Regulation of C9ORF72 FTD/ALS pathology by Ubiquilin-2

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Submitted on April 15, 2019

A thesis submitted in partial fulfillment of the Degree of Bachelor of Science in Neuroscience with Honors

Abstract

Ubiquilin-2 (UBQLN2) is a homeostatic regulatory protein that functions primarily in the ubiquitin-proteasome system and autophagy to degrade toxic proteins. When mutated, UBQLN2 directly leads to frontotemporal dementia (FTD)/amyotrophic lateral sclerosis (ALS), which are adult-onset neurodegenerative diseases characterized primarily by the presence of trans-active DNA binding protein-43 (TDP-43) inclusions in the cytoplasm. Additionally, in a large subset of hereditary cases, FTD/ALS patients exhibit high levels of atypical translation of an expanded hexanucleotide repeat in the Chromosome 9 Open Reading Frame-72 (C9ORF2) gene; this atypical—or RAN—translation, results in the production of aggregation-prone dipeptide repeats (DPRs). To better understand how UBQLN2 regulates these disease proteins, we expressed disease-associated DPRs or different forms of TDP-43 while also either overexpressing or knocking down UBQLN2 in a cellular model and found that UBQLN2 significantly decreased full-length, wildtype and C-terminally truncated TDP-43. However, TDP43 mutations reduced the ability of UBQLN2 to regulate these disease proteins. Preliminary assessment of human disease tissue revealed non-significant decreases in UBQLN2 levels in C9FTD females, highlighting the need for further investigation into sex-specific UBQLN2 changes in C9FTD. To evaluate the role of UBQLN2 in regulating disease pathology *in vivo*, C9ALS/FTD mouse models were generated using adeno-associated virus (AAV) injection of the hexanucleotide repeats in wildtype, UBQLN2 transgenic and UBQLN2 knockout mice, laying the groundwork for future studies that will measure levels of DPRs and TDP-43. In summary, our data suggest that UBQLN2 is an important modulator of aggregation-prone proteins in neurodegenerative disease and support further investigation to better understand the basis of this modulation.

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Scientific Acknowledgements

I would like to thank each person who has contributed to the success of this thesis. This work would not have been possible without the tremendous guidance and support found in the Henry L. Paulson lab. I would like to formally thank Dr. Paulson for allowing me to learn and grow in a lab with the best science has to offer and for the opportunity to complete this thesis. I would also like to acknowledge the help provided to me by other lab members including Stephanie Pistorius, a graduate student in the lab, Dr. Lisa Sharkey, Research Investigator, and undergraduates Jordan Gregory and Emily Crowley for both their technical and emotional support. Most importantly, I'd like to thank my mentor, Dr. Julia Gerson, a postdoctoral fellow in our lab, for her dedication to my success and my sanity. It is her guidance that has made me the scientist I am today; for that, I am forever indebted to her. Additionally, I'd like to thank Dr. Natalie Tronson for acting as my co-sponsor throughout this project and for her helpful guidance during this process. The experiments presented in this thesis were done in close collaboration with Dr. Gerson, who has granted me permission to include data she previously collected and analyzed. Contributions to this paper are clearly outlined in the table on the next page:

Table of Contributions:

Figure #	Person who conducted experiment
3	Julia Gerson injected pups; I collected brain tissue and homogenized/fixed
4	Julia Gerson transfected cells; I ran WB and analyzed data
5	Julia Gerson transfected cells; I ran WB and analyzed data
6	Julia Gerson transfected cells and I ran WB (no data analysis shown)
7	Julia Gerson transfected cells; I ran WB and analyzed data
8	Julia Gerson transfected cells and ran WB; I analyzed data
9	I ran WB and analyzed data; tissue was generously donated
10	Julia Gerson injected pups with AAV, I collected tissue, homogenized, ran WB, and analyzed

Personal Acknowledgements

I would like to thank my parents for their endless support throughout my collegiate career. To my mom, who willingly listens to lab stories through the phone as I walk home from work each day. And to my dad, who is a constant reminder that hard work reaps large rewards. Lastly, thank you to my best friend, Eric, who inspires me and supports me in every stage of my journey. You all fill my life with love.

Introduction

Neurodegenerative Disease

In a society with an increasing aging population, more people than ever are affected by neurodegenerative disease. As a result of population-wide health behavior changes, lifespans have been increasing at a steady rate over the last few decades. In fact, according to the United States Census Bureau, by 2030 the aging population is expected to outnumber children for the first time in the country's history (Iriondo and Jordan, 2018). As age is the number one risk factor for the most neurodegenerative diseases, it is projected that more than 12 million Americans over the age of 65 will experience some type of neurodegenerative disease by 2030 if no therapeutic intervention is developed (Iriondo and Jordan, 2018).

A hallmark of the most common age-related neurodegenerative diseases—including Alzheimer's disease, Parkinson's disease, and the amyotrophic lateral sclerosis (ALS) and frontotemporal dementia spectrum (ALS/FTD)—is dysregulation of protein homeostasis in the form of accumulation of aggregated, misfolded or mislocalized proteins. Often in these disorders, functional proteins lose their stable conformation and misfold or in the case of natively unfolded proteins become aberrantly folded. This may occur due to some form of cellular stress or changes in the cellular environment (Sami et al, 2017). ALS/FTD can be both inherited and sporadic, though most cases are sporadic (Picher-Martel et al, 2015). While the causes of heritable disease inform our understanding of ALS/FTD mechanisms, the underlying driver of initial protein dysregulation in sporadic disease is unknown. Likely, a combination of environmental factors culminates in decreased disease protein stability, causing proteins to accumulate into aggregates that inhibit normal cell processes. Following initial aggregation, a cascade of malfunction occurs often beginning with synaptic dysfunction and eventually leading to cell death. A common factor in the aggregation process involves the posttranslational adoption of abnormal β-sheet conformation (Lim and Yue, 2015), which is a remarkably stable protein structure found in proteins associated with neurodegenerative diseases. The danger of proteins with β-sheet conformation lies in their ability to tightly stack together to form large, stable protein fibrils that disrupt normal cellular processing (Lim and Yue, 2015). However, in recent

years small, soluble oligomeric intermediates of neurodegenerative disease proteins have been recognized as particularly toxic species as well (Sengupta et al, 2016). A better understanding of the function of these aggregation-prone proteins and the cellular processes involved in their regulation and clearance will help elucidate key mechanisms in disease and accelerate the development of treatments in an area of science where none currently exists. Further study could also help target therapeutic intervention prior to the cell death associated with neurodegenerative diseases. Current treatments seek only to relieve the symptoms of the disease rather than stopping the actual progression or onset.

In a healthy cell, mechanisms exist to help mitigate the accumulation of toxic species of proteins and prevent the progression of disease. Two primary mechanisms are the ubiquitin-proteasome system (UPS) and autophagy pathway (Nandi et al, 2006, Dantuma and Bott, 2014, Lopez et al, 2015). Three main types of autophagy have been described in mammals: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) (Lopez et al, 2015). Macroautophagy acts in a bulk-removal fashion to degrade misfolded proteins via autophagosomes while microautophagy acts to directly engulf cytoplasmic components in the lysosome (Lopez et al, 2015). In contrast, CMA degrades toxic substrates in a specific, targeted manner via chaperone proteins and lysosomes (Lopez et al, 2015). Chaperones can also help refold proteins that have adopted a toxic conformation and return them to a normal state or assist with their degradation process. In the UPS, ubiquitinated proteins are flagged for degradation by ATP-dependent 26S proteasomes (Nandi et al, 2006). Ubiquitins can be linked to form a polyubiquitin chain and through its enzymatic activity, the UPS is able to selectively degrade proteins flagged for removal while leaving properly folded proteins intact (Nandi et al, 2006). The movement of ubiquitinated substrates to the proteasome can be facilitated by ubiquitin-proteasome shuttling proteins, including Ubiquilin-2 (UBQLN2).

Ubiquilin-2 as a regulatory protein

UBQLN2 is a protein quality-control protein implicated in both the UPS and autophagy. It is one member of a family of Ubiquilin proteins that are widely expressed in mammals. This universally expressed family includes Ubiquilin-1, Ubiquilin-2, and Ubiquilin-4, as well as the testes-specific Ubiquilin-3. All Ubiquilins are characterized by a ubiquitin-like domain (UBL)

near the N-terminus, a ubiquitin-associated domain (UBA) near the C-terminus, and a variable range of Sti1 repeats in the middle region of the protein (Marín, 2014; Figure 1).

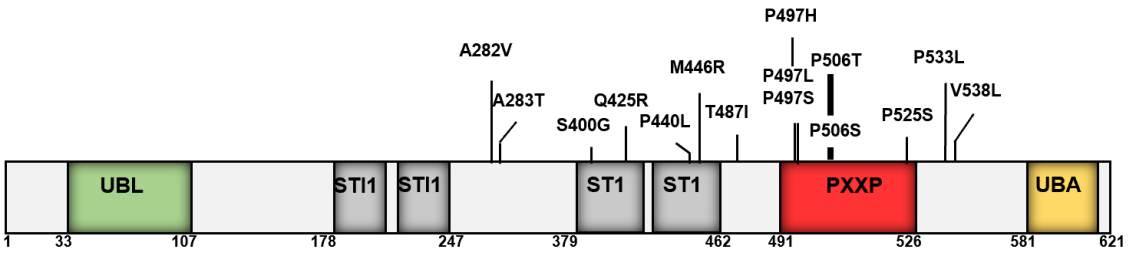

Figure 1. UBQLN2 structure and mutations that are found primarily in middle region of the protein

These domains dictate the function of the Ubiquilins, which physically associate with the proteasome via the UBL domain and with polyubiquitinated proteins via the UBA domain (Figure 2). Thus, the UBA domain binds to a polyubiquitinated chain covalently attached to a misfolded protein, allowing UBQLN2 to shuttle the protein targeted for degradation to the proteasome. The proteasome then breaks down the misfolded protein into peptides that are released back into the cell to be recycled for a different purpose (Dantuma and Bott, 2014, Figure 2).

In addition to UBQLN2's role as a UPS shuttle protein, the Sti1 repeats located in the middle region are structurally like the heat shock protein Sti1 and likely allow UBQLN2 to interact with molecular chaperones to further regulate protein folding and degradation (Zhang et al, 2014). Unique among the Ubiquilins is UBQLN2's proline-rich (PXX) domain, a collagen-like domain that may mediate protein-protein interactions (Zhang et al, 2014). Importantly, mutations found in the PXX domain and Sti1 repeats of UBQLN2 have been shown to cause ALS/FTD (Figure 1, Marín, 2014, Alexander et al, 2018). Overexpression of mutant UBQLN2 has been shown to directly lead to disease phenotypes in mouse models including cognitive and motor deficits, as well as biochemical characteristics similar to human disease pathology including ubiquitinated inclusions in the brain and accumulation of trans-active DNA binding protein-43 (TDP-43) in the cytoplasm (Le et al, 2016). Toxicity associated with UBQLN2 mutations may be mediated by a loss of function mechanism whereby the ubiquitin-proteasome

system is less able to properly degrade and control toxicity in the brain (Le et al, 2016). Alternatively, mutations in UBQLN2 may also lead to a gain in toxicity potentially by enhancing the intrinsic aggregation potential of UBQLN2 (Le et al, 2016).

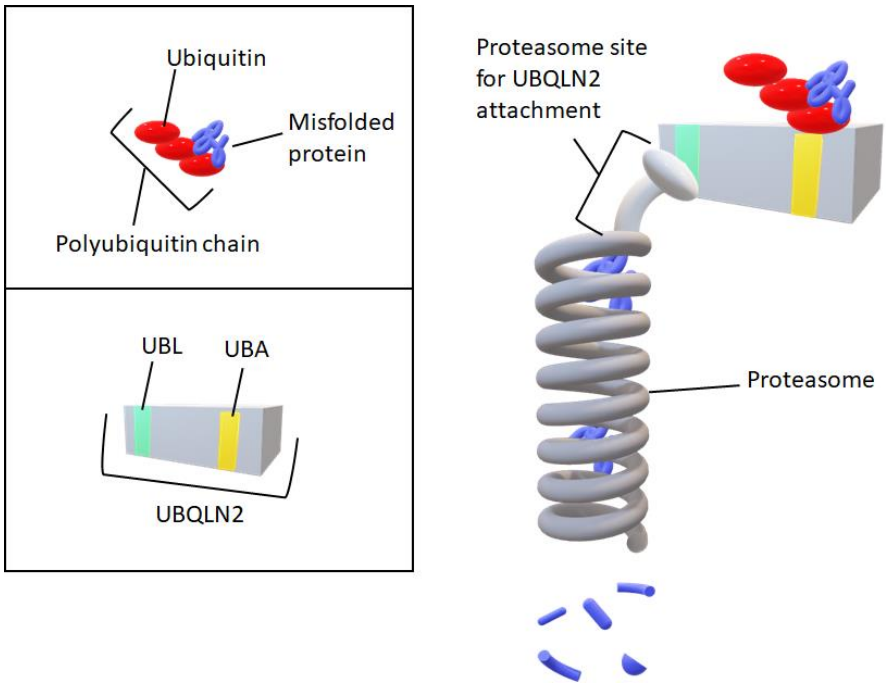

Figure 2. Ubiquilin-2 functionally interacts with the ubiquitin-proteasome system. The UBA domain interacts with poly-ubiquitin chains conjugated to proteins flagged for degradation, while the UBL domain binds with the proteasome allowing ubiquitinated proteins to be degraded and recycled.

Frontotemporal Dementia (FTD)/Amyotrophic Lateral Sclerosis (ALS) Pathophysiology

FTD/ALS are adult-onset diseases that are either hereditary or sporadic in origin. In ALS, large upper and lower motor neurons undergo extreme degeneration that typically results in death within three to five years of diagnosis (Ajroud-Driss and Siddique, 2015). About 10% of all reported ALS cases are genetic and the other 90% occur sporadically (Picher-Martel et al, 2015).

Disease pathology for ALS/FTD is characterized most commonly by four main distinct protein aggregates: TDP-43, dipeptide repeat proteins (DPRs) encoded by the C9ORF72 repeat

expansion, superoxide dismutase 1 (SOD1), and fused in sarcoma (FUS). The most important FTD/ALS markers, TDP43 and DPRs, are discussed in greater detail below. Misfolding of the protein SOD1 occurs due to dominant-acting missense mutations that lead to a toxic gain of function associated with endoplasmic reticulum stress, though SOD1 aggregates are occasionally present in sporadic forms of disease as well (Ajroud-Driss and Siddique, 2015). Another FTD/ALS marker is the RNA-binding protein, FUS. The role of FUS typically is to regulate transcription via gene promotor binding and contribute to mRNA stability (Ederle and Dormann, 2017). FUS functions primarily in the nucleus as a transcription regulator but can temporarily translocate to function in the cytoplasm to regulate mRNA stability, trafficking, and translation (Ederle and Dormann, 2017). FUS has liquid-like properties that play a key role in its ability to travel smoothly between the nucleus and cytoplasm. In disease, however, aggregated FUS loses its liquid-like properties and mislocates into the cytoplasm because of point mutations in the nuclear localization signal or defective arginine methylation during post-translation modification (Ederle and Dormann, 2017).

The most abundant FTD/ALS disease marker is TDP-43, which forms inclusions in the cytoplasm. Normally functioning TDP-43 is an RNA-binding protein that regulates transcription by binding to transcription regulators like FUS (Ederle and Dormann, 2017). After neuronal cell injury, TDP-43 translocates to the cytoplasm where it induces the formation of stress granules (SGs), which are dynamic structures formed as a result of cellular stress (Ajroud-Driss and Siddique, 2015, Alexander et al, 2018, Protter and Parker, 2016). SGs contain untranslated mRNAs that allow for binding of many mRNA binding proteins (Protter and Parker, 2016). When functioning normally, SGs are both useful and important to the cell; however, in disease, TDP-43 severely mislocates to the cytoplasm and forms aggregates, often with the SGs, that interrupt normal cellular functioning.

In FTD/ALS patients, TDP-43 is phosphorylated and cleaved, forming aggregates in the cytoplasm and markedly decreasing the amount of normal nuclear TDP-43 (Ajroud-Driss and Siddique, 2015). Both mutated and truncated TDP-43 fragments are associated with toxicity, heightened aggregation, and mislocalization to the cytoplasm. The short, truncated TDP-43 fragment (sTDP43) is an isoform found only in disease and is thought to play a key role in forming protein aggregates (Wang et al, 2013). The sTDP43 fragments, which include a glycine-rich region and a truncated form of an RNA-recognition motif (RRM2), form fibrils and

cytoplasmic inclusions in neuronal cells (Wang et al, 2013). Both full length TDP-43 and sTDP43 aggregates prevent TDP-43 from returning to the nucleus and stress granules from breaking down as should occur in a normally functioning cell.

Furthermore, key pathogenic mutations in TDP-43 have been linked to the nuclear-to-cytoplasmic translocation seen in disease. The TDP-43 missense mutations M337V and A315T specifically cause ALS/FTD (Mutihac, 2015, Wang, 2015). In a primary motor neuron cell culture model, M337V mutants led to TDP-43 mislocalization and spontaneously formed cytoplasmic inclusions (Mutihac, 2015). While TDP-M337V mutants induce cytoplasmic stress only, A315T mutants instead induce endoplasmic reticulum (ER) stress, which in turn leads to ER-stress mediated apoptosis (Wang et al, 2015). By forcing ER-regulated cell death into overdrive and significantly driving up levels of the stress protein marker GRP-78 and autophagy marker LC3, A315T mutants may alter protein homeostasis that leads to ALS/FTD (Wang et al, 2015).

C9ORF72 hexanucleotide expansion in FTD/ALS

The most important genetic cause of ALS/FTD is the expanded GGGGCC (G_4C_2) hexanucleotide repeat in the gene known as chromosome-9-open-reading-frame-72 (C9ORF72). This hexanucleotide expansion causes dominantly inherited ALS/FTD. Of the 10% of ALS cases that are familial, approximately 30% are caused by the expanded G_4C_2 hexanucleotide repeat, making it the most common genetic cause of FTD/ALS (Picher-Martel et al, 2015). Healthy individuals range from having 2-23 G_4C_2 noncoding repeats, whereas disease patients have upwards of 66 repeats (DeJesus-Hernandez, 2011). This expansion occurs upstream of the C9ORF72 protein coding region, found in intron 1 (Mori et al, 2013). The presence of this expanded hexanucleotide promotes a type of noncanonical protein translation that occurs randomly without an AUG start codon. This type of translation is referred to as repeat-associated non-AUG (RAN) translation. Recent data suggest that the presence of the G_4C_2 repeats induce a cellular stress response that includes the formation of stress granules (Green et al, 2017). An integrated stress response globally suppresses normal translation while simultaneously enhancing the RAN translation response. As a result of RAN translation across the repeat in three different reading frames, a group of toxic peptides are produced called Dipeptide Repeats (DPRs). Their

production creates cellular stress that further favors RAN translation, potentially creating a feedforward loop in the creation of neurotoxic proteins (Green et al, 2017).

Five distinct DPRs are generated from unconventional RAN translation: glycine-alanine (GA), glycine-arginine (GR), proline-alanine (PA), proline-arginine (PR), and glycine-proline (GP) (Freibaum and Taylor, 2017). GA-DPR is the most easily observed in brains of patients with C9FTD/ALS (Freibaum and Taylor, 2017). Interestingly, mass spectrometry analysis indicates that GA-DPR binds to UBQLN1 and UBQLN2 (May et al, 2014). While GA-DPR is toxic when expressed at high levels, it only minimally disrupts the cell when expressed at lower levels (Freibaum and Taylor, 2017). Thus, the GA-DPR appears to only be toxic above a certain physiological threshold. Meanwhile, arginine-containing DPRs, such as GR and PR, are highly toxic given their high charge and polarity (Freibaum and Taylor, 2017). Moreover, GR and PR are highly localized in the nucleolus where they act to disrupt RNA-binding proteins (Freibaum and Taylor, 2017). Therefore, it is perhaps not surprising that GR and PR DPRs are associated with TDP-43 inclusions as well as stress granules (Freibaum and Taylor, 2017). The presence of DPRs markedly decrease the dynamic nature of stress granules. Lastly, little is known about the negative consequences of PA and GP DPRs. Research to date suggests that these two DPRs are relatively inert and do not contribute significantly to disease pathology (Freibaum and Taylor, 2017). Importantly, DPRs are thought to accumulate years before TDP-43 aggregates and can serve as potential new, early markers in diagnosing C9ALS/FTD (Baborie et al, 2015).

Ubiquilin-2 Pathology in FTD/ALS

The role of UBQLN2 in regulating key protein clearance pathways suggests that it may play a critical role in modulating protein changes associated with FTD/ALS. Recent studies show that UBQLN2 functionally acts by suppressing the formation of stress granules, which arise due to protein misfolding or halted translation, while simultaneously increasing the fluidity of FUS (Alexander et al, 2018). This increased fluidity reduces the number of FUS-containing stress granules typical of ALS (Alexander et al, 2018). This property of UBQLN2 suggests that it may normally play a key role in preventing disease onset by interacting with stress granule regulation, as well as its known function as a ubiquitin-proteasome shuttle factor.

In healthy cells, UBQLN2 acts as a regulator of protein homeostasis; however, under certain conditions associated with disease, UBQLN2 may exert its own toxicity. Overexpression of both WT and mutant UBQLN2 demonstrates that UBQLN2 is intrinsically aggregation-prone, as seen when co-transfected with TDP-43 in a Neuro2A cell model (Picher-Martel et al, 2015). UBQLN2 aggregates are dynamic and grow significantly in size over time. However, while WT UBQLN2 is especially fluid, mutated UBQLN2 forms less fluid, aggregate-like structures (Sharkey et al, 2018). ALS disease pathology shows colocalization of UBQLN2 cytoplasmic inclusions with TDP-43 puncta. It is unclear whether aggregation of UBQLN2 could increase the cytoplasmic accumulation of TDP-43 or if dysfunction in UBQLN2 clearance of TDP-43 underlies the colocalization of UBQLN2 in TDP-43 disease aggregates (Picher-Martel et al, 2015). Regardless, the coexistence of these proteins in disease aggregates reaffirms that UBQLN2 plays an important role as a homeostatic regulator while also suggesting its own potential to induce toxicity in certain contexts. UBQLN2's properties suggest it may function only within a certain "Goldilocks" range, such that too much can lead to UBQLN2 aggregation but too little prevents homeostatic regulation of other proteins. Because mutations that lead to malfunction in UBQLN2 are sufficient to cause disease, it is possible that wildtype UBQLN2 plays a role in modulating ALS/FTD proteins (Zhang et al 2014). Furthermore, UBQLN2-positive inclusions are found in several neurodegenerative diseases including both hereditary and spontaneous FTD/ALS, indicating UBQLN2 is a regulatory protein worthy of investigation for its role in neurodegenerative disease (Picher-Martel et al, 2015). While the exact mechanism of UBQLN2 in neurodegeneration is still unknown, it is clear that UBQLN2 is associated with FTD/ALS in some capacity that should be explored further.

Current disease model strengths and weaknesses

In order to develop new and effective treatments for FTD/ALS, researchers must use controllable models to simulate disease. Each model offers its own strengths and weaknesses. For initial experiments, cellular models offer simple and easily replicable means to study disease proteins *in vitro*. Since FTD/ALS pathology is characterized by misfolding and aggregating proteins, this can be a good way to understand protein changes at a basic level. And while cell models offer good insight into mechanistic questions, they can also inform future experimental

directions with increased translational impact. Immortalized cell lines, such as Human Embryonic Kidney 293 (HEK293) cells are helpful for initial experiments but do not function in the same way as neurons (Damme et al, 2017). We used the HEK293 model to initially address the potential regulation of FTD/ALS proteins by UBQLN2. While Neuro2a cells and induced pluripotent stem cell-derived neurons (iNeurons) can model neuronal cells more accurately, they still only remotely model disease features (Damme et al, 2017). Therefore, for our second line of studies, we chose to use rodent models, which offer great benefits in the area of FTD/ALS research. Mice act as a disease model that share high degree of genetic identity and brain circuitry with humans, and modern neuroscience techniques allow mice to be genetically manipulated with relative ease. While they are more expensive to house than invertebrates, like the fruit fly (*Drosophila melanogaster*) and nematode (*C. elegans*), mice offer compelling disease models when studying neurodegenerative disease—especially for preclinical modeling. However, overexpression of transgenes to replicate disease pathology remains a troubling confounding variable (Damme et al, 2017). To date, there is only one ALS model that accurately replicates important hallmarks of C9ALS/FTD in mice, including TDP-43 and DPR pathology. This model is generated via adeno-associated virus (AAV) intracranial injection of the hexanucleotide repeat, leading to widespread induction of DPR production as well as TDP-43 cytoplasmic inclusions (Chew et al, 2015). No other model has shown such a complete picture of C9ALS/FTD with DPRs, TDP-43, and nuclear RNA foci, as well as behavioral phenotypes, making it an excellent route to study interaction of UBQLN2 with C9ALS/FTD *in vivo*.

Thesis Goals

The recent discovery of numerous mutations in UBQLN2 that directly lead to ALS/FTD, as well as studies showing colocalization of UBQLN2 with disease aggregates in genetic and sporadic FTD/ALS, prompted us to test UBQLN2's role as a modulator of ALS/FTD disease pathology. While studies have shown that UBQLN2 specifically interacts with TDP-43, its role in the most common genetic cause for ALS/FTD—the C9ORF72 hexanucleotide expansion—has yet to be investigated. Thus, we aimed to come to a better understanding of UBQLN2's ability to regulate TDP-43 pathology and DPRs. Further, this study, and future studies building from them, aim to better understand how dysregulation of UBQLN2 itself may contribute to disease. To accomplish

these goals, I used multiple disease models ranging from HEK293 cells to transgenic mice and analysis of human disease tissue. The experimental design was guided by the idea that UBQLN2 can mitigate disease pathology given its role as a regulator for the UPS and autophagy. We hypothesized that in disease models, overexpression of UBQLN2 would lead to decreased features of disease pathology whereas knocking out UBQLN2 would cause disease proteins to aggregate more readily. HEK293 cells were transfected to express poly-GA DPRs or different forms of TDP-43 while UBQLN2 was overexpressed or knocked-down to assess the functional interaction of UBQLN2 with these proteins. These data guided the direction of subsequent *in vivo* transgenic mouse models. Using C9ORF72 AAV, transgenic UBQLN2 mice and UBQLN2 knockout mice were induced to express expanded hexanucleotide repeats aimed at measuring the effect of UBQLN2 on FTD/ALS disease phenotypes. Preliminary results suggesting UBQLN2 may be decreased in female C9FTD brains serve as a guide for future studies that will specifically investigate sex-dependent changes to UBQLN2 in C9ALS/FTD. This study serves as a starting point for future projects aimed to more fully understand C9ALS/FTD and its relationship to UBQLN2. Only through increased understanding of basic disease mechanisms will scientists be able to develop more effective therapeutic intervention and translational research to better serve human neurodegenerative disease populations.

Materials and Methods

Cell Transfection

Human embryonic kidney 293 (HEK293) cells were cultured in high glucose DMEM, supplemented with 10% FBS, 10 mM Glutamine and 100 U/ml penicillin/streptomycin. Cells were transfected with either pCMV4-UBQLN2, UBQLN2 siRNA, or empty vector/control siRNA in combination with pGW1-WT-TDP43, pGW1-TDP43-A315T, pGW1-TDP43-M337V, pCMV-GFP, or pGw1-EGFP-sTDP43 using Lipofectamine-2000 according to the manufacturer's instructions. The pCMV4-FLAG-UBQLN2 plasmid (p4455 FLAG-hPLIC-2; Addgene plasmid # 8661) was a gift from Peter Howley (Kleinjnen et al, 2000). The pGW1-

CMV-EGFP-TDP43 plasmid was a gift from Sami Barmada (Barmada et al, 2010). Control empty vector plasmid for cell transfection experiments was pCMV-HA, and pCMV-GFP was used as a control for UBQLN2 clearance experiments. Dharmacon SMARTpool siGENOME siRNA against UBQLN2 and MISSION siRNA Universal Negative Control (Sigma) were used for UBQLN2 knockdown experiments. Cells used for Western Blot analyses were collected in a cocktail of 0.5% Triton-X 100 in PBS with protease inhibitor (catalog no. 11873580001; Sigma Aldrich).

Mouse Models

This study was completed in a facility approved by The Institutional Animal Care and Use Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Approval number PRO00008139; authorized Principle Investigator: Henry Paulson). In order to induce widespread neuronal transduction of a repeat expansion of either 2 or 66 G_4C_2 repeats derived from C9ORF72, viral injection using an adeno-associated virus (AAV) was administered to a control group of wildtype mice and two experimental groups: UBQLN2 Knockout mice (KO) and wildtype UBQLN2 transgenic mice (UBQLN2), as previously described in Sharkey et al (2018). UBQLN2 KO mice were generated via a CRISPR deletion of a single base pair in the *Ublqn2* gene sequence to eliminate expression of the UBQLN2 protein. UBQLN2 KO mice were a gift from Lisa Sharkey (Henry Paulson lab, University of Michigan). The pups were taken from their cages on post-natal day 0 (p0) for injection and were randomly assigned an injection group as outlined below in Table 1. AAVs were provided courtesy of Peter Todd, University of Michigan. The AAVs were constructed with two different viral vectors—$(G_4C_2)_2$ or $(G_4C_2)_{66}$ repeats—lacking an ATG start codon, to allow for two distinct groups of robust expression in the central nervous system (CNS) of the mice as described in Chew et al (2015).

Table 1

WT UBQLN2 Tg and Non-Tg Littermates	UBQLN2 KO and Non-Tg Littermates
2 sham pups (saline control)*	1 sham pup (saline control)*
2 pups $(G_4C_2)_2$ injections*	5 pups $(G_4C_2)_2$ injections*
15 pups $(G_4C_2)_{66}$ injections*	16 pups $(G_4C_2)_{66}$ injections*

*does not reflect the number of pups that survived to 2-month collection date (actual survival rate for UBQLN2 KO cages: 4 $(G_4C_2)_{66}$ pups, 5 $(G_4C_2)_2$ or saline control pups; actual survival rate for WT UBQLN2 Tg cages: 2 $(G_4C_2)_{66}$ pups, 1 saline control pup)

Table 1. *Experimental injection plan for both Tg UBQLN2 and UBLQN2 KO mice.*

Hemizygous WT UBQLN2 transgenic mice were bred with C57-Bl/6 non-transgenic mice in order to produce litters containing hemizygous 615 mice and non-transgenic littermate controls for this study. The UBQLN2 KO mice were bred using either two homozygous KOs or a hemizygous and homozygous pair to produce offspring homozygous for the KO gene. These mice were courtesy of Lisa Sharkey (Henry Paulson Lab, University of Michigan).

Human Disease Brain Tissue

Frozen brain tissue from the pons and medial frontal gyrus (MFG) was obtained from human donors with C9ORF72 FTD and non-C9ORF72 FTD, as well as age-matched controls, from the Michigan Brain Bank (University of Michigan, Ann Arbor, MI, USA). Brain tissue was collected with patient consent and approved by the Institutional Review Board of the University of Michigan. Protocols were followed and in line with Declaration of Helsinki principles. Neuropathologists examined and diagnosed samples.

Neonatal Viral Injections

Neonatal viral injections were performed as described in Kim et al (2014). Pregnant mice were monitored daily to prepare for a P0 injection on newborn pups. Injections were administered as

soon as pups started nursing in lateral ventricles between the lambda suture and each eye. Using a 10 uL injection syringe, 2 uL of AAV was bilaterally injected into the intracerebroventricular (ICV) space while pups were cryoanesthetized. Pups were placed on a heating pad until they regained normal color and movement and were then returned to the cage. Pups were monitored daily for 10 days. Tissue was collected at a 2-month time point based on preliminary data courtesy of Katelyn Green (Peter Todd Lab, University of Michigan) that showed robust neuronal expression of the AAV construct at 3 months of age (Figure 3).

A.

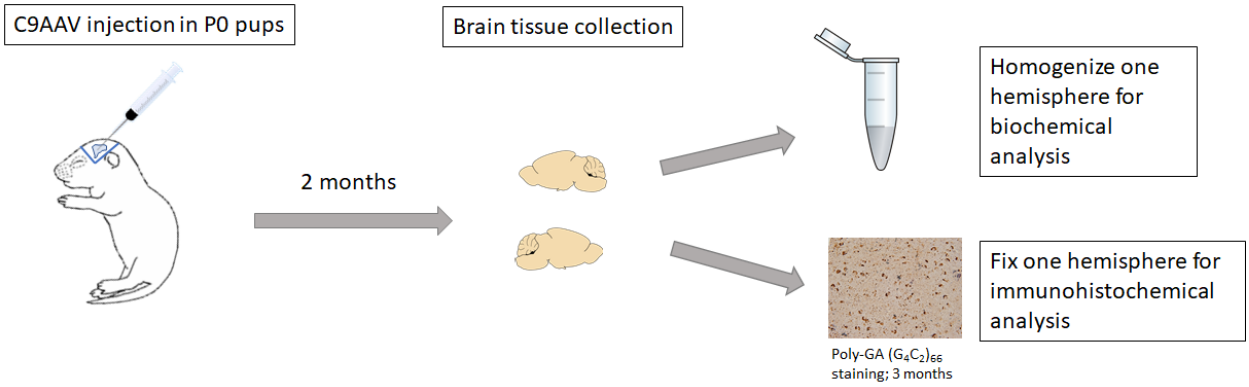

Figure 3*. Injection plan: Injection in lateral ventricles between the lambda suture and each eye in P0 pups to ensure spread of AAV throughout the brain. Mice were aged for 2 months and brain tissue was collected. Brains were cut in half to utilize for both biochemical and immunohistochemistry analysis. One hemisphere was homogenized and used for preliminary Western blot analysis. The other half was fixed for sectioning and immunohistochemistry. Preliminary immunohistochemistry data courtesy of Katelyn Green, Peter Todd Lab, University of Michigan. Imaging shows tissue collected at 3 months of age, stained for poly-GA DPRs. P0 pups were injected with AAV-9 with a* $(G_4C_2)_{66}$ *hexanucleotide repeat.*

Genotyping

DNA was collected from tails and lysed using Qiagen DNeasy blood and tissue kit. Once DNA was isolated, it was amplified using a PCR reaction (KO: LMS, 615: UB2GO). For UBQLN2 transgenic mice, standard PCR genotyping was utilized with the following primers: Ub2 Geno Fwd (GGACTACAAGGACGATGACAACG) and Ub2 Geno Rvs (CTGCATCTGTGGATTAGCCATAATGAG), as well as the internal control primers

oIMR7339 (GTAGGTGGAAATTCTAGCATCATCC) and oIMR7338 (CTAGGCCACAGAATTGAAAGATCT). The PCR product was then run on an agarose gel and imaged to determine genotype. For UBQLN2 KO mice, DNA was amplified using standard PCR with the following primers: Indel seq fwd (GAGCCCAAAATCATCAAAGTCAC) and Indel seq rvs: (AGCTGCTGCTGCATCTGGTTCTG). DNA samples were then sent for Sanger sequencing to determine the presence of a single base pair deletion that indicates protein knockout. Confirmation of protein KO was done via Western Blot analysis.

Mouse Brain Tissue Harvesting

Mice were perfused with PBS and brain tissue was collected 2 months after viral injection. After harvesting, brains were divided into 2 hemispheres; one hemisphere was used for sectioning and the other flash frozen for biochemical analysis.

Brain Tissue Homogenization

Mouse and human brain lysate were prepared using a bead homogenizer in cold phosphate-buffered saline (PBS) with Sigma Aldrich protease inhibitor (PI) cocktail (1 tablet: 50 mL) in an Eppendorf safe-lock microcentrifuge tube. 150 uL of sample was removed from the tube after homogenization and added to a tube with 200 uL of radioimmunoprecipitation assay (RIPA) buffer, a harsh extractant of total protein. After vortexing and a 5-minute water sonication, samples were centrifuged for 30 minutes at 13200 rpm at 4^{o}C. The remaining sample was added to 200 uL of PBS-PI cocktail, vortexed, and centrifuged for 10 minutes at 10000 rpm at 4^{o}C.

The supernatant of both fractions was removed to be aliquoted and stored at -80^{o}C for later use. To homogenize the PBS insoluble fraction, the PBS pellet was rehomogenized with more PBS-PI cocktail and spun again for 10 minutes. Supernatants were discarded. The remaining pellet was homogenized in cold 1% sarkosyl (N-Lauroylsarcosine sodium) in PBS with protease inhibitor cocktail, vortexed for 30 seconds each, and incubated at room temperature for 1.5 hours. Samples were then water sonicated for 5 minutes and centrifuged for 30 minutes at 13200 rpm at 4^{o}C. Supernatants were aliquoted and stored at -80^{o}C.

Western Blot Analysis

Brain tissue and HEK293 cells were analyzed via Western Blot analysis on a precast NuPAGE 4-12% Bis-Tris gels (Invitrogen) for SDS-PAGE. Approximately 50 ug of total protein was loaded for each sample and samples were transferred to a nitrocellulose membrane. Loading amounts were calculated using a bicinchoninic acid (BCA) assay. Membranes were subsequently blocked in 10% nonfat dry milk in Tris-buffered saline tween (TBS-T) buffer overnight at 4ºC. Next, membranes were blocked in 5% nonfat dry milk in TBS-T buffer for one hour in either anti-UBQLN2 (Novus Biologicals; 1:2000), anti-TDP-43 (Protein Tech; 1:1000), anti-green fluorescent protein (GFP) (Thermo Fisher, 1:5000), anti-FLAG (Sigma Aldrich, 1:2000) or anti-GAPDH (EMD Millipore; 1:5000). Membranes were washed, blocked in either anti-mouse or anti-rabbit secondary antibody in 5% milk, and developed.

Experimental Design and Statistical Analysis

Experiments carried out in this study were randomized and blinded whenever possible. All Western Blot analyses were repeated in triplicate and statistical analysis used the mean of each group of replicates. The graphs shown are representative of triplicate mean values. The cutoff for significance level used was standard $p \leq 0.05$. All analyses compared three or more groups and thus utilized a one-way ANOVA test and Tukey post-hoc test (Graphpad Software Inc., San Diego, CA, USA). Any p-values reported in figures that represent a significant relationship were generated by the post-hoc test. Insignificant relationships reported in figures show the overall ANOVA p-value. Data were analyzed using Graphpad Prism version 7.0 (Graphpad Software, Inc., San Diego, CA, USA).

Results

UBQLN2 decreases levels of ALS disease protein, TDP43, in HEK293 cells

To determine if UBQLN2 regulates proteins associated with ALS disease pathology, HEK293 cells were transfected to express wildtype full-length TDP-43 with or without overexpressed UBQLN2 or “knocked down” UBQLN2 via siRNA. We found that coexpressed UBQLN2 significantly decreased levels of PBS-soluble TDP-43 (Figure 4). Conversely, UBQLN2 knock

down significantly increased levels of insoluble TDP-43, particularly high molecular weight TDP-43.

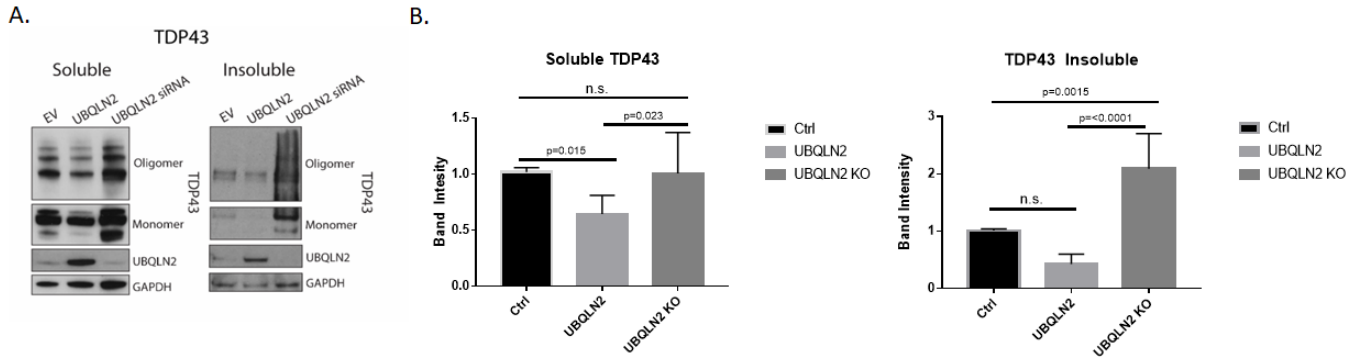

Figure 4. *Coexpressed UBQLN2 decreases levels of wildtype full-length TDP-43 in both soluble and insoluble fractions of HEK293 cells. Soluble TDP-43 levels are significantly lower when UBQLN2 is overexpressed. Insoluble TDP-43 is significantly increased as a result of UBQLN2 siRNA knock-down (n=7 for each experiment).*

Next, we investigated whether UBQLN2 had the same effect on other forms of TDP-43 associated with disease, including C-terminally truncated short TDP-43 (sTDP43) fragments that have been shown to co-localize with full length TDP-43 cytoplasmic aggregates (Feneberg et al, 2018). To study this, we transfected HEK293 cells to express sTDP-43, with either overexpressed or knocked down UBQLN2. UBQLN2 overexpression significantly decreased sTDP43 levels; in contrast, there was no difference in sTDP43 levels when UBQLN2 was knocked down via UBQLN2 siRNA (Figure 5). The sTDP43 fragments proved to be highly insoluble and could only be visualized by Western Blot when extracted using urea. Thus, identical volumes of samples were loaded.

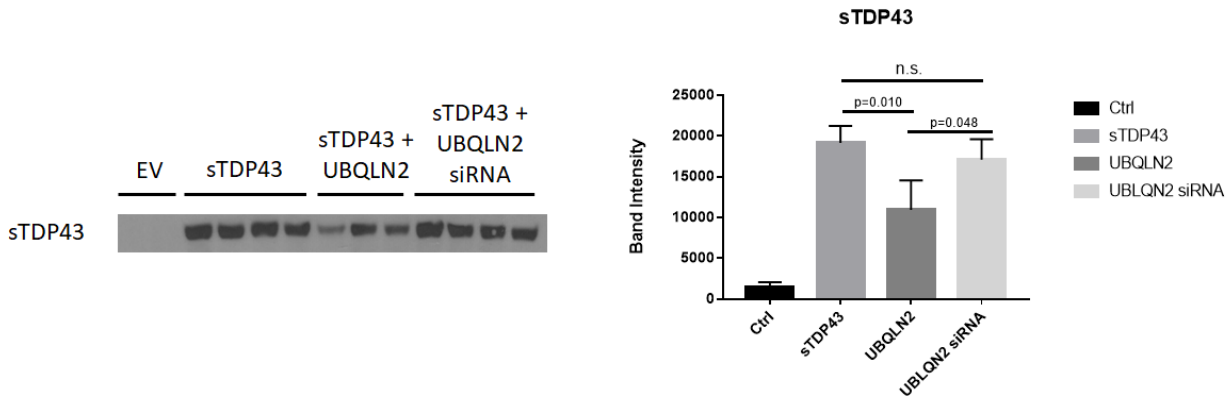

Figure 5. *UBQLN2 significantly decreases levels of the truncated TDP-43 form, sTDP43, in HEK293 cells. Unlike full-length TDP-43, sTDP43 levels are not significantly increased when UBQLN2 is knocked down (n=4).*

To further determine if UBQLN2 is differentially able to regulate disease forms of TDP-43, we expressed two different disease-linked TDP-43 point mutations in HEK293 cells: the A315T and M337V mutants implicated in ALS/FTD. Limited preliminary results (n=1) are reported here and suggest further research should be done to better understand the selectivity of UBQLN2 for disease proteins. Based on Western blot data, UBQLN2 appeared to be unable to regulate mutant forms of TDP-43 (Figure 6) in the same way it regulated wildtype full-length (Figure 4) and truncated TDP-43 (Figure 5). UBQLN2 overexpression and knockdown did not lead to the same robust effect on the soluble and insoluble fractions for either mutant form of TDP-43, and in fact appeared to elevate monomeric and high molecular weight M337V TDP43. This suggests potential selectivity in UBQLN2's ability to regulate disease proteins, though future studies with a proper number of replicates will be needed to confirm this result and further probe this hypothesis.

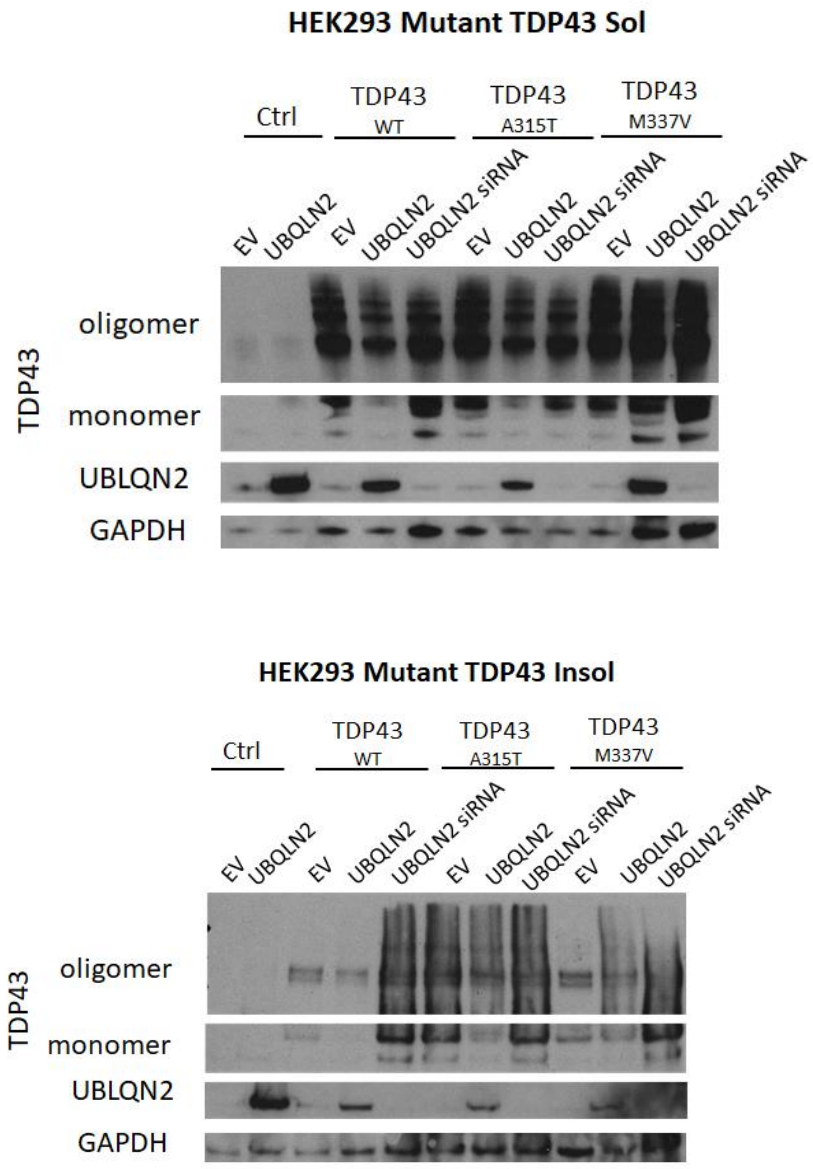

Figure 6. *UBQLN2 does not regulate mutant forms of TDP-43 to the same degree as wildtype TDP-43 in HEK293 cells. UBQLN2 does not robustly decrease mutant TDP-43, nor does UBQLN2 knockdown lead to consistent robust increase in mutant TDP-43, as seen with wildtype TDP-43 (n=1).*

UBQLN2 does not affect DPR levels in HEK293 cells

Given this promising data that UBQLN2 does, in fact, interact with TDP43, we decided to investigate its ability to regulate C9-associated ALS/FTD-specific disease proteins, as ALS/FTD

is known to be associated with TDP43 accumulation. To do this, a plasmid expressing the $(G_4C_2)_{70}$ hexanucleotide repeat expansion tagged with FLAG was transfected into HEK293 cells to induce the formation of GA DPRs (Figure 7); the plasmid was tagged with a FLAG epitope to label the GA-DPR. (Plasmid constructs are described in Green et al, 2017.) In HEK293 cells expressing GA DPR($_{70}$), UBQLN2 was either coexpressed or knocked down via siRNA. Since DPRs in this experiment are produced indirectly by RAN translation, the GA DPR expression level was relatively low. Preliminary data show that both overexpressed UBQLN2 and UBQLN2 knockdown did not significantly alter levels of GA DPR in HEK293 cells, though further experimentation is needed to confirm this preliminary result. Western blot data suggests that UBQLN2 overexpression may have a small effect on DPR levels, though the effect does not appear to be as strong as on TDP43.

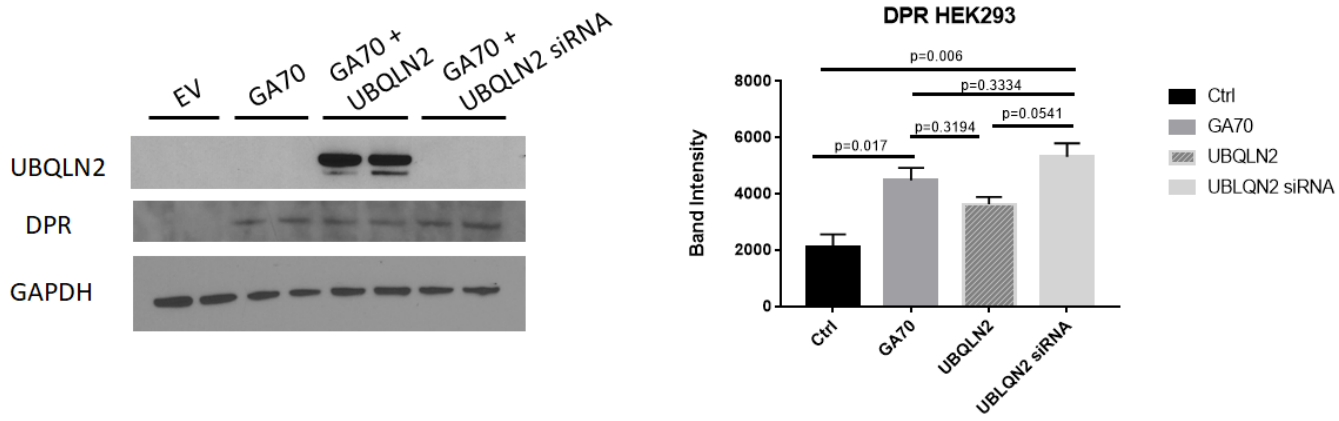

Figure 7. *In HEK293 cells, UBQLN2 exhibits a non-significant trend toward lowering levels of the poly-GA DPR. While there is a significant increase in DPR levels when UBQLN2 is knocked down compared to when UBQLN2 is overexpressed, there is no significant change when compared to control (n=2 for each group).*

UBQLN2 does not broadly regulate all overexpressed proteins and acts in a disease-specific manner

In order to confirm the specificity of UBQLN2 for disease proteins, rather than generalized regulation of any overexpressed protein, HEK293 cells were transfected with the reporter protein GFP (Figure 8). GFP levels were unaffected by overexpression or knockdown of UBQLN2 ($p = 0.72$), showing that UBQLN2 acts on specific disease proteins.

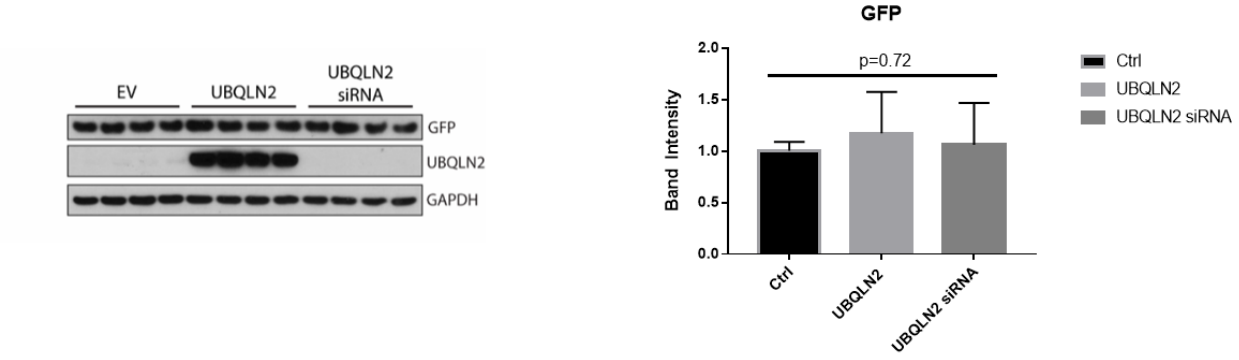

Figure 8. *Levels of the reporter protein GFP are unchanged with varying UBQLN2 expression (n=6). The p-value shown here is for the overall ANOVA rather than individual post-hoc analysis p-values comparing each category.*

UBQLN2 Analysis in C9 FTD human tissue suggest further research in sex-specific changes are needed

To investigate changes in UBQLN2 associated with C9FTD human disease pathology, post-mortem tissue from confirmed C9FTD patients, non-C9 FTLD-TDP patients, and age-matched controls was collected. PBS-soluble fractions from the medial frontal gyrus (MFG) and the pons were analyzed for all groups. No statistically significant differences were observed for levels of UBQLN2 in the MFG, but upon analysis of differences broken down by sex, variability in UBQLN2 levels between male and female samples became clear, with female C9FTD brains showing non-significant decreases in UBQLN2, suggesting further research will be needed to gauge whether sex differences exist that are specific to disease with appropriate sample sizes (Figure 9A). Similarly, for the pons tissue, no statistically significant change in UBQLN2 levels were observed between control, C9FTD, and non-C9FTD-TDP groups. Again, insufficient sample sizes for measuring differences in disease and controls broken down by sex suggest that

further research may reveal important new information on UBQLN2 in disease (Figure 9B). These preliminary results provide reasoning for further investigation using an appropriate number of replicates to account for potential error.

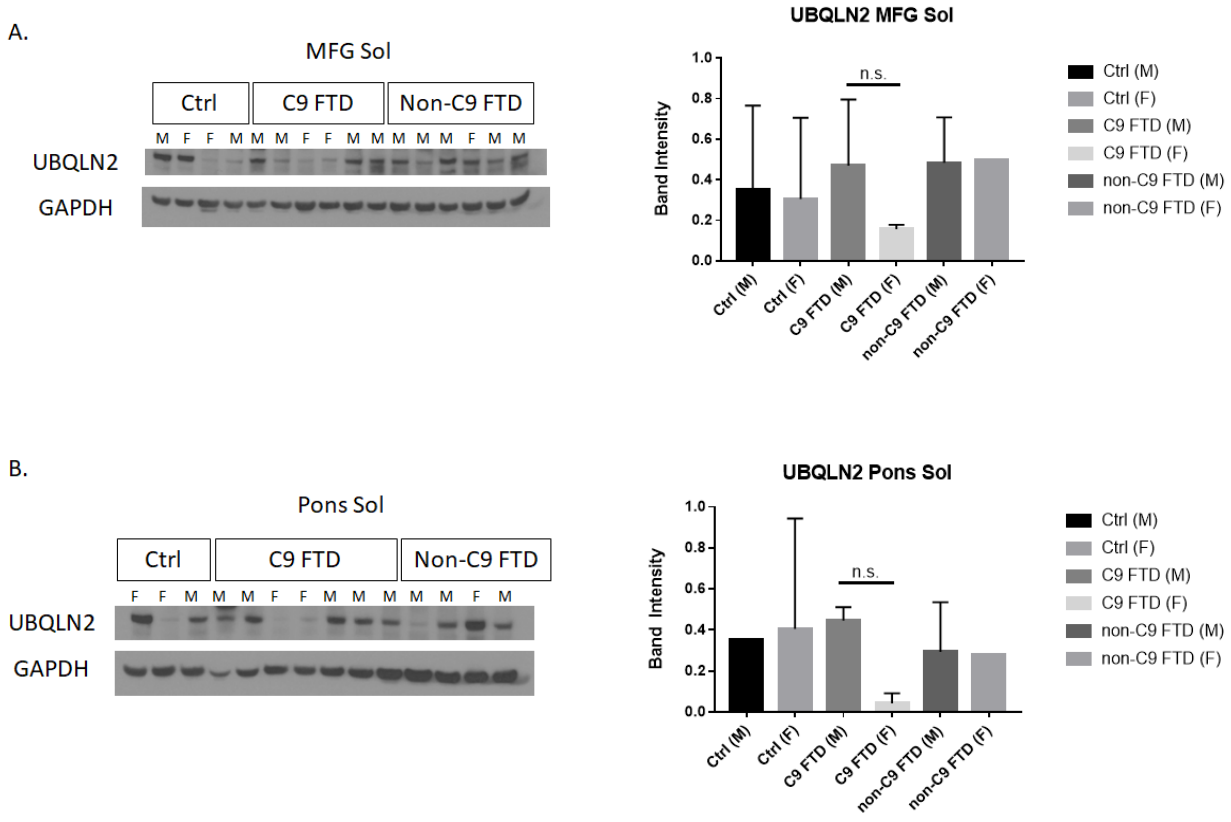

Figure 9A. *Western blot for human MFG tissue and analysis. Soluble MFG tissue of C9FTD, non-C9FTD, and age-matched control patients were homogenized and probed with anti-UBQLN2 via Western blot. No statistically significant changes in UBQLN2 levels were observed between any groups. However, a sex-specific trend was observed for UBQLN2 levels within the C9FTD group only.* ***Figure 9B****. Western blot for human pons tissue and analysis. Soluble pons tissue of C9FTD, non-C9FTD, and age-matched control patients were homogenized and probed with anti-UBQLN2 via Western blot. While no statistically significant changes were observed in UBQLN2 levels between groups, a sex-specific trend in UBQLN2 levels for C9FTD, but not non-C9FTD, was seen.*

Ongoing Analysis of C9ORF72 FTD Modeling in UBQLN2/UBQLN2 Knockout Mice

C9AAV injections were completed using a protocol in which the experimenter was blinded to genotype. As UBQLN2 knockout genotyping is prone to error, confirmation of genotypes for two-month-old injected mice was retrospectively determined via Western blot. Uninjected-age-matched controls, C9AAV-injected mice from a UBQLN2 KO cage, and C9AAV injected mice from a UBQLN2 transgenic cage were compared. Western blot data confirmed the successful knockout of UBQLN2 protein for 5 of the 9 potential UBQLN2 KO samples (Figure 10). Future studies will assess DPR and TDP-43 pathology by immunohistochemistry.

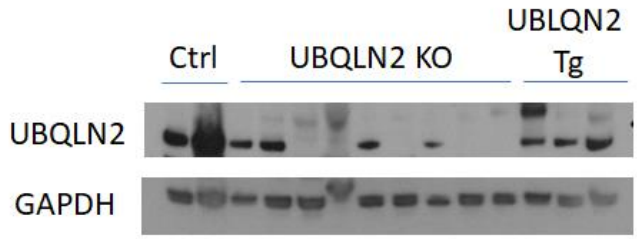

Figure 10. *2-month-old tissue samples from C9AAV injected mice were run on a Western blot to confirm the expected genotypes of the mice. UBQLN2 levels were easily identifiable for all groups and UBQLN2 protein KO was confirmed for 5 of the 9 potential samples loaded. Uninjected age-matched controls were compared to the two injection groups.*

Discussion

Increasingly, UBQLN2 is being studied for its potential role as a key modulator of misregulated proteins in neurodegenerative disease (Zhang et al 2014). While UBQLN2 mutations have been implicated previously in causing ALS/FTD, little research has clarified wildtype UBQLN2's ability to mitigate ALS/FTD disease pathology (Le et al 2016). Furthermore, there are no studies dedicated exclusively to understanding how UBQLN2 might specifically regulate C9ALS/FTD since the discovery of DPRs in 2011. C9ORF72 mutations account for the majority of genetically-linked ALS cases. Thus, one of the primary goals of this thesis was to determine if UBQLN2 plays a role in this subset of disease pathology. Using a cellular model, we investigated UBQLN2's ability to modulate expression and aggregation of

FTD/ALS proteins implicated in C9ALS/FTD, namely TDP-43 and DPRs. We then performed preliminary studies evaluating the role of UBQLN2 in disease using both mouse models and human disease tissue. Our results show that UBQLN2 differentially regulates TDP-43 levels depending on the isoform and mutation status of the protein. Further, we provide unexpected evidence suggesting a decrease in UBQLN2 in female C9FTD brains, highlighting a need for additional studies into sex-specific changes to UBQLN2 in C9FTD.

Our results showing that UBQLN2 reduces full-length TDP-43 and sTDP43 support other studies showing that UBQLN2 is able to modulate FTD/ALS disease proteins (Cassel and Reitz 2013, Williams et al 2012). Because UBQLN2 mutations cause disease, much of the research investigating the role of UBQLN2 in ALS/FTD has focused on mutated UBQLN2. However, UBQLN2's function in protein homeostasis suggests a better understanding of wildtype UBQLN2 in the context of disease is needed. Previous data show that UBQLN2 colocalizes with TDP-43 deposits in human neurodegenerative disease, prompting the question: how does UBQLN2 regulate TDP-43 in the context of FTD/ALS and when there are disease-linked mutations in TDP-43? Our results suggest that UBQLN2 has little to no effect on mutant TDP-43 levels compared to its robust regulation of WT TDP-43 in cellular models, which indicates that UBQLN2 is unable to interact with all forms of aggregated or disease-associated proteins. However, it is known that UBQLN2 binds with high affinity to the C-terminal, glycine-rich region of TDP-43 (Cassel and Reitz 2013). Since both TDP-43 point mutations studied here, M337V and A315T, are located in this glycine-rich region, it is possible that UBQLN2 may be less able to modulate these TDP-43 mutants due to a physical inability to bind to the protein. Further study will be needed to better understand direct interactions between UBQLN2 and TDP43, as well as other disease proteins. Moreover, the inability of UBQLN2 to alter levels of overexpressed GFP supports the idea that UBQLN2 acts in a selective manner on specific proteins, not any overexpressed target. These data are consistent with previously published studies that show UBQLN2 interacting with protein inclusions in specific disease settings. UBQLN2 has been shown to colocalize with Huntington's Disease (HD) inclusions but not spinocerebellar ataxia 3 (SCA3) aggregates (Zeng et al 2015). Moreover, UBQLN2 has been shown to associate with α-synuclein but does not colocalize to α-synuclein-containing Lewy bodies to the same degree as HD inclusions (Rutherford et al 2013). As UBQLN2 does not act broadly on all expanded or aggregated proteins, further elucidating its specific protein-protein

interactions could provide information relevant to targeted therapeutic intervention for these fatal diseases.

Given UBQLN2's ability to modulate levels of wildtype TDP-43, we sought to determine if UBQLN2 had a similar impact on a C9FTD/ALS linked polypeptide: poly-GA DPR. Preliminary results did not reveal a significant UBQLN2 effect on poly-GA levels; however, a trend toward increased DPR with UBQLN2 knockdown versus overexpressed UBQLN2 was observed. Replicating this experiment will be necessary to attain adequate power to determine whether an actual difference exists. Given the limitation of cellular models and previously published data showing that UBQLN2 co-aggregates with poly-GA inclusions, it will be important to continue to investigate this possible relationship in vivo, using a mouse model in which poly-GA—as well as the other DPRs—are expressed via AAV delivery of expanded C9ORF72 to the CNS (May et al 2014). Poly-GA is the most common DPR in C9FTD/ALS and is therefore a useful starting point in this investigation (Freibaum and Taylor 2017). However, poly-GR and poly-PR are highly unstable due to their charged and polar arginine residues and are the most toxic DPRs (Freibaum and Taylor 2017). Poly-GR and poly-PR DPRs have been shown to associate with TDP-43 positive RNA granules indicating the need for investigation of these DPRs in the context of C9FTD/ALS (Freibaum and Taylor 2017). Expression patterns of these highly toxic proteins in the context of UBQLN2 alterations will also be explored in future work. Additionally, accumulation of DPRs is more easily observed via immunohistochemical staining, which will also be carried out in future experiments.

Human tissue samples made available to us through the Michigan Brain Bank allowed us to investigate the role of UBQLN2 in FTD tissue. We measured UBQLN2 levels in age-matched control, C9 FTD, and non-C9 FTD-TDP tissue. Though our experiment did not have the necessary power to determine with certainty, C9FTD females may have lower levels of UBQLN2 compared to all other FTD groups. This could highlight a previously unobserved phenomenon in which UBQLN2 levels differ based on sex only in the C9FTD subset of the disease. A likely contributor to our insignificant results is the inconsistency found when analyzing human tissue compared to laboratory models, as well as our small sample size of available tissue for each sex. Despite the limitations to using cell and mouse models, the ability to ensure proper control conditions for each experiment is a benefit. The unique environmental and genetic makeup of each person impact the body in many ways, adding confounding variables

that are not typically a factor for laboratory models. The level of variability we detected between male and female samples in UBQLN2 levels suggest that further study with adequate sample sizes will be needed to determine if UBQLN2 is altered in C9FTD in a sex-specific manner and highlights the need for further research on the subject. Since UBQLN2 is an X-linked gene, it is not entirely surprising that sex differences may be observed (Deng et al 2011). A possible explanation for this trend could be that UBQLN2 expression differs based on ineffective X-inactivation such that the pattern of UBQLN2 expression is potentially more highly concentrated—leading to a disruption in the stability and solubility of the protein. Because only soluble fractions were studied here, it is possible UBQLN2 is more highly concentrated in the insoluble fraction for C9FTD females compared to males as a result of ineffective X-inactivation. Future studies will investigate the levels of insoluble or aggregated UBQLN2 to further probe these potential differences.

Future Directions

In summary, there are many questions that this project has primed us to investigate moving forward such as how UBQLN2 works to regulate disease proteins *in vivo*. Primarily, we will complete further studies using C9AAV-injected UBQLN2 transgenic and knockout mouse models in order to study UBQLN2 protein regulation *in vivo*. As previously mentioned, the C9AAV-injected mouse model is useful because of its robust phenotype both biochemically and behaviorally. This model exhibits a more complete picture of disease pathology compared to classic transgenic models. Despite this powerful tool, little work has been done to identify changes in UBQLN2 levels or UBQLN2 interactions with hallmarks of C9FTD/ALS. Therefore, we sought to better understand this area in our own studies.

Throughout the course of the C9AAV project we experienced many roadblocks that can be addressed in the future. First, an unexpected breeding issue arose for the Tg UBQLN2 group that severely impacted our experimental timeline. For unknown reasons we found that Tg UBQLN2 mice were both less likely to breed than other groups and typically had smaller litters. This limited our number of injected Tg UBQLN2 mice. Conversely, the UBQLN2 KO mice bred readily and often but faced their own set of challenges. UBQLN2 KO genotyping using Sanger Sequencing turned out to be problematic and prone to contamination due to the high-volume

PCR needed to identify all UBQLN2 KO samples in the lab. Thus, genotyping for this group needed to be confirmed post mortem via Western blotting to identify samples with complete protein knockout. Unfortunately, this meant we were unable to set up homozygous knockout breeding pairs with certainty. A new genotyping protocol is in progress to reduce this inefficient breeding method. After injection of the C9AAV, many injected pups contracted encephalitis around 1-month-old, which is a common side effect of the AAV-9 injection used here (Murlidharan et al 2014). Additionally, attrition in healthy injected mice can occur due to difficulties in maternal acceptance of pups removed at P0 and returned to the breeding cage. Thus, future experiments will be completed with greatly increased sample sizes to account for attrition to fully explore UBQLN2's interactions with C9ALS/FTD pathology in vivo.

Future studies will investigate the relationship of UBQLN2 and C9FTD/ALS in great depth, expanding upon the cohort of samples I have begun to characterize here. C9AAV-injected Tg UBQLN2 and UBQLN2 KO mice will be analyzed for disease pathology biochemically via Western blot and through immunohistochemical staining, since most previous studies analyzing DPR pathology use a staining method that allows for easier visualization than in Western Blot. This further investigation will also delve into TDP-43 pathology in a robust C9ALS/FTD mouse model under different UBQLN2 expression conditions. I hypothesize that UBQLN2 KO will increase levels of both TDP-43 and DPR aggregates. I am particularly interested in studying how arginine-containing DPRs are regulated by UBQLN2 since few studies in mammalian models have investigated these polypeptide species despite being considered the largest contributor to C9ALS/FTD toxicity (Freibaum and Taylor 2017). It will be interesting to determine the relative toxic contribution of each DPR species in differing UBQLN2 contexts *in vivo* since poly-GA and poly-GP are typically more abundant but do not contribute to cell death as much as poly-GR (Chew et al 2015, Freibaum and Taylor 2017). This will help elucidate how UBQLN2 interacts with DPRs and if it associates with certain DPRs more readily. In the C9AAV model, all DPR inclusions are reported to be ubiquitin-positive, indicating the potential of UBQLN2 modulation for these aggregates through its role as a ubiquitin-proteasome shuttle protein (Chew et al 2015).

An additional area for prospective study is the role of UBQLN2 in human FTD tissue. Currently, much of the published data surrounding the relationship between UBQLN2 and ALS/FTD focuses specifically on mutated UBQLN2's ability to cause FTD/ALS or simply the presence of wildtype UBQLN2 inclusions with aggregates in disease, without further

investigation of UBQLN2's regulatory function. (Williams et al 2012, Le et al 2016, Borroni et al 2018). Thus, the role of WT UBQLN2 as a modulator in disease is much less clear and offers an area for investigation, particularly how varying levels or solubility of WT UBQLN2 in disease tissue may impact disease progression. Given our demonstration of WT UBQLN2's ability to modulate specific disease proteins like TDP-43, there is reason to probe deeper into a connection between ALS/FTD and WT UBQLN2 levels. In conjunction with the C9AAV project that will investigate this in mice, our promising human data shows a potential sex-specific difference in C9FTD UBQLN2 levels, indicating that further study is needed in this area as well. From our preliminary human tissue data, we suggest that a sex-specific difference may exist in C9FTD only based on observed levels of WT UBQLN2, with females showing potentially lower UBQLN2 levels than males. A meta-analysis of current FTD/ALS research indicates that there is a higher prevalence of females carrying the C9ORF72 hexanucleotide expansion in ALS patients, despite ALS being generally more common in males (Curtis et al 2017). However, the same meta-analysis also indicates no sex-specific differences in C9FTD patients. Despite this, research into sex-specific differences for C9ALS/FTD based on our preliminary data is still relevant given that many ALS patients are later given a comorbid FTD diagnosis. Additionally, a major limitation of the review is the lack of available sex-segregated information for the C9FTD subtype (Curtis et al 2017). This only encourages our work to study this widely uninvestigated area of sex-differences in C9FTD patients. Our data showing a potential sex-specific difference in UBQLN2 levels for C9FTD is consistent with previously reported ALS research and gives reason to better understand how UBQLN2 might play a role in this higher prevalence of female hexanucleotide repeats.

In summary, we established that UBQLN2 expression decreases levels of both WT TDP-43 and sTDP43, demonstrating UBQLN2's ability to regulate components of FTD/ALS pathology. Additionally, we demonstrated the selective nature of UBQLN2's regulatory function for intracellular disease proteins through its inability to regulate overexpressed GFP. These results highlight the importance of studying UBQLN2 in the context of FTD/ALS. Furthermore, data presented for human disease tissue prompts the investigation into a relationship between UBQLN2 levels varying by sex for the C9FTD/ALS subset. Moving forward, the relationship between UBQLN2 and C9FTD/ALS will be studied *in vivo* to better understand the function of UBQLN2 in disease regulation. Further study into disease pathology regulation by UBQLN2

will ultimately lead to an increased understanding of FTD/ALS mechanisms necessary to develop early stage intervention and disease prevention therapies.

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