IDENTIFYING NOVEL MODIFIERS OF FUS-ASSOCIATED TOXICITY IN A *DROSOPHILA* MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease, and is characterized by the loss of both upper and lower motor neurons. Recently, mutations in genes that encode for RNA-binding proteins have been linked to ALS pathology, suggesting that perturbation of RNA processing may be affiliated with disease pathogenesis. Mutations of the gene Fused in Sarcoma (*FUS*), which codes for the protein FUS, have been linked to both familial and sporadic forms of ALS. FUS is a DNA/RNA-binding protein that plays critical roles in RNA processing including RNA trafficking and alternative splicing.

Using a *Drosophila melanogaster* model for FUS-associated ALS that was developed by our laboratory, we performed an unbiased genetic screen to identify dominant modifiers of ALS-associated neurodegeneration. Unexpectedly, we identified muscleblind (*mbl*), the *Drosophila* homolog of human muscleblind-like (*MBNL*) as a strong suppressor of FUS-mediated neurodegeneration *in vivo*. We found that RNAi-mediated knockdown of endogenous *Drosophila* mbl rescues neurodegenerative phenotypes such as retinal degeneration, reduced life span and neuromuscular junction defects caused by ALS-associated mutations in *FUS*. We validated our findings in a mammalian primary cortical neuron system and found that depleting endogenous muscleblind-like strongly suppressed dendritic morphological defects and toxicity.

Interestingly, we found in both human embryonic kidney cells and primary cortical neurons that muscleblind protects against FUS toxicity by reducing pathogenic incorporation of mutant FUS into cytoplasmic stress granules.

Taken together, our data suggests an unexpected role of mbl in FUS-mediated neurodegeneration and demonstrates that muscleblind is a regulator of toxicity associated with mutant FUS in *Drosophila* and primary cortical neurons. The public health relevance of this project lies in the fact that ALS is a fatal disease with no cures and only one, marginally effective, treatment. The work presented here addresses this issue by highlighting pathways that are affiliated with disease pathology, and identifying modifiers of toxicity that may be useful as future therapeutic targets.

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PREFACE

It is because of the unconditional love and the unwavering support of God, my family, friends and colleagues that I am able to present my work and complete the graduate program. I have learned that though we may work alone, it is through the help of others that we accomplish our goals. I am forever indebted to those who have made this possible, and although I will acknowledge them here, I will never be able to fully express my eternal gratitude for the sacrifices they made for me during this process.

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ABBREVIATIONS

ALS: Amyotrophic Lateral Sclerosis

MND: Motor neuron disease

FTD: Frontotemporal dementia

HD: Huntington's disease

SMA: Spinal muscular atrophy

AD: Alzheimer's disease

PD: Parkinson's disease

FUS: Fused in sarcoma

SMN: Survival of motor neuron

SOD1: Superoxide dismutase

TDP-43: TAR DNA-binding protein 43

VCP: Valosin-containing protein

MATR3: Matrin 3

SALS: Sporadic ALS

FALS: Familial ALS

ASO: Antisense oligonucleotide

SG: Stress granule

RNP: Ribonucleoprotein

P-bodies: Processing bodies

RAN: Repeat-associated non-ATG

OMIM: Online Mendelian Inheritance in Man

WT: Wild type

dSOD1: *Drosophila* SOD1

hSOD1: Human SOD1

BMAA: β-N-methylamino-L-alanine

Rab5: Ras-related in brain 5

VAPB: VAMP (vesicle-associated membrane protein)-associated protein B

ER: Endoplasmic reticulum

MSP: Major sperm protein

hVAPB: Human VAPB

dVAP: *Drosophila* VAPB (DVAP33-A)

NMJ: Neuromuscular junction

Dscam: Down syndrome cell adhesion molecule

PtdIns4P: Phosphatidylinositol 4-phosphate

Osbp: Oxysterol binding protein

dTDP-43: *Drosophila* TDP-43

hTDP-43: Human TDP-43

HIV-1: Human immunodeficiency virus 1

FTLD: Frontotemporal lobar degeneration

TBPH: TAR DNA-binding protein-43 homolog

dIAP: *Drosophila* inhibitor of apoptosis

EP: Enhancer promoter

HDAC6: Histone deacetylase 6

BRP: Bruchpilot

IBMPFD: Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia

SCA2: Spinocerebellar ataxia type 2

RRM: RNA recognition motif

NLS: Nuclear localization signal

FUS/TLS: Fused in sarcoma/ translocated in liposarcoma

NES: Nuclear export signal

EGFR: Epidermal growth factor receptor

MAPK: Mitogen-activated protein kinase

PRMT: Protein arginine methyltransferase

CNS: Central nervous system

ITPR1: inositol-1,4,5-trisphosphate receptor type 1

FXTAS: Fragile X-associated tremor/ataxia syndrome

TAF15: RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa

EWSR1: EWS RNA-binding protein 1

DPR: Dipeptide-repeat protein

hnRNP: Heterogeneous nuclear ribonucleoproteins

EcR: Ecdysteroid receptor

RRM1: RNA recognition motif 1

CAC: Cacophony

mbl: Muscleblind

MBNL1: Muscleblind-like

SSI: Site-specific integration

1.0 INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal, late-onset neurodegenerative disease caused by loss of upper and lower motor neurons, ultimately resulting in loss of muscle function and subsequent paralysis and death. Unfortunately, despite numerous advances in our understanding of the disease over the past few decades, on both clinical and molecular levels, it is still largely unclear why motor neurons die in patients with ALS. As a result, no cures or effective treatments currently exist. With death typically occurring within 2-5 years following diagnosis due to respiratory failure, ALS is a ruthless disease and effective therapies are desperately needed.

1.1 ALS AND NEURODEGENERATION – A DISEASE SPECTRUM

Neurodegenerative diseases, whether researched in the lab or diagnosed in the clinic, are becoming increasingly difficult to differentiate. The more we understand neurodegeneration the more we unveil a continuous spectrum of overlapping phenotypes, etiologies and clinical outcomes. The days of dividing neuronal cell death into discrete diseases with unique causes are behind us, and we are now presented with a difficult task of understanding, diagnosing and treating conditions that have blurred distinctions between them. One consolation to this reality, however, is that

diseases with overlapping clinical outcomes often share genetic or molecular causes. As a result, understanding the pathogenesis of one disease holds the promise for developing cures or treatments for a number of others.

Amyotrophic lateral sclerosis is no exception. ALS is primarily characterized by the loss of upper and lower motor neurons and is the most common form of motor neuron disease (MND), a disease spectrum that also includes progressive muscular atrophy, progressive bulbar palsy, Charcot-Marie-Tooth disease, hereditary spastic paraplegia and primary lateral sclerosis[1-3]. Frontotemporal dementia (FTD) is a disease that affects the frontotemporal lobe of the brain thereby causing cognitive impairment in the affected individuals. Although ALS and FTD are arguably two distinct diseases[4], more recently they have been viewed as part of a larger spectrum of neurodegeneration, with motor neuron disease on one end, and FTD on the other[5]. Therefore, patients who appear to exhibit strictly motor neuron defects fall into the category of MND, whereas patients with cognitive or behavioral defects fall into the category of FTD[5]. In the middle of this spectrum are patients with a mix of both phenotypes, leading to diagnoses of ALSbi (ALS with a behavioral impairment) or ALSci (ALS with a cognitive impairment)[5,6]. As many as 50% of ALS patients also exhibit clinical symptoms of FTD, such as cognitive impairments and degeneration of the frontotemporal lobe[7-12].

Regardless of the clinical presentations or diagnostic distinctions between ALS and FTD, however, these two conditions share common characteristics on a molecular level that may be critical for understanding their pathogenesis and developing a cure.

Namely, mutations of several genes that have been linked to ALS also give rise to FTD[3,6].

Similarities between ALS and other neurodegenerative diseases do not stop with the MND-FTD spectrum. From a clinical perspective, diagnosing ALS is complicated by phenotypes that overlap with Huntington's disease (HD), spinal muscular atrophy (SMA), Alzheimer's disease (AD) and Parkinson's disease (PD)[2,13]. Similar clinical presentations of these diseases are not random, however. As with ALS and FTD, genes that are associated with ALS have also been linked to these other diseases. *TDP-43*, one of the first genes linked to ALS, may also be involved in the pathogenesis of both AD and PD[14-16]. A hexanucleotide (GGGGCC) repeat expansion within *C9orf72*, currently the most common cause of ALS[3], is believed to be the cause of Huntington disease phenocopy syndrome[17] and is found in patients diagnosed with AD and PD[18,19]. Furthermore, not only do mutations in the gene, *FUS* (fused in sarcoma), cause ALS, but FUS protein interacts with SMN (survival of motor neuron), the protein responsible for SMA. As a result, researchers believe that aberrant FUS function may be involved in a subset of SMA patients[20]. With an average of approximately 30,000 cases in the US every year (www.als.org), ALS, at least from a clinical perspective, falls into the rare disease category. From a molecular perspective, however, understanding its pathogenesis and developing treatments and cures, will have implications for treating a number of devastating diseases worldwide.

1.2 DIAGNOSES AND CLINICAL PRESENTATIONS OF ALS

Once an individual begins to show signs of ALS, they are likely to die within 2-5 years[3,21-25]. During that time, their quality of life will decline as they lose mobility, due to degeneration of their upper and lower motor neurons. In most cases, the ultimate cause of death is respiratory failure. Disease onset is either bulbar, respiratory or limb, and can be subdivided into eight distinct clinical phenotypes: classic Charcot type, pure upper motor neuron, pure lower motor neuron, respiratory, pyramidal, flail arm, flail leg and bulbar[26,27]. Approximately 65% of patients are diagnosed with limb onset and 30% are diagnosed with bulbar onset. A small percentage of patients (5%) are diagnosed with respiratory onset[27]. Respiratory onset and limb onset have the worst and best prognoses, respectively[28].

In patients with a limb onset of ALS, the first sign of the disease is an asymmetric weakening of the muscles in the affected limb. This weakness spreads outward, moving to the adjacent limbs. As a result, it is hypothesized that the factors contributing to neuronal cell toxicity are capable of moving between adjacent cells, causing progressive cell death from the site of initial onset[5,27].

A bulbar onset is indicative of a more aggressive form of the disease. Bulbar muscles are the first to show signs of weakness, affecting the individual's ability to swallow and speak. Limb weakness typically follows, but marks the final stages of the disease and means that the individual only has a few months left[5,27].

Though rare, respiratory onset ALS is a devastating prognosis, even as ALS standards go. The mean survival is only 1.4 years, and death can even occur before the individual experiences limb or bulbar muscle weakness[5].

1.3 CONTRIBUTING FACTORS OF ALS

1.3.1 Genetics

ALS was first identified and described clinically in 1869[29], and again in 1874[30]. It wasn't until nearly 120 years later that the first gene, superoxide dismutase 1 (*SOD1*), was linked to ALS[31]. In the past 20 years, mutations in several genes have been linked to causing the disease, including *FUS*[32-35], *TARDP* (HIV trans-activating DNA response element DNA binding protein)[36,37], *VCP* (valosin-containing protein)[38], *C9ORF72*[39-41] and *MATR3* (Matrin 3)[42]. Most cases of ALS (about 90%) occur randomly throughout a given population and are classified as sporadic ALS (SALS). These individuals do not have a family history of ALS. The other 10% are classified as familial ALS (FALS) based on a family history of ALS, and therefore, an apparent inheritance of the disease[3,43]. To date, nearly 70% of FALS cases can be traced to an identified, typically autosomal dominant, genetic mutation or abnormality. Only approximately 10% of SALS cases have a known genetic cause[3]. These familial and sporadic distinctions are purely for identifying inheritance, as SALS and FALS patients are clinically indistinguishable[44].

1.3.2 Environment

Unfortunately, genetic discoveries thus far have not led to significant improvements in patient treatment or life expectancy. As previously stated, approximately 30% of FALS cases still have unknown causes despite showing

Mendelian inheritance patterns within families, and only approximately 10% of SALS cases have a known genetic cause[3]. This disparity between the total incidence of ALS worldwide and the percentage of cases that can be attributed to genetic mutations has prompted researchers to look at potential environmental (non-genetic) causes of the disease. Epidemiological studies of ALS have also provided support for an environmental basis of the disease. Specific ethnic populations within the Kii Peninsula of Japan, Guam, and western New Guinea have historically had incidence rates as many as 50 times that of Caucasians worldwide[45,46]. Although these differences may still be explained by genetic variation in different populations, many researchers over the years have explored environmental differences. Interestingly, by the 1980s, the incidence rates of ALS in Guam had decreased to levels roughly equivalent to those of the United States[47]. The large decrease from their peak in the 1950s is believed to be due, at least in part, to environmental factors, such as improvements in calcium and magnesium deficiencies in affected individuals' diets[48,49].

Overall, numerous environmental factors have been studied, including electromagnetic fields, cyanotoxins, traumatic brain injury, heavy metal exposure and pesticides[50]. Unfortunately, these studies are largely inconclusive, and some are missing epidemiological data to support experiments performed in animal models. The most convincing research links pesticides to ALS[50]. More work is needed to link specific environmental exposures to ALS, whether as a primary cause of the disease, or as a secondary factor in a genetically susceptible individual.

1.3.3 Genetic modifiers of ALS phenotypes

Phenotypic heterogeneity is very common in ALS. Interestingly, even in FALS where a single mutation is inherited and is present in every affected family member, clinical presentation of the disease can vary significantly from person to person[51,52]. These observations have led researchers to suspect that a single mutation cannot be the whole story, and that other factors (whether genetic or environmental in nature) may modify or contribute to the severity of disease outcomes between individuals.

One hypothesis is that ALS is oligogenic, requiring more than one gene mutation to occur. In this case, the combined effect of multiple gene mutations cause neuronal toxicity, the severity of which depends on the deleterious nature of the mutations or the combination of pathways affected. A recent screen of FALS patients revealed mutations in more than one ALS-causing gene in a subset of individuals, supporting this hypothesis[53]. Although this may be true for some cases, it has been argued that more studies are needed to officially label ALS as oligogenic[3].

It is more likely that phenotypic heterogeneity arises from other, non-pathogenic, proteins whose variable expression or altered interactions with ALS-causing proteins mediate toxicity differently from person to person. Several studies, including the work presented in this dissertation, have identified genes that modify classic phenotypes associated with ALS pathology in both cell culture and animal models, including *Epha4*[54], *Pur-alpha*[55,56], *VEGF*[57] and *CNTF*[52]. Identifying proteins that modify the effects of disease-causing mutations highlights common pathways and interactors of ALS-linked proteins that may be directly linked to toxicity. Not only does this provide a

better understanding of the processes that are affected by those mutations, but it also generates new candidates for therapy.

1.4 TREATMENTS

1.4.1 Clinical Trials

Treatment options for patients with ALS are practically non-existent. Currently, the only FDA-approved treatment, Riluzole, an antagonist of glutamate signaling, has been around since 1995. Unfortunately, Riluzole only extends the survival of the individual for 3-6 months, and has no effect on reversing neuronal loss or muscle weakness[43,58]. Since the 1980's nearly fifty randomized controlled clinical trials have been performed using potential therapies for ALS. These trials tested numerous drugs, each targeting one of nearly a dozen suspected mechanisms of disease pathology: viral infection, growth factors, neurotrophic factors, neuroinflammation, oxidative stress, apoptosis, mitochondrial dysfunction, genetic defects, autophagic vesicles/peroxisome, astrocytes and proteinopathy[58]. Unfortunately, none of these, except for two trials utilizing Riluzole[59,60], successfully resulted in a new treatment option. With the resulting lack of pharmacological aid for ALS patients, clinicians are left with treating the symptoms of the disease including depression, loss of mobility/independence and loss of respiratory function, pain, dysphagia, dysarthria and sialorrhea[43,61]. Researchers and clinicians remain undeterred, however. Every year new clinical trials are initiated, utilizing the most recent developments in potential targeted drugs and cell-based

treatments. As research continues to uncover the specific molecular mechanisms behind disease pathogenesis, it may become increasingly apparent that more targeted therapies are necessary, focusing on smaller populations of individuals. Future clinical trials of patients that are affected by the same point mutation, for example, may prove to be more successful than previous trials that encompass a broader ALS target population.

1.4.2 Current and future therapies

Despite a lack of molecular or pharmacological-based treatments, advances in medical devices are addressing some of the needs of ALS patients. Technological developments, such as eye-tracking software and mobile respirators, help with some of the physical aspects of the disease by improving mobility, communication and respiratory function. Facilities such as the Team Gleason House for ALS Residents in St. Margaret's at Mercy in New Orleans, Louisiana utilize innovative technology like eye and movement-tracking devices to restore independence for ALS patients. Residents can perform simple tasks such as opening and closing doors and turning lights on and off by looking at specific locations within the room, or by interacting with monitors at their beds or on their wheelchairs (www.stmmercy.org). Unfortunately, technology has its limitations, and without treatments for ALS on a cellular level, patients inevitably face respiratory failure. Although positive-pressure ventilation helps, it is not a permanent solution, and currently the only other option is a tracheostomy followed by mechanical ventilation. Most ALS patients choose not to undergo mechanical ventilation due its invasiveness and long-term financial burdens[43]. Therefore, although recent

technological advances can improve quality-of-life throughout disease progression, more work needs to be done to improve long-term outcomes for patients.

Hope remains for the next breakthrough in treating ALS. Researchers are currently exploring several different avenues, from surgical procedures to targeted drugs. With the identification of so many gene mutations producing toxic proteins or RNAs, it is not surprising that a prominent goal of therapeutic research is the silencing or rapid turnover of these toxic elements. Drugs that enhance endogenous autophagy, for example, may help with toxic protein turnover, and slow accumulation of insoluble aggregates[55,62]. Work is currently being done with RNA interference, particularly with antisense oligonucleotides (ASOs)[63,64]. These are short nucleotides with homology to their target RNA. Once bound, ASOs tag the RNA for degradation by cellular machinery[61,65,66]. In this form of therapy, the antisense oligonucleotides are injected into the individual intrathecally, and enter neurons on their own, targeting the desired mutant proteins. Early trials showed positive results, at least in terms of tolerance of the procedures, making this a desirable technique for researchers to pursue further[63,65].

These approaches to treating ALS are intended to slow or halt disease progression. They are not intended to prevent disease onset, though there may be some future usefulness in treating cases such as in FALS, where early genetic testing may identify mutations in individuals before they show symptoms of the disease. These possibilities, however, carry with them a host of ethical concerns surrounding genetic testing that would need to be considered and approached cautiously. One area of promising therapeutic research is in utilizing stem cells to reverse the disease process by replacing dead neurons with new ones. Currently, this is being approached through

replacement of neuronal stem cells, mesenchymal stem cells, or induced pluripotent stem cells[61,65]. The advantage to this kind of therapy is that the stem cells can differentiate into glial cells in addition to neurons. The important supporting roles that glial cells, such as oligodendrocytes and astrocytes, play in neuron function have led researchers to suspect that they may be involved in ALS pathogenesis. Studies in ALS animal models have shown that altering mutant protein expression in glial cells can modify disease progression, giving rise to the idea that ALS may be non-cell autonomous[61,67-70]. Therefore, replacing these cells in addition to neurons may prove to be beneficial. Initial studies in stem cell replacement therapy in animal models of ALS have shown some success in modifying pathology[71-75]. Early-phase clinical trials in humans have shown that transplantation can be tolerated by the patients, and modest positive changes in disease progression and patient outcomes have been observed[65,76-79]. These results provide hope that this technology may be a viable treatment option for ALS patients, though later-phase trials are required to verify improvements in clinical outcomes of the disease.

1.4.3 Unconventional therapies

Since a diagnosis of ALS is a death sentence, and life expectancy is so short, it is understandable that patients pursue any lead or rumor of a possible therapy, including options that have little-to-no scientific merit or are even dangerous. Online blogs and websites provide a wealth of unsubstantiated solutions for desperate patients and their families. The validity of claims or supposed testimonies from fellow patients is muddled by a mix of well-intentioned sources with groups that wish to profit from the

fear and desperation of those in need. Add to this a growing online community of ALS support groups, dedicated websites, chat rooms, blogs, etc., and patients are provided with a wealth of conflicting information and opinions.

To combat this growing problem, researchers and clinicians within the ALS community are working hard to provide patients and their families with the resources they need to weed out the facts from the untested opinions and lies. From a clinician's perspective, this begins with maintaining contact with their patients and staying abreast of current clinical trials, as well as the progress of upcoming treatments and medical devices. Their role as an informational resource is as important as their role in treating the disease.

For researchers, helping patients to navigate the ever-expanding sea of misinformation means being more accessible. Clinicians have historically bridged the gap between clinical research and the public, but in an age where technology allows everyone access to the latest scientific developments, it is becoming more imperative that researchers themselves are brought into the conversations. This is being done at ALS centers dedicated to integrating patient care with medical research, as well as at national/international research conferences where ALS patients are invited to participate.

The ALS Association (www.alsa.org) and ALS Untangled (www.ALSUntangled.com), are examples of clinician and scientist-led organizations that inform ALS patients of potential treatment options. The sole purpose of ALS Untangled is to validate "alternative or off-label" treatment claims. Treatments are identified and prioritized by the ALS community, allowing patients and their families to control which

claims will be investigated. It is a critical resource for protecting patients from false hope and ineffective, sometimes life-threatening procedures.

1.5 IMPAIRED CELLULAR PROCESSES: POTENTIAL CAUSES OF NEURONAL TOXICITY

It wasn't until 1993 that the first gene, *SOD1*, was linked to ALS. More than two decades later, researchers are still trying to understand how mutations in ALS-associated genes result in the loss of motor neurons. However, as more causative genes are identified, pathways and functional processes shared by the encoded proteins are emerging. Perturbation of homeostatic processes that utilize ALS-linked proteins, such as RNA processing and stress granule assembly/disassembly may therefore hold the key to understanding how pathogenic mutations cause ALS.

1.5.1 RNA processing

Many ALS-linked genes encode for proteins with RNA-processing functions including transcription regulation, RNA-binding, alternative splicing, trafficking and stability. This has led researchers to suspect that defects in RNA processing may be the cause of ALS. In fact, abnormal RNA metabolism has been implicated in the pathogenesis of several neurodegenerative diseases in addition to ALS[35,80-83], including myotonic dystrophy type 2[84,85], fragile X syndrome[86,87], frontotemporal lobar degeneration[35,83,88,89] and spinal muscular atrophy[90-93]. As a result, aberrant processing

of RNA by RNA-binding proteins provides a pathomechanistic link between these diseases, and has given rise to classes of neurodegeneration termed “RNAopathies” and “RNA binding proteinopathies”[94,95]. However, it is not yet understood how dysfunction of these proteins causes neurodegeneration, making it difficult to develop therapeutic strategies for the treatment of these diseases. One of these proteins, FUS, is the focus of the research presented in this dissertation.

FUS is a primarily nuclear, DNA/RNA-binding protein that was first linked to ALS in 2009[35,83]. Mutations within the *FUS* gene have now been identified in approximately 4% of FALS and 1% of SALS cases[3]. Although these are small percentages, the need for identifying a treatment cannot be overstated. Pathogenic mutations of FUS often lead to juvenile-onset ALS and cause some of the most aggressive cases of the disease[96]. Patients with R521C mutations, for example, have an increased likelihood of developing dropped head syndrome. These individuals lose the ability to hold their heads up, creating additional, significant complications to the already devastating progress of ALS[97,98]. Furthermore, the RNA-binding ability of FUS makes it a good candidate for elucidating disease pathogenesis of all RNA binding proteinopathies. In fact, ALS-associated mutant FUS exhibits similar characteristics to other ALS-linked proteins in patient cells and *in vivo* model systems, including cytoplasmic mislocalization and sequestration into cytoplasmic inclusions[35,36,83,99-101]. Therefore, understanding how mutations in *FUS* lead to the development of ALS in humans will help researchers to understand the role of other RNA-binding proteins in disease pathogenesis.

1.5.2 Stress granules

Accumulation of cytoplasmic foci in motor neurons, now believed to be stress granules (SGs), are a pathological hallmark of ALS. Stress granules are one of three types of ribonucleoprotein (RNP) granule. The other two RNP granules are processing bodies (P-bodies) and transport RNPs. All three play different roles in regulating mRNA in the cell[62,102,103]. Transport RNPs are important for localized translation of mRNAs at sites within the neuron where they are needed, ferrying mRNAs to different cellular locations such as terminal axons[104]. P-bodies and SGs differ in their functions based on their compositions. P-bodies contain proteins that are specific to RNA degradation, and are therefore important for mRNA turnover[105]. SGs, on the other hand, are formed in response to various cellular stresses, and contain RNA-binding proteins as well as proteins that are involved in translation initiation. The sequestration of proteins required for translation allows a cell to assume tighter control over cellular processes in response to stress[62,102,106-108]. In the case of ALS, natural cellular stressors have been proposed as contributors to pathogenesis in patient neurons, including oxidative stress and the endoplasmic reticulum stress response[61].

Observing SGs in the cytoplasm of post-mortem, ALS patient neurons is not enough to label them as pathogenic. The protective role that they play in response to cellular stress would suggest that they are naturally forming as the neurons attempt to cope with the true cause of toxicity. However, research has uncovered certain characteristics of SGs that suggest they may actually be responsible for cell death, regardless of the initial source of stress. SGs are enriched for proteins with low-complexity, or prion-like, domains[62,109]. Prions are proteins with specific sequences that

can spontaneously change conformations, altering the structure and function of the proteins themselves. Most notably, however, prion-like domains can interact with each other, using the conformational changes of one protein as a template for others. This causes the new structure to spread from one protein to another, as they stack onto each other and form fibrils throughout the cell[110,111]. Through this process, these low complexity domains facilitate a liquid-liquid phase separation, in which the granule is essentially a liquid droplet within the cell[112].

Uncontrolled fibrillization can be toxic to cells and has been linked to several neurodegenerative diseases[62,110,113]. In a normal cell, following exposure to stress, proteins with prion-like domains may be used by the cell for their ability to self-aggregate, enabling the formation of the SGs. Under normal conditions, the cell controls this aggregation through protein turnover, via autophagy. However, loss of control over the assembly/disassembly of SGs can lead to over-aggregation and toxicity[62]. This applies to ALS because SGs, by the very nature of their function, are also enriched with RNA-binding proteins[62,109]. Many RNA-binding proteins, including those linked to ALS, have prion-like domains[110] and are known components of SGs[55,114-117]. It has been shown that ALS-causing mutations of FUS enhance liquid-liquid phase separation of developing stress granules, which appear to persist in cells, even after the cellular stress is removed. This prolonged aggregation may cause toxicity by continuing to sequester proteins and mRNAs that are necessary for normal RNA processing[55,62,118].

1.5.3 *C9ORF72* repeat expansions

(GGGGCC) repeat expansions in the first intron of the *C9orf72* gene are currently the most commonly known cause of both FALS and SALS[61]. Due to this frequency, a large majority of current ALS research is focused on this gene. Three leading hypothesis for the pathomechanistic link between these repeats and disease have been proposed. (1) Expansions result in changes to the normal isoforms produced by the gene, thereby causing haploinsufficiency of primary transcripts[39,61,119]. (2) Expansions form stable G-quadruplex structures of c9orf72 RNA that uncontrollably sequester RNA-binding proteins into intracellular foci[3,61,120,121]. (3) Repeat-associated non-ATG (RAN) translation of the expansions generates dipeptide repeat proteins that form stable, insoluble aggregates that are difficult for the cell to remove and result in cellular toxicity[3,61,122,123]. Despite being one of the most studied genes in ALS research today, it is still unclear exactly how *c9orf72* repeat expansions cause the disease. Although researchers have shown evidence that any one of these mechanisms, alone, may be linked to ALS pathogenesis, it is even more likely that cell death is the result of a combination of all three. More work is needed to solve these issues before effective treatments can be developed.

1.5.4 Aberrant glutamate signaling

Transcranial magnetic stimulation, a technique used to measure the activities of the neurons of the motor cortex, has identified aberrant cortical hyperexcitability in ALS patients[61,124]. As a result, a lot of research in ALS has been focused on glutamate

signaling. It is still unclear how this process causes ALS, though it may involve increased oxidative stress, a factor that has also been linked to ALS[125]. Even mutations in the ALS-linked gene, *SOD1*, cause increases in intracellular free-radicals[61,126]. Identification of glutamate-mediated excitotoxicity even led to the development of Riluzole, an antagonist of glutamate signaling. However, due to the modest effects of Riluzole on treating ALS patients, it is likely that excitotoxicity in ALS is merely a symptom of the actual cause of cell death[61].

1.6 PUBLIC HEALTH RELEVANCE

According to the ALS Association, approximately 30,000 Americans currently have ALS, and more than 5500 new cases are diagnosed every year. In Europe, the prevalence is estimated to be 2-4 per 100,000 individuals based on recent studies[127-130]. Unfortunately, since age is such a critical factor for late onset diseases, these numbers are expected to rise due to worldwide improvements in healthcare, and subsequent growth of ageing populations. According to recent reports, the global incidence of ALS is projected to increase nearly 70% over the next 30 years[131]. To make matters worse, a diagnosis of ALS is a death sentence for those who get it. Currently, the most effective treatment, Riluzole, is an FDA approved drug that is only able to extend lifespan by a few months without reducing the clinical symptoms associated with ALS[132]. The absence of any cures or effective treatments is due to a lack of understanding of how ALS-linked proteins cause ALS in the first place. As a result, those who are diagnosed are left without hope. Identifying modifiers of disease-

associated toxicity will elucidate mechanisms and pathways involved in ALS pathogenesis and highlight potential therapeutic targets. To this end, the project presented here directly addresses the need for identifying modifiers to better understand ALS. Our results add clarity to the mechanisms behind pathology, and link this pathology to that of other neurodegenerative diseases. Thus, our findings have implications for more than just ALS.

1.7 SUMMARY

ALS is a complex disease, with potentially numerous factors contributing to onset and progression. Overlapping phenotypes with other neurodegenerative diseases cause challenges in accurate diagnosis of ALS, but provide opportunities for shared therapies between them. The fatality and rapid progression of ALS makes finding a treatment or cure imperative.

Unfortunately, despite some potential treatment options, including stem cell replacement therapy and the development of drugs designed to directly target or alter the effects of mutant proteins, no successful therapies currently exist. This is largely due to the fact that researchers still do not understand how ALS is caused in the first place. Evidence to support an environmental origin is weak, despite being heavily studied. Numerous gene mutations have been directly linked to pathogenesis in humans, suggesting that although environment may play a role in mediating the severity of the disease, it is still a genetic disease. As a result, most ALS researchers, including our group, focus on the cellular and molecular consequences of pathogenic mutations.

To accomplish this, numerous animal models have been developed including fruit flies, yeast, mice, rats and worms. The model used in the study presented in this dissertation is a fruit fly (*Drosophila melanogaster*) model. This is a very versatile model that has been very useful in neurodegenerative research, including ALS. The role that *Drosophila* have played in ALS research is extensively described in the following chapter. It is through models such as this that the consequences of pathogenic gene mutations will be understood, and new, desperately needed therapies will be developed.

2.0 A FRUITFUL ENDEAVOR: MODELING ALS IN THE FRUIT FLY

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2.1 INTRODUCTION

Amyotrophic lateral sclerosis (ALS), also called Lou Gehrig's disease, is an adult onset, fatal, neurodegenerative disorder, for which there is currently no available cure. ALS was first described in the late 1860's and early 1870's by Jean-Martin Charcot [29,30], and a clear understanding of its pathogenesis at cellular and molecular levels is still eluding researchers today, nearly 150 years later. ALS is primarily characterized by the loss of upper and lower motor neurons, and as a result, death is usually due to respiratory failure [45,133,134]. Clinically, ALS can be categorized into three primary types depending on the origin of onset [134]. Approximately 65% of ALS patients are diagnosed with limb onset, 30% with bulbar and the remaining 5% with respiratory onset [134]. In general, bulbar and respiratory have a worse prognosis than limb onset [28]. Occurrence

of the disease is highest between approximately 60 and 85 years of age [128,135-137], with males typically having an overall higher rate of incidence than females [128,138]. Disease progression is very rapid, and the average life expectancy post-diagnosis is 2-5 years [139-144].

Approximately 5% of all ALS cases show signs of inheritance and are referred to as familial ALS (FALS) [145]. The remaining 95% are not inherited, and are referred to as sporadic ALS (SALS). To date, several genes have been linked to both FALS and SALS, including *SOD1* [31,146,147], *ALS2* [148,149], *VAPB* [150,151], *TARDBP* [152-154], *FUS* [32-34,155,156], *VCP* [38], *OPTN* [157-159], *SQSTM1* [160], *UBQLN2* [161], *C9ORF72* [39-41,162], *PFN1* [163], *hnRNPA1* and *hnRNPA2B1* [115], and most recently *MATR3* [42], reviewed in [119,164]. In order to study these genes, researchers have developed animal models that recapitulate several of the cellular and molecular phenotypic features of ALS pathogenesis and neuronal cell death that are observed in human patients. Some of these features include the formation of cellular aggregates [165-171], mitochondrial abnormalities [172-175], axonal abnormalities [174,176], dysfunctions in glutamate signaling and subsequent excitotoxicity [177-179], and oxidative damage [180].

2.2 HISTORY

It was more than 100 years ago that researchers began to realize the usefulness of *Drosophila melanogaster* in genetics research. Shortly after the turn of the 20th century, many key discoveries were made in *Drosophila* that helped shape our understanding of chromosome structure and heredity, reviewed in [181], [182] and [183]. From

there it became instrumental for studying genetic mutations, and today researchers around the world use *Drosophila* as a model system for studying human diseases, including neurodegeneration. Many key discoveries that have helped in advancing the neurodegenerative disease field have been made using flies as a model system including, but not limited to, the identification of candidate genes as causes and therapies of disease [184-186], *in vivo* evidence for RNA-mediated neurodegeneration [187,188], the identification of ER-golgi trafficking inhibition as a contributor to alpha-synuclein-associated toxicity in Parkinson's disease [189] and the identification of a molecular link between the ubiquitin-proteasome system and autophagy [190].

Thomas Morgan, Alfred Sturtevant, Calvin Bridges and Hermann Muller were among the first pioneers of genetic research in *Drosophila*. They worked together in Morgan's "Fly Room" at Columbia University [191] where they studied heredity and clarified the relationships between genes, chromosomes and phenotypes, and ultimately won Morgan the 1933 Nobel Prize in Physiology or Medicine. In 1913, just a few short years after beginning his work with *Drosophila*, Sturtevant published the first ever genetic map [192]. A year later Bridges showed that genes and chromosomes were not separate entities, but that genes existed within chromosomes (a concept that was unclear at the time) [193]. Muller was instrumental in the development of balancer chromosomes that allowed researchers to stably and indefinitely maintain stocks of flies as heterozygotes for a desired mutation that is otherwise lethal to homozygotes [194]. Several years later, Muller also discovered that gene mutations could be caused, as well as intentionally induced, by x-radiation [195]. This finding won him the 1946 Nobel Prize in physiology or medicine.

With the work performed by Morgan and his colleagues, *Drosophila* was thrust to the forefront of genetics research. Researchers moved beyond using fruit flies to understand basic genetic concepts, and began identifying genes and understanding molecular pathways that are relevant to human development and functional processes. Though the importance of these discoveries cannot be overstated, some of the most important milestones in *Drosophila* genetics were those that led to its establishment as a model for studying human diseases. In 1982 Gerald M. Rubin and Allan C. Spradling were the first to use the P transposable element to insert exogenous DNA into the *Drosophila* genome [196]. They used this method of creating transgenic flies to successfully integrate an exogenous gene and rescue a mutant phenotype [197]. In 2000, the fully sequenced *Drosophila* genome was published [198,199]. Since then, it has been estimated that as many as 77% of the human disease-associated genes reported in the Online Mendelian Inheritance in Man (OMIM) database have similar, likely orthologous, genes in *Drosophila* [200,201]. These discoveries laid the foundation upon which modern *Drosophila* models were created. Diseases are now being studied in an animal that is cheaper, faster, and easier to maintain than other models such as mice, rats and primates. The earliest *Drosophila* models of neurodegenerative diseases were developed in the 1990's, and more are created every year [202-207]. In this review, we will highlight the *Drosophila* models that have been developed over the past five years for studying ALS.

2.3 *DROSOPHILA* MODELS OF ALS

2.3.1 Superoxide dismutase (SOD1)

SOD1 is a protein that, under normal conditions, protects cells against oxidative stress by catalyzing the conversion of superoxide anions into oxygen and hydrogen peroxide [208]. As it has been hypothesized that oxidative damage is linked to the aging process [209], *SOD1 Drosophila* models have been used to determine whether altering SOD1 expression levels can extend or shorten lifespans [210-212]. Indeed, overexpression of SOD1 does appear to improve lifespans of *Drosophila*, although this effect appears to be tissue-specific, and does not occur in cells of the nervous system or in muscle cells [213]. SOD1 may also play less of a role in the aging of organisms with naturally longer lifespans, as experiments in ants have shown lower SOD1 expression in queens than in male and worker ants, even though queens live much longer [214]. In 1993, *SOD1* became the first gene responsible for causing ALS [31]. We now know that *SOD1* mutations account for approximately 1% of SALS cases and 12% of FALS cases [119]. After the initial report in 1993, researchers were quick to utilize *Drosophila* to understand how *SOD1* mutations lead to ALS (Table 2.1). By 1995, a fly model was developed to study the effects of mutant SOD1 protein subunits on wild type (WT) and mutant protein dimerization [215]. In more recent years a model was made by comparing transgenic flies overexpressing either *Drosophila SOD1* (*dSOD1*), human *SOD1* (*hSOD1*), or mutant *hSOD1* (A4V or G85R) [216]. Flies expressing WT *hSOD1* or either mutant had a reduced climbing ability compared to *dSOD1* flies, suggesting a motor neuron dysfunction due to expression of either WT or mutant versions of *hSOD1*. The

differences in climbing did not become apparent until day 14, an observation that suggests a progressive loss of motor function in the WT and mutant *hSOD1*-expressing flies. In addition, these flies exhibited reduced synaptic transmission in dorsal longitudinal muscles of the giant fiber motor pathway [216]. Consistent with *SOD1*-associated ALS pathogenesis in mouse models [217-219], the motor neurons developed aggregates of hSOD1 protein. Altogether, this model provided evidence that WT and ALS-causing mutant *hSOD1* are toxic to motor neurons.

ALS is thought to be a non-cell autonomous neurodegenerative disease [67]. Therefore, researchers have also studied non-neuronal cells in animal models of ALS. The *Drosophila* model developed in 2008 was utilized a few years later by another group to examine how different cell-types expressing mutant and WT *hSOD1* are affected by ALS-linked environmental insults [220]. One advantage to using *Drosophila* as a model organism is the ability to direct expression of transgenes to a wide range of specific tissue types using the UAS-gal4 system [221,222]. Flies expressing *hSOD1* specifically in glial cells showed a decrease in lifespan when exposed to the neurotoxin β-N-methylamino-L-alanine (BMAA) compared to *dSOD1*-expressing controls. Interestingly, however, flies expressing either mutant or WT *hSOD1* specifically in motor neurons survived longer than controls when exposed to BMAA. This is an unexpected and intriguing result, considering the role of motor neurons in ALS pathogenesis. Conversely, expression of the *hSOD1-G85R* mutant in either motor neurons or glia reduced *Drosophila* lifespan compared to controls when exposed to a different environmental insult, hydrogen peroxide [223]. In the absence of induced environmental factors, expression of *hSOD1-G85R* in either motor neuron or glial cells led to a

decrease in lifespan, but expression in both cell types simultaneously increased lifespan compared to controls. However, another study showed that expression of the FALS SOD1 mutant, *hSOD1-G41S*, in *Drosophila* motor neurons increases lifespan when compared to controls [224]. Although the differences caused by the G85R and G41S mutants may be due to their locations in different domains of the protein [225], these observations highlight the complexities of studying ALS in animal models, as well as the variability that is observed among different fly models.

Mitochondrial dysfunction is a pathological hallmark of ALS in humans [175,226,227], and may be caused by altered protein activity of *SOD1* mutants [228-230]. ALS-associated mutations can hinder binding of SOD1protein to zinc ions, resulting in cellular toxicity [231,232]. Recently, researchers using transgenic *Drosophila* expressing zinc-deficient hSOD1 obtained evidence supporting a link between mutant *SOD1* and mitochondrial dysfunction [233]. These flies harbored a D83S mutation in a zinc-binding domain of exon 4 that inhibits binding of SOD1 protein to zinc, without affecting binding to its other cofactor, copper. They found that ubiquitous expression of zinc-deficient hSOD1 led to age-dependent locomotor dysfunction that was not present in control flies. However, these flies exhibited normal lifespans and showed no brain or eye degeneration. When they examined the effects of zinc deficiency on mitochondria, they found that mitochondrial structure was altered in flight muscles of mutant flies. ATP production was also reduced in the fly heads compared to controls, but not in fly bodies, possibly suggesting a reduction of ATP production specifically in neurons. In addition, mutant hSOD1 D83S expression resulted in an increased sensitivity to paraquat and zinc exposure based on declines in lifespan of these flies [233]. Taken together, this model

provides evidence that zinc deficiency due to hSOD1 mutations may result in ALS pathogenesis through reduced mitochondrial function.

2.3.2 Alsin

In 2001, mutations of the gene, *ALS2*, were identified in individuals with ALS, specifically ALS2 [148,149]. Alsin (also called ALS2), the protein encoded by *ALS2*, is a guanine nucleotide exchange factor for Ras-related in brain 5 (Rab5) [234]. Since Rab5 is a GTPase involved in the endocytic pathway [235], it has been proposed that loss of Alsin function due to ALS-causing mutations perturb endosomal dynamics, thereby implicating these processes in the pathogenesis of the disease [234].

A *Drosophila* model for Alsin-associated ALS was recently developed using the *Drosophila* homolog of Alsin, dALS2 (Table 2.1) [236]. Consistent with the disease state in ALS2 patients, where mutations of Alsin result in premature cessation of translation and subsequent production of truncated protein products [237], the mutations used in the *Drosophila* model were *dALS2* constructs lacking approximately 30% of their coding sequence. Mutant flies did not show significant phenotypic changes at the NMJ compared to WT controls, apart from a slight increase in the bouton number of abdominal motor axons of adult mutant flies. They did, however, show a significant age-dependent reduction in locomotion when compared to WT flies. This reduction was rescued by ubiquitous WT dALS2 overexpression, suggesting dALS2 loss-of-function as the cause of the phenotype. Interestingly, however, this rescue was not as significantly recapitulated by overexpressing WT dALS2 in motor neurons [236]. It is possible that differences in transgene expression levels between larvae and adult flies, depending on

whether expression is ubiquitous or directed to motor neurons, might explain these results. The locomotor reduction observed with ubiquitous expression, however, may prove to be a useful output in future experiments aimed at identifying modifiers for the dALS2-related phenotype with this model [236].

2.3.3 VAMP-associated protein B (VAPB)

VAMP (vesicle associated membrane protein)-associated protein B (VAPB), encoded by the gene, *VAPB*, localizes to both the endoplasmic reticulum (ER) and mitochondrial-associated membranes, where it is involved in the ER-associated unfolded protein response [238] and ER-mitochondria calcium exchange [239] processes, respectively. A missense mutation in the coding region for the major sperm protein (MSP) domain of VAPB was first identified in human ALS patients in 2004 [150,151]. Since then, *Drosophila* models have been used by researchers to better understand WT VAPB function in various tissue-types, as well as how different ALS-linked mutations in VAPB lead to toxicity and subsequent pathogenesis of the disease.

2.3.3.1 Overexpression and Knockdown

Several *Drosophila* models have been developed to elucidate the function of WT human VAPB (hVAPB) and its *Drosophila* homolog DVAP33-A (referred to as dVAP in this review), encoded by the gene, *DVAP33-A* [99,240-245]. hVAPB and dVAP proteins are structurally and functionally similar, sharing approximately 62% homology, and one protein can compensate for the loss of the other in maintaining normal cellular

processes [240]. In addition, creating equivalent human disease-causing mutations in *dVAP* leads to neurodegenerative phenotypes that mimic those seen when hVAP with ALS-causing mutations are expressed in *Drosophila* [99,240-242,246-248].

Models utilizing either homolog have shown that altering their expression levels in neurons results in functional and morphological changes of the neuromuscular junction (NMJ). Loss of dVAP function in dVAP-null mutant flies exhibit an increase in bouton size, but a decrease of overall bouton number at the NMJ, compared to normal flies. Exogenous expression of hVAPB in dVAP-null flies rescues this loss of boutons, providing evidence to support functional overlap between the two proteins [240]. When dVAP is overexpressed in neurons, bouton size decreases, with a concurrent decrease in the number of vesicles, but the overall bouton number increases [99,240,249]. These changes are also observed in neurons overexpressing hVAPB [240]. Synaptic transmission is also affected by hVAPB/dVAP expression, suggesting a possible role of these proteins in maintaining synaptic functions. Loss of dVAP function in dVAP-null mutants reduces the quantal content at the NMJ by approximately 40%, compared to normal control flies. Conversely, overexpressing dVAP increases the quantal content, consistent with an increase in the bouton number, but the overall quantal size is reduced [240]. It was concluded that quantal changes are a response to the morphological changes occurring at the NMJ in these fly lines, in an effort to keep the synaptic transmission at normal levels [240]. Loss of dVAP function increases the number of presynaptic active zones at the NMJ [240]. Different groups looking at the effects of dVAP overexpression have reported differences in the number of active zones, although these differences may be due to the different techniques used to visualize the NMJ's.

One group, utilizing electron microscopy, has reported no change in the number of active zones upon overexpression of dVAP [240], whereas a second group, utilizing confocal microscopy with antibodies specific to the active zone protein, Bruchpilot, reported a decrease in active zones [99].

Mitochondrial dysfunction has become a hallmark of ALS pathogenesis in humans [175,226,227]. Similarly to a recently developed *SOD1* model [233], dVAP-null mutant flies have morphological defects of their muscle mitochondria [244], suggesting that mitochondrial dysfunction may be a cause of toxicity in VAPB-associated ALS.

Pan-neuronal overexpression of dVAP P58S in *Drosophila* (equivalent to the ALS-causing mutant, P56S, in humans) appears to be toxic, causing a decrease in larval mobility and an increase in larval central neuron cell death [150,151,240] . One possible mechanism for dVAP P58S toxicity is through its ability to induce morphological changes at the NMJ. Similarly to dVAP-null flies, P58S mutant expression in neurons increases bouton size at the NMJ. Not surprisingly, therefore, although WT dVAP is capable of rescuing the bouton phenotype in the dVAP-null background, dVAP P58S is not [99]. dVAP P58S also hinders microtubule organization at the NMJ, although the mutant protein does not appear to have an effect on presynaptic active zones or the number of vesicles [99].

It has been proposed that dVAP P58S might exert toxicity through a dominant negative effect of sequestering normal proteins into cytoplasmic granules [99]. dVAP P58S mutant protein forms ubiquitin-positive, cytoplasmic aggregates in larval neurons and muscle cells [99,241]. Interestingly, WT dVAP is mislocalized from the cell membrane into these aggregates, suggesting that mutant dVAP is capable of recruiting WT protein,

thereby hindering its normal activity in the cell [99,240,241]. Under normal conditions, WT dVAP localizes to the cell membrane, where the N-terminal portion that includes the major sperm protein (MSP) domain is secreted as a ligand for Eph receptors [241]. Secretion of the MSP domain is decreased in cells that are expressing dVAP P58S and are positive for cytoplasmic aggregates [241]. In addition, evidence for a dominant negative effect can be seen on an organismal level. dVAP-null mutant pupae have a reduced eclosion rate, compared to normal controls. Expression of WT dVAP in these pupae rescues the eclosion rate. However, co-expression of WT dVAP with P58S mutant protein in the dVAP-null background does not rescue the phenotype, suggesting that mutant dVAP can suppress WT function, possibly through dimerization of WT and mutant proteins [99].

Another VAPB mutation was identified in FALS patients in 2010, VAPB T46I [246]. A model was subsequently developed to characterize the *Drosophila* homolog of the VAPB mutant, dVAP T48I [246]. Expressing dVAP T48I in adult fly eyes causes structural degeneration not present in control flies, confirming the deleterious nature of the mutation. When WT dVAP is expressed pan-neuronally, neurons of larval brain and nerve fibers show WT protein localized to cell and ER membranes. In contrast, dVAP T48I mutant protein localizes to bright aggregates. Interestingly, mutant dVAP expression in the brain and in muscle cells causes structural changes of the ER, including fragmentation, similarly to what has been observed in ALS patients [247]. The Hsp70 chaperone protein is also found to be elevated in these cells. Taken together, this model has provided evidence that expression of the dVAP T48I mutant leads to toxicity, and has thus provided insight into the nature of the VAPB T46I mutant in

humans. Specifically, these results have identified stress response and morphological changes of the ER as possible modes of mutant protein toxicity in VAPB-related ALS [246].

A *Drosophila* model was recently developed to characterize a third VAPB mutation identified in 2012 in a single FALS patient with an additional *C9ORF72* repeat expansion [248,250]. The human VAPB mutant protein, VAPB V234I, has an equivalent mutation in *Drosophila*, dVAP V260I. The experiments performed in this model focus on comparing overexpression of mutant dVAP with WT dVAP. dVAP V260I and WT dVAP overexpression exhibited similar phenotypic results, with the mutant dVAP producing more severe phenotypes [248]. Expression of both WT and dVAP V260I cause morphological changes at the NMJ including decreased bouton size with increased bouton number, as well as the formation of satellite boutons. The formation of these satellite boutons appears to be the result of alterations in microtubule architecture [248]. On a cellular level, both WT and mutant protein expression form aggregates in muscle cells. These cells also exhibit altered nuclear morphology including elongation, an increase in overall volume and clustering of nuclei with neighboring cells. Interestingly, the dVAP V260I mutant protein localizes to the muscle nuclei [248]. Altered nuclear morphology is also observed in neurons of larval brains, and dVAP V260I protein forms large cytoplasmic, not nuclear, inclusions in these cells. Similarly to dVAP T48I overexpression [246], WT and dVAP V260I expression in muscle cells induces a heat shock stress response, identified by an increase in Hsp70 expression and localization to nuclei [248]. Experiments focused on changes on the organismal level revealed reduced larval locomotion, pupal eclosion rates and altered adult wing posture when WT and

dVAP V260I were neuronally expressed. Altered wing posture was also observed upon expression in muscle cells. When targeted to adult eyes, both WT and mutant protein caused structural degeneration [248]. It has been observed that both WT and mutant protein expression induced the altered phenotypes, with the dVAP V260I mutant producing more severe phenotypes, suggesting a possible gain of mutant protein function. The formation of aggregates in muscle cells and possible protein sequestration, however, may suggest a loss-of-function, so it was suggested that dVAP V260I toxicity may be due to both a loss and a gain-of-function [248].

2.3.3.2 Modifiers of VAPB-Associated Phenotypes

Recently, proteins that interact with endogenous dVAP have been identified, including Down syndrome cell adhesion molecule (Dscam) [245], Suppressor of Actin 1 (Sac1) [242] and Oxysterol binding protein (Osbp). Pan-neuronal, RNAi-mediated knockdown of the endogenous phosphoinositide phosphatase, Sac1, in *Drosophila* causes morphological changes at the NMJ, as well as structural degeneration of the adult eyes [242]. Interestingly, Sac1 and WT dVAP colocalize in larval brains, eye imaginal disks and salivary glands. When the ALS-associated dVAP P85S mutant is overexpressed in neuronal cells of these tissue-types, Sac1 is sequestered into mutant dVAP-induced cytoplasmic aggregates. Furthermore, RNAi-mediated knockdown of dVAP and Sac1in these tissues, as well as overexpression of dVAP P85S mutant protein, elevated expression of phosphatidylinositol 4-phosphate (PtdIns4P). Increased PtdIns4P expression results in degenerative phenotypes at the NMJ [242]. It has therefore been proposed that dVAP P85S mutant toxicity occurs through Sac1

interaction and sequestration into cellular aggregates, resulting in increased expression of PtdIns4P and subsequent morphological changes at the NMJ [242]. Taken together, these results identify Sac1 and PtdIns4P proteins as candidates for involvement in ALS pathogenesis, as well as possible therapeutic targets for developing effective therapies for ALS in the future.

The lipid binding protein, Osbp, interacts with WT dVAP, but not the dVAP P85S mutant [243]. In normal cells, Osbp colocalizes with dVAP at the cell and ER membranes. In dVAP P85S mutant-expressing cells, however, Osbp mislocalizes to cytoplasmic aggregates, similarly to dVAP [243]. Interestingly, in dVAP-null mutants, Osbp mislocalizes to the golgi. Since overexpression of human Osbp (OsbpL8) in ER is sufficient to rescue loss-of-function-associated phenotypes of dVAP-null flies, it has been proposed that the P85S mutation of dVAP incurs a loss-of-function to the protein in disease pathogenesis [243].

2.3.4 TAR DNA-binding protein 43 (TDP-43)

TDP-43 is a 43 kDa protein encoded by the gene *TARDBP*. It was first identified in 1995 as a transcriptional regulator that bound to pyrimidine-rich motifs of the human immunodeficiency virus 1 (HIV-1) regulatory element, TAR [251]. TDP-43 is a primarily nuclear protein, and was first linked to ALS in 2006 when it was identified as a component of ubiquitinated, cytoplasmic granules in the neurons of patients with ALS and FTLD (frontotemporal lobar degeneration) [152]. Shortly thereafter, researchers began identifying several *TARDBP* mutations in FALS and SALS patients [81,252-255]. To date, a total of 47 missense mutations and 1 nonsense mutation in *TARDBP* have been

identified [256], some of which have been directly linked to cytoplasmic mislocalization of the TDP-43 protein [257,258]. We now know that TDP-43 is involved in regulating multiple aspects of RNA metabolism, including alternative splicing [259], mRNA stability [260] and likely mRNA transport [261,262], thus implicating aberrations of these processes in disease pathogenesis.

Several *Drosophila* models for TDP-43-associated ALS have been generated and extensively characterized (Table 2.1). One of the major goals of researchers has been to determine if TDP-43-associated ALS pathogenesis is due to a gain or a loss of protein function. *Drosophila* models have provided evidence to support both hypotheses, and the truth may actually be a combination of the two.

2.3.4.1 Overexpression and Knockdown

TDP-43 is an RNA-binding protein that is highly conserved across organisms, including *Drosophila*. Researchers, therefore, commonly use *Drosophila* models to observe the effects of overexpression or knockdown of either human TDP-43 (hTDP-43) or the drosophila ortholog, dTDP-43, which is encoded by the gene *TBPH* (TAR DNA-binding protein-43 homolog). These gain-of function/ loss-of-function experiments have been helpful for elucidating TDP-43 function as well as validating new models through recapitulation of key phenotypic features of ALS.

It has been demonstrated, with the use of both exogenous and endogenous WT TDP-43 in *Drosophila*, that maintaining normal TDP-43 protein levels in various tissue-types is critical for proper physiological functions of the organism. Pan-neuronal reduction of endogenous *Drosophila* TBPH levels, either through deletion mutants or

through dTDP-43 RNAi, reduces adult fly lifespan and locomotion (typically walking and climbing) [263,264]. Interestingly, co-expressing human TDP-43 rescues these phenotypes, suggesting a possible natural feedback mechanism for maintaining expression levels, that is conserved in these orthologs [263]. Pan-neuronal overexpression of dTDP-43 has also shown a reduction in adult lifespan, as well as a reduction in both adult fly and larval locomotion [264]. In sensory neurons of *Drosophila* larvae, lowered expression of dTDP-43 reduces dendritic branching, and overexpression increases branching [265]. In mushroom bodies of the *Drosophila* brain, both overexpression of human TDP-43, as well as reduced *Drosophila* TDP-43 expression, causes loss of axons and neuronal death [266]. When dTDP-43 is overexpressed in this tissue-type, there is a reduction of lobe size, as well as the formation of TDP-43-positive cytoplasmic aggregates [267].

Since ALS affects motor neurons in humans, several experiments in *Drosophila* models are carried out on larval and adult fly motor neurons. When dTDP-43 levels are decreased specifically in larval motor neurons, dysmorphologies of the NMJ are observed, including reductions in axonal branching [263]. There are, however, differences in reported bouton numbers at the NMJ between different groups. Some have reported a decrease in bouton number when endogenous dTDP-43 levels are reduced via deletion mutants in a *W1118* fly strain [263]. Others have reported an increase in bouton number when using a $y^{1}\ w^{67c23}$ fly strain carrying a different deletion of dTDP-43 [267]. It has been suggested that the conflicting observations may be due to the differences in genetic backgrounds of the flies used in the experiments, as well as the differences in the dTDP-43 deletion constructs [267]. Expression of human TDP-43 in larval motor neurons resulted in reduced axonal branching and fewer synaptic boutons, in addition to

axon swelling, motor neuron cell death and the formation of hTDP-43-positive cytoplasmic aggregates [266,268]. Some groups, however, have reported only small amounts of cytoplasmic hTDP-43, as well as little to no aggregation [269,270]. Therefore, it is possible that a small amount of cytoplasmic TDP-43 is enough to cause toxicity in motor neurons, even without the presence of large aggregates. TDP-43-positive cytoplasmic aggregates, a hallmark of TDP-43-associated ALS in humans [152-154,271], have also been reported in adult *Drosophila* motor neurons overexpressing endogenous TDP-43 [267].

It has previously been shown that overexpressing human TDP-43 in adult fly motor neurons causes a significant reduction in lifespan [269] compared to controls. Interestingly, overexpression of hTDP-43 with either the human caspase inhibitor, P35, or the *Drosophila* homolog, dIAP (*Drosophila* inhibitor of apoptosis), in adult motor neurons does not improve lifespan compared to flies expressing hTDP-43 alone [272]. This result suggests that human WT TDP-43 toxicity may not be due to neuronal apoptosis through programmed cell death mechanisms. However, when overexpressing endogenous *Drosophila* TDP-43 in pupae, caspase inhibition rescues CCAP/bursicon neuron apoptosis, suggesting that in these cells, at this stage of development and using endogenous protein, loss of neurons does occur through programmed cell death [273].

Adult eyes are often used for transgene expression in *Drosophila* models of neurodegenerative diseases to produce easily observable eye phenotypes[274-277]. Death of neurons in the eyes produces degenerative phenotypes that can be seen both inside and on the surface of the eye. These phenotypes can range from structural changes to

loss of pigmentation. Expressing human TDP-43 in adult fly eyes causes age and dose-dependent structural degeneration [266,268,269,278,279]. This degeneration has also been directly correlated with mislocalization of hTDP-43 to the cytoplasm, and the mislocalization alone is sufficient to cause the degeneration [278]. In contrast, however, overexpression of endogenous *Drosophila* TDP-43 in eye imaginal disks of third instar larvae does not appear to cause cytoplasmic mislocalization, despite the presence of observed neurodegenerative phenotypes in adult flies [264].

Muscle atrophy and excitotoxicity from aberrant glutamate signaling are hallmarks of ALS pathogenesis in humans [177-179]. Recently, endogenous *Drosophila* TDP-43 expression has been identified in muscle and glial cells [280]. Specifically knocking down dTDP-43 in adult muscle cells causes an overall reduction in motor function, based on an observed reduction in physical activity, compared to controls expressing normal protein levels. Overexpressing dTDP-43 in larval muscle cells results in protein mislocalization from nuclei into sarcoplasmic aggregates. These larvae also exhibit reduced locomotion and early lethality compared to controls. Targeted overexpression in glial cells is lethal to larvae. However, unlike in muscle cells, knockdown of protein levels in adult glial cells causes motor dysfunction that is only apparent in aged flies [280].

It has also been shown that overexpression and loss of function of dTDP-43 in glial cells and neurons alters the mRNA levels of dEAAT1 and dEAAT2, the *Drosophila* homologs of the glutamate transporters EAAT1 and EAAT2[280]. EAAT2 mRNA has previously been identified as a possible target of TDP-43 [281,282]. This transporter is important for the clearance of glutamate released at the NMJ, and its dysfunction or loss

has been linked to ALS through the identification of increased glutamate levels in the plasma and cerebral spinal fluid of ALS patients, potentially resulting in neuron excitotoxicity [283-290]. Consistent with the disease state in humans, dTDP-43 null mutant flies express lower overall levels of both dEAAT1 and dEAAT2 mRNA compared to controls. WT dTDP-43 decreases mRNA levels when overexpressed in glial cells, but increases mRNA levels when overexpressed in neurons [280].

Recent experiments using *Drosophila* models to study the effects of expressing human TDP-43 in glial cells have provided evidence to support its toxicity in this cell-type. The results have shown that WT hTDP-43, as well as A315T, D169G, G298S and N345K mutants, mislocalize to the cytoplasm when overexpressed [291]. WT and mutant hTDP-43 overexpression in both glial and motor neuron cells also reduces bouton number at larval NMJ's and hinders larval motor function, specifically their ability to turn over after being flipped upside down. When expressed in glial cells, WT and hTDP-43 mutants cause an increase in the number of postsynaptic glutamate receptors, but have no effect on the number of presynaptic active zones. In contrast, when expressed in motor neurons, WT and mutant proteins increase the number of active zones, but only WT hTDP-43 concurrently increases the number of glutamate receptors. The effect of these changes in both cell types is an imbalance in the ratio of active zones to glutamate receptors at the NMJ. This imbalance coincides with the motor dysfunction observed in the larvae, suggesting that this may be the mechanism that leads to toxicity of the overexpressed proteins. It was hypothesized that the increase in both active zone and glutamate receptor number, caused by overexpression of WT hTDP-43 in motor neurons, suggests a different mechanism of toxicity from the mutants in this cell

type [291]. However, the fact that WT and mutant TDP-43 exert similar effects in glial cells makes it difficult to identify the consequences of the ALS-linked mutations. These results suggest that the mutations themselves are not necessary for toxicity in glial cells. Taken together, however, these experiments provide further evidence linking glial and muscle cells to ALS, and support a role of glutamate transport dysfunction in disease pathogenesis.

In addition to studying overexpression and knockdown of WT TDP-43, *Drosophila* models have been used to study ALS-associated TDP-43 mutants. Interestingly, in many of these experiments, mutant and WT toxicity is either not compared, or cannot be distinguished from each other due to having comparable effects. As a result, it is difficult to determine what ALS-associated mutations actually do to normal protein function, and how large of a role they play, if any, in the actual pathogenesis of the disease. Overexpression of human TDP-43 M337V in *Drosophila* eyes causes mild degeneration and shows both full-length TDP-43, as well as a 25kD fragment, similar to what has been identified in FTLD patient samples [152,278]. Dose and age-dependent adult eye degeneration is also seen in flies expressing D169G, G298S and N345K mutants, when compared to non-hTDP-43 expressing control flies [291]. This phenotype is accompanied by axonal aggregation of these mutant proteins in eye neuroepithelium, providing further evidence for a link between the aberrant mislocalization and aggregation of hTDP-43, and neurodegeneration [291]. The *Drosophila* TDP-43 Q367X mutant reduces miR-9a expression in larvae compared to WT TDP-43-expressing flies, and subsequently results in improper specification of sensory organ precursor cells[292]. This suggests that dTDP-43 controls specification

through miR-9a, and that a loss of function through an ALS-associated mutation causes dysregulation of the process [292].

Pan-neuronal expression of A315T, G348C, A382T, G287S, or N390D reduces adult lifespan compared to control flies expressing driver alone, the extent of which varies between different mutants [270]. However, these results do not directly implicate the mutations themselves in toxicity, as WT hTDP-43 overexpression caused the largest reduction in lifespan compared to the mutant and control flies. Interestingly, RNA-binding-deficient hTDP-43 only mildly reduced adult lifespan, essentially rescuing the WT hTDP-43 phenotype [270]. These results were supported by later experiments performed in adult eyes [293]. Overexpression of WT hTDP-43 caused retinal degeneration that was rescued by inhibiting RNA-binding function through both removal and mutation of the RNA recognition motif 1 (RRM1). Degeneration caused by G298S and M337V mutants was also rescued by inhibiting the RNA-binding ability of these proteins. Removal of the nuclear localization signal (NLS) in WT hTDP-43 and G298S and M337V mutants resulted in “forced” cytoplasmic mislocalization when expressed in adult eyes that corresponded to increased degeneration compared to WT hTDP-43 controls with intact NLS’s. When the RNA-binding ability of these proteins was removed, cytoplasmic mislocalization remained, but the degeneration was rescued [293]. Taken together, the experiments performed by these groups provide evidence that the RNA-binding function of TDP-43 may be necessary for toxicity, as mislocalization alone does not appear to be sufficient for retinal degeneration.

Another group has also used human TDP-43 lacking a functional NLS to further study the effects of cytoplasmic mislocalization in disease pathogenesis [294]. The NLS-

mutant hTDP-43 in these experiments causes severe degeneration in adult eyes, as well as reduced adult lifespan when expressed in neurons, and lethality when expressed in larval neurons [294]. Interestingly, increased death of adult flies begins prior to the formation of cytoplasmic aggregates, lending support to the conclusions of others that aggregation of TDP-43 is not necessary for toxicity [269,294]. In contrast, however, studies of FTLD show a link between cellular toxicity and aggregates that are positive for either full-length TDP-43 or the disease-linked, 25kD, C-terminal fragment, TDP-25 [152,295-297]. In fact, it was recently shown in a *Drosophila* model that upregulating endogenous chaperone proteins in various tissue types can reduce TDP-43 and TDP-25 aggregation, a result that coincides with rescue of their ALS-associated phenotypes in the flies [298]. Furthermore, altering expression levels of the stress granule-associated PEK, ROX8 and GADD34 proteins in adult neurons affects TDP-43-associated toxicity [299]. Specifically, knocking down PEK and ROX8, two proteins involved in stress granule formation and the *Drosophila* homologs of mammalian PERK and TIA1, respectively, [300,301] inhibits stress granule formation and subsequently suppresses TDP-43 toxicity. Knocking down GADD34, a phosphatase that prevents the formation of stress granules by dephosphorylating the critical stress granule component, eIF2α, [302] effectively promotes stress granule formation and subsequently enhances TDP-43 toxicity [299]. Therefore, due to the discrepancies in the data obtained by these different groups, it remains unclear as to whether or not cytoplasmic mislocalization and aggregation of TDP-43, a phenotype linked to ALS in humans, is a primary or secondary cause of toxicity.

Drosophila and human TDP-43 overexpression and knockdown flies were utilized to analyze global gene expression changes in various tissue types and stages of *Drosophila* development [272,273,303]. These studies have specifically led to the identification of genes, including *Hey, Nup50* [272] and *Map205* [273], as well as pathways such as Notch [272], Wnt and BMP [303], that may be linked to TDP-43-associated ALS pathogenesis. In a more general sense, they have shed light on the role and importance of TDP-43 in development, and the need for an organism to maintain normal TDP-43 protein levels for physiological functions. Changes in *Drosophila* TDP-43 expression levels in the larval central nervous system (CNS) result in largely non-overlapping gene expression patterns. Specifically, dTDP-43 overexpression leads to downregulation of one set of genes, whereas larvae carrying loss-of-function mutations in dTDP-43 exhibit an upregulation of a different set of genes [303]. However, when dTDP-43 levels are altered ubiquitously at the late pupal stage, there is a significant overlap between upregulated and downregulated genes [273]. Taken together, the results of these studies provide evidence that TDP-43 plays a large role in gene expression that may change throughout *Drosophila* development. Interestingly, a ubiquitous reduction of dTDP-43 in the late pupal stage causes increased expression of the endogenous microtubule associated protein, Map205. Aberrant expression of this protein, in turn, appears to induce cytoplasmic mislocalization of the ecdysteroid receptor (EcR), which may result in a failure to transition from pupal to adult gene expression patterns and subsequent neuronal death [273]. Overall, these *Drosophila* models have identified candidate genes for future study, and suggest that the

pathogenesis of TDP-43-associated ALS is a very complex process, involving many genes and biological processes.

It is still an enigma if WT or mutant TDP-43 toxicity is due to a gain or a loss of function. Observing WT toxicity can be helpful to identify how perturbations of normal TDP-43 levels affect disease pathogenesis. Unfortunately, it is challenging to assess the effects of ALS-associated TDP-43 mutants when WT protein is itself toxic. WT toxicity in and of itself suggests a gain of function. However, loss of function models have also been developed [303]. As mentioned previously, flies overexpressing WT *Drosophila* TDP-43 produce different expression profiles in the CNS during larval development than flies carrying *dTDP-43*-null mutations [303]. Therefore, it is possible that both a gain and a loss of TDP-43 function are toxic, but for different reasons, and through different mechanisms. Taken together, however, *Drosophila* models have shown that WT and ALS-associated mutant TDP-43 expression level changes, cellular mislocalization, and protein functions such as RNA-binding are all factors that may play a role in ALS pathogenesis.

2.3.4.2 Modifiers of TDP-43-Associated Phenotypes

Drosophila has been a useful model organism for performing genetic screens to identify genes and pathways that modify phenotypes associated with neurodegenerative diseases, including spinocerebellar ataxia [184,304,305], Wolfram syndrome [306], Alzheimer's disease [307,308] and Huntington's disease [309]. Screens can be targeted, testing the effects of an array of known genes on a particular phenotype [304,306], or they can be unbiased, looking for any genes that modify a phenotype. Unbiased screens are

typically performed using either deficiency lines that carry heterozygous deletions of mapped regions of the chromosome [310,311], or lines with random enhancer-promoter (EP)-transposable element insertions [184,307]. *Drosophila* at any stage of development can be used, depending on the phenotype of interest [312,313]. Eye degeneration is a commonly used phenotypic output [308,309], although genetic screens have also been performed using survival [310], wing posture [305], climbing [305] defects and axonal and synaptic degeneration [314]. Here we highlight the genes that have been identified as modifiers of TDP-43-associated neurodegeneration in *Drosophila* models of ALS.

Histone deacetylase 6 (HDAC6) is a human class II histone deacetylase that has previously been linked to neurodegenerative diseases [190,315]. Unlike other histone deacetylases, HDAC6 functions primarily in the cytoplasm where it plays a critical role in the clearance of cytoplasmic, ubiquitinated, misfolded proteins [316-318]. Homozygous knockout of *TBPH* in 1st-instar larval brains, as well as heterozygous knockout in adults, shows a reduction in HDAC6 mRNA levels [319]. These results suggest that altering TDP-43 levels in neurons also affects HDAC6 levels, and may therefore result in toxicity by inhibiting the removal of accumulated cytoplasmic proteins in patients with ALS [319]. Another possible mechanism of toxicity is through aberrant synaptic transmission. Bruchpilot (BRP), a protein that is part of the presynaptic dense body and regulates synaptic transmission by gathering synaptic vesicles together for release, was recently identified as a target of HDAC6 deacetylation in the neurons of adult *Drosophila* [320]. Increasing HDAC6 expression reduces BRP acetylation, thereby increasing its binding to vesicles and creating a larger vesicle pool for release. Consistent with the identification of HDAC6 as a target of TDP-43 [319], this phenotype is recapitulated when

human TDP-43 is overexpressed, with a larger effect observed in flies harboring ALS-associated TDP-43-mutations (A315T and A382T) [320]. Conversely, *TBPH*-null mutant flies show an increase in BRP acetylation and subsequently smaller vesicle pools. Following these observations, it was found that reducing HDAC6 levels in the A315T mutant flies rescues the acetylation levels. In addition, reducing HDAC6 expression also rescues motor dysfunction observed in A315T mutant adult flies [320]. Taken together, these results suggest that TDP-43-associated ALS pathogenesis may be due to aberrant synaptic transmission, resulting from increased HDAC6 expression and subsequently reduced BRP acetylation.

Ubiquilin is a cytosolic protein capable of binding both poly-ubiquitinated chains and 19s proteosome subunits. It is therefore believed to play a role in the degradation of misfolded proteins [321]. A *Drosophila* model was developed in 2010 to show the effect of ubiquilin overexpression on TDP-43 associated neurodegeneration. Consistent with other models, overexpression of hTDP-43 in motor neurons reduces the lifespans of adult flies, reduces pupal eclosion rates and causes wing malformations. Co-expression with ubiquilin enhances these phenotypes. Interestingly, however, human TDP-43 in cells co-expressing ubiquilin is almost exclusively nuclear, and is not found in cytoplasmic aggregates, despite the overall enhanced degeneration in these flies. In addition to linking ubiquilin to TDP-associated pathology in a model of ALS, these results provide more evidence that aggregation of mislocalized hTDP-43 is not necessary for progression of the disease [269].

Valosin-containing protein (VCP) is an ATPase that has been linked to the pathogenesis of ALS [38], as well as other neurodegenerative diseases including inclusion

body myopathy associated with Paget disease of bone and frontotemporal dementia (IBMPFD) [322,323]. The *Drosophila* homolog of VCP is encoded by the gene, *ter94*. One mutation of VCP that causes IBMPFD, R155H, has an equivalent mutation in *Drosophila*, dVCP R152H [278]. A drosophila model for TDP-43-associated ALS has revealed that coexpression of dVCP R152H and WT human TDP-43 in fly eyes enhances the structural degeneration and cytoplasmic mislocalization that is observed when WT hTDP-43 is overexpressed alone. This enhancement is also produced when WT *Drosophila* VCP is expressed with the ALS-associated TDP-43 mutant, hTDP-43 M337V. Expression of dVCP alone causes mild degeneration that is suppressed when *Drosophila* TDP-43 is knocked down, and enhanced when dTDP-43 is overexpressed [278]. Taken together, these results identify VCP is a modifier of TDP-43 pathogenesis, and provide *in vivo* evidence linking a single mutation to the pathogenesis of both ALS and IBMPFD.

CAG repeat expansion within the coding region of ataxin-2, *ATXN2*, causes spinocerebellar ataxia type 2 (SCA2) [324-327]. The *Drosophila* homolog of ataxin-2, dAtx2, enhances eye degeneration and decreases adult lifespan when coexpressed with WT human TDP-43 in eyes and motor neurons, respectively [279]. When endogenous dAtx2 levels are reduced, eye degeneration and lifespan improve in flies expressing hTDP-43. It is important to note that altering dAtx2 in these experiments does not alter hTDP-43 levels. Parallel work in cell culture has shown that ataxin-2 and TDP-43 physically interact in an RNA-dependent manner [279]. Thus, ataxin-2 appears to be a modifier of ALS-related TDP-43 toxicity. As with IBMPFD, these experiments have identified a possible link between ALS and SCA2.

Inositol-1,4,5-trisphosphate receptor type 1 (ITPR1), encoded by the gene *ITPR1*, is an IP3 receptor bound to the endoplasmic reticulum, and is involved in controlling the release of intracellular calcium ions [328]. A recent high content RNAi screen in HeLa cells identified ITPR1 as a modifier of TDP-43 cytoplasmic mislocalization [329]. Specifically, knockdown of ITPR1 increased TDP-43 cytoplasmic mislocalization. These results were confirmed in *Drosophila* using the ortholog of *ITPR1*, *Itp-r83A*. Expression of *Itp-r83A* mutants in motor neurons resulted in a very mild increase in cytoplasmic mislocalization of human TDP-43. These mutants also partially rescued adult climbing and lifespan phenotypes associated with hTDP-43 overexpression toxicity. It was thus proposed that TDP-43 overexpression toxicity is due to high nuclear concentrations. The toxic phenotypes are rescued when nuclear concentrations are reduced through the reduction of calcium signaling and subsequent cytoplasmic localization and degradation of TDP-43 [329].

Cacophony, a voltage-gated calcium channel encoded by the gene, *cac*, was recently identified as a potential modifier of *Drosophila* TDP-43 toxicity [303]. Cacophony calcium channels have been identified at presynaptic active zones of the NMJ [330] of *Drosophila*, and play an important role in regulating function, development and plasticity of neurons through calcium signaling [331]. More recent analyses in a *Drosophila* loss of function model of TDP-43-associated ALS have shown that flies homozygous for *Drosophila TDP-43*-null mutations exhibit lower CAC expression levels than control flies [332]. Both pan-neuronal and motor-neuron- specific overexpression of CAC in these mutant flies rescues the crawling defects that have been previously reported [303,332]. In addition, loss of dTDP-43 reduces CAC levels at the NMJ. Furthermore, in normal flies,

WT dTDP-43 associates with CAC mRNA transcripts, and loss of dTDP-43 function in dTDP-43-null flies results in mild alternative splicing of CAC mRNA [332]. Considering the role of CAC at the NMJ, and previous reports linking abnormalities of the NMJ to ALS [263,291], these results provide evidence that CAC dysfunction may be involved in ALS pathogenesis and is a potential therapeutic target.

Recently it has been shown that expression of hTDP-43 can suppress the neurodegenerative phenotypes of a *Drosophila* model of Fragile X-associated tremor/ataxia syndrome (FXTAS) in an RNA-dependent manner. Interestingly, it was found that ALS-causing mutations of TDP-43 enhanced the phenotypes associated with FXTAS. In addition, phenotypic suppression by TDP-43 appears to be through an interaction with Heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1), suggesting a link between these two RNA-binding proteins and FXTAS [333].

2.3.4.3 Posttranslational Modifications

Hyperphosphorylated TDP-43 in cytoplasmic aggregates was reported in neurons of patients with TDP-43-associated ALS [152]. It has been proposed that aberrant phosphorylation of TDP-43 is associated with progression of the disease [154]. Phosphomimetic TDP-43 shows a diffuse pattern in neurons of *Drosophila*. In contrast, phospho-deficient mutants form aggregates in perikarya and dendrites [334]. These results suggest that hyperphosphorylation prevents aggregation of TDP-43, which does not appear to coincide with what is observed in ALS patients. Recently Doubletime (DBT), the *Drosophila* homolog of casein kinase Iε, was identified as a specific TDP-43 kinase that phosphorylates Ser409/410 of WT human TDP-43 as well as Q331K and

M337V mutants [335]. Coexpression of DBT with TDP-43 Q331K in adult fly eyes produces severe retinal degeneration that does not exist when DBT is coexpressed with either WT TDP-43 or the M337V mutant. In addition, only the Q331K mutants form aggregates in neurons when phosphorylated by DBT [335]. It is unclear as to why toxicity and aggregate formation, following phosphorylation by DBT, is specific to TDP-43 Q331K mutant proteins in these experiments. However, in contrast to the earlier phosphorylation study [334], the effects that DBT phosphorylation has on the Q331K mutant more accurately reflect the aggregation and toxicity observed in ALS patients [336,337].

2.3.5 Fused in sarcoma/translocated in liposarcoma (FUS/TLS)

FUS/TLS, often referred to as FUS, is a DNA/RNA binding protein [338,339]. FUS regulates gene transcription [340-342] and, similarly to TDP-43, plays a role in regulating different aspects of RNA metabolism, including nucleocytoplasmic RNA transport [339] and alternative splicing [343-345]. As its name suggests, FUS was originally linked to cancer [346,347]. In more recent years, however, mutations of FUS have been identified in patients with neurodegenerative diseases including FALS [155,156], SALS [32-34] and FTLD [348]. The first *Drosophila* models for FUS-associated ALS were developed in 2011 [349-351] (Table 2.1). Similarly to TDP-43 and SOD1, these models, and those that followed (Table 2.1), have shown that expression of WT or mutant human FUS (hFUS) in various *Drosophila* tissue-types and stages of development recapitulates key pathological features of ALS.

2.3.5.1 Overexpression and Knockdown

Given their functional similarities and mutual link to ALS, it is logical to suspect that FUS and TDP-43 have overlapping mechanisms underlying their toxicity in neurons. FUS overexpression and knockdown in various tissue types has shown similar effects to TDP-43 experiments, and has provided some interesting new insights that may be applicable to TDP-43 pathogenesis as well. Similarly to TDP-43, expression of WT human FUS and ALS-associated mutants (R518K, R521C, R521H, R524S and P525L) in *Drosophila* eyes leads to degeneration that increases with age and expression level [349,350,352]. We and others have also shown evidence that the degeneration is greater in hFUS mutant-expressing flies than in WT-expressing flies [349,350]. Removal of the nuclear export signal (NES) in mutant constructs, thereby containing their localization to nuclei, rescues eye degeneration phenotypes [350]. These results suggest that FUS mutations may confer either a toxic gain of cytoplasmic function, or a loss of nuclear function to the protein. In contrast, when targeted to mushroom bodies, both WT and mutant hFUS (R524S and P525L) cause decreased lobe sizes [349]. Pan-neuronal expression of WT hFUS and R518K, R521C and R521H mutants in adult flies leads to reduced locomotion compared to controls, with mutant locomotion lower than WT flies. In addition, WT and R521H-expressing flies have a shorter lifespan than controls, with mutant flies having shorter lifespans than WT flies [350].

As ALS is primarily a motor neuron disease, researchers in the last few years have focused heavily on studying this cell type in *Drosophila*. It was recently shown that

overexpression of hFUS WT and mutant protein causes presynaptic structural changes at the NMJ of *Drosophila* larvae [353]. Consistent with previous reports [350], bouton number is unaffected. However, contrary to mutant hTDP-43 expression in motor neurons [291], hFUS R521C-expressing larvae do have a reduced number of presynaptic active zones, as well as dysmorphology of the structures that form the active zones [353]. These structural abnormalities are accompanied by reduced synaptic transmission in the NMJ's, a phenotype that is also observed in *Drosophila* FUS-null mutants. Despite these abnormalities, excitability of the neurons and propagation of the action potentials are unaffected in these mutant flies. Therefore, presynaptic abnormalities at the NMJ of motor neurons may in fact be involved in the initial stages of ALS pathogenesis [353].

When expressed in larval motor neurons, WT hFUS cellular distribution is primarily nuclear, whereas ALS-associated mutant hFUS mislocalizes to the cytoplasm [349,350]. These results are consistent with the disease state in ALS-patients [155,156]. There is also a reduction of larval mobility, and an enlargement of the neurons themselves in both WT and mutant hFUS-expressing larvae [349,350,352]. Interestingly, however, it appears that not all ALS-associated hFUS mutants produce the same morphological changes at the NMJ. R521G, R524S and P525L mutants cause a reduction in bouton number, but R518K, R521C and R521H mutants do not [349,350,352].

Over time, there is an accumulation of insoluble, non-aggregated WT hFUS when expressed in adult neurons that coincides with neurodegenerative phenotypes [354]. Reducing insoluble hFUS levels with coexpression of the chaperone protein, HSPA1L, reduces eye degeneration and improves lifespan. WT hFUS is primarily localized in the nucleus of these cells. These results suggest that the toxicity observed from WT hFUS

overexpression is the result of nuclear, not cytoplasmic function [354], consistent with the recently proposed cause of TDP-43-associated toxicity [329].

The *Drosophila* homolog of FUS is cabeza, encoded by the gene *caz*. The two proteins share approximately 53% identity [355]. dFUS-null mutant flies show reduced pupae eclosion, adult lifespan, and adult locomotion compared to controls [351]. Interestingly, expressing WT dFUS, WT human FUS, or ALS-associated human FUS mutants (R522G or P525L) rescues the eclosion phenotype. Adult lifespan and locomotion is rescued by WT dFUS and hFUS, but not by the human FUS mutants [351]. Pan-neuronal expression of dFUS RNAi does not affect adult lifespan, but does inhibit climbing. RNAi knockdown in motor neurons also reduces bouton number and length of synaptic branches at the NMJ [356]. Additionally, knockdown in fly eyes causes external degeneration [357,358]. As with TDP-43, it is still unknown whether FUS toxicity is due to a gain of function or a loss of function. As mentioned previously, some early experiments with human FUS mutants suggest a possible toxic gain of function [349,350]. However, these studies using dFUS show that a loss of function recapitulates ALS-associated phenotypes similarly to overexpression models. Interestingly, both WT dFUS overexpression and null-mutant loss of function are capable of causing similar phenotypes in *Drosophila*, with the exception of motor neuron apoptosis [352]. Overexpression of WT dFUS causes apoptosis of motor neurons, but dFUS-null mutants do not. The conclusion of these results is that although perturbations of dFUS homeostasis are toxic to flies, the mechanisms of toxicity may not always be the same [352]. Taken together, it is possible that, similarly to TDP-43, FUS toxicity is due to a combination of both a loss of nuclear function and gain of cytoplasmic function.

We have recently shown evidence that the RNA-binding ability of FUS is necessary for ALS-associated toxicity in both *Drosophila* and yeast model systems [359]. We expressed WT hFUS and hFUS mutant proteins lacking RNA-binding capabilities in *Drosophila* and found that ALS-associated phenotypes were reduced compared to normal WT and mutant hFUS-expressing flies. Removing RNA-binding function resulted in reduced eye degeneration in adults, improved larval eclosion and mobility, reduced brain atrophy in larvae, reduced cytoplasmic mislocalization, and reduced incorporation into cytoplasmic stress granules following treatment with sodium arsenite [359]. These results are consistent with the previously discussed results showing that RNA-binding may also be necessary for TDP-43-associated toxicity in ALS pathogenesis [270,293].

2.3.5.2 Modifiers of FUS-Associated Phenotypes

In addition to similar pathological phenotypes in ALS, there is evidence that FUS and TDP-43 have similar and potentially overlapping mechanisms of toxicity [360,361]. Our laboratory has previously shown that WT and mutant hTDP-43 modifies the hFUS-associated ALS phenotypes observed in *Drosophila* models. Both WT and ALS-associated mutant hTDP-43 (M337V) enhance WT and hFUS R521H mutant degeneration in fly eyes [350]. Others have shown that *Drosophila* TDP-43 modifies the dFUS phenotype, and the results suggest that *TBPH* and *caz* function in a similar pathway [351].

In addition to modifying TDP-43-associated ALS phenotypes in *Drosophila*, VCP has recently been shown to modify FUS-associated ALS phenotypes as well [357]. RNAi

mediated knockdown of dFUS in adult fly eyes causes external degeneration that can be partially rescued by coexpression with WT dVCP. Conversely, overexpressing dFUS in *dVCP*-null mutant flies enhances the degeneration. dVCP also affects dFUS levels in neurons. Reduction of nuclear levels of dFUS via pan-neuronal, RNAi-mediated knockdown in larvae is enhanced in *dVCP*-null mutant flies compared to knockdown in normal flies. Coexpression with WT dVCP, however, increases nuclear dFUS levels compared to dFUS knockdown alone. Adult flies expressing dFUS RNAi in neurons exhibit reduced climbing capabilities that gets worse with age. This climbing dysfunction is enhanced in *dVCP*-null mutants, but is mildly rescued by overexpressing WT dVCP. Consistent with these results, dVCP also modifies dFUS toxicity at NMJ's [357]. Larvae expressing dFUS RNAi in neurons show a reduction in presynaptic terminal branching and number of boutons. Expressing dFUS RNAi in a *dVCP*-null mutant fly enhances these reductions. Both phenotypes are rescued by coexpression with WT dVCP, with bouton numbers even surpassing control larvae [357]. Not only do these results identify dVCP as a modifier of ALS-associated dFUS phenotypes, they provide further evidence, in addition to TDP-43 studies [278], that VCP may be involved in ALS pathogenesis and may be a viable target for therapy.

Recent evidence suggests that dFUS is involved in the epidermal growth factor receptor (EGFR) signaling pathway through the EGFR-associated genes *rhomboid-1*, *rhomboid-3* and *mirror* [358]. Reduced expression of any one of these genes in adult eyes is sufficient to suppress the eye degeneration observed in dFUS knockdown flies. These results are consistent with previous reports linking perturbations of the mitogen-activated protein kinase (MAPK) signaling pathway to ALS [358,362-368].

2.3.5.3 Posttranslational Modifications

Protein arginine methyltransferases (PRMT's) catalyze the methylation of arginine residues of proteins. This is important for regulation and maintenance of protein function, reviewed in [369,370]. PRMT1 methylates FUS, regulating its function and cellular distribution [371-374]. Using a *Drosophila* model previously developed in our laboratory [350] in combination with mammalian cell cultures, it has been recently shown that PRMT1 interacts with WT hFUS, as well as the ALS-associated hFUS mutant, R521H [375]. Targeted coexpression of PRMT1 RNAi with either WT hFUS or hFUS R521H in *Drosophila* eyes enhances the degeneration that occurs with WT or mutant hFUS alone. The results suggest that reduced arginine methylation of FUS by PRMT1 may be associated with ALS pathogenesis [375].

2.3.6 TAF15 and EWSR1

The structural and functional similarities between TDP-43 and FUS, including their shared RNA-binding abilities, have led to the hypothesis that RNA metabolism plays a critical role in the pathogenesis of ALS [376]. For this reason, a yeast functional screen was recently performed on 133 human RNA-binding proteins, in an effort to identify ALS-causing candidates that exhibit cytoplasmic aggregation and toxicity similarly to TDP-43 and FUS [377]. Of the proteins examined, TAF15 (RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa) and EWSR1 (EWS RNA-binding protein 1) not only exhibited aggregation and toxicity, but are also in the same

protein family as FUS [377-379]. Therefore, TAF15 and EWSR1 were selected as candidates for further study.

TAF15 and EWSR1 are RNA-binding proteins that function in transcriptional regulation [379-381] and alternative splicing [382,383]. Traditionally, *TAF15* and *EWSR1* have been associated with cancer through the formation of fusion genes that result in aberrant transcription [384]. Both genes are linked to extraskeletal mixoid chondrosarcoma [385,386] and acute leukemia [387]. Following their identification in the recent yeast functional screen, *TAF15* and *EWSR1* were found to harbor mutations in patients with SALS [377,378]. *Drosophila* models using human WT and mutant forms of each protein were subsequently developed (Table 2.1) [377,378]. In the TAF15 model, overexpression of WT protein caused eye degeneration and an age-dependent decrease in climbing ability, when expression was targeted to the eyes and motor neurons, respectively, compared to normal control flies. Overexpressing WT TAF15, as well as two different ALS-linked mutants, R408C and G391E, in motor neurons reduces lifespan compared to normal controls, with the mutants exhibiting shorter lifespans than the WT-expressing flies [377].

In the EWSR1 model pan-neuronal expression of WT protein reduces lifespan of the flies, and causes an age-dependent reduction of climbing ability compared to normal controls [378]. Directed expression of human WT EWSR1 to the adult eyes caused dose-dependent structural degeneration. Interestingly, two different ALS-linked mutants, G511A and P552L, produced relatively equivalent levels of degeneration. This group suggests that observing similar effects of both mutant and WT protein expression in the *Drosophila* eyes may represent a limitation of the model, or may be evidence that

mutant EWSR1 is not the cause of pathogenicity in ALS patients [378]. Further experimentation with this model will be necessary to determine the reason for this observation. Taken together, these models have provided *in vivo* data confirming the function of both TAF15 and EWSR1, and further implicate RNA metabolism in disease pathogenesis.

2.3.7 C9ORF72

One of the more recently discovered genes to be linked to ALS is *C9orf72*. It was first identified in ALS patients in 2011, and its toxicity is due to a GGGGCC hexanucleotide repeat expansion within the first intron of the gene [39-41]. A recent study has shown this hexanucleotide repeat expansion of *C9orf72* present in ALS patients in populations all over the world. The combined data showed an overall frequency of approximately 6.3% of SALS cases and 37.6% of FALS cases, making it the most common known genetic cause of SALS worldwide [162]. The first *Drosophila* model for *C9orf72*-associated ALS was developed in 2013 (Table 2.1) [388]. This model shows that expression of 30 riboGGGGCC-repeat constructs recapitulates ALS-associated phenotypes when compared to control flies expressing 3 riboGGGGCC repeats. Expression of 30-repeat constructs in *Drosophila* eyes causes severe degeneration that increases with age and is not seen in control flies. When targeted to motor neurons, the GGGGCC expansion also reduces adult locomotion over time compared to controls that show no effect at all. The RNA-binding protein, Pur α, was concurrently found to bind to the expanded riboGGGGCC repeats in mammalian cell culture experiments performed by this group [388]. Co-expression of Pur α with expanded riboGGGGCC in *Drosophila*

eyes suppressed the degeneration phenotype. In addition, Pur α colocalizes with ubiquitin in cellular inclusions in the flies expressing the expanded riboGGGGCC repeats, but not in controls. Taken together, these results not only add further *in vivo* evidence to support the role of GGGGCC repeats in the pathogenesis of *C9orf72*-associated ALS, but they also provide evidence to support the hypothesis that sequestration of RNA-binding proteins is a mechanism for disease development and progression [388].

There is a growing body of evidence that GGGGCC repeat toxicity may also be due to the production of dipeptide-repeat (DPR) proteins through repeat-associated non-ATG (RAN) translation [122,123,389-394]. Through this process, both the sense and antisence GGGGCC repeat sequences can be translated into five different DPR proteins (proline:arginine, glycine:arginine, proline:alanine, glycine:alanine and glycine:proline/ proline:glycine) that vary based on the reading frame of the transcripts [390]. A *Drosophila* model was recently developed in which adult flies expressing proline:arginine and glycine:arginine DPR proteins in the eyes exhibited eye degeneration that was not present in proline:alanine or glycine:alanine-expressing flies (Table 2.1) [391]. Survival from egg to adult stages was also decreased in the flies expressing the arginine-rich DPR proteins. Adult lifespans are reduced when the arginine-rich DPR proteins are expressed pan-neuronally, compared to the proline:alanine and glycine:alanine-expressing flies [391]. Consistent with the previous *Drosophila* model [388], toxicity is also observed in flies expressing expanded GGGGCC-repeat RNA, compared to controls [391]. These results suggest that both DPR proteins and expanded GGGGCC-repeat RNA are sufficient for cellular toxicity.

2.3.8 Heterogeneous nuclear ribonucleoprotein A1 and A2B1 (hnRNPA1 and hnRNPA2B1)

hnRNPA1 and *hnRNPA2B1* were first linked to ALS in 2013 [115]. hnRNP's are nuclear, RNA-binding proteins involved in mRNA processing [395]. hnRNPA1 and hnRNPA2B1 have been shown previously to bind directly to TDP-43 for mRNA splicing [396]. Mutations within the prion-like domains of *hnRNPA1* and *hnRNPA2B1* were found in familial cases of multisystem proteinopathy and ALS [115]. A newly developed model (Table 2.1) has shown that one of the identified hnRNPA2 mutants, D290V, causes severe degeneration when expressed in indirect flight muscle cells of *Drosophila* [115]. This degeneration is rescued by removing a portion of the prion-like domain. In addition, D290V mutants form cytoplasmic aggregates, whereas WT hnRNPA2, as well as mutant proteins lacking prion-like domain function, are localized to nuclei. These results, in combination with yeast and cell culture data, add *hnRNPA1* and *hnRNPA2B1* to the list of ALS-causing genes, and suggest that toxicity is specifically due to the activity of the prion-like domains of the proteins [115]. These results also imply that other proteins with prion-like domains may be involved in ALS pathogenesis.

2.4 TRANSLATION FROM FLIES TO MAMMALIAN MODELS

Drosophila melanogaster is a very useful organism for modeling human disease. As an animal model, *Drosophila* has an advantage over cell culture models of disease, in that the data obtained is more relevant to a living organism. *Drosophila* are less

expensive to maintain than other animal models such as mice, rats and non-human primates. They also have shorter lifespans than these larger organisms, allowing for faster matings and production of experimental progeny, as well as shorter run-times on lifespan experiments. For these reasons, fruit flies are a fast, inexpensive tool for the initial studies of a disease. However, on a physiological level, *Drosophila* models may not be the best model of choice per se, as the mammalian nervous system is much more complicated than the fly nervous system. Therefore, it is critical that the studies performed in flies are translated and verified in mammals.

Most of the mammalian models of *SOD1*-associated ALS are mice and rats, although dog models have also been developed [397]. ALS-linked *SOD1* mutations have been more thoroughly modeled in rodents than in *Drosophila*. More than 10 of these mutant genes are expressed in mouse models compared to two in *Drosophila*, reviewed in [397]. In both flies and mice, expressing human SOD1 A4V and G85R mutant proteins results in the development of neurodegenerative phenotypes that are reminiscent of those seen in ALS patients, including the formation of SOD1-positive inclusions [216,218,398]. Interestingly, however, SOD1 A4V protein-positive inclusions are only formed in mice when WT human SOD1 is also expressed, an observation not seen in flies [216,398]. SOD1 G85R-expressing mice also show an acceleration of SOD1-positive inclusion formation when WT human SOD1 is concurrently expressed[399]. Although both *Drosophila* and mammalian models will continue to be useful in studying SOD1-associated ALS, the more extensive set of mouse models available will likely make them the most useful to researchers in the near future.

Several mammalian model systems have been generated for TDP-43-associated ALS since TDP-43 has been linked to neurodegenerative diseases. Interestingly, these systems reflect the same gain of function/ loss of function dilemma that exists in *Drosophila* models [293]. Overexpression of WT and ALS-associated mutant human TDP-43 causes loss of neurons in mice [400-402], suggesting a gain of protein function as the cause of the disease. However, loss of endogenous TDP-43 expression in mouse spinal motor neurons causes accumulation of ubiquitinated, cytoplasmic proteins in these cells, as well as cell death, suggesting a loss of protein function as the cause of disease pathogenesis [403]. Furthermore, ALS-like neurodegenerative phenotypes are observed in rats overexpressing ALS-linked mutant human TDP-43, but not WT protein [404]. Although seemingly contradictory, these mammalian models, as with the *Drosophila* models of TDP-43 associated ALS, provide evidence that both a loss and gain of protein function are toxic, although possibly through different mechanisms.

The observation that WT and ALS-linked mutant human FUS are toxic in *Drosophila* [349,350,359] has been confirmed in rats [405,406] and in mice [407]. The toxicity also appears to be greater in the mutant FUS-expressing rats than in the WT-expressing animals [405]. These results are also consistent with work done in ALS-patient skin fibroblasts harboring mutations in the 3' untranslated region of *FUS*, in which these mutations caused overexpression of WT hFUS and are likely the cause of ALS in these patients [408].

GGGGCC hexanucleotide repeat expansion of *C9ORF72* was found to be toxic in *Drosophila* and mammalian cell models, simultaneously [388]. Expression of C9ORF72 with as few as 30 repeats was sufficient to cause ALS-associated phenotypes in the

flies, and cell death of neuro-2a cells, a mouse-derived neuronal cell line [388], and suggested that whatever we learned from a fly model of C9ORF72 could be translated to a mammalian system.

2.5 SUMMARY AND FUTURE DIRECTIONS

Drosophila melanogaster has been established as a useful model organism for studying ALS through its ability to recapitulate phenotypic features of the disease. This, in addition to a fully sequenced genome, has made *Drosophila* models well-suited for unbiased genetic screens that identify modifiers of ALS-associated phenotypes. These screens helped in discovering candidate genes that may be involved in mediating the disease process as well as in modifying neurodegenerative phenotypes. The ability to manipulate the expression of genes with and without ALS-causing mutations in specific tissue types, and at different stages of development, has also been useful for understanding the roles of those genes and tissues in disease progression. In spite of its usefulness in ALS research, however, *Drosophila melanogaster* is a non-mammalian organism and therefore has its limitations as a physiological model system of human disease. It is critical that the knowledge gained from *Drosophila* continues to be translated into mammalian models, followed by validation in human patient samples. The overexpression/knockdown paradigm is overused in *Drosophila*, and it is possible that expressing high levels of human protein in flies may not be completely relevant to the disease process in humans. Translating the knowledge gained from flies into mammalian and human model

systems will turn these insights into a usable understanding of ALS that will lead to effective treatments, and eventually cures.

2.6 ACKNOWLEDGEMENTS

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3.0 MUSCLEBLIND IS A NOVEL MODIFIER OF FUS-ASSOCIATED AMYOTROPHIC LATERAL SCLEROSIS THROUGH MODULATING FUS INTERACTIONS WITH CYTOPLASMIC STRESS GRANULES

3.1 INTRODUCTION

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a devastating, late onset, neurodegenerative disorder that causes the selective loss of upper and lower motor neurons. Loss of these neurons results in progressive paralysis in ALS patients that eventually leads to death, usually by way of respiratory failure[27,45]. The average age of onset of the disease is typically between 50 and 80 years[22,127,128,409,410], and is usually fatal within 2-5 years following diagnosis[21,23-25]. Approximately 5-10% of ALS occurrences are inherited and are termed familial ALS (FALS). In these individuals, the mutations are most often inherited in an autosomal dominant manner[80,411]. The other 90-95% of occurrences do not show a link to genetic inheritance and are referred to as sporadic ALS (SALS). Clinically, however, FALS and SALS patients are indistinguishable[44]. Mutations in the gene, *FUS* (fused in sarcoma), comprise roughly 4% of FALS and 1% of SALS cases[3]. FUS is a nuclear, DNA/RNA-binding protein that functions in several stages of RNA processing, including gene transcription[412], alternative splicing[413,414] and RNA trafficking[3,36,100,101,415-417].

Pathogenic mutations of FUS protein were first identified in ALS patients in 2009 that lead to mislocalization from the nucleus to the cytoplasm, and accumulation into cytoplasmic aggregates[35,83]. These changes to the normal protein dynamics of FUS had been previously identified in ALS patients with mutations in another RNA-binding protein, TDP43[36,37]. In fact, ubiquitin-positive, tau-negative, cytoplasmic inclusions had long been recognized as a pathological hallmark of ALS[418]. To date, several ALS-linked proteins have been identified as components of cytoplasmic inclusions, that are now commonly regarded as stress granules (SGs)[55,114,116,117,417,419-424]. As for FUS-associated ALS, several disease-causing mutations exist within the protein's nuclear localization signal (NLS) potentially hindering its transport into the nucleus, thereby aggregating in the cytoplasm[419,425,426]. Coupled with the presence of an N-terminal prion-like domain, the accumulation of cytoplasmic FUS may provide an opportunity for natural, albeit uncontrolled and toxic, aggregation of the protein into SGs[55,116,117,426,427]. Stress granules are ribonucleoprotein complexes that form membrane-less cytoplasmic structures in response to cellular stress. SGs sequester RNA and RNA-binding proteins to maintain tight control over mRNA processing so that the cell can deal with the stress appropriately[55,102,428]. Under normal circumstances, SGs are dynamic structures that assemble and disassemble in response to the levels of cellular stress. However, research has shown that in the case of ALS, SGs formed by mislocalized proteins, such as FUS, lack the ability to completely disassemble when the stress is gone. The effect is a dysregulation of mRNA processing that may be directly related to cellular toxicity in ALS[55,114].

One interesting, but poorly understood, aspect of ALS is that the age of onset and disease progression can vary significantly between individuals who share a single point mutation in an ALS-causing gene[96]. Not only is this true of SALS patients, but even in cases of FALS, where the same point mutation is present in all affected family members[51]. This suggests that other unknown factors, whether intrinsic or extrinsic, must be contributing to disease pathogenesis. To address this, we sought to identify genetic modifiers of ALS-associated FUS toxicity. We performed an unbiased, whole genome, genetic screen to identify enhancers and suppressors of ALS phenotypes. Here we show that muscleblind, encoded by the *mbl* gene, is a novel modifier of FUS-associated ALS in fruit flies. Specifically, heterozygous loss of muscleblind suppressed eye degeneration caused by overexpression of mutant FUS. RNAi-mediated suppression of endogenous mbl in *Drosophila* neurons suppresses neuromuscular junction (NMJ) defects and increases the lifespans of FUS-expressing flies. We found in mammalian cells that depleting endogenous, human muscleblind-like (MBNL1), the human ortholog of *Drosophila* muscleblind, significantly reduces the cytoplasmic mislocalization of mutant FUS and its subsequent incorporation into stress granules. Interestingly, knocking down mbnl1 suppresses FUS-associated cellular toxicity and defects in dendrite morphology. Like FUS, muscleblind is also involved in RNA processing, including alternative splicing[429], mRNA stability[430] and RNA trafficking[431]. It has already been linked to several diseases including myotonic dystrophy[432], spinocerebellar ataxia[433,434], fragile X syndrome[435] and Huntington's disease[436,437]. However, it has never been linked to ALS. Here we show, for the first time, a mechanistic and functional link between muscleblind activity and FUS-associated

toxicity in ALS *in vivo*. These results point to changes in mRNA processing, through altered levels of functional MBNL1, as an overlapping pathogenesis between ALS and other neurodegenerative diseases. This data specifically provides further insight into the mechanisms behind FUS toxicity and identifies a novel modifier of FUS-ALS that may help in identifying therapeutic targets for ALS.

3.2 RESULTS

3.2.1 Unbiased Genetic Screen identifies muscleblind as a novel modifier of FUS toxicity

We performed an unbiased genetic screen that allowed us to identify novel modifiers (enhancers and suppressors) of FUS toxicity in *Drosophila* eyes **(Fig. 3.1a and Table 3.1)**. Since the *Drosophila* genome is fully sequenced, the modifying deficiency lines provided a set of candidate genes within the deleted regions that may be specifically responsible for modifying mutant FUS toxicity. We identified two, overlapping deficiency lines, Df(2R)Exel6066 and Df(2R)BSC154, that strongly suppressed FUS-mediated degeneration **(Fig. 3.2a)**. We validated our findings by crossing both deficiency lines with flies expressing either WT FUS or one of two ALS-causing mutations. We found that both deficiency lines significantly suppressed FUS-associated degeneration of Drosophila eyes (Fig. 3.2b and 3.2c). The deleted region of the Df(2R)Exel6066 deficiency line contains 44 known and predicted genes **(Table 3.2)**. We obtained all available RNAi lines to map which genes within this deficient region are

responsible for modifying FUS toxicity. We found that specifically knocking *Drosophila* muscleblind, *mbl*, using two independent RNAi lines, was sufficient to suppress FUS toxicity **(Fig. 3.1b and Fig. 3.3).** Importantly, *mbl* also lies within the overlapping Df(2R)BSC154 deficiency region. However, knocking down other genes within the Df(2R)Exel6066 deficiency region, including *CG12699*, a gene that overlaps m*bl*, does not suppress FUS toxicity, suggesting that *mbl* is the gene responsible for modifying toxicity **(Fig. 3.4a – 3.4c)**.

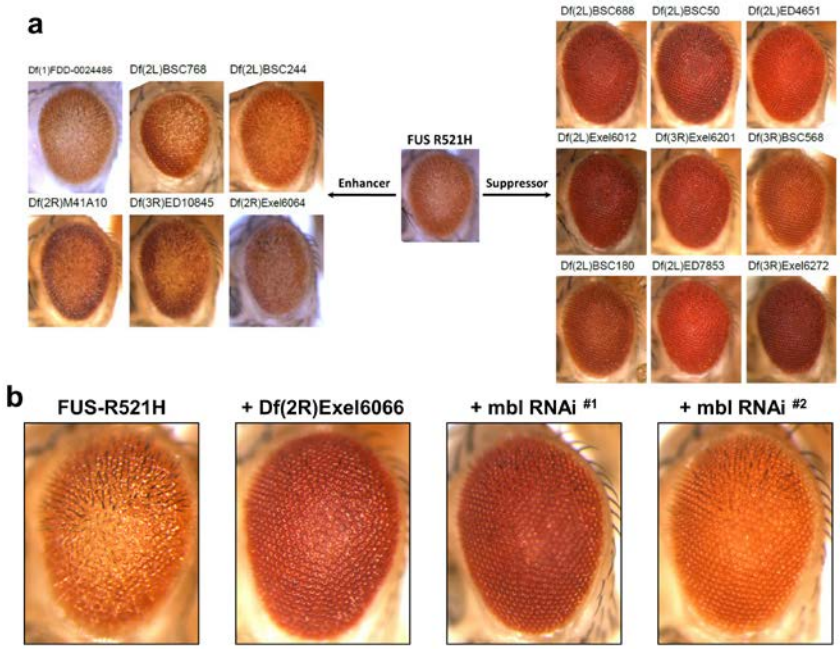


Figure 3.1. Genetic screen performed on a Drosophila model of FUS associated ALS reveals modifiers of FUS toxicity.

(a) Flies from the DrosoDel deficiency kit from the Bloomington *Drosophila* stock center were crossed with flies expressing human, mutant FUS-R521H. Progeny were assessed for modification of the degeneration that is caused by mutant FUS expression in the eyes. Deficiency lines were labeled as either “enhancers” or “suppressors” and flagged for further study. The labels shown are those assigned to the Drosophila lines that are provided in the kit. **(b)** Targeted knockdown of endogenous mbl recapitulates the suppression of FUS toxicity that is caused by the Df(2R)Exel6066deficiency line. Two independent *Drosophila* RNAi lines obtained from the Vienna *Drosophila* Resource Center (#1-105486KK and #2- 28732GD) were crossed with flies expressing human, mutant FUS-R521H in the eyes. Both RNAi lines confirmed that specifically reducing mbl levels in these flies recapitulates suppression of the external eye degeneration that is observed in those flies that are heterozygous for the Df(2R)Exel6066 region. 50-100 flies were analyzed from each group.

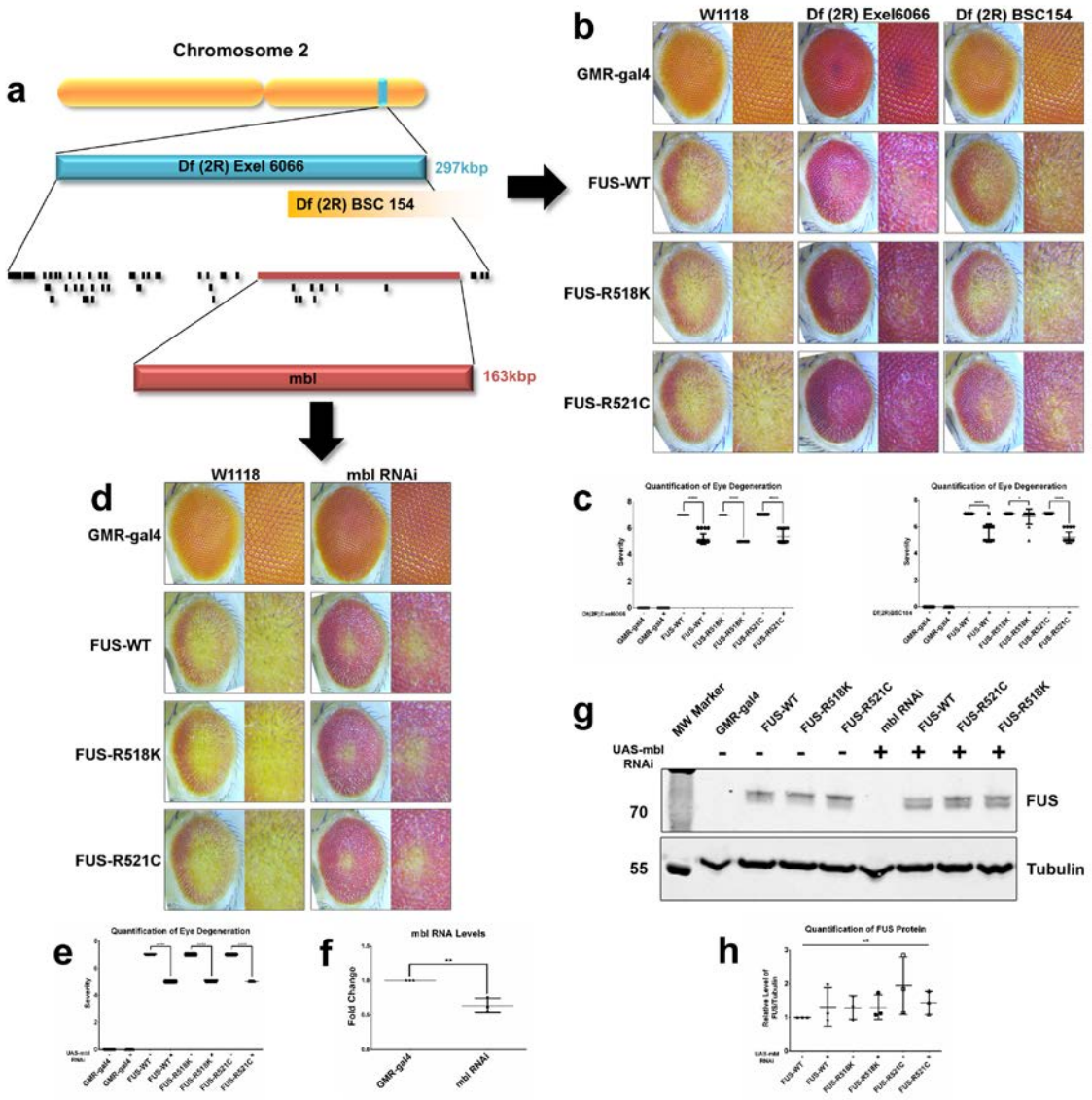


Figure 3.2. Identification of muscleblind as a novel modifier of FUS-associated toxicity in a Drosophila model of ALS.

An unbiased genetic screen performed in our *Drosophila* model of ALS revealed candidate modifiers of toxicity caused by expressing WT and ALS mutant FUS in fly eyes. Further validation confirmed that muscleblind is a novel suppressor of FUS-associated ALS. **(a)** Schematic of the Df(2R)Exel6066 line that suppressed FUS toxicity in the screen, including the relative locations of the genes within the deficient region. This is overlapped by the Df(2R)BSC154 deficiency region, which begins in the middle of the *mbl* gene. **(b)** Representative panel of adult *Drosophila* eyes showing the degeneration caused by tissue-specific expression of FUS-WT and ALS-linked mutant FUS with and without concurrent heterozygous depletion of the genes within the indicated deficiency lines. The UAS-gal4 system with a GMR-gal4 tissue-specific driver was used to drive *FUS* expression in the eyes. 13 – 93 flies were analyzed from each group to provide sufficient sample sizes for measuring degeneration. **(c)** Quantification of the severity of eye degeneration in the flies in (b) shows a significant suppression of FUS-associated toxicity when these flies are crossed with the deficiency lines (****P<0.0001, *P = .0160). **(d)** Representative eye panel of flies expressing the indicated FUS proteins with and without RNAi-mediated knockdown of *Drosophila* muscleblind (*mbl*), the largest gene within the deficient region represented in (a). 17 – 117

flies were analyzed from each group. **(e)** Quantification of the severity of degeneration in (d) confirms a significant suppression of FUS toxicity following depletion of mbl, similar to the WT and mutant FUS-expressing flies, with and without mbl knockdown. n=3 biological replicates of 3 fly heads per group. **(h)** Quantification of FUS protein bands in **(g)**, normalized to tubulin levels, shows equivalent FUS expression in all FUS-expressing groups. In addition, knocking down mbl does not have an effect on the amount of FUS protein in these flies (NS = not significant, p = 0.3578). Statistical significance in (c) and (e) was determined using 2-tailed t-tests for each FUS pair (Mann-Whitney test), but are graphed together for clarity of the figure. 1-tailed T-test was used in (f). One-way ANOVA with Tukey's multiple comparisons test was performed in (h). All quantifications presented are the means ± SD.

Human muscleblind-like has already been linked to myotonic dystrophy[432], fragile X syndrome[435], spinocerebellar ataxia[433] and Huntington's disease[436,437]. In addition, mbl RNA contains long intronic regions. It has already been shown that FUS protein preferentially binds to long intronic regions, and that the RNA from the human orthologs of *mbl*, *MBNL1*, *MBNL2* and *MBNL3* are targets of FUS protein binding[414,438]. Therefore, based on these important links to FUS and neurodegeneration, we chose to pursue *mbl* further as a potential modifier of FUS-associated ALS. We began by using FUS lines with known, site-specific integration (SSI) of the transgenes, and thus equivalent transgene expression, to further validate whether reducing mbl levels is sufficient to suppress FUS toxicity. We crossed WT FUS and two different ALS-linked mutant FUS lines (R518K and R521C) with mbl RNAi, which was also generated through site-specific integration methods. RNAi-mediated knockdown of mbl significantly suppressed the ALS-associated external eye degeneration in all FUS-expressing flies, with approximately 40% knockdown of mbl **(Fig. 3.2d – 3.2f)**. This effect is not due to loss of toxic protein, as knocking down mbl does not reduce FUS protein levels in the flies **(Fig. 3.2g and 3.2h)**. Importantly, depleting endogenous mbl alone in these flies is well tolerated. Next, we sought to determine whether pathogenic mutations of FUS influence endogenous muscleblind expression in ALS-patient cells, as well as in *Drosophila*. We found that ALS-causing mutations of FUS in *Drosophila* or in

ALS patient lymphoblastoid cells do not alter muscleblind expression levels **(Fig. 3.5a and 3.5b)**, suggesting that FUS toxicity is not just due to upregulation of muscleblind expression. Additionally, knocking down endogenous levels of cabeza (caz), the *Drosophila* orthologue of human FUS, does not change muscleblind expression in flies **(Fig. 3.5c)**.

To further rule out the possibility that suppression of FUS toxicity is simply due to gal4 dilution from the additional UAS-mbl RNAi transgenic element in these crosses, we crossed FUS flies with a GFP-expressing control line. UAS-GFP did not suppress eye degeneration **(Fig. 3.6)**, further confirming that suppression was specifically due to knockdown of mbl.

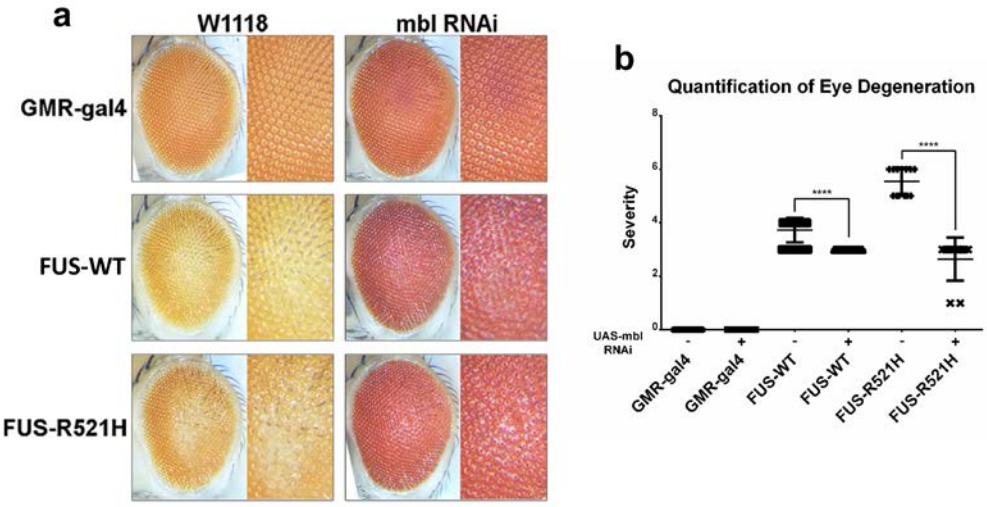


Figure 3.3. Knockdown of endogenous mbl significantly suppresses external eye degeneration in flies expressing either WT or R521H mutant FUS.

Flies expressing either WT or mutant FUS-R521H with the eye-specific GMR-gal4 driver were crossed with mbl RNAi flies, and the progeny were assessed for the severity of external eye degeneration. **(a)** Panel of representative eyes showing suppression of FUS-associated toxicity following targeted knockdown of endogenous mbl. A minimum of 50 flies were assessed from each group. **(b)** Quantification of the severity of degeneration in each group. Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was used to determine significance (**** P<0.0001). The values presented are the means ± SD.

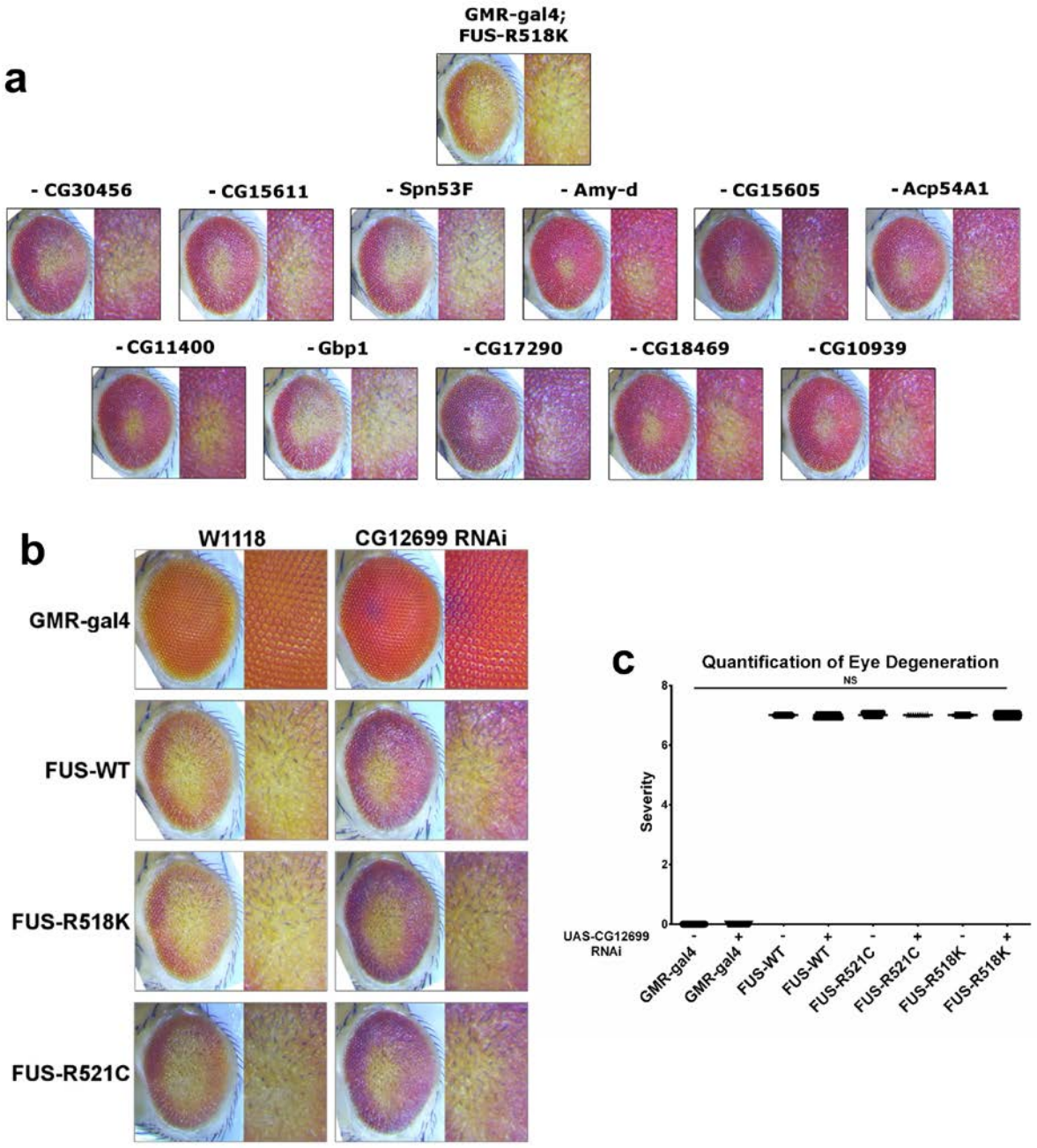


Figure 3.4. Targeted knockdown of other genes within the Df(2R)Exel6066 deficiency region do not show suppression of the external eye degeneration caused by FUS-R518K overexpression.

Flies expressing human FUS in the eyes, under the GMR-gal4 driver, were crossed with RNAi lines targeting other genes that lie within the Df(2R)Exel6066 deficiency region. **(a)** Representative panel of adult *Drosophila* eyes showing similar degeneration caused by expression of FUS-R518K alone (top image) or in combination with RNAi-mediated depletion of the indicated proteins (bottom two rows). A minimum of 20 flies was analyzed from each group. **(b and c)** Targeted knockdown of CG12699, a gene that overlaps mbl in the Df(2R)Exel6066 deficiency region, does not suppress the external eye degeneration caused by overexpression of WT or mutant FUS. Flies expressing either WT or one of two ALS-linked, mutant, FUS proteins (FUS-R518K and FUS-R521C) were crossed with CG12699 RNAi

lines, under the GMR-gal4 driver. **(b)** Representative panel of adult *Drosophila* eyes showing the degeneration caused by tissue-specific expression of FUS-WT and two different forms of mutant FUS, with and without concurrent RNAi-mediated depletion of endogenous CG12699. A minimum of 50 flies were analyzed from each group. **(c)** Quantification of the eye degeneration in (a) confirms that there is no significant difference in the level of FUS-associated external eye degeneration when endogenous levels of CG12699 are reduced in flies. Kruskal-Wallis ANOVA with Dunn's multiple comparison's test was performed (NS = not significant). The values presented are the means ± SD.

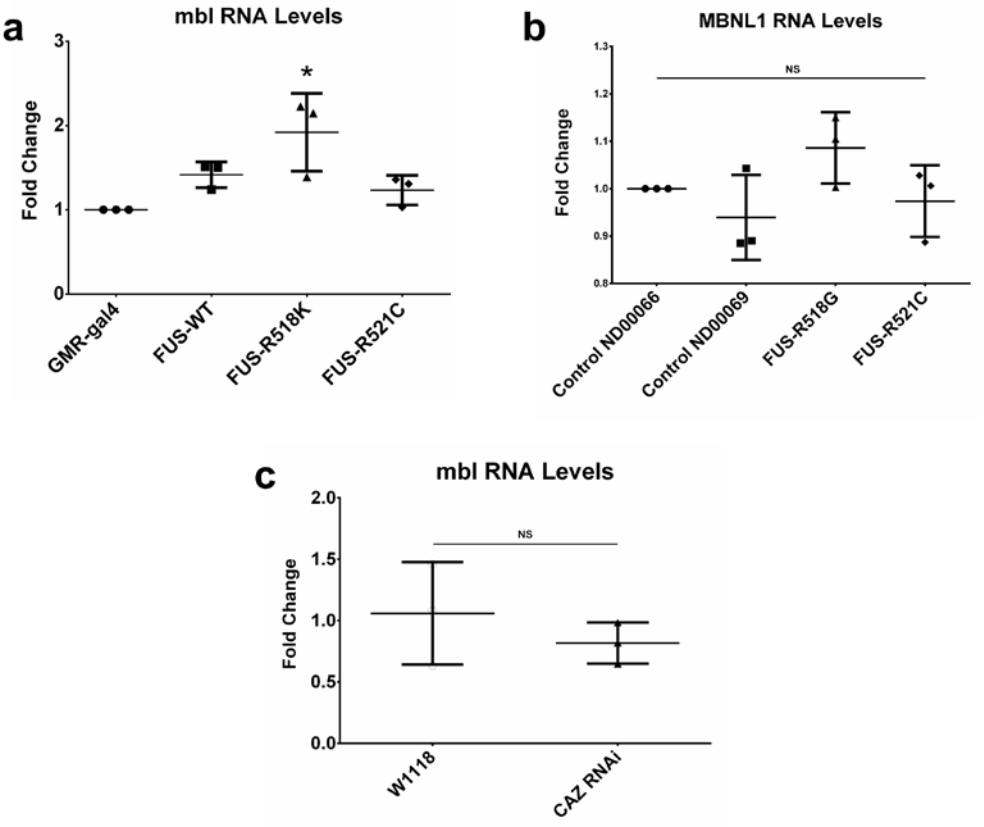


Figure 3.5. Endogenous muscleblind expression levels are unaffected by ALS-causing FUS mutations, or by knockdown of endogenous *caz* in *Drosophila*.

We performed qRT-PCR on *Drosophila* and human samples to determine whether ALS-causing mutations of FUS or knockdown of the endogenous, *Drosophila* ortholog of *FUS*, *caz*, affect muscleblind RNA levels. **(a-c)** Graphs showing muscleblind RNA levels in each group tested. Muscleblind expression remains largely unchanged in all three groups. All qPCRs were performed in triplicate with biological replicates from each group listed in the graphs. The values presented are the means ± SD. **(a)** Endogenous mbl RNA levels in *Drosophila* expressing WT and mutant FUS in eyes, compared to control flies. Only FUS-R518K showed a minor increase in mbl RNA (*P<0.05). **(b)** Endogenous muscleblind-like (MBNL1) RNA levels in two ALS patient lymphoblastoid cell lines with the given FUS mutations show no significant changes compared to two age and sex-matched controls (NS = not significant). **(c)** RNAi-mediated knockdown of endogenous caz has no effect on mbl levels in flies. Statistical analyses in a and b were performed using one-way ANOVA with Tukey's multiple comparisons test. A two-tailed T-test was used in c.

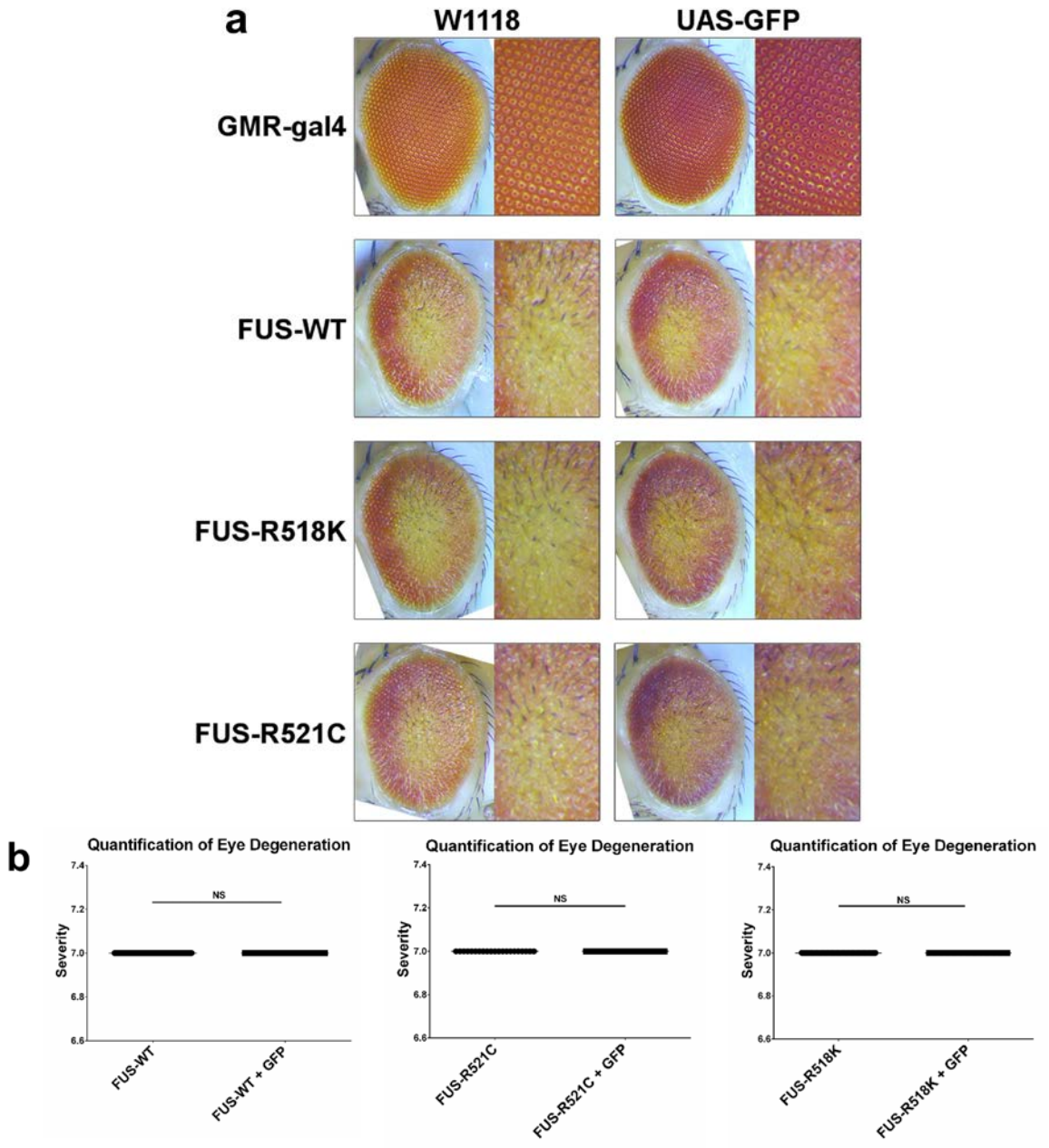


Figure 3.6. FUS-associated toxicity is not suppressed by gal4 dilution.

Flies expressing either WT or one of two mutant FUS proteins (FUS-R518K and FUS-R521C) in the eyes were crossed with flies expressing GFP. **(a)** Representative panel of adult *Drosophila* eyes. The degeneration caused by tissue-specific expression of FUS-WT and two different forms of mutant FUS (first column) is not suppressed by overexpression of GFP (second column) in these flies. **(b)** Quantification of the external eye degeneration from each FUS protein confirms that there is no significant difference in the level of FUS toxicity between these groups. A minimum of 30 flies from each group were analyzed. The values presented are the means ± SD. Mann-Whitney tests were performed for statistical analyses of each comparison (N = NS).

We next asked whether knocking down muscleblind could suppress the degeneration caused by other ALS-linked proteins, or if the effect was specific to FUS-associated ALS. To address this, we crossed the mbl RNAi flies with other lines expressing WT or ALS-linked mutant versions of several proteins (EWSR1, TDP43, dVCP and C9orf72) **(Fig. 3.7)**. Interestingly, we found that knocking down mbl did not suppress the toxicity of any of these proteins except for a mild suppression of EWSR1-WT degeneration. Therefore, the rescue that we observed in our FUS crosses appears to be specific to FUS-associated ALS, but may extend to the FET family of proteins (FUS, EWSR1 and TAF15).

3.2.2 Targeted knockdown of mbl in *Drosophila* neurons suppresses FUS-associated neuronal defects

Since ALS is a disease that primarily affects upper and lower motor neurons, we sought to determine whether knocking down mbl would rescue toxicity in *Drosophila* neurons. We expressed FUS-WT and FUS-R518K mutant in fly motor neurons along with mbl RNAi and analyzed the neuromuscular junctions (NMJ's) of the progeny at the third-instar larval stage, using immunofluorescence and confocal microscopy **(Fig. 3.8a)**. Our results revealed a significant increase in the number of satellite boutons in larvae expressing WT and R518K mutant FUS, compared to controls. Indicating the presence of immature boutons. This morphological defect was significantly rescued when mbl was knocked down in these flies **(Fig. 3.8b)**. However, no changes in the number of mature boutons **(Fig. 3.8c)** were observed. Interestingly, knocking down muscleblind has no effect on *Drosophila* NMJ morphology.

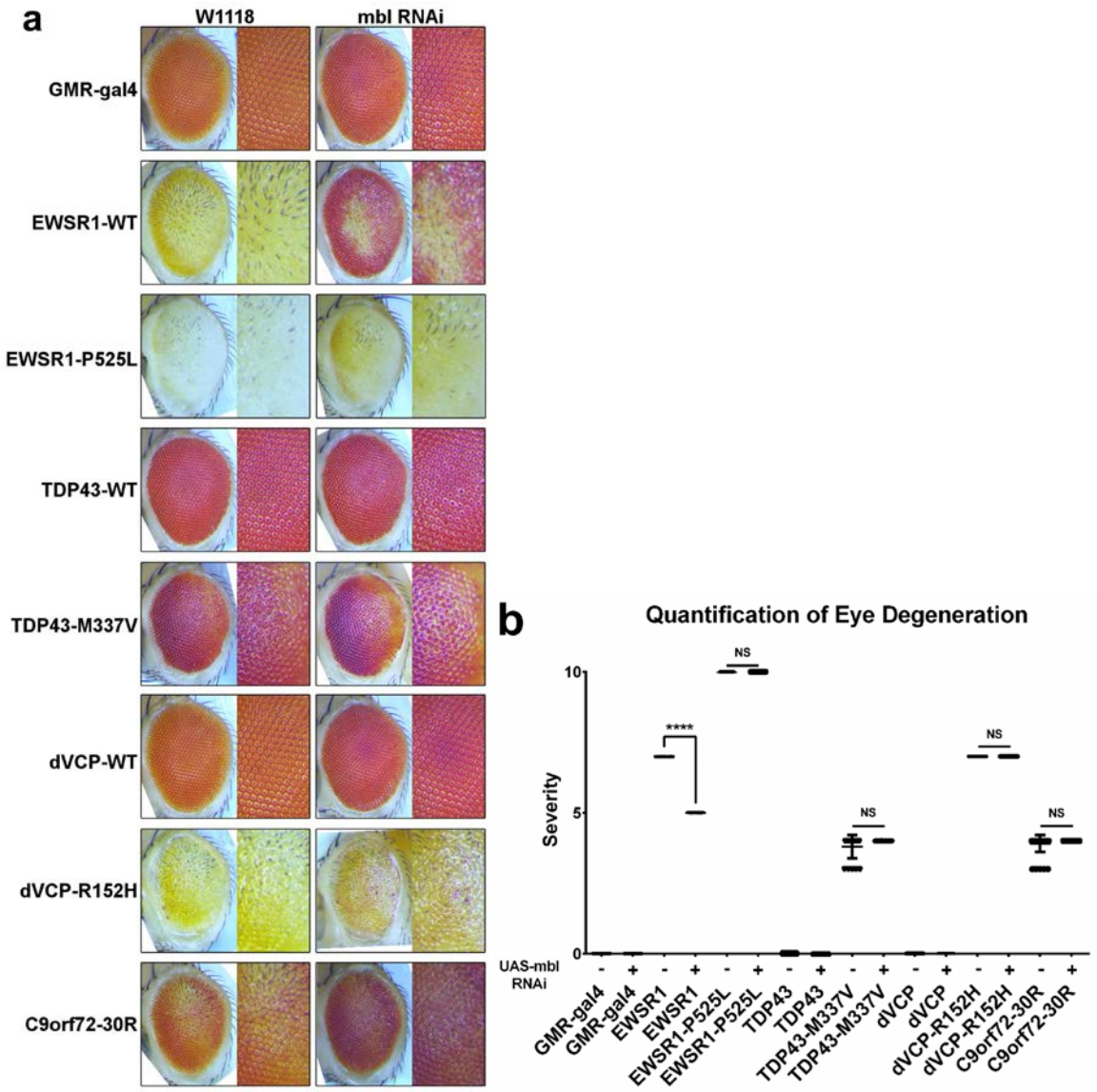


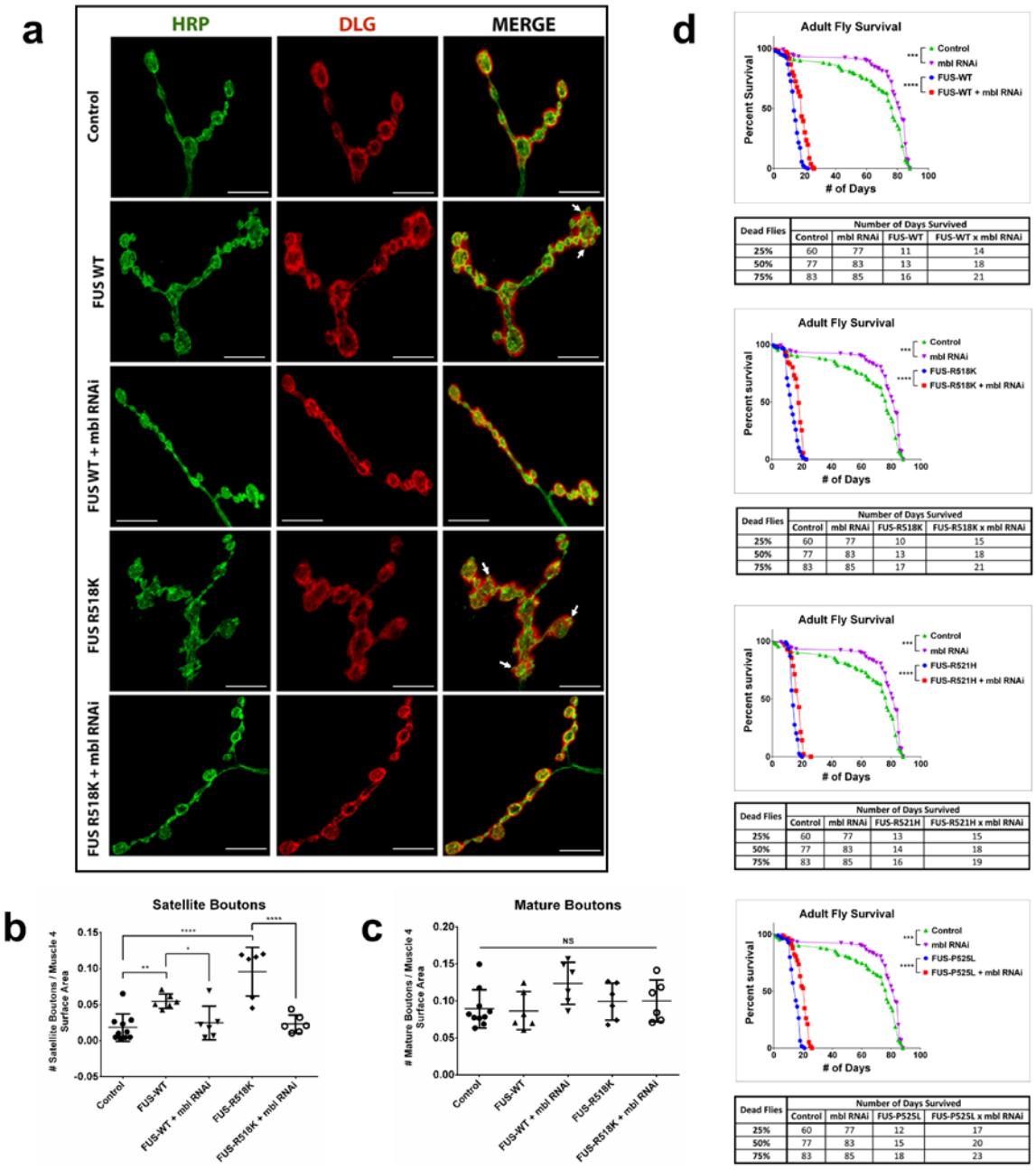
Figure 3.7. Knocking down endogenous mbl does not suppress the external eye degeneration caused by overexpression of other ALS-linked genes in *Drosophila*.

Flies overexpressing WT and ALS-linked mutant versions of human proteins in the eyes were crossed with mbl RNAi-expressing flies, under the GMR-gal4 driver. **(a)** Representative panel of adult *Drosophila* eyes showing the degeneration caused by tissue-specific expression of WT and mutant EWSR1, TDP43, dVCP and C9orf72. The WT and mutant EWSR1, TDP43 and C9orf72-30R images shown are from flies overexpressing human orthologues of these proteins. The WT and mutant dVCP images shown are from flies overexpressing the *Drosophila* ortholog of *VCP*. The *dVCP-R152H* mutant is equivalent to the same mutation in human *VCP* that has been linked to ALS. **(b)** Quantification of the external eye degeneration of the groups represented in (a) confirms that there is no significant change in toxicity of the ALS-linked proteins when endogenous mbl levels are reduced, with the exception of WT EWSR1. The values presented are the means ± SD. A minimum of 20 flies were used for analyses. Mann-Whitney tests were performed for each comparison (N = NS, ****P<.0001).

Since ALS is an adult onset, fatal disease, with a variable disease course, we determined the effect of knocking down mbl on the lifespans of adult flies that are expressing WT or mutant FUS in their neurons using an inducible neuronal driver. Pan neuronal expression of SSI WT and mutant FUS (R518K, R521H and P525L) with the ELAV-GS driver shortens Drosophila lifespan compared to driver-alone controls. However, knocking down mbl in these flies significantly extends their lifespans **(Fig. 3.8d)**. We observed the same effect with both motor neuron-specific (OK371-gal4 driver) and pan neuronal (ELAV-GS driver) expression of random-insertion WT and mutant FUS-P525L flies **(Fig. 3.9)**. Taken together, these results suggest that mbl is a novel modifier of ALS-associated FUS toxicity in neurons.

3.2.3 Targeted knockdown of endogenous MBNL1 reduces mutant FUS cytoplasmic mislocalization and incorporation into stress granules in HEK293T cells

It has been well established that cytoplasmic mislocalization of mutant FUS, and its subsequent accumulation into cytoplasmic stress granules (SGs), is a pathological hallmark of ALS[35,83,421]. It is suspected that these events are in fact responsible for driving the toxicity of the mutant proteins, and lead to the eventual cell death of motor neurons[55,107,417,439]. Therefore, we investigated whether lowering muscleblind-like levels in cells reduces FUS mislocalization and/or incorporation into SGs, thereby suppressing toxicity and cell death.



Dead Flies	Number of Days Survived			
	Control	mbl RNAi	FUS-WT	FUS-WT x mbl RNAi
25%	60	77	11	14
50%	77	83	13	18
75%	83	85	16	21

Dead Flies	Number of Days Survived			
	Control	mbl RNAi	FUS-R518K	FUS-R518K x mbl RNAi
25%	60	77	10	15
50%	77	83	13	18
75%	83	85	17	21

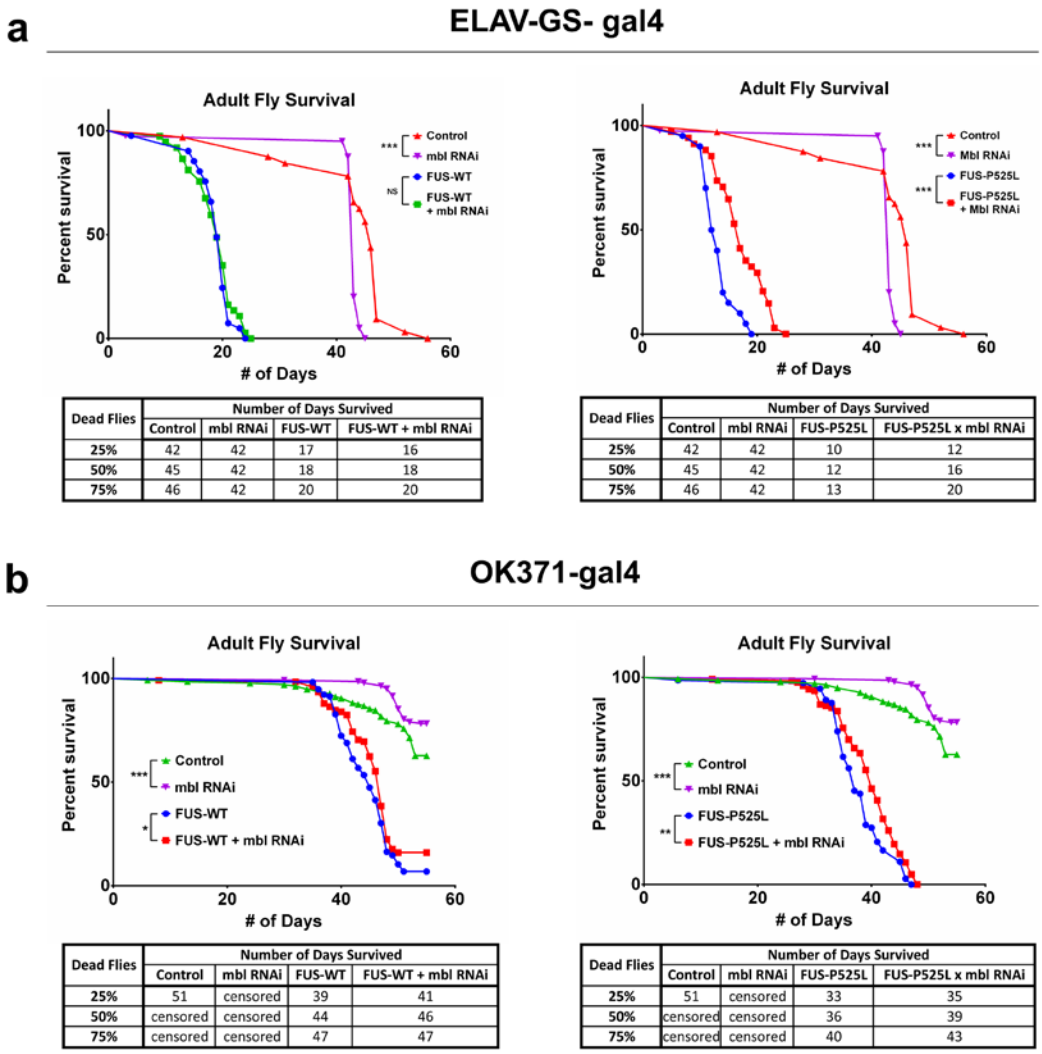
Dead Flies	Number of Days Survived			
	Control	mbl RNAi	FUS-R521H	FUS-R521H x mbl RNAi
25%	60	77	13	15
50%	77	83	14	18
75%	83	85	16	19

Dead Flies	Number of Days Survived			
	Control	mbl RNAi	FUS-P525L	FUS-P525L x mbl RNAi
25%	60	77	12	17
50%	77	83	15	20
75%	83	85	18	23

Figure 3.8. Morphological and lifespan assays in *Drosophila* confirm that mbl is a modifier of FUS toxicity in neurons.

Neuronal expression of WT and ALS-linked mutant FUS causes defects in larval NMJ morphology and adult lifespan, both of which are partially rescued by knocking down endogenous mbl. **(a-c)** Tissue-specific expression of FUS in the motor neurons of third-instar larvae causes morphological defects of NMJ's at muscle 4 from segments A2-A5. **(a)** Immunofluorescence images of NMJ's using presynaptic (HRP) and postsynaptic (DLG) markers. Scale bars = 10µm. Expressing FUS in motor neurons results in an aberrant increase in the number of satellite boutons (white arrows) that is significantly rescued following knockdown of endogenous mbl **(b)**. Mature boutons, however, are unaffected by FUS expression **(c)**. At least 6 NMJ's from at least three different animals were used for analyses. Values presented in B and C are means ± SD. Statistical comparisons were performed with one-way ANOVA

with Tukey's multiple comparisons test for each group (*P<0.05, **P<0.01, ****P<0.0001). (d) Kaplan-Meier survival curves of flies expressing WT FUS and three different mutants pan-neuronally. Knocking down mbl in these flies significantly increases adult lifespan of FUS-expressing flies, as well as controls (***P<0.001, ****P<0.0001). Graphs were generated in GraphPad Prism 6 software. Logrank with Grehan-Breslow-Wilcoxon tests were performed to determine significance. Tables below each graph show how long it took for the given percentage of flies to die. At least 100 flies were assessed from each group.



Dead Flies	Number of Days Survived			
	Control	mbl RNAi	FUS-WT	FUS-WT + mbl RNAi
25%	42	42	17	16
50%	45	42	18	18
75%	46	42	20	20

Dead Flies	Number of Days Survived			
	Control	mbl RNAi	FUS-P525L	FUS-P525L x mbl RNAi
25%	42	42	10	12
50%	45	42	12	16
75%	46	42	13	20

Dead Flies	Number of Days Survived			
	Control	mbl RNAi	FUS-WT	FUS-WT + mbl RNAi
25%	51	censored	39	41
50%	censored	censored	44	46
75%	censored	censored	47	47

Dead Flies	Number of Days Survived			
	Control	mbl RNAi	FUS-P525L	FUS-P525L x mbl RNAi
25%	51	censored	33	35
50%	censored	censored	36	39
75%	censored	censored	40	43

Figure 3.9. Both motor neuron-specific and pan-neuronal knockdown of endogenous mbl significantly extends the lifespans of flies expressing WT and mutant FUS.

Kaplan-Meier survival curves of random insertion *Drosophila* lines expressing either WT or mutant FUS pan-neuronally (ELAV-GS) **(a)** or in motor neurons (OK371-gal4) **(b)**. RNAi-mediated knockdown of endogenous mbl in these flies significantly extends their lifespans (*P<0.05, **P<0.01, ***P<0.001, NS = not significant). Tables beneath each graph show how long it took for the given percentage of flies to die. At least 100 flies were assessed from each group.

To test this hypothesis, we used human embryonic kidney (HEK293T) cells as a model for investigating changes in FUS dynamics following knockdown of endogenous muscleblind. There are three muscleblind paralogues in humans and in rats, called muscleblind-like 1, 2 and 3 (*MBNL1*, *MBNL2*, and *MBNL3*), whereas only one *mbl* gene exists in *Drosophila*. Expression of these three paralogues is variable in human tissues. MBNL1 and MBNL2 are the most ubiquitously expressed in tissues, including fetal and adult brain, heart, kidney, liver, lung and skeletal muscle. Whereas MBNL3 is not as widely expressed and is found primarily in tissues such as the heart, liver, pancreas and placenta[440]. Since MBNL1 is the paralogue that has been most frequently linked to other diseases, including neurodegenerative diseases[432,433,435-437], and is well conserved with *Drosophila mbl*, we chose to focus on this gene for our studies in mammalian systems[441]. To evaluate the effect of knocking down MBNL1 on WT and mutant FUS localization and SG development in the cell, we began by assessing the effect of FUS expression on endogenous cellular distribution of MBNL1. Untransfected HEK293T cells treated with different external stresses form MBNL1-positive SGs **(Fig. 3.10a)**. Therefore, we transiently transfected HEK293T cells with WT FUS and two different ALS-linked FUS mutants (R518K and R521C) and determined the cellular distribution of FUS and endogenous MBNL1 **(Fig. 3.11)**. In the absence of stress, FUS-WT is primarily localized to the nucleus. As a result, when we expose the cells to sodium arsenite, MBNL1-positive SGs do not contain WT FUS. In contrast, FUS-R518K and FUS-R521C mislocalize to the cytoplasm. In these cells, mislocalized mutant FUS is incorporated into MBNL1-positive, cytoplasmic SGs both with and without stress. The presence of SGs in the untreated cells suggests that ALS-

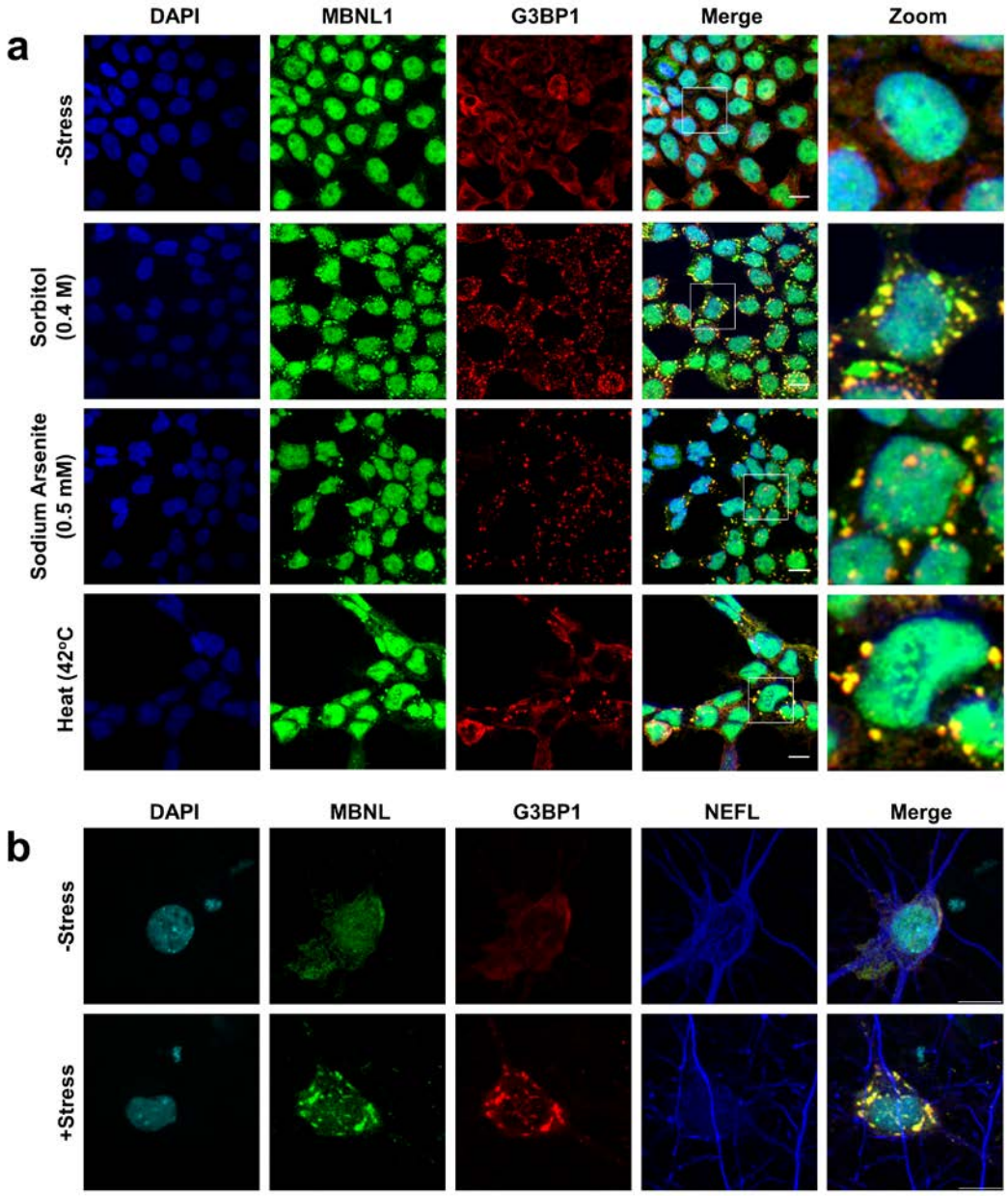


Figure 3.10. Muscleblind-like accumulates into G3BP1-positive stress granules in HEK293T cells and primary cortical neurons following exposure to external stressors.

(a) Representative panel of HEK293T cells visualized by confocal microscopy after cultures were treated with the indicated stress. G3BP1 was used as the marker for cytoplasmic SG formation. Cells exposed to all three types of stress show colocalization of MBNL1 with G3BP1-positive foci, confirming that MBNL1 accumulates in cytoplasmic SGs in these cells. Zoomed images highlight cellular distribution of MBNL1 and colocalization with G3BP1. White boxes outline the locations of the zoomed images. Scale bars = 10 µm (scale bars in zoomed panels = 5µm). **(b)** Representative panel of primary cortical neurons visualized by fluorescence imaging, with or without sodium arsenite treatment. mbnl1 colocalizes with G3BP1 in SGs following stress induction. NEFL was used to highlight the neurons.

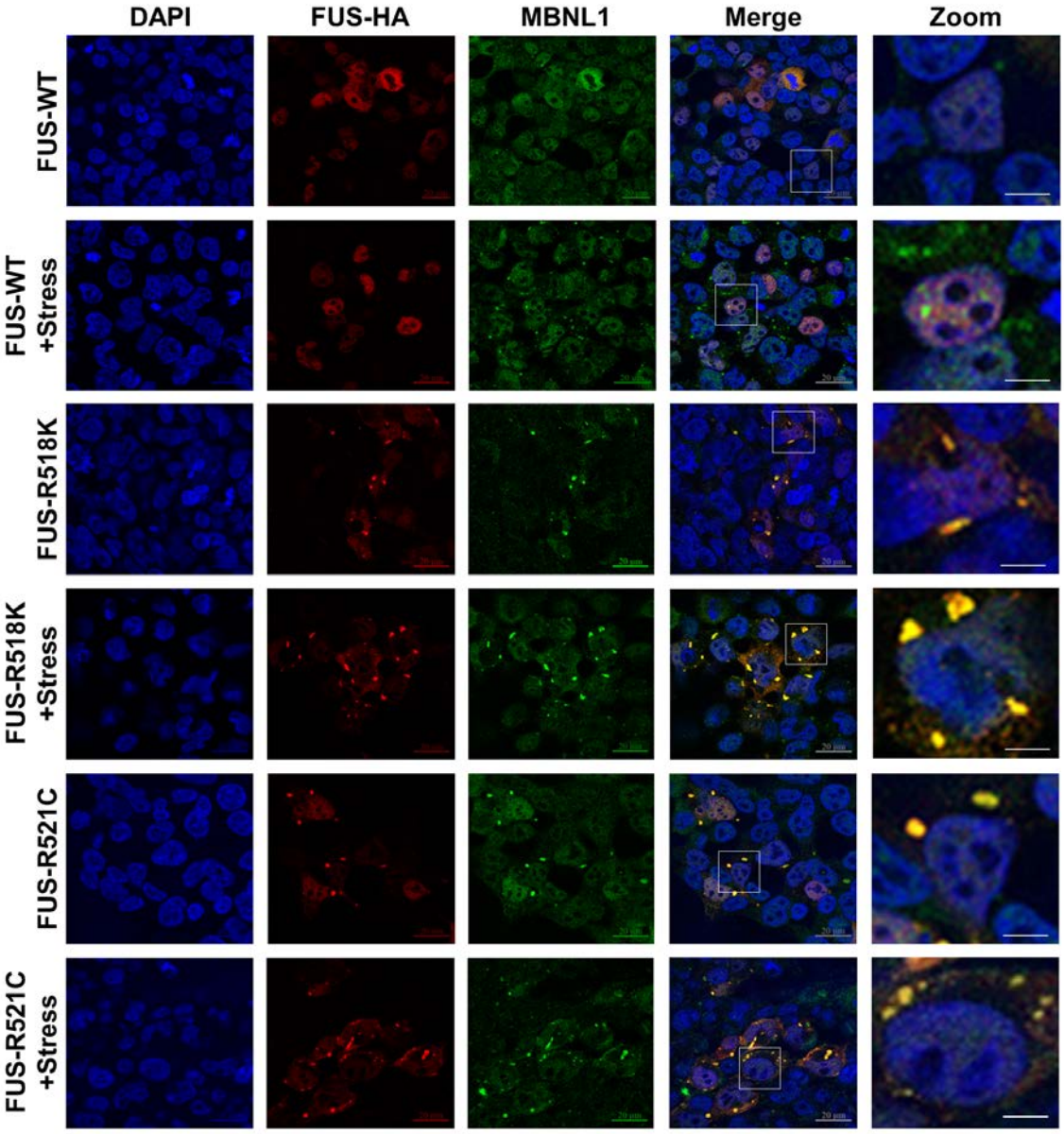


Figure 3.11. ALS-associated mutant FUS, but not WT FUS, mislocalizes to the cytoplasm and incorporates with endogenous MBNL1 protein into cytoplasmic stress granules.

Representative panel of HEK293T cells overexpressing either FUS-WT, FUS-R518K or FUS-R521C and visualized by confocal microscopy. Cells were imaged with and without treatment with sodium arsenite to induce cellular stress. All treatments were stained for both HA-tagged, exogenous FUS and endogenous MBNL1. FUS-WT protein is localized primarily to nuclei in both stressed and unstressed conditions, and therefore is not incorporated into MBNL1-positive, cytoplasmic, stress granules. Both R518K and R521C mutant FUS, however, are mislocalized to the cytoplasm. Cells with cytoplasmic FUS in these groups develop FUS and MBNL1-positive, cytoplasmic, stress granules both in the presence, and in the absence, of sodium arsenite-induced cellular stress. DAPI was used as a nuclear marker. Zoomed images highlight the cellular distribution of MBNL1 and FUS, and the formation of SGs. Scale bars = 20µm (zoomed scale bars = 5µm).

associated mutations of FUS are sufficient for SG formation, even in the absence of additional cellular stress.

We next aimed to determine the effect of MBNL1 knockdown on these cells using short-hairpin RNA (shRNA) that specifically targets all isoforms of MBNL1. After validating that endogenous MBNL1 is knocked down following transient transfection of the shRNA in HEK293T cells **(Fig. 3.12a and 3.12b)**, we co-transfected it with either FUS-WT, FUS-R518K or FUS-R521C **(Fig. 3.14 and Fig. 3.13)**. We found that FUS-WT protein maintains a primarily nuclear localization in both MBNL1 shRNA and scramble shRNA-treated cells **(Fig. 3.13)**. When we added sodium arsenite to these cells to induce stress, the MBNL1-positive, cytoplasmic SGs that developed did not include WT FUS **(Fig. 3.14a and 3.14d)**. In both FUS-R518K and FUS-R521C-expressing cells, however, mutant FUS mislocalization is significantly reduced in the MBNL1 shRNA groups compared to scrambled shRNA controls **(Fig. 3.13)**. The result is a significantly reduced number of MBNL1-positive, cytoplasmic SGs that contain mutant FUS when the cells are stressed with sodium arsenite **(Fig. 3.14b – 3.14c and 3.14e – 3.14f)**. These observations support our hypothesis that lowering MBNL1 levels in the cell reduces mutant FUS mislocalization and subsequent incorporation into SGs. This reversal of the pathological hallmarks of FUS-associated ALS further implicates muscleblind as a modifier of mutant FUS toxicity.

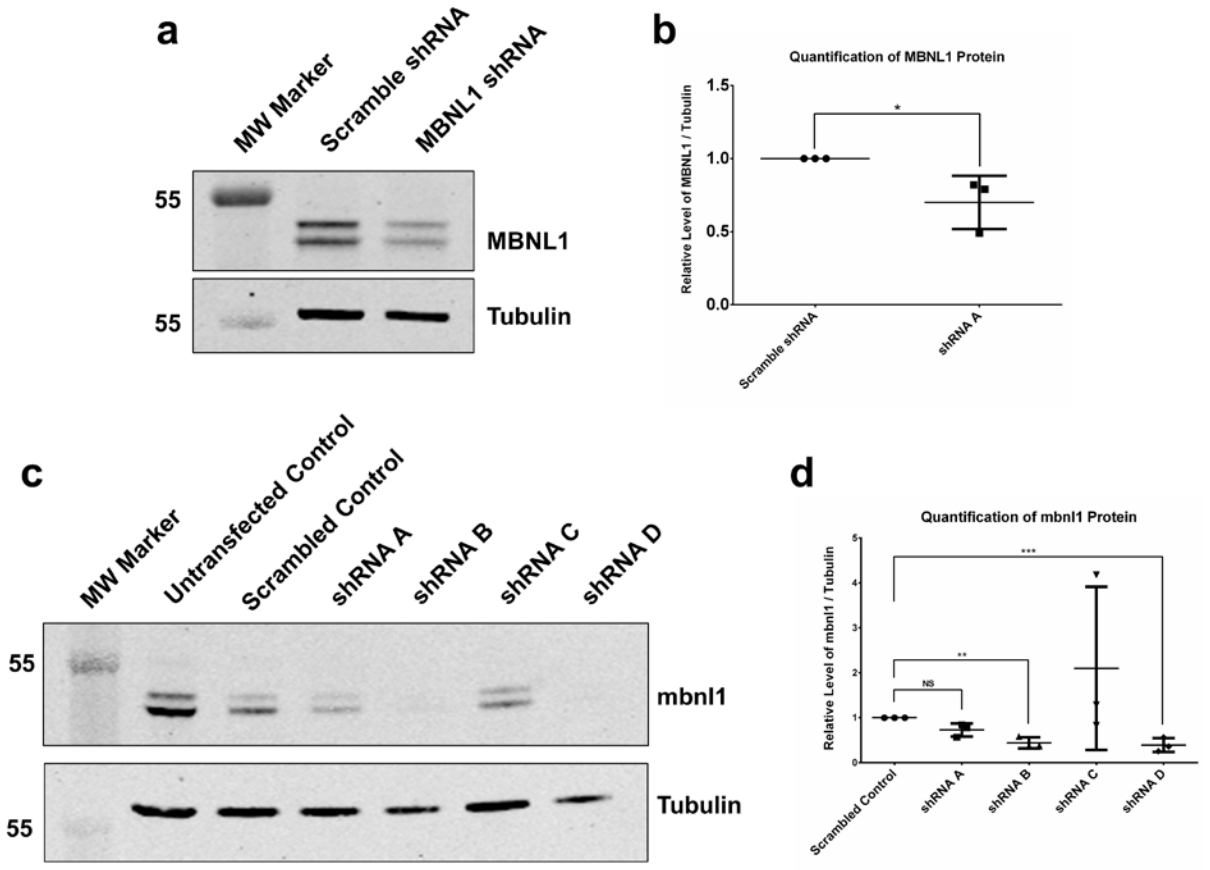


Figure 3.12. HEK293T and N2A cells transiently transfected with muscleblind-like-specific shRNA show significant reductions of muscleblind-like protein.

(a and b) HEK293T cells from ATCC were transfected with MBNL1-specific shRNA from Origene. **(a)** Representative Western blot of endogenous MBNL protein following transient transfection with either scramble shRNA or MBNL1 shRNA. Tubulin was used as a housekeeping protein control. **(b)** Quantification of the MBNL1 protein from the Western blot in (a). The ratio of mbnl1 signal to each sample's corresponding tubulin signal was calculated. MBNL1 protein is significantly reduced in shRNA-treated cells compared to scramble control-treated cells. **(c and d)** Mouse neuro2a (N2A) cells from ATCC were transfected with mbnl1-specific shRNAs from Origene. Representative Western blot for detection of endogenous mbnl1 protein following transfection of either scramble shRNA, or one of four mbnl1-specific shRNAs. Untransfected cells were included as a background control. Tubulin was used as a housekeeping control, as well as for normalizing the mbnl1 protein signals for quantification. **(d)** Quantification of the mbnl1 protein bands in (c). Two of the four shRNA's showed significant knockdown of mbnl1 shRNA. The scrambled, not untransfected, control group was used to determine the effectiveness of the mbnl1-specific shRNAs. Based on these results, shRNA D was selected for use in the experiments in mouse cortical neurons. For all muscleblind Western blots, both bands in the characteristic mbnl1 doublet were measured for quantification. All Western blots were performed in triplicate using biological replicates. The relative protein levels presented in b and d are the means ± SD. Quantifications were performed using Image Studio software (Li-Cor), and statistical analyses were performed using GraphPad Prism 6 software. Statistical significance in (b) was determined by a two-tailed T-test (*P<0.05). Statistical significance in (d) was determined using one-way ANOVA with Dunnet's multiple comparison test (**P<0.01, ***P<0.001, NS = not significant).

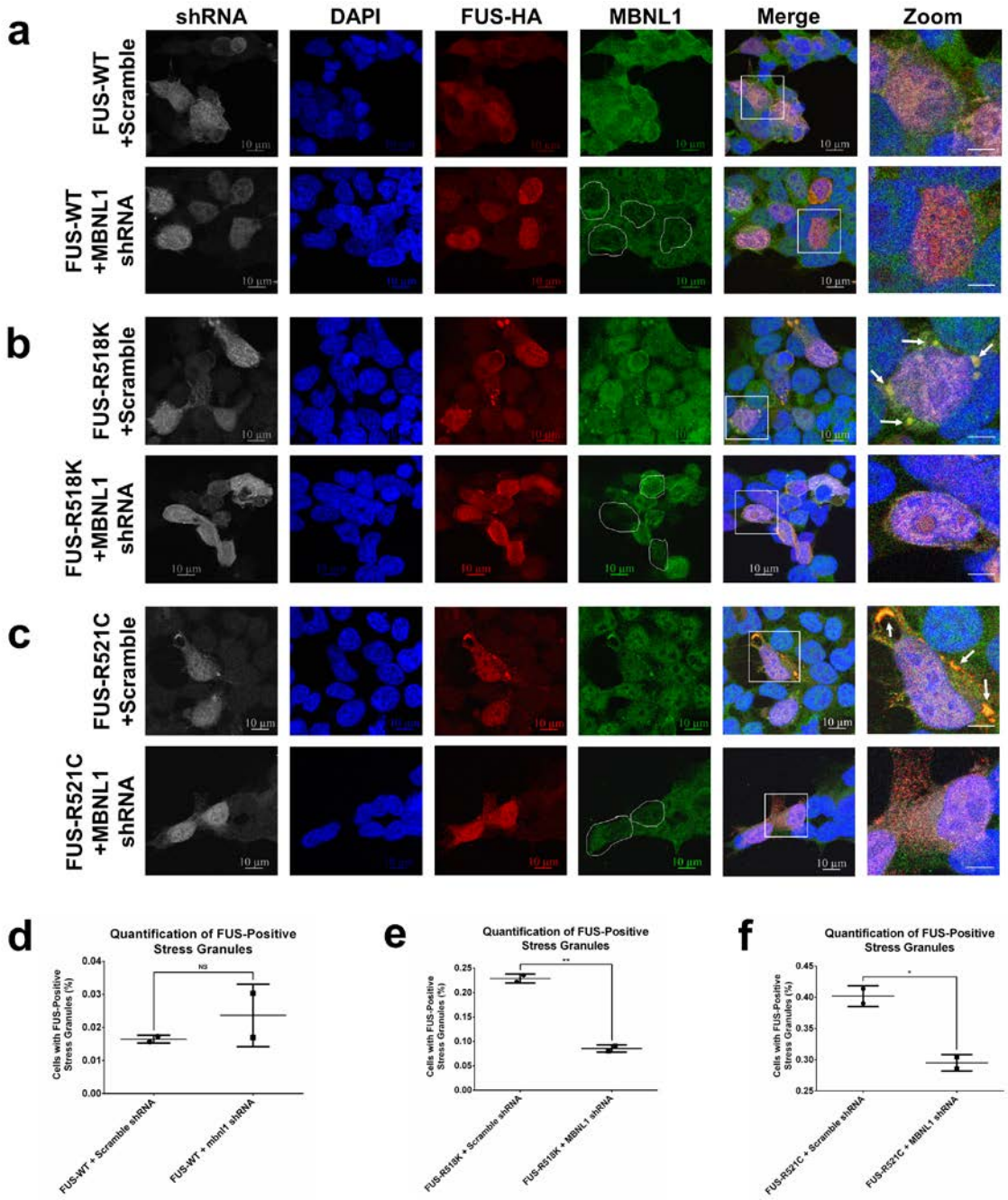


Figure 3.13. shRNA-mediated knockdown of endogenous MBNL1 reduces mutant FUS mislocalization and incorporation into cytoplasmic stress granules in the absence of external cellular stress.

HEK293T cells were co-transfected with one of three FUS constructs (FUS-WT, FUS-R518K or FUS-R521C) and either MBNL1 shRNA or scramble shRNA. Cells were then assessed for cellular distribution of FUS and MBNL1, as well as the presence of naturally-occurring stress granules using confocal microscopy. **(a-c)** Representative confocal images showing FUS and MBNL1 distribution in HEK293T cells. DAPI staining was used to image cell nuclei. Panel is divided into three sections based on the protein being expressed. Labels to the left of the panel identify which groups were transfected with MBNL1 shRNA or scramble shRNA. White outlines in the "MBNL1" images highlight representative

MBNL1 shRNA-transfected cells with reduced MBNL1 signal. White boxes outline the areas magnified in the "zoom" column. Scale bars = 10µm (zoomed scale bars = 5µm). In the absence of artificial exposure to an external stressor, FUS-positive stress granules (white arrows) naturally form in the cytoplasm of mutant FUS-expressing cells, but not in WT-expressing cells. Depleting endogenous MBNL1 reduces FUS-positive SG formation. **(d-f)** Quantification of the percentage of cells with FUS-positive stress granules in each FUS group. Graphs correspond to the representative images in the panel above (a, b or c). A minimum of 50 cells were analyzed for each measurement. Statistical analyses were performed on GraphPad Prism software using two-tailed t-tests (*P = 0.0191, **P = 0.0035, NS = not significant).

3.2.4 Knockdown of endogenous mbnl1 suppresses mutant FUS mislocalization and incorporation into stress granules in primary cortical neurons

To test whether muscleblind can modify FUS toxicity in mammalian neurons, we used rat primary cortical neurons as a model for FUS-associated ALS[442-447]. As with humans, rats have three paralogues of muscleblind, muscleblind-like 1, 2 and 3 (*mbnl1*, *mbnl2*, and *mbnl3*)[440]. We first confirmed that there is significant knockdown of endogenous mbnl1 in mouse cells transfected with mbnl1 shRNA **(Fig. 3.12c and 3.12d)**. Next, we verified that we could successfully transfect rat primary cortical neurons with scrambled and mbnl1-specific shRNAs, and visualize mbnl1 reduction with fluorescence microscopy **(Fig. 3.15a and 3.15b)**. Similarly to endogenous MBNL1 in HEK293T cells, mbnl1 localizes to G3BP1-positive SGs in primary cortical neurons under stress **(Fig. 3.10b)**. Therefore, to test whether depleting endogenous mbnl1 levels reduces mutant FUS mislocalization and incorporation into SGs in our cortical neuron model, we co-transfected either WT or ALS-linked mutant FUS (FUS-R521C) with mbnl1-specific shRNA and scramble shRNA. Using fluorescence microscopy, we visualized FUS distribution with and without knockdown of endogenous mbnl1 **(Fig.**

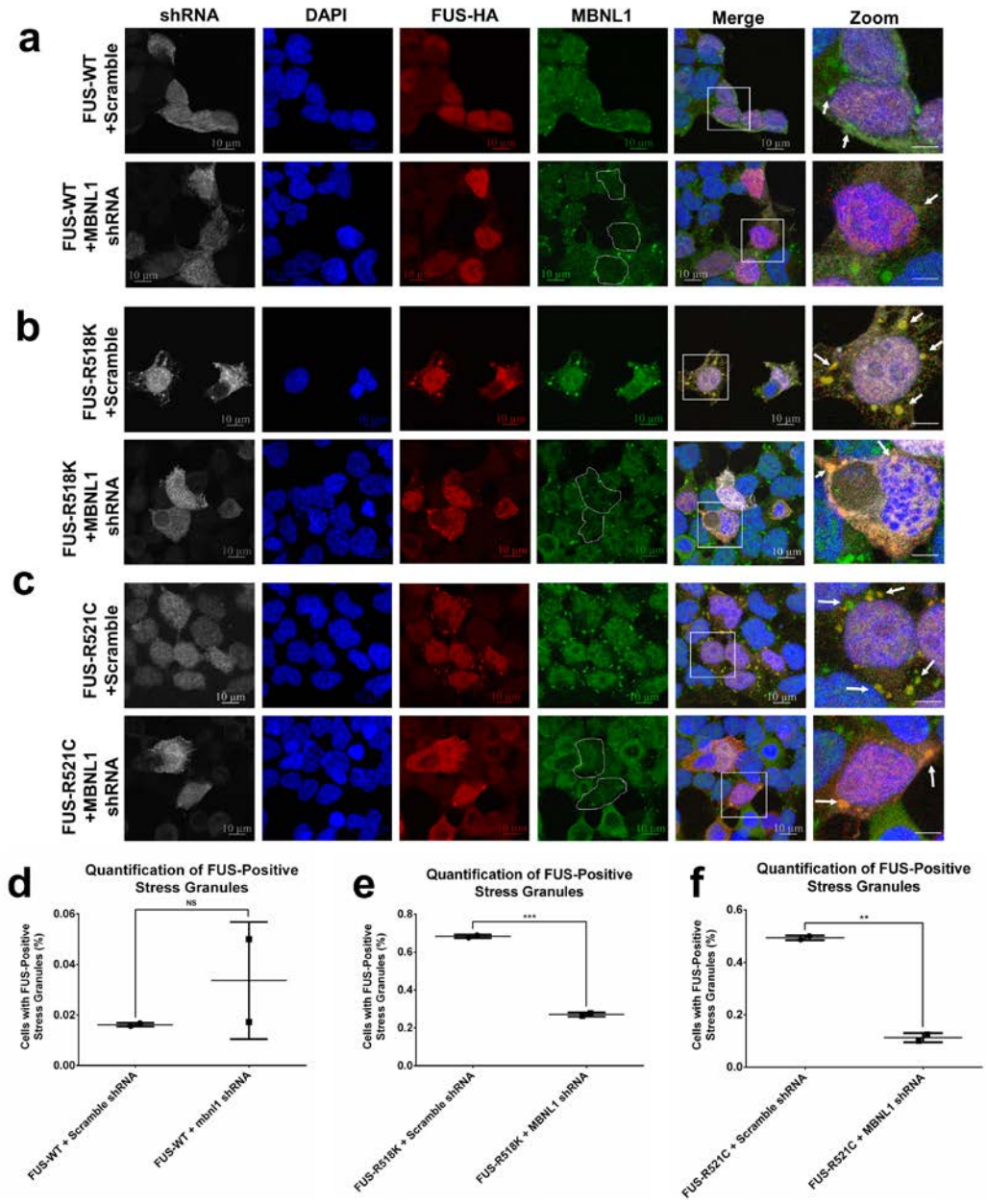


Figure 3.14. shRNA-mediated knockdown of endogenous MBNL1 reduces mutant FUS mislocalization and incorporation into cytoplasmic stress granules following induction of cellular stress.

HEK293T cells were co-transfected with one of three *FUS* constructs (FUS-WT, FUS-R518K or FUS-R521C) and either MBNL1 shRNA or scramble shRNA. Transfected cells were then treated with sodium arsenite as a chemical stress. Following sodium arsenite treatment, cells were assessed for cellular distribution of FUS and MBNL1, as well as the presence of stress granules, using confocal microscopy. **(a, b and c)** Representative confocal images showing FUS and MBNL1 distribution in HEK293T cells. DAPI staining was used to image cell nuclei. Panel is divided into three parts, corresponding to the three FUS proteins being expressed. Labels on the left side of the panel detail which groups were transfected with MBNL1 shRNA or scramble shRNA. White outlines in the "MBNL1" images highlight representative MBNL1 shRNA-transfected cells with reduced MBNL1 signal. White boxes outline the areas magnified in the "zoom" column. White arrows mark representative stress granules. Scale bars = 10µm (zoomed

scale bars = 5 µm. **(d, e and f)** Quantification of the percentage of cells with FUS-positive stress granules confirms that depleting endogenous MBNL1 significantly reduces mutant FUS mislocalization and integration into SGs. Each graph corresponds to the representative panel above (a, b or c). Since all cells with cytoplasmic mislocalization of FUS developed FUS-positive stress granules, this quantification is also representative of the percentage of cells with cytoplasmic mislocalization of FUS. A minimum of 20 cells were analyzed in biological triplicates for each measurement. Values presented in each graph are means ± SD. Statistical analyses were performed on GraphPad Prism software using two-tailed t-tests (***P = 0.0006, **P = 0.0014, NS = not significant).

3.16). We found that in cells transfected with FUS alone, WT FUS is primarily localized to nuclei, whereas FUS-R521C mutants mislocalize to the cytoplasm **(Fig. 3.15c)**.These localization patterns also occur in cells co-transfected with scramble shRNA**,** with no significant change in the percent of neurons with mislocalized FUS **(Fig. 3.16a and 3.16b)**. However, shRNA-mediated knockdown of endogenous mbnl1 significantly reduces mutant FUS mislocalization, with no effect on FUS-WT distribution **(Fig. 3.16a and 3.16b)**. Since the ALS-associated R521C mutation of FUS appeared to induce SGs in these neurons, we used antibody for G3BP1 as a marker for SGs to analyze SG formation in FUS-R521C-expressing cells **(Fig. 3.16c)**. We found that the average number of G3BP1-positive SGs per neuron is significantly reduced in mbnl1 shRNA-expressing cells, compared to scramble shRNA controls **(Fig. 3.16d)**. There is no change, however, in the average size of FUS-positive SGs across all neurons **(Fig. 3.16e)** or the average size of FUS-positive SGs/neuron **(Fig. 3.16f)**, compared to scramble shRNA controls. This rescue appears to be specific to mutant FUS, as we did not observe a change in number or size of SGs in mbnl1 shRNA-expressing neurons without exogenous FUS, compared to scramble controls **(Fig. 3.17)**. Since reducing endogenous mbnl1 levels appears to specifically affect the number of FUS-R521C-positive SGs, and not the size, the data suggests that mbnl1 modifies the incorporation of FUS into stress granules (likely through the rescue of cytoplasmic mislocalization),

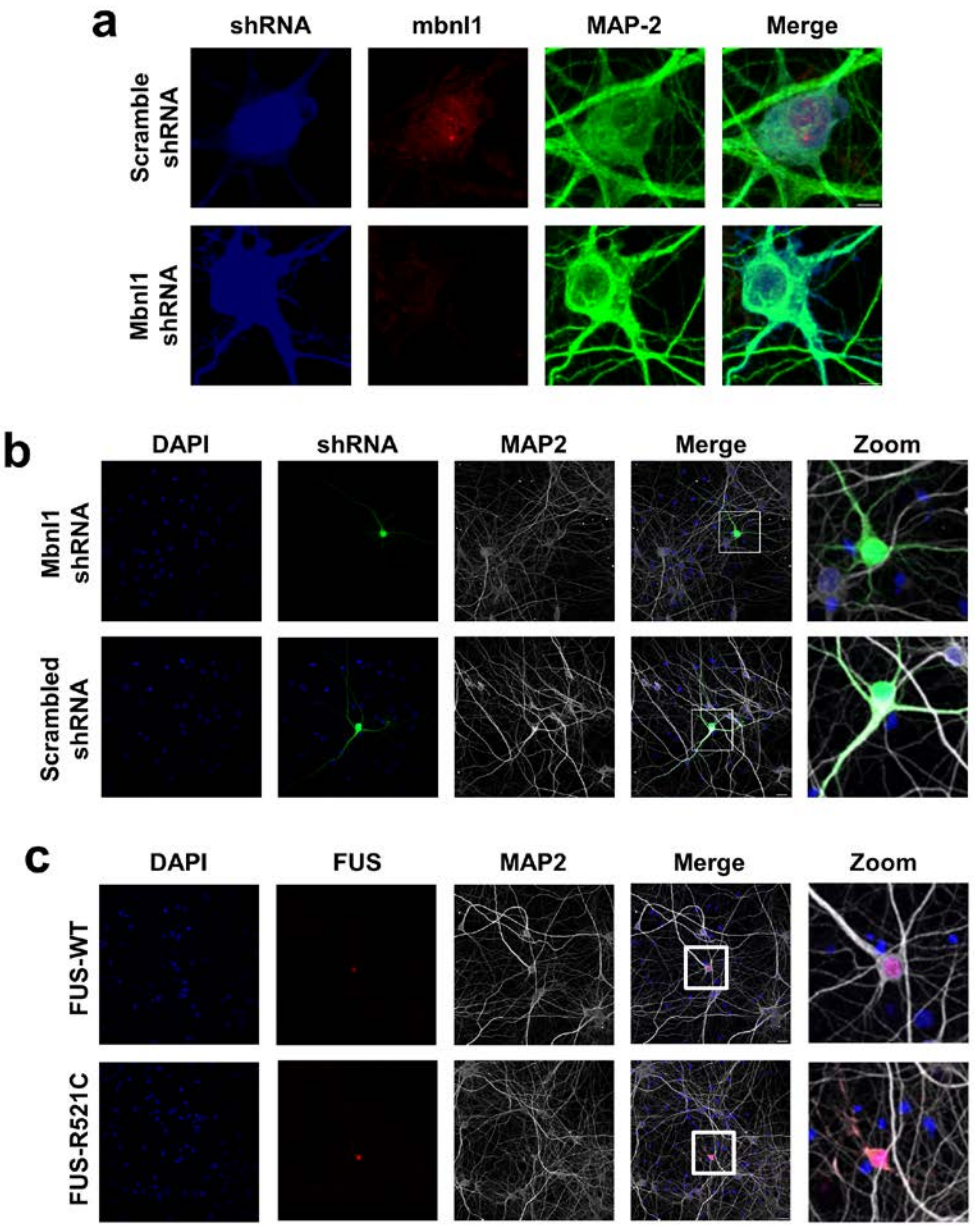


Figure 3.15. Cellular distribution of mbnl1, shRNA and FUS in transiently transfected rat cortical neurons.

(a) Representative fluorescence images of primary cortical neurons transfected with either mbnl1 shRNA or scrambled shRNA. Cells are stained for mbnl1 protein and MAP-2, a neuronal cell marker. mbnl1 protein levels in mbnl1 shRNA-transfected cells are reduced compared to scrambled shRNA controls. **(b and c)** Representative fluorescence images showing the cellular distribution of transiently transfected FUS and shRNAs. Nuclei are stained with DAPI to identify nuclear and cytoplasmic cellular compartments. MAP-2 is used as a marker for neuronal cells. **(b)** mbnl1 shRNA and scramble shRNA are distributed throughout both the nucleus and the cytoplasm. **(c)** Similarly to HEK293T cells, FUS-WT protein is primarily localized in the nucleus, but ALS-linked mutant FUS (FUS-R521C) mislocalizes to the cytoplasm. White boxes indicate locations of zoomed images which highlight cellular distribution of the given transfected elements.

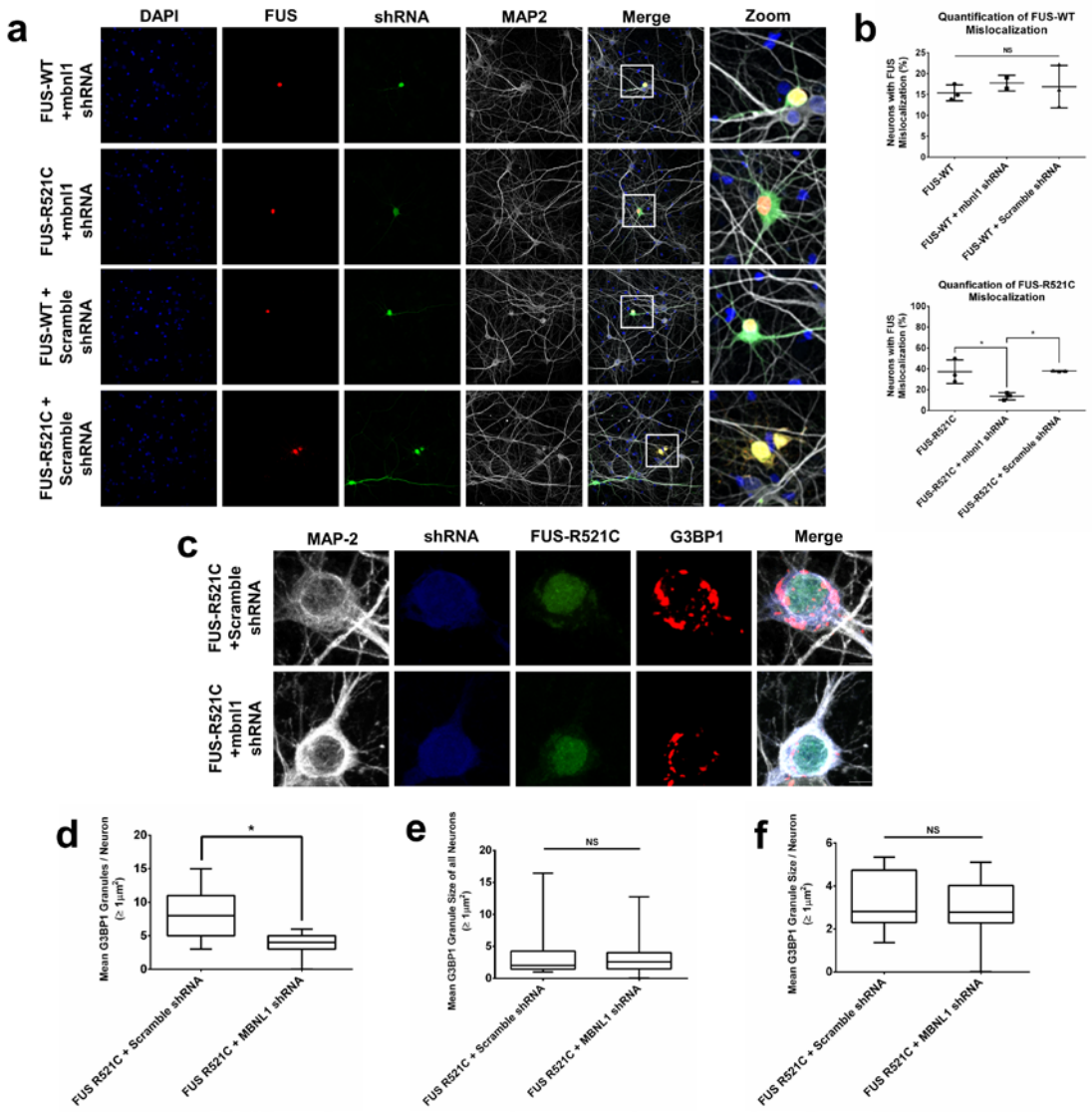


Figure 3.16 shRNA-mediated knockdown of endogenous mbnl1 rescues mutant FUS mislocalization and incorporation into cytoplasmic stress granules in cortical neurons.

We transfected rat, primary, cortical neurons with FUS-WT and FUS-R521C in combination with either mbnl1-specific shRNA or scramble shRNA. FUS-positive cells were then analyzed for cellular distribution of exogenous FUS protein and the extent of SG formation. **(a)** Representative confocal images showing the differences in WT and mutant FUS distribution in cortical neurons. Similarly to what we observed in cells transfected with FUS alone (Fig. 3.15c), scramble shRNA-expressing cells have a nuclear distribution of WT FUS, but exhibit cytoplasmic mislocalization of ALS-linked mutant FUS. DAPI is used for staining cell nuclei and MAP2 is a neuron-specific marker. White boxes designate the sources of the zoomed images which highlight mislocalization of mutant FUS protein in the scramble shRNA-treated cells. **(b)** Quantification of the percent of neurons with FUS mislocalization in each group, including the controls in Figure 3.15c. This analysis reveals a significant rescue of R521C mutant FUS mislocalization in the mbnl1 shRNA-expressing cells that does not occur in the scramble shRNA control group. A minimum of 30 cells were analyzed for each group, in three independent trials. Values presented are means ± SEM. **(c)** Representative confocal images of stress granules in cortical neurons expressing mutant FUS protein and either mbnl1 shRNA or scramble shRNA. MAP2 is used to visualize the neurons and G3BP1 is a marker for stress granules. Cells expressing mbnl1-specific shRNA show fewer

cytoplasmic SGs compared to scramble shRNA controls. Scale bar = 5µm. **(d-f)** Quantification of the number and size of SGs in each group. Only SGs equal to, or greater than, 1µm^2 were used in the quantifications. A minimum of 10-15 cells were analyzed from each group. Values presented are means ± SEM. **(d)** The average number of G3BP1-positive SGs/neuron is significantly lower in mbnl1 shRNA-expressing cells than in scramble shRNA control cells. There is no change between shRNA groups, however, in the average size of FUS-positive SGs totaled across all transfected neurons **(e)** or averaged per neuron **(f)**. All statistical analyses were performed on GraphPad Prism software. One-way ANOVA with Tukey's multiple comparisons test was used in (b) (*P<0.05, NS = not significant). Two-tailed T-tests were used for the comparisons in (d)-(f) (*P<0.05, NS = not significant).

rather than modifying innate SG dynamics, *per se*. These results provide further evidence that muscleblind modifies FUS toxicity through its cellular distribution and interaction with stress granules in the cytoplasm.

3.2.5 Knockdown of endogenous mbnl1 suppresses mutant FUS-induced cell death and morphological defects of primary cortical neurons

To directly test whether knocking down mbnl1 affects FUS toxicity in primary cortical neurons, we looked for changes in lifespan and cellular morphology in FUS-R521C-expressing cortical neurons, with and without endogenous mbnl1 knockdown **(Fig. 3.19)**. Following validation that our mbnl1 and scramble shRNAs could be visualized by fluorescence live imaging microscopy over time **(Fig. 3.18)** and are not toxic to the cells, we performed toxicity assays on neurons co-transfected with either FUS-WT or FUS-R521C and mbnl1 shRNA or scramble shRNA **(Fig. 3.19a – 3.19c)**[448]. Using DRAQ7 as a marker of cell death, we found that neurons expressing WT FUS remained healthy for at least 72 hours following transfection (**Fig. 3.19a)**. Co-transfecting these cells with either mbnl1 shRNA or scramble shRNA had no effect on lifespan, whereas neurons expressing FUS-R521C, however, begin to show signs of cell death through DRAQ7

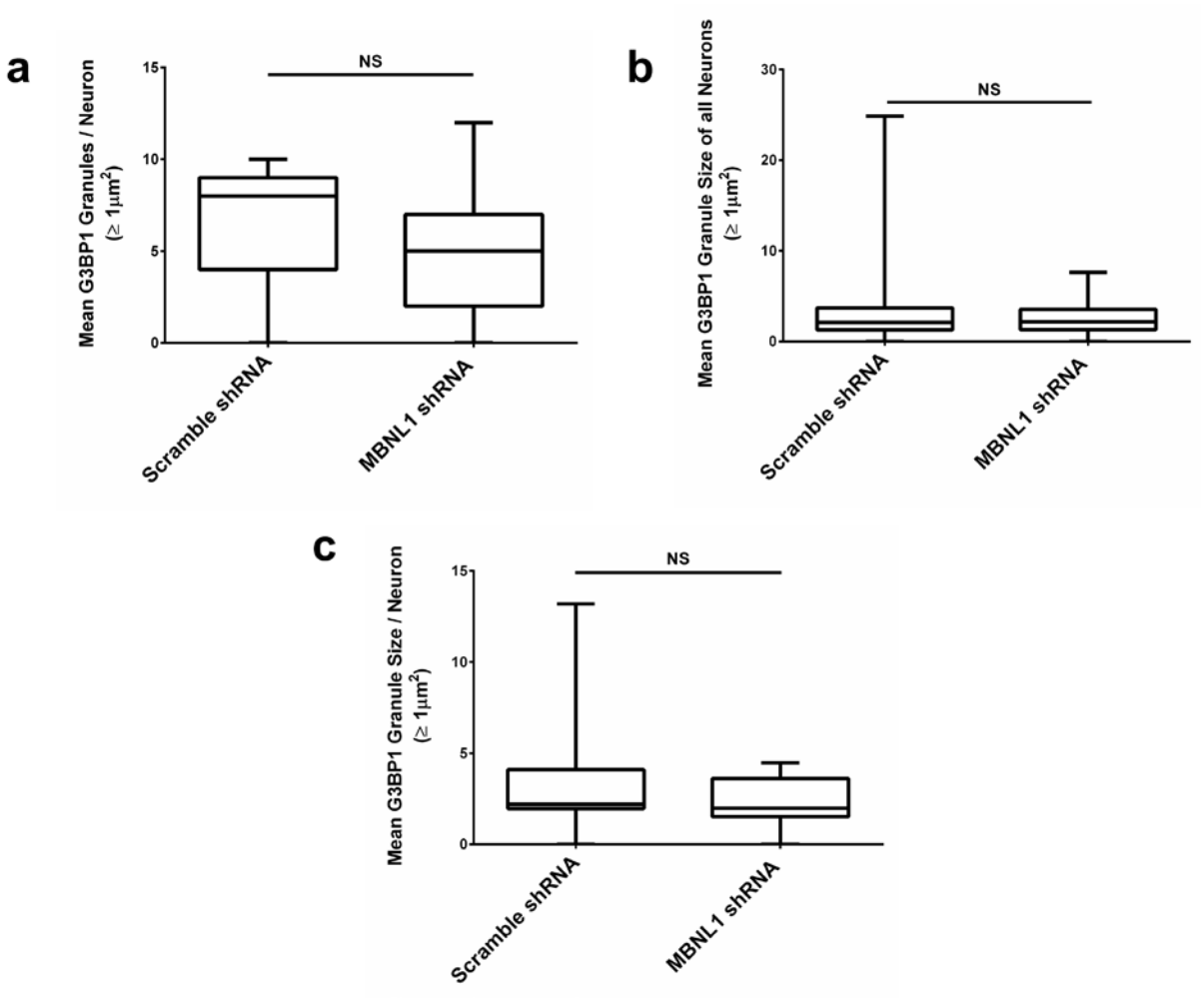


Figure 3.17. Quantifications of SG size and number in cortical neurons expressing mbnl1-specific shRNA or scramble shRNA.

Neurons that were positive for shRNA were analyzed for number and size of SGs. Only SGs equal to, or greater than, 1μm^2 were used in the quantifications. There were no significant differences in the average number of G3BP1-positive SGs/neuron **(a)**, average size of total SGs across all neurons **(b)**, or average size of SGs/neuron **(c)**, between mbnl1 shRNA-expressing neurons and scramble shRNA controls. Statistical analyses were performed on GraphPad Prism 6 software. Values presented are means ± SEM. A minimum of 10-15 cells were analyzed for each group. Two-tailed T-tests were performed for each comparison (NS = not significant).

staining as early as 48 hours following transfection. FUS-R521C-expressing neurons co-transfected with mbnl1 shRNA remain negative for DRAQ7 staining up to 72 hours following transfection. However, neurons transfected with scramble shRNA are positive for DRAQ7 by 48 hours following transfection. Using Kaplan-Meier curves to quantify cell death in these groups, we find no change in the cumulative survival **(Fig. 3.19b)** or associated cumulative risk of death **(Fig. 3.19c)** in FUS-WT-expressing neurons. However, in the FUS-R521C-expressing neurons, knocking down endogenous mbnl1 significantly increases their cumulative survival and significantly reduces their cumulative risk of death, compared to cells expressing mutant FUS alone, or in combination with scramble shRNA.

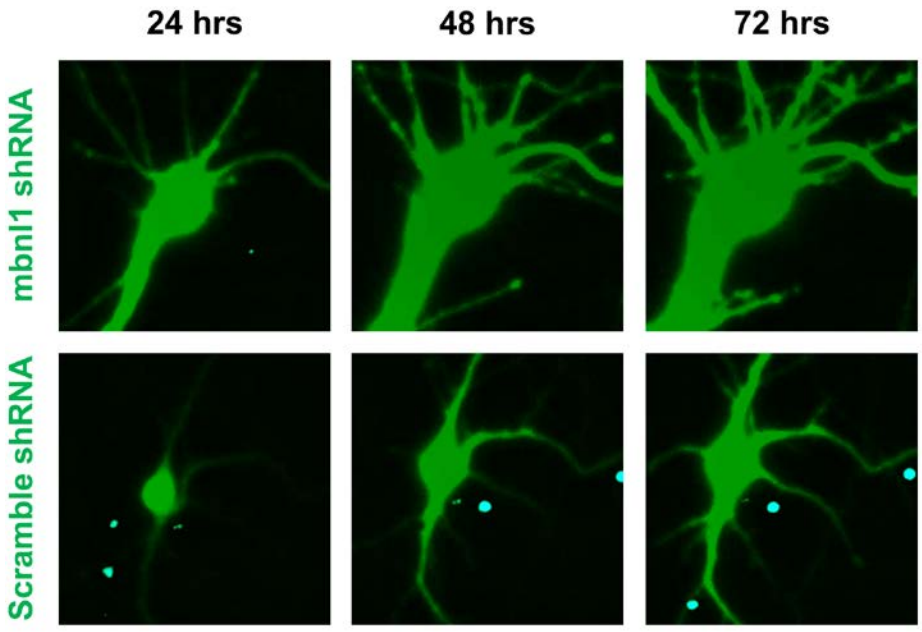


Figure 3.18. Expression of mbnl1 shRNA and scramble shRNA over time in primary cortical neurons is not toxic.

Representative fluorescence images showing clear expression of both mbnl1-specific and scramble shRNA over the course of 72 hours in cortical neurons. DRAQ7 staining within cells (cyan color) is a marker for cell death. These cells are DRAQ7 negative throughout the entire time course, confirming that mbnl1 and scramble shRNAs are not toxic to the cells over time.

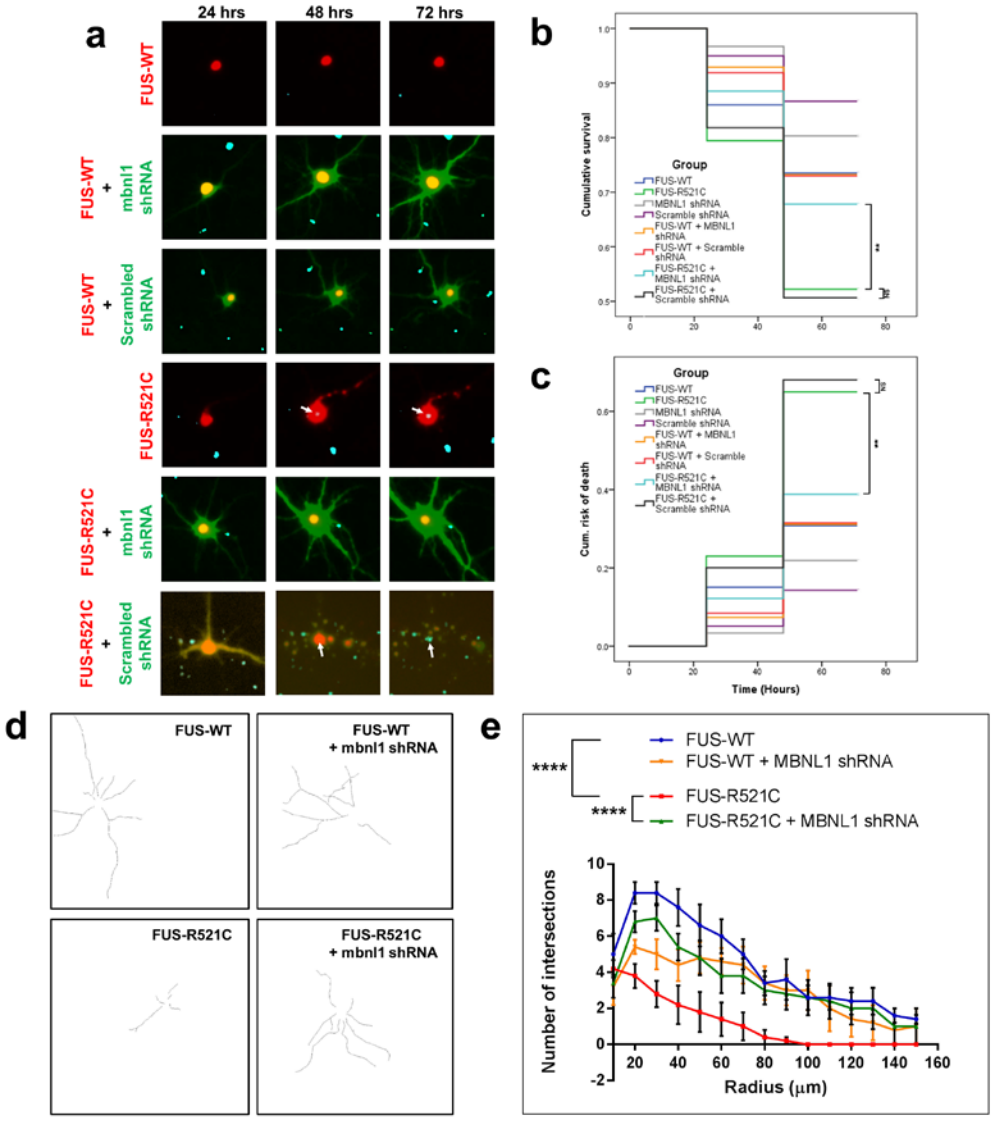


Figure 3.19. Knocking down endogenous mbnl1 suppresses mutant FUS-induced cell death and morphological defects in primary cortical neurons.

Cortical neurons were co-transfected with either FUS-WT or FUS-R521C and mbnl1 shRNA or scramble shRNA and assessed for cell death and morphological changes. **(a)** Representative images, produced through fluorescence live imaging microscopy, showing individual neurons over the course of 72 hours following transfections with *FUS* and shRNAs. Cell death is indicated by positive staining for DRAQ7 (cyan color). DRAQ7 is not present in the FUS-WT-expressing cells, but shows up within 48 hours after transfection with FUS-R521C. This toxicity is rescued in these cells with shRNA-mediated knockdown of MBNL1. Co-transfection with scramble shRNA, however, does not rescue cell death. **(b)** Kaplan-Meier cumulative survival curves for quantifying cell death in the transfected neurons. Transfecting with *FUS-R521C* significantly reduces cell survival compared to *FUS-WT*-expressing cells. Co-transfecting with mbnl1-shRNA, but not scramble shRNA, significantly rescues cell death. A minimum of 40 cells from each group were monitored. Graph was generated with SPSS. Log-rank with Mantel-Cox tests were used for comparisons (**P<0.01, ****P<0.0001, NS = not significant. **(c)** Cumulative risk of death plot that corresponds to the survival analysis. The lower the survival in (b), the higher the cumulative risk of death. **(d-e)** Sholl analysis of dendritic morphology of neurons transfected with *FUS-WT* and *FUS-R521C*, with and without concurrent knockdown of endogenous mbnl1. **(d)** Dendrite tracings for visualization of

changes in branching or elongation. Neurons expressing *FUS-R521C* show reduced branching and elongation of dendrites compared to *FUS-WT*-expressing cells. These changes are rescued when endogenous mbnl1 is knocked down. **(e)** Quantification of the number of intersections at increasing radii from the neuron cell body. Fewer intersections represent loss of dendritic branching, thereby providing a method for quantifying the dendrite tracings. Consistent with the qualitative changes shown in (d), neurons expressing *FUS-R521C* have a significant decrease in the number of intersections compared to the *FUS-WT*-expressing cells. This is rescued when endogenous mbnl1 is knocked down. Statistical analyses were performed in GraphPad Prism 6 software. 5 cells were analyzed from each group. 2-way ANOVA with Bonferroni's, Tukey's and Sideak's multiple comparisons tests were performed on all groups at each radius measured. Asterisks shown come from the radii measurements with the highest significant difference between the groups being compared, and were consistent with all three multiple comparisons tests performed (**P<0.01 and ****P<0.0001).

Next, we performed a Sholl analysis to detect morphological changes of neurons transfected with WT and mutant FUS, with and without knockdown of endogenous mbnl1 **(Fig. 3.19d and 3.19e)**[449]. We did not observe any significant differences in dendrite morphology between cortical neurons expressing FUS-WT alone, or in combination with mbnl1 shRNA. However, cells expressing mutant FUS-R521C showed a noticeable reduction in dendritic branching and elongation compared to WT-expressing cells, that appeared to be partially rescued by co-transfection with mbnl1 shRNA **(Fig. 3.19d)**. Quantification confirmed these results, as FUS-R521C-expressing cells had a statistically significant reduction in the number of intersections at increasing radii from the cell body compared to WT-expressing cells **(Fig. 3.19e)**. However, this was partially rescued by co-transfecting the cells with mbnl1 shRNA. The results from these toxicity and morphological assays suggest that muscleblind is a modifier of behavioral, morphological and pathological defects associated with FUS expression in fly and mammalian models, via regulating FUS distribution and interaction with stress granules.

3.3 DISCUSSION

The apparent heterogeneous nature of ALS complicates our understanding of its pathogenesis. To understand the mechanisms of toxicity of known ALS-causing genes, it is necessary to determine how other genes are involved in these processes, and the molecular changes that alter clinical outcomes from patient to patient. To that end, we performed an unbiased, genetic screen using a *Drosophila* model for FUS-associated ALS to identify modifiers of FUS toxicity *in vivo*. Our screen produced a very useful set of candidate genomic regions that, when one copy is deleted, either enhance or suppress the toxicity caused by mutations in *FUS*. Within one of the heterozygous regions that suppressed degeneration was *mbl*, which we pursued further as a possible modifier of FUS toxicity due to the human ortholog's (*MBNL1*) affiliations with disease. Each deficient region of the screen contains many genes, each of which could be responsible for modifying FUS toxicity. Therefore, we performed an array of assays in *Drosophila* to validate that targeted depletion of mbl, through the use of mbl RNAi, is sufficient to suppress FUS-associated neurodegeneration. We found that targeted knockdown of endogenous mbl in fly eyes reduces the external eye degeneration caused by WT and mutant FUS overexpression. Furthermore, pan-neuronal and motor neuron-specific knockdown of mbl can extend adult fly lifespan and improve larval NMJ morphological defects. Using a rat, primary, cortical neuron model for FUS-associated ALS, we found that shRNA-mediated knockdown of endogenous mbnl1 suppresses cell toxicity and restores dendritic branching. Therefore, we have confirmed with these *in vivo* and mammalian model systems that muscleblind is a modifier of FUS toxicity.

Cytoplasmic accumulation of mutant FUS and its incorporation into stress granules is a well-established, pathological hallmark of ALS. We therefore hypothesized that depleting muscleblind activity was suppressing FUS toxicity through its interaction with stress granules. We found that depleting endogenous muscleblind-like in both models reduces cytoplasmic mislocalization of mutant FUS. Reducing muscleblind-like levels also hinders FUS incorporation into cytoplasmic stress granules, following exposure to chemical stress. These results support our hypothesis that muscleblind regulates critical processes required for FUS toxicity in neurons.

Interestingly, depleting endogenous muscleblind only affects FUS-associated ALS, but does not suppress the toxicity caused by other ALS-linked proteins **(Fig. 3.7)**. This suggests that the relationship between muscleblind and FUS that allows one to affect the other, exists through a common pathway, or another component that is not shared between muscleblind and other ALS-causing proteins. All three members of the FET family of proteins (FUS, EWSR1 and TAF15) specifically bind to RNA from all three human muscleblind-like paralogs in human cells[438]. FUS itself preferentially binds to RNA transcripts with long intronic regions, a characteristic of *Drosophila mbl* that is immediately apparent from its size **(Fig. 3.2a)**[438]. It is possible that this direct interaction between FUS protein and muscleblind RNA is linked to FUS toxicity and depleting muscleblind RNA affects this interaction and suppresses cell death. Further unraveling how MBNL1 modifies FUS toxicity will improve our understanding of how mutations in FUS cause disease pathogenesis in the first place.

There are three *MBNL* (*MBNL1*, *MBNL2* and *MBNL3*) paralogs in humans[450-452], and they all code for DNA/RNA-binding proteins that are involved in RNA trafficking,

stability and alternative splicing[429-431]. In humans and *Drosophila*, muscleblind is essential for proper development and terminal differentiation of neurons[453-455], skeletal muscle[451,452] and photoreceptors[456]. *MBNL1* has been linked to numerous neurodegenerative diseases, and is best known for its involvement in myotonic dystrophy[432-437,457]. In myotonic dystrophy, MBNL1 protein functions normally, but is sequestered into nuclear inclusions due to uncontrolled binding to abnormal CUG repeat expansions in the 3' untranslated region of dystrophia myotonica protein kinase (DMPK) mRNA. Reduced availability of functional MBNL1 in the nuclei of muscle cells prevents the appropriate splicing of the protein's target RNA's, and is thought to be the cause of subsequent muscle-wasting of patients with the disease[451,457,458]. It has even been shown that *upregulating* MBNL1 suppresses the effects of the CUG repeat expansion in myotonic dystrophy[459]. Therefore, not only is it unexpected to discover that muscleblind is a modifier of FUS toxicity, it is surprising that *reducing* endogenous muscleblind levels suppresses toxicity in all three of the models used in our studies. This appears to be the opposite of what is observed in repeat expansion diseases. Interestingly, however, our results are consistent with previous work performed in a *Drosophila* model for spinocerebellar ataxia type 3, in which overexpression of MBNL1 enhanced ataxin-3 induced neurodegeneration[434]. In this work, it was proposed that MBNL1 interacts differently with the CAG repeat expansions of SCA3 than it does with CUG repeat expansions of other diseases. Similar conclusions were made by another group who showed that although MBNL1 is capable of binding to both CAG and CUG repeats, alternative splicing changes are only observed when MBNL1 is bound to CUG expansions[460,461]. From these results, they conclude that sequestration of MBNL1 alone

is not sufficient to cause aberrant changes in splicing patterns. The modification of FUS toxicity that we see in our models, following depletion of endogenous muscleblind, likely occurs through a different mechanism than what is seen in the CUG repeat expansion diseases. This also provides a possible explanation as to why MBNL1 can be involved in the pathogenesis of diseases with different tissue specificities. It has been recently shown that intermediate-length, CAG repeat expansions of the *ATXN2* gene enhance TDP-43-associated toxicity in animal and cellular models of ALS[279]. It is possible that intermediate-length CAG or CUG repeat RNAs act as molecular sponges for muscleblind protein and thereby suppress FUS toxicity. However, this remains to be determined in ALS patient samples harboring pathogenic mutations in FUS.

It is also worth noting that our experiments were performed with a modest knockdown of muscleblind, ranging from approximately 30% to approximately 50% reduction **(Fig. 3.2f and Fig. 3.12)**. This suggests that even small changes in muscleblind activity modify FUS toxicity. Given muscleblind's importance in alternative splicing, this also explains how we suppressed FUS-induced degeneration without causing cell death due to loss of muscleblind.

Here we show a mechanistic link between muscleblind activity and FUS toxicity in both cell culture and animal models of ALS. This is the first time that MBNL1 has been shown to modify ALS disease phenotypes. These results further highlight that MBNL1 is a common player in neurodegeneration, and add ALS to the list of diseases affected by this single factor. Therefore, the work presented here is not just important for understanding ALS, but impacts the field of neurodegeneration as a whole.

3.4 MATERIALS AND METHODS

Drosophila lines

UAS-FUS-WT and FUS-R521H lines generated through random genomic insertion of the transgene (random-insertion lines) were previously described[462]. FUS-WT, FUS-R518K, FUS-R521C, FUS-R521H and FUS-P525L lines generated through site-specific insertion of the transgene (site-specific integration lines) were developed at BestGene Inc. using the (attP2) insertion vector. The mbl RNAi line is from the Vienna *Drosophila* rnai Center (#107778KK). GMR-gal4 and D42-gal4 driver lines were obtained from the Bloomington *Drosophila* Stock Center (www.flystocks.bio.indiana.edu). OK371-gal4 and ELAV-GS driver lines were generous gifts from Thomas Lloyd (Johns Hopkins University School of Medicine) and Haig Keshishian (Yale University), respectively. TDP43, EWSR1 and C9orf72 lines were generous gifts from Diego Rincon-Limas (University of Florida), Nancy Bonini (University of Pennsylvania) and Peng Jin (Emory University), respectively. The dVCP lines were previously described[463].

Genetic screen in Drosophila

The Drosodel Kit used to perform this screen is a collection of more than 450 Drosophila lines from the Bloomington Drosophila Stock Center, each with a heterozygous deletion of a known region of the genome, as previously described[464,465]. By crossing these lines with our model flies that express ALS-linked, mutant, human FUS (FUS-R521H) in the eyes[462], we identified deficiency lines that either enhanced or suppressed FUS-associated degeneration of external eye structure

Eye degeneration experiments in Drosophila

Utilizing the UAS-gal4 system, flies expressing the GMR driver were crossed with flies expressing wild-type and mutant, exogenous, human FUS at 25□C. Images of *Drosophila* eyes were taken from the left eyes of day-1 adult females of the F1 generation, using a Leica M205C dissection microscope with a Leica DFC450 camera. External eye degeneration was quantified using a previously published scoring system[466]. Statistical analyses were performed using GraphPad Prism 6 software.

Western blotting (Drosophila)

Day 1, adult, female heads of the F1 generation were collected from each cross and snap-frozen on dry ice. Three heads were used for each lane of a Western blot. Heads were crushed on dry ice, and allowed to incubate in RIPA buffer (150mM NaCl, 1% NP40, 0.1% SDS, 1% sodium deoxycholate, 50mM NaF, 2mM EDTA, 1mM DTT, 0.2 mM Na orthovanadate, 1x protease inhibitor-Roche #11836170001). Lysates were then sonicated and centrifuged to remove exoskeletal debris. Supernatants were boiled in Laemmli Buffer (Boston Bioproducts, #BP-111R) for five minutes and loaded onto a 4-12%, NuPage Bis-Tris gel (Novex/Life Technologies). Proteins were transferred using the iBlot2 (Life Technologies, #13120134) onto nitrocellulose (iBlot 2 NC Regular Stacks, Invitrogen, #IB23001). Western blots were blocked with milk solution (BLOT-QuickBlocker reagent, EMD Millipore, #WB57-175GM) and incubated in primary antibody overnight. Blots were washed and incubated for one hour in secondary antibody prior to imaging on a LiCor imager (Odyssey CLx). Protein levels were quantified using Image Studio software and statistical analyses were performed with

GraphPad Prism 6 software. All Western blots were run in triplicate using biological replicates.

Western blotting (cells)

HEK293T and Neuro2a (N2A) cells from ATCC were grown up in advanced DMEM culture media (Gibco, #12491-023) containing 10% FBS (Biowest, #S01520) and 1x Glutamax (Gibco, #35050-079). Protein lysates were made from cultures by adding 1x Laemmli (Boston Bioproducts, #BP-111R) in RIPA buffer (150mM NaCl, 1% NP40, 0.1% SDS, 1% sodium deoxycholate, 50mM NaF, 2mM EDTA, 1mM DTT, 0.2 mM Na orthovanadate, 1x protease inhibitor-Roche #11836170001). Samples were then boiled for five minutes and loaded onto a 4-12% NuPage Bis-Tris gel (Novex/Life Technologies). Proteins were transferred onto nitrocellulose (iBlot 2 NC Regular Stacks, Invitrogen, #IB23001) using the iBlot2 (Life Technologies, #13120134). Blots were blocked with milk solution (BLOT- QuickBlocker reagent, EMD Millipore, #WB57-175GM) and incubated overnight in primary antibody solution. Blots were then washed and incubated in secondary antibody solution for one hour, followed by imaging on a LiCor imager (Odyssey CLx). Quantification of protein levels was performed using Image Studio software. All Western blots were run in triplicate using three individual lysates from cultured cells.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR)

RNA was extracted in triplicate from either *Drosophila* tissues or cultured cells using Trizol (Ambion, #15596026) in a phenol-chloroform extraction. Following addition of

samples to Trizol, chloroform was added and samples were centrifuged. The upper aqueous layer was isolated and RNA was precipitated with isopropanol and pelleted through centrifugation. RNA pellets were then washed with 75% ethanol, centrifuged and allowed to air-dry. RNase-free water was used for resuspending RNA. RNA samples were quantified on a NanoDrop ND-1000 spectrophotometer, and purity was verified using 260/280 and 260/230 ratios. Samples were also run on a 1% agarose gel with ethidium bromide to verify the quality of the RNA through the absence of degradation. RNA samples were then used to make cDNA with the BioRad iScript Select cDNA Synthesis Kit (#170-8897) in a Thermo Hybaid, Omn-E PCR machine. Three separate RNA extractions from each experimental group were used to make cDNA, with a control lacking reverse transcriptase to confirm the absence of genomic DNA contamination. All cDNA samples were run on a 96-well plate (Applied Biosystems, #4306737) on an Applied Biosystems, 7300 Real Time PCR System, using the BioRad iQ Supermix (#170-8862). Primers were obtained from Integrated DNA Technologies (IDT) using their PrimeTime qPCR Assays (www.idtdna.com). α-tubulin and GAPDH were used for *Drosophila* and human housekeeping controls, respectively. C(T) values were recorded and analyzed with the comparative C(T) method as previously described[467] using GraphPad Prism 6 software for statistical analyses.

NMJ analyses and immunofluorescence (Drosophila)

Third-instar, wandering larvae from the F1 generation were rinsed in ice-cold phosphate-buffered saline (Lonza, #17-512F) and dissected along the dorsal midline. All tissues except the brain and nerves were removed to expose the muscles and

neuromuscular junctions (NMJ's). The dissected larval pellet was fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature. The larval pelts were given a wash with PBS followed by blocking with 5% Normal Goat Serum (NGS) (Abcam, #AB7681)in 0.1% PBST (0.1% Triton X-100 in PBS). Following blocking, the larval pelts were probed with primary antibodies overnight at 4□ C. They were then washed several times with 0.1% PBST followed by incubation with secondary antibodies for 2 hours at room temperature, subsequently followed by washes with 0.1% PBST. Larvae were then mounted onto slides using Prolong Gold (Invitrogen, #P36930) mounting media. Confocal images were acquired using Olympus IX81, Fluoview FV1000, confocal microscope and a 60x oil objective was used to image the NMJs. Both primary and secondary antibody solutions were prepared in 5% NGS in 0.1% PBST. For the analyses, NMJs innervating muscle 4 on segments A2-A5 were imaged and analyzed for synaptic bouton quantification. Mature boutons are defined as boutons that are included in a chain of 2 or more boutons. Satellite boutons are defined as a single bouton that is not included in a chain of boutons, and instead, sprouts off of a mature bouton or branch. Statistical analyses were performed with GraphPad Prism 6 analysis software.

Drosophila lifespan experiments

Motor neuron driver (OK371-gal4)- The OK371-gal4, motor neuron-specific driver was crossed with human FUS-WT and mutant-expressing flies generated through random insertion of the transgene. Crosses were performed at 25□C and day 1 female F1 progeny were removed from cross vials and placed in fresh vials containing standard

Drosophila food at 20 flies/vial. The number of dead flies from each cross were counted each day, and the data was used to generate a Kaplan-Meyer survival curve on GraphPad Prism 6 statistical software. Logrank with Grehan-Breslow-Wilcoxon tests were performed to determine statistical significances.

Pan-neuronal driver (ELAV-GS)- The ELAV-GS, pan-neuronal driver was crossed with human FUS-WT and mutant-expressing flies generated through either random insertion or site-specific integration of the transgene. Crosses were performed at 25□C and day 1 female F1 progeny were removed from cross vials and placed in fresh vials containing standard *Drosophila* food supplemented to contain 20uM RU486 (mifepristone- Cayman Chemicals, #10006317). 20 flies were placed in each vial and the number of dead flies from each cross were counted daily. The data was used to generate a Kaplan-Meyer survival curve on GraphPad Prism 6 software. Logrank with Grehan-Breslow-Wilcoxon tests were performed to determine statistical significances.

Immunocytochemistry and confocal microscopy

HEK293T cells- HEK293T cells from ATCC were cultured on coverslips coated with Poly-L-Lysine (Sigma, #P4832). Transfections were performed with Turbofect transfection reagent (Thermo Scientific, #R0531), using the manufacturer's protocol. MBNL1 shRNA and scramble shRNA was received from Origene (#TL303325). Cells were washed, incubated in primary antibody, washed again, and incubated in secondary antibody as previously described[55]. Coverslips were then mounted onto

slides with Prolong Gold (Invitrogen, #P36930) mounting media. Cells were imaged with a Zeiss LSM 710 confocal microscope as previously described[55].

Primary neuronal cultures- Primary neuronal cultures were fixed in 4% (volume/volume) paraformaldehyde/sucrose in PBS for 10 minutes at room temperature. Neurons were then permeabilized with 0.25% triton X-100 in PBS for 10 minutes at room temperature and blocked in 10% Horse serum + 2% BSA in PBS for 1 h at room temperature, incubated with primary antibodies at 4 °C overnight, secondary antibodies at room temperature for 1 h and mounted using prolong-diamond anti-fade mounting media with DAPI (Invitrogen). Neurons were washed with PBS three times between each step. Cells were imaged using 40X or 60X objectives of a confocal microscope (Olympus FV1000). Z stack images were acquired and projected onto one single image.

Primary neuronal cultures and transfections

Primary cortical neurons were prepared from E17.5 rat embryos as previously described[468]. Cortical neurons were plated at a density of 125,000/well on poly-D-lysine coated 24-well plates or 12 mm coverslips. Neurons were transfected using Lipofectamine 2000 at DIV 12 with 1 µg of total DNA/well at a ratio of 2:1.

Neuronal toxicity and analysis

For neuronal survival analysis, we used time lapse live cell fluorescence microscopy as previously described[448]. Briefly, neurons were transfected on DIV 12 with 750 ng of mcherry tagged FUS and 250 ng of GFP tagged mbnl1 or scramble shRNA constructs

(Origene, #TL510761). After 24 hours, neurons were labeled with 3uM DRAQ-7 dye (Biostatus) which incorporates in to nuclei of dead cells[469]. Transfected neurons were tracked with an inverted Nikon Eclipse microscope (Nikon Eclipse Ti) equipped with a 20× objective lens (numerical aperture 0.45) and an Andor Zyla sCMOS camera. The same neurons tracked at 24 hours were subsequently tracked at 48 hours and 72 hours. Illumination was provided by a LED light source light (X-cite). During image acquisition, neurons were kept at 37°C, 5% CO_2. All movements of the stage were controlled with software controlled motors. Coordination of fluorescence excitation and emission filters, stage movements, focusing, and imaging acquisition was performed with NIS Elements software (Nikon). Acquired images were analyzed by image J. Dead cells were identified and scored by direct visualization of DRAQ7 positive neurons. At least 100 transfected cells were scored per condition.

Sholl analysis

To analyze dendrite morphology, neurons were stained against the dendritic protein MAP-2. Fluorescence images were acquired, and neurites were traced using simple neurite tracer plugin (Image J). Dendritic arborization was analyzed using Sholl analysis[449] by counting the number of intersections of dendrites with concentric circles centered on the cell body whose radius increased at regular steps of 10 μm. Sholl analysis was performed with ImageJ. The number of intersections was plotted as a function of the radial distance from the soma. At least 5 neurons were analyzed per condition.

Stress granule induction and analysis

Cellular stress granule (SG) assembly was induced in cultured neurons by sodium arsenite as previously described[55]. Briefly cells were treated with 500 μM sodium arsenite for 90 minutes at 37□C. The cells were then fixed and incubated with the appropriate antibodies (anti-G3BP1 and anti-TIA1). SG number per cell was assessed by using the ImageJ analyze particle plugin. G3BP1 puncta above the size threshold of 1 μm^2 were included for analysis.

Antibodies

Drosophila Western blots- For primary antibodies, the following concentrations were used: anti-FUS (Bethyl Labs, #A300-302A, 1:2000); anti-MBNL1 (Millipore, MabE70, 1:2000); anti-mbnl1 (Fisher, #MA1-16967, 1:1000). For secondary antibodies, DyLight fluorescent antibodies (Thermo Fisher) were used at a concentration of 1:10,000.

Drosophila NMJs- For primary antibodies, the following concentrations were used: Alexa Fluor 488-conjugated goat anti-HRP (Jackson ImmunoResearch, #123-545-021, 1:200); mouse anti-DLG 4F3 (DSHB, 1:100). For secondary antibodies, the following antibody concentrations were used: Alexa Fluor 647-conjugated anti-Phalloidin (Invitrogen, #A22287, 1:250); goat anti-mouse Alexa Fluor 546 (Invitrogen, #A11030, 1:500).

HEK293T and N2A cell cultures- For primary antibodies, the following concentrations were used: anti-G3BP1 (Protein Tech, #13057-2-AP, 1:25); anti-HA7 (Sigma, #H3663,

1:500). For secondary antibodies, Alexa-Fluor fluorescent antibodies (Invitrogen) were used at a concentration of 1:500.

Primary neuronal cultures- anti-MAP2 (Milipore, #AB5622, 1:500); anti-FUS (Proteintech, #11570-1-AP, 1:200); anti-TIA1 (Santa Cruz, #SC-1751, 1:250); anti-G3BP1 (Proteintech, #D5444, 1:100); anti-MBNL1 (Millipore# MABE70, 1:100); anti-NEFL (Millipore).

qPCR Primers

All primers for qPCR were obtained from Integrated DNA Technologies (IDT) using their online PrimerQuest primer design tool (www.idtdna.com). IDT PrimeTime qPCR Assays were used as the primer/probe solutions. See **Table 3.3** for a complete list of primers and probes used for qPCR assays.

Cell Lines

HEK293T (#CRL-3216) and Neuro-2A (N2A, #CCL-131) cell lines were freshly obtained from ATCC for this study, and were maintained and utilized at passages 2-5.

Statistics

Drosophila- For eye degeneration experiments, severity of degeneration was quantified as previously described[466]. Statistical comparisons between groups were performed with either Mann-Witney t-tests or Kruskal-Wallis tests with Dunn's multiple comparisons, as indicated. For lifespan experiments Kaplan-Meier survival analyses

were carried out using GraphPad Prism 6 software. All other comparisons between groups in all graphs were made using either T-tests or by one-way ANOVAs with Tukey's or Dunnet's multiple comparisons tests, as indicated. $P<0.05$ was considered statistically significant.

HEK293T and N2A cell cultures- Comparisons between groups in all bar graphs were made using GraphPad Prism 6 software and were performed with either T-tests or by one-way ANOVAs with Tukey's or Dunnet's multiple comparisons tests, as indicated. $P<0.05$ was used to determine statistical significance.

Primary neuronal cultures- For survival analysis, death was defined by the time at which a neuron was last imaged devoid of nuclear DRAQ7 signal. Kaplan-Meier survival and Cox proportional hazards analyses were carried out using SPSS 19.0 software, while other statistical analysis was carried out using GraphPad Prism 6. Differences in Kaplan-Meier curves were assessed with the log-rank test. Comparisons between experimental groups in bar graphs were done by one-way ANOVA or t-test as indicated. Data are expressed as average ± sem. $P<0.05$ was considered statistically significant.

3.5 ACKNOWLEDGEMENTS

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4.0 SUMMARY AND FUTURE DIRECTIONS

Even though the first genetic cause of ALS was identified more than two decades ago, it is still unclear how pathogenic mutations cause ALS. However, as more genes are linked to the disease, commonalities in gene function begin to emerge. These commonalities highlight specific cellular processes, such as RNA processing and autophagy, that may be disrupted due to genetic mutations that affect protein function. How these mutations affect these processes, however, is still unknown. FUS is an RNA/DNA-binding protein with critical functions in RNA processing. To date, numerous mutations of *FUS* have been identified in ALS patients. FUS-ALS is typically more aggressive than ALS resulting from mutations of other genes, with earlier ages of onset and shorter disease courses[96]. This, in addition to functional overlaps with other ALS-linked proteins such as VCP, TDP-43 and HNRNPA1, make FUS a particularly desirable candidate for treatment of the disease. Not only would treating FUS-ALS benefit ALS patients with some of the worst prognoses, but treatments for one RNA-binding protein may also be applicable to the others. To this aim, our laboratory developed a *Drosophila melanogaster* model for understanding FUS-associated ALS[417,462]. *Drosophila* are a useful organism for modeling diseases. They have been particularly helpful for studying neurodegenerative diseases, including ALS (reviewed in Chapter 2). Fruit flies are inexpensive to work with and they have short lifespans which

allows for quick turn-around times for experiments. Their genome is fully sequenced and several genetic tools are available for working with *Drosophila*, such as RNAi lines for knocking down genes of interest and the UAS-gal4 system for tissue-specific gene expression. We have utilized this model in the work presented in this dissertation.

Understanding the root causes of ALS is complicated by the fact that it is a complex, heterogeneous disease. Therefore, different pathways may be affected in different individuals depending on their gene mutation. Furthermore, pathology is likely mediated by additional factors, such as mutations in other genes, environmental exposures or the health of the individual. This is particularly apparent in FALS cases where family members with the same point mutation exhibit differences in disease onset and progression[51]. Identifying the factors that modify ALS pathology will therefore help us to understand the molecular pathways that are affected by pathogenic mutations of ALS-causing genes.

To this end, we performed an unbiased genetic screen with our *Drosophila* model for FUS-associated ALS to identify genetic modifiers of FUS toxicity. Our screen identified muscleblind as a novel modifier of FUS toxicity in our model. Specifically, depleting endogenous levels of muscleblind in flies suppressed behavioral and morphological defects caused by mutant FUS. We validated our findings in primary rat cortical neurons where knocking down endogenous muscleblind-like suppresses mutant FUS-associated toxicity and dendrite morphological defects. Additional work in mammalian cell culture models aimed at understanding the mechanism behind the ability of muscleblind to mediate FUS toxicity revealed that lowering muscleblind protein levels reduces the amount of FUS that mislocalizes to the cytoplasm and incorporates

into stress granules. Cytoplasmic mislocalization and accumulation into stress granules are pathological hallmarks of mutant FUS in postmortem neurons of ALS patients, and reducing these appears to be the mechanism by which muscleblind mediates toxicity.

Therefore, in this present study, we have identified muscleblind as a modifier of FUS toxicity in multiple ALS models. Muscleblind mediates the effects of FUS mutations by rescuing aberrant localization of the mutant protein and hindering its interactions with stress granules in neurons. Additional work is needed to understand how this occurs. Altered stress granule dynamics is a popular topic in ALS research right now, particularly with respect to the interplay between autophagy and the improper turnover of these cytoplasmic aggregates[55,62]. Live-cell imaging would be an effective way to observe the effects of depleting muscleblind on stress granules. Specifically, observing SGs before and after exposure to cellular stress would show whether knocking down muscleblind hinders the assembly, or promotes the disassembly, of the aggregates. This would elucidate the specific pathways associated with SGs that are affected by FUS mutations.

FUS protein preferentially binds to RNAs with long intronic sequences[414]. *Drosophila* mbl RNA contains long introns. It has also been previously shown that human muscleblind-like RNAs are targets of FUS[438]. It is possible that in our work presented here, depleting muscleblind RNA levels is reducing direct interactions with FUS protein, thereby affecting nuclear/cytoplasmic transport of FUS or integration of FUS into cytoplasmic SGs. These interactions can be validated through fluorescence *in situ* hybridization (FISH) analyses of muscleblind RNA and immunofluorescence of FUS protein. Changes in the amount of interaction can be quantified by immunoprecipitation

of FUS followed by RT-qPCR of any bound muscleblind RNA. These experiments would help define whether muscleblind mediates FUS toxicity through direct or indirect mechanisms.

To explore an indirect mechanism for suppression, RNA sequencing analyses can be performed in our *Drosophila* model to identify global changes in gene expression in flies expressing mutant FUS, with and without concurrent depletion of endogenous mbl. RNA sequencing may highlight components of specific pathways, such as the autophagy pathway, that are affected by knocking down muscleblind and would be directly related to the results of our current work.

In the work presented here, total muscleblind RNA levels were reduced via RNA interference. Additional experiments in *Drosophila* using mbl mutants that remove or alter protein function[470-472] would help to elucidate whether it is muscleblind protein or muscleblind RNA that is important for mediating FUS toxicity. These would also help to specify whether loss of a specific mbl function is sufficient for suppression, or if total loss of function is required.

With no cures and marginally effective treatments currently available to ALS patients, there is a great need for new therapies. One strategy that has grown in popularity in recent years is the use of antisense oligonucleotides to target and knock down mutant RNA in neurons (see chapter 1.4.2). Since we have shown that knocking down endogenous muscleblind has a protective effect in neurons, this technology would be worth pursuing as a potential therapy for FUS-associated ALS. In this case, however, muscleblind, not mutant FUS, would be the target.

In ALS patients, single point mutations of FUS do not affect everyone in the same way. Since muscleblind is a modifier of FUS toxicity, it is possible that endogenous muscleblind-like plays a role in different outcomes for patients with FUS-ALS. Muscleblind-like proteins bind to CAG and CUG RNA repeats. Although this property has been shown to potentially contribute to repeat expansion diseases such as spinocerebellar ataxia and myotonic dystrophy, no work has been done to show a correlation with ALS. It is possible that moderate-length repeats of genes in FUS-ALS patients sequester endogenous muscleblind-like proteins to different extents, based on the size of the repeat, thus depleting them to different levels. This may contribute to changes in disease severity and progression in these individuals. If true, then introducing CAG and CUG repeats into our FUS models should mimic the suppression that we see when knocking down endogenous muscleblind. Sequencing ALS patients for known repeat expansions would confirm these results in humans. It may even be possible to perform linkage analyses in FALS cases to "link" repeat lengths with severity of the disease in each affected family member.

In this project, we successfully utilized *Drosophila* and mammalian cell culture models to identify muscleblind as a novel modifier of FUS-associated toxicity in ALS. We determined that muscleblind mediates cell death by altering cytoplasmic mislocalization of FUS protein, as well as its ability to aggregate into stress granules. We have laid the foundation for new experiments focusing on the pathological relationship between FUS and muscleblind. This also links ALS to other neurodegenerative diseases that are affected by muscleblind, and thus our work is beneficial to entire field of nerodegeneration.

APPENDIX: TABLES

Table 2.1 ALS *Drosophila* Models Developed Since 2008.

Table 2.1 ALS *Drosophila* Models Developed Since 2008		
Disease	**Phenotype**	**Reference**
SOD1	Expression of WT or mutant hSOD1 caused climbing defect, reduced synaptic transmission and hSOD1 aggregation in motor neurons.	Watson *et al.* 2008
	Ubiquitous expression of zinc-deficient SOD1 results in locomotor dysfunction, altered mitochondrial structure & reduced ATP levels.	Bahadorani *et al.* 2013
Alsin	Age dependent reduction in adult locomotion in flies homozygous for mutant dALS2 compared to WT controls.	Takayama *et al.* 2014
VAPB	dVAP-null mutant flies exhibit morphological defects at the NMJ, quantal content and size and the number of presynaptic active zones.	Chai *et al.* 2008
	WT dVAP overexpression exhibit morphological changes at the NMJ. dVAP P58S mutant protein expression causes morphological changes at the NMJ and induced the formation of cytoplasmic aggregates.	Ratnaparkhi *et al.* 2008
	Only N-terminal-WT dVAP that includes an MSP domain is cleaved at the cell membrane and secreted as a ligand for Eph receptors.	Tsuda *et al.* 2008
	dVAP T48I mutant protein expression causes external eye degeneration and elevated Hsp70 expression in brain.	Chen *et al.* 2010
	dVAP-null mutant flies exhibit morphological defects of muscle mitochondria.	Han *et al.* 2012
	Endogenous dVAP protein interacts with Down syndrome cell adhesion molecule (Dscam).	Yang *et al.* 2012
	dVAP P85S mutant protein sequesters Sac1 in cytoplasmic granules, increasing PtdIns4P levels, causing degenerative phenotypes.	Forrest *et al.* 2013
	Oxysterol binding protein (Osbp) interacts with WT dVAP. Osbp localizes to cytoplasmic aggregates in dVAP P85S mutant-expressing cells, and localizes to golgi in WT dVAP-expressing cells.	Moustaqim-Barrette *et al.* 2014
	dVAP overexpression caused eye degeneration, wing posture, pupal eclosion rates, protein aggregation and defects at the NMJ.	Sanhueza *et al.* 2014
TDP-43	Reduced lifespan, adult locomotion, axonal branching at neuromuscular synapses and reduction in the number of synaptic boutons upon reduced expression of endogenous dTDP-43.	Feiguin *et al.* 2009
	Reduction of dendritic branching of larval sensory neurons upon reduced expression of WT hTDP-43 or WT dTDP-43. Increase of dendritic branching upon overexpression of dTDP-43.	Lu *et al.* 2009

Table 2.1 Continued

	Reduced HDAC6 mRNA levels in *TBPH* knockout flies.	Fiesel *et al.* 2010
	Axon loss and neuronal death with hTDP-43 overexpression and dTDP-43 knockdown in mushroom bodies. Overexpressing hTDP-43 in motor neurons causes cytoplasmic accumulation of TDP-43 aggregates, cell death, neuron swelling and NMJ morphological defects.	Li *et al.* 2010
	hTDP-43 overexpression in flies caused age-dependent eye degeneration, reduced lifespan and adult wing malformations.	Hanson *et al.* 2010
	Mild degeneration caused by dVCP is enhanced by coexpression with dTDP-43, and suppressed with knockdown of dTDP-43. Coexpression of either dVCP R152H with WT hTDP-43 or WT dVCP with hTDP-43 M337V causes enhanced eye degeneration.	Ritson *et al.* 2010
	Flies exhibit suppressed ALS-associated phenotypes when expressed TDP-43 mutants lack RNA-binding ability.	Voigt *et al.* 2010
	dAtx2 overexpression enhances eye degeneration and decreases adult lifespan when coexpressed with WT hTDP-43.	Elden *et al.* 2010
	Larvae expressing dTDP-43 in neurons as well as larvae carrying dTDP-43 null mutants exhibit changes at the NMJ. Overexpression in mushroom bodies leads to a decrease in lobe size, as well as the formation of TDP-43-positive cytoplasmic aggregates.	Lin *et al.* 2011
	Hyperphosphorylation of hTDP-43 reduces TDP-43 cytoplasmic aggregation in neurons. Hypophosphorylation increases aggregation.	Li *et al.* 2010
	hTDP-43 NLS-deficient mutants cause severe degeneration in adult eyes, lethality when expressed in larval neurons, and reduced adult lifespan when expressed in adult neurons.	Miguel *et al.* 2011
	Expressing hTDP-43 A315T mutant causes eye degeneration. Expressing either WT or A315T mutant TDP-43 causes mislocalization and aggregate formation in axons of eye imaginal disks, with WT TDP-43-expressing flies exhibiting more severe aggregation.	Estes *et al.* 2011
	Upregulation of endogenous chaperones reduces TDP-43 and TDP-25-associted phenotypes, including cellular aggregation.	Gregory *et al.* 2012
	Larvae overexpressing dTDP-43 produce a different expression profile in *Drosophila* CNS than larvae carrying dTDP-43-null mutants.	Hazelett *et al.* 2012
	Expression of *Drosophila Itp-r83A* mutants in motor neurons causes a mild increase of hTDP-43 cytoplasmic mislocalization, as well as a partial rescue hTDP-43-associated lifespan and climbing defects in adult flies.	Kim *et al.* 2012
	Overexpressing human TDP-43 in adult motor neurons causes a reduction in lifespan that is not rescued by expression of the human caspase inhibitor, P35, or the *Drosophila* homolog, dIAP.	Zhan *et al.* 2013
	Pan neuronal overexpression and reduced expression of dTDP-43 both result in a reduction of adult lifespan, as well as reduced adult and larval locomotion. Overexpression of dTDP-43 in third instar larvae eye imaginal disks does not	Diaper *et al.* 2013b

Table 2.1 Continued

	result in cytoplasmic mislocalization of the protein.	
	Reducing dTDP-43 protein levels in glial and muscle cells causes movement disorders in adult flies. Overexpressing dTDP-43 resulted in early lethality and formation of sarcoplasmic aggregates. Altering dTDP-43 protein levels in glia and neurons also alters dEAAT1 and dEAAT2 mRNA levels.	Diaper *et al*. 2013a
	In glial cells, WT hTDP-43 as well as A315T, D169G, G298S and N345K mutants mislocalize to the cytoplasm, reduce larval motor function, reduce bouton number at the NMJ, and increase the number of postsynaptic glutamate receptors.	Estes *et al*. 2013
	WT hTDP-43 and G298S and M337V mutants cause retinal degeneration in adult eyes. WT and mutant proteins lacking a NLS undergo cytoplasmic mislocalization that corresponds to severe retinal degeneration.	Ihara *et al.* 2013
	Altering levels of stress-granule associated proteins, ROX8, PEK and GADD34, causes changes in TDP-43 toxicity in neurons of adult flies. Inhibiting stress granule formation reduces toxicity.	Kim *et al.* 2014
FUS	Expression of WT hFUS and mutant hFUS (R524S and P525L) causes eye degeneration in adult flies. Neurons for both WT and mutant are enlarged, bouton number is reduced, and larval locomotion is decreased.	Chen *et al.* 2011
	Conditional expression of WT hFUS and mutant hFUS (R518K, R521C and R521H) in adult neurons reduces lifespan and locomotion. hFUS R521C mutants have a reduced number of presynaptic active zones at the NMJ.	Lanson, Jr. *et al.* 2011 Shahidullah *et al.* 2013
	dFUS-null mutant causes reduced adult lifespan and locomotion, and reduced pupae eclosion when expressed pan-neuronally. Coexpression with dFUS or hFUS rescues all phenotypes.	Wang *et al.* 2011
	Cytoplasmic accumulation of insoluble, non-aggregated hFUS occurs in neurons over time, and corresponds to neurodegenerative phenotypes. Reduction of insoluble FUS with the chaperone protein reduces phenotypes.	Miguel *et al.* 2012
	RNAi knockdown of dFUS in neurons causes reduced adult climbing, bouton number and synaptic branches.	Sasayama *et al.* 2012
	Expression of WT dFUS causes motor neuron (MN) apoptosis. dFUS-null mutants do not cause MN apoptosis.	Xia *et al.* 2012
	Perturbing the RNA-binding ability of WT and mutant FUS strongly suppresses toxicity, including eye degeneration, larval eclosion and mobility and cytoplasmic mislocalization into stress granules.	Daigle *et al*. 2013
TAF15	Expression of WT human TAF15 in *Drosophila* eyes and neurons causes eye degeneration and locomotor dysfunctions respectively.	Couthouis *et al.* 2011
EWSR1	WT and mutant (G511A and P552L) EWSR1 expression induce degeneration in adult eyes, reduced life span and climbing ability.	Couthouis et al. 2012
C9orf72	30-repeat expansion of riboGGGGCC causes eye degeneration, reduced adult locomotion, and colocalization of the RNA-binding protein, Pur α, with ubiquitin in cellular	Xu *et al.* 2013

Table 2.1 Continued

	inclusions.	
	Flies expressing arginine-rich dipeptide-repeats exhibit eye degeneration, reduced eclosion rate, and reduced adult lifespan.	Mizielinska *et al.* 2014
hnRNPA2	hnRNPA2 D290V mutant expression causes degeneration of indirect flight muscles and mislocalizes to cytoplasmic aggregates. Removal of prion-like domain function rescues these phenotypes.	Kim *et al.* 2013

Table 3.1 Summary of the deficiency lines identified in our screen as modifiers of mutant FUS toxicity.

Deficiency Line	Bloomington Stock #	Enhance (E) / Suppress (S)	Chromosome
Df(1)Exel7468	7768	**S**	1
Df(1)ED6584	9348	**S**	1
Df(1)ED6712	9169	**S**	1
Df(2R)Exel6066	7548	**S**	2
Df(2L)BSC688	26540	**S**	2
Df(2L)BSC50	8469	**S**	2
Df(2L)ED4651	8904	**S**	2
Df(2L)Exel6012	7498	**S**	2
Df(2L)BSC180	9610	**S**	2
Df(2L)ED7853	24124	**S**	2
Df(2L)BSC209	7739	**S**	2
Df(3R)Exel6201	7680	**S**	3
Df(3R)BSC568	25126	**S**	3
Df(3R)Exel6272	7739	**S**	3
Df(3R)BSC137	9497	**S**	3
Df(3L)Exel8104	7929	**S**	3
Df(1)BSC834	27886	**E**	1
Df(1)BSC772	26869	**E**	1
Df(1)FDD-0024486	23295	**E**	1
Df(2L)BSC768	26865	**E**	2
Df(2L)BSC244	9718	**E**	2
Df(2R)M41A10	741	**E**	2
Df(2R)Exel6064	7546	**E**	2
Df(3R)ED10845	9487	**E**	3

Table 3.2 List of the Drosophila genes located within the genomic region that is spanned by the Df(2R)Exel6066 deficiency line.

Gene	VDRC RNAi Lines Available?	Site-Specific Integration RNAi Lines?
CG30456	**Yes**	**Yes**
CG15611	**Yes**	**Yes**
Amy-p	Yes	No
CR45272	No	No
Spn53F	**Yes**	**Yes**
Amy-d	**Yes**	**Yes**
CG15605	**Yes**	**Yes**
Cda9	Yes	No
CR45273	No	No
Acp54A1	**Yes**	**Yes**
CG11400	**Yes**	**Yes**
CR44391	No	No
Gbp1	**Yes**	**Yes**
Gbp2	Yes	No
CG43103	No	No
CG43107	No	No
CR44386	No	No
snoRNA:U3:54Aa	No	No
tRNA:Leu-AAG-1-2	No	No
tRNA:Leu-AAG-1-3	No	No
snoRNA:U3:54Ab	Yes	No
CG17290	**Yes**	**Yes**
CG17287	Yes	No
CG30458	Yes	No
CG30457	Yes	No
CG10953	Yes	No
CR44387	No	No
CG44388	No	No
CR44389	No	No
CR45323	Yes	No
CG10950	Yes	No
CG43237	No	No
CR46235	No	No
CG18469	**Yes**	**Yes**
CG12699	**Yes**	**Yes**
Mbl	**Yes**	**Yes**
CR43660	No	No
CG43272	Yes	No
CG43108	No	No
CR45997	No	No
CR43661	No	No
CR44344	No	No
tRNA:Ala-TGC-1-1	No	No
tRNA:Ala-TGC-2-1	Yes	No

Table 3.2 Continued

CG10939	**Yes**	**Yes**

Table 3.3 List of primers used for qPCR analyses.

Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe (5'-3')
mbl (*Drosophila)*	ACTTGCATTCGGTGTCCTG	GTTGTCAATATGAACAGCCTGC	CAACGGCAAGGATTCGCGCT
FUS (Human)	CACAGACTCAATTGTAACATTCTCA	GGAGGCAGAGGTGGCAT	AGGCCTTGCACAAAGATGGTGTTG
alphatub84b (*Drosophilia*)	CCTCGAAATCGTAGCTCTACAC	ACCAGCCTGACCAACATG	TCACACGCGACAAGGAAAATTCACAGA
MBNL1 (Human)	TGCGTCCATTTATCTCCAACT	GAGTAATCGCCTGCTTTGATTC	TGCAAATATCTTCATCCACCCCCACA
GAPDH (Human)	TGTAGTTGAGGTCAATGAAGGG	ACATCGCTCAGACACCATG	AAGGTCGGAGTCAACGGATTTGGTC

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