Curriculum Vitae for Kathleen M. Gilpin

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Education

University of Maryland School of Medicine, Baltimore, MD

- Doctor of Philosophy, Neuroscience, 2015
- Advisor: Dr. Mervyn Monteiro
- Discovered that ubiquilin-2 binds to hnRNP proteins, but that mutations in either protein reduce that interaction. Mutations in ubiquilin-2 also lead to subcellular mislocalization and increased turnover of hnRNPA1 and increased cellular death.

Naval War College, Newport, RI

- Master of Arts in National Security and Strategic Studies, 2014
- Joint Professional Military Education certification
- Studied the politics and culture of the Korean peninsula

Holy Apostles College, Cromwell, CT

- Master of Arts in Theology and Bioethics, Expected 2015
- Certified in Catholic Bioethics through curriculum taught by National Catholic Bioethics Center (2013)
- Studying the moral theological principles of material cooperation in abortion by scientific researchers

Rochester Institute of Technology, Rochester, NY

- Bachelor of Science in Biopsychology, *cum laude*, 2005.

Fellowships

Ethics Fellow (Aug 2013-July 2014)

- Anne Arundel Medical Center, Annapolis, MD
  - Consult with physicians and patients on cases with ethical dilemmas to provide individualized, patient-centered recommendations in the Emergency Department, Intensive Care Unit, and Maternal Fetal Nursing Ward. Participated and led monthly Ethics Committee meetings.
  - Member of the Patient Advocacy Team

Ethics Fellow (June 2013-September 2014)

- University of Maryland Medical Center
  - Member of Ethics Education committee that organized conference on the pros/cons of the Maryland Medical Orders for Life Sustaining Orders (MOLST) form.

Publications

Gilpin, K. M., Chang, L., Monteiro, M. J. (2015). ALS-linked mutations in ubiquilin-2 or hnRNPA1 reduce interaction between ubiquilin-2 and hnRNPA1. *Human Molecular Genetics.* doi: 10.1093/hmg/ddv020

Gilpin, K. M. (2014) "Commentary on *In Utero* Twins and Their Common Ethical Dilemmas." *Mid-Atlantic Ethics Committee Newsletter*. Winter 2014.

Gilpin, K. F., Friel, D. M., Friel, J. J. (2015) "The Origin of Life—a Three Discipline Approach" *Submitted.*

Gilpin, K. M. (2015) "The Moral Principles Underlying Material Cooperation with Abortion for Biomedical Scientists" *Submitted.*

Abstracts

Gilpin K. M., Chang L., Monteiro M. J. Construction of a Ubiquilin-2 Interaction Network Affected in Amyotrophic Lateral Sclerosis. ASCB. Dec 2012.

Poster Presentations

Gilpin K. M., Chang L., Monteiro M. J. Construction of a Ubiquilin-2 Interaction Network Affected in Amyotrophic Lateral Sclerosis. Poster session presented at: Annual Meeting of American Society for Cell Biology; 2012 Dec 15-19; San Francisco, CA.

Gilpin K. M., Chang L., Monteiro M. J. Construction of a Ubiquilin-2 Interaction Network Affected in Amyotrophic Lateral Sclerosis. Poster session presented at: 17th Annual University of Maryland Program in Neuroscience Retreat; 2013 Jun 6; Baltimore, MD.

Academic Essays

Gilpin K. M., Friel J. J. "Evolution: How the Catholic Church and Science Coincide" Nov 2012

Laboratory Techniques

Mammalian cell culture	Protein biochemistry
Immunocytochemistry	Subcellular fractionations
Cloning	Cell death assays
Yeast two-hybrid screening	Protein purification

Research Interests

- Protein-protein interactions in neurodegenerative diseases
- Prion diseases
- Bioethics

Professional Society Memberships

- Alzheimer's Association (2009-present)
- American Association for the Advancement of Science (2009-present)
- American Society for Bioethics and the Humanities (2013-present)
- American Society for Cell Biology (2012)
- Catholic Medical Association, Baltimore Guild (2012-present)
- Catholic Medical Student Association, University of Maryland SOM (2012-present)
- National Catholic Bioethics Center (2012-present)
- US Rowing (2001-2009)

Honors

Invited Speaker

- Theology on Tap Northeast (Philadelphia) October 2012
- Theology on Tap Fells Point (Baltimore) April 2013
- Medical Grand Rounds, Anne Arundel Medical Center (Annapolis) June 2014

Awards

- Travel award from ASCB (2012)
- Travel award from University of Maryland Graduate Student Association (2012)
- Program in Neuroscience student training grant (2009-2011)
- American Association for the Advancement of Science fellowship (2009-2011; 2014-present)
- Navy and Marine Corps Commendation Medal (2012 & 2014)
- Navy and Marine Corps Achievement Medal (2007, 2009, 2010)
- RIT President's Award recipient for excellence in academics & athletics (2004 & 2005)
- Honor Graduate from University of Rochester Reserve Officer Training Corps (2005)

- Excellence in Leadership, Service, and Ethics award recipient (2003)

Extracurricular Activities

- UMB Student training committee (2009-2014)
- UMB Graduate Student Association (2011-2014)
- Graduate School Newsletter Editor-in-Chief (2012-2014)
- Maryland Alzheimer's Association Outreach Ambassador (community education) (2009-2014)
- Baltimore and Annapolis Rowing Clubs (2009-2014)

ABSTRACT

Title of Dissertation: ALS-linked Mutations in Ubiquilin-2 Reduce Interaction with Heterogeneous Nuclear Ribonucleoproteins.

Kathleen M. Gilpin, Doctor of Philosophy, 2015

Dissertation Directed by: Mervyn Monteiro, Professor, Department of Anatomy and Neurobiology

ALS-linked mutations in ubiquilin-2 and some members of the heterogeneous nuclear ribonucleoprotein (hnRNPs) family are implicated in ALS. Most mutations in ubiquilin-2 are missense mutations that occur in and around a PXX repeat motif located in the central domain of the protein. The PXX motif is not present in other ubiquilins, suggesting it might have some specialized function. However, neither the function of the PXX motif nor the mechanism by which mutations in ubiquilin-2 lead to ALS is known. The present study was designed to screen a yeast two-hybrid library using the central domain of ubiquilin-2 to identify proteins whose binding to ubiquilin-2 was affected by ALS-mutations in ubiquilin-2. Three such interactors were identified and all were members of the hnRNP family—hnRNPA1, hnRNPA3 and hnRNPU. The interacting region in each of these proteins was their C-terminal glycine rich-domain, which is the domain most frequently mutated in other RNA-binding proteins also known to be linked to ALS, such as TDP-43 and FUS/TLS. The subsequent experiments focused my studies on hnRNPA1 because a mutation (D262V) in this protein has been linked to ALS. Immunoprecipitation and

pulldown studies confirmed that ubiquilin-2 interacts with wild type (WT) hnRNPA1 proteins. However, binding of ubiquilin-2 proteins containing any of five different ALS mutations (P497H, P497S, P506T, P509S, P525S) to WT hnRNPA1 was weaker than that of WT hnRNPA1 as compared with WT ubiquilin-2. Moreover, hnRNPA1 containing the D262V mutations did not bind WT ubiquilin-2. Overexpression of the ubiquilin-2 mutants in NSC-34 cells increased cell death and, for several of the mutants, this coincided with increased translocation of hnRNPA1 to the cytoplasm. Knockdown of ubiquilin-2 decreased the cytoplasmic accumulation of hnRNPA1 protein, in part because it increased turnover of the protein. The discoveries that ubiquilin-2 interacts with hnRNP proteins and that mutations in either protein disrupt that interaction suggest a connection between proteostasis and RNA metabolism. Based on the results presented here, it is also tempting to speculate that decreased interaction between ALS-linked mutations of ubiqiuilin-2 and hnRNPA1 may contribute to the neurodegeneration characteristic of this catastrophic condition.

ALS-linked Mutations in Ubiquilin-2 Reduce Interaction with Heterogeneous Nuclear Ribonucleoproteins

By:

Kathleen Marie Gilpin

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2015

-----DEDICATION-----

To Brigid Doohan Friel
“Knowledge is no burden”

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To past and present Monteiro lab members, Dr. Janet Ugolino, Lydia Chang, Valeria Albornoz, Rebecca Danner, Dr. Laura Dosanjh, and Cara Rothenberg. Thank you for the engaging conversations, teaching me new techniques, and the level of camaraderie only achievable in a lab environment, when 90% of what you do fails.

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To my mother, Patricia Friel, whose encouragement of my endeavors never ceased, even when it involved paint chips. I am honored to be your daughter and to have grown into a strong, capable, and confident woman by following your lead.

To my siblings, Joseph & Elizabeth Friel, Christine Friel, and Father David Friel, for your Catholic witness in my life and teaching me to be a good, holy person both in and out of the lab. Thank you for always being proud of my accomplishments.

To my simply amazing husband, Seth, who deserves a PhD of his own in encouragement and patience. Our belief that a little bit of sacrifice now will pay dividends in the future has made these last three years of a long-distance marriage worth it. It is an accomplishment we can be proud of. I am the luckiest girl in the world to be your best friend and I cannot wait for all the good things to come in our future.

To God, above all else, for giving me the gifts of intelligence, determination, and courage—without which I would not be here today. Thank you for sending your Holy Spirit and His enlightenment, even though at times I thought it was slow in coming.

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AA	Amino Acid
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-isoxazolepropionic acid
ANOVA	Analysis of Variance
APP	Amyloid Precursor Protein
BSA	Bovine Serum Albumin
C9orf72	Chromosome 9 Open Reading Frame 72
cDNA	Complementary Deoxyribonucleic Acid
CHOP	Transcription Factor C/EBP Homologous Protein
CHX	Cycloheximide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ER	Endoplasmic Reticulum
ERAD	ER-Associated Degradation
fALS	Familial Amyotrophic Lateral Sclerosis
FBS	Fetal Bovine Serum

FTLD	Frontotemporal Lobar Dementia
FUS/TLS	Fused-in-Sarcoma/Translated-in-Sarcoma
GFP	Green Fluorescent Protein
GRR	Glycine Rich Region
HD	Huntington's Disease
hnRNA	Heterogeneous Nuclear Ribonucleic Acid
hnRNP	Heterogeneous Nuclear Ribonucleoprotein
IP	Immunoprecipitation
kDa	Kilodalton
LMN	Lower Motor Neuron
miRNA	Micro-Ribonucleic Acid
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
NMDAR	N-Methyl-D-Aspartate Receptor
ONPG	σ-nitrophenyl-β-D-galactopyranoside
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease

PLB	Protein Lysis Buffer
PBS	Phosphate Buffered Saline
RRM	RNA-recognition motif
sALS	Sporadic Amyotrophic Lateral Sclerosis
SD	Standard Deviation
SEM	Standard Error of the Mean
siRNA	Short Interfering Ribonucleic Acid
snRNP	Small Nuclear Ribonucleoprotein Particles
SOD1	Superoxide Dismutase-1
TDP-43	Transactive Response DNA-binding protein 43
UBA	Ubiquitin-associated Domain
UBL	Ubiquitin-like Domain
UBQLN	Ubiquilin
UMN	Upper Motor Neuron
UPR	Unfolded Protein Response
UPS	Ubiquitin Proteasome System
UTR	Untranslated Region

VCP	Valosin-Containing Protein
WT	Wild Type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Y2H	Yeast Two-Hybrid

CHAPTER 1: BACKGROUND

Section 1.1: Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that results in the loss of both upper and lower motor neurons and was first described by Jean-Martin Charcot in 1874 (Rowland, 2001) (Robberecht, 2013) (Ling, 2013). Famously, Lou Gehrig suffered from this devastating disease, which is estimated to affect one in every 50,000 people. Roughly 90-95% of ALS cases are sporadic, while the remaining 5-10% is inherited through familial gene mutations. To date, over 20 genes have been implicated in this disease (Deng, et al, 2011) (Ferraiuolo, 2011) (Renton, 2013). The most common ALS-associated genes are listed in Table 1. Regrettably, the disease is rapidly progressive and most patients are fatally paralyzed 2-5 years after diagnosis (Mehta, 2014).

The sporadic and familial forms of the disease have similar clinical manifestations that can be broken down into signs and symptoms arising from upper motor neuron degeneration, or bulbar-onset ALS, and those arising from lower motor neuron neurodegeneration, or limb-onset ALS (Rotunno, 2013) (Mulder, 1986) (Wijesekera, 2009). If only the lower motor neurons are degenerating, the condition is called progressive spinal muscular atrophy, whereas, if only the upper motor neurons are affected, the disease is termed primary lateral sclerosis (Rowland, 2001). The diagnostic criteria for ALS are met only when both upper and lower motor neurons are affected.

Table 1: List of Common Genes Researched in Conjunction with ALS (modified from Amyotrophic Lateral Sclerosis Online Genetics Database) (Abel, 2012)

Gene Name	Gene Abbreviation	Chromosomal Location
Ataxin 2	ATXN2	12q23-q24.1
Chromosome 9 Open Reading Frame 72	C9orf72	9p21.2
Chromatin Modifying Protein 2B	CHMP2B	3p12.1
Cu/Zn Superoxide Dismutase 1	SOD1	21q22.11
Fused-in-Sarcoma/Translated in Sarcoma	FUS/TLS	16p11.2
Heterogeneous Nuclear Ribonucleoprotein A1	hnRNPA1	12q13.1
Neurofilament, Heavy Polypeptide 200kDa	NEFH	22q12.1-q13.1
Optineurin	OPTN	10p13
Profilin-1	PFN1	17p13.3
Sequestosome 1	SQSTM1	5q35
TAR DNA Binding Protein	TDP-43	1p36.22
Valosin-Containing Protein	VCP	9p13
Vesicle-Associated Membrane Protein-Associated Protein	VAPB	20q13.33
Ubiquilin-2	UBQLN2	Xp11.21

Upper motor neuron (UMN) degeneration is a loss of neurons in the motor cortex. Clinical signs of UMN degeneration include alterations in muscle tone, hyperreflexia, and autonomic nervous system dysfunction such as urinary urgency (Kinsley, 2001). Lower motor neuron (LMN) degeneration refers to loss of motor

neurons innervating muscles. Clinical signs of LMN degeneration include muscle wasting, hyporeflexia, muscle cramps, and muscle twitches (Kinsley, 2001) (Handy, 2011) (Piccione, 2014). In pedigrees with no family history of ALS, the average age on onset is 56, but this age decreases by 10 years in families with histories of ALS (Kinsley, 2001). X-linked ALS, with mutations in ubiquilin-2, frequently manifests as juvenile onset. Since ALS combines signs and symptoms of both UMN and LMN degeneration, classic manifestation of the disease begins with lower extremity weakness, progresses to weakness of the upper body, and finally ends with paralysis of the breathing muscles which eventually results in death (Rubin, 2012).

In addition to motor neuron phenotypes, there is high incidence of comorbidity with frontotemporal lobar dementia (FTLD). As the name suggests, FTLD is the result of neuronal degeneration in the frontal and temporal lobes and is the most common form of dementia second only to Alzheimer's (Cardarelli, 2010) (Ferrari, 2011). The estimates of comorbidity of ALS and FTLD vary greatly in literature, but the average seems to be ~50% (Lomen-Hoerth, 2002) (Murphy, 2007) (Ringholz, 2005). Clinical symptoms of FTLD include marked changes in personality, social behavior, and decision making (Cardarelli, 1010) (Neary, 2005) (Janssens, 2013). Despite wide differences in clinical manifestation, ALS and FTLD share pathological features in that they both stain positively for ubiquitin in inclusions in upper motor neurons and for TDP-43 in inclusions in lower motor neurons (Wijesekera, 2009) (Ferrari, 2011).

Unfortunately, treatment for ALS is palliative only. There are no cures and the only drug approved for the treatment of ALS is the moderately effective riluzole, which in some cases can extend life span by a few months through delaying the need for ventilator support (Wijesekera, 2009). Some controversy surrounds the mechanism by which riluzole acts, but the prevailing school of thought is that the effectiveness of riluzole is linked to its ability to inhibit NMDA receptors in neurons (thereby reducing potential excitotoxicity) (Bensimon, 1994) (Song, 1997).

Insights into how riluzole works helped lead researchers to uncovering details regarding the etiology of ALS. The fact that riluzole inhibits NMDA receptors indicates that perhaps excitotoxicity plays a prevalent role in many ALS cases. Indeed, other lines of evidence support this notion. Motor neurons have two structural characteristics that would make them sensitive to the toxic effects of rising intracellular calcium concentrations. The first is their low calcium buffering capacity and the second is a proportionately large number of calcium-permeable AMPA receptors (Van Den Bosch, 2006). While these are undoubtedly required for the proper functioning of motor neurons in healthy controls, it is not hard to imagine how the delicate balance of calcium concentrations could be offset and would contribute to a disease-forming state. In fact, there are multiple studies that offer evidence to support this hypothesis, including one that demonstrated that ionotrophic kainic acid and metabotropic quisqualic acid receptors in motor neurons are sensitive to excessive glutamate and another that showed a portion of ALS patients have increased levels of

glutamate in blood plasma (Saroff, 2000) (Rothstein, 1993) (Andreadou, 2008). There is also evidence that patients with ALS have increased expression of glutamate synthetase in their platelets, which is the enzyme responsible for catalyzing the conversion of glutamine to glutamate (Bos, et al, 2006). Finally, another piece of evidence supporting the excitotoxicity hypothesis is that cerebrospinal fluid (CSF) from ALS patients induces cytotoxicity in cultured motor neurons, which can be blocked by NBQX, an AMPA/kainate receptor antagonist (Sen, 2005).

The second prevailing theory for the cause of ALS, unrelated to the mechanism of riluzole, is oxidative stress. Perhaps the most prominent indicator for this theory is the fact that 20% of familial ALS cases (fALS) are linked to mutations in the copper/zinc superoxide dismutase 1 gene (SOD1), of which there are over 100 (Rosen, 1993) (Banci, 2008). Interestingly, alterations in SOD1 expression levels have been found in sporadic ALS (sALS) cases as well (Gagliardi, 2010). As the first mutation discovered to cause ALS and the gene mutated in the most widely used mouse model of ALS currently available, SOD1 has advanced our understanding of the important role of oxidative stress in ALS (Kunst, 1997). In the cell, SOD1 is responsible for destroying free radicals by dismutating superoxide radicals (O_2^-) to oxygen (O_2) and hydrogen peroxide (H_2O_2) (Fridovich, 1975). Post-mortem tissue samples from both familial and sporadic ALS patients showed extensive immunoreactivity to 3-nitrotyrosine levels, which is indicative of peroxynitrite-induced oxidative stress. Correspondingly, 3-nitrotyrosine was also elevated in CSF samples from living

sporadic ALS patients (Barber, 2006) (Tohgi, 1999). The DNA of sporadic ALS patients also indicated oxidative damage as measured by 8-hydroxy-2'-deoxyguanosine (8-OHdG) in both the spinal cord and the CSF (Barber, 2006).

Naturally, these two theories are not mutually exclusive, nor do they rule out the possibility of other influential factors in the etiology of ALS. The cause and effect in these theories is also difficult to determine. Are the oxidative damage signals just a consequence of the disease or truly the cause of it? Much work remains to determine the cause of the ALS, or, more likely, the combination of causes.

Section 1.2: Link between familial ALS and genes encoding ubiquilins and RNA binding proteins

Studying the known genes/mutations of fALS permits researchers to extrapolate the data to hopefully apply it to sALS as well. This works particularly well since the sporadic and familial forms present with such similar clinical manifestations, as already discussed (Mulder, 1986) (Rotunno, 2013). Mutations in several genes involved in diverse cellular functions have been linked to familial ALS, suggesting defects in a wide variety of cell targets are linked to ALS. However, of the more than 20 known genes, many can be categorized into two large groups: those that affect RNA metabolism and those that affect protein homeostasis (proteostasis). The genes in which problems in RNA metabolism are directly or indirectly linked to ALS include chromosome 9 open reading frame 72 (C9orf72), Transactive Response DNA binding protein (TDP-43), angiogenin, fused in sarcoma/translocated in sarcoma (FUS/TLS), Senataxin, and hnRNPA1

(Renton, 2011) (Ling, 2013) (Robberecht, 2013) (Kim, 2013) (Lagier-Tourenne, 2010) (Gellera, 2007) (Hirano, 2011). The genes linked to dysregulation of proteostasis include ubiquilin-2, SQSTM1 (p62), and valosin-containing protein (VCP) (Robberecht, 2013) (Zhang, 2014) (Seibenhener, 2004) (Wojcik, 2006).

The first mutation linked to ALS was discovered in the SOD1 gene in 1993 (Rosen, 1993). For many years, no other genes were discovered in connection to ALS. Then, in 2008, TDP-43 and FUS/TLS were both discovered to be genes implicated in fALS. Together, they account for approximately 20% of all fALS cases (Renton, 2013). Both are DNA/RNA binding proteins and both have strong ties in RNA processing and metabolism (Lagier-Tourenne, 2010). Interestingly, both also have glycine rich regions in their C-terminal regions, in which there are over 40 mutations in TDP-43 and 15 mutations in FUS/TLS that are linked to fALS (Gendron, 2013) (Sun, 2014) (Corrado, 2010) (Lagier-Tourenne, 2010) (Pesiridis, 2009) (Vance, 2009) (Kwiatkowski, 2009). It is also intriguing that FUS/TLS contains an additional 16 ALS-linked mutations in its nuclear localization signal, suggesting a possible problem with nuclear import of RNA-binding proteins (Lagier-Tourenne, 2010). Two important genes linked to fALS in 2011 include C9orf72 and ubiquilin-2 (Deng, 2011) (Renton, 2011). The function of C9orf72 is unknown as yet, but there can be no denying that the C9orf72 hexanucleotide expansion $(GGGCC)_n$ will be discovered to play a crucial role in the pathogenesis of ALS as it alone has been found in 30% of fALS cases, the highest percentage of any ALS-associated gene (Renton, 2013). It accounts for nearly 4% of all ALS cases (DeJesus-Hernandez, 2011) (Renton, 2011).

Whereas healthy controls have one or two C9orf72 sequence repeats, the repeats in ALS patients can number in the hundreds (Al-Chalabi, 2013).

Most recently, a mutation in the prion-like domain of hnRNPA1 (D262V) was linked to ALS and multisystem proteinopathy, although rarely (Kim 2013) (Calini, 2013). Using a structural algorithm called ZipperDB, the research group predicted that the mutation in hnRNPA1 would induce formation of steric zippers, which are known to compose the spine of an amyloid fibril. When they tested this prediction using thioflavinT immunofluorescence, the found that the D262V mutation in hnRNPA1 did increase fibrillization and was also associated with increased inclusions. (Kim, 2013) Like TDP-43 and FUS/TLS, heterogeneous nuclear ribonucleoproteins (hnRNPs) are DNA/RNA binding proteins, mutations in which increase localization of the proteins to stress granules (cytoplasmic structures responsible for protecting stalled translational machinery and mRNA). The discovery of the hnRNPA1 mutation strengthened the potential role of RNA metabolism in the pathophysiology of ALS.

Mutations in two distinct isoforms of ubiquilin have also been linked to ALS. A mutation identified in ubiquilin-4 was recently suggested to contribute to the pathophysiology of ALS due to the inability of the mutant ubiquilin-4 to participate in macroautophagy (Yan, 2013). Moreover, six mutations in ubiquilin-2 have been identified in dominantly inherited X-linked juvenile ALS (Deng, 2011) (Fahed, 2014). Five of the known missense mutations (P497H, P497S, P506T, P509S, P525S) mutate proline residues and map to the PXX domain of ubiquiln-2 (Deng, 2011). The sixth lies just upstream of the PXX

domain (T487I) (Fahed, 2014). The mutations and the PXX domain are shown in Figure 1.

The five point mutations located within the PXX domain were discovered by studying a five generation family with ALS, including 19 affected individuals. The disease transmitted in a dominant fashion with reduced penetrance in females. There was no evidence of male-to-male transmission of the disease, so they screened the family with markers from the X chromosome and discovered the five point mutations in the ubiquilin-2 gene, which is located on the X chromosome.

Other groups have investigated ubiquilin-2 in conjunction with ALS rates in various populations, including French, French-Canadian, Irish, Italian, and the Dutch. (Millecamps, 2012) (Daoud, 2012) (McLaughlin, 2014) (Gellera, 2013) (van Doormaal, 2012). Ubiquilin-2 mutations were associated with a few cases of familial ALS among the Italians. The research group in Italy also reported that ubiquilin-2 mutations were not associated with sALS.

Familial ALS is a disease with many genes involved, but there are numerous mutations in RNA binding proteins such as TDP-43, FUS/TLS, and hnRNPA1. As will be discussed in detail in Chapter 3, I discovered an interaction between ubiquilin-2 and three RNA binding proteins of the heterogeneous nuclear ribonucleoprotein (hnRNP) family, suggesting that perhaps the theories of proteostasis and RNA metabolism converge in ALS.

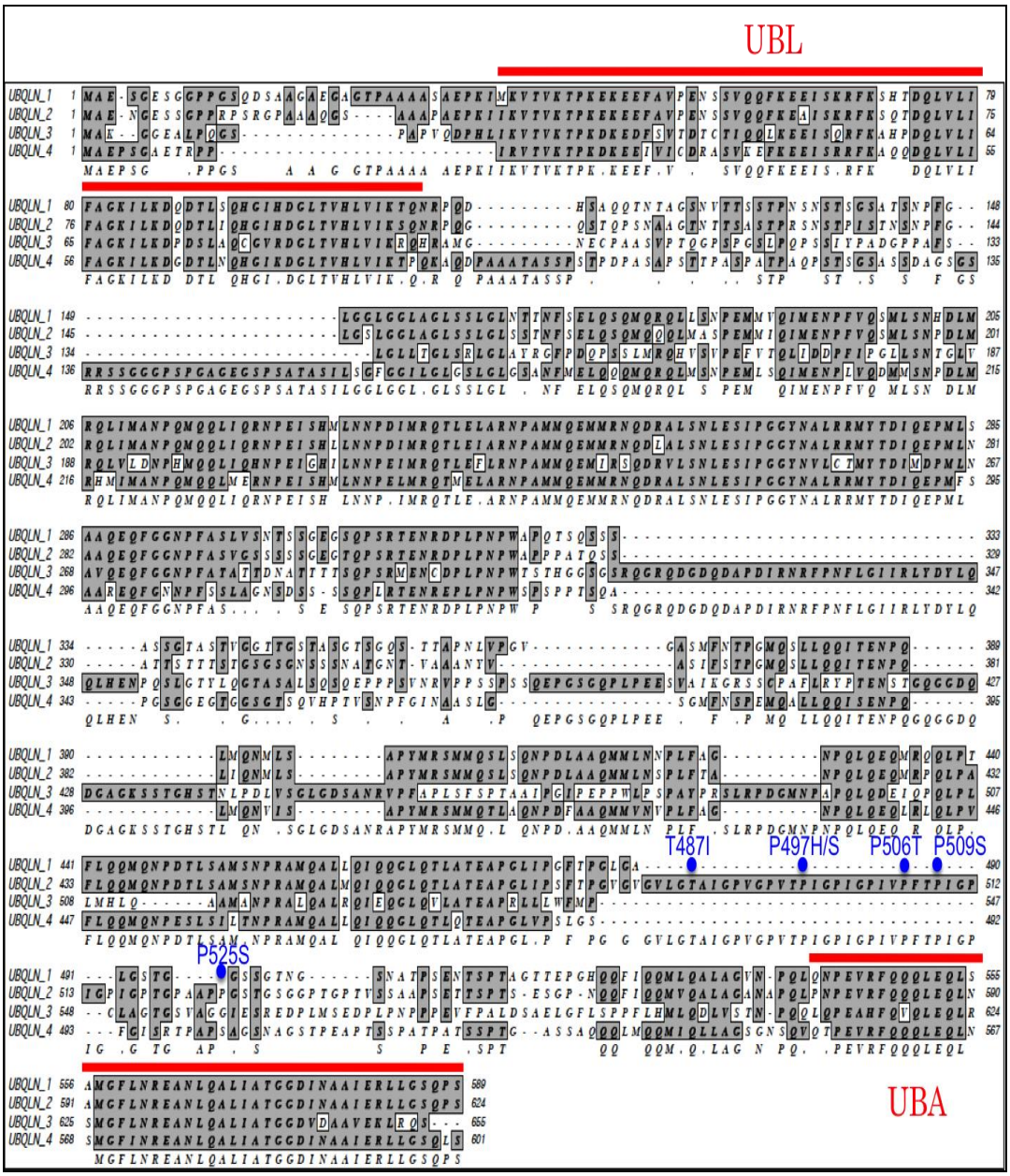


Fig 1. Comparison of ubiquilin isoforms in humans to show homology and the PXX region present only in ubiquilin-2. Ubiquilin-2 missense mutations are marked with blue dots. The N-terminal UBL domain and C-terminal UBA domain (labeled in red) bind the proteasome and poly-ubiquitin chains, respectively.

Section 1.3: Structure and function of ubiquilin proteins

Ubiquilins have been implicated in many neurodegenerative diseases, to include Alzheimer's (AD), Parkinson's (PD), and Huntington's (HD) (Mah, 2000) (Fahed, 2014) (Wang, 2006) (Doi, 2004) (Rutherford, 2013). In humans, four isoforms of ubiquilin are expressed (ubiquilin 1, 2, 3, 4). A comparison of the isoforms can be seen in Figure 1. Although other mammals express ubiquilin-5 and ubiquilin-6, they are not found in humans. Ubiquilin-1 was first discovered in 2000 and is by far the most well characterized (Mah, 2000). Isoforms 1 and 2 are ubiquitously expressed in all major tissues, with the most prominent expression in the brain, muscle, and kidneys. Isoform 4 displays reduced expression in those tissues and isoform 3 is expressed only in the testes (Conklin, 2000). Ubiquilin-1 and 2 are also well conserved among species. In particular, evolutionary conservation for both isoforms is prominent in chimpanzee, dog, cow, mouse, and rat (Williams, 2012).

Despite some differences among tissue expression, chromosome location, and conservation among species, all ubiquilins share a common domain structure. As seen in Figure 1, the proteins are ~600 amino acids long and contain C-terminal ubiquitin-like (UBL) and N-terminal ubiquitin-associated (UBA) domains that flank a more variable central domain (Ko, 2004) (Kleijnen, 2003) (Massey, 2004). While the UBL domain binds the S5a subunit of the 19S proteasome, the UBA domain binds proteins with polyubiquitin chains. In this way, ubiquilins are capable of linking proteins targeted for degradation with cellular degradation machinery (Mah, 2000) (Zhang, 2014). As one might expect

with such important domains, there is a high degree of homology of these domains among the ubiquilin isoforms (Mah, 2000) (Marin, 2014). The central domain has not been as well characterized, but it is known that there are four STI1 domains responsible for binding heat shock chaperones (Wu, 2002) (Ficklin, 2005) (Laessle, 1997).

Although the unique function of individual ubiquilin proteins is not known, there is ample evidence that indicates the proteins function in protein quality control (Mah, 2000) (Massey, 2005) (Lim, 2009) (Miller, 2005) (Elsasser, 2005) (El Ayadi, 2013). A significant part of ubiquilin's role is to regulate the turnover of misfolded proteins in endoplasmic reticulum-associated degradation (ERAD). Since the ER is the site of protein folding, proteins that improperly fold in the ER are retrotranslocated to the cytoplasm for disposal. To accomplish this process, an ER-membrane protein, erasin, recruits p97/VCP and ubiquilin to the channel opening and, once bound to the polyubiquitinated misfolded protein, ubiquilin recruits the proteasome (Lim, 2009) (Liang, 2006). This process is diagrammed in Fig 2.

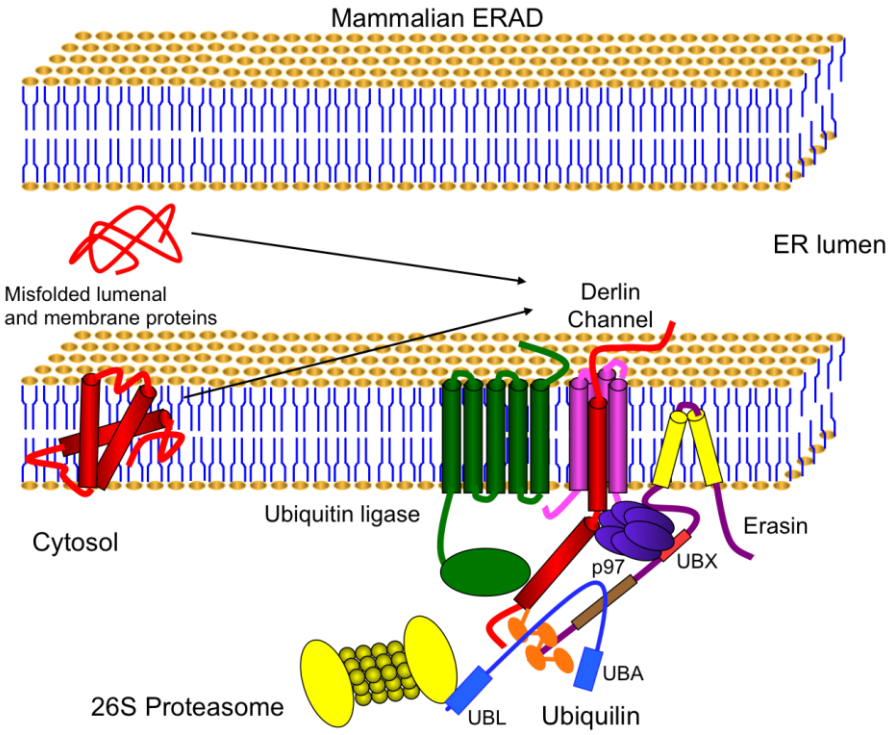


Fig 2. Model of ubiquilin's role in ERAD. Erasin forms a complex with p97 which recruits ubiquilin. Ubiquilin then links the polyubiquitinated misfolded protein to the proteasome for degradation.

The ubiquitin proteasome system (UPS) is not the only pathway in which ubiquilins function. They are also involved in autophagy (Rothenberg, 2010). Although still a process charged with degradation, autophagy differs from the UPS in that organelles are degraded in lysosomes. In other words, autophagy offers the cell a method of degrading cellular material otherwise too large for degradation by the proteasome. To accomplish autophagy, the cell must isolate the cargo targeted for degradation in a double-membrane structure called an autophagosome and deliver the autophagosome to the lysosome. There, it will fuse with the lysosomal membrane so that acid hydrolases can access and degrade

the cargo (Glick, 2010). Ubiquilin-1's involvement in macroautophagy was confirmed when it was discovered that it binds to LC3 in the cytoplasm, but that knockdown of ubiquilin-1 was associated with decreased levels of the mature form of LC3, or LC3-II (Rothenberg, 2010) (Ravikumar, 2009). This maturation and recruitment of LC3-II to the autophagosomal membrane is a hallmark of autophagy (Tanida, 2008).

One of the most promising discoveries of ubiquilin-1 is its protective effect within the cell. In two separate studies, overexpression of ubiquilin-1 was shown to be protective against mutant huntingtin proteins with polyglutamine expansions and also against starvation-induced cell death stemming from mutations in presenilin proteins (Wang, 2006) (Rothenberg, 2010).

There is some evidence that also supports the role of ubiquilin-2 in protein quality control because studies have shown that ALS-linked ubiquilin-2 mutants slow down the turnover of UPS substrates (Deng, 2011). Additionally, the interaction between mutant ubiquilin-2 and UBXD8, an endoplasmic reticulum (ER) membrane protein responsible for retrotranslocating proteins targeted for ERAD out of the ER, is impaired. Therefore, this suggests ubiquilin-2 interrupts ERAD (Xia, 2014). Both of these early findings coincide with the known functions of ubiquilin-1 (Lim, 2009) (Rothenberg, 2010). Spinal cord tissue from a patient with the T487I mutation showed that ubiquilin-2 colocalized with ubiquitin in neuronal inclusions and frequently with TDP-43 as well (Williams, 2012). The function of ubiquilin-2 is the subject of ongoing investigation in this field.

Despite all that is known about ubiquilin-1, many questions still remain. For instance, it is unknown what signals ubiquilin-1 to shift between the autophagic and ubiquilin-proteasome degradation pathways, or even if those signals are contained within the ubiquilin protein itself. Of course, understanding the precise roles that ubiquilin-1 and ubiquilin-2 play in protein degradation and general cellular functioning could lead to novel therapies to treat neurodegenerative diseases.

Section 1.4: Structure and function of heterogeneous ribonucleoproteins

The heterogeneous nuclear ribonucleoprotein family is large and structurally diverse (Martinez-Contreras, 2007). The hnRNP family is organized by letters corresponding to their size in kilodaltons (kDa) that range from hnRNPA1 (36 kDa) to hnRNPU (120 kDa). There are over 25 unique polypeptides that form complexes with small nuclear ribonucleoproteins (snRNPs) to associate with heterogeneous nuclear ribonucleic acid (hnRNA) (Pinol-Roma, 1988) (Dreyfuss, 1993) (Martinez-Contreras, 2007) (Krecic, 1999) (Dreyfuss, 1993). Some hnRNPs perform a shuttling function between the nucleus and cytoplasm, but all hnRNPs function in the nucleus at a minimum. In fact, hnRNPs are thought to be nearly as abundant in the nucleus as histones (Kiledjian, et al, 1994).

Although hnRNPs are ubiquitous proteins, their expression in different tissues varies (Kamma, 1995). For example, most hnRNPs are expressed heavily in neurons and many are expressed heavily in oocytes, but only some are expressed in muscles of the digestive tract (Kamma, 1995).

Like ubiquilins, the family of hnRNPs share a common domain structure. Consistent with their shuttling function, hnRNPs possess both a nuclear localization signal (NLS) and a nuclear export signal (NES), collectively termed the M9 domain (Siomi, 1995) (Michael, 1995) (Martinez-Contreras, 2007). The NLS does not correspond to classical nuclear localization signals and the NES permits nuclear export only in a temperature-dependent manner (Chaudhury, 2010) (Michael, 1995). Consistent with their association with RNA, each hnRNP has at least one RNA-recognition motif (RRM), with the majority of hnRNPs having two or more. Many hnRNPs also have GRRs and RGGs (arginine-glycine-glycine motifs). Specifically, hnRNPA1 has two RRMs followed by one GRR and an RGG box. hnRNP-like proteins, such as TDP-43 and FUS/TLS, also contain RRMs (Ku, 2009) (Liu, 2012). hnRNPU does not have an RRM, but is capable of binding to RNA through a RGG domain at its C-terminus (Dreyfuss, 1993). Table 2 summarizes the characteristics of hnRNPs studied throughout the remainder of the dissertation.

Table 2: Characteristics of hnRNPs Studied (Saccone, 1992) (Mori, 2013) (Modified from Dreyfuss, 1993 and Krecic, 1999)

Name	RRM Domains	Other Domains	Preferred Binding Sequence	Shuttling Capacity	Function	Chromosome
hnRNPA1	**2x**	**1x RGG 1x M9**	**UAGGG**	**Yes**	**Splicing Nuclear export Telomere Biogenesis**	**12**
hnRNPA3	**2x**	**1x RGG 1x M9**	**GGGGCC**	**Yes**	**mRNA cytoplasmic trafficking**	**2**
hnRNPU	**0**	**1x RGG 1x NLS**	**Unknown**	**No**	**Nuclear retention**	**1**

There is a variety of functions in which the hnRNPs are involved, some of which are redundant among the proteins. For example, both hnRNPA1 and hnRNPA3 bind to the *cis*-acting response element, A2RE, which is necessary for mRNA cytoplasmic trafficking (Ma, 2002) (Shan, 2003). Since the hnRNP family is large and the remainder of the dissertation will focus on hnRNPA1, hnRNPA3, and hnRNPU, this discussion will similarly pertain to only those three proteins.

By virtue of being the most studied, hnRNPA1 has the most defined roles. This protein plays a key role in alternative splicing. The alternative splicing of the transcripts of G-protein α, amyloid precursor protein (APP), fibroblast growth factor 2, cyclin D1, insulin receptor gene, and apoptotic peptidase activating factor-1 (apaf-1) have all been shown to be regulated by hnRNPA1 (Pollard, 2002) (Donev, 2007) (Bonnal, 2005) (Talukdar, 2011) (Jo, 2008). A common way that hnRNPA1 regulates alternative splicing is by modulating the 5' splice site. Under the right conditions, such as low pyrimidine content upstream of the 5' splice site, hnRNPA1 promotes the skipping of short exons (Mayeda, 1993). The hnRNPA1 ortholog, hrp48, binds to a P-element-specific inhibitor and U1 snRNP to form a complex, thus sterically prohibiting spliceosome formation at the 5' splice site (Siebel, 1994). Approximately one half of all hnRNPs have been shown to be involved with alternative splicing or have had this function proposed for them, including hnRNPU (Martinez-Contreras, 2007) (Xiao, 2012). Notably, hnRNPA3 has yet to be implicated in alternative splicing (Martinez-Contreras, 2007).

Most hnRNPs also serve as shuttles to and from the nucleus. Before hnRNA is processed to mature mRNA, hnRNPs and snRNPs form a complex with the newly formed hnRNA. This association serves two roles. The first is that it prevents the hnRNA from degrading (Pinol-Roma, 1992). The second is to shuttle mRNA from the nucleus to the cytoplasm where it can be safeguarded in stress granules (Dreyfuss, 1993) (Guil, 2006) (McDonald, 2011). hnRNA form abundant complexes with specific nuclear proteins in order to organize nascent RNA transcripts, regulate gene expression, and export mRNA from the nucleus to the cytoplasm (Krecic, 1999) (Swanson, 1995) (Swanson, 1988) (Weighardt, 1995).

Whereas hnRNPU is localized only to the nucleus, hnRNPA1 is capable of shuttling between the nucleus and cytoplasm (Pinol-Roma, 1991). Although predominantly located in the nucleus, hnRNPA1 localizes to the cytoplasm under conditions of stress (Chaudhury, 2010) (Pinol-Roma, 1992). Subcellular localization of hnRNPA1 can also be regulated by p38 MAP kinase signalling pathways (Shimada, 2009). Pinol-Roma and Dreyfuss concluded hnRNPA1 shuttles actively between the nucleus and cytoplasm (Pinol-Roma, 1991). The presence of hnRNPA1 and other hnRNPs in the cytoplasm, although less studied, suggests they function in RNA metabolism, but it is not yet known how.

Despite being continuously studied for more than two decades, new functions for hnRNPs continue to be discovered. As such, it is important to consider the possibility that the implications of the interaction between hnRNPs and ubiquilin-2 outlined in this dissertation could very well involve one of the roles not presently known.

Section 1.5: Role of proteostasis in neurodegenerative diseases

The proper functioning of a protein depends heavily on its conformation and misfolded proteins that are not properly degraded run the risk of damaging cellular processes, thereby causing cell death. The cell, therefore, has a difficult and crucial task of protein maintenance through a process called protein homeostasis, or proteostasis.

Among the multitude of risk factors for neurodegenerative diseases, age remains the best predictor by far (Douglas, 2010). Unfortunately, processes in proteostasis begin to break down with an increase in age. Some clues that proteostasis is extremely influential in age-related diseases comes from the involvement of proteins like heat shock proteins and several transcription factors, which have been shown both to promote longevity and to play a role in maintaining proteostasis (Morley, 2003) (Ogg, 1997) (Greer, 2005) (Morley, 2002) (Hsu, 2003).

Defined as the competition between protein folding and degradation, protein quality control is a significant part of proteostasis (Balch, 2008). In neurodegenerative diseases such as Parkinson's, Huntington's, and Alzheimer's

disease are diseases in which misfolded proteins aggregate without successful clearance by the cells and thus cause proteotoxicity (Balch, 2008) (Morley, 2002). The toxic aggregates disrupt normal cellular processes. The mechanism by which these aggregates cause toxicity is not well understood, but discovering ways to maintain effective proteostasis despite old age is an area of research capable of providing relief to millions of people.

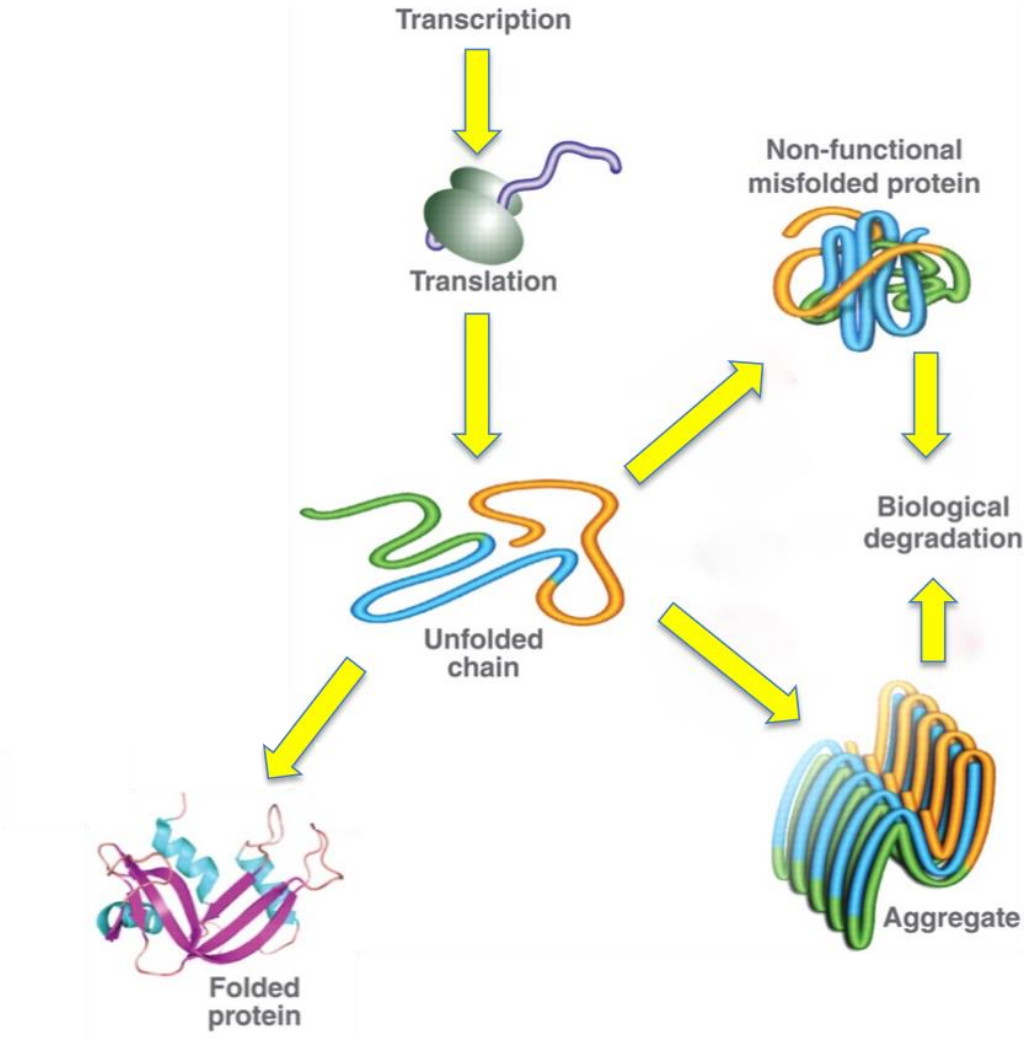


Fig 3. Proteostasis network (Modified from Balch, 2008)

As the DNA is transcribed and the mRNA translated, the result is usually a properly folded protein that either begins its function immediately or continues being processed for post-translational modifications. However, mistakes can occur anywhere in that chain of events and, if they go uncorrected, the result can be a misfolded protein. Once the cell identifies a misfolded protein, attempts are made to degrade it and recycle the components. The degradation of misfolded proteins relies on the ubiquitin proteasome system and macroautophagy, as discussed in Section 1.3, but the degradation can go awry when the misfolded proteins aggregate (Fig 3).

Much work remains in this field. It would be extremely beneficial to figure out why aggregates cannot be cleared, if aggregates somehow signal cell death, and if an exogenous process could disband the aggregates, thus permitting them to be cleared.

Section 1.6: Role of RNA in neurodegenerative diseases

Traditionally, progressive neurodegeneration in numerous diseases has been associated with aberrant accumulation of misfolded proteins. In recent years, though, increasing evidence suggests RNA metabolism malfunctions are involved in the pathophysiology of neurodegenerative disorders. In Huntington's disease, for example, trinucleotide repeat expansions in the coding regions of RNA are translated into a protein with abnormal lengths of glutamine (Huntington's Disease Collaborative Group, 1993). Even in non-coding regions of the RNA, though, gain-of-function toxicity can occur. A problem of

untranslated region (UTR)-binding proteins has been linked to diseases with triplet repeats such as fragile X tremor ataxia syndrome and myotonic dystrophy (Gallo, 2005). Also, when the 3'-UTR of the mRNA that encodes the light neurofilament protein is overexpressed, mice develop motor neuron disease (Nie, 2002). This is an important finding because accumulation of neurofilament in dying motor neurons has been observed in patients suffering from sporadic or familial ALS with SOD1 mutations (Bruijn, 2004).

The UTR is not the only source for thinking RNA is involved in neurodegenerative diseases. Since so many RNAs are subjected to alternative splicing, it is logical that aberrant splicing might contribute to disease. To confirm a problem with alternative splicing of mRNAs in ALS, a study was done with a neuronal intermediate filament protein called peripherin. Two of the three alternative splice variants are normal isoforms, whereas the third, Per 61, is not and is expressed in brains of mutant SOD1 mice, which suggests the possibility that SOD1 plays a role in alternative splicing of peripherin genes (Gallo, 2005).

Finally, the down regulation and up regulation of non-coding RNA like microRNA (miRNA) offers the cell an opportunity to regulate gene expression at the post-transcriptional level. miRNAs have been linked to AD because they can induce tau phosphorylation and increase levels of amyloid precursor protein (APP), to PD because they regulate the expression of α-synuclein, and to HD because they impair transcription throughout the brain (Banzhaf-Strathmann, 2014) (Bicchi, 2013) (Bian, 2011) (Rege, 2013) (Buckley, 2010). In ALS, miRNAs have been shown to play multiple roles throughout the progression of

the disease. In the beginning stages, miRNA machinery degrades and processed miRNAs are no longer present. When the machinery is deleted in a mouse model, the mice die from an aggressive form of ALS (Morel, 2013). Towards the end of the disease, miRNAs are responsible for the down regulation of glutamate transporter (GLT1) expression (Morel, 2013). Interestingly, RNA-binding proteins TDP-43 and FUS/TLS are required for the biogenesis of some miRNAs (Buratti, 2010) (Morlando, 2012).

We know the end result is degeneration of nervous system tissue, but the link between RNA and neurodegeneration is not clear-cut. Several hypotheses are under investigation though. Transcriptional regulation, mRNA splicing, RNA-binding protein distribution, and signal transduction pathways are all potential mechanisms through which disruption in RNA processing could influence neurodegeneration (Todd, 2010).

Section 1.7: Goals and rationale

Since the five point mutations in the PXX domain of ubiquilin-2 provide such strong evidence of the protein's involvement in ALS, an important, unresolved question is how the mutations in ubiquilin-2 ultimately lead to ALS. Thus the overarching hypothesis for testing this question was that **mutations in ubiquilin-2 which are known to cause amyotrophic lateral sclerosis disrupt normal protein interactions, thereby increasing cell death.**

In specific aim one, it was prudent to search for other proteins with which ubiquilin-2 interacted to arrive at a better understanding of ubiquilin-2's function in ALS. To accomplish this aim, a yeast-two hybrid screen of a human

complementary DNA (cDNA) encoded library was conducted in order to build a network of proteins which interacted with ubiquilin-2. After discovering ubiquilin-2 interacts with the GRR of three distinct members of the hnRNP family, those interactions were further characterized through a series of biochemical assays. The site of interaction was important because the GRR is the putative site of protein-protein interaction in other RNA binding proteins like TDP-43 and FUS/TLS (Buratti, 2005). The identities of proteins known to interact with the GRR of the RNA binding proteins are poorly understood and this research project is the first to uncover the link between the GRR and ubiquilin-2. The binding between ubiquilin-2 and hnRNPs was confirmed using GST-pulldowns and co- immunoprecipitations.

Specific aim two centered on elucidating the biological relevance of the ubiquilin-2-hnRNP interaction. One of the main functions of hnRNPs is to shuttle mRNA between the nucleus and cytoplasm, and since ubiquilins are known to be present in both compartments as well, it stood to reason that ubiquilin-2 could be playing an important role in this shuttling process and that perhaps the mutations were interfering with ubiquilin-2's normal role. Therefore, after characterizing the interaction, the research shifted focus to assessing the impact of ubiquiln-2 mutations on the subcellular distribution of hnRNPs using subcellular fractionations. There were subtle differences in the subcellular localization of hnRNPA1 in cells transfected with WT and mutant ubiquilin-2, but none reached statistical significance.

Since the biochemical experiments in specific aim one indicated that the mutations caused reduced binding when compared to WT ubiquilin-2, ubiquilin-2 was knocked down to mimic the reduced binding effect. The concomitant reduction in levels of hnRNPA1 indicated ubiquilin-2 played a role in the stability of hnRNPA1, so hnRNPA1 turnover following ubiquilin-2 knockdown was also measured after the addition of cycloheximide to stop protein synthesis. hnRNPA1 turned over more quickly under conditions of ubiquilin-2 knockdown as compared with scramble control conditions.

Since hnRNPA1 is involved in so many functions throughout the cell, cell viability was tested under the supposition that the instability of hnRNPA1 in cells transfected with mutant ubiquilin-2 would negatively impact the cell. Indeed, abnormal nuclear morphology cell death assays displayed an increased percentage of cell death in cells transfected with mutant ubiquilin-2.

CHAPTER 2: MATERIALS AND METHODS

Yeast Two-Hybrid

The yeast two-hybrid (Y2H) procedure described previously was used (Stabler, 1999) and takes advantage of the modular nature of transcription factors. Three yeast plasmids, each of which is deficient in one amino acid required for growth, were used to grow the bait and prey proteins. The bait was fused to the DNA-binding domain of LexA in a pEG202 vector encoding histidine under an ADH1 promoter. The prey was galactose inducible and was fused to the B42 transcriptional activation domain (encoded by *E. coli* but capable of activating transcription in yeast) in a pJG4-5 vector encoding tryptophan under the Gal1 promoter (Ma, 1987) (Brent, 1985). The pSH18-34 vector contained the uracil selecting marker and encoded the reporter gene, LacZ, whose expression is under the control of the LexA operator. In this system, the central domain of WT ubiquilin-2 (amino acids 108-556) was used as the bait protein, a human fetal brain cDNA library was used as the prey, and the downstream reporter genes were leucine and β-galactosidase. The PCR primers for WT ubiquilin-2 were:

5’ GGCACTGTTCGAATTCCCTCAGGGCCAGTCCACGCAG 3’

5’ GCCTGACATCCTCGAGTCAGTTGGGTCCAGATTCTGATGT 3’

The resulting fragment was double digested with EcoRI and XhoI (sites underlined in the PCR primers above) and ligated to the corresponding sites of the pEG202 LexA fusion plasmid. When the three plasmids were transformed into EGY48 yeast lacking uracil, histidine, and tryptophan, they allow for selection of clones in which the bait and prey proteins interact on –U-H-W-L plates. The

auto-transactivation assay was performed to ensure that the reporter genes were activated only when the bait and prey were present. Interactors based on growth in the absence of leucine and which displayed a deep blue color when grown on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) plates were selected for sequencing. X-gal concentration was 20 mg/mL. Prey plasmids were isolated from the clones using an RPM yeast isolation kit (MP Biomedicals, Santa Ana, CA) and the DNA sequence of their cDNA inserts was determined by the Biopolymer-Genomic Core Facility at the University of Maryland, Baltimore. The sequences were compared with entries in the GenBank/EMBL/DDBJ databases using the BLAST search program. Sequences with homology were aligned using MacVector Software (MacVector Inc, Cary, NC) to identify the overlap between the cDNAs and the corresponding full-length open reading frame that were retrieved from the BLAST search. Candidate clones were rescreened for their strength of interaction, in triplicate, with each of the baits mentioned below using liquid culture assays by the enzymatic conversion of σ-nitrophenyl-β-D-galactopyranoside (ONPG) to o-nitrophenol and D-galactose by β-galactosidase. The baits used were: the original WT ubiquilin-2 bait, and five identical baits to it, but containing the P497H, P497S, P506T, P509S or P525S ALS mutations. Since false positives are a risk in Y2H experiments, an empty vector and an unrelated lamin C bait were also included.

Lithium Acetate Transformation for Yeast

Yeast cells were grown in dropout medium overnight (425 mL distilled water, 3.35 g yeast nitrogen base without amino acids, 1 g dropout powder, 2% carbon source (glucose or galactose/raffinose), 1x amino acids) at 30°C with gentle shaking. Cultures were diluted 1:10 for 4-5 h at 30°C with gentle shaking until the OD_{600} was approximately 0.8. Cultures were centrifuged at 3500 rpm for 15 min. The supernatant was discarded and the pellet was resuspended in sterile water and washed three times. Cells were resuspended in 1X LiAc/TE (Tris + EDTA) transformation buffer. 1 µg DNA, 80 µg carrier DNA, 70 µl dimethyl sulfoxide (DMSO), 100 µl of competent yeast (KC8 cells), and 600 µl of 40% PEG4000 were added to the resuspended cells, vortexed, and incubated for 30 min at 30°C with gentle shaking. Cells were then heat shocked for 15 min at 42°C, followed immediately by incubation on ice for 2 min. Cells were centrifuged at 10,000 rpm for 30 s. The supernatant was discarded and the pellet resuspended in 500 µl of sterile water. 100 µl of the cells were spread onto plates lacking the appropriate auxotrophic marker and incubated at 30°C for 3-4 days.

β-galactosidase Liquid Culture Assays

Overnight cultures (~16 h) of yeast transformations (WT and mutant ubiquilin-2 baits in pEG202 vector and hnRNPs in pJG4-5 vector) using glucose as the carbon source were grown at 30°C with gentle shaking. The cultures were vortexed, 2 ml were diluted into 8 ml of selective media with galactose as carbon source and were incubated at 30°C with gentle shaking until the OD_{600} was

between 0.5 and 1.0. OD_{600} was recorded for 1 ml of culture. The remainder of the culture was aliquoted in triplicate, centrifuged for 10 mins at 13,000 rpm, resuspended in Z-buffer (16.1g $Na_2HPO_47H_2O$, 5.5g $NaH_2PO_4H_2O$, 0.75g KCl, 0.246g $MgSO_47H_2O$) and frozen at -80°C overnight. On the following day, frozen cultures were subjected to 4x cycles of freeze/thawing in liquid nitrogen and at 37°C, respectively, to lyse the cells. 700 µl of Z-buffer + β-mercaptoethanol were added to each reaction tube. 160 µl of ONPG in Z-buffer was then added to the reaction tubes, which were monitored in a 30°C incubator until the solution turned yellow. The reaction was stopped by changing the pH with the addition of 1M Na_2CO_3 and the elapsed time was recorded. Control was a tube with Z-buffer alone. Cultures were centrifuged for 10 min at 14,000 rpm, the OD_{420} was measured and recorded, and the β-galactosidase units were calculated.

Immunoprecipitations

HeLa cells were transfected with WT and mutant ubiquilin-2 constructs using Lipofectamine LTX (Life Technologies, Grand Island, NY), washed in 1x phosphate buffered saline (PBS), and collected in RIPA buffer (0.2% NP40, 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM Peflabloc, 5 µM Leupeptin). Lysates were sheared with a 25-gauge needle and centrifuged at 5000 rpm for 5 min. Supernatant was blocked by adding it to Protein A/G sepharose bead slurry (GE Healthcare, Little Chalfont, United Kingdom) and rotated for 30 min. The lysates were recovered by centrifugation at 3000 rpm for 5 min and the

supernatant was incubated with 5µl of antibody together with fresh Protein A/G beads for 1.5 h. All incubations were carried out at 4°C with gentle rotation. The beads were recovered by centrifugation and washed five times with RIPA buffer. The proteins were eluted from the beads by incubation with SDS-sample buffer at 100°C for 5 min. Equal portions of the eluted supernatants were typically separated by SDS-PAGE and immunoblotted for different proteins.

Immunoblotting

Cells were collected in our standard protein lysis buffer (0.5% SDS, 0.5% NP40, 0.5% sodium N-lauroylsarcosine, 50 mM Tris pH 6.8, 150 mM NaCl, 20 mM EDTA, 1 mM EGTA, 25 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM Pefabloc (AEBSF, Boehringer Mannheim), 1 mM leupeptin, and 1 mM aprotonin) and sheared by passing the lysates 20 times through a 25-gauge needle. Protein concentrations were determined by the bicinchonic acid assay (Thermo Fisher Scientific, Waltham, MA). Lysates were prepared with SDS sample buffer and heated for 5 min at 100°C prior to loading. Equal amounts of protein were separated on either 8.5 or 10% SDS-PAGE gels and the proteins then transferred onto a 0.45 µm PVDF membrane (Millipore, Billerica, MA) for 3 h at 200 mA using the Mini-Trans Blot cell system (BioRad, Berkeley, CA). The membranes were then incubated with appropriate primary antibodies. The following primary antibodies were used: mouse monoclonal antibodies to hnRNPA1, hnRNPU (both from Santa Cruz Biotech., Santa Cruz, CA), ubiquilin (Invitrogen, Grand Island, NY), HIS (GE Healthcare), lamin B (MatriTect, Cambridge, MA), Myc (prepared

in-house) rabbit antibodies to HA, GST, and ubiquilin-2 (all generated in-house), caspase-3 (Cell Signaling Technology, Beverly, MA), Tom20 (BD Biosciences, San Jose, CA), and goat antibodies to actin (Santa Cruz). All primary antibodies were used at a dilution of 1:1000 except for ubiquilin-2, which was used at 1:250. Binding of primary antibodies was detected by incubation with appropriate secondary horseradish peroxidase-conjugated antibodies using the SuperSignal West Pico system (Thermo Fisher Scientific) and the chemiluminescence signal was captured using the Fluoro-Chem M imager (Protein Simple, Santa Clara, CA). The following secondary antibodies were used: goat anti-mouse (Thermo Fisher Scientific), goat anti-rabbit (Pierce Biotechnology, Rockford, IL), and rabbit anti-goat (Pierce Biotechnology). All secondary antibodies were used with a 1:3000 dilution. The intensity of different bands was quantified using AlphaView software (Protein Simple).

Cell Culture, Immunocytochemistry, and Cell Death Assays

HeLa and NSC-34 cells were maintained at 37°C in Dulbecco's Modified Eagle Media (DMEM) with 4.5g/l glucose, L-glutamine, and sodium pyruvate (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO) with 5% CO_2. Cells were transiently transfected with plasmid DNA using Lipofectamine LTX (Life Technologies) for 5 to 6 h in Opti-Mem reduced serum medium (Life Technologies). For cellular stress experiments, HeLa cells were treated with sorbitol for 2 h at a final concentration of 600 mM. For cell death quantification, HeLa cells were incubated with

Hoescht 33342 at a concentration of 1 µg/mL for 15 min. Images were then captured using a Leica DM IRB microscope using a Leica PL APO 10X lens. Quantification of cell death was performed using iVision-Mac software (BioVision Technologies, Exton, PA) based on abnormal nuclear morphology (Ugolino, 2011). The results from this protocol were confirmed using trypan blue assays. In these assays, HeLa cells were transfected with WT or mutant ubiquilin-2 constructs. 24h later, media was collected, cells were harvested through centrifugation, and 0.4% trypan blue stain solution in 1x PBS. Resuspended cells were vortexed before filling a hemocytometer and counting the cells with dark blue staining (Janicki, 1997).

For analysis of siRNA-mediated knockdown of ubiquilin-2, NSC-34 cells were transfected with 20 nM SMARTpool siRNAs (GE Healthcare, Dharmacon, Lafayette, CO) designed to specifically target mouse ubiquilin-2 using DharmaFect 1 reagent (Thermo Fisher Scientific) for 24, 48 and 72 h. Following these times, the cells were lysed and processed for immunoblotting as described above. Mock and scrambled siRNA transfections were conducted in parallel and lysates from these cultures were collected at the 72 h time point.

Subcellular Fractionation

HeLa cells were collected in 1x PBS, centrifuged for 2 min at 10,000 rpm, and re-suspended in 500 µl HB buffer (10 mM Tris pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 protease inhibitor cocktail pellet, 5 mM NEM) for 15 min on ice. Triton X-100 was added to a final concentration of 0.2 % before vortexing and

centrifuging for 10 min at 1000g. The cytoplasmic fraction (supernatant) was collected and adjusted to a final salt concentration of 200 mM. The pellet was resuspended in 250 µl of buffer C (10 mM Tris pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 protease inhibitor cocktail pellet, 5 mM NEM, 400 mM NaCl, 0.4% Triton X-100), vortexed every 5 min at 4°C for 30 min total, and then centrifuged for 15 min at 20,000g. The soluble nuclear fraction (supernatant) was collected and diluted with equal volume of HB buffer. The remaining pellet (insoluble nuclear fraction) was re-suspended in 500µl of our standard protein lysis buffer and sheared using a 25-gauge needle. Samples were mixed with SDS sample buffer and heated for 5 min at 100°C. Equal volumes of the three fractions were then separated by SDS-PAGE and proteins were detected by immunoblotting.

Cloning and bacterial expression of proteins

Full-length human WT ubiquilin-2 and human WT ubiquilin-1 cDNAs were cloned in-frame downstream of GST. The recombinant plasmids were transformed into *Escherichia coli* BL21(DE3) and the fusion proteins were induced with 0.4 mM IPTG for 4 h at 37°C. HIS-tagged hnRNPA1 protein was expressed in bacteria following cloning of the entire human hnRNPA1 isoform A open-reading frame (320 amino acids) in the pET-21a vector under the T7 promoter (Novagen, Billerica, MA). The ALS-linked D262V hnRNPA1 mutant was generated by site-directed mutagenesis of the resulting plasmid using standard PCR procedures. The mutation was verified by DNA sequencing. The plasmids were transformed into *E. coli* BL21(DE3) and protein expression was

induced with 1.0 mM IPTG for 4 h at 37°C. Bacteria were lysed by sonication and frozen at -20°C until needed.

HIS-tag protein purification

Bacterial pellet containing induced HIS-hnRNPA1 protein was thawed, resuspended in lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol, 10 mM imidazole, 0.1% NP40), and sonicated at 30% output two times for 2 min. The lysate was centrifuged at 35,000 rpm for 1 h at 15°C in the 70Ti rotor. The supernatant was collected and incubated with pre-equilibrated nickel-NTA agarose beads for 4 h at 4°C with gentle rotation. The mixture was loaded onto a 10 ml column (Pierce Biotechnology), washed with 2 column volumes of Buffer A (50 mM Tris pH 8.0, 350 mM NaCl, 10% glycerol, 30 mM imidazole) and 2 column volumes of Buffer B (50 mM Tris pH 8.0,10% glycerol, 30 mM imidazole) and the bound HIS-tagged proteins were eluted with 50 mM Tris pH 8.0,10% glycerol, 250 mM imidazole. The eluted proteins were dialyzed against 20 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol buffer and frozen in small aliquots at -80°C.

GST-ubiquilin purification and GST-pull-down assays

Bacterial pellets containing induced GST-ubiquilin fusion proteins under the lac promoter were thawed, resuspended in lysis buffer (20 mM Tris pH 8.0, 350 mM NaCl, 10% glycerol, 1 mM EDTA), and sonicated to reduce their viscosity. The lysates were centrifuged at 35,000 rpm for 1 h at 4°C in the 70Ti

rotor and the resulting supernatants incubated with pre-equilibrated glutathione agarose beads (Sigma) for 2 h at 4°C with gentle rotation. The mixture was poured onto a column and after extensive washing the GST proteins were eluted from the column with 20 mM Tris 8.0, 10% glycerol, 1 mM EDTA, and 10 mM glutathione. The purified GST-fusion proteins were dialyzed in buffer composed of 20 mM Tris pH 8.0, 150 mM NaCl, and 10% glycerol for 3 h at 4°C. Purified GST-alone, GST-ubiquilin-1, and GST-ubiquilin-2 were stored until needed at -80°C. Equal amounts of each GST purified protein (4 µg) were incubated with the same amount of different ligands (either ^{35}S-methionine *in vitro* transcribed and translated hnRNPA1 or hnRNPU proteins, or HIS-hnRNPA1, or HIS-hnRNPA1 containing the D262V mutation) in 800 µl 2% Chaps buffer (20 mM Tris pH 8.0, 150 mM NaCl, 2 mM $MgCl_2$, 10% glycerol, 2.0% Chaps). 70 µl of equilibrated glutathione agarose beads were added and the proteins were incubated for 2 h rotating at room temperature. The mixture was centrifuged again at 5000 rpm for 5 min and the supernatant was discarded. The beads were washed four times with 1x PBS with 2% Tween-20 and 200 mM KCl and one time with 1x PBS, re-suspended in the SDS sample buffer, and heated for 5 min at 100°C. Equal volumes of the pulldown assays were separated by SDS-PAGE and proteins were detected by immunoblotting or, in the case for detection of ^{35}S-labeled proteins, the gel was dried and subject to phosphoimage analysis.

Analysis of protein turnover

NSC-34 cells were transfected with 20 nM siRNAs designed to specifically target mouse ubiquilin-2 or a non-targeting scramble siRNA using DharmaFect 1 reagent for 72 h. Cycloheximide was added to the cultures to a final concentration of 100 μM to inhibit new protein synthesis and protein lysates were collected at time points of 0, 2, or 4 h thereafter. The cells were lysed and processed for immunoblotting as described above. Equal amounts of protein in lysates from the different time points were separated by SDS-PAGE. The rate of turnover was calculated from at least three independent experiments and Microsoft Excel was used to draw a line connecting the average values through each time point after normalization to zero hours.

In-vitro transcription and translation

The same procedure as described previously (Lim, 2009) was used to prepare *in vitro* transcribed and translated ^{35}S-labeled hnRNPA1 and hnRNPU proteins. The plasmid template used for this synthesis contained cDNAs encoding the complete open-reading frame for each of the hnRNP proteins.

Statistical Analysis

Experiments were performed a minimum of three times except where otherwise indicated. Student's t-test and ANOVAs with post-hoc tests were performed using Microsoft Excel and GraphPad Prism software.

CHAPTER 3: CHARACTERIZATION OF THE INTERACTION BETWEEN UBIQUILIN-2 AND hnRNPA1

Published: **Gilpin, K. M.,** Chang, L., Monteiro, M. J. (2015). ALS-linked mutations in ubiquilin-2 or hnRNPA1 reduce interaction between ubiquilin-2 and hnRNPA1. *Human Molecular Genetics.* doi: 10.1093/hmg/ddv020

Section 3.1: Ubiquilin-2 interacts with hnRNPs

Since the six ALS-linked missense mutations of ubiquilin-2 map to the central domain of the protein, a yeast two-hybrid screen was conducted using the entirety of ubiquilin-2's central domain as the bait protein based on the postulation that the central region binds proteins and that the mutations interfere with those interactions. A human human brain cDNA library was used as prey to screen for proteins that bound to ubiquilin-2. The UBA and UBL domains were purposefully excluded from the screen because isolation of ubiquitinated proteins or subunits of the proteasome was out of the scope of present work.

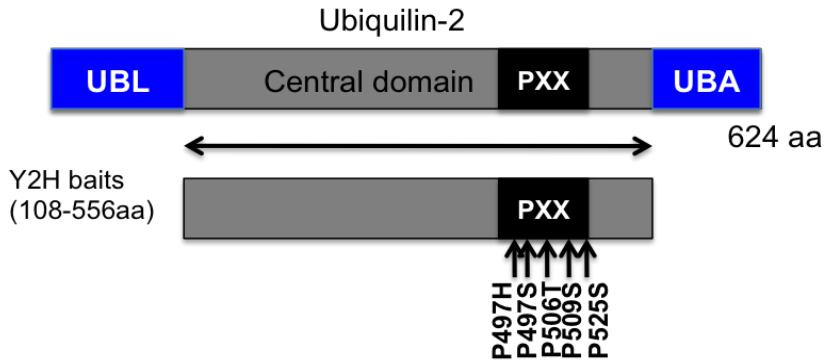


Fig 4. Ubiquilin-2 baits used in yeast two-hybrid. (A) Schematic of human ubiquilin-2 (top) and the baits used for yeast two-hybrid studies (Y2H) (bottom). Six bait proteins were generated in total with one containing the WT sequence and five carrying either the P497H, or P497S, or P506T, or P509S, or P525S mutations in the PXX domain.

The Y2H system takes advantage of the fact that transcription factors are modular in nature and can activate downstream reporter genes if the transcription factor's activation domain and the binding domain are in close enough proximity to each other. Since the bait protein (ubiquilin-2's central domain) is fused to the binding domain and the prey protein (cDNA-encoded library) is fused to the activation domain, the transcription factor can activate transcription of the two reporter genes, leucine and β-galactosidase. Selection of yeast colonies occurs through a series of steps using different vectors, which are designed with auxotrophic marker genes. The Y2H system utilizes leucine and β-galactosidase as reporter genes, thereby allowing selection of yeast colonies for further characterization on the basis of growth in the absence of leucine and the presence of dark blue color when grown on plates that incorporated X-gal, which is one of the substrates of β-galactosidase (on plates with galactose/raffinose as the carbon source) (Fig 5).

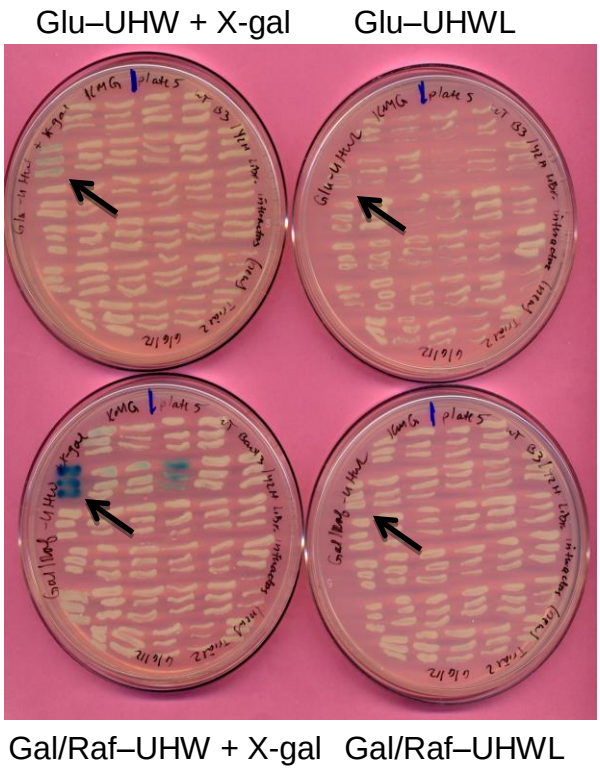


Fig 5. Yeast two-hybrid selection criteria plates. Plates showing interactors that were selected for further characterization (an example is indicated by the arrows) are those that had robust growth on medium lacking uracil (U), histidine (H), tryptophan (W), and leucine (L) and which displayed a dark blue color in the presence of X-gal with galactose/raffinose (Gal/Raf), but not glucose (Glu), as the carbon source.

Plasmid DNAs recovered from the colonies that best met the selection criteria of the reporter genes were submitted for sequencing to the University of Maryland's core sequencing facility. Subsequent BLAST searches on the 41 sequences returned enabled identification of proteins that interact with ubiquilin-2. The hits were then organized into an interaction network based on cellular function (Fig. 6).

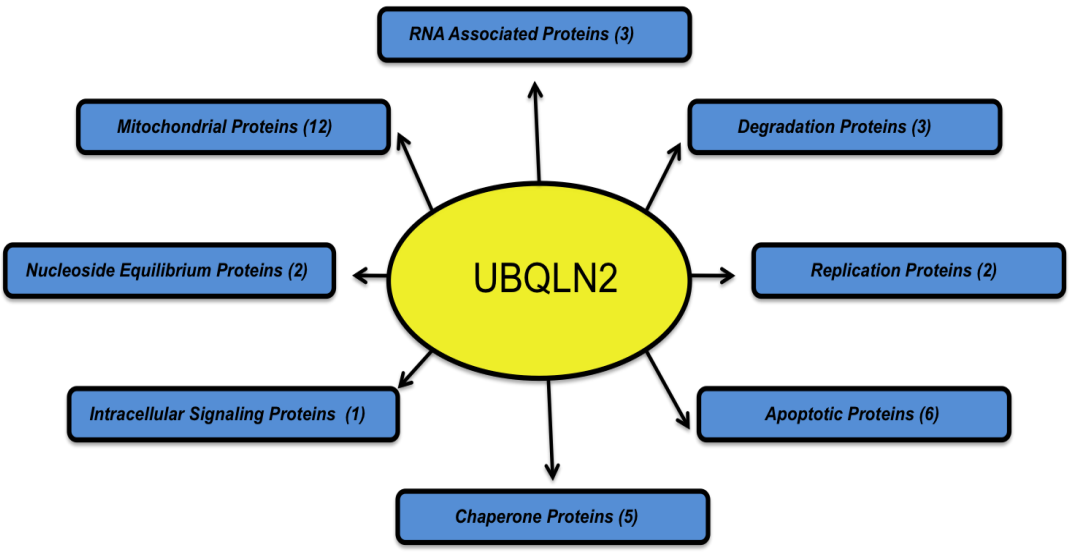


Fig 6. Interaction Network for Ubiquilin-2. Recovered DNA was sequenced and used in BLAST searches to identify the protein interactors. Those interactors were then organized by cellular function.

Although the results presented above deomonstrate that ubiquilin-2 interacts with proteins involved in a wide variety of cellular functions, subsequent experiments were focused on the three RNA associated proteins because they belong to the same family of proteins—that of heterogeneous nuclear ribonucleoproteins. More specifically, the proteins were hnRNPA1, hnRNPA3, and hnRNPU1 (hereafter called hnRNPU) (Dreyfuss, 1993) (Dreyfuss, 2002). Additionally, since mutations in these and other RNA binding proteins have been implicated in ALS pathogenesis (Da Cruz, 2011) (Lanson, 2012), subsequent experiments were focused on the interaction of these RNPs with WT and mutant ubiquilin-2. In particular, experiments were limited to hnRNPA1 because it carries a mutation that had been linked to ALS (Kim, 2013). During alignments of the recovered sequence with the known sequence obtained from BLAST searches, three recovered clones aligned with the 3' end of the ribonucleoproteins,

which contains their glycine-rich regions (Fig 7). Specifically, recovered clone #37 overlapped amino acid (AA) 219-320 of hnRNPA1, clone #67 overlapped AA 262-378 of hnRNPA3, and clone #5 overlapped AA 724-806 of hnRNPU. The regions of overlap do not match exactly the glycine-rich regions. This is because the cDNA library is composed of fragments of the human genome, so recovered clones are rarely full length proteins.

The function of the glycine-rich region is unknown, but it is present in most hnRNP proteins, albeit in different locations (Pesiridis, 2009). Interestingly, it is the site where most ALS mutations map in the hnRNP-related proteins TDP-43 and FUS (Pesiridis, 2009) (Lagier-Tourenne, 2010) (Da Cruz, 2011).

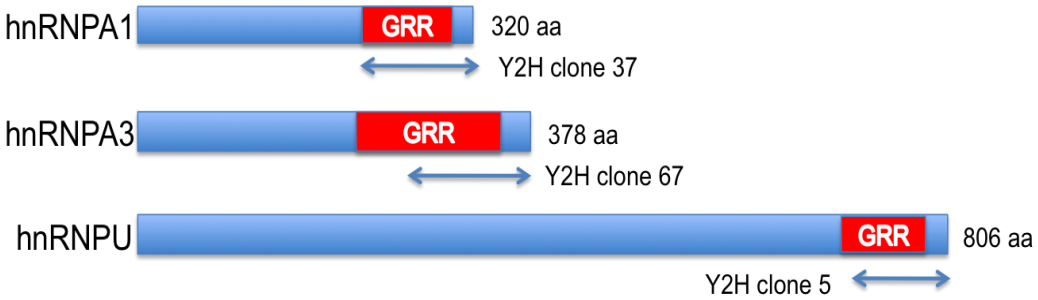


Fig 7. Sequence alignments of three clones from the Y2H cDNA library screen showing the region of overlap with the open reading frames of hnRNPA1, hnRNPA3, and hnRNPU. The glycine-rich region (GRR) in each hnRNP protein is highlighted in red. Note the region found to interact with ubiquilin-2 in the Y2H clones was mainly with the GRR in each hnRNP.

Section 3.2: The strength of interaction with hnRNPA1 varies among ubiquilin-2 mutants

I next examined whether the ALS-linked mutations in ubiquilin-2 affect its binding to hnRNPs by performing liquid β-galactosidase assays to measure the strength of interaction in yeast of the recovered hnRNPA1, A3, and U preys with ubiqulin-2 baits containing either the WT sequence or each of the five different ALS mutations. For each prey, interaction was also measured with the empty vector and with a random, unrelated lamin-C bait, as controls (Fig 8). In the liquid β-galactosidase assay, expression of the recovered prey yeast colonies was induced in media with leucine and with galactose/raffinose as the carbon source. Then, ONPG was added as a colorimetric substrate, and β-galactosidase units were calculated using the following equation:

$$(1{,}000 \times OD_{420})/(t \times V \times OD_{600})$$

where: t = minutes of incubation with ONPG before turning bright yellow

V = 0.1 ml x concentration factor (5)

$OD_{600} = A_{600}$ of 1 ml of culture

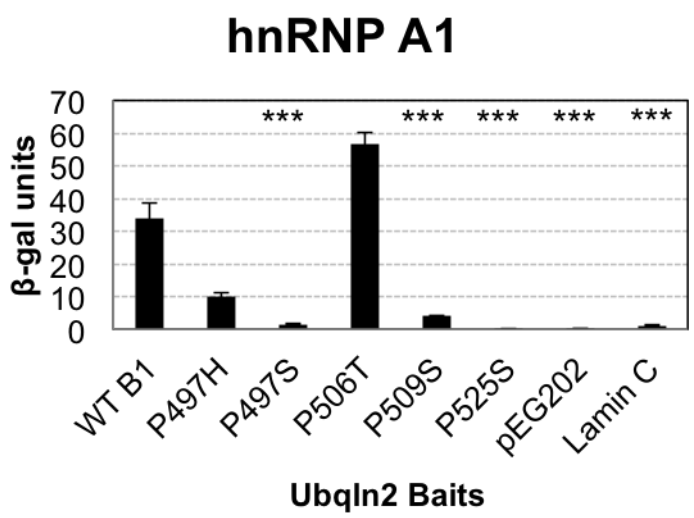
OD = optical density at wavelength of either 600 or 420 nm

A = absorbance at wavelength of 600 nm

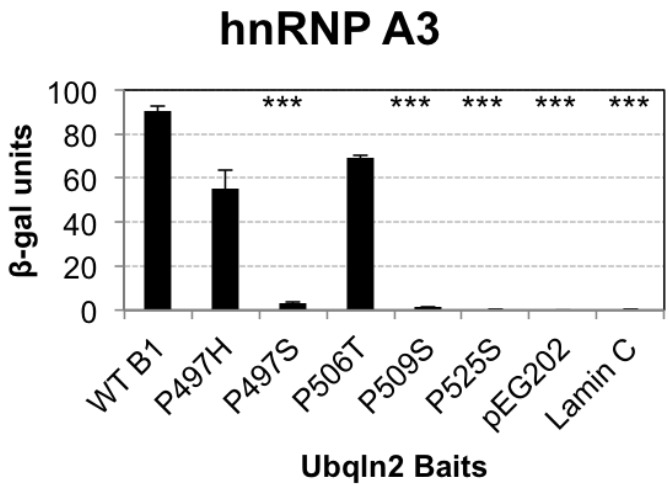
β-galactosidase units are defined as the amount of enzyme that hydrolyzes 1 μM of ONPG to o-nitrophenyl and D-galactose per minute per cell (Miller, 1972). In this case, a dilution factor of 5 was also computed because of the amount of Z-buffer the cultures were resuspended in before freezing in liquid nitrogen.

As depicted in Figure 8, all of the hnRNP proteins failed to interact with either of the control baits, as expected. On the other hand, all three hnRNP proteins did interact with the WT ubiquilin-2 baits, but to varying degrees. hnRNPA3 demonstrated the strongest interaction (88 β-gal units), followed by hnRNPA1 (35 β-gal units), and then by hnRNPU (7.5 β-gal units). The important finding in this assay, though, is that all three hnRNPs displayed a general trend of decreased interaction with the mutant ubiquilin-2 constructs as compared with WT ubiquilin-2. One exception was the P506T mutant. This mutant appeared to interact more strongly with hnRNPA1 and hnRNPU proteins than did the WT ubiquilin-2 bait, although there was no statistical significance. The exact reason for why P506T acted abberantly as compared with other mutant ubiquilin-2 constructs is not known, but it is likely due to a spurious interaction which developed due to the portion of the cDNA-encoded library prey protein recovered. WT ubiquilin-2 binding to TDP-43 (Fig 8D) was also confirmed as previously reported (Cassel, 2013).

A


hnRNP A1
β-gal units
70
60
50
40
30
20
10
0
***
***
***
***
***
WT B1
P497H
P497S
P506T
P509S
P525S
pEG202
Lamin C
Ubqln2 Baits

B


hnRNP A3
β-gal units
100
80
60
40
20
0
***
***
***
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WT B1
P497H
P497S
P506T
P509S
P525S
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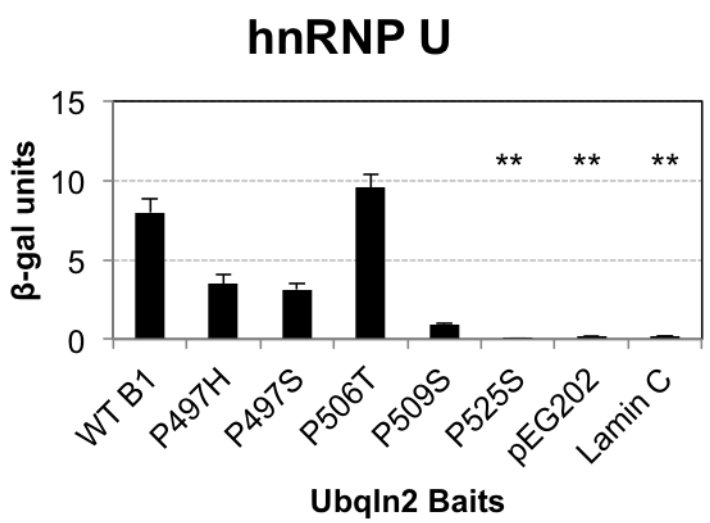
Lamin C
Ubqln2 Baits

C



D

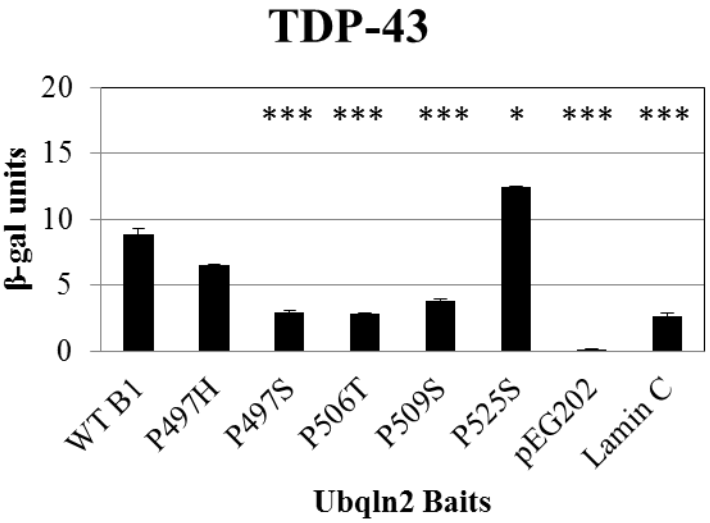


Fig 8. Mutant ubiquilin-2 constructs have reduced interaction with hnRNPs. (A-C) Y2H β-galactosidase liquid culture interaction assays comparing hnRNPA1, hnRNPA3, hnRNPU, and TDP-43, with different baits (the first six baits are WT and the five ubiquilin-2 mutants, followed by pEG202, the empty vector control, and another lamin C control). The β-galactosidase units were normalized to the WT bait. Several of the ubiquilin mutants interacted less strongly with the hnRNP preys. (A) One-Way ANOVA ($F_{7,23}$ =10.17, p=<0.0001) (B) One-Way ANOVA ($F_{7,23}$ =16.74, p=<0.0001) (C) One-Way ANOVA ($F_{7,23}$ =6.25, p=0.0012) (D) One-Way ANOVA ($F_{7,23}$=42.31, p=<0.001). Tukey's post-hoc tests: one star: p<0.05; two stars: p<0.01; three stars: p<0.0001. Data shown as mean ± SEM.

Section 3.3: The interaction occurs in vitro

Since the Y2H used a cDNA-encoded library as the prey, only fragments of hnRNP proteins were recovered. Therefore, I needed to confirm the interaction of full-length hnRNPs with ubiquilin-2. To assess the direct and specific interaction of hnRNP proteins with ubiquiln-2, GST-pulldowns were performed by incubating *in vitro* transcribed and translated ^{35}S-methionine radiolabeled hnRNPA1 and hnRNPU proteins with either GST-ubiquilin-1 or GST-ubiquilin-2 fusion proteins, or with GST protein alone. Due to poor expression, the hnRNPA3 protein could not be assayed in this and other experiments, so it was not studied further. The assays revealed that hnRNPU protein was preferentially pulled down by GST-ubiquilin-2, but not by GST-ubiquilin-1 or by the control GST alone (Fig 9A). This preferential binding is quantified in Fig 9B.

By contrast, hnRNPA1 was pulled down to different degrees by the different GST proteins, including by the control GST alone. Despite varying the salt concentration of the wash detergent, there was a fair amount of background binding of hnRNPA1 to GST-alone. The background binding appeared in all subsequent experiments, which suggested it is a "sticky" protein. GST-ubiquilin-2 pulled down five-times more hnRNPA1 than did GST-alone. GST-ubiquilin-1, though, only pulled down about one half as much as GST-ubiquilin-2 (Fig 9B).

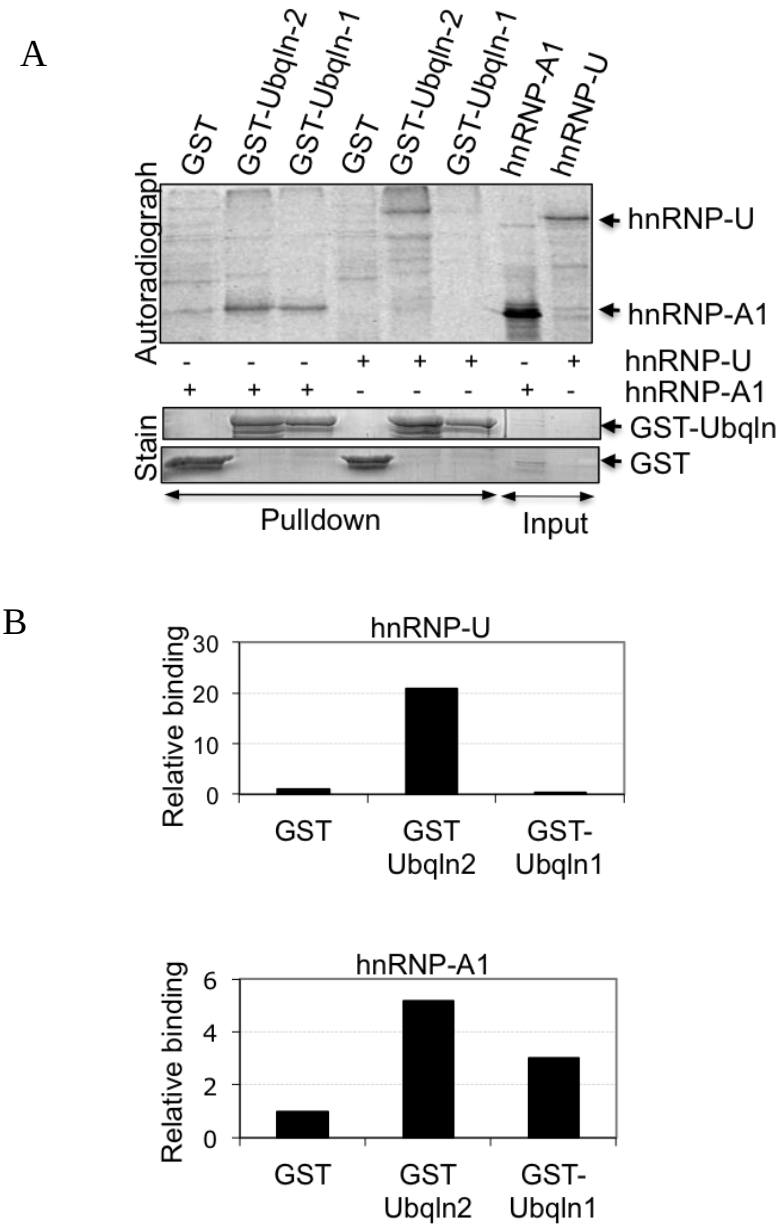
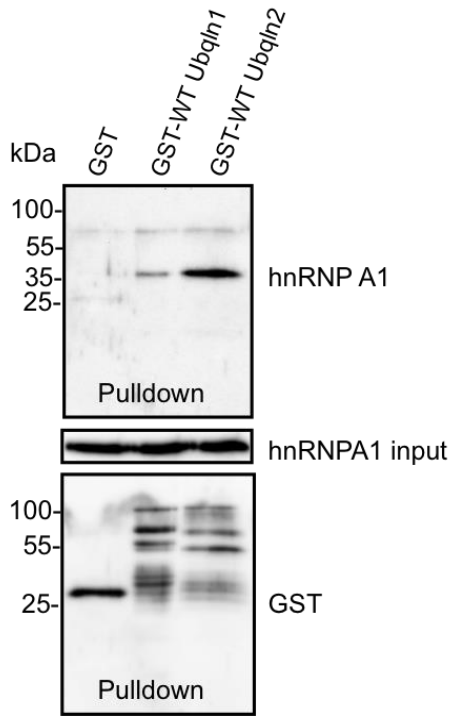


Fig 9 (Previous page). Confirmation of ubiquilin-2 interaction with hnRNP proteins. (A) GST (lanes 1 & 4), GST-WT-ubiquilin-1 (lanes 2 & 5), and GST-WT-ubiquilin-2 (lanes 3 & 6) were incubated with either in vitro translated and ^{35}S-radiolabeled full-length hnRNPA1 (lanes 1 to 3) or hnRNPU (lanes 4 to 6) and the bound proteins were separated by SDS-PAGE together with 1/10 of the inputs (lanes 7 and 8, respectively). The gel was stained with Coomassie Blue, dried and an autoradiograph prepared. Top image is the autoradiograph, and the next two panels show the recovery of GST-ubiquilin and GST proteins. (B) Quantification of the GST-pulldown results for hnRNPA1 and hnRNPU proteins shown in A. Experiment performed only one time.

To establish whether the binding between hnRNPA1 and ubiquilin-2 proteins is direct and to further confirm their binding, the GST-pulldown assay was repeated using purified bacterially-expressed HIS-hnRNPA1 as ligand. These pulldown assays revealed preferential binding of HIS-hnRNPA1 and GST-ubiquilin-2, but not with GST-ubiquilin-1 or GST alone (Fig 10A and B). This is especially impressive considering that the relative amount of GST-ubiquilin-2 in the reaction was less than GST-ubiquilin-1. Taken together with data from Figure 9, the conclusion is clear that hnRNPA1 and hnRNPU interact with ubiquilin proteins directly and with specificity for ubiquilin-2.

A



B

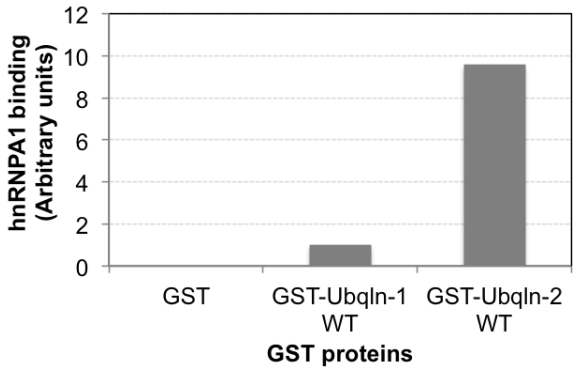
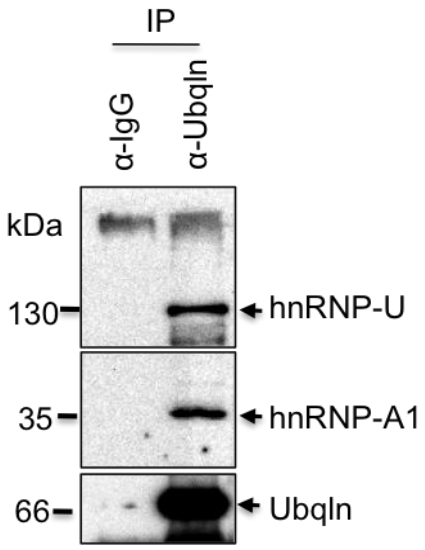


Fig 10. (Previous page) GST-pulldown experiments to examine binding of recombinant full-length HIS-hnRNPA1 with GST-alone, GST-WT ubiquilin-1, or GST-WT ubiquilin-2. (A) GST alone (lane 1), GST-WT ubiquilin-1 (lane 2) or GST-WT ubiquilin-2 (lane 3) were incubated with hnRNPA1. The pulldown of hnRNPA1 (top blot) and input protein (middle panel) were detected by immunoblotting using anti-hnRNPA1 antibody. A parallel blot was probed for the GST pulldown protein (bottom panel). (B) Densitometry analysis of the pulldown results shown in A. Experiment performed only one time.

After confirming that hnRNPs bind with ubiquilins *in vitro,* I wanted to determine if endogenous ubiquilin and full-length hnRNP proteins bind in a complex. To assess this, ubiquilin-2 was immunoprecipitated from HeLa cells using a pan-ubiquilin antibody and the precipitates were examined for the presence of hnRNPA1 and hnRNPU proteins by immunoblotting. As shown in Fig 11A, both hnRNPA1 and hnRNPU were co-immunoprecipitated with ubiquilin, but not with the control immunoprecipitation lacking the ubiquilin antibody. The results confirmed binding between endogenous ubiquilin proteins and hnRNPA1 and hnRNPU proteins.

A


IP
α-IgG
α-Ubqln
kDa
130
hnRNP-U
35
hnRNP-A1
66
Ubqln

B

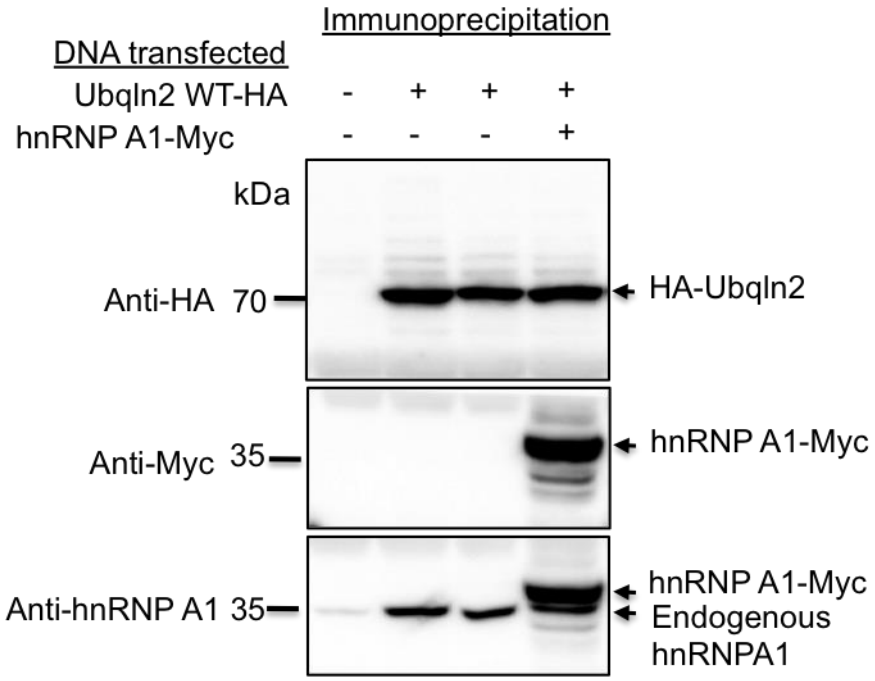

Immunoprecipitation
DNA transfected
Ubqln2 WT-HA - + + +
hnRNP A1-Myc - - - +
kDa
Anti-HA 70
HA-Ubqln2
Anti-Myc 35
hnRNP A1-Myc
Anti-hnRNP A1 35
hnRNP A1-Myc
Endogenous
hnRNPA1

Fig 11. (Previous page) Confirmation of ubiquilin-2 binding with hnRNPA1 in cells and demonstration that the ALS mutations have reduced hnRNPA1 binding. (A) Ubiquilin proteins from HeLa cells were immunoprecipitated with either control whole mouse IgG antibody or with a monoclonal pan-specific ubiquilin antibody. Equal portions of the precipitations were examined for immunoprecipitation of ubiquilin (bottom panel) and for co-immunoprecipitation of hnRNPA1 (middle panel) or hnRNPU (top panel) by immunoblotting. (B) HeLa cells were either mock transfected or transfected with WT HA-tagged ubiquilin-2 cDNA by itself or together with Myc-tagged full-length hnRNPA1 cDNA. 24 h after transfection proteins were immunoprecipitated using an antibody against HA and the precipitates examined by immunoblotting with the different antibodies shown. A control immunoprecipitation was also conducted where the HA antibody was omitted, showing the specificity of co-immunoprecipitation of hnRNPA1 with HA-tagged ubiquilin-2. Experiment performed only one time.

Because multiple ubiquilin proteins are expressed in HeLa cells (ubiquilin-1, 2, and 4 proteins), specificity of hnRNPA1 binding to ubiquilin-2 was tested. The Y2H gave a preliminary affirmative and the GST-pulldowns indicated that purified proteins interact, but I desired to confirm the specificity of interaction with the full length proteins in a mammalian cell system. To address this, HeLa cells were either mock transfected or transfected with expression plasmids encoding the full-length HA-tagged ubiquilin-2 by itself, or together with full-length Myc-tagged hnRNPA1. The expressed HA-tagged ubiquilin-2 protein was immunoprecipitated from the cells using an anti-HA antibody and the precipitates were examined for binding of endogenous and/or the exogenously expressed hnRNPA1 protein. The blots showed that endogenous hnRNPA1 co-immunoprecipitates with HA-tagged ubiquilin-2 (Fig 11B). The specificity of this co-immunoprecipitation can be seen by the almost complete absence of the hnRNPA1 signal in cells in which HA-ubiquilin-2 was not expressed. Further

confirmation that hnRNPA1 forms a complex with HA-ubiquilin-2 was seen by the co-immunoprecipitation of Myc-tagged hnRNPA1 protein with HA-ubiquilin-2 (Fig 11B, last lane).

Section 3.4: Discussion

In this chapter, I showed that ubiquilin-2 binds to hnRNPs and that ALS-linked mutations in ubiquilin-2 alter that interaction *in vitro*. In the GST-pulldown assays, hnRNPU and hnRNPA1 displayed stronger binding with ubiquilin-2 than with ubiquilin-1. One of the most plausible reasons for this preferential binding is the PXX domain unique to the ubiquilin-2 sequence. This region might facilitate hnRNP protein binding. An experiment not performed here, but one that should be done in the future is to determine if each of the four human ubiquilin isotypes display any preferential binding with different hnRNP proteins. An indication that ubiquilin-2 can bind several different hnRNP proteins comes not only from my yeast two-hybrid results showing three different hnRNPs interact with ubiquilin-2, but also from a recent paper in which the authors demonstrated that ubiquilin-2 binds to the C-terminus of TDP-43 (Cassel, 2013). Examination of the portions of the hnRNP proteins recovered in our Y2H screen revealed they all contain the glycine-rich domain, suggesting it is likely the domain responsible for binding ubiquilin-2. Especially telling was the interaction seen with hnRNPU, where interaction was found with the C-terminal 1/10 end of the protein, exactly where the glycine-rich domain is located. This novel interaction could be important as many ALS-linked mutations in FUS/TLS and TDP-43 map to the glycine-rich domains of each protein (Lagier-Tourenne,

2010). It will be interesting to see if ubiquilin-2 binds FUS/TLS and TDP-43 through the glycine-rich regions.

Finally, since hnRNPs form complexes with snRNPs and hnRNA, it will be interesting to look for other proteins in the hnRNP complex. My research identifies ubiquilin-2 as a direct interactor with hnRNP A1 and U, but a search for other proteins involved with the hnRNP complex would paint a more complete picture of the mechanistic reason for such an interaction. In fact, it is possible that some proteins among the remainder of the 41 interactors of ubiquilin-2 identified through the Y2H screen are co-factors in the complex and should be studied in more detail.

In subsequent chapters, and using the overarching hypothesis as a guide, experiments will be performed to determine if the WT ubiquilin-2-hnRNPA1 interaction is direct and specific, to test if mutations in ubiquilin-2 disrupt the normal interaction, and to assess the interaction's physiological relevance within the cell.

CHAPTER 4: DISRUPTION OF UBIQUILIN-2 AND hnRNPA1 INTERACTION WHEN MUTATIONS ARE PRESENT IN EITHER PROTEIN

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Section 4.1: Mutant ubiquilin-2 proteins have reduced binding to hnRNPA1

After determining that ubiquilin-2 binds to hnRNPA1, I next investigated whether the ALS-linked mutations in ubiquilin-2 affected binding to full-length hnRNPA1. To examine this, HeLa cells were transfected with constructs encoding HA-tagged WT-ubiquilin-2 or each of the P497H, P497S, P506T, P509S and P525S mutations. Mock transfection was used as a control. An anti-HA antibody was used to immunoprecipitate the HA-tagged ubiquilin-2 proteins and the precipitates were immunoblotted for hnRNPA1 in order to assess the levels of interaction in the presence of ubiquilin-2 mutations (Fig 12). The ratio of hnRNPA1 protein co-immunoprecipitated relative to HA-ubiquilin-2 protein precipitated was then calculated for each immunoprecipitation. From the quantification, it can be concluded that the ubiquilin-2 mutations bound significantly less hnRNPA1 protein than did WT ubiquilin-2 (Fig 12B).

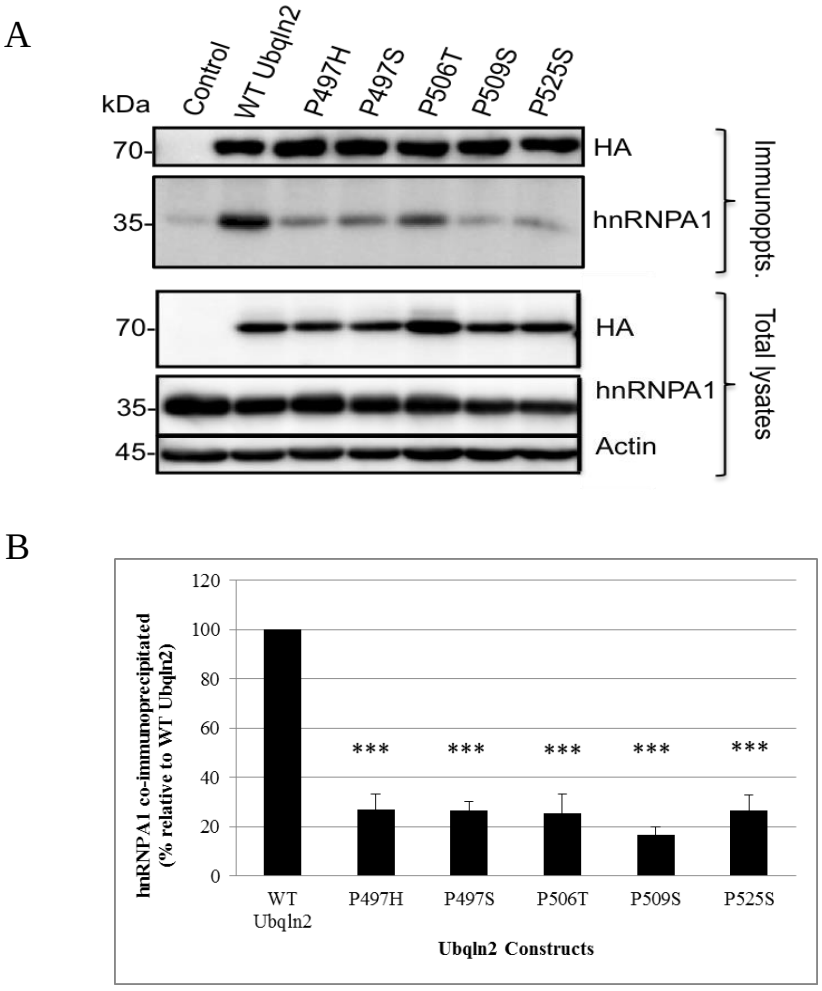
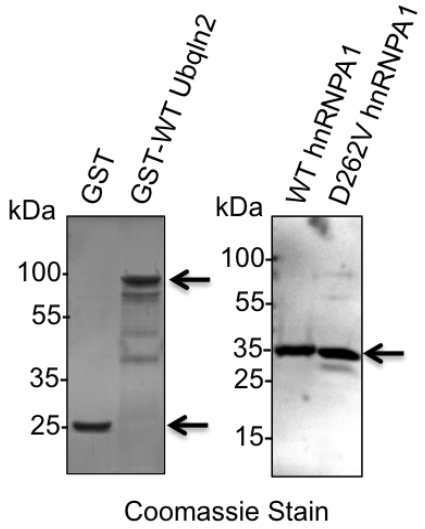


Fig 12. Co-immunoprecipitation to confirm ubiquilin-2 mutants demonstrate reduced binding to full length hnRNPA1. (A) HeLa cells were mock transfected, transfected with WT, or transfected with the ALS mutant HA-ubiquilin-2 constructs shown and proteins immunoprecipitated from the cultures with an anti-HA antibody. The precipitates were examined for co-immunoprecipitation of hnRNPA1. The three bottom panels are total lysates used in the experiment probed for expressed HA-tagged WT ubiquilin proteins, endogenous hnRNPA1 and actin as a loading control. (B) Graph showing densitometry of the co-immunoprecipitated hnRNPA1 band normalized to HA tagged ubiquilin-2 precipitated. The results revealed that all of the ubiquilin-2 mutants had reduced binding to hnRNPA1. One way ANOVA: $F(6,2)=44.97$, $p<0.0001$. Bonferroni multiple comparisons post-hoc test: three stars: $p<0.001$. Data are shown as the mean ± SEM

Section 4.2: Mutant hnRNPA1 demonstrates reduced binding to ubiquilin-2

Recently, a mutation in the C-terminus of hnRNPA1 (D262V) was discovered and linked to ALS (Kim, 2013). This leads one to wonder if the mutation in hnRNPA1 would have the same effect as the ubiquilin-2 mutations on reducing the interaction between hnRNPA1 and ubiquilin-2. To assess this, recombinant HIS-tagged WT hnRNPA1 and recombinant HIS-tagged D262V hnRNPA1 were purified and equivalent amounts of each were used to examine binding to WT ubiquilin-2 by GST pulldown assays (Fig 13A). I subtracted the background binding to GST-alone from the binding to GST-WT ubiquilin-2 and normalized to levels of binding with WT hnRNPA1. By doing this, I saw that mutant hnRNPA1 does alter the binding with ubiquilin-2. The results support the idea that ALS-linked mutations in either hnRNPA1 or ubiquilin-2 have the same effect: they reduce interaction between the two proteins (Figs 13B and C).

A



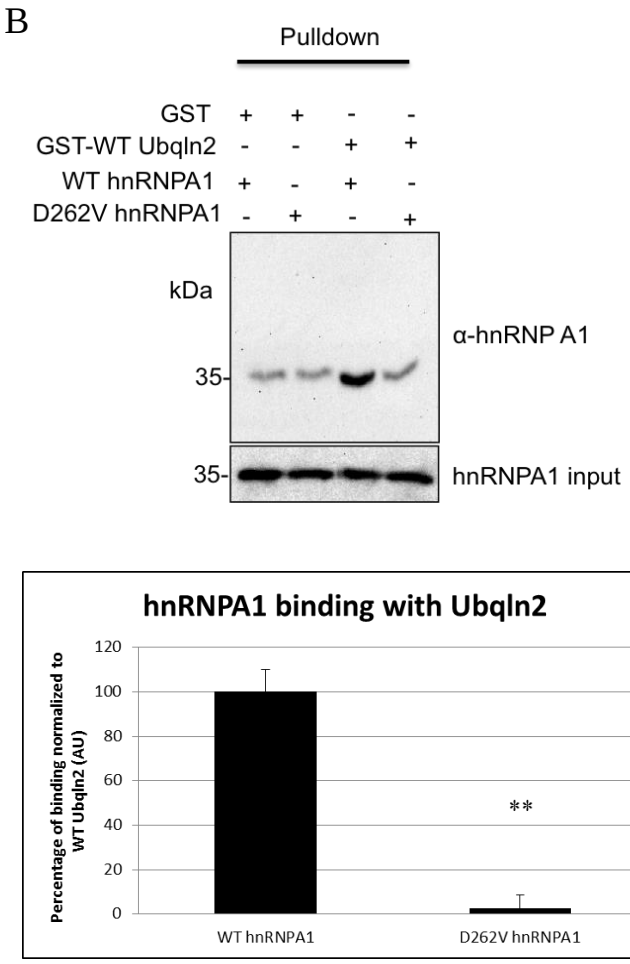
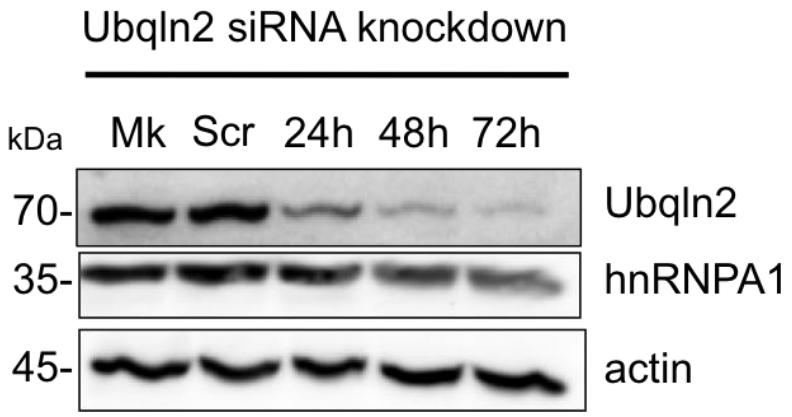


Fig 13. hnRNPA1 carrying the ALS D262V mutation has reduced binding to ubiquilin-2. (A) Coomassie stained gel showing the purified recombinant proteins used in the pulldown studies described in B & C. Left hand panel is the GST proteins (lane 1: GST alone; lane 2: GST-WT Ubiquilin-2) and the right hand panel is the HIS-tagged hnRNPA1 proteins (lane 1: WT HIS-hnRNPA1; lane 2: HIS-hnRNPA1 carrying the D262V). The arrows indicate the positions of the full-length fusion proteins in the different lanes. (B) GST pulldown studies comparing binding of WT HIS-hnRNPA1 and HIS-hnRNPA1 D262V with GST alone and GST-ubiquilin-2. Equal amounts of the HIS tagged proteins were analyzed for binding the GST proteins. Although both hnRNP proteins displayed non-specific binding to the GST proteins, HIS-tagged hnRNPA1 bound more strongly to GST-ubiquilin-2 than GST alone. (C) Quantification of the binding studies shown after subtracting the background binding to GST alone. The D262V mutation abolishes binding to ubiquilin-2. Unpaired t-test: $t(4)=5.879$, $p=0.0042$. Data shown are the mean ± SEM.

Section 4.3: Knockdown of ubiquilin-2 reduces hnRNPA1 levels

To understand the significance of the ubiquilin-2 interaction in a model that better represents ALS, ubiquilin-2 was knocked down in a mouse motor neuron cell line (NSC-34) using siRNA specific for mouse ubiquilin-2 for 24, 48, or 72 h and examined the cells for cell viability and hnRNPA1 accumulation (Fig 14). Cells transfected with the ubiquilin-2 specific siRNAs showed a robust, time-dependent knockdown of ubiquilin-2, compared to cells that were mock transfected or transfected with a scrambled siRNA (Figs 14A and B). Interestingly, knockdown of ubiquilin-2 led to a reduction of hnRNPA1 levels, suggesting ubiquilin-2 promotes stability and/or translation of hnRNPA1. Although siRNA can sometimes have off-target effects, this risk is mitigated by using SMARTpool siRNA, which consists of four separate siRNAs targeting ubiquilin-2.

A



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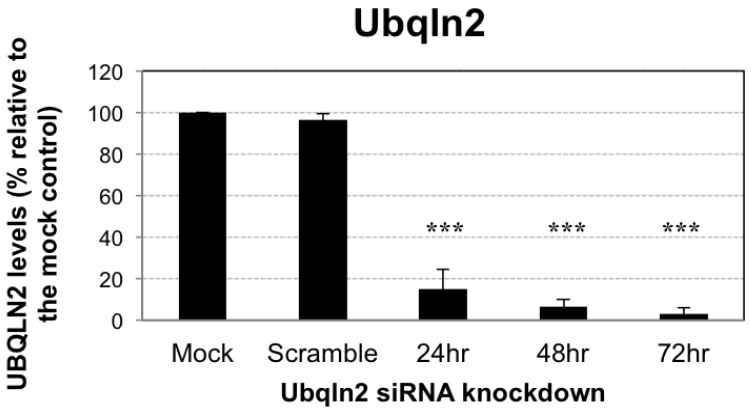

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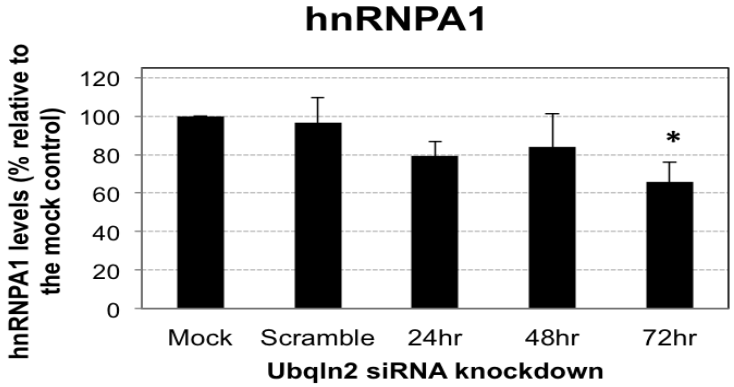
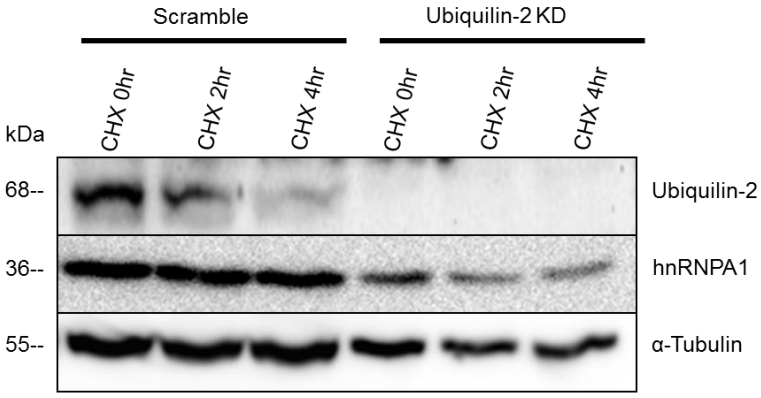


Fig 14. Knockdown of ubiquilin-2 leads to a reduction in hnRNPA1 levels. (Previous page) (A) NSC-34 cells were mock transfected (lane 1), or transfected with control scramble siRNAs that do not target any known gene (lane 2), or with siRNA that were designed to specifically knockdown ubiquilin-2 for 24h (lane 3), 48h (lane 4), or 72h (lane 5). Equal amounts of protein from the cultures were immunoblotted with antibodies specific for ubiquilin-2, hnRNPA1 and actin, which was used as loading control. The lysates from the mock (Mk) and scrambled (Scr) knockdown cultures were obtained from 72 h cultures. (B) Quantification of knockdown of ubiquilin-2 in at least three similar experiments. Repeated measures one way ANOVA for ubiquilin-2: $F_{(4,14)}=84.68$, $p<0.0001$. Bonferroni post-hoc tests: three stars indicates $p<0.0001$. (C) Quantification of hnRNPA1 levels in the knockdown experiments. Knockdown of ubiquilin-2 resulted in a statistical reduction in hnRNPA1 levels at 72 h. Repeated measures one way ANOVA for hnRNPA1: $F_{(4,14)}=3.29$, $p=0.07$. Dunnett's multiple comparisons post hoc test: one star indicates $p<0.05$. Data are shown as mean ± SEM.

I tested the effect of ubiquilin-2 knockdown on hnRNPA1 stability by treating NSC-34 cells with cycloheximide following 72h of ubiquilin-2 knockdown. hnRNPA1 again showed a concomitant reduction in expression in cells treated with ubiquilin-2 siRNAs, despite displaying stability under cycloheximide treatment in scramble controls. (Fig 15A) Additionally, turnover of hnRNPA1 was increased in cells transfected with ubiquilin-2 siRNAs. The reduction reached significance in cells treated with cycloheximide for 4 h. (Fig 15B) This data offers evidence that ubiquilin-2 plays an important role in moderating hnRNPA1 stability.

A



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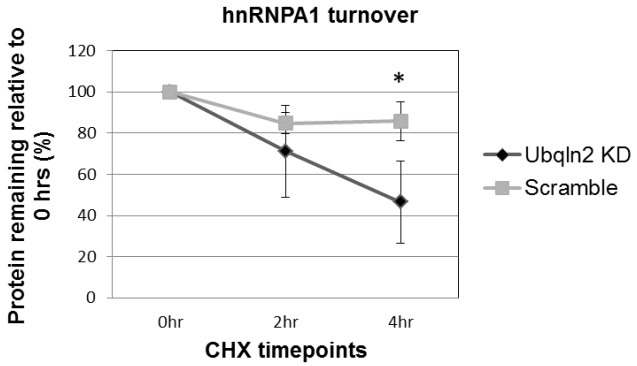


Fig. 15. (Previous page) Knockdown of ubiquilin-2 increases hnRNPA1 turnover. (A) NSC-34 cells were transfected with non-targeting scramble control or siRNAs targeting ubiquilin-2. At 72 h post-knockdown, cells were treated with cycloheximide for 0, 2, or 4 h. Equal amounts of protein from the cultures were immunoblotted with antibodies specific for ubiquilin-2, hnRNPA1 and α-tubulin, which was used as loading control. (B) Quantification of hnRNPA1 turnover with and without ubiquilin-2 siRNAs relative to time point 0 from three independent experiments. Data are shown as mean ± SEM.

Section 4.4: Discussion

ALS-linnked mutations in ubiquilin-2 and hnRNPA1 have the same affect: they reduce interaction between the proteins. The loss of interaction caused by mutation in either protein, the decreased expression levels, and the increased turnover of hnRNPA1 in ubiquilin-2 knock down cells underscore the importance of the interaction and alludes to an important functional role the interaction plays in ALS.

Because ubiquilin proteins function in protein clearance pathways, it is possible that the WT ubiquilin-2 interaction with hnRNPA1 enhances hnRNPA1 degradation. In this case, the prediction would be that the loss of interaction resulting from mutations in ubiquilin-2 would in turn result in increased hnRNPA1 levels. Instead of the increase, though, there was little, if any change (Fig 12A-total lysates). Indeed, the results from the knockdown of ubiquilin-2 paradoxically led to a decrease in hnRNPA1 levels. This suggests ubiquilin-2 interaction might be required to stabilize hnRNPA1 levels and a turnover

experiment confirmed just that.

The precise method of how ubiquilin-2 stabilizes hnRNPA1 remains to be determined. The central domain of ubiquilins, which was the region of ubiquilin-2 used to identify interaction with hnRNPA1, has been speculated to play a more regulatory role compared to the UBL and UBA end domains (Ford, 2006). It will be interesting to discover what, if any, regulatory role the central domain plays in the interaction of ubiquilin-2 and hnRNPA1 and what role it plays in governing hnRNP function.

CHAPTER 5: EFFECT OF MUTATED UBIQUILIN-2 ON hnRNPA1 SUBCELLULAR LOCALIZATION AND CELL DEATH

Published: **Gilpin, K. M.,** Chang, L., Monteiro, M. J. (2015). ALS-linked mutations in ubiquilin-2 or hnRNPA1 reduce interaction between ubiquilin-2 and hnRNPA1. *Human Molecular Genetics.* doi: 10.1093/hmg/ddv020

Section 5.1: Mutant ubiquilin-2 disrupts subcellular localization of hnRNPA1

Since several hnRNP proteins, including hnRNPA1, shuttle between the cytoplasm and nucleus and ubiquilin proteins also segregate between the two compartments, I reasoned that the reduction in binding when mutations in ubiquilin-2 are present could affect the normal subcellular localization of either protein (Dreyfuss, 2002) (Pinol-Roma, 1992) (Mah, 2000). To answer this question, I performed subcellular fractionations in which HeLa cells transfected with cDNAs encoding either WT ubiquilin-2 or each of the five ALS mutations (all HA-tagged) were biochemically fractionated into cytosolic (C), soluble nuclear and mitochondrial (N), and insoluble pellet (P) fractions using differential centrifugation and variation in the concentration of Triton-X100 for extraction. The identity of the pellet fraction is not known, but could represent aggregated protein. Equal fraction volumes were probed by SDS-PAGE to determine the relative proportion of hnRNPA1 and ubiquilin-2 protein in each fraction (Fig 16A). Caspase-3, Tom-20, and Lamin B were used as controls to monitor the quality and consistency of the fractionations. The controls partitioned differently, but in a manner consistent with their distinct subcellular locations and/or their biochemical properties. Caspase-3 is a cytosolic protein involved in intracellular

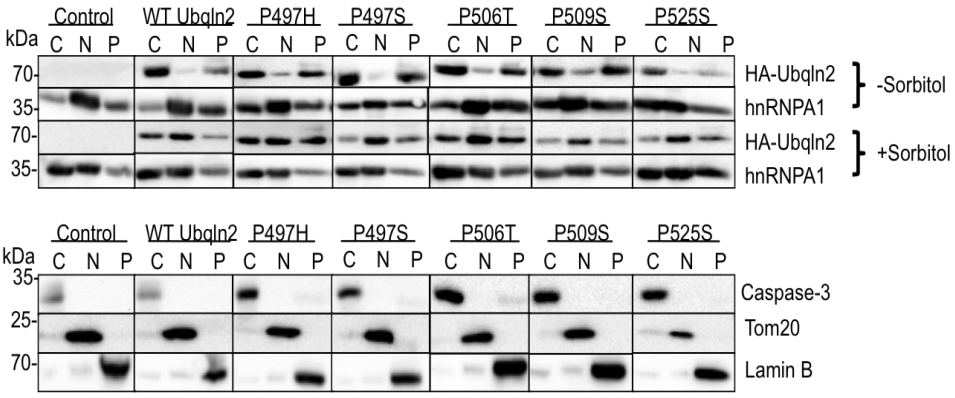
signaling cascades, so this served as a marker for the cytoplasmic fraction. Tom-20 is an outer mitochondrial membrane protein and therefore was a control for the soluble nuclear and mitochondrial fraction. Lastly, Lamin B is a protein associated with intermediate filaments, half of which are insoluble, so this served as a control for the insoluble pellet. Importantly, the profile of the fractionation of the controls was the same across all sets, demonstrating the reproducibility of the fractionations.

Just as a pattern emerged in the subcellular localization of the control proteins, there is a pattern visible in the HA-tagged WT ubiquilin-2 and mutant proteins. The highest amounts of the proteins were present in the cytoplasm (C), followed by slightly lesser amounts in the insoluble fraction (P), and finally the least amount was found in the soluble nuclear fraction (N). This pattern in the immunoblots is visually represented in Fig 16B (–sorbitol), in which only the cytoplasmic fractions are depicted for simplicity. The soluble nuclear/mitochondrial and insoluble pellet fractions can be visualized in the immunoblots. After comparison of the fractionations in cells transfected with WT or mutant ubiquilin-2, it can be concluded that the ubiquilin-2 mutants partition in a similar manner to WT ubiquilin-2 by this assay.

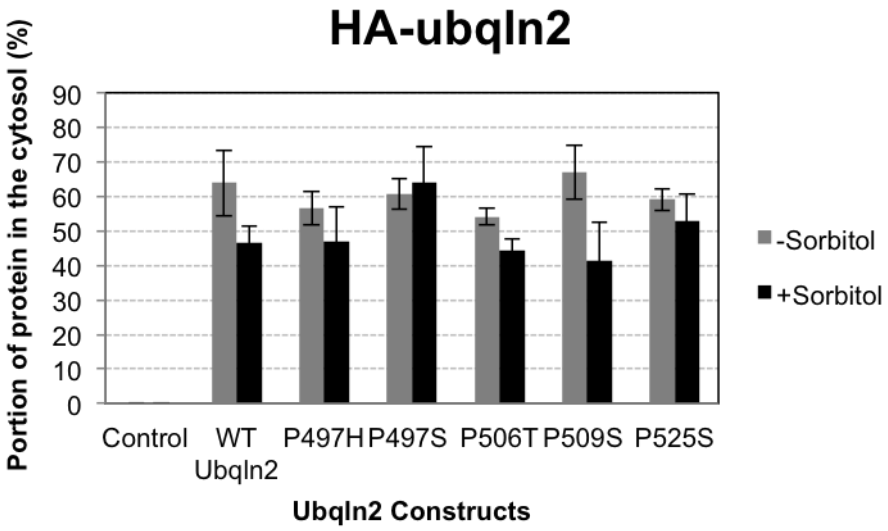
In contrast, fractionation of endogenous hnRNPA1 protein was found to be altered in the cells transfected with four of the five ubiquilin-2 mutants, as evidenced by a more hnRNPA1 protein present in the cytoplasm of cells transfected with mutant ubiquilin-2 as compared to that seen in mock-transfected cells, or those transfected with WT ubiquilin-2 (Fig 16C -sorbitol).

We next examined whether expression of the ALS ubiquilin-2 mutants also affects hnRNPA1 shuttling. hnRNPA1 protein shuttles from its predominantly nuclear location to the cytoplasm in cells exposed to osmotic shock (i.e. treatment with 600 mM sorbitol) (Allemand, 2005). Therefore, I treated transfected HeLa cells with 600 mM sorbitol for 2 h and compared the quantification of hnRNPA1 translocation to the cytoplasm to non-treated cultures (Fig 16C +sorbitol). The change in distribution of the HA-tagged ubiquilin-2 proteins was also quantified (Fig 16B +sorbitol). Despite there not being a statistically significant difference between cells transfected with WT and mutant ubiquilin-2, treatment with sorbitol resulted in an increase and a decrease of HA-ubiquilin-2 in the soluble nuclear fraction (N) and in the cytoplasmic fraction (C), respectively. The distribution of hnRNPA1, however, changed in an opposite manner. In cells transfected with either control or WT ubiquilin-2, sorbitol treatment resulted in increased amounts of hnRNPA1 in the cytoplasm, as expected (16C, +sorbitol) (Allemand, 2005). When we compare the cells transfected with WT ubiquilin-2 to those transfected with mutant ubiquilin-2, there is little difference in the overall accumulation of hnRNPA1 in the cytoplasmic fraction (16C, +sorbitol). However, net translocation to the cytoplasm was reduced for the same four mutants that displayed increased cytoplasmic translocation when cultured under normal growth conditions (16C, comparing the –sorbitol and +sorbitol bars for each transfection).

A


Control
WT Ubqln2
P497H
P497S
P506T
P509S
P525S
kDa
C N P
70-
35-
HA-Ubqln2
hnRNPA1
-Sorbitol
+Sorbitol
25-
Caspase-3
Tom20
Lamin B

B


HA-ubqln2
Portion of protein in the cytosol (%)
90
80
70
60
50
40
30
20
10
0
Control
WT Ubqln2
P497H
P497S
P506T
P509S
P525S
Ubqln2 Constructs
-Sorbitol
+Sorbitol

C

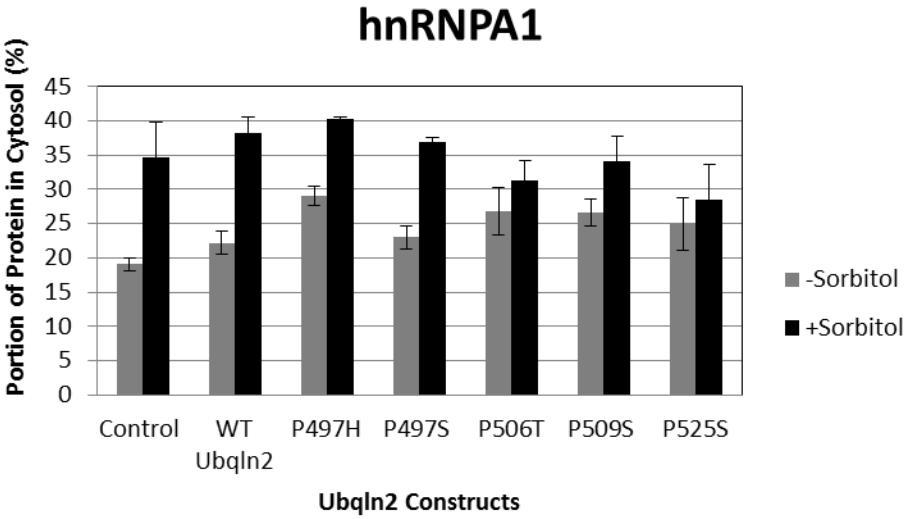

hnRNPA1
Portion of Protein in Cytosol (%)
45
40
35
30
25
20
15
10
5
0
Control
WT Ubqln2
P497H
P497S
P506T
P509S
P525S
Ubqln2 Constructs
-Sorbitol
+Sorbitol

Fig 16. (Previous page) Overexpression of ubiquilin-2 mutants results in increased subcellular localization of hnRNPA1 in the cytoplasm. (A) HeLa cell cultures were either mock transfected, or transfected with HA-tagged WT or mutant ubiquilin-2 cDNAs and 24 hr later were fractionated to proteins in the cytoplasm (C), nuclear and mitochondria (N) and insoluble pellet (P) fractions. The fractions were adjusted to the same volume and equal volumes of the three fractions were immunoblotted with the antibodies shown. Antibodies against caspase-3, Tom20, an outer mitochondrial protein (for lack of a good nuclear extractable protein), and lamin B were used as controls to ensure proper fractionation in the cultures. (B + C) Graphs showing quantification of HA-tagged ubiquilin-2 and endogenous hnRNPA1 proteins in the cytoplasm, which was calculated by dividing the signal in the C fraction by the total signal (C+N+P). The results revealed non-significant increased amounts of hnRNPA1 in the cytoplasm in the cultures transfected with the ubiquilin-2 mutants, but not with the WT ubiquilin-2 construct, compared to the mock transfected control. Two way ANOVA revealed that, for HA ubiquilin-2 and hnRNPA1, there was no alteration in distribution of the WT and mutant proteins in the different fractions. ($F_{6,24}$ =3.78, p<0.6413) and ($F_{6,24}$=17.36, p<0.118) respectively. Data are shown as mean ± SEM.

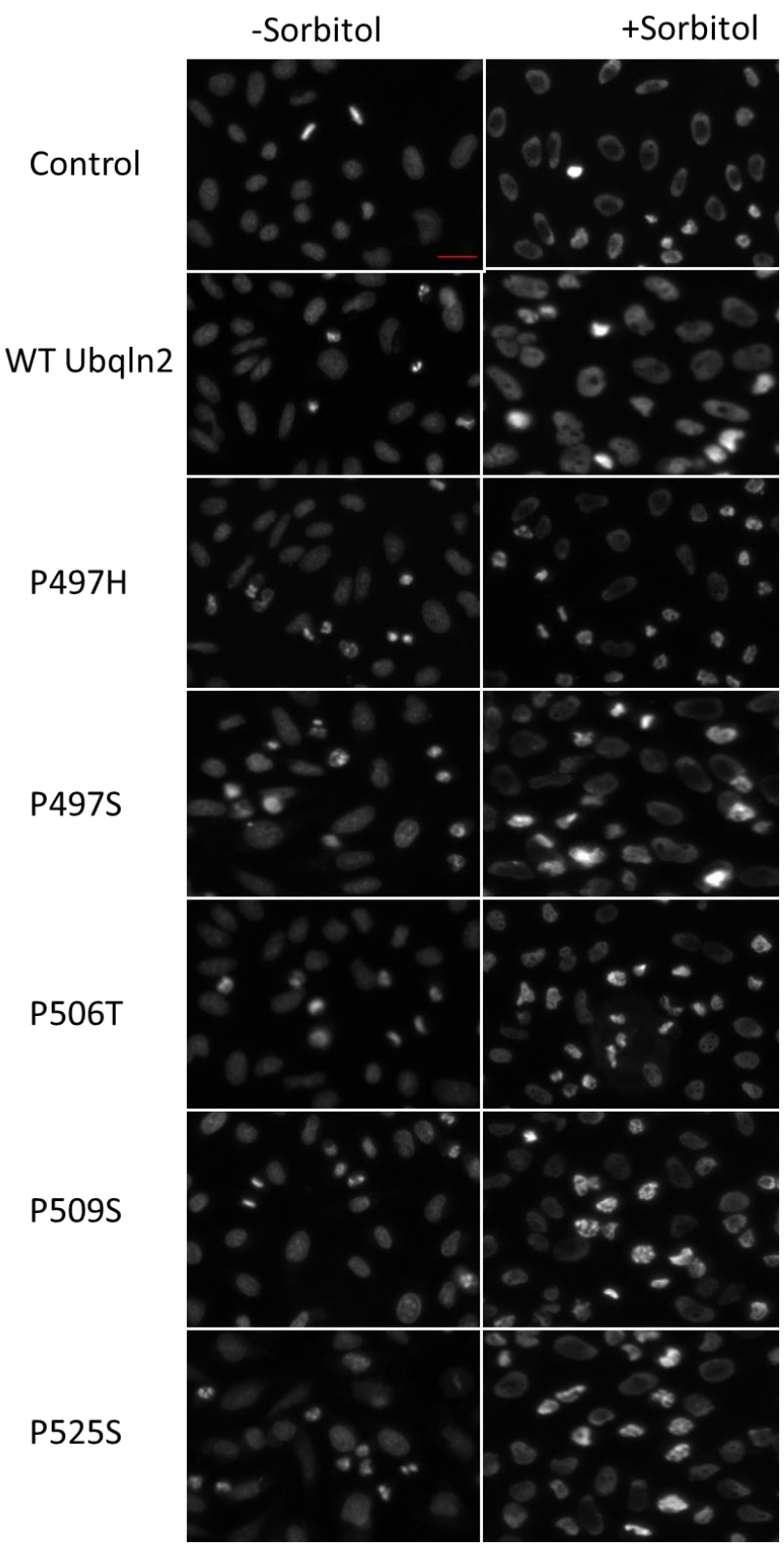
Section 5.2: Mutant ubiquilin-2 increases cell death

A prediction of my hypothesis was that the disruption in normal protein interactions would result in increased cell death. To test this, cell death assays were performed in which HeLa cells were transiently transfected with HA-WT-ubiquilin-2 or each of the five mutated HA-ubiquilin-2 constructs and stained with Hoescht for 15 min (Fig 17). The transfection efficiency in these cultures typically ranged between 85-95%. Thus, any estimate of cell death would be a slight underestimate considering a small percentage of cells were untransfected. Since sorbitol is a known osmotic stressor and induces cell death via the apoptotic signalling cascade, observation of abnormal nuclear morphology is a valid method to assess cell death (Koyamaa, 2000). Therefore, abnormal nuclear

morphology was used to quantify cell death in cultures treated with vehicle or with 600 mM sorbitol for 2 h. The quantification revealed no difference between the mock transfection control and WT ubiquilin-2, but overexpression of all the ubiquilin-2 mutants increased cell death compared to mock transfected cells, irrespective of whether they were treated with sorbitol or not (Fig 17B). Sorbitol treatment did increase the percentage of cell death, though, consistent with osmotic shock causing greater insult to the cells.

I performed trypan blue exclusion assays, as well, to confirm the results from the abnormal nuclear morphology experiments. In this setup, the trypan blue dye is excluded from viable cells with intact cellular membranes, but is taken up by dead or dying cells. As shown in Fig 17C, the general trend seen with abnormal nuclear morphology holds true in the trypan blue exclusion assay.

A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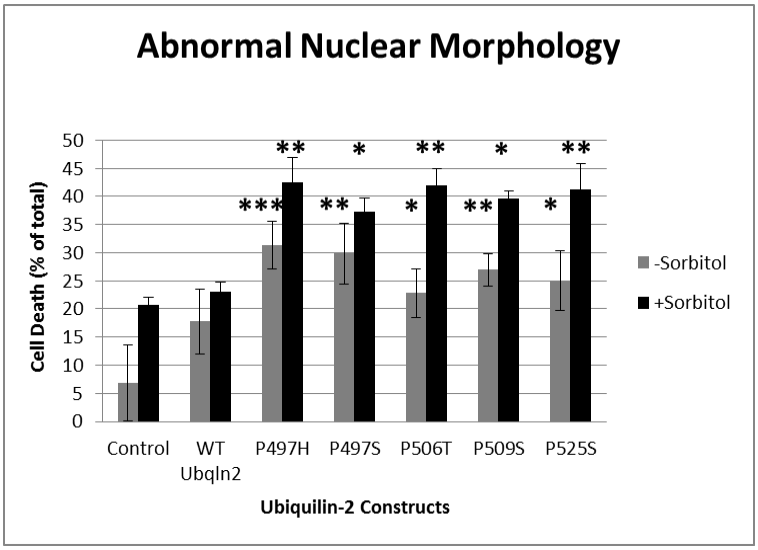
+Sorbitol
Control
WT Ubqln2
P497H
P497S
P506T
P509S
P525S

B



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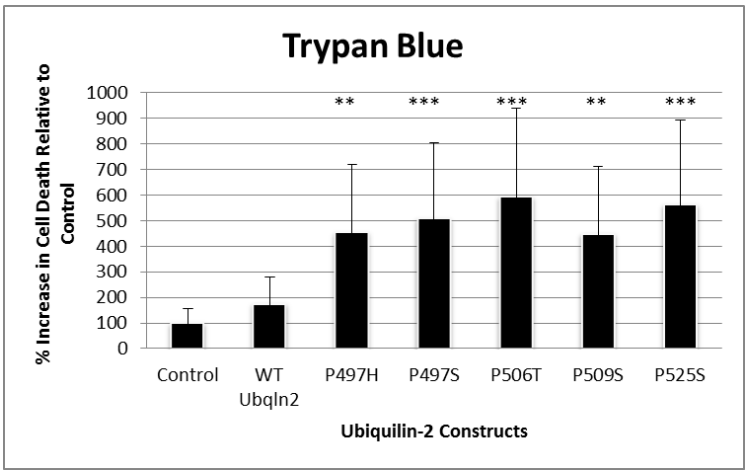


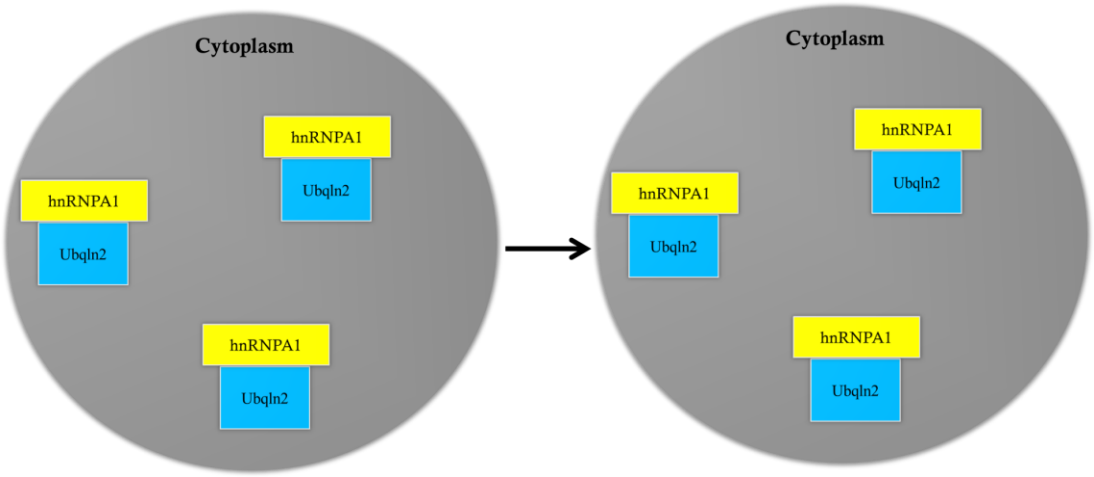
Fig 17. Overexpression of ubiquilin-2 carrying ALS mutations increases cell death. (A) (Previous page) HeLa cells were transfected with WT or mutant ubiquilin-2 constructs, treated with or without 600mM sorbitol for 2hrs, and stained with Hoescht. Bar = 15 μm. (B) Quantification of total number of cells with abnormal nuclear morphology. Two-way ANOVA showed a significant main effect for genotype ($F_{6,30}$ =7.73, $p<0.0001$) and stress ($F_{6,30}$=27.90, $p<0.0001$). Bonferroni post-hoc tests compared each of the mutant constructs in either non-stress or stress conditions to the respective control. One star indicates $p<0.05$; two stars indicate $p<0.01$, three stars indicate $p<0.001$. (C) Trypan blue exclusion assays indicate increased cell death among cells transfected with ubiquilin-2 mutant constructs. One-way ANOVA showed a significant main effect for genotype ($F_{6,20}$=12.16, $p<0.0001$). Bonferroni post-hoc tests compared each of the mutant constructs to the control mock transfection. Two stars indicate $p<0.01$, three stars indicate $p<0.001$. Data are shown as mean ± SEM.

Section 5.3: Discussion

The data in this chapter demonstrates that the mislocalization of hnRNPA1 is not the mechanism by which mutations in ubiquilin-2 increase cell death.

Although there was an increase of hnRNPA1 in the cytoplasmic fraction in cells transfected with mutant ubiquilin-2, the result failed to reach statistical significance. This leaves several possibilities open for how the mutations in ubiquilin-2 eventually lead to cell death. It seems clear that WT ubiquilin-2 binds to hnRNPA1 and stabilizes it, whereas the mutations in ubiquilin-2 caused reduced binding and therefore reduced stability of hnRNPA1, which ultimately triggers cell death. (Figs 18A & B) Since hnRNPA1 is necessary for many cellular activities, the reduced stability likely triggers cell death due to the dysfunction of one of its many roles. The function tested in these experiments was hnRNPA1's nucleo-cytoplasmic transport of mRNA, but the data from the subcellular fractionation indicates there is no significant alteration in hnRNPA1 localization. Other possibilities that should be tested include hnRNPA1's involvement in alternative splicing, transport to the stress granules, and telomere length regulation.

A



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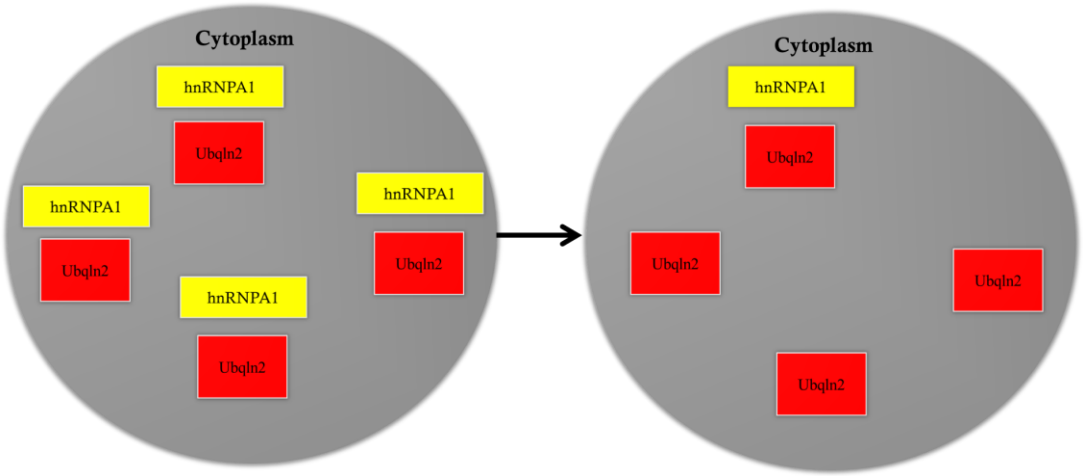

Fig 18. Schematics of putative consequences of loss of ubiquilin-2-hnRNPA1 interaction. (A) WT ubiquilin-2 binds to hnRNPA1 and stabilizes it. (B) Mutations in ubiquilin-2 reduce binding with hnRNPA1, thereby contributing to its instability and resulting in decreased amounts of hnRNPA1.

Despite the fact that the subcellular fractionation data was not significant, the results clearly indicate ubiquilin-2 interacts with hnRNPA1 in both the cytoplasm and nucleus. I performed preliminary double immunofluorescence microscopy experiments to pinpoint the cellular compartment in which the interaction predominantly took place, but could not do so because hnRNPA1 staining was localized and spread throughout the nucleus and ubiquilin-2 staining was spread throughout the whole cell. Any overlap, therefore, in the two staining patterns would naturally appear colocalized and would not give a precise location. Further complicating this effort is that the amount of the two proteins that are bound together at any one time is likely to be small. This is evident by comparing the subcellular distribution of ubiquilin-2 and hnRNPA1 in the cytoplasmic and nuclear fractions, which were quite discordant (Fig 16). Additionally, I found little variance in the fractionation patterns of WT and mutant ubiquilin-2 despite the differences in their capacity to co-immunoprecipitate hnRNPA1 (Figs 16 & 12). The results suggest that only a small fraction of ubiquilin-2 and hnRNPA1 protein pools are bound to each other at any one time.

It is also possible that the ubiquilin-2 mutants induce stress. This idea stems from studies that have shown hnRNPA1 translocates to the cytoplasm under conditions of stress, which classically has been demonstrated following exposure to osmotic shock (Allemand, 2005). Even though overall translocation of hnRNPA1 into the cytoplasm in cells transfected with the mutants compared to mock transfected cells showed only minor differences, the results suggest stress response is intact in the cells, and does not eliminate the possibility that cells

expressing the ubiquilin-2 mutants are more sensitized to stress. Indeed, quantification of cell death in the cell cultures transfected with the ubiquilin-2 constructs indicated that overexpression of all of the mutants induced an increase in cell death compared to mock-transfected cells, which is supportive of the idea that the mutants are more toxic. Furthermore, the percentage of cell death was greater for the cultures transfected with mutant ubiquilin-2 and exposed to osmotic shock, which suggests that overexpression of the mutants sensitize cells to stress.

CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

My research provides direct evidence of a novel interaction between ubiquilin-2 and hnRNP proteins and demonstrates that disruption of those normal protein interactions is caused by ALS-linked mutations in ubiquilin-2. Through biochemical assays, I showed that ubiquilin-2 binds to hnRNPA1 and hnRNPU *in vitro*, but that mutated ubiquilin-2 reduces the strength of this interaction. Mutations in ubiquilin-2 result in no alterations to the normal subcellular localization of hnRNPA1 and its translocation to the cytoplasm, but are associated with an increase in cell death. Finally, knock down of ubiquilin-2 leads to reduced expression of hnRNPA1 and increased turnover.

There are several avenues by which to continue this research. Perhaps the most obvious experiment would be to characterize the interactions between ubiquilin-2 and the other 38 interactors isolated through the Y2H screen. Of particular interest are the 12 clones recovered whose functions correspond to mitochondria. Proteins have long been implicated in neurodegenerative diseases and RNA metabolism is gaining a lot of traction in recent years, but I suspect mitochondrial defects also play an important role.

There are other extensions worthy of pursuit too. To begin with, it would be interesting to investigate the interaction of ubiquilin-2 with the remaining members of the hnRNP family. Moreover, investigating the constitution of the insoluble pellet in the subcellular fractionations would lead to a more precise understanding of where ubiquilin-2 and hnRNPs interact. Also, since hnRNPs

form complexes on hnRNA with snRNPs, it is quite likely that ubiquilin-2 is only one part of that complex and uncovering cofactors might lend insight into the mechanistic importance of the ubiquilin-hnRNP interaction.

One important answer that eluded me is which domain within hnRNPA1 binds to ubiquilin-2. Despite testing this question in two different experiments, I was unable to locate a precise amino acid sequence within hnRNPA1 that was necessary and sufficient for binding ubiquilin-2. Since the glycine-rich region in other RNA binding proteins, like TDP-43 and FUS/TLS, is a primary region for protein-protein interactions and since my research showed the C-terminus of hnRNPs interact with ubiquilin-2, it seemed a reasonable assumption that the GRR of hnRNPs was the site responsible for binding to ubiquilin-2 (Pesiridis, 2009) (Cartegni, 1996). To test this assumption, GST-pulldowns were performed with HIS-tagged GRRs of hnRNPA1, hnRNPU, and TDP-43. The purified GST-ubiquilin proteins did not bind with the isolated GRRs of the RNA binding proteins despite multiple variations of wash stringency. (data not shown) A series of hnRNPA1 deletion mutants were also generated to ensure a segment of amino acids required for interaction in the GRR clones was not excluded. Co-immunoprecipitations verified that the deletion of the GRR does weaken the strength of interaction with ubiquilin-2, but all three deletion mutants (full, medium, and short) showed some degree of interaction (data not shown). A systematic deletion mutant approach is required to slowly narrow in on the specific hnRNPA1 sequence that supports the interaction with ubiquilin-2. In the

interim, though, my data indicate the region with the strongest binding to ubiquilin-2 is AA 1-193.

HeLa cells were used throughout this work for multiple technical reasons, including robust expression, high transfection efficiency, and resiliency under stress, but it would be worthwhile to repeat the beginning experiments in cell lines more representative of ALS. Now that the ubiquilin-2 and hnRNP interaction has been established in HeLa cells, the next step is to replicate the finding in a mouse motor neuron line such as NSC-34.

Upon completion of that, the future studies of this interaction must involve a mouse model of ALS. To date, several transgenic mouse models exist, including those that express mutant SOD1 (several genotypes) and TDP43 (A315T) (Shibata, 2001) (Wegorzewska, 2009). Most recently, a mouse model of the P497H mutation in ubiquilin-2 was generated, but the information gleaned from testing the remaining ubiquilin-2 mutations would certainly advance the understanding of ubiquilin-2's involvement in the pathogenesis of ALS (Gorrie, 2014).

Just what critical function the interaction plays is yet to be determined. Indeed, one obvious consequence of the altered distribution of hnRNPA1 caused by the ubiquilin-2 mutants is the effect that it might have on hnRNPA1 function. Since my data show that ubiquilin-2 and hnRNPA1 interact in both the nucleus and the cytoplasm, albeit to varying degrees, an example list of possible additional consequences include alterations in stress granule formation, intracellular signaling cascades, chaperone protein roles, cytoplasmic-nuclear

shuttling of hnRNPA1 or hnRNPA1-mediated RNA binding. The altered distribution might affect these functions, leading to disease. Additional experiments are required to characterize the proper function of hnRNPA1 in the presence of ubiquilin-2 mutations.

Clarifying the functional, mechanistic role this interaction plays in ALS pathogenesis is crucial to harnessing the interaction's ability to deliver therapeutic options for the juvenile, X-linked ALS as well as, hopefully, other forms of sporadic and familial ALS. The experiments presented in this dissertation make clear the interaction of ubiquilin-2 with hnRNPA1 and the negative consequences that occur when mutations are introduced into either protein. Studying this interaction in more depth and determining the specific role it plays within the nucleus is critical to helping us better understand the pathogenesis of ALS. It will be exciting to continue unraveling the importance of the ubiquilin-2-hnRNPA1 interaction.

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