolated from our screen. By identifying genetic modifiers, we may be able to solidify the relationship between SOD1 and other ALS-causal genes sand provide novel insights into mechanisms behind the selective degeneration of motor neurons in ALS.

3.8 Figures and Tables

Figure 3.1. Screening and analysis strategies for identifying suppressors stress-induced glutamatergic neuron degeneration in *sod-1G85R*. (A) Schematic representation of the dye-filling assay used to assess oxidative stress-induced glutamatergic neuron degeneration. At least 20 animals were scored for intact PHA/PHB neurons per genotype/treatment using a fluorescent dissection scope (Baskoylu reference). Without, all animals have 2 PHA and 2 PHB neurons that take up dye in the tail (photo, right). Loss of processes or cell death can prevent dye-filling. An animal was scored as suppressed if 3 or 4 of the PHA or PHB neurons were intact. On average, after exposure to oxidative stress 10% of *sod-1G85R* animals have intact PHA/PHB neurons (graph, right). WT animals exhibit little to no degeneration. (B) Two screening strategies were used to isolate *sod-1G85R* suppressor lines: Pooled F1 Strategy and Clonal F1 Strategy. Nine suppressor lines were identified through the Pooled F1 Strategy, while 36 candidate lines were isolated with the Clonal F1 Strategy. The number of F1s screened in each strategy is listed (left). At least three putatives were singled from lines with greater than 25% intact F2 animals. Putatives were retested and those showing suppression were serially passaged (3 animals per generation) and frozen for long-term storage. Lines were thawed and retested. Only lines that retained suppression are reported. (C) Suppressor lines were sent for whole genome sequencing along with *sod-1G85R*. The CloudMap program, developed by the Hobert lab, was used to analyze raw sequence data. Using the unmapped mutant workflow, single nucleotide polymporphisms (SNPs) were identified in the suppressor lines. As mutations present in the original line do not cause suppression of the dye-filling defect, the SNPs present in the original *sod-1G85R* strain were then subtracted from suppressor lines with the variant subtraction workflow. Finally, SNPs that caused changes leading to early stops, potential frame shifts, altered splice junctions, or missense amino acid changes. On average, there were 50 of these mutations per line. (D) To further eliminate non-causal mutations 11 suppressor lines were backcrossed to 2x through *sod-1(tm776); hjSi20; vsIs48*. The putative suppressor was maintained by screening for suppression of the dye-filling defect after each backcross step. Two independent isolates from each candidate line was sent for whole genome sequencing. SNPs were mapped using CloudMap and mutations present in both parental *sod-1G85R* and *sod-1(tm776); hjSi20; vsIs48* were subtracted. Mutations in sibling lines were compared and potential causal mutations are defined as mutations that were present in both strains.

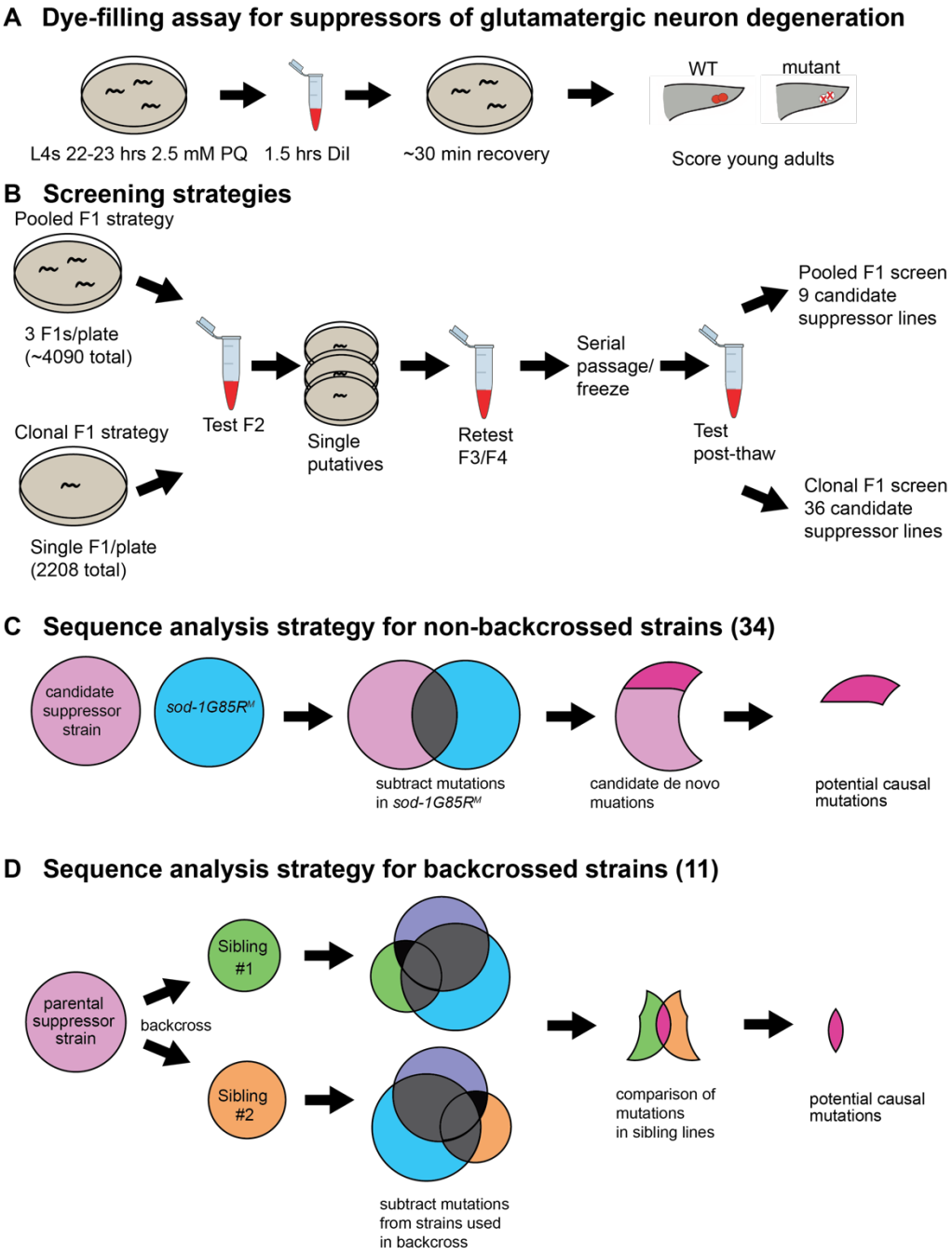

A Dye-filling assay for suppressors of glutamatergic neuron degeneration
WT
mutant
L4s 22-23 hrs 2.5 mM PQ
1.5 hrs DiI
~30 min recovery
Score young adults
B Screening strategies
Pooled F1 strategy
3 F1s/plate
(~4090 total)
Test F2
Single
putatives
Retest
F3/F4
Serial
passage/
freeze
Test
post-thaw
Pooled F1 screen
9 candidate
suppressor lines
Clonal F1 strategy
Single F1/plate
(2208 total)
Clonal F1 screen
36 candidate
suppressor lines
C Sequence analysis strategy for non-backcrossed strains (34)
candidate
suppressor
strain
sod-1G85R
subtract mutations
in sod-1G85R
candidate de novo
muations
potential causal
mutations
D Sequence analysis strategy for backcrossed strains (11)
parental
suppressor
strain
backcross
Sibling
#1
Sibling
#2
subtract mutations
from strains used
in backcross
comparison of
mutations
in sibling lines
potential causal
mutations

Figure 3.2. Summary of the lines screened and variation of suppression. (A) Summary of the screen for glutamatergic neuron degeneration using the pooled and clonal strategy. The average suppression of glutamatergic neuron degeneration after thawing in 45 lines sent for whole genome sequencing. Two or three plates were scored for each line with at least 20 animals counted. Candidate suppressor lines that were sequenced had at least 25% of animals with intact neurons to pass the post-thaw rescreen.

A

	Pooled Strategy	Clonal Strategy	Total
F1 animals screened	4090	2208	6289
Haploid chromosomes screened	8180	4416	12586
Haploid genomes screened	3.27	1.76	5.04
Singled putatives	278	290	568
Serially passaged/frozen lines	37	98	135
Candidate lines	9	36	45

B

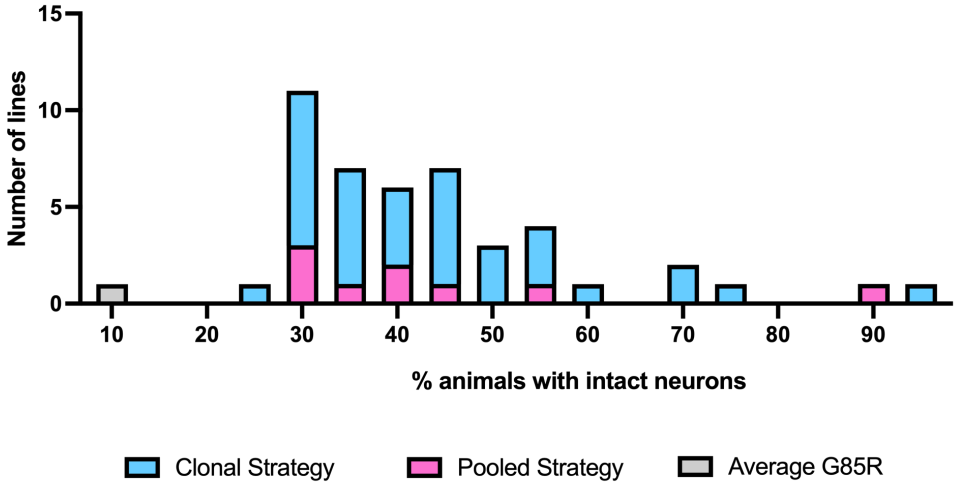


Figure 3.3. ***imph-1* is a suppressor of glutamatergic and cholinergic neuron degeneration.** Suppression observed in the post-thaw rescreen in the lines with a mutation in *imph-1* (A). Two-trials were completed for each line during rescreen. Lines passed if two out of three trials had greater than or equal to 25% of animals with intact neurons. Loss of *imph-1* decreases stress-induced (B) PHA/PHB glutamatergic neurons and (C) cholinergic neuron degeneration in *sod-1G85R* animals. *imph-1(lf)* does not result in degeneration of either glutamatergic or cholinergic neurons after oxidative stress exposure. Additionally, we observed that *imph-1(lf)* suppressed *sod-1(-)* associated glutamatergic neuron degeneration (C). Combined results are from three or four independent trials, n=12 animals per trial. Error bars indicate +/- SEM. Two-tailed t-test. * $P < 0.05$. Location and mutations generated through random mutagenesis and protein alignments detailing the conservation of *imph-1* to IGF2BPs are located in Figure S3.1.

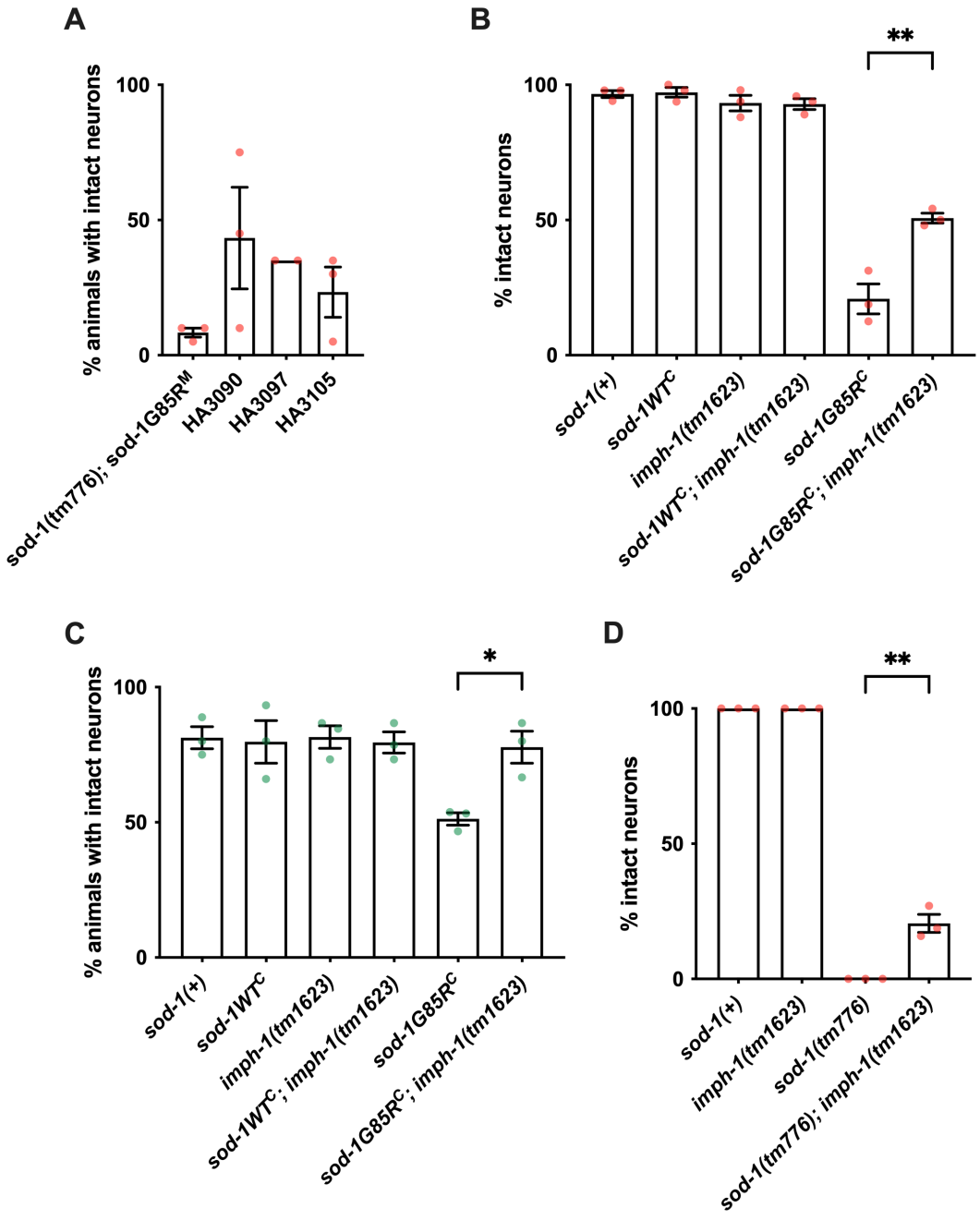

A
% animals with intact neurons
100
50
0
sod-1(tm776); sod-1G85R^M
HA3090
HA3097
HA3105
B
**
% intact neurons
100
50
0
sod-1(+)
sod-1WT^C
imph-1(tm1623)
sod-1WT^C; imph-1(tm1623)
sod-1G85R^C
sod-1G85R^C; imph-1(tm1623)
C
*
% animals with intact neurons
100
50
0
sod-1(+)
sod-1WT^C
imph-1(tm1623)
sod-1WT^C; imph-1(tm1623)
sod-1G85R^C
sod-1G85R^C; imph-1(tm1623)
D
**
% intact neurons
100
50
0
sod-1(+)
imph-1(tm1623)
sod-1(tm776)
sod-1(tm776); imph-1(tm1623)

Figure 3.4. Loss of select stress granule associated genes do not modify stress-induced glutamatergic neuron degeneration in *sod-1G85R* animals. Select stress granule genes, *G3BP1/gtbp-1* and *TIA1/tiar-1* and *tiar-2* were examined. These genes were tested for suppression (22 hrs of 2.5 mM paraquat exposure) as well as enhancement (4 hrs 2.5 mM parquat exposure) of stress induced degeneration of PHA/PHB glutamatergic neurons. Combined results are from three or four independent trials, n > 30 per genotype.

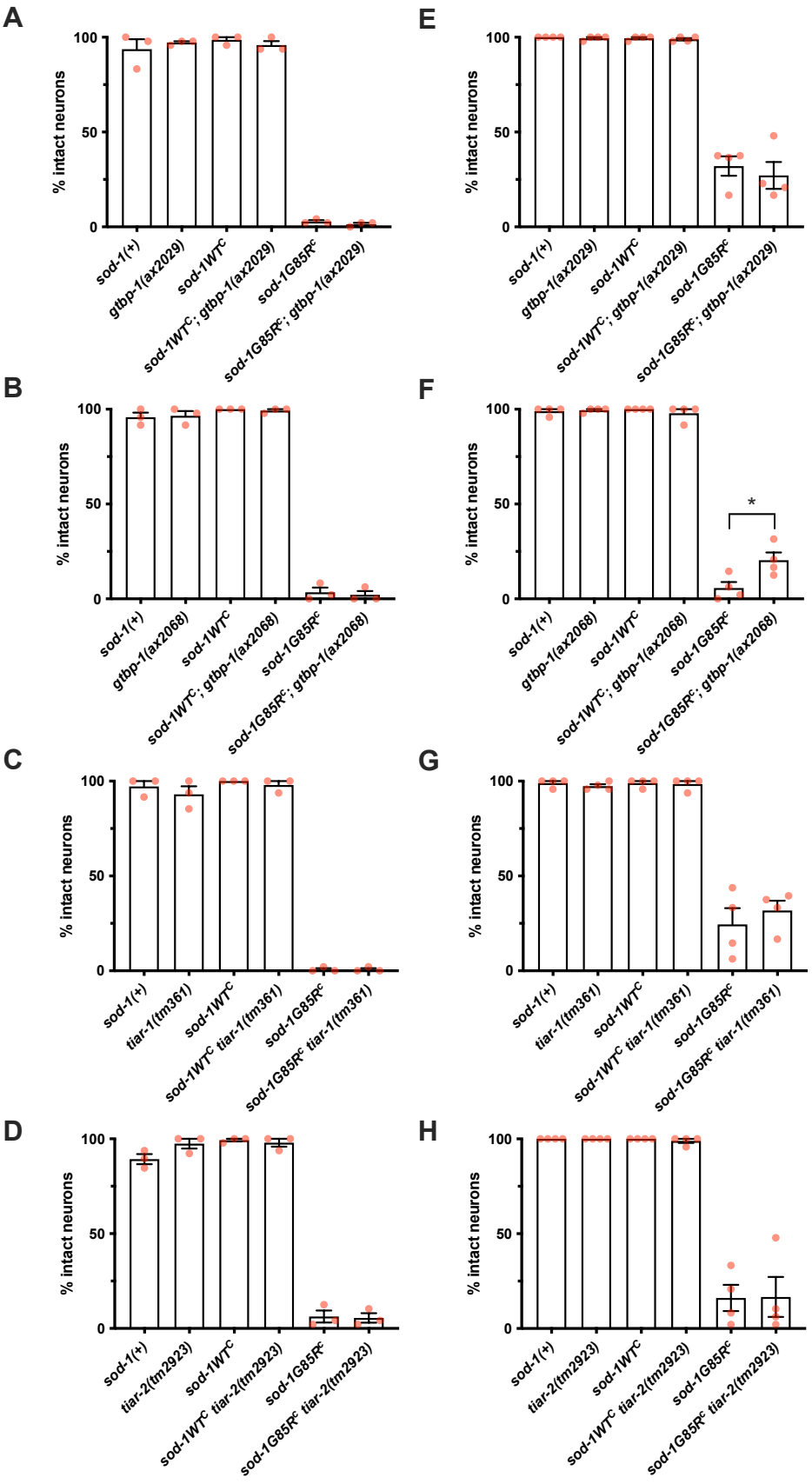

A
B
C
D
E
F
G
H
% intact neurons
100
50
0
sod-1(+)
gtbp-1(ax2029)
sod-1WT^c
sod-1WT^c; gtbp-1(ax2029)
sod-1G85R^c
sod-1G85R^c; gtbp-1(ax2029)
gtbp-1(ax2068)
sod-1WT^c; gtbp-1(ax2068)
sod-1G85R^c; gtbp-1(ax2068)
tiar-1(tm361)
sod-1WT^c tiar-1(tm361)
sod-1G85R^c tiar-1(tm361)
tiar-2(tm2923)
sod-1WT^c tiar-2(tm2923)
sod-1G85R^c tiar-2(tm2923)
*

Figure 3.5. *imph-1(lf)* confers resistance to oxidative stress in *sod-1G85R* mutant animals. Lifespan of mutant animals was assessed at 25˚C from L4 (larval stage 4) until death on 2.5 mM paraquat. Fresh 2.5 mM paraquat plates were made before animals were transferred to avoid progeny contamination. n=30-40 animals in four independent trials. When compared with the respective controls, *imph-1(lf), sod-1WT; imph-1(lf)*, and *sod-1G85R; imph-1(lf)* animals had an increase in median survival time. Censored animals were included in the analysis up until the day they were censored. Log-rank test: * P<0.0001 (GraphPad Prism, La Jolla, CA).

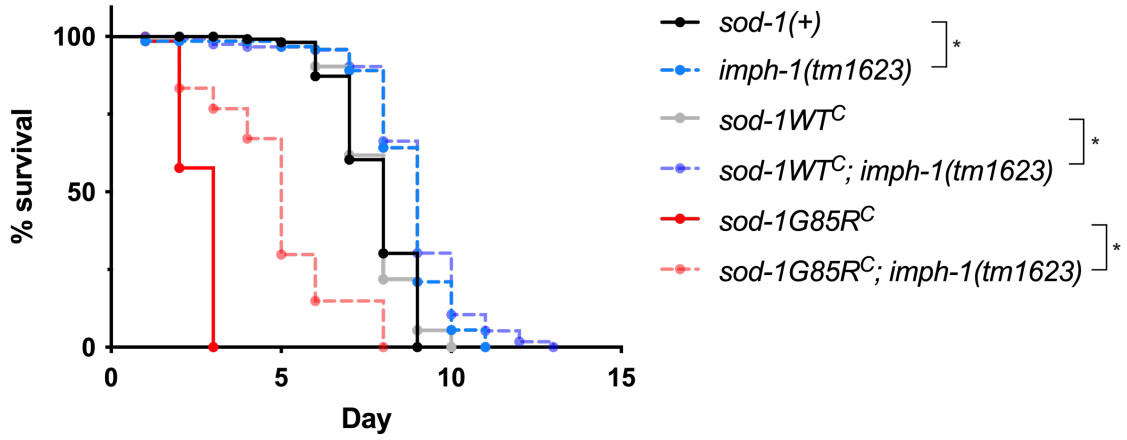


Figure 3.6. *imph-1(lf)* may act as a cross-disease modifier. (A) A schematic representation of *smn-1* gene and the corresponding deletion alleles. *smn-1(ok355)* is a 975 bp deletion that also removes promoter regions in a nearby gene, *klp-16*. *smn-1(rt248)* is an 8 bp deletion causing an early stop. Both of these models exhibit a shortened lifespan. Survival was cored at 20˚C and graphs presented here were broken up for clarity (B) controls, (C) *smn-1(rt248)* vs. *smn-1(rt248); imph-1(tm1623*), and (D) *smn-1(ok355)* vs. *smn-1(ok355); imph-1(tm1623)*. Statistical analysis was completed with a log-rank test: * P<0.0001 (GraphPad Prism, La Jolla, CA).

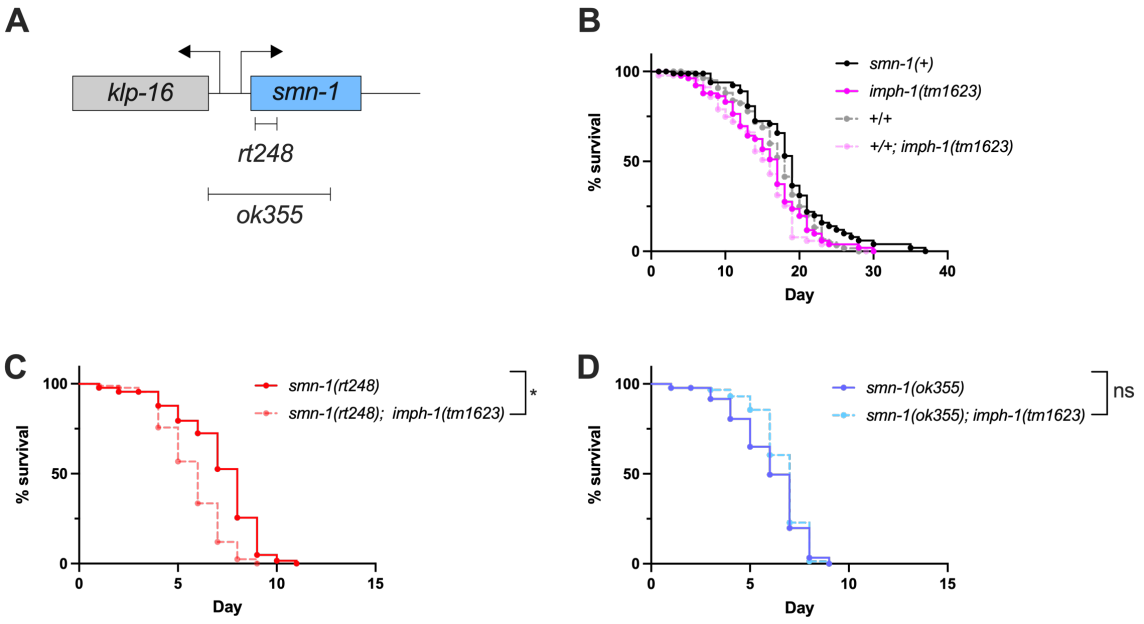


Figure 3.7. Identification of *phy-3* as a candidate suppressor. (A) Whole genome sequencing of two independently isolated backcrossed lines derived from the same original mutagenized line revealed an island of SNPs on chromosome V. After recombination mapping of the candidate suppressor, a likely candidate was identified as *phy-3*. (B) A preexisting deletion allele*, ok199*, was used to test if loss of *phy-3* suppressed glutamatergic neuron degeneration under dissection scope. PHY-3/P4HA forms complexes with PDI-1/*PDI*, thus we tested for enhancement of glutamatergic neuron degeneration. (C-D) Two alleles of *pdi-1* were tested observed that *pdi-1(lf)* does not enhance glutamatergic neuron degeneration in *sod-1G85R* or *sod-1(-)* animals.

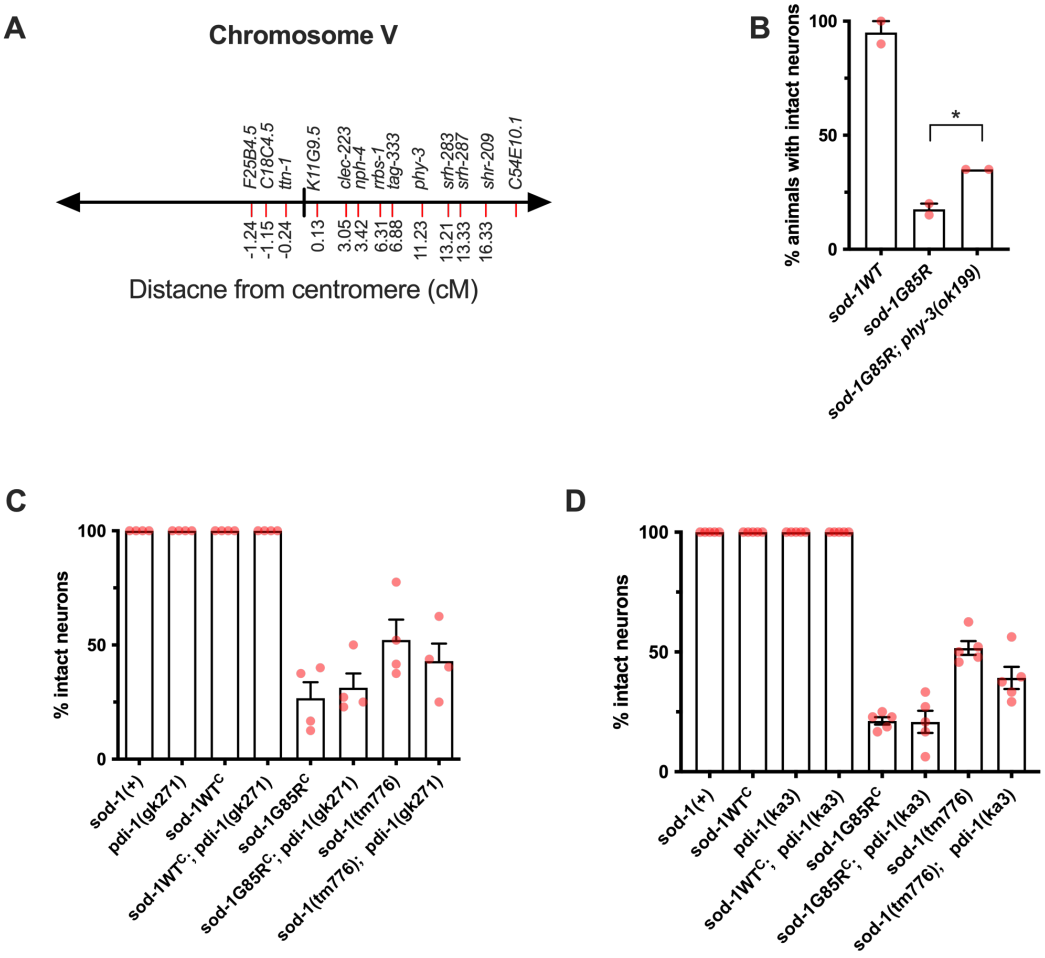


Figure 3.8. Schematic representation of overlap of candidate suppressors. Homozygous non-synonomous exconic mutations caused by random mutagenesis in suppressor strains were compared with hits from an RNAi screen for modifiers of aggregation in a hSOD1G93A overexpression model. Only seven genes were identified with SNPs from the forward genetic screen for suppressors of glutamatergic neuron degeneration that also modified aggregation.

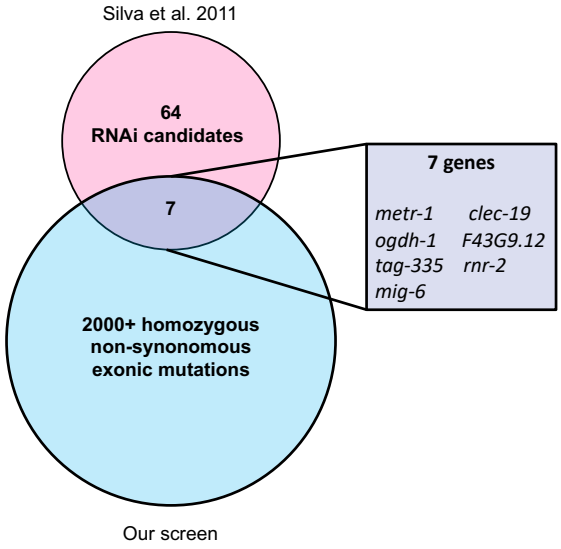


Table 3.1. List of strains.

Strain Name	Genotype
N2	Wildtype, referred to as *sod-1(+)*
HA2281	*sod-1(tm776) II, referred to as sod-1(-)*
HA2986	*sod-1(rt448[WTC] II; pha-1(+) III, referred to as sod-1WTC*
HA2329	*sod-1(rt449[G85RC] II; pha-1(+) III, referred to as sod-1G85RC*
HA2619	*sod-1(tm776) II; unc-119(+) III; rtSi001 [sod-1p::sod-1(WT)];cb-unc-119(+)] IV, referred to as sod-1WTM*
HA2426	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV, referred to as sod-1G85RM*
HA2720	*sod-1(tm776) II; hjSi20 [myo-2p::mCherry::unc-54 3'UTR]; vsIs48 [unc-17p::GFP]*
HA2840	*sod-1(rt448[sod-1WT C]) II*
HA2942	*sod-1(rt451[sod-1G85R C]) II*
HA3487	*imph-1(tm1623) III 4x*
HA3488	*sod-1(rt448[848593WT]) II; imph-1(tm1623) III*
HA3489	*sod-1(rt451[G85R]) II; imph-1(tm1623) III*
HA3494	*imph-1(tm1623) III; vsIs48[unc-17p::GFP]*
HA3495	*sod-1(rt448[848593WT]); imph-1(tm1623) III; vsIs48[unc-17p::GFP]*
HA3496	*sod-1(rt451[G85R]) II; imph-1(tm1623) III; vsIs48[unc-17p::GFP]*
HA3503	*phy-3(ok199) V 4x*
HA3504	*sod-1(rt448[848593WT]) II; phy-3(ok199) V*
HA3505	*sod-1(rt451[G85R]) II; phy-3(ok199) V*
HA3620	*sod-1(tm776) II; imph-1(tm1623) III*
HA4008	*pdi-1(gk271) III 4x*
HA4009	*sod-1WTC II; pdi-1(gk271) III*
HA4010	*sod-1G85RC II; pdi-1(gk271) III*
HA4012	*pdi-1(ka3) III 4x*
HA4013	*sod-1WTC II; pdi-1(ka3) III*
HA4014	*sod-1G85RC II; pdi-1(ka3) III*
HA4050	*gtbp1-(ax2029) IV 4x*
HA4051	*sod-1WTC II; gtbp-1(ax2029)*
HA4052	*sod-1G85RC II; gtbp-1(ax2029)*
HA4053	*gtbp-1(ax2068) IV 4x*
HA4054	*sod-1WTC II; gtbp-1(ax2068)*
HA4055	*sod-1G85RC II; gtbp-1(ax2068)*
HA4056	*tiar-1(tm361) II 4x*
HA4057	*tiar-1(tm361) sod-1WTC II*
HA4058	*tiar-1(tm361) sod-1G85R II*
HA4062	*tiar-2(tm2923) II 4x*
HA4063	*sod-1WTC tiar-2(tm2923) II*
HA4064	*sod-1G85RC tiar-2(tm2923) II*
LX929	*vsIs48 [unc-17p::GFP]*
QP220	*unc-60(m35) dpy-11(e224) rol(sc148) V*
HA3920	*smn-1(ok355)/tmC18[dpy-6(tmIs1236)] I*
HA3902	*smn-1(ok355)/tmC18[dpy-6(tmIs1236)] I; imph-1(tm1623) III*
HA3900	*smn-1(rt248)/tmC18[dpy-6(tmIs1236)] I*
HA3901	*smn-1(ok355)/tmC18[dpy-6(rt248)] I; imph-1(tm1623) III*
HA3903	*+/tmC18[dpy-6(tmIs1236] I*
HA3904	*+/tmC18[dpy-6(tmIs1236] I; imph-1(tm1623) III*

Table 3.2. **Mutagenized *sod-1G85R* lines suppressor lines.** Although there are many *de novo* mutations generated in these strains, the allele name rt### refers to the allele generated in the suppressor gene.

Strain Name	Genotype
HA2843	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt277*
HA2844	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt270*
HA2867	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt274*
HA2868	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt279*
HA2869	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt276*
HA2870	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt278*
HA2871	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt271*
HA2872	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt272*
HA2873	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt269*
HA2875	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt273*
HA2876	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt275*
HA2957	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt283*
HA2958	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt284*
HA2959	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt285*
HA2960	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt286*
HA2961	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt287*
HA2962	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt288*
HA2963	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt289*
HA2967	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt290*
HA2968	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt291*
HA2969	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt292*
HA2970	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt293*
HA2971	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt294*
HA2972	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt295*

HA2973	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt296*
HA2974	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt297*
HA2975	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt298*
HA2976	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt299*
HA2977	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt300*
HA3001	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt301*
HA3002	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt302*
HA3003	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt303*
HA3004	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt304*
HA3005	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt305*
HA3006	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt306*
HA3007	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt307*
HA3008	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt308*
HA3023	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt309*
HA3024	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt310*
HA3025	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt312*
HA3026	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt313*
HA3027	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt314*
HA3028	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt311*
HA3029	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt315*
HA3030	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt316*
HA3041	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt319*
HA3042	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt320*
HA3043	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt321*
HA3044	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt322*
HA3045	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt323*
HA3046	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt324*

HA3047	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt325*
HA3048	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt326*
HA3050	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt328*
HA3052	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt329*
HA3053	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt330*
HA3054	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt331*
HA3055	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt332*
HA3056	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt333*
HA3057	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt334*
HA3058	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt335*
HA3059	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt336*
HA3060	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt337*
HA3061	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt338*
HA3062	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt339*
HA3063	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt340*
HA3064	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt341*
HA3065	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt342*
HA3066	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt343*
HA3067	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt344*
HA3068	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt345*
HA3069	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt346*
HA3070	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt347*
HA3071	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt348*
HA3072	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt349*
HA3073	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt350*
HA3074	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt351*
HA3075	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt352*

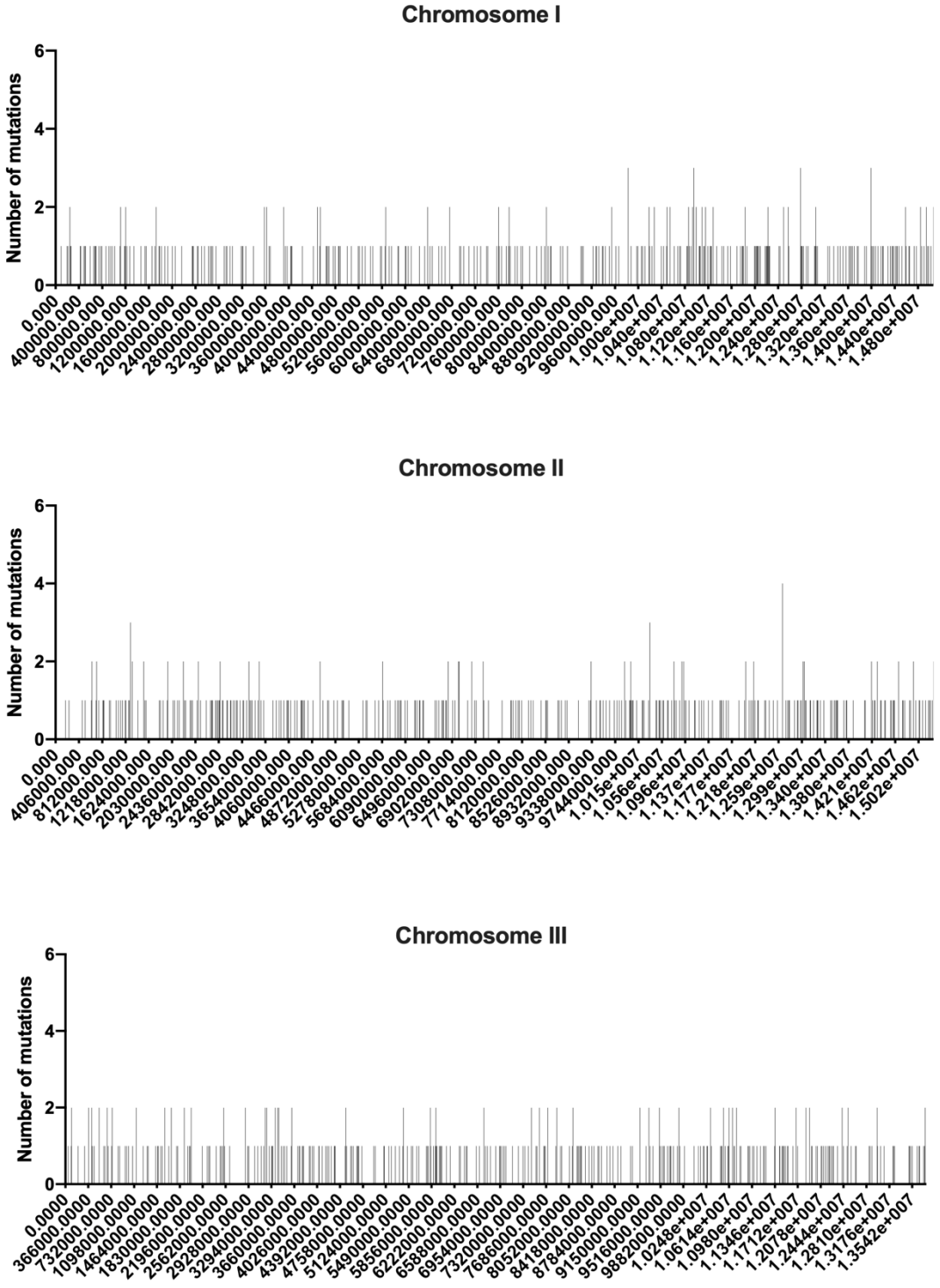
HA3076	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt353*
HA3077	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt354*
HA3078	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt355*
HA3079	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt356*
HA3079	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt356*
HA3080	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt357*
HA3081	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt358*
HA3082	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt359*
HA3083	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt360*
HA3084	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt361*
HA3085	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt362*
HA3086	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt363*
HA3087	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt364*
HA3088	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt365*
HA3089	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt366*
HA3090	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt367*
HA3091	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt368*
HA3092	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt369*
HA3093	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt370*
HA3094	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt371*
HA3095	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt372*
HA3096	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt373*
HA3097	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt374*
HA3098	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt375*
HA3099	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt376*
HA3100	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt377*
HA3101	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt378*

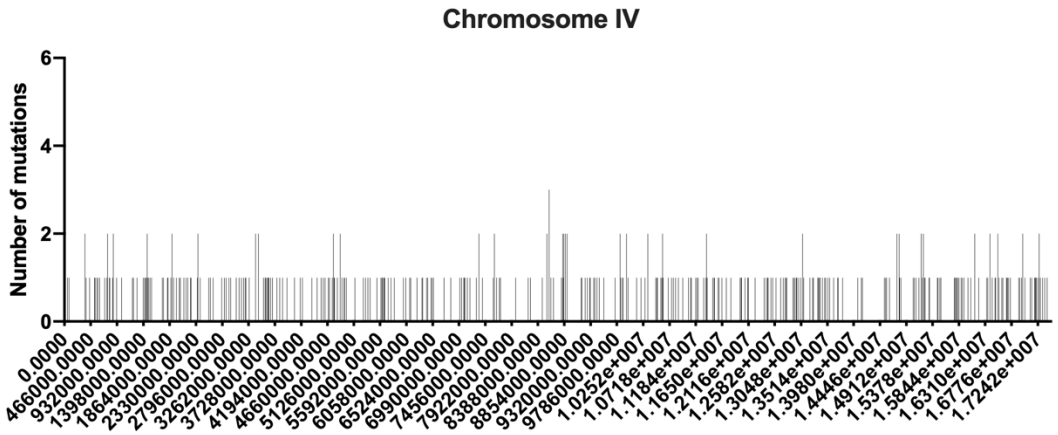
HA3102	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt379*
HA3103	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt380*
HA3104	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt381*
HA3105	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt382*
HA3106	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt383*
HA3109	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt385*
HA3110	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt386*
HA3111	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt387*
HA3112	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt388*
HA3113	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt389*
HA3114	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt390*
HA3115	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt391*
HA3116	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt392*
HA3118	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt394*
HA3119	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt395*
HA3120	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt396*
HA3121	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt397*
HA3122	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt398*
HA3123	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt399*
HA3124	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt404*
HA3125	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt400*
HA3126	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt401*
HA3127	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt402*
HA3128	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt403*
HA3129	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt405*
HA3131	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt406*
HA3132	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt407*

HA3133	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt408*
HA3134	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt409*
HA3135	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt410*
HA3136	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt411*
HA3137	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt412*
HA3138	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt413*
HA3142	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt415*
HA3143	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt416*
HA3144	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt417*
HA3145	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt418*
HA3146	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt419*
HA3148	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt421*
HA3149	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt422*
HA3150	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt423*
HA3151	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt424*
HA3152	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt425*
HA3153	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt426*
HA3210	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt430*
HA3211	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt431*
HA3212	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt432*
HA3213	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt433*
HA3214	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt434*
HA3215	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt435*
HA3216	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt436*
HA3217	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt437*
HA3218	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt438*
HA3223	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt439*

HA3224	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt440*
HA3240	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt441*
HA3241	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt442*
HA3153	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt426*
HA3210	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt430*
HA3211	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt431*
HA3212	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt432*
HA3213	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt433*
HA3214	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt434*
HA3215	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt435*
HA3216	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt436*
HA3217	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt437*
HA3218	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt438*
HA3223	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt439*
HA3224	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt440*
HA3240	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt441*
HA3241	*sod-1(tm776) II; unc-119(+) III; rtSi006 [sod-1p::sod-1(G85R)];cb-unc-119(+)] IV; vsIs48 [unc-17::GFP] X; rt442*

Supplemental Figure 3.1 Collective positions of *de novo* non-synonymous exonic mutations in randomly mutagenized *sod-1G85R* suppressor lines. These candidate exonic SNPs are found throughout the genome. Graphs were generated for each chromosome with 2000 bp bins that is roughly equivalent to the average length of a coding gene.




Chromosome IV
Number of mutations
6
4
2
0
0.0000
466000.0000
932000.0000
1398000.0000
1864000.0000
2330000.0000
2796000.0000
3262000.0000
3728000.0000
4194000.0000
4660000.0000
5126000.0000
5592000.0000
6058000.0000
6524000.0000
6990000.0000
7456000.0000
7922000.0000
8388000.0000
8854000.0000
9320000.0000
9786000.0000
1.0252e+007
1.0718e+007
1.1184e+007
1.1650e+007
1.2116e+007
1.2582e+007
1.3048e+007
1.3514e+007
1.3980e+007
1.4446e+007
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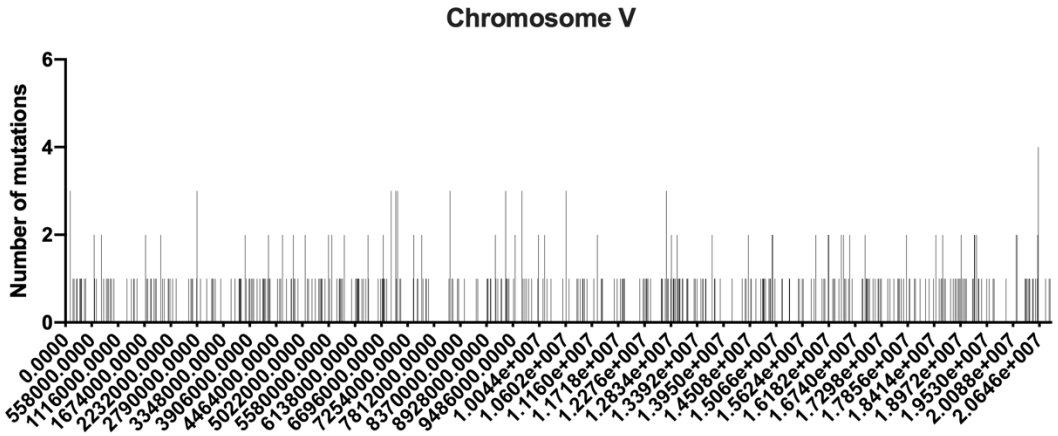
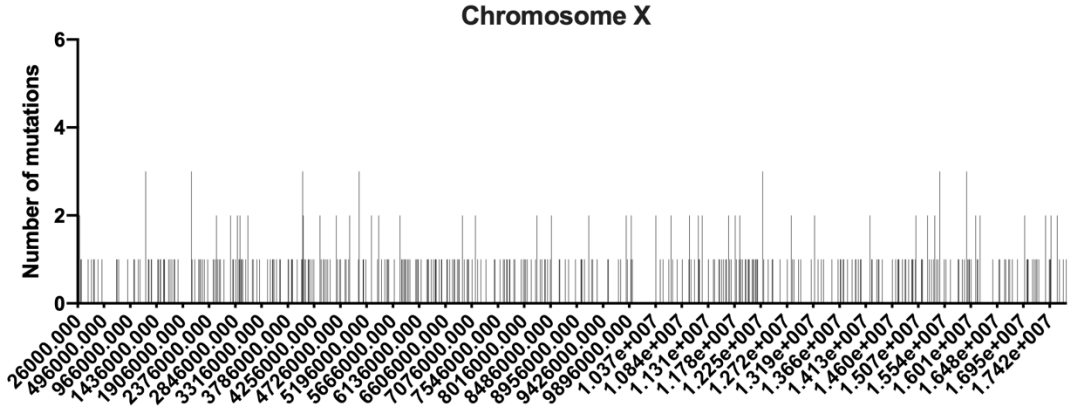
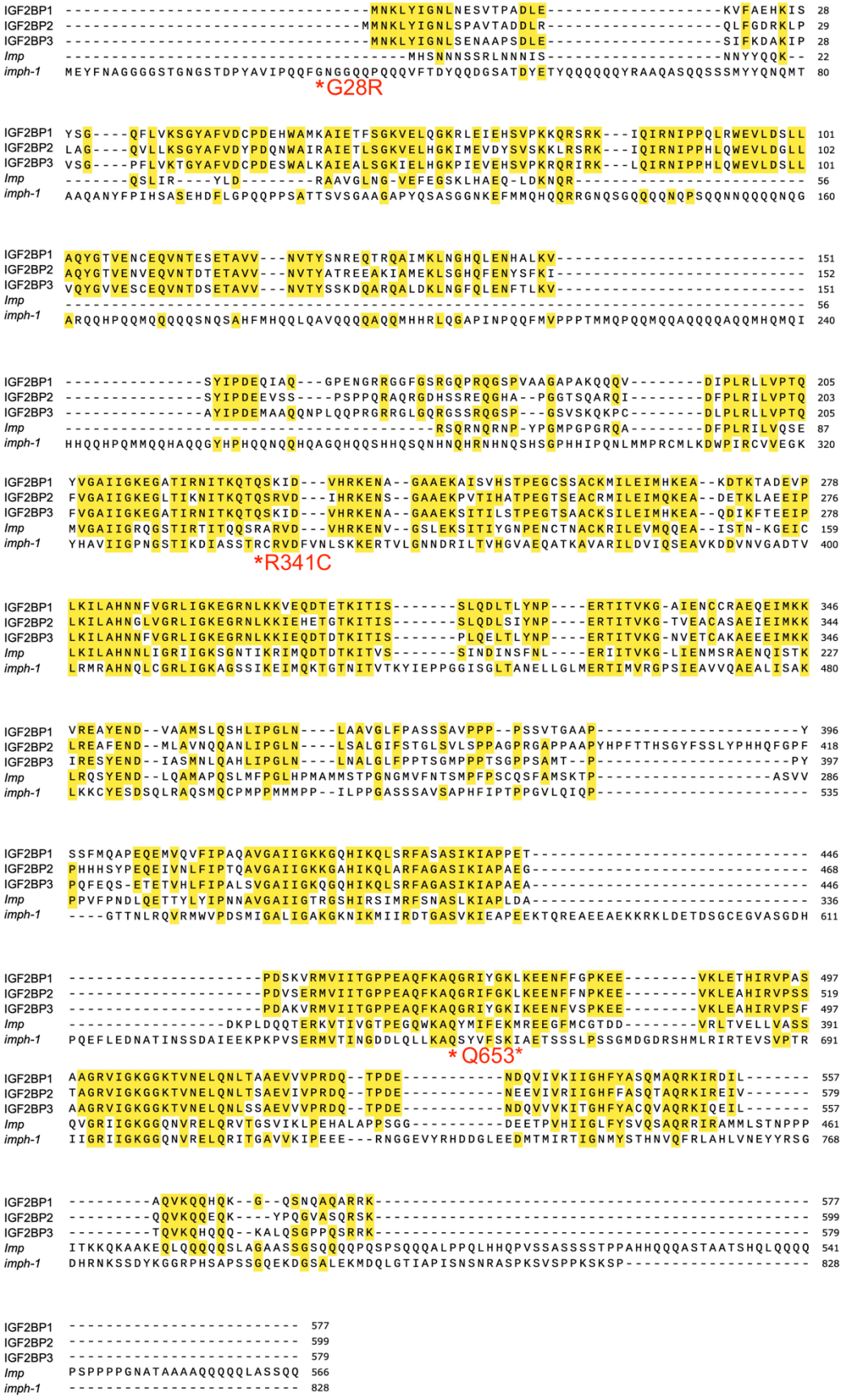
1.5844e+007
1.6310e+007
1.6776e+007
1.7242e+007


Chromosome V
Number of mutations
6
4
2
0
0.0000
558000.0000
1116000.0000
1674000.0000
2232000.0000
2790000.0000
3348000.0000
3906000.0000
4464000.0000
5022000.0000
5580000.0000
6138000.0000
6696000.0000
7254000.0000
7812000.0000
8370000.0000
8928000.0000
9486000.0000
1.0044e+007
1.0602e+007
1.1160e+007
1.1718e+007
1.2276e+007
1.2834e+007
1.3392e+007
1.3950e+007
1.4508e+007
1.5066e+007
1.5624e+007
1.6182e+007
1.6740e+007
1.7298e+007
1.7856e+007
1.8414e+007
1.8972e+007
1.9530e+007
2.0088e+007
2.0646e+007


Chromosome X
Number of mutations
6
4
2
0
26000.000
496000.000
966000.000
1436000.000
1906000.000
2376000.000
2846000.000
3316000.000
3786000.000
4256000.000
4726000.000
5196000.000
5666000.000
6136000.000
6606000.000
7076000.000
7546000.000
8016000.000
8486000.000
8956000.000
9426000.000
9896000.000
1.037e+007
1.084e+007
1.131e+007
1.178e+007
1.225e+007
1.272e+007
1.319e+007
1.366e+007
1.413e+007
1.460e+007
1.507e+007
1.554e+007
1.601e+007
1.648e+007
1.695e+007
1.742e+007

Supplemental Figure 3.2. Protein alignment of IGF2BPs and orthologs in *D. melanogaster (Imp)* and *C. elegans* (*imph-1*). *imph-1* is homologous to IGF2BP family of RNA binding proteins. The KH domains are highly conserved between all species. The RNA recognition motifs (RRMs) found in IGF2BPs are not conserved in the *C. elegans* and *D.* melanogaster orthologs. Additionally, there is a low complexity region/domain near the N-terminus. MUSCLE algorithm was used for this alignment. Red asterisks denote mutations generated through random mutagenesis in the screen for glutamatergic neuron degeneration.


IGF2BP1
IGF2BP2
IGF2BP3
Imp
imph-1
*G28R
*R341C
Q653

Supplemental Table 3.1. Survival data from independent replicates relating to Figure 3.5. Lifespan of animals was assessed on oxidative stress at 25˚C (2.5 mM paraquat). Worms were transferred at least every other day to avoid progeny contamination. MST is the median survival time, n(x) is the number of animals that had a recorded death and the number of animals censored. MST was determined using the statistical analysis software GraphPad Prism (La Jolla, CA).

	T1		T2		T3		T4		Pooled	
Genotype	**MST**	**n(x)**	**MST**	**n(x)**	**MST**	**n(x)**	**MST**	**n(x)**	**MST**	**n(x)**
sod-1(+)	und.	4(26)	7	7(23)	8	10(20)	8	15(25)	8	36(94)
imph-1(tm1623)	9	25(5)	9	18(12)	9	24(6)	10	13(27)	9	94(36)
sod-1WT	und.	3(27)	7	10(2)	8	15(15)	8	6(34)	8	34(96)
sod-1WT; imph-1(tm1623)	9	20(10)	9	20(10)	9	18(12)	9	25(15)	9	83(47)
sod-1G85R	2	12(18)	3	4(26)	3	5(25)	3	12(28)	3	33(97)
sod-1G85R; imph-1(tm1623)	8	9(21)	und.	6(24)	und.	4(26)	5	14(26)	5	33(97)

Supplemental Table 3.2. Survival data from independent replicates relating to Figure 3.6. Lifespan of animals was assessed at 20˚C. As *smn-1* alleles larval arrest, a balancer tmC18 was used. The +/+ genotype was derived from the balancer background to serve as a control. MST is the median survival time, n(x) is the number of animals that had a recorded death and the number of animals censored. MST was determined using the statistical analysis software GraphPad Prism (La Jolla, CA).

	T1		T2		T3		Pooled	
Genotype	**MST**	**n(x)**	**MST**	**n(x)**	**MST**	**n(x)**	**MST**	**n(x)**
smn-1(+)	19	21(9)	18	17(13)	18	18(12)	19	56(34)
+/+	19	16(14)	13	20(10)	17	20(10)	17	56(34)
imph-1(tm1623)	16	23(7)	17	16(14)	18	26(4)	18	65(25)
+/+; imph-1(tm1623)	15	18(12)	16	19(11)	16	25(5)	16	62(28)
smn-1(ok355)	7	17(13)	6	22(8)	6	25(5)	6	64(26)
smn-1(ok355); imph-1(tm1623)	7	25(5)	6	28(2)	7	24(6)	7	77(13)
smn-1(rt248)	8	26(4)	7	20(10)	8	25(6)	8	70(20)
smn-1(rt248); imph-1(tm1623)	6	22(8)	6	27(3)	5	26(4)	6	75(15)

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CHAPTER FOUR

Conclusions, implications, and future directions

4.1 Discussion

Mutations in a variety of genes encoding functionally diverse proteins cause the selective degeneration of motor neurons and lead to the neuromuscular disease Amyotrophic Lateral Sclerosis (ALS). Although there are promising treatments in clinical trials for patients with ALS, there are only two approved treatments only extend lifespan of patients by a couple of months. The lack of effective treatments can be partly attributed to the inherent clinical and genetic variability in the ALS patient population. Thus, understanding factors that influence disease risk or severity will facilitate treatment development (Figure 4.1). Many of the molecular and genetic underpinnings of ALS can be revealed by complementary studies on pathological changes observed caused by mutations in ALS-linked genes and through the identification of modifiers through genetic screens or genome wide association studies (GWAS). This multifaceted approach should reveal mechanisms behind the selective vulnerability of motor neurons in ALS patients.

4.2 Conclusions from meta-analysis of genetic modifiers of ALS

We have compiled a list of 727 modifier genes with human orthologs that modified ALS-associated defects in model organisms or were identified as modifier or risk genes through GWAS (Yanagi et al., 2019). We took a gene ontology bioinformatic approach to identify potential dysregulated pathways from a compiled list of genetic modifiers. We observed that many cellular pathways are dysregulated in ALS mutants including RNA metabolism, protein homeostasis, nucleocytoplasmic transport. Although we only analyzed genetic modifiers for the specific ALS-associated gene or modifiers of multiple ALS-associated defects, the lists of modifiers should be examined together. This analysis would begin to identify common pathways that are dysregulated independent of the ALS-causal gene.

Genetic modifiers identified and analyzed in Chapter Two predominately used overexpression models where ALS-associated phenotypes were observed. To a lesser extent, modifiers were found through both GWAS and candidate gene driven approaches. As technological advances in molecular biology have allowed for the generation of single-copy models of ALS (Baskoylu et al., 2018; Şahin et al., 2017). Identification of modifiers in single-copy models will complement studies previously done in overexpression models and provide valuable insight into mechanisms underlying ALS-associated defect.

Additional modifiers of ALS identified post-2017

Since the list of genetic modifiers in (Yanagi et al., 2019) was compiled, numerous other studies have revealed additional modifiers of ALS (Table 4.1). Notably, in 2018, a CRISPR-based screen for modifiers of C9orf72 toxicity in cultured cells (Kramer et al., 2018). Interestingly, previous modifiers were identified, such as nuclear import/export factors and genes involved in RNA metabolism. These high-throughput CRIPSR-based screens will likely become more common as molecular tools develop.

Additionally, a recent study has completed an unbiased forward genetic screen for suppressors of locomotion defects observed a *C. elegans* SOD1 overexpression model (Lu et al., 2019). From this study, L3MBT1/*lin-61* was identified as a strong suppressor of locomotion defects in *C. elegans*, protects against degeneration in *D. melanogaster* models of C9orf72, and against proteotoxicity in cell models (Lu et al., 2019). The results presented in this study suggest that mutations in SOD1 cause excess proteotoxicity that can be ameliorated by loss of L3MBT1/*lin-61*.

Cross-model and cross- species examination of modifiers may reveal common pathological mechanisms

Although hundreds of modifiers of ALS are described, very few have been tested cross-species and cross-model. As there is high conservation observed in many of these modifier genes from single-cell organisms to mammals, common mechanisms most likely lead to degeneration. Further, analysis can be done on modifiers of specific disease-associated defects. Aggregation of proteins is observed in many neurodegenerative diseases (Blokhuis et al., 2013; Soto and Pritzkow, 2018). Further many genetic screens for modifiers of aggregation have been done in other neurodegenerative disease models (Silva et al., 2011; Zhang et al., 2010). Interestingly, many of the genetic modifiers of ALS-associated defects identified through forward or reverse genetic screens examined aggregation as a primary pathological defect. Although these aggregates likely differ in composition, it would be interesting to examine if similar factors can modify aggregation defects in various disease models. The analysis of phenotype-specific defects may reveal consistent genetic factors underlying disease states and may yield common mechanisms behind different neurodegenerative diseases.

Meta-analysis of genetic modifiers as a resource for the ALS research community

Finally, although number the genes linked with ALS and genetic modifiers of ALS continues to grow, we hope that this curated list can be beneficial to the ALS research community. The array of genetic modifiers that have been identified thus far act in many cellular and biological processes. These databases of genetic modifiers may shed light on if there are converging or parallel pathways that result in neurodegeneration.

4.3 Conclusions from identification of suppressors of glutamatergic neuron degeneration in sod-1G85R mutant animals

Previous screens completed in *C. elegans* models of ALS focused on understanding the phenotype-genotype relationship by using RNAi to investigate candidate genes in overexpression models. Since it is likely that both loss and gain of function mechanisms contribute to ALS pathogenesis (Baskoylu et al., 2018; Saccon et al., 2013; Şahin et al., 2017; Wang et al., 2009), complement the screens already completed in overexpression models. From our forward genetic screen, we identified that *imph-1(lf)* suppresses both glutamatergic and cholinergic neuron degeneration and suggest common mechanisms may lead to both in *sod-1G85R* mutant animals (Figure 3.4). We also observed that *imph-1* confers oxidative stress resistance to *sod-1G85R* animals.

Does *imph-1(lf)* suppress other defects in *sod-1G85R?*

We still do not know the extent of *imph-1* suppression. *imph-1(lf)* suppresses both loss- and gain-of-function defects in *sod-1* mutant animals. Further, *imph-1(lf)* confers resistance to oxidative stress. However, it remains unclear if *imph-1(lf)* suppresses locomotion or aggregation defects observed in *sod-1G85R* (Baskoylu et al., 2018). We have, thus far, primarily examined if *imph-1(lf)* suppresses in the *sod-1G85R* model. As discussed in the previous section, there is a lack of cross-model and cross-species testing of genetic modifiers of disease. Thus, one remaining question is to what extent disease-associated defects does *imph-1(lf)* suppress? Does *imph-1(lf)* suppress in other *sod-1* models, such as cholinergic neuron degeneration in *sod-1G93A* or *sod-1A4V*? Further, does *imph-1(lf)* modify ALS-associated defects in models of FUS, TDP43, or G4C2 expansions in C9orf72? Recently, some studies have shed light on the potential of IGF2BPs modification cross-species. A recent screen in *D. melanogaster* identified *imph-1* as a candidate suppressor of overexpression models of FUS and TDP43 (Kankel et al.,

2020). In C9orf72 cell culture models, IGF2BP1 was observed to modify dipeptide toxicity (Kramer et al., 2018). Together, these data suggests that there is a strong likelihood that *imph-1* will modify defects in other models. If *imph-1(lf)* is confirmed to modify in multiple models of ALS, it may suggest that there are common downstream factors that lead to disease associated phenotypes. Testing modifiers across different models and various disease-associated defects is of paramount importance, since ALS is genetically and clinically heterogeneous.

Does IGF2BP/*imph-1* modify in multiple models of neurodegenerative disease?

There are many genetic and pathological hallmarks shared between ALS and other neurodegenerative disease. Spinal muscular atrophy (SMA) shares clinical and genetic hallmarks with ALS (Bowerman et al., 2018). Both patients diagnosed with SMA or ALS ultimately have motor neuron degeneration. Further, mutations in VAPB have been identified in patients diagnosed with ALS as well as SMA. These data suggest that common mechanisms may underly distinct neurodegenerative diseases. To test the hypothesis that common mechanisms underly related neurodegenerative diseases, we tested if *imph-1(lf)* modified in *C. elegans* models of SMA. Interestingly, we observed that *imph-1(lf)* enhanced defects associated with *smn-1(rt248)* but had no effect on *smn-1(ok355)* (Figure 3.6). The *rt248* is an 8 bp deletion causing an early stop in the *smn-1* gene whereas the *ok355* allele is a 975 bp deletion that also removes some of the promoter region of a nearby gene, *klp-16* (Briese et al., 2009; Dimitriadi et al., 2016). It is unclear if this can explain some of the differences observed between modification in *C. elegans* models of SMA models. Further experiments are needed to validate if *imph-1* does modify defects in models of SMA and additional defects such as locomotion, pumping, or defects in neuromuscular junction signaling

*imph-1/*IGF2BPs and other RNA binding proteins in ALS

Although previous screens have identified IGF2BPs or orthologs of IGF2BPs as modifiers, none have investigated the mechanisms behind the modification of ALS-associated defects. Since IGF2BPs are RNA binding proteins, found in RNA granules, and RNA granule dynamics are dysregulated in many models of ALS, we decided to investigate if stress granules (SGs). Components of stress granules (SGs) do not seem to genetically interact with *sod-1G85R* (Figure 3.5). However, this does not mean RNA granules are not disrupted in SOD1 ALS. There are hundreds of proteins and RNAs that compose stress granules (Buchan, 2014; Buchan and Parker, 2009). Thus, additional proteins may be compensating for a lack of either G3BP1/*gtbp-1* or TIA1/*tiar-1/2*. Alternatively, SGs may not be the critical RNA granule subtype in *sod-1G85R* animals. Instead, neuronal transport granules may be disrupted. Further experiments should address if loss of one granule gene is insufficient for modification of glutamatergic neuron degeneration. Additionally, future experiments should examine if other granule components, such as STAU1/*stau-1 or* eIF4e/ife-2, modify neurodegeneration (Keiper et al., 2000; Ren et al., 2016). Additionally, dynamics of RNA granules could be dysregulated in SOD1 ALS (Lee et al., 2020). GFP fusion proteins can be used to visualize SG and other granule proteins dynamics through fluorescence recovery after photobleaching or localization experiments (Andrusiak et al., 2019).

Links between RNA homeostasis and oxidative stress

These two potential mechanisms, dysregulation of RNA homeostasis through RNA granules and oxidative stress, are not mutually exclusive. SGs form in response to oxidative stress and are composed of RNA binding proteins. Other types of cellular stress are observed in SOD1 models, including endoplasmic reticulum (ER) stress (Nishitoh et

al., 2008). The ER is critical for protein processing and folding and excess stress generated from protein misfolding ER may overload protein degradation machinery to degeneration. Studies could begin to separate these mechanisms by assessing if other stressors also cause degeneration in *sod-1* models and if *imph-1* suppresses these defects. Additionally, examining if *imph-1* modifies non-stress driven defects would also provide into which mechanisms contribute to ALS.

4.4 Future directions

Multi-omic approaches, classical genetics, and GWAS for modifier identification

As more genes become linked to ALS, types of network analysis become more powerful. Transcriptomic and proteomic studies of ALS, in both model organisms and from patient samples, provide unique insight into the interactome. Using these approaches, recent studies have investigated the protein interactome in patients with ALS to identify common pathways that may be dysregulated (Dervishi et al., 2018; Mao et al., 2017). Transcriptional and translational changes These -omic studies should be used to complement genetic screens in order to have a more complete picture of mechanisms that underly neurodegeneration.

Mechanisms behind *imph-1* suppression

As ALS predominately effects the glutamatergic and cholinergic motor neurons, identifying where *imph-1* is acting to suppress neurodegeneration is important. Is *imph-1* acting cell-autonomously or non-cell-autonomously? We are currently investigating if expression of IGF2BPs in neurons is sufficient to restore neurodegeneration in *sod-1G85R; imph-1(tm1623)* mutant animals. Interestingly the fly and worm orthologs of IGF2BPs do not contain the RNA recognition motifs (RRMs). Additional future experiments

should address which of the domains in IGF2BPs are essential for suppression of stress-induced glutamatergic neuron degeneration.

Alternative approaches to determining how *imph-1* suppresses ALS-associated defects should investigate known mRNA targets. Some of IGF2BPs known targets are β-actin and IGF2. IGF2BPs bind β-actin mRNA and facilitate the transport to the leading edge of growth cones during development and can promote axon remodeling (Medioni et al., 2014; Zhang et al., 2001). IGF2 signaling has been shown to be essential in protecting against oxidative damage (Martín-Montañez et al., 2017). Additionally, IGF2 is a candidate biomarker that has been identified in ALS patients (Chen et al., 2016). These suggest a larger role for IGF2BPs and IGF2 signaling in ALS pathogenesis.

Future experiments should address if *imph-1* is suppressing ALS-associated defects through IGF2 signaling. In *C. elegans* there is only one gene encoding an ortholog of insulin-like receptor (IGFR), *daf-2*, and a multitude of insulin-like proteins. To investigate the role of insulin-like signaling in *sod*-1G85R, we can assess if loss of *daf*-2 modifiers stress-induced glutamatergic and cholinergic neuron degeneration. It is likely that *daf-2* will modify defects in *sod-1G85R* animals. *daf-2(lf)* suppressed locomotion defects in *hSOD1G85R* neuronal overexpression model (Boccitto et al., 2012).

Role of *imph-1* in the oxidative stress response

Oxidative stress is thought to be a key driving factor behind the degeneration of motor neurons (Barber et al., 2006). IGF2BPs have many targets, some of which are oxidative stress response genes (Samuels et al., 2020). If IGF2BPs are acting as translational repressors, loss of IGF2BPs would free mRNA targets to be translated by protein machinery, thus protecting the cell. Additional experiments can examine

transcriptional and translational changes in *sod-1* mutant animals and if these defects are restored in an *imph-1(lf)* background.

Identification of additional suppressors of glutamatergic neuron degeneration

Many other suppressor genes in from this screen have yet to be identified. Our lab has begun investigating some of these genes, including genes that overlap with previously identified modifiers of aggregation. *phy-3/P4HA* is a promising candidate for further study as a suppressor of glutamatergic neuron degeneration. P4HA colocalizes with protein disulfide isomerases, which are proposed as genetic risk factors for ALS (Perri et al., 2017; Woehlbier et al., 2016). Additionally, we have identified seven candidate genes from a previous RNAi screen (Silva et al., 2011) that have exonic SNPs in suppressor lines generated from our screen. However, other suppressor lines remain. As cholinergic neuron degeneration is a key pathological mechanism in ALS, suppressor lines should be prioritized if cholinergic neuron degeneration is observed. Additionally, further backcrossing and mapping of SNPs would aid in the identification of the causal suppressor gene in the remaining lines.

4.5 Overall conclusions and perspectives

The cellular and molecular mechanisms behind motor neuron degeneration in ALS remains unclear. The clinical and genetic heterogeneity observed in the ALS patient population suggests additional genetic factors play key roles in the development, progression, and pathogenesis of ALS. Identifying the pathways that underly the selective neurodegeneration in ALS can be revealed by understanding genetic modifiers of the disease. With the many technological advances, identifying modifier genes of complex neurodegenerative diseases, such has ALS, has facilitated the understanding of

molecular pathways that may contribute to disease. However, many questions remain. Why are motor neurons specifically vulnerable to degeneration in ALS patients? How do the functionally diverse set of ALS-causal genes cause the same disease? Finally, is there a common pathway or are there multiple pathways that lead to neurodegeneration?

Our findings in from a forward genetic screen for suppressors of glutamatergic neuron degeneration suggest a larger role for RNA binding proteins in SOD1 ALS. There are inherent caveats when identifying genetic modifiers identified through forward genetic screens. The efficacy and ultimate potential for these genetic modifiers to become targets for therapies depends on cross-species conservation. Thus, it is imperative for genetic modifiers of any disease to be rigorously tested in different models, especially cross-species, and if they modify varying phenotypes associated with the disease of interest. Although many questions remain about why motor neurons are particularly vulnerable to degeneration, the identification of genetic players through genetic analysis can provide insight into pathogenic mechanisms and novel targets for the development of therapies.

4.6 Tables and Figures

Table 4.1 A selection of papers published since 2017 containing genetic modifiers of ALS. With the recent technological advances, increases and availability of whole genome sequencing and gene editing technology, additional modifiers that influenced ALS-associated defects in model organisms or through GWAS studies have been identified. While much focus of investigation is on the four ALS-causal genes (SOD1, FUS, TDP43, and C9orf72), screens for modifiers of ALS-linked genes have also been completed.

ALS gene	Description	Reference
C9orf72	CRISPR inactivation screen for modifiers of dipeptide toxicity in cell culture models	(Kramer et al., 2018)
SOD1	Candidate gene investigation revealed Parkin as a modifier in hSOD1G93A mouse model	(Palomo et al., 2018)
C9orf72	Identified proteins with altered nuclear-cytoplasmic localization in C9orf72 cells using mass spectrometry and examined if defects in *D. melanogaster* models were modified	(Ortega et al., 2020)
TDP43	RNAi screen for RNA interacting proteins that modify TDP43-assocaited defects	(Berson et al., 2019)
SOD1	Screen for suppressors of locomotion of *Psnb-1::hSOD1G85R* in *C. elegans*	(Lu et al., 2019)
FUS and TDP43	Screen for modifiers of *hFUSR521C* and *hTDP-43M337V* associated degeneration	(Kankel et al., 2020)
FUS	Screen for modifiers of pupal lethality defects associated with loss of *caz*	(Mallik et al., 2018)
CHMP2B	Screen for modifiers of retinal degeneration in *D. melanogaster*	(Lu et al., 2020)

Figure 4.1 A potential pipeline for treatment development: from identification of genetic modifiers to novel targets. Treatment development for patients with ALS face many challenges. This includes the inherent clinical and genetic heterogeneity in the disease, but also physical challenges such getting drugs or other small molecules into the nervous system. A potential workflow with the ultimate goal of identifying novel targets for drug development is to (1) identify modifiers through genome wide screens in model organisms complemented with GWAS studies in patients, (2) test these modifiers cross-species, and (3) identify novel targets or cellular pathways for drug development and clinical trials.

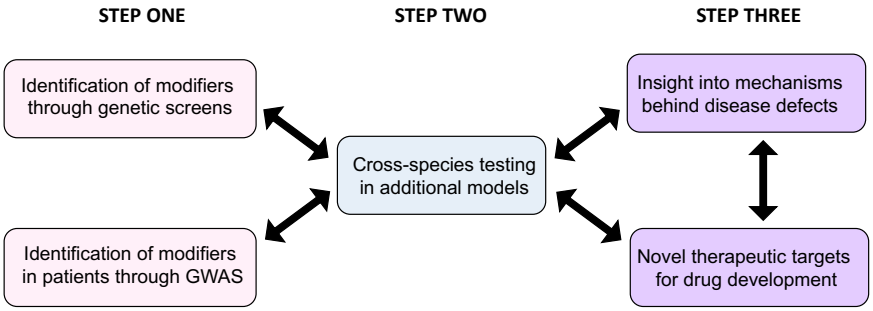
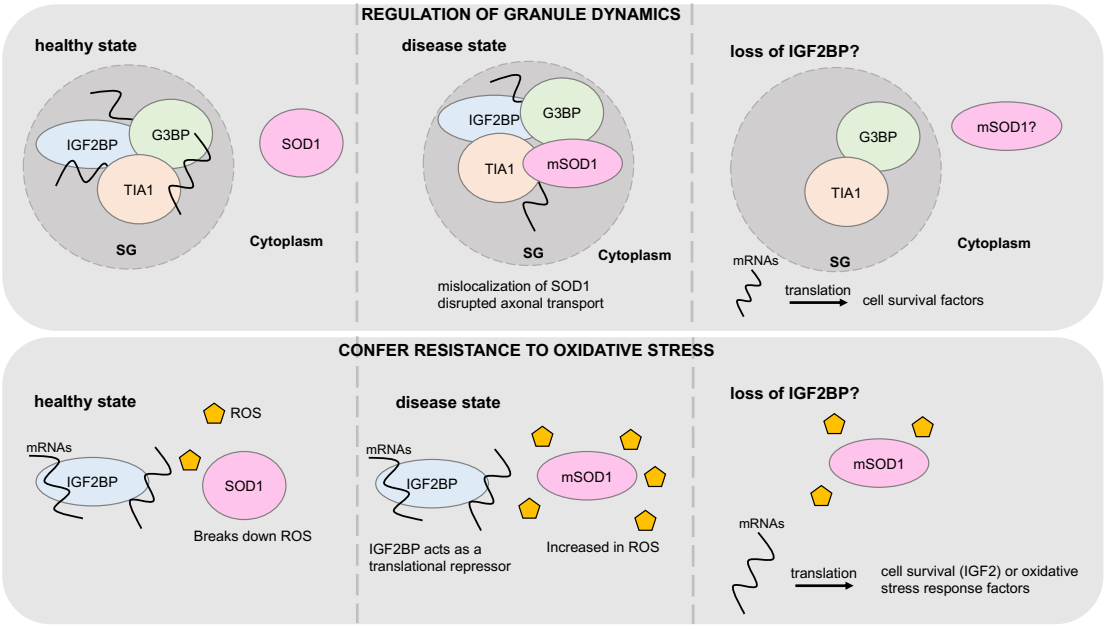


Figure 4.2 Potential mechanisms of IGF2BP/*imph-1* suppression of *sod-1G85R* associated defects. Mutations in SOD1 have been reported to localize with RNA binding proteins commonly found in stress granules (SGs). Thus, one proposed mechanism is that mutations in SOD1 can disrupt granule dynamics. This novel interaction between mutant SOD1 and RNA binding proteins could prevent the release of specific RNAs for translation that required to respond to exogenous and cellular stress. Loss of IGF2BPs may restore proper granule function and localization and allow for the proper response to stress. Alternatively, mutant SOD1 could fail to breakdown excess superoxide radicals leading to excess oxidative stress. Loss of IGF2BPs act as translational repressors could facilitate the translation of cell survival or oxidative stress response factors and rescue defects.



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Appendix

Genetic interactions in a *C. elegans sod-1* ALS model: glutamatergic neuron degeneration

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Contributions
JFO, KSY, and ACH conceptualized experiments done, reviewed, and edited drafts presented in this chapter. JFO and KSY curated data presented in this chapter. JFO wrote the original draft for the results section. ACH acquired funding.

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Results

Amyotrophic lateral sclerosis (ALS) is a fatal degenerative motor neuron disease. While the mechanisms underlying motor neuron death in ALS are not well understood, mutations in over 25 genes can cause this disease (Marangi and Traynor 2015). It remains unclear which, if any, of these genes act in the same disease-associated pathway(s), or if they act in the same pathway(s) as genes associated with the related disorder, frontotemporal dementia (FTD) (Ling, Polymenidou, and Cleveland 2013). The first ALS-causing gene to be identified was superoxide dismutase 1 (*SOD1*), a regulator of cytoplasmic redox homeostasis (Rosen et al. 1993). We can begin to construct a pathway for neurodegeneration through *SOD1* by identifying genes whose loss of function (LOF) modifies the level of degeneration in a *C. elegans SOD1* ALS model. This will contribute to our understanding of whether ALS/FTD genes act in a single or multiple pathways to cause disease.

To undertake this analysis, we introduced LOF alleles for *C. elegans* orthologs of ALS or FTD genes into a *C. elegans* knock-in model of *SOD1* ALS (Baskoylu et al. 2018). Previously, we used CRISPR/Cas9 to introduce a point mutation corresponding to the ALS-causing *SOD1 G85R* variant (Rosen et al. 1993)into a conserved residue in the endogenous *C. elegans sod-1* gene (Baskoylu et al. 2018). *sod-1(rt448)* contains the G85R mutation and *sod-1(rt449)* is the corresponding control strain containing silent edits; these are referred to herein using the corresponding human patient allele nomenclature, *sod-1G85R*^{C} and *sod-1WT*^{C} (Baskoylu et al. 2018). *sod-1G85R*^{C} animals exhibit glutamatergic neuron degeneration following exposure to mild oxidative stress. To begin exploring genetic interactions in this model, we selected several genes with existing deletion alleles (Figure 1A); deletion alleles were crossed into the *sod-1G85R*^{C}

background to investigate their effect on *sod-1G85R* glutamatergic neuron degeneration. ALS/FTD-causing gene orthologs tested include: *figo-1,* ortholog of factor-induced gene 4 (*FIG4*), a regulator of $PI(3,5)P_2$, a phosphoinositide phosphatase involved in endosomal signaling and trafficking (Chow et al. 2009); *sqst-1,* an ortholog of human sequestosome 1 (*SQSTM1*), which encodes the SQSTM1/p62 cargo protein involved in selective autophagy (Fecto et al. 2011); *ubql-1*, ortholog of ubiquilin-2 (*UBQLN2*), a carrier in the ubiquitin/proteasome system (Deng et al. 2011); *ptl-1,* ortholog of microtubule-associated protein tau (*MAPT*), a player in microtubule assembly and dynamics also implicated in Alzheimer's disease and various dementias (Fang et al. 2013; Rademakers, Cruts, and van Broeckhoven 2004); and *daao-1*, ortholog of D-amino acid oxidase (*DAO*), among whose substrates is D-serine, an endogenous neurotransmitter and co-agonist of NMDA receptors (Mitchell et al. 2010).

Loss of glutamatergic neurons was quantified by dye-filling four glutamatergic neurons (PHAR/L and PHBR/L) in the tail with exposed sensory endings (Figure 1B). After mild oxidative stress with paraquat (22 hours, 2.5mM), the majority of these neurons in *sod-1G85R*^{C} animals are unable to take up dye and are presumed to have degenerated (Baskoylu et al. 2018). *figo-1(tm5202); sod-1G85R*^{C} animals exhibited increased dye-filling compared to *sod-1G85R*^{C} (Figure 1C), indicating loss of *figo-1* partially suppresses glutamatergic neuron degeneration in *sod-1G85R*^{C} animals. *sod-1G85R*^{C}*; sqst-1(ok2892), ubql-1(tm1574); sod-1G85R*^{C}*, sod-1G85R*^{C}*; ptl-1(tm543),* and *sod-1G85R*^{C}*; daao-1(tm3673)* animals exhibited no significant difference in dye-filling compared to *sod-1G85R*^{C} (Figure 1D-G). LOF alleles did not show stress-induced neuron degeneration in combination with *sod-1WT*^{C} (Figure 1C-G).

Based on these results, *figo-1* and *sod-1* may lie in the same genetic pathway. *sod-1* LOF is predominantly responsible for glutamatergic neuron degeneration in *sod-1G85R*^{C} animals (Baskoylu et al. 2018). As *figo-1* LOF suppressed glutamatergic neuron degeneration in the *sod-1G85R*^{C} model, *figo-1* likely lies downstream of *sod-1*. The epistatic relationship observed here will need to be confirmed with other deletion alleles of *figo-1* before potential mechanisms of *FIG4* and *SOD1* interaction are investigated. *figo-1* may make neurons more robust or specifically resistant to *sod-1*-related glutamatergic neuron loss. Additionally, *SOD1* ALS-related alterations in endosomal activity have been reported (van Dis et al. 2014; Xie et al. 2015) and may be influenced by the indirect role of *FIG4* in endosomal signaling and trafficking. Further examination of epistatic relationships between *SOD1* and other ALS/FTD-causing genes can yield a complete pathway of *SOD1* action, as well as elucidate whether ALS/FTD is a single disease consisting of one or more pathways or perhaps even multiple diseases with shared characteristics.

Methods

C. elegans strains

All strains were maintained using standard methods (S. Brenner 1974) and constructed using the *C. elegans* Bristol variety N2 as a wild-type parent strain. All strains were backcrossed to N2 at least 4 times before use.

List of *C. elegans* strains (Strain name: *genotype*)

- HA2986: *sod-1(rt448[WT*^{C}*]) II; pha-1(+) III* (abbreviated *sod-1WT*^{C})
- HA3299: *sod-1(rt449[G85R*^{C}*]) II; pha-1(+) III* (abbreviated *sod-1G85R*^{C})
- HA3261: *figo-1(tm5202) I*
- HA3266: *sqst-1(ok2892) IV*
- HA3352: *ubql-1(tm1574) I*
- HA3402: *ptl-1(tm543) III*
- HA3361: *daao-1(tm3673) IV*
- HA3788: *figo-1(tm5202) I; sod-1WT*^{C} *II*
- HA3789: *figo-1(tm5202) I; sod-1G85R*^{C} *II*
- HA3509: *sod-1WT*^{C} *II; sqst-1(ok2892) IV*
- HA3510: *sod-1G85R*^{C} *II; sqst-1(ok2892) IV*
- HA3516: *ubql-1(tm1574) I; sod-1WT*^{C} *II*
- HA3517: *ubql-1(tm1574) I; sod-1G85R*^{C} *II*
- HA3996: *sod-1WT*^{C} *II; ptl-1(tm543) III*

- HA3997: *sod-1G85R*^{C} *II; ptl-1(tm543) III*
- HA3795: *sod-1WT*^{C} *II; daao-1(tm3673) IV*
- HA3796: *sod-1G85R*^{C} *II; daao-1(tm3673) IV*
- FX19472: *tmIn10 [mIs14 spy-10(e128)] II*, used in strain construction

Dye-filling assay

To quantify loss of glutamatergic sensory neurons, we followed a procedure outlined in (Perkins et al. 1986). Animals raised to larval stage 4 were transferred to plates containing 2.5 mM paraquat (Acros Organic, #1910-42) for 22 hours at 25C with minimal light exposure. After the incubation period, 2 mg/mL DiI (Fisher DiIC18(5) D307) was added. After 1.5 hours, animals were transferred back to regular NGM plates, then immobilized with 30 mg/mL 2-3-butanedione monoxime (BDM, Sigma) in M9 buffer and mounted on 2% (vol/vol) agar pads. Fluorescent neuronal cell bodies in the tail were counted under 63x objective on a Zeiss Axioplan2. Animals with three or four phasmid neurons that fill with dye were scored as intact (Figure 1B).

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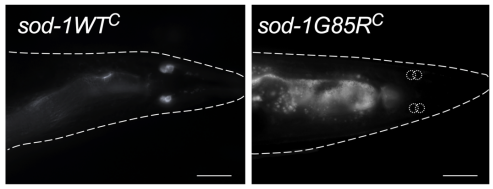
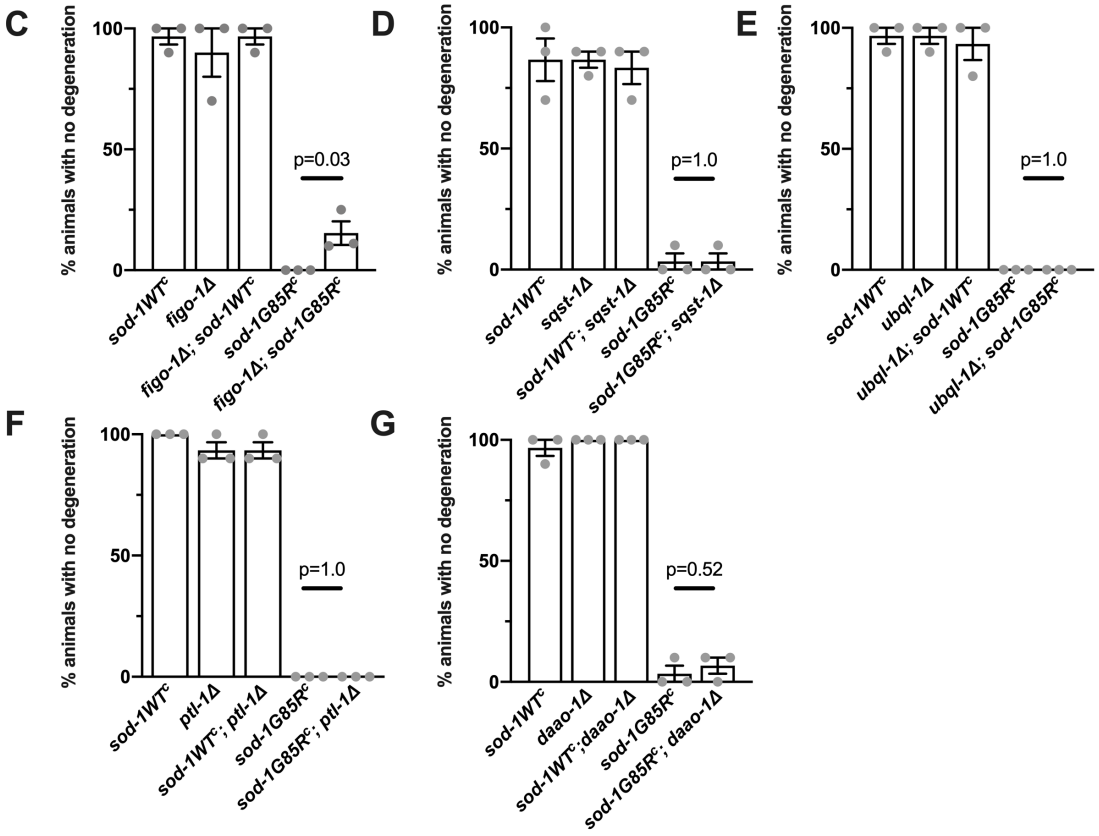
Figures

Figure 1. Assessment of genetic interactions
(A) ALS genes of interest (left) and corresponding deletion alleles in *C. elegans* orthologs (right). (B) Representative images from *sod-1WT^C* (left) and *sod-1G85R^C* (right) after 22 hours of paraquat exposure. Animals with three or four phasmid neurons that fill with dye are scored as intact. Animals are outlined, fluorescence in the gut is visible in each image, and location of missing neurons is outlined in *sod-1G85R^C*. 63x magnification, scale bar 20μm. (C) Glutamatergic neuron degeneration: after 22 hours of paraquat exposure, an increased percentage of *figo-1(tm5202); sod-1G85R^C* double mutant animals were intact, compared to *sod-1G85R^C* animals. Three independent trials totalling n=30 animals, two-tailed t-test, p=0.03. Dots represent the average from each trial of 10 animals. Error bars indicate standard error of the mean. All data were collected blind to genotype. (D-G) Glutamatergic neuron degeneration as described in C for other genotypes.

A

Human	*C. elegans* allele
FIG4	*figo-1(tm5202) I*
SQSTM1	*sqst-1(ok2892) IV*
UBQLN2	*ubql-1(tm1574) I*
MAPT	*ptl-1(tm543) III*
DAO	*daao-1(tm3673) IV*





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