

TDP-43 and FUS in Amyotrophic Lateral Sclerosis: From Animal Models to Disease
Mechanisms

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ABSTRACT

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Amyotrophic lateral sclerosis (ALS) is an aggressive neurodegenerative disease in which motor neurons selectively degenerate, leading to paralysis and death. Rare causal mutations in *FUS* and *TARDBP* implicated RNA binding proteins and RNA metabolism in ALS disease mechanisms. The absence of faithful animal models has impeded precise understanding of the impact of ALS mutations on all functions of ALS-associated proteins. In my graduate studies, I used a novel, animal model of FUS-ALS to explore gain of function disease mechanisms and observed specific, aberrant interactions between mutant FUS and other RNA binding proteins including hnRNP U. Genetic experiments indicate loss of hnRNP U is toxic to motor neurons, suggesting mutant FUS toxicity may result from hnRNP U sequestration and loss of function. In a parallel series of experiments, I also generated novel knock-in mouse models of ALS expressing pathogenic *TARDBP* mutations to address the flaws of existing model systems and to study the functional consequences of disease-related mutations. We demonstrate that the ALS mutant alleles TDP-43^{M337V} and TDP-43^{G298S} are fully functional and are insufficient to cause age-dependent motor neuron pathology, indicating that physiological levels of mutant TDP-43 are alone insufficient to initiate disease. This model enables future exploration of the interaction between genetic and environmental factors that lead to TDP-43 toxicity in ALS and related disorders. Collectively, our findings suggest a gain of function mechanism of toxicity in which

mutations and aging, with other factors, alter the local concentration of RNA binding proteins, leading motor neurons to degenerate.

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DEDICATION

This work is dedicated to those suffering from degenerative diseases and their families and friends who care for them.

Chapter 1: Introduction

Amyotrophic lateral sclerosis (ALS), commonly known as Lou Gehrig's disease in the United States is a progressive, mostly adult onset, motor neuron disease leading to paralysis and death. In 90% of ALS patients, there is no known family history, and in these sporadic cases, the cause of disease is mostly unknown. The 10% that are familial has led to the identification of a growing number of genes in which mutations have been found to cause the disease, and spontaneous mutations in these same genes account for a small percentage of sporadic ALS cases. Clinical and pathological similarities between sporadic and familial ALS suggest that mutations in a variety of genes in addition to unidentified environmental causes converge in common mechanisms of neurodegeneration, however the nature of these mechanisms is still unknown. Riluzole is the only approved therapeutic for the disease and its benefit is modest, extending the lifespan of patients by only 3-6 months (Rowland & Shneider 2001). The absence of effective therapeutics almost 150 years after the description of the disease necessitates further research into mechanisms of disease.

1.1 Early ALS research

Jean-Martin Charcot first described ALS in 1874, and his description of the disease remains largely unchanged from how we know ALS today. He described progressive paralysis accompanied by muscle rigidity and atrophy in the absence of sensory loss, resulting in death in 2 or 3 years. He also noted that onset was associated with a focality, with symptoms starting in the limb or bulbar regions and in some cases

on just one side of the body, and certain muscles such as the rectum were spared. Perhaps most impressive was his ability to correlate clinical observations with pathological observations upon autopsy. In this way he named the disease Amyotrophic lateral sclerosis, after the observed muscle atrophy (amyotrophy) and weakness associated with the hardening of the lateral columns of the spinal cord (lateral sclerosis) (Rowland & Shneider 2001; Rowland 2001).

Ensuing research described many pathological features of the disease, which include motor neuron loss, gliosis, aberrant inclusions, and neurofilament accumulation (Hirano et al. 1967; Schiffer et al. 1996; Earle et al. 1969; Leigh et al. 1988). These and other observations led to many early theories on the mechanisms of neurodegeneration including toxic environmental factors, metabolic defects, immunologic abnormality, viral infection, heavy metal toxicity, axonal transport defects, DNA damage, and aging (Mitsumoto et al. 1988). Through the course of ALS research, excitement for some of these mechanisms has waned, while others remain active areas of study.

ALS is diagnosed clinically by signs of upper and lower motor neuron degeneration accompanied by a progressive spread of symptoms and the absence of evidence suggesting other diseases (Brooks et al. 2000). On a pathological level, ALS is diagnosed by loss of upper and lower motor neurons and the degeneration of the corticospinal tract, and in addition presence of ubiquitinated, cytoplasmic inclusions (Geser et al. 2010).

Clinical observations suggest a multifactorial etiology of ALS with family history, sex, and age as strong risk factors (Oskarsson et al. 2015). Family history led to the identification of causal mutations in familial ALS, which are now known to occur *de novo*

in sporadic cases. ALS is also ~1.6 times more common in men than women, indicating sex as a risk factor (Mitsumoto et al. 1988). With few exceptions, ALS is an adult onset disorder, with onset typically above the age of 40 (Rowland & Shneider 2001). This is true in both familial and sporadic ALS, suggesting age as a precipitating factor even in the presence of a genetic mutation. Environmental factors are also implicated from high ALS prevalence in Guam that cannot be explained by founder effects (McGeer et al. 1997) and additional epidemiological studies (Oskarsson et al. 2015). Lastly, focal onset of muscle weakness that progresses contiguously in the brain and spinal cord suggests that certain factors selectively induce neurodegeneration locally, even in the presence of ubiquitous causal mutations (Ravits et al. 2007). Together, these data paint a complex picture of ALS as a disease with multiple underlying causes.

1.2 SOD1 in ALS

A major breakthrough in ALS research came in 1993 with the identification of causal mutations in 20% of familial ALS cases in *Superoxide Dismutase 1 (SOD1)* (Rosen et al. 1993), which encodes a ubiquitously expressed enzyme that breaks down reactive oxygen species. This changed the landscape of ALS research as it allowed scientists to create animal models of the disease and therefore move from clinical and post mortem descriptive analyses to mechanistic experimentation. The clinical and pathological similarities between familial and sporadic ALS suggested that modeling the effects of SOD1 mutations may lead to discoveries that one could generalize to ALS as a whole.

SOD1 animal models

SOD1 animal models were generated immediately after the discovery of causal mutations. In general, overexpression of mutant, but not wild type, SOD1 leads to a progressive motor phenotype that recapitulates key features of ALS in humans, including motor neuron loss, premature death, and SOD1 aggregation, with varying ages of disease onset and death (Turner & Talbot 2008). Further experiments determined the motor neuron degeneration was associated with a toxic gain of function and not an SOD1 loss of function, as expression of mutant protein led to a motor phenotype independent of enzyme activity (Gurney et al. 1994).

SOD1 models permitted the identification of molecular and anatomical changes that were early, presymptomatic events in the disease course. Defects in the neuromuscular junction (NMJ) were identified as precursors to motor neuron loss, providing evidence in support of a “dying-back” hypothesis in which the axon retracts from the NMJ until ultimately its cell body degenerates in the spinal cord (Fischer et al. 2004). Endoplasmic reticulum (ER) stress was identified as an early marker of disease, and fast-fatigable motor neurons were revealed to be preferentially vulnerable, dying before those innervating slow-twitch muscles (Frey et al. 2000; Pun et al. 2006; Saxena et al. 2009).

Additionally, SOD1 genetic studies implicated both cell autonomous and non-cell autonomous mechanisms of neurodegeneration. Expression of mutant SOD1 in motor neurons seems to govern disease onset, and expression in microglia, astrocytes, oligodendrocytes, Schwann cells, and potentially other cell types also affects the course of motor neuron disease (Yamanaka, Chun, et al. 2008; Boillée et al. 2006; Clement et

al. 2003; Lee et al. 2012; Ferraiuolo et al. 2016; Lobsiger et al. 2009; Yamanaka, Boillee, et al. 2008). In addition, specific mechanisms have been proposed in which astrocytes and microglia release factors that are toxic to motor neurons and induce their degeneration (Nagai et al. 2007; Frakes et al. 2014).

SOD1 models *in vitro* and *in vivo* have implicated a number of mechanisms in ALS pathogenesis including excitotoxicity, oxidative stress, ER stress, mitochondrial dysfunction, axon transport defects, prion-like propagation of aggregates, and glial toxicity (Fig. 1.1) (Hayashi et al. 2016). The most aggressive SOD1 mouse model reaches end stages of disease by 4 months (Gurney et al. 1994) and is thus an experimentally tractable system in which to explore genetic modifiers and ALS treatments targeting each of these mechanisms.

Shortcomings of the SOD1 model

Many genetic experiments and therapeutic trials in SOD1 mice revealed modifiers of the disease phenotype, however thus far none have translated into clinical success in patients (Mitsumoto et al. 2014; Turner & Talbot 2008). Many factors may explain the lack of translation from SOD1 mouse models to clinical benefit. There is evidence that confounding variables such as gender, transgene copy number, and other factors impacted the results and when controlled for, erase the phenotypic benefit of many compounds (Scott et al. 2008). Additionally, there is a need for improved preclinical testing, clinical trial design, and ALS biomarkers to overcome the challenges associated with ALS heterogeneity and diagnosis (Gordon & Meiningner 2011; Ittner et al. 2015).

Close inspection of SOD1 animal models suggests their design may also introduce experimental artifacts that underlie the absence of translatable therapeutics. First, SOD1 transgenic mice that display a motor neuron phenotype rely on dramatic protein overexpression, which in some cases is 20-fold higher than endogenous protein levels (Julien & Kriz 2006). Many models have described a dose dependence of the phenotype, whereby levels of overexpression correlate with phenotypic severity (Gurney et al. 1994; Bruijn et al. 1997). In animals overexpressing wild type SOD1, an overt motor neuron phenotype is absent, however in old mice there is indication of pathology (Dal Canto & Gurney 1994). An SOD1 knock-in mouse, in which mutations were inserted directly into the *SOD1* genetic locus to avoid overexpression artifacts, did not show any heterozygous phenotype, and the homozygous phenotype was shown to arise from loss of SOD1 function (Joyce et al. 2014). Taken together, these data indicate that overexpression is itself toxic, and while it conveniently accelerates the phenotype, it may also lead to molecular changes that are unrelated to ALS, thereby confounding ALS studies.

In addition to suboptimal model design, the discovery of TDP-43 pathology in 2006 dissociated SOD1-ALS from the rest of ALS cases. We now know that TDP-43 inclusions are present in almost all cases of ALS, suggesting that it is a common downstream mediator of neurodegeneration. However TDP-43 pathology is notably absent in patients with *SOD1* mutations (Mackenzie et al. 2007). While ALS patients with *SOD1* mutations are similar to other sporadic and familial patients clinically, the absence of TDP-43 pathology in these patients suggests that SOD1 causes neurodegeneration by a distinct disease mechanism. Therefore, mechanistic insights

gained from SOD1 animal models may not be broadly relevant in ALS. Subsequent genetic advances identified ALS-causing mutations in many other genes, implicating new mechanisms and pathways in the degenerative cascade and enabling the development of novel genetic systems to compare to SOD1 models and to test potential therapeutics.

1.3 Genetics in ALS

That mutations in *SOD1* cause only 20% of familial ALS cases suggested mutations in additional genes also cause the disease. The development of high-throughput DNA sequencing technology led to the rapid identification of mutations in more than 20 new ALS-related genes (Table 1.1), including *TARDBP* (encoding TDP-43) and *Fused in Sarcoma (FUS)*, and a significant advance in 2011 identified a repeat expansion in *C9orf72*, the gene most commonly associated with familial and sporadic ALS and frontotemporal dementia (FTD) (Al-Chalabi et al. 2012; Robberecht & Philips 2013; Guerreiro et al. 2015). These genes span broad functional categories including enzymes, and other proteins involved in RNA metabolism, proteostasis, neuronal excitation, and cellular transport. Genetic advances have also emphasized the multifactorial nature of ALS as not all ALS mutations are fully penetrant, suggesting additional factors are required to lead to disease onset (Borghero et al. 2014). Additionally, genetics advances have strengthened the connection between ALS and other neurodegenerative diseases.

ALS as a spectrum disorder

ALS is clinically and pathologically related to FTD, and the discovery of new genes associated with both disorders supported the idea of ALS as a spectrum disorder whose mechanisms overlap with those of other neurodegenerative diseases. Mutations in *TARDBP*, ubiquilin-2, and valosin-containing protein (VCP) cause both ALS and FTD, and the most common genetic cause of both diseases is a hexanucleotide repeat expansion in *C9orf72* (Morris et al. 2012). TDP-43 and FUS aggregates are also found in both diseases (Morris et al. 2012), demonstrating that ALS and FTD can both manifest from the same underlying causes and disease processes.

Additionally, other ALS-related genes have been implicated in other diseases. For example, angiogenin is related to Parkinson's disease, senataxin to ataxia oculomotor apraxia type 2, and VCP to Paget's disease of bone and inclusion body myopathy; repeats in ataxin-2 cause spinocerebellar ataxia type 2, and optineurin mutations cause glaucoma and ataxia (Al-Chalabi et al. 2012). Together these observations suggest that ALS may be part of a larger disease spectrum, and therefore understanding mechanisms of neurodegeneration in ALS may be broadly applicable to a host of other diseases.

RNA metabolism as a common theme

While genetic developments have been useful in implicating new mechanisms in ALS, they also raise questions as to how seemingly different underlying causes can lead to the same presentation of motor neuron disease. One theme that arose from the genetic discoveries is that of RNA metabolism. Many newly identified causal genes including *TARDBP* and *FUS* encode RNA binding proteins, suggesting that

dysregulated RNA metabolism may be a common disease mechanism. The discovery of *C9orf72* repeat expansions as the most common genetic cause further implicated RNA toxicity as a mechanism, as repeat expansions in other diseases are pathogenic through RNA toxicity mechanisms (Kanadia et al. 2003; Sellier et al. 2010). Additionally, the near-ubiquitous presence of aggregates composed of RNA-binding-protein TDP-43 in ALS cases suggested RNA metabolism plays a central role in the disease.

As this thesis will focus on the role of TDP-43 and FUS, it is important to first consider the known roles of these two RNA binding proteins in the cell and in disease.

1.4 TDP-43 in ALS

As described below, TDP-43 aggregates are found in almost all ALS cases and mutations in *TARDBP* also cause disease, suggesting TDP-43 plays a central role in ALS. Here I will survey TDP-43 literature including pathology in ALS, protein structure and function, animal models, and disease mechanisms in order to provide rationale for the generation of the TDP-43 knock-in model.

TDP-43 mutations and pathology

TDP-43 was identified as the major protein component of insoluble, ubiquitinated inclusions in both ALS and frontotemporal lobar degeneration (FTLD) in 2006, changing the focus of ALS research. One report identified TDP-43 inclusions in the spinal cord of ALS cases and the brain of FTD and Alzheimer's disease cases, and indicated that TDP-43 was abnormally phosphorylated (Arai et al. 2006). A second report identified

TDP-43 ubiquitinated inclusions in the brain and spinal cord of FTD and ALS cases with C-terminal TDP-43 fragments (CTFs), phosphorylated TDP-43, and nuclear exclusion of affected neurons (Neumann et al. 2006). Further characterization of TDP-43 pathology revealed widespread distribution of hyperphosphorylated TDP-43 and 25 KDa and 35 KDa CTFs in brain and spinal cord neurons and glia of both ALS and FTD cases, and there was a greater degree of pathology in the more clinically affected regions (Geser et al. 2009; Neumann et al. 2007; Schiffer et al. 1996). TDP-43 inclusions also contain other proteins such as the stress granule marker TIA-1 (Liu-Yesucevitz et al. 2010). TDP-43 pathology is found in almost all sporadic and familial cases of ALS, with the exception of those with *SOD1* or *FUS* mutations.

This discovery also supported the idea of ALS and FTD as a mechanistically related spectrum of disorders, referred to as multisystem TDP-43 proteinopathies (Geser et al. 2009). TDP-43 pathology was also observed in many other diseases including Parkinson's disease, Huntington's disease, Alzheimer's disease, myopathies, Pick's disease, and others, suggesting broad relevance of TDP-43 to disease (Nakashima-Yasuda et al. 2007; Schwab et al. 2008; Arai et al. 2009; Küsters et al. 2009; Lin & Dickson 2008).

Not only does TDP-43 aggregate in ALS but *TARDBP* is also mutated in ~4% of familial and ~1.5% of sporadic ALS and in some cases of FTD (Mackenzie et al. 2010). Over 50 *TARDBP* mutations have been linked to ALS, most of which fall within the C-terminal prion-like domain, suggesting they may affect the aggregation propensity of the protein. Clinically, *TARDBP* mutations are associated with typical, adult onset ALS, and there are no significant differences in disease manifestation associated with specific

mutations. The identification of TDP-43 pathology and *TARDBP* mutations suggested that age, mutations, and other factors can have a similar effect on TDP-43 behavior, which ultimately cause the protein to behave in a pathogenic manner.

TDP-43 structure and function

TARDBP, located on human chromosome 1, encodes the highly conserved protein TDP-43 of 43 KDa, which is composed of 414 amino acids. The gene contains 6 exons and encodes 11 mRNAs through alternative splicing whose functions are mostly unknown (Wang et al. 2004). TDP-43 is similar in structure and function to many heterogeneous nuclear ribonucleoproteins (hnRNPs), enabling its many roles in RNA metabolism (Figure 1.2) (Buratti & Baralle 2008; Sun & Chakrabartty 2017). It has an N-terminal nuclear localization signal and a nuclear export signal within an RNA binding domain, allowing it to shuttle between the nucleus and cytoplasm to execute its various functions. The two RNA recognition motifs (RRM1 and RRM2) enable TDP-43 to bind to (UG)_n- and (TG)_n- enriched RNA and DNA, respectively (Buratti & Baralle 2001; Ayala et al. 2005). The C-terminal domain is glycine rich and structurally disordered, resembling the yeast prion-like domain, and work implicates this and other low complexity domains in interactions with other RNA binding proteins (Gitler & Shorter 2011; Buratti et al. 2005; D'Ambrogio et al. 2009; Kato et al. 2012). The C-terminal domain is particularly aggregation prone and additionally contains almost all ALS-linked *TARDBP* mutations (Johnson et al. 2007). Together the domain structure of TDP-43 foreshadows its functions in DNA and RNA metabolism and implicate the low complexity domain and protein-protein interactions in TDP-43 toxicity in ALS.

Like most hnRNPs, TDP-43 is an essential protein that is necessary for cell survival, and this is supported by TDP-43 elimination studies. TDP-43 knockout mouse embryos die before implantation between embryonic day 3.5 and 6.5, elimination of the TDP-43 homolog in *Drosophila* results in pupal lethality or reduced lifespan, and knockdown in zebrafish results in motor axon targeting defects and motor deficits (Wu et al. 2010; Sephton et al. 2009; Hazelett et al. 2012; Kabashi et al. 2009). Postnatal elimination of TDP-43 results in dramatic loss of body fat and death in 9 days, elimination from motor neurons results in motor neuron death, and elimination or knockdown in cell culture also led to cell death (Chiang et al. 2010; Wu et al. 2012; Iguchi et al. 2013). TDP-43 is robustly expressed ubiquitously from early developmental stages and maintains lower expression levels in the nervous system through adulthood (Huang et al. 2010; Sephton et al. 2009). Together, these data suggest essential roles for TDP-43 in embryonic and postnatal cell survival and function.

In addition to a general requirement for TDP-43, a neuronal requirement has also been described. TDP-43 is expressed ubiquitously throughout the central nervous system and involved in neurite and dendrite outgrowth, motor function, NMJ maintenance, and axon targeting (Iguchi et al. 2009; Sephton et al. 2012). At the RNA level, TDP-43 regulates many RNAs involved in neuronal development and function, which together suggests an important role for TDP-43 in the nervous system (Sephton et al. 2011). On a molecular level, TDP-43's role in embryonic, postnatal, and neuronal survival likely relates to its various functions in RNA processing (Figure 1.3) (Ratti & Buratti 2016).

TDP-43 regulates gene expression on many levels through its interactions with proteins, DNA, and species of RNA. It was first described as a DNA binding protein that represses human immunodeficiency virus type 1 gene expression, and it has been further implicated as a transcriptional repressor through binding to the SP-10 gene (Ignatius et al. 1995; Acharya et al. 2006). TDP-43 has been implicated in transcription less concretely through interactions with subunits of a complex that bind to RNA polymerase II and initiate transcription and with an exonuclease that is involved in transcription termination (Lehner & Sanderson 2004; Sato et al. 2004), however future work will be required to determine the specific role of TDP-43 in transcription.

TDP-43 also binds to RNA, and early work showed that it regulates splicing of the human cystic fibrosis transmembrane conductance regulator gene (Buratti et al. 2001). Subsequently, many direct splicing targets of TDP-43 were identified including other splicing factors and its own mRNA (Polymenidou & Lagier-Tourenne 2011; Tollervey et al. 2011), and it was shown to interact with other proteins involved in splicing (Freibaum et al. 2010). TDP-43 knockdown led to many splicing changes at loci in which TDP-43 does not bind (Polymenidou & Lagier-Tourenne 2011), and recently a novel role for TDP-43 was identified in the repression of nonconserved cryptic exons in a cell type specific manner (Ling et al. 2015). Together, these data suggest TDP-43 plays a direct role in splicing through interactions with specific mRNAs and an indirect role through the regulation of mRNAs that encode splicing factors and interactions with proteins.

TDP-43 interacts with additional species of RNA that can affect gene expression. It is a component of the Drosha complex (Gregory et al. 2004), which is involved in

microRNA (miRNA) processing, and its functional role comes from observation of dysregulation of many miRNAs upon TDP-43 knockdown (Buratti et al. 2010). It also regulates expression of long noncoding RNAs (lncRNAs) whose functions are mostly unknown but thought to include transcriptional and post-transcriptional regulation of cellular processes. Its interaction with lncRNA NEAT1 suggests a role for TDP-43 in assembly of nuclear paraspeckles, which may contribute to transcriptional regulation (Tollervey et al. 2011).

The interactions between TDP-43 and species of RNA allow TDP-43 to regulate RNA stability and localization. TDP-43 binding regions are abundant in the 3' untranslated region (UTR) of genes (Sephton et al. 2011; Colombrita et al. 2012), a region known to regulate RNA stability, and TDP-43 has been shown to affect the stability of specific RNAs including the low molecular weight neurofilament subunit (Strong et al. 2007). TDP-43 also binds to many mRNAs involved in synaptic function and is actively transported bidirectionally in axons with other RNA binding proteins and specific RNAs, which suggests a role for TDP-43 in localizing mRNA in highly polarized cells like neurons and in promoting synaptic plasticity (Alami et al. 2014; Narayanan et al. 2013).

TDP-43 also binds to a region in its own highly conserved 3' UTR to execute its essential role in the regulation of its expression levels in a negative feedback loop, in a manner similar to other RNA binding proteins. By one mechanism, the 3' UTR binding promotes RNA instability and exosome mediated degradation and by another promotes alternative splicing of an intron within the 3' UTR, leading to nonsense mediated decay (Ayala et al. 2011; Polymenidou & Lagier-Tourenne 2011). Support for this role comes

from observation of equivalent TDP-43 levels in the mouse whether TDP-43 is expressed in a hemi- or homozygous state, downregulation of endogenous mouse TDP-43 upon human TDP-43 overexpression, and increased TDP-43 levels in embryonic stem cells upon deletion of the 3' UTR (Stribl et al. 2014; Sephton et al. 2009; Igaz et al. 2011; Xu et al. 2010). In mice hemizygous for TDP-43, age related motor deficits occur, suggesting that aging may impair TDP-43 autoregulation (Kraemer et al. 2010). Together these data indicate that TDP-43 is a highly regulated protein, and natural or artificial factors affecting TDP-43 autoregulation and expression may be toxic.

In addition to regulation at the transcriptional level, TDP-43 also regulates gene expression at the translational level. TDP-43 acts as a translational repressor in RNA granules within hippocampal dendrites and additionally colocalizes with other RNA regulatory proteins in an activity dependent manner (Wang et al. 2008). TDP-43 associates with translational machinery generally and with stalled ribosomes under conditions of cellular stress (Freibaum et al. 2010; Higashi et al. 2013). Two of TDP-43's specific targets have been identified, Map1b and Rac1, which implicate TDP-43 in neuronal functions (Godena et al. 2011; Majumder et al. 2012), and identification of more targets will further elucidate specific functions of TDP-43.

Lastly TDP-43 is involved in the cellular stress response through localization to stress granules. Stress granules reversibly form in response to cellular stress such as heat, oxidative stress, and glucose deprivation and serve to sequester RNAs and halt translation so that the cell can direct energy and resources towards survival. TDP-43 is recruited to stress granules upon exposure to various stressors and may affect stress granule assembly, morphology, and resulting cell viability (Colombrita et al. 2009;

McDonald et al. 2011; Higashi et al. 2013). Stress granule dynamics are mediated by reversible interactions between RNA binding proteins in their low complexity domains, and through the accumulation of stress these interactions may lead to irreversible aggregate formation.

In summary, TDP-43 has an essential cellular function that may relate to its multifaceted role in the regulation of gene expression. Understanding the role of TDP-43 in ALS therefore requires understanding how mutations and aging lead to changes in TDP-43 behavior and function.

TDP-43 models of ALS

In order to dissect TDP-43 mechanisms of neurodegeneration it is first necessary to model the age-dependent consequences of modifying TDP-43 expression through elimination and mutation. Many groups have generated animal models eliminating TDP-43 and expressing mutant and wild type forms, and the results and conclusions are described below.

TDP-43 knockout models

TDP-43 reduction and elimination has been used as a technique to study the normal role of TDP-43 and its role in disease. Mice hemizygous for TDP-43 with one wild type and one knockout allele maintain the same protein levels as in wild type mice due to TDP-43 autoregulation and do not present with a neurodegenerative phenotype (Kraemer et al. 2010). Groups have shown that TDP-43 elimination constitutively results in early embryonic lethality, establishing a critical role for TDP-43 in development

(Sephton et al. 2009; Wu et al. 2010). Postnatal elimination of TDP-43 resulted in rapid death and metabolic defects 9 days after TDP-43 elimination, establishing a critical role for TDP-43 in postnatal survival (Chiang et al. 2010). Lastly, TDP-43 reduction by ubiquitous expression of a silencing microRNA led to paralysis and motor neuron loss in the brain and spinal cord, and death at approximately 10 weeks (Yang et al. 2014). Interestingly, TDP-43 was not lost in motor neurons themselves, suggesting non-cell autonomous contributions to cell death. Elimination of the TDP-43 homolog from *Drosophila* and zebrafish was also toxic, and elimination from *C. elegans* yielded confusing results with increased lifespan in addition to increased sensitivity to stress (Wang et al. 2011; Kabashi et al. 2009; Vaccaro et al. 2012; Feiguin et al. 2009). These data broadly suggest a neuronal sensitivity to TDP-43 levels and emphasize the toxicity of ubiquitous reduction.

To overcome the lethality of TDP-43 elimination, a cell type specific elimination strategy was used in two studies, which report motor neuron specific elimination of TDP-43 using a conditional knockout strategy. Using cre recombinase driven by the homeobox transcription factor Hb9 promoter, TDP-43 elimination in motor neurons resulted in progressive motor weakness and motor neuron loss, culminating in death at 10 months (Wu et al. 2012). While the authors suggest motor neuron specificity of the Hb9:Cre, previous studies indicate cre is expressed in a wider variety of cells (Hess et al. 2007). A different study uses cre driven by the vesicular acetylcholine transporter (VAcHT) promoter to eliminate TDP-43 in approximately 50% of motor neurons, leading to late onset motor deficits and motor neuron loss (Iguchi et al. 2013). Together, these studies suggest that TDP-43 is required for long term motor neuron survival, however

they do not necessarily implicate TDP-43 loss of function in ALS. The toxicity that results from TDP-43 elimination may simply be due to the absence of an essential gene for cell survival.

Non-mammalian model systems

The advantages of using non-mammalian model systems stem from their ease of generation and use, and as a highly conserved protein, TDP-43 seems to behave similarly in these model systems as in rodents and humans.

A yeast model of TDP-43 proteinopathy found that TDP-43 expression resulted in TDP-43 cytoplasmic aggregation, insolubility, and toxicity in a mutation dependent manner such that ALS mutations exacerbated the phenotypes (Johnson et al. 2009). Additionally, experiments indicated that the C-terminal domain was necessary for cytoplasmic aggregation and toxicity, and the C-terminal domain and RNA recognition motifs together were sufficient for cytoplasmic aggregation and toxicity (Johnson et al. 2007; Johnson et al. 2009). An advantage of the yeast model system is the ability to rapidly screen for genetic modifiers. Such a screen revealed the homolog of ataxin-2 as a TDP-43 modifier, and this led to the identification of intermediate polyglutamine expansions in ataxin 2 as a novel risk factor for ALS (Elden et al. 2010).

Expression of mutant or wild type TDP-43 in *Drosophila*, *C. elegans*, and zebrafish resulted in variable mutation- and dose- dependent phenotypes which included death, paralysis, movement abnormalities, and axonal defects (Wegorzewska & Baloh 2011; Kabashi et al. 2009). TDP-43 pathology was also variable, leading to insoluble TDP-43, soluble CTFs, or neither depending on the model, and whether

toxicity depends on cytoplasmic localization is still unclear. As with the yeast model, these systems have enabled exploration of genetic interactions amongst ALS proteins and have implicated a common pathway with TDP-43 and FUS but not SOD1 (Kabashi et al. 2011; Wang et al. 2011).

Mammalian model systems

Mammalian model systems are often preferred for validating results from non-mammalian systems and modeling complex diseases like ALS. While the experiments are longer, the closer resemblance to humans suggests the systems may be more disease relevant. In addition many therapeutics are first tested in mammalian model systems before progression to clinical trials, thus effective mammalian model systems are necessary for mechanistic analysis and therapeutic identification. Many groups have generated mouse models expressing different forms of mutant and wild type TDP-43, and they will be described below based on the expression pattern of TDP-43 (Table 1.2). Additionally, rat and non-human primate models have been generated and they will be described at the end.

Ubiquitous TDP-43 overexpression

The murine prion promoter (mPrp) allows transgene expression in the central nervous system, including in neurons and astrocytes. Three published models use mPrp-driven human TDP-43 expression and the results are variable. In the first model, mutant TDP-43 at 3-fold endogenous levels led to motor deficits by 13 weeks and death by 22 weeks, accompanied by upper and lower motor neuron loss, gliosis, aggregates,

and CTFs (Wegorzewska et al. 2009). Another model described a motor phenotype resulting from expression of mutant and wild type TDP-43 at 2.5-fold endogenous levels, resulting in death by 4-8 weeks, gliosis, aggregation, and CTFs. Importantly, decreased expression of mutant TDP-43 resulted in the absence of a phenotype (Xu et al. 2010; Xu et al. 2011). A different model described 3-4 fold overexpression leading to an expression-level- and mutation- dependent motor phenotype, resulting in premature death, gliosis, TDP-43 aggregation, and CTFs (Stallings et al. 2010).

One group used recombination mediated cassette exchange to express human mutant TDP-43 from the endogenous TDP-43 locus to achieve more physiological expression, however absence of the 3' untranslated region (UTR) led to 3-fold protein overexpression (Stribl et al. 2014). Mice displayed mild motor neuron loss and motor defects, TDP-43 insolubility and aggregation, and metabolism defects, however the motor neuron phenotype was not progressive. The metabolism defects here resemble those associated with TDP-43 elimination, and in addition the authors observe downregulation of mouse wild type TDP-43. Together these observations suggest the phenotype arose as a result of loss of TDP-43 function. The authors did not generate a similar wild type control mouse, complicating the interpretation of the results.

Perhaps the most promising published TDP-43 model described a mutation-dependent phenotype with “1-1.5”-fold TDP-43 overexpression (Arnold et al. 2013). Closer examination of TDP-43 levels revealed 1.5-3-fold overexpression, with the only motor phenotype present upon 3-fold overexpression. Nonetheless, authors described a motor phenotype upon expression of TDP-43^{Q331K} but not TDP-43^{WT} or TDP-43^{M337V}, accompanied by motor neuron loss and neuromuscular junction defects. Interestingly,

absent was TDP-43 mislocalization, aggregation, insolubility, and CTFs. Concerning about this model, however, is that the phenotype stopped progressing and stabilized by 10 months, and only 1 of the mutants led to a phenotype. The authors did not speculate about these concerns and thus readers are left wondering about the relevance to ALS.

Use of bacterial artificial chromosome (BAC) transgenesis has allowed expression of human TDP-43 under the control of its own promoter, and therefore expression levels and patterns should more closely mimic those of endogenous TDP-43 (Swarup et al. 2011). This approach yielded 3-fold overexpression of wild type and mutant TDP-43 and resulted in a motor phenotype, gliosis, TDP-43 aggregation, and CTFs. Some of these phenotypes were exacerbated with expression of mutant protein.

Neuronal TDP-43 overexpression

The Thy1.2 promoter has been used to overexpress wild type and mutant human TDP-43 almost exclusively in neurons (Caroni 1997), albeit with line to line variability of expression patterns (Feng et al. 2000). In two models, overexpression of wild type TDP-43 led to an expression-level-dependent motor phenotype, and in some cases premature death, motor neuron loss, gliosis, TDP-43 aggregation, and CTFs (Shan et al. 2010; Wils et al. 2010). In one report, *TARDBP* mutation exacerbated pathology (Janssens et al. 2013). An unpublished report from Philip Wong's group described mild overexpression of TDP-43 accompanied by a late onset motor neuron phenotype, and this phenotype was similar upon expression of mutant or wild type TDP-43 (Tsao et al. 2012). Additionally, overexpression of CTFs led to age dependent increase in CTFs and cognitive decline (Caccamo et al. 2012).

The most recent published TDP-43 model used the neurofilament heavy chain promoter to drive expression of TDP-43 without its nuclear localization signal (TDP-43^{ΔNLS}) in the brain, spinal cord, and some astrocytes in order to recapitulate the cytoplasmic aggregation of TDP-43 present in ALS patients (Walker et al. 2015). Authors observe neuronal loss and muscle denervation, motor defects, premature death, TDP-43 insolubility, and rare TDP-43 aggregates, and these pathologies were reversible upon turning off the transgene. The artificiality of the system, however, questions the ALS relevance. The authors expressed a form of TDP-43 that is not observed genetically in patients in order to force the cytoplasmic mislocalization and aggregation of TDP-43. Moreover, authors did not use overexpression controls upon analyzing motor neuron pathology, making it difficult to determine whether it is cytoplasmic mislocalization or overexpression itself leading to pathology. Interestingly, authors observed rare aggregation despite widespread motor neuron loss thus dissociating aggregation from motor neuron pathology.

Forebrain TDP-43 Overexpression

The CamKII promoter has been used to overexpress wild type and modified mouse and human TDP-43 in the cortex and hippocampus. Overexpression of mouse TDP-43 led to behavioral and motor deficits, premature death, gliosis, motor neuron loss, TDP-43 aggregation, and CTFs (Tsai et al. 2010). Another model observed a motor phenotype, premature death, neuronal loss, gliosis, and CTFs upon overexpression of human TDP-43^{ΔNLS} (Igaz et al. 2011). In comparison, overexpression of wild type led to a similar phenotype with later onset, however as expression was

almost 8 fold higher in TDP-43^{ΔNLS} mice, the phenotype may be more expression-level- rather than TDP-43^{ΔNLS}- dependent. Lastly, another group reported expression-level- dependent premature death, neuronal loss, and TDP-43 aggregation upon overexpression of wild type human TDP-43 (Cannon et al. 2012). Toxicity of mutant TDP-43 was also expression-level-dependent such that high overexpression led to brain size abnormalities, however low expression led to the absence of a phenotype. Interestingly, the toxicity was completely independent of protein aggregation (D'Alton et al. 2014).

TDP-43 overexpression in the rat

Overexpression of human TDP-43 in the rat using a BAC transgenesis approach led to a motor phenotype and premature death that was absent with wild type overexpression (Zhou et al. 2010). As a result, the authors generated tet-inducible transgenics expressing mutant TDP-43 in neurons under the control of a CMV promoter, leading to rapid development of a motor phenotype, premature death, gliosis, neuronal loss and rare TDP-43 aggregation. Motor neuron specific expression resulted in a more pronounced motor neuron phenotype, however with less TDP-43 aggregation (Huang et al. 2012). Tet-inducible transgenics were compared to nontransgenic controls instead of wild type overexpressors, thus questioning whether experiments were properly controlled.

TDP-43 overexpression in a non-human primate

Viral overexpression of human wild type TDP-43 in monkeys led to progressive motor weakness, TDP-43 mislocalization, and motor neuron loss (Uchida et al. 2012). Comparison to a rat overexpression system revealed that mislocalization was not required for presentation of the phenotype. TDP-43 was overexpressed 20 times higher than endogenous levels in both systems.

Animal models have emerged as an important tool with which to study the consequences of genetic mutations, elucidate disease mechanisms, and test potential therapeutics. While animal models changed the landscape of ALS research, their discoveries have not yet translated into clinical success. Close inspection of the mammalian models described above suggests many caveats associated with these systems.

These models highlight the importance of TDP-43 levels to its role in the cell: both elimination and overexpression of mutant or wild type forms are toxic. While TDP-43 is normally able to autoregulate through binding to its 3' UTR, this ability is eliminated upon expression of TDP-43 from cDNA in the aforementioned transgenics without key elements of the 3' UTR that are involved in autoregulation and mRNA processing (Stribl et al. 2014). This caveat prevents meaningful analysis of the effects of *TARDBP* mutations in these systems. Indeed, the 3' UTR has been recently shown to play an important role in translation and protein-protein interactions in addition to RNA stability (Mayr 2016). Thus to model the age-dependent effects of *TARDBP* mutations on long term motor neuron survival a less artificial approach is required in which TDP-43 expression levels and patterns are not disrupted.

We also observe an interesting dissociation in some of these model systems between TDP-43 aggregation and neurodegeneration. While aggregation of TDP-43 is present in some model systems, it is notably absent or minimal in others in which neurodegeneration is still observed, in some cases dramatically (Arnold et al. 2013; D'Alton et al. 2014; Walker et al. 2015). This opens the possibility that aggregation may not be driving neurodegeneration but either acting as an amplifier, an innocent bystander, or a protective event late in the disease and thus when present in model systems may primarily be a product of protein overexpression.

Taken together, these data suggest the importance of observing the effects of *TARDBP* mutations in a physiological context in which we can determine the effects of the mutations on the normal properties of the protein and on age-dependent motor neuron health.

TDP-43 mechanisms of neurodegeneration

TDP-43 is an essential cellular protein with many described functions, and additionally it aggregates in ALS. These data suggest two non-mutually-exclusive general mechanisms by which TDP-43 might lead to neurodegeneration: loss of function and gain of function.

TARDBP mutations and aging may have the effect of interfering with normal TDP-43 function in the cell, and this loss of TDP-43 function may lead to motor neuron disease. Support for this hypothesis comes from observations of TDP-43's functions, the effects of ALS-mutations on protein function, and observations from ALS patients. TDP-43 has a role in tightly controlling its own expression through an autoregulatory

mechanism involving RNA transcription, splicing and 3' end processing, and loss of this function results in toxic overexpression. TDP-43 affects splicing of cryptic exons, which are shown to be dysregulated in ALS patients. TDP-43 also has critical roles in survival, as elimination embryonically or postnatally in the mouse results in lethality. This lethality may indicate a role for TDP-43 function in ALS, however it may also simply result from the absence of an essential gene. Work in zebrafish model systems indicates that mutant TDP-43 cannot fully rescue the defects associated with TDP-43 homolog elimination, suggesting that the protein does not retain full function (Kabashi et al. 2009). Other observations from patients indicate nuclear exclusion and cytoplasmic aggregation of TDP-43 are hallmarks of ALS pathology, suggesting absence from the nucleus and/or sequestration in cytoplasmic aggregates may lead to TDP-43 loss of function. Despite this evidence, however, whether TDP-43 loss-of-function causes neurodegeneration in ALS remains uncertain.

Alternatively, or in addition, the mutations and aging may not impair normal protein function but may instead confer a gain of function toxicity on TDP-43, which may relate to the formation of abnormal assemblies. Observations from patients indicate that TDP-43 is hyperphosphorylated, ubiquitinated, cleaved into CTFs, and mislocalized to the cytoplasm, and the toxic gain of function may relate to any of these properties. TDP-43 aggregation is thought to arise from the structurally disordered C-terminal domain due to its low complexity and resemblance to yeast prion domains, and this particularly aggregation prone region is a primary component of ALS-associated TDP-43 inclusions (Sun & Chakrabartty, 2017). ALS mutations cluster in the C-terminal domain and studies suggest that these mutations may increase the aggregation propensity of the

protein and disrupt the normal interactions between TDP-43 and components of ribonuclear granules that regulate a variety of cellular processes, thus contributing to the formation of abnormal assemblies and neurotoxicity of TDP-43 (Conicella et al. 2016; Gopal et al. 2017). Additionally, the increased propensity to aggregate may lead to TDP-43 sequestration and a secondary loss of TDP-43 function. The near-ubiquitous TDP-43 pathology suggests aggregates play a role, however the absence of aggregation in many motor neuron disease models complicates our understanding of the importance of aggregation.

Differentiating between these disease mechanisms is critical for the development of effective therapeutics, however the absence of genetically faithful animal models in combination with the complexity of TDP-43 pathology has impeded clear understanding of TDP-43-mediated neurodegeneration. Work in this thesis will address these problems in order to better understand the consequences of *TARDBP* mutations.

1.5 FUS in ALS

Mutations in Fused in Sarcoma (FUS, also known as Translated in Liposarcoma-TLS) were linked to ALS in 2009 (Kwiatkowski et al. 2009; Vance et al. 2009). As described below, FUS is both structurally and functionally related to TDP-43, suggesting overlapping disease mechanisms involving RNA metabolism.

FUS mutations and pathology

Over 70 mutations in *FUS* are linked to ALS, accounting for ~4% of familial ALS and less than 1% of sporadic ALS (Deng et al. 2014). Many of the mutations fall in the

C-terminal nuclear localization signal (NLS), causing FUS to mislocalize from the nucleus into the cytoplasm (Figure 1.4). Many mutations that are not in the NLS fall in the N-terminal prion-like domain and, similar to the case of TDP-43, increase the aggregation propensity of FUS (Nomura et al. 2014). Unlike *TARDBP* mutations, certain *FUS* mutations, such as P525L, are associated with increased pathogenicity and cause some of the only forms of juvenile onset, aggressive cases of ALS, while others cause the typical adult onset form of the disease (Deng et al. 2014). Interestingly, the pathogenicity of the mutation correlates with the degree of cytoplasmic mislocalization of FUS (Dormann et al. 2010).

On a pathological level, patients with *FUS* mutations have insoluble FUS aggregates in the cytoplasm of neurons and glia, sometimes accompanied by nuclear clearance of FUS (Rademakers et al. 2011). These aggregates are ubiquitinated and contain p62 and stress granule markers but interestingly do not contain TDP-43 (Dormann et al. 2010; Vance et al. 2009). Some reports indicate the presence of FUS in aggregates in sporadic ALS patients, however others suggest FUS only aggregates when *FUS* mutations are present (Vance et al. 2009; Deng et al. 2010). Therefore the relevance of FUS to sporadic ALS remains uncertain.

Like TDP-43, FUS is also connected to FTD and other neurodegenerative diseases. *FUS* mutations themselves do not seem to solely cause FTD but are found in some ALS patients that also present with FTD, and in addition FUS aggregates are found in a subset of FTD cases (Deng et al. 2014). These patients generally present with early onset, sporadic FTD without motor features, and the aggregates have similarities and differences to those found in ALS. Like in ALS, in FTD FUS aggregates

are neuronal, and glial, insoluble, TDP-43 negative, and ubiquitin positive. Unlike in ALS, in FTD FUS aggregates with other proteins from the FET family and nuclear import protein Transportin (Neumann et al. 2011; Neumann et al. 2012). FUS aggregates have also been found in other diseases such as Huntington's and spinocerebellar ataxias (Lagier-Tourenne et al. 2010; Lattante et al. 2013). Together, these data suggest that FUS, like TDP-43, plays a broad role in neurodegeneration, however FUS may act through distinct, disease-specific mechanisms.

FUS structure and function

FUS, located on human chromosome 16, encodes the highly conserved FET family protein FUS of 75 KDa, which is composed of 526 amino acids. Like TDP-43, FUS is similar in structure and function to many other hnRNPs (Figure 1.4). It has an N-terminal low complexity domain that mediates self-assembly and interactions with other proteins and a C-terminal NLS that facilitates import into the nucleus (Schwartz et al. 2013; Kato et al. 2012; Dormann et al. 2010). In between are RRM, RGG, and zinc finger domains that bind RNA, DNA, and proteins to mediate aspects of FUS function. FUS was initially identified as part of a fusion protein in tumors in which the FUS sequence acted as a transcriptional activator for transcription factor CHOP (Crozat et al. 1993; Rabbitts et al. 1993), and since, many roles in regulating gene expression have been ascribed to FUS (Figure 1.5).

Like TDP-43 and other hnRNPs, FUS is a predominantly nuclear and ubiquitously expressed DNA and RNA binding protein that affects gene expression on many levels. On a transcriptional level, FUS binds to chromatin to initiate transcription

(Yang et al. 2014) and binds to and regulates phosphorylation of RNA polymerase II to enable elongation (Schwartz et al. 2012). In addition it binds to transcription factors, such as Spi-1 and others, to both activate and repress transcription (Hallier et al. 1998; Uranishi et al. 2001).

FUS is also involved in mRNA processing through interactions with proteins and many species of RNA. FUS binds to RNA, primarily at introns, and FUS elimination alters the splicing and expression of many mRNAs (Lagier-Tourenne et al. 2012; Rogelj et al. 2012). FUS is involved in the maintenance and formation of gems, subnuclear structures with roles in RNA processing, and has been reported to bind to components of the spliceosome and other hnRNPs (Yamazaki et al. 2012; Behzadnia et al. 2007; Blokhuis et al. 2016; Wang et al. 2015; Kamelgarn et al. 2016). Together, these data implicate FUS in mRNA splicing directly by interacting with RNA and indirectly by interacting with other RNA binding proteins. FUS also interacts with the Drosha complex, miRNAs, and lncRNAs, and regulates circular RNA biogenesis, which ultimately influence gene expression (Gregory et al. 2004; Modigliani et al. 2014; Lagier-Tourenne et al. 2012; Errichelli et al. 2017).

Like TDP-43, FUS has an important role in regulating its own protein levels through a negative feedback loop. Its autoregulation may involve miRNA suppression of FUS or nonsense mediated decay as a result of exon skipping (Modigliani et al. 2014; Zhou et al. 2013). By whichever mechanism, it seems FUS expression levels are tightly controlled.

FUS also shuttles from the nucleus into the cytoplasm where it affects RNA stability and localization and the cell's stress response. Evidence in support of its role in

stability comes from its interaction with RNA at the 3' UTR and other factors controlling 3' end processing, and from specific transcripts whose stability FUS regulates (Colombrita et al. 2012; Lagier-Tourenne et al. 2012; Rogelj et al. 2012; Udagawa et al. 2015). FUS also associates with RNA granules that can affect gene expression in a context-dependent manner. Activity dependent localization of FUS with RNAs to the synapse can regulate local translation and spine remodeling (Fujii et al. 2005; Fujii & Takumi 2005), while localization to stress granules ensures that a cell is focused primarily on survival in response to exogenous stressors (Andersson et al. 2008).

In addition to its RNA processing functions, FUS is involved in the cell's DNA damage response. FUS binds both single and double stranded DNA and is recruited to DNA upon lesion (Baechtold et al. 1999). It interacts with other DNA damage proteins such as HDAC1 at sites of DNA damage and promotes homologous recombination during break repair (Wang et al. 2013). In addition, FUS knockout mice show genomic instability, chromosomal breakage, and increased sensitivity to radiation (Hicks et al. 2000; Kuroda et al. 2000).

Lastly, FUS has important functions in development and neurons. Its elimination resulted in lethality in *Drosophila* and embryonic lethality with immune defects in the mouse that was dependent on the mouse genetic background, and knockdown in zebrafish led to motor deficits (Wang et al. 2011; Hicks et al. 2000; Kuroda et al. 2000; Kabashi et al. 2009; Kabashi et al. 2011). In addition, FUS elimination resulted in abnormal dendritic spine morphology and density, suggesting a role for FUS in synaptic function (Fujii et al. 2005).

In summary, FUS has many overlapping roles with TDP-43 in the regulation of gene expression and in development and function. Effective animal modeling has further elucidated the role of FUS function in long term motor neuron survival and ALS.

FUS animal models

Many models expressing human wild type and mutant forms of FUS have been generated and analyzed in order to determine the role of FUS in ALS. Similar to TDP-43 models, overexpression and transgenic models have yielded confusing results due to the toxicity of overexpression of wild type FUS and the variability of the phenotypes. Recent work, however, established two different and effective mouse models of FUS-ALS that lead to similar conclusions about disease mechanisms.

FUS expression from the *MAPT* locus (Sharma et al. 2016)

Work from our lab established a series of mice expressing human wild type or mutant FUS in a controlled manner from the *MAPT* genetic locus to avoid overexpression and insertion effects. Modest FUS expression led to mutation dependent motor neuron loss, NMJ defects, FUS mislocalization, and motor defects with cell autonomous and cell non-autonomous contributions to the phenotype. Notably absent were cytoplasmic FUS inclusions, such as those observed in end stages of disease in patients. Interestingly, phenotypic severity correlated with clinical severity of the mutations, and the expression of wild type FUS did not lead to a phenotype, suggesting that the model faithfully reproduces the effects of *FUS* mutations.

Additional experiments assessed the mechanism by which *FUS* mutations cause ALS. As described previously with respect to TDP-43, mutations may cause neurodegeneration by a loss of the normal *FUS* function, or the mutations may lead to a gain of toxic function. In order to distinguish between these mechanisms, authors asked whether *FUS* function was required for long term motor neuron survival. While *FUS* is an essential protein in development, its postnatal requirement is unknown. Surprisingly, postnatal loss of *FUS* did not cause any motor neuron pathology, suggesting that *FUS* is not required for long term motor neuron survival and loss of *FUS* function is insufficient to cause ALS.

FUS knock-in models

Insertion of *FUS* mutations into the endogenous mouse *FUS* genetic locus is an effective strategy to overcome the toxicity associated with overexpression and the random insertion in typical transgenic approaches and has the advantage of authentic expression of mutant protein. Insertion of a cre-dependent truncation mutation, modeled after a case of sporadic ALS (DeJesus-Hernandez et al. 2010), led to *FUS* cytoplasmic mislocalization and perinatal lethality of homozygotes in a similar manner to *FUS* knockout animals (Scekic-Zahirovic et al. 2016; Scekic-Zahirovic et al. 2017). However, unlike *FUS* knockout animals, knock-in homozygotes had motor neuron loss and knock-in heterozygotes presented with an age-dependent, progressive motor neuron phenotype resembling ALS. In addition, both cell autonomous and cell-non autonomous expression of mutant *FUS* contributed to the phenotype. Together, these data suggested that the mislocalized *FUS* leads to motor neuron death through a toxic gain

of function, and the absence of FUS inclusions in these mice suggest aggregation is not a necessary event. Similar unpublished work from our lab has led to the same conclusions. FUS knock-in mice display dose-dependent toxicity upon comparison of hemizygous and homozygous mutants, suggesting that the mutants are associated with a toxic gain of function (Drs. Alexander Lyashchenko & Bea Blanco, personal communication).

These animal models demonstrate the advantages of expression of mutant protein in a controlled, stereotyped, and physiologically relevant manner. Both models report a similar progressive phenotype without FUS aggregation and with involvement of motor neurons and other cell types, and both lead to the conclusion that the mutations are associated with a gain of function toxicity.

FUS mechanisms of neurodegeneration

Models described above suggest *FUS* mutations are associated with a gain of function toxicity and importantly, that aggregation is not a necessary component of motor neuron loss.

Recent work implicates the low complexity domain of specific RNA binding proteins including FUS as a mediator of toxicity in ALS. Low complexity domains, or regions with little diversity in amino acid composition, are enriched in DNA and RNA binding proteins and may have an important role in regulating gene expression (Kato et al. 2012; Han et al. 2012). The low complexity domain mediates the formation of RNA granules, membrane-less organelles involved in spatially and temporally localizing

proteins and RNA within in the cell. RNA granules are dynamic such that proteins are exchanged freely between the granules and the cytoplasm, causing a context-dependent phase separation into liquid compartments, somewhat like oil and vinegar. Certain factors such as protein and salt concentration, temperature, and RNA can drive the phase separation and the formation of RNA granules (Guo & Shorter 2015). These domains, however, are also prone to cause aberrant protein-protein interactions and protein aggregation. The prevalence of the low complexity domain in ALS proteins in combination with the dysregulation of RNA as an emerging theme suggested their mechanistic role in ALS.

Subsequent work showed that ALS-mutations in a number of genes alter the dynamics within these granules to promote phase separation and polymerization. Two groups showed *FUS* mutations in the low complexity domain and in the nuclear localization signal promote the polymerization of FUS into a solid state from a liquid state in a protein concentration-, temperature- and time- dependent manner (Patel et al. 2015), and this polymerization can ultimately become irreversible (Murakami et al. 2012). Additionally ALS-causal mutations in *heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1)* accelerated hnRNP A1 fibrillization in a concentration-dependent manner, and resulting amyloid-like fibrils could then cross-seed fibrillization of wild type protein (Kim et al. 2013; Molliex et al. 2015). Importantly, this work dissociated fibrillization and phase separation, as hnRNP A1 can fibrillize without phase separating, and non-fibrillizing mutants can phase separate (Molliex et al. 2015; Lin et al. 2015). Recent work also showed that ALS mutations in *TARDBP* cause granules to become less mobile, more viscous, and disrupt self-interaction, though unclear in this work was

whether mutations promote TDP-43 polymerization (Conicella et al. 2016; Gopal et al. 2017). Lastly, there is accumulating evidence that *C9orf72*-repeat-expansion-derived dipeptide repeats, especially those containing arginine, phase separate into membrane-less organelles with other proteins containing low complexity domains and possibly disrupt RNA granule dynamics as a result (Boeynaems et al. 2017; Lee et al. 2016; Lin et al. 2016).

Together, these data suggest that ALS mutations in *FUS* and other genes can alter phase separation of RNA binding proteins and therefore RNA granule dynamics. How and whether these changes lead to motor neuron degeneration is still unclear, necessitating further research into the precise gain of function mechanisms associated with *FUS* mutations.

1.6 Summary and aims of this thesis

The importance of RNA binding proteins in ALS has become clear through observation of near-ubiquitous TDP-43 aggregates, mutations in *TARDBP*, *FUS*, and other RNA binding proteins, and the ALS-causal hexanucleotide repeat expansion in *C9orf72*. Additionally, the structural and functional similarities between TDP-43 and *FUS* suggest that they may lead to overlapping disease mechanisms, however these mechanisms have yet to be identified.

The generation of a faithful animal model of ALS as a result of *FUS* mutations permits further inquiry into gain of toxic function disease mechanisms, and I hypothesize that these mechanisms relate to aberrant protein-protein interactions. Therefore, in Chapter 2, I will determine the differences in protein-protein interactions associated with

FUS mutations in the mouse model of ALS and subsequently determine whether these interactions may be mechanistically relevant. I will then describe experiments to determine the functional role of a candidate *FUS*-interactor in both motor neuron health and disease.

A barrier to understanding TDP-43-dependent mechanisms of motor neuron disease is the absence of a faithful genetic model. Therefore, in Chapter 3, I will present the generation of a novel TDP-43 knock-in model in which ALS-causal mutations were inserted directly into the mouse endogenous *TARDBP* genetic locus. I will then describe experiments to determine the consequences of these mutations on the normal function of the protein and on long term motor neuron survival.

In Chapter 4 I will draw conclusions from the work presented in this thesis, and in Chapter 5 I will describe experimental methods and procedures used in the completion of this work.

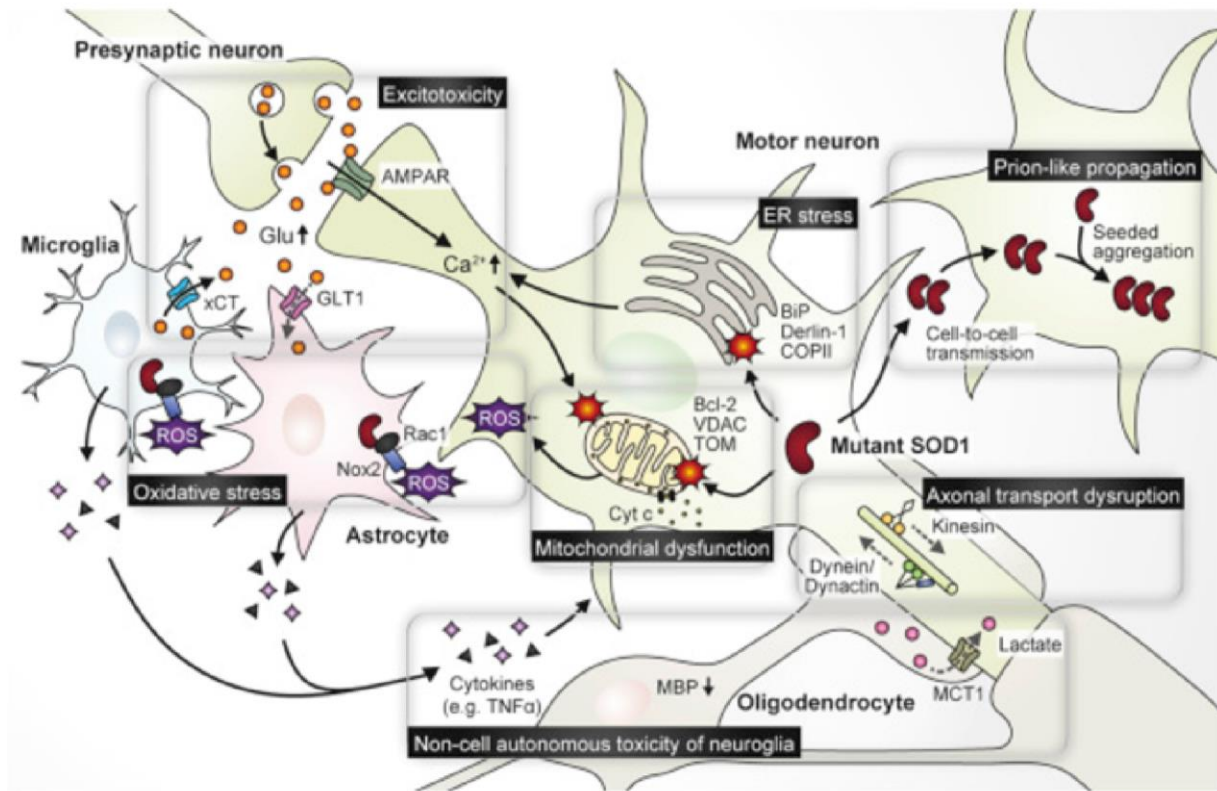


Figure 1.1: Overview of proposed neurotoxic mechanisms in SOD1 mediated ALS. (Hayashi, Homma, and Ichijo, 2016)

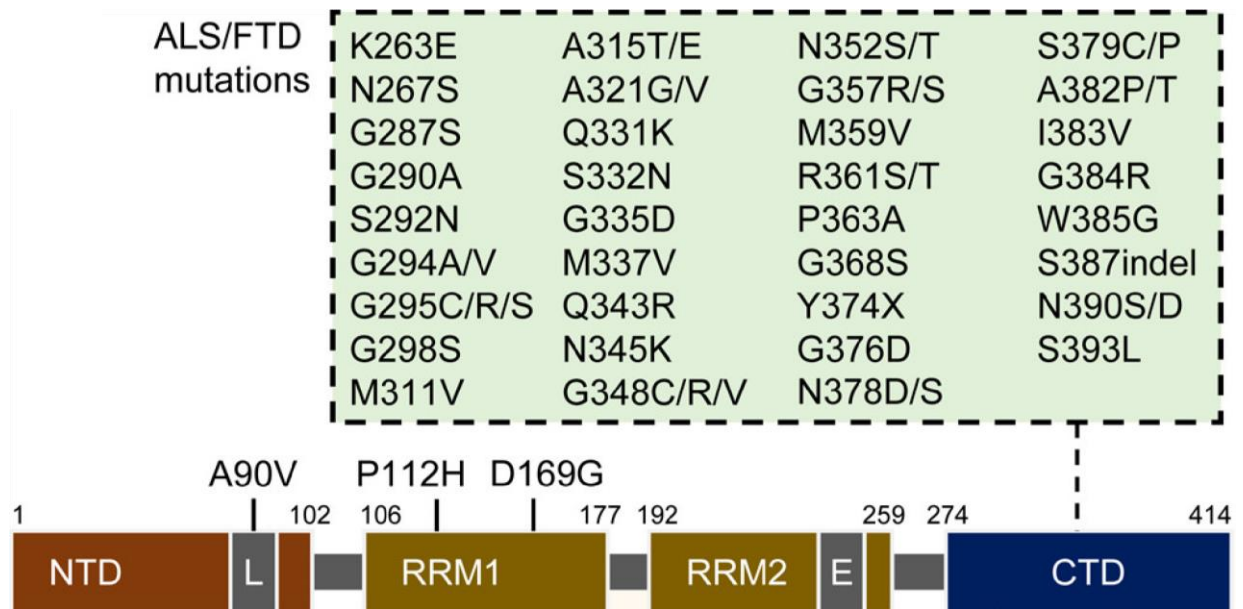


Figure 1.2: TDP-43 domain structure and ALS mutations.

TDP-43 contains a nuclear localization signal (L) within the N-terminal domain (NTD), nuclear export signal (E), 2 RNA recognition motifs (RRM1 & RRM2), and a low complexity C-terminal domain (CTD). Mutations are primarily found in the CTD. Numbers indicate amino acid position. (Adapted from Sun and Chakrabarty, 2017).

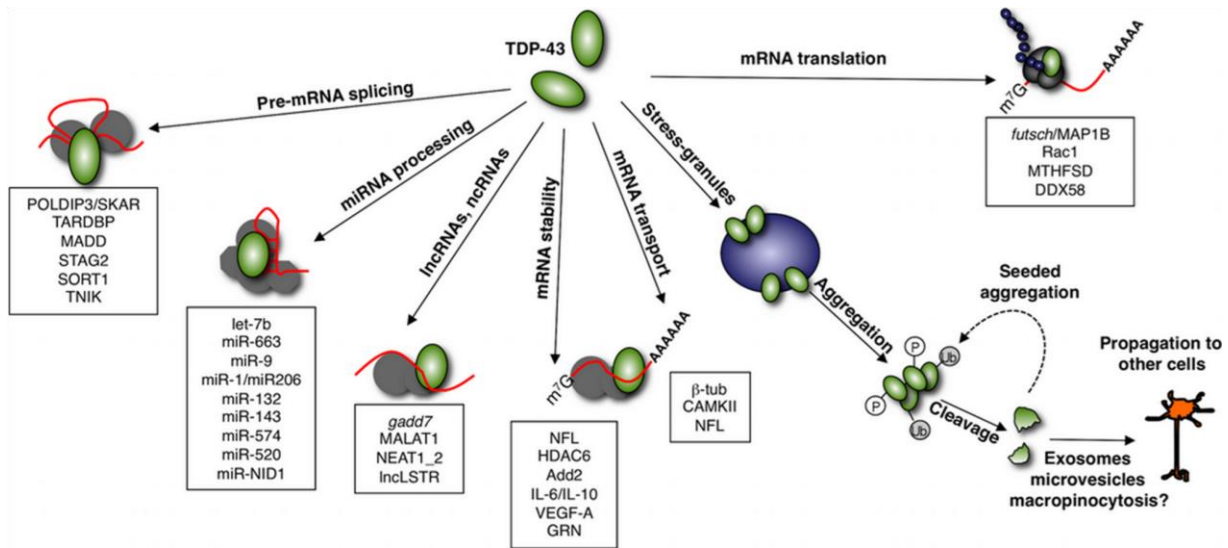


Figure 1.3: TDP-43 functions in RNA metabolism.

TDP-43 regulates many aspects of RNA processing through interactions with different species of RNA and proteins. Boxes indicate RNA targets that have been validated experimentally. Consequences of stress granule formation, such as aggregation and subsequent propagation, are also indicated. Taken from Ratti & Buratti, 2016.

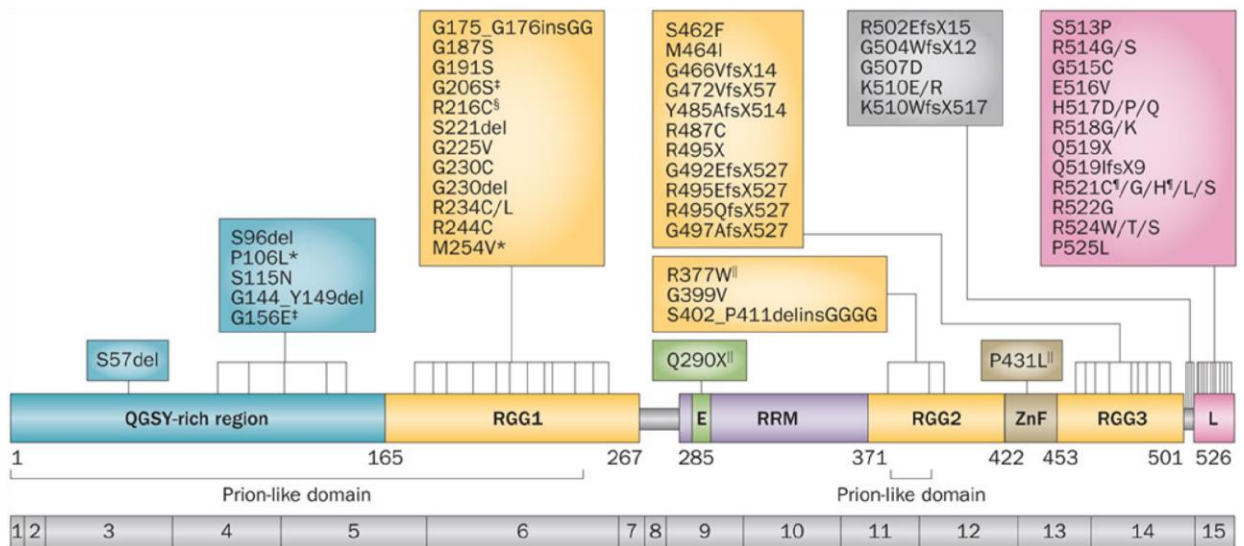


Figure 1.4: FUS domain structure and ALS mutations.

FUS has 15 exons (gray), encoding a protein of 526 amino acids. It has an N-terminal low complexity domain (QGSY-region), 3 RGG motifs, an RNA recognition motif, nuclear export (E) and import (L) signals, and a zing finger domain (ZnF). ALS mutations are located in most domains, but in particular within the nuclear localization signal. (Taken from Deng et al. 2014).

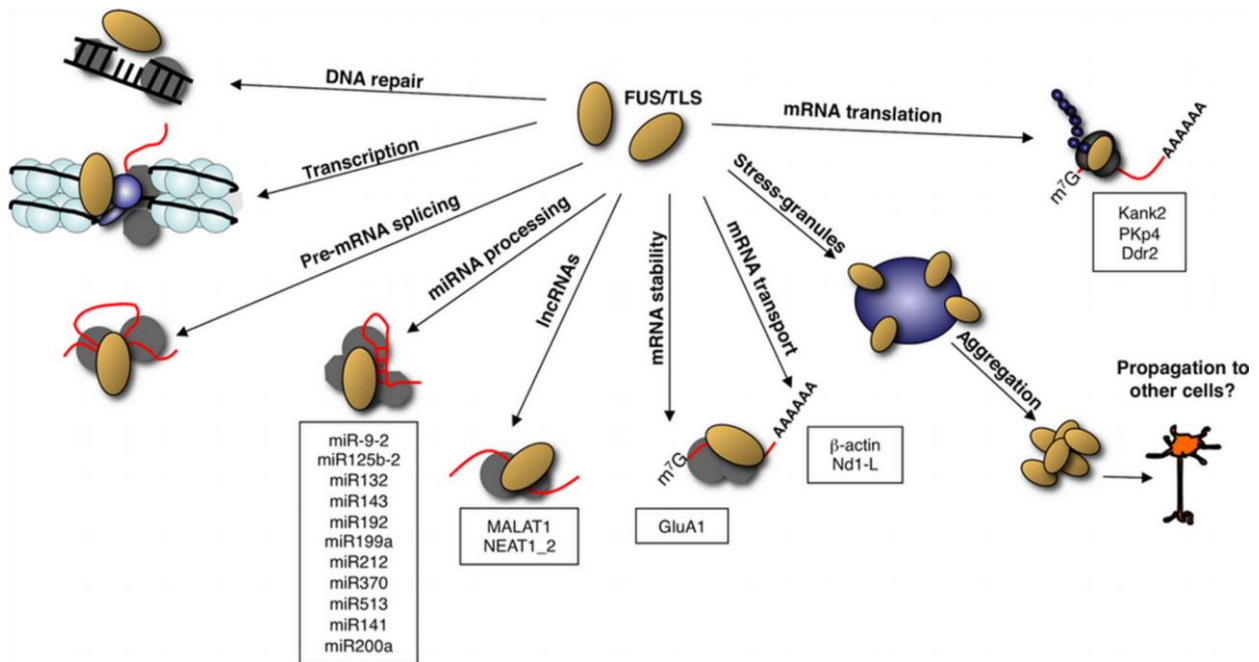


Figure 1.5: FUS functions in DNA and RNA metabolism.

FUS regulates many aspects of DNA and RNA processing through interactions with different species of RNA, DNA, and proteins. Boxes indicate RNA targets that have been validated experimentally. Consequences of stress granule formation, such as aggregation and subsequent propagation, are also indicated. Taken from Ratti & Buratti, 2016.

Gene	Pathway
<i>C9orf72</i>	Toxic RNA and/or dipeptide aggregation
<i>FUS</i>	DNA/RNA metabolism
<i>EWSR1</i>	DNA/RNA metabolism
<i>TARDBP</i>	DNA/RNA metabolism
<i>SETX</i>	DNA/RNA metabolism
<i>MATR3</i>	DNA/RNA metabolism
<i>TAF15</i>	DNA/RNA metabolism
<i>HNRNPA1</i>	DNA/RNA metabolism
<i>HNRNPA2B1</i>	DNA/RNA metabolism
<i>ANG</i>	DNA/RNA metabolism
<i>GLE1</i>	DNA/RNA metabolism
<i>SPG11</i>	DNA damage repair
<i>SQSTM1</i>	Autophagy
<i>UBQLN2</i>	Autophagy
<i>VCP</i>	Autophagy
<i>OPTN</i>	Autophagy
<i>CHMP2B</i>	Autophagy
<i>TBK1</i>	Autophagy, inflammation
<i>FIG4</i>	Intracellular trafficking
<i>ALS2</i>	Intracellular trafficking
<i>VAPB</i>	Intracellular trafficking
<i>DCTN1</i>	Intracellular trafficking
<i>PRPH</i>	Cytoskeleton
<i>PFN1</i>	Cytoskeleton
<i>NEFH</i>	Cytoskeleton
<i>SIGMAR1</i>	Endoplasmic reticulum lipid rafts
<i>ERLIN2</i>	Endoplasmic reticulum lipid rafts
<i>DAO</i>	Excitotoxicity
<i>SOD1</i>	Enzyme, toxic aggregation
<i>CHCHD10</i>	Mitochondrial function
<i>ERBB4</i>	Dysregulation of neuregulin-Erb4 pathway
<i>ARHGEF28</i>	Interaction with NEFL
<i>PNPLA6</i>	Neurite outgrowth and process elongation

Table 1.1: Mutations in genes of diverse functions cause ALS.
(Adapted from Robberecht and Philips, 2013 and Guerreiro et al. 2015)

Species	Promoter	TDP-43 protein	Expression level	Molecular Phenotype					Cellular Phenotype			Motor Phenotype			Reference
				Mislocalization	Aggregation	Ubiquitination	Phosphorylation	CTFs	Denervation	MN loss	Gliosis	Age of onset (Days)	Survival (Days)	Description	
Mouse	mPrp	A315T	3	++	-	A	ND	Y	0	20%	Y	90	154	gait	Wegorzewska et al. 2009
	mPrp	A315T	3	+	+	ND	ND	-	20%	0	Y	ND	ND	-	Herdeyn et al. 2014
	mPrp	WT	3	ND	ND	D	ND	Y	0	ND	Y	ND	>330	-	Stallings et al. 2010
	mPrp	A315T	4	-	++	A	++	Y	0	ND	Y	ND	75	weakness, gait	Stallings et al. 2010
	mPrp	WT	2.5	+	++	D	++	Y	ND	0	Y	21	45	clasp, tremors, gait	Xu et al. 2010
	mPrp	M337V	2.7	+	++	D	+++	Y	ND	0	Y	21	30	clasp, tremors, gait	Xu et al. 2011
	mPrp	WT	1	-	-	-	-	-	0	0	Y	NA	-	-	Arnold et al. 2013
	mPrp	M337V	1	-	-	-	-	-	0	0	Y	300	-	rotarod	Arnold et al. 2013
	mPrp	Q331K	1.5	-	-	-	-	-	30%	35%	Y	90	-	tremors, weakness	Arnold et al. 2013
	endogenous	A315T	3	++	++	A	-	-	ND	10%	ND	90	-	gait	Stribl et al. 2014
	Thy 1.2	WT	2	++	++	D	+	Y	ND	25%	Y	20	<30	paralysis, clasp	Wils et al. 2010
	Thy 1.2	M337V	1.8	++	++	D	++	Y	ND	Y	Y	12	17	clasp, tremors, gait	Janssens et al. 2013
	Thy 1.2	WT	3.6	-	++	D	ND	-	0	ND	Y	16	-	clasp, tremors, gait	Shan et al. 2010
	Thy 1.2	25 kDa CTF	4.7	-	-	-	-	Y	ND	ND	ND	-	-	-	Caccamo et al. 2012
	CamKII (i)	WT	3	ND	++	D	++	Y	ND	Y	Y	ND	60	ND	Cannon et al. 2012
	CamKII (i, P21)	WT	3	ND	++	A	++	Y	ND	Y	Y	ND	>360	ND	Cannon et al. 2012
	CamKII	mouse WT	2	++	++	A	-	Y	ND	25%	Y	180	495	clasp, rotarod	Tsai et al. 2010
	CamKII (i)	WT	0.8	-	+	-	+	-	ND	20%	Y	75	ND	clasp	Igaz et al. 2011
	CamKII (i)	ΔNLS	7.9	+++	+	A	+	-	ND	50%	Y	7	180	clasp	Igaz et al. 2011
	BAC	WT	3	++	++	A	ND	-	5%	0	Y	294	ND	rotarod	Swarup et al. 2011
	BAC	A315T	3	+++	+++	A	ND	Y	ND	0	Y	266	ND	rotarod	Swarup et al. 2011
	BAC	G348C	3	+++	+++	A	ND	Y	10%	0	Y	252	ND	rotarod	Swarup et al. 2011
	NEFH (i)	ΔNLS	>10	++	+++	A	+++	-	60%	50%	Y	14	72	clasp, tremors, rotarod	Walker et al. 2015
NEFH (i)	WT	ND	-	-	ND	+	ND	ND	ND	ND	ND	ND	ND	Walker et al. 2015	
NA	constitutive KO								ND	ND	ND	ND	-	-	Wu et al. 2009
NA	constitutive KO								ND	ND	ND	ND	E6.5	-	Sephton et al. 2010
NA	constitutive KO								ND	ND	ND	ND	E7.5	-	Kraemer et al. 2010
NA	conditional KO								ND	ND	ND	ND	9	-	Chiang et al. 2010
	Hb9-Cre conditional KO								ND	40%	Y	100	300	rotarod, kyphosis	Wu et al. 2012
	VaChT-Cre conditional KO								40%	Y	Y	350	-	tremor, weakness	Iguchi et al. 2013
Rat	BAC	WT	ND	++	-	D	D	Y	ND	0	-	NA	>200	-	Zhou et al. 2010
	BAC	M337V	ND	ND	ND	ND	ND	ND	ND	Y	ND	15	20	weakness, paralysis	Zhou et al. 2010
	CMV (i)	M337V	ND	++	+	D	D	Y	40%	12%	Y	40	55	weakness, paralysis	Zhou et al. 2010
	NEF (i)	M337V	2	ND	-	A	ND	ND	0	20%	ND	14	ND	weakness, paralysis	Huang et al. 2012
	ChAT (i)	M337V	3.5	++	-	A	ND	ND	0	60%	Y	7	12	weakness, paralysis	Huang et al. 2012

Table 1.2: TDP-43 rodent models.

Levels are expressed as fold change compared to endogenous mouse TDP-43 expression levels. “TDP-43 protein” refers to the human protein unless noted. NA – not applicable, ND – not described, Y – yes, dash indicates absence, (i) – inducible system, rating system ranges from + (rarely seen) to +++ (frequently seen). A indicates presence of the molecular phenotype in aggregates, and D indicates diffuse staining. Adapted from Lehrer, H. (2015). “Investigating the role of the RNA binding protein TDP-43 in Amyotrophic Lateral Sclerosis using animal and cell-based models of disease” (Doctoral dissertation).

Chapter 2: Gain of function mechanisms of FUS toxicity

2.1 Introduction

FUS mutations are associated with a gain of function toxicity and importantly, aggregation is not a necessary component of resulting toxicity. Therefore we focus on three differences between mutant and wild type *FUS* in ALS.

First, mislocalization seems to be an essential component of disease. Mutations cause the protein to mislocalize from the nucleus to the cytoplasm in animal models, recapitulating a key feature of human pathology. Importantly, the degree of mislocalization seems to correlate with the severity of the disease clinically (Dormann et al. 2010), suggesting it is mechanistically relevant.

Second, unpublished work from our lab suggests that *FUS* mutations cause the protein to adopt an abnormal protein conformation. Wild type *FUS* is recognized by *FUS* antibodies that do not recognize mutant *FUS*, and the reverse is also true (Drs. Alex Lyashchenko and Bea Blanco, personal communication). This indicates that the mutations alter protein folding and cause different epitopes to be exposed, leading to an altered protein conformation

Third, accumulating work indicates that *FUS* mutations affect its interactions within RNA granules with other proteins containing low complexity domains. Studies recapitulating the formation of RNA granules in a cell-free environment identified a biological role for the low complexity domain in reversibly forming RNA granules by interacting with RNA and other RNA binding proteins (Kato et al. 2012). Subsequent work showed that ALS-mutations in a number of genes, including *FUS*, alter the

dynamics within these granules to promote liquid-liquid phase separation and polymerization. *FUS* mutations in the low complexity domain and in the nuclear localization signal promote the polymerization of *FUS* into a solid state from a liquid state in a protein concentration-, temperature- and time- dependent manner, and this polymerization can ultimately become irreversible (Patel et al. 2015; Murakami et al. 2012).

Together, these observations indicate that mutations affect the interactions between *FUS* and other cellular proteins due to compartmental and conformational differences, and as a protein's role in the cell is in large part determined by the proteins with which it interacts, I hypothesize that changes in protein-protein interactions are a proximal event in mutation associated toxicity.

2.2 Results

Mutant FUS protein interactors identified by mass spectrometry

Observed differences between mutant and wild type (WT) FUS, such as protein localization, protein conformation, and behavior in membrane-less structures, may cause mutant and WT FUS to interact with different proteins inside the cell, and we hypothesized that changes in protein-protein interactions as a result of FUS mutations are a proximal event in mutation-associated toxicity. Thus to identify gain of function mechanisms of FUS toxicity it is first necessary to identify changes in protein-protein interactions as a result of FUS mutations.

In order to identify FUS-interacting proteins that may have mechanistic relevance, we used the novel animal model of FUS-ALS generated in the Shneider lab in which mutant and wild type myc-tagged hFUS cDNA was expressed from the *MAPT* genetic locus (Sharma et al. 2016). Importantly, a clinically severe P525L-mutation resulted in more severe motor neuron (MN) pathology in comparison to a clinically typical R521C-mutation, and expression of hFUS^{WT} did not result in any MN pathology. We co-immunoprecipitated hFUS^{WT} or hFUS^{P525L} from the spinal cord of P90 animals, an age at which MN pathology is evident, and used a myc antibody so that only hFUS and not endogenous mouse FUS would be immunoprecipitated (Fig. 2.1A). To control for non-specific interactions between the antibody or beads and the protein lysate, we used a WT C57BL/6J non-myc-expressing mouse as a control. Upon co-immunoprecipitation, we visualized hFUS interacting proteins using SDS-PAGE followed by a silver stain. In comparison to the WT negative control, many proteins were

co-immunoprecipitated from hFUS-expressing spinal cord, and interestingly differences between hFUS^{WT} and hFUS^{P525L} were also apparent (Fig. 2.1B). Specifically, many proteins seemed to interact with mutant, but not wild type hFUS. Thus with confirmation of differences in protein-protein interactions, we used mass spectrometry (MS) to identify the proteins bound to hFUS.

By MS we identified 22 proteins that interact with hFUS^{P525L} (Table 2.1). Likely due to differences in protein levels between hFUS^{WT} and hFUS^{mutant} (Fig. 2.1C), there was insufficient quantity of hFUS immunoprecipitate to identify interactions by MS with hFUS^{WT} above the C57BL/6J background. Previous work established that differences in protein levels were a consequence of the effect of mutations on protein stability, as RNA levels are equivalent across mutant mouse lines despite differences at the protein level (Sharma et al. 2016). Nevertheless, we proceeded to validate MS-identified interactions with hFUS^{P525L} and test the presence of the interaction with hFUS^{R521C} and hFUS^{WT}.

FUS interactions are mutation dependent

Upon testing 8 MS-identified interactions with hFUS^{P525L}, we found that all co-immunoprecipitated (Fig. 2.1C): hnRNP U, SFPQ, Caprin1, hnRNP R, hnRNP Q, hnRNP K, Ddx1, and endogenous mouse FUS. Interestingly, we found that the proteins also interacted with hFUS^{R521C} but not hFUS^{WT}, and that the strength of the interaction correlated with the severity of the mutation: interactors were more abundant upon co-immunoprecipitation with the clinically-severe P525L mutant than the clinically-typical R521C mutant. Important to note is that the strength of the interaction also seems to correlate with the protein levels, suggesting that the aberrant interactions may simply be

a result of increased protein stability. However, as the increased protein levels and stability result from the mutations found in ALS patients, the aberrant interactions may indeed indicate changes in protein-protein homeostasis in patients and may therefore be mechanistically relevant.

FUS-hnRNP U interaction is RNA-independent

The most abundant mutant-FUS interactor was RNA-binding protein heterogeneous nuclear ribonucleoprotein U (hnRNP U). hnRNP U has documented roles in neurological deficits such as seizure and intellectual disability (Thierry et al. 2012) and importantly has previously been connected to ALS. hnRNP U was shown to bind in an ALS-mutation-dependent manner to Ubiquilin-2 (Gilpin et al. 2015) and bind in an RNA-dependent manner to TDP-43 (Suzuki et al. 2015). In addition, hnRNP U expression modulated TDP-43 toxicity (Suzuki et al. 2015). Thus for the relevance to neurological deficits and ALS, we identified hnRNP U as a candidate mediator of mutant-FUS toxicity.

We next wanted to reciprocally validate the interaction between hnRNP U and mutant-FUS, and so we performed a co-immunoprecipitation with an hnRNP U antibody and found that we selectively precipitate mutant-FUS (Fig. 2.1D). Again, this may result from mutation-dependent changes in protein stability, and therefore represents protein homeostasis found in ALS patients. Lastly, we wanted to further characterize the interaction and wondered whether it was RNA-dependent, as FUS interacts with proteins in both RNA-dependent and RNA-independent manners. Upon treatment of the lysate with RNase A, we determined that mutant-FUS and hnRNP U still interact (Fig.

2.1D). These data suggest that hnRNP U interacts specifically with mutant FUS in an RNA-independent manner.

Reduction of FUS interactor hnRNP U ubiquitously is pathological

How might the interaction between hnRNP U and FUS lead to MN degeneration? In order to answer this question, we first wondered whether hnRNP U has a normal role in cells and specifically MNs. Previous work shows that hnRNP U reduction *in vivo* constitutively and specifically from the heart is lethal (Roshon & Ruley 2005; Ye et al. 2015) and elimination *in vitro* from neurons causes cell death (Suzuki et al. 2015); however its role *in vivo* in MNs has not yet been described.

Using a conditional hnRNP U knockout mouse (Fig. 2.2A) (Ye et al. 2015), we first generated a knockout allele and observed mice hemizygous for hnRNP U. As detected by western blot, reduction of hnRNP U in hemizygotes was mild and not statistically significant (Fig. 2.2B); however the mild reduction had detrimental effects. In crossing WT to hemizygotes, Mendelian ratios predict 50% of offspring will be WT and 50% will be hemizygotes. In actuality, 63% were WT and 37% hemizygotes (N = 110 animals), suggesting that the hnRNP U knockout allele is maladaptive and selected against embryonically. Additionally, the hemizygotes that were born were smaller than their WT littermates (Fig. 2.2C) and this size differences persisted into adulthood, indicating that hnRNP U reduction is pathological (Fig. 2.2D).

Loss of hnRNP U in motor neurons results in premature death, denervation, and motor neuron abnormalities

To determine the role of hnRNP U in motor neurons specifically, we crossed a ChAT-Cre deleter line to the hnRNP U conditional knockout mouse. In comparison to a hemizygous control, hnRNP U was efficiently eliminated from 100% of MNs (Fig. 2.3A). In addition to being smaller than hemizygous littermate controls (Fig. 2.3B), MN knockouts had a reduced lifespan (Fig. 2.3C). At P30, MN knockout mice reached “endstage” and were no longer able to right themselves when placed on their side or back and were therefore euthanized.

We next asked whether MN pathology, such as MN loss and denervation, was evident following hnRNP U elimination and contributed to the shortened lifespan. We counted ChAT positive MNs in the ventral horn of L5 spinal cord at endstage, and we observed no statistically significant difference in L5 MNs (Fig. 2.4A). However upon analysis of the muscle, we observed denervation of three ALS-susceptible muscles in MN knockouts: the diaphragm, tongue, and tibialis anterior (TA) (Fig. 2.4B). Lastly, visualization of lumbar MNs with MN marker ChAT and nuclear envelope marker nup50 revealed that hnRNP U elimination caused morphological abnormalities in >30% MNs (Fig. 2.4C). Nuclei did not appear spherical and instead were misshapen and eccentric. Together, these data suggest that hnRNP U has an essential role in MNs and its elimination causes morphological and functional abnormalities that lead to death.

HnRNP U is not a component of FUS aggregates in ALS patients

As a key hallmark of ALS in patients is protein aggregation, we asked whether hnRNP U was a component of FUS aggregates. Using spinal cord tissue from a

FUS^{P525L/WT} patient, immunocytochemical results suggest that hnRNP U is not present in FUS aggregates (Fig. 2.5), however further biochemical experiments are needed as aggregation may prevent antibody recognition of proteins.

2.3 Discussion and future experiments

Mutant FUS interacting proteins

The generation of a faithful ALS mouse model in our lab permitted further analysis of gain of toxic function mechanisms associated with *FUS* mutations. We reasoned that mutations result in compartmental and conformational differences between wild type and mutant *FUS* that cause mutant *FUS* to interact with different proteins than the wild type. As a protein's role is in large part determined by the other proteins with which it interacts, we hypothesized that changes in protein-protein interactions were a proximal event in mutation-associated toxicity, and therefore to understand toxic gain of function mechanisms we first need to understand differences in protein-protein interactions. We used an unbiased mass spectrometry approach to identify 22 proteins that interact with P525L-mutant *FUS*, and subsequent validation indicated that these are aberrant interactions, likely due to mutation-dependent changes in protein conformation and stability.

Most of the mutant-*FUS* interactors are RNA binding proteins with roles in RNA splicing, processing, and stabilization. Many of the proteins are ribosomal proteins, consistent with *FUS* localization to polysomes and its role in local protein synthesis (Belly et al. 2005), and many are involved in various steps of RNA processing including mRNA granule transport, splicing, miRNA maturation, and translation. In addition, many interactors are components of RNA granules, specifically stress granules.

Comparisons with other data sets reveal similarities and differences in protein interactions and common cellular pathways. The first paper to publish a wild type *FUS*

interactome used a HeLa overexpression system and revealed that most FUS interactors are RNA binding proteins, many of which are involved in RNA splicing, and interact with FUS in an RNA-dependent manner (Yamazaki et al. 2012). Significantly, FUS interacted with spinal muscular atrophy protein survival motor neuron (SMN) and TDP-43, however subsequent work in our lab has failed to reproduce these interactions (Dr. Bea Blanco, personal communication), suggesting that it may be an artifact of the overexpression system. 5 interactions with wild type FUS were identified as mutant-FUS interactors in our system (Table 2.2).

Additional studies have identified interacting proteins with mutant and wild type FUS. In one HEK293 cell overexpression system, most FUS interactors were related to metabolism and protein degradation, as well as RNA metabolism (Wang et al. 2015). 6 proteins were also found in our dataset including hnRNP U, and SFPQ showed an increased interaction with mutant FUS as we see in our system. Another system expressed mutant and wild type FUS in a neuronal cell line and identified FUS interactors that were largely DNA/RNA binding proteins with roles in RNA metabolism (Blokhuys et al. 2016). 9 proteins overlapped with those in our system including hnRNP U, and 8 of these proteins were also interactors of TDP-43 and ALS-related protein Ataxin-2, suggesting these proteins may be involved in common ALS downstream mechanisms. Interestingly, no interaction between FUS and SMN was identified, despite the authors reporting an interaction in a previous publication (Groen et al. 2013). Lastly, a group used FUS overexpression in HEK293T cells to identify RNA-dependent and RNA-independent interactors with mutant and wild type FUS, identifying many of both that were almost exclusively related to RNA metabolism (Kamelgarn et al. 2016). 8

proteins overlapped with those in our system including hnRNP U, and interestingly the interaction with hnRNP U was independent of RNA, consistent with the results reported in this thesis.

These data therefore are largely consistent with other reports of FUS interacting proteins. 8 mutant-FUS interacting proteins (hnRNP U, hnRNP R, hnRNP K, Fxr1, G3bp2, SFPQ, Ddx1, and Caprin1) were common to at least 3 datasets including that presented in this thesis, suggesting they are bona fide interactors. 9 identified proteins are novel interactors, and future work will be required to determine the function of these interactions.

In comparison to other work described above, this mass spectrometry experiment identified a small number of FUS-interacting proteins (22 vs. 112, 157, 166, or 124). This is likely due to the low expression levels of hFUS from the *MAPT* locus in comparison to endogenous FUS levels (Sharma et al. 2016). In addition, hFUS is only expressed in Tau-expressing cells, and together these suggest the myc-tagged protein is not abundant in the cell lysate and we therefore identified only the most abundant FUS interactions. While the number of interactors is low, the fidelity seems high, as 8 out of 8 tested interactions from the top and bottom of the list were successfully validated.

Particularly interesting is the correlation between the amount of the interactor co-immunoprecipitated and the clinical severity of the mutation. For example, hnRNP U was more abundant in the precipitate of the clinically severe juvenile onset P525L-mutant as compared to the clinically typical adult onset R521C-mutant, and no hnRNP U was present in the WT precipitate. While these differences may simply result from

differences in protein stability and therefore protein levels (Sharma et al. 2016), the differences are ultimately mutation-dependent, whether the result of altered binding affinity or protein levels, and therefore may be mechanistically relevant.

Possible disease mechanisms

The aberrant interactions between mutant FUS and the RNA binding proteins described above suggest a few possible disease mechanisms that may lead to motor neuron loss.

Many of the interacting proteins including Caprin 1, Fxr1, and Ddx1, and in particular those common to multiple data sets, are components of stress granules. Stress granules are membrane-less subcellular structures that form in response to cell stress and serve to ensure that a cell is focusing resources on that which is needed to survive and combat the stress. In addition, FUS and TDP-43 aggregate with other stress granule proteins in ALS patients, suggesting their role in disease. Mutant FUS and other stress granule proteins may therefore promote degeneration in a few ways. These aberrant interactions in stress granules may promote abnormal protein fibrillization, which may eventually lead to aggregation. Protein fibrils or aggregates may be toxic through unidentified gain of function mechanisms and/or in addition may sequester RNA and proteins, inducing toxicity through the loss of function of specific RNAs and proteins.

Relatedly, many of the protein interactors harbor a low complexity domain, including hnRNP U, SFPQ, and Caprin 1. The low complexity domain is important in the manifestation of toxicity associated with *FUS* and other ALS mutations, possibly through

altered phase separation of RNA granules. The aberrant interaction between mutant FUS and other proteins with low complexity domains may therefore lead to degeneration through altered phase separation of FUS and its interacting proteins. While still unclear the specific toxic consequence of altered phase separation, possibilities include similar protein aggregation and sequestration of RNA binding proteins and mRNAs as was described above.

One way to test these hypotheses is to determine the context of the aberrant protein-protein interactions. Are these interactions occurring within cellular complexes such as stress granules? Are these interactions changing the solubility (and therefore propensity to aggregate) of the FUS complexes? Biochemical experiments, such as sucrose gradients and solubility fractionation will be able to probe whether these interactions are occurring within particular complexes in specific cellular compartments in order to ultimately determine disease mechanisms.

An attractive hypothesis from these models is that mutant FUS sequesters specific RNA binding proteins through these aberrant interactions, and it is the loss of function of specific RNA binding protein(s) that is ultimately toxic. Support for this sequestration hypothesis comes from mechanistically understood diseases such as Myotonic Dystrophy in which nuclear RNA foci sequester muscleblind and it is the loss of function of these proteins that is pathogenic. The sequestration hypothesis has also become popular in ALS due to observation of RNA foci associated with the hexanucleotide repeat expansion in *C9orf72*, the most common genetic cause of ALS. These foci are hypothesized to sequester RNA binding proteins, and an attractive

hypothesis is that the same proteins are sequestered by mutant FUS and by RNA foci, providing a common downstream disease mechanism.

To further explore disease mechanisms, we took a candidate approach to determine whether a specific interaction was disease relevant. Due to its previous connection to ALS research and abundance as a mutant-FUS interactor, we pursued the relevance of hnRNP U to ALS. We showed that it was reciprocally pulled down with myc (pulling down human FUS) and hnRNP U antibodies, and then we showed that the interaction was independent of RNA. As previous reports have shown, many FUS interactions are RNA-dependent, and some are RNA-independent (Kamelgarn et al. 2016). The RNA independence could suggest that FUS and hnRNP U bind directly to each other as opposed to associating in the same ribonucleoprotein complex, and binding assays could determine whether this is the case.

Future work will determine the relevance of interactors in humans and further characterize the nature of these interactions. We will seek to validate these mutation-dependent interactions in humans using ALS-patient-derived spinal cord samples and induced pluripotent stem cells. We did not find hnRNP U to be a component of FUS aggregates, and as FUS-dependent motor neuron disease does not depend on protein aggregation, this observation does not preclude mechanistically-relevant protein mislocalization. Cytoplasmic mislocalization may cause a loss of protein function, and while FUS loss of function is insufficient to cause ALS, the loss of other RNA binding proteins may indeed be sufficient. Therefore we will determine whether the aberrant hnRNP U-FUS interaction occurs in the cytoplasm or the nucleus through subcellular

fractionation experiments. Mislocalization of RNA binding proteins may also alter RNA granule dynamics, leading to motor neuron death by a still undetermined mechanism.

HnRNP U is essential ubiquitously and in motor neurons

In addition to uncovering a potential role for hnRNP U in ALS, we also investigated its role generally and in motor neurons. Using genetic experiments, we show that hnRNP U reduction is toxic as hnRNP U hemizygotes, despite non-statistically-significant reduction in hnRNP U levels, are not born at Mendelian ratios and are smaller than controls, suggesting that the knockout allele is maladaptive. Additionally, we present the first evidence *in vivo* that hnRNP U elimination in motor neurons is toxic. HnRNP U motor neuron knockouts die around P30, have denervation of the tongue, diaphragm, and tibialis anterior muscles, and have abnormal nuclear morphology in motor neurons. Further work will characterize whether hnRNP U elimination in motor neurons results in the same cell type vulnerabilities as in ALS. As the diaphragm, tongue, and tibialis anterior are all vulnerable in ALS, analysis of ALS-spared motor neurons such as the oculomotor neurons or the less vulnerable soleus will determine whether hnRNP U elimination is generally toxic, or whether it is selectively toxic in a way that suggests ALS relevance.

Still unknown is the mechanism by which hnRNP U reduction is toxic, however several essential functions for hnRNP U have previously been described. HnRNP U is a critical cellular protein, like many other hnRNPs. Its expression 2 to 5 times lower than wild type led to morphological abnormalities and death by embryonic day 11.5 (Roshon & Ruley 2005). Selective elimination from the heart resulted in abrupt death around

postnatal day 14 due to heart failure, with features comparable to those in humans with a heart condition due to a non-hnRNP U genetic mutation (Ye et al. 2015). *In vitro*, its reduction from motor neuron cell line NSC34 led to caspase cleavage, indicating cell death (Suzuki et al. 2015). Together, these data indicate hnRNP U is an essential protein that may be required ubiquitously or in specific cell types and not just in motor neurons. Future experiments using conditional knockout animals and different cre lines will determine whether motor neurons are preferentially vulnerable, which may explain cell type specificity in ALS, or whether there is a general requirement for hnRNP U.

Many specific roles for hnRNP U have been described. HnRNP U was initially identified as an abundant RNA and DNA binding protein and a component of the nuclear matrix, suggesting its roles in maintaining nuclear architecture and regulating transcription (Romig et al. 1992; Dreyfuss et al. 1984). HnRNP U is also cleaved by caspase 3 during apoptosis resulting in a loss of DNA binding and detachment from the nuclear matrix, suggesting hnRNP U may be involved in the nuclear breakdown during apoptosis (Göhring et al. 1997; Kipp et al. 2000). It is recruited to sites of DNA damage and plays an important role in base repair (Hegde et al. 2012; Berglund & Clarke 2009; Britton et al. 2009; Britton et al. 2014), and in addition binds to intronic DNA to regulate aspects of development (Zhao et al. 2009). Thus through interactions with DNA and other proteins, hnRNP U has diverse functions in regulating gene expression and nuclear organization.

It has similarly diverse roles in regulating gene expression at the RNA level through protein-protein and protein-RNA interactions. Firstly, it regulates RNA polymerase II-mediated transcription through interactions with transcription factors,

actin, and RNA polymerase II itself (Kukalev et al. 2005; Kim & Nikodem 1999). It also binds to RNA through its RGG domain, and through interactions with noncoding RNAs and other splicing factors it regulates mRNA stability and alternative splicing (Kiledjian & Dreyfuss 1992; Xiao et al. 2012; Hacısuleyman et al. 2014; Liu & Dreyfuss 1996; Yugami et al. 2007). Indeed, its elimination in the heart results in widespread defects in alternative splicing (Ye et al. 2015).

HnRNP U is primarily nuclear, but biochemical and immunocytochemical experiments suggest it also localizes to the cytoplasm (Howell et al. 2004). It translocates to the cytoplasm upon stimulation of toll-like receptors, implicating it in the proinflammatory cellular response (Zhao et al. 2012), however cytoplasmic expression and functions in other cell types have not yet been described.

While work has been done to describe hnRNP U's normal role in the cell, there is less known about its potential role in disease. It is a candidate for a deleted gene causing intellectual disability, developmental delay, and seizures (Caliebe et al. 2010; Thierry et al. 2012), and it has previously been connected to ALS through interactions with ubiquilin-2 and TDP-43 (Gilpin et al. 2015; Suzuki et al. 2015). Together, these data suggest that hnRNP U plays a role in ALS and other neurological deficits, however further work will be required to better define the potential disease mechanisms.

As described above, hnRNP U has diverse functions in regulating gene expression and nuclear architecture, and hnRNP U elimination is likely toxic due to functional effects on one or more of these processes. As motor neuron elimination results in nuclear morphological abnormalities, we hypothesize that the toxicity in motor neurons relates in part to hnRNP U's role in maintaining nuclear architecture. We

expect deeper understanding of hnRNP U function will enable better understanding of its role in disease.

Role of hnRNP U-FUS interaction

Work presented in this thesis demonstrates *FUS* mutations cause an aberrant interaction between FUS and hnRNP U. Together with the data that hnRNP U elimination in motor neurons is toxic, we propose a sequestration model in which the mutant FUS - hnRNP U interaction prevents hnRNP U from executing its normal function, leading to toxicity by a loss of hnRNP U function (Fig. 2.6). This hypothesis leads to two predictions: decreasing hnRNP U levels would aggravate mutant FUS-dependent toxicity and increasing hnRNP U levels would rescue mutant-FUS dependent toxicity. Future experiments will entail genetically testing these predictions by crossing an hnRNP U knockout allele into the FUS mouse model of ALS generated in the Shneider lab and by injecting virally encoded hnRNP U into the cerebrospinal fluid to increase protein levels in motor neurons.

The observation that hnRNP U elimination causes an abnormal nuclear morphology suggests defects in the nuclear architecture may ultimately be the cause of neurodegeneration. One function of the nuclear matrix is to connect the nucleus to the cytoplasm both structurally and functionally, enabling nucleocytoplasmic transport and proper nuclear positioning. Two complexes- the nuclear pore complex and the LINC complex- serve these functions and are intimately connected to lamins and the nuclear membrane in which they reside.

Additional research supports role of hnRNP U in nucleocytoplasmic transport defects. The abnormal nuclear morphology associated with hnRNP U elimination in motor neurons also resembles that in laminopathies such as Hutchinson Gilford Progeria Syndrome in which a nuclear envelope residing lamin is mutated (Liu et al. 2011; Zhang et al. 2011; Miller et al. 2013). Lamins maintain the structure of the nuclear envelope and associate with both the nuclear pore and LINC complexes such that the interaction between these complexes facilitates nucleocytoplasmic transport (Jahed et al. 2016). The specific function of hnRNP U in the nuclear matrix is unclear, however it is known to interact Lamin B, one of the primary components of the nuclear envelope (Lobov et al. 2001), and future work will determine whether FUS mutations perturb this interaction.

Recent work also suggests defects in nucleocytoplasmic transport through the nuclear pore complex are a key pathological mechanism associated with the ALS-causal repeat expansion in *C9orf72*. Two unbiased screens identified proteins involved in the nuclear pore complex as modifiers of C9orf72-mediated toxicity (Jovičić et al. 2015; Freibaum et al. 2015). Additionally, the nuclear envelope was disrupted in cellular and animal models of ALS as well as ALS patients, and studies using iPS lines derived from ALS patients verified the nucleocytoplasmic transport defects (Zhang et al. 2015; Freibaum et al. 2015; Jovičić et al. 2015; Kinoshita et al. 2009).

Taken together, these data suggest that hnRNP U sequestration by mutant FUS may have the downstream consequence of altering nucleocytoplasmic transport by perturbing the nuclear matrix in a manner similar to *C9orf72* repeats, further implicating nucleocytoplasmic defects as a common disease mechanism in ALS.

The work presented in this Chapter suggests novel aberrant interactions between mutant FUS and other RNA binding proteins that may ultimately lead to understanding gain of function mechanisms associated with *FUS* mutations, and in addition that have enabled better understanding of the role of RNA binding protein hnRNP U in motor neurons. We suggest that aberrant FUS-RNA binding protein interactions may result in toxicity due to alterations in stress granule dynamics, RNA granule phase separation, and/or sequestration of RNA binding proteins, specifically hnRNP U. These areas of research remain active areas of study in the lab today, and future work will empirically test the potential disease mechanisms described above. Together, these data suggest the potential sensitivity of a cell (and perhaps specifically motor neurons) to changes in the concentration of RNA binding proteins and highlight the value in carefully generated animal models in order to learn about normal protein function and disease mechanisms.

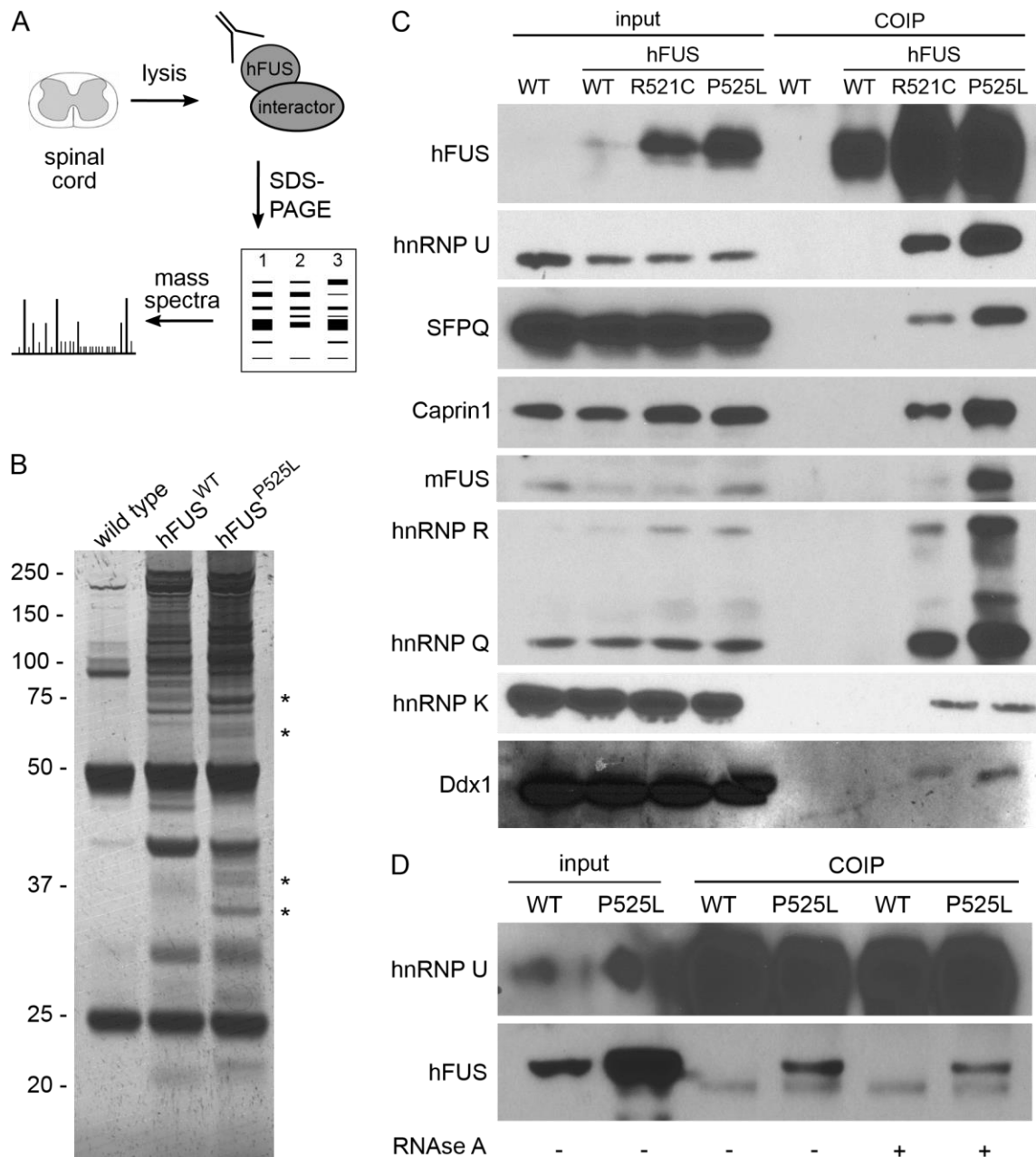


Figure 2.1: Identification and characterization of hFUS interactors.

A. Schematic of mass spectrometry (MS) experiment. Using myc antibody, human FUS was co-immunoprecipitated with interacting proteins from mouse spinal cord, and interactors were identified by MS. B. Silver stain of samples submitted for MS with size markers in kDa on the left. Bands in wild type (WT) lane represent background. Examples of differences between hFUS samples are indicated with asterisk (*). C. Co-immunoprecipitation with myc antibody reveals validation of MS-identified interactions with P525L-mutant hFUS and altered interactions with R521C-mutant or WT- hFUS. WT lane represents non-myc-expressing, C57BL/6J mouse. mFUS- mouse FUS; hFUS- human FUS. D. Co-immunoprecipitation with hnRNP U antibody from hFUS-WT or hFUS-P525L expressing mice reveals interaction with mutant protein, and interaction remains in the presence of 10 μ g/ 500 μ L RNase A treatment of lysate.

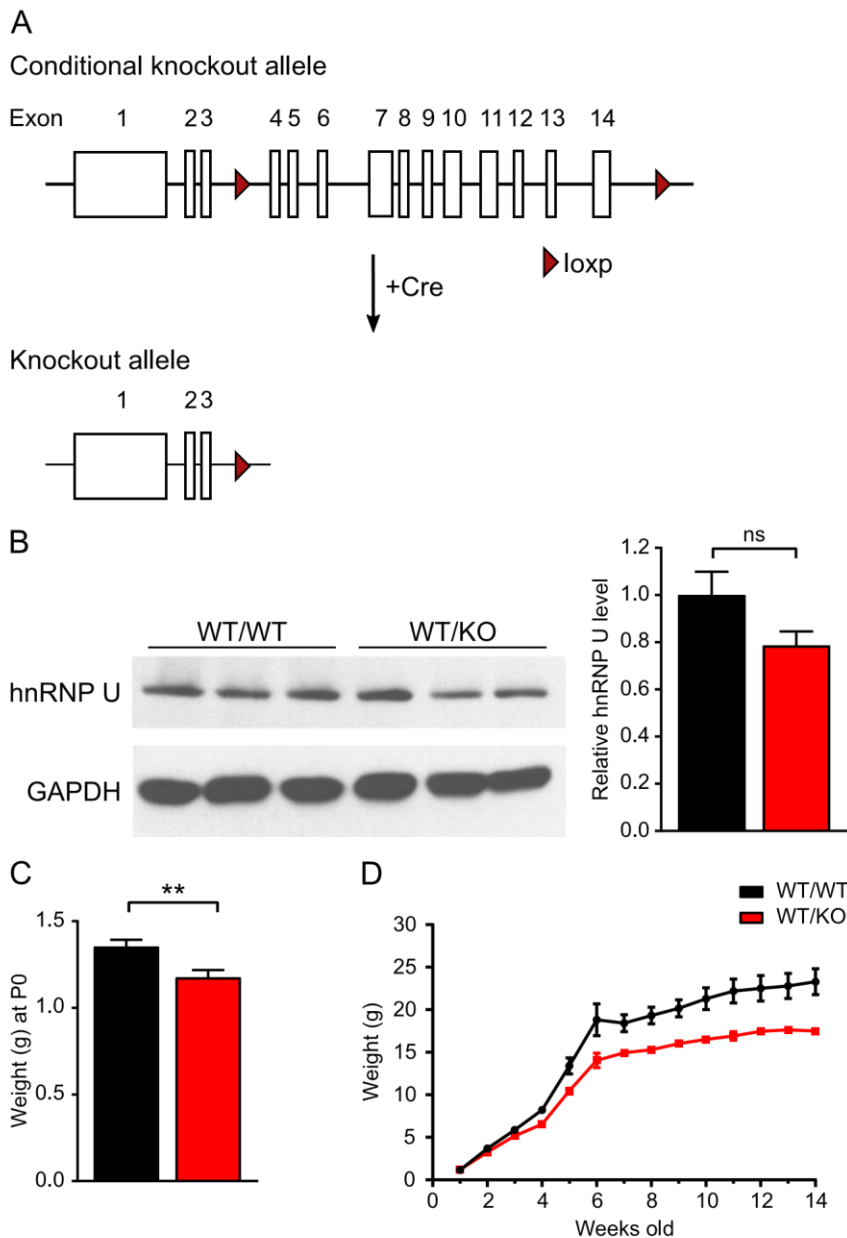


Figure 2.2: HnRNP U reduction ubiquitously is pathological.

A. Schematic representing hnRNP U conditional allele. Co-expression of the conditional knockout allele with cre recombinase leads to the excision of exons 4 through 14, and thus no hnRNP U protein is produced. B. HnRNP U hemizygous animals, expressing 1 wild type (WT) and 1 knockout (KO) allele, display a non significant, slight reduction in hnRNP U protein levels in the brain, quantified on the right relative to GAPDH levels. C. HnRNP U hemizygous animals are smaller than WT littermates at P0. (N = 11-21; **P<0.01 using *t*-test). D. HnRNP U hemizygous animals are smaller than WT littermates into adulthood. (N = 2-5). (For B, C, and D error bars represent s.e.m.).

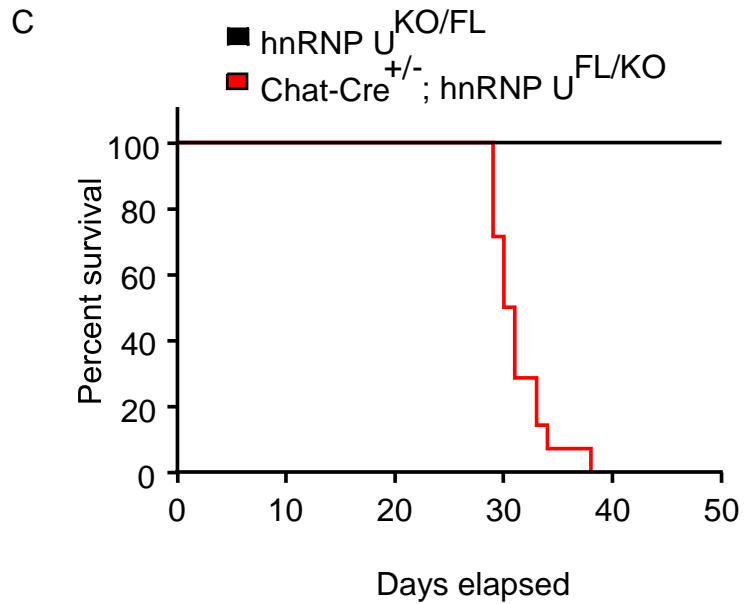
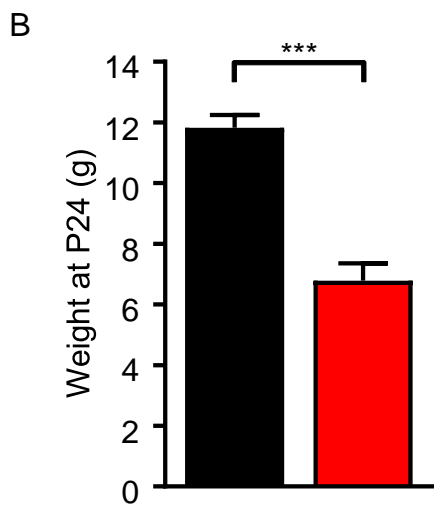
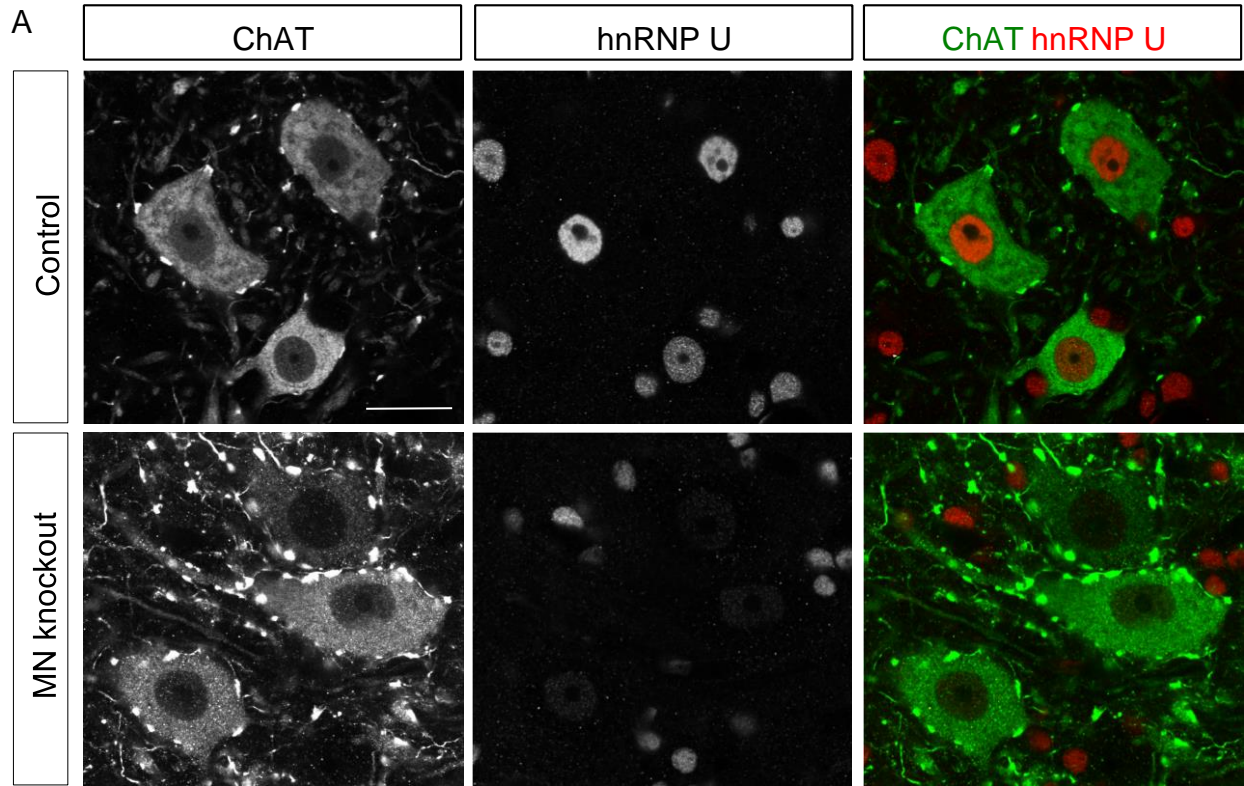


Figure 2.3: HnRNP U elimination from motor neurons results in premature death. A. Sections of lumbar spinal cord stained with MN marker ChAT and hnRNP U from control (hnRNP U^{KO}) or MN knockout (Chat-Cre^{+/-}; hnRNP U^{FL/KO}) animals. Scale bar – 25 μm. B. MN knockouts (red) are smaller than controls (black) at P24. (N = 5-8; ***P<0.001 using *t*-test. Error bars represent s.e.m.). C. Shorter lifespan of MN knockouts compared to control. Kaplan-Meier plot showing the cumulative probability of survival of indicated genotypes. (N = 14-19 per genotype; log-rank test = 40.29; ***P<0.001).

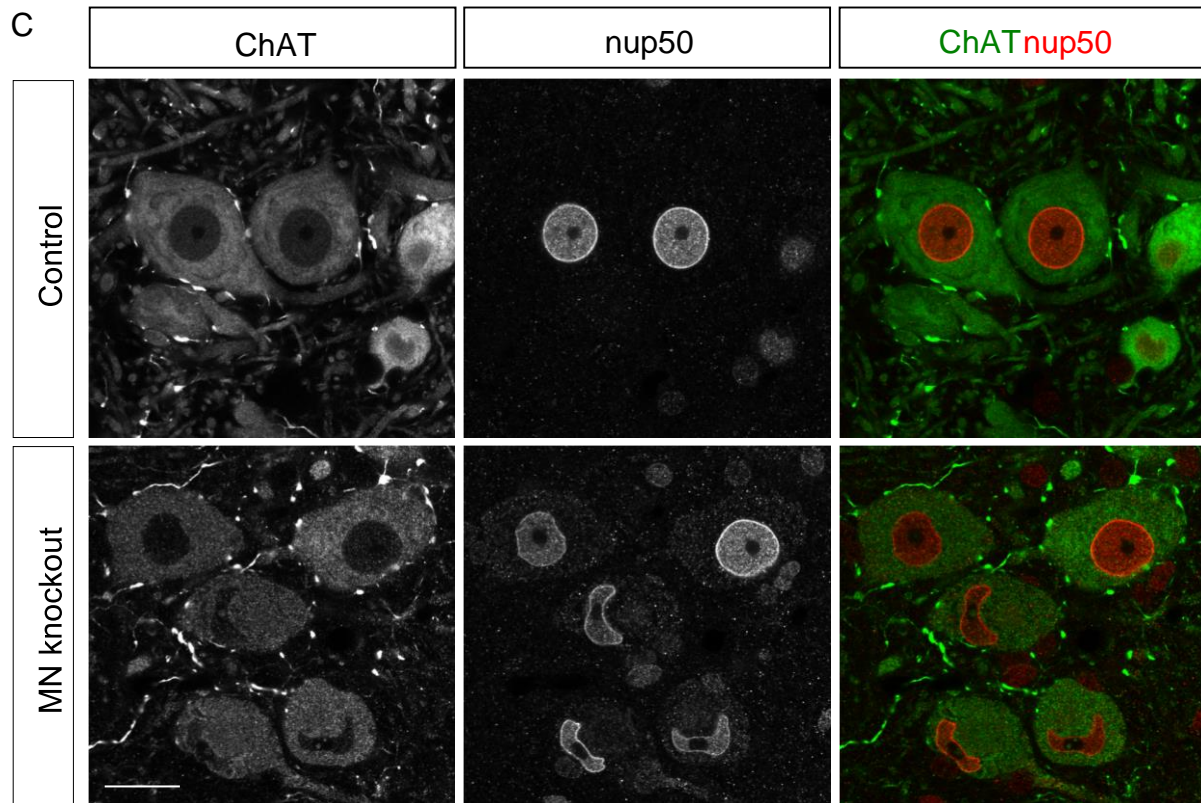
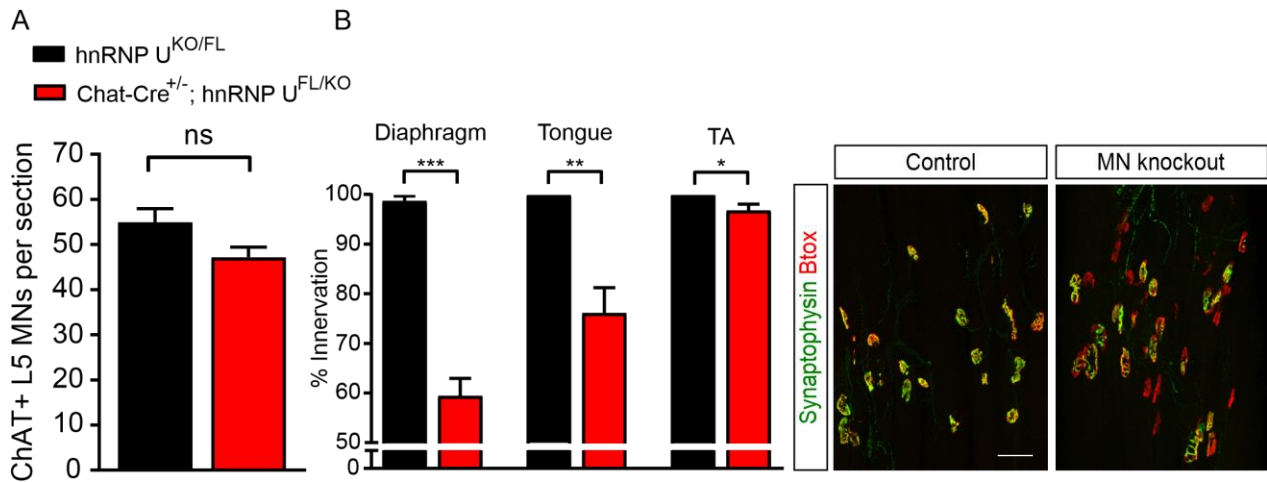


Figure 2.4: Loss of hnRNP U from motor neurons (MNs) causes denervation and MN abnormalities.

A. Loss of hnRNP U in MNs does not cause L5 MN loss by endstage (P30). B. Loss of hnRNP U in MNs causes loss of innervation (denervation) of the diaphragm, tongue, and TA muscles at endstage. Example of diaphragm muscle stained with presynaptic marker synaptophysin (green) and postsynaptic marker tetramethylrhodamine-conjugated alpha-bungarotoxin (Btox). Scale bar, 50 μ m. C. Loss of hnRNP U in MNs causes abnormal nuclear morphology. Lumbar MNs stained with MN marker ChAT and nuclear envelope marker nup50. Scale bar, 25 μ m. (For A and B, N = 4-6; ***P<0.001, **P<0.01, *P<0.1 using *t*-test. Error bars represent s.e.m.).

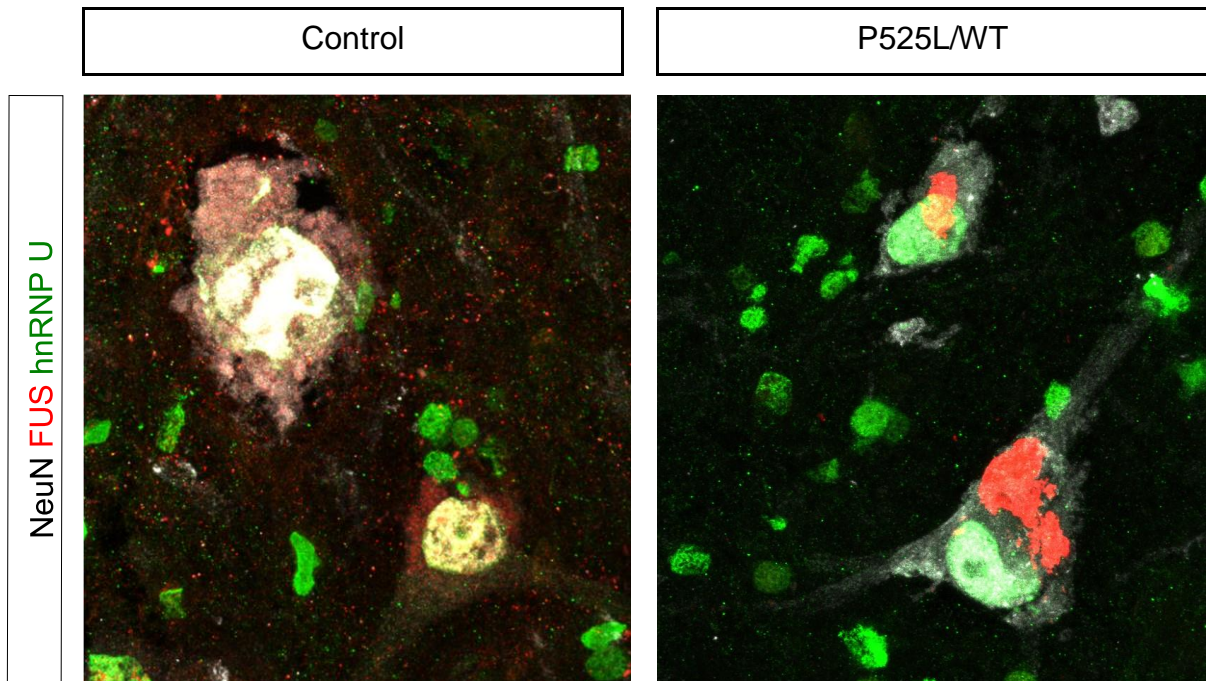


Figure 2.5: HnRNP U does not colocalize with FUS aggregates in FUS patients.

Staining neurons with neuronal marker NeuN, FUS, and hnRNP U from an ALS patient with a P525L mutation in comparison to a control reveals that hnRNP U remains nuclear in mutant-FUS patients and does not localize to cytoplasmic aggregates.

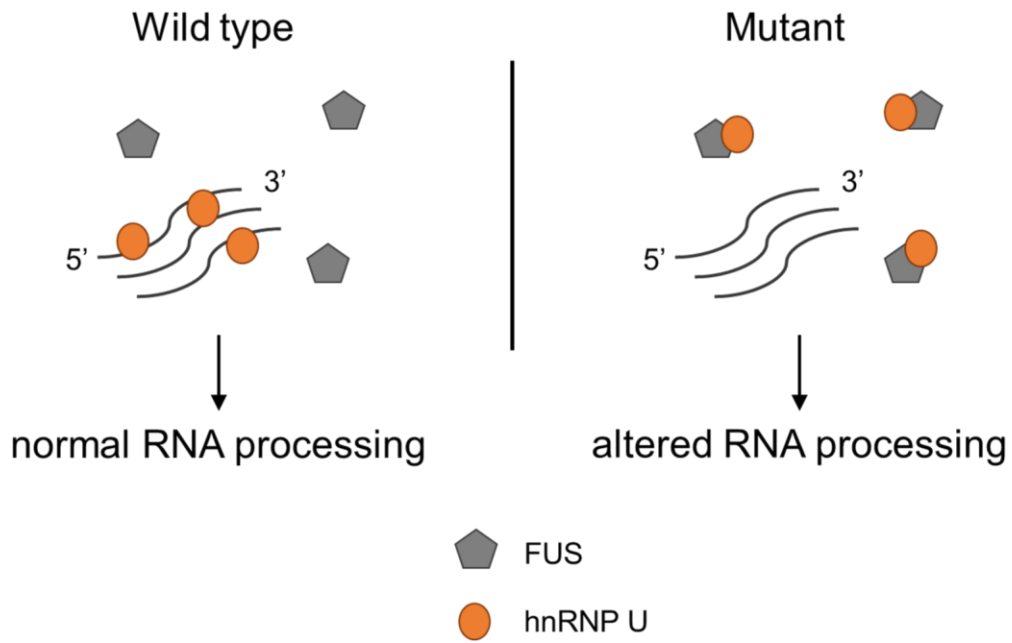


Figure 2.6: Model for hnRNP U-dependent toxicity.

Under normal circumstances, hnRNP U executes its RNA-processing functions in the cell. When mutant FUS is present, however, FUS aberrantly interacts with hnRNP U, leading to hnRNP U sequestration, loss of function, and subsequent neurodegeneration. This model predicts modulating hnRNP U levels would modulate the FUS-ALS neurodegenerative phenotype.

Protein	hFUS-P525L	WT
hnRNP U	44	0
hnRNP R	15	0
hnRNP Q	14	0
FUS	10	0
hnRNP K	8	0
Fxr1	7	0
Dsg1a	7	2
Rtcb	6	0
Rbmx11	6	0
Habp4	6	0
G3bp2	4	0
Rps2	4	0
Rps4l	4	0
Rpl23a	4	0
SFPQ	3	0
Fbl	3	0
Cnp	2	0
Ddx1	2	0
Caprin1	2	0
Ubap2l	2	0
Serbp1	1	0
Pde4d	1	0

Table 2.1: Proteins identified by mass spectrometry as mutant-FUS interactors.

22 proteins were identified as hFUS-P525L interactors, most of which were RNA binding proteins. Proteins are listed with the number of spectral counts identified (a marker for abundance). WT refers to a non-myc-expressing, C57BL/6J animal.

Protein	Yamazaki et al.	Wang et al.	Blokhuis et al.	Kamelgarn et al.
hnRNP U		X	X	X
hnRNP R	X		X	X
hnRNP Q	X		X	
FUS	X			
hnRNP K		X	X	X
Fxr1			X	X
Dsg1a				
Rtcb				X
Rbmx11				
Habp4				
G3bp2	X		X	X
Rps2				
Rps4l				
Rpl23a				
SFPQ		X	X	
Fbl				
Cnp				
Ddx1		X	X	X
Caprin1	X		X	X
Ubap2l		X		
Serbp1		X		
Pde4d				

Table 2.2: Proteins identified as FUS interactors in other data sets.

Comparison of hFUS-P525L interactors identified in this thesis (left column) with 4 other data sets reveals a number of proteins common in multiple systems (indicated by 'X').

Chapter 3: The TDP-43 knock-in model

3.1 Introduction

TDP-43 inclusions are found in almost all ALS patients, and *TARDBP* mutations have been identified as causal for the disease, suggesting TDP-43 plays a central mechanistic role. Understanding the age-dependent mechanisms that underlie the toxicity of wild-type (WT) TDP-43 in ALS and related TDP-43 proteinopathies, and the effects of ALS-associated *TARDBP* mutations on this process will have important implications for the development of novel therapies for these disorders.

TDP-43 is a DNA/RNA binding protein that functions in many aspects of RNA metabolism including splicing, translation and transport (Ratti & Buratti 2016). The functional sequestration of TDP-43 in nuclear and cytoplasmic aggregates and the nuclear exclusion of TDP-43 in ALS and related disorders may result in a loss-of-function that underlies TDP-43 toxicity. Alternatively, ALS-related mutations or the aberrant processing of wild-type TDP-43 may lead to toxicity through a novel gain of function mechanism, possibly related to protein aggregation.

Differentiating between loss and gain of function mechanisms is made difficult by our incomplete understanding of the functional consequences of *TARDBP* mutations. Studies of the effects of ALS-related mutations on TDP-43 function have also been complicated by the use of overexpression systems in which TDP-43 - with or without ALS-associated mutations - is toxic in a dose dependent manner (McGoldrick et al. 2013). These models fail to reproduce key features of ALS, raising concerns about their relevance to ALS, and suggest that a more faithful genetic model is needed to

understand the role of normal and mutant TDP-43 function in motor neuron development, function and survival in health and disease.

Here we report the generation of two TDP-43 knock-in mouse lines in which we introduced the ALS-causing mutation M337V or G298S into the mouse *TARDBP* gene to express mutant TDP-43 from the endogenous mouse locus, recreating the ALS patient genotype. This knock-in approach to model TDP-43-ALS in the mouse overcomes the issues of overexpression that may complicate phenotypic analysis, and makes it possible to study the consequence(s) of disease-related mutation on TDP-43 function and the dose-dependent effect of mutant TDP-43 on motor neuron survival.

3.2 Results

Generation of TDP-43 knock-in mice

To determine the effects of *TARDBP* mutations on normal TDP-43 function and to model the toxicity of mutant TDP-43 faithfully, we recreated the human ALS genotype in the mouse by introducing ALS-associated mutations into the mouse *TARDBP* gene. Two causal mutations - M337V, one of the most common TDP-43 mutations, and G298S, associated with a shorter disease progression (Corcia et al. 2012), were each used to generate novel TDP-43 knock-in (TDP-43^{KIN}) mouse lines (Fig. 3.1).

Mutant TDP-43 is not associated with loss of function

In the TDP-43 knock-in mice, an ALS allele replaces the endogenous (WT) gene, which makes it possible to address the effects of the mutation on protein function. TDP-43 is critical for cell survival both during development and in adulthood, as demonstrated respectively by the lethality associated with TDP-43 elimination from the embryo or postnatally. First, to determine whether the ALS-associated mutations M337V or G298S have any effect on the critical function(s) of TDP-43, we asked whether either of these alleles could overcome the lethality associated with the loss of TDP-43. Using a TDP-43 knockout (TDP-43^{KO}) allele (Sephton et al. 2009), we were able to generate both hemizygous (TDP-43^{KIN/KO}) and homozygous (TDP-43^{KIN/KIN}) animals with each of the M337V and G298S knock-in alleles in the expected Mendelian ratios. In all cases, the animals appeared normal at birth, indistinguishable from wild-type and heterozygous (TDP-43^{KIN/WT}) littermate controls. This early experiment

demonstrated that the M337V and G298S mutant alleles, causally related to ALS in patients, are both fully functional in the mouse in that they are able to rescue the lethality associated with TDP-43 loss-of-function, without any obvious functional consequences.

We next looked in the mutant mice to see whether these ALS-causing *TARDBP* mutations affect the level or pattern of TDP-43 expression *in vivo*. Analysis of TDP-43 protein and RNA levels of homozygous mice revealed that the mutations have no effect on RNA (Fig. 3.2A) or protein levels (Fig. 3.2B). TDP-43 is a predominantly nuclear protein, and its mislocalization to the cytoplasm in ALS has been implicated in the neurotoxicity of TDP-43. To determine whether ALS-associated mutations in *TARDBP* directly cause cytoplasmic mislocalization of TDP-43, we analyzed TDP-43^{M337V} and TDP-43^{G298S} homozygous mice and observed that TDP-43 remains nuclear and does not mislocalize to the cytoplasm as a consequence of the mutations alone (Fig. 3.2C). One essential function of TDP-43 is in autoregulation of its own expression and precise maintenance of cellular TDP-43 levels. As even a modest excess of wild-type TDP-43 is toxic in a variety of systems (McGoldrick et al. 2013), any defect in the autoregulatory function of TDP-43 could lead to TDP-43 accumulation and toxicity. We used the hemi- and homo-zygous mutants to assess the consequences of the mutations on TDP-43's ability to regulate its own expression. Although only one allele is active in the hemizygous (TDP-43^{KO/WT}) mutants, TDP-43 protein levels are equivalent to that in wild-type (TDP-43^{WT/WT}) animals as a consequence of autoregulation (Fig 3.2D). Similarly we observed equivalent amounts of TDP-43 in all knock-in mutant animals compared to

WT and hemizygous KO controls (Fig. 3.2D), demonstrating that the M337V and G298S mutations do not impair TDP-43 autoregulatory function.

Heterozygous mutations are insufficient to cause motor neuron pathology

In ALS patients, *TARDBP* mutations typically cause late (adult) onset motor neuron disease, so we next asked about the age-dependent consequences of the M337V and G298S knock-in mutations on long term motor neuron health in heterozygous mice, which copy the human ALS genotype. We looked for key pathological hallmarks of ALS, including motor neuron loss, muscle denervation and aggregation of TDP-43. Using an antibody against choline acetyl transferase (ChAT), we visualized and counted motor neurons in lumbar levels 4 and 5. At 1, 1.5 and 2 years of age, we observed no difference in the average number of motor neurons in heterozygous TDP-43^{M337V} and TDP-43^{G298S} mice (TDP-43^{M337V/WT} or TDP-43^{G298S/WT}) in comparison to wild-type controls (Fig. 3.3B).

In ALS patients, fast-fatigable motor neurons are preferentially affected, and denervation of the neuromuscular junction (NMJ) in corresponding muscles precedes spinal motor neuron loss (Dengler et al. 1990; Fischer et al. 2004). We therefore examined the integrity of the neuromuscular junctions of the relatively vulnerable tibialis anterior (TA) muscle, which is predominantly innervated by fast-fatigable motor neurons, using an antibody against synaptophysin (syn) to label motor axon terminals, and a fluorescent conjugate of α -bungarotoxin (BTX) to label the post synaptic side of the neuromuscular junction. At 1, 1.5, and 2 years of age, we found no increased

denervation of the TA endplate in heterozygous knock-in mice relative to wild-type controls (Fig. 3.3A).

Lastly, since TDP-43 inclusions are found in the large majority of ALS patients in both sporadic and familial cases, we looked for evidence of TDP-43 pathology in the spinal cord of TDP-43^{M337V} and TDP-43^{G298S} heterozygous mutants. Using antibodies against TDP-43 and ChAT, we found no evidence of abnormal TDP-43 aggregation in these knock-in mice at 2 years of age, and the localization remained nuclear (Fig. 3.3C). Together, these data demonstrate that in the TDP-43^{M337V} and TDP-43^{G298S} heterozygous knock-in mutants that faithfully model the human ALS genotype, we find no evidence of motor neuron degeneration.

Homozygous mutations are insufficient to cause motor neuron pathology

The mechanisms by which dominant mutations in *TARDBP* cause ALS are not known, but many ALS-associated alleles are not fully penetrant in humans, and so it is perhaps not surprising that the equivalent mutations in the knock-in mice are alone insufficient to cause motor neuron degeneration. In this age-dependent disease, several factors – environmental and genetic - likely contribute to the onset and progression of disease. One determinant of disease onset may be levels of TDP-43 expression, as an excess of even wild-type TDP-43 is toxic. In this threshold model of disease, local increases in protein concentration on a cellular level may underlie toxicity, and mutant forms of TDP-43 may more easily reach the critical threshold required to elicit motor neuron pathology. Given that the mouse TDP-43^{M337V} and TDP-43^{G298S} alleles both appear to be fully functional, we asked whether higher levels of mutant TDP-43 in

homozygous knock-in animals could elicit a motor neuron phenotype. At 1, 1.5, and 2 years of age, we observed no excess motor neuron degeneration (Fig. 3.4B), TA denervation (Fig. 3.4A) or mislocalization and aggregation (Fig. 3.4C) in the TDP-43^{M337V} or TDP-43^{G298S} homozygous knock-in animals compared to wild-type controls. Although all of the TDP-43 in these homozygous animals was ALS mutant protein, twice the physiological level of mutant TDP-43 was insufficient to cause motor neuron degeneration.

TDP-43 remains soluble in knock-in mutants

Although we found no evidence of motor neuron degeneration in the TDP-43 knock-in mice, we looked for other abnormalities that may suggest early signs of TDP-43 toxicity in motor neurons. At end-stage in ALS patients, TDP-43 becomes insoluble (Fig. 3.5A) and cytoplasmic inclusions form in degenerating neurons and surrounding glia (Neumann et al. 2006; Arai et al. 2006). These changes in TDP-43 localization and solubility likely precede motor neuron degeneration, so we looked in the knock-in mice for biochemical evidence of early TDP-43 pathology.

We carried out fractionation experiments to separate out the soluble, sarkosyl-soluble, and sarkosyl-insoluble proteins from the brain of 2-year-old knock-in animals. Upon probing for TDP-43, we observe no differences in TDP-43 solubility in the knock-in animals as compared to wild-type controls (Fig. 3.5B).

Knock-in animals do not show gliosis

In other SOD1 and FUS models of ALS and in ALS patients, motor neuron degeneration is accompanied by an inflammatory response in the ventral horn of the spinal cord (Bruijn et al. 1997; Sharma et al. 2016; Schiffer et al. 1996). To look for evidence of astrocytosis and microgliosis in the TDP-43^{KIN} mice, we stained the lumbar spinal cord of TDP-43^{M337V} and TDP-43^{G298S} hetero- and homo-zygous mutants with antibodies for glial fibrillary acidic protein (GFAP) and Iba1, specific for astrocytes and microglia respectively. At 2 years of age we observe no differences in GFAP or Iba1 immunoreactivity in knock-in animals as compared to wild-type controls (Fig. 3.5C), demonstrating no evidence of cellular inflammation in this model.

Mouse and human TDP-43 are similarly toxic

While a great advantage of the knock-in system is expression of mutant TDP-43 from the endogenous locus, the differences between mouse and human TDP-43 may make the mutant mouse protein less pathogenic and underlie the absence of pathology. The proteins are >96% identical with only 16 amino acid differences, however, most differences cluster in RRM2 and the low complexity domain, two regions which are important in TDP-43 aggregation (Fig. 3.6A). To address this caveat, I performed toxicity experiments in yeast as described previously to determine whether mouse and human TDP-43 were similarly toxic (Johnson et al. 2009). TDP-43 was expressed in yeast under the control of a galactose inducible promoter such that when yeast were grown in glucose there was no TDP-43 expression, however upon growth in galactose,

TDP-43 was robustly expressed. Using spotting assays, both mouse and human TDP-43 caused similar toxicity upon growth in galactose (Fig. 3.6B). Additionally, the growth rates of the yeast upon expression of mouse or human TDP-43 were significantly slower than an empty vector control but not different from each other (Fig. 3.6C), suggesting mouse TDP-43 is similarly toxic in comparison to human TDP-43.

3.3 Discussion

TDP-43 inclusions are a common pathological feature of nearly all patients with sporadic and familial forms of ALS. This, together with the identification of causal mutations in *TARDBP* strongly support the idea that TDP-43 plays a key role in the pathogenesis of ALS and other TDP-43 proteinopathies (Geser et al. 2009), but the mechanism of TDP-43-dependent neurodegeneration remains elusive.

More than 15 mouse models have been reported in which wild type and ALS mutant forms of human TDP-43 are exogenously expressed (McGoldrick et al. 2013; Arnold et al. 2013; Walker et al. 2015; Stribl et al. 2014). Though a neurodegenerative phenotype is observed in these animals, the disease relevance of these models to ALS is uncertain. Differences in the level and pattern of TDP-43 expression in these mice have led to considerable variability in motor phenotypes. In some mice, premature death is accompanied by motor neuron loss and denervation, and others it is not. In some but not others, a motor phenotype is accompanied by TDP-43 aggregation and misprocessing. Survival phenotypes range from less than 3 weeks to upwards of 1 year. Additionally, in some but not all systems, the overexpression of wild type TDP-43 leads to a similar motor phenotype as that observed with the overexpression of mutant TDP-43, suggesting that overexpression of TDP-43 is itself toxic in a way that may not be disease relevant. The normal function of TDP-43 to regulate its own protein levels through a negative feedback loop underscores the importance of maintaining TDP-43 levels within a precise physiological range. Therefore, any effort to model TDP-43

pathology by ectopic overexpression of this protein will override this autoregulation, possibly resulting in phenotypes unrelated to human motor neuron disease.

Similarly, TDP-43 loss-of-function models may not reproduce the disease phenotype, and the lethality associated with widespread TDP-43 elimination in development (Wu et al. 2010) or postnatally (Chiang et al. 2010), or the cell autonomous effect of TDP-43 elimination in motor neurons (Iguchi et al. 2013; Wu et al. 2012) may simply reflect the loss of a critical regulatory protein, whether or not that occurs in ALS in the course of disease. Despite the abundance of systems, the role of TDP-43 activity in motor neuron survival and the effect of ALS-causing *TARDBP* mutations on physiological or toxic functions of TDP-43 has not been determined.

To overcome the potential artifacts associated with overexpression of exogenous TDP-43 *in vivo* and produce an unbiased animal model, we generated a novel knock-in mouse expressing ALS-mutant TDP-43. In this model, we inserted ALS-associated mutations into the mouse *TARDBP* genetic locus so that heterozygous TDP-43^{KIN} mutants express TDP-43 at a physiological level and in a pattern that mimics ALS patients with the equivalent mutations. The great advantage of this system is that it makes possible a genetic approach to assess the consequence of ALS-associated mutations on normal TDP-43 function when crossed to the knockout allele or in the homozygous mutants, and permits the study of increasing dosage of mutant TDP-43 on motor neuron survival.

Using this mouse, we first demonstrated that the TDP-43^{M337V} and TDP-43^{G298S} alleles are fully functional since in the hemizygous mutants (TDP-43^{M337V/KO}, for example), both rescue the embryonic and postnatal lethality of TDP-43 loss of function.

These ALS-causing *TARDBP* mutations also do not influence TDP-43 protein or RNA levels, change cellular localization of this nuclear protein, or interfere with the autoregulation of TDP-43 levels that underlie its critical role in cell survival. Importantly, homozygous mutant mice are indistinguishable from wild type mice, suggesting that the mutant protein retains normal activity.

These data examine *in vivo* for the first time in the mouse the effects of *TARDBP* mutations on properties of the protein in the absence of wild type protein, which confounds data interpretation in previous studies. Interestingly, these data are inconsistent with observations in zebrafish, in which mutant TDP-43 expression could not rescue the motor defects associated with TDP-43 reduction (Kabashi et al. 2009). This discrepancy may indicate differing roles of mutations on TDP-43 function between vertebrates and invertebrates, and perhaps specifically in development, or may be a result of the overexpression approaches used to express TDP-43 in zebrafish. Due to the increased similarity of mice to humans and the cleaner genetic approach, the results from the knock-in mouse model system are likely more relevant to humans and disease.

These data demonstrate that the mutations themselves do not affect TDP-43 function, providing evidence against a primary loss of TDP-43 function in ALS. While loss of function does not seem to be a direct consequence of mutations themselves, it may be a secondary consequence of protein aggregation and TDP-43 sequestration, and therefore we cannot rule out loss of function as a downstream disease mechanism. This suggests *TARDBP* mutations cause a gain of function toxicity, perhaps through protein aggregation and/or secondary loss of function due to sequestration of TDP-43 or other proteins.

Both time (age) and mutations seem to have similar effects on TDP-43 pathogenic behavior, and therefore understanding the long-term consequences of the mutations on motor neuron health will likely be relevant to ALS broadly. In older heterozygous and homozygous mice, we report an absence of motor neuron pathology, as determined by motor neuron loss, denervation, TDP-43 aggregation and insolubility, and gliosis. One explanation for the absence of pathology is the flaws with the mouse model system. As aging is an established risk factor for ALS, it is possible that a certain amount of time is required for pathology to accumulate to a pathological level, and the mouse lifespan (approximately 2-3 years) is simply not long enough. In support of this hypothesis, juvenile onset, aggressive ALS mutations result in a motor phenotype when mice are between 1 and 2 years old, and therefore adult onset mutations may not lead to pathology within the mouse lifespan (Scekic-Zahirovic et al. 2017). A second explanation is that differences between mouse and human TDP-43 mute the toxicity of mouse TDP-43. Expression studies in yeast modeled precisely after those previously reported (Johnson et al. 2009) indicate that mouse TDP-43 is as toxic as human TDP-43, however future mutation analyses will be required to determine whether any of the mouse-human differences more subtly influence the biophysical properties of the protein.

Alternatively, the mutations by themselves may be insufficient to precipitate motor neuron pathology, which is perhaps a likely explanation given the multitude of factors causing ALS in humans, and the additional factors causing disease in humans may be absent in this model system. Age, mutations, and sex are the biggest known risk factors, however the vast majority of ALS cases are still an etiological mystery.

These data suggest a stressor threshold hypothesis of motor neuron degeneration in ALS in which genetic mutations confer a predisposition in an individual to ALS but additional stressors, such as age, environmental toxins, and genetic modifiers, are required to reach the pathological threshold. The threshold hypothesis is also consistent with many observations from ALS patients. Most ALS cases onset in middle or old age suggesting age as a precipitating factor, however absence of ALS from the majority of the world's population suggests it cannot be the only contributing factor. Additionally, ALS typically presents with focal onset, suggesting that external factors can change the local environment on a cellular level to induce selective degeneration, despite ubiquitous mutations. An advantage of this knock-in model is the ability to model the multifactorial nature and variability associated with ALS.

Future experiments will focus on the combination of external ALS relevant stressors with mutations to determine to which stressors mutations confer vulnerability. Strong candidates are genetic modifiers, which have previously been associated with the human disease. In addition, the genetic background of the mouse can greatly modulate ALS phenotypes in SOD1 mouse models, and unpublished work from our lab suggests this is true for human TDP-43 as well. A different TDP-43 animal model expressing human TDP-43 cDNA from the endogenous *MAPT* genetic locus yielded a paralysis phenotype by 18 months on a mixed C57BL/6 and Ola129 background, and this phenotype was completely abolished on a pure C57BL/6 background (Drs. Aarti Sharma and Helaina Lehrer, personal communication). Through crossing knock-in alleles onto other genetic backgrounds we will determine whether certain modifiers in

combination with *TARDBP* mutations are sufficient to reveal a phenotype, and further sequencing experiments could permit the identification of the modifying alleles.

Additionally, epidemiological data suggests environmental toxins as additional risk factors in ALS (McGeer et al. 1997; Oskarsson et al. 2015). Therefore, through a collaboration with Dr. Diane Re in the Environmental Health and Sciences department, we are asking whether *TARDBP* mutations can combine with environmental factors to reveal a susceptibility of motor neurons. From preliminary experiments, knock-in motor neurons *in vitro* either from primary culture or differentiated from embryonic stem cells seem more susceptible to heavy metal toxicity than wild type, and whether this is true *in vivo* in the mouse and relevant to ALS remains to be determined (Dr. Diane Re, personal communication).

The presence of TDP-43 inclusions in ALS and the ubiquitous presence of protein aggregates in neurodegenerative disease in general suggests aggregation may be a common downstream mediator of disease through a prion-like mechanism by which TDP-43 adopts an abnormal protein conformation, templating further TDP-43 aggregation and spreading from cell to cell. This prion-like mechanism would also explain the focality of ALS and regional spread, as TDP-43 aggregates would induce aggregation in neighboring cells and contiguously spread. Indeed *in vitro* experiments have documented the seeding ability of aggregated TDP-43. Insoluble patient-derived TDP-43 can trigger intracellular TDP-43 aggregation and insolubility *in vitro*, which can be propagated (Smethurst et al. 2016; Shimonaka et al. 2016; Furukawa et al. 2011; Nonaka et al. 2013). Still missing are *in vivo* experiments like those done with α -synuclein in Parkinson's disease (Luk, V. Kehm, et al. 2012; Luk, V. M. Kehm, et al.

2012) or Tau in Alzheimer's disease (de Calignon et al. 2012), in which inoculation with aggregated TDP-43 in one brain or spinal cord region induces wild type or mutant protein to convert into a prion-like form and results in spreading neurodegeneration. I would predict that inoculation into even wild type animals would result in a propagating neurodegenerative phenotype, but that endogenous knock-in mutant TDP-43 would accelerate disease spread.

On a molecular level, a threshold that precipitates pathology may be reached from local increases in TDP-43 concentration resulting from alterations in the dynamics of RNA granules. Protein-protein interactions within these granules are mediated by interactions between the low complexity domains, and interestingly most ALS-causal *TARDBP* mutations fall within that domain and may increase aggregation propensity, suggesting they may influence TDP-43 interactions within RNA granules. Through phase separation experiments, it has been shown that ALS mutations in *FUS* and *hnRNP A1*, and *C9orf72* change the dynamics of RNA granules and may promote time-dependent protein fibrillization within RNA granules in a concentration dependent manner. The downstream consequences of this altered phase-homeostasis may include protein aggregation and the sequestration of RNA binding proteins, and we hypothesize this to be true with regards to TDP-43 as well. Exciting work recently demonstrated the formation of TDP-43-containing liquid-like granules in neurons *in vitro*, whose structure was dependent on localization within the axon, density, and aging (Gopal et al. 2017). M337V and G298S *TARDBP* mutations increased the viscosity of these granules and altered transport dynamics, providing an *in vivo* correlate of altered liquid-liquid phase separation and suggesting that decreased molecular mobility within RNA granules may

contribute to disease pathogenesis. Together, these data demonstrate the exquisite sensitivity cells in general, and perhaps motor neurons in particular, to their internal concentration of RNA binding proteins, and we suggest ALS-stressors act through altering these local concentrations to precipitate pathology. This knock-in model provides an ideal *in vivo* system in which to explore ALS-relevant factors that combine with age and mutations to alter the dynamics of RNA binding proteins and lead to TDP-43 dependent neurodegeneration.

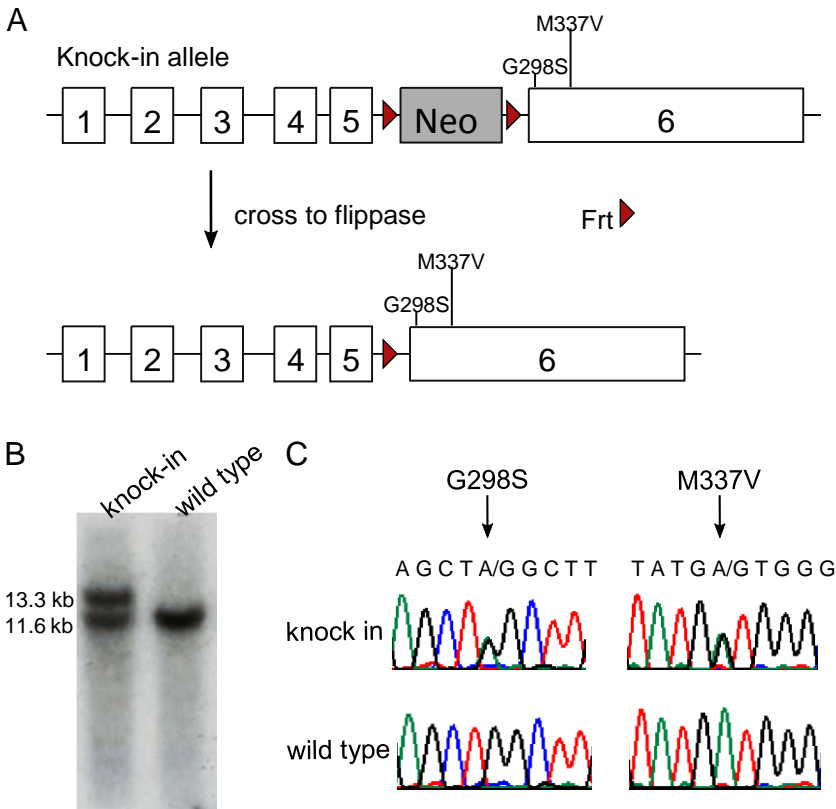


Figure 3.1: Generation of TDP-43 knock-in mice.

A. M337V or G298S knock-in point mutations were inserted into the endogenous mouse *TARDBP* genetic locus with a neomycin cassette flanked by Frt sites for selection, which was removed by crossing to flippase mice. B. Southern blot analysis of targeted embryonic stem cell clones following BamHI digestion and hybridization with an external probe. Presence of Neo cassette yielded larger product (13.3 kb) than the wild type (11.6 kb). C. Sequencing of knock-in cells reveals a double peak, indicating the heterozygous presence of the point mutations.

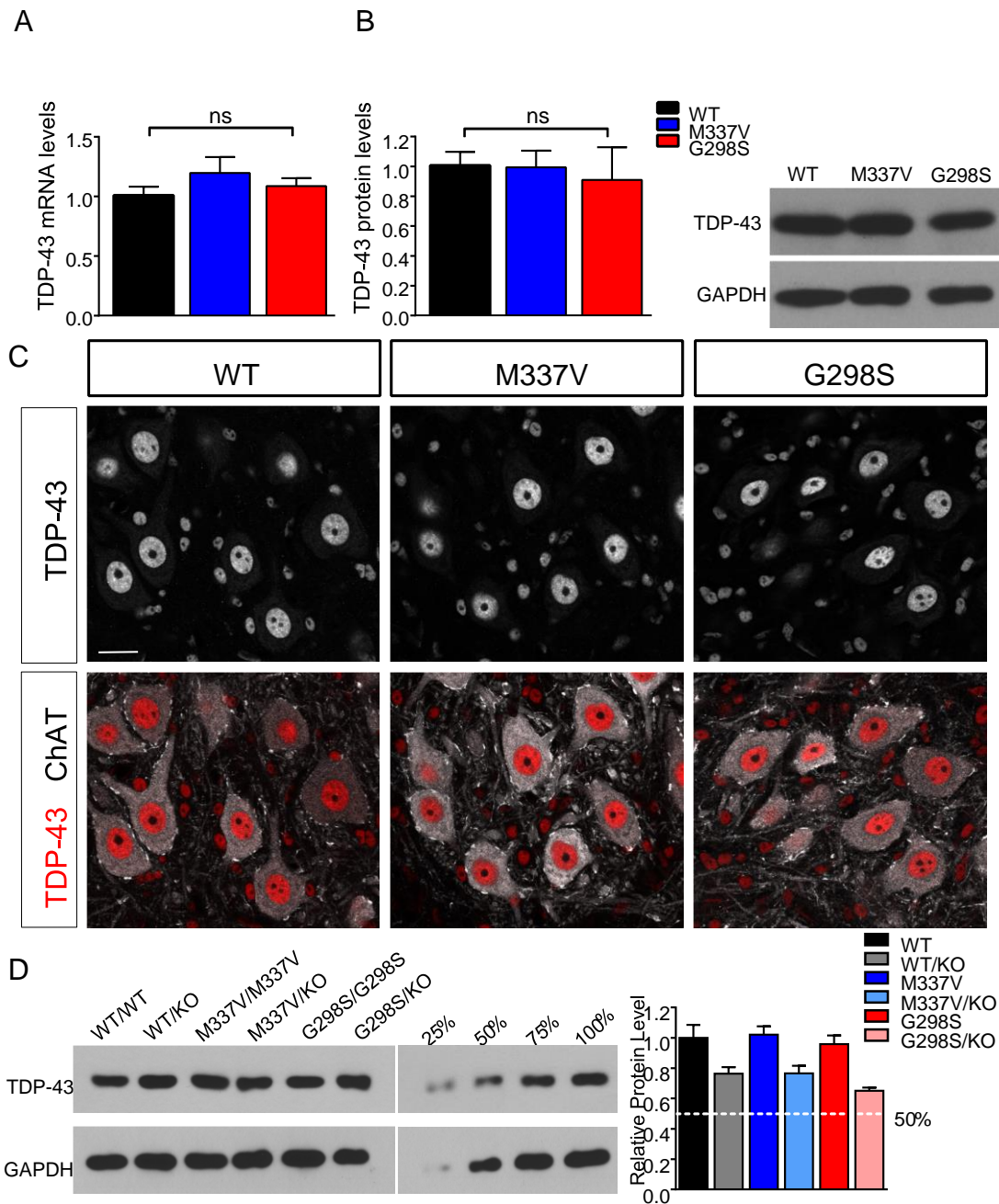


Figure 3.2: Knock-in mutations do not affect normal TDP-43 properties.

A. Quantitative RT-PCR of TDP-43 from spinal cord extracts of knock-in homozygous mice in comparison to GAPDH. B. Western blot analysis of TDP-43 levels from spinal cord extracts of knock-in homozygous mice, quantified relative to GAPDH loading control. C. Confocal images of L5 spinal cord cross-sections of wild type (left) or homozygous M337V (center) and G298S (right) animals immunostained with anti-TDP-43 and anti-ChAT antibodies. Scale bar, 25 μ m. D. Western blot analysis of TDP-43 levels from spinal cord extracts of homozygous or hemizygous (knockout = KO) wild type and mutant TDP-43 animals, with protein titration to semi-quantify signal difference. Protein levels quantified on the right relative to GAPDH. (For A, B, and D, N = 3 and error bars represent s.e.m.).

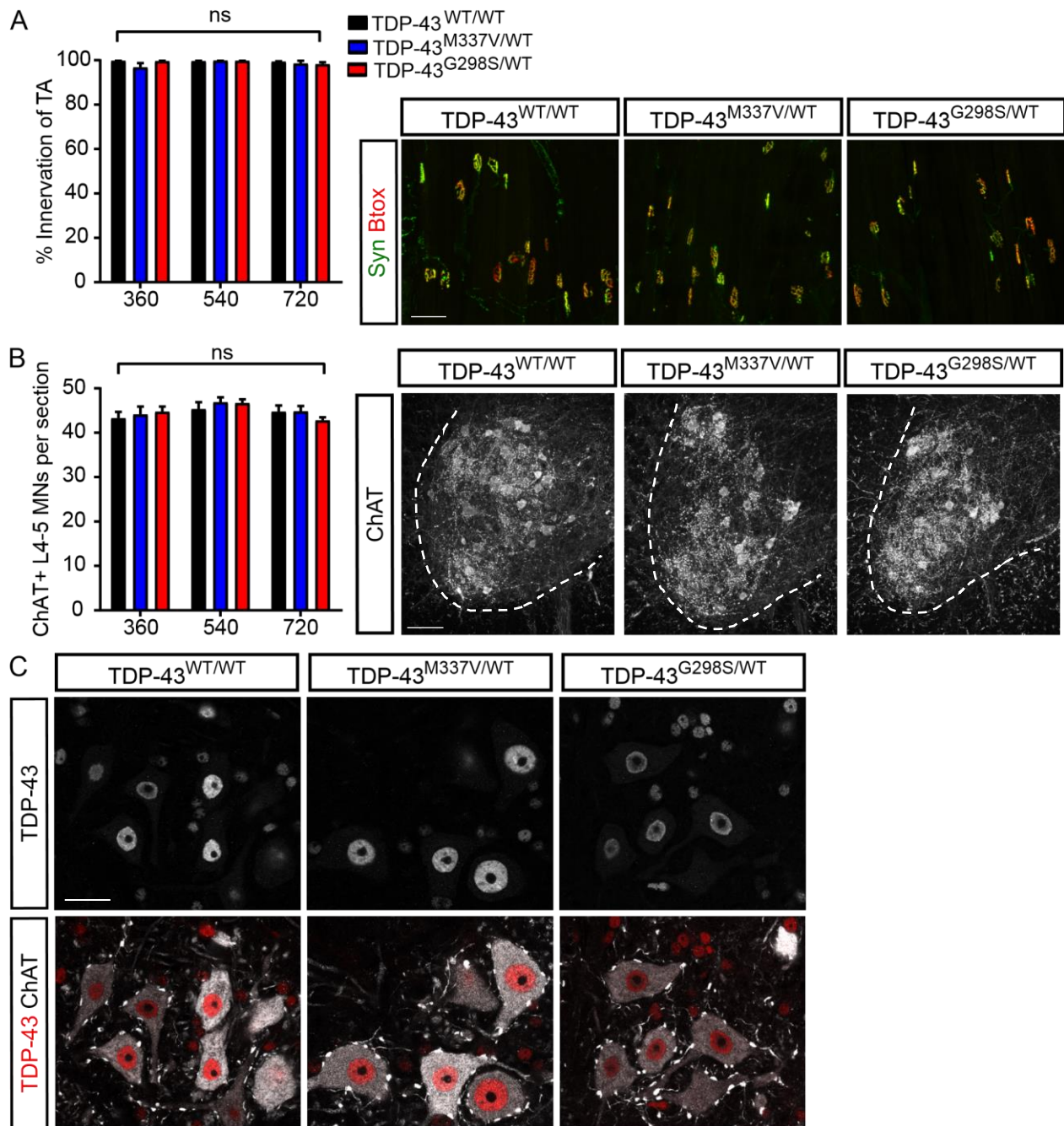


Figure 3.3: Heterozygous knock-in mutants do not display ALS pathology.

A. Representative images of tibialis anterior (TA) muscle immunostained with anti-synaptophysin (syn) antibody and tetramethylrhodamine-conjugated alpha-bungarotoxin (Btox), and percent innervation quantified over 2 years. Scale bar, 100 μ m. B. Representative images of L4-5 spinal cord immunostained with anti-ChAT antibody, and average MN number quantified over 2 years. Scale bar, 100 μ m. C. Representative images of wild type (left) or heterozygous M337V (center) and G298S (right) L4-5 spinal cord immunostained with anti-TDP-43 and anti-ChAT antibodies from 2 year old mice. Scale bar, 25 μ m. (For A and B, N = 3-4 and error bars represent s.e.m.).

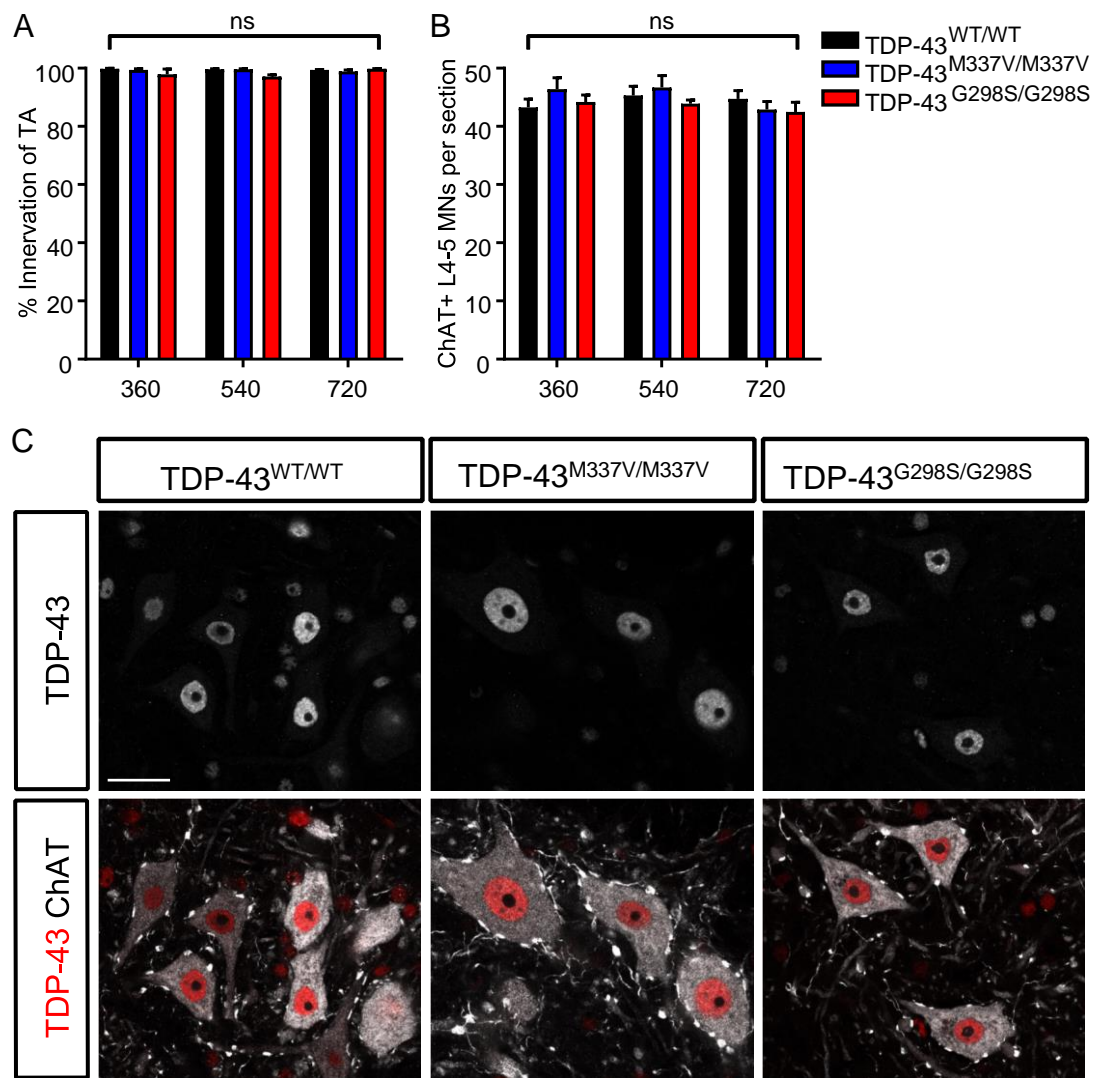


Figure 3.4: Homozygous knock-in mutants do not display ALS pathology.

A. Quantification of TA muscle innervation over 2 year period. B. Quantification of average MN number per L4-5 spinal cord section over 2 year period. C. Representative images of wild type (left) or homozygous M337V (center) and G298S (right) L4-5 spinal cord immunostained with anti-TDP-43 and anti-ChAT antibodies from 2 year old mice. Scale bar, 25 μ m. (For A and B, N = 3-4 and error bars represent s.e.m.).

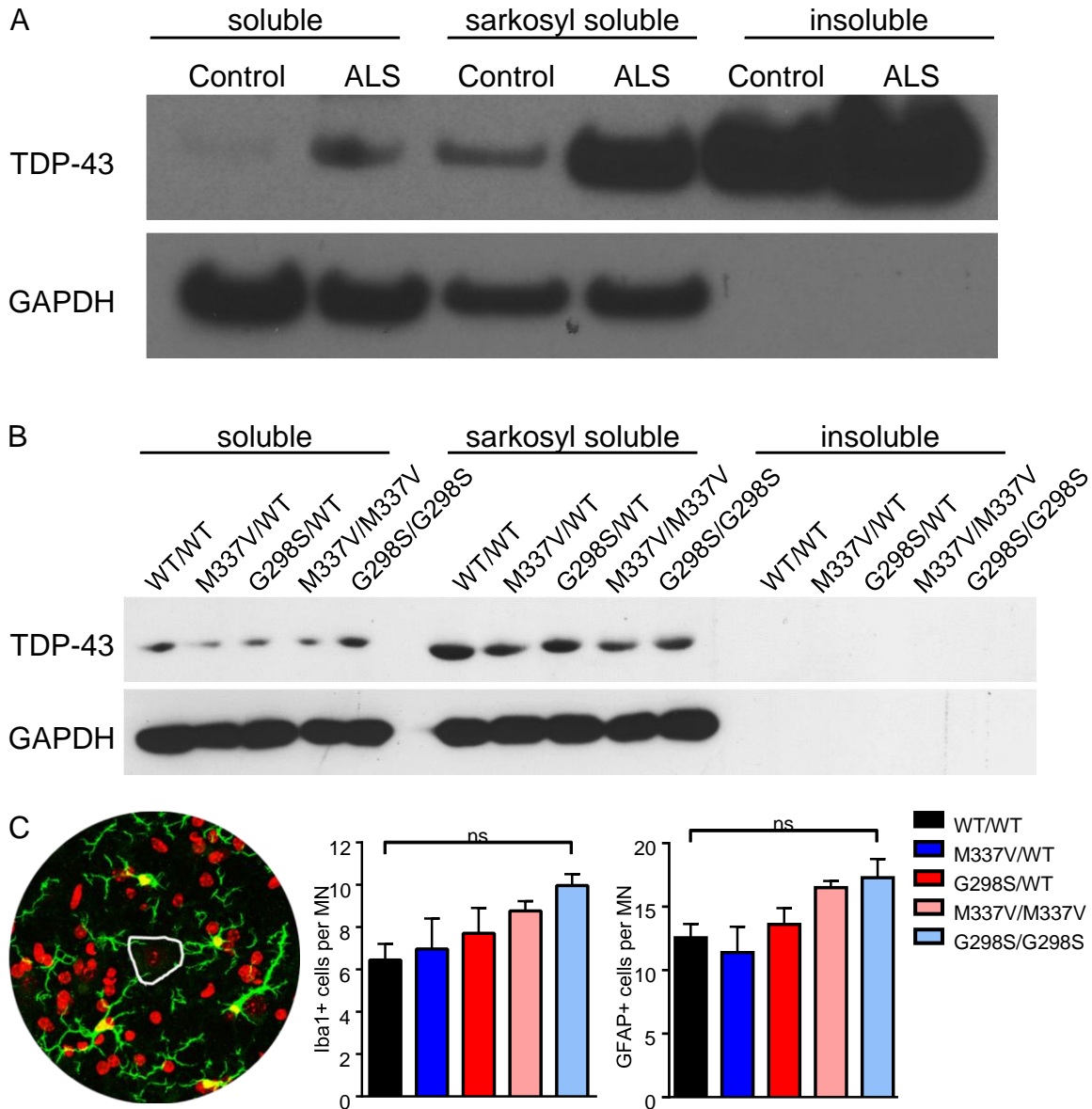


Figure 3.5: Other ALS indicators absent from knock-in mice.

Western blot analysis of TDP-43 levels in soluble, sarkosyl soluble, and insoluble protein fractions from an ALS patient with TDP-43 pathology and an age matched control (A) and 2 year old heterozygous and homozygous knock-in brain (B, N = 3). C. Glial reactivity (microglial marker Iba1 in green, DAPI in red, and MN outlined in white) within 100 μ m of lumbar MNs from 2 year old heterozygous and homozygous knock-in animals. Microglia quantified in the center and astrocytes quantified on the right. (N = 3 and error bars represent s.e.m.).

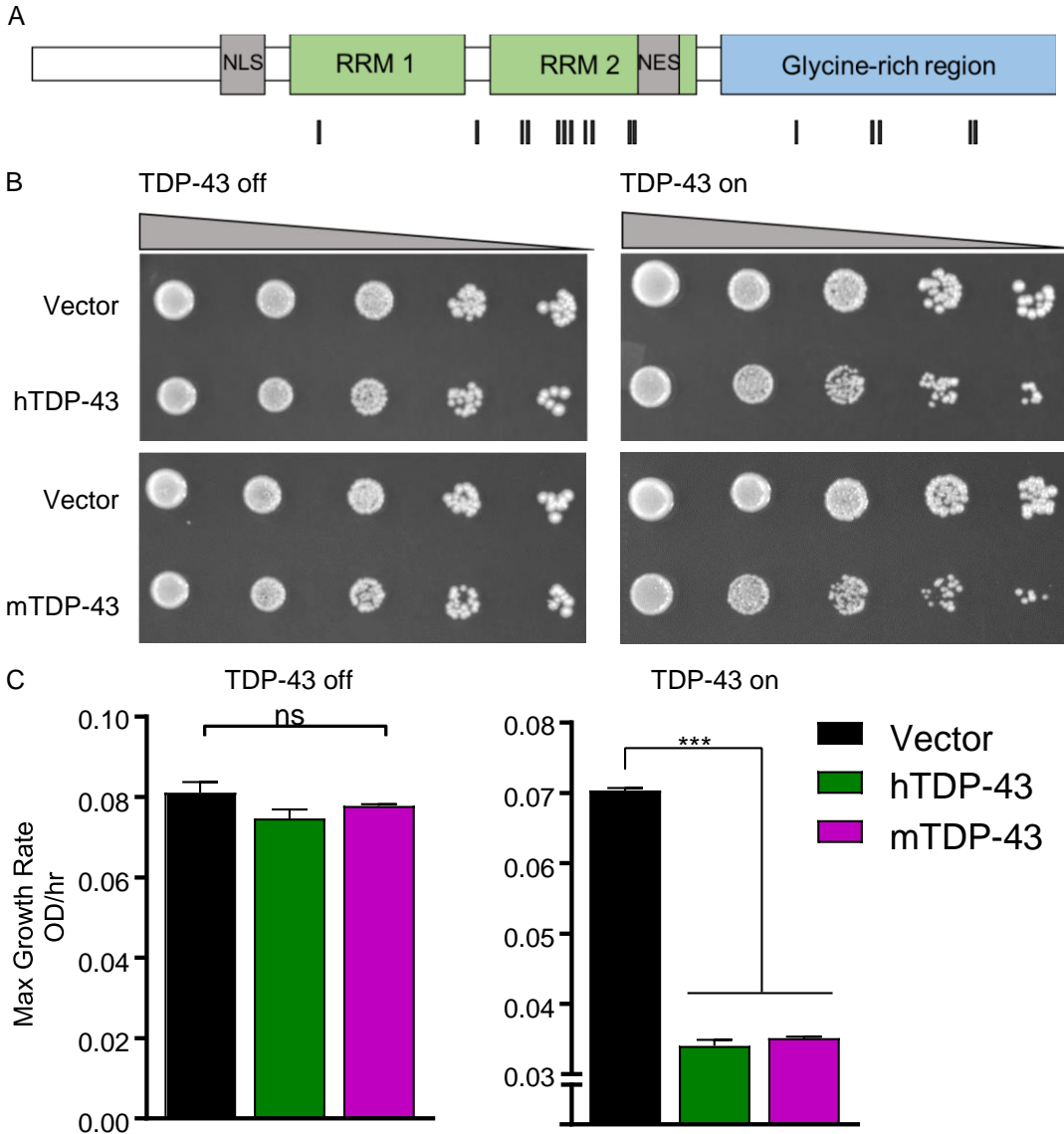


Figure 3.6: Mouse and human TDP-43 both cause toxicity in yeast.

A. There are 16 amino acid differences between mouse and human TDP-43 (indicated by gray bars), and most localize to RRM2 and the low complexity domain, regions that have been implicated in protein aggregation. B. Spotting assays upon expression of TDP-43 or an empty vector in yeast reveal both mouse and human TDP-43 cause toxicity. C. Growth rate did not differ between yeast expressing mouse or human TDP-43. (For B and C, N = 3; for C, ***P<0.001 using one way ANOVA and error bars represent s.e.m.).

Chapter 4: Conclusions

One of the fundamental questions in ALS is how a diverse set of genetic and environmental factors leads to a stereotyped, adult onset, neurodegenerative disorder primarily involving motor neurons. The genetics alone implicate a range of cellular processes that may be mechanistically involved, raising the question of whether various insults converge on common disease pathways or whether they initiate independent neurodegenerative mechanisms. Addressing this question requires systematic analysis of the consequences of ALS mutations to identify common or disparate disease processes, and this often occurs in animal model systems.

Through the generation of animal models of ALS, we in the ALS field have learned valuable lessons about effective genetic approaches, with *FUS* mouse models best exemplifying these lessons. Early *FUS* rodent models of ALS involved overexpression by transgenic approaches, leading to toxicity due to protein overexpression that was not mutation-specific and associated with variable motor phenotypes (Huang et al. 2011; Verbeeck et al. 2012; Mitchell et al. 2013; Shelkovernikova et al. 2013). In contrast, more precise knock-in genetic approaches yielded two independent *FUS* models displaying a progressive and consistent ALS phenotype and leading to the same conclusion that *FUS* mutations are associated with a toxic gain of function (Sharma et al. 2016; Scekcic-Zahirovic et al. 2017; Scekcic-Zahirovic et al. 2016). The controlled and physiological expression in these latter systems enabled unambiguous analysis of the consequences of *FUS* mutations, leading

to identification of relevant disease pathology and strong conclusions about disease mechanisms.

While animal models of ALS have led to significant discoveries about disease mechanisms, many aspects of the disease have yet to be modeled in a mouse. In humans, ALS onsets in either bulbar, upper limb, or lower limb regions. In contrast, in mice, ALS has always onset in the lower limbs. In humans, there is regional spread of pathology to neighboring muscle groups, however no such observation has been recorded in mice. These data reveal potential mouse-human differences or a failure to model the complexity of ALS disease onset and progression. In addition, some mutations, such as ones in *TARDBP*, are incompletely penetrant in humans, however the same is not true in the mouse. Lastly, genetic-environment interactions contribute to disease in ALS, however these interactions have also yet to be modeled in the mouse. Future modeling efforts will be required to understand these important features of ALS pathogenesis.

Through work presented in this thesis, we identified the aberrant protein-protein interactions that result from *FUS* mutations and may contribute towards FUS gain of function toxicity ALS. These interactions between FUS and other RNA binding proteins may lead to motor neuron loss by sequestering specific RNA binding proteins, in general altering RNA metabolism, and/or leading to the formation of toxic and abnormal protein assemblies. From a therapeutic perspective, given that FUS is not required for long term motor neuron survival (Sharma et al. 2016), perhaps the most effective way to treat FUS-ALS would be to eliminate FUS. Antisense oligonucleotides are currently in use to alter or reduce expression of mutant proteins and may be a possible therapeutic

avenue in this case. Alternatively, or in addition, other RNA binding proteins whose function may be altered as a result of *FUS* mutations, such as hnRNP U, could be modulated in similar ways, and systematic analysis of these other *FUS*-interacting-proteins will determine whether modulation is in fact therapeutic.

In contrast to *FUS*, the more than ten different transgenic rodent models of TDP-43 are hindered by the toxicity associated with overexpression of exogenous protein, which obscures analysis of the consequences of *TARDBP* mutations and therefore limits the utility of these systems for the study of ALS. Here we present the generation of the first rodent model system in which mutant TDP-43 is expressed at physiological levels under the control of endogenous regulatory elements. Interestingly, we found that the mutant protein is fully functional but alone is insufficient to cause motor neuron degeneration in ALS. Considering that even the aggressive *FUS* mutations associated with juvenile-onset ALS are associated with a late onset phenotype in the mouse, the absence of motor neuron pathology in the TDP-43 knock-in mice is perhaps not surprising. Nevertheless, the system makes it possible to study perturbations that reveal the increased vulnerability associated with *TARDBP* mutations. That the mutant protein is fully functional suggests *TARDBP* mutations are associated not with a primary loss of function but a gain of toxic function, which may ultimately lead to a secondary loss of function and/or other pathogenic consequences. In addition, the insufficiency of mutant alleles to cause motor neuron pathology further suggests the importance of maintaining TDP-43 levels within a physiological range: 2-fold overexpression is highly toxic, however “normal” mutant expression levels are not by themselves pathogenic.

The TDP-43 knock-in model suggests a threshold model of ALS in which many factors contribute to disease onset. This is supported by the observation that some ALS-related *TARDBP* mutations are not fully penetrant in families, and differences in background strain can influence the motor phenotype associated with a particular mutant TDP-43 transgene (Drs. Aarti Sharma and Helaina Lehrer, personal communication). We conclude from these observations that – to a greater or lesser degree – “causal” ALS mutations increase the likelihood of initiating the pathogenic mechanisms that lead to motor neuron degeneration in ALS. By themselves, specific mutations may be insufficient to cause disease and require additional stressors – innate/genetic and/or acquired/environmental. The *FUS* alleles used here meet the threshold to initiate pathology, but the TDP-43 knock-in alleles do not, indicating differences in pathogenicity associated with the mutations. We expect this model to be a useful tool for the ALS field to identify genetic modifiers and environmental stressors that combine to reach the critical threshold.

How might combinations of stressors lead to TDP-43-dependent motor neuron disease? The presence of aggregates in ALS patients suggests they may be mechanistically relevant and facilitate disease propagation. If this is the case, the dissolution of protein aggregates may improve motor neuron survival, and protein disaggregases and remodeling enzymes have proven effective in suppressing aggregate-dependent toxicity (Jackrel et al. 2014). Indeed, this hypothesis is attractive for many reasons. First, it can explain the regional spread of ALS as local protein assemblies would propagate first to neighboring cells, then radially outward. Second, this mechanism implicates other functional categories of ALS-causal genes. Many ALS-

related genes encode proteins involved in protein homeostasis including optineurin and valosin-containing protein. Loss of cellular functions in protein degradation and autophagy, for example, may lead to a similar accumulation of abnormal protein assemblies, thus initiating the propagating aggregation. Third, this mechanism is consistent with the threshold hypothesis of degeneration, as environmental factors and aging may impair the clearance of abnormal protein assemblies and bring the protein load over the necessary threshold to lead to toxicity. In addition, while a mutation may be one factor that brings a motor neuron closer to the threshold, age related changes with environmental factors may be sufficient to induce ALS in the sporadic cases. Testing this proposed mechanism will require seeding experiments like those done in Alzheimer's (de Calignon et al. 2012) and Parkinson's disease (Luk, V. M. Kehm, et al. 2012; Luk, V. Kehm, et al. 2012) in which aggregated proteins, such as TDP-43 or FUS purified from ALS patients, are injected into a mouse to determine whether the aggregates can propagate and cause motor neuron pathology.

While the mechanism described above explains many clinical, pathological, and genetic observations in ALS, protein aggregation is not a necessary component of some animal models of ALS, questioning its mechanistic importance. In the ALS-relevant mouse models of FUS-ALS and in some TDP-43 models of ALS, motor neuron degeneration is observed despite the absence of protein aggregation. Therefore while protein aggregation may be sufficient to propagate motor neuron pathology, it does not seem a necessary stressor in early stages of the disease process to reach the critical toxic threshold.

Alternatively, or in addition, the toxicity of TDP-43 may relate to aberrant protein-protein interactions in a similar manner to mutant FUS. An attractive hypothesis is that similar RNA binding proteins, such as hnRNP U, are perturbed by both *FUS* and *TARDBP* mutations and abnormal TDP-43 assemblies. These aberrant interactions may be sufficient to sequester RNA binding proteins and thus lead to degeneration through an aggregation-independent mechanism, in which protein aggregation is an accelerating factor, an innocent bystander, or a protective event late in the disease. If this is the case, it will be necessary to determine whether it is the sequestration of specific RNA binding proteins or alteration of RNA binding protein concentrations as a whole that contributes to motor neuron pathology. In contrast to FUS, TDP-43 is required for postnatal motor neuron survival and overexpression leads to toxicity. Therefore altering TDP-43 expression is likely not a viable therapeutic strategy. In this case, modulating the levels of specific RNA binding proteins using antisense oligonucleotide therapy may correct RNA metabolism defects and improve survival outcomes.

Precise understanding of the consequences of ALS-related mutations and faithful modeling of contributing genetic and environmental factors leading to disease will elucidate disease mechanisms in ALS and related disorders, and it is our hope that better understanding of disease mechanisms will lead to development of effective therapeutics.

Chapter 5: Materials and Methods

Generation of mice and mouse genetics

All procedures were performed in accordance with the National Institutes of Health Guidelines on the Care and Use of Animals and approved by the Institutional Animal Care and Use Committee at Columbia University.

To generate knock-in mice, we cloned the mouse *TARDBP* gene from BAC clone RP23-291O2 into the targeting vector using recombineering methods. M337V or G298S mutations and a neomycin resistance cassette were inserted using overlap extension PCR and restriction cloning. The targeting construct was then electroporated into C57BL/6N embryonic stem cells and individual clones were picked and screened. Approximately 35% of clones had successfully undergone homologous recombination, as screened by Southern analysis and sequencing. Positive clones were injected into mouse blastocysts to generate chimeras, and germline transmitters were identified by genotyping and sequencing F1 pups. Mice were back-crossed to C57BL/6J to establish and maintain the lines.

Pgk1-FLPo (Stock #011065), Protamine-Cre (Stock #003328), and ChAT-IRES-Cre (Stock #006410) mice were purchased from the Jackson Laboratory. Protamine-Cre and ChAT-IRES-Cre were back-crossed to C57BL/6J for 6 generations. HnRNP U floxed mice were a generous gift from Dr. Tom Maniatis (Ye et al. 2015), and hnRNP U knockout allele was generated by crossing hnRNP U floxed mice to Protamine-Cre, selecting males with both alleles, and crossing back to C57BL/6J. $\tau^{\text{ON}}\text{hFUS}$ mice were generated as described previously (Sharma et al. 2016).

Genotyping primers are noted in Table 5.1.

Tissue preparation for immunohistochemical analysis

Mice were anesthetized with intraperitoneal injections of ketamine (100 mg/kg, Ketaset, Pfizer) and xylazine (10 mg/kg Anased, Lloyd Laboratories). They were then transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 (PFA). Muscles were dissected immediately after perfusion and washed in 1x phosphate buffered saline, pH 7.4 (PBS). Muscles were prepared for cryosectioning by equilibrating in a sucrose gradient (10%-20%-30% sucrose in 0.1M PB) followed by embedding in O.C.T. compound (Sakura, Torrance, CA) and freezing at -20°C. Muscle was sectioned on cryostat at thickness of 40 µm and stored at -20°C. Spinal columns were fixed in PFA overnight and washed in 1x PBS. Spinal cord segments were embedded in 4% agarose and transverse sections were cut at a thickness of 75 µm using a vibratome.

Immunocytochemistry of spinal cord sections

Free floating spinal cord sections were blocked in 5% normal donkey serum in tris buffered saline, pH 7.4, with 0.5% Triton X-100 (TBS-T) for 1 hour at room temperature. Sections were incubated with primary antibodies overnight at room temperature (goat anti-ChAT, 1:250, Millipore; rabbit anti-TDP-43, 1:1000, Proteintech; rabbit anti-hnRNP U, 1:1000, abcam; rabbit anti-nup50, 1:1000, Proteintech). Sections were washed 6 X 20 minutes with TBS-T and incubated in secondary antibodies for 3 hours at room temperature (donkey anti-goat Alexa 647, donkey anti-rabbit Alexa 555, donkey anti-rabbit Alexa 488 all at 1:1000). Sections were washed 6 X 20 minutes and mounted on microscope slides in Fluoromount G (Southern Biotech, Birmingham, AL,

USA). Slides were imaged using SP5 and SP8 Leica confocal microscopes (Leica Microsystems, Wetzlar, Germany).

MN counts

Lumbar level 4 and lumbar level 5 (L4 and L5) segments were identified, sectioned, and stained as described previously. For adults (> 2 months old) a confocal z-series was imaged for every fourth section at 20X magnification with a step size of 2.0 μm . For pups, L5 section was counted. MNs (labelled with anti-ChAT) in the lateral motor column were outlined in the confocal plane of the nucleolus and counted using ImageJ.

Muscle innervation analysis

Tibialis anterior, diaphragm, and tongue muscles were dissected, equilibrated in sucrose gradient (10%, 20%, 30%), and embedded in O.C.T compound. Cryosections were cut serially on a freezing microtome (Leica CM 3050S) at thickness of 40 μm and stained with antibodies against synaptophysin to identify the presynaptic terminal (rabbit anti-synaptophysin, 1:500, Zymed) and tetramethylrhodamine-conjugated α -Bungarotoxin (1:1000, Invitrogen) to identify the post-synaptic acetylcholine receptors at the NMJ. A confocal z-series was imaged at 10X magnification with a step size of 10 μm . Synapses were identified with α -Bungarotoxin and subsequently innervation was determined by synaptophysin colocalization (presence or absence). At least 1000 NMJs were counted per animal.

Measurement of GFAP and Iba1 positive cells

Lumbar motor neuron sections were stained with ChAT, GFAP (mouse anti-GFAP, 1:400, sigma), and Iba1 (rabbit anti-Iba1, 1:5000, Wako). A confocal z-series

was imaged as previously described. A motor neuron was identified in ImageJ and a circle of radius 100 μm was drawn around the motor neuron. GFAP and Iba1 positive cells were counted within the circle in the z-planes containing the motor neuron nucleus. At least 10 cells were averaged per animal.

Protein extraction and quantification

Brain and spinal cord samples were homogenized in Tris-Glycine SDS sample buffer using first a 23G and then a 27G syringe. Samples were sonicated 3X 5 seconds at 4°C and centrifuged for 20 minutes at 20,000 RCF at 4°C. Supernatant was kept as lysate. Lysate was denatured for 10 minutes at 95°C and quantified using the RC DC Protein Assay kit (Bio-Rad). Lysate (10-20 μg) was run on a 10% SDS-PAGE gel and transferred onto nitrocellulose membrane using a TE77x semidry transfer unit (Hoefer). Immunoblots were probed with antibodies (Table 5.2) in 4% milk in tris buffered saline, pH 7.4, with 0.1% Tween. Antibody was detected by incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) followed by chemiluminescence using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). Signal was detected with autoradiography using LucentBlue X-Ray film, and relative protein levels were quantified using ImageJ.

RNA extraction and quantification

Work space in laminar flow hood and all materials were cleaned with RNaseZap. Brain and spinal cord samples were homogenized in Trizol using first a 23G and then a 27G syringe. RNA was isolated using Direct-zol RNA MiniPrep kit (Zymo Research) and quantified using a nanodrop. cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Fermentas). For RT-qPCR, each sample was measured in

triplicate using a Mastercycler ep Realplex4 (Eppendorf) PCR system and Power SYBR Green PCR Master Mix (ABI). TDP-43 levels were normalized to GAPDH. Primers are indicated in Table 5.1.

Solubility fractionation

Protocol was followed as described (Jo et al. 2014). Brain samples were homogenized in soluble buffer and centrifuged at 50,000 RCF for 20 minutes at 4°C. Supernatant was removed as soluble fraction and pellet was resuspended in RAB buffer. Sample was vortexed and incubated at 4°C overnight with rotation. Sample was then centrifuged at 200,000 RCF for 30 minutes at 12°C and the supernatant was removed as the sarkosyl soluble fraction. Pellet was resuspended in RAB buffer, homogenized with 27G needle, and kept as insoluble fraction. Equivalent portions were aliquoted, added to 2X sample buffer, denatured at 95°C, and run on SDS-PAGE gel as previously described.

Co-immunoprecipitation

Spinal cords were lysed on ice for co-immunoprecipitation (COIP) with 800 µL of lysis buffer containing 50 mM Tris-HCl pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and supplemented with Pierce protease and phosphatase inhibitor tablets (Gal et al. 2007). Samples were homogenized with 16G, 23G, and 27G needles and rotated for 30 minutes at 4°C. Samples were subsequently spun down at 20,000 RCF at 4°C, and supernatant was removed as lysate. Protein concentration was quantified using Bradford protein assay (Bio-Rad), and protein was then added to beads in a total volume of 500 µL. For RNase treatment, lysate was pretreated for 45 minutes on ice with 10 µg RNase A before mixing with beads.

While samples were lysing, 40 μ L Protein G Dynabeads per COIP were washed with 0.02% Tween in PBS. They were subsequently resuspended in 500 μ L 0.02% Tween in PBS with COIP antibody (mouse anti-myc, 4 μ L per COIP, Millipore or rabbit anti-hnRNP U, 5 μ L per COIP, Bethyl) and rotated at room temperature for 45 minutes to crosslink antibody to the beads. Beads were then washed 3X with lysis buffer and stored on ice until adding lysate. COIPs were then rotated overnight at 4°C. In the morning, COIPs were washed 4X with cold lysis buffer. Beads were then resuspended in 1X LDS with reducing agent, heated at 70°C for 15 minutes, and samples were then run on a gel as described above. For western, antibodies were used as described in Table 5.2. For visualizing proteins, Silver Stain Plus Kit (Bio-Rad) and colloidal blue staining kit (Invitrogen) was used. For mass spectrometry, proteins were stained with colloidal blue, excised, digested, and analyzed by the Columbia University Proteomics Core Facility. Proteins that interact with hFUS were identified by spectral counts.

Mouse weights and survival

For weights, mice were weighed between 9AM-11AM. To determine if a mouse was “end stage”, they were placed on their back or side, upon which their reflex is to right themselves on 4 paws. When they were too weak to right themselves in 15 seconds or less, they were considered “endstage” and euthanized.

Patient Samples

Patient samples were obtained from the Columbia Brain Bank with written consent from patients prior to autopsy.

Immunocytochemistry of patient samples

Spinal cord samples were obtained from the Columbia Brain Bank, embedded in pre-chilled O.C.T., and sections were cut on a freezing microtome at a thickness of 20 μm . Sections were either stored at -80°C or used for staining. For staining, sections were washed with PBS for 5 minutes and then fixed with 4% paraformaldehyde at room temperature for 15 minutes. Sections were then washed 3 X 5 minutes with PBS, blocked with 5% normal donkey serum diluted in TBS-T and incubated with primary antibodies in TBS-T at 4°C overnight. Primary antibodies used were rabbit anti-hnRNP U, 1:1000, abcam; guinea pig anti-FUS, 1:16,000, generous gift from Susan Morton; and mouse anti-NeuN, 1:1000, Millipore. Sections were washed 3 X 10 minutes with TBS-T at room temperature and incubated with secondary antibodies for one hour at room temperature (donkey anti-rabbit Alexa 488 at 1:1000, donkey anti-mouse Alexa 647 at 1:1000, donkey anti-guinea pig Cy3 at 1:500). Sections were then washed 3 X 10 minutes with TBS-T and mounted with Fluoromount G.

Yeast strains

Yeast protocols were followed as described (Johnson et al. 2009) and conducted with help from Dr. Rio Higuchi-Sanabria in Dr. Liza Pon's lab. Yeast were grown in rich medium (YPD) or in synthetic media without uracil with 2% glucose, raffinose, or galactose. Mouse and human full length TDP-43 were cloned into the pRS416GAL vector and transformed into BY4741.

Yeast spotting assay

Yeast were grown at 30°C in raffinose media overnight until reaching the log phase. Cultures were then serially diluted 10-fold and spotted onto plates containing either glucose or galactose and grown at 30°C for 3 days.

Yeast growth rate assay

Growth rate was measured as described previously (Vevea et al. 2015) with help from Dr. Liza Pon's lab. Growth curves were measured with an automated plate reader (Tecan; Infinite M200, Research Triangle Park, NC). Strains were grown to mid-log phase in raffinose media and diluted to OD₆₀₀ of 0.07. 10 µL of diluted yeast were added to wells in a 96-well plate containing 200 µL of either glucose or galactose media, and samples were plated in quadruplicate. Yeast were grown at 30°C and OD₆₀₀ measurements were made every 20 minutes for 3 days. The maximum growth rate was estimated using the greatest change in OD₆₀₀ over a 4 hour interval.

Statistical analysis

For all statistical analysis Graph Pad Prism 6 software was used. Statistical analysis of mean differences between groups was performed using one- or two-way ANOVA followed by a Bonferroni *post hoc* analysis.

Primer	Target	Sequence	Use
5' TDP KIN probe FWD	Mouse genomic	CAGCCAGTCAGATATATTTTAATACC	Southern probe
5' TDP KIN probe REV	Mouse genomic	ATAAAACCAAACCCATGGACATAC	Southern probe
3' TDP KIN probe FWD	Mouse genomic	CTTGAAAATGTTGATTCTTGAGC	Southern probe
3' TDP KIN probe REV	Mouse genomic	AGCAAGCTAAAGTTATTCTTGACAG	Southern probe
TDP seq FWD	TARDBP	TGCTGTGTTGATATCTGGGTTGATC	Sequencing
TDP seq REV	TARDBP	TCTACCTAACCTAATAACCAACCTAC	Sequencing
TDP KIN Neo FWD	Neo cassette	GTATAGGAACTTCGTCGACGTC	Genotyping
TDP KIN FWD	TARDBP	GTTGGTCATTTGTCCCAGGGCTG	Genotyping
TDP KIN REV	TARDBP	GGAAAAACAGGCAATTTACTTAAAC	Genotyping
TDP FWD	TARDBP	GATAAGGTTGCCAGTCTCTTTGTG	qPCR
TDP REV	TARDBP	TTCCACTTCTTTCTAACTGTCTATTGC	qPCR
GAPDH FWD	GAPDH	AATGTGTCCGTCGTGGATCTGA	qPCR
GAPDH REV	GAPDH	GATGCCTGCTTCACCACCTTCT	qPCR
hnRNP U FWD	hnRNP U	TCAGCAGCGAATGGTATCAA	Genotyping
hnRNP U FLOX REV	hnRNP U	GAAAGCAGCCATGTTTTACTT	Genotyping
hnRNP U KO REV	hnRNP U	TTCCAATTATGCCAATTCC	Genotyping

Table 5.1: Primers used in this study.

Protein	Host	Source
TDP-43	rabbit	Proteintech 10782-2-AP
GAPDH	mouse	Millipore MAB374
hnRNP U	rabbit	Abcam ab2066
myc	mouse	Invitrogen 46-0603
SFPQ	rabbit	Abcam ab38148
Caprin 1	rabbit	Proteintech 15112-1-AP
hnRNP R	mouse	Livio Pellizzoni
hnRNP Q	mouse	Livio Pellizzoni
hnRNP K	mouse	Livio Pellizzoni
Ddx1	rabbit	Livio Pellizzoni

Table 5.2: Antibodies used for western blotting.

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