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Review Article

Structure, dynamics and functions of UBQLNs: at the crossroads of protein quality control machinery

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Cells rely on protein homeostasis to maintain proper biological functions. Dysregulation of protein homeostasis contributes to the pathogenesis of many neurodegenerative diseases and cancers. Ubiquilins (UBQLNs) are versatile proteins that engage with many components of protein quality control (PQC) machinery in cells. Disease-linked mutations of UBQLNs are most commonly associated with amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), and other neurodegenerative disorders. UBQLNs play well-established roles in PQC processes, including facilitating degradation of substrates through the ubiquitin–proteasome system (UPS), autophagy, and endoplasmic-reticulum-associated protein degradation (ERAD) pathways. In addition, UBQLNs engage with chaperones to sequester, degrade, or assist repair of misfolded client proteins. Furthermore, UBQLNs regulate DNA damage repair mechanisms, interact with RNA-binding proteins (RBPs), and engage with cytoskeletal elements to regulate cell differentiation and development. Important to the myriad functions of UBQLNs are its multidomain architecture and ability to self-associate. UBQLNs are linked to numerous types of cellular puncta, including stress-induced biomolecular condensates, autophagosomes, aggresomes, and aggregates. In this review, we focus on deciphering how UBQLNs function on a molecular level. We examine the properties of oligomerization-driven interactions among the structured and intrinsically disordered segments of UBQLNs. These interactions, together with the knowledge from studies of disease-linked mutations, provide significant insights to UBQLN structure, dynamics and function.

Introduction

Cells must maintain an intricate protein homeostasis to maintain proper function and survival. The protein flux of the cell must remain in balance despite being continuously challenged by limited protein folding capacity [1], environmental stress and aging. Maintenance of protein homeostasis is particularly important in neurons due to their unique morphology and long lifespan [2]. Dysregulation of homeostasis is associated with neurodegenerative diseases such as Amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), Alzheimer's disease (AD) and frontotemporal dementia (FTD) [3–5]. Cells have developed protein quality control (PQC) mechanisms to surveil proteome balance, facilitate protein folding, and respond to accumulation of protein aggregates.

Due to the highly complex and crowded nature of the cellular environment, substrate targeting in the PQC pathways can be challenging. Cells rely on shuttle proteins that have dual capability to interact with substrate and PQC components to facilitate this process, closing the gap between substrates and degradation machineries [6]. Ubiquilins (UBQLNs) are multifaceted shuttle proteins, as they can chaperone misfolded proteins, but also facilitate degradation of substrates through the ubiquitin–proteasome system (UPS), autophagy, and endoplasmic-reticulum-associated protein degradation (ERAD) pathways [7–10]. UBQLNs are invoked during cellular stress responses, as evident by their localization into stress granules, a stress-induced membraneless organelle consisting of arrested translation

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machinery, mRNAs, and proteins [11–13]. UBQLNs also localize to cellular aggregates and aggresomes to help the cell sequester misfolded protein into separate locations [14,15]. Understanding the structure, dynamics, and function of UBQLNs is therefore essential to elucidating PQC mechanisms in cells.

Mutations in the human UBQLN proteins have been reported to associate with or cause a variety of diseases, including neurodegenerative disorders and cancers. The most well-characterized pathological mutations are extensively related to neurodegenerative diseases, specifically ALS, FTD, and AD [15–21], among others such as Brown–Vialetto–Van Laere syndrome (BVV LS) [22]. Additionally, changes in the protein expression levels of UBQLNs are associated with diseases such as HD [23], breast, lung, and gastric cancers [24–27]. While the underlying molecular mechanisms associated with disease mutations of UBQLNs remain unknown, many studies show that mutant UBQLN pathology often involves compromised PQC mechanisms (UPS and autophagy), and/or formation of disease-related aggregates [12,15,28–32].

To date, five human UBQLN proteins are known (1, 2, 3, 4, and L). Among these five UBQLN proteins, UBQLN3 and UBQLNL are specific to testes [33,34], whereas UBQLN1, 2 and 4 are widely expressed in all tissues [35]. Notably, UBQLN2 expression is elevated in the nervous system whereas UBQLN1 and UBQLN4 are relatively evenly expressed throughout all tissue types [35–37]. Given the ubiquitous expression of these three paralogs, this review will focus on elucidating the biochemical and biophysical properties of UBQLN1, UBQLN2, and UBQLN4.

In the first part of this review, we summarize the structural properties of the individual domains in UBQLNs, and how these domains interact with each other to drive UBQLN oligomerization and self-assembly. UBQLN's ability to self-assemble is critical to its localization into intracellular puncta, including membraneless organelles, autophagosomes, and aggregates. Next, we summarize the functional roles of UBQLNs in cells, with special emphasis on PQC mechanisms. We elaborate on insights gained from disease-linked mutations on UBQLN functions. Combining current knowledge, we explore how regulation of oligomerization may contribute to UBQLN functionality and cross-talk among the different PQC pathways. Finally, we highlight future directions to elaborate UBQLNs' roles in neurodegenerative diseases and cancers.

Structure of UBQLNs

UBQLN1, UBQLN2 and UBQLN4 are widely expressed in humans and comprise 589, 624, and 601 amino acids respectively (Figure 1A). UBQLN1 is 74% identical with UBQLN2, while UBQLN4 is 60% identical with UBQLN1. UBQLNs are found in the nucleus and cytoplasm of cells, appearing either diffuse in these compartments or organized into puncta. UBQLN4 localizes to the nucleus and the ER [38], while UBQLN2 has been observed in the nucleus and cytoplasm depending on cell state [28,37,39]. UBQLN1 appears to be cytosolic [40,41]. Interestingly, overexpression of UBQLNs typically produce numerous punctate structures throughout the cell [17,42,43].

All three UBQLNs share similar domain architecture, consisting of a N-terminal Ubiquitin (Ub)-like (UBL) domain, a C-terminal Ub-associated (UBA) domain and two STI1 regions in the middle (Figure 1A). The UBL-UBA construct of the UBQLNs is similar to other Ub-binding protein shuttles, such as Rad23B/HR23B and Ddi1 [44,45]. Located between the two folded UBL and UBA domains, the middle region is largely intrinsically disordered (Figure 1B). UBQLN2 contains a unique proline-rich region (PXX), where neurodegenerative disease-related mutations are found disproportionately. This multidomain architecture of UBQLNs enables a rich pool of diverse binding partners that include PQC components, such as the proteasome, autophagic proteins, misfolded proteins, and ubiquitinated substrates. The largely intrinsically disordered segments confer flexibility and dynamics to UBQLNs that contribute to functionality as we will discuss below.

Ubiquitin-like (UBL) domain

The UBL domain is highly conserved among the UBQLNs, especially between UBQLN1 and UBQLN2 (Figure 2). As its name implies, the UBL domain is structurally similar to Ubiquitin (Ub) despite low sequence identity. The canonical hydrophobic patch formed by Ub residues L8, I44, V70 is mostly conserved in the UBL domain (UBQLN2 residues T39, I75, V101). However, the electrostatic surface potential differs between UBQLN UBL and Ub (Figure 3A). Consequently, the UBL domain and Ub share some common binding partners, such as the Ub-binding shuttle protein hHR23b and proteasome subunits, but with different binding affinities [46].

The UBL domain interacts directly with subunits of the proteasome, the main proteolytic machinery of the cell (Figure 4A). The proteasome is composed of a 20S catalytic core and can be capped with different

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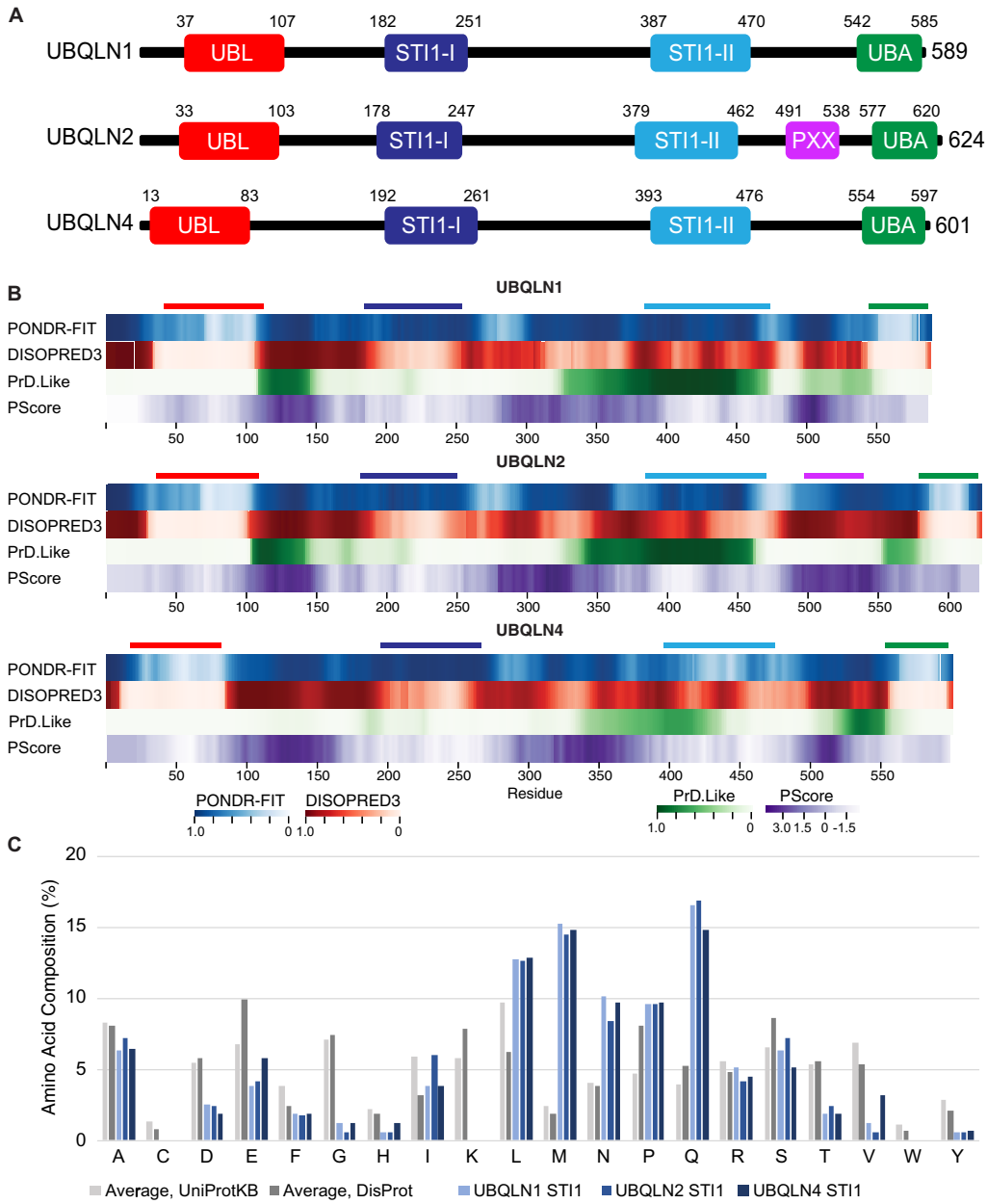

Figure 1. Domain architecture and sequence characteristics of UBQLNs.

(**A**) Domain architecture map of UBQLN1, UBQLN2 and UBQLN4 with UBL, STI1-I, STI1-II, PXX, and UBA domains colored as red, dark blue, blue, magenta, and green, respectively. PXX is a proline-rich region unique to UBQLN2. (**B**) Predictions for UBQLN disorder, prion-like propensity, and phase separation propensity. PONDR-FIT [96] and DISOPRED3 [97] predict intrinsically disordered regions. PONDR-FIT is a meta-predictor that produces prediction scores by combining results from a series of algorithms; DISORPRED3 is a separate program trained on conserved sequence features of intrinsically disordered regions, identified by missing residues in high-resolution X-ray structures [96,97]. For both programs, higher values represent protein regions likely to be disordered. PLAAC identifies prion-like domains (PrD.like) consisting of certain hydrophobic residue patterns that can speed amyloid formation [98]. Phase-separation propensity scores (PScore) identifies intrinsically disordered protein sequences that may drive liquid–liquid phase separation (LLPS), mainly by pi–pi contact contributions [109]. The overall PScores for UBQLN1, 2 and 4 are 2.91, 3.24 and 3.7, respectively. (**C**) Comparison of the amino acid compositions between all proteins (structured and unstructured) in the UniProt database [168], disordered proteins and domains in the DisProt database [169], and STI regions found in UBQLN1, 2, and 4.

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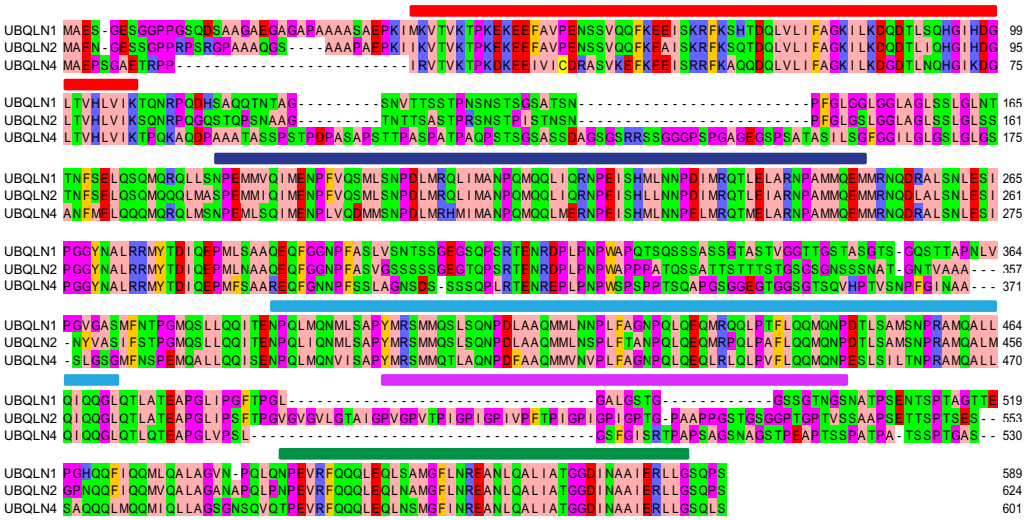

Figure 2. Sequence alignments of the UBQLNs.

Sequence alignment of UBQLN1, UBQLN2, and UBQLN4 performed using T-Coffee [170]. UBL STI1-I, STI1-II, PXX, and UBA domains are colored as red, dark blue, blue, magenta, and green, respectively. Visualized with Jalview [171], and residues colored according to this scheme: beige for hydrophobic, blue for positive, red for negative, green for polar, magenta for prolines and glycines, and yellow for aromatic amino acids.

regulatory subunits, such as the 19S regulatory cap [47]. The UBQLN UBL domain binds directly to 19S cap proteasomal receptors Rpn3, Rpn13, and Rpn10 (Figure 3B) [7,48,49]. These proteasomal receptors also recognize polyubiquitinated substrates. Notably, the Rpn13 and Rpn10 proteasome subunits preferentially interact with the UBQLN UBL over Ub. However, the binding preference is modest as the interaction between the UBL and Rpn13 ($K_d \sim 10$ μM) is only three-fold stronger than the interaction between Rpn13 and K48-linked diUb [48,49]. Notably, Rpn10 binding preference favors polyUb over UBL in the presence of long K48-linked polyUb chains [50].

The UBQLN UBL domains interact with the two ubiquitin-interacting motifs (UIMs) of Rpn10 and the pleckstrin-like receptor for ubiquitin (PRU) domain of Rpn13 (Figure 3D) [48,49]. In Rpn10, both UIMs bind to UBQLN2 UBL, however, the first UIM exhibits 25-fold higher affinity indicative of preferred occupancy at this site [49]. First found in Rpn10, the UIM is not unique to the proteasome [51,52]. In fact, other cellular components recruit UBQLN proteins via UBL–UIM interactions as well. For example, epidermal growth factor substrate 15 (EPS15), an adaptor protein involved both in secretion and endocytosis [53], interacts with UBQLN1 via its UIM, and this UBL–UIM interaction recruits EPS15 into UBQLN1-positive aggregates *in vivo* [42]. The co-chaperone human neuron specific DNAJ-like protein 1a (HSJ1a) and ataxin-3 rely on their UIMs to bind UBQLN1 and consequently regulate the formation of aggresomes [14]. Aggresomes accumulate misfolded proteins to minimize their toxicity in the cytosol [54].

Ubiquitin-associated (UBA) domain

The Ubiquitin-associated domain is a small (~45 amino acid) helical domain, named for its ability to bind Ub. This domain is highly conserved, especially among proteins that are involved in ubiquitination and degradation processes. For example, UBA domains with similar structure are found in Ubiquitin ligase (E3) HUWE1, a substrate-specific ubiquitination enzyme, and the UPS shuttle hHR23b that interacts directly with ubiquitinated substrates (Figure 3C) [55,56]. Sequences of the C-terminal UBA domains found in all UBQLN proteins are over 93% identical. The α1 helix residues (UBQLN1 Met-557 and Phe-559) and α3 helix residues (UBQLN1 Ile-576, Ile-580, and Leu-584) form the binding interface with the hydrophobic patch on Ub, consisting of Leu-8, Ile-44, and Val-70 [57,58]. These UBQLN UBA residues are conserved across all UBQLNs (Figure 3C), and the same residues are involved in UBQLN2 UBA interactions with Ub [13]. Indeed, the tight binding affinity between UBA and Ub ($K_d \sim 1$–5 μM) is relatively unchanged in the context of polyUb chains, indicative of UBQLN's ability to interact with either monoUb or polyUb tags on ubiquitinated substrate proteins. As the

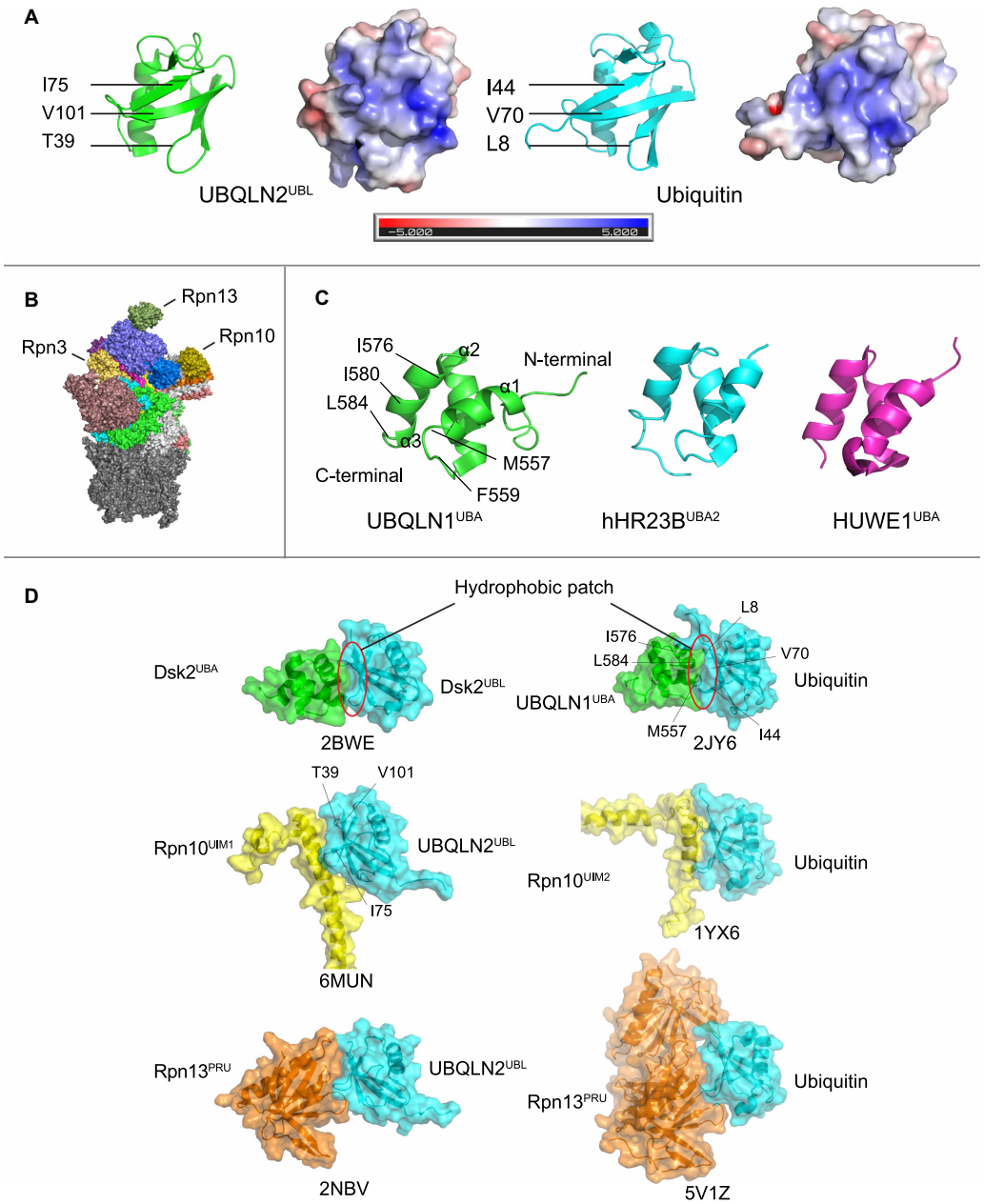

Figure 3. Known structures and binding interfaces of UBQLN domains.

(**A**) Structural comparison of UBQLN2 UBL to Ubiquitin (Ub). Color represents the surface charge electrostatic potential (±5 kT/e). (**B**) 19S proteasome regulatory subunits that interact with UBQLNs (labeled), including Rpn3, Rpn10/S5a and Rpn13. Shown is the yeast 19S regulatory cap (PDB: 4CR2). (**C**) Structural comparison between UBA domains found in different Ub-binding proteins. (**D**) Structural comparison between the Ub-binding pattern/domains and Ub/UBL proteins. Ub/UBL domains are colored cyan, with Ub-binding domains/motifs colored in green, yellow, and orange.

same residues on Ub are used to interact with proteasomal receptor Rpn10 [59], competition exists for ubiquitinated substrates between the UBQLN proteins and the proteasome. Substrates could be transferred from UBQLNs to the proteasome for processing, consistent with UBQLN shuttle functionality.

Aside from targeting polyubiquitinated substrates to the proteasome for degradation, evidence exists that the UBA domain plays a protective role for certain proteins such as anti-apoptotic BCL2-like protein BCLb, Insulin-like growth factor-1 receptor (IGF1R), and ER-associated membrane protein extended synaptotagmin 2

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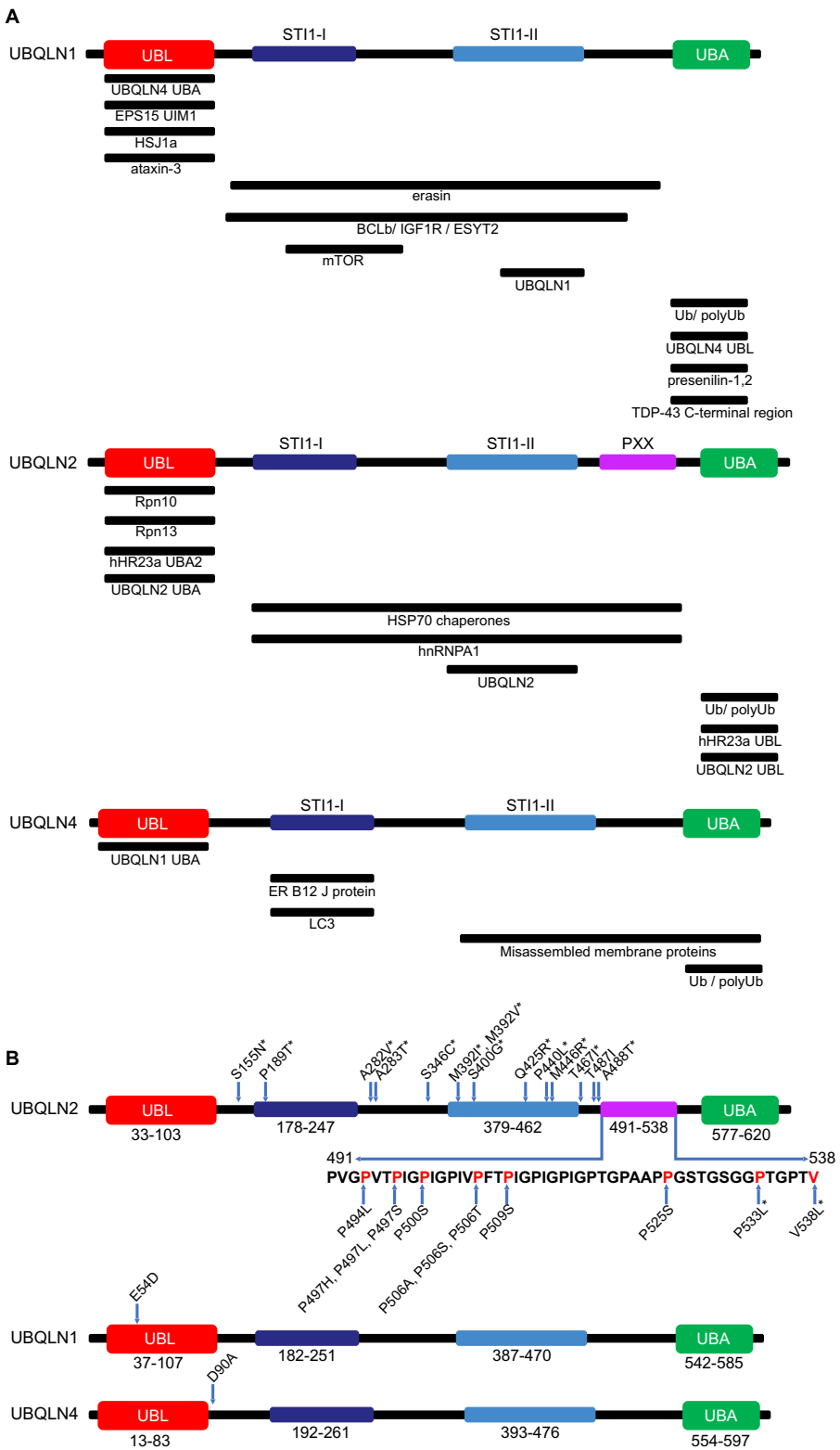

Figure 4. Binding partners and known disease-linked mutations of UBQLN proteins. Part 1 of 2

(**A**) Interactions between UBQLNs and binding partners involve different domains of the proteins. The UBL domain interacts with proteasome subunits [7,48,49], UIM-containing proteins like EPS15, ataxin-3 and HSJ1a [14], and UBA-domain containing proteins hHR23a and UBQLNs [9,46,105,172]. The UBA domain interacts with mono- and polyUb [7,9,13], TDP-43 [146,149],

Figure 4. Binding partners and known disease-linked mutations of UBQLN proteins. Part 2 of 2

presenilins [17,104], and UBL-domain containing proteins like hHR23a and UBQLNs [9,46,105,172]. The central region of the UBQLNs including the two STI1 regions, linkers and PXX region (UBQLN2 only) is involved in binding to a variety of partners, such as erasin [8], mTOR [36]; HSP70 chaperones [28,66], ER membrane B12 J protein [130]; IGF1R, ESYT2, and BCLb [40], and misassembled membrane proteins [67]. (**B**) Mutations are indicated by arrows; all are linked to neurodegenerative diseases (see Table 2). The UBQLN2 PXX region harbors most of the mutations, followed by the STI1-II region. UBQLN1 and UBQLN4 each carry one known missense mutation. Asterisks represent mutations that were detected in patients with neurodegenerative diseases, but the underlying mechanism of action is unknown.

(ESYT2) [40,60]. This is presumably due to the UBA binding to Ub and blocking polyUb chain elongation on substrate proteins. Therefore, the UBA domain could also prevent substrate polyubiquitination and subsequent degradation [60]. The UBA domain of Dsk2 (budding yeast homolog of UBQLNs) also protects Dsk2 from proteasomal degradation, presumably due to the stable structure of the UBA domain resisting the initiation of Dsk2 degradation at the proteasome, resulting in release of Dsk2 [60]. It is notable that UBQLNs are capped by folded domains on both the N- and C-terminus.

Although less well-studied, the UBA domain interacts with the Rpn10/S5a subunit and possibly the 20S core of proteasome [61]. These interactions could be the result of direct binding to components of the proteasome, in combination with indirect binding through proteasome-bound polyubiquitinated proteins [61]. The interactions may bring the substrate close to the proteasome, facilitating the transfer of substrate to the proteasome.

STI1 regions

Two STI1 regions, namely, STI1-I and STI1-II, are found in UBQLN1, 2, and 4. Other nomenclature defines a total of four STI regions, such that STI1 and STI2 compose STI1-I, while STI3 and STI4 compose STI1-II [4,41]. The STI1 regions are conserved among the UBQLNs, with >94% sequence identity between UBQLN2 and 1, and ∼ 82% between UBQLN4 and UBQLN1 for both STI1-I and STI1-II. The STI1 regions of UBQLNs were named from stress-induced phosphoprotein 1 or HSP70/HSP90 organizing protein (STI1/Hop), which is a co-chaperone protein that mediates heat shock response of the HSP70 genes [62]. The UBQLN STI1 regions resemble the amino acid sequence of the two aspartate/proline-rich domains, DP1 and DP2, of STI1/HOP. DP1 and DP2 domains regulate glucocorticoid receptor activation [63,64]. Interestingly, while STI1/HOP DP1 and DP2 domains are largely composed of α-helices, the STI1 regions are predicted to be intrinsically disordered, despite the sequence similarities (Figure 1B) [64].

Functions of the STI1 regions are much less understood when compared with the UBL or UBA domains. Recent studies reveal that the STI1 regions interact with PQC components (e.g. HSP70 chaperone, autophagosome-associated LC3), contributing to UBQLNs' role in mediating PQC [28,65]. Additionally, STI1 regions in UBQLNs may serve as interaction hubs, or 'cargo decks' for binding partners. Proteomics studies on UBQLNs have determined a number of client proteins that bind to the middle region of UBQLNs (Figure 4A). For example, UBQLN2 STI1 regions interact with HSP70 family proteins Stch [66]; UBQLN1 STI1 interacts with mammalian target of rapamycin (mTOR) [36]; UBQLN4 STI1-II recognizes and binds misassembled membrane proteins [67]; UBQLN4 STI1-I interacts with autophagy protein LC3 [9]; UBQLN1 STI1-I and STI-II mediates its binding to anti-apoptotic BCL2 protein BCLb, insulin-like growth factor 1 receptor (IGF1R) and receptor tyrosine kinase ESYT2 [41]. Importantly, UBQLNs can either facilitate the degradation of the clients through the interactions (misassembled membrane proteins), or stabilize them (BCLb, IGF1R, ESYT2) [41,67,68].

We speculate that the ability of the STI1 regions of UBQLNs to interact with such a diverse set of proteins is due to its unique amino acid composition and intrinsically disordered nature. The STI1s resemble low-complexity domains (LCDs), defined as regions where only a few types of amino acids make up its composition. Met and Gln residues (along with Leu, Asn, and Pro) are overrepresented in the STI1 regions (Figure 1C). These residues mediate hydrophobic and polar interactions, such as hydrogen bonds and π–π interactions. Interestingly, the STI1-II regions of UBQLNs exhibit prion-like characteristics (Figure 1B). Proteins with prion-like sequences, such as ALS-linked TDP-43 and FUS exhibit tendency to form pathological β-sheet rich aggregates in cells, and also mediate interactions to form biomolecular condensates/membraneless organelles (Box 1). In line with these observations, the middle region of UBQLN2 (without the UBL and UBA domains) is enough to recruit UBQLN2 into stress granules, a type of membraneless organelle [12]. The UBQLN4 STI1-II region is the least

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Box 1. Membraneless organelles and protein aggregates

Biomolecular condensates, or sometimes referred to as membraneless organelles, selectively enrich macromolecular components such as protein and RNA in comparison with the surrounding milieu [69,70]. Unlike their membrane-bound counterparts, condensates are not surrounded by a lipid bilayer, thus rendering them dynamic, liquid-like, and allowing them to exchange content with their surroundings rapidly [69]. These biophysical properties confer condensates the ability to form and dissipate on a much faster time scale than membrane-bound organelles [69]. For example, cytoplasmic stress granules rapidly assemble in response to external stressors, such as oxidative stress, heat shock or proteasomal inhibition.

Liquid–liquid phase separation (LLPS) is hypothesized as the underlying biophysical mechanism that leads to the formation of biomolecular condensates [71–73]. LLPS is a thermodynamic process by which macromolecules demix into at least two phases: a dense droplet phase consisting of a high concentration of selective proteins, DNA, or RNA in equilibrium with a dilute phase where the concentrations of these macromolecules are much lower. An important driver of LLPS under physiological conditions is high multivalency, i.e. the ability to noncovalently interact with other components at multiple sites [74–77]. Multivalency arises from long, intrinsically disordered segments (sometimes enriched in certain amino acids in so-called low complexity domains) and/or many folded domains that are connected by linkers (e.g. poly-SUMO scaffold proteins interacting with poly-SIM substrates [75,78–80]). Condensates form when macromolecules reach concentration thresholds, above which the components phase separate. Notably, this behavior is not straightforward for condensates assembled from heterotypic interactions [81,82].

Recent studies suggest that LLPS is on pathway to the formation of insoluble aggregates or protein inclusions that are characteristic of neurodegenerative disorders [83,84]. Condensates with disease related-mutated components can undergo liquid-to-solid phase transitions over time [85–87]. Therefore, the distinction between condensates and aggregates is blurred, and past studies of intracellular 'puncta' or 'foci' will need to reexamine their biophysical properties.

prion-like, and this same region is predicted to be the least disordered of all UBQLN STI1 regions according to PONDR-FIT calculations (Figure 1B). The structured nature of UBQLN4's STI1-II region has been shown to be functionally significant for misfolded protein recognition [67]. As we will describe below, the STI1-II region drives the self-assembly and oligomerization of UBQLN2, complementing the prion-like predictions [13].

Proline-rich region is unique to UBQLN2

Of all UBQLN proteins, the UBQLN2 paralog is the only one to contain the proline-rich (PXX) repeat region, which is located between the STI1-II region and UBA domain. Notably, the PXX region harbors most of the UBQLN-associated disease-linked mutations, specifically linked to ALS and FTD, among others [15,19,20,88–91]. The amino acid sequence in the PXX region mimics that of collagen with a high representation of Pro and Gly residues, with mostly hydrophobic residues in between. Additionally, the sequence of the PXX region resembles elastin and elastin-like polypeptides (ELPs). ELPs are a class of self-interacting biopolymers that has been widely studied from a biomaterials perspective as they undergo temperature-based phase transitions to form condensates [92–94]. Despite the repeating proline residues, the PXX region has a low tendency to form polyproline II helical structure, as predicted by the PPIIPRED program [95]. Using NMR spectroscopy, our lab has found that the PXX domain is intrinsically disordered [13]. Binding to the PXX region may alter polyproline helix propensity, although no binding partners for this region are yet known. Interestingly, the PXX domain participates in the self-interactions of UBQLN2, thus contributing to UBQLN oligomerization (see below) [13].

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Linkers

A significant portion of UBQLN sequence consists of linker regions that connect the various domains described above. The linkers are predicted to be disordered by both the PONDR-FIT and the DISOPRED programs (Figure 1B) [96,97]. Being as long as 130 residues (between STI1-I and STI1-II regions) or as short as 30 amino acids (between STI1-II and PXX), the linkers likely impart flexibility among the domains and permit simultaneous binding to multiple components, e.g. proteasome and ubiquitinated substrates. Interestingly, parts of the linker regions exhibit prion-like characteristics, according to the PLAAC prediction algorithm [98] (Figure 1B). These prion-like segments likely contribute to self-interaction and potentially mediate the accumulation of the UBQLNs into aggregates. In UBQLN4, the hydrophobic region between UBL and STI1-I region interacts with small hydrophobic (SH) protein of mumps virus [99]. While the exact consequence of this binding is unclear, it was speculated that the virus disturbs normal cellular function of UBQLN4, resulting in an anti-apoptotic function. Residues between the STI1-II and UBA domain (residues 550–570 in UBQLN2) are prion-like, and also exhibit some α-helical propensity [13]. Mutations in this segment alter UBQLN2 self-association and ability to phase separate and form condensates [100]. These findings are reminiscent of transient α-helical structure in ALS-linked TDP-43 that contributes to its oligomerization, phase separation and normal functions [101].

UBQLN domains interact with each other to drive oligomerization

Oligomerization is important for the functionality of UBQLNs and many other shuttle proteins [102,103]. UBQLNs form homo- and hetero-dimers, although the K_d of dimerization remains unknown. Specifically, each of the UBQLNs (1, 2, and 4) forms homodimers [9,11,104]. UBQLN1 or UBQLN2 can form heterodimers with UBQLN4 [9]. Studies have found that, in addition to dimeric forms, UBQLN1 and UBQLN2 can exist in oligomeric assemblies of various sizes; disease mutations may perturb this property [11,104].

UBQLN oligomerization is mainly dependent upon the STI1-II region of the proteins [13,41,104]. Using a UBQLN2 construct that removed residues 379–486, which contains the entire STI1-II region (residues 379–462), we found that UBQLN2 can be converted into monomeric form [13,100]. Our NMR study on UBQLN2 450–624 found that residues 450–470 exhibited exchange broadening, consistent with their role in driving self-association. We also determined that a C-terminal construct of residues 487–624, which lacks the STI1-II region was also monomeric, at least up to 500 μM [13]. Additionally, other regions outside the STI1-II region contribute to oligomerization of UBQLN2. Using NMR paramagnetic relaxation enhancement (PRE) experiments that provide proximity information between an installed spin label and nearby residues, we found that the UBA domain transiently interacts with the C-terminal portion of the STI1-II region [11]. Additionally, we showed that disease-linked mutations in the PXX region of UBQLN2 also increase oligomerization propensity in a C-terminal construct of UBQLN2 (residues 450–624). Interestingly, the effect is recapitulated in full-length UBQLN2 for at least the P506T mutation [29]. Native-PAGE experiments showed P506T forming high-molecular mass aggregates versus the largely monomeric wild-type UBQLN2 in soluble mouse brain lysates [29]. However, *in vitro* experiments of purified full-length UBQLN2 mutants show that PXX mutations do not enhance oligomerization, so it is likely that the situation in cells is more complex and/or that the N-terminal part of UBQLNs further regulate oligomerization propensity [28].

UBA–UBL interactions also influence UBQLN oligomerization [13]. The UBQLN2 UBA domain interacts directly with its UBL domain with a weak binding affinity ($K_d \sim 175$ μM). The UBA–UBL interaction can occur intramolecularly, between members of the UBQLN family (e.g. UBQLN1 UBA domain binds to UBQLN4 UBL domain) [9,105], or between UBQLN UBA and other UBL-containing proteins (e.g. UBQLN2 UBA domain binds hHR23a UBL domain [46]). However, it is not entirely clear what types of inter-domain interactions contribute to the oligomerization of the UBQLNs and what types counteract it. An increase in oligomerization of UBQLN2 was observed when the UBA domain was deleted [104], suggesting that the UBA-involving interactions may actually down-regulate oligomerization instead.

UBQLN2 oligomerization is linked to liquid–liquid phase separation

We recently showed that UBQLN2's oligomerization propensity is linked to its liquid–liquid phase separation (LLPS) behavior *in vitro* and its recruitment to biomolecular condensates in cells [13]. UBQLN2 self-assembles into liquid-like, stress-induced condensates [13,29,39]. In addition, endogenous UBQLN2 colocalizes with stress granules. These observations are fully consistent with UBQLN2 undergoing LLPS in cells.

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Oligomerization drives LLPS of many other protein systems, including heterochromatin protein 1 (HP1a), nucleophosmin (NPM1), and nuclear speckle-associating protein SPOP, among others (Box 1) [106–108].

In the test tube, UBQLN2 undergoes a phase transition into forming dynamic, round, protein-dense puncta, as temperature is increased to near physiological conditions [13]. This phase behavior is called LCST (lower critical solution temperature), as there is a temperature below which the protein solution does not phase separate. LLPS of UBQLN2 relies on the oligomerization-driving STI1-II region as well as the UBA domain. Removal of STI1-II abrogated LLPS for all conditions tested, and deletion of the UBA domain significantly increased the protein concentration necessary for UBQLN2 LLPS. However, the UBL domain, STI1-I and PXX regions all affect UBQLN2 LLPS. To better understand the molecular basis of UBQLN2 LLPS, we adopted the 'stickers and spacers' framework used to describe associative polymers. 'Stickers' are residues involved in the multivalent interactions that provide the driving forces for LLPS, whereas 'spacers' are the regions between stickers and do not significantly drive LLPS [82,100]. We classified segments of UBQLN2 residues as either stickers or spacers according to concentration-dependent NMR data on a C-terminal construct consisting of residues 450–624 [100]. To test these classifications, we selected three 'sticker' positions (497, 506, 564) and two 'spacer' positions (525, 538). Consistent with our expectations, mutations in the stickers, but not spacers, substantially alter UBQLN2 oligomerization and phase separation [100]. ALS-linked disease mutations in the PXX domain also affected UBQLN2's LLPS profile by decreasing the protein concentration and temperature required for phase separation, while also promoting a liquid-to-solid transition that sees UBQLN2 droplets solidifying into aggregate-like structures *in vitro* [11,100].

Interestingly, the PXX domain, which is unique to UBQLN2, is not essential for LLPS [13]. UBQLN2 retains LLPS behavior when the PXX domain is removed, although the LCST phase transition is not as sharp as for full-length UBQLN2 [13]. As none of the other UBQLNs contain the PXX segment, we speculate that UBQLN1 and UBQLN4 also undergo LLPS. Using the phase separation predictor from the Forman-Kay laboratory [109], all three UBQLN paralogs scored close to the phase separation threshold, with UBQLN4 having the highest overall PScore (Figure 1B). Interestingly, residues in all linker regions and the UBQLN2 PXX domain exhibit the highest phase separation propensity. Further evidence that supports LLPS of UBQLNs is that UBQLN1 and UBQLN4 localize in intracellular puncta, as we will discuss below [14,17,42].

Physiological functions of UBQLNs

UBQLNs are truly versatile, multifaceted proteins that carry out diverse cellular functions (Figure 5A). UBQLNs participate in multiple PQC pathways, including the ubiquitin–proteasome system (UPS), autophagy, and endoplasmic-reticulum-associated protein degradation (ERAD). Additionally, UBQLNs exhibit molecular chaperone functions to prevent specific protein substrates from misfolding. UBQLNs also participate in DNA/RNA metabolism, cell differentiation/development and DNA damage response. We examine the role of UBQLNs in these pathways below and summarize how different UBQLN paralogs mediate these functions in Table 1.

UBQLNs target proteins for degradation via UPS

The ubiquitin–proteasome system (UPS) is a vital protein degradation mechanism in eukaryotes. Its proper functions rely on many components, including enzymes (E1, E2, E3) responsible for the ubiquitination of substrates, shuttle proteins that direct ubiquitinated substrates to the proteasome, and ultimately the proteolytic activity of the proteasome. Ubiquitination is a post-translational modification that covalently attaches mono- or polyUb chains onto protein substrates at specific positions (often lysines). These Ub markers act as signaling tags for various cellular pathways. K48-linked polyUb is the common tag for proteasomal degradation; K63-linked polyUb, on the other hand, signals for DNA repair, trafficking, and autophagy [110]. However, these signals are not exclusive, as evidence exists that K48-linked chains can associate with autophagy and K63-linked chains with UPS [111,112].

UBQLN1, 2 and 4 bind proteasomal subunits and ubiquitinated proteins via their UBL and UBA domains, respectively (Figures 4A and 5B). Therefore, UBQLNs are characterized as shuttle proteins that transport ubiquitinated substrates to the proteasome. Consistent with their shuttle functionality, UBQLNs transiently associate with the proteasome, primarily via Rpn10 and Rpn13 receptors [48,113]. Indeed, two disease-linked mutations, UBQLN1 E54D and UBQLN4 D90A, impair proteasomal degradation (Table 2) [21,22]. The positions of these mutations are in or near the UBL domain, which could lead to decreased binding to the proteasome. Curiously, neither E54D nor D90A is near the binding interface between UBL and proteasomal receptors (Rpn10, Rpn13). As described above, the UBA domain binds both monoUb and polyUb chains. Studies of the isolated UBQLN

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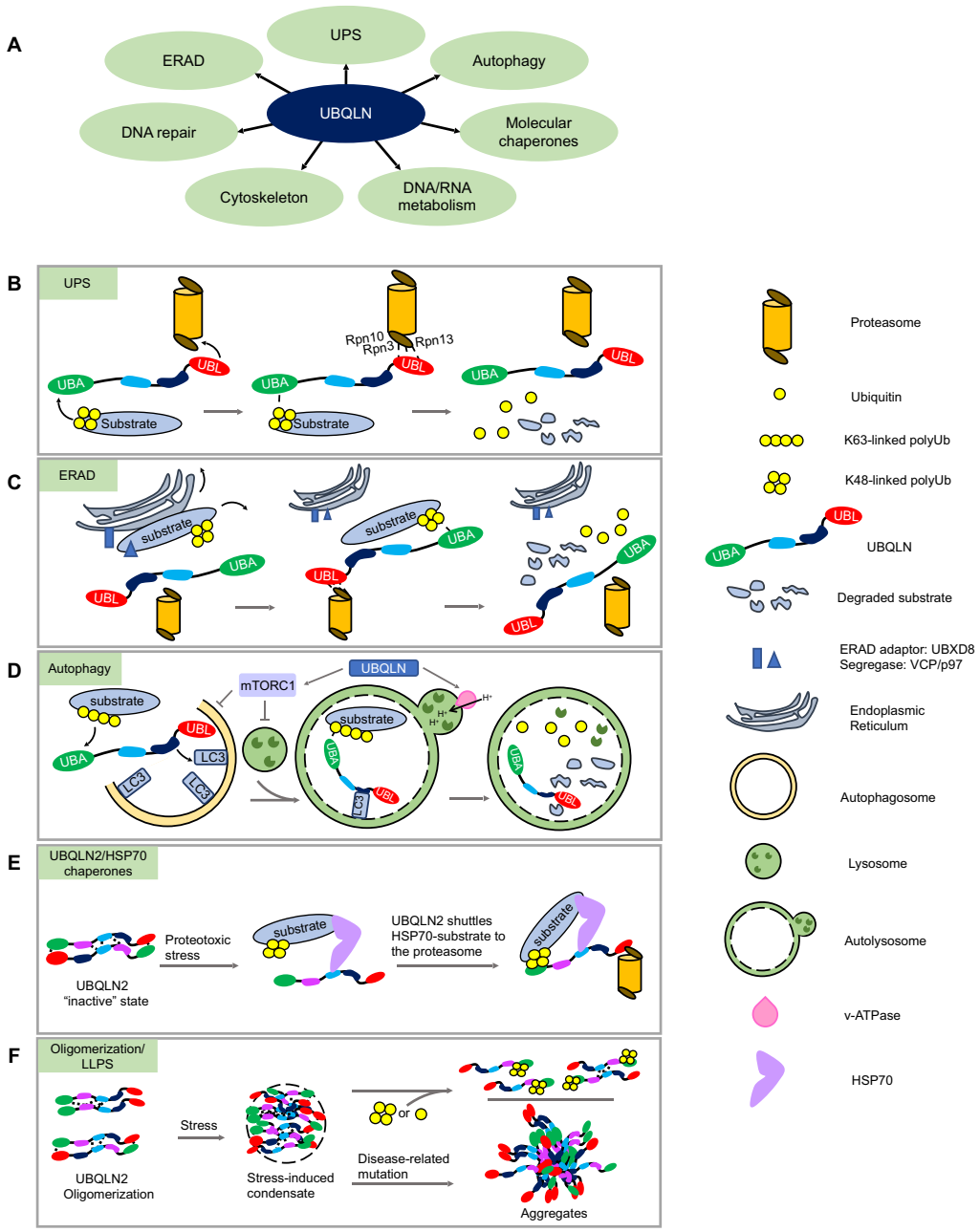

Figure 5. Physiological functions of UBQLNs.

(**A**) Schematic representation of UBQLN physiological functions. (**B**) UBQLNs target proteins for degradation via UPS. UBQLNs shuttle K48-linked polyUb-tagged substrates to the proteasome via UBA-polyUb interactions and UBL-proteasome interactions [7,48,49]. (**C**) UBQLNs form complexes with ERAD adaptors such as VCP/p97 and UBXD8 to facilitate the extraction of ERAD substrates from the ER, then transport the substrate to the proteasome for degradation [8,127]. (**D**) UBQLNs regulate autophagy at several steps. UBQLN interacts with macroautophagy/autolysosome component LC3 and mediates autophagosome maturation [9,65,122]. UBQLN stabilizes V-ATPase, which is essential for lysosome acidification and autophagic degradation [123]. UBQLN also regulates autophagic flux by interacting with mTOR [124]. (**E**) UBQLN2 assists HSP70 chaperone machinery. Under non-stress conditions, UBQLN2 adopts an 'inactive' state. Upon proteotoxic stress, UBQLN2 is activated and binds to substrate-bound HSP70. UBQLN2 then shuttles the complex for degradation [28,65]. (**F**) Tuning of UBQLN oligomerization likely plays a role in PQC pathways. UBQLN2 oligomerizes under physiological conditions and self-assembles into liquid-like condensates upon stress [13]. UBQLN2 condensates are disassembled by ubiquitin or K48-linked polyUb [13]. Introduction of a disease-linked UBQLN mutation may cause the formation of aggregates [29].

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Table 1 Functions of UBQLN proteins

	UBQLN1	UBQLN2	UBQLN4
UPS	Shuttle ubiquitinated substrates to proteasome for degradation [7].		
Autophagy	Involved in TDP-43 containing aggresome [146,173]. Modulates autophagosome formation and acidification [124]. Regulates autophagic flux through mTOR signaling [124].	Stabilize v-ATPase, promote lysosome acidification and autophagic degradation [123].	Involved in autophagy via UBQLN1 interaction [9].
ERAD	Forms complex with ER-localized VCP/p97 and erasin, involved in ERAD [8].		Assists in ER-to-cytosol escape of nonenveloped virus SV40 [130]. Recognizes mislocalized transmembrane domain proteins and targets them for proteasomal degradation [67].
Molecular chaperone	Interacts with molecular chaperone HSP70 [28,66]. Chaperones APP by binding and preventing its aggregation [10]. Chaperones mitochondrial membrane proteins [135].		
Cytoskeleton and cell development	UBQLN1 and 2 interact with IAP, and mediate the interaction of vimentin-containing intermediate filaments with plasma membrane [37]. Implicated in postsynaptic growth by maintaining Ub level through interactions with Leon [142].		Regulates motor axon morphogenesis [21].
Aggregate/ aggresome related		Promotes Huntingtin inclusion body formation and degradation through autophagy [120]. Is recruited and sequestered by polyQ expanded protein aggregates [153]. Promotes TDP-43 and p62 aggregation [39]. Modulates TDP-43 level and triggers mislocalization of endogenous TDP-43 from nucleus to cytoplasm [149].	
Other functions	Implicated in endocytosis through interactions with Eps15 [42].	Drives NF-κB (transcription factor, implicated in inflammation) activity [39].	Balances between DNA damage repair pathways [38].

UBA domain indicate that the UBA domain has no binding preference to any specific polyUb chains [58,114], unlike the UBA domains of hHR23 proteins or p62 [115,116]. However, a recent study on full-length UBQLN1 suggests that it has a binding preference for K63-linked polyUb chains (over K48) [114]. PolyUb chain preference needs to be examined in the context of the full-length UBQLN proteins.

Whereas the UBL domain predominantly interacts with the proteasome, other parts of UBQLN appear to modulate UBL-proteasome interactions. ALS-linked mutations in the PXX region of UBQLN2 (P497H, P497S, P506T, P509S, and P525S) interfere with UBQLN's interaction with the proteasome (Table 2). The mutants interact less effectively with Rpn10 of the regulatory cap, as shown by *in vitro* experiments using GST-fusion

Table 2 Disease mutations of UBQLNs Part 1 of 2

Mutation[2]	Phenotype[3]	Bioinformatic predictions by silico analysis[4]	Structure, function and pathology studies	Sticker or spacer[5][13]	References
S155N[1]	SALS without dementia	Benign by PolyPhen-2 and SIFT			[174]
P189T[1]	SALS without dementia	Damaging protein function by PolyPhen-2 and SIFT			[174]
A282V[1]	Sporadic FTD	Pathological by MutationTaster, PolyPhen, SIFT, and AGVGD			[89]
A283T[1]	SALS	Pathological by MutationTaster, PolyPhen, SIFT, and AGVGD			[89]
S346C[1]	FTD	Neutral by PMUT and SIFT			[175]
M392I[1]	SALS and Madras-type motor neuron disease (MND)				[176]
M392V[1]	SALS	Damaging by Polyphen-2, tolerated by SIFT			[177]
S400G[1]	SALS	Neutral by PMUT and SIFT			[175]
Q425R[1]	SALS	Pathological by MutationTaster, PolyPhen, SIFT, and AGVGD			[89]
P440L[1]	SALS	Pathologic by PMUT, tolerated by SIFT			[175]
M446R[1]	FALS	Pathological by PMUT, non-neutral by SNAP			[20]
T467I[1]	Family history of dementia, without ALS	Tolerated by SIFT		Sticker	[178]
T487I	FALS without dementia		Colocalization with ubiquitin and frequent colocalization with TDP-43 and FUS in postmortem spinal cord tissue.	Sticker	[148]
A488T[1]	SALS	Deleterious by SIFT		Sticker	[88]
P494L	FALS/FTD	Deleterious by SIFT	Expression of P494L impairs autophagy in the lymphoblast and HSP70 binding	Sticker	[88]
P497H	ALS with or without dementia	Pathological by PMUT, non-neutral by SNAP [20]	Expression of P497H alters ubiquitin binding in cells [179], impairs UPS [15,179] and exacerbates TDP-43 pathology in rats [179]. UBQLN2 P497H was hyper-ubiquitinated in cells [179]. It also reduces interaction with FUS, impairs its function in regulating FUS–RNA complex dynamics and stress granule formation [12]. Besides these, P497H impairs the association with UBXD8 and disrupts ERAD [128]. Overexpression drives TDP-43 pathology	Sticker	[12,15,20,30,31,39, 128,157,179,180]

Continued

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Table 2 Disease mutations of UBQLNs Part 2 of 2

Mutation[2]	Phenotype[3]	Bioinformatic predictions by silico analysis[4]	Structure, function and pathology studies	Sticker or spacer[5][13]	References
			in neuronal cells [39,180], causes defective autophagy and ALS-like phenotypes including cognitive deficits and neuronal loss in rats [31,157].		
P497L	ALS with or without dementia		Gliosis exists in corticospinal tracts of carriers' brain.	Sticker	[19]
P497S	FALS with or without dementia	Deleterious by SIFT [88]	Expression of P497S impairs autophagy in the lymphoblast and HSP70 binding [88]. It impedes autophagy by reducing autophagosome acidification in mouse model [122].	Sticker	[15,88,122]
P500S	FALS	Deleterious by SIFT		Spacer	[88]
P506A	FALS and spastic paraplegia (SP)	Deleterious by SIFT	Expression of P506A impairs autophagy in the lymphoblast and HSP70 binding.	Sticker	[88]
P506S	ALS with or without dementia	Pathological by PMUT, neutral by SNAP [20]. Not tolerated by SIFT, benign by PolyPhen [91]	Frequent localization in neuronal cytoplasmic inclusions in the dentate gyrus of postmortem tissue [90].	Sticker	[20,90,91]
P506T	ALS with or without dementia		Expression of P506T increases ubiquitination level [30], reduces HSP70 interaction [28] and impairs UPS [15]. It reduces interaction with FUS, impairs its function in regulating FUS–RNA complex dynamics and stress granule formation [12]. P506T also increases UBQLN2 aggregation propensity [29], alters droplet morphology [29], causes cognitive deficits [28] and increases neuronal death [29] in mice.	Sticker	[12,15,28–30]
P509S	ALS		P509S mutant shows similar ubiquitination level and solubility compared with WT [30].	Spacer	[15,30]
P525S	ALS		P525S mutant shows slightly higher ubiquitination level and solubility compared with WT. Expression of P525S increases neuronal toxicity [30].	Spacer	[15,30]
P533L[1]	FALS	Pathological by PMUT, non-neutral by SNAP		Spacer	[20]
V538L[1]	SALS	Neutral by PMUT and SNAP [20]	NMR analysis shows V538 was in spacer region, which does not affect phase separation dramatically [13]. Turbidity assay of V538L shows it behaves almost the same as WT using UBQLN2 450–624 construct [100].	Spacer	[13,20,100]
UBQLN1 E54D	Brown–Vialetto–Van Laere syndrome (BVVLS)	Not functionally deleterious by PMUT, PolyPhen and SIFT	It forms cytosolic aggregates with TDP-43. Expression of UBQLN1 E54D impairs UPS.		[22]
UBQLN4 D90A	FALS		Expression of UBQLN4 D90A reduces proteasomal efficiency. It also impairs normal motor axon morphogenesis in mouse neurons and zebrafish.		[21]

[1]pathology needs further validation.
[2]Mutations are in UBQLN2 unless otherwise noted.
[3]SALS = sporadic ALS, FALS = familial ALS.
[4]Bioinformatics prediction results are from the corresponding literature references (last column).
[5]Residue designated as 'sticker' or 'spacer' based on LLPS results specified in ref. [13] using the UBQLN2 450–624 construct.

constructs of UBQLN2 [32]. The ALS-linked mutations do not appear to negatively affect UBQLN2's binding to ubiquitinated substrates, nor does the expression of UBQLN2 mutants impact overall proteasome activity. Rather, the mutants are defective in transporting ubiquitinated substrates to the proteasome due to decreased affinity [32].

Ub also affects the ability of UBQLN2 to phase separate and form biomolecular condensates, with potential implications for how LLPS contributes to UPS function [13]. We found that noncovalent Ub binding to the UBA domain of UBQLN2 drives disassembly of UBQLN2 condensates *in vitro* (Figure 5F, top). Importantly, noncovalent interactions between UBQLN2 and K48-linked tetra-Ub, the canonical signal for proteasomal degradation, disassemble UBQLN2 condensates. We speculate that Ub-mediated phase transitions of UBQLN2 also occur in cells, enabling a mechanism where UBQLN2 could shuttle ubiquitinated proteins out of condensates or membraneless organelles.

UBQLNs interface with multiple autophagy components

Autophagy is another major degradative pathway for cellular components. Autophagy complements the UPS in degrading cytosolic proteins, protein aggregates, RNA, and organelles in bulk. Macroautophagy is the most studied autophagy in mammalian cells, and it relies on the formation of autophagosomes, double membrane-bound structures that fuse together with lysosomes [117]. Microautophagy engulfs intracellular proteins and organelles in lysosomes, while chaperone-mediated autophagy (CMA) depends on chaperones like Hsc70 to transport the substrates into lysosome. In all types of autophagy, the acidic environment and the cocktail of hydrolases provided by the lysosomes ensure substrate degradation [118]. Autophagy is an important stress-response mechanism that promotes cell survival, especially during times of starvation [118]. Accordingly, autophagy must remain tightly regulated to maintain homeostasis. Several proteins share the regulatory responsibility, with the mechanistic target of rapamycin (mTOR) signaling playing a key role [119].

Compared with UPS, the molecular mechanisms of UBQLNs in autophagy are less understood. Nonetheless, UBQLNs associate with autophagy components and autophagy substrates (Figures 4A and 5D). UBQLN2 and yeast UBQLN/Dsk2 interact with mutant Huntingtin protein, facilitating its clearance via autophagy [120]. UBQLN2 reduces accumulation of mutant Huntingtin in mouse models of Huntington's disease [121]. UBQLN1, UBQLN2, and UBQLN4 bind to macroautophagy/autolysosome component 1A/1B-light chain 3 (LC3) and facilitate the process in which LC3-I matures to LC3-II [9,65,122]. LC3-II then initiates formation and elongation of the autophagosome [118]. Indeed, an ALS-linked mutant of UBQLN2 (P497H) causes a decreased LC3-II to LC3-I ratio [31]. Interestingly, the mutation also reduces expression levels of several autophagy proteins, including LC3, p62, ATG7 and a lysosome/CMA protein LAMP2a over time, implying additional autophagy mechanisms that involve UBQLN2 [31]. Notably, UBQLN1 itself is a substrate of both macroautophagy and CMA, and the CMA-targeting motif (KFERQ) is present in all of UBQLN1, 2, and 4 [65]. The degradation of UBQLN1 by the two autophagy pathways could pose important regulatory implications. Recently, another mechanism by which UBQLN2 contributes to autophagy was identified. A study on five ALS-linked mutants of UBQLN2 revealed that UBQLN2 interacts with the proton pump V-ATPase to protect it from degradation, and promotes stable V-ATPase formation [123]. Proper V-ATPase function is essential for lysosome acidification and autophagic degradation [122,123]. Loss of UBQLN2 or ALS-linked mutation (P497S) results in reduced autophagosome acidification [122]. In addition, three other UBQLN2 disease-linked mutants (P494L, P497S, and P506A) were found to disturbed autophagic degradation in lymphoblast [88].

Importantly, UBQLN1 interacts with and modulates the activity of the mechanistic target of rapamycin (mTOR) [124], whose kinase activity inhibits autophagosome formation and lysosome biogenesis [119,125]. Thus, UBQLN1 may indirectly regulate autophagic flux [124]. Another mechanism by which UBQLNs may participate in autophagy is through their association with polyUb chains. As mentioned above, UBQLN1 exhibits a binding preference for K63-linked polyUb chains over K48 [58]. As K63-linked polyUb chains are often associated with autophagy, UBQLN1 interactions with these chains could be important for regulating autophagy [126].

UBQLNs associate with ERAD adaptors

Nascent membrane protein folds into their native conformation in ER. Despite the assistance of a variety of cellular factors, a significant fraction of newly synthesized proteins ends up misfolded [1]. The endoplasmic reticulum-associated degradation (ERAD) pathway prevents potential toxicity stemming from accumulation of

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misfolded proteins. ERAD components form complexes to recruit machinery to ubiquitinate the misfolded protein, and then direct it to the cytosolic proteasomes for degradation. UBQLNs participate in ERAD mainly by facilitating the translocation of ERAD substrate from the ER to the proteasome [8]. Specifically, UBQLN1 forms a complex with the segregase, Valosin-containing protein (VCP/p97), and UBX-containing protein UBXD2/erasin (Figure 5C). The complex facilitates the extraction of ERAD substrates from the ER [8]. Additionally, UBQLN2 interacts with another ERAD adapter, UBXD8, that possesses similar domain architecture as erasin [127,128]. ERAD is disturbed when the interaction between UBQLN2 and UBXD8 is impaired by UBQLN2 pathogenic mutation P497H [128]. In addition to ERAD substrates extraction, UBQLN2 is also involved in ER–Golgi trafficking. P497H and another ALS-linked mutation, P506T, have been found to inhibit protein transport from ER to the Golgi in neuronal cells, resulting in disorganized and fragmented Golgi [129].

While most transmembrane proteins are successfully integrated into the ER in the co-translational process, a noteworthy portion of transmembrane proteins frequently fail to reach the ER [1]. UBQLN4 complements ERAD by recognizing the exposed hydrophobic transmembrane domains of those proteins that fail to integrate into the ER and targets these proteins for degradation [67]. The STI1-II region of UBQLN4 is essential for transmembrane domain recognition. Notably, the STI1-II region is also involved in binding a nonenveloped polyomavirus, Simian Virus 40 (SV40). UBQLN4 interacts with the structural coat protein, VP1 of SV40, captures the virus at the ER, and facilitates the penetration of the virus through the ER into the cytosol [130]. It was proposed that the virus developed affinity to UBQLN4, therefore exploiting the ER-associated functions of UBQLN4 to infect cells.

UBQLNs assist chaperone machinery and stabilize client proteins

Molecular chaperones assist and maintain proper folding and prevent aggregation of cellular proteins, playing a critical role in maintaining homeostasis. HSP70 chaperones participate in the folding and assembling of newly synthesized proteins, as well as refolding of misfolded and aggregated proteins [131–133]. UBQLN1 and UBQLN2 interact with HSP70 chaperones, as discussed above [28,66]. As a co-chaperone, UBQLN2 builds a connection between client-bound HSP70 and the proteasome, facilitating the degradation of misfolded and aggregated proteins (Figure 5E) [28]. Disease-linked mutants of UBQLN2 disrupt this interaction [28].

Aside from working with HSP70s, the UBQLNs themselves exhibit molecular chaperone and/or protein stabilization functions. How UBQLNs delineate between degradation and stabilization pathways for specific client proteins remains unclear. UBQLN1 stabilizes amyloid precursor protein (APP) upon exposure to elevated temperature, and prevents the toxic aggregation of proteolytic fragments of APP [10]. Moreover, the UBA directly interacts with presenilin 1 and 2, which form the catalytic core of the APP processing γ-secretase complex [17,134]. As previously mentioned, UBQLN1 also stabilizes client proteins like BCLb, IGF1R and ESYT2 [41,68]. This function, however, should be distinguished from its chaperone activity. Instead, UBQLN1 blocks polyubiquitination of the clients, therefore protecting the clients from proteasomal degradation.

The versatility of UBQLN1 is further highlighted in its relationship with mitochondrial membrane proteins [135]. As a molecular co-chaperone, cytosolic UBQLN1 binds to the transmembrane domains of these proteins and shields them from aggregation during their time in the cytosol. Meanwhile, the UBL domain of UBQLN1 remains bound to its own UBA as long as the substrate remains unmodified. In the event that the mitochondrial insertion fails and the membrane protein lingers in the cytosol, UBQLN1 recruits ubiquitination machinery to modify the substrate, and then guides the mitochondrial protein to the proteasome for degradation [135]. A separate study further confirmed that UBQLN1's role in mitochondrial membrane protein integration is indispensable. Absence of UBQLN1 leads to accumulation of mitochondrial proteins in the cytosol [136].

UBQLNs associate with the cytoskeleton to impact cell differentiation and development

UBQLN1 and 2 were first identified as human proteins linking integrin-associated protein with cytoskeleton 1 and 2 (hPLIC1 and hPLIC2) [37,137]. UBQLN1 and 2 interact with integrin-associated protein (IAP), which regulates the interaction between vimentin-containing intermediate filament and plasma membrane [37]. Overexpression of UBQLN1 alters the organization of vimentin in cells [37]. UBQLN1 and 2 colocalize with the cytoskeleton, which is an interlinking protein filament system essential for the growth, differentiation, function, and shape of cells [37,138]. UBQLN2 distributions vary between undifferentiated and differentiated mammalian cells. In undifferentiated cells, perinuclear fiber localization of UBQLN2 has been observed, contrasting

the extensive cytosolic fiber staining observed in differentiated cells [137]. However, the functional significance of UBQLN-cytoskeleton interactions remain largely unclear. One study showed that UBQLN2 safeguards cytoskeletal integrity by forming a complex with phosphoinositide phosphatase myotubularin 1 (MTM1). The complex recognizes and guides misfolded intermediate filament proteins to the proteasome for degradation [139]. For example, UBQLN2 interacts with vimentin and mediates the degradation of misfolded vimentin [139]. In this manner, UBQLNs use their PQC functionality to protect misfolded proteins from aggregating. Moreover, UBQLN4 has been shown to activate ERK signaling, a crucial pathway for cell differentiation and apoptosis [140].

UBQLNs facilitate neuronal morphogenesis, which is crucial for the functions of neurons [141]. In Drosophila, in association with the deubiquitinase USP5/leon, dUBQLN regulates cellular Ub level during neuronal development, and disruption of this regulatory mechanism causes axon termini to not extend properly [142]. In mammals, UBQLN4 is linked to motor axon morphogenesis. The ALS-associated D90A mutant in UBQLN4 causes abnormal morphology with more neurites and compromised viability [21].

UBQLNs also associate with aggresomes, perinuclear structures that contain ubiquitinated misfolded proteins transported via microtubules [14,143]. The cytoskeleton reorganizes and forms a cage-like, vimentin-containing structure around the aggresome [54]. UBQLN1 transports aggregates to the aggresome at the microtubule-organizing centres (MTOC), and UBQLN1 itself is also found in the aggresome [14]. Taken together, evidence suggests that UBQLNs, through the association with the cytoskeleton, are involved in the formation of aggresomes.

UBQLNs interact with DNA/RNA binding proteins

In response to DNA double strand breaks (DSBs), cells resort to two major DNA damage response pathways: the faster but error-prone nonhomologous end joining (NHEJ), and the slower but more accurate homologous recombination repair (HHR) pathway [144]. The balance between the two is essential for ensuring efficient repair. Upon detection of DNA damage in the nucleus, UBQLN4 is recruited to the damage sites where it undergoes phosphorylation at residue S318. Phosphorylated UBQLN4 mediates the Ub-dependent degradation of double-strand break repair protein MRE11, which is a HHR regulator [38]. Therefore, overexpression of UBQLN4 shifts the NHEJ: HHR balance towards NHEJ. On the other hand, the UBQLN4 loss-of-function mutation that creates a premature stop codon, R326X, leads to an increased HHR-driven response. Thus, both mutation and change in expression levels of UBQLN4 modulate UBQLN4's regulation of DNA damage repair pathways.

UBQLNs also participate in regulating DNA/RNA metabolism, as proteomics studies reveal interactions between UBQLNs and DNA/RNA-binding proteins including hnRNPA1, FUS, and TDP-43 [12,145,146]. Interestingly, the central region of UBQLNs mediate interactions with hnRNPA1, and data suggest that UBQLN2 stabilizes hnRNPA1 [145]. UBQLN2 regulates the formation of FUS–RNA complexes and also promotes their dynamics [12]. UBQLN2 and TDP-43 colocalize into protein inclusions found in brains of ALS patients or transgenic mouse, although the interactions between UBQLNs and TDP-43 remain unclear [15,147–149]. The pathological hallmarks of TDP-43 usually include mislocalization of the protein from nucleus to cytoplasm, formation of toxic C-terminal TDP-43 (residues 218–414), and protein aggregation, among others [150–152]. Mutations in UBQLNs compromise normal function of these RBPs. ALS-linked UBQLN2 mutations P497H, P497S, P506T, P509S, and P525S reduce UBQLN2 binding to hnRNPA1 and altered subcellular distribution of hnRNPA1 protein [145]. In addition, UBQLN2 P497H or P506T mutations impair the interaction between UBQLN2 and FUS, as well as disrupt FUS–RNA complex dynamics [12]. Studies found that expression of WT UBQLN2 [15], UBQLN2 P497H [31], P506T [29], T487I [148] and UBQLN1 E54D [22] result in the mislocalization of TDP-43 and accumulation in the cytoplasm. Much remains to be determined regarding the role of UBQLNs in modulating the function of RBPs. It must be noted that each of these RBPs also undergo LLPS and colocalize with biomolecular condensates in cells. Therefore, UBQLNs may further regulate RBP LLPS, as recently demonstrated for FUS [12].

Is tuning of UBQLN oligomerization at the crossroads of PQC pathways?

Given UBQLNs' versatility to function in multiple protein degradation pathways, what are the signals that direct UBQLNs to these pathways? We hypothesize that manipulation of UBQLN oligomerization may play a role. Abundant cell-based evidence highlights how UBQLNs self-assemble into puncta of different biophysical

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characteristics, i.e. liquid-like vs. solid-like, membraneless vs. membrane-bound. These puncta include stress-induced biomolecular condensates, membrane-bound autophagosomes, aggresomes, and insoluble aggregates/inclusions [9,14,17,39,120,153]. As we discussed above, the STI1 regions mediate both UBQLN self-association and interactions of several binding partners with UBQLNs. Therefore, competition likely exists between client interactions and oligomerization. One hypothesis is that UBQLNs adopt an 'inactive' state, possibly employing head-to-tail UBL-UBA interactions to render it dimeric (Figure 5F). Self-association of UBQLNs through the STI1 regions could prevent unnecessary protein–protein interactions between UBQLNs and their binding partners. Upon activation with a client protein, for example, UBQLNs could interface with HSP70 or other chaperones, potentially changing the UBQLN oligomeric state. Coupled with the knowledge that at least one UBQLN (UBQLN2) can use oligomerization to drive self-assembly into liquid-like biomolecular condensates, oligomerization likely plays a substantial role in regulating UBQLN functionality.

Recently, it was made clear that shuttle protein oligomerization is closely related to the pathway selection between UPS and autophagy, specifically for two Ub-binding shuttle proteins in yeast: Dsk2 (yeast UBQLN homolog) and Cue5 [154]. Unlike UBQLNs, Dsk2 only exists in monomeric or dimeric forms and mainly directs its clients to the proteasome. In contrast, Cue5 oligomerizes extensively and directs clients to autophagy. Using designed UBL-UBA constructs with a middle oligomerization-capable FKBP domain, Lu et al. elegantly illustrated that oligomerization-capable UBL-UBA proteins do not facilitate proteasomal degradation, but rather promote autophagic degradation, and vice versa [154]. In a similar manner, tuning of UBQLN oligomerization may be used to determine UBQLN participation in the two degradation pathways. Oligomerization could be controlled by at least three ways. First, as we discussed above, the oligomerization-driving STI1-II domains of the UBQLN paralogs exhibit different extents of disorder and prion-like characteristics and could determine the propensity for each UBQLN to oligomerize. Second, it has been established that noncovalent interactions between the adaptor protein and its clients could affect the oligomerization propensity of the adaptor, which could apply to UBQLNs as well [104,155]. Third, disease-linked mutations perturb oligomerization and aggregation propensity both *in vitro* and *in vivo* (Table 2) [4,11,29,30]. Mutations on UBQLNs likely act in a position-dependent manner to affect oligomerization.

Is UBQLN aggregation functional?

The involvement of UBQLNs in aggregates could either be physiological or pathological. On the one hand, UBQLNs may associate with aggregates to exercise their physiological functions in promoting degradation via PQC pathways. For example, through their shuttling activities in either the proteasomal or autophagic degradation, the UBQLNs could drive clearance of aggregates [120]. UBQLNs directly interact with non-ubiquitinated parts of client proteins, such as how UBQLN1 UBL binds to the UIMs of EPS15 to promote colocalization into aggresomes [42]. As mentioned above, UBQLN2 is involved with the HSP70 system to promote proteasomal degradation of insoluble ubiquitinated protein aggregates [28]. Aggregates are first solubilized by HSP70–HSP110 disaggregase activity, followed by UBQLN2 binding to ubiquitinated protein and the proteasome. On the other hand, UBQLNs may become trapped in aggregates due to STI1-driven association with the aggregated client protein and/or UBA interactions with the polyUb tag on the aggregated protein. Indeed, UBQLNs are commonly found in Ub-containing inclusions commonly associated with neurodegenerative disorders: UBQLN1 is found in Ub-rich cytoplasmic aggregates upon proteasome inhibition as well as in ALS-associated TDP-43 aggregates [42,146], UBQLN2 associates with ubiquitinated inclusions of ALS patients [15], and UBQLN4 is also found in stress-induced Ub-positive aggregates [67]. Sequestration of UBQLNs in aggregates could result in compromised functions of the UBQLNs, as UBQLNs would not be available to participate in degradation of other proteins. For example, UBQLN2 can be sequestered into polyubiquitinated, mutant huntingtin and ataxin-3 aggregates, which presumably reduces the cellular level of available UBQLN2 and compromises proteostasis [153].

Aside from interacting with aggregated proteins, UBQLNs possess an intrinsic ability to aggregate. This is not surprising given that UBQLNs self-associate. However, UBQLNs likely autoregulate aggregation via their unique UBL-UBA domain architecture. A recent study shows that the UBA domain of UBQLN2 intrinsically aggregates into amyloid-like fibers, while the UBL domain does not [29]. These observations correlate with the number of observed puncta in neuronal cells via transfection experiments using different UBQLN2 deletion constructs. An increased number of UBQLN2 intracellular puncta/aggregates were observed with UBQLN2 ΔUBL, whereas the number of aggregates and puncta were significantly decreased in neurons expressing UBQLN2 ΔUBA [29]. Interestingly, these results are consistent with our lab's UBQLN2 LLPS observations, whereby removal of the UBA domain increased the protein concentration required for LLPS, and removal of

the UBL domain had the opposite effect [13]. The countering effects of the UBL and UBA domains on aggregation propensity suggest that UBQLNs are poised to promote or inhibit aggregation when engaged with binding partners.

Ub binding interferes with UBQLN aggregation given that UBA interacts with Ub. Indeed, studies have suggested that UBQLN2 aggregation, puncta formation and neuronal toxicity are regulated by Ub binding. For example, the Ub-binding-deficient UBQLN2 mutant L619A was more prone to form puncta than wild-type UBQLN2 in neurons and increases neuronal death, suggesting that the L619A mutation increases UBQLN2 aggregation propensity with deleterious consequences [29]. In contrast, in a separate study, the same mutation (UBQLN2 L619A) prevents both WT and mutant UBQLN2 (P506T or P497H) from forming puncta/foci in HEK293 cells [28]. Similarly, the F594A substitution, which also abolishes Ub binding, reduces UBQLN2 P497H aggregation propensity and toxicity in Drosophila neurons [30]. Whether Ub positively or negatively regulates aggregation propensity will likely depend on the client protein, Ub chain types, and other protein–protein interactions involving UBQLNs.

Increasing evidence suggests that disease-linked mutations in UBQLN2 promote aggregation, although there is no clear consensus. In a C-terminal construct of UBQLN2 containing residues 450–624, ALS-linked mutations in the PXX region significantly perturb oligomerization propensity and promote LLPS-induced aggregation [11]. Some of these mutants formed aggregates *in vitro* over time, especially at positions 497 and 506 [11,100]. However, the effects are amino-acid specific, as P497H and P497S variants produce amorphous aggregates whereas P497L droplets exhibit more gel-like consistency. UBQLN2 P506T impairs droplet morphology in transgenic mice by forming irregularly shaped puncta when compared with WT puncta [29]. Interestingly, those same mutants that significantly impacted LLPS cause ALS at an earlier age, and vice versa; this suggests a connection between LLPS behavior and ALS disease pathology [156]. Consistent with these findings, disease-linked mutations P506T or P497H increases UBQLN2 aggregation propensity in cultured cells and in animal models [29,157]. In contrast, introduction of some of these disease-linked mutations (P497H, P506T, P509S, P525S) in recombinant full-length UBQLN2 did not increase aggregation propensity of UBQLN2 [28]. There may be other factors that modulate the effects of UBQLN disease-linked mutations on aggregation propensity in cells. Kim et al. showed that UBQLN2 P497H, but not P506T, P509S, P525S, was hyper-ubiquitinated in HEK 293T cells, and that P497H solubility decreased compared with WT. This indicates that both the type of the substitution and position determine the effect of mutation [30]. It must be noted that the relationship between protein aggregation and cellular toxicity is not clear. In some cases, the propensity of UBQLN2 variants to form aggregates correlates with the risk of neuronal death [29]. In other cases, cellular toxicity does not necessarily correspond with UBQLN aggregation propensity [30].

UBQLN expression levels are linked to disease

Organization of UBQLNs into punctate structures in cells is exquisitely sensitive to UBQLN protein expression level. This is not surprising as increased expression of UBQLNs will promote UBQLN self-assembly. Indeed, numerous studies reveal that overexpression or knockdown of even wild-type UBQLN can exert deleterious effects [29,43]. Overexpression of UBQLN2 leads to impairment of protein degradation by UPS and autophagy [61]. Overexpression of UBQLN2 P497H causes pathological UBQLN2 accumulations, neuronal loss, cognitive and behavioral deficits, as well as impaired autophagy in transgenic mice [157,158]. Other studies confirm that overexpression of UBQLN2 P506T or P497S is associated with early deficits and more severe neurotoxicity in mice [147,158]. Overexpressing UBQLN1 in the eye of Drosophila induced age-dependent retinal degeneration, and the expression of an Alzheimer's Disease-associated splicing variant of UBQLN1, UBQ-8i, exacerbates the degeneration [16,159]. Meanwhile, knockdown of UBQLNs may also cause detrimental effects to cellular functions, such as increased cell death and symptoms associated with Huntington's disease [23]. Knockdown of either wild-type or mutant (P497H) UBQLN2 caused accumulation of ERAD substrate, the null Hong Kong variant of α-1-antitrypsin (NHK) [128]. Moreover, knockdown of UBQLN2 increases turnover and degradation of hnRNPA1, impairing proteostasis and RNA metabolism in cells [145]. Dysregulation of UBQLN expression level is also linked to cancers. UBQLN1 expression positively correlates with patient overall outcome and survival rate in invasive breast carcinoma [27], gastric adenocarcinoma [24], and lung adenocarcinoma [26]. UBQLN2 shows similar prognostic potential in hepatocellular carcinoma (HCC) [160] and osteosarcoma [161]. However, UBQLN4 expression levels are increased in certain neuroblastomas and melanoma [38] and connected to worse outcomes.

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Clearly, UBQLN protein expression levels contribute to UBQLNs' roles in PQC and in the progression of several diseases. Although regulatory mechanisms for UBQLN expression levels remain largely unknown, studies have identified a micro-RNA (miRNA), MIR-155, to be a down-regulator of UBQLN1 and UBQLN2 protein expression [162]. MIR-155 has been studied for its connection to cardiovascular diseases and multiple cancers, as MIR-155 levels are elevated in certain cancer cells [163]. miRNAs regulate protein expression at the post-transcriptional level by targeting the mRNAs and inhibiting their translations [164]. Post-translationally, UBQLNs' ability to enter condensates and aggregates suggests that sequestrations of UBQLNs in these compartments could also directly affect their protein levels in the cells.

Future directions and unanswered questions

In this review, we highlighted current evidence that unambiguously positions UBQLNs at the crossroads of protein quality control pathways. The ability of UBQLNs to either promote the degradation or stabilization of their clients/substrates, and that dysregulation of these proteins cause diseases from ALS to cancers, reveal the importance of UBQLNs in cells. However, as much as we have learned about these proteins over the last twenty years, UBQLNs present many more questions for future studies.

What is the correlation between UBQLN biochemical properties (i.e. oligomerization, LLPS and aggregation) and physiological functions? UBQLN2 oligomerization and LLPS underlie UBQLN2 aggregation propensity. To address this point, identification of the 'stickers' that drive LLPS is essential [100]. Some of these sticker residues reside in the domains employed for proteasome or ubiquitin binding, thus interactions with these proteins likely modulate phase separation and oligomerization. Likewise, introduction of disease-related mutations interferes with protein degradation pathways and affect UBQLN2 aggregation propensity. Future experiments are needed to clarify the relationship among UBQLN oligomerization, LLPS, and aggregation.

How is oligomerization regulated by protein–protein interactions with PQC machinery, chaperones, and client proteins? Interestingly, oligomerization of other chaperones can regulate their activity with clients [165,166]. This applies to the functionality of HSP70 and small HSP chaperones. Does UBQLN oligomerization regulate degradation pathway selection, particularly between UPS and autophagy? There is likely a regulatory system that aids the decision-making process, presenting a very curious topic for future studies. Do UBQLNs use oligomerization to control localization into membraneless organelles or other subcellular compartments? Along the same lines, why do UBQLNs aggregate with some proteins, but prevent aggregation of others?

Do post-translational modifications (PTMs) of UBQLNs further modulate their physiological functions in cells? Despite the relative lack of information regarding PTMs on the UBQLNs, studies have found evidence for phosphorylation on UBQLNs [36,38], and ubiquitination on mutant UBQLN2 [30]. While there is little known about the modification on other UBQLNs, ubiquitination of UBQLN2 likely has functional significance and a huge influence on its biophysical properties. On the other hand, phosphorylation may affect protein–protein interactions of the UBQLNs as well as self-interactions. We found that amino acid substitutions that mimic phosphorylation substantially alter UBQLN2 LLPS, regardless of position where the substitution was installed [100]. Moreover, phosphorylation has been shown to change the LLPS profile of other proteins that undergo LLPS such as FUS [167]. Therefore, it is likely that phosphorylation on the UBQLNs also changes its LLPS behavior, which could then tune the UBQLN-associated membraneless organelles inside the cell. Notably, many disease-linked mutations in the PXX region of UBQLN2 are to residues that can be phosphorylated (e.g. His, Ser, Thr) (Table 2). Additionally, we speculate that PTMs could help UBQLNs decide between stabilizing their binding partners or facilitating their degradation by autophagy or the proteasome.

The versatility of UBQLNs in acting across many PQC pathways has offered insights into how the individual pathways (UPS, autophagy, and ERAD) work and also illuminated on the cross-talk and connections among the various components of the complex PQC system in cells. However, the diverse functions of UBQLNs have often made it difficult to pinpoint exactly how disease-linked mutations in UBQLNs cause disease. We emphasize the need for additional biochemical and biophysical studies of UBQLNs to address the effects of mutations on a structural and dynamical level. The multidomain architecture of UBQLNs offers a rich combination of structured and intrinsically disordered domains that interact with each other to promote self-assembly and oligomerization. Regulation of these inter-domain interactions is at the core for how UBQLNs reorganize into intracellular puncta such as biomolecular condensates, autophagosomes, aggresomes, and aggregates. Cells are exquisitely sensitive to the expression levels of UBQLNs, as dysregulation often results in disease. We now need to determine the signaling mechanisms that modulate UBQLN self-assembly into different subcellular structures, and how this translates to UBQLN functionality in cells. We expect that improved knowledge of UBQLN

structure and function will illuminate on disease mechanisms associated with UBQLNs in neurodegenerative diseases and cancers.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; CMA, chaperone-mediated autophagy; ELPs, elastin-like polypeptides; EPS15, epidermal growth factor substrate 15; ERAD, endoplasmic-reticulum-associated protein degradation; FTD, frontotemporal dementia; HD, Huntington's disease; HHR, homologous recombination repair; IAP, integrin-associated protein; IGF1R, Insulin-like growth factor-1 receptor; LLPS, Liquid–liquid phase separation; NHEJ, nonhomologous end joining; PQC, protein quality control; PTMs, post-translational modifications; RBPs, RNA-binding proteins; SV40, Simian Virus 40; UBA, Ub-associated; UBL, Ubiquitin (Ub)-like; UBQLNs, Ubiquilins; UIMs, ubiquitin-interacting motifs; UPS, ubiquitin–proteasome system.

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