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Biochemical fractionation of induced pluripotent stem cell derived motor neurons from an ALS patient with the Glycine-298-Serine TDP-43 mutation

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Thesis

BIOCHEMICAL FRACTIONATION OF INDUCED PLURIPOTENT STEM CELL DERIVED MOTOR NEURONS FROM AN ALS PATIENT WITH THE GLYCINE-298-SERINE TDP-43 MUTATION

by

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Dedications

This graduate thesis is dedicated to my loving grandparents Bicky and Lou Goldszer. Their ever-present guidance was instrumental in my decision to return to higher education and further my career in medicine. I also thank my parents Richard Goldszer and Irene Grace for doing everything in their power to see my dreams to actuality, and Lane Zajac, my moral compass and best friend.

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ABSTRACT

Transactive response DNA-binding protein (TDP-43), and fused in sarcoma/translocated in liposarcoma (FUS/TLS) form protein aggregates in amyotrophic lateral sclerosis (ALS) and fronto-temporal lobar degeneration (FTLD). The sequencing of the TDP-43 gene *TARDBP* in a large patient population has shown more than 40 missense mutations that are now known to cause disease. The effect of genetic mutation on protein aggregation, and the pathogenesis of ALS is the focus of this study. In order to determine the effect of the *TARDBP* Glycine-298-Serine (G298S) missense mutation on protein aggregation in disease, induced pluripotent stem cells (iPSCs) were reprogrammed from control and G298S mutant fibroblasts, and differentiated into motor neurons using defined factors, and fractionated to determine the soluble and insoluble TDP-43 burden. There was an increase in insoluble TDP-43 in the ALS-patient-derived motor neuron lysates over a normal control, but the significance could not be assessed because of the small

sample size. A toxicity assay using fluorescence activated cell sorting showed an unexpected trend towards healthier control neurons. Future studies should include quantified immunohistochemical analysis of motor neurons and use novel pharmaceuticals to attempt to correct aberrant TDP-43-mediated RNA processing.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
EDTA	Ethylenediaminetetraacetic acid
eIF2α	Elongation initiation factor 2-alpha
FACS	Fluorescence activated cell sorting
fALS	Familial amyotrophic lateral sclerosis
FL	Full length
FTLD	Frontotemporal lobar degeneration
FUS/TLS	Fused in sarcoma/translocated in liposarcoma
G298S	Glycine-298-serine
HRP	Horseradish peroxidase
iPSC	Induced pluripotent stem cell
kDa	Kilodalton
KO	Knockout
LDH	Lactate dehydrogenase
M337V	Methionine-337-valine
mRNA	messenger ribonucleic acid
MSC	Mesenchymal stem cell

PVDF	Polyvinylidene difluoride
PAGE	Polyacrylamide gel electrophoresis
RRM	RNA recognition motif
sALS	Sporadic amyotrophic lateral sclerosis
STEMCCA	Stem cell cassette
SDS	Sodium dodecyl sulfate
SG	Stress granules
SOD	Copper/Zinc Superoxide dismutase
TDP-43	Transactive response DNA binding protein 43

Introduction

Amyotrophic lateral sclerosis (ALS) is a degenerative motor neuron disease first described in 1869 by Jean-Martin Charcot. This devastating disease is characterized by the selective loss of upper and lower motor neurons (Brodtmann and Kiernan 2013), and the pathologic accumulation of the TAR-DNA Binding Protein (TDP-43) and fused in sarcoma/ translocated in liposarcoma (FUS/TLS) (Neumann et al., 2006). ALS is genetically and pathophysiologically related to frontotemporal lobar degeneration (FTLD), as both disorders can be caused by the *C9ORF72* hexanucleotide repeat (DeJesus-Hernandez et al., 2011, Renton et al., 2011), and ubiquitinated, phosphorylated TDP-43 aggregation, and mislocalization from the nucleus to the cytoplasm are seen in both (Arai et al., 2006, Neumann et al., 2006). Clinically, this relationship is also evident and it has been estimated that up to 50% of ALS patients experience cognitive symptoms, suggesting frontal lobe impairment (Ringholz et al., 2005). TDP-43 is composed of 414 amino acids, is highly conserved evolutionarily (Ayala et al., 2005), and ubiquitously expressed, with enrichment in brain and kidney (Ayala et al 2011). In healthy controls the protein is restricted to the nucleus, where it has a role in mRNA processing including transcript packaging, splicing, and export (Da Cruz and Cleveland 2011). As an RNA-binding protein it is promiscuous, and has binding sites within one in three mRNA transcripts in brain for mouse and human (Da Cruz and Cleveland 2011).

When both muscle atrophy, and hyper-responsive tendon reflexes (indicating a loss of descending inhibition) are present in a given limb, ALS can be diagnosed with confidence (Kandel et al., 2012). The disease presents in late adulthood, with a prevalence of 1-2 in 100,000 worldwide and affects more men than women with a ratio of around 1.4 to 1 (Beghi et al., 2006). Pasinelli and Brown (2006) reported that 90% of ALS cases are sporadic (sALS). Of the familial cases, around 20% have a mutation in the gene encoding the Cu/Zn superoxide dismutase, *SOD1* (Pasinelli and Brown 2006), and 23 – 46% have a characteristic hexanucleotide repeat expansion in an intronic sequence of the *C9ORF72* gene (Renton et al., 2011). Mutation in *TARDBP* – the gene encoding TDP-43 protein, is rare in comparison, represented in only ~5% of ALS patients including sporadic and familial variants (fALS) (Mackenzie et al., 2010). Still, insoluble TDP-43 protein is a hallmark feature of the disease post-mortem, and studying rare mutations could inform therapies applicable to ALS patients as a whole.

ALS is progressive and unremitting; survival times longer than five years without respiratory support (tracheotomy and mechanical ventilation) are rare (Vitacca and Vianello 2013). Without a cure, current approaches focus on respiratory care and the minimization of discomfort, as motor function and autonomy are lost (Bae et al., 2012). Despite this grim picture, novel research strategies have an exciting target with the identification of mislocalized,

aggregated TDP-43 protein, and mutations in a number of disease-related genes including *TARDBP* (see table 1).

Table 1: Genetics of human ALS. From Da Cruz and Cleveland, 2011
***Includes sALS cases**
****Gene has since been identified as *C9orf72***
+Only reported in a single publication

Locus	Gene	Protein	Mutations	Proportion of inherited ALS	Discovery date
21q22.1	*SOD1*	Cu/Zn superoxide dismutase	>150*	20%	1993
9p13.2-21.3	Unknown	Unknown	Unknown	20%**	Unknown
1q36	*TARDBP*	TDP-43	>40*	5%	2008
16p11.2	*FUS*	FUS/TLS	>40*	4%	2009
9p13.3	*VCP*	Valosin-containing protein	5	1–2%+	2010
10p15-p14	*OPTN*	Optineurin	1	1–2%+	2010
6q21	*FIG4*	PI(3,5)P(2)5-phosphatase	5*	1%+	2009
12q24	*DAO*	D-amino acid oxidase	1	1%+	2010
14q11	*ANG*	Angiogenin	>10*	<1%	2006

ALS presents complicated post-mortem features, and increasingly genetics are informing new definitions of disease. In one study of ALS pathology all sALS cases had ubiquinated TDP-43 in the cytoplasm, but only 25% of fALS cases with the SOD1 mutation had detectable TDP-43 inclusions (see Fig. 1) (Maekawa et al., 2009). When Maekawa's group looked at a patient with a regional disease with features of ALS and Parkinson's disease, "Guam ALS-PD complex," a surprising finding was a TDP-43 positive inclusion in a neuron that co-localized with phosphorylated tau. Like TDP-43, tau is mislocalized in neurodegenerative disease, including Alzheimer's disease, the most common

cause of dementia in the elderly (Evans et al., 1989). This finding highlights the connection between stress granule formation, pathologic protein aggregation, and neurodegeneration.

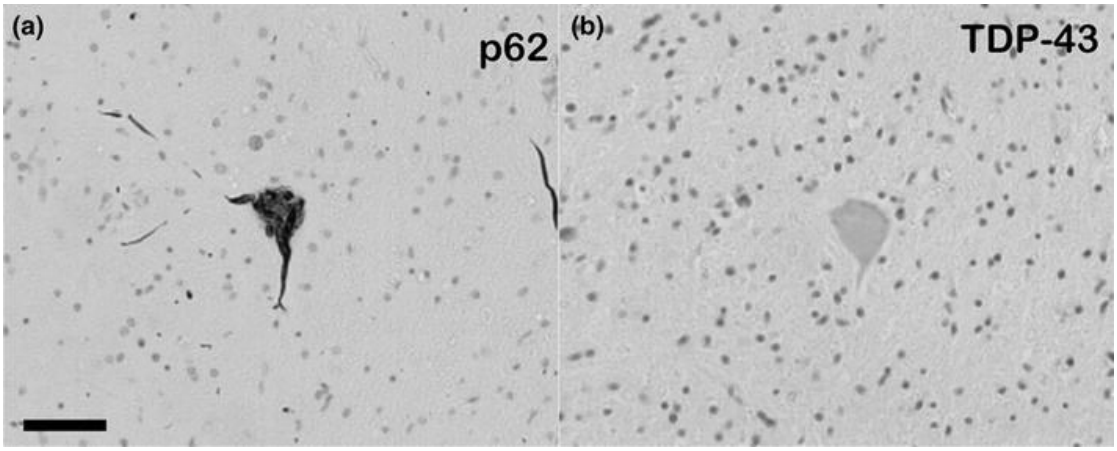

Figure 1: p62 and TDP-43 Labeling of a Motor Neuron in the Ventral Horn of an SOD1 Mutant ALS Patient. From Maekawa et al., 2009

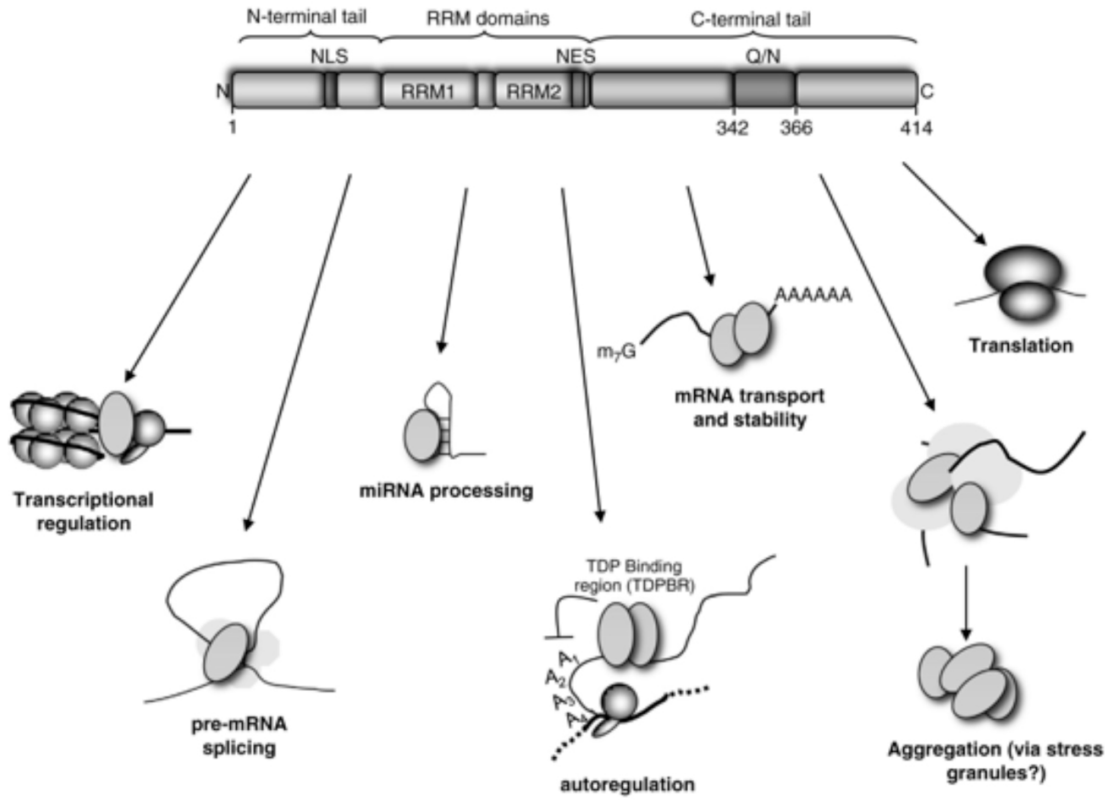

Figure 2: TDP-43 Structure and Function, From Budini et al. 2012

Before it was known that TDP-43 was the pathologically accumulating protein in ALS and FTLD, it was known for its ability to bind nucleic acids (Ou et al., 1995). RNA recognition motifs (RRMs) 1 and 2 confer this property, with 1 being the more important (See Figure 2) (Buratti, E., & Baralle, F. E., 2001). Normally, TDP-43 acts in the nucleus to regulate gene splicing with the use of these RRMs, but in sALS, the protein is mislocalized from the nucleus to the cytoplasm. The “clear” nuclei seen when staining ALS brain tissue for TDP-43 indicates nuclear TDP-43 function is impaired. An alternative hypothesis is that the aggregated TDP-43 protein in the cytoplasm is the principle culprit, and causes cell death by impairing normal energy metabolism, or activating mediators of apoptosis such as caspase 3 (Liu-Yesucevitz et al., 2010). Evidence pointing away from this hypothesis is taken from the lack of TDP-43 in the cytoplasm of fALS cases with *SOD1* mutation, as well as a number of animal models of motor neuron disease. For example, two mouse strains with ALS-associated genetic mutations in *TARDBP* had severe motor impairment without aggregation or mislocalization of TDP-43 (Cleveland et al., 2013). Studies demonstrating the essential, positive function of TDP-43 support this “loss-of-function” model. TDP-43 KO mice do not survive embryogenesis, (Sephton et al., 2010) and heterozygotes have motor impairments without obvious TDP-43 pathology (Kraemer et al., 2010). More recently, Cre-Lox recombination was used to knock out TDP-43 selectively in murine motor neurons, causing age-related motor impairments (Iguchi et al., 2013). Also not all TDP-43 labeled

aggregates are harmful; in fact stress granules are demonstrably cytoprotective (Arimoto et al., 2008, Kedersha et al., 2000). Stress granules are structures that form to sequester harmful RNAs as a part of the response to decreased nutrition, oxidative stress, or other mild stressors, and are initiated in part by the phosphorylation of eIF2α (see figure 3). Indeed, stress granule protein knockouts and inhibition of eIF2a phosphorylation render cells more vulnerable to acute stress (Jiang et al., 2003), and TDP-43 is intimately linked to the stress response (Vaccaro et al., 2012).

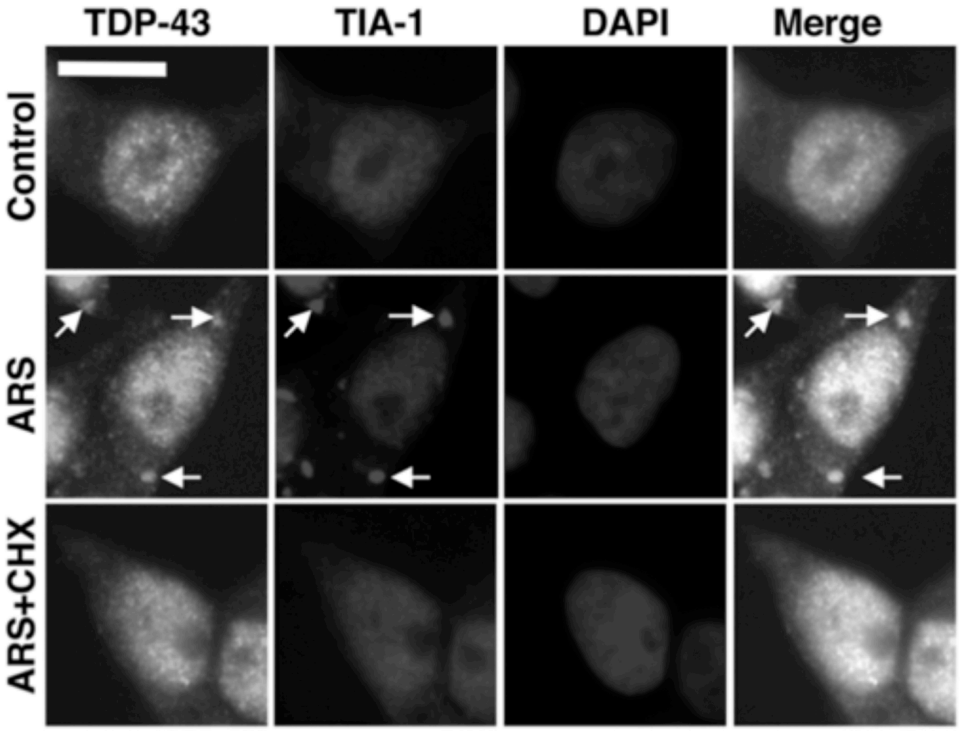

Figure 3: Stress Granules in a Neuroblastoma Cell line are Induced by Arsenite (ARS) and Blocked by Cycloheximide (CHX)
From Liu-Yesucevitz et al., 2010

While disruption of *TARDBP* was not shown to cause any aggregation in Cleveland's 2013 study, a different alteration in the gene, in the region required for nuclear export, was associated with insoluble inclusion formation in the cytoplasm (Winton et al., 2008). Other ALS causing *TARDBP* mutations also increased insoluble TDP-43, and increased the formation of stress granules in the cytoplasm for a neuroblastoma cell line (Liu-Yesucevitz et al., 2010). Stress granules are physiologically normal, reversibly bound complexes of protein and RNA. TDP-43 is a central player in stress granule formation, regulating levels of, and binding to other RNA-binding proteins associated with stress granules such as TIA-1 and G3BP (Mcdonald et al., 2011). Mutant forms of TDP-43 are more likely to aggregate either because of added sites making homo, or hetero-dimerization more likely, or because of modifications preventing movement back into the nucleus (Wolozin 2012). TDP-43 is aggregation prone; even native conformations can probably dimerize, and it possesses a domain with some homology to the prion protein, which causes Creutzfeldt-Jakob disease (King et al., 2012). Protein mislocalization appears to be a common mechanism in neurodegenerative disease. In ALS, TDP-43 and FUS leave the nucleus and form aggregates in the cytoplasm; stress can also cause tau to leave the axon and appear in dendrites and the soma, where it interacts with RNA binding proteins (Hoover et al., 2010).

The G298S mutation in particular is pathogenic in ALS. The perfect segregation of mutant *TARDBP* with disease in fALS pedigrees supports it as the

cause of disease (Van Deerlin et al., 2008). The mutation is in the glycine-rich domain, which regulates the splicing of several genes, and in which other mutations are also particularly pathogenic (Van Deerlin et al., 2008). In 2008 pathology was presented for a family with this mutation demonstrating TDP-43 positive lesions in neurons and glia in multiple cortices, the brainstem, and limbic regions (See Figure 4).

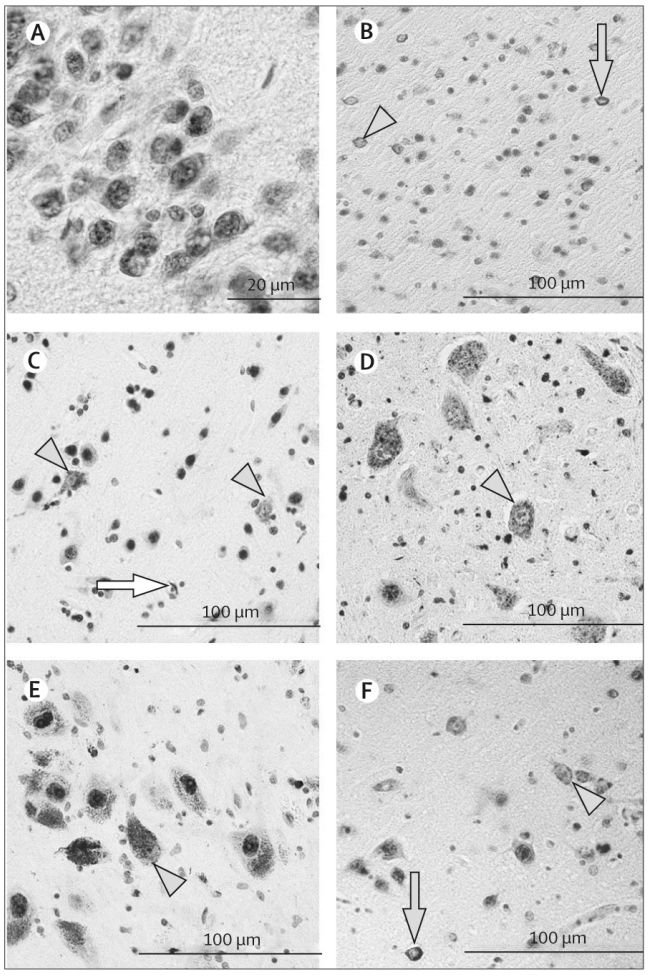

Figure 4: Pathology of G298S Mutant *TARDBP* ALS Patient. Inclusions are Shown in Hippocampus (A) Entorhinal Cortex (B) Temporal Cortex (C) Hypoglossal Nucleus (D) Substantia Nigra (E) and Amygdala (F) From Van Deerlin et al., 2008.

In order to further the understanding of protein aggregation in human disease, other model systems have been utilized including *C. elegans, Drosophila*, and *Danio rerio*. An elegant set of zebrafish studies used genetic knockdown, and replacement of the missing protein or another protein in the pathway, to see if motor impairments persisted. Because the addition of FUS could rescue TDP-43 knockdown, but not vice versa, it was inferred that TDP-43 is earlier in the pathway supporting motor function. Similarly, because SOD was not able to rescue the knockdown of either TDP-43 or FUS, it was reasoned that it is part of a separate pathway. Indeed, the expression of mutant *SOD1* enhanced the motor deficits induced by mutant FUS, indicating they act separately to cause dysfunction (Kabashi et al., 2011). To illustrate the likelihood that different genetic profiles cause motor disease by different mechanisms, the Cleveland group demonstrated *TARDBP* mutation could produce motor neuron disease in mice without aggregates in the cytoplasm, nuclear clearance of TDP-43, or an increase in insoluble TDP-43 (Cleveland et al., 2013). Similarly, a strain of *SOD1* mutant mice exhibited motor impairments without TDP-43 mislocalization or ubiquitination (Robertson et al., 2007). The different pathways towards motor impairment in animal models, and the desire to study ALS-linked mutations in human cells has led to the search for alternative approaches, including the use of induced pluripotent stem cells. One major limitation for iPSC modeling is the loss of network and biochemical signals within a mammalian central nervous system. Additionally, generating iPSCs from a large number of

cell donors is expensive, and labor-intensive, which has led to a number of initial studies with low subject numbers (the experiment presented here has only one subject in each treatment group). One partial solution for the labor cost is the development of the STEMCCA vector. By constructing a polycistronic vector with all four factors necessary to reprogram cells back to a pluripotent stage, a great deal of experimental time is saved, with minimal cost to efficiency (Somers et al., 2010). Using this technology, IPSCs can be generated from human ALS patients with a known genetic mutation, and manipulated *in vitro*. A group differentiated stem cells from an ALS patient with the M337V TDP-43 mutation into motor neurons and saw increased soluble and insoluble TDP-43, as well as decreased cell survival (Bilican et al., 2012). A critique of this study is that only three subjects were sampled, limiting statistical power. Even if limitations such as small sample sizes can be overcome, stem cells are unlikely to be useful as therapeutics. Among proposed treatments for ALS, Feng and Gao (2012) reviewed current paradigms in stem cell treatment as they relate to ALS, and concluded that major challenges to stem-cell therapy exist including maintaining immunocompatibility, accessing the sites of damage, controlling the spread of injected cells, and generating appropriate cell types needed for disease amelioration. Currently, mesenchymal stem cell (MSC) therapy has merit on the basis that cells excrete trophic factors or immuno-modulatory chemokines. It is unlikely that transplanted stem cells or stem cell-derived motor neurons would be able to integrate into local circuitry and replace previously lost cells (Mazzini et

al., 2012). Still, the use of induced pluripotent stem cells allows for large-scale drug screening, and provide a controlled system to discover the effects of a specific genetic mutation such as the *TARDBP* G298S substitution.

TDP-43 auto-regulates its expression by binding to and destabilizing its own mRNA via a negative feedback loop with an auto-regulatory site near the middle of the amino acid sequence also called the TDP binding site (Buratti and Baralle 2011). Intact C-terminal domain and RRM1 are necessary for autoregulation to occur properly (Buratti and Baralle 2011). Because many TDP-43 mutations that cause ALS are in the C-Terminal domain, it follows that loss of autoregulation could underlie the excess cytoplasmic protein seen in disease. Disease-associated mutations could also make pathologic processing of TDP-43 more likely; phosphorylation and cleavage distinguish toxic TDP-43 from physiologically beneficial forms (Gendron et al., 2013). In other diseases of protein aggregation such as Alzheimer's disease (AD), insoluble aggregates form from post-translationally modified targets (Larson et al., 2012). In fact, these diseases share more than just a disease mechanism; in one study 75% of AD patients had TDP-43 positive inclusions at autopsy (Amador-Ortiz et al., 2007). Although *TARDBP* mutation is a rare cause of disease, its relevance to SGs and insoluble aggregate formation highlight its importance in elucidating the pathogenesis of ALS.

Discovered in a large percentage of ALS and FTD cases in 2011, the *C9ORF72* hexanucleotide repeat expansion indicates these diseases are linked

not only clinically and pathologically, but also genetically. Although it has no described function, RT-PCR analysis shows C9ORF72 is largely cytoplasmic and is expressed in a variety of tissues (DeJesus-Hernandez et al., 2011). Indeed the pedigree analysis from Renton et al., (2011) shows families burdened with both ALS and FTD, often in the same generation. DeJesus-Hernandez et al. (2011) saw that individuals with FTD that did not have the repeat in *C9ORF72* were very unlikely (1.4%) to have a relative with ALS, where the rate for FTD patients with the repeat was 31 percent. Furthermore, because the repeat is in an intronic sequence, the likelihood is that it interferes with cell function by the accumulation of non-coding RNA, bolstering the theory of ALS pathogenesis related to disturbed RNA processing (the loss of function model) (Renton et al., 2011). This finding indicates that the hexanucleotide repeat contributes to disease pathogenesis by interfering with normal TDP-43 function, in turn making protein aggregation or mislocalization more likely. In order to develop efficacious drugs for ALS it is imperative to discover the common pathways by which motor neurons degenerate as a result of mutant *C9ORF72, TARDBP, or SOD1*.

SOD1 mutation is much more common than *TARDBP* mutation in ALS (but less common than the *C9ORF72* repeat) and may cause disease by a separate mechanism. The Cu/Zn superoxide dismutase is partially responsible for free radical scavenging in the cell, and reactive oxygen species and impaired energy output are common pathways known to precipitate apoptosis (Freeman et al., 2000). The fundamental pathologic outcome in ALS is the apoptotic death of

upper and lower motor neurons, and cell death in ALS could be caused by deficits in free radical scavenging associated with aging coupled with mutated or otherwise impacted SOD activity caused by *SOD1* mutation, or subsequent to another ALS-linked protein alteration. Yet another theory of neural death in ALS points to the endpoint of glutamatergic excitotoxicity. Riluzole, the only approved drug for ALS, interferes with glutamatergic neurotransmission by blocking voltage gated sodium channels (Sierra Bello et al., 2012), although it has met with somewhat limited success (Miller et al., 2012). Ultimately, the cure for this complex disease will most likely derive from animal and iPSC modeling, and also large drug screens as well as human genetics studies to discover the causes of misregulated protein aggregation, and motor neuron death.

Objectives

1. Illustrate the role of TDP-43 in the normal cell and in disease with immunohistochemistry against TDP-43, and its binding partners G3BP and TIA-1, in a control cell population, and mutant TDP-43 cell population of iPSC-derived motor neurons.
2. Measure soluble and insoluble TDP-43 burden in disease using biochemical fractionation and quantitative western blot, on iPSC derived motor neuron lysates from normal control humans, and ALS patients with the G298S mutation.
3. Induce protein aggregation and stress granule formation prior to protein quantification using stressors such as salubrinal and arsenite, and novel pharmaceuticals at physiologic doses to correct protein and mRNA homeostasis, with the goal of identifying an effective ALS therapeutic.

METHODS

iPSC Motor Neuron Differentiation

Collection of fibroblasts from ALS and control patients, culturing of fibroblasts, reprogramming via STEMCCA vector insertion, differentiation into motor neurons, and assessment of motor neuron phenotype were carried out by the Murphy lab according to their published protocol (Somers et al., 2010). The STEMCCA vector contains a polycistronic message with the four transcription factors OCT4, KLF4, SOX2, and cMYC. At the reprogramming stage there is approximately 1% efficiency. After the selection of an appropriate clone there is a 30 day maturation process to generate motor neurons. Neural induction media is used containing low concentration sonic hedgehog, ascorbic acid, cAMP, and brain derived neurotrophic factor. Motor neuron morphology and phenotype were assessed with the TUJ1 stain.

Immunohistochemistry

Cells were grown in 6 mm 96 well dishes in neural differentiation media. Following treatment with a stressor such as arsenite, staurosporine, or no treatment, cells were rinsed with PBS and fixed in 4% paraformaldehyde 15 min. Fixative was rinsed off and cells were permeabilized with triton-X100 15 min. A blocking buffer containing PBS and 5% BSA was used 1 hr. Primary antibodies were mouse anti-TDP-43 (1:2000, Novus biological) and rabbit anti G3BP (1:2000, sigma). Secondary antibodies were Dylight 488 conjugated donkey

anti-rabbit IgG as well as Dylight 549 conjugated donkey anti-mouse IgG (1:2500, Jackson ImmunoResearch). Primary antibodies were diluted in blocking solution and cells were incubated overnight at 4 C. Cells were washed three times in PBS and then secondary antibody was applied for 1 hr in darkness. Cells were rinsed three times in PBS, and DAPI was applied to visualize nuclei at 1:20000 between washes in dilute triton. For imaging, exposure times were consistent across conditions and treatments, for each filter setting.

Hoechst-Propidium Iodide Assay

In order to assess toxicity of compounds, cell survival was measured using the Hoechst-propidium iodide FACS assay. These experiments were conducted at the Murphy laboratory by our collaborator Shirley Nah.

Biochemical Fractionation

To examine the effect of the G298S TARDBP mutation on solubility profiles of TDP-43, sequential protein extractions were performed. iPSC derived motor neurons were washed twice in cold PBS and lysed in salt solution containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2mM EDTA, and 1x Halt protease inhibitor cocktail (Thermo Scientific). Cells were scraped into pre-chilled microcentrifuge tubes and rotated at 4°C for 30 minutes before the lysates were transferred to cold glass ultracentrifuge tubes. Samples were centrifuged 30 min at 100,000xg and 4°C, and the supernatants were collected as the salt soluble fraction. To protect against contamination between fractions, pellets were re-suspended and re-centrifuged three times for 30 min at 100,000xg and 4°C.

Every wash was labeled and saved for future assessment of contamination. Salt insoluble pellets were solubilized in ice cold triton solution containing 50 mM tris HCl, pH 7.5, 150mM NaCl, 2mM EDTA, and 1% Triton-X100, as well as 1x Halt protease inhibitor cocktail (Thermo Scientific) and spun 30 min at 100,000xg and 4°C. The Triton soluble fraction was collected and again three washes were conducted to prevent crossing over. The Triton insoluble pellet was frozen overnight and resuspended in SDS (sodium dodecyl sulfate) solution containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2mM EDTA, 1% SDS, and 1x Halt protease inhibitor cocktail (Thermo Scientific) the following morning. After spinning 30 min at 100,000xg 4°C the supernatant was collected as the SDS soluble (second insoluble) fraction, and the pellet was washed three times as described previously. The remaining pellet was solubilized in urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-HCl and 1x Halt protease inhibitor cocktail, pH 8.5). BCA protein assay (Pierce) was conducted to measure protein concentrations following fractionation, and for each fraction, total protein concentrations for the control and ALS derived motor neurons were made equivalent using the appropriate residual buffer, except for the urea fraction, in which BCA was not possible. Next, the salt soluble, Triton soluble (insoluble), SDS soluble (second insoluble), and urea fractions were analyzed by western blot.

Immunoblot

For immunoblots, gradient PAGE was used on a 10-well, 4-12% Tris-Glycine gel (Novex). 10ug of protein was loaded per lane, except for urea sample lanes, where BCA was not possible and a concentration of .5ug/uL was assumed for a loading volume of 20uL. Gels were separated 1.5 hours at 70 Volts and transferred to PVDF at 4°C for 2 hours at 40V on ice. Primary antibodies were polyclonal rabbit anti TDP-43 (1:1000, ProteinTech 10782) or monoclonal mouse anti actin (1:20000, Millipore 1501) in TBS-T plus 5% BSA. Signal was detected using horseradish peroxidase conjugated secondary directed against rabbit or mouse (1:10000, Abcam), developed for 2 seconds.

Quantification of Western Blot and Statistical Analyses

For quantification of the western blot Image J software was used. The modal value was subtracted from all pixels as background and an equivalent area was counted for all lanes. Pixel intensity multiplied by area was used to generate an integrated intensity value. Presented values are corrected for total protein in the sample by dividing by the actin value for that fraction and treatment group.

RESULTS

A western blot showed three distinct bands for TDP-43 and one clear band for actin (See Figure 5). The three bands for TDP-43 were at approximately 25 kDa, 35 kDa, and 43 kDa. When controlling for total protein loaded by dividing integrated intensity of TDP-43 signal by integrated intensity for actin, the insoluble TDP-43 in the ALS derived, mutant *TARDBP* motor neuron lysates was greater than control, for full-length TDP-43, as well as the 25, and 35 kDa fragments.

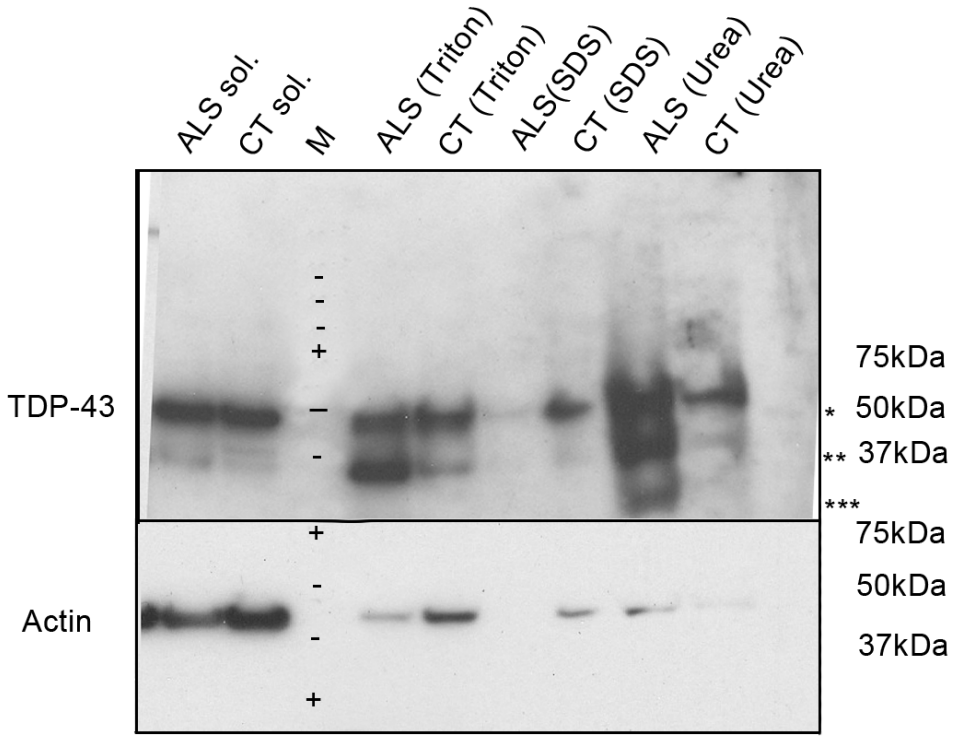

Fig. 5: iPSC Motor Neuron Lysates WB For TDP-43
ALS – G298S Mutant ALS Patient Sample
CT – Control Patient Sample
Sol. – Salt Soluble Fraction,
Triton – Triton Soluble Fraction,
SDS – SDS Soluble Fraction,
Urea – Urea Soluble Fraction.
Primary Antibodies are Indicated on Left,
Molecular Weights are Indicated on Right
***:TDP-43**
****:35kDa Fragment**
*****:25kDa Fragment**

For the soluble fraction (Figure 6), full length TDP-43 was increased 50% from the control patient-derived motor neuron lysate values, from 1.4 to 2.1 (arbitrary units are a ratio between the integrated intensity of the measured band and actin) and 137% from 0.312 to 0.738 to for the 35 kDa signal. There was no detectable signal for the 25 kDa species in the soluble fragment.

In the first insoluble fraction (the Triton soluble fraction) (Figure 7), full-length TDP-43 was increased in the disease neuron lysates 148% from 4.2 to 10.4. The 35 kDa cleavage product was increased nearly five fold, from 2.2 to 12.8. The 25 kDa product was increased 23 fold, from 0.05 to 1.2. The 25 kDa fragment was cropped for this fraction to simplify presentation (Figure 5), as a result of the curvature of the bands on this blot.

For the second insoluble fraction (SDS soluble fraction) (Figure 8), the trend of actin corrected intensity being higher in the ALS patient-derived motor neuron lysates continued. The full-length signal was increased more than four fold in the mutant lysates, from 12 to 67. For the 35 kDa signal the pattern repeated but this time the increase was 11 fold, from 3 to 36. For the 25 kDa signal there was an even larger increase in the disease cell line from 0.4 to 7.6, representing an 18 fold change.

In the urea soluble fraction (the most insoluble fraction: Figure 9), the trend reversed, with the actin-corrected integrated intensity of the control group TDP-43 being higher than in the disease group. Full-length TDP-43 was reduced

from 77 to 19 in the disease lysates, and smaller reductions were seen in the 25 and 35 kDa cleavage products.

There was a significant decrease in Actin intensity between the salt soluble fraction and all subsequent fractions (Figure 10). The variability between actin samples was large, with coefficients of variation ranging from 20% for the soluble fraction to 125% for the SDS fraction. Still the decrease from the salt soluble actin integrated intensity to all subsequent fractions reached significance with a P value of 0.04, 0.01, and 0.01 for the Tukey test and 0.01, 0.004 and 0.004 for Fisher's test of least significant difference. There was no significant difference between any of the insoluble fractions for actin intensity, with p values all > 0.05 regardless of the statistical test applied.

When averaging the Triton and SDS soluble fractions, the trend continued for increased insoluble TDP-43 in the mutant ALS motor neurons (Figure 11).

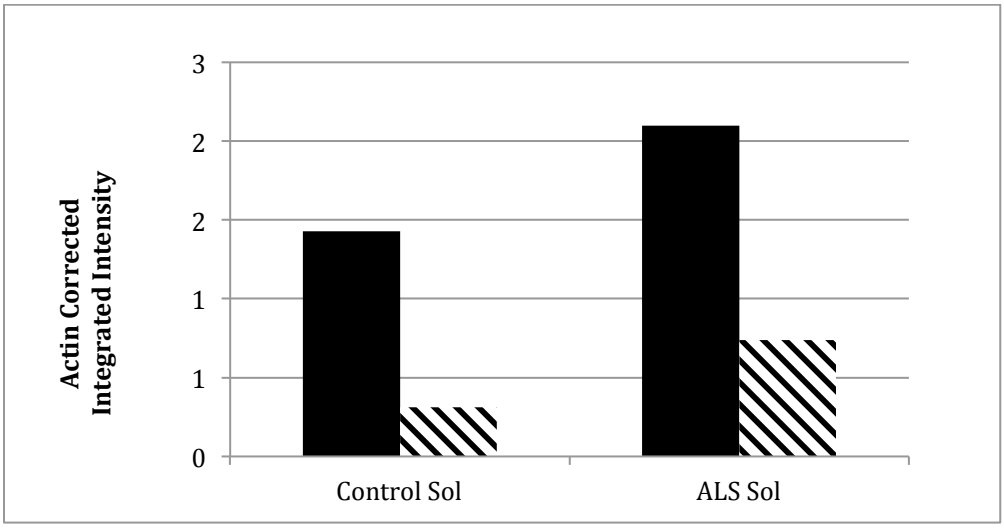

Figure 6: Soluble TDP-43 and Cleaved Products for a Disease (ALS) and Control (BU) Strain of iPSC Derived Motor Neurons

- **Full Length TDP-43 \ 35 kDa Fragment**

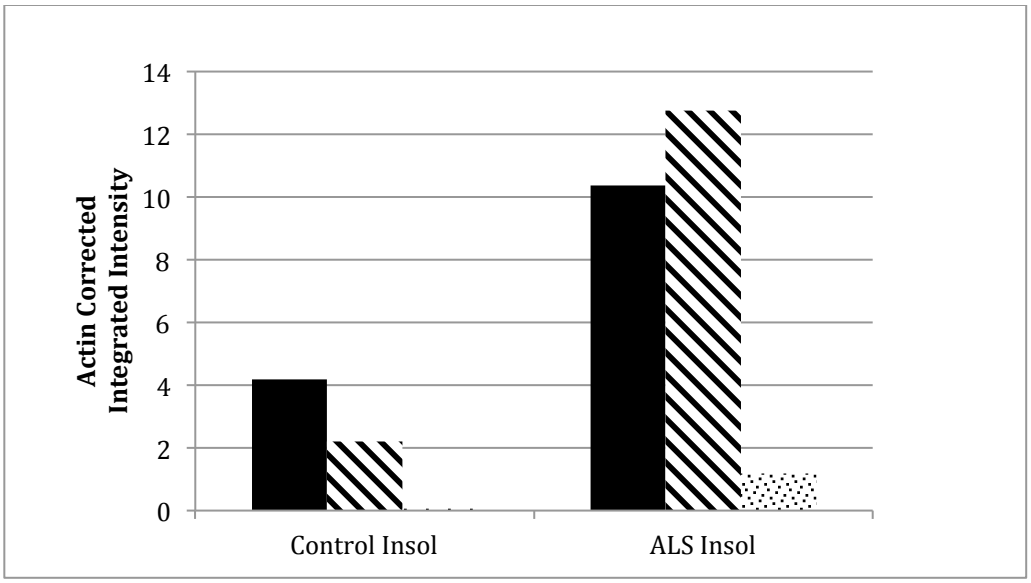

Figure 7: Triton Soluble (Insoluble) TDP-43 and Cleaved Products for a Disease (ALS) and Control (BU) Strain of iPSC Derived Motor Neurons
■ Full Length TDP-43 \ 35 kDa Fragment . 25 kDa Fragment

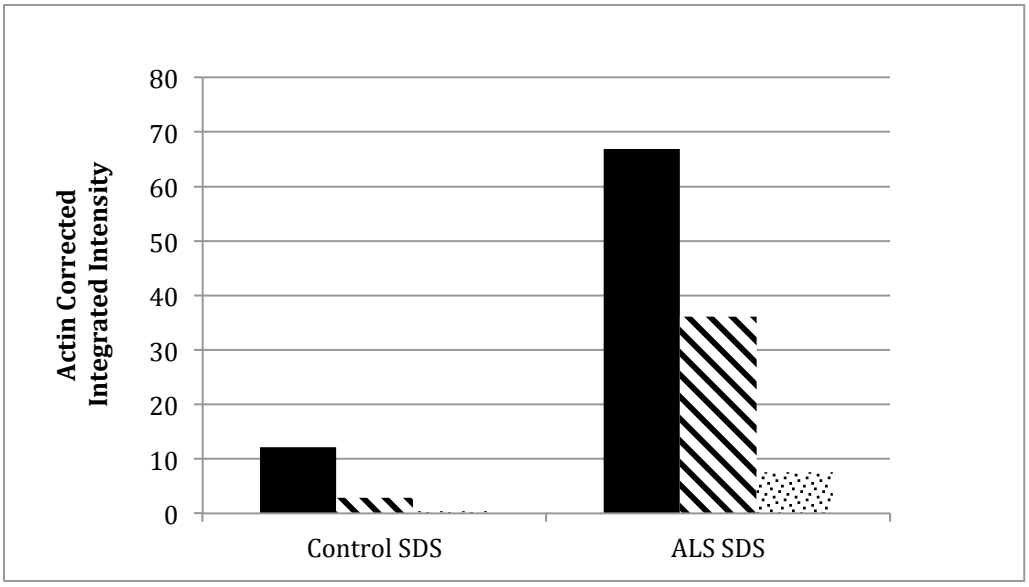

Figure 8: SDS Soluble (Insoluble) TDP-43 and Cleaved Products for a Disease (ALS) and Control (BU) Strain of iPSC Derived Motor Neurons
■ Full Length TDP-43 \ 35 kDa Fragment . 25 kDa Fragment

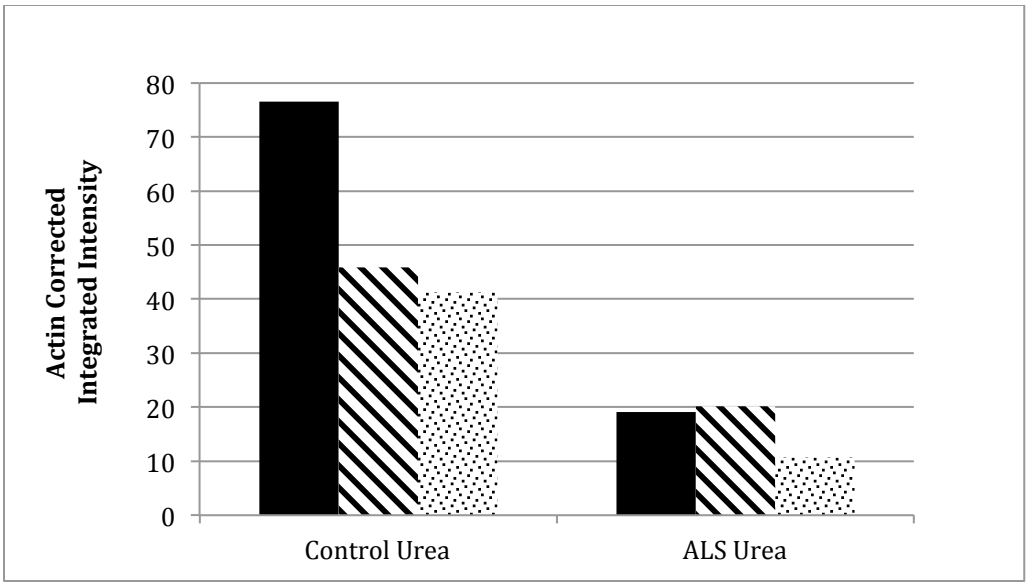

Figure 9: Urea Soluble (Insoluble) TDP-43 and Cleaved Products for a Disease (ALS) and Control (BU) Strain of iPSC Derived Motor Neurons
■ Full Length TDP-43 \ 35 kDa Fragment . 25 kDa Fragment

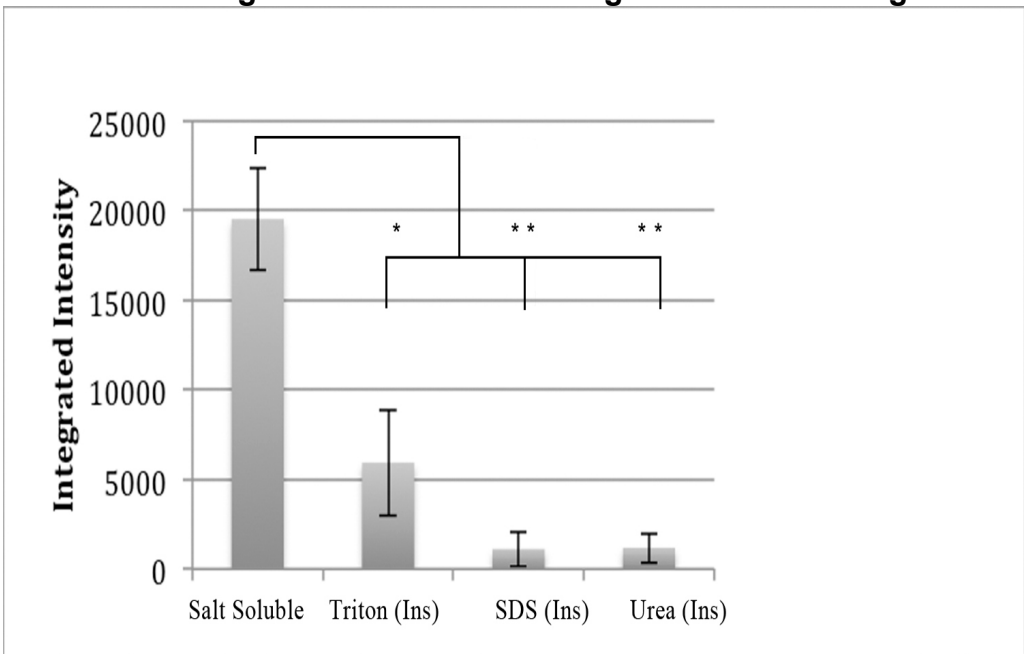

Figure 10: Actin Integrated Intensity for Four Fractions Mean Values from Both Disease (ALS) and Control (BU) Strains, Error Bars Represent Standard Error of the Mean * p< 0.05 **p< 0.005

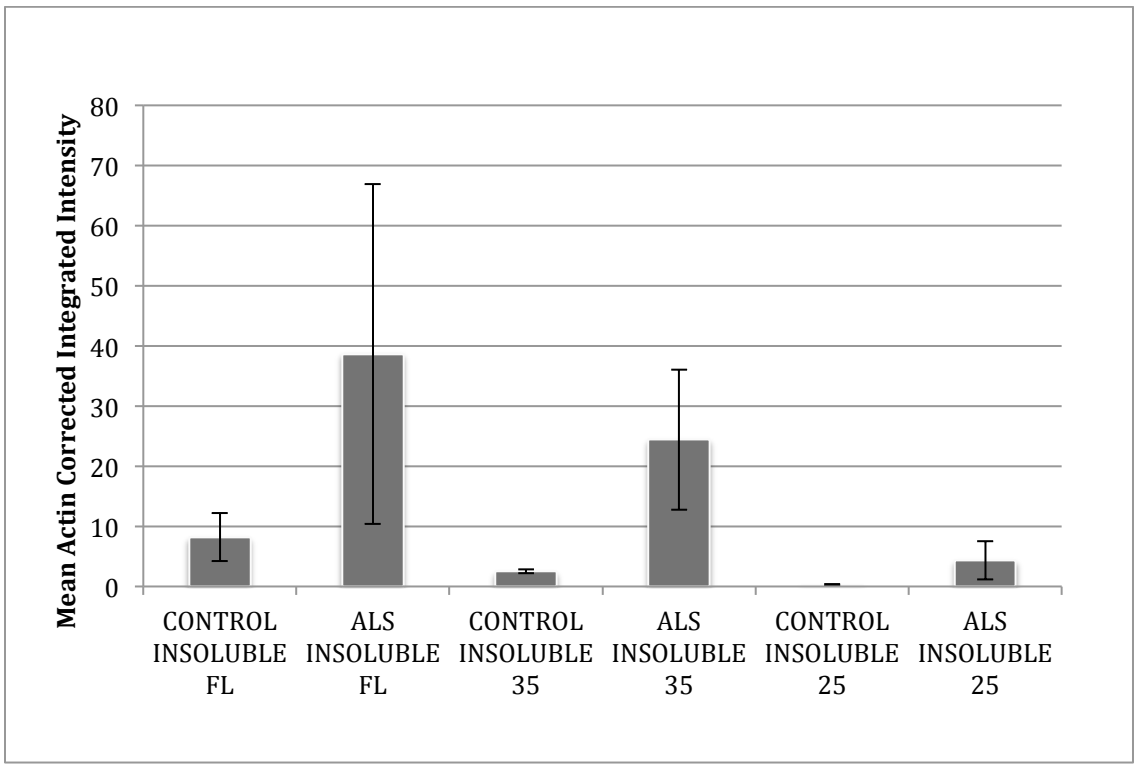

Figure 11: Total Insoluble TDP-43 (Triton and SDS Soluble Fractions) for a Normal Control and G298S Mutation-Bearing ALS Patient. Error Bars Represent Standard Error of the Mean

Immunohistochemistry was used in control and ALS-derived motor neurons to assess morphology and attempt to quantify inclusions in the cytoplasm. TDP-43 was predominantly nuclear, but was also seen in the cytoplasm. G3BP was predominantly cytoplasmic. Arsenite treatment was effective at inducing a few inclusions, some of which showed colocalized TDP-43 and G3BP.

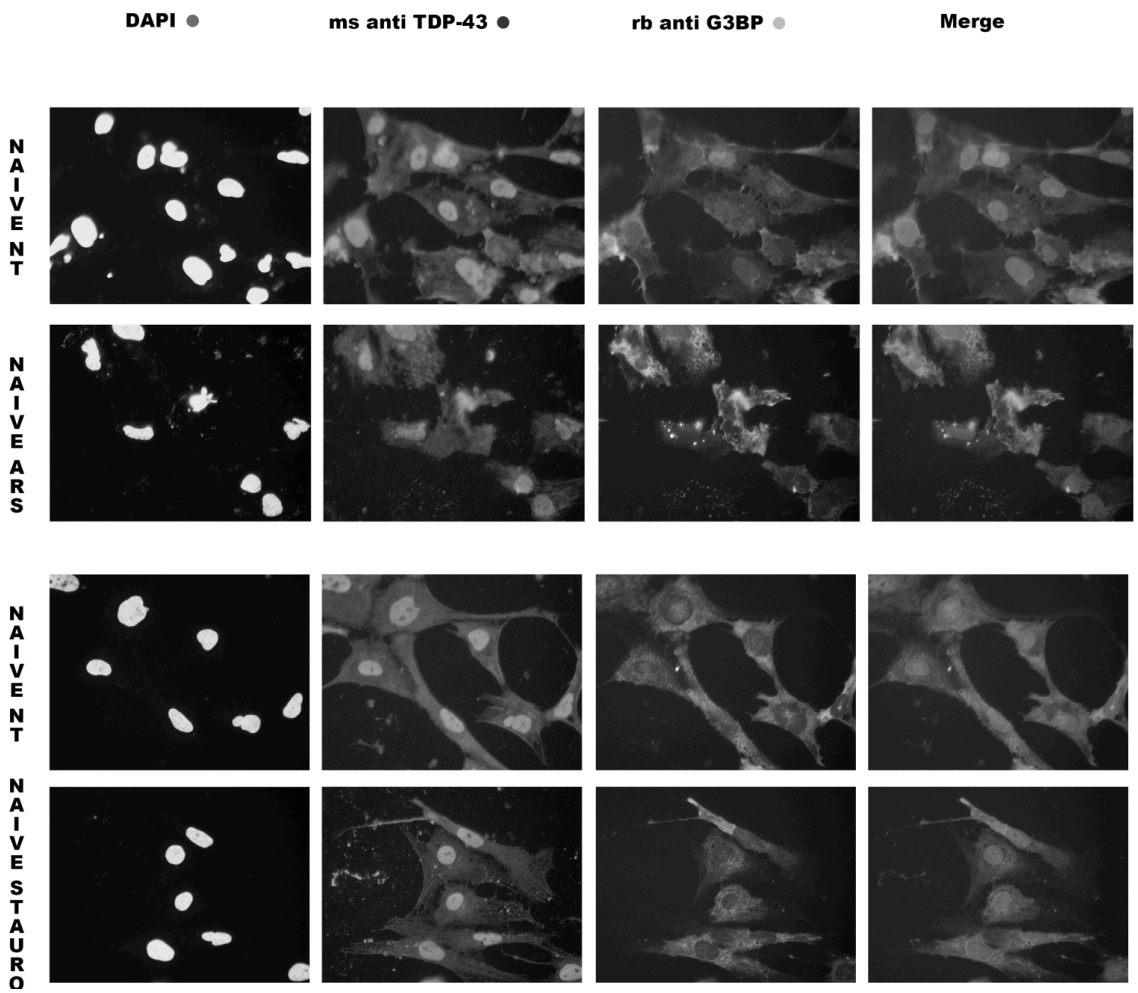

Figure 12: Control iPSC Derived Motor Neurons Treated with Arsenite or Staurosporine, and Stained with Antibodies Against G3BP and TDP-43 to Demonstrate Inclusion Formation

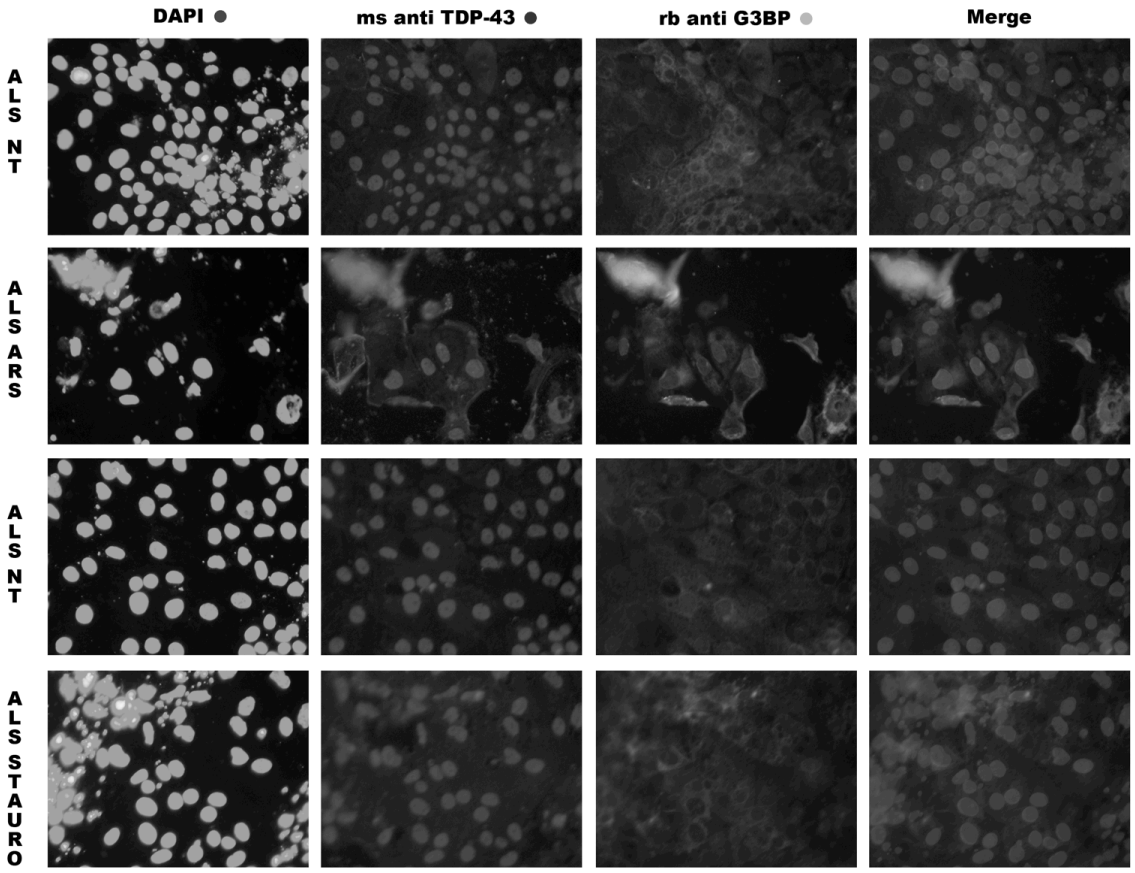

Figure 13: ALS iPSC Derived Motor Neurons Treated with Arsenite or Staurosporine, and Stained with Antibodies Against G3BP and TDP-43 to Demonstrate Inclusion Formation

FACS analysis of cell survival showed a trend towards increased survival in the disease cell line. The only condition for which the ALS derived cells were less likely to survive (relative to control) was the lowest dose of arsenite (10μM).

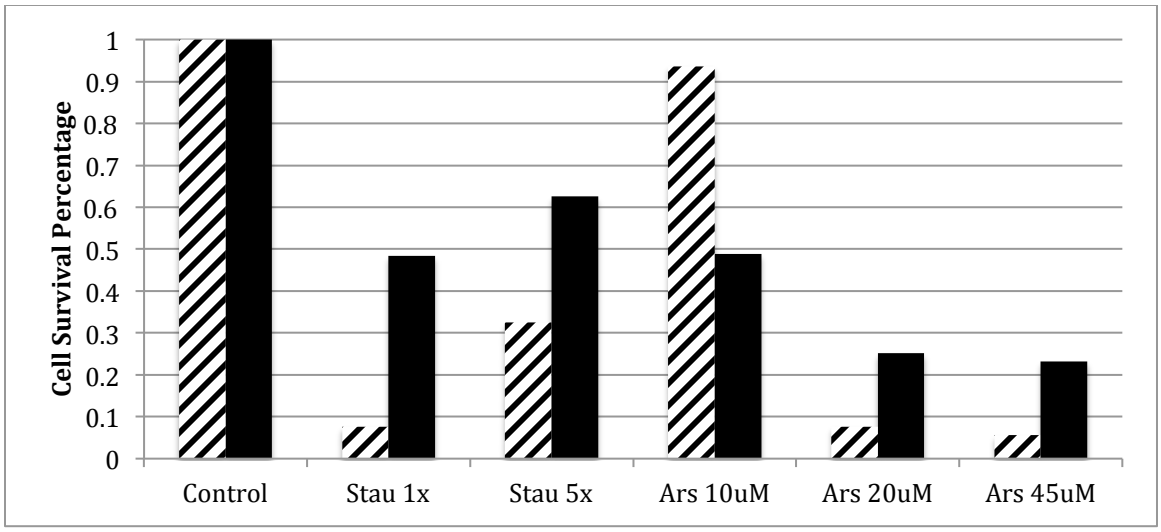

Figure 14: iPSC-Derived Motor Neuron Survival with Staurosporine and Arsenite Treatment Normalized to Control.

Disease Free / ALS ■ Stau: Staurosporine Ars: Arsenite

DISCUSSION

1) How is the mutation in *TARDBP* acting to increase protein in the cytoplasm?

The G298S mutation was associated with an increase in insoluble TDP-43 in these experiments. When controlling for total protein concentration by dividing integrated intensity of TDP-43 signal by actin signal, the ratio for the mutant ALS-derived motor neurons was higher than control for every fraction except for urea. These findings support the hypothesis that TDP-43 mutations cause RNA misregulation, destabilization, and transport of TDP-43 into the cytoplasm, where it may form insoluble aggregates.

The Triton soluble fraction of TDP-43 in this experiment is related most directly to the stress granule burden. TDP-43 in insoluble aggregates should separate into the SDS soluble fraction (Liu-Yesucevitz et al., 2010). Because there was more TDP-43 in the ALS mutant cell lysates in both of these fractions, it follows that there were more stress granules, and more insoluble TDP-43 aggregates in the ALS mutant cells, although this difference would be easier to detect using immunohistochemistry. Cleaved forms of TDP-43 contribute to inclusion formation in the cytoplasm (Liu-Yesucevitz et al., 2010, Wang et al., 2013), but full-length TDP-43 is the most prevalent form in human neuronal inclusions post-mortem (Igaz et al., 2008). It follows then that full-length TDP-43 in the SDS soluble fraction suggests insoluble aggregate burden, and full-length,

and cleaved forms of TDP-43 in the Triton soluble fraction indicate stress granules in the cytoplasm. For the Triton soluble fraction, the 25 kDa fragment showed the greatest increase in intensity for the mutant cells (Figure 7). For Liu-Yesucevitz et al., (2010) the 25 kDa fragment of TDP-43 was associated with toxicity and cell death, through its activation of caspase 3. Differences between control and mutant TDP-43 for full-length TDP-43 were the smallest. The predominant difference seemed to be in insoluble aggregate increase for mutation-bearing cells, as full-length TDP-43 was more than doubled in the Triton soluble (first insoluble) fraction, and increased more than four fold for the SDS soluble (second insoluble) fraction. Still, with a 23 fold increase in the 25kDa cleavage product for the *TARDBP* mutant cells (Figure 7), for the Triton soluble fraction, the TDP-43 cleavage fragments showed the largest differences. Because there were large effects for both the stress granule-related measures, and the insoluble aggregate-related measures, the indication is that G298S mutant iPSC-derived motor neurons had more highly insoluble TDP-43 aggregates, and more stress granules than control cells. This finding fits with the hypothesis that mutation in *TARDBP* causes protein aggregation, by mislocalizing TDP-43 to the cytoplasm, or by affecting TDP-43 aggregation propensity by making protein-protein, or protein-mRNA interaction more favorable.

A 2012 study verified these findings of increased detergent-insoluble TDP-43 in iPSC-derived motor neuron populations from ALS patients with mutant

TARDBP (Egawa et al., 2012). Of particular interest, they measured mRNA levels for patients with three different mutations including the G298S missense mutation. They found that motor neurons from the patient with the G298S mutation (and the other two mutations they quantified) had increased levels of TDP-43 mRNA, which would fit with our finding of increased insoluble TDP-43 for the lysates from G298S mutant cells. In addition they measured vulnerability of the motor neurons to arsenite and other stressors, and used antibodies directed at TDP-43, which allowed them to see mislocalization of the protein to the cytoplasm. A major strength of our study was that no arsenite or other stressor was used, allowing our interpretation to focus on the genetic difference, as opposed to the response to a chemical stimulus.

2) Are the insoluble aggregates toxic or is the toxicity in disease mediated by some other means?

Toxicity associated with G298S mutation was assessed using the hoechst-propidium iodide fluorescence-assisted cell separation (FACS) method. Our hypothesis was that more cell death would be seen in mutant *TARDBP* neurons. An interpretation would be that insoluble aggregates are toxic to these cells, provided that the finding of increased insoluble TDP-43 reflects the presence of aggregates in the cytoplasm. Contrary to our expectations we saw a robust trend for more cell survival in the ALS mutant iPSC strain (Figure 14).

Incorporating the finding of increased insoluble TDP-43 in these cells, it suggests TDP-43 aggregates are not toxic, and disease-related changes in ALS are mediated by other means. An alternate explanation would be that the inclusions are indeed toxic, but other genetic factors distinguishing the two cell lines are outweighing their impact on survival.

3) What is the TDP-43 burden related to stress granules as opposed to insoluble aggregates?

By using immunohistochemistry it was possible to assess the presence of stress granules, as opposed to other types of protein aggregates. G3BP is a stress granule associated protein, and a known binding partner of TDP-43. G3BP and TDP-43 colocalization was seen in both disease and control cell lines (Figure 12 and 13), indicating that stress granules were present in both populations. TDP-43 in the most insoluble fraction suggests that highly insoluble protein aggregates were also present in both species. Still, the trend was towards more insoluble TDP-43 in the disease cell line. It would be interesting to know if the toxicity seen in disease was related more to the stress granules or the insoluble aggregates. Using a combination of stress granule inhibitors such as cycloheximide, and the FACS assay for cell survival, it would be possible to measure the toxicity related primarily to insoluble aggregates, as opposed to stress granules.

Other Questions

1) As a binding partner to both RNA and numerous proteins, the effects of TDP-43 are often mediated by the elements with which it interacts. One binding partner of particular interest is HDAC6, because it represents a site of potential pharmacologic manipulation. HDAC6 promotes the formation of aggresomes, or ribonucleoprotein complexes used for sequestering unnecessary protein and RNA in the cytoplasm. Blockade or deletion of HDAC6 has been seen to slow disease progression in an SOD1 mutant mouse model of ALS (Taes et al., 2013). FUS and TDP-43 are actually binding partners, and also compete for a binding site on HDAC6 (Kim et al., 2010), which indicates how mutant FUS and TDP-43 might act together or separately to cause familial forms of ALS and FTLD. One interesting experiment would be to measure HDAC6, as well as stress granule formation and insoluble TDP-43, in a *TARDBP* mutant model such as G298S motor neurons. While blockade of HDAC6 works to prevent motor degeneration in an SOD1 mutant model of disease, this pathway might not be effective for all ALS cases as the SOD1 models do not tend to accumulate TDP-43 and could therefore be distinct in their pathogenesis.

2) Like protein aggregation, it appears with TDP-43 that excess, as well as insufficient function can cause cellular irregularities and motor impairment. In *drosophila* the TDP-43 KO results in a paralytic phenotype (Feiguin et al., 2009), while overexpression of TDP-43 causes motor impairment and early mortality in mice (Xu et al., 2010). *TARDBP* mutation can also cause increased, or

decreased TDP-43 function, and mediate toxicity in either case (Cleveland et al., 2013, Liu-Yesucevitz et al., 2010). While increased insoluble TDP-43 in G298S mutant iPSC-derived motor neuron lysates suggests mutation in TDP-43 can increase aggregate formation, other studies (Cleveland 2013, Igaz 2011) point to the loss of TDP-43 mRNA splicing function in the nucleus as being central to toxicity in ALS. Our observation of increased insoluble TDP-43 aggregates, without increased toxicity, fits with this thinking.

3) When averaging the Triton and SDS soluble fractions the trend continued for increased insoluble TDP-43 in the mutant ALS motor neurons (Figure 11). This combined measurement approximates the SDS soluble, or sarkosyl soluble fraction. Other studies (Liu Yesucevitz et al., 2010, Egawa et al., 2012) saw increased insoluble TDP-43 in the "sarkosyl soluble" fraction with *TARDBP* mutant cells, supporting the hypothesis that the genetic mutation confers increased susceptibility to aggregation. Still, by separating a Triton soluble fraction it was possible to make statements regarding the type of aggregates affecting this model, such as stress granules, or insoluble protein aggregates.

4) Imaging data are essential to demonstrate morphology and biochemical features of protein aggregates in the cytoplasm. Stress granules (SG) are regulated by TDP-43 with the association of the proteins G3BP and TIA-1 (McDonald et al., 2011) and these proteins can be used to label SGs, and could also be targets for therapy. In order to determine if there was an increase in

stress granule formation in mutant TDP-43-motor neurons the cells immunolabeled for TDP-43, TIA, and G3BP could be used and putative SG could be counted using confocal or traditional fluorescence microscopy. This approach was attempted for the study described here, but the number of SGs was too low to measure a statistically significant difference. One solution to this problem is to use a stressor such as arsenite or salubrinal to increase the number of stress granules to a measurable number. Again, this was attempted, however the long-term stress conditions (24 hours) used were not effective at generating SG in these experiments (Figure 12,13). Yet another approach would be to use a short term, high concentration dose (1 hr. at 500μM), which was effective at generating stress granules even in control cells for Liu-Yesucevitz et al., (2010). Still, the ideal experimental model would have the only variable as genetic mutation between groups, and no treatment other than a novel pharmaceutical included for a treatment group.

Limitations and Future Direction

1. Protein concentrations were not adjusted prior to fractionation, and there was no measurement of TDP-43 in the unfractionated sample to use as a comparator. Instead, total protein concentration was measured and adjusted for each fraction using the bicinchoninic acid (BCA) method following the separation of all four fractions. The urea solubilized pellet protein concentration was not

measured because it is not compatible with BCA. Still the actin was assessed for each fraction and should give a reliable estimate of total protein in the sample. By dividing integrated intensity of each band by the actin band for that fraction the amount of total protein loaded for each group should be included in the measurement.

2. It is not possible to generate variability estimates for individual fractions for full length, 25kDa, and 35kDa fragments of TDP-43 signal, because only one measurement exists for each group and fraction. Because there is no variability estimate it is also not possible to assess significance of this finding. Still the directionality was consistent for each fraction other than the urea, for which there was no protein concentration correction made prior to separation with western blot. With only one subject it is difficult to make a conclusion regarding this finding, but it is still interesting that the directionality matches what would be expected given the previous studies on iPSC fractionation with *TARDBP* mutation (Bilican et al., 2012). One method to assess variability was to combine the western results for the Triton soluble and SDS soluble fractions for each species (FL, 25kDa, and 35kDa) as a type of “sarkosyl soluble” approximation. For this combination, the variability was fairly large for the ALS cells, but was moderate in the control measurements. In order to make a more confident assessment of the insoluble TDP-43 burden in this system, a number of subjects should be included, and multiple measurements made for each

subject (Deo et al., 2012). This will allow both biologic and experimental variability to be assessed.

3. There was an increase in the corrected integrated intensity for the urea fraction in the control cells, contrary to the pattern detected in the other fractions and our hypothesis. The finding would be of great interest as the urea fraction contains the most insoluble aggregates, which cannot be dissolved in SDS. Dividing by the actin intensity should have worked to correct for protein loaded, but the signal was practically undetectable in the control urea fraction. Because this correction was used the urea control corrected integrated intensity was quite large, and could be inaccurate. An alternative explanation is that there were more insoluble aggregates of TDP-43 in the control cells, but this would not fit with the current hypothesis and previous findings in the field (Liu-Yesucevitz et al., 2010).

4. Loading variability for the western blot was quite high, which could be seen in the error of the actin replicates (Figure 10). Still it was possible to detect significant differences in actin (total protein) as the fractionation proceeded. In order to generate a more accurate loading control multiple actin measurements should be conducted. The pooling of the insoluble fractions for quantification purposes did not give a measurement of the loading variability because it incorporates measures for separate fractions. Also, because actin migrates to the same region where TDP-43 is detected on PAGE, it is not possible to run on the same blot with the HRP detection method. To assess

protein loading on the quantified blot, a different standard protein such as tubulin could be used, or one could incorporate fluorescent secondary antibodies and a detection system capable of resolving two fluorophors of differing wavelengths.

5. Pellets were frozen following the removal of the triton soluble layer and the three subsequent washes, and the SDS fraction and urea fraction were separated on the following day. Freezing protein in the middle of a fractionation is not ideal as it could affect protein aggregation.

6. Further characterization of immunolabeled motor neurons could include cell counting, and measurement of cell processes such as somal volumes or dendritic or axonal length. The possibility of co-culturing motor neurons with iPSC-derived glia has also not escaped consideration. As knowledge of ALS grows, so does the interest in astrocytes, which can serve as a buffer for toxic concentrations of glutamate, potassium, and many other chemicals and ions. In fact astrocytes are also burdened by TDP-43 in human ALS and some animal models as well (Van Deerlin et al., 2008).

7. Biochemical fractionation for TDP-43 is typically conducted in two stages, as the sarkosyl (SDS) soluble, and sarkosyl insoluble fractions. This was not sufficient for the current investigation, because TDP-43 localizes in stress granules as well as insoluble aggregates in the cytoplasm, and the addition of the Triton soluble fraction allowed a statement regarding the size of each. With only two fractions it would not be possible to distinguish the SDS soluble from the Triton soluble and therefore make a statement regarding the fraction

corresponding to stress granules. Having all four fractions was therefore a major asset and worth the labor-intensive process. Stress granules are increasingly recognized as key modulators of pathology in ALS, and other related diseases of protein aggregation.

Conclusions

In order to determine the effect of a mutation in *TARDBP* on protein aggregation, iPSC-derived motor neurons were reprogrammed and differentiated from a patient positive for the G298S mutation, and a normal control. The cells were lysed in high salt buffer, and a sequential fractionation was conducted, consisting of four steps of progressively increasing detergent strength. Following the fractionation, protein concentrations for the disease and control groups were made equivalent, and a western blot was run. The blot showed a clear trend toward increased insoluble TDP-43 in the disease cell line, for full length TDP-43, and also the 25 and 35 kDa cleavage products. These differences were interpreted as an increase in stress granule and insoluble cytoplasmic aggregate burden in the G298S mutant cells. Future studies should include immunohistochemical quantification of cellular inclusions, more replicates assessing toxicity to allow for variability estimates, and additional cell lines to increase statistical power.

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