ABERRANT LOCALIZATION OF FUS/TLS AND TDP-43 TRIGGERS MISFOLDING OF HUMAN SOD1

by

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease caused by the progressive death of motor neurons. Recent studies report the presence of misfolded Cu/Zn superoxide dismutase (SOD1) in all ALS cases, and link the RNA-processing proteins, fused in sarcoma/translated in liposarcoma (FUS/TLS) and TAR-DNA binding protein 43 (TDP-43) to ALS. The phenotypes of SOD1-FALS and non SOD1-FALS and SALS, including cases with mutant and translocated FUS/TLS and TDP-43, are clinically indistinguishable. Therefore, **we hypothesized that FUS/TLS and TDP-43 gain new pathological functions upon their aberrant localization to the cytosol, which could lead to their participation in the misfolding of SOD1** in cells, as both events, independently, are associated with ALS pathology. **We further hypothesized that nuclear depletion of FUS/TLS and TDP-43 is associated with altered proteostasis and misfolding of SOD1**. Although several studies demonstrated a possible cause of ALS in the cytosolic trapping and aggregation of the predominantly nuclear FUS/TLS and TDP-43, no association with the misfolding of SOD1 has been previously explored. We used immunofluorescence microscopy and immunoprecipitation studies to demonstrate that expression of cytosolic FUS/TLS variants, but not wild type, is associated with misfolding of SOD1. Additionally, we find that over-expression of human wild-type and expression of cytosolic mutant TDP-43 are associated with the misfolding of SOD1. Although we find that both cytosolic FUS/TLS and TDP-43 are associated with presence of misfolded SOD1, only partial co-localization of the proteins can be determined, suggesting a possible transient association, which is consistent with independent propagation of SOD1 misfolding through a competent template. Our immunoprecipitation data also demonstrate for the first time a physical interaction between the pathogenic FUS/TLS and misfolded SOD1. We also used immunoblotting and immunoprecipitation studies to get preliminary data demonstrating that nuclear depletion of FUS/TLS and TDP-43 using siRNA leads to over-expression of SOD1 and its misfolding. These observations demonstrate for the first time a relationship between the presence of misfolded SOD1 and cytosolic expression of FUS/TLS and TDP-43 and their nuclear depletion. Both studies suggest

that all three key proteins implicated in ALS may participate in a common pathogenic process.

Preface

The hypotheses and study designs required for the studies in this thesis were fully researched and developed by Edward Pokrishevsky with approval by Dr. Neil Cashman. Other Cashman Lab members involved in this study were: Dr. Leslie I. Grad, Ms. Jing Wang and Mr. Masoud Yousefi.

Edward Pokrishevsky was responsible for formulating the original hypothesis and the overall design of this project (with the guidance of Dr. Cashman), and performed all the involved cloning of wild type and mutant FUS and TDP-43 constructs. Edward carried out all HEK293 and SH-SY5Y cell culture work, from initial seeding and maintenance to transfections. Edward performed all immunofluorescence staining, microscopy and image acquisition work. Edward performed the majority of immunoprecipitations in Chapter 2, all of the immunoprecipitations in Chapter 3, all of the immunoblotting in both chapters, SOD1 expression studies in the absence of FUS and TDP-43, and all final data analyses and quantifications. Edward also genotyped mouse embryos prior to preparation of primary neural cultures, carried out their transfections and stained for immunofluorescence studies. Finally, Edward wrote the entire thesis and the manuscript based on Chapter 2.

Dr. Grad was responsible for instructing Edward Pokrishevsky on how to perform and interpret immunoprecipitations studies, and performed part of the immunoprecipitations in Chapter 2. Ms. Wang prepared primary neural cultures and performed some of the genotyping of the mouse embryos prior to preparing primary neural cultures. Mr. Yousefi assisted in choosing and performing the proper statistical analysis on the immunoprecipitation data.

A version of Chapter 2 is submitted as a manuscript for peer review as: *Aberrant Localization of FUS and TDP-43 is Associated with human SOD1 misfolding.* Edward Pokrishevsky, Leslie I. Grad, Neil R. Cashman.

A version of section 1.7.2 was included in Generalization of the Prion Hypothesis to Other Neurodegenerative Diseases: An Imperfect Fit. Will C. Guest, J. Maxwell

Silverman, Edward Pokrishevsky, Megan A. O'Neill, Leslie I. Grad, and Neil R. Cashman. Accepted by Journal of Toxicology and Environmental Heath (JTEH) Part A.

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List of Abbreviations

α	Anti
aa	Amino acid
Ab	Antibody
ALS	Amyotrophic lateral sclerosis
bp	Base pairs
ΔX	Missing sequence X
DIV	Days *in vitro*
DMEM	Dulbecco's modified eagle medium
ΔNLS	Missing NLS
DOC	Deoxycholate
DSE	Disease specific epitope
ev	Empty vector
ESL	Electrostataic loop
fALS	Familial amyotrophic lateral sclerosis
FBS	Fetal bovine serum
FUS	Fused in sarcoma / Translated in liposarcoma
HEK293	Human embryonic kidney cells (HEK293FT)
hnRNP	Heterogeneous nuclear ribonucleoproteins
HRP	Horseradish peroxidase
HSP	Heat shock protein
IB	Immunoblot
IF	Immunofluorescence
Ig	Immunoglobulin
IP	Immunoprecipitation
mIgG	Mouse immunoglobulin
NES	Nuclear export signal
NGS	Normal goat serum
NLS	Nuclear localization signal
O_2^-	Superoxide radicals
PBS	Phosphate buffer solution

PBST	0.3% Triton X-100 in phosphate buffer solution
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PVDF	Polyvinylidene difluoride
PK	Protein kinase
rIgG	Rabbit immunoglobulin
ROS	Reactive oxygen species
rpm	Revolutions per minute
RRM	RNA recognition motif
sALS	Sporadic amyotrophic lateral sclerosis
SDS	Sodium dodecylsulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SOD1	Cu/Zn superoxide dismutase 1
SY5Y	SH-SY5Y human neuroblastoma cells
TDM	Template directed misfolding
TDP-43	TAR DNA-binding protein 43
WB	Western blot
wt	Wild-type

List of Amino Acid Codes

One letter code	Three letter code	Name of amino acid
A	Ala	alanine
C	Cys	cysteine
D	Asp	aspartic acid
E	Glu	glutamic acid
F	Phe	phenylalanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lysine
L	Leu	leucine
M	Met	methionine
N	Asn	asparagine
P	Pro	proline
Q	Gln	glutamine
R	Arg	arginine
S	Ser	serine
T	Thr	threonine
V	Val	valine
W	Trp	tryptophan
Y	Tyr	tyrosine

Acknowledgments

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I am thankful to other members of my committee, Dr. Yu-Tian Wang and Dr. Weihong Song. I thank them for finding the time in their busy schedules to meet with me and guide me through my project.

My thanks also go to members of the Cashman Lab. First and foremost, my gratitude goes to Dr. Leslie I. Grad. This project would not have been possible without him. I thank him for instructing me and for helping me with the extensive work required in my studies. I also wish to express my sincere gratitude and appreciation to Dr. Anat Yanai, who has taught me most of what I know about project design, many of the techniques used in this project, and for being a friend. This project would not have been possible without the help of Ms. Jing Wang, who unconditionally provided me with primary neural cultures for my studies, and Mr. Masoud Yousefi, who always found time to assist me with the required statistical analysis. Thank you goes also to all the other members of the Cashman Lab who believed in me and were willing to discuss the project at any time of their busy schedule: Dwayne Ashman, Li Li, Changiz Taghibiglou, Judith Silverman, Megan O'Neill, Ebrima Gibbs and Maja Filipovic.

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I would like to thank the Canadian Institutes of Health Research (CIHR) for awarding me with the Frederick Banting and Charles Best Canada Graduate Scholarships – Master's Award, PrioNET for the travel award to present my data at the Society of Neuroscience (SFN) annual meeting, Brain Research Centre for financing my Live Cell Microscopy course, and for Dr. Cashman's financial resources to support my work.

Finally, I would like to express my loving thanks to my wife, parents, sister, grandmother, and all of my dear friends, without whose support I would have never gone this far.

Dedication

This thesis is dedicated to my beloved parents, sister and grandmother: Alla, Semion, Karin and Tanya Pokrishevsky, and to my dear partner in life Oana Pokrishevsky for their endless love, support, encouragements and understanding.

"Medical science has proven time and again that when the resources are provided, great progress in the treatment, cure, and prevention of disease can occur."

Michael J. Fox

1. Introduction

1.1. Amyotrophic lateral sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS), more commonly known as Lou Gehrig's disease, is characterized by weakening and subsequent atrophy of muscles of the limbs, as well as muscles involved in speech, swallowing, and respiration, due to the degeneration of motor neurons that innervate these muscles (Trail, Nelson et al. 2004). More than a century after Dr. Jean-Martin Charcot first described it in 1869, ALS remains an incurable neuromuscular disease despite many efforts at elucidating its etiology and discovering a treatment for it. ALS afflicts approximately 30,000 individuals in North America, with 5,000 new cases reported per year (Van Den Bosch, Van Damme et al. 2006). Half of the affected individuals die within 3 years, with survival over 5 years being less than 20% (Cleveland 1999). It has been proposed that ALS is an autoimmune disease as antibodies reacting *in vitro* with gangliosides were found in sera of majority of ALS patients (Smith, Siklos et al. 1996), although it is not clear whether this is indeed the case (Drachman and Kuncl 1989). An excitotoxic theory of ALS pathogenesis has been also proposed (Van Den Bosch, Van Damme et al. 2006). According to the excitotoxic pathological process in ALS, abnormal functioning of glutamate receptors in motor-neurons results in high levels of Ca^{2+}, which increases the risk of mitochondrial damage and production of reactive oxygen radicals (Jaiswal, Zech et al. 2009). However, the more recent perception is that ALS belongs to a family of neurodegenerative disorders, including Alzheimer's and Parkinson's diseases, and the prion disorders, in which aggregated misfolded proteins are pathogenically implicated (Thompson and Barrow 2002; Cashman and Caughey 2004; Ramos and Ferreira 2005).

ALS involves the degeneration of both upper and lower motor neurons, which leads to weakening and eventual atrophy of muscles, ultimately resulting in death (Chen, Meininger et al. 2009). Electromyographic studies have shown that healthy neurons can partially reinnervate the denervated muscles in the early stages of ALS, as the afflicted neurons do not all die simultaneously (Bek, Kasikci et al. 2009). Cytologically,

degenerating neurons exhibit loss of dendrites, formation of cytoplasmic inclusions and derangement of the cytoskeleton (Leung, He et al. 2004). The disease is remarkably specific for motor neurons, leaving ALS patients to suffer with their sensory, emotional and cognitive abilities largely unaffected (Gu, Farina et al. 2009). This selectivity could be attributed to the motor neural high content of the Cu/Zn Superoxide Dismutase protein, which has a long half life, and is subject to occupational hazard of detoxifying mitochondrial superoxide (Pardo, Xu et al. 1995).

ALS possibly represents a collection of very closely related disorders resulting from numerous causes, which may be all due to a common pathological process. There are three recognized forms of ALS: non inherited sporadic ALS (SALS), inherited familial ALS (FALS), and Guamanian ALS, a nearly extinct form of ALS observed in Guam and the Trust Territories of the Pacific (Tandan and Bradley 1985; Stone 1993). Despite the absence of apparent hereditary components in SALS, which accounts for 90% of all ALS cases, several environmental factors have been linked to it. Head injuries, cigarette smoking and exposure to heavy metals have all been linked to detrimental effects on the human body, however only recently have these factors been associated with sporadic ALS (Schmidt, Kwee et al. 2010). Familial ALS has a genetic component and accounts for ~10% of all ALS cases. Many genes have been linked to FALS, but SOD1, TAR-DNA binding protein 43 (TDP-43), and Fused in Sarcoma/Translated in Liposarcoma (FUS/TLS) are widely acknowledged as the most well-known proteins implicated in this disease (Lagier-Tourenne and Cleveland 2009). Clinically, FALS cases are very similar to SALS (Shaw 2010), however the mean age of onset for FALS occurs about 10 years earlier than for SALS (Valdmanis and Rouleau 2008). As with other neurodegenerative diseases, given the predisposition and the proper environmental conditions, the probability of developing familial or sporadic form of ALS increases with age (Riggs 1998) (**Figure 1**). Gender has been documented to be a factor in the likelihood of developing ALS, with male:female ratio of 3:2 (Pasinelli and Brown 2006). Oxidative stress is also thought to play a role in ALS, either as a consequence of genomic mutations leading to defective elimination of free radical (Barber, Mead et al. 2006), or as a standalone cause (Mitsumoto, Santella et al. 2008).

Cases of Guamanian ALS were diagnosed between 1950s and 1960 in Guam with an incidence rate of 50 times higher than anywhere else in the world (Hirano, Malamud et al. 1966). Uniquely, people suffering from Guamanian ALS, also presented symptoms consistent with Parkinson and Alzheimer diseases. Postmortem analysis of 63% of Guamanian ALS patients tested, showed evidence of neuronal loss and de-pigmentation of the substantia nigra, a characteristic of Parkinson's disease (Esiri, Lee et al. 2004). Neurofibrillary tangles, a postmortem characteristic of Alzheimer's disease, were also present in these patients, although in a wider distribution of degenerating neurons (Guiroy, Mellini et al. 1993). Although the frequency of Guamanian ALS among indigenous peoples originally suggested a familial component, it is now widely believed that diet consisting of cycad nut flour is responsible for the high incidence of Guamanian ALS (Spencer 1987; Chen, Craig et al. 2002).

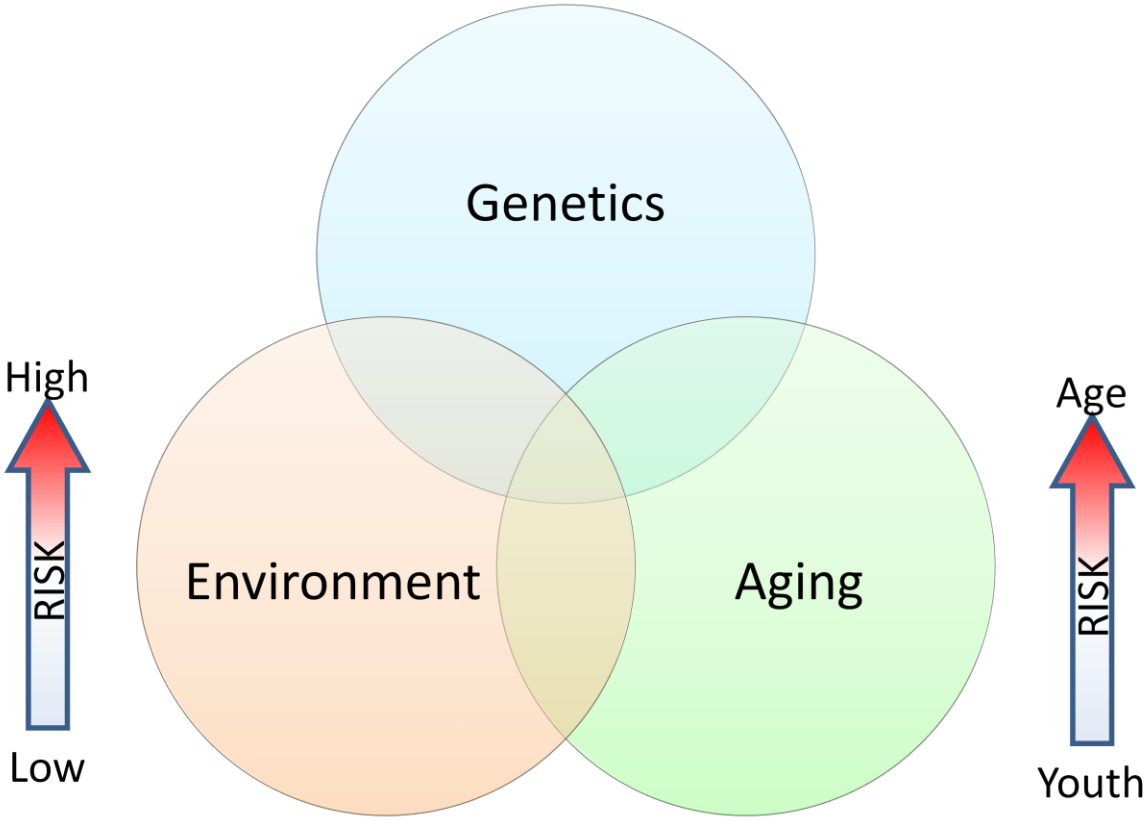

Figure 1: Factors to consider in ALS

As is the case with several other neurodegenerative diseases, ALS is believed to occur due to a convergence of factors. Genetic variation in genes including Cu/Zn Superoxide Dismutase (SOD1), Fused in Sarcoma/Translated in Sarcoma (FUS/TLS), TAR DNA binding protein 43 (TDP-43) causes ALS in individuals. Late disease onset age, in the 50s, indicates that the risk of getting ALS increases as we age. Gender has also been documented as a risk factor for sporadic ALS (Pasinelli and Brown 2006). Several environmental factors increase the risk of getting this disease: immunological response, oxidative stress (Barber, Mead et al. 2006), head trauma (Schmidt, Kwee et al. 2010), smoking (Schmidt, Kwee et al. 2010), and exposure to diets rich in heavy metals (Spencer 1987).

1.2. Genes linked to fALS: Cu/Zn superoxide dismutase

Mitochondrial respiration is required for most living organisms to maintain healthy cell processes and in order to survive. However, several byproducts generated during this process, such as superoxide, a reactive oxygen species, can be damaging to cellular macromolecules such as nucleic acids, lipids and proteins (Nohl 1994). Superoxide radicals are also generated by immune cells, primarily neutrophils, to fight intruding pathogens (West, Sinclair et al. 1983). Due to the highly toxic nature of these radicals, nearly all organisms living in the presence of oxygen, express isoforms of superoxide dismutase, a group of enzymes that form the first line of defense against superoxide radicals(Halliwell 1978). An important member of the superoxide dismutase family is the Cu/Zn Superoxide Dismutase (SOD1).

About 20% of fALS and 3% of sALS cases are associated with mutations in the gene encoding SOD1 (Rosen 1993; Sreedharan, Blair et al. 2008), an abundant and ubiquitously-expressed soluble cytosolic protein. When properly folded and fully functional, SOD1 enzyme is an intracellular antioxidant that is extremely efficient at catalyzing the neutralization of free radicals (Aricioglu, Bozkurt et al. 2001). As shown in **Figure 2**, in one complete cycle, SOD1 catalyzes the conversion of two superoxide radicals, O_2^-, into molecular oxygen and hydrogen peroxide (Fridovich 1978). Later, peroxidases and catalases in the cell convert the less harmful hydrogen peroxide into water and oxygen (Proctor and Reynolds 1984).

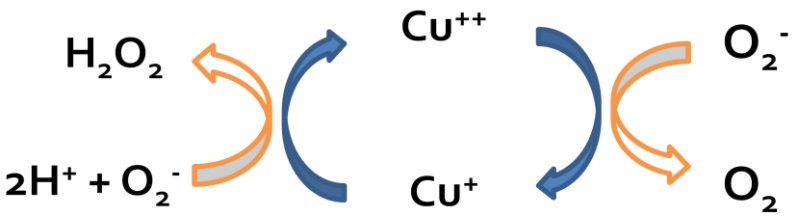

Figure 2: Activity of SOD1

A Copper ion, lying within the active site of SOD1, dismutes superoxide in the cytoplasm. It catalyzes the conversion of 2 highly reactive superoxide molecules into oxygen and hydrogen peroxide.

Although SOD1 is only 154 amino acid long, over 100 distinct mutations have been identified in all five exons (Banci, Bertini et al. 2008). In order to structurally stabilize the enzyme and to allow its catalytic activity, SOD1 binds zinc and copper co-factors. Specifically, the catalytic copper is found in the active site and is bound by four histidines, His46, His48, His63 and His120 when oxidized and His46, His48 and His120 when in the reduced form (Rakhit and Chakrabartty 2006). In its native form, SOD1 is a homo-dimer.

1.2.1. SOD1 misfolding in fALS and sALS

Proteins, and enzymes in particular, need to form proper secondary, tertiary and quaternary structures in order to function properly. Various factors, such as heat, mutations in coding regions or lack of chaperone activity, can lead to protein misfolding. Evidence from recent years indicates that protein misfolding is involved in the pathological mechanisms of a number of neurodegenerative diseases, including prion diseases, Parkinson's disease, Alzheimer's disease and Huntington disease (Soto 2003; Soto and Estrada 2008; Guest, Silverman et al. 2011). Although misfolded proteins tend to form aggregated deposits, it is not yet established whether it is the gain of neurotoxic function of the misfolded proteins and their aggregates, or the lack of physiological protective functions that cause these diseases (Winklhofer, Tatzelt et al. 2008). It is possible that a combination of gain and loss of function due to protein misfolding leads to disease pathogenesis (Pesiridis, Lee et al. 2009; Kabashi, Lin et al. 2010; Xu, Gendron et al. 2010).

As many fALS linked mutations in SOD1 confer one or more toxic functions on the protein, the misfolding of SOD1 has been an important topic in recent years as a causative factor for ALS (Rosen 1993; Kerman, Liu et al. 2010). Recent studies by Grad, *et al.* (unpublished), Forsberg, *et al.*, and Bosco, *et al.* have shown that oxidized and misfolded SOD1 is present in both FALS and SALS patient samples by using conformation-specific antibodies that detect only misfolded SOD1 (Bosco, Morfini et al. 2010; Forsberg, Jonsson et al. 2010). In other words, mutant SOD1 and aberrantly oxidized (or otherwise misfolded) wild type SOD1 share conformational epitopes that are not present in the natively folded wild type SOD1. Therefore, although genetically

mutated SOD1 is absent from SALS, misfolding of wild type SOD1 could be adequate to initiate and propagate the pathogenesis of ALS through a common biochemical pathway involving misfolded SOD1.

1.2.2. Specific antibodies against misfolded SOD1

Our laboratory identified immunological epitopes that become exposed upon misfolding or metal depletion, yet are inaccessible to antibody binding in the context of its native structure, and are potentially involved in SOD1 aggregation. Antibodies were raised against linear peptides composed of amino-acid sequences corresponding to two disease specific epitopes (DSEs): an oxidized version of a previously identified DSE (Rakhit, Robertson et al. 2007) – the dimer interface epitope (DSE1a), and a segment of the SOD1 electrostatic loop (ESL; DSE2), a structural element that is extruded in amyloid-like fibrils and nanotube crystal structures of misfolded SOD1 (Elam, Taylor et al. 2003).

Mouse monoclonal antibodies against two such hypothesis-driven epitopes, which have been subsequently experimentally validated, will be used in these studies: 10C12 and 3H1 (**Figure 3**). 10C12 was raised against a linear peptide (DSE1a) corresponding to dimer interface residues 145-151, with cysteic acid in the place of a cysteine at position 146. It was reasoned that natively dimeric SOD1 becomes monomerized, therefore exposing its dimer-interface domain (Rakhit, Crow et al. 2004; Rakhit, Robertson et al. 2007), with subsequent reduction of the exposed Cys57-Cys146 intrachain disulfide bond, followed by irreversible oxidation of the free sulfhydyl cysteine at Cys146. 3H1 was raised against the linear peptide, DSE2 corresponding to SOD1 residues 125-142, which comprise a segment of the SOD1 electrostatic loop, a region that becomes unstable during SOD1 misfolding (Elam, Taylor et al. 2003; Strange, Antonyuk et al. 2003; Vande Velde, Miller et al. 2008). **Figure 4** shows the specificity of 3H1 antibody in detecting misfolded SOD1 in cells transfected with a plasmid expressing either human wild type SOD1 or the misfolded mutant G93A SOD1. Since 10C12 does not perform well in immunofluorescence staining in our hands, only 3H1 is used for immunocytochemistry stains, while both antibodies are used in immunoprecipitation studies.

Figure 3: SOD1 DSE epitopes

Illustration of the primary structure of SOD1 and the regions recognized as disease specific epitopes, DSE1a and DSE2. 10C12 and 3H1 are the antibodies raised against DSE1a and DSE2, respectively. Adapted from Grad *et al.* (2011).

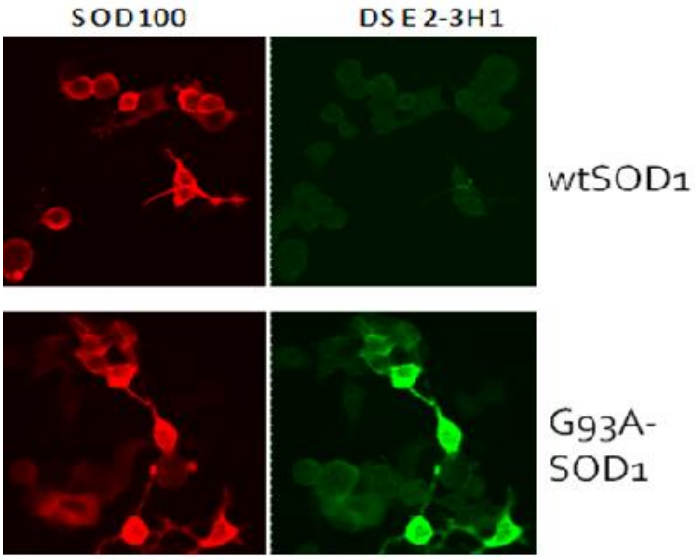

Figure 4: DSE antibody specificity

Staining of human wtSOD1 and G93A-SOD1 transfected NSC-34 cells with the pan-SOD1 polyclonal antibody, SOD100, and 3H1. While SOD100 detects the endogenous human SOD1 in all cells, the bright fluorescence staining corresponds to the presence of the transfection-driven SOD1. 3H1 antibody identifies only misfolded forms of SOD1. Transfection of wtSOD1 alone (top left), does not cause misfolding of SOD1, as demonstrated by the absence of 3H1 staining (top right). G93A-SOD1, a common mutation in FALS patients, also transfects well into NSC-34 cells (bottom left), and causes SOD1 misfolding, as detected by 3H1 staining (bottom right).

1.3. Genes linked to fALS: TAR DNA-binding protein-43

Although SOD1 was the first specific gene to be linked to ALS, mutations in SOD1 only account for 20% of all fALS cases. The formation of ubiquitin-positive inclusions is a common feature of many neurodegenerative diseases (Lansbury and Lashuel 2006), however the mechanism of formation and the composition of these inclusions remained unknown until recently. In 2006, Neumann *et al.* found another protein linked to FALS: TAR DNA-binding protein 43 (TDP-43). TDP-43 associates with ubiquitin-positive, tau- and α-synuclein- negative inclusions, which are hallmarks of ALS (Neumann, Sampathu et al. 2006). TDP-43, consisting of 414 amino acids, is predominantly a nuclear protein and a major constituent of neuronal and glial cytoplasmic inclusions in SALS patients (Arai, Hasegawa et al. 2006; Davidson, Kelley et al. 2007; Lagier-Tourenne and Cleveland 2009). TDP-43 inclusions are now recognized as a defining characteristic in ALS cases where SOD1 is not mutated (Lagier-Tourenne and Cleveland 2009). TDP-43-positive inclusions are also observed in other neurodegenerative disease, including Alzheimer's (Amador-Ortiz, Lin et al. 2007), Huntington's (Schwab, Arai et al. 2008) and Parkinson's (Nakashima-Yasuda, Uryu et al. 2007) diseases. As shown in **Figure 5**, TDP-43 contains several RNA binding domains: RRM1, RRM2, arginine/ glycine rich domains, and the C-terminal glycine-rich domain, where most ALS linked mutations occur. Although the roles of TDP-43 in cell metabolism are not fully known, it is involved in gene regulation, mRNA splicing and localization, as well as having a purported role in neuronal activity responses in the dendrites of hippocampal neurons (Wang, Wu et al. 2008; Johnson, Snead et al. 2009). Disease-associated TDP-43 is abnormally cleaved into the cytotoxic 25- and 35-kDa C-terminal fragments (Rutherford, Zhang et al. 2008), and becomes hyperphosphorylated, ubiquitylated and prone to aggregation (Kim, Shanware et al. 2010), possibly due to its C-terminal protein interaction domain. A total of three dozen mutations, which account for ~3% of fALS and ~1.5% of sALS, have been identified to date in ALS patients (Lagier-Tourenne and Cleveland 2009). Given that TDP-43 inclusions are recognized as a common characteristic of most ALS patients, it is of significant interest to determine the functional role of TDP-43 in disease pathogenesis.

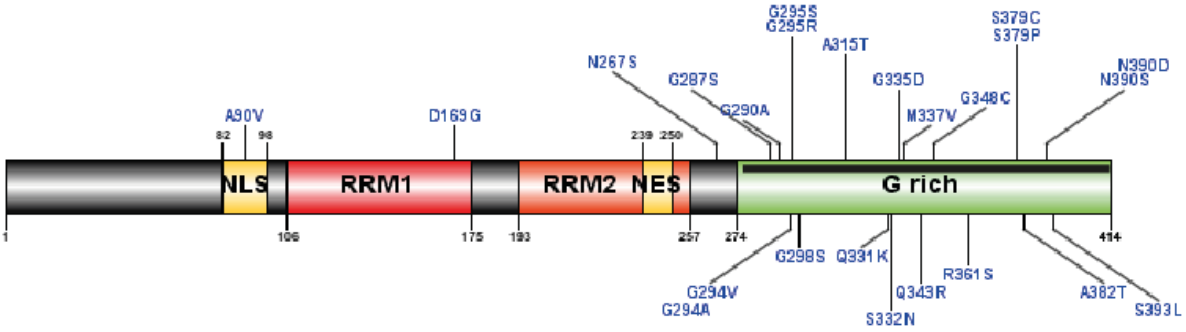

Figure 5: TDP-43

A schematic representation of TDP-43 and its functional domains: two RNA/DNA Recognition Motifs (RRM1 and RRM2) and one protein-protein interaction G-rich domain. To function as a shuttling protein, TDP-43 also contains nuclear localization and nuclear export sequences. Most mutations in this protein occur in the G-rich domain, which is the predicted yeast prion like domain (black bar, more on TDP-43 as a yeast prion on page 18). Data compiled from www.uniprot.com.

1.4. Genes linked to fALS: fused in sarcoma / translated in liposarcoma

The discovery of the association between TDP-43 and ALS inspired researchers to search for other ALS-linked DNA and RNA binding proteins. In 2009, back-to-back studies identified mutations in a functionally related protein, fused in sarcoma, originally named translated in liposarcoma (FUS/TLS or FUS), that were linked to ALS (Kwiatkowski, Bosco et al. 2009; Vance, Rogelj et al. 2009). Mutations in FUS have been identified in 3-5% of non-SOD1, non-TDP-43 FALS and ~1% of SALS cases (Hewitt, Kirby et al. 2010; Rademakers, Stewart et al. 2010). To properly bind DNA and RNA, the 526 amino acid long FUS protein harbors several important domains: a RNA recognition motif (RRM domain), and a zinc finger domain surrounded by two arginine/glycine-rich (RGG) regions (Morohoshi, Ootsuka et al. 1998). In addition, FUS contains a long N-terminal glutamine/glycine/serine/tyrosine (Q/G/S/Y)-rich region, followed by a glycine-rich domain (**Figure 6**). These domains are proposed to be involved in protein-protein interactions (Lagier-Tourenne and Cleveland 2009; Pesiridis, Lee et al. 2009). Although predominantly nuclear, FUS continuously shuttles between the nucleus and cytoplasm via its nuclear export and non-classical C-terminal nuclear localization signals (Zinszner, Sok et al. 1997; Zakaryan and Gehring 2006; Dormann, Rodde et al. 2010). Although, the precise functions of FUS have yet to be characterized, its structural similarity to TDP-43, along with recent studies focused on TDP-43 and FUS functions, suggest an involvement in transcription regulation, RNA and microRNA processing, and DNA repair, as well as regulation of neuronal spine morphology (Fujii, Okabe et al. 2005; Mackenzie, Rademakers et al. 2010).

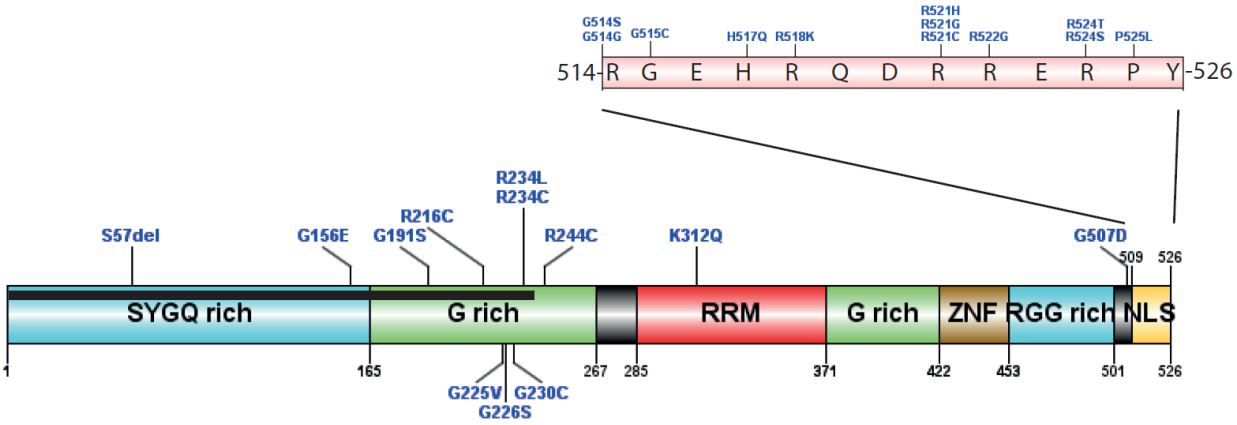

Figure 6: FUS

Schematic representation of the FUS protein. FUS contains a RNA Recognition Motif (RRM), G- and SYGQ-rich domains, and nuclear localization and export signals. Most mutations in this protein occur in the nuclear localization signal, some of which significantly affect its proper predominantly nuclear localization. Other common mutations fall in the G- and SYGQ- rich domains, which are the predicted yeast prion like regions (black bar, more on FUS as a yeast prion on page 18). Data Compiled from Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009; and www.uniprot.org.

1.5. Genes linked to fALS: others

In addition to mutations in SOD1, TDP-43 and FUS mutations in proteins such as Alsin (Gene: ALS2), VAPB (vesicle associated membrane protein B), and Senataxin (Gene: SETX) have been linked to cases of ALS (Daoud, Valdmanis et al. 2009). Alsin, which is comprised of three guanine nucleotide exchange factor domains, is expressed in a variety of tissues and plays a role in endosome trafficking. Alsin promotes neurite outgrowth in neuronal cultures, and may play some neuroprotective role against the cytotoxicity of mutant SOD1 (Hadano, Kunita et al. 2007). Although the normal function of VAPB is not clear, it has been shown to take part in the unfolded protein response, a reaction of the endoplasmic reticulum to the accumulation of misfolded proteins (Suzuki, Kanekura et al. 2009). Mutations in VAPB predispose motor neurons to prematurely die due to ER stress (Suzuki, Kanekura et al. 2009). Senataxin is a putative DNA / RNA helicase involved in the coordination of transcriptional events and DNA repair (Suraweera, Lim et al. 2009). Considering the multitude of different proteins with widely-varying functions implicated in its pathogenesis, ALS is clearly a complex disease with many potential contributing factors.

1.6. Similarities and interactions between FUS and TDP-43

In addition to being genetic causes for ALS, FUS and TDP-43 also share similar structural and functional features (**Figure 7**). Structurally, both proteins are similar to other heterogeneous ribonucleoproteins (hnRNP), containing RNA recognition motifs (RRM; RRM in FUS; RRM1 and RRM2 in TDP-43), nuclear localization signals, and G-rich regions. While most mutations in TDP-43 occur in its G-rich domain, the majority of the mutations in FUS occur in its C-terminal nuclear localization signal (NLS). However, gene mutations in these proteins cause convergent ALS phenotypes in humans, which led Kim *et al.* to discover that FUS and TDP-43 physically bind in a common biochemical complex (Kim, Shanware et al. 2010). FUS and TDP-43 can bind RNA, as well as single and double stranded DNA, and are both involved in RNA processing, including transportation, localization and alternative splicing (Lagier-Tourenne, Polymenidou et al. 2010; Strong 2010). Improper alternative splicing can also be a major cause of neurological diseases (Dredge, Polydorides et al. 2001; Grabowski and Black 2001; Tazi, Bakkour et al. 2009). As we now understand it, damage in transportation of cargoes through the axon can display axonal pathologies including abnormal accumulation of proteins and organelles in multiple neurological diseases, such as Alzheimer's disease and Parkinson's disease, as well as in ALS (Roy, Zhang et al. 2005; De Vos, Grierson et al. 2008). In neurons, FUS and TDP-43 actively shuttle mRNAs from the nucleus to the cytoplasm, and to the dendrites for local translation (Sutton and Schuman 2006; Wang, Wu et al. 2008). Therefore, FUS and TDP-43 might be regulating translation and transportation of mRNA of proteins essential for promoting neuronal survival and preventing cellular death in ALS.

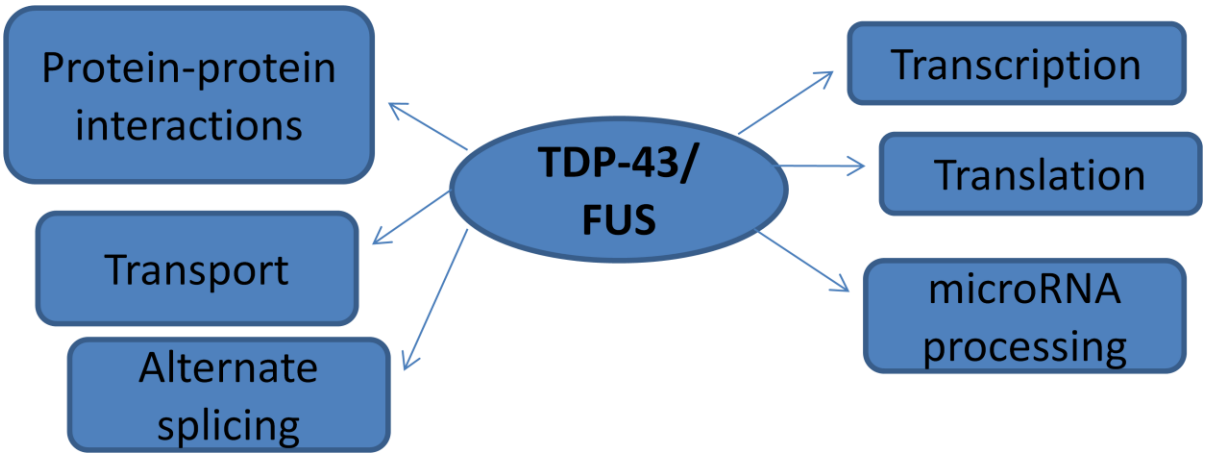

Figure 7: Functions of FUS and TDP-43 in the cell

FUS and TDP-43 form a cellular biochemical complex responsible for binding and processing of RNA and DNA. Recent research suggests their involvement in DNA transcription, RNA translation, alternate splicing, microRNA processing, and the transportation of mRNA for local translation.

1.7. ALS as a prion-like disease

The clinical observation that ALS propagates systematically, in a spatio-temporal manner, through the neuroaxis (Ravits and La Spada 2009) led some to hypothesize a prion-like mechanism of propagation for ALS. The activity of SOD1, FUS and TDP-43 as prion-like proteins in ALS is discussed below.

1.7.1. SOD1 as a prion-like molecule

The field of infectious diseases that included certain viruses, bacteria, fungi, protozoa and parasites, was revolutionized 30 years ago, when prions were first discovered (Prusiner 1982). For the first time, it was shown that that a nucleic acid-independent exogenous infectious agent can transmit disease via a principle of template protein misfolding. Although the exact mechanism of action remains elusive, we know that in order for prion diseases to propagate, a misfolded version of the prion protein must come into direct contact with native form, and misfold it via a process called template directed misfolding (TDM) (Paramithiotis, Pinard et al. 2003). Misfolded prion protein can be viewed as a transmissible infectious agent that catalyzes conversion of the normal prion protein to a pathological form. Transmittable prion diseases can affect various mammals including humans (Kuru, fatal familial Insomnia, and Creutzfeldt-Jakob disease), and cattle (bovine spongioform encephalopathy, also known as the mad cow disease) (Collinge 2001). Recently, several neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Huntington's disease and ALS have been proposed to be re-categorized as prion-like diseases (Cushman, Johnson et al. 2010).

SOD1, a major player in fALS, has been shown to monomerize and assume misfolded conformations that can rapidly form amyloid-like fibrils resembling those formed by misfolded prion proteins (Kelly 1997; Chia, Tattum et al. 2010). Mutant, misfolded SOD1 has also been shown to be efficiently exported and imported by cells (Sendtner 2006; Gomes, Keller et al. 2007; Munch, O'Brien et al. 2011). Additional data

from the Cashman Lab[1] show that wtSOD1 protein can be induced to misfold by misfolded mutant SOD1 within living cells and that this misfolding can be propagated intercellularly as well (Cashman, Grad et al. 2009). Genetic mutation is an obvious mechanism by which protein misfolding can occur, however other factors can also be responsible, including, alternative splicing (Romero, Zaidi et al. 2006), cellular stress (Vabulas, Raychaudhuri et al. 2010) or non-native protein-protein interactions such as those arising from gain or loss of function of improperly localized proteins (Huang and Liu 2010).

1.7.2. FUS and TDP-43 as yeast-like prions[2]

Although the existence of mammalian prions was known since the early 1980's, it was not until 1994 that prions were identified in *Saccharomyces cerevisiae* (Wickner 1994). Unlike human PrP that is typically recognized as a causative agent for neurological diseases (Collinge 2001), it is contentious as to whether the yeast prion can cause disease or is merely an adaptation mechanism to environmental stresses (Halfmann, Alberti et al. 2010). However, it is accepted that both yeast and human prion proteins can convert between structurally and functionally two different states, with one or more of these states being infectious (Paramithiotis, Pinard et al. 2003; Halfmann, Alberti et al. 2010). To screen for more prion-like protein sequences in yeast, Alberti *et al.* developed a hidden Markov Model algorithm based on experimentally known yeast prion sequences (Alberti, Halfmann et al. 2009), but not those for human PrP or fungal HET-s prions. Screening the entire database of known human proteins using this hidden Markov Model algorithm ranks FUS and TDP-43 15th and 65th, respectively, as predicted prions (Cushman, Johnson et al. 2010). Cushman *et al.* used FoldIndex, a

[1] *Intermolecular transmission of SOD1 misfolding in living cells.* Leslie I. Grad, Will C. Guest, Anat Yanai, **Edward Pokrishevsky**, Megan A. O'Neill, Ebrima Gibbs, Valentyna Semenchenko, Masoud Yousefi, David Wishart, Steven S. Plotkin, and Neil R. Cashman. Currently under review at Proceedings of the National Academy of Sciences (PNAS).

[2] A version of this text was included in *Generalization of the Prion Hypothesis to Other Neurodegenerative Diseases: An Imperfect Fit*. Will C. Guest, J. Maxwell Silverman, **Edward Pokrishevsky**, Megan A. O'Neill, Leslie I. Grad, and Neil R. Cashman. Accepted by Journal of Toxicology and Environmental Heath (JTEH) Part A.

bioinformatics tool that predicts intrinsic unfolding of a certain amino acid sequence (Prilusky, Felder et al. 2005), to determine the regions in FUS and TDP-43 that are prone to misfolding.

Not surprisingly, the C-terminal region in TDP-43, specifically amino acids 277-414, was highlighted as most prone to misfolding (Cushman, Johnson et al. 2010). This terminus includes the glycine-rich domain, that is proposed to be involved in protein-protein interactions (Mousavi and Hotta 2005). The vast majority of mutations identified to date in TDP-43 fall within the glycine-rich domain. These mutations may represent an additional destabilizing stress on a region that is already prone to misfold. Mediated via its C-terminus, TDP-43's spontaneous aggregation into structures *in-vitro* have been reported to resemble TDP-43 deposits in degenerating neurons in ALS (Johnson, Snead et al. 2009). In neuroglioma cells, the 25-kDa C-terminal TDP-43 fragment resulting from caspase-3 cleavage is predicted to be a prion-like domain using the hidden Markov Model algorithm. The cleaved 25-kDa fragment can induce cell death through a toxic gain of function, via formation of toxic, insoluble and ubiquitin positive aggregates (Zhang, Xu et al. 2009). Conformation-specific antibodies against C-terminal fragment of TDP-43 confirmed its presence in pathological inclusions of human TDP-43 proteinopathies (Rutherford, Zhang et al. 2008; Zhang, Xu et al. 2009). Therefore, the presence of inclusions containing the toxic 25kDa C-terminal fragment of TDP-43 is detrimental to proper cellular functional and survival.

In FUS, the N-terminal region, specifically amino acids 1-239, was predicted as the region prone to misfolding (Cushman, Johnson et al. 2010). This part of the protein contains both glycine- and Q/G/S/Y-rich domains. Although several mutations exist in the Q/G/S/Y-rich domain in FUS, most mutations occurs in the C-terminal NLS, which was not predicted as misfolding prone by the FoldIndex tool. Mutation in the NLS may lead to the inability of transportin, a protein that mediates the shuttling of FUS (Dormann, Rodde et al. 2010), to properly bind and transport FUS. Dormann *et al.* also showed that if cultured cells expressing a primarily cytoplamic mutant of FUS, P525L, were heat shocked at 44°C, FUS joins cytoplasmic aggregates that are associated with

cellular responses to stress, also known as stress granules. Although the exact functions of stress granules are not well understood, some have proposed that these granules are formed as part of a mRNA stabilizing mechanism in times of stress (Buchan and Parker 2009), as well as to redirect all the available cellular resources to counter the stress (Anderson and Kedersha 2008). Merging the findings that 1) FUS ranks high in its similarity to yeast prions using the hidden Markov Model, 2) the proposal that yeast prions might be part of an adaptation to stress mechanism, and 3) the experimental finding that cytoplasmic FUS aggregates into stress granules, we speculated that FUS aggregation might be a eukaryotic cellular adaptation mechanism to stress.

1.8. Aims of thesis

Although predominantly nuclear, FUS and TDP-43 function as nucleo-cytosolic shuttling proteins that can temporarily reside in the cytoplasm. However, some pathological and experimental variants of FUS and TDP-43 with mutations in the NLS remain in the cytoplasm. Furthermore, stress conditions can trigger mislocalization of FUS and TDP-43 into the cytoplasm resulting in potential aggregation. We hypothesize that there are at least three ways in which aberrant distribution of FUS and TDP-43 could impact on the structural integrity of SOD1 (**Figure 8**):

i) **Gain of function.** Both FUS and TDP-43 contain glycine-rich domains for protein-protein interaction. Therefore, their presence in the cytoplasm could lead to misfolding of SOD1 via direct interaction at the protein level or through indirect biochemical pathways involving these proteins;

ii) **Loss of function.** The loss of FUS and TDP-43's DNA/RNA processing properties may prevent the production of some "Protein X" or "miRNA Y" that is required for proper regulation of SOD1 expression or folding;

iii) A combination of **gain and loss of function** as the result of the mislocalization.

We have investigated hypothesis **i)** by over-expressing wild-type or NLS-mutated FUS and TDP-43 proteins in cell line cultures and primary mouse neural cultures from human wild-type (wt) SOD1 Tg mice, and analyzed for human wtSOD1 misfolding using immunofluorescence and immunoprecipitation. We have investigated hypothesis **ii)** by down-regulating FUS and TDP-43 in cell models, followed by immunoblotting to assess differences in SOD1 proteostasis, and determine wtSOD1 misfolding by immunoprecipitation.

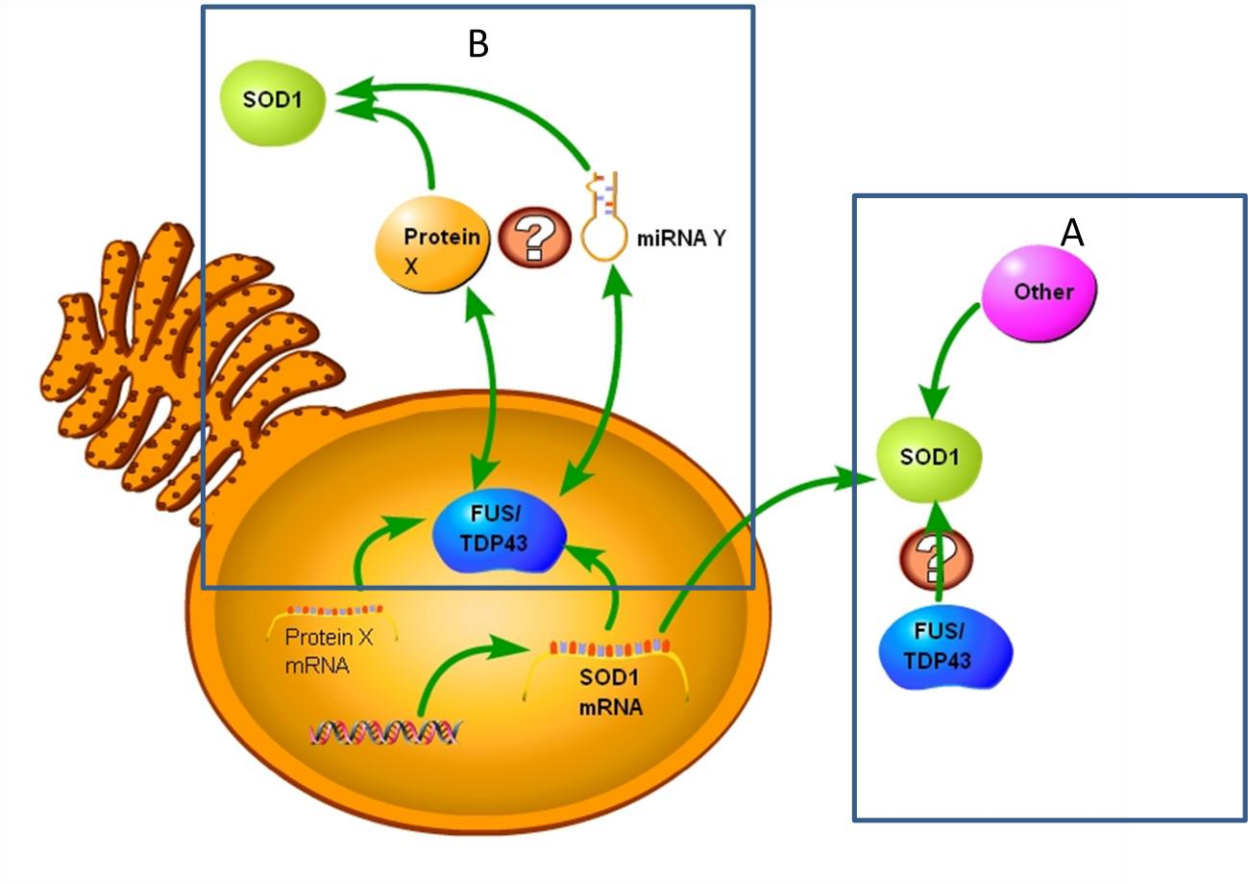

Figure 8: Hypothesis

Schematic representation of possible gain and loss of function of mislocalized FUS and TDP-43, as can be detected by misfolded SOD1. Once extra-nuclear (A), FUS and TDP-43 mislocalize, they might cause misfolding of SOD1 through direct protein-protein interactions, or through indirect pathways involving these proteins. Additionally, the absence of FUS and TDP-43 from the nucleus might lead to a loss of function that further affects the expression and proper folding of SOD1 (B). This could happen through a lack of processing of intermediate proteins or miRNA that regulate SOD1 folding and expression, or the clearance of its misfolded form.

2. Effects of Cytoplasmic FUS and TDP-43 on SOD1 Misfolding

2.1. Introduction

Protein folding is an essential cellular process during which linear polypeptides are organized into defined three-dimensional structures required for correct protein functioning (Lee and Tsai 2005). The process of folding often requires the assistance of molecular chaperones and begins co-translationally: the N-terminal portion begins to fold as the C-terminal portion is being translated (Basharov 2000). Proteins that do not assume the proper conformation are said to be misfolded, and can participate in aberrant protein-protein interactions, as well as induce cellular stress by overloading the proteolytic machinery, which might lead to formation of cytotoxic aggregates and apoptosis (Stefani and Dobson 2003; Ali, Kitay et al. 2010). The accumulation of misfolded protein participates in the pathology of several other neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, frontotemporal dementia and Huntington's disease (Selkoe 2003). Although the precise etiology of ALS remains unknown, certain proteins have been identified that undergo changes in cellular distribution, cytotoxic aggregation and conformational changes (Lagier-Tourenne and Cleveland 2009; Bosco, Morfini et al. 2010; Munoz 2010).The significance of understanding what causes these proteins to misfold, including SOD1 in ALS, is immense (Sharma, Ding et al. 2008). Although SOD1 is known to be a highly stable homodimeric protein (Banci, Bertini et al. 2009), it undergoes misfolding under various conditions, such as genetic mutations that result in less stable SOD1 isoforms (Rakhit, Cunningham et al. 2002).

In ALS, mutations in the gene encoding the Cu/Zn superoxide dismutase (SOD1), a ubiquitously-expressed free-radical defense enzyme, can cause the normally stable homodimeric protein (Banci, Bertini et al. 2009), to adopt an aberrant conformation and monomerization and subsequent aggregation (Rakhit, Cunningham et al. 2002). However, recent studies have also detected misfolded SOD1 in both familial and sporadic forms of the disease in which SOD1 mutation is excluded (Bosco, Morfini et al. 2010; Forsberg, Jonsson et al. 2010), suggesting non-native conformers of SOD1 participate in a common pathological mechanism in all types of ALS. In addition to

SOD1 misfolding in ALS, two other well-studied proteins are associated with ALS and appear to be prone to mislocalization and subsequent aggregation: the RNA-processing enzymes fused in sarcoma (FUS), originally named TLS (translocated in liposarcoma), and TAR-DNA binding protein 43 (TDP-43) (Lagier-Tourenne, Polymenidou et al. 2010). Understanding the mechanisms and consequences of protein misfolding in ALS, and other neurodegenerative diseases, may lead to novel effective therapies and diagnostics.

FUS/TLS and TDP-43 are primarily nuclear proteins that form a biochemical complex together (Kim, Shanware et al. 2010), and are involved in transcription, translation, splicing, nucleo-cytoplasmic shuttling, RNA transport for local translation, and stress granule formation (Lagier-Tourenne, Polymenidou et al. 2010). These proteins belong to the family of heterogeneous nuclear ribonucleoproteins (hnRNP) and are known to interact with other members of the family through their G-rich domains (Pesiridis, Lee et al. 2009). Under pathological circumstances, these resident nuclear proteins can be trapped in the cytosol and potentially form abnormal protein associations in this aberrant locale. Phenotypes of ALS involving FUS/TLS and TDP-43 mutations are clinically indistinguishable from those with SOD1-linked mutations, and wild-type FUS/TLS and TDP-43 aggregation can be observed in SALS without a recognized genetic component (Neumann, Rademakers et al. 2009; Shaw 2010). In this study, we tested the hypothesis that FUS/TLS and TDP-43 gain new pathogenic functions upon localization to the cytosol, which subsequently lead to the misfolding of SOD1 in cells. Moreover, since mutant and wild-type SOD1 misfolding can be propagated (Cashman, Grad et al. 2009), we propose that SOD1 misfolding by FUS/TLS and TDP-43 could result in a “productive template” which serves as a final common pathway for motor system degeneration in ALS.

In order to experimentally test the association of SOD1 misfolding with pathological cytosolic mutants of FUS/TLS, the naturally occurring point P525L (Kwiatkowski, Bosco et al. 2009; Dormann, Rodde et al. 2010) and truncation R495x (Bosco, Lemay et al. 2010) mutations were generated. For TDP-43, previous studies have determined that a substitution of 3 amino acids (K82A, R83A, K84A) in its nuclear localization signal (NLS)

results in mislocalization of TDP-43 to the cytosol (Winton, Igaz et al. 2008), providing a test mutant for our study.

To avoid steric hindrance effects due to large tagging probes, the small hemagglutinin (HA)-tag, derived from amino acids 98-106 of human influenza hemagglutinin (Hua, Sakai et al. 1995), was used to label the amino-termini of the transfection-driven wild-type and mutant proteins so as to distinguish from endogenous proteins. HA-tagging has been successfully used in other studies for detection of exogenous FUS/TLS (Vance, Rogelj et al. 2009; Dormann, Rodde et al. 2010) and TDP-43 (Kim, Shanware et al. 2010; Seyfried, Gozal et al. 2010). We used the HA-tagged FUS/TLS and TDP-43 to show for the first time that expression of either exogenous cytosolic mutant FUS/TLS or TDP-43 leads to the presence of misfolded SOD1, as detected by SOD1 misfolding-specific antibodies directed against a disordered electrostatic loop (Vande Velde, Miller et al. 2008; Israelson, Arbel et al. 2010), or monomerized and oxidized SOD1. Furthermore, overexpression of wild-type TDP-43, but not wild-type FUS/TLS, is also associated with SOD1 misfolding. Although our data demonstrate a direct link between expression of ALS-linked FUS/TLS proteins and SOD1 misfolding, only partial co-localization is observed between the proteins, suggesting that the physical binding between misfolded SOD1 and cytoplasmic FUS/TLS is transient. Our immunoprecipitation data also demonstrate a physical interaction between the pathogenic FUS/TLS and misfolded SOD1. Our finding are consistent with the independent propagation of SOD1 misfolding (Cashman 2010), and suggest that all three key proteins implicated in ALS participate in a common pathological process.

2.1.1. Aims of chapter

The principal hypothesis for this chapter was that FUS and TDP-43 gain new pathological functions upon their mislocalization to the cytosol, which could associate with the misfolding of SOD1 in cells, as both events, independently, are associated with ALS pathology. This hypothesis was tested in cultured human neuroblastoma (SH-SY5Y) cells, human embryonic kidney (HEK293) cells, and primary neural cells from human wtSOD1 expressing mice. As has been previously discussed, point P525L mutation and truncation R495x mutation in FUS, as well as the three amino acid

substitution mutant (K82A, R83A, K84A) in TDP-43, cause their respective proteins to remain primarily in the cytoplasm instead of localizing to the nucleus. Therefore the **first aim** of this chapter was the cloning of the genes coding for wild-type and mutant FUS and TDP-43 into a pCINeo expression vector.

Following adequate expression in human cell lines, the presence of misfolded SOD1 was analyzed in cell cultures that expressed mutant FUS and TDP-43, and compared to cultures transfected with wtFUS, wtTDP-43, and empty vector. Specifically, the effects of cytosolic FUS (**second aim**), and TDP-43 (**third aim**) on SOD1 misfolding were explored using immunofluorescence and immunoprecipitation.

2.1.2. Study design

Original FUS and TDP-43 plasmids (Plasmid #21827, AddGene, Cambridge, MA; MHS1010-98051213, Open Biosystems, Huntsville, AL), were amplified and modified using PCR-based site-directed mutagenesis, ligated into a pCINeo expression vector, and transformed into chemically competent DH5-alpha *E.Coli* bacteria. Cloned mutant and wild type genes were confirmed correct by sequencing and large quantities were purified using a low endotoxin plasmid DNA maxi-prep kit (Invitrogen, Carlsbad, CA) for cell transfections. All clones contained a short N-terminally fused HA-tag in order to distinguish between transfection-driven FUS and TDP-43, and the resident endogenous versions.

For the second and third aims, immortalized human neuroblastoma cells (SH-SY5Y) and human embryonic kidney cells (HEK293) were used. SH-SY5Y cells offer a physiologically relevant neuronal-like characteristic, but have an inherently slower growth rate and lower transfection efficiency. Alternatively, HEK293 cells are widely used as a model that provides rapid cellular growth and high exogenous protein expression levels. Transfected cell cultures were analyzed using immunoprecipitations (IP) followed by immunoblotting (IB). IPs utilized our misfolded-SOD1 specific monoclonal antibodies, 3H1 and 10C12. These antibodies are conformation-specific, and therefore cannot be used to detect misfolded SOD1 from protein separated using typical denaturing electrophoresis methods as the structure of the epitope becomes lost upon denaturation of the protein prior to immunoblot detection. We therefore utilize

these antibodies in immunoprecipitation experiments of native cell lysates prior to denaturing gel electrophoresis. Following IPs and IBs, the amounts of 3H1 and 10C12 - immunoprecipitable SOD1 were quantified, compared to SOD1 pull down by a mIgG control, and expressed as a fraction of total immunoprecipitable SOD1 (using a pan-SOD1 antibody, SOD100 (Stressgen Bioreagents, Ann Arbor, MI), which detects natively folded and misfolded SOD1). The presence of misfolded SOD1 in cultures that had been transfected with mutant FUS or TDP-43 was compared to cells transfected with wtFUS or wtTDP-43, as well as cells containing only the pCINeo expression vector (referred to as “empty vector”, or “ev”). Cells were transfected for 48h, as this was determined to be the point of maximal protein expression. All immunoblots were probed with the pan-SOD1 antibody.

In addition to IPs, SH-SY5Y and HEK293 cells were also analyzed by immunofluorescence (IF) microscopy. Cells were grown and transfected on glass cover slips, fixed 48h post transfection, permeabilized and stained with the relevant antibodies. To establish native localization of FUS and TDP-43, non transfected cells were stained with: 3H1, αFUS or αTDP-43, and Hoechst33342. Slides from FUS and TDP-43 transfected cells were stained with: 3H1 and αHA antibodies, and Hoechst33342 nuclear counterstain. As a final step, IF microscopic analysis of primary neural cultures was performed. Primary cultures were prepared from human wtSOD1 Tg C57 BL/6 mice and transfected at 5 DIV (days-in-vitro). Primary neural cells were stained as described above. All samples were collected using an Olympus FV1000 confocal microscope using FV1000 ASW.

2.2. Materials and methods

This study involves gene mutagenesis, cloning, immunofluorescence microscopy, immunoprecipitations, protein gel electrophoresis and immunoblotting. Below is the description of the experimental procedures used in this study.

2.2.1. pCINeo backbone

Expression of exogenous proteins in cell cultures has become an essential tool in understanding how proteins work. In order to express an exogenous protein, the DNA encoding the protein of interest is cloned into a mammalian expression vector and subsequently transfected into cells. The choice of expression vector is very important; the chosen plasmid in this study is the pCINeo mammalian expression vector (**Figure 9**, Promega, Madison, WI,USA), which contains the cytomegalovirus (CMV) promoter that constitutively drives the expression of cloned DNA inserts in mammalian cells (Li, Wang et al. 2003). Another advantage of pCINeo is the presence of the neomycin phosphotransferase gene that acts as a selective marker in mammalian cells to generate stably expressing cell lines (Rees, Coote et al. 1996; Yague, Higgins et al. 2004). The plasmid also contains an ampicillin resistance gene. The genes of interest for this study were cloned between *XhoI* and *XbaI* restriction sites (highlighted in **Figure 9**) in the multiple cloning site region of the plasmid. The chosen endonuclease restriction enzymes are known to be efficient and compatible for double digestion reactions.

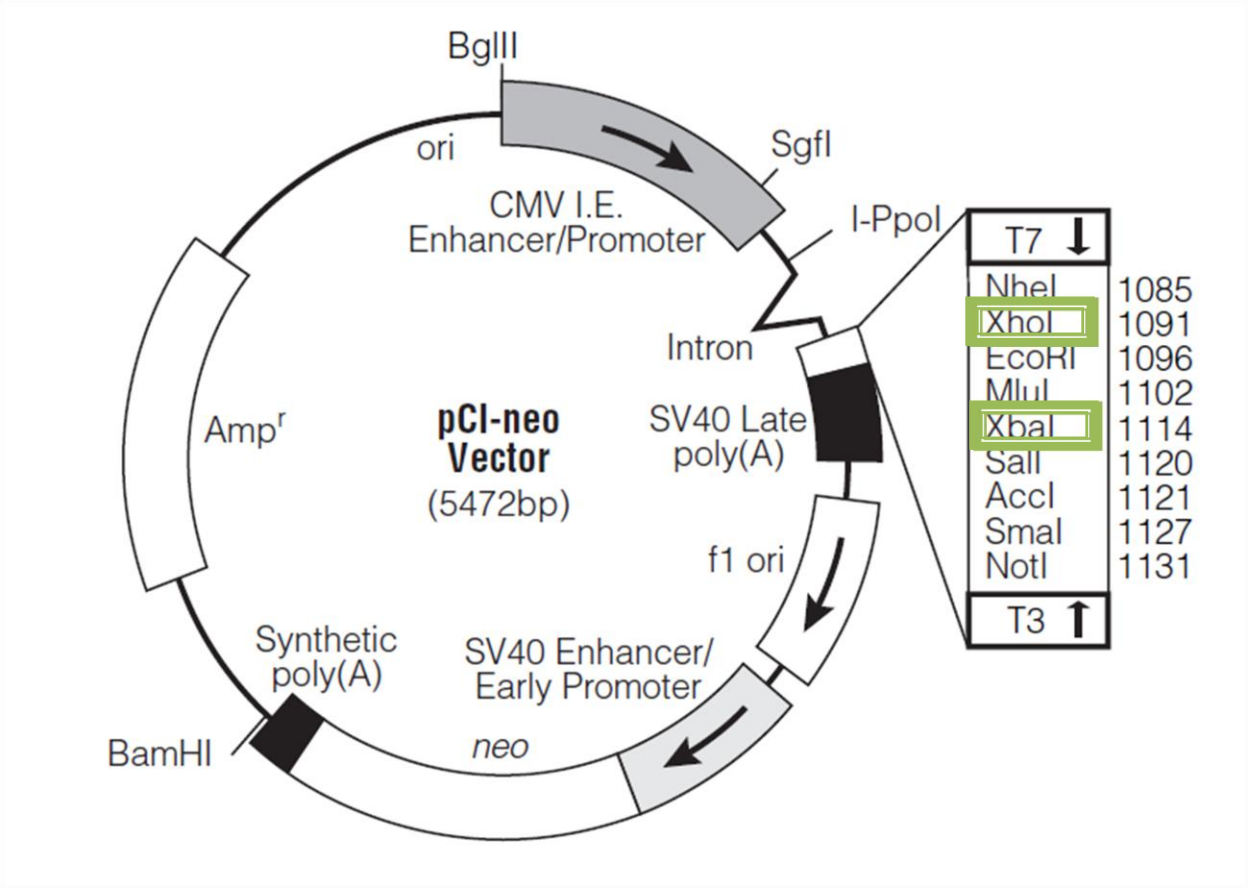

Figure 9: pCINeo expression vector

A mammalian expression vector that carries the ubiquitous human cytomegalovirus (CMV) promoter. This plasmid contains a neomycin phosphotransferase gene, a selectable marker for mammalian cells. The highlighted *XhoI* and *XbaI* restriction enzyme digestion sites will be used for cloning of FUS and TDP4-43 into the plasmid.

2.2.2. Mutagenesis and cloning approach

The methodology used for cloning of wild type and mutant FUS and TDP-43 into pCINeo is described below. Protocols for restriction digests, ligation, and transformation of chemically competent DH5-alpha *E.Coli* cells are modified versions of the ones described in Molecular Cloning: A Laboratory Manual (Sambrook, Fritsch et al. 1989) and are outlined in Appendix I: Cloning protocols. All nonsense and missense mutagenesis was performed via the polymerase chain reaction (PCR) and are thoroughly described below.

In order to facilitate accurate and efficient mutagenesis, PCR oligonucleotide primers were designed with at least 10 base pairs complementary to the DNA template. All primers have 40-60% GC content and have G or C at both ends to prevent breathing of ends due to strong G-to-C binding. Each forward and reverse oligonucleotide that binds to either end of the template contains a restriction enzyme digestion site on its 5' or 3' end for ease of cloning. TDP43_F_HA and FUS_F_HA are the forward primers for TDP-43 and FUS, respectively. They contain the *XhoI* digest site, as well as a long 5' end containing the sequence encoding the HA-tag. This tag is inserted between the start codon of the protein and the second amino acid (**Figure 10** and **Table 1**). TDP43_R and FUS_R are the reverse primers for TDP-43 and FUS, respectively, and contain *XbaI* digest sites on their flanging ends. The annealing temperature of all PCR reactions was determined by averaging the melting temperature of both primers and subtracting 1°C from it. This method of determining the annealing temperature was chosen, to get sufficient primer-template hybridization with low non-specific binding. At the end of each cloning procedure, the constructs were sequenced and carefully compared to the theoretical sequences. Final plasmid maps can be found in Appendix II: Maps of pCINeo HA-TDP-43 and pCINeo HA-FUS.

Primer name	Sequence 5'→3'
TDP43_F_HA	CCG *CTC GAG* GCC ACC **ATG** TAC CCA TAC GAT GTT CCA GAT TAC GCT TCT GAA TAT ATT CGG
TDP43_R	CCT AGC TAG *TCT AGA* CTA CAT TCC C
TDP43_82-84_F	CCA AAA GAT AAC GCA GCA GCA ATG GAT GAG ACA GAT GC
TDP43_82-84_R	GCA TCT GTC TCA TCC ATT GCT GCT GCG TTA TCT TTT GG
FUS_F_HA	CCG *CTC GAG* GCC ACC **ATG** TAC CCA TAC GAT GTT CCA GAT TAC GCT GCC TCA AAC GAT TAT ACC
FUS_R	CTA G*TC TAG A*TT AAT ACG GCC TCT CC
FUS_R495X_R	CTA G*TC TAG A*TT AGA AGC CTC CAC GGT C
FUS_P525L_R	CTA G*TC TAG A*TT AAT ACA GCC TCT CCC TGC G

Table 1: TDP-43 and FUS PCR primers

All the primers that were used for cloning wild type and mutated FUS and TDP-43 gene sequences are listed. **Bold faced** sequence represents the first Met of the sequence in forward primers. Underlined sequence represents the sequence for the HA-tag. *Italicized* sequence represents XhoI and XbaI restriction digest sites.

In order to generate the missense mutations in TDP-43 (K82A, R83A, and K84A), two additional primers were designed. TDP43_82-84_F contains 12bp before and 17bp after the mutations. TDP43_82-84_R is the reverse complement of TDP43_82-84_F. Mutagenesis was performed in two PCR cycles (**Table 2**). The first cycle included two reactions: i) amplifying the region upstream of the mutation by using primers TDP43_F_HA and TDP43_82-84_R as forward and reverse primers, respectively; ii) amplifying the rest of the DNA sequence using TDP_82-84_F and TDP43_R as forward and reverse primers, respectively. The products from both above reactions were purified and were added as the template for the second PCR cycle performed using the TDP43_F_HA and TDP43_R primers. Since the flanking overlapping ends of the primers complement each other, the two pieces of DNA were joined as soon as they were incubated together in the second PCR cycle. The amplification of the wtTDP-43

was performed by using TDP43_F_HA and TDP43_R as forward and reverse primers, respectively.

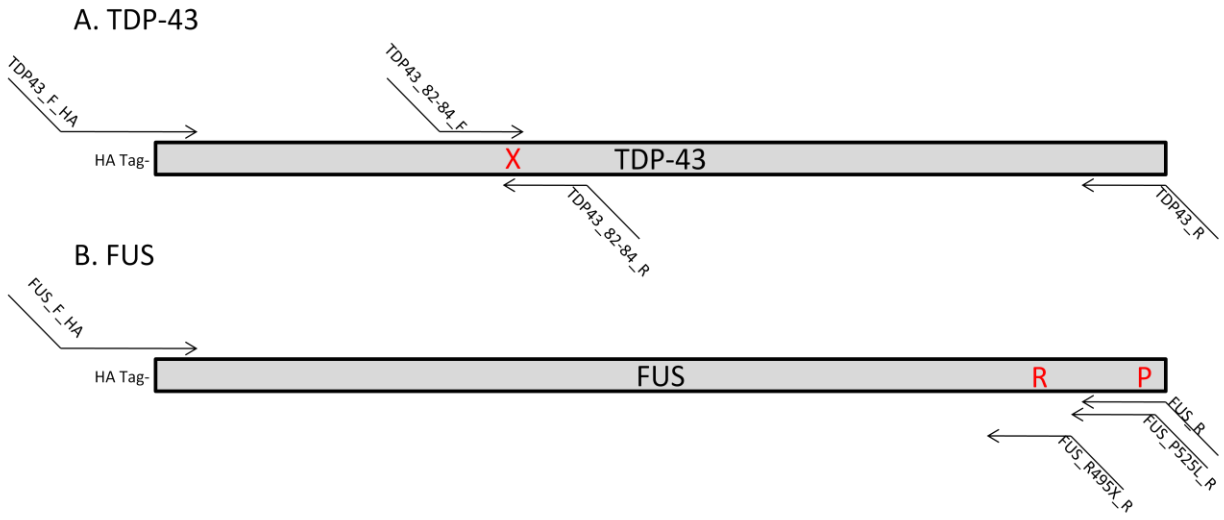

Figure 10: Cloning strategy

Schematic representation of cloning strategy of TDP-43 (A) and FUS (B). To generate a triple mutant TDP-43 (K82A, R83A, and K84A), 4 primers were used in two separate PCR reactions. Wild type TDP-43, as well as wild type FUS and its mutants, were generated using only 2 primers per reaction. X indicates the location of the 82-84aa missense mutations. P and R indicate location of P525L and R495x mutations, respectively.

Mutagenesis of FUS DNA sequence was more straightforward as P525L and R495x mutations occur near the end of the sequence. To generate wtFUS, P525L-FUS and R495x-FUS constructs, three one-cycle reactions (**Table 2**) were prepared. All reactions included the FUS_F_HA primer to introduce the HA-tag to the FUS protein. In order to generate wtFUS, P525L-FUS and R495x-FUS, FUS_R, FUS_P525L_R, and FUS_495x_R reverse primers were used, respectively.

Ingredient	Amount (First cycle)	Amount (Second cycle)
Forward primer (10µM)	1µl	1µl
Reverse Primer (10µM)	1µl	1µl
Template DNA I (1mg/ml)	1µl	1µl
Template DNA II	-	1µl
Deoxynucleotide mix (10nm)	1µl	1µl
High Fidelity Taq polymerase	0.5µl	0.5µl
5x PCR buffer	10µl	10µl
Water	35.5µl	34.5µl

Table 2: PCR reaction

Amounts and concentrations of reagents used in all PCR reactions are listed. Expand High Fidelity reagents from Roche Diagnostics (Mannheim, Germany) were used for the PCR. Amounts of ingredients in the mixture differed based on whether it was a one cycle reaction (in the case of wtFUS, P525L-FUS, R495x-FUS and wtTDP-43), the first cycle of two (in the case of mutagenesis of TDP-43), or the second cycle of TDP-43 mutagenesis.

2.2.3. Tissue culture and DNA transfection

HEK293 and SH-SY5Y cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 10U/ml penicillin, 10 U/ml streptomycin and 2 mM L-glutamine. Cells were transiently transfected using Lipofectamine LTX (Invitrogen, Carlsbad, CA), which offers low cytotoxicity and high transfection efficiency, according to manufacturer's instructions. Cells were plated the day prior to transfection and grown to 50-90% confluency. Per well of a 24-well plate, 0.5 µl of 1 mg/ml plasmid DNA and 1.25µl of Lipofectamine LTX were mixed in 100µl OptiMEM. This mix was incubated for 30 minutes at room temperature and added drop-wise to the cultures. For immunofluorescence, cells were cultured and transfected on glass cover-slips (#1.5). For immunoprecipitation experiments, cells were cultured in 10 cm tissue culture-treated plates, and the transfection reagent volumes were multiplied by 24.

2.2.4. Mouse primary spinal cord culture

Pregnant C57 BL/6 female mice (Strain name: B6SJL-Tg(SOD1)2Gur/J; Stock no. 002297; Jackson Laboratories, Bar Harbor, ME) were sacrificed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC). Primary cerebral cortical cultures were prepared from 12-day to 14-day fetal mice using minor modification of an established technique (Anderson, Potter et al. 2004). Prior to preparation of neural cultures, each embryo was genotyped (refer to section 2.2.5). Cervical, thoracic and lumbar- regions of the spinal cord were dissected out in Ca^{2+}/ Mg^{2+}-free Hanks Balanced Salts (GIBCO BRL, Grand Island, NY). Meninges were removed and the tissue was transferred to 0.25% trypsin (GIBCO BRL, Grand Island, NY) and digested at 37°C for 15 min. Tissue was then resuspended in DMEM (GIBCO BRL, Grand Island, NY) plus 10% fetal bovine serum (GIBCO BRL, Grand Island, NY) and triturated 4-6 times through the fire-polished tip. The supernatant was centrifuged at 200 x g for 45 sec. Pelleted neural cells were resuspended in Neurobasal media (GIBCO BRL, Grand Island, NY), B27(GIBCO BRL, Grand Island, NY), 2 mM L-glutamine (Sigma, Saint Louis, MO) and seeded at a density of 2 x 10^5 cells/well onto poly-D-lysine (Sigma, Saint Louis, MO) coated #1.5 coverslips in 24-well plates. These cultures were maintained in serum-free Neurobasal-B27 medium, and one-half of the medium was replaced on day 3 or 4 by equal volume of fresh medium. Cells were transfected at 5 DIV using Lipofectamine LTX with Plus reagent (Invitrogen, Carlsbad, CA).

2.2.5. Embryo genotyping

To mimic the scenario of association between human wtSOD1, and FUS and TDP-43 in primary neural cells, neural cultures were prepared from human wtSOD1 expressing C57 BL/6 Tg mouse embryos. Tissue was collected in 200µl of lysis buffer (0.45% Nonidet P-40, 0.45% Tween 20, 50mM KCl, 10mM Tris-HCl in water, pH 8.8) with 2 µl of Proteinase K (Invitrogen, Carlsbad, CA), and shaken at 60°C at 350rpm for 2h. The sample was then incubated at 96°C for 10 min to inactivate the Proteinase K. Immediately upon inactivation, the PCR mixture was prepared using 2 µl of lysis buffer containing DNA for genotyping. Two sets of primers were used for the PCR reaction: 5'-CATCAGCCCTAATCCATCTGA and 5'-CGCGACTAACAATCAAAGTGA (hwtSOD1

specific primers); 5'-TGGACAGGACTGGACCTCTGCTTTCCTAGA and 5'-TAGAGCTTTGCCACATCACAGGTCATTCAG (internal mouse control). The PCR cycle used was as described in Appendix I: Cloning protocols, with the exception of using 58°C as the annealing temperature. Lastly, the resulting amplified DNA was run on a 1.5% agarose gel: the presence of two bands indicates a positive transgenic embryo (internal control and h-wtSOD1 specific bands).

2.2.6. Immunofluorescence

In order to detect specifically misfolded SOD1 disease specific epitope (DSE) monoclonal antibody 3H1, was used (Vande Velde, Miller et al. 2008; Cashman, Grad et al. 2009). SH-SY5Y and HEK293 cells were plated on glass cover-slips in a 24-well plate one day prior to transfection. Cells were transfected as described above, and processed for immunofluorescence microscopy 48 h post transfection as follows: Cells were washed once with ice-cold PBS and fixed in 4% paraformaldehyde (in PBS, pH 7.4) for 15 min at room temperature. Fixed cells were then washed/permeabilized twice for 5 min in PBST (0.3% Triton-X 100 in PBS) and incubated with 2 µg/ml mouse monoclonal antibody 3H1, as well as 10 µg/ml rabbit αTDP-43, αFUS or αHA-tag polyclonal Ab, diluted in PBST with 2% normal goat serum, for 1h at room temperature. Cells were then washed twice in PBST and incubated with appropriate secondary antibody conjugated to Alexa Fluor 488 (green) or Alexa Fluor 647 (red) dyes (Invitrogen, Carlsbad, CA; both diluted 1:1000) for 1 h at room temperature in the dark. For DNA staining, bisBenzimide H33342 trihydrochloride (Hoechst 33342) was used at 2 µg/ml (in PBST). Hoechst33342-stained cells were incubated for 15 min at room temperature after the first wash of secondary antibody, followed by two more washes with PBS. These secondary Alexa antibodies were chosen to prevent bleed-through. (Spectra are shown in Appendix III) Cells were mounted on a glass slide in a drop of Fluoromount-G (SouthernBiotech) and allowed to dry overnight before imaging. Images were viewed and captured on Olympus FluoView FV1000 microscope (Olympus Canada) using FV1000 ASW software. Slides were stored at 4°C.

2.2.7. Immunoprecipitation

Transfected cells growing on 10 cm dishes were washed twice in ice-cold PBS and collected by centrifugation (5 min at 1,000 x g, 4 °C). Cell pellets were lysed in 300 µl lysis buffer (PBS, 0.5% sodium deoxycholate (DOC), 0.5% Triton X-100 and 1x complete, EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) for 2 min on ice, followed by centrifugation for 5 min at 1,000 x g , 4 °C. Lysate was removed to a fresh tube. For immunoprecipitation experiments, 100 µl cell lysate was added to 0.65 ml microfuge tubes. 10 µl of antibody-coupled M-280 Tosyl-activated magnetic Dynabeads (Invitrogen, Carlsbad, CA) were added to each tube and mixed. Tubes were incubated for 3 h at room temperature with constant rotation. Beads were then washed three times with 150 µl RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.5% DOC, 0.1% SDS) with brief vortexing in-between washes, and boiled in SDS sample-buffer containing 1% β -mercaptoethanol for 5 min. 1 µl of lysate was added directly into SDS sample buffer, boiled and used as a pre-IP control.

2.2.8. Preparation of antibody-coupled magnetic Dynabeads

175 µl beads were washed twice in 1 ml PBS and resuspended in a final volume of 1 ml PBS. 80 µg DSE monoclonal antibody, or 50 µg pan-SOD1 (SOD100; Assay Designs, Ann Arbor, MI) polyclonal antibody were added to beads and incubated for 24 h at 37°C with constant rotation (100 µg mouse IgG2a, and rabbit IgG were coupled to beads as negative controls). Coupled-beads were then washed twice in 1 ml PBS with 0.1% BSA and incubated in 1 ml blocking buffer (0.2 M Tris-HCl, pH 8.5, 0.1% PBS) for 4 h at 37°C with constant rotation. Beads were then washed again in 1ml PBS with 0.1% BSA, resuspended in a final volume of 500µl of PBS and stored at 4°C.

2.2.9. Immunoblotting and quantification

Boiled samples were analyzed on 4-20% acrylamide Tris-Glycine precast gels for 2 h at 125 V (Invitrogen, Carlsbad, CA). Proteins were electrophoretically transferred to PVDF membrane for 90 min at 25 V, blocked with 5% milk in Tris-buffered saline, 0.1% Tween-20 (TBST) for 30min and incubated with 1 µg/ml pan-SOD1 antibody (SOD100; Assay Designs, Ann Arbor, MI) in 5% milk-TBST overnight at 4°C with constant rocking.

Membranes were washed with TBST followed by 1 h incubation with donkey anti-rabbit IgG, horseradish peroxidase linked whole antibody (GE Healthcare, Buckinghamshire, UK) diluted 1:5,000 in 5% milk-TBST. Membranes were then developed with ECL-Plus chemiluminescent substrate (GE Healthcare, Buckinghamshire, UK) and visualized using a VersaDoc Imager (Bio-Rad Laboratories, Hercules, CA); signal intensities were quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA). All images were acquired with no digital or biological signal saturation. For accurate quantification, bands were digitally zoomed in and surrounded by a rectangular box that was later quantified by the Quantity One software with comparison to the background. Finally, to produce accurate results 5 SOD1 bands were quantified in each set of samples: mIgG, rIgG, SOD100, 3H1 and 10C12. In order to determine the percentage of misfolded SOD1 in each sample, quantified misfolded SOD1 immunoprecipitate was compared to mIgG control, and normalized to total immunoprecipitable SOD1 in the respective sample. To test our results for statistical significance, without any assumption regarding the distribution underlying our small samples we applied nonparametric tests (Kruskal-Wallis and Mann-Whitney-U tests). The significance thresholds were also adjusted for multiple comparisons by the Bonferroni correction to maintain the familywise error rate and keep the alpha level at 0.05.

2.3. Results

Both FUS and TDP-43 are nuclear proteins that play an important role in RNA metabolism (Lagier-Tourenne and Cleveland 2009). The vast majority of SH-SY5Y and HEK293 cells, as well as primary neural cells, clearly express nuclear-localized FUS and TDP-43, with no detection of misfolded SOD1 (**Figure 11**). However in our cell cultures, <0.5% of cells display extranuclear FUS. It is reported that a small population of cells undergo spontaneous cell death under normal culture conditions (Ludewig, Graf et al. 1995; Pregi, Vittori et al. 2006), which could potentially cause FUS mislocalization. In 55% of these cytosol FUS-positive cells, cytosolic misfolded SOD1 can be detected by the SOD1 misfolding specific antibody, 3H1 (**Figure 12**), which was raised against a linear peptide corresponding to the SOD1 electrostatic loop (Vande Velde, Miller et al. 2008; Cashman, Grad et al. 2009); extension of the loop during misfolding permits 3H1 binding to the protein. Lack of a structured electrostatic loop, which is necessary for functional activity (Sigel, Sigel et al. 2006), can contribute to oxidative stress within the cell.

In order to examine the effects of aberrantly localized FUS and TDP-43 on the folding of human wild-type SOD1 (wtSOD1), wild-type and mutant FUS and TDP-43 constructs were successfully cloned and expressed in the human cell lines neuroblastoma SH-SY5Y and mesenchymal HEK293 cells (**Figure 13a** and **b**, respectively). Consistent with other reports, we observe that wtFUS and wtTDP-43 localize in the nucleus (**Figure 14a**, **d**; **Figure 15a**, **c**; **Figure 11a**, **d**). In order to experimentally test the association of SOD1 misfolding with pathological cytosol-localizing mutants of FUS, the point mutation P525L (Kwiatkowski, Bosco et al. 2009; Dormann, Rodde et al. 2010) and truncation mutation R495x (Bosco, Lemay et al. 2010) were generated. For TDP-43, previous studies have determined that a substitution of 3 amino acids (K82A, R83A, K84A) in its nuclear localization signal (NLS) results in mislocalization of TDP-43 to the cytosol (Winton, Igaz et al. 2008), providing a test mutant for our study. To test the effect of FUS localization on SOD1 misfolding, we immunostained cells with our SOD1 misfolding specific antibody, 3H1. To recapitulate as best possible the potential effect of FUS and TDP-43 on human wtSOD1 in primary neural cells, neural cultures were prepared from E12-14 human wtSOD1-expressing

transgenic C57BL/6 (need the JAX designation) mouse embryos. We find that in SH-SY5Y, HEK293 and the abovementioned neural cultures, expression of extranuclear FUS mutants, R495x and P525L, result in the presence of misfolded SOD1 in the cytosol as detected by reactivity with 3H1 (**Figure 14**). Arrowheads in **Figure 14b**-**c** and **e**-**f** point to transfected cells and human wtSOD1 expressing primary neural cells, which show exogenously-expressed cytosolic mutant FUS with significant detection of misfolded SOD1, compared to non-transfected cells in the same fields of view. In primary neural cells, significant amounts of mutant FUS and misfolded SOD1 are detectable in the cytosol (**Figure 14e**-**f**). Cultured cells and primary neural cells transfected with the wtFUS (**Figure 14a**, **d**) display nuclear FUS, without detectable misfolded SOD1. We conclude that the presence of cytosolic FUS is associated with misfolding of SOD1 in the cytosol. In addition, we conducted a similar study using human wild-type- and mutant- TDP-43 (cytosolic; ΔNLS-TDP-43). Immunofluorescence of transfected SH-SY5Y cells and human wtSOD1-expressing primary neural cells using either TDP-43 construct, shows the presence of misfolded SOD1, as detected by the SOD1-misfolding conformation-specific antibody 3H1 (**Figure 15**).

A quantitative analysis of the effect of cytosolic FUS and TDP-43 on SOD1 misfolding was performed by quantitative immunoprecipitation of non-denatured lysates from transfected SH-SY5Y (**Figure 16a**) and HEK293 (**Figure 17a**) cells followed by immunoblot detection. For these experiments, we added a second conformational-specific misfolded SOD1 antibody, 10C12, raised against a linear peptide corresponding to the dimer interface region, with the cysteine residue at position 146 modified to cysteic acid to capture oxidation of misfolded SOD1. Specific immunoprecipitation of conformation-specific 3H1 and 10C12 antibodies were controlled by background pull-down of the mIgG control. To ensure that observed results are not due to stress caused by the transfection and culture, immunoprecipitation experiments include a pCINeo empty vector control (ev) in which cells are transfected with a non-coding DNA. SOD1 misfolding specific monoclonal antibodies, 3H1 and 10C12, detect significant levels of misfolded SOD1 in cultured cells transfected with cytosolic mutants R495x- and P525L-FUS (3H1: $p=0.009$, $p=0.002$ and 10C12: $p=0.006$, $p=0.001$, respectively, in SH-SY5Y cells; compared to ev). No misfolded SOD1 was detected in cells transfected with

wtFUS (3H1: p=0.5 and 10C12: p=0.7 in SH-SY5Y cells; compared to ev). These data demonstrate that accumulation of extranuclear FUS is associated with misfolding of SOD1. Quantification of SOD1 misfolding due to FUS expression in SH-SY5Y and HEK293 cells is summarized in **Figure 16b**-**c** and **Figure 17b**-**c**. Similar data was obtained for cells transfected with wild-type or ΔNLS-TDP-43, demonstrating misfolded SOD1 in the cytosol of SH-SY5Y (**Figure 16a**, **d**-**e**; 3H1: p=0.002, p=0.001 and 10C12: p=0.001, p=0.001, respectively in SH-SY5Y cells; compared to ev), and HEK293 (**Figure 17a**, **d**-**e**). SH-SY5Y cells transfected with a control protein, enhanced green fluorescent protein (eGFP), show no misfolded SOD1 (**Figure 18**).

Having identified the association between the presence of aberrant cytosolic FUS and TDP-43, and misfolded SOD1, we next explored whether they physically interact in cells. As determined from high magnification images of transfected neural cells (**Figure 19a**-**b**), both the αHA-tag and mAb 3H1 detect cytosolic mutant FUS and misfolded SOD1, respectively. Only partial co-localization of misfolded SOD1 with mutant cytosolic FUS is observed. When these proteins do not overlap, we detect mainly cytosolic mutant FUS in the vicinity of the nucleus, and misfolded SOD1 adjacent to the process plasma membrane. We further used immunoprecipitation beads coated with total and conformation-specific SOD1 antibodies, followed by immunoblotting and probed with αHA-tag antibody to show co-immunoprecipitation of FUS with SOD1. We determined that exogenous wild-type and mutant FUS co-immunoprecipitated with total SOD1; however, only cytosolic mutant FUS, but not wtFUS, was co-immunoprecipitated with misfolded SOD1 (**Figure 19c**). Whereas interactions between TDP-43 and SOD1 have been established before (Volkening, Leystra-Lantz et al. 2009; Higashi, Tsuchiya et al. 2010), the finding of the direct interaction between cytosolic mutant FUS and SOD1 is a novel observation.

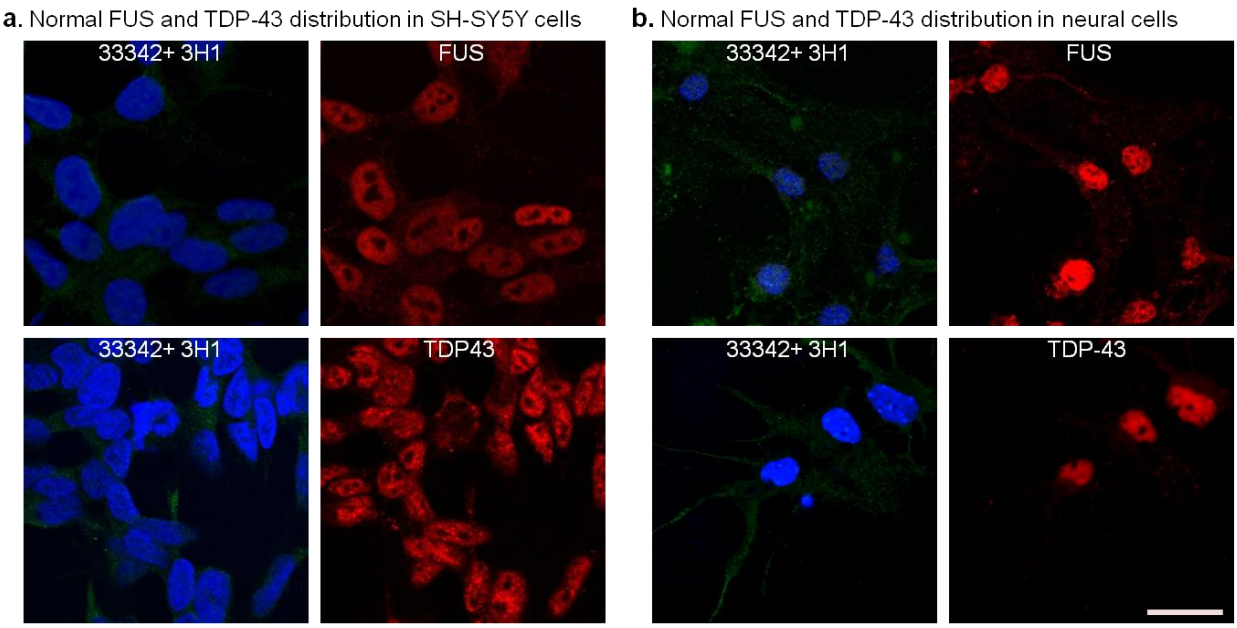

Figure 11: Normal distribution of FUS and TDP-43 in the cell

Untreated human neuroblastoma SH-SY5Y (**a**) cells and primary neural cells (**b**) probed for misfolded SOD1 (green) and Hoechst 33342 nuclear counterstain (blue). Staining of the respective cells against FUS and TDP-43 (top, bottom), shows completely nuclear localization of these proteins with no detectable misfolded SOD1. Scale bar, 20µm.

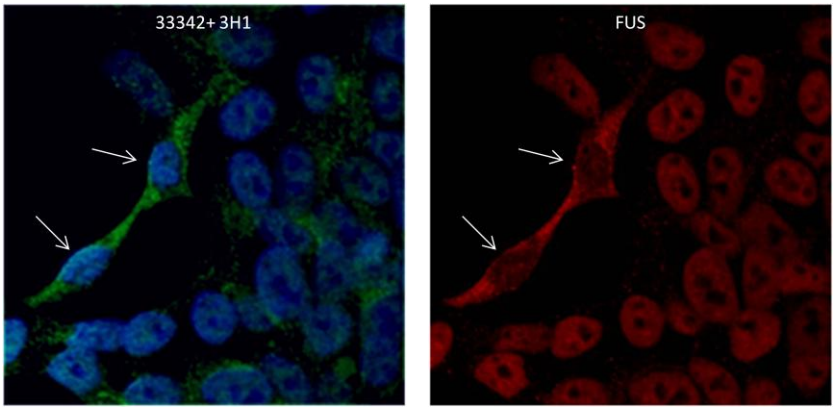

Figure 12: Abnormal distribution of FUS in untreated human neuroblastoma cells

Arrowheads point to untreated SH-SY5Y cells exhibit an abnormal cytoplasmic localization of FUS, estimated to occur in under 0.5% of cells. Nearly 55% of cells showing mislocalized FUS, also clearly stain with an antibody against misfolded SOD1 in the cytosol (green). Hoechst 33342 (blue) stains the nucleus, where FUS is normally found, as can be observed from other cells in the field of view.

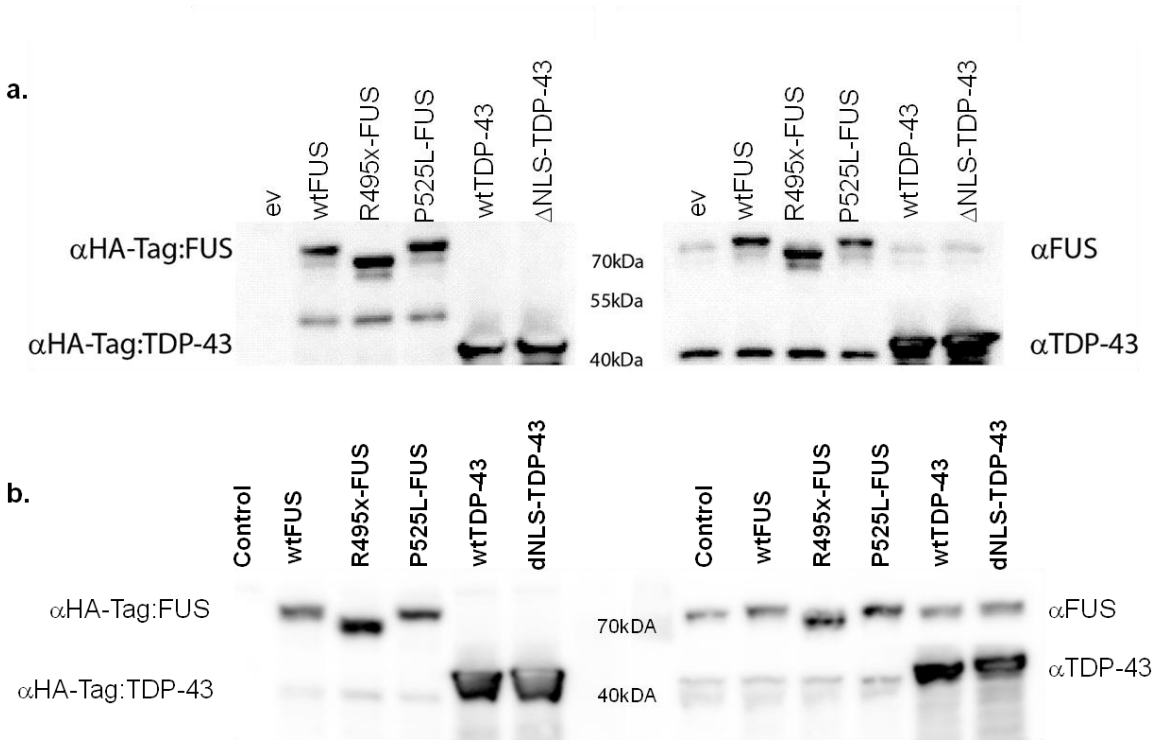

Figure 13: Expression of FUS and TDP-43 vectors in cultured cells

Expression of cloned constructs of wild-type-, R495x- and P525L-FUS, as well as wild-type and ΔNLS-TDP-43 in SH-SY5Y and HEK293 cells (**a** and **b**, respectively). The immunoblots on the left were developed with αHA-tag antibody, showing only the exogenous proteins in these samples. The control pCINeo vector does not react with the antibody, while both exogenous FUS and TDP-43 are detected as 45kDa and 70kDa bands, respectively. Top and bottom portions of the immunoblot on the right were developed using αFUS and αTDP-43 antibodies, respectively. Endogenous FUS and TDP-43 are detected in all samples; however stronger signals are detected in the transfected samples.

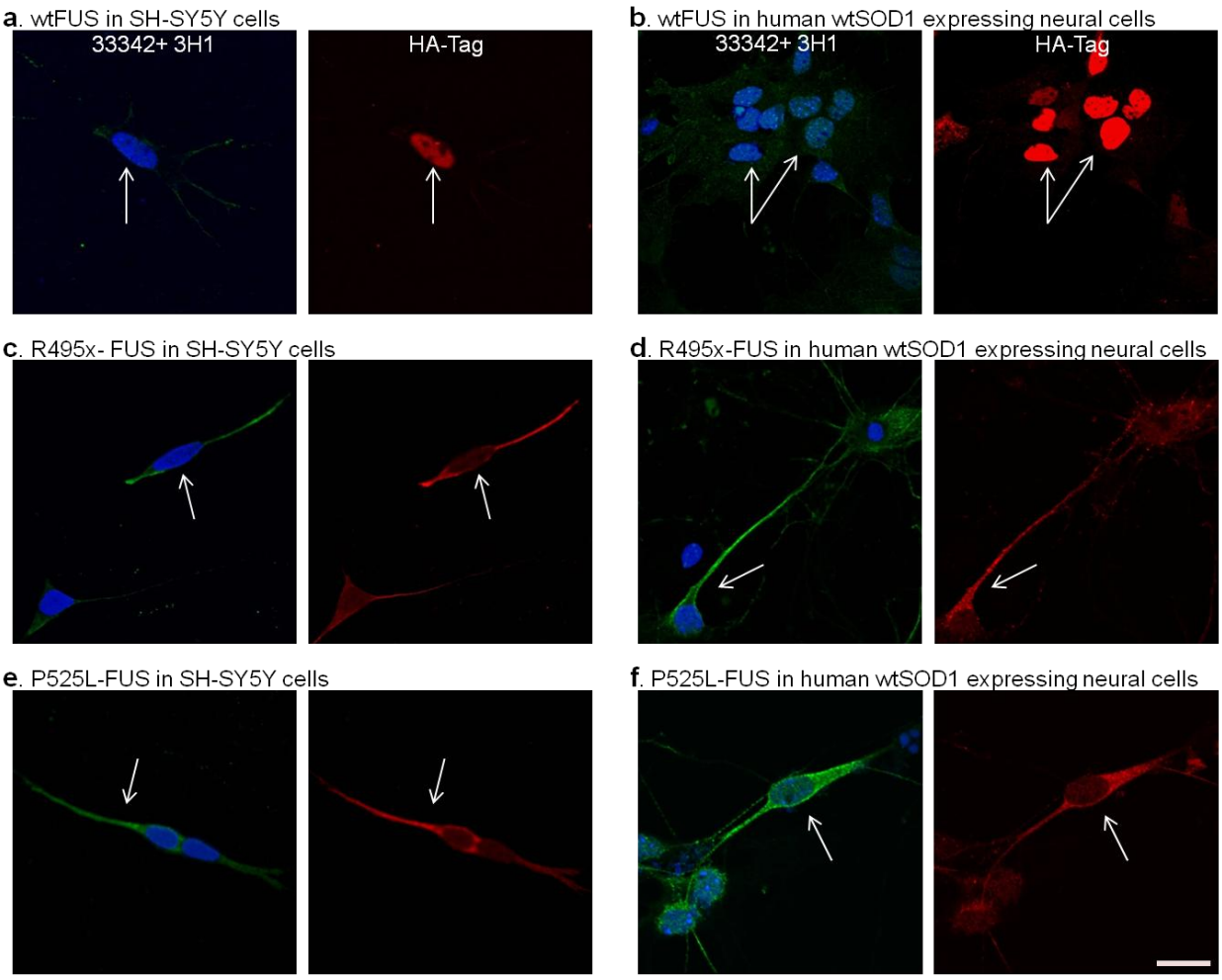

Figure 14: Expression of wt, cytoplasmic R495x-FUS and P525L-FUS in SH-SY5Y cells and in primary neural cells from human wtSOD1Tg mice

SH-SY5Y cells and primary neural cells stained against HA-Tag (red), misfolded SOD1 (green), and Hoechst33342 nuclear counterstain (blue). Human wild-type FUS localizes in the nucleus and no misfolded SOD1 is detected (**a**, **d**). Both of the truncated variant, R495x-FUS, and point mutation variant, P525L-FUS, localize in the cytosol and trigger misfolding of SOD1, as can be detected by the positive staining of 3H1 (**c**, **d** and **e**, **f** respectively). Exogenous FUS was detected using the N-terminal HA-tag. Scale bar, 20µm.

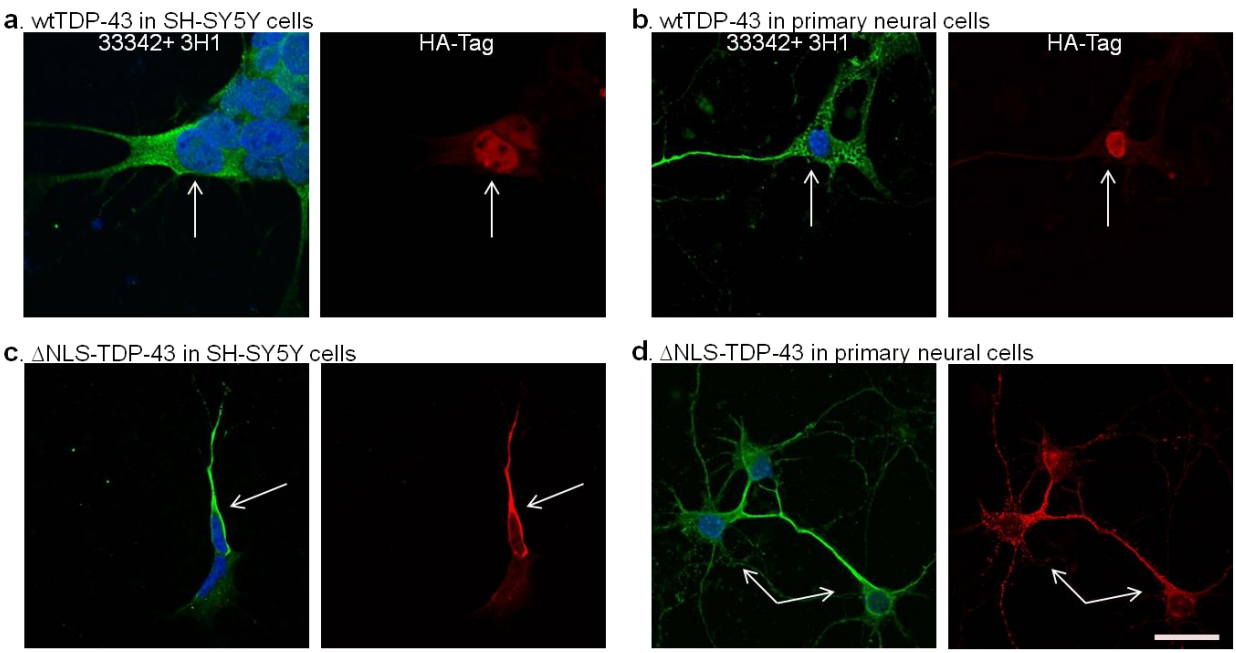

Figure 15: Human wt- and ΔNLS-TDP-43 in human neuroblastoma cells and human wtSOD1 Tg mouse neural cells

SH-SY5Y cells and primary neural cells stained against HA-tag (transfected TDP-43;red), misfolded SOD1 (green), and Hoechst33342 (blue) nuclear counterstain. Human wtTDP-43 localizes in the nucleus (**a**, **c**) while the mutant ΔNLS-TDP-43 localizes in the cytosol (**b**, **d**). Both variants of TDP-43 are associated with the misfolding of SOD1, as detected by 3H1 reactivity. Scale bar, 20µm.

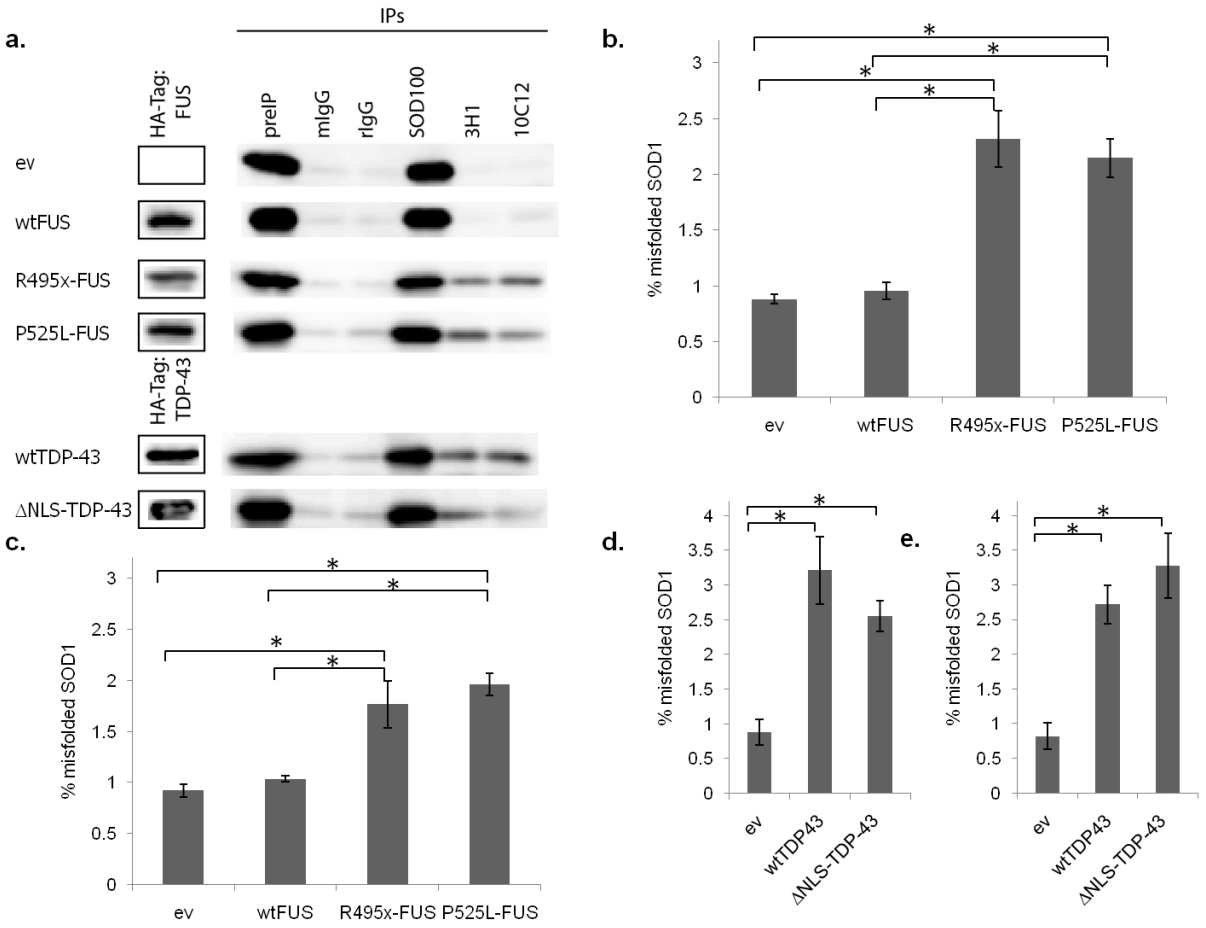

Figure 16: Immunoprecipitations and quantifications of pCINeo, human wt, R495x and P525L-FUS, as well as wt- and ΔNLS-TDP-43, in SH-SY5Y cells

Representative immunoblots of immunoprecipitations (**a**). SOD1 proteins from transfected (48h) and untransfected SH-SY5Y cell lysates were precipitated using pan-SOD1 antibody, SOD100 (rabbit), and SOD1 misfolding-specific mouse monoclonal antibodies, 3H1 and 10C12. rIgG was used as isotype control for SOD100, and mIgG was used as isotype control for DSE antibodies. Blots were probed with pan-SOD1 antibody. Framed bands show FUS, and TDP-43 transfection efficiency of the indicated construct, as was detected by probing with αHA-tag antibody. (**b**-**e**) show percentage of immunoprecipitable misfolded SOD1 (out of the total precipitable SOD1) using 3H1 (**b**, **d**) and 10C12 (**c**, **e**) from lysates of transfected SH-SY5Y cell cultures. Significant differences are indicated (*, $p<0.01$). N=5 for wtFUS, R495x- and P525L-FUS. N=6 for ev, wtTDP-43 and ΔNLS-TDP-43. Error bars represent s.e.m.

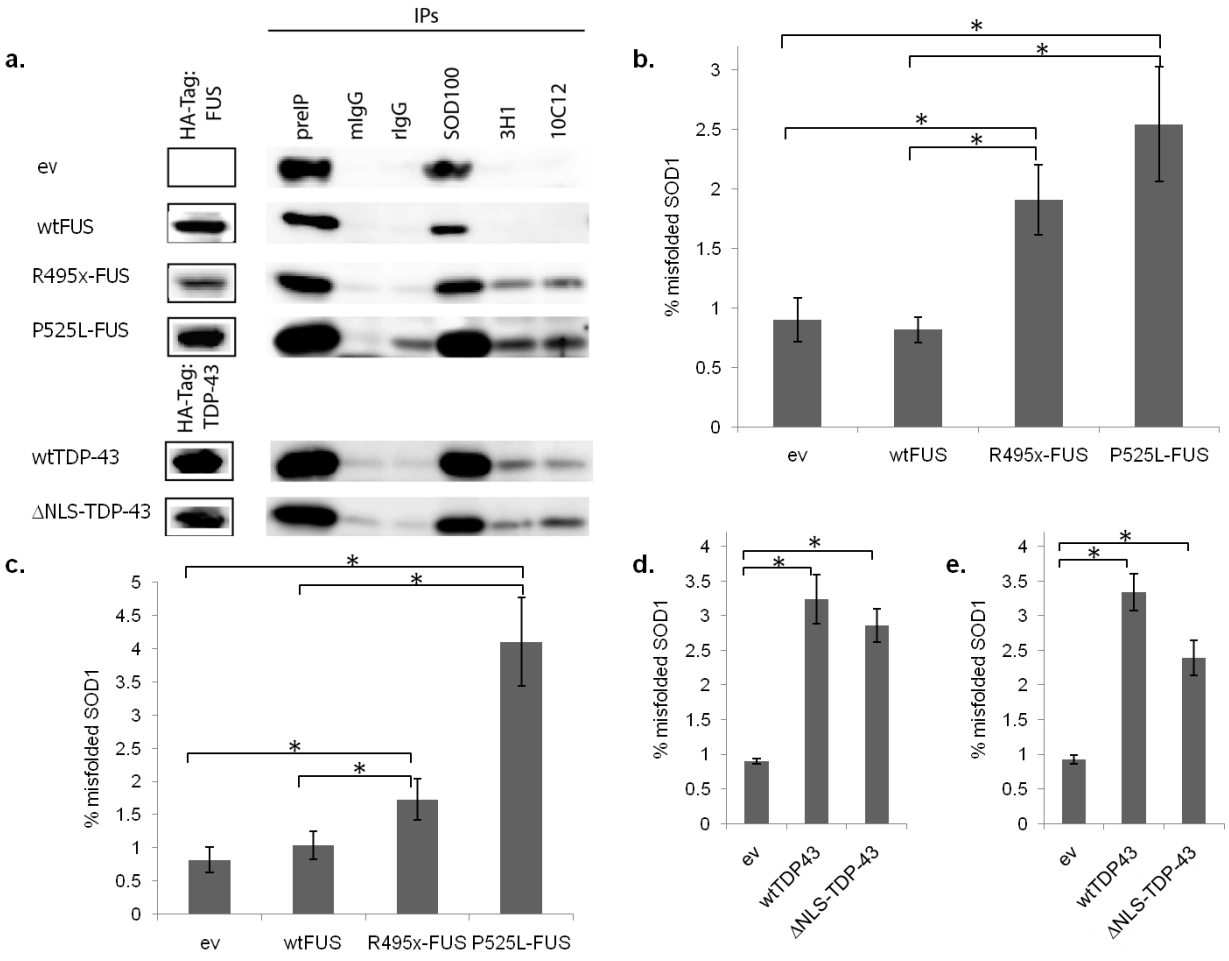

Figure 17: Immunoprecipitations and quantifications of pCINeo, human wt, R495x and P525L-FUS, as well as wt- and ΔNLS-TDP-43, in HEK293 cells

Representative immunoblots of immunoprecipitations (**a**). SOD1 proteins from transfected (48h) and untransfected SH-SY5Y cell lysates were precipitated using pan-SOD1 antibody, SOD100 (rabbit), and SOD1 misfolding-specific mouse monoclonal antibodies, 3H1 and 10C12. rIgG was used as isotype control for SOD100, and mIgG was used as isotype control for DSE antibodies. Blots were probed with pan-SOD1 antibody. Framed bands show FUS, and TDP-43 transfection efficiency of the indicated construct, as was detected by probing with αHA-tag antibody. (**b**-**e**) show percentage of immunoprecipitable misfolded SOD1 (out of the total precipitable SOD1) using 3H1 (**b**, **d**) and 10C12 (**c**, **e**) from lysates of transfected SH-SY5Y cell cultures. Significant differences are indicated (*, *P*<0.01). N=5 for wtFUS, R495x- and P525L-FUS. N=6 for ev, wtTDP-43 and ΔNLS-TDP-43. Error bars represent s.e.m.

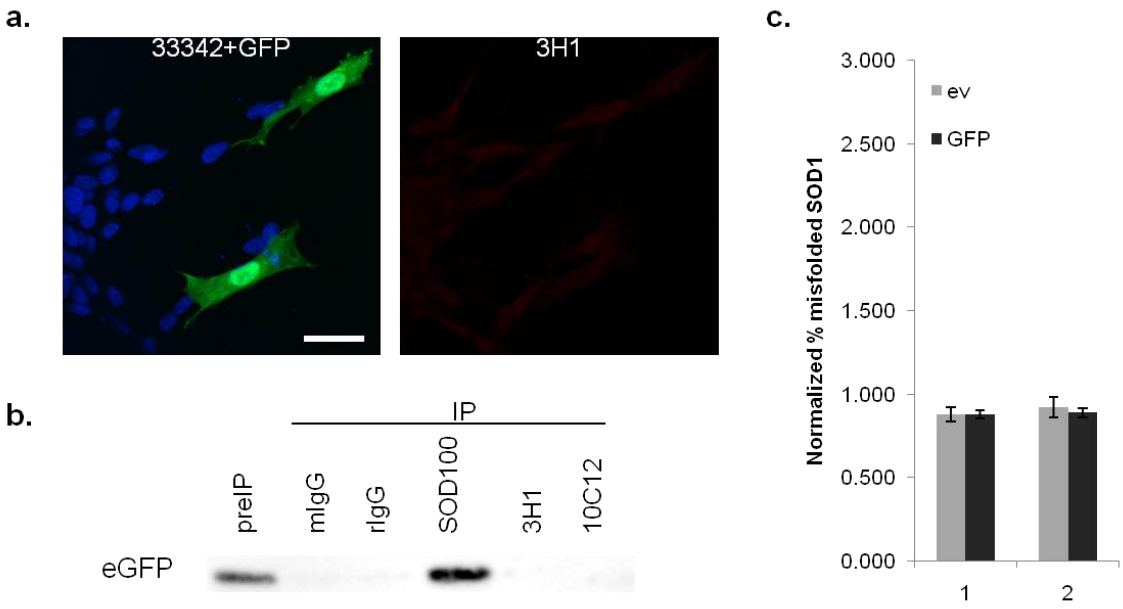

Figure 18: Expression of GFP in SH-SY5Y cells

(**a**) Human neuroblastoma cells transiently expressing the exogenous GFP protein (green) do not contain misfolded SOD1, as is evident by staining with 3H1 (red). (**b**) Immunoprecipitations of SOD1 from SH-SY5Y cultures that were transfected with the GFP vector, show pull-down of total SOD1 using the pan-SOD1, SOD100 antibody, but no presence of misfolded SOD1, as is seen by lack of pull-down of both 3H1 and 10C12. (**c**) Presence of normalized % of misfolded SOD1 in eGFP transfected cultures is comparable to ev control. Error bars show s.e.m. Scale bar, 20 µm.

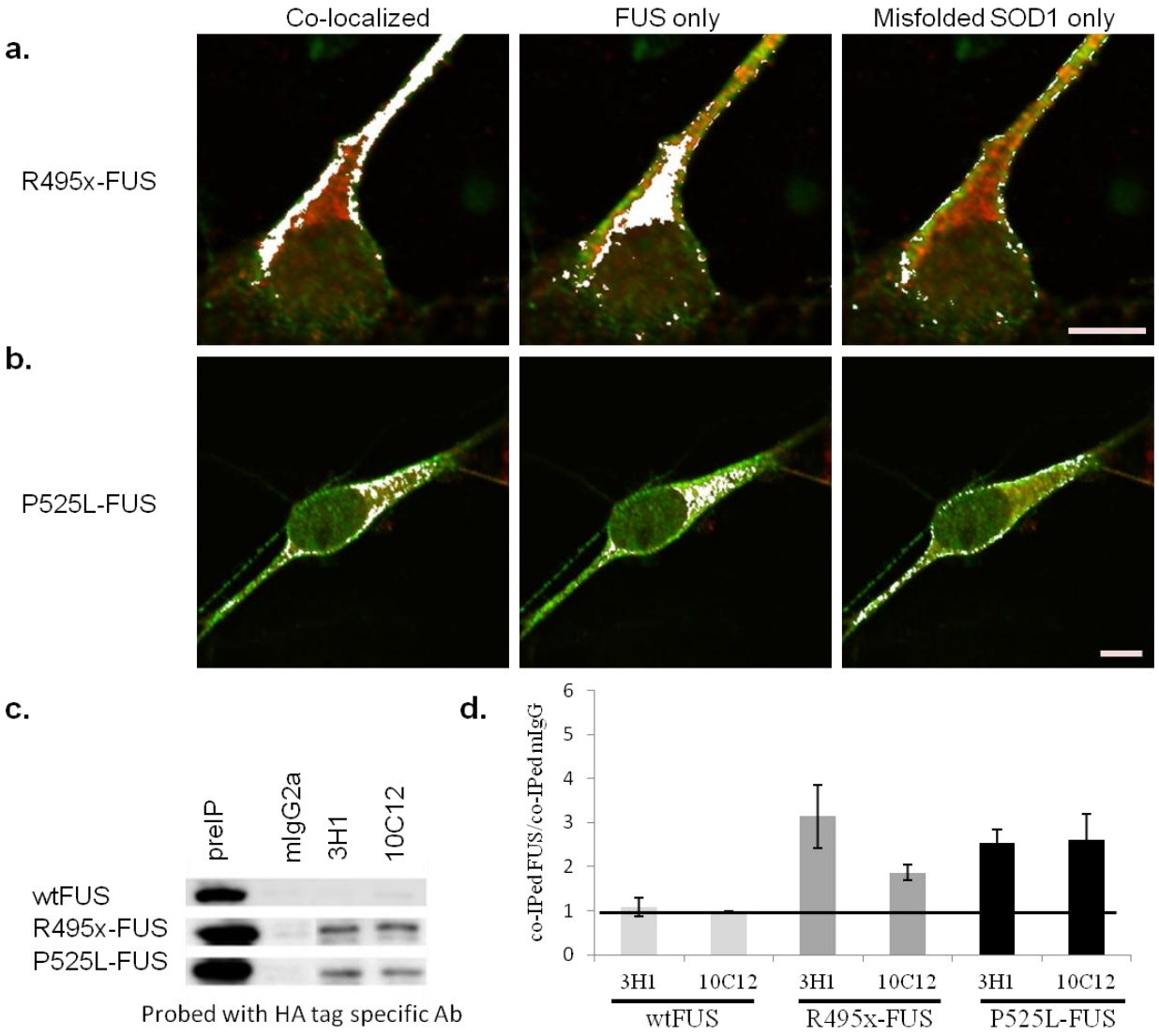

Figure 19: Co-localization and co-immunoprecipitation of exogenous FUS with SOD1

Co-localization analysis of individual neural cells from human wtSOD1 Tg mice that express R495x-FUS (**a**) and P525L-FUS (**b**), shows three regions: co-localized FUS and misfolded SOD1, FUS but not misfolded SOD1 (FUS only), and misfolded SOD1 but not FUS (misfolded SOD1 only). Pearson's coefficients for cytosolic co-localization of FUS and misfolded SOD1 in (**a**) and (**b**) are 0.63 and 0.69, respectively, indicating a certain degree if overlap. Representative immunoblots (**c**) of co-immunoprecipitation (**d**) of HA-tagged wild-type and mutant FUS using beads coated with the indicated SOD1 antibodies. Immunoblots were probed using antibody specific to HA-tag. The

summarizing graph shows the fold difference between the co-immunoprecipitated FUS using conformation-specific SOD1 antibodies, and the appropriate IgG control (N=4 each). Error bars indicate s.e.m. Scale bar, 5µm.

2.4. Discussion

Progressive degeneration of motor neurons is the main feature in ALS. Motor neurons possess the highest concentration of the long lived SOD1, and are subject to occupational hazard of detoxifying mitochondrial superoxide (Pardo, Xu et al. 1995; Rakhit, Cunningham et al. 2002). Three key proteins that have been implicated in ALS pathogenesis to date are SOD1, TDP-43 and FUS. Clinical and neuropathological similarities between familial ALS (FALS) and SALS cases, along with recent studies demonstrating the presence of misfolded SOD1 in both mutant SOD1-FALS and non-SOD1-associated SALS (Bosco, Morfini et al. 2010), suggest that disease pathology from different forms of ALS likely converge into a common biochemical pathway. FUS and TDP-43 are primarily nuclear proteins that can form a biochemical complex required for protein transcription, translation and alternative splicing (Rosen 1993; Kim, Shanware et al. 2010; Lagier-Tourenne, Polymenidou et al. 2010). Pathological TDP-43 can accumulate in the neuronal cytosol in many sporadic neurodegenerative diseases (e.g. Alzheimer's disease), in which it may be a marker of cell stressors. However, when genetically modified, TDP-43 and FUS cytoplasmic aggregation are associated with limited number of heritable syndromes, including ALS and Frontotemporal Lobar Degeneration (FTLD) (Guest, Silverman et al. 2011; Wilson, Dugger et al. 2011). Perhaps this pattern of sporadic and familial involvement is most consistent with the notion that mutations in either of these RNA binding proteins can induce a propagated toxic process that can transmit from cell to cell in the nervous system, whereas wtTDP43 aggregation observed in sporadic disease is a consequence of cell stress induced by that toxicity. Given the recent findings that misfolded SOD1 can be detected in SALS as well as FALS-SOD1 (Bosco, Morfini et al. 2010; Forsberg, Jonsson et al. 2010), that mutant and wtSOD1 can propagate as a misfolding template from cell to cell, and our current report that cytosolic localization of FUS or TDP-43 is associated with SOD1 misfolding, an attractive speculation is that propagated SOD1 misfolding could represent a final common molecular pathway of all types of ALS – both cause and consequence of TDP-43 and FUS aggregation.

Previous studies suggest a cytosolic gain of functions is incurred in the presence of pathological forms of FUS and TDP-43 in the cytosol (Lagier-Tourenne and Cleveland

2009; Kim, Shanware et al. 2010). Although the gain of function mechanism of cytoplasmic FUS and TDP-43 remains mostly uncharacterized, studies have shown that cytosolic FUS and TDP-43 are recruited into stress granules, RNP structures used by the cell to temporarily store translationally arrested mRNA, upon cellular stress (Dormann, Rodde et al. 2010; Liu-Yesucevitz, Bilgutay et al. 2010). In this study, we clearly show that expression of pathological, extra-nuclear FUS is associated with the presence of misfolded SOD1, while over-expression of wtFUS carries no such consequences. This is consistent with other observation of over-expression of human mutant, but not wild-type, FUS leading to progressive paralysis resembling ALS in rats (Huang, Zhou et al. 2011). We also show that over-expression of wild-type and cytosolic TDP-43 is associated with elevated levels of misfolded SOD1, compared to transfection using an empty vector control or an expression of GFP control. We show the distribution of these associations using immunofluorescence in human neuroblastoma SH-SY5Y cells and primary neural cells from human wtSOD1 Tg C57 BL/6 mice. The qualitative data is supported by statistically significant quantitative data from immunoprecipitations using SOD1-misfolding specific antibodies. Both 3H1 and 10C12 detected misfolded SOD1 in immunoprecipitation studies of non-denatured lysates once cytosolic FUS, as well as wild-type and cytosolic TDP-43, were expressed in cells. This finding implies that presence of pathogenic FUS or TDP-43 is associated with misfolded SOD1. To this end, our findings using immunoprecipitations also imply that misfolded SOD1 is aberrantly oxidized and monomerized, as shown by SOD1 immunoprecipitation using the 10C12 monoclonal antibody, characteristics that have been linked to misfolded SOD1 (Rakhit, Crow et al. 2004). Importantly, the misfolding of SOD1 can contribute to a toxic build up of oxidative radicals, as dimeric SOD1 structure is required for stability and activity of this protein that can no longer function properly in its monomerized and oxidized form. Using co-immunoprecipitation studies, we further find that R495x- and P525L-, but not wild-type, FUS physically interact with misfolded SOD1 protein. Furthermore, several studies have shown that misfolding of SOD1 propagates in a prion-like fashion, where the misfolded protein acts as a template for conversion of the normal protein (Cashman 2010; Munch, O'Brien et al. 2011). To spread the infection from cell-to-cell, misfolded SOD1 is now known to passage between cells, whereas

neither FUS nor TDP-43 are known to do so (Cashman 2010; Munch, O'Brien et al. 2011). However, it is poorly understood what misfolds the original SOD1 protein. In light of our results, we propose that SOD1 misfolding is triggered by physical interaction of normal SOD1 with pathogenic forms of FUS and TDP-43, which can form a productive template for the ongoing SOD1 misfolding. Alternatively, the presence of pathogenic FUS or TDP-43 may trigger an indirect and uncharacterized pathway that results in the misfolding of SOD1.

Although the initial finding of association between mislocalized FUS and presence of misfolded SOD1 in untreated SH-SY5Y cells was surprising, spontaneous cell death or differentiation may account for this. A recent study has shown that FUS has characteristics of a putative tumor suppressor, and promotes apoptosis in prostate cancer cells (Brooke, Culley et al. 2011). It was also reported that a small population of cells undergo spontaneous cell death under normal culture conditions (Ludewig, Graf et al. 1995; Pregi, Vittori et al. 2006). Speculatively, once the cell has committed itself to death, maintaining functional SOD1 is not a high priority, resulting in detectable levels of misfolded SOD1. An alternative explanation for this association might arise from cell differentiation occurring spontaneously in a small population of SH-SY5Y cells. As with more well studied proteins, such as tau (Uberti, Rizzini et al. 1997) and protein kinase C (Leli, Shea et al. 1993), FUS localization and expression patterns might be modified during SH-SY5Y cell maturation into terminally differentiated neuron-like cells, compared to neuroblastoma cells.

Consistently with other studies showing neuronal toxicity of over-expressed human wild-type and cytosolic mutant TDP-43 (Ash, Zhang et al. 2010; Barmada, Skibinski et al. 2010), our data shows that both wtTDP-43 and ΔNLS-TDP-43 associate with SOD1 misfolding. The mechanism by which wild-type TDP-43 contributes to neuronal toxicity and death is unknown. In order to properly clear misfolded and aggregated proteins, the cell employs several heat shock proteins (HSP), including the SOD1-associated HSP-B8 (Crippa, Sau et al. 2010) and HSP70 (Koyama, Arawaka et al. 2006), which recent work shows to be involved in decreasing aggregation and increase solubility of mutant TDP-43 (Crippa, Sau et al. 2010; Estes, Boehringer et al. 2011), suggesting a potential

chaperone titration mechanism. Such overwhelming, could prevent HSP-B8 and HSP70 from handling additional unfolded proteins in the form of misfolded SOD1, leading to failure of cellular processes revealing disease symptoms (Waxman 2007). Furthermore, FUS and TDP-43 form a biochemical complex that modulates expression of histone deacetylase (HDAC) 6, a protein required for clearance of misfolded proteins (Boyault, Zhang et al. 2007; Kim, Shanware et al. 2010). Both cytosolic mutant FUS and TDP-43 have previously been suggested to sequester endogenous FUS (Neumann, Rademakers et al. 2009; Vance, Rogelj et al. 2009) and TDP-43 (Winton, Igaz et al. 2008), respectively, therefore depleting the nucleus of the respective protein. The nuclear depletion of these proteins could result in lack of modulation of HDAC6 expression, consequentially leading to the buildup of misfolded SOD1, as well as TDP-43 aggregates.

Although primarily nuclear, FUS actively shuttles nucleotides between the nucleus and the cytoplasm, where it dissociates from its cargo, and imported quickly back into the nucleus by transportin (Kino, Washizu et al. 2010). If transportin is not able to re-import FUS into the nucleus, such as in the cases for mutants R495x and P525L, extra-nuclear FUS can sequester essential cytoplasmic mRNA using its RNA recognition motif (RRM) domain, thus preventing translation. A precedent for such gain of function activity of mislocalized FUS was demonstrated for hnRNP A1, where its cytoplasmic mutant delayed and inhibited viral mRNA transcription and genome replication (Shi, Huang et al. 2000). Reduced production of chaperones required for proper folding of SOD1, such as HSP-B8 (Crippa, Sau et al. 2010), and HSP70 (Koyama, Arawaka et al. 2006), could lead to the misfolding of the superoxide scavenging SOD1.

With the ever increasing interest in the implication of TDP-43 in ALS, studies have shown that the nucleo-cytoplasm shuttling TDP-43, forms physical interactions with both wild-type and mutant, therefore misfolded, SOD1 (Watanabe, Morita et al. 2008; Volkening, Leystra-Lantz et al. 2009; Higashi, Tsuchiya et al. 2010). Consistent with this, we used co-immunoprecipitations to confirm that both wild-type and cytosolic mutant TDP-43 physically interact with misfolded SOD1. We also find that exogenous cytosolic mutants, but not wild-type, FUS interact with misfolded SOD1. Recent findings

that FUS and TDP-43 form a nuclear biochemical complex, and that TDP-43 overlaps with FUS in the same cytosolic fraction (Kim, Shanware et al. 2010), suggests to us that FUS and TDP-43 can also transiently bind in the cytosol. Although the likelihood of such cytosolic binding is low in normal cells where both RNA binding proteins are primarily in the nucleus, it increases dramatically when FUS is aberrantly trapped in the cytosol. Therefore, we propose that TDP-43 might act as the mediator between FUS and misfolded SOD1, by forming a cytosolic FUS/TDP-43 complex that can transiently bind both wild-type and misfolded SOD1. Furthermore, we hypothesize that physical association between FUS, TDP-43 and wild-type SOD1 that occurs in cells expressing cytosolic mutant FUS, can physically initiate the cascade of SOD1 misfolding.

Collectively, for the first time our work demonstrates a direct association between expression of cytosolic FUS and TDP-43, and misfolding of SOD1, suggesting that all three of these key proteins implicated in ALS associate in a common cellular pathway. We also showed that cytosolic mutants, but not wild-type, FUS interact with misfolded SOD1. This opens a new window of opportunities to study the common underlying disease mechanisms between SALS cases with pathological FUS and TDP-43, and non-SOD1 FALS cases. Further research is necessary to establish the mechanisms in which these proteins interact in the cytosol, and how mislocalized FUS, as well as wild-type- and mutant TDP-43, lead to misfolding of SOD1 in ALS. The identification of a common biochemical pathway may lead us to the development of a single therapeutic against different forms of ALS, regardless of etiology.

3. Loss of Function of FUS and TDP-43 Leads to SOD1 Misfolding

3.1. Introduction

Regulation of proteostasis is crucial and occurs on several levels, including RNA and protein levels. Once the protein biosynthesis or degradation machineries fail to maintain proper expression levels of certain proteins in cells, these proteins might be present in greater or lesser levels than is optimal for cell function. FUS and TDP-43 play an important role in RNA processing, transcription, translation, splicing, stress granule formation, nucleo- cytoplasmic shuttling and RNA transport for local translation (Lagier-Tourenne, Polymenidou et al. 2010). Both proteins are involved in microRNA processing (Buratti, De Conti et al. 2010; Lagier-Tourenne, Polymenidou et al. 2010; Ling, Albuquerque et al. 2010). Although several studies have suggested that aberrant translocation of FUS and TDP-43 leads to loss of function (Pesiridis, Lee et al. 2009; Kabashi, Lin et al. 2010; Kino, Washizu et al. 2010; Xu, Gendron et al. 2010), the effect on SOD1 expression and misfolding has not yet been explored. We hypothesized that nuclear depletion of FUS and TDP-43 leads to abnormal steady state levels of SOD1 and to the misfolding of SOD1. Although TDP-43 itself is thought to not bind SOD1 mRNA (Polymenidou, Lagier-Tourenne et al. 2011), it is known to form a common biochemical complex with FUS and other proteins (Kim, Shanware et al. 2010). The FUS/TDP-43 complex, which co-regultes HDAC6 mRNA, may also bind and regulate SOD1 mRNA processing. Furthermore, FUS and TDP-43 may be indirectly involved in regulating cell content and folding of SOD1 through regulating expression of other genes, which might affect proteostasis of SOD1 and its folding properties.

3.1.1. Aims of chapter

In addition to gain of function in SOD1 misfolding as observed in Chapter 2, we hypothesized that the absence of FUS and TDP-43 may lead to a loss of function relating to proteostasis of SOD1 or its regulation, which could manifest as changes to SOD1 structure and/or a change in cellular SOD1 expression levels. To test this hypothesis, we set two aims: **first**, we studied the change in SOD1 proteostasis; **second**, we analyzed the degree of SOD1 misfolding in a cell culture model at various time-points post-FUS and TDP-43 knockdown.

3.1.2. Study design

Previous studies established that the half-life time of wtTDP-43 is significantly shorter than that of tested mutant TDP-43, yielding estimated half-lives of 12h for the wild type versus 24-48 hours for the mutants (Ling, Albuquerque et al. 2010). In mouse myeloid progenitor 32D cells, the half-life of wild-type and S256A mutant FUS were determined to be in the vicinity of 11 and 1.3 hours, respectively (Perrotti, Iervolino et al. 2000). Therefore, if new copies are not continuously synthesized, concentration of both FUS and TDP-43 are substantially reduced due to protein degradation within 24 hours. Although cell-dependant variation exists, the half-life of SOD1 in cultured cell lines is around 24 hours (Ermak, Cheadle et al. 2004).

The use of small interfering RNA (siRNA) oligonucleotides is one of the primary methods to knockdown proteins in cells (McCoy, Litterst et al. 2010). Short interfering oligonucleotides can be used for studying the effects of gene downregulation, as well as studying interaction networks and signaling pathways. siRNA knockdown of FUS and TDP-43 causes significant drop in their steady state levels about 12 hours post transfection as they are being degraded and no further synthesized. If FUS and TDP-43 play a role in SOD1 expression, an initial effect in SOD1 expression and misfolding should be detectable 24 hours post downregulation of nuclear FUS and TDP-43.

In order to determine whether SOD1 expression levels are modified after the knockdown of FUS and TDP-43, HEK293 cells were used as a cell model. Two siRNA oligonucleotides were used to downregulate each of FUS and TDP-43, as it had been previously determined by our laboratory to maximize the knockdown efficiency. SOD1 expression levels were analyzed 24, 48 and 72 hours post siRNA transfection, normalized to tubulin, a ubiquitous, unrelated cytoskeletal protein that should remain unaffected by the gene knockdown, and compared to cells transfected with identical dosages of non-specific, scrambled, siRNA.

For the second aim of determining whether knockdown of FUS and TDP-43 leads to SOD1 misfolding, HEK293 cells were used. Lysates from transfected cells, under non-denaturing conditions, were analyzed by immunoprecipitation (IP) as both 3H1 and 10C12 SOD1-DSE antibodies used are sensitive to conformational changes, and could

therefore not be used directly to probe immunoblots (IB) from standard SDS-PAGE protein gels. Following immunoblotting, protein pulled down by the SOD1-DSE mouse monoclonal antibodies was quantified and compared to pull-down by mIgG2a isotype control, and expressed as a percentage of total immunoprecipitable SOD1 by a pan-SOD1 polyclonal antibody. The presence of misfolded SOD1 in cell cultures, where both FUS and TDP-43 were knocked down, was compared to pulldown of misfolded SOD1 in cultures transfected with the non-specific, scrambled, siRNA.

3.2. Materials and methods

3.2.1. Downregulation of FUS and TDP-43 in HEK293 cells

FUS and TDP-43 steady-state protein levels were reduced in HEK293 cells using a combination of two FUS and two TDP-43 specific commercially-available siRNA oligonucleotides (Invitrogen, Carlsbad, CA). Per 10cm plate, transfection complexes were prepared as follows: 2.4µl (50mM stock) of each FUS and TDP-43 siRNAs were added to 1.2ml OptiMEM and mixed with 1.2ml OptiMEM containing 40µl Lipofectamine RNAiMAX (Invitrogen). A non-specific siRNA control mixture was also prepared in a similar fashion (Invitrogen, Carlsbad, CA). The transfection complex was incubated for 10min and added to 60% confluent HEK293 cells growing in 8ml of complete growth media. For determining the folding state of SOD1 48h post transfection, cells were lysed for immunoprecipitation as previously described in Chapter 2.

3.2.2. Determination of cellular content of SOD1

HEK293 cells were plated in 6-cm tissue culture dishes and transfected with FUS and TDP-43 siRNA as described above. 24, 48 and 72 hours later the cells were washed and collected in ice-cold PBS, lysed, and 1µl was boiled in SDS sample buffer containing 1% β -mercaptoethanol for 10min. In order to determine expression of SOD1 at different time points following siRNA transfection, the ubiquitous and unrelated tubulin protein, was used for normalization. Gels and immunoblots were run as described in section 2.2.9 and stained for SOD1 and tubulin. After normalizing SOD1 expression at various time points to the expression of tubulin, are shown as multiples of SOD1 expression in cells transfected with equal dosages of siRNA control. For the 0h time point, non-transfected cells were used.

3.3. Results

In our preliminary set of experiments, we observe that double knockdown of FUS and TDP-43 in HEK293 cells leads to 10-70% over-expression of SOD1 (**Figure 20**). As both FUS and TDP-43 become downregulated shortly after siRNA transfection due to their short half-lives, expression of SOD1 starts to increase and reaches nearly a 2-fold increase 48 hours post transfection, following which SOD1 expression levels drop. The eventual decrease in SOD1 expression could stem from reactivation of FUS and TDP-43 synthesis, as levels of siRNA in the cell fall due to cell division and degradation. The duration of gene knockdown is inversely proportional to the rate of cell division and gene expression return to steady-state levels within a week in rapidly dividing cells (Barlett, Kolakowski et al. 2006; Malen, Lillehaug et al. 2009). The transfected siRNA may be destabilized by nucleases (Choung, Kim et al. 2006; Bartlett and Davis 2007), allowing the slow restoration of FUS and TDP-43, which leads to restoration of physiological levels of SOD1 (**Figure 20**).

As has been known for many years, SOD1 is an abundant cellular component of motor neurons and other neurons (Pardo, Xu et al. 1995). Over-expression of wild type human SOD1 in mice leads to motor neuron death, however it remains debatable whether it accelerates motor neuron disease in mice expressing mutant human SOD1 (Jaarsma, Haasdijk et al. 2000; Audet, Gowing et al. 2010). Additionally, over-expression of wild-type SOD1 acceleration disease onset and neuron death in mutant SOD1 Tg mice (Wang, Deng et al. 2009). In this study, we show that over-expression of wtSOD1 in cells leads to significant levels of misfolded SOD1 (**Figure 21**). It has been previously shown that metal-catalyzed oxidation of wild-type SOD1 at physiological concentrations (40μM) results in SOD1 destabilization, dissociation into monomers and aggregation in vitro (Rakhit, Crow et al. 2004). Taken together with the fact that over-expression of wild type human SOD1 in human cell lines causes SOD1 to misfold (**Figure 21**), these lines of evidence suggest the significance of maintaining proper concentration of SOD1 in healthy cells. Due to the important roles of FUS and TDP-43 in cells, nuclear depletion could lead to devastating consequences. In fact, knockdown of FUS and TDP-43 leads to over-expression of SOD1 (**Figure 20**), and as **Figure 22** shows, immunoprecipitation using SOD1 misfolding specific antibodies, 3H1 and

10C12, shows the detection of misfolded SOD1. However, no misfolded SOD1 is detected in samples transfected with equal dosage of scrambled control siRNA. There is 3 times more misfolded SOD1 in FUS and TDP-43 downregulated samples, compared to control samples ($p<0.07$, N=3).

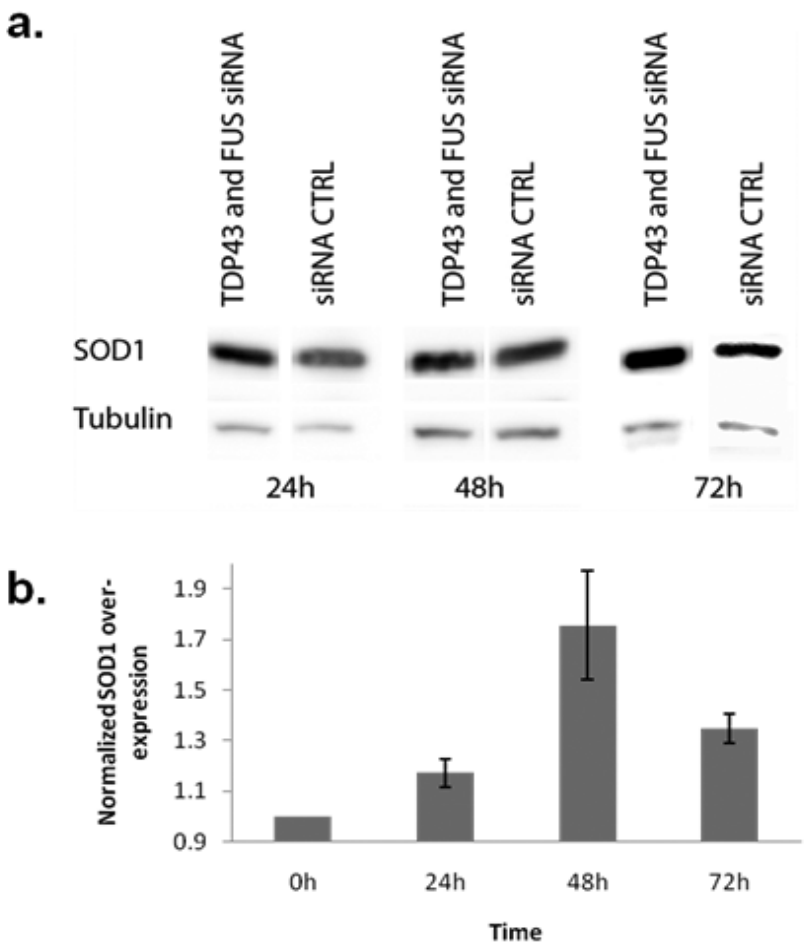

Figure 20: SOD1 over-expression following FUS and TDP-43 downregulation

FUS and TDP-43 were downregulated in HEK293 cells for the indicated period of time, following which cells were collected, lysed and analyzed using immunoblotting for steady state levels of SOD1, and normalized to the expression of tubulin (**a**), an ubiquitous and unaffected control protein. Tubulin expression levels in cells transfected with control and FUS/TDP-43 siRNA are shown for each time-point. Data is quantified in **b**, showing the peak of SOD1 over-expression 48 hours after knockdown of FUS and TDP-43 (N=4 each time point).

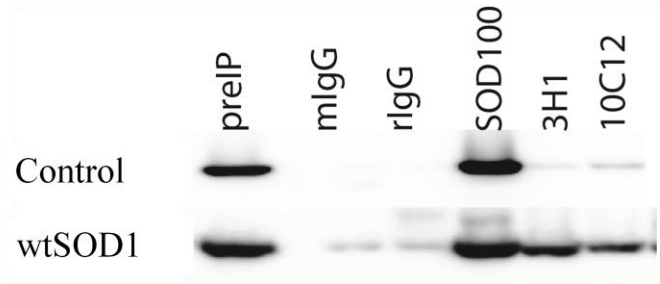

Figure 21: Over-expression of wtSOD1 in HEK293 cells leads to SOD1 misfolding

Comparison of HEK293 cell lysates transfected with empty vector (control) or human wtSOD1 by immunoprecipitations using 3H1 and 10C12, shows that misfolding of SOD1 occurs when excess wtSOD1 is present in cells. SOD100 is a polyclonal pan-SOD1 antibody that immunoprecipitates total SOD1. rIgG and mIgG2a are the isotype controls for SOD100 and 3H1/10C12, respecively.

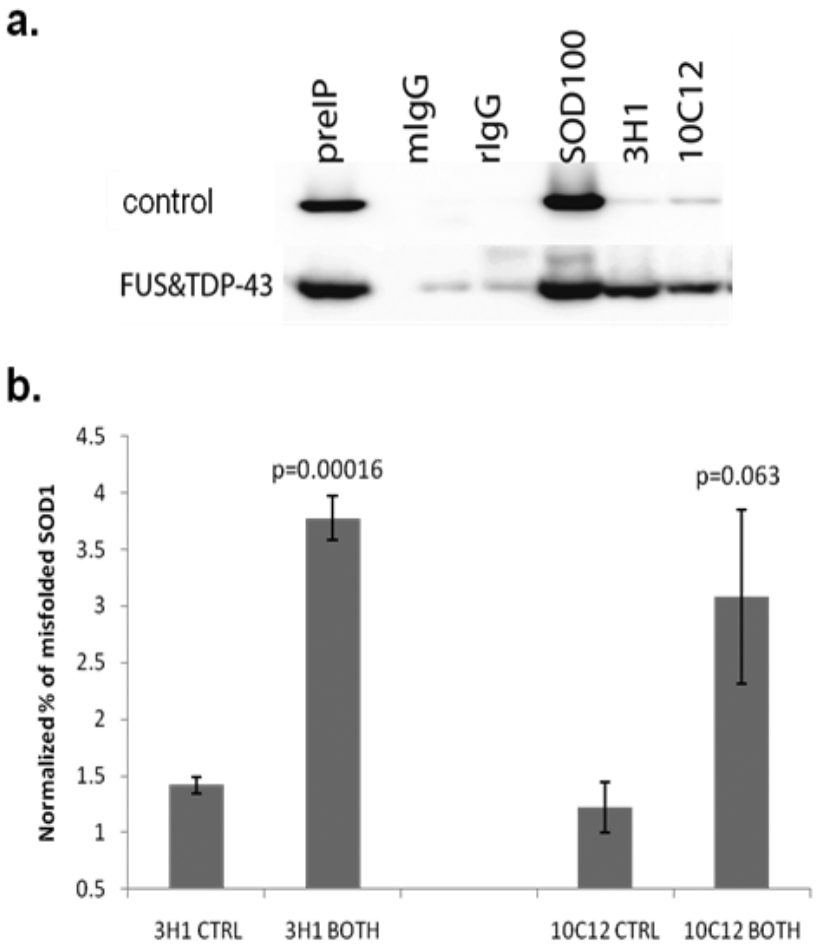

Figure 22: Misfolding of SOD1 post FUS and TDP-43 downregulation

Downregulation of FUS and TDP-43 for 48 hours leads to increased SOD1 misfolding in HEK293 cells using both 3H1 and 10C12 antibodies (**a**), compared to scrabbled control. Data quantification (**b**) indicates 3 fold increase in SOD1 misfolding in samples where both in FUS and TDP-43 were downregulated.

3.4. Discussion

In light of the major roles that FUS and TDP-43 play in RNA processing and shuttling, it comes as no surprise that their downregulation can lead to significant consequences. In our study we demonstrate that downregulation of FUS and TDP-43 carries loss of function effects, as can be determined by both over-expression and misfolding of SOD1. Over-expression of SOD1 in our study confirms and extends the finding that SOD1 mRNA levels are increased in SALS motorneurons (Bergeron, Muntasser et al. 1994). It is for the first time that absence of endogenous FUS and TDP-43 is found to have a profound effect on SOD1, a protein that has been implicated in familial ALS for over a decade.

The significance of expressing functional TDP-43 was demonstrated by Kraemer *et al.* Mice homozygous for loss of TDP-43 are not viable, while heterozygous animals have motor deficits, confirming the essential nature of this protein in motor function and embryogenesis (Kraemer, Schuck et al. 2010). Additionally, mice homozygous for FUS deletion die at birth (Hicks, Singh et al. 2000), and FUS-deficient neurons show abnormal spine morphology (Fujii, Okabe et al. 2005). Downregulation of these essential proteins may lead to a devastating global effect. Although a direct link between FUS/TDP-43 and the proteolysis machinery is yet to be established, FUS and TDP-43 may regulate expression levels of proteins involved in the protein degradtion, thus preventing the build-up of misfolded proteins. In such an instance, small amounts of SOD1 could randomly misfold without being cleared, which would further inhibit the proteasome (Urushitani, Kurisu et al. 2002). This leads for increasing amounts of misfolded SOD1 detectable by immunoprecipitation in our study. As was previously discussed in Chapter 2, overexpression of wild-type SOD1 and its misfolding may also seed the propagation of SOD1 misfolding, which potentiates the effects of toxicity and cell to cell propagation (Cashman 2010; Munch, O'Brien et al. 2011).

Arguably, aberrant RNA processing could be a pathogenic factor for ALS (Lin, Bristol et al. 1998; Chang, Kong et al. 2008). Due to their involvement in protein biosynthesis and RNA trafficking, the RNA processing complex comprised of FUS and TDP-43 may be involved in the processing of SOD1 mRNA. Although previous reports show that

TDP-43 does not bind SOD1 mRNA (Polymenidou, Lagier-Tourenne et al. 2011), no study has explored whether FUS binds SOD1 mRNA. A recent study by Gagliardi *et al.* observed abnormally high levels of SOD1 transcript in the brain stems and spinal cords of SALS patients, as well as below normal soluble SOD1 expression levels, which is proposed to precipitate in insoluble proteinaceous aggregates (Gagliardi, Cova et al. 2010). Our previous studies also show that transient over-expression of wild type SOD1 in cell lines leads to misfolding of SOD1. Together with the necessity of having functional FUS and TDP-43, and their pathological involvement in ALS, we speculate that the complex formed by FUS and TDP-43 might be indirectly involved in regulating SOD1 biosynthesis. In the absence of FUS and TDP-43, steady state levels of SOD1 increase, possibly in response to compensate for increased levels of misfolded SOD1. This hypothesis is supported by previous studies showing that oxidative insult leads to both depletion of nuclear TDP-43 due to its redirection into stress granules (Colombrita, Zennaro et al. 2009) and over-expression of endogenous SOD1 to neutralize the stressor (Cova, Cereda et al. 2006).

Functionally, the biochemical complex that FUS and TDP-43 form was recently shown to co-regulate histone deacetylase (HDAC) 6 mRNA (Kim, Shanware et al. 2010). Kim *et al.* also showed that silencing of either FUS or TDP-43 reduces the expression of HDAC 6, a member of the HDAC family that catalyze the removal of acetyl groups from lysine residues in histones, a process leading to chromatin condensation and transcriptional repression (Bolden, Peart et al. 2006). Additional studies showed that HDAC 6 rescues neurodegeneration by providing an essential link between autophagy and the ubiquitin-proteasome pathway required for the degradation of ubiquitinated proteins (Urushitani, Kurisu et al. 2002; Pandey, Nie et al. 2007). HDAC6 also regulates aggresome formation and cell viability in response to cellular stress due to presence of misfolded proteins (Kawaguchi, Kovacs et al. 2003). Another line of evidence shows that misfolded SOD1 forms ubiquitinated cytoplasmic inclusions (Ilieva, Polymenidou et al. 2009). Considering this, we hypothesize that downregulation of FUS and TDP-43 leads to reduction in HDAC 6's expression, which is required for the removal of misfolded protein. Therefore, detection of high levels of misfolded SOD1 in

FUS and TDP-43 downregulated samples suggests the inability of cells in clearing the misfolded protein.

In conclusion, our study demonstrates that nuclear depletion of FUS and TDP-43 leads to a significant loss of function in the cell. We determined that knockdown of FUS and TDP-43 using siRNA leads to over-expression and misfolding of SOD1. We propose that misfolding of SOD1, which is detected in both FALS and SALS cases, is caused by the reduction in normal FUS and TDP-43 due to their cytoplasmic aggregation. Further research is necessary to determine which of the protein synthesis or cellular degradation system is affected, and whether HDAC6 plays a role in ALS pathogenesis. We will also examine whether downregulation of a single proteins, FUS or TDP-43, leads to over-expression of SOD1 and its misfolding.

4. Conclusion and Future Directions

ALS is a devastating neurodegenerative disease leading to the degeneration of motor-neurons and eventually death. Although the aetiology of ALS is largely unknown, recent studies show that SOD1, FUS and TDP-43 play an important role in disease pathogenesis. Our aim was to show for the first time that a link exists between pathological FUS and TDP-43 and the misfolding of SOD1, which is found in all cases of ALS. Pathological forms of FUS and TDP-43 tend to accumulate in the cytosol instead of their normal locale in the nucleus. Both gain and loss of functions of mislocalization of these proteins were previously proposed, which we studied with the readout being SOD1 misfolding.

We first cloned the DNA of wild type and cytoplasmic versions of both FUS and TDP-43 into the pCINeo expression vector. This plasmid was chosen due to its relatively small size, efficient and ubiquitous CMV promoter, and presence of neomycin selection marker for the generation of stably expressing cell lines. Unlike with the over-expression of human wtFUS, we observe that expression of cytosolic pathological FUS variants in cultured human neuroblastoma SH-SY5Y and human kidney HEK293 cells, is associated with the presence of misfolded SOD1 using both immunofluorescence and immunoprecipitations. We also show a similar effect in transfected spinal cord primary neural cells expressing human wild-type SOD1 (from h-wtSOD1 Tg C57 BL/6 mice). Additionally, we have demonstrated that over-expression of wild type TDP-43, as well as expression of cytosolic mutant TDP-43, leads to SOD1 misfolding in SH-SY5Y cells, HEK293 cells, and primary neural cultures. Immunofluorescence and immunoprecipitation studies also show partial co-localization and co-immunoprecipitation of misfolded SOD1 with transfection-driven pathogenic FUS and TDP-43. Finally, our preliminary studies show that downregulation of endogenous FUS and TDP-43 using specific siRNA oligomers is associated with over-expression and misfolding of SOD1.

Future directions for this project revolve around testing the specific hypotheses mentioned in the individual chapters. In order to establish the phenomena in living organisms, confirming our findings *in vivo* is of utmost interest. Therefore, we will

determine whether wild-type and mislocalized FUS and TDP-43 associate with misfolding of SOD1 in living mice. For such purpose we will cross the human wtSOD1 Tg mouse strain that we are currently breeding in our facility, with other mouse strains that express wild-type and cytoplasmic mutant FUS and TDP-43. Since FUS and TDP-43 transgenic mice are not available through The Jackson Laboratory, we will aim at establishing collaborations with other research groups that carry these mouse strains. Embryos from this cross will be analyzed for motor deficits, and their neurons will be analyzed using immunofluorescence studies at different stages of their lives. Finally, postmortem staining of brain slices will be performed to confirm the presence of misfolded human SOD1 in FUS (cytoplasmic) and TDP-43 (nuclear and cytoplasmic) transgenic and not in the control mice.

Additionally, the biochemical pathway through which FUS and TDP-43 affect the folding of SOD1 will be determined, with hopes of blocking it to prevent the onset of ALS. As others' and our previous work shows, once misfolding of SOD1 has begun, it spreads in a prion-like fashion; therefore, it is best to prevent it from misfolding from the start. Future studies should aim at understanding the mechanism of how mislocalized FUS and TDP-43 cause for SOD1 to misfold. Of particular importance is to study whether the presence of cytoplasmic mutant FUS and TDP-43 saturates the clearance capabilities of HSP-B8 and HSP-70, and make them unavailable for the clearance of misfolded SOD1. The effects of a single downregulation of either FUS or TDP-43 on the expression and misfolding of SOD1 will also be explored. Finally, the hypothesis that downregulation in HDAC 6, due to the nuclear depletion of FUS or TDP-43, leads to accumulation of misfolded SOD1 will be tested via specific inhibition of HDAC 6 with Tubasin (Marcus, Zhou et al. 2005), followed by immunoprecipitations targeting misfolded SOD1. Presence of misfolded SOD1 in HDAC 6-inhibited cultures will be compared to the presence of misfolded SOD1 in normal, untreated cultures. If specific inhibition or downregulation of HDAC 6 leads to the build-up of misfolded SOD1, we will perform further rescue experiments, reintroducing HDAC6, and reevaluate the presence of misfolded SOD1.

In conclusion, our work shows for the first time the association between FUS, TDP-43 and SOD1 that could lead to misfolding of SOD1 under pathological circumstances. We find that both cytosolic expression and nuclear depletion of FUS and TDP-43 associate with SOD1 misfolding via gain and loss of function mechanisms, respectively. We hypothesize that the gain of function mechanisms of the cytosolic variants may: initiate the cytotoxic template directed misfolding of SOD1, which can then be transmitted between cells; work through overloading of the cytoplasm with misfolding-prone proteins that chaperones cannot adequately handle; sequester essential mRNA transcripts, such as HSPs, therefore preventing their translation. Over-expression of wild type TDP-43 has been shown to be destructive before, but here we show that it associates with SOD1 misfolding, which carries devastating consequences. Knockdown of FUS and TDP-43 from the nucleus is detrimental as well. We hypothesized that the loss of function due to the downregulation of FUS and TDP-43 could: prevent proper processing of SOD1 mRNA leading to higher concentration of SOD1 in an attempt to compensate for misfolded SOD1 transcripts; cause reduction in HDAC 6 expression that is required for clearance of misfolded proteins; cause deficient synthesis of proteins involved in the protein degradation system.

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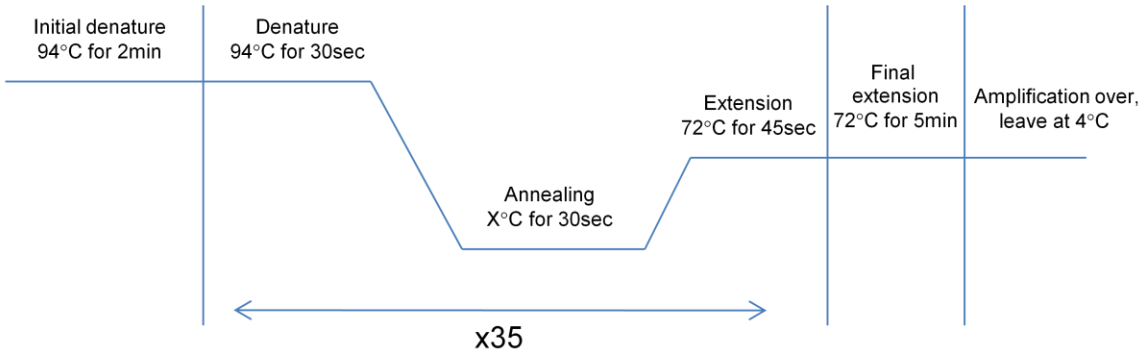
Appendices

Appendix I: Cloning protocols

PCR reaction and annealing temperatures (using Expand High-Fidelity Taq polymerase from Roche Diagnostics, Mannheim, Germany):



Where x is annealing temp for the generation of:	Annealing temperature (in °C):
wtTDP-43	61
ΔNLS-TDP-43	66/ 59 (First cycle, Reaction I/II) 61 (Second cycle)
wtFUS	62
R495x-FUS	64
P525L-FUS	65

Figure 23: PCR reaction and primer annealing temperatures

Upper panel shows the typical PCR cycle used for amplification of wild type and mutant FUS and TDP-43. Bottom panel indicates the annealing temperature required for the specific amplification reaction.

Restriction digest reactions (using enzymes from Fermentas Life Sciences, Burlington, ON):

- 6µl Tango buffer
- 0.75µl XbaI
- 0.75µl XhoI
- (22.5µl of PCR fragment purified from agarose gel) or (2µl of pCINeo backbone plus 20.5µl nucleases free water)
- Incubated at 37°C overnight

Ligation (using Taq Ligase from Roche Diagnostics, Mannheim, Germany):

- 1µl of ligation buffer
- 1µl of Taq ligase
- 0.5µl of purified (from agarose gel) digested plasmid backbone
- 7.5µl of purified (from agarose gel) digested PCR insert

Transformation into DH5α E. coli chemically competent cells (sub-cloning efficiency from Invitrogen, Carlsbad, CA):

- Thawed E. coli cells on ice for 15 minutes or until fully thawed
- Aliquoted 75µl of thawed cells into pre-chilled 1.5ml eppendorf tubes
- Added 5µl of ligation reaction into tube
- incubated on ice for 20 minutes
- Heat shocked for 45 seconds at 42C
- Immediately put on ice for 5 minutes
- Added 700µl LB media
- Incubated at 37°C for 30 minutes with constant shaking

Appendix II: Maps of pCINeo HA-TDP-43 and pCINeo HA-FUS

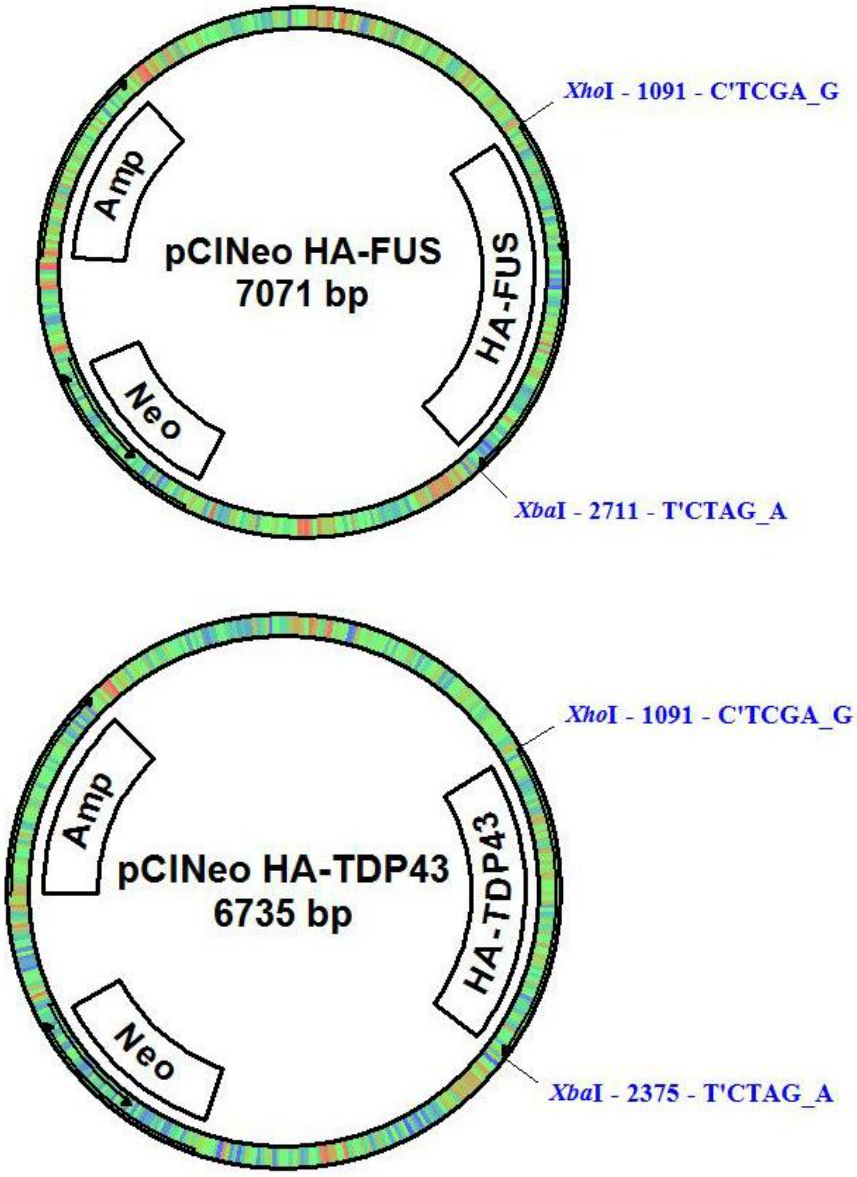


Figure 24: FUS and TDP-43 containing plasmids

Both HA-FUS and TDP-43 were cloned into the multiple cloning site of pCINeo, between *XhoI* and *XbaI* restriction digest sites. The chosen pCINeo plasmid contains ampicillin and neomycin selection markers.

Appendix III: Spectral analysis of Alexa-488 and Alexa-647

Choosing the right fluorophores for immunofluorescence studies is crucial to prevent spectral overlapping and bleed-through. Although partial differentiation between the various spectra is possible using various microscope features, such as filters and sequential image acquisition, it is better to prevent such spectral overlapping by selecting compatible fluorophores. We chose to double stain using Alexa-488 in conjunction with ALexa-647 (Figure 25). The bleed-through using these fluorophores is negligible, therefore preventing detection of artifacts.

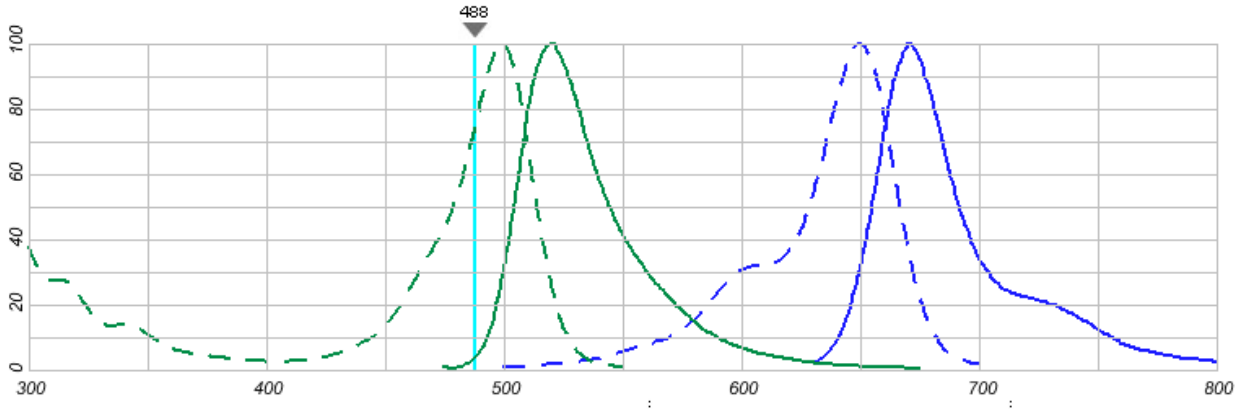


Figure 25: Spectra comparison between Alexa 488 and Alexa 647

Dotted and solid curves represent excitation and emission spectra, respectively. The green spectrum on the left is Alexa-488, and the blue spectrum on the right is Alexa-647. Spectra analysis were generated using Fluorescence SpectraViewer (Invitrogen, Carlsbad, CA).