Induction of wild-type SOD1 misfolding, aggregation and its cell-to-cell propagation

by

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M.Sc., The University of British Columbia, 2011

A THESIS Submitted in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

in

The Faculty of Graduate and Postdoctoral Studies

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

March 2017

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Abstract
Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease characterized by progressive degeneration and loss of motor neurons that appears to spread through the neuroaxis in a spatiotemporally restricted manner. Misfolded Cu/Zn superoxide dismutase (SOD1) has been detected in all ALS patients, despite SOD1 mutations accounting for only 2% of total cases, while the presence of inclusions containing pathological TAR-DNA binding protein-43 (TDP-43) represent a hallmark of all non-SOD1/FUS familial ALS. We previously reported that TDP-43 and FUS can trigger misfolding of human wild-type SOD1 (HuWtSOD1) in living cells, however the mechanisms and consequences are unknown. Here, we used immunocytochemistry, immunoprecipitation and cell viability studies to demonstrate that TDP-43 or FUS-induced misfolded HuWtSOD1 can propagate from cell-to-cell via conditioned media, and seed cytotoxic misfolding of endogenous HuWtSOD1 in the recipient cells in a prion-like fashion. Knockdown of SOD1 using siRNA in recipient cells, or incubation of conditioned media with misfolded SOD1-specific antibodies, inhibits intercellular transmission, indicating that HuWtSOD1 is an obligate seed and substrate of propagated misfolding. Furthermore, we developed several chimeric SOD1-GFP proteins that we validated to aggregate in the presence of pathological SOD1 or TDP-43 seed. We used this assay, along with immunofluorescence, live-cell microscopy and flow cytometry studies, to show that intermolecular conversion of SOD1 by pathological TDP-43 is mediated by tryptophan residues in both proteins. Furthermore, we used the reporter proteins to show that human spinal cord extracts prepared from familial, but not sporadic, ALS patients can trigger SOD1 aggregation in cultured cells. Finally, we used this system to show that small molecules, akin to 5-fluorouridine, can block this intermolecular kindling of SOD1 aggregation, and demonstrated that our assay can be used as a high-throughput tool for screening drugs against induced SOD1 aggregation. Altogether, our studies indicate that pathological TDP-43 and FUS may exert motor neuron pathology in ALS through the initiation of tryptophan-dependent propagated SOD1 misfolding. Furthermore, it is key to recognize that elucidation of the pathogenic role of a simple structural motif in ALS may provide a framework for understanding other neurodegenerative diseases in which propagated protein misfolding is shown to occur.
Preface

Hypotheses and study designs required for the studies in this thesis were researched and developed by Edward Pokrishevsky with approval and guidance from Dr. Neil Cashman. Other Cashman Lab members involved in this study were: Mr. Jeremy Nan, Mrs. Lana Hong, Mrs. Destiny Lu-Cleary, Mrs. Alyssa Nickel, Dr. Leslie I. Grad, Ms. Jing Wang and Mr. Masoud Yousefi.

For all the Chapters, Edward Pokrishevsky was responsible for formulating original hypotheses and the overall designs of these projects (with guidance from Dr. Cashman), and performed all of the necessary molecular cloning of the SOD1, TDP-43, FUS and reporter protein constructs. Together with the help from Mrs. Hong, Mrs. Lu-Cleary, Mrs. Nickel, Edward carried out the HEK293 and SH-SY5Y cell culture work, from initial seeding and maintenance to transfections and other cell manipulations. Edward performed the time-course living cell microscopy, immunofluorescence staining, as well as image acquisition and analysis. Edward performed majority of the immunoprecipitation and immunoblotting studies, and all of the final data analyses and quantifications. Together with Mr. Nan, Edward performed the high-throughput studies utilizing the reporter protein. Edward also genotyped mouse embryos prior to preparation of primary neural cultures, carried out their manipulations and stained for immunofluorescence studies. 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. Finally, Edward prepared all the figures presented in this thesis.

A version of Chapter 2 was published as:

Pokrishevsky, E., Grad, L. I. & Cashman, N. R. TDP-43 or FUS-induced misfolded human wild type SOD1 can propagate intercellularly in a prion-like fashion. Sci Rep 6, 22155, doi:10.1038/srep22155 (2016). Contribution: Conceived and designed the experiments: E.P. (75%) and N.R.C (25%); Performed the experiments: E.P. (80%), L.I.G. (20%); Analyzed the data: E.P. (70%), L.I.G. (15%) and N.R.C. (15%); Wrote manuscript E.P. (75%) and N.R.C. (25%).

Screening of small molecules was performed in collaboration with the ALS Therapy Development Institute. Dr. Steve Plotkin reviewed parts of the introduction.
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Δ – delta, missing.
μ - micro
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<table>
<thead>
<tr>
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<th>Description</th>
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<tr>
<td>5-FUr</td>
<td>5-fluorouridine</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>α</td>
<td>Anti</td>
</tr>
<tr>
<td>aa</td>
<td>Amino Acid</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>bp</td>
<td>Base Pairs</td>
</tr>
<tr>
<td>ΔX</td>
<td>Missing sequence X</td>
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<tr>
<td>DIV</td>
<td>Days <em>in-vitro</em></td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DOC</td>
<td>Deoxycholate</td>
</tr>
<tr>
<td>DSE</td>
<td>Disease Specific Epitope</td>
</tr>
<tr>
<td>fALS</td>
<td>Familial Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FUS</td>
<td>Fused in Sarcoma / Translated in Liposarcoma</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescence protein</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human Embryonic Kidney Cells (HEK293FT)</td>
</tr>
<tr>
<td>hnRNP</td>
<td>Heterogeneous Nuclear Ribonucleoproteins</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat Shock Protein</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
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<tr>
<td>NES</td>
<td>Nuclear Export Signal</td>
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<td>NGS</td>
<td>Normal Goat Serum</td>
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<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
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<tr>
<td>O$_2^-$</td>
<td>Superoxide radicals</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffer Solution</td>
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<tr>
<td>PBSTx</td>
<td>0.3% Triton X-100 in Phosphate Buffer Solution</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>-----------</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>PK</td>
<td>Protein Kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>RRM</td>
<td>RNA Recognition Motif</td>
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<tr>
<td>Ur</td>
<td>Uridine</td>
</tr>
<tr>
<td>sALS</td>
<td>Sporadic Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium DodecylSulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
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<tr>
<td>SOD1</td>
<td>Cu/Zn Superoxide Dismutase</td>
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<tr>
<td>SY5Y</td>
<td>SH-SY5Y human neuroblastoma cells</td>
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<tr>
<td>TDM</td>
<td>Template Directed Misfolding</td>
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<td>TAR DNA-Binding Protein 43</td>
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<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blot</td>
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## List of Amino Acid Codes

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<td>Ala</td>
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<td>cysteine</td>
</tr>
<tr>
<td>D</td>
<td>Asp</td>
<td>aspartic acid</td>
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<td>E</td>
<td>Glu</td>
<td>glutamic acid</td>
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<tr>
<td>G</td>
<td>Gly</td>
<td>glycine</td>
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<td>H</td>
<td>His</td>
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<td>I</td>
<td>Ile</td>
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<td>tyrosine</td>
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Acknowledgements

I would like to express my deepest gratitude to my advisor, Dr. Neil R. Cashman for his expert advice, guidance, encouragements and full support, as well as for believing in me.

I also express my appreciation to my committee members, Dr. Cheryl Wellington, Dr. Yu-Tian Wang and Dr. Max Cynader for finding the time in their busy schedules to meet with me and guide me through my project, as well as our collaborator Dr. Ian R. Mackenzie for all of his help and resources. I would also like to thank Dr. Vincent Duronio for his leadership and guidance throughout the Experimental Medicine program.

My special thanks also go to the current and past members of the Cashman Laboratory. First and foremost, I wish to express my sincere gratitude and appreciation to Dr. Anat Yanai, who has taught me about project design, guided me through many of the techniques used in this project, and for being a friend. My gratitude also goes to Dr. Leslie I. Grad who guided me through parts of these studies and for physically helping with some of the extensive work. I would like to particularly thank the students that helped me generate and acquire the data presented in this thesis: Jeremy Nan, Alyssa Nickel, Destiny Lu-Cleary and Lana Hong. 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 schedules, especially: Dean Airey, Dwayne Ashman, Judith Silverman, Luke McAlary, Ebrima Gibbs, and Sarah Fernando.

I am thankful to Cecily Bernales and Sarah Louadi for their continuous support, encouragement, cheerfulness, and for being good friends.

I would like to thank the sources of funding that allowed me to perform this work, including ALS Canada, Canadian Institutes of Health Research (CIHR), and Brain Canada, Giancarlo and Odette Tognetti Trust Foundation as well as for Dr. Cashman’s financial resources.
Finally, I would like to express my loving thanks to my wife, children, 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 amazing wife Oana Pokrishevsky, our exuberant and kind-hearted little kiddos, Daniel and Maya, and to my beloved parents, sister, and grandmother: Alla, Semion, Karin and Tanya Pokrishevsky for their endless love, support, encouragements and understanding.
Chapter 1: Introduction

Neurodegeneration is an umbrella term for an array of neurological diseases, including amyotrophic lateral sclerosis, Alzheimer’s and Parkinson’s diseases, that are characterized by the progressive structural or functional loss of neurons, which ultimately leads to development of clinical features. The symptoms are typically chronic in nature with profound impacts on the well-being of the affected individuals, as well as their families, friends and caregivers. Although these neurological conditions may affect people of all ages, their prevalence and incidence increases dramatically with age. As life expectancy increases and our population ages, the number of individuals suffering from these disorders, as well as the personal and economic burdens, are expected to rise. The mechanisms that are responsible for these disorder are not yet clear, however oxidation and protein misfolding is a common theme.

1.1 Amyotrophic lateral sclerosis

“Larry was a handsome and healthy man who had a very successful career. We had just gotten married. The world was our oyster,” says Joanne, Larry’s wife. “When we got the diagnosis, it was like a brick wall had hit us. The impact was devastating. There was no hope, no treatments, nothing, and that was the hardest part. We had no control.” 1. Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease is similarly so described by all ALS patients, relatives, and friends. ALS is a terminal motor neuron disease characterized by systematic paralysis of the muscles of the limbs, speech, swallowing, and respiration, due to the progressive degeneration of innervating motor neurons2. 30,000 individuals in North America are currently suffering from the disease, and 5,000 new cases are reported each year3. 2-3 Canadians are lost to ALS every day4. Moreover, less than 20% of the affected individuals survive for longer than 5 years after diagnosis4. Given the early risk of developing ALS, which peaks between 50 and 70 years 5, ALS robs otherwise perfectly healthy individuals of the prime years of their life. Nearly a century and a half since ALS was first described by Dr. Jean-Martin Charcot in 1869, ALS remains incurable, though marginal disease slowing is provided by the drug riluzole, a glutamate release inhibitor6,7. ALS is typically classified into one of two groups: non inherited sporadic ALS (SALS), and genetically linked familial ALS (FALS). Despite the lack of hereditary component in SALS, which accounts for up to 90% of patients, certain environmental risk
factors have been linked to disease; these include head trauma\textsuperscript{8,9}, cigarette smoking\textsuperscript{10}, and dietary factors (e.g. intake of vitamin E)\textsuperscript{11}. The remaining cases of hereditary ALS are caused by mutation to one of several proteins implicated in disease. The first breakthrough in ALS was made in 1993, when Rosen et al. linked mutations in the Cu/Zn superoxide dismutase (SOD1) to FALS. Inheritance of FALS appears to be predominantly autosomal dominant, although some autosomal recessive pedigrees have been described\textsuperscript{12,13}. Similarly to other neurodegenerative diseases, given the predisposition and the proper environmental conditions, the probability of developing familial or sporadic form of ALS increases with age\textsuperscript{14}, with a lifetime risk being as high as 1:400\textsuperscript{15,16}. For an unknown reason, males have been documented to develop ALS more frequently than women with a ratio of 3:2\textsuperscript{17}. A unique subclass of ALS is represented by the cases of Guamanian ALS that were diagnosed in 1950’s in Guam with 50 times higher incidence rate than typical ALS\textsuperscript{18}. This disorder was characterized by patients presenting with not only ALS symptoms, but also those of Alzheimer’s and Parkinson’s diseases. Consistent with the symptoms, post-mortem pathology identified evidence of neuronal loss and de-pigmentation of the substantia nigra, as well as neurofibrillary tangles in a wide distribution of degenerating neurons\textsuperscript{19,20}. It is now widely believed that that the cause of the high incidence rate of the Guamanian ALS is rooted in the consumption of cycad nut flour containing a beta-methylamino-L-alanine (BMAA), a non-proteinogenic amino acid also known to be a neurotoxin\textsuperscript{21,22}. Although the exact mechanism of its toxicity is not well understood, it may act through protein misincorporation leading to protein misfolding and aggregation\textsuperscript{23}, or through the overstimulation of NMDA receptors\textsuperscript{24}.

Pure ALS is remarkably specific for motor neurons, leaving patients to suffer with their sensory, emotional and cognitive abilities largely unaffected\textsuperscript{25}. This selectivity, which spares the equally long sensory neurons, could be attributed to: 1. Motor neuron large size, which potentially requires high cellular metabolism\textsuperscript{26}; 2. Dysfunction of mitochondria required for continuous supply cellular energy\textsuperscript{27,28}; 3. GluR2-lacking AMPA receptors, which renders them permeable to calcium\textsuperscript{29}; 4. Increased threshold for activation of heat shock proteins could result in accumulation of misfolded proteins\textsuperscript{30}; 5. High concentration of misfolding-prone proteins, e.g. SOD1, as part of defense mechanism against oxidative radicals\textsuperscript{31}; 6. Low expression levels of calcium-binding proteins\textsuperscript{32} leading to excitotoxicity. It is important to note, however, that not all
motor neurons are affected equally in disease (e.g. ocular motor neurons); motor neurons resistant to degeneration show distinct gene expression profiles leading to reduced excitotoxicity⁴³.

The elusive and broad nature of disease pathology has resulted in many proposed mechanisms. Early studies have shown that autoimmunity may play a role in disease, as complete sera or immunoglobulins purified from ALS patients may directly lead to motor neuron death in-vitro⁴⁴,⁴⁵, although this cytotoxic effect is controversial⁴⁶. An excitotoxicity mechanism for ALS has also been proposed, according to which malfunctioning glutamate receptors in ALS motor neurons result in higher than normal levels of calcium ions, resulting in increased risk of mitochondrial damage and production of reactive oxygen radicals³,³⁷. However, the more recent perception is that ALS belongs to a subclass of neurodegenerative diseases (which include Alzheimer’s and Parkinson’s diseases) that are characterized by protein misfolding and aggregation³⁸-⁴⁰.

1.2 Genes linked to ALS

1.2.1 Cu/Zn superoxide dismutase

In 1993, Rosen et al. identified the first protein to be implicated in familial ALS: Cu/Zn superoxide dismutase (SOD1), an intracellular protein whose function is to convert highly reactive superoxide radicals into oxygen or the less toxic hydrogen peroxide⁴¹,⁴². SOD1 is highly conserved throughout evolution, and it makes up 1-2% of total neuronal protein content³¹,⁴³. The eukaryotic enzyme is a 32-kDa homodimer, where each monomer contains one copper within its active site, one stabilizing zinc, and one disulfide bond. The dimer is held together by hydrophobic interactions which significantly decrease its hydrophobic surface area thus increasing solubility. Due to its metallation and dimerization, SOD1 is one of the most stable proteins known⁴⁴, which allows it to function properly under extremely oxidizing conditions without excessive self-oxidation and unfolding. Mutations in the 153 amino-acid long SOD1 gene occur in 20% of familial ALS patients and have also been detected in a subset of sporadic ALS patients⁴⁵. To date, over 180 mutations have been identified⁴⁶, with considerable phenotypic heterogeneity across the various mutations. While homozygosity for D90A causes ALS with slow disease progression (mean survival of 13 years for diseased patients), heterozygosity for
A4V gives rise to an aggressive form of ALS with typical mean survival of less than one year after onset\(^{47-49}\). Functionally, mutations in SOD1 do not necessarily deem the enzyme inactive; while activity is only slightly reduced in D90A or L117V heterozygous patients, activity is significantly disturbed in patients with G85R or G93A variants of SOD1\(^{50}\). Genetic variations of SOD1 display a number of thermodynamic signatures conducive to misfolding, including decreased thermal stability, increased susceptibility to mechanical deformation, increased propensity to monomerize, decreased affinity for metals, and accelerated unfolding under stress conditions\(^{51,52}\). This can lead to the formation of a pool of misfolded SOD1 protein species in the cell, potentially through the depletion of zinc or copper under stress\(^{53,54}\). Notably, recent studies have identified misfolded and oxidized SOD1 in all cases of ALS, including SOD1-linked familial ALS, and cases in which SOD1 mutations are excluded, such as non-SOD1 linked familial ALS and sporadic ALS\(^{55-58}\). However, these findings remain controversial as other studies find no misfolded SOD1 in sporadic disease\(^{59}\), which could be explained by the fact that detectable levels of misfolded SOD1 in sporadic disease are relatively low, and may be missed due to technical differences of processing the tissue. Interestingly, one should note that while mutant SOD1 in SOD1-FALS deposits as intracellular aggregates, misfolded wild-type SOD1 seems to be soluble.

### 1.2.2 TAR-DNA binding protein 43

The formation of ubiquitin-positive inclusions is a common feature of many neurodegenerative diseases\(^{60}\), however the mechanism of formation and the composition of these inclusions in ALS and frontotemporal lobar dementia (FTLD) remained unknown until recently. Two independent discoveries in 2006 identified pathological TAR-DNA binding protein 43 (TDP-43) as a major protein in ubiquitin-positive, tau- and α-synuclein- negative inclusions, hallmark structures in both FTLD-TDP (subtype of FTLD with TDP-43 inclusions) and in ALS without SOD1 mutations\(^{61,62}\). Disease-associated TDP-43 is abnormally cleaved into the cytotoxic 25- and 35-kDa C-terminal fragments\(^{63}\), and becomes hyperphosphorylated, ubiquitinated and prone to aggregation\(^{64}\). TDP-43 immunoreactivity has been also observed in inclusions found in Huntington’s disease\(^{65}\), Parkinson’s disease\(^{66}\), and nearly 20% of Alzheimer’s disease cases\(^{67}\), which further implicates TDP-43 in a wide spectrum of neurodegenerative diseases.
Functionally, TDP-43 is an important regulatory protein in the nervous system: it binds over 6,000 pre-mRNA sequences, affects the splicing patterns of 965 mRNAs and affects expression levels of another 600 mRNAs.\(^6^8\) Due to its substantial role in transcription regulation, some propose that the loss of function of pathological TDP-43 in disease can cause neuronal loss due to altered proteostasis of proteins that rely on TDP-43 for their synthesis.\(^6^9\) Others believe that cytoplasmic mislocalization of pathological TDP-43 is toxic to neurons, through mitochondrial dysfunction, ER stress, or potentially by leading to the formation of cytotoxic misfolded SOD1 protein, which could implicate both proteins in a common pathological pathway in ALS.\(^5^5,7^0-7^2\)

Structurally, TDP-43 is made up of 414 amino acids and it belongs to the heterogeneous ribonucleoprotein family. In addition to its nuclear localization and export domains that enable it to shuttle sequences of nucleic acids from nucleus to cytoplasm, TDP-43 also contains two RNA recognition motifs (RRM1 and RRM2), and an glycine-rich C-terminal domain through which it interacts with other proteins.\(^7^3,7^4\)

### 1.2.3 Fused in Sarcoma

The linkage of TDP-43, an RNA/DNA binding protein, to ALS inspired researchers to search for additional nucleic acid binding proteins that may play a role in ALS. In 2009, two back-to-back studies identified that a structurally and functionally similar protein, FUsed in Sarcoma (FUS; originally termed translocated in liposarcoma, TLS), is also implicated in disease.\(^7^5,7^6\) Mutations in FUS have been identified in 3-5% of non-SOD1 or TDP-43 FALS and in ~1% of SALS cases.\(^7^7-7^9\) Histopathological analysis of these cases identified distinctive FUS positive and TDP-43 negative inclusions, however immunoprecipitation and immunohistochemistry studies confirmed the presence of misfolded wild-type SOD1 in ALS with FUS mutations.\(^5^5,7^5,7^6\) The 526 amino acid long FUS protein is composed of several domains for proper functioning: nuclear localization and export signals (NLS and NES, respectively), RNA recognition motif (RRM1), zinc finger motif, SYGQ (serine, tyrosine, glycine and glutamine)-rich domain, and multiple RGG (arginine, glycine and glycine)-repeat regions. The SYGQ motif was proposed to play a role in protein-protein interaction, while the RRM domain along with the zinc finger motif are important for RNA recognition.\(^8^0-8^3\) Owing to its non-canonical NLS, FUS is predominantly localized to the nucleus, although it can be continuously shuttled between cytoplasm and nucleus to deliver nucleic acids to dendritic translation, and assemble into stress granules.\(^8^4-8^9\) Although
the physiological functions of FUS are not yet well understood, emerging evidence indicates that it may be involved in the response to DNA damage, splicing and maturation of RNAs, as well as regulate neuronal plasticity and maintenance of dendritic integrity\textsuperscript{89-93}.

1.3 \textbf{Other major genes and proteins implicated in ALS}

Missense mutations in SOD1 were linked to ALS in 1993, and while it took nearly 15 years to identify additional genes linked to ALS, TDP-43 and FUS, advancement in sequencing technologies and better understanding of disease mechanisms has led to a discovery of a multitude of new genes that cause disease when mutations are present. In 2010 it was first shown that mutations in optineurin (OPTN) can cause ALS in Japanese families\textsuperscript{94}. Although it remains to be established how OPTN cause disease phenotype and clinical features, it has been shown to be a key player in various physiological processes, including membrane trafficking and protein secretion\textsuperscript{95}. This discovery was quickly followed up by mutations in another gene encoding the ATP-driven chaperone valosin-containing protein (VCP) using whole-exome sequencing\textsuperscript{96}. Recent studies have shown that VCP interacts with various proteins to help process ubiquitin-labeled protein degradation or recycling by the proteasome, and it has also been shown to play a role in endosome sorting and protein degradation through the autophagy machinery\textsuperscript{97}, both of which are affected in ALS\textsuperscript{98,99}. Deficiency in VCP can cause profound mitochondrial uncoupling, leading to lower ATP production, which might disrupt homeostasis and send neurons into a deadly loop\textsuperscript{100,101}. In 2011, mutations in UBQLN2, encoding the ubiquitin-like protein ubiquilin 2 responsible for proteasome-mediated degradation, were shown to cause dominant X-linked ALS with both juvenile and adult onset\textsuperscript{102}. The biggest discovery in ALS genetics in recent years, however, was made in 2011, when two back-to-back studies identified a massive hexanucleotide repeat expansion in \textit{C9ORF72} as the cause of chromosome 9p21–linked ALS\textsuperscript{103,104}: while typical people carry up to several dozen GGGGCC repeats in the non-coding region of exon 1, ALS patients may carry several thousand repeats. The repeat expansion is now accepted to be the most common cause of familial ALS (~25%), and it accounts to ~7% of the sporadic cases in people of European descent\textsuperscript{105}. Despite significant efforts of elucidating how the repeat expansion can cause disease, the mechanism is not clear, however evidence points towards several potential mechanisms: 1. Disruption of RNA metabolism through RNA sequestration as RNA foci are observed in fibroblasts and iPSC-differentiated neurons from
C9ORF72 ALS patients\textsuperscript{106}; 2. Happloinsufficiency in C9ORF72 protein which may regulate membrane traffic and is found to be downregulated in zebrafish models of ALS\textsuperscript{107,108}; 3. Formation of aggregating di-peptide repeat proteins that can self-translate through repeat associated non-ATG (RAN) translation, which have been extensively observed in cerebellum, hippocampus and the neocortex from ALS patients but not healthy individuals\textsuperscript{109,110}; and 4. Contributing to telomere instability and disrupting RNA processing through formation of highly stable G-quadruplexes\textsuperscript{111}. In 2012, missense mutations in profilin 1 (PFN1), which is essential to proper formation of filamentous actin required for cytoskeletal formation, were found to cause ALS\textsuperscript{112}. Most recently, in 2015 whole-exome sequence led to identification of ALS-associated mutations in TBK1, which encodes TANK-binding kinase-1 that is known to phosphorylate a number of proteins involved in autophagy, including optineurin and p62\textsuperscript{113}.

1.4 Overlap with frontotemporal dementia

Although classically regarded to as a strictly motor neuron disease, ALS is now considered to be a multi-systemic condition that may affect non-motor regions in the brains that lead to cognitive impairment\textsuperscript{114}. Frontotemporal dementia (FTD), the second most common cause of dementia after Alzheimer’s disease, is characterized by progressive neuronal loss in the frontal and temporal cortices leading to personality and behavioral changes. Nearly 50% of ALS patients show subtle deficits in cognition, and nearly 15% of ALS patients meet the criteria for diagnosis with FTD\textsuperscript{115,116}. Increasing evidence demonstrates that the partial overlap between ALS and FTD is not observed only in clinical features, but also in pathophysiology: pathological TDP-43 characterizes all non-SOD1 ALS and nearly half FTD cases, and the majority for ALS-implicated proteins can also be mutated in FTD, with the striking exception of SOD1\textsuperscript{116}. A further molecular link between the two neurological conditions was shown to exist with the finding of C9ORF72 expansion mutations in over 20% and 10% of familial ALS and FTD, respectively\textsuperscript{104}. Naturally occurring mutations in other proteins, such FUS, UBQLN2 and VCP, can cause both diseases with different degrees of probability, all of which suggest the existence of a clinical and pathophysiological continuum where ALS and FTD are found at opposite extremes. Despite establishing that wild-type SOD1 can misfold in the presence of pathological TDP-43, FUS or C9ORF72 in ALS\textsuperscript{55,56,58,117}, and that ALS and FTD have overlapping pathologies, the presence of misfolded SOD1 has not been formally studied in pure FTD despite
the fact that pathological TDP-43 or FUS can lead to wild-type SOD misfolding in cultured cells. However, it should come as no surprise if oxidized and misfolded SOD1 is detected in FTD brains in the near future; Choi et al. have found oxidized and aggregated SOD1 in brains from Alzheimer’s and Parkinson’s diseases\textsuperscript{118}, suggesting that SOD1 pathology is not a unique feature in ALS only, although it may be the only disorder where it acquires propagative properties.

1.5 Protein folding, misfolding and aggregation

As one of the essential molecular components of life, proteins play a crucial role in cellular structure, metabolism, communication, and defense against invading organisms. Depending on their intended function, proteins can vary in size and three-dimensional structure from basic units of secondary structure, i.e. alpha helix and beta sheet, to the more complex globular, fibrous, or transmembrane structures. In eukaryotes, proteins typically assemble into multi-domain structures stabilized by disulfide bonds or non-covalent interactions. The dynamics in proteins allows a certain degree of structural flexibility and conformational change, while maintaining normal functionality\textsuperscript{119,120}. However, if the native conformation is significantly altered, or changed within critical domains, a protein might lose its ability to function properly or it may even gain toxic, potentially infectious, properties\textsuperscript{121-123}. Under healthy conditions, when a protein is misfolded, it is shuttled by the cell to the chaperone machinery for proof-reading followed by repair through protein refolding, or clearance through protein-degradation pathways\textsuperscript{124,125}. Despite tightly-regulated cellular control however, under certain conditions a build-up of misfolded protein can overwhelm the proteostatic machinery, resulting in loss of homeostasis of native proteins, as well as further accumulation of misfolded species. This generally leads to protein aggregation, fibrillation, and eventually cell death\textsuperscript{126-129}.

The genetic code within each cell is an essential determinant of the primary amino acid sequence of every protein in the cell. The amino acid sequence is in turn essential for dictating the three dimensional structure and function of every protein, as well as the steps required for achieving this conformation and function. Consequently, during the protein folding process, each protein attains a well-defined native structural ensemble by conformational search towards the minimal free energy\textsuperscript{130-132}. As the ribosomal machinery begins to translate nucleotide sequences into unfolded chains of amino acids (Figure 1), molecular chaperones stabilize the newly synthesized
chains and assist in their proper folding into either natively or intermediately folded states\textsuperscript{125,133}. For most proteins, this process entails folding the nascent polypeptide into intermediate secondary structures, which are then refolded into tertiary structure, potentially forming quaternary multimers with other folded protein units. In contrast, 35-45\% of the eukaryotic proteome is estimated to be fully or partially disordered under physiological conditions\textsuperscript{134}; these partially unfolded proteins are typically responsible for critical steps in regulatory processes and cell cycle and are fully functional despite lacking tertiary or often even secondary structure\textsuperscript{135-137}.

Aging, genetic factors (i.e. mutations and polymorphisms) and environmental factors (including oxidative stress and macromolecular crowding) can lead to changes in protein folding and result in, or catalyze, protein misfolding\textsuperscript{123,138,139}. Such dysregulation could then lead to the buildup of toxic oligomers, inclusion bodies, or aggregates containing the aberrantly folded proteins. The myriad of conformations that are not properly folded are referred to as “misfolded states”; a protein in such a state is said to be a “misfolded protein”. Some misfolded proteins acquire the capacity to trigger or seed misfolding in a propagated fashion, conceptually similar to prion infection.

In the tightly-controlled environment of the cell, misfolded proteins are dealt with by two primary mechanisms: I. Repair using chaperone protein complexes; II. Clearance using degradation pathways. Upon the detection of a misfolded protein, specific and highly conserved molecular chaperones are recruited to refold the misfolded protein back into its native conformation and a fully functional protein\textsuperscript{140}. If this attempt fails in otherwise healthy cells, clearance of the misfolded proteins through chaperone-mediated autophagy or activation of ubiquitin-dependent degradation is initiated\textsuperscript{141,142}. Direct activation of intracellular protein degradation and scavenging (autophagy, ubiquitin-proteasome system and endoplasmic reticulum associated protein degradation) can also occur independently of chaperone mediation\textsuperscript{143,144}.

Protein misfolding is a common feature in stressed cells\textsuperscript{145-147} (Figure 1). With aging, the intracellular repair and scavenging mechanisms become less efficient, contributing to accumulation of misfolded proteins and the potential formation of aggregates\textsuperscript{148-151}. It is important to note that although most non-native conformations are not “prion-like” (i.e. capable
of recruiting or seeding), they can still be harmful to the cell through pathways involving loss-of-function, mitochondrial dysfunction, defective axonal transport or clogging of the clearance mechanisms. Studies have also shown that a small subset of disease-implicated protein conformations are very stable, aggregation-prone, and trigger or seed protein aggregates, becoming a toxic nuisance to the cell\textsuperscript{152,153}. Some of the misfolding-prone proteins in neurodegenerative diseases are discussed below.

Initial interest in studying and monitoring the formation of toxic protein conformations was sparked by the observation of insoluble amyloid structures in brains of individuals with neurodegenerative diseases\textsuperscript{154,155}. Amyloid structures, or more amorphous aggregates, are abnormal fibrous and proteinaceous deposits, which are generally dominated by beta sheet substructures. These formations consist primarily of misfolded proteins but can also sequester metastable proteins\textsuperscript{156-158}. The proteins found in aggregates in different amyloid diseases can include misfolded wild-type sequences in sporadic cases or sequences with genetic variability in familial cases\textsuperscript{159}.Aggregate formation has been proposed to occur either through deposition of monomeric misfolded protein into a large inclusion (seeded aggregation), or through indirect deposition of monomeric misfolded proteins into smaller inclusions (oligomers), which then undergo combination to a nascent aggregate\textsuperscript{160}. Although protein aggregates represent a distinct pathological feature in diseased tissue, it is not clear whether all or some of these inclusions are toxic, or perhaps even protective \textsuperscript{152,159,161,162}. On one hand, a growing body of evidence suggests that protein aggregates increase cell death and exhibit cytotoxicity through proteasomal inhibition or by disruption of the integrity of the plasma membrane\textsuperscript{152,163,164}. Some protein aggregates acquire the capacity of prion-like infection, supported by experimental evidence showing propagation of pathogenic aggregates comprising host protein from their initial point of inoculation\textsuperscript{165,166}. On the other hand, aggregates could also play a protective role in disease. Due to the toxicity and infectious nature of misfolded monomers and oligomers\textsuperscript{167,168}, an attractive hypothesis is that living cells sequester monomers and oligomers into large intracellular inclusion bodies in order to hinder their toxicity and ability to spread the disease\textsuperscript{169,170}. The study of misfolded proteins has increasingly grown as aggregate formations have been associated with over 20 human diseases, including the prion diseases\textsuperscript{147,171-173}. 
Figure 1: The cycle of protein folding, misfolding and refolding.

Ribosomal translation of nucleotides produces unfolded polypeptide chains that are quickly self-assembled or chaperoned into the native or partially-folded states. The polypeptide chains may also be degraded if they had formed disordered aggregates or erroneous intermediates structures. Genetic and environmental triggers of neurological disorders, such as oxidative stress, may lead to protein misfolding and/or aggregation. The majority of these misfolded conformations are benign and leading to a loss-of-function, and processed by cells via the refolding or degradative machineries, i.e. ubiquitin-proteasome system or autophagy. However, an important subset of
misfolded protein conformations is stable, aggregation-prone, and toxic to the cell and its surroundings. Some conformations in this toxic subset are also infectious and may lead to the irreversible formation of fibrils, which can grow in size by recruiting wild-type, unfolded and misfolded proteins in a template directed or seeded-polymerization fashion. Thickness of arrows correlates with probability to assume the pointed out state.
1.6 The Prion concept

The breakthrough Prusiner hypothesis in 1982 that proteins could function as an infectious agent has fundamentally changed our view of infectious agents\textsuperscript{174}. For the first time, it was shown that proteinaceous infectious particles, dubbed prions, are responsible for the disease of scrapie in sheep and goats, as well as experimental models of scrapie. By the late 20\textsuperscript{th} century, prions had been implicated in several fatal human neurological disorders, such as kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS) and fatal familial insomnia (FFI). Importantly, the transmittance of some of these prions and the resulting diseases in mammals can occur through dietary exposure or other routes of inoculation with infected tissue. Genetically, the human prion protein is encoded by the prion protein gene (PRNP) located on chromosome 20. Mutations and polymorphisms in PRNP are responsible for familial prion disease, and play an important role in determining the susceptibility or resistance of individuals to prion disease\textsuperscript{175}-\textsuperscript{178}. Once translated into its polypeptide form, the prion protein can exist in multiple isoforms: the natively folded, α helix-rich, cellular prion protein (PrP\textsuperscript{C}), or the misfolded, β sheet-rich, pathological strains collectively referred to as the scrapie isoform of the prion protein (PrP\textsuperscript{Sc})\textsuperscript{179,180}.

Although it is now commonly accepted that misfolded prion protein can replicate by misfolding the natively folded PrP\textsuperscript{C}, the mechanism and its cellular compartment remain enigmatic. To date, two possible mechanisms of prion replication have been proposed: template directed misfolding and seeded polymerization\textsuperscript{181}. The template directed misfolding model asserts that monomeric PrP\textsuperscript{Sc} is kinetically inaccessible; it is likely thermodynamically less stable than the natively folded protein. In the context of misfolded PPrP\textsuperscript{Sc} however, structural transformation of native PrP can be induced (templated). Multimeric interactions are thus likely in the initial spontaneous oligomer formation process. The PrP\textsuperscript{Sc} oligomer becomes a physical template for the misfolding of the native PrP\textsuperscript{C}. Once converted to PrP\textsuperscript{Sc}, it can further propagate by catalyzing prion replication and forming aggregates and fibril structures. The alternative model, seeded polymerization, proposes that PrP\textsuperscript{Sc} compatible conformations are occupied by the monomer, but with low probability. When these conformations encounter misfolded oligomer, they are accreted onto the misfolded substrate. This scenario may be more applicable to oligomerization of proteins lacking native structure such as α-synuclein and Aβ peptide, or if oligomerization is
initiated from the globally unfolded state. This leads eventually to fibril elongation, which may eventually break up into multiple oligomeric seeds which become the new recruiters.

1.6.1 Neurodegenerative disease from the prion perspective
Recent discoveries elucidate molecular details of other human neurodegenerative diseases indicate that a number of non-prion proteins tend to obey prion-like intra- and inter-cellular propagation mechanisms within a single organism. These diseases include Alzheimer’s disease, amyotrophic lateral sclerosis, Parkinson’s disease, Huntington’s disease, and the tauopathies (Table 1).

1.6.1.1 Alzheimer’s disease
Alzheimer’s disease (AD) is the most common cause of late-life dementia. The disease has two characteristic pathological features in the human brain: extracellular accumulation of amyloid-β (Aβ) peptides into fibrillar plaques, and intracellular accumulation of neurofibrillary tangles consisting of hyperphosphorylated tau protein. Inheritable mutations in amyloid precursor protein (APP), or γ-secretase genes, account for less than 5% of total AD cases, and generally manifest long prior 65 years of age. In these early-onset cases, the causal mutations act to increase the production of Aβ peptides, particularly for the most toxic Aβ42 species. However, for the remaining 95% of sporadic AD cases, failure of Aβ clearance is thought to lead to the progressive accumulation of Aβ monomer, oligomers, and plaques.

Although the etiology of AD remains elusive, over a decade of research increasingly implicates Aβ oligomers (AβO), rather than free Aβ monomers or insoluble fibrils, as the primary toxic molecular species responsible for synaptotoxicity and neurodegeneration. Several studies support a robust correlation between AβO concentration and the extent of synaptic loss and severity of cognitive impairment, as well as triggering of tau abnormalities associated with irreversible neuronal damage. Structurally, Aβ monomers are intrinsically disordered, existing in a structurally diverse ensemble possessing conformations including a minority of partial α-helical or β-sheet rich conformations. Amyloid-β oligomers, on the other hand, have been suggested to exist as beta barrels, triangular beta-sheet structures, low beta-sheet pentamer disc formations, and globulomers. The self-assembly of Aβ monomers into
higher order structures *in-vitro* is studied in the literature\(^{204,205}\). The revised amyloid cascade hypothesis, which was first proposed by Hardy and Higgins in 1992\(^{206}\), stipulates that when monomeric A\(\beta\) is generated by \(\beta\)- and \(\gamma\)-secretase cleavage of amyloid precursor protein (APP), A\(\beta\) rapidly forms soluble, toxic A\(\beta\)Os, which convert to less toxic \(\beta\)-amyloid fibrils\(^{187,207,208}\). We propose that the A\(\beta\) structural flux may not be linear and irreversible, as outlined by the cascade hypothesis, but cyclical as under certain conditions mature fibrils may become a source of oligomers. Considering the delayed onset of cognitive defects relative to the appearance of plaques\(^{209}\), it is possible that A\(\beta\)Os reach critical toxic concentrations by “saturation” of the fibril incorporated-mediated detoxification.

Certain similarities between the behavior of A\(\beta\) in Alzheimer’s disease and PrP in the prion diseases have led to speculations and discussion of the prion-like nature of A\(\beta\) (e.g.\(^{210-213}\)), although the lack of similarity in native structure would strongly suggest that a seeded polymerization mechanism likely accounts for aggregation of A\(\beta\). Like infectious prion protein, A\(\beta\)-containing inocula prepared from synthetic material, or from AD human or mouse model brains, can “seed” AD pathology *in-vivo*\(^{213-215}\). The data suggests that the most efficient seed of AD pathology in mouse models are structured soluble species, consistent with small A\(\beta\) oligomers as opposed to insoluble fibrils or plaques, which can act as seeds but in a significantly longer time frame\(^{213-216}\). These experiments are examples of a modified interpretation of the prion hypothesis whereby seeded polymerization of A\(\beta\) monomers into oligomers accounts for AD pathology\(^{204,217}\).

### 1.6.1.2 Parkinson’s disease

After Alzheimer’s disease, Parkinson’s disease (PD) is the second most common adult onset neurodegenerative disease. As the most common movement disorder, its key clinical features are tremor at rest (uncontrollable shaking), excessive muscle rigidity, postural instability, and bradykinesia (slow movement)\(^{218}\). These symptoms can be primarily attributed to the progressive degeneration of dopaminergic neurons, leading to a substantial, yet unexplained, asymmetry of clinical symptoms. In addition to motor symptoms, longitudinal studies suggest that the majority of PD patient may eventually develop cognitive deficits including dementia, psychosis, and sensory disturbances\(^{219}\). The pathology of PD involves massive degeneration of dopamine-containing neurons in the midbrain, and is characterized by the accumulation of
distinct proteinaceous inclusions in dopaminergic neurons in the substantia nigra pars compacta (SNc) region. These hallmark inclusions are divided into two groups based on their cellular localization: neuronal perikarya (Lewy bodies), and neuronal processes and axons (Lewy neurites). These are filamentous structures composed of ubiquitin, α-synuclein, and abnormally phosphorylated neurofilament proteins. In dementia with Lewy bodies— the second most common cause of dementia— presynaptic α-synuclein aggregates cause neurodegeneration through synaptic dysfunction. While Lewy bodies were first believed to be toxic and cause disease, these inclusions may indeed be bystanders or play a cytoprotective role by grouping all of the misfolded and aberrantly processed proteins. In support of this, several molecular and morphological similarities between Lewy bodies and aggresomes, part of the protective response machinery, have been observed.

α-Synuclein is a highly conserved protein with an unknown function, although it is likely involved in vesicular release and synaptic function in the central nervous system. It is an abundant presynaptic brain protein, and it was identified as a major component of Lewy body fibrils. The 140 amino acid α-synuclein protein is an intrinsically disordered protein; clustering analysis indicates that monomeric α-synuclein is conformationally diverse. Both NMR-derived and directly simulated ensembles exhibit transient long-range tertiary interactions between N- and C-termini, which are thought to play a protective role against aggregation. Such sequestering interactions are consistent with force spectroscopy measurements indicating long-range, intermolecular interactions. Upon binding negatively charged cellular membranes, α-synuclein can adopt N-terminal α-helical structures.

Considering their rapid degeneration in PD, restoring a population of healthy nigral dopaminergic neurons is viewed as a potential treatment for PD. Indeed, evidence shows that intrastriatal grafts of human embryonic mesencephalic dopaminergic neurons into PD patients significantly improve their clinical features for up to 3 years post transplantation. Positron emission tomography (PET) studies demonstrated that the grafted neurons can survive, grow, integrate and release dopamine for over 10 years postsurgery. Surprisingly, postmortem analysis of the transplant recipients identified Lewy-like inclusions that stained positive for α-synuclein and ubiquitin in the grafted neurons. These inclusions were similar to other inclusions.
observed in the host brain and they stained positively to an antibody that recognizes a phosphorylated version of α-synuclein (Ser129), a key posttranslational modification detected in PD with Lewy bodies\(^\text{230}\). Together, these findings strongly suggest that the inclusions found in the grafted neurons are in fact Lewy bodies, which were induced to form by the ongoing misfolding and seeding of endogenous α-synuclein in the host tissue. *In-vivo* studies have confirmed the transmission, albeit with relatively low frequency, of α-synuclein from PD transgenic mouse striatal tissue to the healthy engrafted dopaminergic neurons, as well as the endocytotic spread of α-synuclein between neuronal cells triggering the formation of Lewy-like inclusions\(^\text{232}\). Since the transmitted inclusions were small, the authors in this study speculated that endogenous α-synuclein from the engrafted cells was actively seeded to form the larger aggregates. The intercellular spread of α-synuclein was elegantly demonstrated by expressing GFP or DsRed tagged α-synuclein proteins in two separate populations of neuroblastoma cells initially expressing only one of the above constructs\(^\text{232}\). Co-culturing these cell populations resulted in the gradual increase in double labeled cells and detection of colocalized aggregates, suggesting the seeding effect of transmitted α-synuclein. Finally, Luk *et al.* have demonstrated that a single intrastriatal inoculation of wild-type non-transgenic mice with synthetic α-synuclein fibrils initiates intercellular transmission of pathogenic α-synuclein and the formation of Lewy-like bodies in anatomically interconnected regions\(^\text{166}\). These inclusions resulted in the progressive degeneration of dopaminergic neurons in the SNc, but not in the adjacent ventral tegmental region, which recapitulates PD in rats. Additionally, intracerebral inoculation of sarkosyl-insoluble α-synuclein from brain homogenates of patients with dementia with Lewy bodies induced the pathological hyperphosphorylation of α-synuclein in wild-type mice\(^\text{233}\). This study also demonstrated that while synthetic human α-synuclein fibrils injected into wild-type mice were not detectable one week later, endogenous mouse α-synuclein started accumulating three months post inoculation further suggesting the species-specific, prion-like nature of α-synuclein.

### 1.6.1.3 Taupathies

Taupathies are a subclass of neurodegenerative diseases with filamentous inclusions. The best known member in this class of neurological disorders is Alzheimer’s disease, where hyperphosphorylated microtubule-associated *tau* protein accumulates in insoluble neurofibrillary
tangles\textsuperscript{234}. Although these inclusions are commonly found within the cytosol of neurons and glial cells, they can also be observed as ghost tangles in the extracellular space corresponding to the location where the host neuron has died\textsuperscript{234,235}. Rarer neurological disorders in this class include Pick’s disease, progressive supranuclear palsy (PSP), chronic traumatic encephalopathy (CTE), and FTLD.

Tau pathology typically begins in a single locale, from where it spreads to other, disease-specific, regions of the brain\textsuperscript{236}. de Calignon \textit{et al.} described the propagation of tau pathology in a mouse model by selectively expressing the FTD-linked pathogenic human mutant P301L-tau only in a fraction of neurons from layer-II of the entorhinal cortex (EC), a tissue that exhibits a profound loss in mild AD\textsuperscript{237,238}. Tau pathology was first observed in transgene-expressing neurons, but quickly affected adjacent neurons without the transgene, and further spread from the original EC regions to synaptically connected brain regions involved in AD, such as the dentate gyrus and cornu ammonis fields of the hippocampus. The transmissibility of tau pathology is further supported by the notion that injection of brain extract from P301L-tau transgenic mouse into the brain of human wild-type transgenic mice induces misfolding of the human tau protein and its assembly into filaments, which spread from the original site of injection\textsuperscript{239}. In cell culture, extracellular tau aggregates, but not monomers, are actively endocytosed and seed the formation of new intracellular fibrils containing wild-type tau, which can be further propagated intracellularly\textsuperscript{240}.

1.6.1.4 Huntington’s disease

Huntington’s disease (HD) is an autosomal dominant neurodegenerative condition leading to the preprogrammed death of neurons in the basal ganglia, and the striatum in particular. The disease is predominantly familial with a small sporadic component. Unlike Alzheimer’s and Parkinson’s diseases, HD belongs to the class of triplet expansion diseases, featuring long trinucleotide expansion mutations. As such, the disease-specific huntingtin gene contains an expanded number of CAG repeats, resulting in an abnormal huntingtin protein with a long stretch of polyglutamine residues (polyQ). Clinical studies established full penetrance of disease for more than about 40 repeats and reduced penetrance for the 36-39 repeat range; in this latter range, penetrance is positively correlated with the length of the expansion, while age of onset is inversely correlated
to the length of expansion\textsuperscript{241,242}. In a mammalian cell culture model, pathologic polyQ proteins interact with non-pathological polyQ proteins, and recruit them into insoluble and detergent-resistant aggregates\textsuperscript{243}. Furthermore, aggregates made up of exogenous recombinant polyQ fibrils can be internalized by mammalian cells, where they are sequestered in the aggresome along with cytoplasmic chaperones and components of the ubiquitin-proteasome system\textsuperscript{244}. In order to test whether the internalized fibrils remained in a vesicle or became directly exposed to the cytoplasm, an intracellular seeding experiment was conducted by Ren \textit{et al}\textsuperscript{244}. In this study, mammalian cells in culture were engineered to express a reporter gene of a fluorescence protein fused with huntingtin protein encoding 25 glutamines. The fusion protein was expressed diffusely within the cells. However, when the cells were incubated with medium containing aggregates made up of huntingtin with the 44 polyQ expansions, the reporter was redistributed into puncta pattern which colocalized with the internalized protein. Control cultures expressing the reporter genes, but not exposed to polyQ expansion containing fibrils, did not show similar reporter redistribution.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Cultured cells</th>
<th>Intercellular transmissio n</th>
<th>Animal model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Induction of wild-type misfolding and seeding</td>
<td>Resistance to proteases</td>
<td>Induction of wild-type misfolding and seeding</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spread from point of inoculation</td>
</tr>
<tr>
<td>PrP</td>
<td>Yes (^{245})</td>
<td>Yes (^{245})</td>
<td>Yes in humans (^{245})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased (^{245}) or</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>decreased (^{246}) depending on disease/ state</td>
<td></td>
</tr>
<tr>
<td>Amyloid-β</td>
<td>Induces seeded aggregation (^{247})</td>
<td>No.</td>
<td>Induces seeded aggregation (^{214,2}^{215,250})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amyloid -β are extracellular</td>
<td></td>
</tr>
<tr>
<td>α-synuclein</td>
<td>Induces seeded aggregation of native protein (^{232})</td>
<td>Yes (^{232})</td>
<td>Yes (^{230-232})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased in mutant protein (^{251})</td>
<td></td>
</tr>
<tr>
<td>Huntingtin (PolyQ)</td>
<td>Induces seeded aggregation of native protein (^{243,244})</td>
<td>Yes (^{244})</td>
<td>NA</td>
</tr>
<tr>
<td>SOD1</td>
<td>Induces misfolding of native protein (^{117})</td>
<td>Yes (^{253})</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Misfolded protein is protease sensitive (^{117})</td>
<td></td>
</tr>
<tr>
<td>Tau</td>
<td>Induces seeded aggregation of native protein (^{256,257})</td>
<td>Yes (^{240})</td>
<td>Yes (^{239})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sensitive to proteases resulting in nucleating fragments (^{258})</td>
<td></td>
</tr>
<tr>
<td>TDP-43</td>
<td>Induces misfolding, seeded aggregation of native protein (^{259})</td>
<td>Yes (^{260}/No^{26})</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Digestible into &lt;20kDa C-term fragments (^{259})</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Transmissibility properties of proteins implicated in neurodegenerative diseases.

NA: Not Available.
1.6.2  Amyotrophic lateral sclerosis

The exact molecular mechanism that causes ALS is not known, however literature implicates a variety of mechanisms that may drive disease progression, including altered RNA metabolism, mitochondrial dysfunction, ER or oxidative stress. However, multiple parallels between transmissible spongiform encephalopathy (TSE) diseases featuring prion protein biology and ALS have been observed in patient and in-vivo disease models. ALS pathology may begin at a single focal, or multifocal sites, and appears to spread through the neuroaxis in a spatiotemporal manner\textsuperscript{262,263}. This systematic spread of disease is consistent with a prion-like propagation of misfolded protein in disease, for which several protein candidates have been proposed in ALS.

1.6.2.1  Prion-like properties of SOD1

How misfolded SOD1 might cause ALS (or be a consequence of the disease) is not yet fully understood; however, a number of toxic properties have been studied. For example, misfolded SOD1 might inhibit proteasomal function, or transform into a net producer of superoxide and nitrogen radical species\textsuperscript{264,265}. The net gain-of-function result is the extensive oxidative damage to intracellular structures, proteins, lipids and DNA, as well as the loss of the cell’s ability to clear misfolded proteins resulting in their accumulation and fibrillation\textsuperscript{266}.

Co-expression of human wild-type SOD1 with mutant SOD1 accelerated onset of disease in mouse models of disease, suggesting that wild-type SOD1 can be misfolded via intermolecular conversion initiated by the mutant SOD1. This finding implies that wild-type SOD1 can also misfold and contribute to the growth of the aggregates and neurotoxicity in disease\textsuperscript{267-269}. The first biochemical evidence demonstrating that misfolded SOD1 has prion-like properties observed that human mutantSOD1-containing aggregates can traverse between neuronal mouse cells, triggering the self-perpetuating growth of these aggregates\textsuperscript{270}.

Confirmation of the intermolecular conversion of wild-type SOD1 into a misfolded state was produced in a cell culture model\textsuperscript{117}. In this study, human SOD1\textsuperscript{G127X} or SOD1\textsuperscript{G85R}, two naturally occurring mutations in familial ALS, were over-expressed in human cell lines. Immunocytochemistry and immunoprecipitation studies revealed that transfected cells not only expressed the misfolded exogenous protein, but also contained a significant proportion of the
endogenous wild-type SOD1 protein in a misfolded form. The change in the conformational state was established through remarkable difference in protease sensitivity and access to binding of misfolding-specific antibodies that detect an exposed electrostatic loop or an oxidized dimer-binding interface, two epitopes that are inaccessible in the native conformation. The necessary distinction between endogenous and exogenous misfolded SOD1 was achieved using the SOD1$^{G127X}$ mutant, which contains a premature stop codon preventing the translation of antibody binding epitopes, and SOD1$^{G85R}$, which carries a different charge than wild-type SOD1 and is therefore distinguishable via migration on polyacrylamide gel electrophoresis and immunoblotting. Furthermore, once misfolded, wild-type SOD1 can propagate intercellularly via exosome-dependent and -independent mechanisms. Importantly, monomeric species of SOD1 have also been detected extracellularly, suggesting a potential mechanism of transmission of misfolded SOD1.

Reminiscent of the species barrier concept in prion transmissibility, overexpression of human wild-type SOD1 in mice expressing human SOD1$^{G85R}$, but not mouse SOD1$^{G86R}$, accelerates motor neuron disease onset. Furthermore, substituting the single, solvent exposed, human tryptophan (Trp32) with a serine (Ser32) was sufficient to ablate the intermolecular conversion of human SOD1 by mutant constructs (SOD1$^{G85R}$ or SOD1$^{G127X}$) in HEK293 cells. Notably, tryptophans are used rarely in polypeptide architecture but they play a crucial role in protein stabilization and folding. Interestingly, we also showed that TDP-43-induced misfolded SOD1 can traverse intercellularly and trigger misfolding in primary spinal cord cultures from HuWtSOD1 transgenic mice, but not in BL6 control mice. Taken together, these findings suggest that the human Trp32 plays an important role in stabilizing the misfolded and infectious SOD1 particle, and could represent a useful target for potential ALS therapies.

More recently, transmissibility of SOD1 in-vivo has been demonstrated; intraspinal injection of injection of SOD1$^{G85R}$-YFP transgenic mice with homogenates prepared from paralyzed mice expressing SOD1$^{G93A}$ can induce ALS symptoms and produce widespread spinal inclusion pathology. A second passage of homogenates from injected mice into newborn SOD1$^{G85R}$-YFP transgenic mice induces disease by 3 months of age. Additionally, injecting
spinal homogenates from paralyzed SOD1 mice into the sciatic nerve of adult SOD1\textsuperscript{G85R}-YFP mice produces a spreading ALS-like disease 3 months after injection.

1.6.2.1.1 **SOD1 misfolding-specific antibodies**
A novel subclass of antibodies includes antibodies designed to be specific to epitopes that are buried within the properly folded proteins, however become exposed when the protein loses its structural integrity. Our lab has identified two hypothesis driven immunological epitopes that become exposed in the misfolded or metal depleted SOD1. The disease specific epitopes (DSEs) are: an oxidized version of a previously identified DSE normally buried within the dimer interface of SOD1\textsuperscript{275}, and a segment of the SOD1 electrostatic loop that is extruded in amyloid-like fibrils and nanotube crystal structures of misfolded SOD1\textsuperscript{276}. We then raised misfolding-specific monoclonal antibodies against these epitopes, 3H1 and 10C12, targeting the electrostatic loop and dimer interface in misfolded SOD1, respectively. Both of these antibodies target the C-terminus of the misfolded SOD1 protein.

1.6.2.2 **TDP-43 and FUS as prions**
Screening of the entire database of known human proteins using a hidden Markov Model algorithm, designed to detect prion-like sequences in yeast, identified both TDP-43 and FUS to be likely prion proteins in which the C-terminus and N-terminus were highlighted as misfolding-prone, respectively\textsuperscript{80}. The prion-like nature of the C-terminal domain has been also confirmed \textit{in-vitro}. Udan-Johns \textit{et al.} observed that both endogenous and fluorescent-tagged wild-type human TDP-43 undergo reversible aggregation in the nucleus in response to heat shock\textsuperscript{277}. The same study confirms that deletion of the C-terminal prion-domain markedly reduced aggregation of TDP-43, and that replacement of the prion domain in TDP-43 with a prion domain from TIA-1 recapitulates the heat shock induced aggregation.

Nonaka \textit{et al.} further demonstrated TDP-43 to possess prion-like properties and play a central role in TDP-43 proteinnopathy\textsuperscript{259}. This study determined that detergent-insoluble isolation of filamentous TDP-43 from ALS and FTLD-TDP brains can act as seeds in human neuroblastoma SH-SY5Y cells overexpressing wild-type human TDP-43, resulting in hyperphosphorylated and
uniquitinated TDP-43. The induced pathogenic TDP-43 formed cytotoxic aggregates in a self-templating manner, with aggregation of full length TDP-43 preceding the C-terminal cleavage of the protein by caspases. Interestingly, although treatment of the detergent-insoluble pellet with proteinase K degrades TDP-43 to <20kDa fragments, their seeding ability is retained, further suggesting that the full-length protein is not necessary for disease pathogenesis. Finally, induced accumulation of TDP-43 leads to proteasomal dysfunction, which can drive the cytoplasmic accumulation of TDP-43 aggregates in a positive-feedback manner, resulting in the accumulation of additional misfolded proteins. Interestingly, our own studies did not identify pathological TDP-43 to traverse from transfected cells to untreated HEK293 cells, which could be attributed to the fact that our recipient cells were not “primed” by TDP-43 overexpression that might be required for efficient seeding to occur.

Similarly to TDP-43, prion-like domains were identified in FUS using bioinformatics approaches. FUS possesses a high degree of self-aggregation in-vitro, as FUS can spontaneously form filamentous structures reminiscent of inclusions found in ALS patients. In-vitro studies show that spontaneously formed inclusions made up of mutant FUS can effectively seed aggregation of soluble wild type FUS. Furthermore, nuclear mutations of FUS also facilitate the formation of intranuclear inclusions in rat hippocampal neurons while triggering toxicity. Intriguingly, Murakami et al. demonstrated that FUS phase transition can occur between monomeric, liquid drop and hydrogel states. FUS mutations can induce a further phase change into non-reversible fibrillar hydrogels that sequester other ribonucleoproteins. Nonetheless, intracellular transmissibility for FUS has not been shown, in either of the abovementioned experiments; our own experiments show that pathological FUS does not propagate from transfected cells to untransfected recipient cells through incubation with conditioned media.

1.7 Intercellular propagation of protein misfolding in neurodegeneration and ALS

Before cell death ensues in any neurodegenerative diseases, pathology spreads from cell-to-cell and region-to-region. The mechanism of this spreading pathology has long been considered to be simple diffusion of aggregates (fibrils or oligomers) following cell death. However, recent evidence suggests that export and uptake of pathological proteins, such as PrP, Aβ, and SOD1,
may be mediated by a pathological hijacking of extracellular vesicles\textsuperscript{283-285}. One subclass of such vesicles, called exosomes, has been termed the “Trojan Horses of Neurodegeneration”\textsuperscript{286}. A rapidly growing body of work has demonstrated that extracellular vesicles, secreted by virtually all mammalian cells, as well as non-mammalian and bacterial cells, play functional roles in both health and disease\textsuperscript{287}. Cells of the nervous system, including neurons\textsuperscript{288}, oligodendrocytes\textsuperscript{289}, astrocytes\textsuperscript{290} and microglia\textsuperscript{291}, have been shown to release vesicles of various sizes and different specific functions. Exosomes are endocytically derived membrane nanovesicles, 30-100 nm in diameter, that are secreted by eukaryotic cells as a means of releasing / secreting RNA, proteins, and lipids\textsuperscript{287,292}. During endocytosis, the plasma membrane invagination (outside-in) gives rise to early endosomes, the limiting membrane of which undergoes another round of invagination (inside-out) to form the intraluminal vesicles, which give the endosome a multivesicular appearance\textsuperscript{293,294}, referred to as a multivesicular endosome (MVE), which can fuse with the plasma membrane to release the intraluminal vesicles as exosomes\textsuperscript{295}. Another class of vesicle commonly released by cells are the microvesicles, which are generally larger (0.5-2 μm in diameter) than exosomes and are formed by budding or blebbing off of the cell surface (for a comprehensive review of the various types of vesicles secreted by cells see\textsuperscript{287,293,296}). Exosomes and microvesicles can interact with neighboring cells by various mechanisms, including direct fusion with recipient plasma membrane, endocytosis and fusion with endosomal membrane, receptor-mediated endocytosis and phagocytosis\textsuperscript{296}. The uptake of exosomes and exosome-associated proteins could shuttle pathological proteins between cells, as observed for the transfer of Wnt proteins between synapses via exosome-like vesicles\textsuperscript{297}. Furthermore, infectious prion protein can be actively transported into the extracellular environment through association with exosomes\textsuperscript{298}.

Luminally exposed amyloid forming proteins, such as APP and PrP, are both secreted on exosomal vesicles\textsuperscript{298-300}. Since extracellular A\textsubscript{β} has pathological consequences, it is possible that exosomes carrying A\textsubscript{β} function as “Trojan horses”, facilitating the release and subsequent uptake of these pathogenic peptides and playing a role in AD progression. Processing of amyloid precursor protein (APP) by β- and γ-secretase generates A\textsubscript{β} peptide in early endosomes. A\textsubscript{β} is then released from the cells in association with exosomes\textsuperscript{299}. Moreover A\textsubscript{β} oligomers have been detected in the intracellular space of primary human neurons\textsuperscript{301,302}. Together these findings
suggest that ILVs/exosomes could both be the site of APP cleavage and Aβ monomer formation as well as act as seeds for Aβ oligomerization, intracellularly and extracellularly. However, the definitive experiment demonstrating exosome mediated cell-to-cell transfer of Aβ has not been conducted.

Notably, recent data has expanded our understanding of the potential functional roles played by exosomes and extracellular vesicles in AD. Kyongman An and colleagues found that exosomes harvested from healthy human cerebrospinal fluid (CSF) can block the synaptotoxicity of both Aβ from AD patient brain and synthetic Aβ assemblies303. Additionally, when neuron-derived exosomes were placed into culture along with Aβ, the exosomes significantly promoted the uptake and clearance of the Aβ by cultured microglia, in what was suggested to be a protective strategy304. Moreover, an independent group has recently shown that microvesicles secreted by activated microglia (harvested from human CSF) are toxic to neuronal cultures, and that their toxicity was the result of 1) the microvesicles conversion of Aβ into soluble toxic oligomers from extracellular insoluble inert fibrils and 2) the uptake of toxic Aβ by microglia and subsequent trafficking to the cell surface and secretion of the toxic Aβ forms in associated with microvesicles305. This work suggests that microvesicles could represent an additional class of extracellular vesicle responsible for cell-to-cell transfer of toxic Aβ species in AD.

In the case of prion protein, experiments with cells in-vitro have found that exosomes can participate in the cell-to-cell transfer of prion protein during prion disease298,306-308. However, the experiments that would unambiguously demonstrate a functional role for exosomes in delivering infectious prions to naïve cells have not yet been performed or published. Moreover, the role of other secreted vesicles, i.e. microvesicles, has not been explored.

In addition to PrP and Aβ, tau, α-synuclein, TDP-43 and SOD1 have all been found to associate with exosomes. It was previously reported that α-synuclein is secreted in association with exosome release by neuronal cell lines, and that these vesicles cause neuronal death upon uptake by naïve cells309. However more recent work has shown exosome-associated α-synuclein to account for less than 1.5% of the total α-synuclein secreted by these neuronal cells310. Nevertheless, exposure to α-synuclein was observed to induce exosome release from
microglia\textsuperscript{311}, and while it is early yet for conclusion, this finding may suggest a secondary pathogenic role for exosomes, in light of the work on microglia-derived vesicles in the context of AD\textsuperscript{305}. Likewise, the role of non-exosomal secreted vesicles in α-synuclein and PD pathology are topics needing further investigation.

SOD1, one of the proteins associated with ALS, is a well-established cargo of mammalian exosomes\textsuperscript{312}. When investigation into SOD1 secretion via vesicular mechanisms began, a mouse spinal cord neuroblastoma hybrid cell model (NSC34) of ALS was found to secrete exosomes associated with both wild type and mutant SOD1\textsuperscript{313}. More recently, our group has found that misfolded SOD1 (both mutant and wild type) is associated with the outer leaflet of exosomes secreted by these same NSC34 mouse cells, and that the exosomes were capable of delivering misfolded SOD1 to naïve cells through macropinocytosis\textsuperscript{271,313}. Moreover, mutant SOD1 has been shown to induce exosome release from astrocytes\textsuperscript{314}, which have been known for some time to secrete motor neuron-specific toxic material\textsuperscript{315}. Additionally, the intermolecular transmission of misfolded human SOD1 could occur through release and uptake of naked aggregates that trigger seeded aggregation: aggregates composed of ALS-causing mutant protein can penetrate into cells through macropinocytosis and nucleate seeding of cytosolic mutant SOD1 protein\textsuperscript{253,270,316}. In the case of TDP-43, which was recently classified as a prion-like protein, it has been suggested that exosomes may contribute to the release of intracellular TDP-43 inclusions and provide the necessary delivery vehicle for their intercellular spread\textsuperscript{259}. Ding et al. also showed that exposure of glioblastoma cells to CSF from ALS/FTD generates TDP-43 aggregates through uptake of exosomes and tunnel-like structures\textsuperscript{317}. Here again with ALS associated proteins, the question of the roles played by other extracellular vesicles looms large.

1.8 Aims of thesis

The goals of this thesis are to advance our current understanding of SOD1 biology, and to develop better ways to treating ALS. Basic biology will be advanced through the following aims:

- We previously established that pathological TDP-43 and FUS can trigger misfolding of SOD1 in living cells. In Chapter 2, we tested if TDP-43 or FUS-induced misfolded SOD1 can propagate intercellularly and cause cell toxicity, as well as trigger additional rounds of SOD1 misfolding in the recipient cells. We also explored methods for blocking this
intermolecular transmission through treatment with SOD1-specific siRNA and misfolding-specific antibodies.

- Although the seeding of SOD1 in a prion-like fashion has been reported in recent years, the assays used are often technically cumbersome. Here, we aimed to generate several SOD1-GFP chimeric proteins to be used as reporter proteins for SOD1 misfolding and aggregation in living cells. This assay is used to assess the intermolecular kindling of SOD1 aggregation in a tryptophan-mediated fashion, and the seeding of SOD1 aggregation by extracts prepared from SOD1-FALS spinal cords. We also used this assay to test if tryptophan-binding small molecules can block the intermolecular aggregation.

- The exact mechanisms how TDP-43 aggregates and triggers the misfolding of SOD1 are unknown. We have previously shown that tryptophan residue at position 32 in SOD1 is essential for the intermolecular misfolding of SOD1. In Chapter 4, we explored if tryptophan residues play a role in TDP-43 self-aggregation and in the intermolecular conversion of SOD1 through homophilic interaction between tryptophan residues.

The ultimate goal of this thesis is to identify new therapeutic targets and drugs to treat ALS. As such, the following aims are incorporated in Chapters 2-4:

- Develop a medium/high-throughput assay for screening small drugs for blocking SOD1 or TDP-43 induced misfolding of SOD1.

- Test if tryptophan 32 residue in SOD1 is an effective target for SOD1 therapeutics by targeting it with known small molecules.

- Establish if solvent exposed tryptophans in TDP-43 contribute to its self-aggregation and play a role in inducing intermolecular misfolding and aggregation of SOD1, and can therefore be translated into new therapeutic targets aiming to block tryptophans containing pockets in TDP-43.
Chapter 2: TDP-43 or FUS-induced misfolded human wild-type SOD1 can propagate intercellularly in a prion-like fashion

2.1 Summary
Amyotrophic lateral sclerosis (ALS), which appears to spread through the neuroaxis in a spatiotemporally restricted manner, is linked to heritable mutations in genes encoding SOD1, TDP-43, FUS, C9ORF72, or can occur sporadically without recognized genetic mutations. Misfolded human wild-type (HuWt) SOD1 has been detected in both familial and sporadic ALS patients, despite mutations in SOD1 accounting for only 2% of total cases. We previously showed that accumulation of pathological TDP-43 or FUS coexist with misfolded HuWtSOD1 in patient motor neurons, and can trigger its misfolding in cultured cells. Here, we used immunocytochemistry and immunoprecipitation to demonstrate that TDP-43 or FUS-induced misfolded HuWtSOD1 can propagate from cell-to-cell via conditioned media, and seed cytotoxic misfolding of endogenous HuWtSOD1 in the recipient cells in a prion-like fashion. Knockdown of SOD1 using siRNA in recipient cells, or incubation of conditioned media with misfolded SOD1-specific antibodies, inhibits intercellular transmission, indicating that HuWtSOD1 is an obligate seed and substrate of propagated misfolding. In this system, intercellular spread of SOD1 misfolding is not accompanied by transmission of TDP-43 or FUS pathology. Our findings argue that pathological TDP-43 and FUS may exert motor neuron pathology in ALS through the initiation of propagated misfolding of SOD1.

2.2 Introduction
Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by degeneration of both upper and lower motor neurons, leading to progressive paralysis in muscles of the limbs, speech, swallowing and respiration. Nearly 90% of ALS cases are sporadic (SALS) with no known Mendelian genetic component, while the remaining 10% of cases are hereditary in a primarily autosomal dominant fashion. In familial ALS (FALS), the presence of any one of over 180 inherited mutations in the gene that encodes Cu/Zn superoxide dismutase (SOD1; http://alsod.iop.kcl.ac.uk/), a cytosolic scavenger of the superoxide anion radical, can lead to misfolding of the protein and to its toxic gain of function. Additionally, multiple studies have detected misfolded forms of human wild-type SOD1 (HuWtSOD1) protein in SALS and FALS in the absence of SOD1 mutations, suggesting that non-native conformers of SOD1
may play a key pathological role in all cases of ALS. However, the presence of misfolded SOD1 in sporadic disease remains a controversial topic as not all conformation specific antibodies can equally detect the aberrant forms of wild-type SOD1 in sporadic disease, which could be attributed to the differing epitope specificity and affinity of the antibodies employed. The disease can also be caused by mutations in either TAR-DNA binding protein 43 (TDP-43), or fused in sarcoma (FUS; originally designated translocated in liposarcoma, TLS), as well as other proteins (reviewed in ). Both TDP-43 and FUS are predominantly nuclear RNA binding proteins that are redistributed in a mutually exclusive fashion to the cytosol in FALS, where they form insoluble inclusions. Furthermore, post-mortem immunohistochemistry shows that all known cases of SALS, as well as non-SOD1 and non-FUS-FALS, contain neuronal and glial wild-type (wt)TDP-43 inclusions. The clinicopathological similarities among all types of ALS, as well as the co-presence of mislocalized TDP-43 or FUS along with misfolded SOD1 in pathology, led us to determine that aberrant cytoplasmic localization of TDP-43 or FUS triggers misfolding of HuWtSOD1 in cell culture models.

ALS pathology may begin at a single focal or multifocal sites; however, disease appears to spread through the neuroaxis in a spatiotemporal manner. This systematic spread of disease is consistent with a prion-like propagation of misfolded protein in disease, for which several protein candidates have been proposed in ALS. Insoluble TDP-43 from diseased brains has been reported to induce TDP-43 pathology in neuroblastoma cells that overexpress wtTDP-43 as detected by TDP-43 hyperphosphorylation, ubiquitination and aggregation. SOD1 also can display prion-like properties: we have established a cell culture system in which endogenous HuWtSOD1 was induced to misfold in the presence of misfolded mutant SOD1 ‘seed’. Subsequent studies demonstrated that misfolded HuWtSOD1 can be propagated intercellularly via exosome-dependent and independent mechanisms. Thus, induced misfolding of HuWtSOD1 by mutant SOD1 constructs expressed via transient cell transfection acquires propagation competency, and the capability to confer its misfold indefinitely on subsequent cell culture passages, even after the initial mutant protein is no longer detectable due to dilution and degradation. In-vivo studies have also recently demonstrated the presence of paralysis-associated seeded aggregation of fluorescently tagged SOD1G85R in neonatally inoculated mice. Here, we asked whether TDP-43 or FUS-induced misfolded HuWtSOD1 also acquires
the prion-like property of seeding HuWtSOD1 propagated misfolding that can be passaged from cell culture to cell culture via conditioned media.

2.3 Materials and methods

2.3.1 Cell culture

Human embryonic kidney cells (HEK293FT; ATCC, Manassas, VA) were cultured in complete Dulbecco’s Modified Eagle Medium (DMEM) containing 10% FBS, 10 U/ml penicillin, 10 U/ml streptomycin and 2 mM L-glutamine (Life Technologies, Carlsbad, CA). For immunoprecipitation and immunofluorescence studies, recipient cells were grown in 10 cm and 24-well tissue culture-treated plates, respectively. Cells were transiently transfected with TDP-43 or FUS plasmid DNA using Lipofectamine LTX (Life Technologies, Carlsbad, CA), according to the manufacturer’s instructions. For the transfection of SOD1-siRNA, RNAiMaX reagent (Life Technologies, Carlsbad, CA) was used according to manufacturer’s instructions. Biological repeats refer to experiments that were performed on different weeks with cells of different passage. Cell handling, transfections, media transfer and biochemical analyses were also performed separately for each experiment to ensure true biological repeat.

2.3.2 Preparation of conditioned media and antibody blocking

For induction of SOD1 misfolding in HEK293 cells, 48 h post transfection, conditioned media was collected from the transfected cells, and centrifuged at 1,000 x g for 5 min to remove floating cells and cell debris from the media. Clarified conditioned media were then supplemented with 25% of fresh complete DMEM media, and placed onto recipient cells for a 20 h incubation. For antibody blocking experiments, the conditioned media was prepared as described above, and subsequently incubated with 20 µg/ml of 3H1 or mIgG2a isotype control for 30 min at 37°C with constant rotation, prior to adding it to recipient cells. For induction of human SOD1 misfolding in primary spinal cord cultures, competent misfolded SOD1 ‘seed’ was concentrated via ultracentrifugation at 100,000 x g for 1 h, as we previously determined that the majority of misfolded SOD1 is present in the pellet fraction of ultracentrifuged media. The isolation was performed to minimize the transfer of DMEM growth media to the primary cultures that are cultured in complete Neurobasal media (Life Technologies, Carlsbad, CA; supplemented with neuronal supplements [StemCell Technologies, Vancouver, BC], 10 U/ml
penicillin, 10 U/ml streptomycin and 2 mM L-glutamine). Pellets were resuspended in complete Neurobasal media, and added to spinal cord cultures for a 20 h incubation.

2.3.3 Immunoprecipitation

Cells incubated with conditioned media were grown on 10 cm tissue culture-treated plates, and lysed in 400 µl of lysis buffer (0.5 % Triton X-100, 0.5 % sodium deoxycholate in PBS, and EDTA-free protease inhibitors, pH 7.4). For immunoprecipitation experiments, 100 µl of cell lysate was mixed with 10 µl of antibody-coupled M-280 Tosyl-activated magnetic Dynabeads® (Life Technologies, Carlsbad, CA). Tubes were incubated for 3 h at room temperature with constant rotation. Beads were then washed 3 times with 150 µl of RIPA buffer (150mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with brief agitation between washes, and boiled in loading sample buffer containing 1% β-mercaptoethanol. Pre-IP consists of 1 µl lysate boiled in sample buffer.

2.4 Results
2.4.1 TDP-43 or FUS-induced misfolded HuWtSOD1 can propagate intercellularly and seed cytotoxic misfolding of endogenous HuWtSOD1 in recipient cells

Earlier work has established that aggregates composed of ALS-causing SOD1 mutants can nucleate aggregation of the same soluble mutant SOD1 protein in-vitro and in-vivo254,270, and that induction of HuWtSOD1 misfolding via transient transfection with mutant SOD1 triggers the propagation of HuWtSOD1 misfolding for at least 5 passages253. To test if conditioned media from TDP-43 and FUS-transfected HEK293 cells can transmit HuWtSOD1 misfolding, we first utilized primary spinal cord cultures containing motor neurons, astrocytes and microglia from transgenic E12-E14 mice over-expressing HuWtSOD1. These transgenic animals were used, as mouse wild-type SOD1 does not participate in propagated protein misfolding253, which is due to the lack of a key tryptophan residue at codon 32117. We used mixed spinal cord cultures as ALS is a non-cell autonomous disease, in which neuronal and non-neuronal cells of the nervous system participate in pathogenesis326. For the immunodetection of misfolded HuWtSOD1, we used a mouse monoclonal misfolded SOD1-specific antibody (3H1), generated against an extended electrostatic loop in misfolded SOD1 that is not antibody-accessible in its wild-type native conformation117. To induce misfolding of HuWtSOD1 in transfected cells, we used the
naturally occurring R495x and P525L mutations of FUS, and an experimentally designed TDP-43 mutation with a dysfunctional nuclear localization signal (ΔNLS)\(^5\); exogenous TDP-43 and FUS were fused to HA-tag at their amino terminus. Conditioned media from the transfected cells was collected and concentrated, and overlaid on the primary cultures at 7 DIV (Figure 7). Our results show that primary spinal cord cultures incubated for 20 h with neural growth media containing the pellet fraction of HEK293-conditioned media from FUS\(^{R495x}\), FUS\(^{P525L}\), wtTDP-43 and TDP-43\(^{ΔNLS}\), but not empty vector control or wtFUS transfected cells, display significant neurite immunoreactivity for misfolded SOD1 by immunocytochemistry (Figure 2), despite similar transfection efficiency of the various constructs in HEK293 cells (Figure 8). Background staining observed in primary cultures incubated with conditioned media from empty vector or wtFUS transfected cells could be attributed to newly translated HuWtSOD1, which may take hours to properly fold, become metalated, disulphide-oxidized and dimerized\(^3\). Incubation of spinal cord cultures from the non-transgenic littermates with conditioned media from TDP-43 or FUS transfected cells does not induce misfolding of SOD1 (Figure 9). This finding is consistent with induction and intercellular propagation of HuWtSOD1 misfolding by the TDP-43 or FUS-induced misfolded HuWtSOD1.

To measure the extent of HuWtSOD1 transmitted misfolding, we performed quantitative immunoprecipitations on human HEK293 cells that were incubated with conditioned media collected from TDP-43 or FUS transfected HEK293 cells. Lysates of recipient cells were immunoprecipitated using magnetic beads coupled to misfolded SOD1-specific antibodies, 3H1 and 10C12 (Figure 3a). The latter antibody is a mouse monoclonal misfolded SOD1-specific antibody raised against an oxidized epitope within the SOD1 dimer interface, which is normally buried within the native SOD1 homodimer\(^5\). Consistent with the immunocytochemical observations in primary spinal cord cultures, 20-40% of the total immunoprecipitable HuWtSOD1 in HEK293 lysates was misfolded in cells that had been incubated with media from FUS\(^{R495x}\), FUS\(^{P525L}\), wtTDP-43 or TDP-43\(^{ΔNLS}\) transfected cells, but not empty vector control or wtFUS conditioned media (Figure 3b, c).

To rule out the possibility that the immunoprecipitation signal in recipient HEK293 cells was merely due to passive uptake of misfolded HuWtSOD1 from conditioned media, we selectively
knocked-down endogenous SOD1 in the recipient cells using SOD1-siRNA (Figure 3e inset) prior to incubating recipient cells with conditioned media for 20 h (Figure 3d). Quantitative immunoprecipitation demonstrated that SOD1-knockdown HEK293 cultures show a highly significant 15-25 fold drop in misfolded HuWtSOD1 compared to non-knockdown HEK293 cells when incubated with conditioned media originating from mutant FUS, as well as wild-type and mutant TDP-43, transfected cells (Figure 3e, f). Immunoblots in Figure 2d were transformed to clearly demonstrate the lack of immunoprecipitatable misfolded SOD1 in cultures with downregulated endogenous levels of SOD1. The ability of SOD1-siRNA to significantly reduce detectable misfolded SOD1 in recipient cells is consistent with the requirement of endogenous HuWtSOD1 as substrate for a prion-like conformational conversion process.

We also sought to determine if seeded conversion of HuWtSOD1 might prove to be toxic. Recipient cells incubated for 20 h with conditioned media from TDP-43 or FUS transfected cells were analyzed for viability using the MTT assay (Figure 10). Our results show that there is a modest but statistically significant reduction in cell viability in those cells incubated with conditioned media originating from FUSR495x, FUSP525L, wtTDP-43 or TDP-43ΔNLS transfected cells. We used this data to estimate cell death to be between 13-15\% when the conditioned media originated from misfolded-SOD1 producing cells, when compared to empty vector control.

2.4.2 SOD1 misfolding-specific antibodies inhibit propagated misfolding of HuWtSOD1

We previously established that pre-incubation of conditioned media from SOD1G85R or SOD1G127x-transfected cells with either misfolding-specific or pan-SOD1 antibodies inhibits transmission of propagated HuWtSOD1 misfolding in-vitro. To test the activity of 3H1, a potent inhibitor or propagated misfolding, in TDP-43 and FUS-induced HuWtSOD1 intercellular transmission, conditioned media derived from HEK293 cells transfected with FUSR495x, FUSP525L, wtTDP-43 or TDP-43ΔNLS were pre-incubated with 20 µg/ml of 3H1 antibody or mouse IgG2a isotype control (Figure 4a). A significant reduction of 50-80\% misfolded HuWtSOD1 was observed in HEK293 lysates incubated with 3H1-treated conditioned medium when analyzed by quantitative immunoprecipitation using 3H1 and 10C12, as compared to conditioned medium incubated with an isotype control (Figure 4b, c). Although the neutralization effect is not complete, the efficiency is comparable to the level of inhibition of
intercellular transmission of mutant SOD1-induced misfolded HuWtSOD1 using 3H1 \(^{253}\). Additionally, the ability of misfolded SOD1 specific antibodies to block transmission of protein misfolding is indicative of the presence of electrostatic loop-exposed misfolded SOD1 seed in the respective conditioned media.

### 2.4.3 No propagation of TDP-43 or FUS pathology to recipient cells in the timeframe of SOD1 misfolding

We used our HEK293 cell culture model (Figure 7), which supports the propagated misfolding of HuWtSOD1, to study the potential propagation of TDP-43 (Figure 5) or FUS (Figure 6) pathology from transfected cells to untreated recipient cells in the same experimental timeframe as propagated-misfolding of SOD1 occurs. Consistent with other studies, we confirmed pathological TDP-43 in transiently transfected HEK293 cells to be hyperphosphorylated, mislocalized and fragmented \(^{62}\) (Figure 5a, b). While transient over-expression of wtTDP-43 is predominantly localized to the nucleus with several cytoplasmic punctae, transfection of the mutant TDP-43\(^{ΔNLS}\) results in nearly exclusive cytoplasmic localization and aggregation (Figure 5a, b). Transfection of an empty vector control does not alter the predominantly nuclear localization of TDP-43. Immunofluorescent staining using a phospho-TDP-43 antibody reveals hyperphosphorylation of TDP-43 exclusively in cytoplasmic inclusions in HEK293 cells transfected with TDP-43\(^{ΔNLS}\), and to a significantly lesser extent with wtTDP-43 (Figure 5b). However, these TDP-43 phenotypic features were not transmitted to recipient cell cultures following a 20 h incubation with conditioned media; neither pan-TDP-43 nor phospho-TDP-43 antibodies detected mislocalized or hyperphosphorylated TDP-43, respectively, in recipient cells following a 24 h incubation with conditioned media (HEK293 cellsFigure 5c, d; Primary mouse spinal cord cultures: Figure 11). Immunoblotting analysis (Figure 5e) confirmed our immunocytochemistry findings; HEK293 transfection of wild-type and mutant TDP-43, but not empty vector, results in: (1) hyperphosphorylation of full size TDP-43 (blue arrowheads; and (2) partial fragmentation of TDP-43 into 35 kDa and 25 kDa bands (black arrowheads). These signs of pathological TDP-43 are not observed in recipient cell cultures when incubated with conditioned media. Additionally, probing the immunoblots using an HA-tag antibody shows significant protein expression in cells transfected with TDP-43 constructs, but no expression in the incubated cells, indicating that the conditioned media contains no active residual
lipofectamine reagent and that the transfection-encoded TDP-43 protein does not transmit to recipient HEK293 cell cultures (Figure 5a,c,e).

Furthermore, we found that while mutant FUS is predominantly localized to the cytoplasm of transiently transfected HEK293 cells (Figure 6a, b), this pathology is not transmitted to untreated recipient cell cultures when incubated with conditioned media from FUS$^{R495x}$ or FUS$^{P525L}$ transfected cells (Figure 6c, d), in the same timeframe as misfolding of SOD1 occurs. Transfected wild-type FUS localizes predominantly in the nucleus, and does not trigger FUS mislocalization or aggregation in incubated cells (Figure 6a, b). Cell fractionation into cytoplasmic and nuclear fractions (Figure 6e) shows that transfection of HEK293 cells with wild-type or mutant FUS drives expression of the construct-encoded proteins in both nucleus and cytoplasm (although immunocytochemistry reveals cytoplasmic aggregates only with mutant FUS; Figure 6a, b), while no exogenous protein is detectable in either fraction in cultures incubated with the indicated conditioned media (Figure 6). Furthermore, immunofluorescence and cell fractionation of recipient cells reveals no increase in cytoplasmic, or decrease in nuclear, endogenous FUS, indicative of absence of transmission of pathological FUS mislocalization in-vitro. Expression levels and localization of TDP-43 and FUS are not affected by the incubation of cells with conditioned media. Notably, mutations in either TDP-43 or FUS can be found in ALS, but only wtTDP-43 pathology is observed in sporadic ALS$^{324}$; we find in the present study that pathological wtTDP-43, but not wtFUS, is associated with propagation-competent SOD1 misfolding.
Figure 2: TDP-43 and FUS-induced misfolded HuWtSOD1 propagates from transfected cells to untreated spinal cord cultures.

Primary spinal cord cultures containing neurons (including motor neurons) and astrocytes prepared from human HuWtSOD1 transgenic mice were incubated for 20 h with conditioned media from transfected HEK293 cells, and stained for misfolded SOD1 (green) using misfolded SOD1-specific antibody, 3H1, and counterstained using Hoechst 33342 (blue). The source of the media is indicated for each panel. Primary cultures incubated with conditioned media from FUS^{R495X}, FUS^{P525L}, wtTDP-43 and TDP-43^{ANLS} show an increase in the presence of cytoplasmic misfolded SOD1, as compared to cells incubated with conditioned media from cells transfected with empty vector control and wtFUS. Scale bar: 75 µm.
Figure 3: Propagation of misfolded HuWtSOD1 depends on endogenous HuWtSOD1 substrate.
Representative immunoblots of quantitative immunoprecipitation of SOD1 proteins from untransfected (a) or SOD1-siRNA transfected (d) HEK293 cells incubated for 20 h with conditioned media from TDP-43 or FUS-transfected HEK293 cells. Immunoprecipitation studies were performed using a rabbit polyclonal pan-SOD1 antibody, SOD100, and misfolded SOD1-specific mouse monoclonal antibodies, 3H1 and 10C12. Mouse IgG2a isotype control was used as negative control, and blots were probed with pan-SOD1 antibody. Lysate pull-down signals from 3H1 (b) and 10C12 (c) were normalized to total immunoprecipitable SOD1 in each lysate and expressed as a percentage of total SOD1. We used non parametric one-way ANOVA to established statistical significance between cells incubated with conditioned media from FUS^{R495x}, FUS^{P525L}, wtTDP-43 and TDP-43^{ΔNLS} transfected cells as compared to cells incubated with conditioned media from empty vector transfected cells. Two tailed Student’s t-test was used to demonstrate statistically significant reduction in detectable misfolded SOD1 between SOD1-siRNA treated (e, f) and the corresponding untreated (b, c) recipient cell cultures incubated with the same conditioned media. Inset in (d) confirms downregulation of SOD1 using an immunoblot probed with pan-SOD1 and actin (load control) antibodies.*, p < 0.05 ; **, p < 0.01.
; ****, p < 0.0001. Number of biological repeats was 9 for empty vector and wtFUS, and 16 for the other constructs.
Figure 4: Intercellular propagation of HuWtSOD1 misfolding is inhibited by misfolded SOD1-specific antibodies.

(a) Representative immunoblots of SOD1 immunoprecipitations from recipient cells incubated for 20 h with conditioned media that were pre-incubated with either 20µg/ml 3H1 or mIgG2a isotype control. Immunoprecipitation studies were performed using pan-SOD1 antibody (SOD100), and misfolded SOD1-specific mouse monoclonal antibodies, 3H1 and 10C12. Blots were probed with pan-SOD1 antibody. Immunoprecipitation using 3H1 (b) and 10C12 (c) were quantified and expressed as a fraction of total immunoprecipitable SOD1 in each lysate. Statistical significance between the ability of 3H1 vs. isotype control to inhibit induction of SOD1 misfolding for specified conditioned media was established using a two-tailed Student’s t-test (*, p < 0.05; **, p < 0.01; ***, p < 0.001). Graph bars represent 12 biological repeats per construct.
Figure 5: No intercellular propagation of TDP-43 pathology is detected in recipient cell cultures.

Immunofluorescent staining of HEK293 cells transfected with TDP-43 using HA tag (yellow, a), or pan-TDP-43 (green, b) and phosphorylated-TDP-43 (red, b) antibodies shows an increase in TDP-43 mislocalization, cytoplasmic accumulation, and hyperphosphorylation (yellow punctae in ‘b’ correspond to colocalization). Pathological forms of TDP-43 are detected in significantly greater amounts when cells are transfected with TDP-43ΔNLS, but can be also detected with wtTDP-43. Immunofluorescent staining of HEK293 cells incubated for 20 h with conditioned media from TDP-43 transfected cells shows no signs of transmission of transfected (e) or pathological (d) TDP-43. In immunoblotting analysis of cell lysates, fragmentation (black arrowheads), hyperphosphorylation (blue arrowheads) and expression of exogenous TDP-43 are all present in the transfected, but not incubated cells (e). Abundance of FUS and SOD1 is not
affected in either transfected or incubated cells, when compared to actin load control. Empty vector and TDP-43 panels for each antibody are cut-outs from the same gel. Scale bar: 50 µm.
Figure 6: FUS pathology does not propagate between cell cultures through conditioned media.

Immunofluorescent staining of HEK293 cells transfected with wtFUS, FUSR495x or FUSP525L using HA tag (red, a) or pan-FUS (green, b) antibodies shows mislocalization and aggregation of the exogenous FUS mutants. Cells transfected with mutant FUS contain cytoplasmic, and often aggregated protein, while exogenous wtFUS localizes in the nucleus. A similar staining performed on recipient HEK293 cell cultures that were incubated for 20 h with conditioned media from FUS transfected cells reveals no transmission of exogenous protein (c) or of pathology as can be seen by the lack of mislocalized or aggregated FUS (d) in the recipient cells. Cells were counterstained with Hoechst33342 (blue). Additionally, cells transfected with empty vector control, wild-type or mutant FUS, and cells incubated with conditioned media from cells transfected with the indicated construct, were fractionated into cytoplasmic and nuclear fractions (e). Actin was used as loading control, while LaminB1 and SOD1 were used as nuclear and cytoplasmic purity controls, respectively. Scale bar: 50 µm.
2.5 Discussion
An increasing body of evidence supports the notion that neurodegenerative disorders, including Alzheimer and Parkinson diseases, and ALS, can spread between contiguous and projection regions via the intercellular transmission of aggregated misfolded proteins and/or extracellular vesicles\textsuperscript{46,165,254,328,329}. Previous reports have shown that aggregates composed of ALS-causing SOD1 mutants are taken up by cells, where they trigger the nucleation and aggregation of soluble mutant SOD1\textsuperscript{270}. Also, overexpression of mutant SOD1 in cells can kindle the misfolding of endogenous wild-type SOD1\textsuperscript{117}, which can propagate intercellularly and trigger additional rounds of misfolding of wild-type SOD1 in recipient cells\textsuperscript{253}. Although cytotoxic mutations in SOD1 represent only a fifth of familial ALS cases, we and others reported on the presence of misfolded SOD1 in sporadic ALS and non-SOD1 familial ALS\textsuperscript{55,56,58,253}, potentially implicating misfolded SOD1 as a pathogenic molecule in all types of ALS. We previously reported that pathogenic FUS and TDP-43, the latter of which is present in all SALS in its wild-type isoform, as well as non-SOD1 and non-FUS FALS\textsuperscript{324}, trigger the intracellular misfolding of HuWtSOD1 in the same cells\textsuperscript{55} potentially through physical interaction between the aberrantly mislocalized and aggregated TDP-43 or FUS and HuWtSOD1. Alternatively, the kindling of SOD1 misfolding could occur through indirect mechanisms. Disease-implicated forms of TDP-43 and FUS have been recently reported to be associated with mitochondrial impairment\textsuperscript{330,331}, which could lead to free radical generation and subsequent SOD1 misfolding through protein oxidation\textsuperscript{332}. Other potential indirect mechanisms of SOD1 misfolding might include titration of chaperone proteins, or bulk saturation of clearance mechanisms such as proteosomal or autophagic degradation; in this latter regard, pathological TDP-43 and FUS are cleared via the proteasome\textsuperscript{333}, thus reducing efficient clearance of misfolded SOD1\textsuperscript{334}.

Here, we show for the first time that TDP-43 or FUS-induced misfolded HuWtSOD1 acquires the prion-like property of intercellular transmissibility and induction of endogenous HuWtSOD1 misfolding in recipient cells. The intercellular transmission of human mutant or misfolded wild-type SOD1 is likely to occur through release of naked aggregates by dying cells, which are taken up by macropinocytosis and can trigger seeded aggregation\textsuperscript{253,270,316}, or through the release of disease-associated exosomes containing intraluminal and surface-associated misfolded SOD1\textsuperscript{253,313}. Following uptake of exosomes from the extracellular environment, the release of
misfolded SOD1 into the recipient cells might occur through direct fusion of exosomal membrane with the plasma membrane, or by intraluminal fusion of the exosomes with the endosomal membrane following endocytosis. We further find that knockdown of SOD1 expression in recipient cells prior to incubation with conditioned media leads to significant inhibition in the propagation of misfolded HuWtSOD1, consistent with HuWtSOD1 being the seed and substrate for the propagation of misfolding. This finding is consistent with the fact that in SOD1$^{G93A}$ and SOD1$^{G37R}$ experimental mouse models of ALS, suppression of mutant human SOD1 synthesis using adeno-associating virus encoding small hairpin RNA against mutant SOD1 led to improved motor performance, as well as a delay in disease onset and progression. Taken together, this work indicates that the propagation of SOD1 misfolding is an active and independent process, which can occur in the absence of TDP-43 or FUS pathology. Our observation that propagated misfolded SOD1 is toxic to mesenchymal HEK293 cells may also provide insight into possibly pertinent mechanisms of neurodegeneration in ALS. Although transmissible TDP-43 or FUS-induced misfolded SOD1 triggers only a 13-15% decrease in HEK293 viability, we expect toxicity to be more marked in the more vulnerable end-mitotic motor neurons degenerating in disease. The cytotoxicity of the soluble TDP-43 or FUS-induced misfolded SOD1 is also consistent with reports showing similar toxicity of soluble mutant SOD1 to CHO cells.

Familial, and to a significantly lesser degree sporadic, ALS can be partially explained by the presence of pathological mutations in a variety of proteins; recent research has shown that heritable mutations in some of these genes, for instance C9ORF72, VCP, VAPB, and ataxin 2, are linked to the pathophysiological hallmark of abnormal cytoplasmic wtTDP-43 accumulation in disease. Other disease-implicated proteins that are essential for proteostasis, such as P62, ubiquilin 2, and optineurin, are also known modifiers of TDP-43 aggregation. Since misfolded SOD1 is detectable in both sporadic and familial ALS, including in spinal cords of patients with the C9ORF72 expansion mutation, we now propose that in all SALS, non-SOD1 and non-FUS FALS, pathological aggregation of wtTDP-43 triggers a prion-like cycle of propagated misfolding of HuWtSOD1.
Given reports that TDP-43 may act as a prion-like protein by inducing its own nucleation, mislocalization and propagation\textsuperscript{259,277}, could HuWtSOD1 misfolding be secondary to TDP-43 or FUS pathology? Here, we provide a system to disentangle the relative contribution of TDP-43 or FUS and HuWtSOD1 to the prion-like intercellular transmission of propagated protein misfolding in ALS. Importantly, in this model, the recipient cell cultures were not primed to accept protein pathology by overexpressing the relevant proteins; rather, recipient cells expressed normal loads of endogenous SOD1, TDP-43 and FUS. We find that during the timeframe in which HuWtSOD1 misfolding propagates to recipient cells, no pathological TDP-43 or FUS is transmitted, indicating that spread of TDP-43 or FUS pathology is not necessary for the transmission of HuWtSOD1 misfolding. Neuronal cytoplasmic TDP-43 aggregation is also observed in non-ALS disorders such as Alzheimer’s disease\textsuperscript{118}, where it is clearly not sufficient to induce the ALS syndrome. Even in frontotemporal dementia (FTD) associated with C9ORF72 mutation, TDP-43 pathology can be observed in spinal motor neurons without evidence of motor neuron cell death\textsuperscript{344}, suggesting the existence of a molecular “second hit,” which could be the induction of propagated misfolding of HuWtSOD1. Moreover, transgenic mouse models expressing mutant and wtTDP-43 frequently do not exhibit the robust motor neuron disease observed in transgenic human mutant SOD1 mice, or in SOD1-FALS patients\textsuperscript{345}. Notably, HuWtSOD1, which can trigger an ALS-like syndrome in mice, is completely competent to engage in propagated misfolding with itself, but is unable to do so with mouse SOD1 substrate\textsuperscript{117,253,346}. These finding suggest that humans possess a critical vulnerability to SOD1 misfolding propagation (such as Trp32\textsuperscript{117}) which is lacking in transgenic mouse models of TDP-43 or FUS pathology, supporting the idea that human SOD1 misfolding, induced by mutation or seeded propagation, is necessary and sufficient to cause the human ALS syndrome.

We report here that neutralization of the propagating TDP-43 or FUS-induced misfolded HuWtSOD1 using SOD1 misfolding-specific antibodies reduces the induction of protein misfolding by 50-80\%, implying that misfolded HuWtSOD1 is a key participant in the intercellular transmission particle \textit{in-vitro}. Passive immunization of SOD1\textsuperscript{G93A} mice with anti-human SOD1 antibody, or other monoclonal antibodies specific to misfolded forms of SOD1, have been shown to reduce the burden of misfolded SOD1 in the spinal cord and prolong the survival of antibody-treated mice\textsuperscript{347,348}. Furthermore, active immunization using SOD1 exposed
dimer interface (SEDI) peptide in SOD1G37R transgenic mice reduced the accumulation of misfolded SOD1 in the spinal cord, and increased survival by an average of 40 days. Together, our data strengthens the viability of an immunological therapy approach against misfolded SOD1 in familial and sporadic ALS.

2.6 Supplementary information

2.6.1 Supplementary materials and methods

2.6.1.1 Immunocytochemistry

Cells were placed on glass cover-slips (#1.5) in a 24 well plate prior to transfection or incubation with conditioned media. For immunofluorescence study, cells were washed twice with ice-cold phosphate buffer saline (PBS) and fixed in 4% paraformaldehyde (in PBS, pH 7.4) for 15 min at room temperature. Fixed cells were then washed once with PBS, permeabilized for 10 min using PBSTx (0.3% Triton X-100 in PBS), and blocked for 30 min with incubation buffer (2% normal goat serum in PBS, filtered). Cells were then incubated with the following primary antibodies: 2 µg/ml anti SOD1 misfolding-specific mouse monoclonal 3H146,55, 1 µg/ml rat anti HA tag (Roche Diagnostics, IN), 10 µg/ml rabbit anti TDP-43 (ProteinTech Group Inc., Chicago, IL), 10 µg/ml rabbit anti phosphor-(409/410) TDP-43 (ProteinTech Group Inc., Chicago, IL), 10 µg/ml mouse anti FUS (ProteinTech Group Inc., Chicago, IL) diluted in incubation buffer for 1 h at room temperature. Cell were then washed twice in PBS, and incubated with appropriate secondary antibody conjugated to Alexa Fluor-488 or 647 fluorescent dyes (Life Technologies, Carlsbad, CA; 1:1000 dilution) for 1 h at room temperature in the dark. The cells were washed with PBS, and DNA was counterstained using 2 µg/ml Bis-benzimide H33342 trihydrochloride (Hoechst 33342) for 5 min. Following 2 final washes the cells were mounted on a glass slide in a drop of Fluoromount-G (SouthernBiotech, Birmingham, AL). Confocal images were captured using Leica TCS SP8 microscope (Leica Canada) using the LAS-X software. Images were acquired at the same settings and not subjected to any further image processing.

2.6.1.2 Immunoblotting and quantification

Cell lysates were prepared by incubating pelleted cells with a mild lysis buffer containing 0.5% sodium deoxycholate, 0.5% Triton X-100 and EDTA-free protease inhibitor cocktail (in PBS; for analysis using phosphoTDP-43, phosphatase inhibitors were added) for 2 min on ice, followed
by centrifugation for 5 min at 1,000 x g at 4°C. Prepared samples were boiled in sample buffer containing 1% β-mercaptoethanol, and analyzed on 4-20% Tris-Glycine gels (LifeTechnologies, Carlsbad, CA). Proteins were then transferred to a PVDV membrane, blocked (Tris-buffer saline, 0.1% Tween-20 (TBST) with 3% BSA for phosphor-(409/410) TDP-43 or 5% skimmed milk for all other proteins) for 1 h, and incubated with 1 µg/ml SOD100 (Assay Designs, Ann Arbor, MI), 10 µg/ml TDP-43, 10 µg/ml phosphor-(409/410) TDP-43, 10 µg/ml FUS (Abcam, Cambridge, MA), 5 anti-HA (Abcam, Cambridge, MA), 0.5 µg/ml of Lamin B1 (Abcam, Cambridge, MA), or 1 µg/ml actin (ABM Inc., Richmond, BC) antibodies overnight at 4°C. Membranes were then washed twice in TBST and incubated with anti-mouse or rabbit IgG horseradish peroxidase linked whole antibody (GE Healthcare, Buckinghamshire, UK) diluted 1:10,000 in blocking buffer for 1 h at room temperature. Membranes were developed using SuperSignal West Femto chemiluminescence substrate (Thermo Scientific, Waltham, MA), and visualized using VersaDoc Imager (Bio-Rad Laboratories, Hercules, CA) with no digital or biological signal saturation. The densitometry of western blot bands was quantified using Quantity One software. Percentage of misfolded SOD1 was calculated by quantifying specific immunoprecipitation of 3H1 or 10C12 using densitometry, subtracting pull-down with mIgG2a isotype control, and normalizing to total immunoprecipitable SOD1.

2.6.1.3 Nuclear and cytoplasmic extraction
Cells were grown in 6 cm plates one day prior to either transfection for 48 h or incubation for 24 h with conditioned media. On collection day, cells were washed twice in cold PBS, collected in a microcentrifuge tube, and spun for 5 min at 1,000 x g. Cell pellets were lysed on ice in cytoplasmic extract (CE) buffer (10 mM HEPES, 10 Mm KCl, 10 mM EDTA, 1.5 mM MgCl₂, 0.4% NP40, 1mM DTT and protease inhibitors; adjusted to pH 7.9 and filtered), and centrifuged for 5 min at 14,000 x g. Supernatants were removed to fresh tubes and centrifuged for an additional 10 min, resulting in cytoplasmic fractions. Nuclei containing pellets were washed once in buffer CE, and incubated in nuclear extract (NE) buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT and protease inhibitors; adjusted to pH 7.9 and filtered) on ice for 90 minutes with brief vortexing every 20 minutes. Mixtures were then centrifuged for 5 min at 14,000 x g, and supernatants containing nuclear fractions were transferred to fresh tubes.
2.6.1.4 Mouse primary spinal cord culture
Experiments involving animals were conducted according to the Canadian Council on Animal Care guidelines and have been approved by the Animal Care Committee of the University of British Columbia. Pregnant C57 BL/6 female mice (Strain: B6SJL-Tg(SOD1)2Gur/J, Stock:002297; Jackson Laboratories, Bar Harbor, ME) were sacrificed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC). Primary spinal cord cultures were prepared from 12–14 day fetal mice using minor modification of an established protocol\textsuperscript{349}. Following embryo genotyping for human wtSOD1, cervical, thoracic and lumbar-regions of the spinal cord were dissected out in Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-free Hanks Balanced Salts (Life Technologies, Carlsbad, CA). Meninges were removed and the tissue was transferred to 0.25\% trypsin (Life Technologies, Carlsbad, CA) and digested at 37°C for 15 min. Tissue was then resuspended in DMEM (Life Technologies, Carlsbad, CA) plus 10\% fetal bovine serum (Life Technologies, Carlsbad, CA) and triturated 4–6 times through a fire-polished tip. The supernatant was centrifuged at 200 × g for 45 sec. Pelleted neural cells were resuspended in Neurobasal media, B27 supplements, 2 mM L-glutamine (all from Life Technologies, Carlsbad, CA) and seeded at a density of 2 × 10\textsuperscript{5} cells/well onto poly-D-lysine (Sigma, Saint Louis, MO) coated #1.5 coverslips in 24-well plates. Cultures were maintained in serum-free Neurobasal-B27 medium, and one-half of medium was replaced on day 3 with equal volume of fresh medium. Cells were incubated with conditioned media at 7 DIV.

2.6.1.5 Cell viability
Cells were grown in a 96-well plate and incubated overnight with conditioned media. Stock 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT; Sigma-Aldrich, MO) was prepared at 5 mg/ml in warm PBS and filtered, added directly to culture media to a final concentration of 0.5 mg/ml, and incubated for 3 h. Absorbance was read at 570 and 650 nm. For every biological MTT repeat, 4-8 technical repeats were performed.

2.6.1.6 Statistical analysis
We tested every set of data for Gaussian distribution. For normal distributions we used the parametric one-way ANOVA test. Otherwise, without any assumption regarding the distribution underlying our sample sets, we applied nonparametric Kruskal-Wallis test. 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.
2.6.2 Supplementary figures

Figure 7: Experimental flow chart.

HEK293 cells were transfected for 48 h, following which the conditioned media was collected and pre-cleared. Afterwards, conditioned media was either placed for an additional 20 h on untreated recipient cells for determining the ability of TDP-43 or FUS-induced misfolded SOD1 to induce further rounds of HuWtSOD1 misfolding (A). In (B), conditioned media was pre-treated with a misfolding specific antibody, 3H1, prior to placement on fresh cell cultures. Alternatively, conditioned media was placed on SOD1-siRNA treated cells for determining whether HuWtSOD1 is an obligate substrate for misfolding (C).
Cell cultures were transfected with the indicated construct for 48 h, following which they were lysed using RIPA buffer (50mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, protease inhibitors in water) and analyzed for the presence of the exogenous protein. Immunoblots were probed using FUS or TDP-43 antibodies (top two rows) to visualize total proteins in lysates, or with HA-tag (middle two rows) for the detection of transfection-encoded proteins. Actin was used as loading control.
Figure 9: TDP-43 and FUS-induced misfolded HuWtSOD1 does not kindle the misfolding of mouse SOD1.

Primary spinal cord neural cultures prepared from wild-type mice were incubated for 20 h with conditioned media from transfected HEK293 cells, and stained for misfolded SOD1 (green) using misfolded SOD1-specific antibody 3H1 and counterstained using Hoechst 33342 (blue). The source of the media is indicated for each panel. Scale bar: 75 µm.
Figure 10: Misfolded SOD1-containing media are cytotoxic to recipient HEK293 cells.

Cell viability was determined by using a colorimetric assay, MTT, on recipient cells that were incubated with conditioned media from TDP-43 or FUS transfected cells for 20 h. When compared to cells incubated with conditioned media from cells transfected with empty vector control, a significant 13-15% reduction in cell viability (***, p < 0.001) is established in cells incubated with conditioned media from mutant FUS, as well as wild-type and mutant TDP-43, but not from wtFUS or empty vector transfected cells. Number of biological repeats is 13. Error bars represent s.e.m.
Figure 11: No propagation of TDP-43 pathology to primary spinal cord cultures.

Primary spinal cord cultures were incubated with conditioned media from empty vector, wild-type or mutant TDP-43 transfected cells. 20 h following incubation, cells were fixed and stained using pan (top; green) or P409/410 phospho-TDP-43 antibody (bottom; green). No mislocalization, aggregation or hyperphosphorylation of TDP-43 is detectable in these incubated cultures. Scale bar: 50 µm.
2.7 Acknowledgements
We thank ALS Canada, the Canadian Institutes of Health Research, Brain Canada and the Giancarlo and Odette Tognetti Trust Foundation for the funding to undertake these studies. We also thank Jing Wang and Masoud Yousefi for their technical assistance and help with statistical analysis, respectively.
Chapter 3: Induction of fluorescently-tagged SOD1 aggregation in a tryptophan-dependent fashion

3.1 Summary

Mutant SOD1 can confer its misfolding on wild-type SOD1 inside living cells; the propagation of misfolding can also be transmitted intercellularly. Our laboratory has previously reported that tryptophan (Trp) at position 32 of SOD1 is required for SOD1 self-recognition during this prion-like conversion. Recent studies identified fluorescently-tagged SOD1G85R as a promiscuous substrate that is highly prone to misfold and aggregate in-vivo by a variety of templates. To test its effectiveness in cell cultures, we used several SOD1-GFP reporter proteins with A4V, G37R, G85R, or G93A mutations in the SOD1 moiety. We then co-expressed these reporter proteins along with pathological SOD1 or TDP-43 proteins in HEK293 cells, to find that both forms of inducer proteins can trigger the aggregation of our reporter proteins; however the efficiency of induced aggregation and their sizes depends on the inducer as well as the substrate. The intermolecular induction of SOD1 aggregation was shown to be mediated through tryptophan 32 using immunofluorescence and live cell microscopy. Next, we used the reporter proteins to show that human spinal cord extracts prepared from familial ALS patients can trigger aggregation of the reporter proteins in cultured cells. Finally, we used this system to show that small molecules, akin to 5-fluorouridine, can be used to block this intermolecular aggregation of SOD1, and demonstrated that our assay can be used as a high-throughput tool for screening drugs against induced aggregation of SOD1. This innovative assay is a robust “prion activity” aggregation fluorescent reporter assay that can assess intermolecular propagation of SOD1 misfolding. The importance of Trp32 has been validated via this assay, and would be adapted to screen small molecules predicted to bind the Trp32 pocket, thus blocking propagated SOD1 misfolding.

3.2 Introduction

Over the past decade, mounting evidence demonstrated that ALS acts as a prion-like disease, with induced SOD1 misfolding playing a key role in this process: (1) aggregates composed of ALS-causing mutant SOD1 can penetrate inside cells and nucleate aggregation of soluble wild-type SOD1 protein; (2) misfolded endogenous HuWtSOD1 induced by mutant SOD1 can propagate intercellularly, and (3) spinal cord injection of homogenates prepared from paralyzed SOD1G93A mice can induce widespread spinal inclusion pathology.
As described in the Introduction chapter, mutant SOD1 commonly aggregates in SOD1-FALS, but also misfolds in sporadic cases. While SOD1 normally forms a homodimer, it can be kindled to misfold by breaking down into monomers that are often oxidized. Once SOD1 is misfolded, its hydrophobic chains become exposed, therefore driving its self-aggregation, a process potentially mediated through cysteins. Despite showing that SOD1 can be triggered to misfold by mutant SOD1, or by pathological TDP-43 or FUS, this approach involves cumbersome immunoprecipitations and immunofluorescence studies. It was recently shown that intra-spinal injection of SOD1\textsuperscript{G85R}-YFP mice with spinal cord homogenates prepared from paralyzed SOD1\textsuperscript{G93A} transgenic mice can trigger disease in the recipient mice, and trigger aggregation of SOD1\textsuperscript{G85R}-YFP. Based on this finding, we sought to generate a fluorescence-based assay using reporter proteins to screen for induced aggregation of SOD1. Following careful consideration, we generated a series of fusion proteins with a C-terminal AcGFP tag: (1) wtSOD1; (2) SOD1\textsuperscript{A4V}; (3) SOD1\textsuperscript{G37R}; (4) SOD1\textsuperscript{G85R}; and (5) SOD1\textsuperscript{G93A}. AcGFP was chosen due to its monomeric nature and similar fluorescence profile to the commonly used eGFP enabling us to use microscopes with a common 488 filter sets. Additionally, GFP is better suited than YFP for co-immunofluorescence studies with red dyes due to better spectral resolution.

In this Chapter we established the reagents, tools, protocols and optimal assay conditions to monitor induction of SOD1 misfolding and aggregation in living cells through the use of fluorescently-tagged mutant SOD1. We find that co-expression of mutant SOD1 with low doses of fluorescently-tagged SOD1 induces aggregation of the reporter protein, which does not aggregate on its own or in the presence of empty vector control. Importantly, we also demonstrated that homogenates prepared from familial ALS spinal cord tissue with SOD1 mutations induce more aggregation than homogenates prepared from sporadic ALS, Alzheimer’s disease or MSA tissue. Also, we used this system to demonstrate that tryptophan at position 32 plays an important role in cross-seeding of SOD1, and demonstrated that this residue can represent a therapeutic target for ALS: Trp-32 binding small molecules, including 5-fluorouridine, can effectively block induced aggregation of SOD1. Furthermore, we adapted our fluorescence-based assay for a high-throughput screen of drugs for targeting the induced SOD1 aggregation process in living cells. Finally, we find that the levels of induced misfolding and
aggregation of SOD1 depend on the nature of the inducer and substrate, as some inducers and reporter proteins appear to show better induction.
Figure 12: Schematic representation of fluorescence-based SOD1 aggregation assay.

HEK293 cells are pre-plated at 50-60% confluency. Cells are then co-transfected with the appropriate GFP-tagged reporter protein and SOD1 or TDP-43 inducer, and incubated under cell culture conditions for 48 h, following which they are analyzed and/or fixed using 4% PFA. Although first signs of protein expression are detected 8 h after transfection, induced aggregation typically occurs between 24-48 h post transfection, making 48 h an ideal time point for cell analysis.
3.3 Methods and materials

3.3.1 Cell culture

Human embryonic kidney cells (HEK293FT; ATCC, Manassas, VA) were cultured in complete Dulbecco’s Modified Eagle Medium (DMEM) containing 10% FBS, 10 U/ml penicillin, 10 U/ml streptomycin and 2 mM L-glutamine (Life Technologies, Carlsbad, CA). For immunofluorescence studies, cells were grown in 24 well plates with cover slips or black 96 well plates with glass bottom. To test the potency of the various tissue homogenates to seed aggregation of the SOD1-GFP protein in living cells (plasmids were a gift from Elizabeth Fisher\(^{352}\)), we transfected pre-plated HEK293 cells with the chimeric reporter protein using Lipofectamine LTX (Life Technologies, Carlsbad, CA), according to manufacturer’s instructions. 4 h later, homogenates were mixed with the appropriate volume of Lipofectamine 2000 (Life Technologies, Carlsbad, CA) in serum reduced media (Life Technologies, Carlsbad, CA), and added drop-wise to the cells. The cells were then incubated for 48 h in a 37°C humidified incubator supplemented with 5% CO\(_2\).

3.3.2 Tissue extraction and addition to cells

We perform tissue extraction on the following tissues: 4 SOD1-FALS (A4V, disease duration: 2 years; D90A, disease duration: 17 years; D93S, disease duration: 6 years; I113T, disease duration: >10 years), 3 SALS, 2 Alzheimer’s disease (AD), one Multiple System Atrophy (MSA), and one healthy control. We chose AD and MSA as negative controls as both of these disorders have been studied for their prion-like features\(^{353,354}\), and their neurodegenerative nature that presents overall stress conditions. Tissue homogenates were prepared by first cutting ~0.1g of flash frozen human spinal cord tissue (C- or T-spine) and adding it to 9-parts of cold PBS supplemented with protease inhibitors (Roche Diagnostics, IN). Each tissue was then homogenized 3x for 20 sec with 40 sec breaks (on ice), and sonicated once for an additional 15 sec. Homogenized and sonicated tissue was spun down at 1,000 x g for 5 min and the supernatant was aliquoted into fresh tubes. Total protein concentration in each homogenate was determined using a standard BCA assay, and adjusted between the samples in order to ensure that equal amount of protein is later added to the cell cultures. Homogenized tissue was stored in -80°C and each aliquot was only used for one experiment. Homogenates were then briefly
mixed with Lipofectamine 2000 (Life Technologies, Carlsbad, CA) in serum reduced media, and added dropwise to the cells 4 h following reporter protein transection.

3.3.3 Immunofluorescence
The protocol for immunofluorescence staining was described in detail in Section 2.6.1.1 of this thesis. Here, I would like to note that representative images for reporter protein fluorescence refer to average fields of view with the main considerations for acquisition being even distribution of cells within the field, good transfection efficiency, and presence/absence of aggregates. The images do not show the “best” field in the wells/cover slips. Number of repeats refers to biological repeats with independent cell culture, transfections and staining. For all figures were different constructs are compared to each other, data acquisition settings were kept constant.

3.3.4 Inclusion counting algorithm using microscopy
Images were acquired using an inverted AxioObserver microscope (Carl Zeiss AG, Germany) with a motorized stage, and exported as a high resolution JPG files. For these studies, we used the Zen microscope software (Carl Zeiss AG, Germany) to prepare a 96 well template with four global focus points. The template was calibrated for every new plate, and each focus point was adjusted in order to determine the focus plane of the plate. The software, together with the Definite Focus (Carl Zeiss AG, Germany), ensured that each well is then in focus. In order to quantify induced aggregation of SOD1 using our reporter protein, we developed an ImageJ-based algorithm that reliably counts inclusions based on the area that inclusions occupy and normalized to total expressed GFP. Briefly, to count inclusions and their area the algorithm first converts each image into 8-bit images, performs a local background subtraction, thresholds based on pre-set settings, and counts the number of inclusions based on a predefined range in size. In parallel, another algorithm estimates the area all chimeric protein fluorescence with certain intensity levels by using threshold and inclusion counting. The percentage of aggregation is reported as a ratio of area of inclusions divided by total area of fluorescence.

3.3.5 Inclusion counting using flow cytometry
In order to quantify the number of cells with induced aggregation of reporter protein, flow cytometry was utilized. To overcome the challenge that flow cytometry may not distinguish between diffused cytoplasmic GFP, and aggregated GFP, we briefly permeabilized the cells
using cold PBS supplemented with 0.03% saponin, and performed a quick wash in cold PBS. Cells were then stored on ice until analysis on LSRII (BD Biosciences). Data analysis was performed using FlowJo.
3.4 Results

3.4.1 Induction of reporter protein aggregation through protein expression

In order to validate the aggregation properties of the chimeric reporter proteins, we had co-transfected the reporter protein along with non-tagged SOD1 or TDP-43 protein and monitored for induced aggregation of the GFP-tagged SOD1 over time using fluorescence microscopy.

We find here that mutant SOD1 (A4V, G85R, and G127x) and pathological TDP-43 can trigger the aggregation of mutant (A4V, G37R, G85R, and G93A), but not wild-type, SOD1-based reporter proteins (Figure 13, Figure 14 and Figure 15). We performed blinded manual counts to confirm the induced aggregation and to determine how many of the co-transfected cells contain aggregated reporter protein. We find that the effectiveness of induced aggregation depends on the reporter protein used (Figure 16): 10-15% of cells co-transfected with SOD1\textsuperscript{A4V}-GFP and mutant SOD1 or TDP-43 contain induced fluorescent aggregated compared to 1-3% in empty vector controls; 14-25% of cells co-transfected with SOD1\textsuperscript{G37R}-GFP and mutant SOD1 or TDP-43 contain induced fluorescent aggregated with SOD1\textsuperscript{G127x} and TDP-43\textsuperscript{ΔNLS} being the most effective compared to 2-3% in empty vector controls; 20-26% of cells co-transfected with SOD1\textsuperscript{G85R}-GFP and mutant SOD1 or TDP-43 contain induced fluorescent aggregated compared to 2-4% in empty vector controls; and 17-27% of cells co-transfected with SOD1\textsuperscript{G93A}-GFP and mutant SOD1 or TDP-43 contain induced fluorescent aggregated compared to 2-5% in empty vector controls. The statistical significance for each inducer compared to its empty vector control is indicated in Figure 16.

In order to assess induced aggregation of the reporter protein in larger populations of cells in an independent fashion, we developed an algorithm that quantifies the area occupied by aggregated reporter protein and normalized it to total reporter protein expression in each micrograph (Figure 17). This algorithm relies on 3 important sequential steps; local background subtraction used to emphasize the inclusions, threshold analysis to select the bright inclusions, and particle counting based on a pre-set size. Using our algorithm (Figure 18), we find that: 3-5% of total fluorescent reporter protein in cells co-transfected with SOD1\textsuperscript{A4V}-GFP and mutant SOD1 or TDP-43 is aggregated compared to <1% in empty vector controls; 4-6% of total fluorescent reporter protein in cells co-transfected with SOD1\textsuperscript{G37R}-GFP and mutant SOD1 or TDP-43 is aggregated
compared to 1-2% in empty vector controls; 4-6% of total fluorescent reporter protein in cells co-transfected with SOD1\textsuperscript{G85R}-GFP and mutant SOD1 or TDP-43 is aggregated compared to <1% in empty vector controls; and 4-6% of total fluorescent reporter protein in cells co-transfected with SOD1\textsuperscript{G93A}-GFP and mutant SOD1 or TDP-43 TDP-43 is aggregated compared to 1-2% in empty vector controls.

To further quantify induced aggregation using unbiased technologies, we sought to utilize flow cytometry analysis to count cells with induced inclusions of the reporter protein. However, since flow cytometry detects whole cell fluorescence, aggregated and soluble forms of the reporter protein may not be easily distinguished. Therefore, we used a mild reversible detergent (0.03% saponin in cold PBS) to partially permeabilize the transfected cells immediately prior to analysis, which results in the leak of soluble GFP out of the cells, while retaining the intracellular inclusions and the cell morphology unharmed (demonstrated using live cell microscopy in Figure 19\textsuperscript{355}). Importantly, Figure 19 also demonstrates that this quick pre-treatment does not result in morphological changes in the cell.
Figure 13: Wild-type SOD1-GFP cannot be efficiently seeded by mutant SOD1.

GFP-tagged wtSOD1 was co-transfected with either empty vector control (FUW) or mutant SOD1 (G85R or G127x). Microscopy analysis performed 48 h post transfection indicates that while wtSOD1-GFP expresses well, it cannot be triggered to aggregate by mutant SOD1. Scale bar: 100µm (N=5).
Here, we co-transfected HEK293 cells with an array of SOD1-based reporter proteins (A4V, G37R, G85R, G93A) and various mutant SOD1 (A4V, G85R, G127X) for 48 h prior to fixation and subsequent analysis. We find that regardless of the exact mutation in the inducer or reporter protein, all tested mutant SOD1 constructs can trigger aggregation of the abovementioned reporter proteins. However, the levels of induced aggregation depend on the nature of the particular inducer and reporter proteins. Scale bar: 200 µm (N=5).
Figure 15: Phenotype of induced aggregation of reporter protein by TDP-43\textsuperscript{ΔNLS} is dependent on the specific SOD1 mutation in the reporter protein.

TDP-43\textsuperscript{ΔNLS} was co-transfected with our array of fluorescent reporter proteins (wt, A4V, G37R, G85R, G93A) for 48 h in HEK293 cells. While TDP-43\textsuperscript{ΔNLS} cannot induce the aggregation of wtSOD1-GFP, it is capable to trigger aggregation of all the mutant-SOD1 reporter proteins, albeit with differential efficiencies. Scale bar: 100µm (N=5).
HEK293 cells were co-transfected for 48 h with an array of reporter proteins (A4V, G37R, G85R, G93A), and either mutant SOD1 (A4V, G85R, G127X or its empty vector control, FUW) or TDP-43 (or empty vector control, pCINeo). Manual counts were performed on PFA-fixed cells immediately after the experimental end-point. Percentages of cells with inclusions were quantified by counting the number of cells with reporter protein inclusions out of total transfected cells expressing GFP. Counts were performed by a blinded individual on 3 independent experiments, with over 200 GFP-expressing cells counted per construct in each experiment.
Figure 17: Quantification of GFP inclusions using a computerized algorithm.

This flowchart provides a schematic representation of our ImageJ-based automatic counting process, which occurs in two separate steps: counting of the inclusions, and counting of total reporter protein fluorescence. At first, the image is converted to 8-bit black & white image and
local background is subtracted to emphasize the inclusions and reduce background. Threshold is then set to identify inclusions only, followed by particle counter that identifies only particles that had been thresholded and are of a pre-determined size. The combination of local background subtraction, threshold analysis and size estimation ensures identification of inclusions only. In parallel, another algorithm quantifies total GFP based on a similar process but with higher threshold and more permissive size to capture entire cells. Once all the parameters are adjusted based on a representative image in each folder (experiment), the rest of the folder is analyzed in a batch-form using the same set of optimized parameters.
Figure 18: Algorithm counts of induced inclusions of reporter protein by mutant SOD1 or TDP-43.

HEK293 cells were pre-plated in 96-well black plates with optical bottom and co-transfected for 48 h with an array of reporter proteins (A4V, G37R, G85R, G93A), and either mutant SOD1 (A4V, G85R, G127X or its empty vector control, FUW) or TDP-43 (or empty vector control, pCINeo). The percentage of reporter protein in an aggregated form was calculated by using our algorithm to quantify the area occupied by aggregated reporter protein and express it as a fraction of total expressed GFP. The experiment was repeated 5 times (biological repeats) with each construct being replicated 3 times in each experiment (technical repeats).
Figure 19: Partial cell permeabilization using saponin.
HEK293 cells co-transfected with SOD1^{G85R}-GFP and SOD1^{G85R} for 48 h were partially permeabilized using 0.03% saponin in cold PBS for up to 80 sec. While this brief treatment retains cell morphology and intracellular aggregates consisting of the reporter protein, it allows soluble fluorescence protein to exit the cell. Scale bar: 100µm.
3.4.2 Subtypes of misfolded and aggregated SOD1

Mutations in SOD1 gene were first shown to cause familial ALS (SOD1-FALS) over 20 years ago\textsuperscript{356}. Since then, \textit{in-vivo} and \textit{in-vitro} studies have collectively shown that mutations in nearly every amino acid in SOD1 can trigger its misfolding and cause toxicity. As technologies evolved and conformation-specific antibody were raised, multiple studies have detected misfolded forms of human wild-type SOD1 protein in SALS and FALS in the absence of SOD1 mutations\textsuperscript{55,56,58}, suggesting that non-native conformers of SOD1 may play a key pathological role in all cases of ALS. However, the presence of misfolded SOD1 in sporadic disease remains a controversial topic\textsuperscript{320,321} as not all conformation specific antibodies can equally detect the aberrant forms of wild-type SOD1 in sporadic disease, which could be attributed to the different epitope specificity and affinity of the antibodies employed. Alternatively, SOD1 may be kindled to misfold into various three-dimensional conformations leading to a pool of misfolded SOD1 types (or species). Ultimately, since conformation-specific antibodies are raised against specific sequences as well as structural contexts, it is likely that SOD1 misfolding-specific antibodies recognize different subsets of misfolded SOD1 species, with some antibodies recognizing larger pools.

The notion of subsets of misfolded protein species in neurodegenerative diseases is not new. Among the many unique features that made the prion field so novel, is the presence of several strains, which possess the same chemical composition but differ in their three dimensional structures and pathological profiles. However, the presence of a multitude of misfolded wild-type SOD1 species has not been formally studied. In this Chapter, we studied the structural loosening of endogenous SOD1 that is triggered to misfold by SOD1, TDP-43 or FUS. Furthermore, we developed a series of fluorescence-based reporter proteins to study the ability of different mutant SOD1 proteins to induce intermolecular SOD1 aggregation. We used this fluorescence assay to compare the kinetics, number and size of the induced aggregates formed by different inducers and reporter proteins.

In addition to the differences in the amounts of induced aggregation described in Chapter 3.2.1, we also observed that induced aggregation of the various reporter proteins greatly depends on the inducer and substrate used: while SOD1\textsuperscript{G37R}-GFP forms smaller inclusions than SOD1\textsuperscript{A4V, G85R} or
G93A)-GFP regardless of the inducer, TDP-43ΔNLS triggers smaller induced aggregates than mutant SOD1 in all reporter proteins used (Figure 14, Figure 15, and Table 2).

Structural differences between natively folded and misfolded proteins can also be studied by assessing sensitivity or resistance of the protein to proteases, which naturally target exposed poly-peptide backbones. We previously published that while wtSOD1 is extremely resistant to protease digestion (tested up to 100 µg/ml), if it is triggered to misfold by mutant SOD1 (either G85R or G127x), it acquires extreme sensitivity to as little as 1 µg/ml. We used two misfolded SOD1 specific antibodies, 3H1 and 10C12, to show that while TDP-43 (wild-type or ΔNLS)-induced misfolded SOD1 is partially sensitive to 1 µg/ml, approximately 15-20% of it remains resistant to this treatment suggesting that it is in a different conformation and a part of a structure (Figure 20).
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**Table 2: Inclusion sizing (manual assessment)**

Semi-quantitative analyses of the induced reporter protein aggregates were performed in a blinded fashion in 3 biological repeats. No significant inclusions were observed when wtSOD1-GFP was used as the reporter protein, or when the empty vector controls (FUW for mutant SOD1, pCINeo for TDP-43) were used as inducers. + small inclusions; ++ medium inclusions; +++ large inclusions.
Figure 20: SOD1 and TDP-43 induced misfolded SOD1 possess different proteinase K sensitivity.

Most of the mutant SOD1-induced misfolded SOD1 is proteinase sensitive; however, only a fraction of the pathological FUS/TDP43-induced misfolded SOD1 is proteinase sensitive. Here, HEK293 cell cultures transfected with pathological SOD1 or TDP-43 for 48 hours. Following lysis, proteinase K (PK) digestion was performed using 1µg/ml of PK for 30 minutes at 37C on half of each lysate (other half being the control). After digestion, protease inhibitors were added and misfolded SOD1 was immunoprecipitated using pan-SOD1 antibody, SOD100, or SOD1 misfolding-specific mouse monoclonal antibodies, 3H1 and 10C12. rIgG and mIgG were used as isotype control for SOD100 and misfolding-specific antibodies, respectively (representative
immunoblots presented in (A). The amounts of immunoprecipitable misfolded SOD1 was quantified using densitometry and summarized in the form of bar graphs presented in (B). The amounts of PK resistant SOD1 or TDP-43 induced misfolded SOD1 is presented in (C) as can be detected by 3H1 and 10C12. N=4 and 7 (for SOD1 and TDP-43, respectively). *: p<0.05.

1 Representative immunoblots from SOD1^{G85R} and SOD1^{G127X} induced misfolded SOD1 were included in a published manuscript. Ref: 117Grad, L. I. et al. Intermolecular transmission of superoxide dismutase 1 misfolding in living cells. Proc Natl Acad Sci U S A 108, 16398-16403, doi:10.1073/pnas.1102645108 (2011).
3.4.3 Propagation of SOD1 aggregation from human homogenates to cells in culture

Over the past decade, an increasing number of *in-vivo* and *in-vitro* studies have identified prion-like mechanisms contributing to the spread of ALS pathogenesis from its initial focus/foci sites. In these studies, it was observed that aggregates composed of mutant SOD1 can penetrate inside cells through macropinocytosis and nucleate aggregation of soluble cytoplasmic mutant SOD1 protein, and that overexpression of mutant SOD1 protein in human cells can trigger the misfolding of endogenous SOD1 in the transfected cells. These studies have also demonstrated that once SOD1 is triggered to misfold and/or aggregate inside cells, it can propagate intercellularly by hijacking the exosomal machinery or through macropinocytosis. However, it was not until 2014, when Ayers et al. demonstrated for the first time that mutant SOD1 can be transmitted *in-vivo*. In this study, spinal cord extracts were prepared from paralyzed mice expressing SOD1 and injected intra-spinally into mice expressing SOD1-G85R-YFP, which produced motorneuron disease in the recipient mice as well as widespread spinal inclusion pathology. Most recently, the same group used organotypic spinal cord slice cultures prepared from paralyzed mice expressing SOD1-G93A and injected intra-spinally into mice expressing SOD1-G85R-YFP, which produced motorneuron disease in the recipient mice as well as widespread spinal inclusion pathology. We also tested 3 reporter proteins as possible substrates for induction of SOD1 aggregation, by using a chimeric SOD1-GFP protein with G37R, G85R or G93A mutations in the SOD1 moiety.

As we show in Figure 21, despite some variability in their potency, homogenates prepared from SOD1-FALS spinal cord tissues can trigger significantly more aggregation (approximately 40%) of SOD1-G37R-GFP following 48 h incubations, when compared to homogenates from non-ALS patients. Extracts prepared from SALS spinal cord tissue can apparently trigger limited amount of induced aggregation of the SOD1-G37R-GFP reporter protein (approximately 15%), however it is not statistically significant. Additionally, homogenates prepared from SOD1-FALS tissue can also trigger significantly more induced aggregation of G85R and G93A-based reporter proteins.
when compared to extracts prepared from non ALS patients (approximately 30 and 20%, respectively). For the latter reporter proteins, extracts prepared from SALS cannot induce more reporter protein inclusions than non ALS controls. Interestingly, these studies suggest that SOD1$^{D90A}$ and SOD1$^{G93S}$ are the best suited inducers in this system.
Figure 21: Homogenates prepared from familial ALS spinal cord tissue induce SOD1 aggregation.

A) Homogenates from human spinal cord tissue were incubated with HEK293 cells pre-transfected with the indicated reporter protein (G37R, G85R or G93A-based). The cells were imaged 48 h post treatment and analyzed for the presence of induced aggregation using our algorithm. Representative immunocytochemistry micrographs demonstrating induced aggregation of SOD1\textsuperscript{G85R}-GFP in cells incubated with the indicated homogenate. B) Summary of the effect of FALS, SALS and non-ALS control tissue homogenates on the reporter proteins. Bar graphs represent the percentage of reporter protein in inclusion form out of total reporter protein (area). Statistical significance was established using one way ANOVA followed by Dunnett’s test for multiple comparisons. C) Induced aggregation of the reporter protein using the individual homogenates grouped in (B). Each homogenate was tested 8 times with 2 technical repeat per run. *** p < 0.001, * p < 0.05. Scale bar: 40 µm.
3.4.4 Induced SOD1 aggregation is mediated through tryptophan residue at location 32

Although the exact mechanism how intermolecular propagation of SOD1 misfolding occurs is not known, we have previously shown that human mutant SOD1 cannot induce the misfolding of mouse SOD1, and demonstrated that tryptophan at position 32 (Trp32) is crucial for the conversion of human SOD1 and for its propagated protein misfolding. Here, we used the fluorescence-based SOD1 aggregation assay to test if Trp32 is required for both inducer and reporter protein for induced aggregation to occur. For this, we used SOD1\textsuperscript{G85R} and SOD1\textsuperscript{G85R/W32S} inducers, and generated another reporter protein based on SOD1\textsuperscript{G85R}-GFP where Trp-32 was replaced with serine (SOD1\textsuperscript{G85R/W32S}-GFP), a substitution capable of blocking induction of SOD1 misfolding. We find that SOD1\textsuperscript{G85R}, but not SOD1\textsuperscript{G85R/W32S}, induces the aggregation of SOD1\textsuperscript{G85R}-GFP (Figure 22A). Additionally, Trp32 also plays a role in the reporter protein substrate as mutant SOD1 can induce SOD1\textsuperscript{G85R}-GFP, but not SOD1\textsuperscript{G85R/W32S}-GFP, to aggregate (Figure 22A). The inability of SOD1\textsuperscript{G85R/W32S} to induce aggregation of the reporter protein is especially important in light of the fact that both SOD1\textsuperscript{G85R} and SOD1\textsuperscript{G85R/W32S} express well in HEK293 cells and appear to be at least partially misfolded (green and red staining against pan-SOD1 and 3H1, respectively, in Figure 22B). It is interesting to note, however, that not all exogenous SOD1 protein is misfolded, as some cells show protein expression without detectable misfolded SOD1. Furthermore, we performed live cell microscopy on cells that were co-transfected with reporter protein and either SOD1\textsuperscript{G85R} or SOD1\textsuperscript{G85R/W32S}, and found that the presence of tryptophan significantly increases the rate of induced aggregation (Figure 23). We then performed linear regression on the aggregate growth phase of the curves using the G85R-based reporter protein (from 6 to 20 h) to find that the rate of induced aggregation by SOD1\textsuperscript{G85R} is 0.08 while that of SOD1\textsuperscript{G85R/W32S} is 0.04 percent of inclusion growth per hour (Figure 23A). Aggregation rate induced by empty vector control is 0.005. We chose to focus on SOD1\textsuperscript{G85R} as an inducer instead of SOD1\textsuperscript{G127x} as the latter contains a novel tryptophan at its C-terminus formed due to the G127x shift mutation. We also confirmed that the G37R and G93A-based reporter proteins respond similarly to the presence of tryptophan in the inducing mutant SOD1 (Figure 23B). These results are consistent with our previous findings showing that Trp32 mediates the intermolecular conversion of endogenous SOD1 by mutant SOD1 or pathological TDP-43. Together, these data further strengthens the key role that tryptophan 32 plays in the pathogenesis of SOD1 in ALS.
Figure 22: Induced SOD1 aggregation is mediated through tryptophan 32.

A) HEK293 cells were co-transfected with either SOD1\textsuperscript{G85R} or empty vector together with SOD1\textsuperscript{G85R}-GFP or SOD1\textsuperscript{G85R/W32S}-GFP reporter protein for 48 h, following which the cells were imaged. The results show that SOD1\textsuperscript{G85R} can trigger aggregation of the reporter protein in a tryptophan-32 dependent manner (N=4). (B) HEK293 cells were transfected for 48 h with either SOD1\textsuperscript{G85R} or SOD1\textsuperscript{G85R/W32S} and stained using pan-SOD1 antibody (green) or 3H1, misfolded SOD1 specific antibody (red). Nuclei were counterstained using Hoechst33342 (blue). Scale bars: 100 and 50 µm (A and B, respectively; N=3).
Figure 23: Tryptophan 32 contributes to cross seeding of SOD1 aggregation.
(A) HEK293 cells were co-transfected with the SOD1G85R-GFP along with either SOD1G85R, SOD1G85R/W32S or empty vector. 24 h post transfection, the cells were subjected to live cell microscopy under cell growth conditions (37°C, 5% CO₂, humidity). Images were acquired every 30 min for a period of 20 h, following which the images were analyzed using our aggregation-counting algorithm and plotted above. (B) The experiment was duplicated with SOD1G37R-GFP (blue) or SOD1G93A-GFP (red) as reporter proteins. Regardless of the reporter protein used, SOD1G85R can trigger seed faster and greater number of inclusions than SOD1G85R/W32S. Time-point h=0 corresponds to when we started imaging, which occurred approximately 20-24 h post transfection.
3.4.5 Inhibition of tryptophan-mediated mutant SOD1 induced aggregation of SOD1 using small molecules

In addition to potentiating aggregation and cytotoxicity of SOD1, our laboratory reported that Trp32 of SOD1 is required for SOD1 self-recognition during prion-like conversion\textsuperscript{117,359}. A recent study has shown through crystal-structure analysis that 5-fluorouridine (5FUr), a chemotherapy agent, can bind at Trp32 \textsuperscript{360}, leading us to hypothesize that small molecules binding at or near the Trp32 site can block SOD1 template-directed misfolding. For this study, we tested if 5-fluorouridine, or its precursor uridine, can block the intermolecular conversion of SOD1 by mutant SOD1. We first showed that 1 µM 5-FUr can dramatically decrease induced aggregation of the reporter protein (Figure 24A). Next, we found using flow cytometry that as little as 0.5 µM of 5-FUr applied to the cells 4-6 h after transfection with SOD1\textsuperscript{G127x} and SOD1\textsuperscript{G85R} along with SOD1\textsuperscript{G85R}-GFP reporter protein can significantly block induced aggregation by nearly 3-fold judging by the shift of mean GFP fluorescence (Figure 24B, D). In fact, 5-FUr can block induced aggregation of reporter protein in a dose-dependent manner with near significance for both 5 and 10 µM (p=0.056 and 0.061, respectively). Here, we also tested the ability of uridine, a natural metabolite and a precursor for 5-FUr, in blocking induced aggregation of reporter protein using our algorithm counts. We found that while uridine can indeed block the induced aggregation, it requires much higher concentrations (over 100 µM) than 5-FUr (Figure 24C). We also performed a time-course live cell microscopy study where we transfected cells with SOD1\textsuperscript{G85R} and reporter protein to show that the presence of 0.5 or 1 µM 5-FUr significantly reduces the build-up of inclusions (Figure 24E). We performed linear regression on the aggregate growth phase of the curves (from 10 to 48 h) to find that the rate of induced aggregation without 5-FUr is 36 inclusions/hour, and drops to 12 inclusions/hour when 5-FUr is added. A quick immunoblotting analysis of reporter protein content in lysates of cells from (D) shows that 0.5 or 1 µM 5-FUr, or 50 µM uridine do not decrease the total load of reporter protein in the cells (Figure 24F).
Figure 24: 5-fluorouridine, and with a lower efficacy uridine, can block seeding of SOD1 aggregation.

HEK293 cells were co-transfected with SOD1
\(^{G127x}\) and SOD1
\(^{G85R}\)-GFP for 6 h prior to the addition of 5-fluorouridine or uridine to the final indicated concentration. The cells were first imaged for induced aggregation in the presence of 1 µM 5-FUr (A), and further analyzed using...
flow cytometry 48 h after transfection (B). Control samples contain transfection reagent along with small molecule vehicle at a volume equal to that of 10 µM 5-FUr, the highest tested concentration of 5-FUr (N=8). (C) Percentage of reporter protein in the aggregated form in cells co-transfected with SOD1G127x and SOD1G85R-GFP and treated with uridine (N=4). (D) HEK293 cells were incubated with the indicated final concentration of 5FU-r or Ur 6 h post transfection with SOD1G85R and SODG85R-GFP (N=8). (E) HEK293 cells were co-transfected with SOD1G85R and SODG85R-GFP in the presence of 5-FUr (green, purple) or vehicle control (red). Images were acquired every 30 min for a period of 20 h, following which the images were analyzed using our aggregation-counting algorithm and plotted above. (F) Representative immunoblot of HEK293 cells were lysed 48 h post co-transfection with SOD1G127x and SOD1G85R-GFP. Lysates were separated using SDS-PAGE and membranes probed with anti GFP and load control, tubulin, antibodies. *** p < 0.001, ** p < 0.01, * p < 0.05.
3.4.6 High-throughput screen of small molecules for the inhibition of induced SOD1 aggregation

The ultimate goals of this thesis were to gain a better understanding of the biology behind SOD1 misfolding and aggregation, and to develop an assay for an effective screening of small molecules targeting the tryptophan-dependent intermolecular conversion of SOD1. To transform the fluorescence-based induced SOD1 aggregation assay into high-throughput, we adapted the assay to 96-well plate format plates. We opted to use black plates with optical bottom designed specifically for fluorescence studies to ensure that we get technically sound micrographs with minimal cross-talk between wells. For automatic data acquisition, we used an inverted microscope with a motorized stage for which we generated a 96 well template that reflects the dimensions of our plate. Additionally, we added several “anchoring” focus points that form a focal plane used to automatically focus every image. 20 images are then acquired per well and stitched into a single high-resolution micrograph representing the entire well. Finally, images are analyzed in batches using our aggregate counting algorithm. The proof-of-principal screen was performed using 110 small compounds provided to us by ALS Therapy Development Institute (Figure 25). The library included small molecules that are known to bind at or around Trp32 or otherwise hypothesized to block SOD1 aggregation, as well as an array of negative controls. During this screen, we remained blinded throughout performing the experiment and its analysis. The inducer for these experiments was SOD1^{G127x} and the reporter protein utilized was SOD1^{G85R}-GFP.
Figure 25: High-throughput screening of small molecules against induced aggregation of SOD1.

HEK293 cells were co-transfected with SOD1^{G85R}-GFP and SOD1^{G127x} for 6 h prior to the addition of 110 compounds to a final concentration of 10 µM. Cells were incubated for 48 h and imaged using an inverted microscope with a motorized stage and a chamber to keep cells at 37°C, 5% of humidified CO₂. Images were then analyzed using our algorithm and data was reported as a percentage of aggregated reporter protein. Black and red horizontal lines indicate the levels of aggregated reporter protein in cells without any additives or in cells incubated with vehicle control, respectively (N=3).


3.5 Discussion

Similar to other neurodegenerative disorders including Alzheimer’s and Parkinson’s diseases, ALS is considered by many to possess prion-like pathological mechanisms that cause the spread of pathology beyond its initial focal point(s)\(^{262,263}\). Although the nature of the particle that spreads the pathology remains unknown, based on the arguable evidence that misfolded SOD1 can be identified in all cases of ALS even in the absence of mutation in its gene\(^{55,56,58,59,117,320,321}\), and that SOD1 possess prion-like characteristics\(^{117,253,261}\) we argue that SOD1 could act as the final molecule responsible for spread of disease. Therefore, we focused here on studying the intermolecular transmission of SOD1 pathology in greater detail using a fluorescence-based assay that we developed.

Given that fluorescently-tagged SOD1\(^{G85R}\) has been identified to aggregate \textit{in-vivo} when SOD1\(^{G85R}\) mice were injected with SOD1\(^{G93A}\) mouse spinal cord extracts\(^{254}\), and that the G85R mutation forms a metal-deficient monomer, we generated a SOD1\(^{G85R}\)-GFP fusion protein. In order to study the effects of other mutations in SOD1 on the induced aggregation of the reporter protein, we used reporter proteins based on wild-type, as well as A4V, G37R and G93A-SOD1. While wild-type SOD1 form a stable homodimer that is not expected to seed easily, the chimeric protein containing SOD1 mutation is expected to aggregate more readily. With the exception for the enzymatically inactive G85R, the other chosen mutations are as enzymatically active as the wild-type protein\(^{361}\). Unlike both A4V (the most clinically severe and common SOD1 mutation found in FALS in North America\(^{362}\)) and G93A (possess a moderate propensity to aggregate with very short disease duration), G37R has a lower propensity to form inclusions and a higher disease duration\(^{363}\).

Our validation and optimization of the fluorescence-based assay has been fruitful from early on. We found that mutant SOD1 (A4V, G85R or G127x) and pathological TDP-43, but not empty vector vehicle control nor mutant SOD1 lacking Trp32, can all trigger significant aggregation of the tested reporter proteins, regardless of the specific mutation. Although this finding was first confirmed using manual counts, we opted to generate an automated algorithm in order to avoid human biases and errors. The key features in this algorithm are local background subtraction that emphasizes induced inclusions that may be normally hard to detect, threshold used to select inclusion based on the presumption that chimeric proteins in inclusion form fluoresces stronger than in their soluble form due to its higher concentration in a small space, and particle counting.
based on size where noise and overall brightly-fluorescing cells are eliminated due to size constraints. This algorithm confirmed our manual counts showing the ability of mutant SOD1 and pathological TDP-43 to trigger SOD1 misfolding. Intriguingly, both methods of counting show that the levels of induced aggregation is comparable between pathological SOD1 and TDP-43, which is consistent with our previous findings that both proteins can trigger comparable amounts of misfolded SOD1\textsuperscript{55,117}. Furthermore, we find that all mutant-based reporter proteins can be induced to aggregate, however the degree of induced aggregation seems to depend on the exact construct. While a reporter protein based on SOD1\textsuperscript{A4V}, a mutation with a high propensity to aggregate, is found to aggregate in 10-15\% of cells co-expressing pathological SOD1 or TDP-43, this number nearly doubles (15-30\%) for the other reporter protein (G37R, G85R and G93A-based). We also found differences in the average sizes of the induced aggregates, with SOD1\textsuperscript{G37R} forming smaller inclusions, consistent with previous finding showing that despite its propensity to unfold, SOD1\textsuperscript{G37R} does not aggregate to the same extent as SOD1\textsuperscript{G93A} mutation\textsuperscript{364}. These data suggest that individual SOD1 mutations can lead to conformational differences between the various chimeric proteins, which may enable them to be induced to aggregate more easily, despite their respective propensities for self-aggregation. We also found that wtSOD1-based reporter protein appears to be stable and proves not to be an efficient substrate for seeded aggregation of SOD1 by either mutant SOD1 or pathological SOD1 within the timeframe of the transfection (48 h). Although this finding may be slightly concerning as the endogenous non-tagged wild-type protein can be efficiently misfolded by either mutant SOD1 or pathological TDP-43\textsuperscript{55,117}, we hypothesize that the GFP moiety might partially stabilize the chimeric protein or mask the epitope required for conversion.

Despite the ability of mutant SOD1 and TDP-43 to trigger comparable levels of induced endogenous misfolded SOD1 and reporter protein aggregates, our immunoprecipitation studies using SOD1-misfolding specific antibodies show that while over 95\% of SOD1-induced misfolded SOD1 is protease sensitive, approximately 15-20\% of TDP-43 induced misfolded is resistance to a similar treatment. This implies that multiple structure of misfolded SOD1 may exist: the structurally loosened molecules that are protease-accessible, and the more stable molecules (or potentially as part of a structure) and less protease-accessible. Together with abovementioned findings that SOD1\textsuperscript{G37R}-GFP aggregates are different in size, these data further
suggest that not all misfolded SOD1 molecules share the same conformation. Our detection of several strains of misfolded SOD1 is supported by recent findings in-vivo that demonstrated the two different strains of SOD1 particles can transmit ALS-like disease in mice with strain-specific phenotypes differing in disease progression rates, end-stage aggregate levels and histopathology.350,365

Based on the recently confirmed prion-like properties of SOD1 in vitro and in vivo, we determined whether human spinal cord homogenates from SOD1-FALS or SALS could induce aggregation of SOD1G37R-GFP, SOD1G85R-GFP and SOD1G93A-GFP in living cells. We found that all three reporter proteins can be significantly aggregated by SOD1-FALS homogenates, dependent on the specific SOD1 mutation in the homogenate. Interestingly, little difference was observed in the induced aggregation of all three reporter proteins, suggesting that G85R is not the only permissive substrate for conversion. Consistent with recent findings that SOD1-A4V homogenate can trigger aggregation of SOD1G85R-YFP in organotypic spinal cord slice tissue,358 we find that SOD1-A4V can also trigger the aggregation of our reporter proteins in HEK293 cell culture. Similar to the SOD1-A4V, a homogenate from SOD1-G93S spinal cords triggered aggregation of all of the reporter proteins, consistent with the high propensity of the non-dimer interface mutant G93S to aggregate in patients.366 Curiously, the SOD1-D90A homogenate appears to be the most potent inducer of reporter protein aggregation, despite the relatively slow disease progression (disease duration of 17 years), and the apparent small load, of aggregates in this patient. A recent study examined the effect of overexpressing the human SOD1-D90A mutation in mice, and found that SOD1-D90A can co-exist in two strains, with one strain forming more fragile and fragmentation-prone aggregates, as well as greater pathogenicity causing an earlier disease onset and faster progression.365 It is possible that the human SOD1-D90A spinal cord homogenate used in this study was enriched in the aggressive strain, which would explain its rapid nucleation of the reporter proteins. In our assay, spinal cord homogenates from SOD1-I113T can also induce aggregation of the reporter protein; however the efficiency depends on the reporter protein employed. The third most common mutation in SOD1, I113T, is known to have an incomplete penetrance and is disruptive to SOD1 homodimerization on the molecular level.367,368 Our observation that SOD1G37R-GFP and SOD1G93A-GFP, but not SOD1G85R-GFP, are triggered to aggregate in the presence of this homogenate is consistent with
the conformational selection mechanism previously proposed for prions. In this model, conversion and aggregation would be most efficient when the conformational ensemble of the input mutation (in this case SOD1-I113T) overlaps with the conformational ensemble of the reporter proteins (G37R and G93A). If this notion applies to SOD1 aggregation, it is intriguing to speculate that the input ensemble of SOD1-I113T possesses conformational states which are dissimilar to G85R, which is natively a misfolded monomer. Interestingly, we find that clinical duration of disease or the loads of misfolded SOD1 in the SOD1-FALS tissues do not directly correlate with the amount of induced reporter protein aggregation in cells exposed to the corresponding tissue homogenates. We propose several reasons for this lack of apparent correlation. First, regardless of its specific tissue source, mutant SOD1 seed in the homogenate may be sufficient to trigger seeding of the chimeric reporter protein, which then follows a patient-independent aggregation. Secondly, normally stable SOD1 aggregates in SOD1-FALS may break into the more volatile SOD1 oligomers during the extraction protocol. Lastly, mutant SOD1 in patients may form different aggregate strains with varying seeding properties, some of which are more favourably extracted during our extraction protocol.

Our results confirm the previous finding that spinal cord homogenates from SALS patients cannot efficiently induce aggregation of the reporter protein in living cells despite the controversial presence of misfolded wild-type SOD1 in these tissues reported by some groups, but not others. The only reporter protein that exhibited a minimal visual apparent response to SALS homogenates was SOD1-G37R-GFP, a destabilized mutation that has been previously suggested to aggregate as a different strain. This notion is further supported by the discovery that the epitope surrounding glycine at position 37 (inclusive) is a fibril forming segment, and that G37R substitution disables fibril formation. There are potentially two distinct types of SOD1 misfolding: 1) aggregation-prone mutant SOD1 that is present in FALS-SOD1 patients; and 2) misfolded wild-type SOD1 found in sporadic ALS without obvious ubiquitinated SOD1 inclusions. Previous studies showed that mutant SOD1 inclusions prepared in vitro can seed insoluble aggregation of cytosolic soluble mutant SOD1, consistent with our current findings that mutant SOD1 seeds from FALS-SOD1 homogenates lead to aggregation of the reporter proteins. Wild-type misfolded SOD1 is present in cells expressing mutant SOD1 or TDP-43 as a non-aggregated soluble molecular species, and acquires the property of propagation...
between cells in a template directed fashion \textsuperscript{253,261}. We do not find this type to be an effective seed for aggregation in our system.

The next aspect of this work was to hone down on the region responsible for this intermolecular induction of SOD1 aggregation. Our previous work showed that tryptophan residue at position 32 is crucial for the induced misfolding of SOD1\textsuperscript{55,117,261}. We show here that SOD1\textsuperscript{G85R} can indeed trigger the aggregation of SOD1\textsuperscript{G85R}-GFP but not SOD1\textsuperscript{G85R/W32S}-GFP reporter protein. Furthermore, we show that SOD1\textsuperscript{G85R} induces faster aggregation of the reporter proteins than SOD1\textsuperscript{G85R/W32S}, all of which are consistent with Trp32 playing an important role not only in induced misfolding of SOD1, but also in its induced aggregation\textsuperscript{117,359}. An important notion is that both SOD1\textsuperscript{G85R} and SOD1\textsuperscript{G85R/W32S} express well in cells and can be detected using a SOD1 misfolding-specific antibody implying that although the misfolded SOD1 molecule are present in both cases, only G85R mutant is in the right conformation to act as an effective seed. This further advances our understanding of misfolded SOD1 species, as it demonstrates that not all species of misfolded SOD1 acquire the infectious property required for effective seeding. This results could also explain the absence of induced aggregation by SALS tissue (despite the likely presence of misfolded SOD1); although this could be due to lower levels of misfolded SOD1 in SALS tissue compared to FALS, akin to SOD1\textsuperscript{G85R/W32S} it is possible that the misfolded SOD1 in SALS is of a different strain that can be detected by SOD1 misfolding-specific antibodies but has a different transmission profile. Despite the apparent need for tryptophan at position 32 to be present for induced aggregation and misfolding of human SOD1 to occur, SOD1 in other species can aggregate without tryptophans; for example, despite dogs and mice lacking Trp32, dogs can develop canine degenerative myelopathy with SOD1 mutations\textsuperscript{371}, while mice carrying SOD1\textsuperscript{G86R} develop disease that features SOD1 inclusions\textsuperscript{372}. Although the mechanism for SOD1 aggregation in these species is not known, one could speculate it to involve homophilic attraction and self-seeding of mutant SOD1 since the mutant protein is constitutionally expressed. It is important to note, however, that despite mice expressing either human SOD1\textsuperscript{G85R} or mouse SOD1\textsuperscript{G86R} developing ALS-like disease featuring SOD1 aggregates, additional overexpression of human wtSOD1 exacerbates disease only in the humanized mice \textsuperscript{268,273}. 
Finally, the last portion of this Chapter was dedicated to potential therapeutics aiming to block the intermolecular induction of SOD1-GFP aggregation by mutant SOD1. As we continue to establish the importance of tryptophan residue at position 32, we hypothesized that the pocket containing this residue could be used as a therapeutic target targeting the mechanisms of cross seeding of SOD1 molecules. A recent study has shown through crystal-structure analysis that 5-FUr, a chemotherapy agent, binds at Trp32. Here, we demonstrated that intermolecular seeding of SOD1 aggregation can be blocked by 5-FUr in a dose dependant manner. In fact, as little as 0.5 µM of 5-FUr, a clinically-relevant concentration, lowers the induced aggregation by over 60% to levels comparable to empty vector control. Additionally, we tested the effect of uridine, a non-fluorinated precursor of 5-FUr, for its ability to block the intermolecular seeding and found that although it does have a certain degree of dose-response effect, it requires 100’s of µM for the effect to be significant. Exactly how the interaction between Trp32 and 5-FUr inhibits the intermolecular seeding is not known. Original studies using 5-FUr suggested that its mechanism of action is the stabilization of SOD1 dimer which prevents its monomerization and inhibits aggregation\textsuperscript{373,374}, however single crystal X-ray diffraction demonstrated that 5-FUr does not in fact bind at the dimer interface of SOD1\textsuperscript{360}. Instead, 5-FUr binds directly at Trp32 pocket, with the fluorouracil group engaging in aromatic stacking with Trp32 \textsuperscript{360}, and forms non-covalent bonds between the molecule and the 3D landscape surrounding of Trp32. Given the important role that Trp32 plays in the intermolecular induction of SOD1 misfolding and aggregation, we hypothesize that inhibition of Trp32 by 5-FUr in either seed or substrate SOD1 can physically block their interaction as well as the spread of SOD1 pathology. Alternatively, the intermediate filament protein vimentin is known to bind uridine phosphorylase \textsuperscript{375}. Since uridine phosphorylase can bind intracellular 5-FUr \textsuperscript{376}, it is possible that this binding could lead to cytoskeletal re-organization potentially affecting extracellular release or uptake of misfolded SOD1.

Despite the usability of our novel fluorescent-based assay for studying induced SOD1 aggregation for advancing the basic biology of SOD1 pathogenesis, we sought to adapt this assay to a high-throughput format, where it can be used to screen libraries of small molecules aiming to disrupt the process of intermolecular induction of SOD1 aggregation. For this, we opted to use 96 well plates that allow us to screen up to 60 compounds per plate, in conjunction with an
inverted fluorescence microscope fitted with a motorized stage. Image analysis was later performed using our aggregate-counting algorithm. Our initial blinded screen validated the usability of this assay in a high-throughput fashion, where we identified several small molecules that reduce induced aggregation of SOD1 at a concentration of 10 µM. It was later disclosed that one of the hits was in fact 5-fluorouracil (5-FU), a compound that is structurally similar to 5-FU, but without the ribose ring. This result is not entirely surprising as fluorouracil, the Trp32-binding moiety of 5-FU, is also present in 5-FU. The identified molecule, 5-FU, is an FDA approved drug used for treating cancer, where it aids in the disruption of cell proliferation of rapidly dividing cancerous cells through inhibition of RNA and DNA synthesis. Additionally, 5-FU is known be an effective penetrant of the blood-brain-barrier, which could makes its application relatively non-invasive and technically simple. Owing to its disturbance of RNA/DNA metabolism, and its similarity to 5-FU, even at low concentration 5-FU represents a toxic insult to the cells. The mechanism of action of 5-FU has also been ascribed to the inhibition of thymidylate synthase, which normally catalyzes the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate. Over the years, uridine has been shown to mitigate toxicities associated with these mechanisms while not affecting its activity. However, more recently, uridine triacetate has been approved by the FDA as a drug used to counteract the severe toxicity of 5-FU in patients undergoing chemotherapy. In our case, neither the DNA/RNA misincorporation nor the inhibition of thymidylate synthase is necessary, as 5-FU is designed to inhibit SOD1 conversion through aromatic π-stacking with Trp32. It is important to note that both 5-FU and 5-FUc are effective at blocking the mechanism causing induced aggregation of SOD1 without significantly affecting the levels of the reporter protein; even though these drugs may be slightly toxic to the rapidly dividing HEK293 cells, the fact that they are FDA approved reassures us that they can be safely used in humans. As a workaround of providing the decrease in seeding of SOD1 while partially protecting from cytotoxicity, a combination treatment using 5-FU/5-FUc together with either uridine or uridine triacetate could be used.

In this Chapter, we developed a series of SOD1-based chimeric reporter proteins that we validated to aggregate in living cells in the presence of mutant SOD1 originating from protein overexpression studies. Using this assay, we established that spinal cord extracts prepared from
SOD1-FALS, but not SALS, can induce aggregation of the reporter proteins in living cells, suggesting template directed misfolding akin to the prion protein. Next, we confirmed that induced aggregation of SOD1 depends on Trp32 and concluded the Chapter by showing that drugs targeting the Trp32-containing pocket in SOD1 can block induced aggregation and spread of SOD1 pathology. This work not only introduces new and effective tools for studying induced SOD1 aggregation, but it further confirms the Trp32-dependant prion-like seeding ability of SOD1, which can be blocked using small molecules. We also hope that the high-throughput assay can be used to identify additional small molecule capable of blocking the spread of SOD1 pathology, or dissolve pre-existing SOD1 inclusion.
Chapter 4: Induced aggregation of SOD1 by TDP-43 is mediated by tryptophan residues

4.1 Summary

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease characterized by progressive degeneration and loss of motor neurons, with no effective therapy. Misfolded Cu/Zn superoxide dismutase (SOD1) has been detected in all ALS patients, despite SOD1 mutations accounting for only 2% of total cases, while the presence of inclusions containing pathological TDP-43 represent a hallmark of all non-SOD1 familial ALS. We previously reported that pathological TAR-DNA binding protein-43 (TDP-43) can trigger the propagated misfolding of human wild-type SOD1 (HuWtSOD1) in living cells. However, the mechanisms how TDP-43 kindles the misfolding of SOD1, and how it aggregates, are unknown. Here we show that the intermolecular conversion of HuWtSOD1 by TDP-43, and its self-aggregation, are mediated through tryptophans. We found that cytoplasmic mutant TDP-43 (TDP-43ΔNLS), but not its tryptophan-less version (Trpless-TDP-43ΔNLS), induces misfolding and aggregation of SOD1 in living cells. Furthermore, we narrowed down the culprit tryptophans for the intermolecular seeding of SOD1 to be tryptophans at position 68 and 113. We also found that aggregation of pathological TDP-43 itself is dependent on the presence of tryptophans. Finally, we blocked TDP-43 induced misfolding and aggregation of SOD1 using 5-fluorouridine, a small molecule binding tryptophan-32 in SOD1. Our results demonstrate for the first time the mechanism how two major proteins implicated in ALS, TDP-43 and SOD1, may interact under pathological conditions. This work points to a treatable mechanism for sporadic and familial ALS, and highlights tryptophans in both proteins as therapeutic targets in blocking propagated misfolding of SOD1, and cytoplasmic aggregation of TDP-43. Furthermore, it is key to recognize that elucidation of the pathogenic role of a simple structural motif in ALS may provide a framework for understanding other neurodegenerative diseases in which propagated protein misfolding is shown to occur (e.g., Alzheimer's and Parkinson's diseases).

4.2 Introduction

ALS is characterized by progressive paralysis of the muscles of the limbs, speech, swallowing and respiration leading to eventual death often within 2-5 years. The familial form of the disease can be caused by hereditary mutation to one of several proteins, including SOD1 and TDP-
43,356,381. However the pathological forms of the wild-type versions of these proteins have also been identified in the sporadic cases: ubiquitinated TDP-43 inclusions have become a hallmark of all non-SOD1/FUS ALS, while evidence suggests that misfolded wild-type SOD1 is present in all ALS cases. The apparent propagatory nature of familial and sporadic ALS throughout the neuroaxis point the way to certain infectious particles that may spread the ALS pathology from its initial focus/foci sites. Recent studies in cells and in-vivo demonstrated that SOD1 is a prion-like protein: (1) aggregates composed of ALS-causing mutant SOD1 can penetrate inside cells and nucleate aggregation of soluble wild-type SOD1 protein; (2) misfolded endogenous HuWtSOD1 induced by mutant SOD1 can propagate intercellularly; and (3) spinal cord injection of homogenates prepared from paralyzed SOD1G93A mice can induce widespread spinal inclusion pathology. Furthermore, our own research has shown that pathological TDP-43 can trigger cytotoxic propagated misfolding of HuWtSOD1 in cultured cells, suggesting a potential mechanism in sporadic ALS.

The co-presence of pathological TDP-43 along with misfolded SOD1 in sporadic ALS led us to discover that pathological TDP-43 can in fact trigger the misfolding of endogenous wild-type SOD1 in transfected cells lines, or in spinal cord primary cells from HuWtSOD1 transgenic mice. However the mechanism for this remained unknown. Our previous findings that tryptophan residue (Trp) at position 32 is crucial for the conversion and propagated misfolding of SOD1, led us to investigate the possibility that Trps in TDP-43 play a role in the intermolecular conversion of SOD1, as well as its self-aggregation. For a remarkably underrepresented amino acid, it is striking to find 6 such residues in native human TDP-43 spanning many of its domains (1 in N-terminal domain, 2 in RRM1, 3 in Gly-rich domain). Here, we hypothesized that the intermolecular conversion of HuWtSOD1 by pathological TDP-43 is mediated by homophilic tryptophan interactions in the respective proteins.

In order to evaluate the effect of tryptophan residues on the induced conversion of SOD1, we performed PCR-mediated mutagenesis to systematically replace single or multiple tryptophan residues in TDP-43ΔNLS (predominantly forming cytoplasmic inclusions) into serine residues. Here, we show that overexpression of Trp-containing, but not Trp-less, version of TDP-43ΔNLS in living cells triggers its self-aggregation as well as induction of fluorescently-tagged SOD1G85R-. 

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AcGFP reporter protein that acts as an inducible substrate for SOD1 aggregation. We also narrowed down the tryptophans that are responsible for cross-seeding of SOD1 to Trp68 and Trp113, neither of which can trigger the effect alone. Finally, we showed that small molecules, akin to 5-fluorouridine, can block the induced aggregation of SOD1, without affecting the self-aggregation of TDP-43, likely through binding to the substrate molecules.

4.3 Methods and materials
Please refer to Chapters 2.3 and 3.3.

4.4 Results
4.4.1 Tryptophan residues play a role in self-aggregation of TDP-43
In order to explore the potential effect of the tryptophan residues on the self-aggregation of TDP-43, we used PCR mutagenesis to swap all individual tryptophan residues to serines and generated a tryptophan-less (Trpless) construct by replacing all of the tryptophan residues with serines (Figure 26 & Table 3). All of our TDP-43 constructs are N-terminally tagged with hemagglutinin (HA)-tag\textsuperscript{55}. Our immunocytochemistry studies using HA (exogenous TDP-43) and 409/410 phospho-TDP-43 (common hyperphosphorylation features in familial and sporadic ALS\textsuperscript{382}) specific antibodies revealed that although transfection of both TDP-43\textsuperscript{ANLS} and Trpless-TDP-43\textsuperscript{ANLS} leads to protein expression, however, the profile seems to differ. While TDP-43\textsuperscript{ANLS} forms inclusion bodies, many of which are also phosphorylated, Trpless- TDP-43\textsuperscript{ANLS} does not form inclusions, nor do we observe more phosphorylation than in cells transfected with empty vector control (Figure 27). Next, we found that single tryptophan to serine mutation do not result in a decrease in TDP-43 inclusions.
Figure 26: List of TDP-43ΔNLS mutations.

Schematic representation of select tryptophan to serine mutations tested in this Chapter. TDP-43 contains a grand total of 6 tryptophan residues: 1 in the N-terminus, 2 in the RRM1 domain, and 3 in the Gly-rich domain. Here, we used PCR mutagenesis to generate single tryptophan to serine substitutions (Table 3), as well as a series of combination mutations, including: all 6 Trp converted to serine (Trpless), all Trps in N-terminus (Nterm), all Trps in Gly-rich domain (GLY), both Trps in RRM1 domain (RRM1), and Trps at residues 68 and 113 (W68/113S). All of the above mutations were performed on the TDP-43ΔNLS background.
<table>
<thead>
<tr>
<th>Tryptophan residue number(s)</th>
<th>Name</th>
</tr>
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<tbody>
<tr>
<td>68</td>
<td>W68S-TDP-43ΔNLS</td>
</tr>
<tr>
<td>113</td>
<td>W113S-TDP-43ΔNLS</td>
</tr>
<tr>
<td>172</td>
<td>W172S-TDP-43ΔNLS</td>
</tr>
<tr>
<td>334</td>
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<tr>
<td>385</td>
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<td>68 &amp; 113 &amp; 172 &amp; 334 &amp; 385 &amp; 412</td>
<td>Trpless-TDP-43ΔNLS</td>
</tr>
</tbody>
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**Table 3: List of TDP-43 mutations in tryptophan residues.**

A comprehensive list of single and combination constructs that we generated, where tryptophan residues were replaced with serine residues.
Figure 27: Tryptophan residues in TDP-43\textsuperscript{ANLS} play a role in its aggregation and hyper-phosphorylation.
HEK293 cells were transfected for 48 h with either empty vector control, TDP-43\textsuperscript{ΔNLS}, Trpless-TDP-43\textsuperscript{ΔNLS}, or W68/113S-TDP-43\textsuperscript{ΔNLS}. Following fixation, the cells were co-stained with phosphorylated TDP-43 at sites 409/410 (green) and HA (green) antibodies. Scale bar: 50 µm (N=3).
4.4.2 Intermolecular homophilic interaction between tryptophan residues triggers SOD1 aggregation

Our next goal was to investigate the effect that tryptophans in TDP-43\textsuperscript{ANLS} have on its cross-seeding of SOD1. We first show that TDP-43\textsuperscript{ANLS}, but not its Trpless variant, triggers misfolding of the endogenous HuWtSOD1 in HEK293 cells (Figure 28A) as can be concluded by the staining using SOD1 misfolding-specific antibody, 3H1 (green). Next, we co-transfected HEK293 cells with our SOD1\textsuperscript{G8SR}-GFP reporter protein with either trp-containing or Trpless TDP-43\textsuperscript{ANLS}, and found using fluorescence microscopy that the presence of tryptophan in TDP-43 is essential for triggering SOD1 seeding (Figure 28B). We also confirmed these results using flow cytometry, where we found a significant increase in the mean GFP fluorescence (4-fold) and number of cells with inclusions (3-fold) in cells co-transfected with reporter protein and TDP-43\textsuperscript{ANLS} versus its Trpless version or empty vector control (Figure 28C-D). We also demonstrated that the induced reporter protein aggregates can be precipitated into P1 following a 1 h spin at 100,000 x g (Figure 28E). Furthermore, we used time-course microscopy to visualize the induced aggregation of the reporter protein in living cells transfected with reporter protein and either empty vector control, TDP-43\textsuperscript{ANLS} or Trpless- TDP-43\textsuperscript{ANLS} (Figure 28F). We performed a linear regression of the aggregate rapid-growth phase (approximately 8-30 h after data acquisition or 24-48 h post transfection) and found that while TDP-43\textsuperscript{ANLS} triggers the reporter protein to aggregate at a rate of 0.11 area inclusions per hour, the rate is reduced to 0.07 and 0.05 for Trpless- TDP-43\textsuperscript{ANLS} and empty vector control, respectively. These data show that the rate of induced aggregation reaches a plateau at 48 h post transfection, consistent with our previous findings showing that once generation of misfolded SOD1 is initiated, it plateaus at ~20% of cellular SOD1\textsuperscript{117}.

After establishing that Trpless- TDP-43\textsuperscript{ANLS} cannot induce SOD1 aggregation, we explored which tryptophan residues contribute the most to the intermolecular seeding of SOD1. Using manual counts (Figure 29A), algorithm counts (Figure 29B), and flow cytometry analysis (Figure 29C-D), we show while TDP-43\textsuperscript{ANLS} is the most effective at triggering SOD1 aggregation, and its Trpless version is the poorest at this, other constructs fall in between in terms of seeding ability. We find the W68/113S- TDP-43\textsuperscript{ANLS} mutation to be of particular interest as it seems to be as poor of an inducer of SOD1 aggregation as Trpless- TDP-43\textsuperscript{ANLS}.
In order to study the intermolecular homophilic interaction between the tryptophan residues in TDP-43 and Trp32 in SOD1, we studied if TDP-43ΔNLS or Trpless-TDP-43ΔNLS can trigger the aggregation of SOD1$^{G85R}$-GFP or SOD1$^{G85R/W32S}$-GFP. We find that TDP-43 can induce the aggregation of SOD1, but only in the presence of tryptophan residues in both protein (Figure 30A). Conversely, we found that neither Trp-containing nor Trpless- TDP-43ΔNLS can induce aggregation of SOD1$^{G85R}$-GFP (Figure 30B).
Figure 28: TDP-43\textsuperscript{ΔNLS}, but not Trpless-TDP-43\textsuperscript{ΔNLS}, can trigger aggregation of SOD\textsuperscript{G85R}GFP.

A) Immunocytochemistry staining reveals that transfection of HEK293 cells with TDP-43\textsuperscript{ΔNLS} (red) stains stronger for misfolded SOD1 (green) than transfection with Trpless-TDP-43\textsuperscript{ΔNLS}. B) Induced aggregation of reporter protein can be observed 48 h after its co-transfection with TDP-43\textsuperscript{ΔNLS} but not Trpless- TDP-43\textsuperscript{ΔNLS}, suggesting the importance of tryptophan residues in the conversion process. C) Representative histograms of flow cytometry on cell that were co-transfected for 48 h with reporter protein and TDP-43 inducer, and briefly permeabilized using 0.03%. Histograms show GFP fluorescence. D) Analysis of the flow cytometry experiments showing the shift in the overall mean GFP fluorescence, and percentage of cells with inclusions, in cells co-transfected with reporter protein and one of the indicated constructs (N=11). E) Representative immunoblotting of precipitation of TDP-43\textsuperscript{ΔNLS}-induced reporter protein aggregates following a 1 h spin at 100,000 x g. Probed with GFP antibody. F) A time-course algorithm count of aggregates in living cells from 24 to 48 h post co-transfection with reporter protein and indicated construct. Images were acquired every 20 minutes. . Time-point h=0
corresponds to when we started imaging, which occurred approximately 20-24 h post transfection
Figure 29: The effect of tryptophan substitutions in TDP-43^ANLS on SOD1 aggregation.

HEK293 cells were co-transfected with various TDP-43ANLS constructs and SOD1^G85R-GFP reporter protein for 48 h prior to cell analysis. Here, we used manual counts (A) and algorithm counts (B) to assess the percentage of cells with inclusions out of total transfected cells (N=6), and the percentage of area occupied by aggregated GFP out of total GFP (N=8), respectively. We also used flow cytometry to assess the percentage of cells with inclusions by counting their frequency (C), and the shift in the mean GFP fluorescence (D) (N=8).
Figure 30: Self aggregation and induced aggregation of SOD1 by TDP-43ΔNLS is mediated through tryptophan residues.

HEK293 cells were co-transfected for 48 h with TDP-43ΔNLS or Trpless-TDP-43ΔNLS together with SOD1G85R-GFP (A) or SOD1G85R/W32S-GFP (B). Reporter protein is observed in the green channel, while red corresponds to staining using HA antibody targeting exogenous TDP-43. Scale bar: 50 µm (N=6).
4.4.3  5-fluorouridine can block induced aggregation of SOD1 by TDP-43, but not TDP-43’s self-aggregation

We demonstrated in Chapter 3 that 5-fluorouridine (FUr) can prevent induction of reporter protein aggregation when induced by mutant SOD1. Here, we expanded on this original finding by using fluorescence microscopy to demonstrate that 1 and 5 µM 5-FUr can dramatically reduce induced aggregation of SOD1 by TDP-43, without affecting the self-aggregation of TDP-43 (Figure 31). Furthermore, we used flow cytometry to show that as little as 0.5 µM 5-FUr can significantly block induction of SOD1-based reporter protein aggregation by TDP-43$^{\Delta NLS}$, a response that is dose dependent (Figure 32A-B; **** p<0001). We also used live cell microscopy to show that induced aggregation of reporter protein by TDP-43$^{\Delta NLS}$ is reduced in the presence of 5-FUr, an effect that is magnified by increasing the concentration of 5-FUr from 1 to 5 µM (Figure 32C). However, even in the presence of 5 µM 5-FUr, we still observe more induction of SOD1 aggregation than in empty vector control. Furthermore, we also find that 5-fluorouracil (FU) can lower the levels of TDP-43 induced SOD1 aggregation, however with lower efficiency; while 5 µM has a lower effect than 5 µM of 5-FUr, 1 µM 5-FU did not seem to lower induced aggregation (Figure 32D).

Next, we used our high-throughput assay to test the effect of 5 µM of 5-FU or 5-FU on the various TDP-43$^{\Delta NLS}$ constructs with tryptophan to serine substitutions (Figure 33). While we find that both small molecules greatly reduce induced aggregation of SOD1, 5-FUr is consistently more potent than 5-FU at 5 µM final concentration. It should be noted that the peaks corresponding to Trpless- and W68/113S- TDP-43$^{\Delta NLS}$ are nearly identical.
Figure 31: 5-FUr can block the cross seeding of SOD1, without disturbing self-aggregation of TDP-43.

HEK293 cells were co-transfected with TDP-43ΔNLs and SOD1G85R-GFP prior to adding of 1 or 5 µM 5-FUr. Following 48 h incubation, induced aggregates were visualized using light microscopy in the presence or absence of 5-FUr (top). Drug vehicle was added to control cells at the volume equivalent to that of 5 µM 5-FUr. The cells were also fixed, and stained using an HA-tag specific antibody to detect TDP-43ΔNLs in transfected and treated cells (bottom). Scale bars: 100 µm (top), 20 µm (bottom) (N=3).
Figure 32: Induced aggregation of SOD1 by TDP-43^ANLS can be blocked by 5-fluorouridine and 5-fluorouracil.

5-FUr or 5-FU was added to HEK293 cells at the indicated final concentration 4-6 h following a co-transfected with TDP-43^ANLS and SOD1^G85R-GFP. A) Representative histogram showing the total GFP fluorescence of cells that were transfected for 48 h and briefly permeabilized using...
0.03% saponin immediately prior to flow cytometry analysis. B) Analysis of the flow cytometry experiments showing the shift in the overall mean GFP fluorescence in cells co-transfected with reporter protein mutant TDP-43 and treated with 5-FUr (N=4-13). Trpless column refers to co-transfection of reporter protein with Trpless- TDP-43ANLS provided for comparison purposes. Live cell microscopy was performed in cells transfected with reporter protein and TDP-43ANLS, and subjected to the indicated final concentration of 5-FUr (C) or 5-FU (D) 4 h after transfection. Data acquisition began 16 h post transfection (h=0 in plots), and images were taken every 30 min for 24 h. Each experiment was repeated 3 times. ****: p<0.0001.
Figure 33: Both 5-fluorouridine and 5-fluorouracil are effective at blocking induced aggregation of SOD1 by an array of W→S TDP-43ANLS mutations.

HEK293 cells were pre-plated in a 96 well plate, co-transfected with SODG85R-SOD1 and the indicated version of TDP-43ANLS (color-coded), and incubated with 5 µM of 5-FUr or 5-FUc 4 h later. 48 h post-transfection, the cells were imaged as described in Chapter 3.4.6 and analyzed using our algorithm. Y-axis represents the area occupied by aggregated GFP as a percentage of total GFP (N=4).
4.5 Discussion

While build-up of inclusion composed of TDP-43 is a hallmark of sporadic ALS, several studies have identified the presence of misfolded SOD1 in sporadic ALS using SOD1 misfolding-specific antibodies. SOD1 has also been shown to misfold in the presence of pathological TDP-43 in cell cultures; however, the mechanism is unclear. Here, we used immunofluorescence staining, live cell microscopy and flow cytometry to demonstrate that self-aggregation of TDP-43, and the intermolecular interaction between TDP-43 and SOD1 is mediated by tryptophan residues in both proteins. Previously, tryptophan residues have been found to play a fundamental role in promoting aggregation of myoglobin peptides through its inherent tendency to form interactions, and TDP-43 oligomers were observed to cross-seed Alzheimer’s amyloid-β, setting the precedence for the tryptophan-mediated TDP-43 cross seeding of SOD1 we observe here. The importance of tryptophan residues, with their large hydrophobic surface, to the aggregation should come as no surprise as aromatic groups have been shown to form π-stacking interactions in amyloid fibrils.

We determined that tryptophan residues at position 68 and 113 are important for cross-seeding of SOD1. The importance of these residues is of particular curiosity on the scientific level and may lead to the identification of new therapeutic molecules targeting the cross-seeding process. Given that aggregated TDP-43 can trigger misfolding and aggregation of SOD1, but only in the presence of its tryptophan residues, and that aberrant assembly of RRM1 is linked to the pathogenic conversion of TDP-43, we tested whether tryptophan residues in the RRM1 domain are linked to SOD1 conversion. The RRM1 domain spanning from residue 106 to 176 contains tryptophan residues at positions 113 and 172, both of which are surface exposed according to the protein data bank. The requirement for the presence of both Trp68 and Trp113 in pathological TDP-43 to induce aggregation of SOD1 is only partially explained by the Trp113 residue in RRM1. Our results demonstrating the involvement of N-terminal tryptophan residues (especially Trp68) is also consistent with the involvement of amino acids 1-105 in TDP-43’s oligomerization in-vitro, and the formation of an intermolecular interaction domain spanning amino acids 3-183. Both tryptophan residues (68 & 113) are found in the region spanning the N-terminus of TDP-43 and are part of a domain that facilitates intermolecular interaction. Furthermore, the N-terminus of TDP-43 has been recently shown to encode a well-structured
novel ubiquitin-like fold found in equilibrium with its unfolded state\textsuperscript{389}; binding of this domain to single stranded DNA shifts the equilibrium towards increasing the folded population. Cytoplasmic accumulation, akin to those formed by TDP-43\textsuperscript{ANLS}, are not likely to be associated with nucleic acids due to their nuclear exclusion, which may lead to unfolding of the N-terminus and exposure of the tryptophan residues. The cross seeding of SOD1 could then occur through π-stacking interactions between the N-terminal tryptophan residues in TDP-43 and the single tryptophan residue in SOD1 that drives its misfolding and aggregation.

Interestingly, we also found that swapping of all 6 tryptophan residues in TDP-43 to serine results in a protein that cannot longer aggregate, and our inability to narrow down the effect to specific tryptophan suggest that all tryptophan work in synergy to trigger self-aggregation. Since establishing TDP-43 inclusion as a major histopathological feature in sporadic ALS, numerous studies attempted to identify the exact mechanism how the aggregates form. Original studies showed that C-terminal TDP-43 fragments have a high propensity to aggregate, and are therefore critical for the protein’s self-aggregation\textsuperscript{390,391,392}. This dogma has been challenged recently with studies showing that the C-terminus alone is not enough for efficient self-aggregation of TDP-43, and that the N-terminus and RRM1 may in fact be required for this process\textsuperscript{386,393,394}, all of which suggests that the various regions in TDP-43 are synergistically involved in TDP-43 self-aggregation. In light of this, it is not surprising that we find that only replacement of all tryptophan residues in TDP-43\textsuperscript{ANLS} ablates its self-aggregation.

Finally, we show here that the cross-seeding of SOD1 by TDP-43 may be blocked using small molecules such as 5-fluorouridine and 5-fluorouracil, which can also block the seeding of SOD1 by its mutant version. Given that these molecules engage in aromatic stacking with tryptophan 32 in SOD1 \textsuperscript{360}, we predict that they interact with the Trp32 residue in the substrate and block induction through direct interference. This is consistent with our finding that 5-FUr in fact does not block the self-aggregation of TDP-43, which also indicates that TDP-43 aggregation alone is not enough to trigger SOD1 seeding. The low concentration of the compound required not only indicates the high affinity of 5-FUr, and to a smaller degree 5-FU, to SOD1, but also demonstrates that the cross seeding processes may be blocked pharmacologically as part of a therapy. Our findings that tryptophan residues are also important in TDP-43 self-aggregation, as well as the identification of Trp68 and Trp113 as being the triggers of SOD1 aggregation, offers
new potential targets to explore in the prevention of disease. Furthermore, it is key to recognize that elucidation of the pathogenic role of a simple structural motif in ALS may provide a framework for understanding other neurodegenerative diseases in which propagated protein misfolding or cross-seeding is shown to occur (e.g., Alzheimer's and Parkinson's diseases).
Chapter 5: Conclusion and future direction

Amyotrophic lateral sclerosis is a devastating neurodegenerative disease leading to the degradation of motor neurons and eventual death. The disease appears to spread from its initial focus point(s) throughout the neuroaxis in a spatiotemporal fashion. While pathophysiological inclusions of TDP-43 is a hallmark of all ALS without SOD1 or FUS mutations, misfolded SOD1 has been observed in all cases of ALS. We previously observed that pathological TDP-43 and FUS can trigger misfolding of wild-type SOD1; however the mechanism and the consequences of this induction have not been explored.

Here, I established that once SOD1 is kindled to misfold by pathological TDP-43 or FUS, it changes its biochemical properties (e.g. becomes susceptible to low concentrations of proteinase K), and can spread from cell-to-cell where it interacts with endogenous SOD1 and converts it in a template directed misfolding fashion. Using primary spinal cord cultures from HuWtSOD1 transgenic mice, I demonstrated that the substrate for induced SOD1 aggregation is only human SOD1 (i.e. not mouse), likely attributed to its single tryptophan residue at position 32. To the best of our knowledge, this was also the first time that spread of misfolded HuWtSOD1 was demonstrated to be cytotoxic. I also found that the intercellular spread is restricted to SOD1 in our system, as I do not detect signs of pathological TDP-43 or FUS in cells incubated with conditioned media from TDP-43 or FUS transfected cells. I then used our novel SOD1-based inducible aggregation assay to demonstrate that the interaction between TDP-43 and SOD1 is mediated by tryptophan residues in both proteins, and specifically isolated Trp68 and Trp113 as the culprit residues in TDP-43 causing the misfolding of SOD1. Moreover, I find that the tryptophan residues in TDP-43 appear to be also important for the self-aggregation of TDP-43 as conversion of all tryptophan to serine ablates its cytoplasmic accumulation.

The work in this thesis was not limited to studying the induction of SOD1 misfolding by pathological TDP-43. Using several reporter proteins, I demonstrated that not all misfolded SOD1 molecules are alike and while some can be induced to aggregate more easily, others may form inclusions of different sizes, all of which is also dependent on the inducer used (SOD1 vs. TDP-43). The idea of different misfolded SOD1 strains was also confirmed using proteinase K treatments that demonstrated a subpopulation of TDP-43 induced misfolded SOD1 to be resistant
to the treatment. Furthermore, I demonstrated using the reporter protein assay that fluorescently-tagged SOD1 can be triggered to aggregate intracellularly using spinal cord extracts prepared from SOD1-FALS, but not SALS, further suggesting subspecies of misfolded SOD1.

Finally, and perhaps most importantly, the work presented here explored several routes targeting the intermolecular template directed misfolding of SOD1, as well as its cell-to-cell transmission, using several methods including a novel fluorescence-based assay. I first demonstrated that immunodepletion of misfolded SOD1 using SOD1 misfolding-specific antibodies from conditioned media collected from cells transfected with TDP-43 or FUS dramatically decreases the spread of SOD1 pathology, which indicates that the misfolded SOD1 molecules are accessible in the extracellular environment and as such can be targeted using specific antibodies or small molecules. Not surprisingly, I also find that knockdown of SOD1 prior to incubation with misfolded SOD1-containing media reduced the load of induced misfolding, further confirming SOD1 to be the substrate for misfolding. In fact, reduction of SOD1 is tolerated well in mice and humans, opening the door to its plausible use as a therapeutic. I also find that pharmacological intervention targeting the pocket containing the lone tryptophan residue in SOD1 (e.g. using 5-fluorouridine) can be an effective method for blocking its cross-seeding. We believe that our novel fluorescence-based assay should be used to explore in greater-detail the effectiveness of other small molecules that bind at the SOD1 tryptophan-containing pocket (e.g. L-methionine, aniline, and quinazoline), and combination treatments that include 5-FUr or 5-FU along with uridine derivatives. Furthermore, I believe that 5-FU should be tested as an inhibitor of SOD1-aggregation in animal models of ALS, starting with well established model such as SOD1 with pathological SOD1 inclusions. Given that 5-fluorouridine engages in aromatic stacking with tryptophan 32 in SOD1, we predict that these molecules bind to this pocket in the substrate protein and therefore inhibit the intermolecular conversion of SOD1. Our fluorescence-based assay was also optimized to be used as a tool for high-throughput screening of small molecules targeting the intermolecular aggregation of SOD1. We strongly believe that these methods for blocking induced misfolding and aggregation can represent a valid therapeutic for not only SOD1-FALS, but other familial and sporadic cases of the disease with SOD1 pathology.
The data presented in this thesis advances our current understanding of SOD1 biology, all of which led us to propose an updated model for SOD1 pathogenesis as illustrated in Figure 34. The cascade may begin with an irreversible insult to SOD1 itself, and could come in a form of mutations to the gene that encodes SOD1, oxidative stress, post translational modification, or decreased activity of the clearance machineries including the ubiquitin-proteasome or autophagy-lysosome pathways. However, misfolding of SOD1 could also be triggered by pathological TDP-43 or FUS. Regardless of the initial insult, once misfolded SOD1 seed is formed, it can interact with wild-type SOD1 and template its misfolding in a prion-like fashion. As the concentration of misfolded SOD1 molecules increases, they can assemble into oligomers or larger aggregates, a process that can be further facilitated by pathological TDP-43. For cell-to-cell propagation, monomeric misfolded SOD1 can be loaded into exosomes and secreted into the extracellular environment, where it is picked up by other cells and trigger additional rounds of SOD1 misfolding. Concurrently, misfolded SOD1 can be released into the extracellular environment in the form of oligomers or aggregates, potentially through association with aggresomes. Adjacent cells can then internalize the aggregates through processes such as macropinocytosis. Once internalized, the aggregates escape the vesicular lumen and trigger additional rounds of SOD1 misfolding/seeding. Therapeutics targeting the spread of SOD1 misfolding can target either the template directed misfolding process inside the cells (e.g. use of small molecules to block induced aggregation or misfolding of SOD1, or SOD1-siRNA to lower the concentration of the substrate) or in the extracellular environment (e.g. targeting misfolded SOD1-loaded exosomes and aggregates using SOD1 misfolding-specific antibodies).
Figure 34: Model describing the propagated misfolding of SOD1 in ALS, and potential treatments.

SOD1 misfolding can be induced directly by mutation in the SOD1 gene, or through excessive oxidation, post translational modifications or decreased clearance of misfolded protein (1). Once SOD1 seed is formed, it can act as a template for misfolding of neighboring SOD1 molecules (2), leading to the generation of oligomers and larger aggregates (3). In cases where SOD1 does not initiate disease, it can also be triggered to misfold by pathological TDP-43 (4) or FUS (5), both of which are nuclear ribonucleoproteins that tend to form cytoplasmic inclusions in SALS, and FUS-FALS, respectively. Once mislocalized, both TDP-43 and FUS can induced SOD1 misfolding (6). Interestingly, cytoplasmic inclusions of TDP-43 can facilitate oligomerization and aggregation of SOD1 (7). Regardless of the trigger, once oligomers and aggregates have
formed, cells can exchange these structures with neighboring cells through their release and uptake (8) that triggers additional rounds of SOD1 misfolding in the recipient cells. Release of monomeric misfolded SOD1 could also occur through exchange of exosomes carrying the misfolded SOD1 seed (9). I find that both intracellular and the intercellular spread of SOD1 misfolding can be blocked. Small molecules, akin to 5-FUr targeting the tryptophan residue in SOD1, can effectively block oligomerization and aggregation of SOD1. The cell-to-cell spread can blocked by either targeting SOD1 aggregates or exosomes carriers while there are in the extracellular space by SOD1 misfolding-specific antibodies, or by using SOD1-siRNA to reduce the concentration of available SOD1 substrate in the recipient cells.
I recognize that our major limitations of studying the interaction between TDP-43 and SOD1 are the use of overexpressed proteins and the use of HEK293 cells. To complement studies of TDP-43 mutant transfection studies unavoidably accompanied by overexpression of TDP-43, CRISPR/Cas9 can be used to generate mutant TDP-43 knock-in human cell lines in HEK293 cells. Based on our overexpression studies, these knock-ins should include TDP-43ANLS, Trpless-TDP-43ANLS and W68/113S-TDP-43ANLS to study the induction of HuWtSOD1 misfolding at endogenous levels. The knock-in lines should be evaluated for induction of SOD1 misfolding using immunoprecipitation and immunocytochemistry for misfolded HuWtSOD1 and mutant TDP-43 protein inclusions. To study cell-to-cell propagation of misfolded SOD1, media from knock-in lines can be collected and placed onto fresh HEK293 cells. Concurrently, the concern of using HEK293 cell line can be mitigated by generating human induced pluripotent stem (hiPS) cells that can be readily differentiated into motor neurons for studies in ALS. These motor neurons generate extensive axonal networks, and are functional as they possess the ability to fire action potentials. Although the initial test if mutations in TDP-43 can trigger propagated-misfolding of SOD1 in hiPS cells can take place in hiPS cells prepared from a naturally occurring TDP-43M337V cell, the ultimate experiment would be to mutate the tryptophan residues in TDP-43 using CRISPR/Cas9 in motor neuron cells derived from healthy individuals. These cells can then be analyzed for the presence of induced misfolded SOD1 and a propagating SOD1 seed.

Additionally, the interaction between TDP-43 and SOD1 should be validated in-vivo using models such as zebrafish and transgenic mice. The advantage of using transgenic zebrafish with GFP-filled primary motor neurons is their transparency, allowing for monitoring and quantifying axonopathy. In order to assess how these interactions impact primary motor neurons, TDP-43 (specifically TDP-43ANLS, Trpless-TDP-43ANLS and W68/113S-TDP-43ANLS) and human wild-type SOD1 can be delivered to zebrafish via microinjection of encoding mRNA. Following in-vivo studies and the assessment of axonopathy, SOD1 misfolding can be determined by biochemical studies, including immunohistochemistry and immunoprecipitation, using SOD1 misfolding-specific antibodies. Additionally, immunocytochemistry and immunoblotting can be used to detect fragmented, hyper-phosphorylated, aggregated, and mislocalized TDP-43 in the same samples. Once the effect in zebrafish is established, these can also be used to screen small
molecules for blocking the intermolecular interaction between the proteins in hopes to reduce axonopathy. Additionally, the cross-seeding can also be studied in transgenic mice. It was recently demonstrated that when transgenic mice with deoxycycline (Dox)-suppressible human TDP-43\textsuperscript{ANLS} express the transgenic protein, these form hyperphosphorylated cytoplasmic inclusions, and eventually lead to brain atrophy, and progressive motor impairments leading to death\textsuperscript{401}. To test if TDP-43\textsuperscript{ANLS} can trigger misfolding of HuWtSOD1 \textit{in-vivo}, I propose to cross the two mouse strains and characterize the resulting double transgenic mouse model for general activity, survival, and body weight after turning off the suppression of human TDP-43\textsuperscript{ANLS} by removing Dox from their diet at 5 weeks\textsuperscript{401}. Following mouse sacrifice and tissue collection, the loads of misfolded SOD1 and pathological TDP-43 will be assessed using immunocytochemistry and immunoprecipitations.

The cross-seeding of SOD1 by TDP-43 mediated by their respective tryptophan residues represents a novel mechanism of protein-protein interaction that could trigger the spread of pathologies in neurodegenerative diseases. This mechanism also provides new therapeutic targets aiming to block self-aggregation and the cross-seeding observed in these disorders.
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Brockington, A. et al. Unravelling the enigma of selective vulnerability in neurodegeneration: motor neurons resistant to degeneration in ALS show distinct gene


Kim, S. H., Shanware, N. P., Bowler, M. J. & Tibbetts, R. S. Amyotrophic lateral sclerosis-associated proteins TDP-43 and FUS/TLS function in a common biochemical
complex to co-regulate HDAC6 mRNA. J Biol Chem 285, 34097-34105, doi:M110.154831 [pii]
10.1074/jbc.M110.154831 (2010).


10.1126/science.1166066 (2009).


10.1038/nature08971 (2010).


10.1073/pnas.0911829107 (2010).


10.1038/nm1782 (2008).


Emmanouilidou, E. et al. Cell-produced alpha-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival. *J Neurosci* 30, 6838-6851, doi:30/20/6838 [pii]


Elden, A. C. *et al.* Ataxin-2 intermediate-length polyglutamine expansions are associated with increased risk for ALS. *Nature* **466**, 1069-1075, doi:10.1038/nature09320 (2010).


Appendices

Appendix A  pCINeo backbone

Figure 35: pCINeo expression vector.
A mammalian expression vector that carries a ubiquitous CMV promoter, as well as ampicillin and neomycin resistance genes.
Expression of exogenous proteins in cell cultures has become an essential tool in understanding how proteins work and study their pathways. In order to express an exogenous protein, the DNA encoding the protein of interest (TDP-43, FUS or SOD1) was 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 35, Promega, Madison, WI, USA), which contains the cytomegalovirus (CMV) promoter that constitutively drives the expression of cloned DNA inserts in mammalian cells. 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. 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 the multiple cloning site region of the plasmid.