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Evidence for the unique pathological classification of mutant SOD1-ALS

(Spine Title: Genotype – Phenotype Correlations in ALS)

(Thesis Format: Integrated Article)

by

Brian Andrew Keller

Graduate Program in Pathology

A thesis submitted in partial fulfillment of the requirements for the degree of:

Master of Science

School of Graduate and Postdoctoral Studies

The University of Western Ontario – London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO
SCHOOL OF GRADUATE AND POSTDOCTORAL STUDIES

CERTIFICATE OF EXAMINATION

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The thesis by

Brian Andrew Keller

entitled:

Evidence for the unique pathological classification of mutant SOD1-ALS

is accepted in partial fulfilment of the requirements for the degree of

Master of Science

Date	Chair of the Thesis Examination Board

ABSTRACT

Amyotrophic lateral sclerosis [ALS] is a neurological disease characterized by the selective loss of motor neurons [MNs] and death due to respiratory failure usually occurs within 3-5 years of symptom onset. Mutations in the genes encoding TAR DNA binding protein of 43 kDa [TDP-43], fused in sarcoma/translocated in liposarcoma [FUS/TLS] and Cu/Zn superoxide dismutase [SOD1] have all been associated with ALS. Never before has a link been made between the variant of ALS and intraneuronal pathology. This thesis has sought to (1) analyze the immunohistochemical expression of TDP-43, FUS/TLS, SOD1, Rho Guanine nucleotide exchange factor [RGNEF], and 5 other ALS-associated proteins across multiple variants of ALS, and (2) characterize the immunohistochemical expression of RGNEF for the first time. Here, I show that **mutant SOD1-ALS is a pathologically unique variant of ALS**. This finding may be used in the identification of novel families that may be at risk for developing ALS.

KEYWORDS

Amyotrophic lateral sclerosis, NFL mRNA, TDP-43, RGNEF, p62, mutant SOD1-ALS

CO-AUTHORSHIP

Chapter 2: Manuscript entitled "Evidence for the unique pathological classification of mutant SOD1-ALS" currently being prepared for submission.

1. Keller, BA: Helped to design study, performed all immunohistochemistry, microscopy, tissue scoring, data analysis, statistical analysis, and wrote the manuscript.
2. Volkening, K: Supervised work, edited the manuscript.
3. Ang, LC: Verified data analysis and microscopy techniques.
4. Radamakers, R: Performed genotyping.
5. Strong, MJ: Designed study, supervised all aspects of the work, edited the manuscript.

Chapter 3: Manuscript entitled "Amyotrophic lateral sclerosis is a disease of altered RNA metabolism characterized by the aberrant expression of RGNEF and other RNA binding proteins" currently being prepared for submission.

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2. Volkening, K: Supervised work.
3. Droppelmann, C: Performed C-Myc-RGNEF transfections.

4. Strong, MJ: Designed study, supervised all aspects of the work, edited the manuscript.

DEDICATION

This thesis is dedicated to the thousands of people living with ALS around the world. It is also dedicated to their families, who work to take care of their loved ones, and who are so generous with their time and donations in an effort to help the progression of research and cure this disease.

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Although I have only been here at Robarts for a short time, I've formed relationships with so many people, all of whom have helped in some way throughout my Masters and their contributions to this thesis are huge.

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To my supervisory committee – Dr. Lee Cyn Ang, Dr. Arthur Brown, Dr. Steve Pasternak – you all helped my project in different ways, but it was the perfect combination of experience and guidance. Dr. Ang, the hours you spent with me on the microscope were absolutely critical to the success of my project. Dr. Brown, you forced me to look at my project in ways I probably never would have, and I will never forget you telling me that the goal is to make a story, not a catalogue. Dr. Pasternak, helping me with new software has allowed me to make strong arguments that would have been much more difficult otherwise. Thank you all.

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TABLE OF CONTENTS

CERTIFICATE OF EXAMINATION ii

ABSTRACT iii

KEYWORDS iii

CO-AUTHORSHIP iv

DEDICATION vi

ACKNOWLEDGEMENTS vii

TABLE OF CONTENTS x

LIST OF TABLES xiv

LIST OF FIGURES xv

LIST OF APPENDICES xviii

LIST OF ABBREVIATIONS xix

CHAPTER 1: Introduction and Literature Review 1

Background 2

History and Epidemiology of ALS 4

Disease Course and Clinical Spectrum 6

Genetics of ALS 8

Biology and Pathobiology of ALS 13

- TDP-43 14
- FUS/TLS 16
- Mutant SOD1 17
- RGNEF 18
- High Molecular Weight Neurofilament 19
- Peripherin 20
- Ubiquitin 21
- Sequestosome 1 [p62] 23
- Optineurin 24

RNA Metabolism and ALS 25
    Staufen [Transport Granules] 26
    TIA-1 [Stress Granules] 27
    XRN-1 [P-bodies] 27
    L26 [Polysomes] 28
Glutamate Excitotoxicity 28
Oxidative Stress 31
Glial Involvement in ALS 32
    Microglia 33
    Astrocytes 34
Motor Neuronal Death in ALS 35
Rationale and Hypothesis 37
References 39

CHAPTER 2: Evidence for the unique pathological classification of mutant SOD1-ALS 64
Abstract 65
Introduction 66
    Genetics, Function, and Histopathology of ALS-Associated Markers 66
        RNA Binding Proteins 66
            SOD1 66
            TDP-43 68
            FUS/TLS 68
            RGNEF 69
        Other ALS-associated Proteins 70
            Ubiquitin 70
            Sequestosome 1 [p62] 70
            Optineurin 71
            High Molecular Weight Neurofilament [NFH] 71
            Peripherin 72

Clinical Link and Experimental Aims 72
Materials and Experimental Procedures 73
Genotyping and Case Selection 73
Antibodies 75
Immunohistochemistry 75
Protein Expression Analysis 77
Statistical Analysis 79
Results 79
TDP-43 79
FUS/TLS 82
RGNEF 88
SOD1 88
Ubiquitin 88
p62 93
Optineurin 93
pNFH [SMI31] 100
Peripherin 100
Mutant SOD1 Tissues 100
Discussion and Concluding Remarks 106
Acknowledgements 112
References 113

CHAPTER 3: Amyotrophic lateral sclerosis is a disease of altered RNA metabolism characterized by the aberrant expression of RGNEF and other RNA binding proteins 122
Abstract 123
Introduction 124
Materials and Experimental Procedures 128
RGNEF Antibody Development and Optimization 128
Immunohistochemistry 129

Immunofluorescence 130

Protein Expression Analysis 132

Protein Complex Immunoprecipitation/Western Blot 133

Cloning of Full Length RGNEF 133

Transfection and Western Blot 133

Results 134

RGNEF Expression Analysis 134

Discussion and Concluding Remarks 146

Acknowledgements 155

References 157

CHAPTER 4: General Discussion and Conclusions 166

Implications and Future Directions 172

References 174

APPENDICES 177

Appendix A: Use of Human Subjects – Ethics Approval Notice 178

CURRICULUM VITAE 180

LIST OF TABLES

Table 1.1: Genetic Variants of ALS..9

Table 2.1a: Genetic details of the cases used in this study...............................74

Table 2.1b: Information for control cases used in this study...........................74

Table 2.2: Details of use for each antibody in this study..................................76

Table 2.3: Summary of TDP-43 quantification data..83

Table 2.4: Summary of neuronal cytoplasmic inclusions across the variants of ALS evaluated in this study...105

Table 3.1: Details of use for each antibody in this study...............................131

LIST OF FIGURES

Figure 1.1: RNA Metabolism..30

Figure 2.1: Representative TDP-43 expression across all variants of ALS evaluated in this study..81

Figure 2.2: A TDP-43 nucleocytoplasmic redistribution occurs in ALS tissues, but not in healthy controls..85

Figure 2.3: Representative FUS/TLS expression across all variants of ALS evaluated in this study..87

Figure 2.4: Representative RGNEF expression across all variants of ALS evaluated in this study..90

Figure 2.5: Representative SOD1 expression across all variants of ALS evaluated in this study..92

Figure 2.6: Representative ubiquitin expression across all variants of ALS evaluated in this study..95

Figure 2.7: Representative p62 expression across all variants of ALS evaluated in this study..97

Figure 2.8: Representative optineurin expression across all variants of ALS evaluated in this study..99

Figure 2.9: Representative pNFH expression across all variants of ALS evaluated in this study.........102

Figure 2.10: Representative peripherin expression across all variants of ALS evaluated in this study.........104

Figure 3.1: The characterization of the normal intraneuronal expression of RGNEF in neuropathologically normal control tissues.........136

Figure 3.2: A protein-complex immunoprecipitation testing the interaction between RGNEF and L26 in complex followed by a Western Blot.........138

Figure 3.3: The involvement of RGNEF within the RNA metabolic pathway through interactions with RNA granules.........141

Figure 3.4: The co-localization between RGNEF and p62 suggests that RGNEF can be targeted for protein degradation via the proteasome degradation system.........143

Figure 3.5: The co-localization between RGNEF and ubiquitin suggests that RGNEF can be targeted for protein degradation via the ubiquitin-proteasome degradation system.........145

Figure 3.6: A high degree of co-localization can be found between RGNEF and TDP-43 within ALS motor neurons, but not within control motor neurons........148

Figure 3.7: A high degree of co-localization between RGNEF and FUS/TLS within ALS motor neurons, but not within control motor neurons........150

Figure 3.8: The lack of glial RGNEF expression within the human spinal cord........152

LIST OF APPENDICES

Appendix A: Use of Human Subjects – Ethics Approval Notice……………………..178

LIST OF ABBREVIATIONS

*81-84 delCATA	Cytosine-adenine-tyrosine-adenine deletion at positions 81-84
3'UTR	3' Untranslated region of mRNA
3'UTR*41G>A	Guanine substituted for adenine at position 41 of the 3'UTR
A4T	threonine substitution for alanine at the 4th amino acid
A4V	valine substitution for alanine at the 4th amino acid
AHC	Anterior Horn Cell (motor neuron within anterior horn)
ALS	Amyotrophic Lateral Sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropanoic acid
ANOVA	Analysis of variance
BMAA	β-methylamino-L-alanine
C-terminus	Carboxy terminus
C774G	guanine substitution for cytosine at the 774th nucleotide (DNA)
CNS	Central Nervous System
Cu	Copper

D76Y	tyrosine substitution for aspartic acid at the 76th amino acid
DAB	3,3'-diaminobenzidine
DNA	Deoxyribonucleic Acid
EAAT2	Excitatory amino-acid transporter 2
fALS	familial Amyotrophic Lateral Sclerosis
FIG4	Polyphosphoinositide phosphatase gene
FTD	Frontotemporal dementia
FUS/TLS	Fused in Sarcoma/Translocated in Liposarcoma
G37R	arginine substitution for glycine at the 37th amino acid
G85R	arginine substitution for glycine at the 85th amino acid
G93A	alanine substitution for glycine at the 93rd amino acid
GEF	Guanine exchange factor
GFAP	Glial Fibrillary Acidic Protein
H_2O	Water
H_2O_2	Hydrogen Peroxide
hnRNP	heterogenous Ribonucleoprotein

I113T	threonine substitution for isoleucine at the 113th amino acid
IBA-1	Ionized calcium Binding Adaptor molecule 1
LMN	Lower Motor Neuron
MAPT	Microtubule associated protein tau
miRNA	micro Ribonucleic Acid
MN	Motor Neuron
mRNA	messenger Ribonucleic Acid
mt	mutant
N-terminus	Amino-terminus
NCI	Neuronal Cytoplasmic Inclusion
NF	Neurofilament
NF-κB	Nuclear Factor-kappa B
NFH	High molecular weight neurofilament subunit
NFL	Low molecular weight neurofilament subunit
NFM	Intermediate molecular weight neurofilament subunit
NMDA	N-methyl-D-aspartate

O_2	Molecular diatomic oxygen
OPTN	Optineurin gene
p62	Sequestosome 1
pNFH	phosphorylated high molecular weight neurofilament
POAG	Primary Open Angle Glaucoma
PRPH	Peripherin gene
QGSY region	Serine-Tyrosine-Glutamine-Glycine-rich region
R521C	cysteine substitution for arginine at the 521st amino acid
RER	Rough Endoplasmic Reticulum
RGNEF	Rho Guanine Nucleotide Exchange Factor
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RRM	Ribonucleic Acid Recognition Motif
rRNA	ribosomal Ribonucleic Acid
sALS	sporadic Amyotrophic Lateral Sclerosis
siRNA	small interfering Ribonucleic Acid
SETX	Senataxin gene

SMN	Survival motor neuron
SOD1	Cu/Zn Superoxide Dismutase
TARDBP	Transactive Response DNA Binding Protein gene
TDP-43	Transactive Response DNA Binding Protein of 43 kDa
TNF-α	Tumor Necrosis Factor-alpha
UMN	Upper Motor Neuron
VAPB	Vesicle-Associated membrane Protein B
VEGF	Vascular Endothelial Growth Factor
XRN-1	Exoribonuclease 1
Zn	Zinc

Chapter 1: Introduction and Literature Review

Background

Amyotrophic lateral sclerosis [ALS] is a progressive, fatal neurodegenerative disease that involves the selective loss of motor neurons [MNs] of the brain, brainstem, and spinal cord (Logroscino *et al.*, 2008). The death of increasing numbers of MNs leads to muscle paralysis and the arrest of most voluntary movements. The median survival for patients with ALS is 3 to 5 years following symptom onset, and death most often comes as a result of respiratory failure. The only pharmaceutical that has been approved after a Phase III study for the treatment of ALS is riluzole, which is an inhibitor of glutamate excitotoxicity (Bensimon *et al.*, 1994). By delaying the introduction of assisted ventilation, it is known to slow the progression of ALS and increase survival by 3 to 5 months in many patients. With this exception, the management of ALS is palliative and care is focused on symptom relief. There are currently about 3,000 patients across Canada that are living with ALS, and for every new diagnosis, someone dies as a result of the disease (ALS Society of Canada, 2011).

Approximately 90% of ALS cases are sporadic [sALS], while the remaining 10% are familial [fALS]. There are currently 13 known genetic variants of the disease that are largely indistinguishable from one another with respect to clinical phenotype. The mutations that are of major importance to this thesis are in the genes encoding superoxide dismutase 1 [SOD1], TAR-DNA binding protein of 43 kDa [TDP-43], and fused in sarcoma/translocated in liposarcoma [FUS/TLS].

If permission is granted to perform an autopsy when an individual affected by ALS dies, a neuropathologist will validate the diagnosis by microscopic examination of brain and spinal cord tissue. Working with a colleague [Dr. Rosa Radamakers], we evaluated our reservoir of cases for known ALS-associated genetic mutations. Eight percent of cases that were previously thought to be sporadic in origin were found to harbour mutations in either *SOD1*, *TARDBP*, or *FUS/TLS*. In this thesis, I have examined whether spinal cord motor neuron pathology would allow neuropathologists to differentiate the variants of ALS and direct clinicians and researchers to screen patients for new mutations and the possible identification of novel families that may be affected by fALS. Specifically, I have studied the expression of SOD1, TDP-43, FUS/TLS, Rho-Guanine nucleotide exchange factor [RGNEF], phosphorylated high molecular weight neurofilament [pNFH], peripherin, ubiquitin, sequestosome 1 [p62], and optineurin across a panel of cases containing cases of mutant [mt] SOD1-ALS, mtTDP-43-ALS, mtFUS/TLS-ALS, fALS without any known mutations, sALS without any known mutations and neuropathologically normal controls. In doing so, I have tested the **hypothesis** that ***all variants of ALS are pathologically distinct and can be differentiated by spinal cord pathology using a panel of contemporary ALS-associated markers.***

As I will show, there are changes in the expression patterns of several RNA binding proteins as well as several other ALS-associated proteins across the variants of ALS that I have tested. In addition, I will show that cases of mtSOD1-ALS have distinct neuropathological characteristics when compared with other genetic variants of the disease as well as with cases of sALS and fALS without any known mutations. This leads me to propose that mtSOD1-ALS [ALS 1] is a pathologically unique variant of the disease and may possess a unique disease pathophysiology.

History and Epidemiology of ALS

First described by the French neurologist known as the "founder of modern neurology" Jean-Martin Charcot (Charcot and Joffroy, 1869), ALS is classified based on a patient's family history or their habituation within certain hyperendemic foci of ALS. The first and most common form of ALS, sALS, accounts for approximately 90% of cases. It is most commonly seen in adulthood with a peak age of onset in the 6th decade of life (Shoesmith and Strong, 2006). Juvenile forms of the disease are recognized, however, and onset under the age of 20 does occur (Gouveia and de Carvalho, 2007). In addition to sALS, approximately 10% of cases come from families with a known history of the disease and are appropriately termed fALS.

There is also a Western Pacific variant of ALS that accounts for a small percentage of cases. This affects people from Guam, the Marianas archipelago of

Micronesia and the Kii peninsula of Japan, and is thought to have an environmental cause. There are currently 2 hypotheses that could explain the increased incidence of ALS in these regions. The first is the presence of β-methylamino-L-alanine [BMAA], a neurotoxin found in the seeds of the cycad, a traditional food staple in the region (Garruto, 2006). The second hypothesis involves neurotoxicity due to the deposition of metals within MNs. This is thought to occur due to a defect in mineral metabolism causing a form of secondary hyperparathyroidism. This is provoked by a chronic nutritional deficiency of environmental calcium and magnesium, which causes an increased gastrointestinal absorption of aluminum and the ultimate neuronal metal deposition (Garruto, 2006). The incidence of the Western Pacific variant of ALS has been decreasing in recent years, which has been attributed to a changing diet due to the Westernization of the region's culture following World War II (Spencer *et al.*, 1987).

ALS is the most common MN disease diagnosis. The worldwide incidence ranges between 1.89 and 1.91 per 100,000 and has a prevalence of approximately 8 per 100,000 (Worms, 2001). There is a gender bias amongst ALS patients with a male-to-female ratio of 1.4:1, but this seems to approach unity after the age of 70 (Shoesmith and Strong, 2006). Recent evidence, however, suggests that the phenomenon of a gender bias is disappearing (Logroscino *et al.*, 2008; Zoccolella *et al.*, 2008). Over one's lifetime, the estimated risk of acquiring ALS ranges from 1 in 1,000 (Traynor *et al.*, 1999) to 1 in 400 (Johnston *et al.*, 2006).

Disease Course and Clinical Spectrum

Death from ALS usually comes as a result of respiratory failure, and usually comes within 3 to 5 years of symptom onset. The first signs of ALS can help physicians to provide a prognosis with respect to the rate of progression. Patients with bulbar onset ALS, in which motor problems first start affecting speech and swallowing, experience a more aggressive disease course with a median survival of 2-3 years, whereas patients presenting with limb onset ALS have a longer survival median of 3-5 years (Logroscino *et al.*, 2008). Survival longer than 5 years is uncommon; the number of those affected managing to survive longer than 5 years ranges from 23-28% (Strong *et al.*, 1991; Chancellor *et al.*, 1993; Testa *et al.*, 2004). Survival longer than 10 years does occur; however, only in approximately 4% of cases (Turner *et al.*, 2002). As death due to respiratory failure is common, patients often require assisted ventilation in the final stages of the disease (Shoesmith and Strong, 2006).

The diagnosis of ALS is based on the El Escorial criteria as defined at "The Clinical Limits of ALS" conference in El Escorial, Spain, 29-31 May 1990. A definitive clinical diagnosis of ALS includes 3 criteria: (1) evidence of upper motor neuron [UMN] degeneration, (2) evidence of lower motor neuron [LMN] degeneration, and (3) progression (Brooks, 1994). The finding of LMN degeneration can be supported by electromyography. Signs of UMN degeneration include spasticity, hyperreflexia, and rigidity, while signs of LMN degeneration include fasciculations, weakness, hypotonia and eventual muscular atrophy and paralysis. To be diagnosed with ALS, it is important to ensure the absence of any other disease process that may mimic ALS. Even with evidence to diagnose a case as clinically definite, a post-mortem neuropathological exam is required to establish absolute certainty. Although the traditional view of ALS has been one in which cognitively intact patients lose motor function, an increasing finding is the presence of both ALS and a frontotemporal syndrome.

Increasingly, ALS is being regarded as a multisystems disorder with non-motor manifestations in addition to the progressive loss of motor function. There are currently 4 accepted classifications for patients suffering from frontotemporal impairment concomitantly with ALS. The 2 most commonly seen diagnoses are ALS with cognitive impairment [ALSci] and ALS with behavioural impairment [ALSbi], which generally encompass mild to moderate frontotemporal impairment. ALS-frontotemporal dementia [ALS-FTD] describes the fulfillment of the Neary criteria for FTD (Neary *et al.*, 1998) in addition to ALS. Finally, ALS-dementia describes, in addition to ALS, the presence of dementia that does not fit the above criteria, such as concomitant Alzheimer's disease or vascular dementia (Strong, 2008). Estimates of cognitive impairment in addition to the motor manifestations typical of ALS range from 3-52% (Strong, 2008), but cases of ALSci and ALSbi have been identified in greater than 50% of patients through neuropsychological testing, with ALS-FTD representing a small fraction of those (Woolley and Jonathan, 2008). In order to establish consensus criteria for the diagnosis of frontotemporal syndromes in

ALS patients, a conference was held in London, Canada in June 2007. Due to this conference, there are now recognized criteria for the identification of non-motor manifestations in ALS patients (Strong *et al.*, 2009). It is hoped that this will help in the development of novel therapies for cognitive impairment in ALS. Recently, evidence has suggested that administration of lithium to individuals with ALS may stop progression of cognitive impairment (Rush *et al.*, 2010). Further work into the efficacy of lithium treatment on cognitive impairment needs to be done, but current results seem promising.

Genetics of ALS

As discussed, approximately 10% of ALS cases are familial. Because the hypothesis on which this thesis is based assumes an understanding of the genetic variants of ALS, what follows is a discussion regarding the known genetic linkages to ALS. A complete list of presently known gene associations is presented in **Table 1.1: Genetic Variants of ALS**.

Approximately 20% of fALS cases are associated with mutations in the *SOD1* gene on chromosome 21 and are known as ALS 1 (Rosen *et al.*, 1993). SOD1 is a 153-amino acid metalloenzyme that forms a homodimer and whose function is to convert toxic superoxide free radicals to H_2O_2, which can then be removed from the cell by other enzymes (Shaw, 2005). To date, there are 153 known *SOD1* mutations that have been associated with ALS (Felbecker *et al.*, 2010). The majority of these mutations are clustered around the edges of the

Genetic Variant	Inheritance Pattern	Chromosome Locus	Gene	Alternative Phenotype
ALS 1	Dominant	21q22.1	SOD1	None
ALS 2	Recessive	2q33	Alsin	Infantile spastic, juvenile PLS
ALS 3	Dominant	18q21	Unknown	None
ALS 4	Dominant	9q34	SETX	CMT or dHMN
ALS 5	Recessive	15q15.1-q21.1	Unknown	None
ALS 6	Dominant	16q12	FUS/TLS	None
ALS 7	Dominant	20p13	Unknown	None
ALS 8	Dominant	20q13.33	VAPB	Late onset SMA
ALS 9	Dominant	14q11	ANG/VEGF	None
ALS 10	Dominant	1p36.2	TARDBP	Possible FTD
ALS 11	Dominant	6q21	FIG4	None
ALS 12	Dominant/ Recessive	10p15-p14	OPTN	None
ALS-FTD	Dominant	17q21.1	MAPT	Disinhibition, dementia

Table 1.1: Genetic Variants of ALS (Strong, 2010; Ticozzi *et al.*, 2011)

protein's β barrel, within the dimer interface, or near one of the zinc-binding superoxide dismutase pockets (Beckman *et al.*, 2001). Many of these mutations are known to affect interactions that are critical to the structural integrity of SOD1's β barrel (Deng *et al.*, 1993). The toxicity of mutant [mt] SOD1 is thought to occur due to a toxic gain-of-function mechanism, which is achieved when Zn^{2+} is lost from SOD1. This is sufficient to cause MN cell death (Estevez *et al.*, 1999). Although wild-type SOD1 cannot bind RNA, mtSOD1 has been shown to interact with RNA and act as a destabilizer (Ge *et al.*, 2005). In addition to being linked to fALS, mutant forms of SOD1 have been shown to be associated with sALS (Robberecht *et al.*, 1996). Interestingly, different *SOD1* mutations have different clinical manifestations with respect to survival following diagnosis. For example, patients with the common A4V mutation experience an aggressive disease course with a survival median of 1.4 years, whereas patients expressing the G37R mutation commonly approach 20 years survival (Cudkowicz *et al.*, 1997).

ALS 10 is caused by mutations in *TARDBP*, the gene encoding TDP-43. Since the discovery of TDP-43-positive ubiquitinated neuronal cytoplasmic inclusions [NCIs] in ALS tissue (Arai *et al.*, 2006; Neumann *et al.*, 2006), much work in the ALS field has centered on the role of TDP-43 in ALS pathogenesis. Since 2008, many *TARDBP* mutations have been found (Kabashi *et al.*, 2008; Kuhnlein *et al.*, 2008; Rutherford *et al.*, 2008; Sreedharan *et al.*, 2008; Van Deerlin *et al.*, 2008; Corrado *et al.*, 2009; Daoud *et al.*, 2009; Del Bo *et al.*, 2009). There are currently 30 known distinct mutations that have been associated with

ALS in 22 unrelated families and in 29 sporadic cases of ALS (Lagier-Tourenne and Cleveland, 2009; Perry *et al.*, 2010). The majority of *TARDBP* mutations are missense mutations that can be mapped to the glycine-rich, C-terminus of the protein, and therefore would be predicted to affect protein-protein interactions.

ALS 6 is caused by mutations in the gene encoding FUS/TLS. The FUS/TLS protein is a 526 amino acid protein encoded for by 15 exons. It is characterized by distinct motifs including: an N-terminal serine-tyrosine-glutamine-glycine-rich region [QGSY region], a glycine-rich region, an RNA-recognition motif [RRM], multiple RGG repeats involved in RNA binding, a C-terminus zinc finger, and a highly conserved extreme C-terminal region (Lagier-Tourenne and Cleveland, 2009). Recently, FUS/TLS was found to harbour mutations that are associated with fALS (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009), the majority of which are missense and cluster in the extreme C-terminus [aa514-525] (Perry *et al.*, 2010). In addition to the C-terminus mutations, there are 2 mutations of note in the glycine-rich region of FUS/TLS, both of which correspond to an insertion or deletion of 2 glycines (Lagier-Tourenne and Cleveland, 2009).

Although less directly related to this thesis, many other known forms of fALS have been attributed to mutations in specific genes. ALS 2, for example, occurs due to mutations in the gene encoding alsin (Yang *et al.*, 2001). A rare autosomal dominant form of juvenile ALS [ALS 4] is associated with mutations in the gene encoding senataxin [SETX] (Chen *et al.*, 2004). ALS 8 is used to describe ALS associated with mutations in the gene encoding vesicle-associated membrane protein B [VAPB] (Nishimura *et al.*, 2004). Vascular endothelial growth factor [VEGF] mutations have been shown to lead to sequence variations that have biological effects in ALS and have been found in families, thus the label of ALS 9 (Lambrechts *et al.*, 2003; Greenway *et al.*, 2004). Mutations have been found in the *FIG4* gene in 6 sALS and 3 fALS patients (Chow *et al.*, 2007). Mutations have also been reported in the gene encoding optineurin in 4 Japanese consanguineous kindreds (Maruyama *et al.*, 2010). Very recently, 10 unique mutations in 15 individuals have been discovered in *SQSTM1/p62* that are associated with ALS, which has led to the suggestion that p62 may be involved in ALS pathogenesis (Personal Communication: Fecto *et al.*, 2011). In addition to the strictly ALS phenotypes, mutations in the *MAPT* gene are associated with a combined ALS and FTD phenotype (Curcio *et al.*, 2002). For a complete list of all known genetic linkages in ALS, please refer to **Table 1.1** above.

In addition to mutations in genes leading to familial forms of ALS, other mutations have been associated with events that may lead to ALS or other ALS-like phenotypes. For example, the altered expression of Excitatory amino-acid transporter 2 [EAAT2] may affect surrounding mRNAs and RNA binding proteins, although its exact role remains to be determined (Strong, 2010). Spinal muscular atrophy has been associated with an abnormal copy number of *SMN* leading to its decreased expression (Corcia *et al.*, 2002; Veldink *et al.*, 2005). Finally, the gene that encodes the intermediate filament protein peripherin, which is known to form NCIs in ALS, can form multiple splice variants as a result of alternative splicing of *PRPH* (Robertson *et al.*, 2003).

Biology and Pathobiology of ALS

Although the exact pathogenic mechanism of ALS has yet to be determined, several contributing factors have been identified. The classical pathology within motor neuron cell bodies includes the presence of intermediate filamentous NCIs. One type of intermediate filament is neurofilament [NF], which is composed of 3 subunits. The 3 subunits are named for their molecular weight and include high molecular weight NF [NFH], intermediate molecular weight NF [NFM], and low molecular weight NF [NFL]. The presence of NF NCIs has been associated with suppression of steady state NFL mRNA levels (Bergeron *et al.*, 1994; Menzies *et al.*, 2002).

The contemporary thinking, however, is that ALS is a disease of altered RNA metabolism. The evidence supporting this includes: (1) RNA binding proteins have been shown to be abnormally expressed in ALS, but not in neuropathologically normal controls, (2) abnormal RNA binding protein expression is related to the suppression of NFL mRNA, and (3) mtSOD1, TDP-43, FUS/TLS and RGNEF [see Chapters 2 and 3], all of which form NCIs in ALS, modulate NFL mRNA stability (Strong, 2010). In addition, some mutations with known associations to ALS occur in the genes encoding RNA binding proteins, which in turn affect RNA-mediated processes. Namely, these include mutations in *SOD1*, *TARDBP*, and *FUS/TLS*, which are detailed in **Table 1.1**.

Interestingly, the abnormal expression of some of the proteins thought to be involved in the pathogenesis of ALS can be targeted for protein degradation through the association with degradative markers. This is important because it is suggestive that protein degradation may be involved in the pathogenesis of ALS. Because ALS is known to be a disease associated with multiple proteins, and because this thesis utilizes various protein expression patterns to draw conclusions, what follows is a discussion of the biology and neuropathology of the proteins involved in this work. Since the first immunohistochemical descriptions of neuropathology in ALS, work centered on the presence of NCIs has been a general focus within the field (Strong *et al.*, 2005). For the purposes of this thesis, NCIs are defined as aggregates or filamentous skein-like protein accumulations within affected anterior horn MNs.

TDP-43

TDP-43 is a 414 amino acid protein whose gene, found on chromosome 1p36.2, contains 1 non-coding and 5 coding exons (Buratti and Baralle, 2001). It contains 2 RNA recognition motifs and a glycine-rich region on the C-terminus that allows it to bind DNA, RNA, and other proteins (Wang *et al.*, 2004). Its role as a dual DNA/RNA binding protein is conducive to its wide array of functions in gene expression. TDP-43 acts to regulate pre-mRNA alternative splicing (Buratti and Baralle, 2001) as well as transcription, and increasing evidence points to its involvement in mRNA transport, stabilization, and degradation, as well as translation, and microRNA [miRNA] biogenesis (Buratti and Baralle, 2008; Warraich *et al.*, 2010). As further evidence to its role in RNA metabolism, TDP-43 has been shown to be a component of RNA granules isolated from rat brain (Elvira *et al.*, 2006). This suggests involvement in RNA trafficking, an important step of gene expression. TDP-43 has also been shown to act as a stabilizer of human NFL mRNA through a direct interaction to its 3' untranslated region [3'UTR] (Strong *et al.*, 2007). Due to the evidence that ALS is an RNA-mediated disorder and because TDP-43 has been shown to be functionally integrated in gene expression, much of the ALS field has targeted it as *the pathological protein* in ALS. Much of this speculation, however, is due to the cellular pathology of TDP-43.

The normal intraneuronal localization of TDP-43 is nuclear with minimal cytoplasmic presence in RNA granules. In ALS tissues, however, the expression of TDP-43 is upregulated approximately 1.5 fold (Mishra *et al.*, 2007; Geser *et al.*, 2008) and there is a transition from nuclear to cytoplasmic localization. Recent work has suggested that this nucleocytoplasmic redistribution is a reversible, normal response to injury (Moisse *et al.*, 2009). TDP-43 has been shown to form NCIs in ALS, but not in healthy controls. These inclusions have been shown to be ubiquitinated in ALS (Arai *et al.*, 2006; Neumann *et al.*, 2006), suggesting that TDP-43-positive NCIs are being targeted for degradation (Strong *et al.*, 2007), although a lack of direct TDP-43-ubiquitin co-localization has been speculated (Sanelli *et al.*, 2007). As discussed above, mutations in

TARDBP have been found in both familial and sALS. Ubiquitinated, TDP-43-positive NCIs have been observed in cases of ALS that do not harbour a mutation in *TARDBP.* Of note is the aberrant phosphorylation of TDP-43 in these NCIs, which has been shown to associate with the pathogenesis of multiple neurodegenerative diseases, including ALS (Fujiwara *et al.*, 2002).

FUS/TLS

Like TDP-43, FUS/TLS is a dual DNA/RNA binding protein that has multiple functions in gene expression. Due to its more recent discovery, less is known about the biological role of FUS/TLS; however, current studies suggest that it is found to associate with the heterogeneous ribonucleoprotein [hnRNP] complex involved in pre-mRNA splicing and the extra-nuclear transport of fully processed transcript (Iko *et al.*, 2004). In addition, FUS/TLS is associated with Drosha [a nuclear RNase III] in the microprocessor complex, suggesting that it likely has a role in miRNA processing (Gregory *et al.*, 2004). As mentioned above, FUS/TLS has multiple motifs that allow it to perform its functions. Given its role in RNA metabolism, the RRM was considered to be crucial for RNA targeting, but this was proven not to be the case by Zinszner and colleagues (Zinszner *et al.*, 1997). In fact, the zinc-finger motif has been shown to outperform the RRM with respect to RNA interaction (Iko *et al.*, 2004).

FUS/TLS is a ubiquitous protein and cellular localization is cell-type dependent (Yang *et al.*, 2010). The typical FUS/TLS expression pattern is similar to that of TDP-43: mainly nuclear, with lower levels seen throughout the cytosol (Andersson *et al.*, 2008). Within the brain and spinal cord of ALS patients, however, FUS/TLS-positive NCIs can be found that are also immunoreactive for TDP-43, ubiquitin, and p62. The same report, which presented convincing evidence for the importance of optimizing staining conditions via antigen retrieval and optimal antibody selection, presented FUS/TLS-positive inclusions in mtFUS/TLS-ALS cases (Deng *et al.*, 2010).

Mutant SOD1

SOD1, as briefly mentioned above, is an enzyme whose sole function is thought to be the catalytic conversion of toxic superoxide free radicals to molecular oxygen [O_2] and hydrogen peroxide [H_2O_2], which is further reduced to H_2O by other enzymes. It is a ubiquitous protein, can be found in any cell in the human body and in any organism above bacteria, and accounts for 0.5-0.8% of the soluble protein in the human brain and spinal cord (Andersen, 2006). The pathological effect of mtSOD1 is not due to a loss of enzyme function, but rather due to a toxic gain-of-function. The toxic properties of SOD1 seem to be gained when Zn^{2+} is lost from the protein, seemingly enough of an assault to cause MN cell death (Estevez *et al.*, 1999). Many mutations within *SOD1* are known to affect the structural integrity of the protein, and as a result, Zn^{2+} is lost and SOD1 becomes toxic (Deng *et al.*, 1993). Past studies characterizing the localization of SOD1 expression within MNs have shown abnormal aggregation within ALS 1 cases (Stieber *et al.*, 2000). Mutant SOD1, but not wild type, has been shown to bind directly to the 3'UTR of NFL mRNA, which is necessary to induce mRNA destabilization (Ge *et al.*, 2005). These findings support the hypothesis that ALS is a disease of altered RNA metabolism and are the grounds for the focus on mtSOD1 throughout this thesis as opposed to wild type SOD1.

RGNEF

RGNEF is a novel protein and the human homologue of the murine p190RhoGEF (Volkening *et al.*, 2010). In the mouse, p190RhoGEF is a stability factor for NFL mRNA through its interaction adjacent the stop codon and extending into the 3'UTR (Canete-Soler *et al.*, 2001). Additionally, p190RhoGEF was shown to interact with NFL subunits that were not polymerized into filaments, an interaction that is associated with decreased NFL mRNA levels and subsequent NFL aggregation (Lin *et al.*, 2005). Early studies on RGNEF have proven its *in vitro* interaction with NFL mRNA isolated from brain and spinal cord in ALS cases, but not in controls (Volkening *et al.*, 2010). More recent work has connected ALS pathology via RGNEF to RhoA signaling cascades (Droppelmann, C *et al.*, Submitted, 2011).

There is evidence of the formation of RGNEF-positive NCIs in ALS cases but not in neuropathologically normal controls. This manuscript has also provided evidence that aberrant RGNEF-positive NCIs can be targeted for degradation through the proteasome degradation pathway due to their co-localization with ubiquitin and p62 [see Chapter 3].

High Molecular Weigh Neurofilament

Neurofilament is one of the major intermediate filaments of the cytoskeletal network of adult MNs. As mentioned, 3 subunits classified by their molecular weight [low, intermediate, high] make up the NF polymer. The presence of NFL is necessary for correct NF assembly and integrity, and stoichiometric ratios between NFL, NFM and NFH are variable (Sakaguchi *et al.*, 1993). In complex, these subunits polymerize into 10 nm structures and run the length of an axon. In mouse models in which the stoichiometric ratio between NFL, NFM, and NFH is disrupted, a motor neuronopathy with phosphorylated NFH [pNFH]-positive NCIs develops (Julien, 1999; Beaulieu *et al.*, 2000). Phosphorylated tails of NFM and NFH extend out from the NF complex; these phosphorylated tails interact with other filaments and axonal proteins, participating in cross-bridge formation and the regulation of interactions with other cytoskeletal elements. Phosphorylation of NF subunits is thought to expand axonal caliber as well as lateral spacing between NF subunits (Nixon *et al.*, 1994).

As mentioned, one of the classic pathological hallmarks of ALS is the accumulation of intermediate filamentous NCIs (Strong *et al.*, 2005). This can manifest itself in the aberrant aggregation of pNFH, which is associated with a down-regulation of steady-state levels of NFL mRNA (Bergeron *et al.*, 1994; Wong *et al.*, 2000; Menzies *et al.*, 2002). The other NF-related classic pathological hallmark in ALS that is of relevance to this thesis is the presence of neuroaxonal enlargements. Smaller axonal enlargements (in the 6-20 µm range) are referred to as globules and are normal (Carpenter, 1968). Larger enlargements, however, referred to as neuroaxonal spheroids and defined as greater than or equal to 30 µm at their smallest diameter, are considered to be part of the pathology of ALS (Carpenter, 1968) and may have a role in the obstruction of axonal transport of various proteins (Strong *et al.*, 2005).

Peripherin

Another neuronal intermediate filament protein, peripherin is a type III intermediate filament protein that is capable of self-assembling to form homopolymeric filamentous networks (Cui *et al.*, 1995). Much like the NF subunits, peripherin shares a similar secondary protein structure including an alpha helix, which is highly conserved across the different types of intermediate filaments (Xiao *et al.*, 2006). After injury, peripherin expression is known to be upregulated in various neuronal types, including MNs (Troy *et al.*, 1990; Wong and Oblinger, 1990). It has been reported that up-regulation of peripherin may be associated with neuronal regeneration (Troy *et al.*, 1990; Wong and Oblinger, 1990). Paradoxically, transgenic mice over-expressing wild type mouse peripherin develop a motor neuronopathy characterized by the development of peripherin-positive NCIs (Beaulieu *et al.*, 1999). In mice, 3 alternative splice variants of peripherin are known [Per 56, Per 58, Per 61] (Robertson *et al.*, 2003). The function of these alternative splice variants is not known, however Per 56 appears normally expressed with Per 58 *in vivo*, whereas Per 61 does not appear to be involved in normal expression of the peripherin gene and actually has proved to be neurotoxic (Robertson *et al.*, 2003). This same study used isoform-specific antibodies to detect Per 61 within MNs of SOD1^{G37R} transgenic mice, but never within MNs of healthy controls or peripherin transgenic mice. This suggests that alternative splicing of peripherin leads to splice variants that may be actively involved in the pathogenesis of ALS.

Like neurofilamentous inclusions, peripherin-positive NCIs may take the form of either neuroaxonal spheroids and/or somatic inclusions. In addition, peripherin-positive NCIs can take the form of Lewy body-like inclusions that are highly immunoreactive with ubiquitin antibodies (He and Hays, 2004). Interestingly, peripherin-positive inclusions are known to be a commonality between sALS and fALS and may represent a point of convergence with respect to disease pathogenesis (Xiao *et al.*, 2006).

Ubiquitin

Ubiquitin is a highly conserved 76 amino acid protein that is ubiquitously expressed. It is an important protein in neurodegenerative diseases because of its association with the proteasome degradation pathway. The proteasome is a large protein complex found universally across eukaryotic cells. It contains proteases whose function is to degrade misfolded proteins into small 7 or 8 amino acid peptides, which are then further degraded to amino acids, which can then be used to synthesize new proteins (Ciechanover, 1994). The significance of this discovery was acknowledged in awarding the 2004

Nobel Prize in Chemistry to Aaron Ciechanover, Avram Hershko, and Irwin Rose for their discovery of ubiquitin-mediated protein degradation. The role of ubiquitin in the process of proteasome-mediated degradation is to label proteins that are to be degraded by the cell with at least 4 ubiquitin molecules (Thrower *et al.*, 2000). This leads to the targeting of the protein for degradation by the proteasome (Haas and Rose, 1982; Haas *et al.*, 1982).

The field of ALS changed in 1991 upon the discovery of ubiquitin-positive NCIs in the extra-motor cortices in patients with ALS (Okamoto *et al.*, 1991). This was a critical finding because of the known relationship between poly-ubiquitin labeling and protein degradation, and confirmed that unidentified proteins were likely involved in the pathogenesis of ALS. Shortly thereafter, the intermediate filament proteins associated with ALS were found to co-localize with ubiquitin-positive NCIs in Betz cells (Sasaki and Maruyama, 1991; Sasaki and Maruyama, 1992). There were, however, many more ubiquitin-positive NCIs, within the same and different cases, that were not immunoreactive for the intermediate filament proteins, and were not morphologically similar to intermediate filament-positive NCIs. Many of these unidentified, ubiquitin-positive NCIs were found to be immunoreactive with TDP-43 (Arai *et al.*, 2006; Neumann *et al.*, 2006), which was the discovery that began the research focus on TDP-43. Now, with the association of other RNA binding proteins, such as FUS/TLS (Deng *et al.*, 2010) and RGNEF [see Chapter 3], with ubiquitin-positive NCIs, and the knowledge that ubiquitin may be associated with the pathogenesis of ALS, ALS is increasingly being regarded as a disease of altered RNA metabolism. The pathological presence of ubiquitin-positive NCIs is evidence for protein degradation via the proteasome complex, and is a crucial pathological marker of ALS.

Sequestosome 1 [p62]

P62 is a 62 kDa, 440 amino acid protein that is known to be expressed in many tissues (Ishii *et al.*, 1996; Rachubinski *et al.*, 1999). P62 has a ubiquitin-binding associated C-terminal domain that has a high affinity for poly-ubiquitin chains (Vadlamudi and Shin, 1998). It is known to act as a scaffold protein in that it can bind many signaling molecules, thus playing a role in linking poly-ubiquitination with various signaling events (Geetha and Wooten, 2002). The link between neurodegeneration and p62 became evident with the finding that elevated levels of neuronal and glial p62 can be found in human tauopathies and synucleinopathies (Kuusisto *et al.*, 2001). Namely, elevated intraneuronal expression of p62 has been found in Alzheimer's disease, Pick's disease, frontotemporal dementia, Parkinson's disease, dementia with Lewy body disease, and multiple system atrophy, in addition to ALS.

P62-positive NCIs have been found in ALS cases, but not in controls. Specifically, p62-positive NCIs have been reported in skein-like inclusions, Lewy body-like inclusions, and basophilic inclusions (Mizuno *et al.*, 2006) and are known to co-localize with ubiquitin. These findings suggest that p62 is important in the protein-proteasome degradation pathway.

Optineurin

Optineurin is a 66 kDa, 574 amino acid protein that has traditionally been associated with primary open-angle glaucoma [POAG] (Rezaie *et al.*, 2002). There have been many reports of mutations in *OPTN* associated with POAG although the mechanisms through which these mutations may lead to POAG are currently unknown. Aberrant optineurin expression causes motor neurons to utilize either the TNF-alpha or Fas-ligand pathway to undergo apoptosis, inflammation, or vasoconstriction (Sarfarazi and Rezaie, 2003). In a Japanese population, 3 distinct mutations in *OPTN* have been associated with ALS: 1 missense autosomal dominant, 1 nonsense autosomal recessive, and 1 deletion leading to an autosomal recessive mutation (Maruyama *et al.*, 2010). Mechanistically, it is likely that the recessive mutations lead to a loss-of-function, while the dominant mutation is association with a gain-of-function.

Optineurin immunostaining has revealed optineurin-positive NCIs in ALS cases, but not in healthy controls. More specifically, NCIs were optineurin-positive in some sALS and fALS cases without any known mutations. In addition, neuritic staining was evident and seemed to be more intense in cases of sALS. Some of these inclusions were ubiquitin and TDP-43-positive as well, suggesting a unifying role for optineurin in disease pathogenesis. Interestingly, optineurin-positive NCIs were also found in cases of ALS 1 (Maruyama *et al.*, 2010). More recent work, however, has suggested that optineurin-positive NCIs are in fact present in a minority of cases and that optineurin does not likely play a role in the pathogenesis of ALS or in any other neurodegenerative disease (Hortobagyi *et al.*, 2011; Sugihara *et al.*, 2011). This conclusion is based upon large studies that looked for *OPTN* mutations in non-Japanese populations as well as immunohistochemical observations.

RNA Metabolism and ALS

One of the key aspects of the Central Dogma of Molecular Biology is RNA metabolism (Crick, 1970). As briefly discussed above, there is considerable evidence suggesting that ALS is a disease of altered RNA metabolism. To summarize, the evidence supporting this is:

1. RNA binding proteins have been shown to be abnormally expressed in ALS;
2. Mutations in genes encoding these RNA binding proteins affect RNA mediated processes such as splicing, transcription, translation, transport, and degradation;
3. Abnormal RNA binding protein expression is related to the suppression of NFL mRNA; and,
4. The 4 RNA binding proteins discussed thus far, mtSOD1, TDP-43, FUS/TLS, and RGNEF, form NCIs in ALS and are all known to modulate NFL mRNA stability.

As the knowledge base of information regarding RNA metabolism grows, it is increasingly regarded as a very dynamic process. It is now known that protein expression is somatotopic in that protein synthesis occurs at variable sites within the cell, responding to cellular protein needs (Strong, 2010). For somatotopic expression to successfully occur, it is necessary for mRNA to be shipped throughout the cell for translation at optimal sites. This occurs via RNP complexes, which can be identified as RNA granules. In general, RNP complexes consist of multiple building blocks: ribosomal subunits, translation factors, decay enzymes, helicases, scaffold proteins, molecular motors, and RNA binding proteins, all of which control the complex' localization, stability and translation of RNA contents (Anderson and Kedersha, 2006). RNP granules exist within a cell in 3 main forms: transport granules, stress granules, and cytoplasmic processing bodies [P-bodies]. Due to the fact that RGNEF has been proven to be an RNA binding protein and given that this thesis will be characterizing RGNEF expression for the first time, what follows is a discussion of several additional relevant proteins.

Staufen [Transport Granules]

Characteristic of transport granules, Staufen is present within RNA granules when the mRNA is translationally silent and being shipped to the site of protein synthesis. It is possible for these granules, which contain all of the machinery necessary for RNA translation, to be shipped to distal axonal sites. Transport granules are a normal part of RNA metabolism and can be found in damaged and healthy cells alike. Staufen is an accepted marker for transport granules, and is used as such within this thesis.

TIA-1 [Stress Granules]

Used as a marker for stress granules, TIA-1 is present within RNP complexes whose core constituents are stalled 48S pre-initiation complexes (Kedersha *et al.*, 2002; Kimball *et al.*, 2003). Pre-initiation complexes are evidence that the initial steps of translation have occurred, but due to some environmental stress, translation was not able to move forward. The induction of stress granules is rapid following the environmental stress and usually occurs within 15-30 minutes (Anderson and Kedersha, 2006). The transformation into a translationally silent granule then occurs via the phosphorylation of a translation initiation factor by a family of stress-activated kinases, and results in polysome disassembly (Anderson and Kedersha, 2006). Although many proteins exist within stress granules, TIA-1 is accepted as the preferred marker for stress granules, and is used as such within this thesis.

XRN-1 [P-bodies]

Found in P-bodies, the 5'-3' mammalian exonuclease XRN-1 is one component of a complex containing decay machinery for each step of the mRNA decay process. Although generally recognized as a state from which mRNA will be degraded and its components used for other purposes, P-bodies share many features in common with stress granules, including a common origin (Strong, 2010), and they actually exist interchangeably with one another (Barbee *et al.*, 2006; Anderson and Kedersha, 2008). XRN-1 is the accepted marker for P-bodies, and as such, is used in this thesis.

L26 [Polysomes]

Polysomes are clusters of ribosomes that bind to molecules of mRNA and work to synthesize protein from the transcript at multiple locations. In a micrograph, polysomes can be visible as coarse granular structures throughout the cell that are the sites of protein synthesis. The most prominent component of the large ribosomal 60S subunit is rRNA, which is held together by proteins, thus allowing it to perform its catalytic function. L26 is a ribosomal protein of the 60S subunit. It has been shown to help bind the 5'UTR of p53 mRNA and to control p53 translation (Takagi *et al.*, 2005). Thus, L26 is the marker of polysomes and translationally active granules used throughout this thesis.

As suggested by the number of proteins discussed thus far, RNA metabolism is a complex, dynamic process with many possibilities for aberrancies to occur. For a schematic of the process of RNA metabolism and how these proteins contribute, please see **Figure 1.1: RNA Metabolism** (Strong, 2010).

Glutamate Excitotoxicity

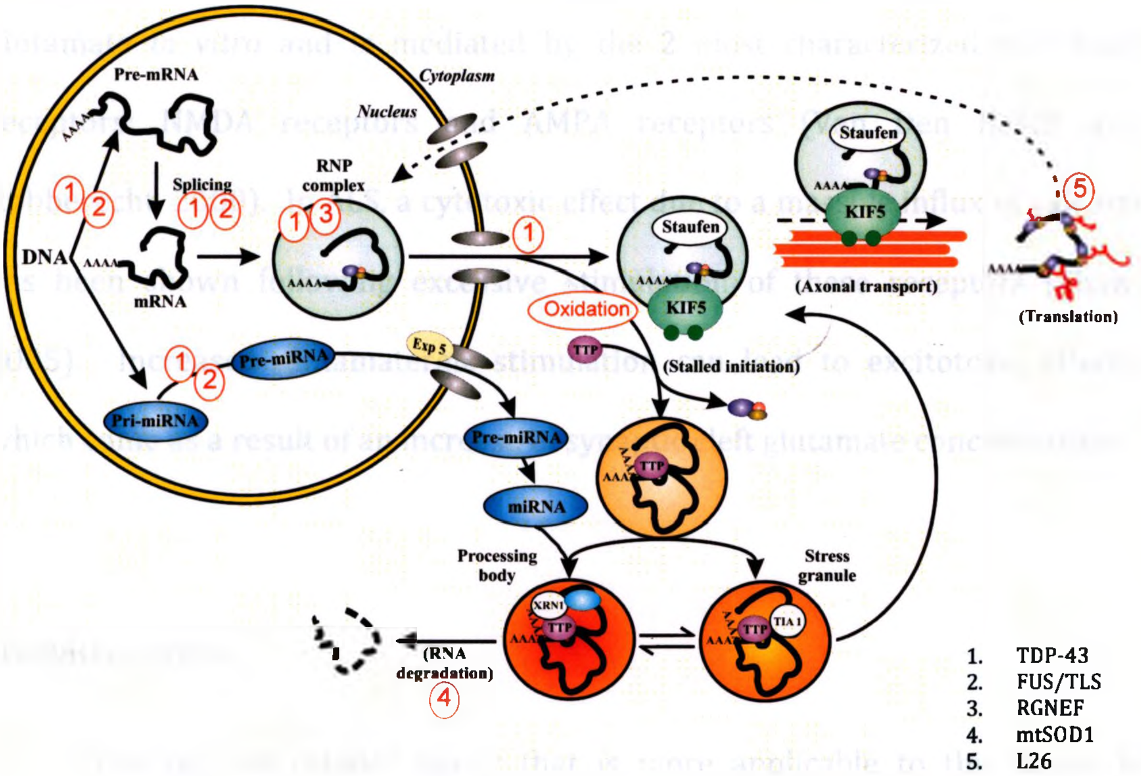
As briefly mentioned, the only pharmaceutical currently used to combat ALS, riluzole works by inhibiting pre-synaptic glutamate release

Figure 1.1: RNA Metabolism.

Due to the important role for RNA metabolism in ALS and throughout this thesis, this schematic outlines the dynamic complexity of the process and how various relevant proteins discussed thus far fit into it.

1. TDP-43 has numerous roles, and is known to be involved in transcription, mRNA splicing, miRNA biogenesis and nucleocytoplasmic shuttling. Consistent with its role in transport, TDP-43 has been found in RNP complexes.
2. FUS/TLS is known to be involved in transcription, mRNA splicing and miRNA biogenesis.
3. RGNEF is a signaling molecule, but is known to be an active protein in RNA metabolism due to its presence in RNA granules [see Chapter 3].
4. Mutant SOD1 is known to be involved in neuronal toxicity due to a gain-of-function mechanism. As discussed, this can result in neuronal death via an RNA degradative mechanism.
5. L26 is a protein found in the large ribosomal subunit, a primary component of polysomes, and an accepted marker of translational activity.

In addition, an example of an environmental stressor is oxidation, which can lead to stalled initiation, and the transformation from transport granules to stress granules or P-bodies. Also included in this figure are all of the accepted unique markers for RNA granules: Staufen, TIA-1, and XRN-1.


Pre-mRNA
Cytoplasm
Nucleus
AAAA
Splicing
RNP complex
DNA
mRNA
Staufen
KIF5
(Axonal transport)
(Translation)
Oxidation
TTP
(Stalled initiation)
Exp 5
Pre-miRNA
Pri-miRNA
miRNA
Processing body
Stress granule
XRN1
TIA 1
(RNA degradation)
①
②
③
④
⑤

1. TDP-43
2. FUS/TLS
3. RGNEF
4. mtSOD1
5. L26

Adapted from: Strong, M.J., J Neuro Sci, 2010, 288: 1-12

(Zinman and Cudkowicz, 2011). Other promising compounds have been tested in clinical trials, although all trials have been negative. The commonality amongst almost all of these compounds that have made it to clinical trials is their targeting of glutamate pathways. L-Glutamate is the most abundant amino acid in the mammalian central nervous system and functions as an excitatory neurotransmitter (McEntee and Crook, 1993). MN death has been induced by glutamate *in vitro* and is mediated by the 2 most characterized glutamate receptors: NMDA receptors and AMPA receptors (Van Den Bosch and Robberecht, 2000). In ALS, a cytotoxic effect due to a massive influx of calcium has been shown following excessive stimulation of these receptors (Shaw, 2005). Increased glutamatergic stimulation can lead to excitotoxic effects, which come as a result of an increase in synaptic cleft glutamate concentration.

Oxidative Stress

The type of cellular injury that is more applicable to this thesis is oxidative stress. Clearly involved in ALS pathogenesis, researchers are unsure whether oxidative stress is a cause of disease pathogenesis or merely an effect of some unknown upstream event. The main cause of oxidative stress is an accumulation of reactive oxygen species [ROS], which are believed to be associated with aging (Beal, 2002; Lenaz *et al.*, 2002; Genova *et al.*, 2004). As described above, SOD1 is a key regulator of ROS accumulation, and has been implicated in ALS pathogenesis. Although some mutations in *SOD1*, such as A4V and G85R, lead to a decrease in enzymatic activity, other mutations, such as G37R (Borchelt *et al.*, 1994) and G93A (Yim *et al.*, 1996), allow SOD1 to retain its full activity. Additionally, SOD1 knockout mice do not develop an ALS phenotype (Reaume *et al.*, 1996). Taken together, these data have led to the currently accepted beliefs that a lack of ROS removal is not sufficient to be cytotoxic, and that a loss of SOD1 activity is not sufficient to cause ALS.

Glial Involvement in ALS

One aspect of innate immunity is inflammation, which is a normal cellular response to environmental injury (Moisse and Strong, 2006). Because antigens do not stimulate it, the innate immune response is generally executed by phagocytes and is non-specific. One type of phagocyte is the macrophage, and the resident macrophage of the central nervous system [CNS] is the microglia. Another type of glial cell that is known to be involved in ALS is the astrocyte. Due to the relationship between cellular injury, inflammation, and ALS, and the subsequent involvement of microglia and astrocytes, what follows is a discussion of glial involvement in ALS. This discussion is pertinent to this thesis because RGNEF expression has never been characterized, and one aspect of its characterization includes a potential involvement in glial cells [see Chapter 3].

Microglia

Microglia act as support cells for neurons within the CNS through the production of anti-inflammatory cytokines, neurotrophins, and growth factors. They can also phagocytose unneeded cellular debris following injury or disease. Some studies have challenged these findings with the identification of the release of pro-inflammatory factors that show microglia enhancing neuronal injury (Banati *et al.*, 1993; Aldskogius and Kozlova, 1998). When MN injury occurs, a morphological transition of microglia follows that is known as "priming" (Perry *et al.*, 2003) and can be a result of neurodegeneration (Cunningham *et al.*, 2005), stress (Johnson *et al.*, 2002), or aging (Godbout *et al.*, 2005). Primed microglia have thickened cell bodies and processes, with a more intense local surveillance.

In ALS, considerable evidence exists for the proliferation and activation of microglia (Lampson *et al.*, 1990; Troost *et al.*, 1990; McGeer *et al.*, 1991; Kawamata *et al.*, 1992; Troost *et al.*, 1993; Wilson *et al.*, 2001; Henkel *et al.*, 2004; Solomon *et al.*, 2006). Importantly for this thesis, these observations have been made in the anterior horn of the spinal cord (Kawamata *et al.*, 1992), which is the location on which the studies in this thesis have focused. The microglial marker used in this thesis is ionized calcium binding adaptor molecule 1 [IBA-1]. IBA-1 has been associated with an inflammatory response, and has been shown to be up-regulated in microglia as a response to injury and in ALS tissues (Moisse *et al.*, 2009).

Astrocytes

Also part of the neuroinflammatory response, the primary role of astrocytes in the CNS is to mediate extracellular glutamate levels, thus mediating the potential for excitotoxic neuronal degeneration. Outnumbering neurons approximately 10 to 1, they also provide trophic support for surrounding neurons (Sofroniew and Vinters, 2010). Astrocytes are able to participate directly in inflammatory reactions. Injured MNs have been found to secrete fibroblast growth factor-1 [FGF-1], which is known to lead to an accumulation of FGF-1 receptors in astrocytic nuclei (Cassina *et al.*, 2005). This accumulation stimulates astrocytic nerve growth factor expression, which was previously linked to MN apoptosis via a p75-dependent mechanism (Cassina *et al.*, 2002; Pehar *et al.*, 2002).

Astrocytes are activated in ALS tissue as well as in mtSOD1 human transgenic mice (Engelhardt and Appel, 1990; Kushner *et al.*, 1991; Kawamata *et al.*, 1992; Engelhardt *et al.*, 1993; Nagy *et al.*, 1994; Schiffer *et al.*, 1996; Hall *et al.*, 1998; Alexianu *et al.*, 2001). As with microglia, astrocytic activation is characterized by a larger cell body and thicker astrocytic processes. Characteristic of astrocyte activation is an up-regulation of the intermediate filament glial fibrillary acidic protein [GFAP] (Philips and Robberecht, 2011), which is the astrocytic marker used within this thesis. This increased astrocytic expression causes a down-regulation of the glutamate transporter EAAT2, which causes a decreased glutamate transport and a possible active release of glutamate (Lin *et al.*, 1998). This leaves MNs more susceptible to excitotoxic damage.

Motor Neuronal Death in ALS

One of the ongoing debates in the field of ALS research is the mechanism by which MN cell death occurs. One of the reasons for this debate is the difficulty associated with catching a MN in the dying process in human tissue because it is seen just at a "snap-shot" in time. Three main types of cell death have been proposed: apoptosis, necrosis, and autophagy. What follows is a brief discussion of each mechanism and its potential role in MN death in ALS.

Apoptosis is a tightly regulated, programmed, energy-dependent process that is specifically designed to switch off unneeded or damaged cells and eliminate them. This occurs through the formation of small cellular fragments called apoptotic bodies that surrounding cells such as macrophages can engulf and degrade. There are several characteristic morphological changes observed of the cell undergoing apoptosis. The first of these is the shrinkage of the cell and the formation of blebs on the cell membrane. At the same time, the nucleus becomes pyknotic before karyorrhexis, marked by a fragmented nucleus, occurs, and apoptotic bodies appear from the fragmented cell. At a molecular level, pro-apoptotic markers can be present (Yoshiyama *et al.*, 1994; Mu *et al.*, 1996; Ekegren *et al.*, 1999; Martin, 1999; Gonzalez de Aguilar *et al.*, 2000; Martin, 2000; Guegan *et al.*, 2002), and anti-apoptotic factors such as the proteins of the Bcl-2 family are down-regulated (Martin, 1999; Vukosavic *et al.*, 1999). These finding have been replicated in human tissue from ALS patients as well as in mouse models of ALS.

Necrosis is cellular death followed by inflammation that is caused by some environmental stress. In contrast to apoptosis, necrosis is not programmed, and is therefore not planned for by the cell. Past studies have revealed necrotic cell death in ALS due to excitotoxic cellular injury (Glazner *et al.*, 2000), and DNA fragmentation, which has been shown to occur in apoptosis as well as necrosis.

Autophagy is a mechanism for eukaryotic cells to degrade intracellular components and its altered regulation can contribute to neurodegeneration. In contrast to ubiquitin-proteasome protein degradation that was discussed earlier and tends to degrade short-lived misfolded proteins (Ciechanover, 2006), autophagy is the lysosomal degradation of organelles and long-lived cytoplasmic proteins (Marino and Lopez-Otin, 2004). Both the inhibition (Hara *et al.*, 2006; Komatsu *et al.*, 2006) and excessive activation (Tsujimoto and Shimizu, 2005) of autophagy can lead to cell death, the former provoking neurodegeneration and the latter causing auto-digestion. Thus, the effects of autophagy on the ALS MN depend on its temporal, spatial, and degree of activation (Pasquali *et al.*, 2009). Microscopic examination of post-mortem human tissue has revealed phenotypic evidence of autophagic MN death (Przedborski, 2004). Further, autophagy has been implicated in *in vitro* models of excitotoxicity-induced MN death (Matyja *et al.*, 2005). In that study, Matyja and colleagues demonstrated a different mode of cell death in excitotoxic MNs, and suggested a cell death continuum including apoptotic-necrotic and apoptotic-autophagic mechanisms. Motor neurons of mtSOD1^{G93A} mice have been shown to contain increased numbers of autophagosomes compared to wild-type mice (Morimoto *et al.*, 2007), suggesting that autophagy is involved in cell death in transgenic mouse models of ALS. Taken together, multiple studies have implicated autophagy as a mechanism involved in cell death in ALS.

Evidence exists in support of each of the mechanisms discussed above. Molecular and morphological findings do not fully support one mechanism in all cells, so a definitive mechanism of cell death has yet to be agreed upon.

Rationale and Hypothesis

It has been demonstrated that ALS is a disease of altered RNA metabolism, and that many proteins are involved in regulating the RNA metabolic process, including the novel NFL mRNA binding protein, RGNEF. In addition to the RNA binding proteins that regulate RNA metabolism, several other proteins have been introduced, all of which have been suggested to play unique roles in the pathogenesis of ALS. Further, several variants of ALS have been introduced. I have sought to examine the relationship between the immunohistochemical expression of various ALS-associated proteins and, for the first time, characterize RGNEF pathology, across multiple variants of ALS. In doing so, I have tested the **hypothesis** that ***all variants of ALS are pathologically distinct and can be differentiated by spinal cord pathology using a panel of contemporary ALS-associated markers.***

In **Chapter 2**, I will demonstrate that ALS is indeed characterized by aberrant RNA binding protein expression, and I will show that mtSOD1-ALS is a pathologically distinct variant of ALS.

In **Chapter 3**, I will show that RGNEF can be aberrantly expressed in ALS tissues, but not in neuropathologically normal controls, and that it is likely involved in the pathogenesis of ALS. I will also show that RGNEF is actively involved in the RNA metabolic pathway and that it does not seem to be involved in neuroinflammation. Finally, I will provide evidence that further supports the hypothesis that ALS is a disease of altered RNA metabolism.

In **Chapter 4**, I will summarize my findings and discuss the implications of this work as well as future directions.

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Chapter 2: Evidence for the unique pathological classification of mutant SOD1-ALS

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Abstract

Recent studies have associated various genetic mutations with amyotrophic lateral sclerosis [ALS], including mutations in the genes encoding superoxide dismutase 1 [SOD1], fused in sarcoma/translocated in liposarcoma [FUS/TLS], TAR DNA-binding protein of 43 kDa [TDP-43], and optineurin. The contribution of these mutations with respect to ALS motor neuron pathology remains to be elucidated. Our aim in this study was to analyze spinal anterior horn cells [AHCs] in patients with identified mutations in *SOD1* [n=5], *FUS/TLS* [n=2], and *TARDBP* [n=2], and compare this with protein expression, at the light microscopy level, in cases of sporadic ALS [sALS] [n=6] and familial ALS [fALS] [n=5] without any known mutations. We performed 3,3'-Diaminobenzidine immunohistochemistry [IHC] using a panel of contemporary ALS-associated markers, which included antibodies against TDP-43, FUS/TLS, SOD1, ubiquitin, sequestosome 1 [p62], optineurin, phosphorylated high molecular weight neurofilament [pNFH], peripherin, and a novel antibody against Rho-guanine nucleotide exchange factor [RGNEF]. With the exception of mutant [mt] SOD1-ALS cases, we observed neuronal cytoplasmic inclusions [NCIs] in all variants of ALS when evaluated for TDP-43, FUS/TLS, RGNEF, peripherin, and pNFH. SOD1-positive NCIs were only observed in cases of mtSOD1-ALS. Common to all variants of ALS, regardless of pathophysiology, were p62-positive NCIs. Our data suggests that mtSOD1-ALS is a pathologically unique variant of ALS and prudent IHC-based pathological analysis of AHCs can aid clinicians in identifying novel families affected by Type 1 fALS.

Introduction

Amyotrophic lateral sclerosis [ALS] is a fatal neurodegenerative disease characterized by the progressive loss of upper and lower motor neurons [MNs] in the brain and spinal cord (Logroscino *et al.*, 2008). Death from ALS usually comes 3 to 5 years after symptom onset due to respiratory failure. Although the cause(s) of ALS have not yet been determined, several mechanisms and/or cellular processes have been identified that likely play a role in disease pathogenesis. These include toxic gain-of-function mutations in the gene encoding superoxide dismutase 1 [SOD1] (Rosen *et al.*, 1993), aberrant neuronal cytoplasmic inclusions [NCIs] of TAR DNA binding protein of 43 kDa [TDP-43] (Arai *et al.*, 2006; Neumann *et al.*, 2006), the subsequent identification of mutations in the *TARDBP* gene in individuals with ALS (Kabashi *et al.*, 2008; Kuhnlein *et al.*, 2008; Rutherford *et al.*, 2008; Sreedharan *et al.*, 2008; Van Deerlin *et al.*, 2008; Corrado *et al.*, 2009; Daoud *et al.*, 2009; Del Bo *et al.*, 2009), abnormal inflammatory processes (Moisse and Strong, 2006), and the formation of insoluble NCIs (Strong *et al.*, 2005). It is likely that the pathogenesis of ALS is multi-factorial and all of these processes play a role.

Genetics, Function, and Histopathology of ALS-Associated Markers

RNA Binding Proteins

SOD1

Approximately 10% of ALS cases occur in a family with a known history of the disease and are thus identified as familial ALS [fALS]. Twenty percent of fALS cases have been found to be associated with mutations in the *SOD1* gene (Rosen *et al.*, 1993) and are thought to cause ALS through a toxic gain-of-function mechanism as opposed to a loss of function of the enzyme (Shaw, 2005). To date, there are 153 known mutations in the *SOD1* gene that have been associated with ALS (Felbecker *et al.*, 2010). The majority of these mutations are clustered around the edges of the protein's β barrel, within the dimer interface, or near one of the zinc-binding superoxide dismutase pockets (Beckman *et al.*, 2001). Many of these mutations are known to affect interactions that are critical to the structural integrity of SOD1's β barrel (Deng *et al.*, 1993). The gain-of-function that causes SOD1 toxicity is achieved when Zn^{2+} is lost from SOD1, which is sufficient to cause MN cell death (Estevez *et al.*, 1999). In addition, mutant SOD1 [mtSOD1] has been shown to bind with the 3' untranslated region of low molecular weight neurofilament [NFL] mRNA and act as a destabilizer (Ge *et al.*, 2005). Similarly, vascular endothelial growth factor mRNA is significantly down-regulated in the spinal cords of $SOD1^{G93A}$ mice (Lu *et al.*, 2007). In the case of the $SOD1^{G93A}$ mouse, mRNA destabilization leads to an increase in the rate of neurodegeneration leading to ALS. Due to the fact that SOD1 exhibits increased RNA binding properties in its mutant form, we have classified it as an RNA binding protein for the purposes of this study.

Past studies characterizing the intraneuronal expression of SOD1 have shown abnormal aggregation within cases of ALS 1 (Stieber *et al.*, 2000). We are unaware of any studies that have reported SOD1-positive NCIs in any other variant of ALS.

TDP-43

TDP-43 is a dual DNA/RNA binding protein that functions in alternative splicing of mRNA as well as other processes related to protein expression (Buratti and Baralle, 2008). In healthy MNs, TDP-43 expression is typically nuclear with minimal cytoplasmic presence in RNA granules. TDP-43 was discovered to be abnormally expressed in ALS when ubiquitinated TDP-43-positive NCIs were observed (Arai *et al.*, 2006; Neumann *et al.*, 2006). Since that time, much work has centered on identifying mutations in the *TARDBP* gene, and since 2008, many *TARDBP* mutations have been found (Kabashi *et al.*, 2008; Kuhnlein *et al.*, 2008; Rutherford *et al.*, 2008; Sreedharan *et al.*, 2008; Van Deerlin *et al.*, 2008; Corrado *et al.*, 2009; Daoud *et al.*, 2009; Del Bo *et al.*, 2009). There are currently 30 distinct mutations that have been associated with ALS in 22 unrelated families and in 29 sporadic cases of ALS (Lagier-Tourenne and Cleveland, 2009; Perry *et al.*, 2010). The majority of *TARDBP* mutations are missense mutations that can be mapped to the glycine-rich, C-terminus of the protein and affect protein-protein interactions.

FUS/TLS

FUS/TLS is also a dual DNA/RNA binding protein that is typically expressed in the nucleus in healthy MNs and functions in transcription, RNA splicing, and transport (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009). The

FUS/TLS protein is a 526 amino acid protein encoded for by 15 exons. It is characterized by distinct motifs including: an N-terminal serine-tyrosine-glutamine-glycine-rich [QGSY] region, a glycine-rich region, an RNA-recognition motif, multiple RGG repeats involved in RNA binding, a C-terminus zinc finger, and a highly conserved extreme C-terminal region (Lagier-Tourenne and Cleveland, 2009). In sALS and non-mtSOD1 fALS, FUS/TLS expression has been identified to be in the form of insoluble NCIs (Deng *et al.*, 2010). Recently, mutant forms of FUS/TLS have been described that are associated with fALS (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009). The majority of ALS-associated mutations are missense mutations and seem to cluster in the extreme C-terminus (aa514-525) (Perry *et al.*, 2010). The only 2 identified mutations in *FUS/TLS* that are not missense mutations are in the C-terminus and involve the insertion or deletion of 2 glycines (Lagier-Tourenne and Cleveland, 2009).

RGNEF

RGNEF is a novel protein that is the human homologue of the murine p190RhoGEF. It has been shown to regulate the stability of human NFL mRNA. Interestingly, this is a direct *in vitro* interaction that appears to occur in lysates from ALS tissues, but not in controls (Volkening *et al.*, 2010). Recent data suggests an abnormal formation of NCIs that can be ubiquitin and p62-positive in ALS, but not in neuropathologically normal controls [see Chapter 3]. This suggests a role for RGNEF in the pathogenesis of ALS.

Other ALS-Associated Proteins

Ubiquitin

Ubiquitin is a common marker of protein degradation through the ubiquitin-proteasome degradation pathway. Schiffer, D. *et al.* observed many clinically aggressive cases of ALS with a high percentage of ubiquitin-positive NCIs (Schiffer *et al.*, 1991). Further, ubiquitin-positive NCIs are found across many variants of ALS, suggesting that protein ubiquitination is an early-stage event in the pathogenesis of ALS.

Sequestosome 1 [p62]

Sequestosome 1 [p62] is a protein that is involved in intracellular signaling, and is thus able to interact with numerous signaling molecules. It has been reported to bind in a non-covalent manner to ubiquitin, which suggests an important role for it in the ubiquitin-proteasome degradation pathway (Vadlamudi *et al.*, 1996; Shin, 1998). Further, it has been identified in ubiquitin-positive NCIs in cases of ALS with cognitive impairment [ALSci] of a broad clinicopathological spectrum (Nakano *et al.*, 2004). Interestingly, recent work has identified 10 unique *SQSTM1* mutations in 15 individuals with ALS (Personal Communication: Fecto *et al.*, 2011). We have chosen to evaluate it in this study as an additional marker of proteasomal protein degradation.

Optineurin

Recently, optineurin, a protein traditionally associated with primary open-angle glaucoma (Rezaie *et al.*, 2002) was discovered to be associated with ALS in 4 distinct Japanese families (Maruyama *et al.*, 2010). Three unique mutations were found, all of which cause the protein to lose its ability to inhibit the activation of nuclear factor kappa B (NF-κB). In addition to the pathophysiological effects caused by the mutations, optineurin-positive NCIs were found in these cases. Immunohistochemical staining reported by Maruyama *et al.* revealed optineurin-positive hyaline inclusions and NCIs that were also immunoreactive to antibodies against TDP-43 and ubiquitin in cases of sALS (Maruyama *et al.*, 2010). More recent studies have suggested that optineurin is not likely a pathogenic commonality amongst all variants of ALS (Hortobagyi *et al.*, 2011).

High Molecular Weight Neurofilament [NFH]

High-molecular weight neurofilament [NFH] is 1 of 3 neurofilament [NF] subunits comprising the NF family of intermediate filaments. In the NF complex, the C-terminal tail of NFH is highly phosphorylated, promoting polymerization between NF subunits (Strong, 1999). The antibody used to detect NFH is specific to this phosphorylated form of NFH [pNFH]. NFs are major contributors to the neuronal cytoskeleton. Intraneuronal aggregates of NF proteins have been observed in ALS (Strong *et al.*, 2005). In addition, neuroaxonal spheroids, the largest of which have been suggested to be pathological in ALS (Carpenter, 1968), are pNFH-immunoreactive.

Peripherin

Peripherin, a type III intermediate filament protein, has been shown to abnormally aggregate in the cytosol of anterior horn cells [AHCs] (Migheli *et al.*, 1993). Additionally, inclusions may form within the axon, leading to the presence of neuroaxonal spheroids. Traditionally, these intraneuronal aggregates have been seen as a pathological hallmark in ALS, which is why the expression of peripherin was analyzed in this study.

Clinical Link and Experimental Aims

The clinical diagnosis of ALS is based on a set of international criteria published in 1994 by the World Federation of Neurology (Brooks, 1994). Even a case that has been diagnosed as clinically "definite" for ALS can have a small incidence of misdiagnosis and thus requires pathological examination for absolute certainty (Ross *et al.*, 1998). The brainstem and spinal motor nuclei are examined for loss of lower MNs, and the corticospinal tracts and motor cortex are examined for loss of upper MNs. Some common pathological features that help to confirm the diagnosis are TDP-43-positive and ubiquitin-positive NCIs. **To date, no studies have been completed which discuss the linkage of unique intraneuronal ALS-associated protein expression patterns to**

specific variants of ALS. This study aimed to, first, determine if a link exists between intraneuronal pathology of ALS-associated proteins in AHCs and the variant of ALS, and, second, to determine the most robust and reliable markers of ALS pathogenesis that may be of use to neuropathologists in the pathological diagnosis of specific disease variants while providing genetic information without the need for gene sequencing.

Materials and Experimental Procedures

Genotyping and Case Selection

We performed genotypic analyses on 137 cases of ALS from tissue archives at the London Health Sciences Center. The sequencing was performed at the Mayo Clinic College of Medicine in Jacksonville, FL. Sequencing revealed several cases harbouring genetic mutations known to associate with ALS in cases with previously unidentified mutations. For specific genetic findings, please refer to **Table 2.1a**.

Following this, we selected a panel of 20 ALS cases and 4 neurologically normal controls [**Table 2.1b**]. Of the 20 cases of ALS in our panel, 5 variants of ALS were represented: 5 cases of mtSOD1-ALS [A4T (n=2), I113T (n=2), D76Y], 2 cases mtTDP-43-ALS [C774G, *81-84 delCATA], 2 cases of mtFUS/TLS-ALS [R521C, 3'UTR*41G>A], 5 cases of fALS without any identified mutations, and 6 cases of sALS without any identified mutations [**Table 2.1a**].

Type	Variant	Specific Mutation
Healthy Control	N/A	No Mutation (n=4)
Familial ALS	mtSOD1-ALS	SOD1^{A4T} (n=2)
	mtTDP-43-ALS	TDP-43^{C774G} (n=1) TDP-43$^{81\text{-}84delCATA}$ (n=1)
	mtFUS/TLS-ALS	FUS/TLS$^{3'UTR*41\ G>A}$ (n=1) FUS/TLS^{R521C} (n=1)
	fALS – no known mutations	Unknown (n=5)
Sporadic ALS	mtSOD1-ALS	SOD1^{I113T} (n=2) SOD1^{D76Y} (n=1)
	sALS – no known mutations	Unknown (n=6)

Table 2.1a: Genetic details of the cases used in this study

Control	Age	Sex	Cause of Death
1	67	M	Respiratory Failure (COPD)
2	71	M	Cardiac Arrest
3	61	M	Cardiac Arrest
4	61	M	Renal Failure

Table 2.1b: Information for control cases used in this study

Immunohistochemistry was then performed in triplicate for each case using each antibody. Staining was performed on 3 different cases of neuropathologically normal control tissue in order to determine a normal protein expression pattern.

Antibodies

A goat polyclonal antibody against RGNEF was produced by 21st Century Biochemicals [260 Cedar Hill Street, Marlboro, MA, USA 01752] using the immunogenic peptide sequence 1567-INEALVQMSFNTFNKLNPSV-1586. All other antibodies used in this study were obtained commercially and immunoreactivity was optimized with antigen retrieval techniques and titres **[Table 2.2]**.

Immunohistochemistry

Six-micrometer sections of formalin-fixed, paraffin-embedded spinal cord tissue were warmed at 63°C for 30 minutes and rehydrated through an ethanol series to 1xPBS with 5-minute washes [xylenes, 1:1 xylenes:ethanol, 100% ethanol, 95% ethanol, 75% ethanol, 50% ethanol, 1xPBS]. Following rehydration, antigen retrieval was performed as specified in **Table 2.2** using a 2100 Retriever chamber [Pick Cell Laboratories, Lelystad, Netherlands]. Sections were washed 3 times for 5 minutes each using 1xPBS and then treated for 5 minutes at room temperature with 3% H_2O_2 to block endogenous peroxidase activity. After 3 additional 5-minute washes with 1xPBS, the sections were blocked for 60 minutes in 5% bovine serum albumin [Roche

Antibody	Titre	Source	Secondary	Antigen Retrieval
TDP-43 Rabbit Polyclonal	1:500	Proteintech [Cat # 10782-2-AP]	Biotinylated Rabbit IgG [Vector, Cat # PK-6101]	Tris-EDTA [0.05% Tween 20], pH 7.4
FUS/TLS Rabbit Polyclonal	1:90	Proteintech [Cat # 11570-1-AP]	Biotinylated Rabbit IgG [Vector, Cat # PK-6101]	Sodium Citrate [10mM Sodium Citrate, 0.05% Tween 20], pH 6
RGNEF Goat Polyclonal	1:500	21st Century Biochemicals [Antigen: Ac-INEALVQMSFNT-FNKLNPSVC-N]	Biotinylated Goat IgG [Vector, Cat # PK-6105]	Sodium Citrate [10mM Sodium Citrate, 0.05% Tween 20], pH 6
SOD1 Rabbit Polyclonal	1:500	Enzo Lifesciences [Cat # ADI-SOD-100]	Biotinylated Rabbit IgG [Vector, Cat # PK-6101]	None
Ubiquitin Mouse Monoclonal	1:500	Millipore [Cat # MAB-1510]	Biotinylated Mouse IgG [Vector, Cat # PK-6102]	None
p62 Rabbit Polyclonal	1:1000	Enzo Lifesciences [Cat # PW 9860]	Biotinylated Rabbit IgG [Vector, Cat # PK-6101]	Sodium Citrate [10mM Sodium Citrate, 0.05% Tween 20], pH 6
Optineurin Rabbit Polyclonal	1:120	Abcam [Cat # ab79110], Abcam [Cat # ab23666]	Biotinylated Rabbit IgG [Vector, Cat # PK-6101]	None
SMI 31 [pNFH] Mouse Monoclonal	1:30,000	Sternberger Monoclonals [Cat # SMI-31R]	Biotinylated Mouse IgG [Vector, Cat # PK-6102]	Tris-EDTA [0.05% Tween 20], pH 7.4
Peripherin Rabbit Polyclonal	1:500	Millipore [Cat # Ab1530]	Biotinylated Rabbit IgG [Vector, Cat # PK-6101]	Tris-EDTA [0.05% Tween 20], pH 7.4

Table 2.2: Details of use for each antibody in this study

Diagnostics, Mannheim, Germany] in water. Following blocking, the sections were incubated with antibodies against TDP-43, FUS/TLS, RGNEF, SOD1, ubiquitin, p62, optineurin, phosphorylated NFH [pNFH] or peripherin for 20 hours at 4°C. The sections were then washed 3 times for 5 minutes each in 1xPBS and then incubated with the appropriate biotinylated secondary antibody for 60 minutes at room temperature [Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA] [**Table 2.2**] at a titre of 1:500. Following 3 additional washes of 5 minutes each in 1xPBS, the tissues were incubated with the avidin/biotin enzyme complex solution [Vectastain kits], washed in 1xPBS 3 times for 5 minutes each and treated with 3,3'-diaminobenzidine [Sigma-Aldrich Canada, Oakville, ON]. Nuclear counterstaining was performed using Modified Harris Formula Haematoxylin [Ricca Chemical Company, Arlington, TX]. Sections were dried, mounted with Cytoseal 60 mounting medium [Richard-Allan Scientific, Kalamazoo, MI], observed using a BX45 light microscope [Olympus, Markham, ON], and photographed with a Q Color 3 camera [Olympus, Markham, ON].

Protein Expression Analysis

Given the nature of our study, we evaluated protein expression qualitatively. Qualitative observations were made for all proteins and involved noting the presence or absence of protein-specific NCIs. Based on the literature, we defined NCIs as skeins and/or aggregates, which are hypothesized to be insoluble and are thus considered to be involved in the pathogenesis of ALS.

Pathological neuroaxonal spheroids were described as greater than 30 µm at their smallest diameter.

Semi-quantitative observations were made for all non-RNA binding proteins. For expression patterns evaluated in a semi-quantitative manner, a score of + denotes the presence of less than 10 pathological inclusions per spinal cord section [both anterior horns], ++ denotes the presence of 10 or more pathological inclusions per spinal cord section, and – denotes the absence of pathological inclusions.

A quantitative analysis was only made on tissues examined for TDP-43 immunoreactivity. All AHCs with evidence of a nucleolus were included in the quantification. Each AHC was analyzed twice: once noting the presence or absence of nuclear staining, and once noting the presence or absence of cytoplasmic staining. The percentage of counted cells that showed a nuclear expression pattern and the percentage of counted cells that presented with a cytoplasmic expression pattern were calculated for each case. The cases were then grouped by variant of ALS, and the mean nuclear and cytoplasmic expression levels were calculated using these figures. Based on past studies that highlighted the importance of the phenomenon, the degree of TDP-43 nucleocytosplasmic redistribution was calculated for each variant of ALS (Moisse *et al.*, 2009).

All tissues across the panel of antibodies were analyzed in a blinded manner. One individual that was observing slides independently for all proteins examined further confirmed the methods used for the original analysis. A clear consensus was reached for every slide that was examined.

Statistical Analysis

A one-way ANOVA was performed with a Tukey-Kramer multiple comparisons post-hoc test in order to determine the significance of the nucleocytoplasmic redistribution of TDP-43 observed between all variants of ALS.

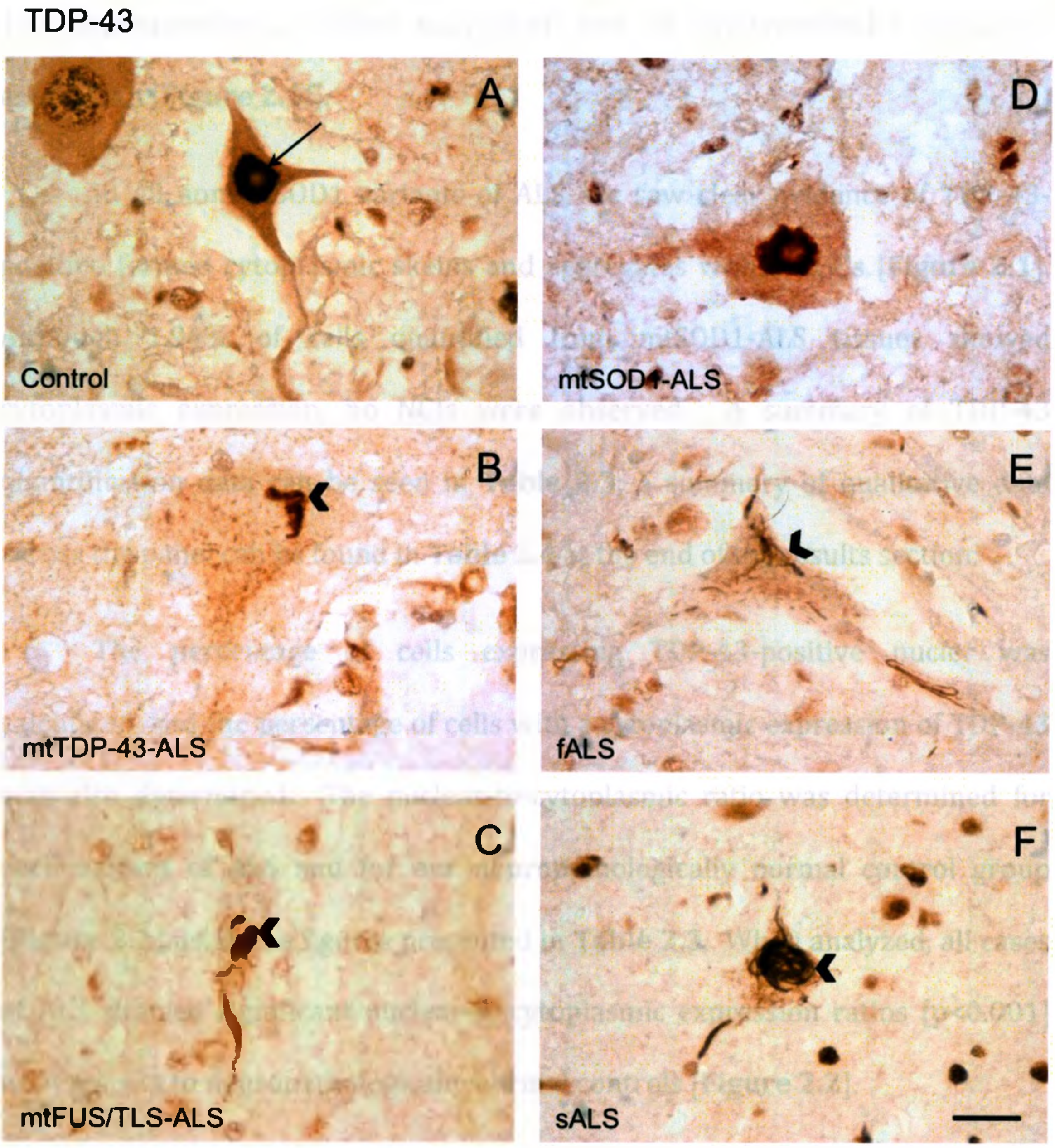
Results

TDP-43

In spinal cord tissues examined for TDP-43 expression, 198 AHCs were included from 3 neuropathologically normal control cases [**Figure 2.1a**]. Of these, 195 had a nuclear expression pattern, and 3 showed cytoplasmic TDP-43 expression, while no formed inclusions were detected. In mtSOD1-ALS tissues, 206 AHCs were counted with 199 showing a nuclear expression pattern and 4 with a cytoplasmic expression pattern [**Figure 2.1d**]. In mtTDP-43-ALS tissues, 112 AHCs were counted, of which 97 cells presented with a nuclear expression pattern, while 12 showed a cytoplasmic expression pattern [**Figure 2.1b**]. In mtFUS/TLS-ALS tissues, 88 AHCs were counted. Of these cells, 78 showed a nuclear expression pattern, while 4 showed evidence of cytoplasmic expression [**Figure 2.1c**]. In spinal cord tissues of fALS cases without known

Figure 2.1: Representative TDP-43 expression across all variants of ALS evaluated in this study.

Staining using an antibody against TDP-43 [Rabbit Polyclonal, Proteintech (Cat # 10782-2-AP), 1:500, 20 hours, 4°C] was done and NCIs were seen in all non-mtSOD1 variants of ALS. Healthy controls presented almost exclusively with intense nuclear staining [A, represented by arrow]. Similar to data from healthy controls were the expression patterns found in AHCs of mtSOD1-ALS cases [D]; no TDP-43-positive NCIs were seen in cases of mtSOD1-ALS throughout our study. NCIs [represented by arrow heads throughout this figure] were commonly seen in all non-mtSOD1 genetic variants of ALS in addition to sALS and fALS cases without known mutations. Non-fibrillary NCIs were typically seen in cases of mtTDP-43-ALS [B], whereas the presence of skein-like NCIs were seen in cases of mtFUS/TLS-ALS [C], fALS without known mutations [E], and sALS without known mutations [F]. Of note is the possibility for an NCI to contain both fibrils and more compacted TDP-43 aggregates, seen in this figure in the case of mtFUS/TLS-ALS [C]. Although only AHCs in which a nucleolus was evident were included in the quantitative analysis, the cells included in this figure provide representative examples of AHC pathology. The scale bar represents 20 μm.


TDP-43
A
Control
D
mtSOD1-ALS
B
mtTDP-43-ALS
E
fALS
C
mtFUS/TLS-ALS
F
sALS

mutations, 348 AHCs were counted. Of these, 293 showed nuclear staining, while 47 showed cytoplasmic staining [**Figure 2.1e**]. In spinal cord tissues from cases of sALS without known mutations, we counted a total of 189 AHCs, 153 demonstrated a nuclear expression and 28 demonstrated cytoplasmic expression [**Figure 2.1f**].

In all non-mtSOD1 variants of ALS, we saw clear evidence of TDP-43-positive formed cytoplasmic skeins and aggregates within AHCs [**Figure 2.1**]. Although 1.94% of cells quantified from mtSOD1-ALS tissues showed cytoplasmic expression, no NCIs were observed. A summary of TDP-43 quantification data can be seen in **Table 2.3**; a summary of qualitative data across the panel can be found in **Table 2.4** at the end of the results section.

The percentage of cells expressing TDP-43-positive nuclei was calculated, and the percentage of cells with a cytoplasmic expression of TDP-43 was also determined. The nuclear-to-cytoplasmic ratio was determined for each variant of ALS and for our neuropathologically normal control group [**Figure 2.2**] using the figures presented in **Table 2.3**. When analyzed, all cases of ALS showed significant nuclear-to-cytoplasmic expression ratios [$p<0.001$] with respect to neuropathologically normal controls [**Figure 2.2**].

FUS/TLS

Only qualitative observations were made for spinal cord tissues examined for FUS/TLS immunoreactivity. Control tissue showed FUS/TLS-positive nuclear staining [**Figure 2.3a**], which was also the most common type

Type	AHCs Counted #	Nuclear Expression #	Nuclear Mean*	Cytoplasmic Expression #	Cytoplasmic Mean*	Nuclear Mean: Cytoplasmic Mean	NCIs?
Control	198	195	0.977	3	0.01	97.67	No
mtSOD1-ALS	206	199	0.96	4	0.025	38.40	No
mtFUS/TLS-ALS	88	78	0.933	4	0.053	17.50	Yes
mtTDP-43-ALS	112	97	0.878	12	0.093	9.49	Yes
fALS, no known mutations	348	293	0.834	47	0.143	5.85	Yes
sALS, no known mutations	189	153	0.828	28	0.127	6.54	Yes

Table 2.3: Summary of TDP-43 quantification data

* Means were calculated by averaging the percentage of cells with nuclear or cytoplasmic expression from each case within control and ALS variants.

Figure 2.2: A TDP-43 nucleocytoplasmic redistribution occurs in ALS tissues, but not in healthy controls.

Cells with clear evidence of a nucleolus [A, B, arrows] were scored. Each cell received a score for nuclear expression and cytoplasmic expression. A is representative of a motor neuron with nuclear expression, while B is representative of a motor neuron with cytoplasmic expression. When the ratio of the mean of cells expressing nuclear TDP-43 staining to the mean of cells expressing cytoplasmic TDP-43 staining is calculated, there is evidence of a statistically significant [$P<0.001$] intracellular nucleocytoplasmic redistribution occurring in every variant of ALS examined [C]. Means were calculated by averaging the percentage of cells with either a nuclear or cytoplasmic expression from each case across each variant of ALS and controls. When further analyzing the data, the ratio calculated from mtSOD1-ALS cases was significantly higher than that of cases of mtTDP-43-ALS [$P<0.01$], and fALS and sALS without any known mutations [$P<0.001$]. Interestingly, the difference in the redistribution ratios calculated for cases of mtSOD1-ALS and mtFUS/TLS-ALS were not found to be statistically significant; however, there does appear to be a trend approaching significance. Statistical analysis was done using a one-way ANOVA with a Tukey-Kramer Multiple Comparisons post-hoc test.

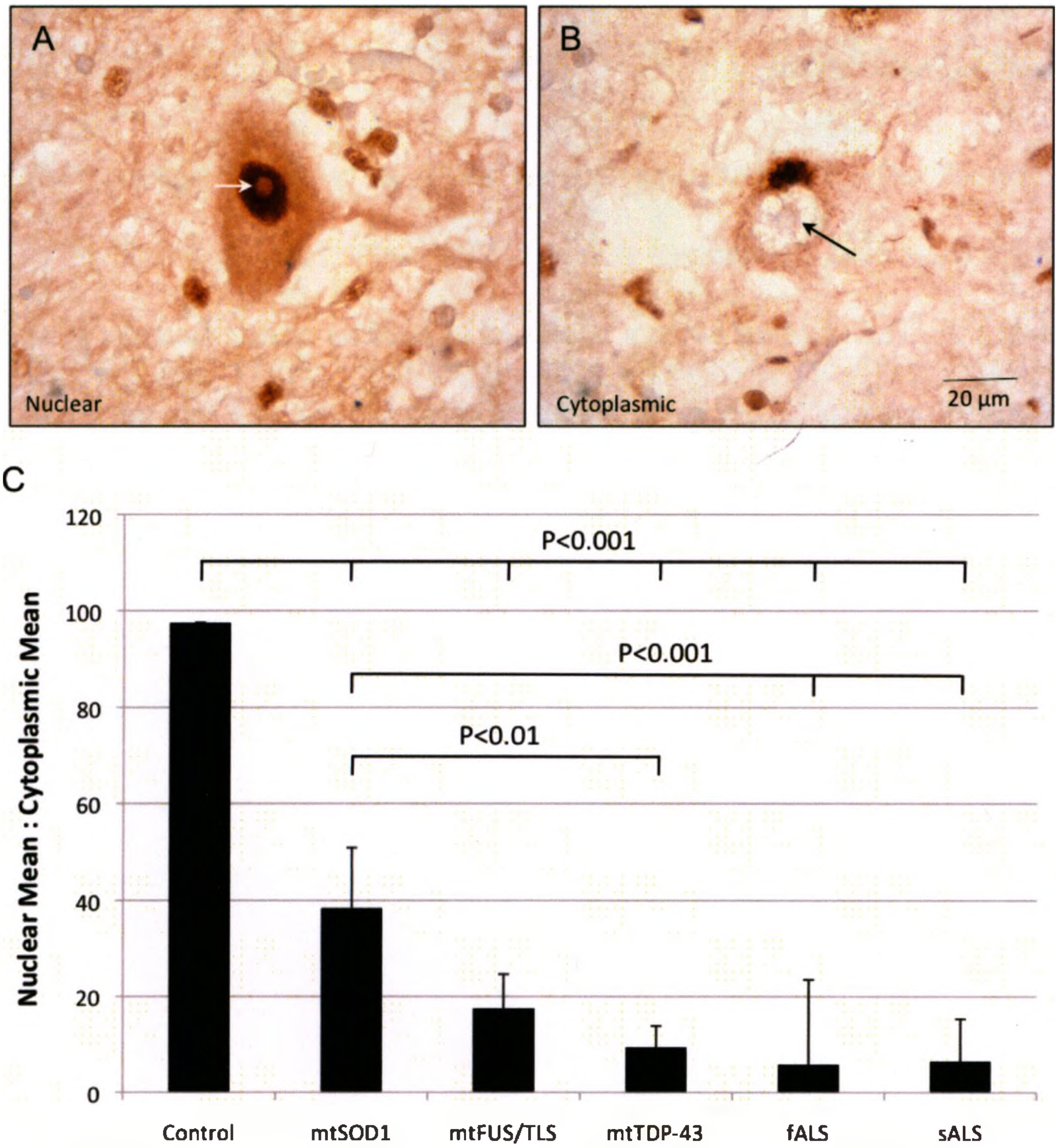
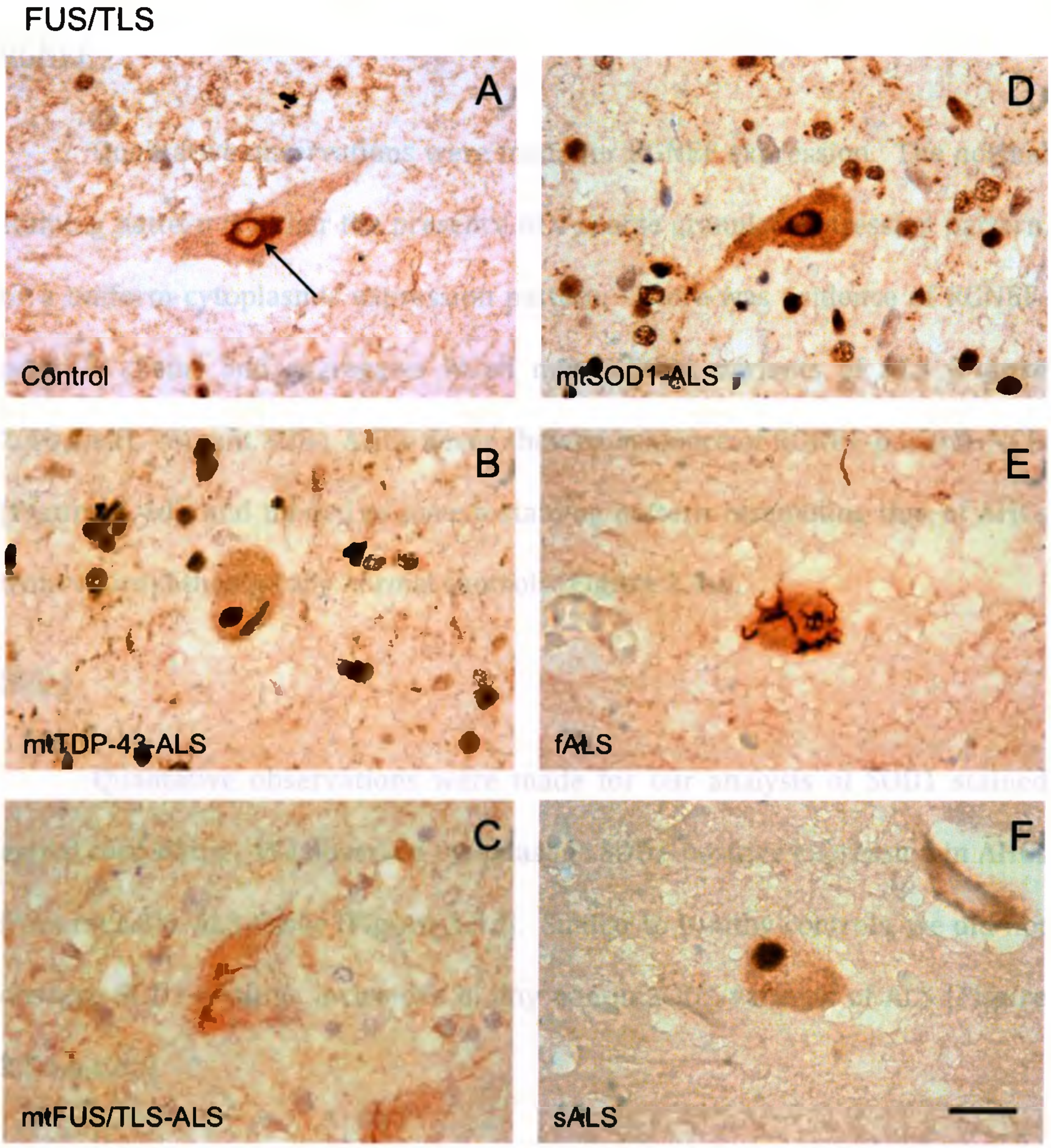

A
Nuclear
B
Cytoplasmic
20 µm
C
Nuclear Mean : Cytoplasmic Mean
120
100
80
60
40
20
0
P<0.001
P<0.001
P<0.01
Control
mtSOD1
mtFUS/TLS
mtTDP-43
fALS
sALS

Figure 2.3: Representative FUS/TLS expression across all variants of ALS evaluated in this study.

Immunohistochemistry was performed using an antibody against FUS/TLS [Rabbit Polyclonal, Proteintech (Cat # 11570-1-AP), 1:90, 20 hours, 4°C]. The typical FUS/TLS staining pattern observed in healthy motor neurons is almost exclusively nuclear. This is represented by an arrow and can be seen in A. Similarly, mtSOD1-ALS cases did not appear to have any type of NCIs [D]. NCIs were seen in all non-mtSOD1 variants of ALS. Interesting to note is the typical filamentous expression pattern observed in cases of mtFUS/TLS-ALS [C] as well as in cases of fALS without any known mutations [E]. Also commonly seen amongst cases of mtTDP-43-ALS [B] and cases of sALS without any known mutations [F] are densely packed FUS/TLS-positive NCIs. The scale bar represents 20 μm.


FUS/TLS
A
Control
D
mtSOD1-ALS
B
mtTDP-43-ALS
E
fALS
C
mtFUS/TLS-ALS
F
sALS

of FUS/TLS immunoreactivity observed in mtSOD1 tissue [**Figure 2.3d**]. Evidence of FUS/TLS-positive skeins and aggregates in all non-mtSOD1 variants of ALS was found [**Figure 2.3b,c,e,f**].

RGNEF

Qualitative observations were made for RGNEF expression. The normal staining pattern is either the presence of a coarse granular expression pattern, or a uniform cytoplasmic expression pattern. There was evidence of RGNEF-positive skeins and aggregates in all non-mtSOD1 variants of ALS [**Figure 2.4b,c,e,f**]. Mutant SOD1 AHCs never showed evidence of RGNEF-positive NCIs [**Figure 2.4d**], and tended to have a staining pattern resembling that of AHCs from neuropathologically normal controls [**Figure 2.4a**].

SOD1

Qualitative observations were made for our analysis of SOD1 stained spinal cord tissue. We observed cytoplasmic SOD1-positive expression in AHCs from mtSOD1-ALS tissue [**Figure 2.5d**]. Similar to healthy controls, we did not observe SOD1-positive inclusions in any non-mtSOD1 variants of ALS [**Figure 2.5a,b,c,e,f**].

Ubiquitin

Our data for ubiquitin staining of spinal cord tissue was collected in a semi-quantitative manner. We did not observe any cytoplasmic ubiquitin-positive NCIs in our neuropathologically normal control group or in our

Figure 2.4: Representative RGNEF expression across all variants of ALS evaluated in this study.

An immunohistochemical analysis was performed using a novel antibody against RGNEF [Goat Polyclonal, 21 Century Biochemicals, 1:500, 20 hours, 4°C]. The typical staining pattern observed in AHCs of healthy controls is weakly cytoplasmic [A]. Interestingly, another very commonly observed RGNEF expression pattern within healthy controls and ALS is that of coarse granular polysomes. Polysomes are clusters of ribosomes that synthesize proteins from multiple sites on a transcript [arrows, D] [see Chapter 3]. RGNEF-positive polysomes are commonly seen across all variants of ALS, and was the only type of intraneuronal pathology seen in mtSOD1-ALS cases in this study [D]. Within the AHCs of other types of ALS, however, can be observed RGNEF-positive NCIs. Filamentous NCIs were found in mtFUS/TLS-ALS cases as well as in the sALS and fALS cases without any known mutations [C, E, and F]. Cases of mtTDP-43-ALS did not contain filamentous RGNEF-positive inclusions, but there was evidence of RGNEF sequestration into dense cytoplasmic aggregates [B]. The scale bar represents 20 μm.

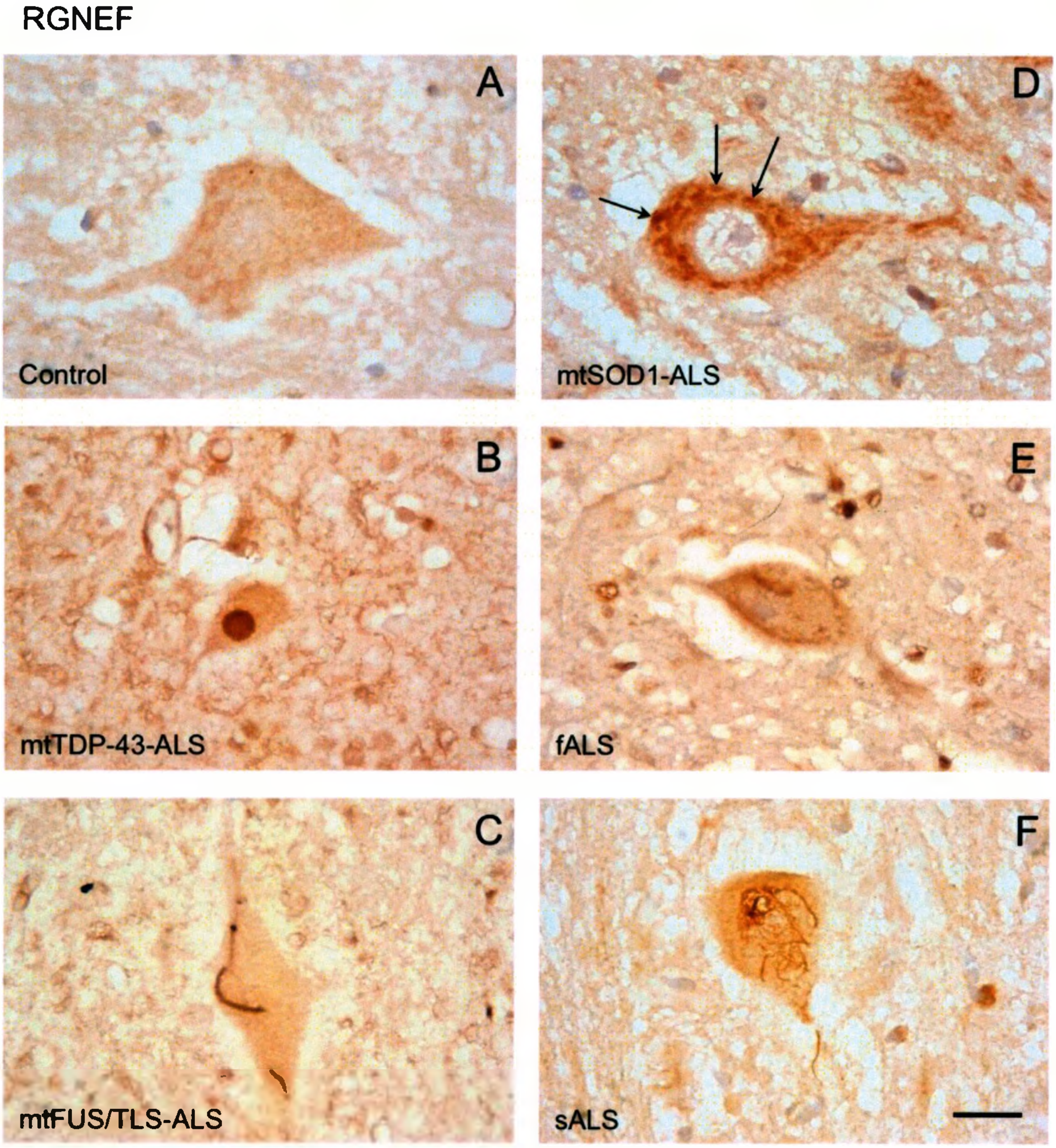
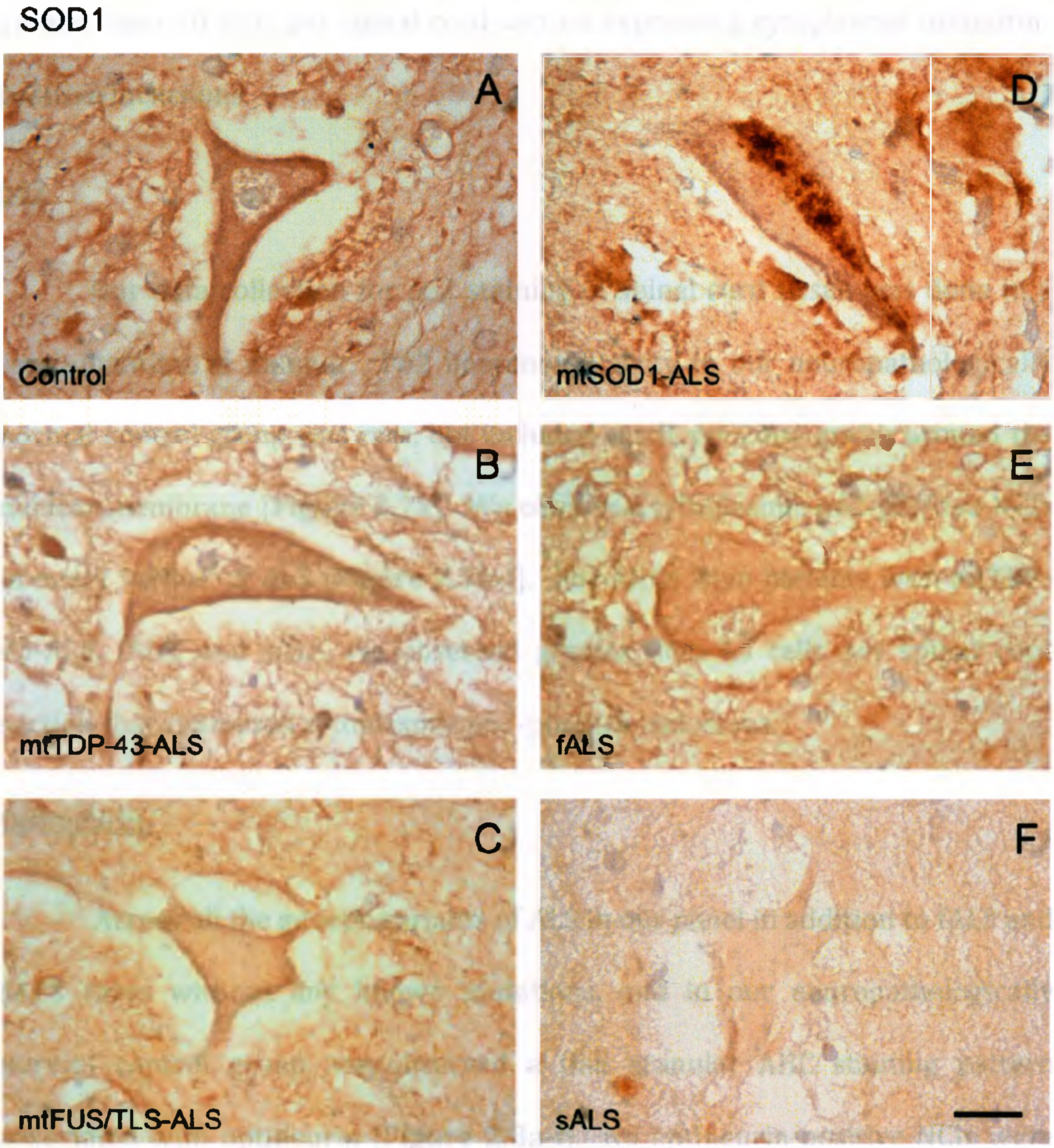

RGNEF
A
Control
D
mtSOD1-ALS
B
mtTDP-43-ALS
E
fALS
C
mtFUS/TLS-ALS
F
sALS

Figure 2.5: Representative SOD1 expression across all variants of ALS evaluated in this study.

An expression pattern analysis was done using an antibody against SOD1 [Rabbit Polyclonal, Enzo Lifesciences (Cat # ADI-SOD-100), 1:500, 20 hours, 4°C], the results of which support previously published studies. SOD1-positive NCIs were never observed in healthy control cases, in mtTDP-43-ALS, in mtFUS/TLS-ALS, or in fALS and sALS cases without any known mutations. SOD1 expression was evident, however, in cases of mtSOD1-ALS. The most commonly observed SOD1-positive pathology within AHCs of mtSOD1-ALS cases is diffuse intraneuronal non-filamentous inclusions, which can be seen in D. The scale bar in this figure represents 20 μm.


SOD1
A
Control
D
mtSOD1-ALS
B
mtTDP-43-ALS
E
fALS
C
mtFUS/TLS-ALS
F
sALS

mtFUS/TLS group [**Figure 2.6a,c**]. We observed cytoplasmic ubiquitin-positive NCIs in the mtTDP-43, mtSOD1, fALS, and sALS variants of ALS [**Figure 2.6b,d,e,f**]. For AHCs in the mtTDP-43 genetic variant of ALS, we observed greater than 10 cells per spinal cord section expressing cytoplasmic ubiquitin-positive inclusions.

p62

Our data collection for p62 staining of spinal cord tissue was done in a semi-quantitative manner. P62 immunoreactivity in our neuropathologically normal control group was rare, but included small, punctate grains around the nuclear membrane [**Figure 2.7a**]. We observed cytoplasmic p62-positive NCIs in every variant of ALS [**Figure 2.7b-f**]. For AHCs from patients with mtTDP-43-ALS, fALS, and sALS, we observed greater than 10 cells per spinal cord section that expressed cytoplasmic p62-positive inclusions.

Optineurin

Across all the genetic variants of ALS in our panel in addition to fALS and sALS cases without any known mutations, and in our neuropathologically normal control group, we observed a fine granular AHC staining pattern associated with optineurin [**Figure 2.8a-f**]. No optineurin-positive NCIs were observed in any MN analyzed.

Figure 2.6: Representative ubiquitin expression across all variants of ALS evaluated in this study.

An immunohistochemical analysis was done using an antibody against ubiquitin [Mouse Monoclonal, Millipore (Cat # MAB-1510), 1:500, 20 hours, 4°C]. Typical staining in healthy control cases was non-existent [A]. No ubiquitin-positive NCIs were observed in motor neurons of cases of mtFUS/TLS-ALS [C], which may be due to a case selection bias, as these have been reported in other studies (Deng *et al.*, 2010). Ubiquitin-positive NCIs were observed in mtTDP-43-ALS cases [B], mtSOD1-ALS cases [D], fALS cases without known mutations [E] and sALS cases without known mutations [F]. Like the NCIs observed using antibodies against other proteins, both filamentous [arrows] and densely aggregated [arrow heads] NCIs were observed throughout our panel. The scale bar represents 20 μm.

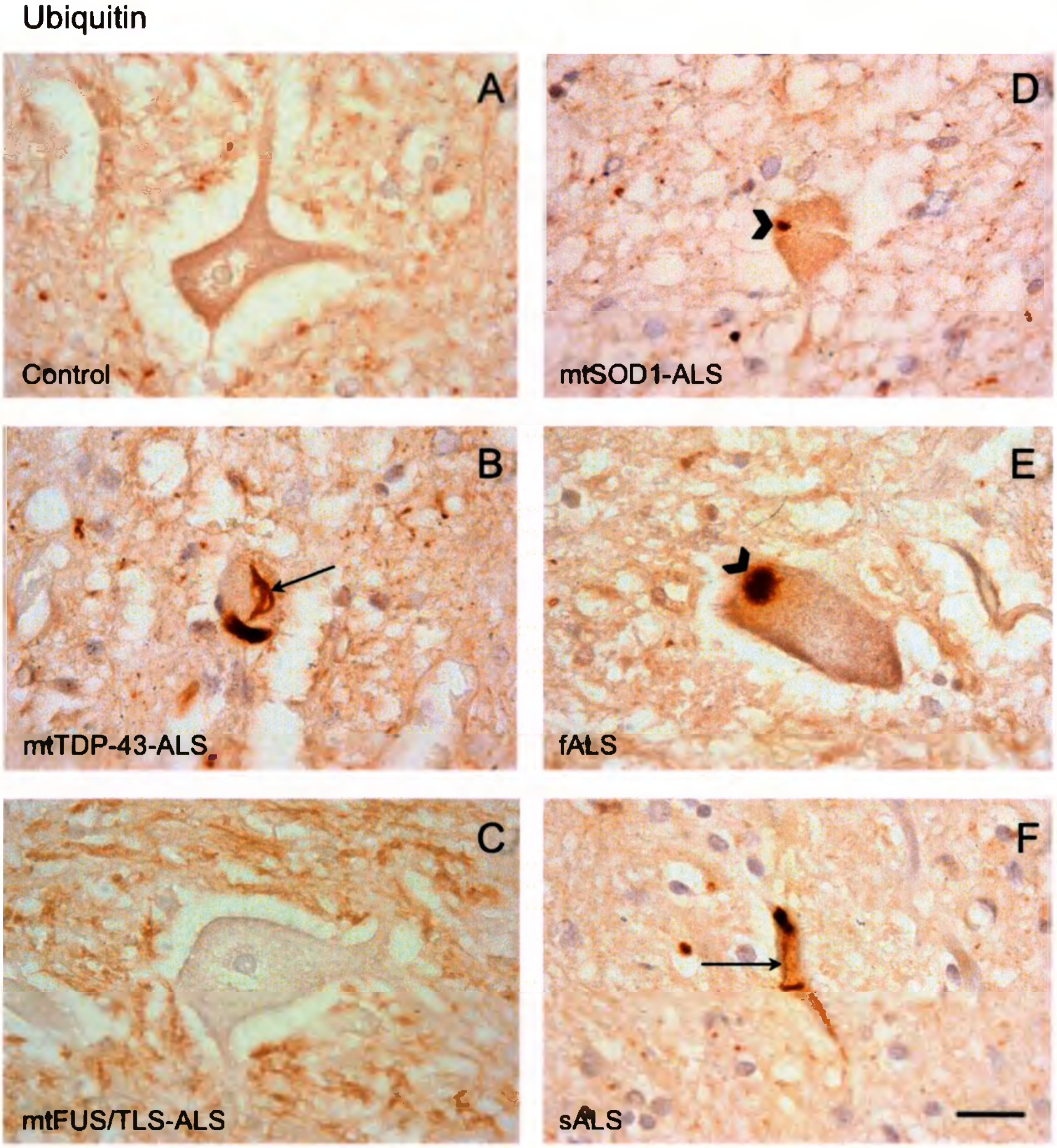

Ubiquitin
A
Control
D
mtSOD1-ALS
B
mtTDP-43-ALS
E
fALS
C
mtFUS/TLS-ALS
F
sALS

Figure 2.7: Representative p62 expression across all variants of ALS evaluated in this study.

Immunohistochemistry was performed using an antibody against p62 [Rabbit Polyclonal, Enzo Lifesciences (Cat # PW9860), 1:1000, 20 hours, 4°C] and NCIs were commonly observed in all genetic variants of ALS analyzed, as well as in cases of fALS and sALS without any known mutations. As observed in NCIs using antibodies against other proteins, NCIs can take the form of both/either filamentous [arrow] and/or densely aggregated inclusions [arrow head]. In neuropathologically normal control tissues, p62 immunoreactivity was rarely observed, but if it was present, it was expressed faintly in the nucleus [A]. The scale bar represents 20 μm.

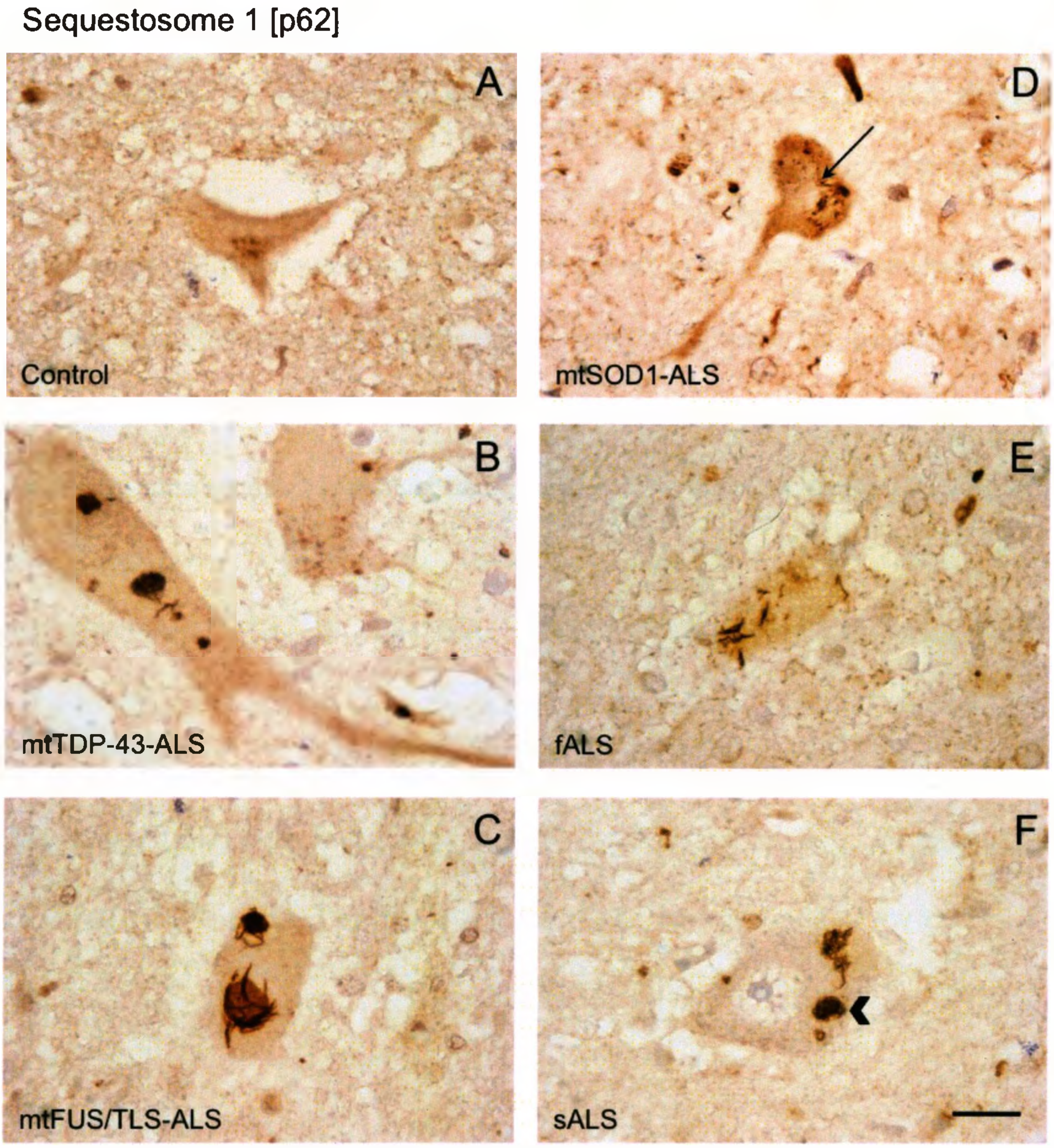
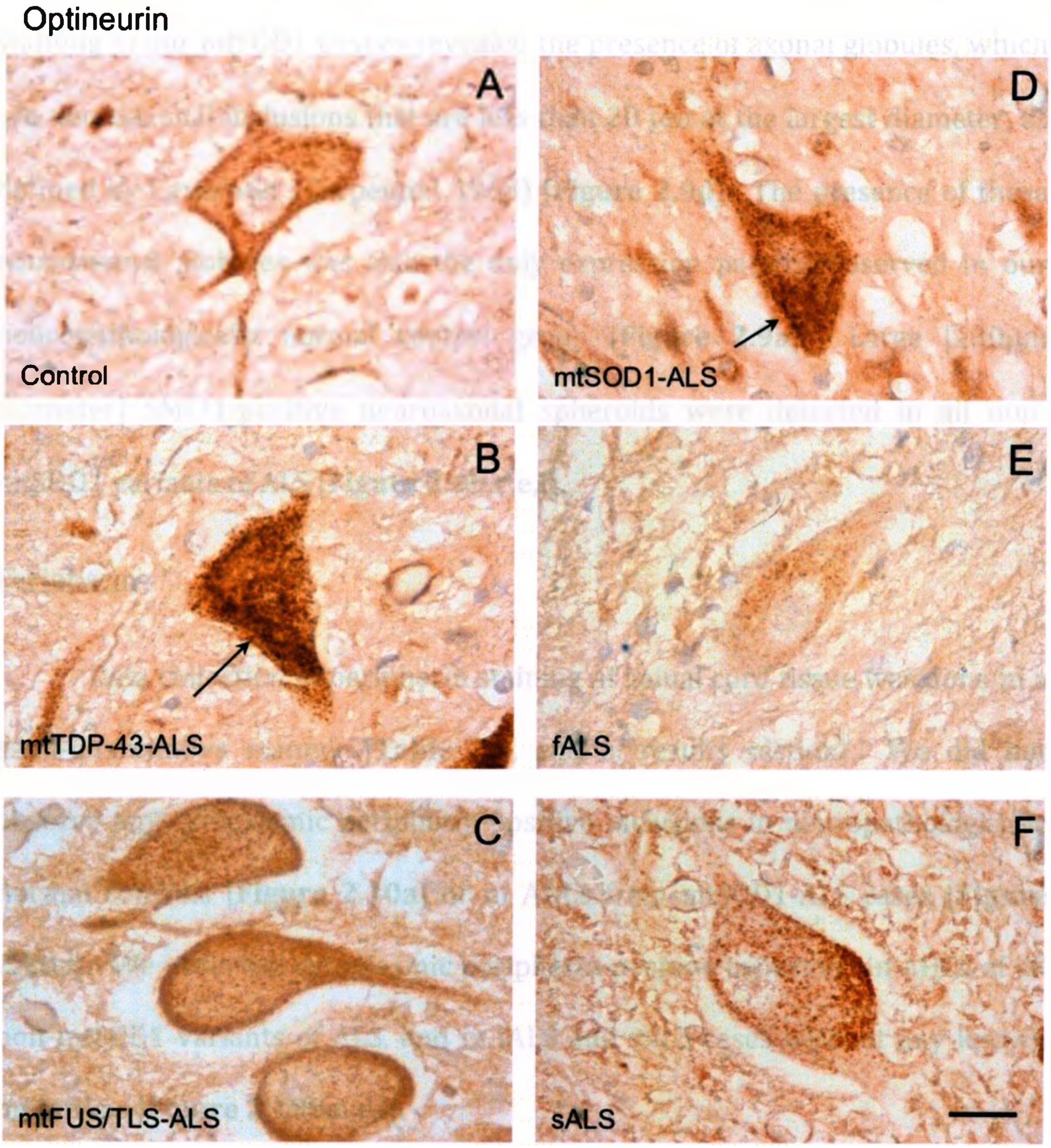

Sequestosome 1 [p62]
A
Control
D
mtSOD1-ALS
B
mtTDP-43-ALS
E
fALS
C
mtFUS/TLS-ALS
F
sALS

Figure 2.8: Representative optineurin expression across all variants of ALS evaluated in this study.

An expression analysis was performed using immunohistochemical methods with 2 antibodies against optineurin [Rabbit Polyclonal, Abcam (Cat # ab79110 & ab23666), 1:150, 20 hours, 4°C]. An analysis of the expression patterns revealed an identical fine granular staining pattern across all genetic variants of ALS, both fALS and sALS without any known mutations, and neuropathologically normal controls. Although the fine granules could occasionally be more densely packed [B, D, arrows], the pattern was observed to be identical in every case. Of note is the observation that the fine granules could often be seen concentrated in a perinuclear and/or a pericellular fashion [A]. This was a phenomenon observed in AHCs from both healthy control and ALS cases. The scale bar represents 20 μm.


Optineurin
A
Control
D
mtSOD1-ALS
B
mtTDP-43-ALS
E
fALS
C
mtFUS/TLS-ALS
F
sALS

pNFH [SMI31]

Our data analysis of pNFH [SMI31] staining of spinal cord tissue was done in a semi-quantitative manner [**Table 2.4**, end of results section]. SMI31 staining in our mtSOD1 tissues revealed the presence of axonal globules, which are neuroaxonal inclusions that are less than 20 μm at the largest diameter, as defined by Carpenter (Carpenter, 1968) [**Figure 2.9d**]. The presence of these neuroaxonal globules was also the only expression pattern observed in our neuropathologically normal control group [**Figure 2.9a**]. Large [≥30μm diameter] SMI31-positive neuroaxonal spheroids were detected in all non-mtSOD1 variants of ALS [**Figure 2.9b,c,e,f**].

Peripherin

Data collection of peripherin staining of spinal cord tissue was done in a semi-quantitative manner [**Table 2.4**, end of results section]. We did not observe any cytoplasmic peripherin-positive inclusions in neuropathologically normal controls [**Figure 2.10a**] or in AHCs from mtSOD1-ALS cases [**Figure 2.10d**]. We observed cytoplasmic peripherin-positive inclusions in AHCs of all non-mtSOD1 variants of ALS, and in fALS and sALS cases without any known mutations [**Figure 2.10b,c,e,f**].

Mutant SOD1 Tissues

As mentioned above, expression patterns for most proteins across our panel were found to be unique in mtSOD1 variants of ALS when compared with

Figure 2.9: Representative pNFH expression across all variants of ALS evaluated in this study.

An immunohistochemical analysis was performed using an antibody against phosphorylated high molecular weight neurofilament [Mouse Monoclonal, Sternberger Monoclonals (Cat # SMI-31R), 1:30,000, 20 hours, 4°C]. A classical pathology of ALS, consistent with the definition of Carpenter (Carpenter, 1968), is the presence of neuroaxonal spheroids that have been defined as axonal inclusions greater than or equal to 30 μm. Large neuroaxonal spheroids were seen in the non-mtSOD1 genetic variants of ALS [B, C], as well as in fALS and sALS cases that have no known mutations [E, F]. Also defined by Carpenter are neuroaxonal globules, which are axonal inclusions smaller than 20 μm. These were commonly observed in healthy control cases [A] as well as in cases of mtSOD1-ALS [D]. The scale bar represents 20 μm.

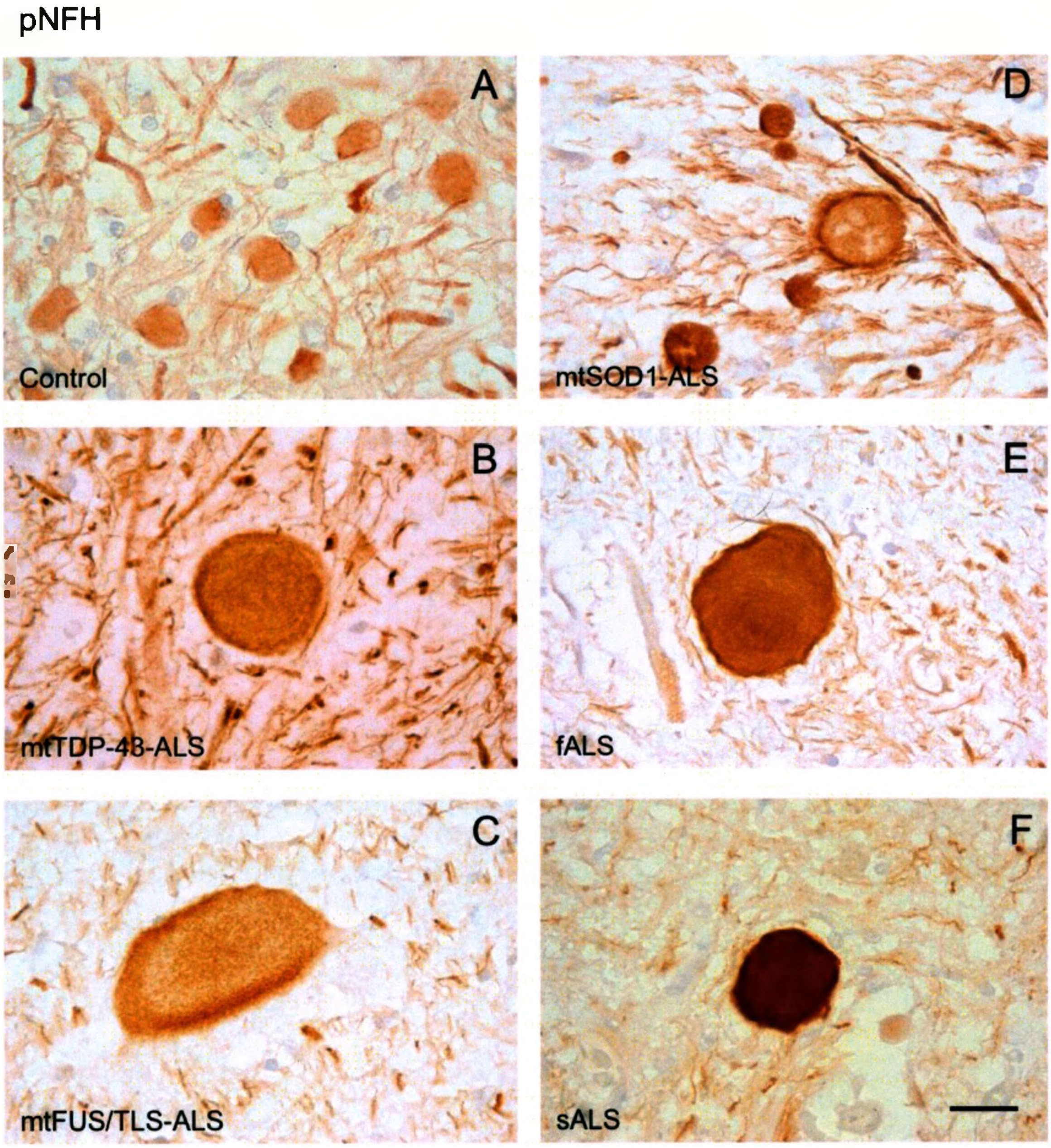
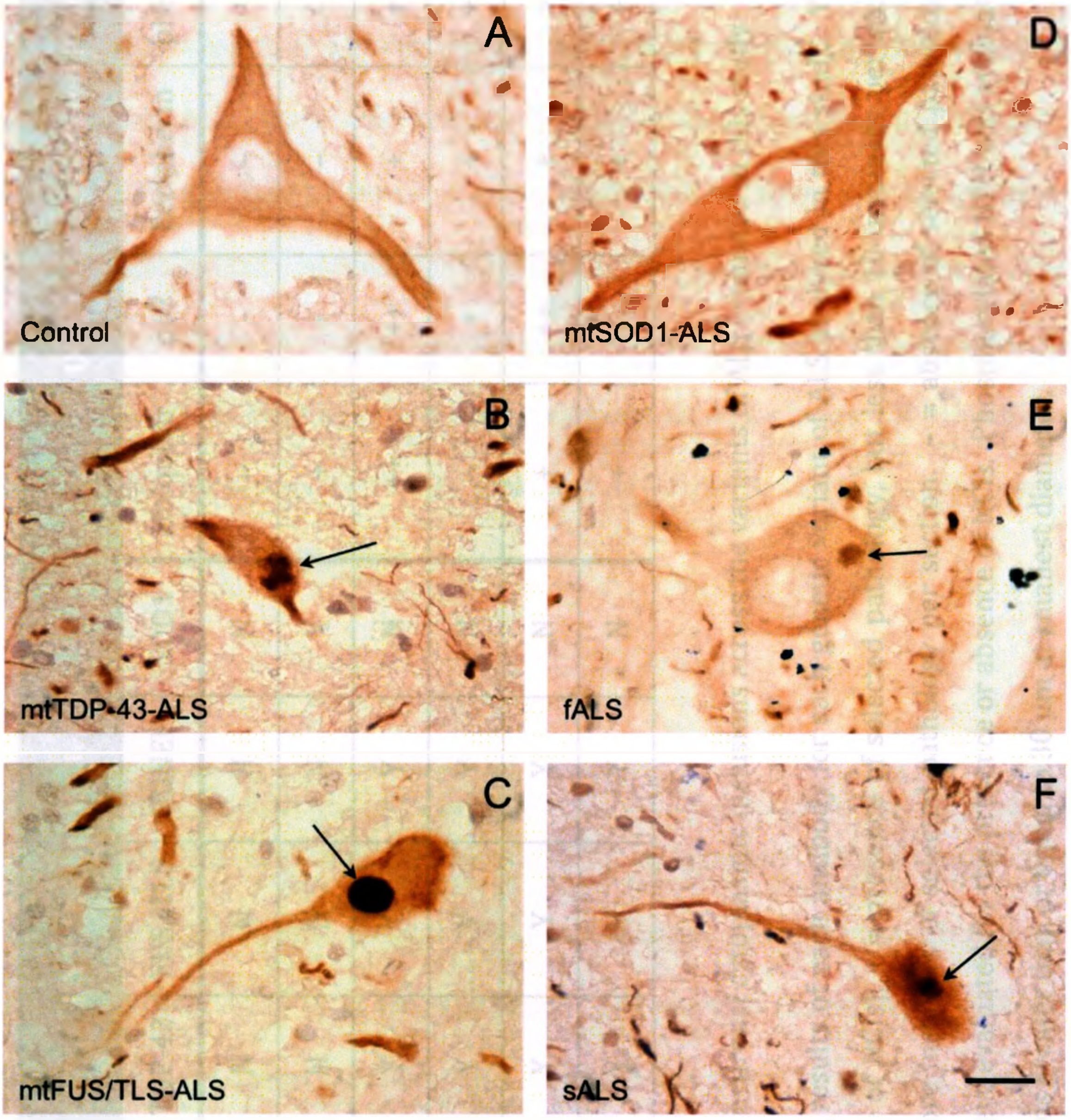

pNFH
A
Control
D
mtSOD1-ALS
B
mtTDP-43-ALS
E
fALS
C
mtFUS/TLS-ALS
F
sALS

Figure 2.10: Representative peripherin expression across all variants of ALS evaluated in this study.

Immunohistochemistry using an antibody against peripherin [Rabbit Polyclonal, Millipore (Cat # Ab1530), 1:500, 20 hours, 4°C] was performed and an analysis revealed the presence of NCIs in the non-mtSOD1 genetic variants of ALS [B, C] as well as in cases of fALS and sALS without any known mutations [E, F]. Unlike NCIs observed using antibodies against other proteins, peripherin-positive NCIs were only observed to be densely aggregated, and never filamentous. These dense cytoplasmic aggregates are highlighted throughout this figure with arrows. No peripherin-positive NCIs were evident in mtSOD1-ALS cases [D] or in neuropathologically normal controls [A]. The scale bar represents 20 μm.

Peripherin


A
Control
D
mtSOD1-ALS
B
mtTDP-43-ALS
E
fALS
C
mtFUS/TLS-ALS
F
sALS

	RNA Binding Proteins				Other ALS-Associated Proteins				
Variant	TDP-43	FUS/TLS	RGNEF	SOD1	Ubiquitin	p62	Peripherin	pNFH	Optineurin
Control	N	N	N	N	–	–	–	–	–
mtSOD1-ALS	N	N	N	Y	+	+	–	–	–
mtTDP-43-ALS	Y	Y	Y	N	++	++	+	+	–
mtFUS/TLS-ALS	Y	Y	Y	N	–	+	+	+	–
fALS, no known mutations	Y	Y	Y	N	+	++	+	+	–
sALS, no known mutations	Y	Y	Y	N	+	++	+	+	–

Table 2.4: Summary of neuronal cytoplasmic inclusions across the variants of ALS evaluated in this study.

A summary of staining results of each antibody for each genetic variant of ALS analyzed in this study. Y = yes [presence of NCIs], N = no [absence of NCIs], + = presence of selected pathology, but less than 10 instances per section [both anterior horns]. ++ = 10 or more instances of selected pathology per section. – = absence of selected pathology. All "Other ALS-Associated Proteins" were evaluated for the presence or absence of NCIs. Phosphorylated NFH was evaluated for the presence of neuroaxonal spheroids greater than or equal to 30 μm at smallest diameter.

other variants of ALS and controls. Antibodies against the RNA binding proteins TDP-43, FUS/TLS, and RGNEF did not label any NCIs, and expression patterns for these proteins matched those seen in neuropathologically normal controls. SOD1-positive inclusions were detected in mtSOD1 tissues, but not in any other genetic ALS variants, or in neuropathologically normal controls. Ubiquitin and p62-positive NCIs were observed in mtSOD1-ALS tissues, perhaps marking SOD1-positive inclusions for proteasome degradation. There was no evidence of any differential optineurin expression in mtSOD1-ALS cases. The intermediate filament proteins peripherin and pNFH revealed the presence of pathological NCIs only in non-mtSOD1 variants of ALS. Large pNFH-positive neuroaxonal spheroids were not observed in mtSOD1-ALS cases, nor were peripherin-positive NCIs.

Discussion and Concluding Remarks

With the discovery of the mtSOD1 association with a significant proportion of fALS cases, there was a concrete target to attempt to unravel the pathogenesis of ALS. With the subsequent discovery of mutations in *TARDBP* and *FUS/TLS* in some fALS families, the field began to target RNA binding proteins as contributors to ALS pathogenesis. After optimizing antigen retrieval, titres, and protocols for all of the antibodies used in this study, we examined our panel of diverse genetic variants of ALS as well as cases of fALS and sALS without any known mutations with a selection of 9 ALS-associated protein markers that are a contemporary focus of ALS research. We have determined that mtSOD1-ALS is a pathologically unique variant of the disease in that there is differential protein expression seen between mtSOD1-ALS and the other variants of ALS analyzed in this study. This is important given the linkage of these RNA binding proteins to familial forms of ALS and the subsequent association of ALS to altered RNA metabolism. Although mtSOD1-ALS has a differential expression of RNA binding proteins, it is important to consider the RNA binding properties of the mutant form of SOD1, and thus the classification of mtSOD1-ALS as a disease of altered RNA metabolism as well.

As summarized in **Table 2.3**, TDP-43-positive skeins and aggregates were seen across all non-mtSOD1 variants of ALS [**Figure 2.1, Table 2.3**]. This is in contrast to an earlier observation that TDP-43-positive cytoplasmic aggregates can be observed in mtSOD1-ALS tissue (Robertson *et al.*, 2007). The key difference between these studies is that the present study used DAB immunohistochemistry and visualized protein deposition at the light microscopy level, whereas the study by Robertson *et al.* (Robertson *et al.*, 2007) used immunofluorescent staining and visualized protein deposition with confocal microscopy. The level of detection differences in these 2 techniques accounts for this discrepancy. While confocal microscopy is widely available to researchers, it is prohibitive in clinical settings due to time constraints and lack of automated staining and visualization techniques. The techniques employed here could be automated, which would allow for rapid slide processing and give reproducible and easily discernible staining patterns. Our goal was to provide an analytic algorithm that would be widely applicable using routine immunohistochemistry.

We also made the observation that the nucleocytoplasmic redistribution of TDP-43 occurs in all variants of ALS, but occurs at a significantly higher rate in mtTDP-43-ALS, fALS, and sALS when compared to mtSOD1-ALS [**Figure 2.2**]. Although the redistribution results between mtSOD1-ALS and mtFUS/TLS-ALS were not statistically significant, there seems to be a trend towards increased TDP-43 nucleocytoplasmic redistribution in mtFUS/TLS-ALS cases as well [**Figure 2.2**]. This phenomenon has been shown to be a normal response to injury in an axotomy mouse model and is reversible (Wang *et al.*, 2008; Moisse *et al.*, 2009). Another possibility is that this nucleocytoplasmic redistribution is a protein-level manifestation of a cellular repair mechanism. This may suggest that non-mtSOD1 variants of ALS engage in a more severe response to MN injury and will eventually recover, or it may be a pathological response and the initial stages of the formation of NCIs. An important distinction to draw, however, is the difference between cytoplasmic up-regulation of TDP-43, and the presence of cytoplasmic intraneuronal TDP-43-positive inclusions. Where an up-regulation is known to be a normal injury response, the presence of formed inclusions is thought to be pathological. In either case, the divergent pathological expression patterns between mtSOD1-ALS and all other variants of ALS suggest the possibility of divergent pathways of aberrant RNA metabolism linked to disease pathogenesis despite indistinguishable clinical phenotypes.

Further pathological observations of RNA binding proteins made in the present study are in agreement with previously published studies. Firstly, the presence of FUS/TLS-positive cytoplasmic inclusions in all non-mtSOD1 variants of ALS [**Figure 2.3, Table 2.4**] concurred with data presented by Deng *et al.* (Deng *et al.*, 2010). We made note of the importance of antigen retrieval and antibody selection discussed by Deng *et al.* as well as the observation that many FUS/TLS-positive NCIs were also immunoreactive with antibodies against p62, ubiquitin, and TDP-43.

Detection using an antibody against SOD1 revealed immunoreactive inclusions only in mtSOD1-ALS cases [**Figure 2.5**]. This is in line with previous observations that mtSOD1-ALS cases have SOD1-positive NCIs (Shibata *et al.*, 1996; Kato *et al.*, 1999). The lack of SOD1-positive inclusions in cases of sALS without known mutations in the present study could be due to case selection bias, as only 6 such cases were examined, and the finding is known to be uncommon.

Finally, we evaluated a novel RNA binding protein [RGNEF] in this study. Similar to the findings for both TDP-43 and FUS/TLS, in all non-mtSOD1 variants of ALS, we observed RGNEF immunoreactive NCIs. These inclusions have also been found to co-localize with ubiquitin and p62 [see Chapter 3]. This suggests that RGNEF-positive NCIs can be targeted for proteasomal degradation in ALS cases, which is similar to other RNA binding proteins; a common theme thus emerges.

There also was evidence of NCIs of ubiquitin [**Figure 2.6**] across all non-mtFUS/TLS variants of ALS, and of p62-positive inclusions across all variants of ALS [**Figure 2.7**]. As previously discussed, both are markers of protein degradation. The presence of ubiquitin or p62-positive NCIs suggests that the proteins being targeted are being degraded by the proteasome complex. Ubiquitin-positive NCIs have previously been reported in all non-mtSOD1 variants of ALS (Deng *et al.*, 2010). Past studies have determined p62 to be an alternate marker of proteasome degradation, and suggestions have been made regarding its superiority over ubiquitin (Mizuno *et al.*, 2006). Interestingly, we observed p62-positive NCIs more reliably than detection with ubiquitin. For this reason, as well as our observation of more intense immunoreactivity using the antibody against p62 compared with ubiquitin across our panel, we have selected our antibody against p62 to be a more reliable and robust marker of protein degradation than ubiquitin.

Our results revealed no optineurin-positive inclusions across our panel of cases [**Figure 2.8**], in contrast to past work (Maruyama *et al.*, 2010). Maruyama and colleagues noted optineurin-positive inclusions in mtSOD1 variants of ALS and suggested that optineurin may be the unifying protein in seemingly divergent pathways of MN degeneration. Taken with data presented here, we suggest that mtSOD1-ALS is a pathologically distinct variant of ALS. More recent work (Hortobagyi *et al.*, 2011) has found that optineurin does not appear to be a pathologically unifying protein across variants of ALS.

The intermediate filament proteins peripherin and NFH highlighted pathology in all non-mtSOD1 genetic variants of ALS as well as in cases of fALS and sALS without known mutations [**Figures 2.9, 2.10**]. As mentioned above, we analyzed pNFH immunohistochemistry for the presence of large [≥30 μm at smallest diameter] neuroaxonal spheroids (Wohlfart, 1959; Carpenter, 1968). A NF marker is important to this study due to the focus on NFL mRNA binding proteins and the finding that NF subunit stoichiometry can be altered in ALS, but not in controls (Bergeron *et al.*, 1994; Wong *et al.*, 2000; Menzies *et al.*, 2002). Peripherin was found to form NCIs in non-mtSOD1-ALS cases as well as in cases of fALS and sALS without any known mutations. Intermediate filament pathology is a classical hallmark of ALS, and our observation that intermediate filament pathology could only be seen in non-mtSOD1 variants of ALS lends further support to our finding that mtSOD1-ALS is a pathologically unique variant of the disease.

This is the first work to comprehensively focus on the contemporary markers of ALS across a panel of diverse variants of the disease and examine specific proteins for pathology to determine if these variants of ALS can be identified through routine immunohistochemical methods. It appears that cases harbouring mutations in *SOD1* can be identified by their immunohistochemical characteristics: p62-positive NCIs that are negative for the 3 non-mtSOD1 RNA binding proteins. We thus recommend that all ALS cases be studied by immunohistochemistry using these antibodies, and if this pattern is found, then a genotypic analysis should be done to confirm the presence of mtSOD1-ALS.

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Chapter 3: Amyotrophic lateral sclerosis is a disease of altered RNA metabolism characterized by the aberrant expression of RGNEF and other RNA binding proteins

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A version of this chapter is in preparation and will be submitted for publication.

Abstract

Amyotrophic lateral sclerosis [ALS] is a fatal neurological disease in which those affected experience a selective loss of motor neurons, leading to muscle paralysis and death within 3-5 years of symptom onset. The classic pathological hallmark of ALS is the presence of intermediate filament-positive neuronal cytoplasmic inclusions [NCIs] in the anterior horn of the spinal cord. Accompanying this pathology is a decrease in the steady state mRNA levels of low molecular weight [68 kDa] neurofilament [NF] mRNA, which has been hypothesized to alter the stoichiometry of the NF polymer. Thus, proteins regulating the stability of NFL mRNA are of considerable interest. We recently identified RGNEF, a human homologue of the murine p190RhoGEF, which is a protein known to regulate the stability of murine NFL mRNA. Because NFL mRNA binding proteins [ex. TDP-43, FUS/TLS, mtSOD1, 14-3-3] have been shown to form NCIs in ALS, we sought to determine if RGNEF also forms pathological inclusions in ALS. To do so, we developed polyclonal and monoclonal antibodies against RGNEF. We observed RGNEF-positive NCIs only in ALS anterior horn cells [AHCs], and not in healthy controls. RGNEF immunoreactive inclusions were also immunoreactive to p62 and ubiquitin, suggesting that its degradation is also impaired. Similar to TDP-43, we also observed that RGNEF is present in transport granules, stress granules, and processing bodies. We observed that some RGNEF-positive NCIs are also immunoreactive for TDP-43 and FUS/TLS, and a strong degree of co-localization is evident within some cells. Overall, our data indicates that RGNEF is actively

involved in RNA metabolism, can be aberrantly expressed in ALS, and is a possible contributor to disease pathogenesis.

Introduction

Amyotrophic lateral sclerosis [ALS] is a progressive, motor neuron [MN] selective disease that is generally fatal due to respiratory failure within 5 years of symptom onset (Logroscino *et al.*, 2008). The cause(s) of ALS remain to be elucidated, although it is generally accepted that there are multiple factors contributing to its pathogenesis. ALS is also considered to be a multisystems disorder, with the presence of a frontotemporal syndrome observed in up to 50% of patients (Strong, 2010). Approximately 10% of cases are inherited [familial ALS; fALS]. Amongst these cases, the most common genetic mutations are present in the gene encoding Cu/Zn-superoxide dismutase [SOD1] (Rosen *et al.*, 1993). More recently, mutations in the genes encoding TAR-DNA binding protein of 43 kDa [TDP-43] (Kabashi *et al.*, 2008; Rutherford *et al.*, 2008; Sreedharan *et al.*, 2008; Van Deerlin *et al.*, 2008; Corrado *et al.*, 2009; Daoud *et al.*, 2009; Del Bo *et al.*, 2009), and fused in sarcoma/translocated in liposarcoma [FUS/TLS] (Lagier-Tourenne and Cleveland, 2009; Vance *et al.*, 2009; Corrado *et al.*, 2010) have also been identified. Interestingly, TDP-43, FUS/TLS, and mutant [mt] SOD1 are all known to bind RNA and help to regulate its metabolism (Strong, 2010).

A classic pathological hallmark of ALS is the presence of intermediate filamentous aggregates within degenerating MNs. These include aggregates of peripherin (Migheli *et al.*, 1993; Beaulieu *et al.*, 2000; He and Hays, 2004), alpha-internexin (Wong *et al.*, 2000), and neurofilament [NF] (Julien, 1999; Beaulieu *et al.*, 2000; Strong *et al.*, 2005). There are 3 subunits of NF, which correspond to their molecular weights [68 kDa NFL, 160 kDa NFM, 200 kDa NFH]. The key to subunit polymerization to form functional NF is the stoichiometric ratios of NFL, NFM, and NFH with respect to one another. Interestingly, in the case of NF NCIs seen in ALS patients, NFL mRNA levels are significantly suppressed with respect to steady-state mRNA levels of NFM and NFH (Bergeron *et al.*, 1994; Wong *et al.*, 2000; Menzies *et al.*, 2002; Strong, 2010). Further, the RNA binding proteins introduced above, TDP-43 (Buratti and Baralle, 2001; Strong *et al.*, 2007), FUS/TLS (Strong, 2010; Ticozzi *et al.*, 2010), and mtSOD1 (Strong, 2010), are known to interact with NFL mRNA and modulate its stability. Also, both TDP-43 and FUS/TLS have been shown to form ubiquitinated NCIs in ALS, but not in controls, suggesting a possible role for them in the pathogenesis of ALS (Arai *et al.*, 2006; Neumann *et al.*, 2006; Deng *et al.*, 2010).

P190RhoGEF is a protein known to alter the stability of murine NFL mRNA through binding to a destabilizing element in the 3' untranslated region (Canete-Soler *et al.*, 2001). P190RhoGEF can co-localize with NFL aggregates, but not with NFL subunits assembled in functional NF arrays (Lin *et al.*, 2005). Further, the same study showed that aggregation of NFL is associated with the decrease in steady-state levels of NFL mRNA, and that siRNA silencing of p190RhoGEF increases NF polymerization and decreases NFL aggregation. For these reasons, we searched for a human homologue of p190RhoGEF, and discovered RGNEF to be a highly homologous protein (Volkening *et al.*, 2010). Initial findings regarding RGNEF include the expression of its RNA in both brains and spinal cords, and that RGNEF and NFL mRNA interact directly *in vitro* in lysates from ALS-affected spinal cords, but not in lysates from healthy controls (Volkening *et al.*, 2010). RGNEF is the first described link between ALS and an RNA binding protein that is also of an important role in cell signaling via the RhoA signaling cascade (Droppelmann *et al.*, Submitted, 2011). This study will, for the first time, characterize RGNEF expression within human ALS spinal motor neurons.

Increasingly, RNA metabolism is being regarded as a very dynamic process. Gene expression within a MN occurs under somatotopic direction, meaning that translation occurs at the site within the cell where the protein will be immediately needed. For this to occur successfully, several post-transcriptional modifications must first take place – mRNA processing, which includes the addition of a 7-methylguanosine cap to the 5' end of the transcript and the addition of a 3' poly(A) tail, splicing, editing, and stabilization (Bolognani and Perrone-Bizzozero, 2008). Following post-transcriptional modifications, mRNA is packaged into a ribonucleoprotein [RNP] complex that includes some necessary translational machinery, which is then shipped to the site of protein synthesis (Strong, 2010). These RNP complexes, which can be identified as RNA granules, can take on several identities depending on the cell's health and its need for their contents. Transport granules are one such identity. Transport granules are RNA granules that are being directed through the cell to the location of protein synthesis. An accepted molecular marker of these granules is Staufen (Kim and Kim, 2006). On the other hand, when a cell is under stress and translation is inhibited, RNA granules take on a molecule of TIA-1 and can be referred to as stress granules (Kedersha *et al.*, 1999). Lastly, when mRNA is going to be degraded, the RNA granule becomes a mRNA processing body [P-body], characterized by the presence of the exoribonuclease XRN-1 (Chang *et al.*, 2011). Increasing evidence is pointing to the fluid nature of the relationship between stress granules and P-bodies (Kedersha *et al.*, 2005; Barbee *et al.*, 2006; Anderson and Kedersha, 2008).

As reports have indicated, neuroinflammation is a natural aspect of the innate immune response within the nervous system, and innate immunity has been associated with ALS (Moisse and Strong, 2006). Attenuation of inflammation in animal models delays the onset of ALS symptoms and extends survival (Moisse and Strong, 2006). Also, descriptions of microglial (Lampson *et al.*, 1990; Troost *et al.*, 1990; McGeer *et al.*, 1991; Kawamata *et al.*, 1992; Troost *et al.*, 1993; Wilson *et al.*, 2001; Henkel *et al.*, 2004; Solomon *et al.*, 2006) and astrocytic (Engelhardt and Appel, 1990; Kushner *et al.*, 1991; Kawamata *et al.*, 1992; Engelhardt *et al.*, 1993; Nagy *et al.*, 1994; Schiffer *et al.*, 1996; Hall *et al.*, 1998; Alexianu *et al.*, 2001) activation have been well documented in ALS. Thus, it is of considerable interest to understand proteins that may mediate the glial response and improve prognosis for ALS patients. In an effort to determine if RGNEF may be a potential mediator of gliosis in ALS, this study sought to determine the relationship between RGNEF, microglia, and astrocytes. In addition, we have sought to investigate the relationship between RGNEF and the markers of RNA metabolism mentioned above. Finally, we have examined the association between RGNEF and proteasome degradation in ALS cases as well as the association between RGNEF and 2 other ALS-associated, RNA binding proteins: TDP-43 and FUS/TLS.

Materials and Experimental Procedures

RGNEF Antibody Development and Optimization

A goat polyclonal antibody against RGNEF was produced by 21st Century Biochemicals [260 Cedar Hill Street, Marlboro, MA, USA 01752] using the immunogenic peptide sequence 1567-INEALVQMSFNTFNKLNPSV-1586. The antibody specificity was determined by comparing the expression patterns of immunohistochemistry using the RGNEF antibody to staining with the antibody and blocking peptide. Performing immunolabeling with a range of antibody titres optimized sensitivity. The staining results were further optimized using different methods of antigen retrieval.

Immunohistochemistry

Six-micrometer sections of formalin-fixed, paraffin-embedded spinal cord tissue were warmed at 63°C for 30 minutes and rehydrated through an ethanol series to 1xPBS with 5-minute washes [xylenes, 1:1 xylenes:ethanol, 100% ethanol, 95% ethanol, 75% ethanol, 50% ethanol, 1xPBS]. Following rehydration, sodium citrate buffer [10mM Sodium Citrate, 0.05% Tween 20, pH 6] antigen retrieval was performed using a 2100 Retriever chamber [Pick Cell Laboratories, Lelystad, Netherlands]. Sections were washed 3 times for 5 minutes each using 1xPBS and then treated for 5 minutes at room temperature with 3% H_2O_2 to block endogenous peroxidase activity. After 3 additional 5-minute washes with 1xPBS, the sections were blocked for 60 minutes in 5% bovine serum albumin [Roche Diagnostics, Mannheim, Germany] in water. Following blocking, the sections were incubated with a polyclonal antibody against RGNEF [21st Century Biochemicals (Ac-INEALVQMSFNTFNKLNPSVC-N), 1:500] for 20 hours at 4°C. The sections were then washed 3 times for 5 minutes each in 1xPBS and were then incubated with Biotinylated Goat IgG [Vectastain Elite ABC Kit, Vector Laboratories, Cat # PK-6105, Burlingame, CA] for 60 minutes at room temperature at a titre of 1:500. Following 3 additional washes of 5 minutes each in 1xPBS, the tissues were incubated with the avidin/biotin enzyme complex solution [Vectastain kit], washed in 1xPBS 3 times for 5 minutes each and treated with 3,3'-diaminobenzidine [Sigma-Aldrich Canada, Oakville, ON]. Nuclear counterstaining was then performed using Modified Harris Formula Haematoxylin [Ricca Chemical Company, Arlington, TX]. Sections were dried, mounted with Cytoseal 60 mounting medium [Richard-Allan Scientific, Kalamazoo, MI], observed using a BX45 light microscope [Olympus, Markham, ON], and photographed with a Q Color 3 camera [Olympus, Markham, ON].

Immunofluorescence

Formalin-fixed, paraffin-embedded spinal cord tissues were treated for 15 hours with 365 nm ultraviolet light to minimize the autofluorescence of lipofuscin. Tissues were warmed at 63°C for 30 minutes and rehydrated through a series of ethanol washes [xylenes, 1:1 xylenes:ethanol, 100% ethanol, 95% ethanol, 75% ethanol, 50% ethanol, 1xPBS]. Following rehydration, antigen retrieval was performed using sodium citrate solution [10mM Sodium Citrate, 0.05% Tween 20, pH 6] in a 2100 Retriever chamber [Pick Cell Laboratories, Lelystad, Netherlands]. The sections were washed 3 times for 5 minutes each using 1xPBS and then treated for 5 minutes at room temperature with 3% H_2O_2 to block endogenous peroxidase activity. Following 3 additional 5-minute washes in 1xPBS, the sections were blocked for 60 minutes in 5% bovine serum albumin [Roche Diagnostics, Mannheim, Germany] in water. After blocking, the sections were incubated with the appropriate antibodies for 20 hours at 4°C [**Table 3.1**]. The sections were then washed 3 times for 5 minutes each in 1xPBS and were then incubated with the appropriate fluorescent-conjugated secondary antibody for 60 minutes at room temperature at a titre of

Antibody	Titre	Source	Secondary Antibody
RGNEF Goat Polyclonal	1:75 (IF) 1:500 (IHC)	21st Century Biochemicals (Antigen: Ac-INEALVQMSFNT-FNKLNPSVC-N)	Invitrogen, Alexa Fluor® 488, Donkey α-Goat, Cat # A11055
TDP-43 Rabbit Polyclonal	1:500	Proteintech (Cat # 10782-2-AP)	Invitrogen, Alexa Fluor® 555, Donkey α-Rabbit, Cat # A31572
FUS/TLS Rabbit Polyclonal	1:90	Proteintech (Cat # 11570-1-AP)	Invitrogen, Alexa Fluor® 555, Donkey α-Rabbit, Cat # A31572
p62 Rabbit Polyclonal	1:1000	Enzo Lifesciences (Cat # PW9860)	Invitrogen, Alexa Fluor® 555, Donkey α-Rabbit, Cat # A31572
Ubiquitin Mouse Monoclonal	1:500	Millipore (Cat # MAB-1510)	Invitrogen, Alexa Fluor® 568, Donkey α-Mouse, Cat # A10037
L26 Rabbit Polyclonal	1:1000	Abcam (Cat # ab59652)	Invitrogen, Alexa Fluor® 555, Donkey α-Rabbit, Cat # A31572
Staufen Rabbit Polyclonal	1:250	Abcam (Cat # ab50914)	Invitrogen, Alexa Fluor® 555, Donkey α-Rabbit, Cat # A31572
TIA-1 Mouse Monoclonal	1:100	Abcam (Cat # ab2712-250)	Invitrogen, Alexa Fluor® 568, Donkey α-Mouse, Cat # A10037
XRN-1 Rabbit Polyclonal	1:100	Bethyl Laboratories (Cat # A300-443A)	Invitrogen, Alexa Fluor® 555, Donkey α-Rabbit, Cat # A31572
GFAP Mouse Monoclonal	1:500	BD Pharmingen (Cat # 556330)	Invitrogen, Alexa Fluor® 568, Donkey α-Mouse, Cat # A10037
IBA-1 Rabbit Polyclonal	1:1000	Wako Chemicals (Cat # 019-19741)	Invitrogen, Alexa Fluor® 555, Donkey α-Rabbit, Cat # A31572

Table 3.1: Details of use for each antibody in this study

1:500 [**Table 3.1**]. Following 3 additional 5-minute washes in 1xPBS, nuclear counterstaining was performed using Hoechst 33342 [Invitrogen, Cat # H21492] at a titre of 1:1000 in 1xPBS for 7 minutes. Sections were partially dried, mounted with Immu-Mount mounting medium [Thermo Scientific, Kalamazoo, MI] and then observed at 400× magnification and photographed using an LSM 510 Meta Confocal Imaging System [Carl Zeiss Canada Ltd., Toronto, ON].

Protein Expression Analysis

We first characterized the expression of RGNEF within MNs and surrounding cells. We performed double immunolabeling assays using markers for RGNEF and the ribosomal protein L26, which has been shown to be an accurate marker of translationally active polysomes (Singhrao and Nair-Roberts, 2010). After determining the normal expression patterns, we performed further double immunolabeling assays using antibodies against RGNEF and either p62 or ubiquitin, 2 markers of the proteasome-protein degradation pathway. For details of antibody usage, please refer to **Table 3.1**.

We were then interested in the interaction between RGNEF and the ALS-associated, RNA binding proteins TDP-43 and FUS/TLS within anterior horn MNs. To look at the interaction, we performed double immunolabeling experiments using antibodies against RGNEF and either TDP-43 or FUS/TLS. To determine if RGNEF plays an active role in the RNA metabolic pathway, we performed double immunolabeling using markers against transport granules

[Staufen], stress granules [TIA-1], and P-bodies [XRN-1]. For details of antibody usage, please refer back to **Table 3.1**.

Finally, due to the role neuroinflammation plays in neuronal injury and ALS, we aimed to determine the interaction between RGNEF and glial cells in the anterior horn of the spinal cord. We performed double immunolabeling experiments using antibodies against RGNEF and either IBA-1 [microglia] or GFAP [astrocytes]. For details of antibody usage, please refer back to **Table 3.1**.

Protein Complex Immunoprecipitation/ Western Blot

Cloning of full length RGNEF

The full RGNEF coding sequence was cloned by PCR using Pfu DNA polymerase [Fermentas, Burlington, ON, Canada]. The commercial RGNEF clone [GenBank BC157846] MHS1768-99865695 [Open Biosystem] was used as a template. The RGNEF coding sequence was incorporated in the pcDNA3.1-myc-His [Invitrogen, Carlsbad, CA, USA] vector between the Kpn I/Xho I sites.

Transfection and Western blot

HEK293T cells were transfected with the pcDNA-C-Myc-RGNEF or pcDNA3.1-C-Myc-His [control cells] vector using Lipofectamine 2000 [Invitrogen, Carlsbad, CA, USA]. The cells were lysed with an NP40-based lysis buffer, and then a Protein Complex Immunoprecipitation [Co-IP] was performed for 60 minutes at room temperature using an antibody against RGNEF [Goat anti-RGNEF, 21st Century Biochemicals] and Sepharose A [GE Healthcare, Piscataway, NJ] as the protein carrier. Two Western Blots were performed: one using an antibody against L26 [1:2000] detailed in **Table 3.1** above, and one using mouse monoclonal antibody against C-Myc [Cedarlane, Catalogue # CLX229AP, 1:2000].

Results

RGNEF Expression Analysis

We observed 2 very distinct expression patterns for RGNEF in control tissues – a light uniform cytoplasmic expression pattern, and a coarse granular expression pattern [**Figure 3.1**]. To determine if the latter pattern was consistent with the association of RGNEF with translationally active polysomes, we did a double immunolabeling experiment using antibodies against RGNEF and the large ribosomal subunit protein, L26. In **Figure 3.1**, RGNEF is demonstrated to co-localize with L26. To further confirm this association, RGNEF was immunoprecipitated from HEK 293T cells, and a Western Blot using an antibody against L26 was performed. We did not observe that RGNEF co-immunoprecipitated L26 [**Figure 3.2a**]. To confirm the efficacy of the techniques used for the immunoprecipitation and Western Blot, we used an RGNEF antibody to immunoprecipitate C-Myc-RGNEF, and then performed a Western Blot using an antibody against C-Myc [**Figure 3.2b**]. This blot had a positive band at the expected molecular weight. Taken together, these data

Figure 3.1: The characterization of the normal intraneuronal expression of RGNEF in neuropathologically normal control tissues.

Immunolabeling was performed using an antibody against RGNEF [Goat Polyclonal, 21st Century Biochemicals, 1:500 IHC, 1:75 IF, 20 hours, 4°C] and expression in neuropathologically normal control tissues revealed the presence of 2 commonly seen histological features: coarse granular polysomal granules [A, represented by arrows], and light cytoplasmic expression [B]. To test for the presence of RGNEF within translationally active polysomes, a double immunolabeling experiment was performed using an antibody against the large ribosomal subunit marker, L26 [Rabbit Polyclonal, Abcam (Cat # ab59567), 1:1000, 20 hours, 4°C]. Co-localization was evident [C]. The inset in Figure C shows a clear co-localization between RGNEF and L26. All scale bars represent 20 μm.

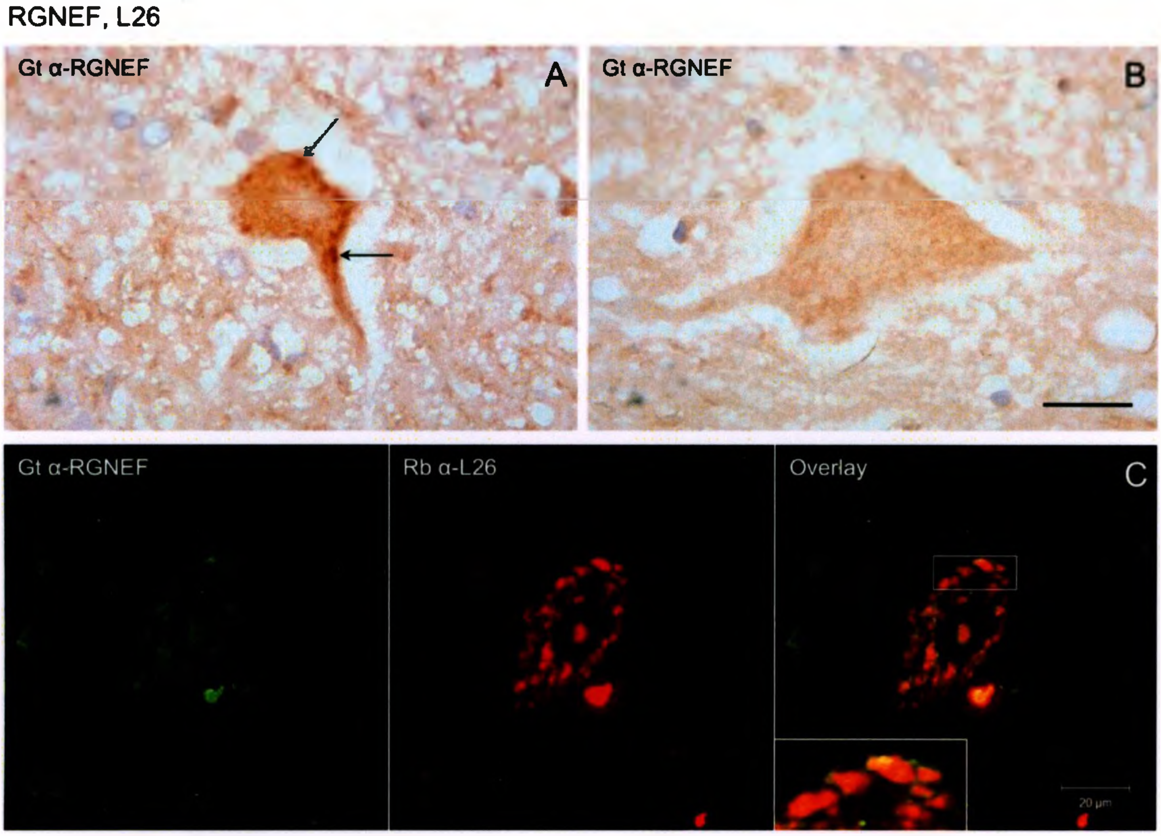
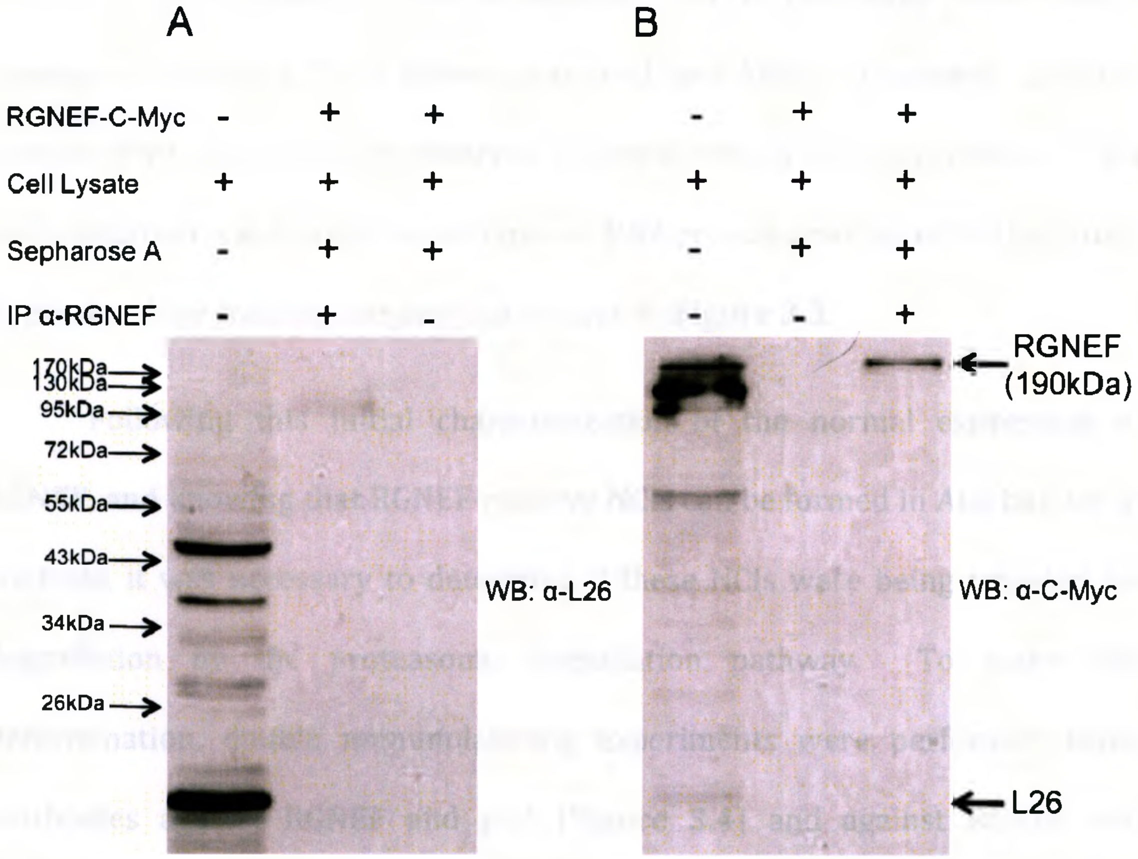

RGNEF, L26
Gt α-RGNEF
A
Gt α-RGNEF
B
Gt α-RGNEF
Rb α-L26
Overlay
C
20 µm

Figure 3.2: A protein-complex immunoprecipitation testing the interaction between RGNEF and L26 in complex followed by a Western Blot.

RGNEF was immunoprecipitated from HEK 293T cells, and a Western Blot using an antibody against L26 was performed [A]. RGNEF was not observed to co-immunoprecipitate L26. As a confirmation that the co-immunoprecipitation and Western Blot protocols were followed correctly, we used an antibody against RGNEF to immunoprecipitate RGNEF-C-Myc from HEK293T cells, and then performed a Western Blot using an antibody against C-Myc [B]. This blot contains a band at the expected molecular weight, confirming the validity of the protocol, and suggesting an indirect interaction between RGNEF and L26.


A
B
RGNEF-C-Myc - + + - + +
Cell Lysate + + + + + +
Sepharose A - + + - + +
IP α-RGNEF - + - - - +
170kDa
130kDa
95kDa
72kDa
55kDa
43kDa
34kDa
26kDa
WB: α-L26
RGNEF (190kDa)
WB: α-C-Myc
L26

suggest that the interaction between RGNEF and L26 is indirect, although they seem to be localized within the same complex.

It is known that RGNEF is an NFL mRNA binding protein (Volkening *et al.*, 2010), so double immunolabeling experiments were performed to determine associations between RGNEF and markers of RNA metabolism. As mentioned the markers of RNA metabolism used in this study were Staufen [transport granules], TIA-1 [stress granules], and XRN-1 [P-bodies]. RGNEF-positive RNA granules were observed in spinal MNs in ALS and controls. This co-localization was evident for all types of RNA granules evaluated in this study. Representative staining samples can be seen in **Figure 3.3**.

Following this initial characterization of the normal expression of RGNEF, and knowing that RGNEF-positive NCIs can be formed in ALS but not in controls, it was necessary to determine if these NCIs were being targeted for degradation by the proteasome degradation pathway. To make this determination, double immunolabeling experiments were performed using antibodies against RGNEF and p62 [**Figure 3.4**] and against RGNEF and ubiquitin [**Figure 3.5**]. The presence of RGNEF-positive NCIs was very often associated with near complete co-localization between RGNEF and p62, and a strong co-localization could also be observed between RGNEF and ubiquitin. This co-localization suggests that aggregated intraneuronal forms of RGNEF are being targeted for degradation via the proteasome pathway.

Figure 3.3: The involvement of RGNEF within the RNA metabolic pathway through interactions with RNA granules.

RGNEF [Goat Polyclonal, 21st Century Biochemicals, 1:75, 20 hours, 4°C] was found to be involved in the RNA metabolism pathway through the association with RNA granules found using double immunolabeling experiments. Co-localization between RGNEF and Staufen [Rabbit Polyclonal, Abcam (Cat # ab50914), 1:250, 20 hours, 4°C] [Figure A], TIA-1 [Mouse monoclonal, Abcam (Cat # ab2712-250), 1:100, 20 hours, 4°C] [Figure B], and XRN-1 [Rabbit Polyclonal, Bethyl Laboratories (Cat # A300-443A), 1:100, 20 hours, 4°C] [Figure C] suggests that RGNEF can be found within transport granules, stress granules, and P-bodies, respectively. The figure insets are selected areas of the figures presented at higher magnification in order to clearly identify the co-localization and the involvement of RGNEF in RNA metabolism. The blue fluorescence staining is nuclear counterstaining using Hoechst 33342. All scale bars represent 20 μm.

RGNEF in RNA Metabolism

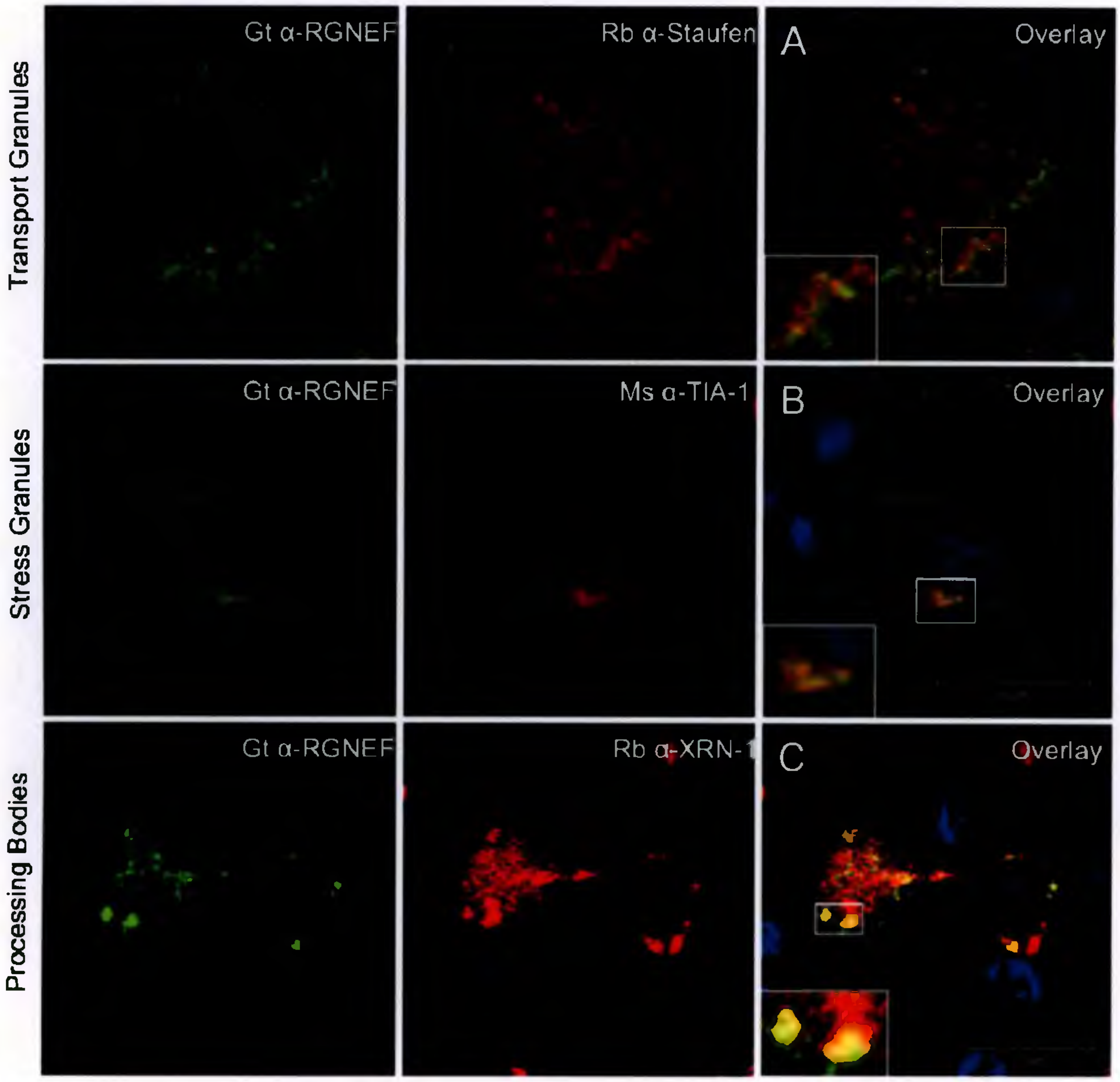

Transport Granules
Gt α-RGNEF
Rb α-Staufen
A
Overlay
Stress Granules
Gt α-RGNEF
Ms α-TIA-1
B
Overlay
Processing Bodies
Gt α-RGNEF
Rb α-XRN-1
C
Overlay

Figure 3.4: The co-localization between RGNEF and p62 suggests that RGNEF can be targeted for protein degradation via the proteasome degradation system.

Double immunolabeling experiments done using antibodies against RGNEF [Goat Polyclonal, 21st Century Biochemicals, 1:75, 20 hours, 4°C] and p62 [Rabbit Polyclonal, Enzo Lifesciences (Cat # PW 9860), 1:1000, 20 hours, 4°C] reveal a high level of co-localization in human spinal MNs. P62 immunoreactivity was not typically present within spinal MNs from healthy control tissue [A]. As described, the converse is true of spinal MNs from ALS patients. RGNEF-positive NCIs can be immunolabeled with p62 [B]. The inset presents a selected area of the figure at high magnification that allows clear visualization of the RGNEF and p62 co-localization. The blue fluorescence staining is nuclear counterstaining using Hoechst 33342. All scale bars represent 20 μm.

RGNEF, p62

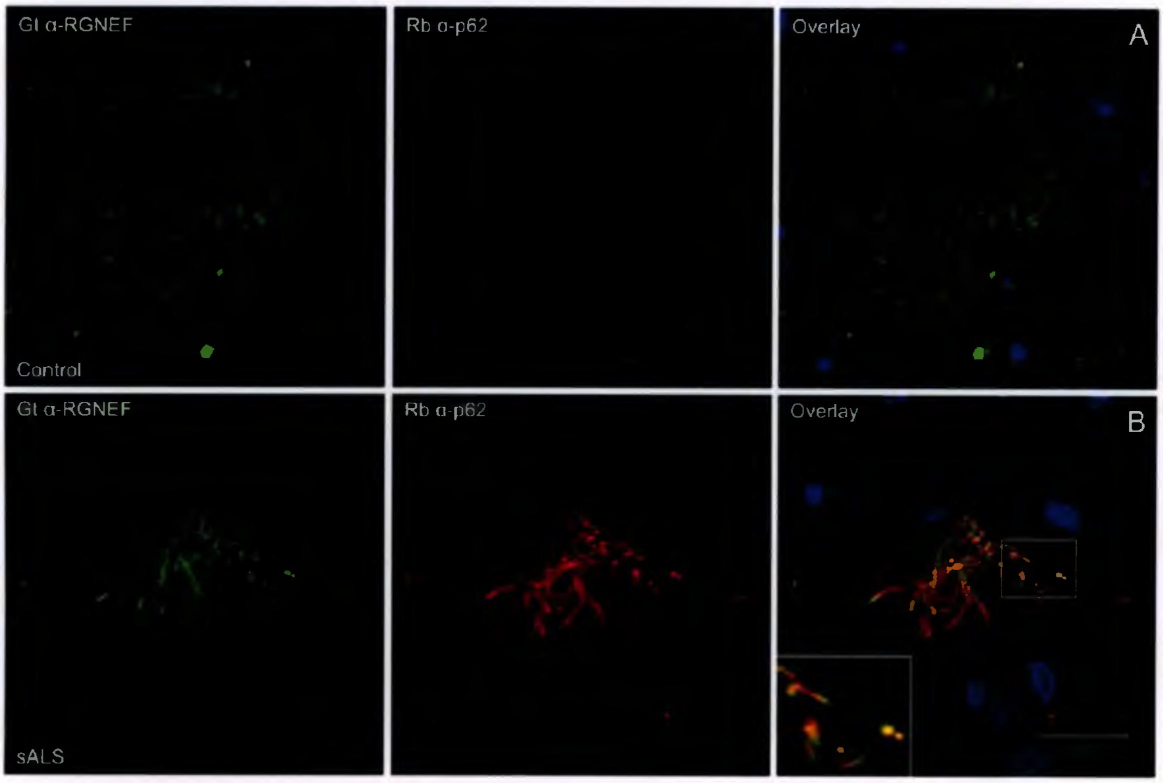
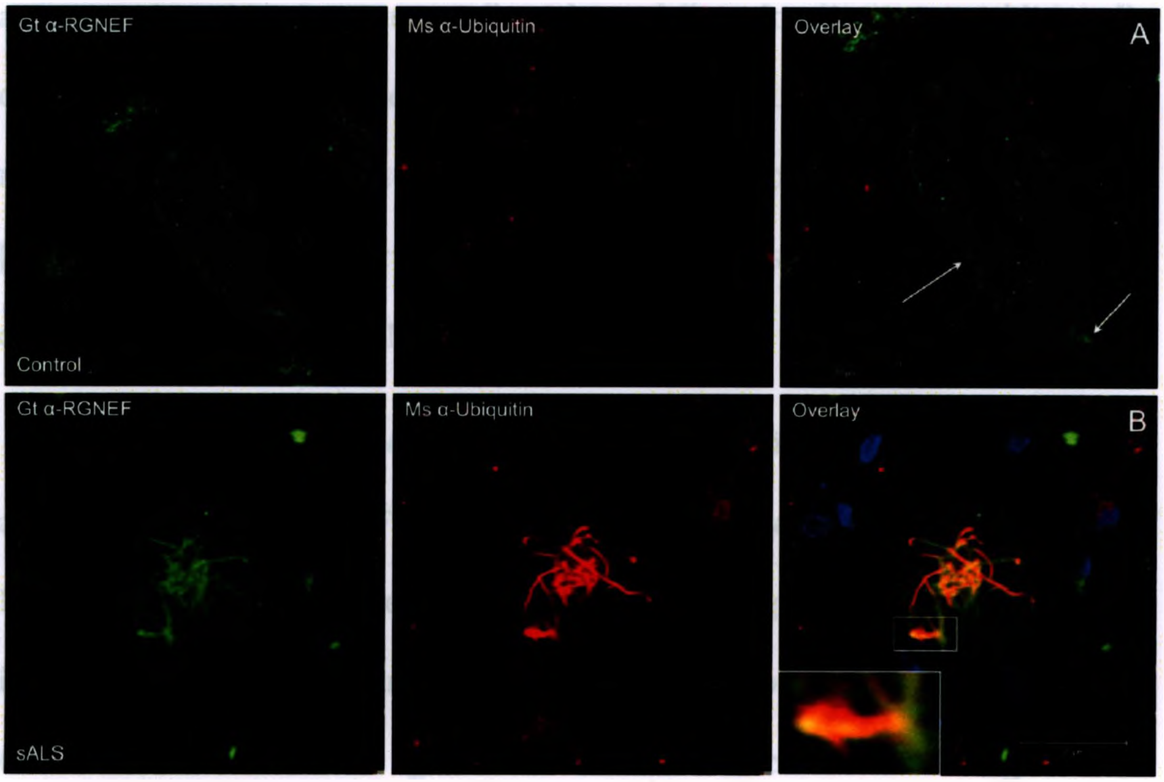

Gt α-RGNEF
Rb α-p62
Overlay
A
Control
Gt α-RGNEF
Rb α-p62
Overlay
B
sALS

Figure 3.5: The co-localization between RGNEF and ubiquitin suggests that RGNEF can be targeted for protein degradation via the ubiquitin-proteasome degradation system.

Antibodies against RGNEF [Goat Polyclonal, 21st Century Biochemicals, 1:75, 20 hours, 4°C] and ubiquitin [Mouse Monoclonal, Millipore (Cat # MAB-1510), 1:500, 20 hours, 4°C] were used in a double immunolabeling experiment in human spinal MNs. In spinal MNs from healthy control tissue, staining patterns reveal RGNEF-positive polysomes [arrows] with no ubiquitin-immunoreactivity [A]. Conversely, spinal MNs from ALS tissues revealed RGNEF-positive NCIs that could be strongly immunoreactive to ubiquitin as well [B]. The inset in this figure highlights a selected area of the RGNEF-ubiquitin co-localization at a high magnification in order to clearly show the interaction between the 2 proteins. The blue nuclear counterstaining was done using Hoechst 33342. All scale bars represent 20 μm.



RGNEF, Ubiquitin

Gt α-RGNEF
Ms α-Ubiquitin
Overlay
A
Control
Gt α-RGNEF
Ms α-Ubiquitin
Overlay
B
sALS

Due to the focus on TDP-43 and FUS/TLS within the field of ALS, and given the presence of RGNEF-positive NCIs, double immunolabeling experiments were done in an effort to determine the relationship between RGNEF and these other NFL mRNA binding proteins. Double immunolabeling was done using antibodies against RGNEF and TDP-43, which revealed clear co-localization [**Figure 3.6**]. It was also possible for RGNEF and TDP-43 to be independently aggregated within the same cell without full co-localization. Additionally, double immunolabeling done using antibodies against RGNEF and FUS/TLS revealed the presence of near complete co-localization between the 2 proteins [**Figure 3.7**]. Analysis focusing on only the brightest 2% of pixels revealed a complex of co-localized RGNEF and FUS/TLS-positive fibrils [**Figure 3.7**].

Analysis of double immunolabeling experiments performed using antibodies against RGNEF and the glial markers revealed no expression of RGNEF within glial cells. Representative photographs of double immunolabeling experiments performed using antibodies against RGNEF and GFAP as well as antibodies against RGNEF and IBA-1 can be seen in **Figure 3.8**.

Discussion and Concluding Remarks

With considerable evidence mounting in support of ALS being regarded as a disease of altered RNA metabolism, adding another player to the short list of RNA binding proteins that can be aberrantly expressed in ALS is critical. The most striking finding from this study is the near complete co-localization

Figure 3.6: A high degree of co-localization can be found between RGNEF and TDP-43 within ALS motor neurons, but not within control motor neurons.

Double immunolabeling experiments were performed using antibodies against RGNEF [Goat Polyclonal, 21st Century Biochemicals, 1:75, 20 hours, 4°C] and TDP-43 [Rabbit Polyclonal, Proteintech (Cat # 10782-2-AP), 1:500, 20 hours, 4°C] in human spinal MNs. In spinal MNs from healthy control tissues, TDP-43 was always localized to the nucleus, and RGNEF was always expressed faintly throughout the cytosol or in a polysomal pattern [A]. The inset in Figure A is a higher magnification view of the clearly defined boundaries and the lack of interaction between nuclear TDP-43 and cytosolic RGNEF. Within ALS tissues, however, spinal MNs can present with RGNEF-positive NCIs that are also immunolabeled with TDP-43 [B]. The inset of this figure provides a higher magnification view of filamentous NCIs that are both RGNEF and TDP-43-positive and seem to be interacting within the MN. Nuclear counterstaining was done using Hoechst 33342 and can be seen in blue. All scale bars represent 20 μm.

RGNEF, TDP-43

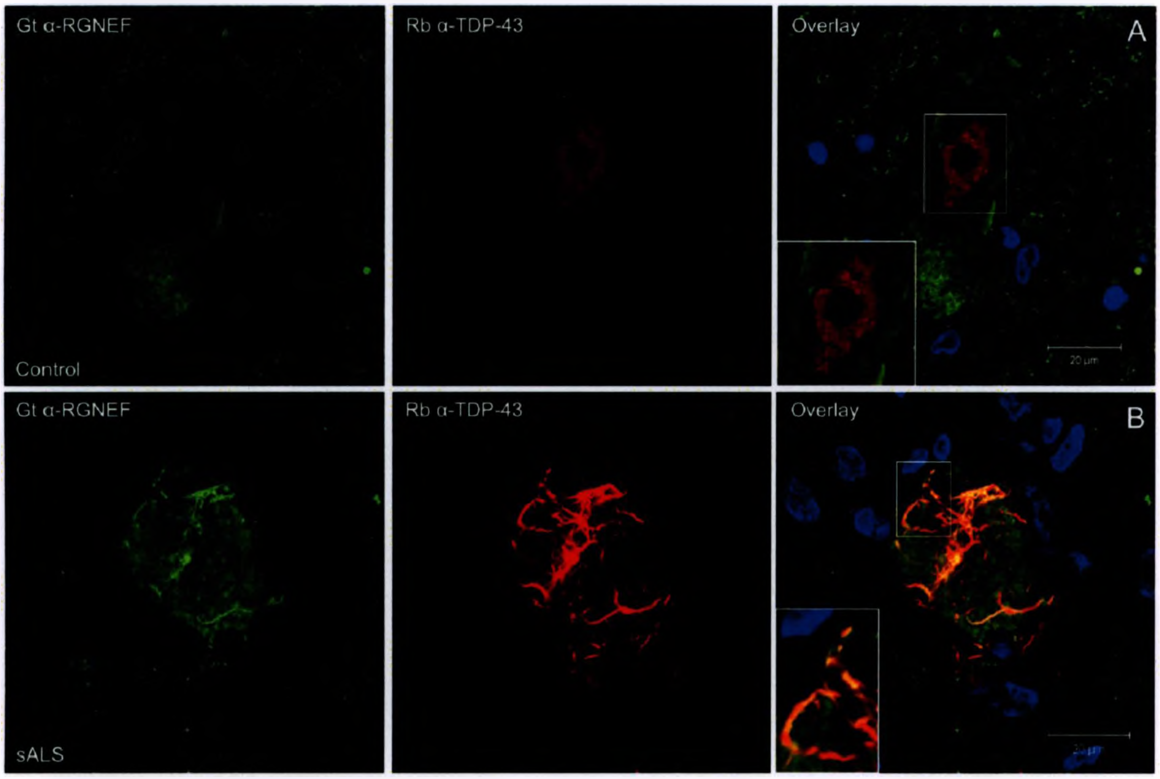

Gt α-RGNEF
Rb α-TDP-43
Overlay
A
20 µm
Control
Gt α-RGNEF
Rb α-TDP-43
Overlay
B
20 µm
sALS

Figure 3.7: A high degree of co-localization between RGNEF and FUS/TLS within ALS motor neurons, but not within control motor neurons.

Antibodies against RGNEF [Goat Polyclonal, 21st Century Biochemicals, 1:75, 20 hours, 4°C] and FUS/TLS [Rabbit Polyclonal, Proteintech (Cat # 11570-1-AP), 1:90, 20 hours, 4°C] were used in double immunolabeling experiments within human spinal MNs. Analysis revealed expected expression patterns in spinal MNs from healthy controls: mostly nuclear FUS/TLS with coarse granular RGNEF expression [A]. ALS tissue, however, contained spinal MNs that revealed high levels of co-localization between NCIs that were positive for both RGNEF and FUS/TLS [B]. This highly co-localized appearance is confirmed throughout multiple optical slices, and when looking at the brightest 2% of pixels of a co-localization channel throughout a Z-stack [B, inset], co-localization is clearly evident. Nuclear counterstaining, which was performed with Hoechst 33342, can be seen throughout this figure in blue. All scale bars represent 20 μm.

RGNEF, FUS/TLS

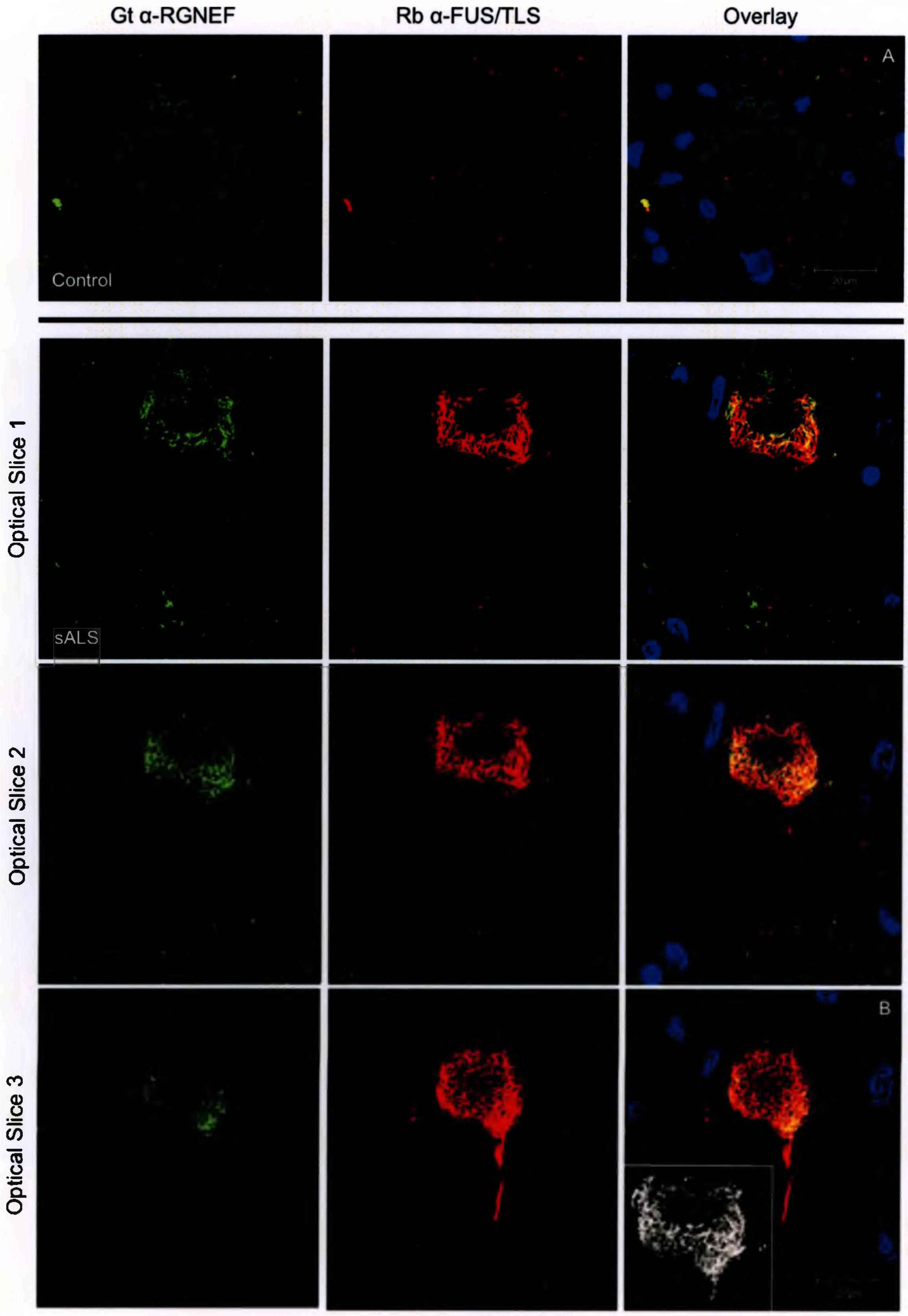
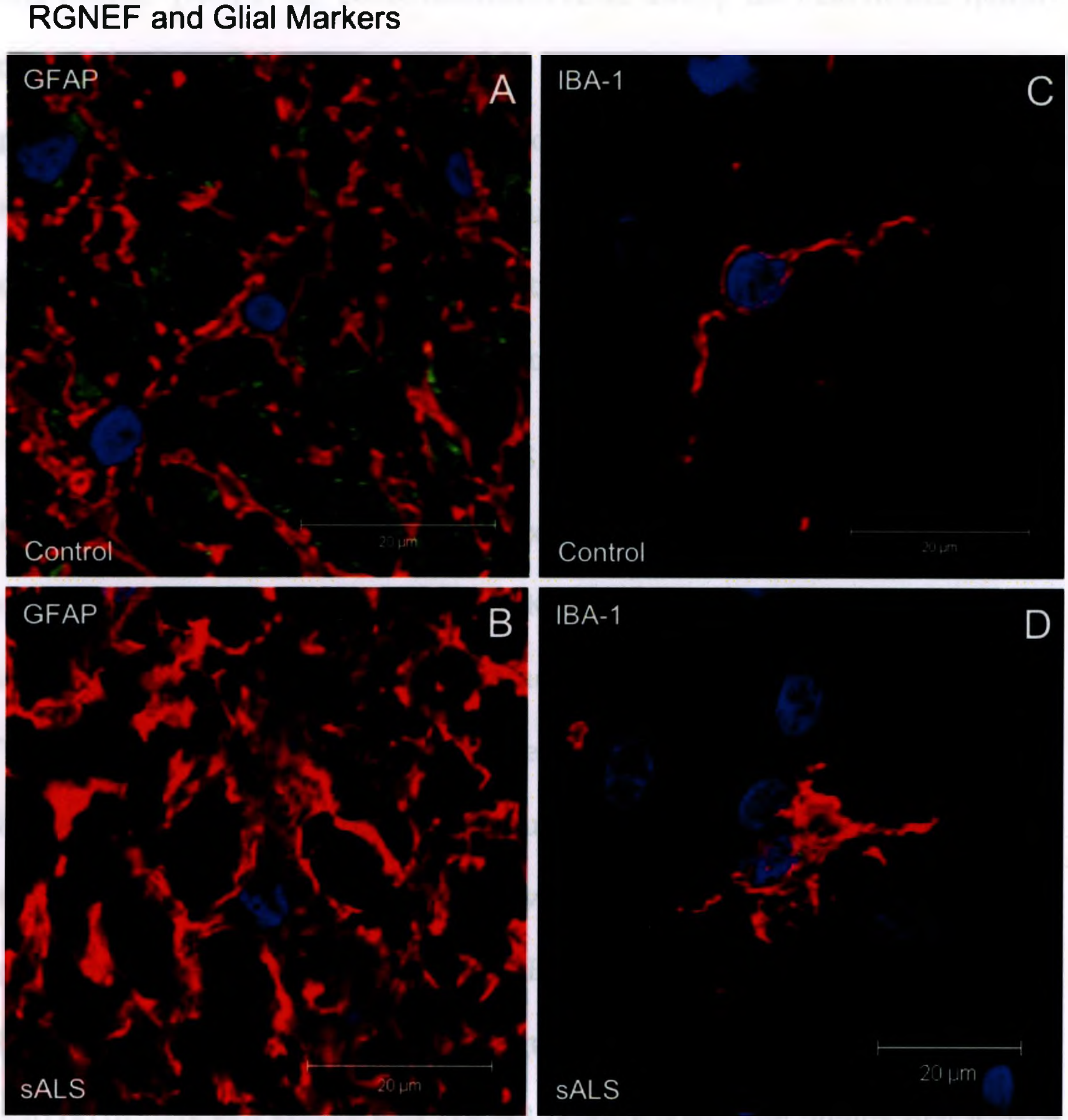

Gt α-RGNEF
Rb α-FUS/TLS
Overlay
A
Control
20 µm
Optical Slice 1
sALS
Optical Slice 2
Optical Slice 3
B

Figure 3.8: The lack of glial RGNEF expression within the human spinal cord.

Double immunolabeling experiments were performed using antibodies against RGNEF [Goat Polyclonal, 21st Century Biochemicals, 1:75, 20 hours, 4°C] [green] and the microglial marker IBA-1 [Rabbit Polyclonal α-IBA-1, Wako Chemicals (Cat # 019-19741), 1:1000, 20 hours, 4°C] [red], as well as between RGNEF [green] and the astrocytic marker, GFAP [Mouse monoclonal α-GFAP, BD Pharmingen (Cat # 556330), 1:500, 20 hours, 4°C] [red] in human spinal MNs. RGNEF is not expressed in astrocytes in tissue from either healthy controls or from ALS cases [A, B]. Additionally, RGNEF is not expressed in microglia in tissue from either healthy controls or from ALS cases [C, D]. All photos in this figure are overlayed and include accurate expression of RGNEF, and either GFAP or IBA-1. The blue fluorescence staining is nuclear counterstaining using Hoechst 33342. All scale bars represent 20 μm.


RGNEF and Glial Markers
GFAP
A
Control
20 µm
IBA-1
C
Control
20 µm
GFAP
B
sALS
20 µm
IBA-1
D
sALS
20 µm

between RGNEF and p62, as well as between RGNEF and ubiquitin. This evidence suggests that RGNEF-positive NCIs are being targeted for protein degradation via the proteasome degradation system. In 2006, when evidence was first presented showing that TDP-43 was being targeted for degradation in this manner (Arai *et al.*, 2006; Neumann *et al.*, 2006), the field of ALS quickly turned its focus to TDP-43 and the possibility of ALS being a TDP-43 proteinopathy. Now, with the knowledge that RGNEF and FUS/TLS-positive NCIs can be ubiquitinated in ALS, but not in controls, we are compiling convincing evidence that the misregulation of RNA is part of the pathogenicity of ALS, as opposed to the misregulation of a single RNA binding protein.

Recent work has shown FUS/TLS and TDP-43 interaction in a *Saccharomyces cerevisiae* yeast model expressing FUS/TLS and TDP-43, neither of which possess a yeast homologue (Kryndushkin *et al.*, 2011). We are not aware of any studies suggesting interaction between FUS/TLS and wild type TDP-43 in human tissue. There is evidence, however, indicating an interaction between mtTDP-43 and FUS/TLS in a human cell line (Ling *et al.*, 2010). This is the first evidence suggesting a convergence of 2 potentially pathogenic pathways in ALS. Interestingly, RGNEF-positive NCIs were shown to very intensely co-localize with FUS/TLS [**Figure 3.7**], and strong co-localization can also occur between RGNEF and TDP-43 [**Figure 3.6**]. The finding that RGNEF and FUS/TLS can be co-localized in NCIs suggests that they may be parts of the same pathogenic mechanism. Another possibility is that the sequestration of these proteins into filamentous inclusions within the cell is a coping mechanism for another cellular injury that may ultimately be leading to MN death. Similarly, the co-localization between RGNEF and TDP-43 suggests that these 2 proteins may be parts of a similar pathogenic mechanism, or that these inclusions represent a coping mechanism activated upon cellular injury. Important to note, too, is the fact that not all areas of an inclusion are necessarily labeled with each marker. Examples of this can be seen either in **Figure 3.6** or **Figure 3.7**, where there are portions of the TDP-43 and FUS/TLS inclusions, respectively, in addition to what is sequestered with RGNEF. This raises the possibility that the aberrant expression of RNA binding proteins is contributing to cell death via a convergent mechanism, but they may also be acting individually in some instances. Regardless of the mechanism of whether or not these inclusions do contribute to the pathogenicity of ALS, they do aggregate, and are certainly part of the pathology of ALS. This raises the question of what upstream trigger is causing RNA binding protein sequestration, and if this trigger may be the insult that has an early role in disease pathogenesis.

Finally, the observation that RGNEF is not expressed in active or inactive glial cells suggests that it does not play a role in the process of neuroinflammation. As mentioned, neuroinflammation is a normal aspect of innate immunity within the nervous system, and it has been shown to be increased in ALS. The priming and subsequent activation of astrocytes and microglia is evidence that this is the case, but the lack of RGNEF association suggests no role for RGNEF in this process.

Taken together, this study has, for the first time, characterized the intraneuronal expression of RGNEF. We have determined that RGNEF can be co-localized with translationally active polysomes within healthy tissues in a coarse granular expression pattern, and that a uniform cytoplasmic presentation is also a normal presentation. Importantly, we have shown strong evidence that RGNEF NCIs can be targeted for degradation within MNs, which is evidence that RGNEF may be involved in ALS pathogenesis. Lastly, the observation that RGNEF-positive NCIs can be co-localized with FUS/TLS and TDP-43-positive NCIs raises exciting possibilities for future work focusing on the role of RGNEF in ALS. This work provides strong reinforcement to the hypothesis that ALS is in fact a disease of altered RNA metabolism, as opposed to a disease of the misregulation of a single RNA binding protein.

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Chapter 4: General Discussion and Conclusions

I have tested the **hypothesis** that ***all variants of ALS are pathologically distinct and can be differentiated by spinal cord pathology using a panel of contemporary ALS-associated markers.*** In the process of testing this hypothesis, I have made several key observations, including:

- That p62 is a more robust immunohistochemical marker of proteasome-mediated degradation in ALS tissues than ubiquitin;
- That all variants of ALS can be characterized by the aberrant expression of RNA binding proteins;
- That the only pathologically distinct variant of ALS is mtSOD1-ALS, which is characterized by a unique protein expression pattern;
- That optineurin NCI formation is not a pathological marker of any variant of ALS;
- That RGNEF-positive NCIs are observed in all variants of ALS with the exception of mtSOD1-ALS, and that these inclusions can be co-localized with ubiquitin and p62; and,
- That RGNEF co-localizes with other ALS-associated, RNA binding proteins including TDP-43 and FUS/TLS in NCIs.

This is the first comprehensive study to analyze the expression of such a large group of ALS-associated proteins across a panel of ALS cases encompassing most of the known variants of ALS. My findings are unique and will have significant implications for our understanding of the pathogenesis of ALS. Beyond this, however, they also have direct clinical implications in the field of neuropathology as it relates to ALS diagnosis confirmation and the identification of novel cases of fALS.

There are several important implications to the finding that mtSOD1-ALS is a pathologically unique variant of ALS. First, this finding ties together pathological observations from other researchers. Other less comprehensive studies have noted disparity between mtSOD1-ALS and other single variants of the disease, but never has a study been designed to determine the existence of a pathologically unique variant of ALS. Second, although a toxic gain-of-function for mtSOD1 is the acquisition of RNA binding properties [destabilizing NFL mRNA], my findings raise the question of whether mtSOD1 can act completely independently of any other RNA binding protein in carrying out this aberrant function. With this question raised, as more work is done into the pathophysiology of ALS, it may be possible that different therapeutic targets could be designed to target different variants of the disease.

Critical to note is the standard practice for neuropathologists at the autopsy of an ALS case, which includes the application of a minimal immunohistochemical panel in order to confirm diagnosis. Never before has immunohistochemical evidence been presented that will give neuropathologists probable cause that they may be dealing with a case of mtSOD1-ALS. With mtSOD1-ALS being the most commonly seen type of fALS, the finding of p62-positive NCIs that are negative for the non-mtSOD1 RNA binding proteins should lead to genotypic analysis in an effort to identify new families that may be prone to fALS.

There were several other objectives met in the process of determining the unique characteristics of mtSOD1-ALS. Firstly, I observed that p62 is a more consistent marker of proteasome protein degradation than is ubiquitin. There have been many indications from the literature that p62 may be a more robust marker of the ubiquitin-proteasome system, although the claim has never been made explicitly (Shin, 1998; Mizuno *et al.*, 2006). Immunohistochemical expression of ubiquitin across the panel studied in this thesis proved more difficult than expression of p62, which resulted in a more precise expression pattern than ubiquitin and highly intense chromogen deposition.

I have also confirmed that ALS can be characterized by the aberrant expression of RNA binding proteins, providing strong support for the hypothesis that ALS is a disease of altered RNA metabolism. Given the main conclusion from this thesis, the most important observation is that altered RNA binding protein expression characterizes all variants of ALS, including mtSOD1-ALS. Although TDP-43, FUS/TLS, and RGNEF NCIs were not evident in mtSOD1-ALS cases, mutations in *SOD1* have been reported to confer RNA binding properties on the protein (Ge *et al.*, 2005; Lu *et al.*, 2007; Li *et al.*, 2009), and more recent studies have reported the ability of mtSOD1 to sequester key regulatory RNA binding proteins (Lu *et al.*, 2009). Given these findings and the knowledge that SOD1 can form cytoplasmic aggregates in mtSOD1-ALS cases, the conclusion can be drawn that ALS is a disease of altered RNA metabolism characterized by an aberrant expression of RNA binding proteins.

Finally, chapter 2 of this thesis presented evidence that the pathological expression of optineurin that has been reported amongst familial variants of ALS in Japan is not evident in either the sALS or fALS cases of our panel that bear no known mutations (Maruyama *et al.*, 2010). This suggests that alterations in optineurin expression or function are likely not contributors to the pathogenesis of ALS. This observation is consistent with recent studies (Hortobagyi *et al.*, 2011; Millecamps *et al.*, 2011; Sugihara *et al.*, 2011). The finding that optineurin pathology is not a consistent feature of either sALS or fALS is important because of initial interest in the development of mtOPTN animal models of ALS, in addition to interest in the development of NF-κB inhibitors being used as a possible treatment in ALS.

There were also several important findings in Chapter 3. Due to the novelty of RGNEF, it was first imperative to characterize its normal expression pattern. Once this was established, it became evident that RGNEF-positive NCIs were present in ALS cases, but never in controls. Importantly, RGNEF-immunoreactive NCIs could also be ubiquitin and p62 positive, providing evidence that RGNEF-positive NCIs are targeted for degradation via the proteasome degradation system. The finding is also significant because it provides yet another RNA binding protein that is both differentially expressed in ALS, but also directly involved in NFL mRNA metabolism. A similar observation regarding the first-discovered of these proteins (Arai *et al.*, 2006; Neumann *et al.*, 2006), TDP-43, created a frenzied research focus surrounding ALS being a TDP-43 proteinopathy. This was followed with the labeling of all non-nuclear TDP-43 immunostaining as "pathological" in ALS. Similar interest was generated by the finding that FUS/TLS-positive NCIs could also be ubiquitinated in ALS cases, but never in healthy controls (Deng *et al.*, 2010). We expect that our observations regarding the deposition of RGNEF immunoreactive aggregates that are also ubiquitinated across all non-mtSOD1 variants of ALS will generate similar interest. More critical, however, is the finding that RGNEF is the first known link between an RNA binding protein that is known to form ubiquitin and p62-positive NCIs in ALS that is also a central molecule in a cell signaling pathway.

Additionally, other important findings from Chapter 3 include the co-localization of RGNEF with both FUS/TLS and TDP-43 immunoreactive NCIs. This is not minor, but rather a very high degree of co-localization between RGNEF and FUS/TLS, as well as between RGNEF and TDP-43. This observation suggests that each of these 3 RNA binding proteins may be sequestered into the same degradative bodies as they await proteasome degradation. It is tempting to hypothesize that this sequestration is into RNA granules that are polymerized into skein-like and aggregate formations. Note also that the co-localization is not absolute as there are clear examples provided where RGNEF forms skeins independent of its association with either TDP-43 or FUS/TLS. This might suggest that there are additional proteins yet to be defined that will also co-localize with RGNEF.

Implications and Future Directions

This work has significant implications with respect to the future of ALS research within my lab and the field in general. First of all, as discussed, it has provided evidence that mtSOD1-ALS is pathologically a distinct variant of ALS. It is my hope that this finding will be applied in neuropathologists' practice in an effort to identify novel families that may be affected by ALS 1.

It is also crucial, however, to gain a more thorough understanding of the upstream causes of ALS so that the problems can be addressed before the aberrant biological processes manifest themselves as insoluble protein deposits. Evidence presented in this thesis suggests that the upstream events may indeed be interactions between RNA and proteins.

With respect to the future directions surrounding RGNEF, there are multiple avenues that could be pursued. Firstly, it will be important to determine whether mutations may be harboured within *RGNEF* that are associated with ALS. As ALS-associated mutations were discovered within *TARDBP* and *FUS/TLS* following the finding of similar intraneuronal pathology, it will be interesting to see if such mutations exist within *RGNEF.* Secondly, we need to understand how RGNEF-mediated RhoA signaling cascades may be altered in ALS, and how they may contribute to the misregulation of RNA and ultimately, ALS pathogenesis. Finally, and perhaps of greatest important to the field of ALS research, will be the need to critically examine the relationship between RGNEF, TDP-43, and FUS/TLS. The possibility of these 3 proteins contributing to ALS pathogenesis concomitantly is an intriguing one, and lends further support to the hypothesis that ALS is a disease of altered RNA metabolism characterized by aberrant expression of RNA binding proteins. It also clearly illustrates the fallacy in defining ALS as an "-opathy" of any single RNA binding protein.

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Appendices

Appendix A: Use of Human Subjects – Ethics Approval Notice

Western

Office of Research Ethics

The University of Western Ontario
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Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. M.J. Strong **Review Level:** Expedited
Review Number: 16852E **Revision Number:** 1
Review Date: May 07, 2010 **Approved Local # of Participants:** 21
Protocol Title: Neuropathological analysis of spinal motor neuron inclusions in sporadic and familial ALS.
Department and Institution: Clinical Neurological Sciences, London Health Sciences Centre
Sponsor: ALS RESEARCH
Ethics Approval Date: May 07, 2010 **Expiry Date:** January 31, 2013
Documents Reviewed and Approved: Additional Co-Investigator - Brian Keller
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. Joseph Gilbert
FDA Ref. #: IRB 00000940

Ethics Officer to Contact for Further Information

☐ Janice Sutherland (jsutherl@uwo.ca)	☐ Elizabeth Wambolt (ewambolt@uwo.ca)	☐ Grace Kelly (grace.kelly@uwo.ca)	☒ Denise Grafton (dgrafton@uwo.ca)

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cc: ORE File
LHRI