UBQLN2 Function and Dysfunction in the Central Nervous System and Synucleinopathies

by

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Dedication

Para Ana, Alba, y Jesus. Por todo lo que han sacrificado por sus hijos.

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Abstract

Protein accumulation and aggregation are hallmarks of many neurodegenerative disorders. The protein alpha-synuclein (α−syn) accumulates in Parkinson's disease and several other age-related diseases, collectively referred to as the synucleinopathies. When phosphorylated at serine 129 (pS129), α−syn aggregates and ultimately forms Lewy bodies in neurons. A better understanding of how α−syn is regulated both normally and in disease would provide insight into the development and progression of Parkinson's disease pathology. The protein quality control factor, Ubiquilin-2 (UBQLN2) has been implicated in multiple neurodegenerative diseases including in Parkinson's disease and other synucleinopathies. However, it is not known whether UBQLN2 regulates α−syn. In this dissertation, I aim to determine if UBQLN2 regulates α−syn in normal and disease states and to elucidate the mechanism by which UBQLN2 acts.

UBQLN2 is associated with synucleinopathies through its presence in Lewy bodies. However, it is unknown whether UBQLN2 is simply sequestered in these inclusions or whether its presence in Lewy bodies reflects a function of the protein in handling α−syn. In chapter two, I use cellular and mouse models to show that UBQLN2 regulates α−syn levels, particularly pS129, and demonstrate that UBQLN2 knock-out leads to total and pS129 α−syn accumulation. Pharmacological inhibition of the proteasome revealed that UBQLN2 targets pS129 for proteasomal degradation. Moreover, in brain tissue from PD and transgenic mice expressing pathogenic α−syn (A53T), endogenous UBQLN2 becomes more insoluble. Collectively, these

studies support a previously unknown role for UBQLN2 in directly regulating pathological forms of α−syn and indicate that UBQLN2 dysregulation in disease may contribute to α−syn mediated toxicity.

In chapter three I describe the unexpected finding that UBQLN2 overexpression in UBQLN2 transgenic mice leads to severe retinal degeneration in a dose-dependent manner. Immunofluorescence of retinas from UBQLN2 transgenic mice overexpressing WT or a pathogenic form of UBQLN2 (P506T) show punctate UBQLN2 expression in the outer retina, whereas endogenous UBQLN2 expression is diffuse and largely contained to the inner retina. I used optical coherence tomography to show that retinal degeneration in mice begins early and is rapid; with high UBQLN2 expression, degeneration began as early as four weeks of age, with a total loss of outer retinal layers by eight weeks of age. Retinal degeneration was less profound in P506T mice; retinas began thinning after six months of age, leading to a robust loss of outer retina cells by nine months of age. Disruption of proteostasis has been linked to multiple retinopathies, and our studies suggest that dysregulation of the protein quality control factor UBQLN2 is deleterious to retinal health. UBQLN2 knock-out did not robustly affect retinal thickness but may negatively impact retinal health at one year of age, suggesting that UBQLN2 is not necessary for proteostasis in the retina. UBQLN2 dysregulation in WT overexpressing and P506T retinas may lead to dysfunctional protein degradation, perhaps through sequestration of key protein quality control factors. These studies introduce the retina as a robust model for studies investigating UBQLN2 function and dysfunction and may have implications for retinal health in neurodegeneration.

Chapter four summarizes key findings from this dissertation and identifies work that is now needed to bolster a mechanistic understanding of UBQLN2 function and dysfunction in the

nervous system. In particular, I describe ongoing studies and future directions that will seek to define the mechanism by which UBQLN2 regulates pathogenic, phosphorylated forms of α−syn (e.g. pS129). This dissertation reveals a novel role for UBQLN2 as a regulator of pathogenic α−syn in disease and establishes the retina as a model for future studies of UBQLN2 function and dysfunction in protein quality control pathways.

Chapter 1 Introduction

1.1 Development and Progression of neurodegenerative diseases

Neurodegenerative diseases are a diverse family of disorders that affect the nervous system and result in a range of symptoms from motor deficits to cognitive decline. They are devastating conditions that result in progressive dysfunction and death of nerve cells and currently have no cure. Many factors contribute to the development of neurodegenerative disease including stress, toxic chemicals, and genetic mutations. However, the greatest risk factor is increasing age.[1] Outside of genetic forms of disease, relatively little is known about the etiology of neurodegeneration. And even in many genetic forms of disease, we do not fully understand the impact of mutations on the disease onset.

A hallmark of many neurodegenerative diseases is the accumulation of proteins in aggregates.[2] Some proteins are highly prone to aggregate and are linked to disease, allowing us to classify most neurodegenerative diseases by the principal protein found in aggregates. For example, tauopathies are diseases in which the microtubule associating protein, tau, forms aggregates; tauopathies include the most common neurodegenerative disease, Alzheimer disease (AD). Synucleinopathies, such as Parkinson's disease (PD) and Lewy body dementia (DLB), are diseases in which the protein α–synuclein (α–syn) accumulates. Other disease-linked proteins include TAR DNA-binding protein 43 (TDP-43) in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), and huntingtin (HTT) in Huntington's disease. It is believed that

protein accumulation in disease is due, in part, to dysfunction in the cellular mechanisms regulating protein homeostasis, which can be promoted by pathogenic mutations in the protein-coding gene.[2] The processes underlying protein dysregulation are not well understood.

1.1.1 Alpha-synuclein is dysregulated in synucleinopathies

PD and DLB share a common pathology, initially presenting different symptoms. PD is the second most common neurodegenerative disease, affecting 1% of individuals over 60 years of age[3, 4] and manifesting with tremors, rigidity, and slowness of movement.[3, 4] Though it is generally considered a movement disorder, there is growing evidence for cognitive dysfunction in PD, even in early stages of disease.[3,5] DLB, on the other hand, initially presents as a cognitive or psychiatric disorder and patients often develop movement dysfunction later in disease.[6]

Both PD and DLB are characterized by the formation of Lewy bodies, which are intraneuronal inclusions made up of pathological forms of the protein α–syn.[4,6-9] Many factors have been linked to the development of PD and DLB such as pesticide exposure[10,11], head injury[12], genetic mutations[4,13-16], and stress.[17] However, we do not fully understand how these risk factors translate to pathology. The accumulation of α–syn into Lewy bodies supports the view that healthy regulation of α–syn is impaired in these diseases.

α–syn is a pre-synaptic protein encoded by the *SCNA* gene.[16] The precise function of α–syn remains uncertain, although recent evidence suggests it plays a role in neurotransmitter release through its interaction with other synaptic proteins.[16] α-syn is considered a natively unfolded, or intrinsically disordered, protein.[16] However, its partial folding into secondary structures, such as alpha–helices and beta-sheets[16,18] is thought to enable its formation into protein fibrils.[18]

Native wild-type α–syn can aggregate and its propensity do so is exacerbated by protein modifications such as mutations and post-translational modifications.[16] The disease-linked A53T and A30P α–syn mutations enhance α–syn aggregation compared to wild-type α–syn.[15,16] Mutations in α–syn may affect the cell's ability to properly regulate the protein or affect its folding in a way that enhances its propensity to aggregate. Once formed, proteins can undergo post-translational modifications such as the addition of ubiquitin or phosphate groups.[16] The addition of a phosphate group to α–syn at serine 129 (pS129) is a post-translational modification that is closely linked to disease.[19-23] In a healthy brain, only 4% of α–syn is phosphorylated at S129.[19] In synucleinopathies, however, over 90% of α–syn is phosphorylated at S129.[19] pS129 α–syn is the major component of Lewy[19], but it is unclear whether α–syn is phosphorylated before Lewy body formation, thereby increasing its propensity to aggregate, or whether it is phosphorylated after aggregation as a potential signal to enhance its degradation.

1.2 Protein quality control is central to proteostasis

Protein homeostasis, also known as proteostasis, is carried out by a series of highly regulated and interconnected cellular processes that are involved in the fate of proteins from their initial synthesis at the ribosome to their clearance from the cell via protein degradative pathways.[24] Proteostasis pathways ensure that proteins achieve and maintain their intended conformation and mediate their degradation once they have reached the end of their lifespan or have folded incorrectly (misfolding; Figure 1-1).[24] Many factors can disrupt cellular proteostasis, including genetic mutations, harsh environment (stress, toxic chemicals), disease processes, and aging itself.[2] As soon as a protein is made, it is monitored by the cell's protein quality control machinery to ensure proper folding and eventual degradation.[2,25,26] Protein misfolding leads to

either re-folding by chaperones or premature elimination via degradative pathways.[2,25,27] In this manner, cells maintain healthy turnover of proteins.

1.2.1 Proper protein folding is essential to cell health

A protein's structural conformation determines its function.[25] As nascent proteins emerge from the ribosome, they are merely a chain of amino acids, which is known as their primary structure. Proteins begin to fold as they emerge from the ribosome,[25,28] forming alpha-helices or beta-pleated sheets held together by hydrogen bonds, thus folding into their secondary structures.[25] The tertiary structure is a three-dimensional conformation resulting from interactions between amino acid side chains within the polypeptide chain.[25] A protein's final conformation determines its function which is specified by the composition and contour of exposed amino acid side chain groups once native folding has been achieved.

Protein misfolding can occur due to errors in polypeptide synthesis or initial folding, or when a protein denatures and re-folds during its lifetime. Molecular chaperones help prevent misfolding by participating in the folding of nascent proteins,[29] and if a protein misfolds, chaperones attempt to correct misfolded proteins.[29] Genetic mutations and physiological stressors increase the occurrence of protein misfolding.[25,29] Misfolding may impair a protein's function, decrease its stability, or precipitate its aggregation,[2,24-26,30] all of which are ultimately harmful to cell health.

Protein aggregation occurs when misfolded proteins persist. Proteins can coalesce in several different conformations ranging from small oligomers to large intracellular inclusions and even extracellular amyloid fibrils. Misfolded oligomers are often inherently toxic due to loss-of-function properties, gain-of-function properties, or both.[30,31] Oligomers are prone to further aggregate, ultimately forming amyloid fibrils. However, in some cases the formation of

amyloid fibrils may stabilize otherwise toxic oligomers into stable structures that could serve a protective purpose[32-34] and even mediate their uptake by select protein degradation pathways.[35,36] The recognition of the role of toxic oligomers in many diseases, including neurodegenerative diseases, has spurred extensive studies that target the process of oligomer formation and clearance as a potential route to disease-preventing therapy.

1.2.2 The Ubiquitin Proteasome System

The ubiquitin proteasome system (UPS) is a key protein degradation mechanism, both in the cytosol and nucleus of cells.[37] As the name suggests, degradation via the UPS involves ubiquitination of targeted proteins which are then recognized and degraded by the proteasome. The UPS is thought to primarily act on soluble proteins[38,39] but can degrade some protein aggregates.[38] To be recognized by the proteasome for degradation, a protein typically undergoes a series of ubiquitin conjugations carried out by E3 ligases. This process results in the addition of a polyubiquitin chain to the protein, enabling its recognition by the proteasome.[39] The 26S proteasome is responsible for protein degradation through the UPS.[37,39] At its core is the 20S proteasome, which is also known as the catalytic particle due to its proteolytic function.[37,39] The 20S proteasome is capped at either one or both ends by the 19S proteasome.[37,39] The 19S is the regulatory particle of the 26S proteasome, mediating translocation of polyubiquitinated proteins into the 20S proteasome for degradation.[37,39] The 19S cap contains a binding site that recognizes ubiquitin chains and interacts with ubiquitin like domains (UBLs) that participate in shuttling substrate proteins to the proteasome.[40,41] The UPS plays an important role in proteostasis, and as such, stimulating UPS activity has been proposed as a potential treatment for proteinopathies. Conversely, dysfunctional UPS activity is deleterious to cells and has been implicated in neurodegeneration.[42]

1.2.3 Autophagy

Proteolysis also takes place in cells through autophagy. Autophagy-mediated protein degradation takes place via the lysosome in a less selective way than the UPS. There are three types of autophagy: macroautophagy, microautophagy, and chaperone mediated autophagy (CMA). In macroautophagy, proteins are trafficked to the lysosome through an intermediary autophagosome, whereas they are engulfed directly into the lysosome in microautophagy. CMA is a selective form of autophagy that requires a transmembrane receptor for protein translocation into the lysosome. (Reviewed in Glick et al., 2003)[43] For the purposes of this dissertation, I will focus on macroautophagy and CMA.

1.2.3.1 Macroautophagy

Macroautophagy occurs in a multistep process. The initiating step for autophagy is the formation of the phagophore, a cup-shaped stretch of membrane. Phagophore formation is regulated by autophagy-related (Atg) proteins that aid in phagophore elongation and the creation and regulation of the autophagosome.[44] The autophagosome is an organelle that encloses the portion of the cytosol that contains substrate proteins for lysosome digestion.[44] Once formed, the autophagosome travels to the lysosome for fusion. Importantly, autophagosome-lysosome fusion enables proteolysis by the lysosome. When fusion occurs, hydrolases degrade the contents of the autophagosome and its inner membrane.[43]

Unlike proteasomal degradation, lysosomal degradation through macroautophagy is partially nonselective. Nonselective macroautophagy occurs when proteins are indiscriminately sequestrated into the autophagosome. However, selective macroautophagy is mediated by LC3, an Atg protein found on the inner membrane of the autophagophore that selectively binds p62[45-47], leading to its degradation by the lysosome. Another form of selective macroautophagy is the

process of aggrephagy, the selective degradation of protein aggregates. Ubiquitination targets protein aggregates for aggrephagy[48] through its interaction with p62, which then serves as a linker between aggregates and the LC3 receptor within the autophagosome.[46] The ability of macroautophagy to degrade aggregates, which the UPS has only limited capacity to do[38], has led to disease modifying therapy approaches that target macroautophagy.

1.2.3.2 Chaperone mediated autophagy

CMA is a highly selective form of autophagy that only degrades proteins that contain the KFERQ or KFERQ-like recognition motifs.[49-51] The KFERQ targeting motif enables its interaction with HSC70 and target it for degradation through the lysosome.[52] Once shuttled to the lysosome, the transmembrane LAMP2-A receptor recognizes the substrate-HSC70 complex.[53,54] CMA protein degradation requires co-chaperones to unfold the protein prior to translocation into the lysosome by LAMP2-A[50], which makes CMA degradation of misfolded and aggregated proteins difficult.[55] However, many disease-linked proteins are known substrates for CMA, including α−syn[55], tau[56], HTT[57], and TDP-43[58], emphasizing the importance of CMA activity in preventing disease, while also implicating it in disease etiology and progression.

1.3 PQC is decreased in neurodegenerative diseases

Proteins accumulate and aggregate in aging and in many neurodegenerative diseases, implicating protein quality control pathways in age-dependent neurodegeneration. Substantial evidence suggests there is a decrease of UPS and autophagy activity with aging, leading to the persistence and aggregation of misfolded proteins.[59] For example, inclusions found in disease contain ubiquitinated proteins[60], suggesting dysfunctional UPS activity. Crosstalk between proteostasis pathways allows for compensatory activity when one pathway is compromised.[61,62]

For example, CMA activity is upregulated in response to normal age-related decrease of macroautophagy, though balanced activity between the two pathways is optimal for cell health. This point is most evident in the retina, where incremental changes in proteostasis activity leads to cell death and vision impairment.[50,63] While CMA activity may compensate for some loss of macroautophagy function in the retina, the reverse mechanism does not exist, and loss of CMA activity is detrimental to retinal health.[50,63]

In addition to the age-related decreases in protein quality control, neurodegenerative diseases directly impact proteostasis.[2,5,13,50] In PD, pathogenic forms of α−syn and LRRK2 interact with HSC/HSP70 and LAMP2-A in a manner that prevents their translocation into the lysosome, resulting in their aggregation at the lysosome and subsequently leading to CMA dysfunction.[55] When mutated, the E3 ubiquitin-ligase PARKIN loses its ability to ubiquitinate target proteins, including α−syn, decreasing their degradation by the UPS.[64] Protein aggregates themselves have a direct negative impact on protein degradation by creating a positive feedback loop where alterations in protein degradation pathways lead to protein aggregation, which in turn leads to further dysfunction in protein degradation.[55] Accordingly, an approach taken to treat neurodegenerative disease is to induce protein degradation pathways. Enhanced understanding of the role that key protein quality control proteins play in degradation is needed to better understand proteostasis dysfunction in disease. To this end, this dissertation focuses on Ubiquilin-2, a protein quality control shuttle protein.

1.4 Ubiquilins, a family of PQC proteins

Ubiquilin (UBQLN) proteins are a family of shuttle factors broadly implicated in protein quality control.[38,65-69] There are 5 known UBQLNs, UBQLN 1, 2, 3, 4, and L. UBQLN 1 and 4 are ubiquitously expressed, while UBQLN2 has limited expression, mainly in muscle and the

central nervous system (CNS).[65] UBQLN 3 and L are exclusively expressed in the testis.[65,70] Of the CNS-expressed UBQLNs, UBQLN2 is most homologous with UBQLN1 (74% homology[65], but all three share similar protein structure[65] (Figure 1-2). UBQLNs all contain a N-terminal ubiquitin like domain (UBL), which facilitates interactions with the 19s cap of the proteasome[65] and a C-terminal ubiquitin-associated domain (UBA) that interacts with ubiquitin on substrate proteins.[65] UBQLNs also contain a series of stress-induced protein 1 (STI-1) motifs that are thought to associate with chaperones.[65] UBQLN2 is unique among the UBQLNs in that it alone contains a proline-rich domain (PXX).[65] While the many potential roles of the PXX in UBQLN2's function are not well understood, one known role of PXX domains is to stabilize protein interactions.[65,71]

1.4.1 UBQLN2 is a shuttle protein for the UPS

UBQLN2 is functionally implicated in the UPS due to its UBA and UBL domains[40,65,72] (Figure 1-3). The UBL domain has been shown to interact with the hRpn10 subunit of the 19S proteasome, which recognizes ubiquitin on substrate proteins[73], and knockdown of hRpn10 results in impaired proteasome-UBQLN2 interactions.[74] The UBA domain has a high affinity for ubiquitin, including poly-ubiquitin, suggesting that UBQLN2 contain a ubiquitin receptor functionality, further supporting the role for UBQLN2 as a shuttle protein for the UPS.

1.4.2 UBQLN2 also mediates lysosomal degradation

Though UBQLN2 has been implicated in autophagy, its potential roles in lysosomal degradation are poorly understood. A study implicating UBQLN2 in macroautophagy revealed that UBQLN2 is found within autophagosomes (Figure 1-3).[75] UBQLN2 has been shown to interact with LC3, a receptor found in the inner membrane of autophagosomes, suggesting that it

may also serve as a shuttle for proteins targeted for degradation through macroautophagy.[76] However, UBQLN2's presence in autophagosomes and its interaction with LC3 may be due to its own clearance by macroautophagy.[75] UBQLN2 has also been indirectly linked to autophagy by studies showing that UBQLN2 depletion leads to increased cell death following starvation-induced stress[77], which is known to activate autophagy as a protective mechanism. Further, studies have revealed that UBQLN2 mutations lead to reduced expression of autophagy proteins, including p62, ATG7, and LAMP2-A.[78] UBQLN STI-1 motifs further support a role for UBQLNs in autophagy due to their known interactions with chaperones[38,75], their cochaperone activity[79], and their interactions with LC3 in autophagosomes[38,75]. All three of the brain expressed UBQLNs contain a KFERQ motif, suggesting they may be substrates of CMA. The presence of the KFERQ in UBQLNs may also enable their shuttling of substrate proteins to the lysosome for degradation via LAMP2-A.

1.5 UBQLN2 is implicated in neurodegenerative disease

The proposed roles for UBQLNs in protein quality control suggest that they are relevant to neurodegenerative disease. The importance of UBQLNs in neurodegeneration has been highlighted by the discovery that mutations in UBQLN2 directly cause ALS and FTD.[80] Interestingly, many of the disease-causing mutation in UBQLN2 are found in the PXX domain[80,81], which is unique to UBQLN2 among the UBQLN proteins. Multiple disease linked UBQLN2 mutations are known to suppress protein degradation. For example, P506T UBQLN2, which causes familial ALS, has been shown to lose its ability to interact with HSC/HSP70 and shuttle aggregates for degradation through the proteasome.[38] P506T and other mutations in UBQLN2 are known to decrease its ability to shuttle ubiquitinated proteins for degradation through the UPS.[67,68] These findings distinguish UBQLN2 among the UBQLN protein family as

a regulator of disease-linked proteins and highlight the importance of understanding normal UBQLN2 function and its role in disease.

UBQLN2 has been linked to multiple neurodegenerative diseases by studies showing that UBQLN2 interacts with protein inclusions in FTD/ALS, and HD, but not taupoathies.[80,82-84] In ALS/FTD, UBQLN2 is known to interact with TDP-43[81,82,85], colocalizing with inclusion in both familial and sporadic forms of disease.[80] Studies have shown that dysregulation of UBQLN2, either by overexpression of WT protein or disease-linked mutations, leads to increased accumulation of TDP-43[85], suggesting that UBQLN2 plays a role in TDP-43 pathology in disease. The principal component of inclusions in HD is the protein HTT. UBQLN2 is known to interact with mutant HTT, but not normal HTT.[38,86] Overexpression of UBQLN2 decreases levels of pathogenic HTT in cellular and mouse models[86,87], while UBQLN2 knock-down leads to an accumulation of insoluble HTT in vitro.[38] These findings support an important role for UBQLN2 in regulating HTT in disease. While UBQLN2 does not colocalize with inclusions in tauopathies[82,84], recent studies show that UBQLN2 itself is altered in tauopathies.[87,88] UBQLN2 dysregulation is associated with subsequent accumulation of pathological, phosphorylated tau.[87]

The only studies implicating UBQLN2 in synucleinopathies report colocalization of UBQLN2 with Lewy bodies in PD and DLB.[82] It is unknown whether UBQLN2 is selectively recruited to inclusions in disease, nor do we know if UBQLN2 regulates levels of normal or pathological α–syn.

1.6 Summary and aims of the dissertation

Neurodegenerative diseases are debilitating, ultimately causing physical and cognitive decline. Altered protein quality control is thought to be central to many age-dependent neurodegenerative diseases due to the formation of proteinaceous inclusions in disease.

However, little is known about how altered proteostasis leads to disease or contributes to disease progression. UBQLN2 has emerged as a potential key regulator of disease-linked proteins, yet little is known about UBQLN2's role normally or in disease states.

The primary goal of this dissertation is to elucidate the function of UBQLN2, both normally and in disease. My central hypothesis is that UBQLN2 functions as a shuttle for the UPS (Figure 1-4). To address this, my dissertation is divided into two aims. The first aim explores the role of UBQLN2 as a regulator of α−syn. Despite its prevalence, little is known about the etiology of PD, particularly the cause of α−syn aggregation in disease. UBQLN2 has been linked to PD through its colocalization with α−syn rich Lewy bodies in post-mortem tissue from patients with PD. Thus, I chose to study the role of UBQLN2 in disease by investigating its function in regulating α−syn in synucleinopathies. These studies are described in chapter two where we use a combination of human disease tissue and cellular and mouse models to elucidate the role of UBQLN2 on α−syn. The second aim of this dissertation is to expound on the normal mechanism of UBQLN2 function. In chapter three, we describe the unexpected finding that UBLQN2 dysregulation leads to robust, early-onset retinal degeneration in UBQLN2 transgenic mice and introduce the retina as a model system for studying UBQLN2 function and dysfunction in protein quality control pathways. Chapter four concludes this dissertation and proposes areas for future study.

1.7 References

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1.8 Figures

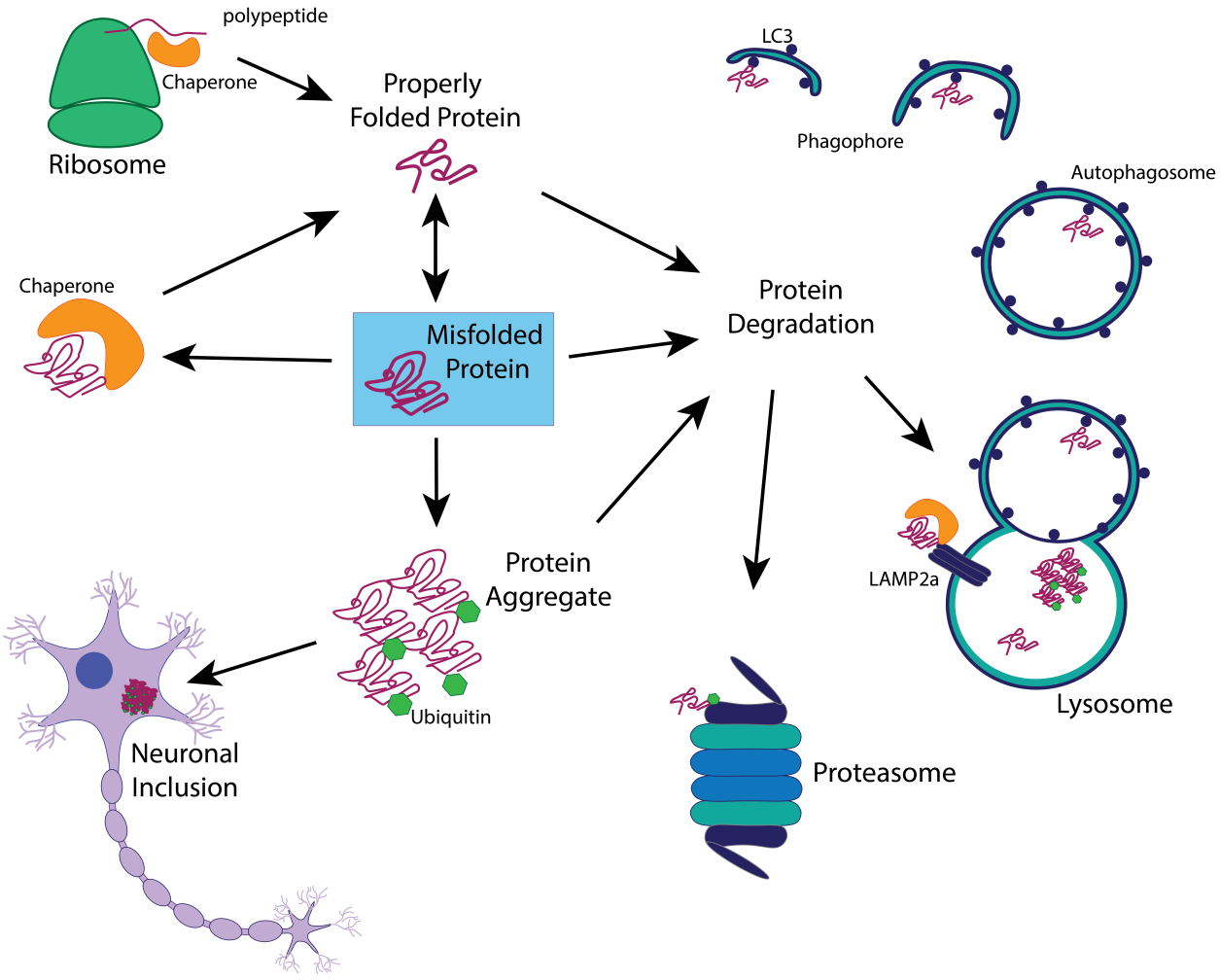

Figure 1-1 Intracellular proteostasis is carried out by highly regulated and interconnected processes.

As nascent proteins emerge from the ribosome, chaperones ensure proper folding of the polypeptides. Once a native conformation is achieved, proteins serve a variety of functions in the cell and at the end of their lifespan are degraded by either proteasomal or lysosomal protein degradation. If a protein adopts a nonnative conformation, chaperones often can restore the protein to its native conformation and function, but if it cannot successfully be refolded, the protein will be targeted for degradation. Misfolded proteins are prone to aggregate, so if misfolded proteins evade degradation, they can form aggregates that ultimately form into neuronal inclusions in cell bodies, nuclei or neuritic processes. Neuronal inclusions formed by specific proteins are characteristic of many age-dependent neurodegenerative diseases, including the synucleinopathies.

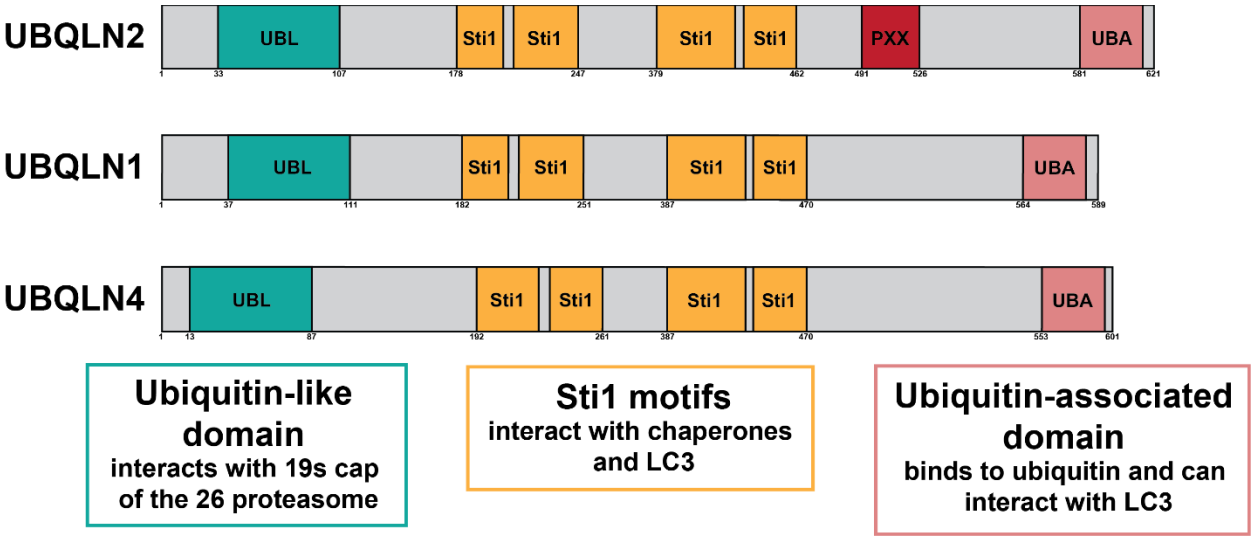

Figure 1-2 Functional domains are conserved among CNS-expressed UBQLNs.

UBQLN1, 2, and 4 all contain an N-terminal UBL domain that facilitates interactions with the 19s cap of the 26s proteasome. They also all contain 4 Sti-1 motifs that interact with chaperones, LC3 in autophagosomes and are thought to enable protein dimerization. Near the C-terminus, there is a UBA domain that binds ubiquitin and is required for liquid-liquid phase separation of UBQLNs. UBQLN2 uniquely contains a PXX domain in which many of UBQLN2's disease linked mutations, such as the P506T mutation employed in this study, are found.

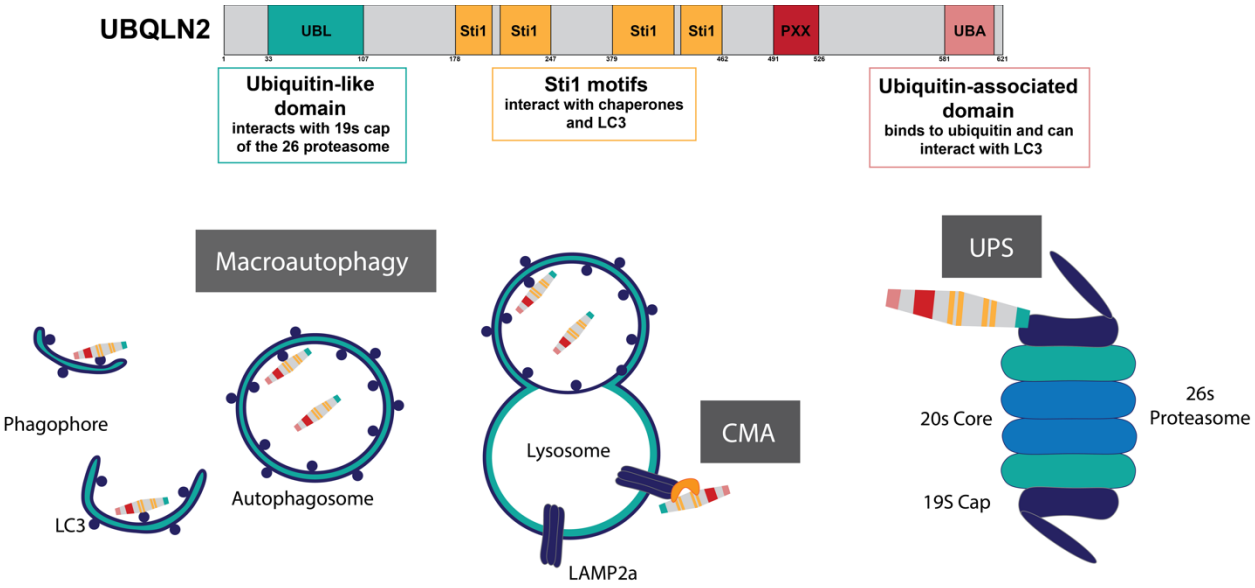

Figure 1-3 UBQLN2 has been implicated in multiple degradation pathways.

UBQLN2's UBA and UBL domain implicate it in the UPS. UBQLN2 is known to interact with LC3 and can be found in autophagosomes, implicating it in macroautophagy. The Sti1 motifs within UBQLN2 are also known to interact with HSC/HSP70. HSC/HSP70 is central to protein degradation by chaperone mediated autophagy (CMA) through its interaction with LAMP2a, a receptor on the lysosome that recognizes substrate proteins and translocates them into the lysosome. UBQLN2 may function as an intermediary protein between HSC/HSP70 and CMA substrates.

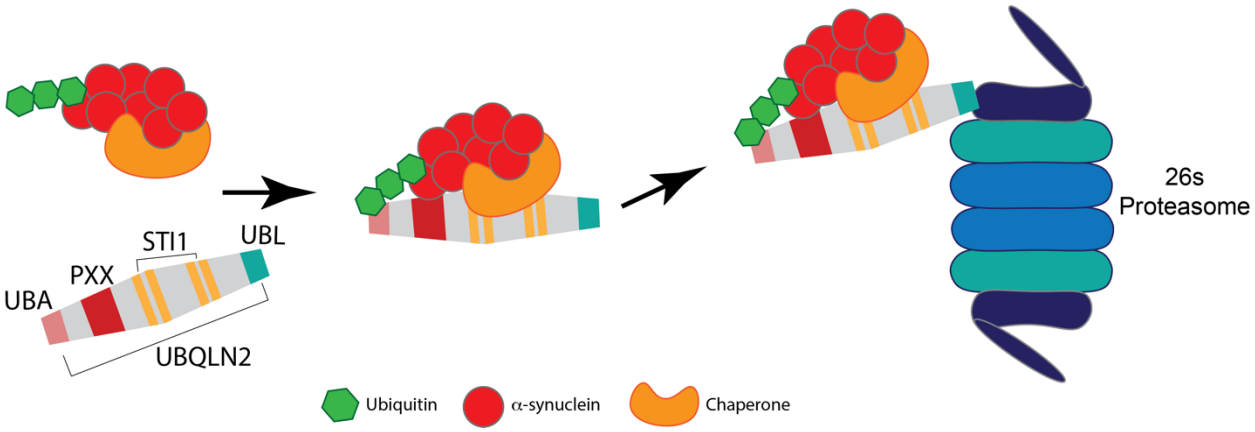

Figure 1-4 Hypothesis of UBQLN2 function in regulating α-synuclein.

α–syn can be degraded by proteasomal and lysosomal degradation. Due to UBQLN2's key function as a shuttle protein for the UPS, we hypothesize that UBQLN2 principally shuttles α–syn (monomer and oligomer) to the proteasome for degradation.

Chapter 2 UBQLN2 Regulates Pathological α-synuclein

2.1 Introduction

Like many neurodegenerative diseases, Parkinson's disease (PD) and Lewy Body Dementia (DLB) are characterized by the accumulation and misfolding of disease-specific proteins.[1] A key pathological feature of PD and DLB is the presence of α-synuclein (α-syn)-rich Lewy bodies (LBs) and Lewy neurites (LNs) in neurons of affected brain regions.[2,3] Several factors contribute to the accumulation of aggregated α-syn in synucleinopathies, including post-translational modifications (such as phosphorylation at serine 129), increased production (duplication or triplication of the *SNCA* gene), missense mutations (e.g., A30P and A53T)[4,5] and altered degradation of α-syn protein.[6] Perturbations in proteostasis have been implicated in the pathogenesis of synucleinopathies.[7] Evidence supports regulation of α-syn by multiple quality control systems, including proteasomal and lysosomal pathways.[6,8,9] The specific aggregation state of α-syn may also influence which pathway(s) it is handled by.

Ubiquilin-2 (UBQLN2), a protein quality control protein, has been implicated in PD and DLB due to its colocalization with accumulated α-syn in PD and DLB post-mortem tissue.[10] UBQLN2 is one member of the family of ubiquilins that function as shuttle proteins for the ubiquitin-proteasome system (UPS).[11] Ubiquilins are similar in structure, containing N-terminus ubiquitin-like (UBL) and C-terminus ubiquitin-associated (UBA) domains that facilitate the shuttling of ubiquitinated substrate proteins to the proteasome for degradation.[12,13] UBQLNs also contain STI1 motifs that may mediate binding to chaperones.[14] Among the UBQLNs, UBQLN2

uniquely contains a proline-rich domain (PXX).[15] The prevailing view is that UBQLNs function predominantly as UPS shuttle proteins but likely participate in other quality control pathways as well, including autophagy.[11-16-18]

The importance of UBQLNs in neurodegenerative disease was underscored by the discovery that mutations in UBQLN2 directly cause frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS).[15,19,20] UBQLN2 also forms liquid condensates and can aggregate *in vitro*[17,21] and *in vivo*[22], with pathogenic mutations increasing UBQLN2 aggregation.[22-26] UBQLN2 also co-localizes with aggregates of disease proteins such as huntingtin in Huntington's disease, and TDP-43 and dipeptide repeat proteins in ALS/FTD.[10,15] Collectively, these results support the view that UBQLN2 contributes to multiple neurodegenerative diseases. Precisely how, however, is poorly understood.

Outside of the colocalization of UBQLN2 with Lewy bodies in PD and DLB, little is known about the role of UBQLN2 in synucleinopathies. For example, it is unknown whether UBQLN2 sequestration into protein aggregates supports a role for UBQLN2 in regulating α-syn, either normally or in disease states. Here, we use human disease tissue and transgenic mouse and cellular models to show that UBQLN2 regulates a key pathogenic form of α-syn, pS129, by targeting it to the proteasome for degradation.

2.2 Results

2.2.1 UBQLN2 insolubility is increased in PD

Previous studies established that UBQLN2 accumulates in proteinaceous aggregates in several neurodegenerative diseases.[10,15] To determine whether UBQLN2 solubility is altered in synucleinopathies, we assessed soluble and insoluble levels of UBQLN2 in protein lysates from

PD and DLB patient cingulate cortex. Compared to age-matched controls, PD patient brain lysates exhibited increased insoluble UBQLN2 ($p=0.03$; Figure 2-1A) and DLB brain lysates exhibited a trend towards increased insoluble UBQLN2 ($p=.07$; Figure 2-1B). These results support previous studies showing that UBQLN2 colocalizes with LBs[10] and thus might be sequestered in insoluble material.

To determine whether a rodent model of PD recapitulates this observed increase in insoluble UBQLN2 in human disease, we analyzed levels of UBQLN2 in whole brains from 10-month-old transgenic mice expressing a pathogenic form of α-syn (A53T) implicated in familial PD.[27] Similar to our observations in human disease tissue, insoluble UBQLN2 was increased in brains from A53T mice compared to non-transgenic (Non-Tg) controls ($p=0.01$; Figure 2-1C). This result suggests not only that the A53T transgenic mouse is a relevant model for the study of UBQLN2 in synucleinopathies, but also that UBQLN2 solubility is affected in A53T mutant transgenic mice.

2.2.2 UBQLN2, and not UBQLN1, preferentially regulates pS129 α-syn

UBQLN2's function as a shuttle protein in protein quality control pathways and its colocalization with various disease protein aggregates[10,15] suggest that it may regulate misfolded disease proteins such as α-syn by altering protein levels. LBs are enriched for pS129[28,29], and based on studies showing that UBQLN2 colocalizes with LBs in disease,[10] we hypothesized that UBQLN2 may regulate α-syn, particularly levels of phosphorylated forms such as pS129. Preliminary studies in HEK-293 cells transiently transfected to express UBQLN2 and α-syn supported the view that UBQLN2 decreases levels of α-syn (not shown). However, HEK-293 cells contain endogenous UBQLNs which can confound analysis. Accordingly, we used UBQLN

triple knockout cells (TKO)[30], which are devoid of UBQLNs 1, 2 and 4 to test the role of UBQLN2, or closely similar UBQLN1, in regulating α-syn, absent any potential compensatory effects of other UBQLNs. We transiently transfected α-syn with plasmids encoding UBQLN1, UBQLN2 or an empty vector (EV) and found that UBQLN2, but not UBQLN1, significantly decreased total α-syn ($p=<0.001$) and pS129 levels ($p=0.03$) compared to EV control (Figure 2-2B). UBQLN1 expression led to an accumulation of pS129 levels ($p=0.01$; Figure 2-2B), but unlike UBQLN2, UBQLN1 has not been broadly implicated in neurodegeneration and does not display altered solubility in PD and DLB[31]. These results point to a preferential effect of UBQLN2 in altering levels of α-syn, including pS129.

UBQLNs function as shuttle proteins for the UPS and form heterodimers that may be important for their function.[32] To assess the role of UBQLN 1 or 2 on α-syn and pS129 protein levels when expressed at closer to endogenous levels and independent of other UBQLNs, we transiently expressed α-syn in TKO UBQLN rescue cells that inducibly express either UBQLN1 or UBQLN2 when treated with doxycycline.[30] These UBQLN rescue lines overexpress UBQLN1 or UBQLN2 to a lesser extent than when transiently transfected, allowing us to assess UBQLN1 and UBQLN2 regulation of α-syn in a more biologically relevant environment. Control cells that express endogenous levels of UBQLN 1, 2 and 4 were also evaluated. In the absence of all UBQLNs, levels of pS129 were significantly increased in TKO cells, and induction of UBQLN2 significantly decreased pS129 levels, compared to TKO cells ($p=0.001$; Figure 2-2C). In contrast, UBQLN1 induction had no effect on pS129 levels compared to TKO cells ($p=0.95$; Figure 2-2C), suggesting UBQLN1 may only cause pS129 accumulation when an overabundance of UBQLN1 is expressed. Total α-syn levels were unchanged in TKO and UBQLN 1 or 2 rescue

cells compared to control cells (p=0.37; Figure 2-2C). These data further support a role for UBQLN2 in regulating pS129 α-syn, if not total α-syn itself.

To further explore UBQLN2 regulation of α-syn, we performed immunofluorescence on HEK-293 cells in which we transiently expressed or knocked down UBQLN2 while over-expressing wild-type α-syn. Immunofluorescence analysis supported the finding that UBQLN2 overexpression decreases levels of α-syn (p=0.004; Figure 2-3A,C). However, α-syn levels were unchanged upon UBQLN2 knock-down (p=0.7; Figure 2-3A,C), perhaps due to compensatory effects of other UBQLNs and the presence of remaining UBQLN2 following partial knock-down. In contrast, levels of α-syn were not altered by UBQLN1 overexpression or knock-down (p=0.96; Figure 2-3B,D). A direct comparison of the number of α-syn-positive cells expressed as a ratio to UBQLN2- or UBQLN1-positive cells again showed far fewer α-syn-positive cells with UBQLN2 than with UBQLN1 (p=0.005; Figure 2-3E), further supporting the conclusion that UBQLN2 preferentially regulates α-syn.

2.2.3 UBQLN2 KO robustly increases the levels of pathogenic pS129 α-syn

The above results showing UBQLN2 regulation of α-syn in cell models (Figure 2-2) and increasing UBQLN2 insolubility in human and mouse brains harboring α-syn accumulation (Figure 2-1) led us to test whether UBQLN2 regulates α-syn in vivo. To assess this, we measured levels of total α-syn in brains of transgenic mice expressing pathogenic α-syn (A53T) crossed to either a transgenic mouse line overexpressing UBQLN2 (Ub2-Hi; Figure 2-4A) or to the UBQLN2 KO mouse (Ub2-KO; Figure 2-4A). Contrary to our in vitro studies (Figures 2-1 and 2-2), A53T mice devoid of UBQLN2 (A53TxUb2-KO) displayed an accumulation of total α-syn compared to A53T control mice (p=0.0003; Figure 2-4B), while A53T mice

overexpressing UBQLN2 (A53TxUb2-hi) showed a trend toward decreased levels of α-syn compared to A53T (n=7; p=0.1). To further assess total α-syn levels in vivo, we performed immunofluorescence to measure levels of α-syn in the deep cerebellar nucleus (DCN), a brain region known to be affected in the A53T mouse model.[27] We did not detect a significant difference in α-syn immunofluorescence between A53T and A53TxUb2-hi (p=0.9) or A53TxUb2-KO (p=0.1) mice (Figure 2-4C).

To determine if UBQLN2 plays a role in handling α-syn in non-disease states, we measured levels of total endogenous murine α-syn in Ub2-hi and Ub2-KO mice as well as in non-transgenic littermate controls by western blot. We did not detect a significant change in total endogenous α-syn in Ub2-Hi (p=0.3) or Ub2-KO (p=0.6) mice compared to controls (Figure 2-4D). To determine whether UBQLN2 and α-syn interact, we performed immunoprecipitation assays which captured an interaction between UBQLN2 and α-syn with reciprocal pull downs using either α-syn or UBQLN2 antibodies (Figure 2-4E-F). These results suggest that UBQLN2 interacts with α-syn, despite not decreasing levels of total α-syn in vivo

The accumulation and deposition of pS129 α-syn into LBs is a hallmark of synucleinopathies.[29] UBQLN2 can also be found in LBs.[10] To determine whether UBQLN2 regulates levels of pS129 α-syn we measured pS129 levels in A53TxUb2-hi and A53TxUb2-KO mice. Western blot analysis revealed robust accumulation of pS129 in brain lysates of A53TxUb2-KO mice compared to controls (p=0.002; Figure 2-5A), while A53TxUb2-hi mice showed no difference in pS129 levels (p=0.96; Figure 2-5A). Further assessment of pS129 levels by immunohistochemistry revealed an accumulation of pS129 in A53TxUb2-KO mice compared to A53T controls (Figure 2-5B). We were also able to detect endogenous levels of murine pS129

in Ub2-hi and Ub2-KO mice despite the generally low amount of pS129 in non-diseased brains[29,33] (Figure 2-5C). We observed a trend towards decreased murine endogenous pS129 in Ub2-hi mice (p=0.2) and no change in pS129 levels in Ub2-KO mice (p=0.8). Together, these results suggest that UBQLN2 regulates pS129 in vivo, both under normal conditions and in disease states.

2.2.4 UBQLN2 targets pS129 a-syn to the proteasome for degradation

To test whether overexpression of UBQLN2 leads to an increased rate of α-syn degradation we used optical pulse labeling to measure alpha-synuclein degradation over time.[34] For this experiment we used human α-syn tagged with Dendra2 (hSyn-Dendra2), a photoconvertible protein whose fluorescence changes from green to red following 405nm light exposure (Figure 2-6A). Rat primary cortical neurons were transfected with hSyn-Dendra2 and either UBQLN2-iRFP or iRFP. Following photoconversion, red fluorescence was tracked over time, allowing us to determine the rate of hSyn-Dendra2 turnover on a single-cell level. UBQLN2 overexpression had no effect on hSyn-Dendra2 decay compared to the iRFP control, suggesting that UBQLN2 does not play a significant role in nonpathogenic α-syn degradation (p=0.6; Figure 2-6B,C). Analysis of photoconverted hSyn-Dendra2 half-life further confirmed that UBQLN2 overexpression does not significantly affect normal α-syn degradation (p=0.28; Figure 2-6D-E). This finding is consistent with our studies suggesting that UBQLN2 has a stronger role in pS129 α-syn regulation.

α-syn and pS129 are regulated by both proteasomal and lysosomal degradation.[35-37] Though UBQLN2 may function in lysosomal protein degradation[16,18], it is thought primarily to play a role in proteasomal degradation[16,22]. To investigate whether UBQLN2 targets α-syn to the

proteasome for degradation, we treated cells transfected to express α-syn and UBQLN2 or EV with either the proteasome inhibitor lactacystin[38] (10 uM) or the proteasome activator Rolipram[39] (50 uM). In the presence of overexpressed UBQLN2, proteasome inhibition led to a robust increase in pS129 ($p=<0.001$) but not total α-syn ($p=1.0$), whereas in the absence of UBQLN2 there was no such effect on pS129 ($p=0.98$) or α-syn ($p=1.0$) levels (Figure 2-7A). Immunofluorescence supported the finding that overexpression of UBQLN2 leads to an accumulation of pS129 when the proteasome is inhibited ($p=0.002$; Figure 2-7B). These results suggest that while α-syn likely can be cleared by multiple, redundant quality control pathways, overexpressed UBQLN2 preferentially targets pS129 to the proteasome for degradation.

2.3 Discussion

Although UBQLN2 has been linked to several neurodegenerative diseases[10,15,31] and is known to accumulate in Lewy bodies in disease[10], whether this ubiquitin-linked quality control protein regulates α-syn under normal conditions or in disease states is unknown. Here we used human disease tissue, transgenic mouse models and cellular models to query whether UBQLN2 regulates α-syn. Our findings support a role for UBQLN2 in regulating levels of a key pathological form of α-syn, namely pS129.

UBQLN2 is intrinsically prone to form condensates and aggregates[10,15,21,25] and accumulates in various neurodegenerative proteinopathies, including Lewy bodies in PD.[10] Our results establish that UBQLN2 is more insoluble in PD, consistent with the previous finding that UBQLN2 colocalizes with aggregates of α-syn in PD.[10] Protein solubility may correlate with functionality, with decreased solubility correlating with decreased normal function. As UBQLN2 is sequestered in protein aggregates in disease, it may lose its normal function of regulating

protein turnover. Conversely, as UBQLN2 solubility decreases, it could also undergo a gain-of-toxic-function.[40] In PD, UBQLN2 may be sequestered in aggregates, precipitating a loss of normal UBQLN2 function and in turn accelerating pS129 accumulation and potentially α-syn toxicity and disease progression.

Of the three brain-expressed UBQLNs, UBQLN2 has been most widely implicated in neurodegenerative diseases[15,31,41] and our results supporting a significant role for UBQLN2, but not UBQLN1, in regulating α-syn are consistent with this concept. The UBQLNs are highly similar proteins except for the PXX domain that is unique to UBQLN2. Among UBQLNs, UBQLN2 and UBQLN1 are most similar in structure, sharing 79% homology[42], but do not appear to share the role of regulating specific disease proteins such as α-syn and tau.[43] The PXX domain may be a driver of UBQLN2's potential function as a regulator of such disease proteins. Of their many functions, proline-rich domains aid in protein binding[44] so conceivably the PXX domain of UBQLN2 favors its interaction with client proteins independent of ubiquitination. Intriguingly, the PXX domain is also the site of most of the pathogenic UBQLN2 mutations that directly cause hereditary neurodegeneration.[15] Further investigations into the role of the PXX and other UBQLN2 domains are warranted to better understand how UBQLN2 shuttles pS129 to the proteasome for degradation.

A recent study assessing the effects of UBQLN2 on another disease-linked protein, tau, reported a greater effect of UBQLN2 on phosphorylated tau than on total tau[43], reminiscent of our finding of a greater effect of UBQLN2 on pS129 α-syn than on total α-syn. Together, these studies support the view that UBQLN2 preferentially affects pathological forms of proteins, such as those that are phosphorylated or aggregated. Conceivably, UBQLN2 regulates protein phosphorylation, but there is no evidence that UBQLN2 regulates kinase or phosphatase

activity.[43] Another possibility is that UBQLN2 preferentially targets aggregated proteins for degradation and disease aggregates are often enriched in phosphorylated proteins. Assessing this possibility in the current studies was complicated by the mild phenotype seen in hemizygous A53T mice and the lack of significant pathology until after 22 months of age.[27] An effect of UBQLN2 on A53T might be captured with the more robust homozygous mouse model. Alternatively, the A53T mutation may alter UBQLN2's regulation of α-syn considering that A53T α-syn is known to alter proteasome activity[45], further complicating UBQLN2's role on α-syn in PD.

UBQLN2 has been implicated in proteasomal and lysosomal degradation pathways[16,18,46], pathways by which α-syn is regulated.[6,9,36,47,48] Since UBQLN2 is primarily considered a proteasome shuttle protein, we sought to determine whether UBQLN2 acts on α-syn at the proteasomal level. In the absence of UBQLN2, pS129 appears to be cleared by other pathways when the proteasome is inhibited, but the presence of UBQLN2 restricts pS129 clearance to the proteasomal pathway. UBQLN2 may sequester pS129 at or near the proteasome, limiting accessibility to other clearance pathways. Our results further indicate that normal (nonpathogenic) α-syn can be regulated by pathways other than the proteasome and that the presence or absence of UBQLN2 does not greatly affect normal α-syn degradation by those pathways. This may reflect a limited role for UBQLN2 in normal α-syn degradation or an ability of UBQLN2 to regulate normal α-syn via multiple clearance pathways. The former is supported by our kinetic studies showing that UBQLN2 does not significantly alter normal α-syn half-life. Further optical pulse labeling kinetic studies employing a phospho-mimetic of pS129 could help determine the basis of UBQLN2's selective effect on this pathogenic form of α–syn.

In summary, we have established a role for UBQLN2 in regulating pathological forms of α-syn by targeting it to proteasomal degradation. This knowledge of the role of UBQLN2 in regulating pS129 sheds light on one potential reason why UBQLN2 is found in Lewy bodies in synucleinopathies: to decrease pS129 levels. Although further studies will be needed to understand the differential roles of UBQLN2 in regulating normal, post-translationally modified, and mutant forms of α-syn, our results here defining UBQLN2 as a regulator of pS129 α−synuclein point to UBQLN2 as a potentially important modifier of synucleinopathies and as a component of altered proteostasis in neurodegenerative disease.

2.4 Methods

2.4.1 Plasmids and siRNAs

The pCMV4-FLAG-UBQLN2 plasmid (p4455 FLAG-hPLIC-2; Addgene plasmid # 8661) and pCS2-FLAG-UBQLN1 plasmid (p4458 FLAG-hPLIC-1; Addgene plasmid # 8663) were gifts from Peter Howley(*114*). The pAAV-MCS-α-synuclein plasmid (pAAV asyn WT; Addgene plasmid # 36055) was a gift from Hilal Lashuel.[50] Control empty vector plasmid for cell transfection experiments was pCMV-HA was used as a control for UBQLN2 clearance experiments. For UBQLN2 and UBQLN1 knockdown experiments, Dharmacon SMARTpool siGENOME siRNA against UBQLN1 and UBQLN2 and MISSION siRNA Universal Negative Control (Sigma) were used. The pGW1-hSyn-Dendra2 plasmid was synthesized by VectorBuilder. Creation of the pGw1-UBQLN2-iRFP and pGW1-iRFP plasmids is described in Sharkey et al, 2018.

2.4.2 Cell Culture

Human embryonic kidney 293 (HEK-293) cells were cultured in high glucose DMEM (Gibco), supplemented with 10% FBS, 10 mM glutamine, and 100 U/ml penicillin/streptomycin. UBQLN1/2/4 triple knockout (TKO)[30], UB1 rescue[30], UB2 rescue, and TREX HEK-293 (TREX) cell lines were cultured in high glucose DMEM, supplemented with 10% FBS, 10 ug/ml blasticidin, and 100 ug/ml hygromycin.

2.4.3 Cell Transfection

Cells were transfected with DNA plasmids using Lipofectamine-2000 according to the manufacturer's instructions. Cells used for downstream Western blot analyses were lysed in RIPA buffer (Thermo Scientific) with protease inhibitor cocktail (catalog no. 11873580001; Sigma Aldrich). Cells used for immunofluorescence analyses were fixed in chilled methanol for 20 minutes at -20˚C prior to staining. For UBQLN rescue experiments, TREX, TKO, UB1 rescue, and UB2 rescue cell lines were treated with 10 ng/mL doxycycline to induce UBQLN 1 or 2 expression in the respective cell lines 24 hours prior to transfection.

2.4.4 Human Disease Brain Tissue

Frozen brain tissue from the cingulate cortex was obtained from subjects with PD, DLB and age-matched control subjects (Table 1) from the Michigan Brain Bank (University of Michigan, Ann Arbor, MI, USA). Brain tissue was collected following a standard protocol in which patients gave informed consent for autopsy and, upon death, a Michigan Brain Bank physician confirmed this existing consent with the next of kin before proceeding.

Protocols were approved by the Institutional Review Board of the University of Michigan and abide by the Declaration of Helsinki principles. Samples were examined at autopsy by neuropathologists for diagnosis.

2.4.5 Mouse Models

This study was conducted in a facility approved by the American Association for the Accreditation of Laboratory Animal Care, and all experiments were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee of the University of Michigan and reported in accordance with ARRIVE guidelines.[51] Mice were housed at the University of Michigan animal care facility and maintained according to U.S. Department of Agriculture standards (12 h light/dark cycle with food and water available *ad libitum*). Twelve-month-old hemizygous UBQLN2 transgenic mice generated in the laboratory[52] and non-transgenic littermates were euthanized with isofluorane, perfused with PBS and brains were removed for analysis. Additionally, we acquired brain from 10-month-old homozygous A53T-mutated α-synuclein M83 mice (B6;C3-Tg-Prnp-SNCA-A53T 83Vle/J; The Jackson laboratory stock #004479)[27], and non-transgenic littermates were a gift from Dr. Rakez Kayed. Both male and female mice were included in analyses.

Hemizygous UBQLN2-high[52] and UBQLN2-KO[52] mice were crossed with hemizygous A53T-mutated α-synuclein M83 mice (B6;C3-Tg-Prnp-SNCA-A53T 83Vle/J; The Jackson laboratory stock #004479).[27] 12 months of age male and female, UBQLN2-high transgenic, UBQLN2 KO, A53T, A53TxUBQLN2-high, A53TxUBQLN2-KO, and non-transgenic littermate controls were included in analyses. Prior to analyses, mice were euthanized with isofluorane and perfused with

PBS prior to removing the brain for analysis. Following dissection, brains were divided sagittally. One half was flash frozen on dry ice and stored at -80C for biochemical studies. The other half was fixed in 4% paraformaldehyde at 4C before undergoing a 10-30% sucrose gradient at 4C until saturated and then flash frozen in OCT and stored at -80C until sectioning. 25 uM sagittal sections were taken from fixed hemispheres using a cryostat. Sections were stored at -20C in cryoprotectant prior to immunofluorescence studies. Both male and female mice were included in analysis.

2.4.6 Western blot analysis

Cells were lysed in RIPA buffer (ThermoScientific) with protease inhibitor cocktail then sonicated for 5 minutes in chilled water before being centrifuged at 10000 rcf for 10 min at 4°C. Cell lysates were loaded (without boiling) on precast NuPAGE 4-12% Bis-Tris gels (Invitrogen) for SDS-PAGE analysis. Gels were subsequently transferred onto nitrocellulose membranes and stained with ponceau then blocked for 1 hour at room temperature with 10% nonfat dry milk in TBS-T buffer. Membranes were then probed overnight at 4°C in anti-Ubiquilin-2 (Novus Biologicals; 1:2,000), anti-FLAG, clone M2 (Sigma, 1:2,000), anti-α-synuclein (Invitrogen; 1:50,000), anti-pS129-α-synuclein (Abcam; 1:3,000), anti-FK1 (Enzo, 1:2,000), or anti-GAPDH (Millipore; 1:5,000) diluted in 5% nonfat dry milk. HRP-conjugated goat anti-rabbit IgG, goat anti-mouse IgG, or goats anti-mouse IgM (1:5,000) secondary antibodies were used for detection as appropriate. EcoBright ECL (Innovative Solution) was used to visualize bands, which were normalized to corresponding ponceau smear. All quantification of immunoblots were performed by densitometric analysis using ImageJ software (National Institutes of Health). Analyses were completed in triplicate and analyzed by one-way ANOVA with the Tukey post hoc test for

multiple comparisons. Representative western blots were cropped for ease of visualization with full un-cropped western blot images included in supplemental figures.

Mouse and human brain samples were homogenized in PBS with protease inhibitor cocktail (catalog no. 11873580001; Sigma Aldrich), using a 1:3 dilution of tissue: PBS (w/v). 100 uL of homogenate was removed and lysed using RIPA buffer. The remaining lysate was centrifuged at 10000 rcf for 10 min at 4°C. Supernatants (PBS-soluble fraction) were collected. Pellets were resuspended in PBS with protease inhibitor cocktail, centrifuged at 10000 rcf for 10 min at 4°C and supernatants were added to the PBS-soluble fractions then aliquoted, snap-frozen, and stored at -80°C until use. Remaining pellet was resuspended in 1% sarkosyl in PBS with protease inhibitor, vortexed for 1 min, and incubated at room temperature for 1 hr then stored at -80ºC overnight. The next day, samples were thawed then water sonicated for 5 min and centrifuged for 30 min at 14000 rpm at 4ºC. Supernatants were collected as insoluble fraction. Brain extracts containing 15 μg of total protein were boiled at 98ºC for 1 min then loaded on precast NuPAGE 4-12% Bis-Tris gels (Invitrogen) for SDS-PAGE analysis. Gels were subsequently transferred onto nitrocellulose membranes and blocked for 1 hour at room temperature with 10% nonfat dry milk in TBS-T buffer. Membranes were then probed overnight at 4°C in anti-Ubiquilin-2 (Novus Biologicals; 1:2000), anti-FLAG, clone M2 (Sigma, 1:2000), anti-α-synuclein (Invitrogen; 1:50,000), anti-pS129-α-synuclein (Abcam; 1:5,000), anti-FK1 (Enzo, 1:1,000) diluted in 5% nonfat dry milk. HRP-conjugated goat anti-rabbit IgG, goat anti-mouse IgG, or goat anti-mouse IgM (1:5000) were used for detection as appropriate. EcoBright ECL (Innovative Solutions) was used to visualize bands, which were normalized to corresponding ponceau smear. All quantification of immunoblots was performed by densitometric analysis using ImageJ software

(National Institutes of Health). Analyses were completed in triplicate and analyzed by one-way ANOVA with the Tukey post hoc test for multiple comparisons.

2.4.7 Immunofluorescence

Cells were fixed in chilled methanol for 20 minutes at -20C then washed in 1X PBS. Cells were then permeabilized with 0.5% triton-X 100 then blocked in 5% goat serum for 1hr prior to incubation in either anti-UBQLN2 (Novus Biologicals; 1:200), anti-UBQLN1 (Novus Biologicals, 1:100), or anti-FLAG (Sigma; 1:300) and anti-α-synuclein (BD Biosciences, 1:250) overnight at 4C. The following day, cells were washed three times in 1X PBS for 10 minutes then incubated in goat anti-rabbit IgG Alexa Fluor 568 (Invitrogen; 1:500) and goat anti-mouse IgG Alexa Fluor 488 (Invitrogen; 1:500) for 1 hour. Cells were then washed three times in 1X PBS for 10 minutes and incubated in DAPI (Sigma) for 5 minutes at room temperature. Cells were washed in 1X PBS for 5 minutes three times then mounted with Prolong Gold Antifade Reagent (Invitrogen). Slides were images using a IX71 Olympus inverted microscope and analyzed for α-syn fluorescence using the cell counter toll in ImageJ.

Brain Sections were washed in 1X PBS at 4C overnight to wash out storage cryoprotectant. The next day, sections were subjected to heated antigen retrieval in 10 mM Citrate Buffer (pH 6) at 80C for 20 minutes then allowed to cool to room temperature for 15 minutes. Sections were then washed in 1X PBS-T three times for 5 minutes each followed by permeabilization in 0.5% triton-X 100. Slices were then incubated in M.O.M. Mouse IgG Blocking Reagent (Vector Laboratories) for 2 hours and then incubated in either anti-α–synuclein (Invitrogen, 1:5,000), UBQLN2 (Novus; 1:250) overnight at 4C. The next day, sections were washed three times in 1X PBS-T and then incubated in secondary antibody then washed three time in 1X PBS-T. Section

were then incubated in DAPI for 15 minutes followed by three more washes in 1X PBS-T. Once on slides, sections were cover slipped using ProLong Gold Antifade Reagent. Images were taken using a Nikon A1 inverted confocal microscope and analyzed for total fluorescence using ImageJ.

2.4.8 Immunohistochemistry

Brain sections were washed with 1X PBS at 4C overnight to was out storage cryoprotectant. The next day, sections were subjected to heated antigen retrieval in 10 mM Citrate Buffer (pH 6) at 80C for 15 minutes then allowed to cool to room temperature for 10 minutes. We then incubated cells in 1% hydrogen peroxide for 30 minutes to quench endogenous peroxidase activity. Sections were washed in 1X PBS for 10 minutes followed by 15 minutes in 0.1% Triton X-100 and then washed for 15 minutes in 0.5% bovine serum albumin. Sections were blocked for 1 hour in 5% normal goat serum then washed in 1X PBS for 10 minutes followed by a 15 min wash in 0.1% Triton X-100. Sections were incubated in anti-pS129 primary antibody (Abcam, 1:1000) dilutes in 0.5% BSA overnight at 4C. The next day, sections was washed in 0.1% Triton X-100 for 15 minutes followed by a 15 minute was in 0.5% BSA and then incubated in secondary antibody for 1.5 hours. Sections were then washing for 15 minutes in 0.1% Triton X-100 followed by a 15-minute wash in 0.5% BSA. Sections were then incubated in ABC buffer (Elite ABC-HRP kit, Vector labs) in the dark for 1 hour. Sections were then washed 3 X 5 mins in 1X PBS. Sections then underwent a DAB reaction using ImmPACT DAB as per manufacturer's instructions (Vector Labs) followed by 3 X 10min washes in 1X PBS. Sections were then mounted on Superfrost Plus slides with 1x PBS and air dried overnight. The next day, sections were rinsed in ultra-pure water (MilliQ) and then dehydrated in 70% ethanol for 1 min, 95% ethanol for 2 mins, and then 2 X 3 minutes in 100% ethanol followed by 3 X 5-minute

incubations in xylenes. Coverslips were then places on sections with DPX mounting media. Once dried, slides were imaged using an Olympus BX51 microscope.

2.4.9 Optical Pulse Labeling

Cortical primary neurons were dissected from rat pups at embryonic day 20. Neurons were plated on maninin/poly-D-lysine coated 96 well plates[53-56] at a density of $1x10^5$ cells/well in NEUMO photostable media supplemented with SOS (Cell Guidance Systems). Neurons were transfected with hSyn-Dendra2 and UBQLN2-iRFP or iRFP plasmids using lipofectamine 2000 as previously described.[53-56]

Imaging of primary neurons was accomplished using a Nikon Eclipse Ti inverted microscope equipped with Semrock filter sets, Sutter Lambda 421 multi-LED light source, and an Andor Zyla 4.2(+) sCMOS camera (Oxford Instruments), enclosed in a custom-built environmental chamber to maintain a temperature of 37C and 5% CO2 levels. All stage movements, filter switching, illumination and image acquisition were controlled through μManager with original code written in BeanShell, as described previously. Image processing and analyses were accomplished through dedicated software written expressly for these purposes.[57] Fluorescence was initially measured 24h following transfection. We then used a 3s pulse of 405 nm light for Dendra2 photoconversion and obtained images again 3h following photoconversion and then every 24h for the following 3d as previously described.[34,53,54] Only cells that survived the entire duration of the experiment were included for further analysis. The half-life of hSyn-Dendra2 in individual neurons was determined by fitting the time-dependent decline in photoconverted (red) hSyn-Dendra2 fluorescence for each cell to a first-order exponential decay curve.

2.4.10 Experimental Design and Statistical Analysis

The accepted level of significance for all analyses was $p \leq 0.05$. All analyses comparing two groups were analyzed using student's t-test and comparisons between three or more groups were completed by one-way ANOVA and the Tukey post-hoc test for multiple comparisons or, when applicable, the non-parametric Kruskal Wallis test. Data are expressed as means +/- SEM. P-values for overall statistical analysis are displayed in analyses that did not show a significant difference and individual post-hoc comparison P-values are displayed for significant analyses. Data were analyzed using RStudio.

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2.6 Figures

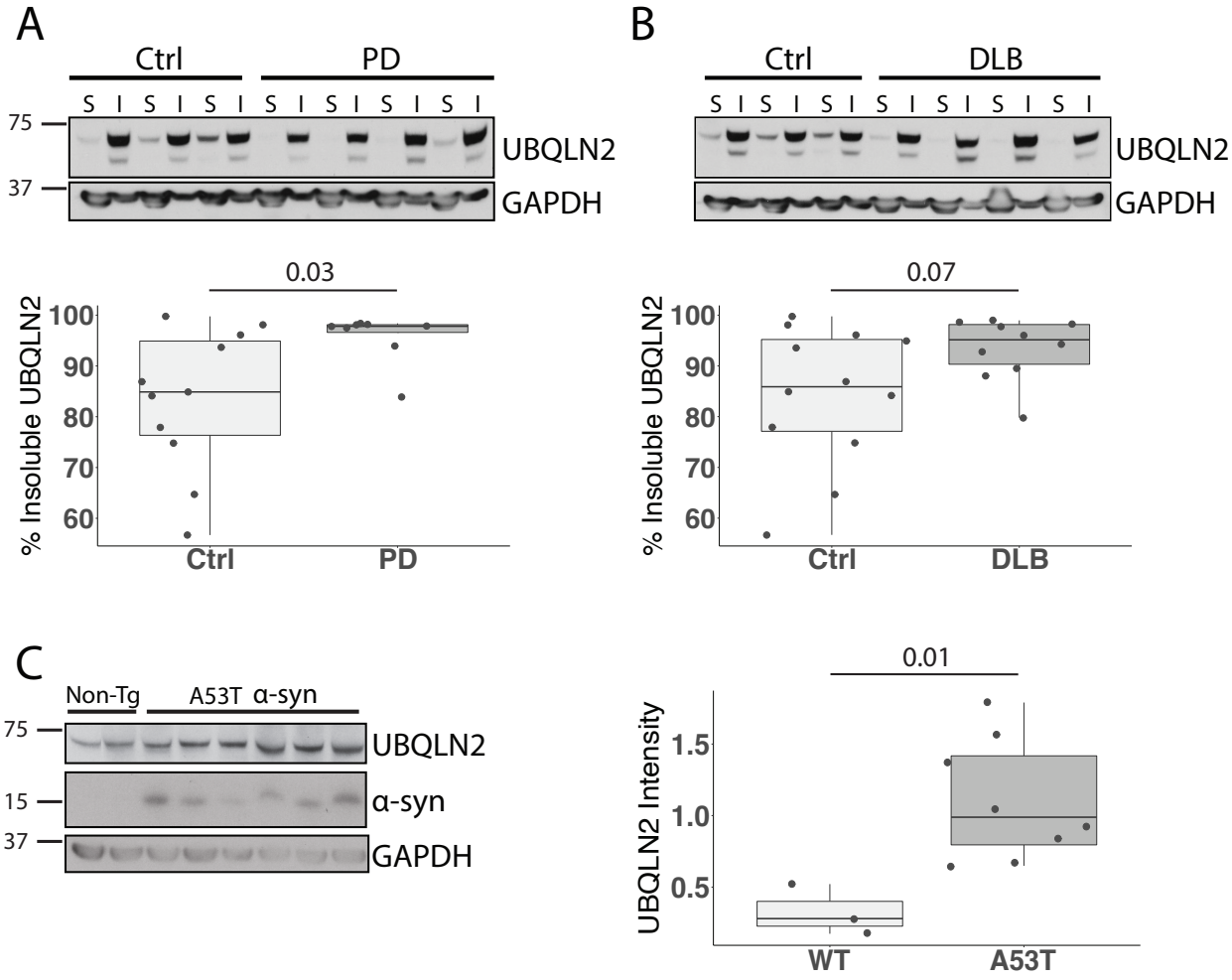

Figure 2-1 UBQLN2 insolubility is increased in Parkinson's disease human tissue and mouse models.

(A-B) Western blots of lysates from post-mortem cingulate cortex of PD and DLB patients show an increase in the percent of UBQLN2 that is insoluble (% Insoluble UBQLN2) in DLB (B; n=10) and PD (A; n=8) compared to age-matched controls. S=PBS soluble; I=PBS insoluble/sarkosyl soluble. (C) Representative western blot of whole brain lysate from mice overexpressing human mutant α–syn A53T solubilized in Triton-X (n=8) shows elevated levels of insoluble UBQLN2 compared to non-transgenic controls (n=3).

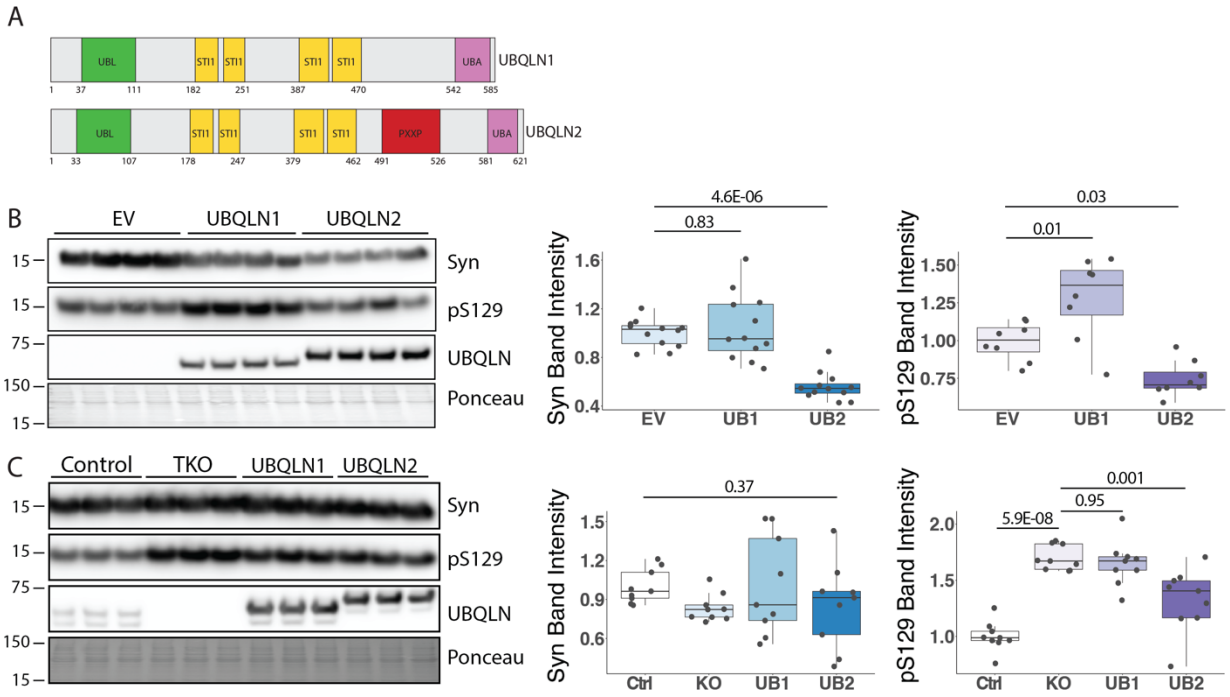

Figure 2-2 UBQLN KO leads to pS129 accumulation while UBQLN2 overexpression decreases levels of pS129 in vitro.

(A) Diagram of UBQLN2 and UBQLN1 protein structure. (B) Representative western blot of UBQLN 1,2, 4 Total Knock out (TKO) cells transfected to express WT α-syn alone or together with UBQLN1 (UB1) or UBQLN2 (UB2). Total α-syn and pS129 levels are decreased by co-expression of UBQLN2 and increased by UBQLN1. (n=8) (C) Representative western blot of TKO, UBQLN1 rescue, UBQLN2 rescue, and control cells transfected with α-syn showing elevated pS129 levels in TKO cells compared to control cells, which is partially reversed by co-expression of UBQLN2 but not UBQLN1. Western blots were cropped to focus on protein(s) of interest. Data were analyzed by ANOVA with the Tukey post-hoc test for multiple comparisons.

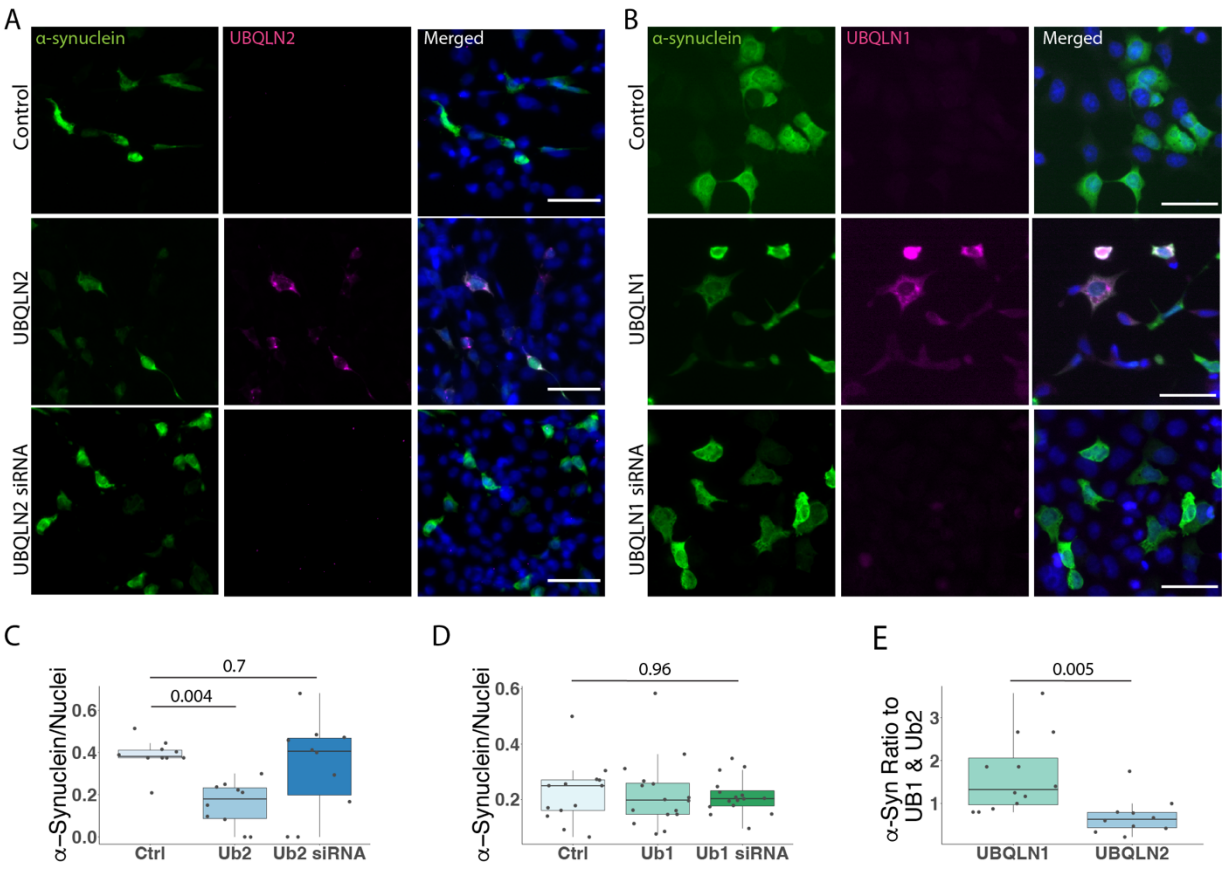

Figure 2-3 UBQLN2, but not UBQLN1, lowers α -syn levels in HEK-293 cells.

(A-D) Representative immunofluorescence images show that the number of α-syn-containing cells are decreased when co-expressed with UBQLN2 (A, C; n=10 field of cells over 3 independent experiments) but not with UBQLN1 (B, D; n=10 field of cells over 3 independent experiments). Scale bar 100 μm. (E) Expressed as a ratio of α-syn positive cells to UBQLN1 and UBQLN2, α-syn is significantly reduced in UBQLN2-expressing cells. Data were analyzed by ANOVA with the Tukey post-hoc test for multiple comparisons.

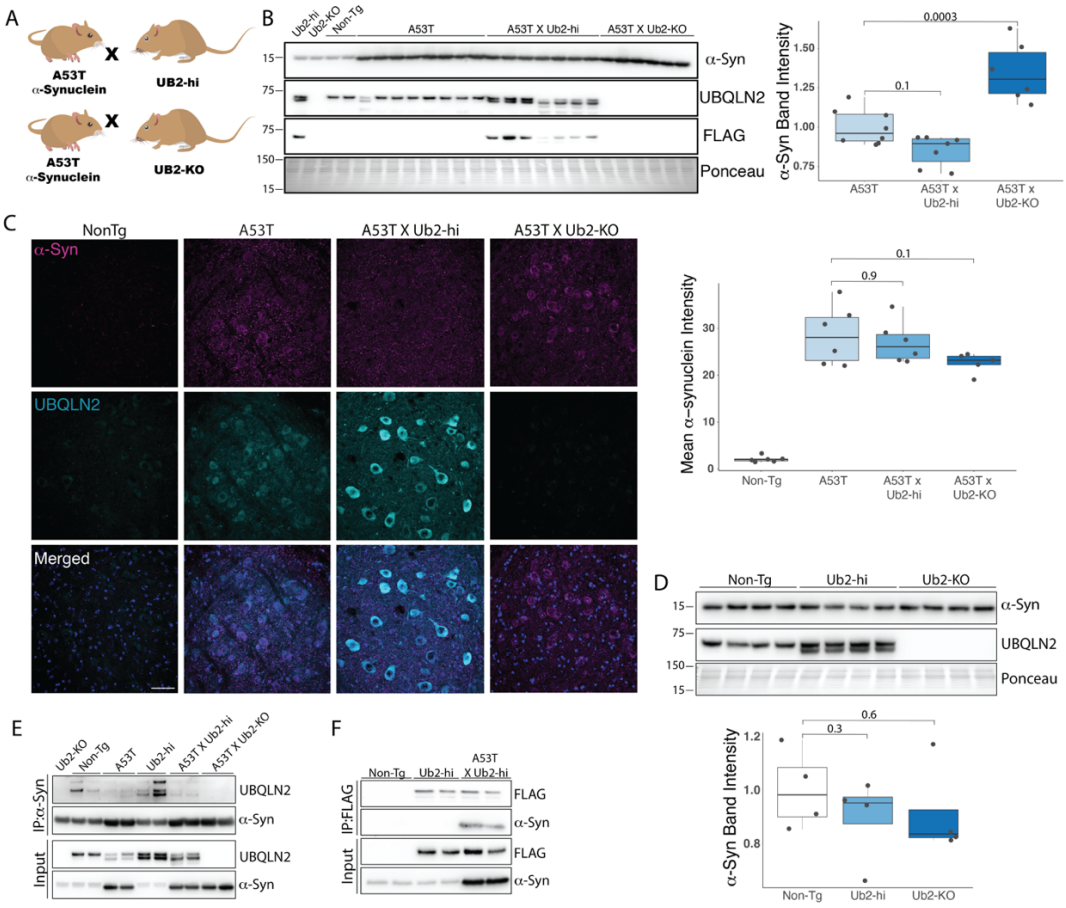

Figure 2-4 UBQLN2 interacts with and may alter α -syn levels in A53T mice.

(A) Schematic depicting breeding strategy to obtain A53TxUb2-hi and A53TxUb2-KO mice. (B) Western blot of total α-syn showing increased levels of α-syn in A53TxUb2-KO mice (n=6), while A53TxUb2-hi (n=7) mice show a trend toward decreased α-syn levels compared to non-transgenic (Non-Tg) controls (n=8). (C) Representative images of total α–syn (magenta) and UBQLN2 (cyan) immunofluorescence in the deep cerebellar nucleus of 12-month-old Non-Tg (n=6), A53T (n=6), A53TxUb2-hi (n=6), and A53TxUb2-KO (n=5) mice and quantification show no significant difference in total α–syn expression between genotypes. (D) Western blot showing total endogenous α-syn levels were unchanged in both Ub2-hi (n=4) and Ub2-KO mice (n=4) compared to Non-Tg controls (n=4). (E) Immunoblot showing endogenous and transgenic UBQLN2 is pulled down when α-syn is immunoprecipitated from mouse brain lysate (n=4). (F) Immunoblot showing α-syn coprecipitates with FLAG-tagged UBQLN2 in A53TxUb2-hi mice when FLAG is immunoprecipitated from mouse brain lysate (n=2).

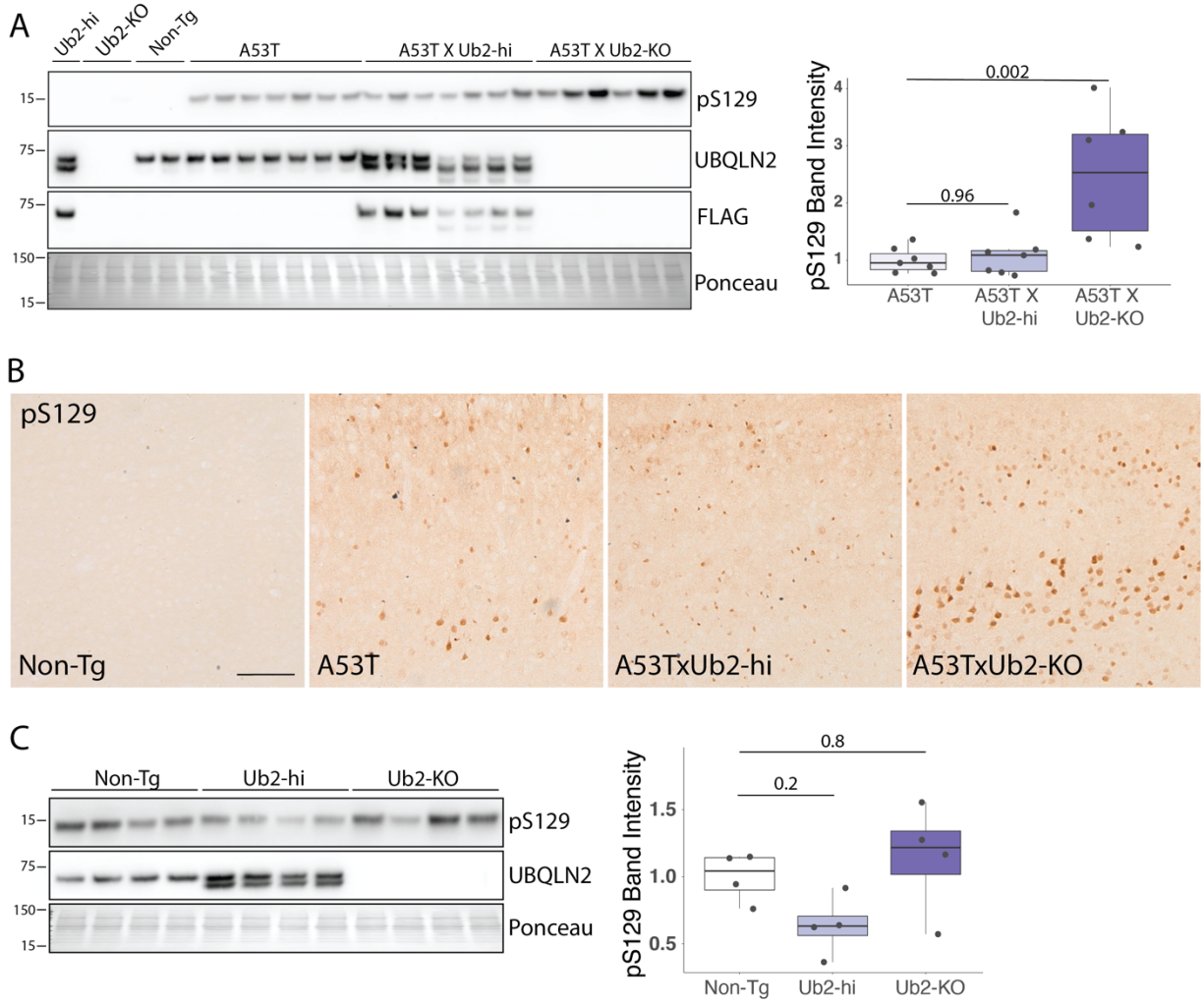

Figure 2-5 pS129 α -syn accumulates in A53T x UBQLN2 KO mice.

(A) Representative blot of total pS129 levels showing accumulation of pS129 in A53TxUb2-KO mice (n=6) and unchanged in A53TxUb2-hi mice (n=7) compared to A53T controls (n=8). (B) Representative images of pS129 expression in the cortex of A53T (n=3), A53TxUb2-hi (n=3) and A53TxUb2-KO (n=3) 12-month-old-mice show increased pS129 expression in A53TxUb2-KO mice. (C) Western blot analysis showing a trend toward decreased endogenous murine pS129 levels in Ub2-hi (n=4) mice and no change in Ub2-KO (n=4) mice compared to Non-Tg controls (n=4). Western blots were cropped to focus on protein(s) of interest. Uncropped Western blots are shown in Supplemental Figure S5).

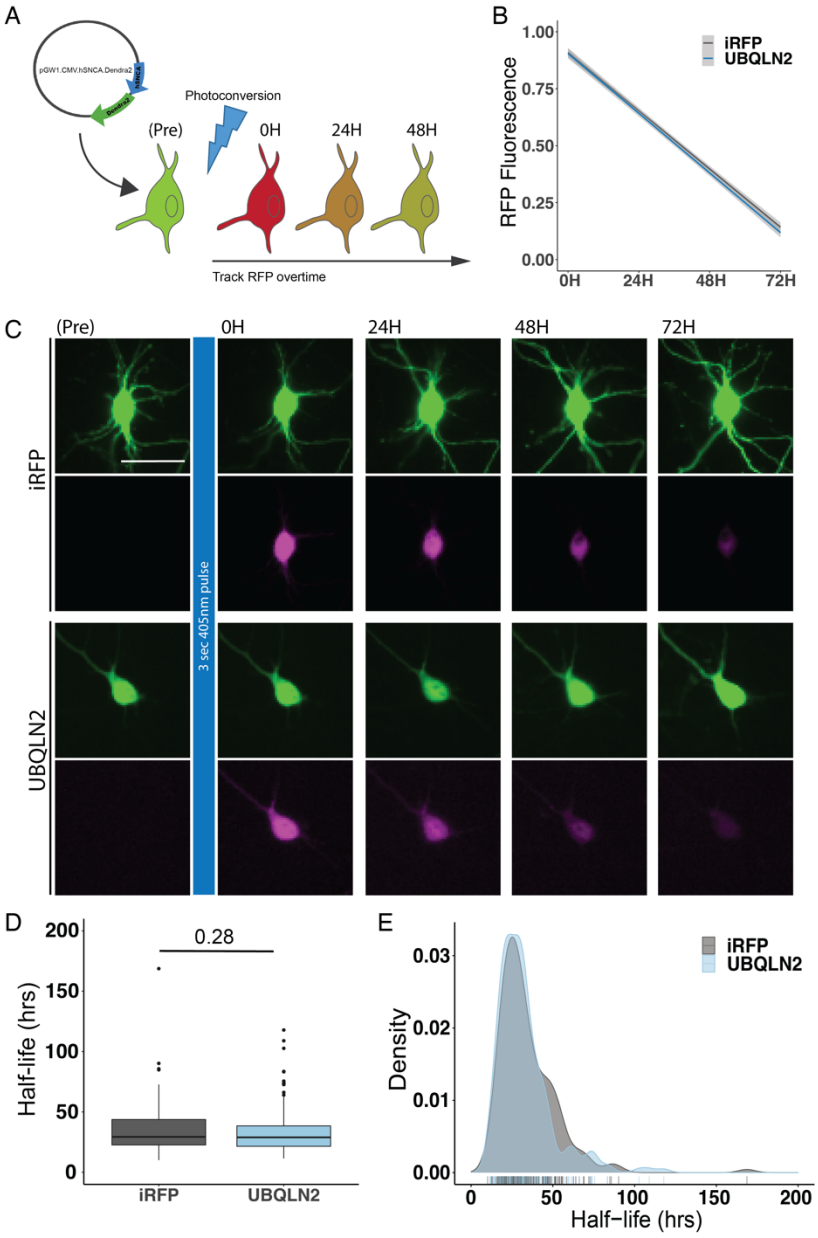

Figure 2-6 Optical Pulse Labeling reveals no effect of UBQLN2 on α -syn clearance in neurons.

(A) Schematic of Optical Pulse labeling experiment. Rat primary cortical neurons were transfected with hSyn-Dendra2 and UBQLN2-iRFP or iRFP. Dendra2 was photoconverted 24h post-transfection with 405nm light, imaged immediately and every 24h thereafter for 3 days. Scale bar = 25um. (B) Optical pulse labeling reveals no change in red fluorescence intensity over time in neurons transfected with UBQLN2 (n=213) compared to control (n=182; p=0.6) (C) Representative images of optical pulse labeling of hSyn-Dendra2 in rat primary cortical neurons transfected with hSyn-Dendra2 and UBQLN2-iRFP or iRFP. (D) Optical pulse labeling in primary rat neurons reveals no change in half-life of hSyn-Dendra2 upon transfection with UBQLN2 vs control (iRFP; p=0.28). hSyn-Dendra2 half-life was determined by measuring the loss of photoconverted hSyn-Dendra2 (RFP) signal over time for each cell. (E) Density plot of hSyn-Dendra2 half-life measurements from individual neurons transfected with control (n=182) or UBQLN2 (n=213). Values were pooled from eight wells per condition, from each of two biological replicates and analyzed by the Kruskal-Wallis test.

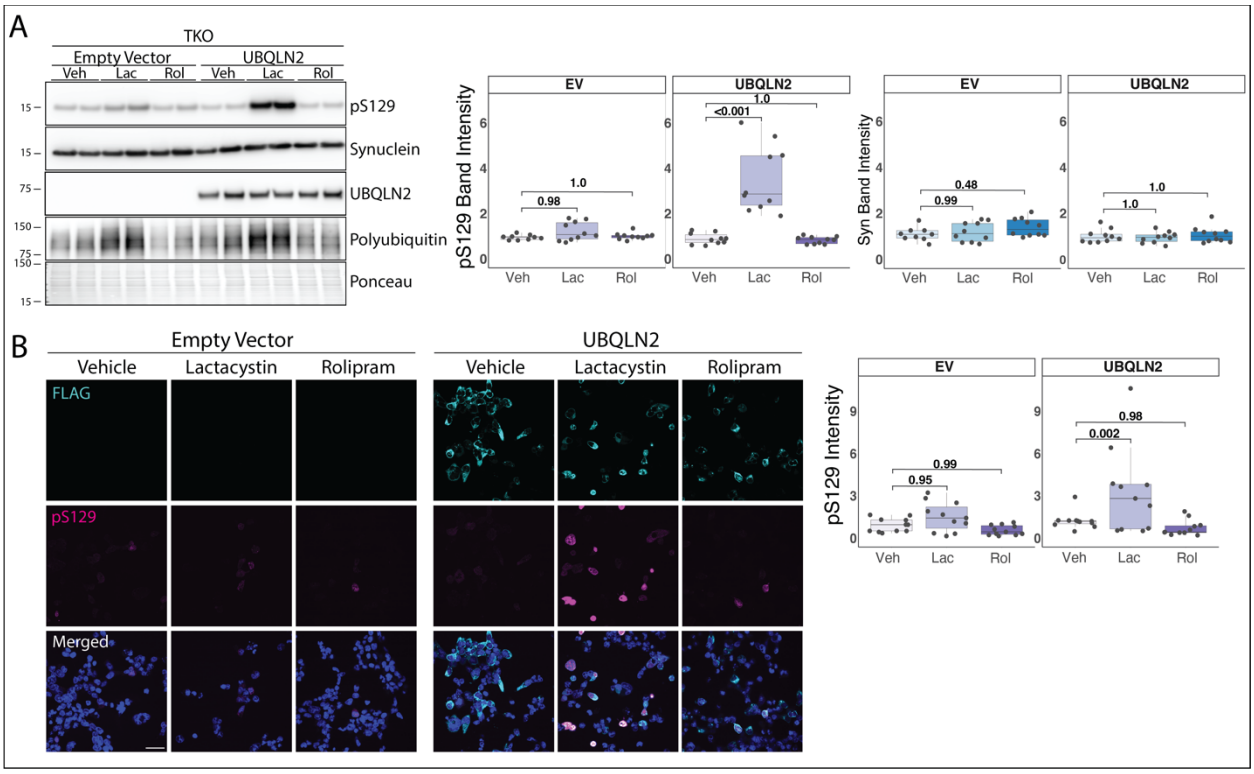

Figure 2-7 UBQLN2 targets pS129 α -syn to the proteasome for degradation.

(A) Representative western blot of TKO cells overexpressing α-syn and transfected with either EV or UBQLN2 and treated with 10 uM lactacystin (Lac), 50 uM rolipram (Rol), or vehicle (Veh). Quantification of total α-syn and pS129 levels shows that pS129 accumulates in cells transfected with UBQLN2 and treated with Lac while total α-syn levels were not changed between groups (n=9-10). Total α-syn and pS129 levels were each normalized to α-syn levels of EV, vehicle-treated cells. Western blots were cropped to focus on protein(s) of interest. (B) Representative images showing pS129 fluorescence in cells transfected with either EV or UBQLN2 and treated with either Veh, Lac, or Rol. Quantification of pS129 fluorescence intensity further supports that pS129 α-syn is increased when treated with Lac and in the presence of UBQLN2 (n=11). pS129 fluorescence intensity was normalized to that of vehicle treated, EV cells. Scale bar = 50 um.

2.7 Table

Synucleinopathy Tissue			
Diagnosis	Sex	Age	PMI (hrs)
Control	Female	83	21
Control	Female	80	19
Control	Male	100	3
Control	Female	96	18
Control	Male	75	9
Control	Male	65	24
Control	Female	83	not recorded
Control	Male	71	4
Control	Female	80	5
PD	Male	78	22
PD	Male	74	14
PD	Female	71	7
PD	Male	86	10
PD	Female	74	6
PDD	Male	81	16
DLB	Male	78	12
DLB	Female	82	10
DLB	Male	84	5
DLB	Female	68	24
DLB	Male	66	8
DLB	Male	86	not recorded
DLB	Male	66	15
DLB	Male	71	5
DLB	Female	80	4
DLB	Female	57	9
DLB	Female	84	6
DLB	Male	87	13
DLB	Male	72	18
DLB	Female	71	12

Table 2-1 Synucleinopathy samples used for analysis of UBQLN2.

Chapter 3 UBQLN2 Overexpression Leads to Robust, Early-Onset Retinopathy

3.1 Introduction

The retina is a complex, highly organized neural tissue that is responsible for turning light into neural signals and enables visual sensation. The retina's complexity includes 5 populations of neurons[1] and 3 types of glia.[2]

Photoreceptors are highly specialized and regulated cells responsible for responding to visual stimuli.[1,3] They contain an inner segment that stores proteins important for photoreceptor function prior to shuttling those proteins into the outer segment, where phototransduction occurs.[3,4] Due to their high energetic demands, photoreceptors are constantly renewing their outer segments, shuttling key proteins and membranes from the inner segment to the base of the outer segment. The tips of the outer segment, which contain the oldest membranes, are phagocytosed and degraded via lysosomes in retinal pigment epithelium cells (RPE).[5,6] Thus, the retina requires strict proteostatic regulation to maintain proper photoreceptor health and function.

The retina is extremely sensitive to changes in proteostatic pathways. Disruptions in proteostasis have been linked to multiple retinopathies[5,7-18], and thus protein quality control pathways have been targeted as treatments for retinopathy. However, retinopathies are a highly heterogenous family of diseases, leading to conflicting results concerning whether altering proteostatic pathways is beneficial or deleterious to retinal health.[4,6-12]

Dysfunction of protein quality control pathways has also been implicated in many neurodegenerative diseases. Since the retina is an extension of the central nervous system, retinal health has been explored as an early biomarker for neurodegenerative disease.[19-21]

Moreover, the highly organized architecture of the retina and its sensitivity to proteostasis dysfunction make it a compelling model organ to study the function of disease-linked proteins and pathways of proteostasis.

Ubiquilin-2 (UBQLN2) is one of a family of protein quality control proteins linked to multiple proteostasis pathways. UBQLN2 has also been implicated in various neurodegenerative diseases[22-24], and a better understanding of UBQLN2's normal function may shed light on its contributions to neurodegeneration. Toward this goal, multiple UBQLN2 transgenic mouse lines have been created and characterized as described in Sharkey et al., 2020. To examine the function of wild-type UBQLN2, mouse lines were created to express wild-type UBQLN2 at either low (Ub2-low) or high (Ub2-hi) levels. Sharkey et al. also created a mouse line expressing the UBQLN2 P506T mutation (P506T), a pathogenic mutation that directly causes neurodegeneration manifesting as ALS/FTD.[22,25] A UBQLN2 knock-out (Ub2-KO) mouse line was also created. Early studies characterizing UBQLN2 transgenic mice suggested that that Ub2-hi mice have vision impairments[26], raising the possibility that excess expression of UBQLN2 is deleterious to the retina. It is unknown how or why excess UBQLN2 expression might lead to vision impairment but understanding this process may elucidate our understanding of normal and abnormal UBQLN2 function.

In this study, we use immunofluorescence and optical coherence tomography (OCT) to show that UBQLN2 overexpression leads to a marked retinopathy that is driven by degeneration of the outer retina.

3.2 Results

3.2.1 UBQLN2 overexpression leads to early loss of photoreceptors

We examined cross sections of retinas at multiple ages to assess changes in retinal layers in UBQLN2 transgenic and UBQLN2 KO mice. Anti-rhodopsin staining revealed that Ub2-hi mice lost all photoreceptors by eight weeks of age, and P506T mice lost all photoreceptors more slowly, by one year of age (Figure 3-1A). In contrast, the photoreceptor layer in Ub2-low mice was comparable to that of Non-Tg mice (Figure 3-1A). Our initial hypothesis was that UBQLN2 high overexpression leads to dysregulation of UBQLN2 that is deleterious to photoreceptor health. However, Ub2-KO mouse photoreceptors appeared intact up to 52 weeks of age (Figure 3-1A). These findings suggest that UBQLN2 overexpression leads to dose-dependent retinal degeneration whereas complete loss of UBQLN2 is tolerated, or compensated for, by the retina.

Earlier studies of UBQLN2 overexpressing mice revealed differences in localization and aggregation of UBQLN2 based on expression levels and mutation state.[27] To examine UBQLN2 expression in the retinas of UBQLN2 transgenic mice, we used immunofluorescence to visualize UBQLN2 in retinal cross sections of 3-week-old mice to capture UBQLN2 expression in the retina before significant degeneration took place. Endogenous UBQLN2 was diffusely expressed at low levels in the inner layers of the retina of Non-Tg mice (Figure 3-1B). UBQLN2 expression in Ub2-low mice was also seen diffusely in cells and spread from the ganglion cell layer to the outer nuclear layer (Figure 3-1B). In Ub2-hi mice UBQLN2 was found throughout the retina, except in the outer segments of photoreceptors. UBQLN2 did not colocalize with rhodopsin, the receptor within photoreceptor outer segments that is responsible for initiating phototransduction[28] (Figure 3-1B). UBQLN2 expression in Ub2-hi was largely diffuse with a small degree of punctate expression in the inner segment of photoreceptors (Figure 3-1C). P506T mice also expressed UBQLN2 throughout the retina, except for photoreceptor outer segments. Unlike the Ub2-hi mice, however, P506T mice did not form observable puncta (Figure 3-1B-C).

These results suggest that UBQLN2 overexpression in the outer layers of the retina is deleterious to photoreceptor health. The early appearance of UBQLN2 puncta in photoreceptors of Ub2-hi mice but not in other Ub2 mouse lines, suggests that the puncta, which may sequester UBQLN2 in a manner that hinders its function, are linked to the process that drives early loss of photoreceptors.

3.2.2 UBQLN2 overexpression leads to robust retinal thinning in Ub2-hi and P506T mice.

Our studies revealed that high UBQLN2 expression is deleterious to retinal health (Figure 3-1). We used optical coherence tomography (OCT) to measure retinal thickness over time and capture the progression of retinal thinning indicative of the severe degeneration in Ub2-hi mice. OCT provides high-definition images of the retina that enable accurate measurements of retinal thickness. With OCT, we can see and measure the various retinal layers that would otherwise be difficult to observe (Figure 3-2). OCT also facilitates measuring retinal thickness at multiple timepoints in an individual mouse to capture progressive degeneration. We used OCT to measure total retina thickness in Non-Tg, Ub2-low, P506T, Ub2-hi and Ub2-KO mice (Figure 3-3). Quantification revealed that Ub2-hi retinas are already thinner than Non-Tg retinas by 4 week of age ($p=0.052$; Figure 3-4) and undergo robust thinning by 8 weeks of age ($p<0.001$; Figure 3-4) with continued further thinning until one year of age. In contrast, P506T retinal thickness is no different from Non-Tg at one, two, and six months of age. However, between six and nine months of age, P506T retina undergoes degeneration, resulting in significant thinning compared to Non-Tg mice ($p<0.001$; Figure 3-4); this thinning continues until twelve months of age. In contrast, Ub2-low retinas did not differ from Non-Tg retinas up to one year of age, the oldest age examined (Figure 3-4). Likewise, Ub2-KO mice did not differ from Non-Tg mice at one year

(Figure 3-4). These data support the view that UBQLN2 overexpression causes retinal degeneration in a dose-dependent and age-dependent manner.

3.2.3 UBQLN2-mediated retinopathy disproportionately affects the outer retina.

Our initial studies using immunofluorescence to assess retinal layers revealed that photoreceptors were no longer present in Ub2-hi mice by eight weeks of age, and in P506T mice by 52 weeks of age (Figure 3-1A). We measured both inner and outer retina by OCT to determine if total retinal thinning is driven by loss of the outer retina where photoreceptors reside. Ub2-hi mice had significantly thinner outer retinas at one month ($p<0.001$; Figure 3-5A), two months ($p<0.001$; Figure 3-5B), and six months ($p<0.001$; Figure 3-5C) of age compared to Non-Tg controls. At twelve months of age, Ub2-hi retinal degeneration was so severe that we were unable to calculate outer or inner retina measurements (Figure 3-3). Similarly, P506T mice had significantly thinner outer retinas at nine months ($p<0.001$; Figure 3-5D) and twelve months of age ($p<0.001$; Figure 3-5E) compared to Non-Tg, but were no thinner than Non-Tg outer retinas at younger ages: one month ($p=1$; Figure 3-5A), two months (p-0.62; Figure 3-5B), and six months old ($p=1$; Figure 3-5C). Ub2-low outer retinas remained unchanged from Non-Tg at all ages ($p=1$; Figure 3-5A-E). Finally, Ub2-KO retinal thickness at twelve months of age displayed a trend towards thinner outer retinas compared to Non-Tg controls ($p=0.22$; Figure 3-5E).

These data indicate that UBQLN2-mediated retinal degeneration likely begins in the outer retina, which is largely comprised of photoreceptors. These findings suggest that while UBQLN2 overexpression is toxic to photoreceptors, the protein itself is not essential to photoreceptor health up to twelve months of age.

3.3 Discussion:

Healthy proteostatic regulation is crucial for healthy retina in which highly-organized and specialized cells require strict proteostasis to ensure proper phototransduction for visual sensation.[4-12] As a regulator of protein turnover, UBQLN2 is thought to play a role in various protein quality control pathways, including those that are crucial to photoreceptor turnover.[29] Here, we show that overexpression of UBQLN2 leads to severe retinal degeneration in a dose-dependent manner and provide evidence that, to a lesser degree, the absence of UBQLN2 may negatively impact retinal health.

Mutations in UBQLN2 have been implicated in neurodegenerative disease[22,23,80], and mutations in the PXX domain, such as P506T, are directly linked to ALS/FTD.[22,31] While our studies show that P506T mice experience severe retinal degeneration by nine months of age, it is unclear whether this degeneration is caused by the degree of overexpression of UBQLN2 in these mice or the pathogenic missense mutation itself. To investigate whether the P506T mutation alters UBQLN2 function in a manner that leads to retinopathy, studies would need to be done in mice that harbor the P506T mutation but do not significantly overexpress the UBQLN2 protein. Because the retina appears to be extremely sensitive to changes in proteostasis caused by the loss or gain of UBQLN2 function, the retina may be an early sign of neurodegeneration in diseases in which UBQLN2 is implicated, such as ALS/FTD. This possibility has not yet been investigated in people known to have UBQLN2 mutations, but retinal changes have been observed in patients with ALS.

Programmed cell death is an important process in retinal development that regularly occurs until eighteen days postnatal.[5,35] To control for this natural flux in the retina, our first OCT measurements were taken between postnatal days 28 and 36. Despite detecting all retinal

layers at three weeks of age (Figure 3-1), OCT analysis revealed that Ub2-hi retinas were already thinner than Non-Tg controls by ~28 days of age. While the retina may begin degenerating immediately after maturation, it is also possible that the toxic effects of high UBQLN2 overexpression may affect photoreceptor development. Autophagy has been linked to developmental programmed cell death[5] in the retina and alterations in UBQLN2 may lead to dysregulation of this key process in retinal development. While UBQLN2 dysregulation is linked to neurodegeneration, it may also affect retina developmental.

The retina is sensitive to alterations in protein quality control, and its response to changes in any protein quality control pathway is not straightforward. There have been conflicting results as to whether activation of autophagy or the proteasome is protective or detrimental to the retina.[6-17] That said, proteostasis is crucial to photoreceptor health. Our studies establish that altered UBQLN2 is detrimental to retinal health, though it is still unclear what UBQLN2's normal function in the retina may be. Overexpressed UBQLN2 may alter normal retinal proteostasis, either through its role in proteasomal or lysosomal degradation. Protein turnover is especially important for photoreceptors, whose outer segments are constantly growing and experiencing degradation by retinal epithelial (RPE) cells.[6] Photoreceptor turnover is a highly regulated process and, as such, RPE cell homeostasis is key to retinal health. Alterations in CMA have been linked to RPE cell death.[6] UBQLN2 has been implicated in CMA activity due to its known interaction with HSC/HSP70, a key chaperone in CMA.[29] Studies have shown that HSC/HSP70 protein levels are decreased in Ub2-hi mice.[26] UBQLN2 may alter CMA activity in RPE cells, perhaps leading to decreased photoreceptor outer segment turnover. Future studies evaluating the PR outer segment length using electron microscopy would help reveal the potential mechanism by which UBQLN2 leads to photoreceptor death. An investigation into

UBQLN2 function and dysfunction in RPE cells, both *in vivo* and *in vitro,* to assess changes in proteostasis within these specialized cells would further clarify whether UBQLN2 overexpression leads to altered turnover of photoreceptor outer segments. A better understanding of the role of UBQLN2 in CMA and other protein quality control pathways would elucidate the mechanism by which UBQLN2 mediates protein turnover and how it may contribute to disease progression.

The retina contains a variety of highly specialized cells, including both neurons and glia.[1,36,37] While it is clear from our studies that the outer retina is most sensitive to changes caused by UBQLN2 overexpression, UBQLN2 may also affect other populations of cells. For example, Muller glia are supporting cells that play a critical role in maintaining cell health. UBQLN2 overexpression might negatively impact photoreceptor supporting cells, causing photoreceptor cell death. Studies of UBQLN2 in neurodegeneration have largely focused on neuronal populations. With its highly organized structure, the retina may provide the opportunity to explore potentially different protein expression patterns or roles of UBQLN2 in neurons versus glia.

Here, we show that excessive UBQLN2 expression leads to severe, early-onset retinopathy. Whether a high level of UBQLN2 expression leads to a toxic accumulation of UBQLN2 or a loss of normal function remains to be determined, but our observations showing a trend towards thinner outer retinas in Ub2-KO mice suggest that normal UBQLN2 function is important for healthy photoreceptor cells. These studies open the door to retina use to study UBQLN2 function and dysfunction and further invite studies into retinal health in neurodegeneration.

3.4 Methods

3.4.1 Animals

This study was conducted in a facility approved by the American Association for the Accreditation of Laboratory Animal Care, and all experiments were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee of the University of Michigan. Mice were housed at the University of Michigan animal care facility and maintained according to U.S. Department of Agriculture standards (12 h light/dark cycle with food and water available *ad libitum*). UBQLN2 transgenic mice were generated and genotyped as previously described.[27] UBQLN2-KO mice were generated using Crispr/Cas9 technology at the University of Michigan Transgenic Animal Core and genotyped as previously described.[26]

3.4.2 Tissue Harvesting

Eyeballs from perfused mice were enucleated and fixed in 4% PFA overnight at 4 degrees. Following fixation, the cornea and the lens were removed to form an eye cup. The eyecup was transitioned through a 10%, 20%, and 30% sucrose gradient, then embedded in Tissue Plus O.C.T. Compound (Fisher HealthCare, 4585). Eye cups were sectioned with a cryostat at 10 um thickness and mounted on Superfrost slides (Fisher Scientific, Pittsburgh, PA).

3.4.3 Immunofluorescence

10 um sections from fixed retinas were washed in PBS, permeabilized with 0.5% Triton-X, followed by 3×10-minute washes in 0.1% Tween20. Sections were then washed with 70% ethanol for 5 mins. Sections were incubated in Autofluorescence Eliminator Reagent (Millipore for 10 minutes) followed by three 1-minute washes and one 5-minute wash in 70% ethanol.

Sections were blocked in 5% goat serum for one hour at room temperature followed by a 30-minute incubation in goat anti-mouse FAB (1:30). Sections were incubated in primary antibodies (anti-rhodopsin, Abcam, 1:250; Anti-UBQLN2, Novus, 1:350) at 4C overnight. The following day, sections were washed in 0.1% Tween20 three times for 10 minutes and incubated in goat-anti-mouse IgG Alexa Fluor 488 (Invitrogen, 1:500) at room temperature for one hour. Sections were then washed in 0.1% Tween20 3×10 minutes and incubated in DAPI (Sigma) for 15 minutes at room temperature. Sections were then washed in 0.1% Tween20 three times for 10 minutes prior to being cover-slipped with Prolong Gold Antifade Reagent (Invitrogen). Slides were imaged using an Olympus confocal microscope.

3.4.4 Spectral Domain-Optical Coherence Tomography

In vivo images of retinal structure were obtained using a spectral domain-optical coherence tomography (SD-OCT) (Bioptigen, Durham, NC). The mice received 0.5% tropicamide drops to stimulate eye dilation and then anesthetized with ketamine and xylazine (50 and 5 mg/kg body weight). Rectangular volumes consisting of 1000 A-scans by 100 B-scans over a 1.4 × 1.4-mm area centered on the optic nerve head (ONH) were taken for visualization of retinal anatomy. Retinal thicknesses were measured at 350 μm from the ONH using the In Vivo Vue Diver analysis software (Bioptigen). Measurements of total retinal thickness from the top of the retinal ganglion cell layer to the retinal pigment epithelium were obtained in nasal, temporal, superior, and inferior regions of the retina. The four measurements were averaged to generate an average total retinal thickness.

3.4.5 Statistics

The accepted level of significance for all analyses was $p \leq 0.05$. All analyses comparing two groups were analyzed using student's t-test, and comparisons between three or more groups were completed by one-way ANOVA and the Tukey post-hoc test for multiple comparisons. Data are expressed as means +/- SEM. P-values for overall ANOVAs are displayed in analyses that did not show a significant difference and individual post-hoc comparison P-values are displayed for significant ANOVAs. Data were analyzed in R Studio.

3.4.6 Acknowledgements:

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3.6 Figures

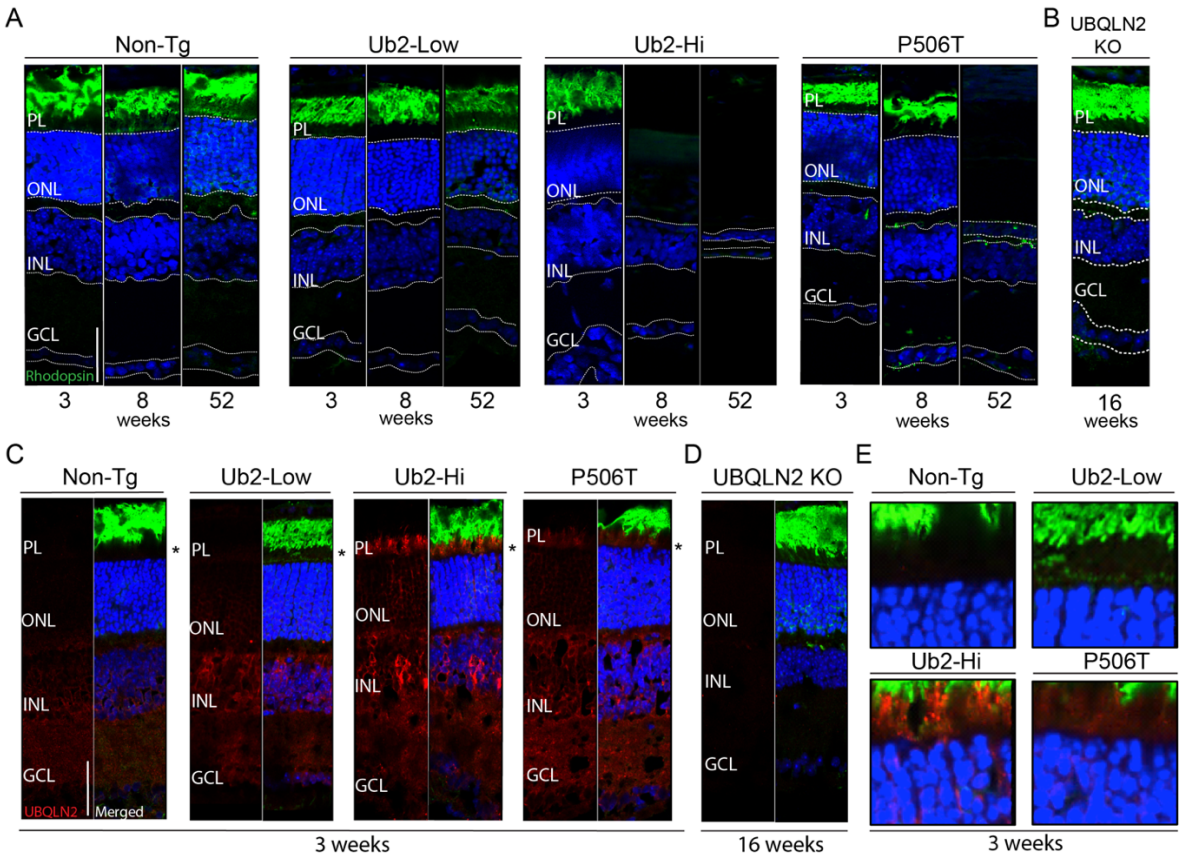

Figure 3-1 High overexpression of UBQLN2 leads to loss of photoreceptors by 8 weeks of age in mice.

(A) Representative images showing all retinal layers present in Non-Tg, Ub2-low, Ub2-hi, and P506T mice at 3 weeks of age. By 8 weeks of age, Ub2-hi mice no longer contain outer retinal layers while Ub2-low, P506T and Non-Tg retinas contain all layers. By 52 weeks, Ub2-hi retinas are fully degenerated and P506T mice have lost the PR layer and nearly all of the ONL while Ub2-low and Non-Tg mice retinas contain all layers. At 16 weeks (n=3-6). PL = Photoreceptor layer, ONL = Outer Nuclear Layer, INL = Inner Nuclear Layer, GCL = Ganglion Cell layer. Scale bar=50uM. (B) All retinal layers are present in Ub2-KO mice at 16 weeks of age (n=3). (C) Representative images of UBQLN2 staining at 3 weeks of age. UBQLN2 expression is diffuse and largely in inner layers of the retina in Non-Tg mice (n=5). Ub2-low retinas express diffuse UBQLN in the ONL, INL, and GCL (n=5). Ub2-hi mice express diffuse UBQLN2 throughout the retina except for outer segments of photoreceptors and contain UBQLN2 puncta in the inner segment of PRs (n=4). P506T express diffuse UBQLN2 throughout the retina except for outer segments of PRs (n=4). (D) Representative images showing no UBQLN2 expression in Ub2-KO mice (n=3). (E) Enlarged view of PR inner segments marked with * in panel C showing punctate UBQLN2 expression in Ub2-hi mice, diffuse UBQLN2 expression in P506T mice and no UBQLN2 expression in Non-Tg and Ub2-low mice.

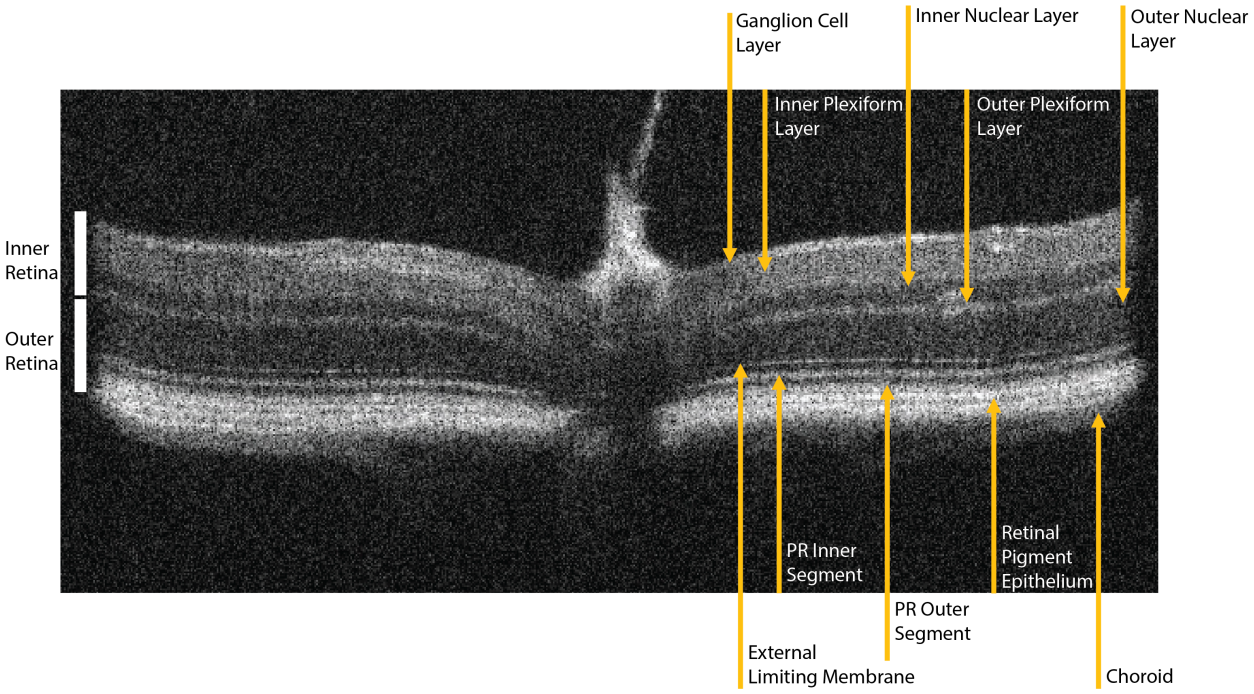

Figure 3-2 Optical Coherence Tomography

Diagram of the retinal cross section obtained through OCT.

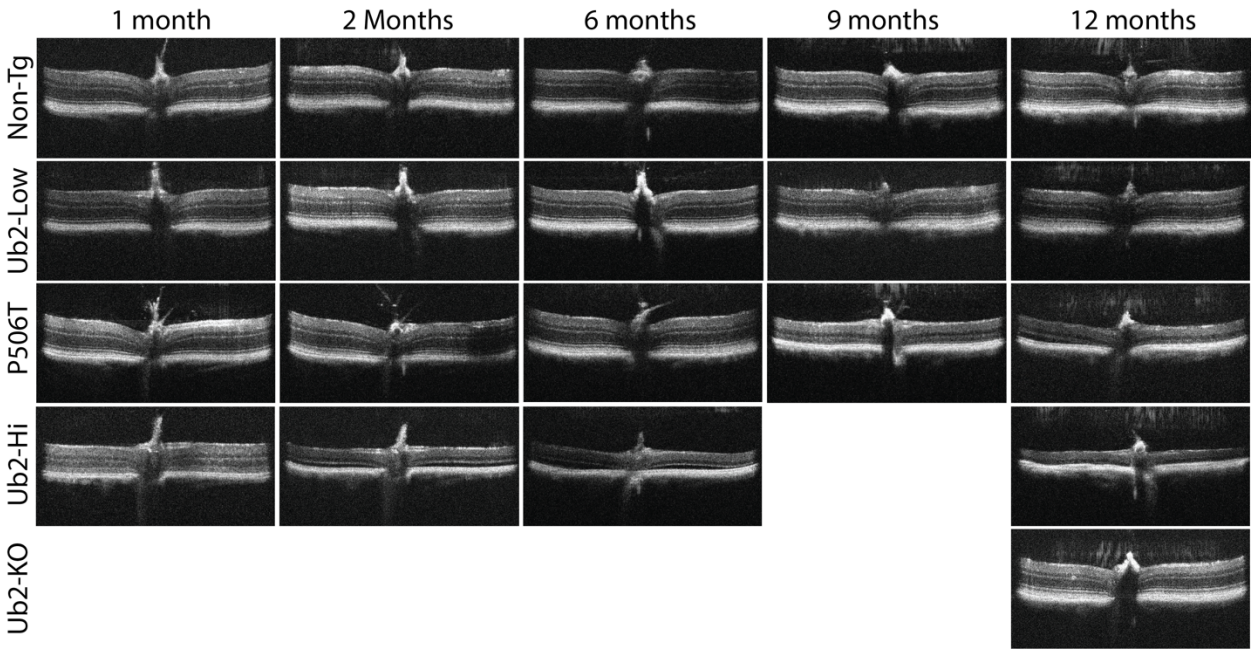

Figure 3-3 Retinal thinning captured by optical coherence tomography.

Representative cross-sectional images obtained through OCT are shown for each genotype at the indicated ages.

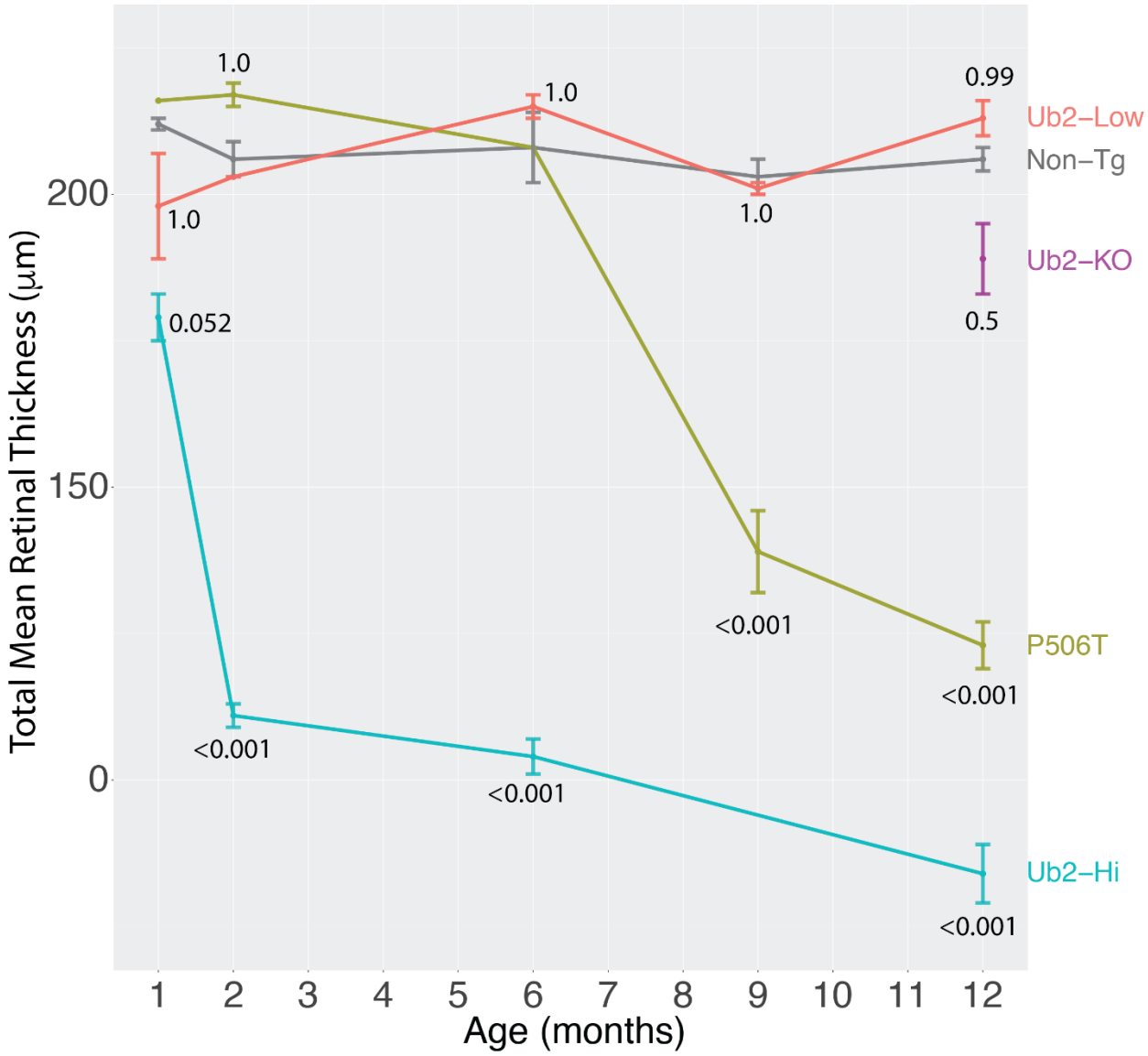

Figure 3-4 Quantification of OCT shows early retinal thinning in Ub2-hi mice.

Quantification of total retina thickness reveals thinning of the retina in Ub2-hi mice as early as 1 month of age (p=0.052) and continuing to thin at 2 (p=<0.001), 6 (p=<0.001), and 12 (p=<0.001) months of age. Retinal thickness in P506T mice was unchanged from Non-Tg at 1 month , 2 month (p=1), and 6 months (p=1) of age but had thinned greatly by 9 months (p=<0.001) and continued to thin up to 12 months of age (p=<0.001). Retinal thickness in Ub2-low mice did not differ fromNon-Tg mice at any age: 1 month (p=1), 2 months (p=1), 6 months (p=1), 9 months (p=0.99), and 12 months of age (p=0.5)

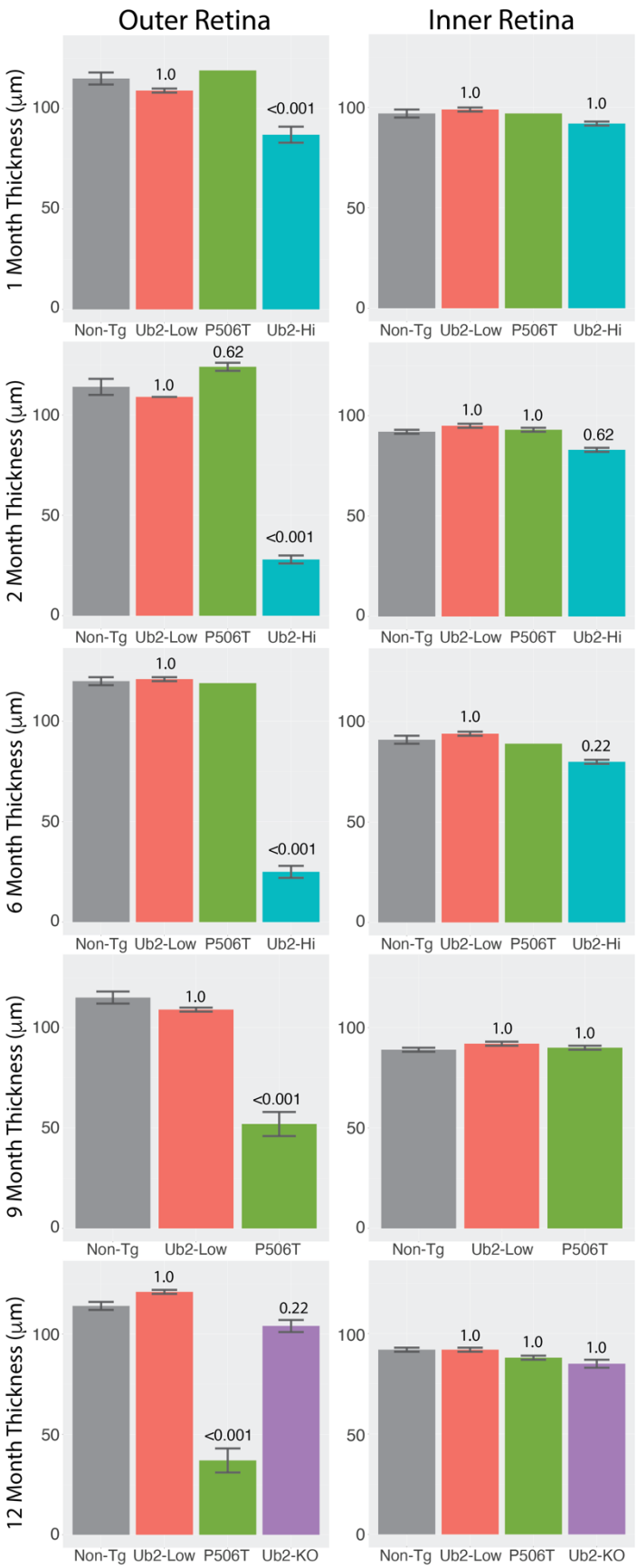

Figure 3-5 Ub2-hi overexpression leads to marked thinning of outer retinal layers.

Quantification of inner and outer retinal thickness in UBQLN2 transgenic and knockout mice.

(A) At 1 month old, Ub2-hi mice have thinner outer retinas than Non-Tg controls ($p<0.001$) while Ub2-low ($p=1$) and P506T mice have outer retinas comparable to Non-Tg. Inner retina thickness was not different between genotypes ($p=1$). Ub2-hi n=7 ; Ub2-low n=5; P506T n=1, Non-Tg n=5

(B) At 2 months old, Ub2-hi mice have thinner outer retinas than Non-Tg controls ($p<0.001$) while Ub2-low ($p=1$) and P506T ($p=0.62$) mice have outer retinas comparable to Non-TG. Inner retina thickness was not different between genotypes ($p_{Ub2\text{-}hi}=0.62$, $p_{ub2\text{-}low,\ P506T}=1$). Ub2-hi n= 7; Ub2-low n=5; P506T n=4, Non-Tg n=5

(C) At 6 months old, Ub2-hi mice have thinner outer retinas than Non-Tg controls ($p<0.001$), while Ub2-low ($p=1$) and P506T mice have outer retinas comparable to Non-TG. Inner retina thickness in Ub2-hi mice show a trend towards a thinner inner retina compared to Non-Tg ($p=0.22$), while Ub2-low and P506T were no different from controls ($p=1$). Ub2-hi n=7 ; Ub2-low n=5; P506T n=1, Non-Tg n=5

(D) At 9 months old, P506T mice have thinner outer ($p<0.001$) retinas compared to Non-Tg controls and comparable inner retinas to controls ($p=1$). Ub2-low mice have outer ($p=1$) and inner ($p=1$) retinas of similar thickness to Non-tg mice. Ub2-low n=7; P506T n=6, Non-Tg n=8

(E) At 12 months old, P506T mice have thinner outer retinas than Non-Tg mice ($p<0.001$) while their inner retinas remained as thick as Non-Tg ($p=1$).

Ub2-KO mice had outer retinas that showed a trend towards thinner retinas compares to Non-Tg controls ($p=0.22$) with inner retinas comparable to Non-Tg ($p=1$). Ub2-low outer and inner retinas were unchanged from Non-Tg ($p=1$). Ub2-low n=3; P506T n=5, Non-Tg n=8, Ub2-KO n=6

Chapter 4 Discussion and Future Directions

4.1 Summary of Contribution

In this dissertation I have established the importance of UBQLN2 homeostasis in protein quality control pathways in neurodegeneration and in normal physiology. Chapter 2 provides evidence for UBQLN2 regulation of a pathological phosphorylated form of α−syn, pS129 α−syn. In vitro, UBQLN2 overexpression leads to decreased pS129 α−syn whereas proteasome inhibition leads to pS129 accumulation. In vivo, the absence of UBQLN2 leads to an accumulation of pS129 in a mouse model of PD. Chapter 3 describes the unexpected finding that UBQLN2 overexpression causes dose-dependent retinal degeneration, suggesting that UBQLN2 homeostasis is critical to neuronal health and introduces the retina as a potentially useful model for investigating UBQLN2 function and dysfunction. While this work describes key observations in UBQLN2's role in protein quality control, in both normal and disease states, there is still much to uncover concerning the mechanism of UBQLN2 function. This chapter outlines ongoing studies and outstanding questions to further elucidate UBQLN2's function as a regulator of protein quality control in healthy and disease states.

4.2 Uncovering UBQLN2's mechanism of action

Previous studies have broadly implicated UBQLN2 in protein quality control, with evidence suggesting a role in both proteasomal and lysosomal degradation.[1-5] In chapter 2, I began to elucidate which protein quality control pathways UBQLN2 uses to regulate α−syn. My studies revealed an accumulation of pS129, but not total α−syn, following proteasome inhibition,

suggesting that, when overexpressed, UBQLN2 handles pS129 via the proteasome and may regulate un-phosphorylated α−syn via other mechanisms. Further studies are required to determine if UBQLN2 regulation of pS129 via the proteasome occurs in a ubiquitin-dependent or independent manner and if UBQLN2 regulates pS129 or normal α−syn via the lysosome. Studies probing the mechanism of UBQLN2 regulation of pS129 are a natural extension of the work described in this dissertation.

UBQLN2 functions as a shuttle protein for proteasomal degradation[2], but is also implicated in macroautophagy.[3,6] The proteasome is known to primarily act on soluble, short lived proteins[7,8], while macroautophagy is thought to primarily degrade insoluble, aggregated proteins.[7,8] However, previous studies suggest that UBQLN2 may be able to regulate aggregated proteins via the proteasome.[2] UBQLN2 is known to regulate many disease-linked proteins, including tau[9], huntingtin[4,10], TDP-43[11-13], and α−Syn, all of which form aggregates in disease. Evidence from chapter 2 and previous studies suggest that UBQLN2 may regulate normal and pathological forms of proteins differently, and a clearer understanding of these mechanisms may enhance our understanding of protein accumulation in disease. To further explore this, future studies will assess pS129 levels in cell and/or brain lysates separated into soluble and insoluble fractions (the latter containing aggregates). In cell models, we can pharmacologically modulate proteasome and lysosome activity[14,15] and evaluate changes in pS129 in soluble versus insoluble fractions. However, these proposed studies may be complicated by the lack of adequate in vitro and in vivo models for PD that recapitulate α−syn fibril formation and toxicity. α−syn does not form insoluble fibrils without seeding or external stressors[16], nor does it significantly form fibrils in transgenic mouse models of PD.[17] We will, however, be able to assess changes in soluble and insoluble pre-fibrillar oligomers.[16-18]

Normal α−syn and pS129 clearance occurs via proteasomal and lysosomal degradation.[19-24] Our studies in Chapter 2 suggest that UBQLN2 handles pS129 via the proteasome. Our results also suggest that UBQLN2 may even sequester pS129 at the proteasome, which may prevent pS129 degradation by the lysosome (Figure 4-1). To expand on this finding, ongoing studies will include immunoprecipitation of cell lysates transfected with UBQLN2 and α−syn and treated with a proteasome inhibitor to determine if we can detect increased interactions between α−syn and the 20s proteasome. This finding would support our hypothesis that UBQLN2 targets pS129 to the proteasome for degradation and sequesters it there when cells are treated with a proteasome inhibitor.

To determine if UBQLN2 also can handle pS129 via macroautophagy, ongoing studies are using pharmacological inhibitors of macroautophagy in UBQLN1,2,4 total knock out cells (TKO) cells overexpressing UBQLN2 and α−syn. These studies would reveal whether UBQLN2 regulates pS129 or normal α−syn via macroautophagy. Dual inhibition of the proteasome and lysosome would allow us to determine if UBQLN2-mediated regulation of pS129 occurs via the proteasome and lysosome. In this experiment, an increase of pS129 that is greater than that seen with single pathway inhibition would indicate that UBQLN2 targets pS129 for degradation by both the UPS and macroautophagy. A potential confounding factor to these studies is potential crosstalk occurring between proteasomal and lysosomal degradation pathways.[7,25,26] Studies have shown that modulating one pathway can indirectly lead to a compensatory response from the other.[7,25,26]

Chaperone mediated autophagy is a specialized form of autophagy that has been implicated in Parkinson's disease[27-31] and is a pathway in which UBQLN2 may play a role. Proteins are selected for degradation by CMA based on the presence of the recognition motif,

KFERQ, which α–Syn contains.[32] UBQLN2 may influence this pathway due to its known interaction with the chaperone HSC/HSP70[2,33], which is a key chaperone in CMA.[28,32] Unfortunately, it is difficult to modulate CMA activity with traditional methods such as pharmacological inhibitors/activators[34] or the use of stress-inducing techniques independent of macroautophagy[35], since both pathways function via the lysosome. An alternative approach is to downregulate LAMP2A[35], the receptor that HSC/HSP70 binds on the surface of the lysosome and which mediates internalization of proteins to be digested. However, the similarity of LAMP2A to other LAMP2 isoforms makes selectively targeting it challenging and LAMP2A knockdown may result in LAMP2B or C compensation.[35] Nevertheless, future studies could use small interfering RNA to downregulate LAMP2A and decrease CMA activity to study its effect on UBQLN2 regulation of pS129. Alternatively, HSC/HSP70 downregulation could also lead to decreased CMA activity, but HSC/HSP70 is not specific to CMA activity and thus downregulating it may cause confounding off-target effects. Though each downregulation approach to decreasing CMA has its caveats, a combined approach might provide sufficient evidence to confirm or refute UBQLN2 regulation of pS129 through CMA.

The retina provides an opportunity to further investigate the role of UBQLN2 in proteostasis pathways.[36] In Chapter 3, we report the unexpected finding that UBQLN2 overexpression leads to dose-dependent retinal degeneration, with the highest level of UBQLN2 expression leading to complete degeneration of photoreceptors by 8 weeks of age. It is likely that, when expressed at high levels, UBQLN2 perturbs proteostasis pathways in a manner that is deleterious to the retina, but the extreme sensitivity of the retina to changes in proteostasis[37-40] makes delineating the cause of UBQLN2-mediated retinopathy difficult. Recent data suggest that increased levels of UBQLN2 inhibit CMA activity.[41] Studies have shown that macroautophagy

decreases with age and this decrease coincides with a compensatory increase of CMA activity.[38] While CMA compensates for disrupted macroautophagy, inhibition of CMA does not result in compensatory activity from macroautophagy and is deleterious to photoreceptors.[28,38] To determine if UBQLN2 suppresses CMA, or alters other proteostasis pathways in the retina, future studies would assess the state of key proteostasis proteins (ubiquitin, p62, LC3, LAMP2a, etc.) in the retina. To capture changes at the protein level that lead to degeneration, samples would be collected from 3-weeks old mice from Ub2-hi mice. We could also assess expression patterns of proteostasis proteins in Ub2-KO and Ub2-low mice compared to control to evaluate how the absence of UBQLN2 or moderate overexpression of UBQLN2 alters protein quality control pathways. UBQLN2 expression in Ub2-hi retina was punctate, particularly in the inner segment of outer receptors. Immunofluorescence will allow us to determine if the puncta observed colocalize with proteostasis proteins, suggesting that they may be sequestered and unable to mediate protein degradation. These studies will enhance our understanding of UBQLN2's participation in protein quality control and may contribute to studies of age-dependent retinopathies, including those involving retinopathies seen in neurodegenerative disease.

Despite the evidence provided by this dissertation and other studies that UBQLN2 handles disease-linked proteins by targeting them for degradation, there is little evidence for UBQLN2 directly mediating their clearance.[2,4,10] A common technique used to assess protein turnover involves pulse-labeling the protein of interest (metabolically or fluorescently) and then biochemically measuring protein levels at specific timepoints. This technique has been used to show that the absence of UBQLN2 leads to substrate accumulation[2,4,10], suggesting a lack of clearance. However, these studies have not shown that UBQLN2 expression increases clearance.

In chapter 2, we conducted an optical pulse labeling experiment[42] to directly measure UBQLN2-mediate clearance of α−syn in individual live neurons overexpressing UBQLN2. For this experiment, we created an α−Syn-Dendra2 construct, which converts from GFP to RFP after exposure to 405nm light. Following photo-conversion, we tracked the RFP signal over multiple days. UBQLN2 overexpression did not affect α−syn half-life, but what we could not capture in this experiment was whether UBQLN2 decreases pS129 half-life. Assessing UBQLN2-mediated clearance of pS129 specifically is crucial to determine UBQLN2's role in pS129 regulation. While we are unable to selectively photo-label pS129, we could transiently express a photo-labeled phosphomimetic form of the protein (S129D or S129A α−syn)[43] that contains aspartic acid instead of serine at amino acid position 129, essentially mimicking the function of a phosphate group at that residue. Future studies could use photo-labeled S129D or S129A phosphomimetic α−syn to define the role of UBQLN2 in pS129 clearance. If UBQLN2 enhances clearance of pS129, but not normal α−syn, this would support the view that UBQLN2 preferentially recognizes and clears pathological (phosphorylated) α−syn over healthy α−syn.

4.3 Determining which of UBQLN2's functional domains are necessary for its function on α−syn

In chapter 2, we describe a function for UBQLN2 in regulating pS129 α−Syn that closely related UBQLN1 does not serve. The UBQLNs all contain a ubiquitin-like domain (UBL), ubiquitin-associated domain (UBA), and a series of stress-induced protein 1 (STI-1) motifs.[44] UBQLN2 is unique among UBQLN proteins in that it contains a proline-rich domain (PXX)[44] just amino-terminal to the UBA domain. While our results in Chapter 2 suggest that the PXX domain is key to UBQLN2's function on pS129, further studies are needed to determine which

functional domains within UBQLN2 are relevant and necessary for its regulation of pathological pS129. Our laboratory has established various UBQLN2 domain deletion constructs (Figure 4-2) that can be used in the studies outlined below.

The C-terminal UBA domain allows UBQLN2 to bind to mono- and poly-ubiquitin, and UBQLN2's function as a shuttle protein for the UPS suggests that the UBA domain is critical to UBQLN2's function. A UBQLN2 UBA deletion construct[45] would allow us to determine if this domain is indeed critical, as we expect. However, the UBA domain may serve as more than a binding site for ubiquitin. UBQLN2 is known to undergo liquid-liquid phase separation (LLPS) that allows it to oligomerize and perhaps allows it easy access to substrate proteins.[45] UBQLN2 interaction with ubiquitin breaks up phase separated condensates, allowing UBQLN2 to shuttle client proteins to the proteasome or lysosome for degradation. Deletion of the UBA domain prevents UBQLN2 from phase separating[45], which hinder its regulatory function on α–syn. ΔUBA UBQLN2 may lead to an accumulation of pS129 due its importance for UBQLN2-mediated clearance through the UPS or by disrupting UBQLN2 function mediated by LLPS. L619A UBQLN2 is a genetically engineered form of UBQLN2 containing a single missense change that markedly inhibits ubiquitin binding by UBQLN2[46] and may allow us to selectively study the role of ubiquitin binding at the UBA in UBQLN2 handling of pS129. However, L619A UBQLN2 may present its own complications with inconsistent results on L619A UBQLN2 toxicity and whether it has a higher propensity to aggregate and cause cell death.[2,46] For this reason, future studies using ΔUBA or L619A UBQLN2 will include Immunocytochemistry to assess the effect of these constructs on pS129 levels on an individual cell basis. Studies exploring the role of the UBA domain may shed light on the importance of both ubiquitin binding and LLPS on UBQLN2-mediated α–syn regulation.

The UBL domain enables UBQLN2's interaction with the proteasome[47,48], however UBL domains also serve as regulators of macroautophagy[49] and thus may be integral to UBQLN2's function in both the UPS and macroautophagy. UBQLN2 UBL deletion constructs would allow us to assess the importance of the UBL domain on UBQLN2's action on α–syn. Considering that UBL domains are implicated in both proteasomal and lysosomal degradation, future studies using the ΔUBL UBQLN2 construct can be coupled with pharmacological modulation of the UPS and macroautophagy to determine in which pathway(s) the UBL domain is necessary for UBQLN2 function.

UBQLN2 contains four STI-1 motifs that are thought to promote UBQLN dimerization and interaction with chaperones (HSC/HSP70).[44] Because of their interactions with HSC/HSP70 the STI-1 motifs may help us better understand any role UBQLN2 plays in chaperone mediated macroautophagy. Our lab has created multiple deletion constructs containing different combinations of STI-1 motifs to study their individual roles in UBQLN2's function (Figure 4-2). Assessing changes in pS129 levels when expressed with the various ΔSTI-1 constructs may reveal whether UBQLN2 interactions with HSC/HSP70 or dimerization, or both, are important for UBQLN2 actions on pS129. Immunoprecipitation studies employing these constructs could also revel which STI-1 motifs (if any) are necessary for UBQLN2-HSC/HSP70 interactions and for UBQLN2 dimerization with other UBQLNs. The functions of STI-1 motifs are still poorly understood; hence these studies would contribute to our understanding of these motifs while also elucidating their contribution to in UBQLN2 function generally and regulation of pS129 specifically.

Within the UBQLN family, only UBQLN2 contains a PXX domain[44], which may enable its role in regulating disease-linked proteins such as pS129 α–syn. In addition to using the

domain deletion approach to study the role of the PXX domain in UBQLN2 function, we can insert the PXX domain into UBQLN1 (Figure 4-2). UBQLN1 is 74% homologous to UBQLN2[44] yet does not share UBQLN2's ability to decrease levels of disease-linked proteins when overexpressed.[9] If the PXX domain is responsible for UBQLN2's regulatory function, then inserting the PXX domain into UBQLN1 will enable UBQLN1 to act as UBQLN2 does on proteins such as pS129. To test this, we would compare pS129 levels in cells expressing full length UBQLN2, full length UBQLN1, ΔPXX UBQLN2, and UBQLN1+PXX. If UBQLN1+PXX does not enable UBQLN1 to decrease pS129, this may suggest that other differences in UBQLN2 structure compared to UBQLN1 are responsible for its ability to regulate pS129. Many of UBQLN2's disease-causing mutations are found in the PXX domain, though it is not fully understood if these mutations lead to a loss of function or gain of toxic function. Nevertheless, UBQLN2 mutations, such as P506T, may show altered regulation of α–syn, suggesting that UBQLN2 mutations affect its ability to handle substrate proteins. Our lab has established a P506T UBQLN2 transgenic mouse[36] and future studies could assess α–syn and pS129 levels in these mice. The importance of studies investigating the function of UBQLN2's PXX domain is underscored by the relevance of this domain in human disease.[50]

There is still much to uncover concerning UBQLN2's functional domains and their role in UBQLN2's function, including as a pS129 regulator. Because UBQLN2 can function in both the UPS and macroautophagy, deleting specific domains may push UBQLN2 towards one pathway over the other, leading to potentially moderate changes in pS129 levels. The potential for this confounding variable would require that we carefully assess indirect changes in proteostasis pathways by monitoring key proteostasis proteins (LAMP2-A, ubiquitin, p62, LC-3, etc.) during these studies. Domain deletion may also prevent proper folding of UBQLN2, which

would alter its functionality and stability. Nevertheless, studies evaluating UBQLN2's individual functional domains will be important as they could elucidate the mechanisms by which UBQLN2 functions in protein quality control and in regulating pS129.

4.4 Implications for models of PD

While there are multiple rodent models of PD, they do not recapitulate the dopaminergic cell loss characteristic of PD and do not develop Lewy body pathology.[17,18] In chapter 2, we show that the absence of UBQLN2 in 12-month-old, hemizygous A53T α–syn mice leads to pS129 accumulation. This finding is significant because it implicates UBQLN2 in regulating pathological α–syn. Hemizygous A53T mice, like the ones used in this study, usually do not begin to display α–syn pathology until ~22 months of age.[51] UBQLN2 KO may drive A53T mice to more closely model Lewy body pathology. Future studies will use 12-month homozygous A53T mice to achieve a more robust effect than was seen in the hemizygous mice and determine if these mice develop Lewy bodies. A more robust phenotype that recapitulates late-stage α-syn may allow us to capture previously undetected effects of UBQLN2.To confirm a role for UBQLN2 in PD, it would be beneficial to replicate the studies in Chapter 2 in multiple mouse models, such as in SCNA triplication[52] mice and the Leucine-rich repeat kinase 2 mutation mouse model[53], considering the robust effect of UBQLN2 on pS129. Expanding these studies to multiple mouse lines will allow us to make broader conclusions about the role of UBQLN2 in Parkinson's disease and may inform us of how, if at all, UBQLN2 contributes to the progression of PD pathology in different disease landscapes.

Ultimately, assessing the role of UBQLN2 on pS129 in induced pluripotent stem cells (IPSCs) from patients with either sporadic or familial Parkinson's disease, would further link UBQLN2 dysfunction to PD. In chapter 2, we provide evidence for dysregulation of UBQLN2 in

synucleinopathies using postmortem brain tissue, however it would benefit our understanding of UBQLN2's role in PD to perform mechanistic studies probing UBQLN2-mediated regulation of pS129 in patient-derived cells. The use of IPSCs in studies would enable us to probe the function of UBQLN2 on endogenous human α-synuclein and pS129 in a disease relevant environment. The results of this work may potentially lead to new targets for treating PD, whether that be UBQLN2 or a downstream target of UBQLN2.

4.5 Concluding remarks

Protein quality control is essential to neuronal health and a better understanding of proteostasis pathways may help us better understand the etiology and progression of many devastating neurodegenerative diseases, including Parkinson's disease. In this dissertation, I described a novel function for the protein quality control protein UBQLN2 in regulating pathological pS129, including in a rodent model of PD, and showed that UBQLN2 homeostasis is critical for photoreceptor health. However, there is significant work to be done to understand how UBQLN2 regulates pS129 and its role in disease. This work, along with the proposed future directions, could lead to important findings about the development and progression of neurodegeneration pathology, particularly in PD.

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4.7 Figures

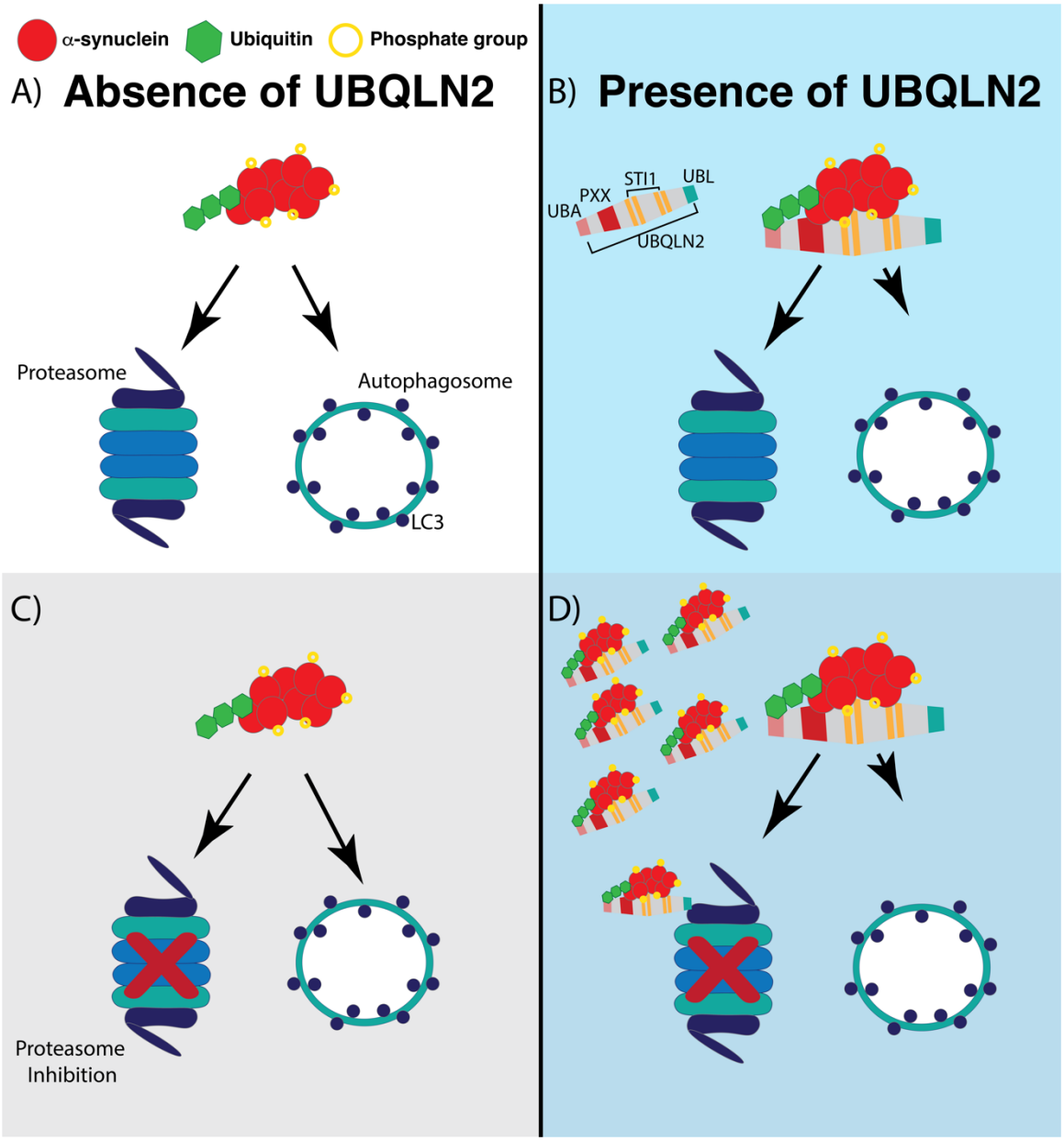

Figure 4-1 Proposed mechanism for UBQLN2 sequestration of pS129 during proteasome inhibition.

A) In the absence of UBQLN2, both the UPS and macroautophagy can regulate levels of pS129. B) When present, UBQLN2 shuttles pS129 to the proteasome and autophagosome for degradation but favors the proteasome. C) In the absence of UBQLN2 and during proteasomal inhibition, macroautophagy continues to regulate pS129 and maintains steady-state levels. D) During proteasome inhibition UBQLN2 sequesters pS129, perhaps even at the proteasome, in a manner that prevents lysosomal degradation.

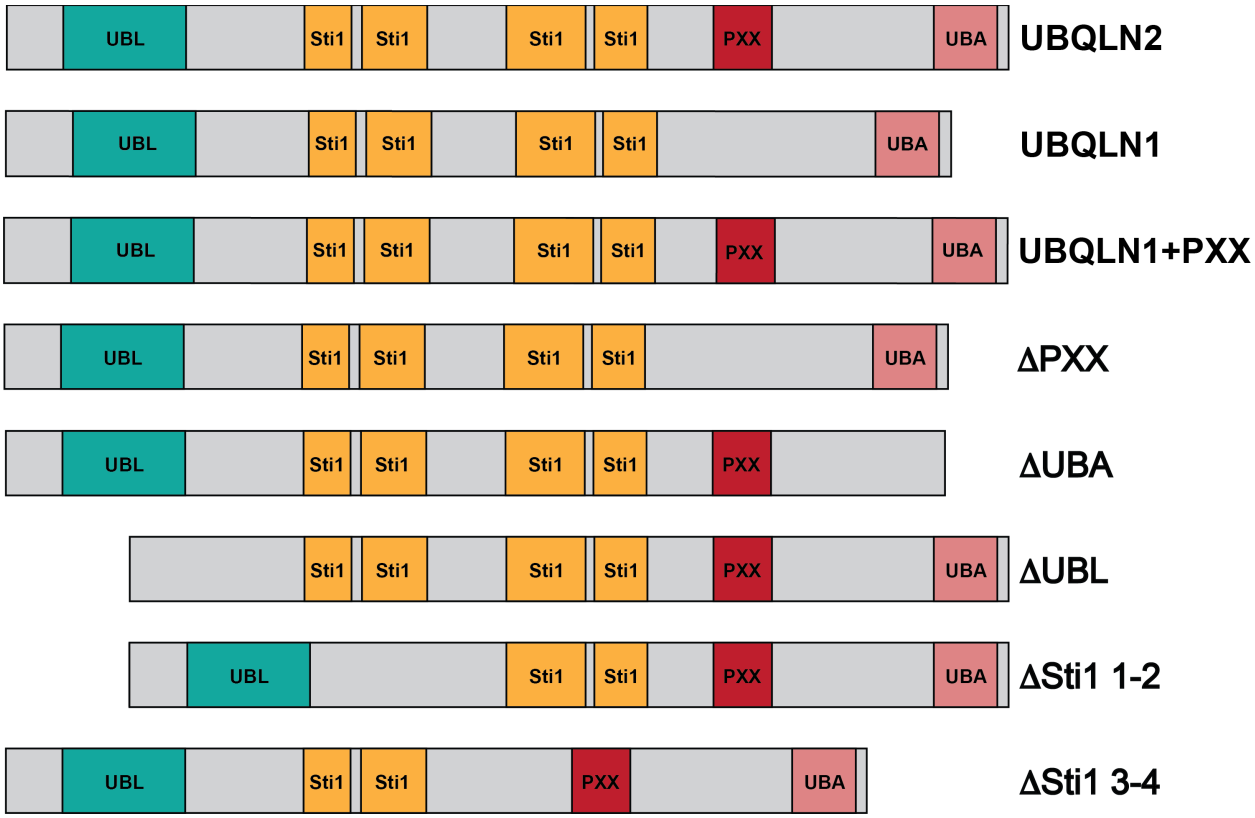

Figure 4-2 Schematic of UBQLN2 and UBQLN1 constructs to be used in experiments outlined in section 4.3.