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MOLECULAR DETERMINANTS OF UBQLN2 LIQUID-LIQUID PHASE SEPARATION MODULATED BY UBIQUITIN

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MOLECULAR DETERMINANTS OF UBQLN2 LIQUID-LIQUID PHASE SEPARATION MODULATED BY UBIQUITIN

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Abstract

Liquid-liquid phase separation (LLPS), the mechanism by which macromolecules demix to form a dense liquid- like phase in equilibrium with a phase dilute of molecules, is hypothesized to be the dominant mechanism governing membraneless organelle formation inside the cell. Stress granules, a type of membraneless organelle that assemble reversibly in response to cellular stress, may be formed through LLPS. Dysregulation of this process may lead to disease aggregates *in vivo.* LLPS is thought to be governed by the occurrence of weak, multivalent interactions between molecules such as proteins and RNA molecules. Multivalent interactions, the proposed driving force governing LLPS, are weak, dynamic forces between multiple sites on different proteins, giving rise to liquid-like properties. Here, we use human UBQLN2 as a model system for studying molecular drivers of LLPS *in vitro*. UBQLN2 contains a domain that binds to ubiquitin, which marks proteins for degradation by the proteasome. Here, we show that ubiquitin interactions with UBQLN2 are altered by UBQLN2 amino acid substitutions.

Executive Summary

UBQLN2 is a human protein involved in protein quality control mechanisms and interacts with the protein ubiquitin, which tags proteins for degradation by the proteasome. UBQLN2 shuttles ubiquitin, along with tagged proteins, to the proteasome. UBQLN2 may also be involved in regulating misfolded proteins (Hjerpe et al., 2016). UBQLN2 is relevant to disease formation as the domain called, Pxx, a region rich in the amino acid proline, which contains many missense mutations related to familial ALS (Deng et al., 2012). Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease which is marked by progressive and rapid deterioration of the spinal cord and brain. A hallmark of ALS is the development of pathological inclusions, aggregates of deposited cellular components such as proteins, inside degenerating motor neuron cells (Taylor, Brown, & Cleveland, 2016). Under stress many eukaryotic cells form what is termed stress granules, a type of membraneless organelle. Stress granules and other membraneless organelles may form by the process of liquid-liquid phase separation.

Liquid-liquid phase separation (LLPS) is the spontaneous de-mixing of a homogenous mixture. Similar to the separation of oil and water, LLPS occurs *in vitro* and hypothesized to occur *in vivo,* where molecules that are thoroughly mixed separate into droplets of a dense phase, with concentration of those molecules outside of the droplets being dilute. UBQLN2 is known to undergo LLPS and may serve as a model system to study the underlying mechanisms of LLPS. Changes in the amino acid sequence of UBQLN2 may help reveal the molecular determinants of UBQLN2 LLPS behavior.

My project is divided into two parts. First, I am investigating the regions of UBQLN2 that are involved in multivalent interactions by altering the amino acid sequences in those regions. Amino acids in these so-called multivalent interaction sites are important for oligomerization and liquid-liquid phase separation in the UBQLN2 protein system, determined by NMR chemical shift data (Dao et al., 2018). I am then investigating how these amino acid alterations affect the modulation of UBQLN2 LLPS by the human protein ubiquitin. Ubiquitin binding is shown to disrupt UBQLN2 LLPS, suggesting that ubiquitin modulates UBQLN2 phase separation (Dao et al., 2018). Substitutions in amino acids of UBQLN2 may affect ubiquitin interaction with UBQLN2, elucidating the role of particular amino acids in UBQLN2's binding affinity for ubiquitin.

In order to obtain these particular constructs of UBQLN2 450C, the protein is expressed and purified in *E. coli* cells through a salting out purification method. The human protein ubiquitin is purified by acid precipitation and buffer exchange. This work then utilizes assays from a UV/Vis Spectrophotometer to analyze the absorbance of *in vitro* samples as a proxy for LLPS of UBQLN2 450C. These results are the basis of all the data that are collected and analyzed. We hypothesize that amino acid substitutions in the UBA domain modulate UBQLN2 LLPS. Furthermore, we hypothesize that binding affinity of particular UBQLN2 450C mutants for ubiquitin is reduced, and this affects how ubiquitin modulates UBQLN2's LLPS behavior.

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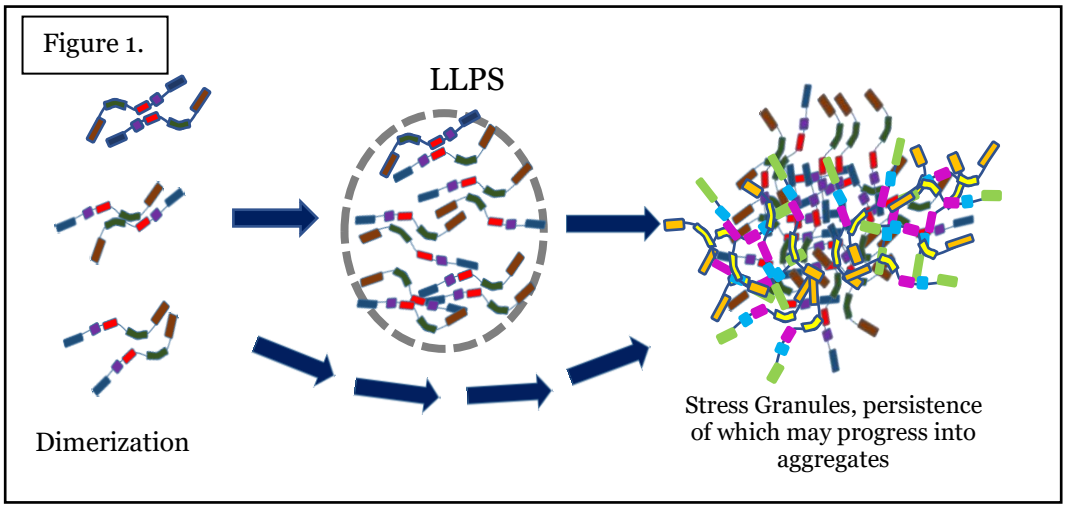
Advice to Future Students

Enjoy the process! Creating a thesis takes a lot of time, effort, and learning, but if you enjoy the time you put into it, it will be so worth it, and the work will be fun.

Introduction

UBQLN2, expressed in most human tissues and especially neurons, is a shuttle protein involved in protein quality control mechanisms in the cell. These quality control mechanisms include proteasomal degradation, binding to ubiquitinated substrates, autophagy, and response to stress (Kleijnen et al., 2000; Mah, Perry, Smith, & Monteiro, 2000; N'Diaye et al., 2009; Rothenberg et al., 2010; Yun Lee & Brown, 2012; Yun Lee, Arnott, & Brown, 2013). There are four isoforms of UBQLNs, including UBQLN2, ubiquitously expressed in humans, found in the cytoplasm and nucleus (Mah et al., 2000). Our lab recently showed that UBQLN2 is found in



stress granules in the cell under physiologically relevant stress conditions, including oxidative stress, heat shock, translation inhibitor stress, and osmotic stress, and experiences liquid-liquid phase separation (LLPS) *in vitro*, which is depicted in Figure 1 (Dao et al. 2018). UBQLN2 has also been shown to exist in disease-linked inclusions in cells such as Lewy bodies in Parkinson's disease, glial cytoplasmic inclusions in multiple system atrophy, and neuronal nuclear inclusions in polyglutamine disease and intranuclear inclusion body disease (Mori et al., 2012).

LLPS may govern the formation of stress granules, which is depicted in Figure 1 on the right. The persistence of stress and stress granules may lead to aggregate formation inside the cell, linking the study of LLPS to the study of neurodegenerative diseases mechanisms such as

those behind the disease amyotrophic lateral sclerosis (ALS) (Taylor et al., 2016). A hallmark of ALS pathology is the presence of proteinaceous inclusions, whose formation may be related to the persistence of stress granules and are an important topic for investigation (Taylor et al., 2016). Stress granules are a type of membraneless organelle. Membraneless organelles are dynamic compartments inside the cell that are unlike other organelles such as mitochondria which are surrounded by lipid membranes. Membraneless organelles have properties somewhere between solids, gels, and liquids and have been referred to as biomolecular condensates (Banani, Lee, Hyman, & Rosen, 2017). Biomolecular condensates have been known about for decades, however their importance for regulation and control of reactions within the cell via concentrating reaction components together or in isolation to increase or decrease reaction rates has only recently been elucidated in the field (Brangwynne et al., 2009). The need for physical models for understanding biomolecular condensate or membraneless organelle formation has opened the door for using theories from polymer chemistry and soft matter physics (Banani, Lee, Hyman, & Rosen 2017). The process of LLPS driven by molecular interactions may be the important organizing principle for assembly and disassembly of membraneless organelles.

LLPS occurs when a homogenous mixture of molecules separates into two distinct phases: a dense phase, containing high concentrations of molecules which may appear as "droplets" and a dilute phase, containing a very low concentration of molecules. The droplets in the dense phase act like liquid including properties such as maintaining the ability to wet surfaces, the ability to undergo fusion and fission, and dripping when exposed to stress (Alberti & Carra, 2018). The molecules that are inside of these droplets, formed by LLPS, include specific proteins such as RNA-binding proteins and non-RNA binding proteins and additionally

RNA molecules. The specific composition and detailed mechanistic drivers of the droplet formation is largely unknown and is a topic of investigation.

The molecular interactions of these molecules participating in LLPS include "multivalent interactions," which are very weak and dynamic interactions, forming and breaking between many molecules on multiple sites on multiple molecules, especially proteins. The multivalent regions have been coined "sticker" regions on some accounts and are separated from each other by "spacer" regions (Lin, Forman-Kay, & Chan, 2018). Studying the molecular grammar behind certain proteins' LLPS behavior, in particular multivalent interactions, may elucidate the effects of particular amino acids or the composition of amino acids on the formation of membraneless organelles such as stress granules. The molecular grammar behind the LLPS behavior of RNA-binding protein FUS were investigated and recently characterized to show that FUS LLPS is governed by its specific amino acid composition (Wang et al., 2018). Here, we use human protein UBQLN2 as a model system to study the particular molecular and amino acid determinants of its specific LLPS behavior. UBQLN2 is particularly relevant to study LLPS as it is a part of the protein quality control system, which may be especially burdened during neurodegenerative disease formation (Chung, Lee, & Lee, 2018).

Two domains of UBQLN2, depicted in Figure 2, are important for the protein's ability to

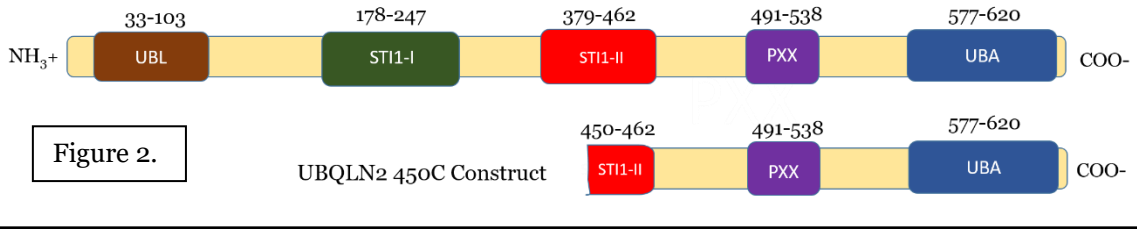


Figure 2.

UBQLN2 450C Construct

shuttle ubiquitin tagged proteins to the proteasome, specifically the UBL and UBA domains. The UBL domain is so named because of its ubiquitin like appearance and it non-covalently interacts

with cellular proteasomes after shuttling ubiquitin tagged proteins. The UBA domain, or the ubiquitin associating domain, non-covalently interacts with ubiquitin during this protein shuttling. UBQLN2 also contains domains within residues 100 to 570 (Figure 2), called ST1I-I, ST1I-II, and Pxx, which were mostly uncharacterized until recent studies completed by Dao et al. 2018. The ST1I-II and Pxx domains are shown to modulate LLPS, while the UBA domain is shown to modulate LLPS as well (Dao et al. 2018). The UBQLN2 450C construct (residues 450 – 624), used in this study here, has similar phase separation behavior as full length (FL) UBQLN2. In NMR studies, the 450-624 construct and the FL construct had many peaks that were superimposed, which indicates chemical and physical environments that are similar for many residues and similar enough to compare results between the shortened construct and FL (Dao et al. 2018).

Multivalent interaction sites exist among residues 505-508 of the Pxx domain, residues 559-571, and residues 592-594 and 616-619 of the UBA domain, observed by NMR studies of UBQLN2 450-624 where peaks were broadened beyond detection, measured by large chemical shift perturbations (CSPs), as concentration increased (Dao et al. 2018). These results strongly indicated that oligomerization mediated by these residues promotes UBQLN2 LLPS. Residues 554-571, 592-594, and 616-619 are located in a region of low-complexity, a term defining a region of a protein with a statistically low number of amino acids. This region contains amino acids that are mostly either hydrophobic, such as A, L, P, V, and I, or polar, such as N and Q. We hypothesize that particular amino acid sequences of these regions define multivalent interactions in UBQLN2 and therefore UBQLN2's phase separation behavior.

These particular results suggest the importance of multivalent interactions and hydrophobicity of amino acids to UBQLN2 LLPS. This has enabled me to predict that UBQLN2

LLPS is decreased by loss of one of the regions that participates in multivalent interactions and that UBQLN2 LLPS would decrease by the reduction of amino acid hydrophobicity at residues 594 and 619. An example amino acid substitution would be a replacement of a leucine with an alanine. This example is one that reduces the hydrophobicity of the UBQLN2 protein based on the Kyte and Doolittle hydropathy scale, defined by the hydrophilic and hydrophobic properties of each of the 20 amino acids (Kyte & Doolittle, 1982). Many others have also examined the 20 amino acids structures and hydrophilic and hydrophobic properties to devise an order of their own (Table 1).

The regions of the protein UBQLN2 containing residues 594 and 619 are both located the interaction site of UBA, which is involved with UBQLN2 shuttling of proteins to the proteasome. Dao et al. recently showed that UBQLN2 LLPS behavior is disrupted when ubiquitin is added to UBQLN2 solutions. These results allow us to predict that amino acid substitutions at these UBA residues may disrupt ubiquitin's ability to modulate UBQLN2's LLPS behavior.

Materials and Methods:

All procedures were completed by direction, with minor adjustments, of protocols from the Castañeda Laboratory, outlined elsewhere (Dao et al. 2018).

Bacterial Culture: The UBQLN2 human gene used for expression was obtained as a gift from Dr. Peter Howley's laboratory in Addgene plasmid 8661. UBQLN2 was moved into the NEBuilder HiFi DNA Assembly Master Mix plasmid called pET24b Novagen (excluding the His tag marker). UBQLN2 450C Δ552-570 (deletion), F594V, F594A, L619A, and L619A F594V mutation constructs were created from the Thermo Scientific Phusion Site-Directed Mutagenesis Kit with the addition of an extra Tryptophan codon at the end of the sequence. *Escherichia coli* Rosetta2 DE3 pLysS cells were used to express all UBQLN2 constructs in LB broth at 37˚Celsius. Large amounts of cells were collected by growing (in the presence of Kan and CAM antibiotics) first a small starter culture (between 10 mL and 20 mL), then transferring to a larger (1 Liter) culture flask, inducing with IPTG, and harvesting at about 12 to 16 hours (all at 37˚C). After harvesting, cells were centrifuged, frozen and lysed, and soluble supernatant was collected after centrifugation at 20,000G and 4˚C for 20 minutes.

UBQLN2 450C Expression and Purification: After cells containing human UBQLN2 450C gene in LB broth were grown and harvested, protein was purified. UBQLN2 450C was used in place of full length because of similar characteristics (see introduction). Bacteria for all constructs were lysed in 50 mM TRIS, 1 mM EDTA pH = 8 buffer, 1 mM PMSF, 1 mM MgCl2, and 0.2 mg/mL DNase. All protein constructs (including mutation constructs) demonstrated some LLPS behavior and were purified by "salting out," by which UBQLN2 precipitated out of solution at the addition of NaCl. Some constructs including 450-624 Δ552-570, 450-624 L619A,

and 450-624 F594A exhibited lower LLPS behavior and required more salt than the WT construct to be purified. Supernatant solution was made to a concentration of 0.5 M – 1 M NaCl during salting out. UBQLN2 droplets (to the visible eye, solution became cloudy) were pelleted by centrifuge and resuspended in 20 mM Na Phosphate 0.5 mM EDTA pH = 6.8 buffer. Another salting out step was performed to remove any leftover contaminants. Leftover NaCl was removed from the protein using the GE HiTrap Desalting column. Concentration of UBQLN2 was determined using a Nanodrop Spectrophotometer. The C-terminal Tryptophan codon enabled protein concentration determination. Purified proteins were checked with SDS-PAGE gel and mass spectrometry and were frozen at -80˚C. Human WT Ubiquitin was expressed in cells and purified as elaborated here (Beal, Deveraux, Xia, Rechsteiner, & Pickart, 1996; Castañeda et al., 2016).

Ubiquitin Expression and Purification: After *Escherichia coli* NiCo21 PET 24b Ub cells containing human 76 amino acid long ubiquitin gene (from plasmid vector PET 24b) in LB broth with Ampicillin or Kanamycin antibiotics were grown and harvested, protein was purified. Bacteria for all constructs were pelleted by centrifugation, then frozen, and lysed in 50 mM TRIS, 1 mM EDTA, pH =8, 1 mM PMSF, 50 µL, 0.4 mg/mL Lysozyme, 1.6 mM $MgCl_2$, and 0.2 mg/mL DNase buffer. Bacteria were frozen, lysed, and pelleted once more, removing protein from supernatant to perform perchloric acid precipitation (pH=1) of ubiquitin on ice. Precipitate was collected and pelleted, with supernatant undergoing dialysis in 50 mM NH_4Ac pH 4.5 by acetic acid buffer overnight. FPLC was conducted using two buffers, 50 mM NH_4Ac pH 4.5 and 50 mM NH_4Ac, 1 M NaCl, pH 4.5, called A and B respectively, in order to perform cation exchange using Hitrap HP sp 5 mL column. Column was washed with 5CV of 100% A buffer, then protein was eluted via increasing percent of B buffer. Buffer exchange was performed to get

protein into 20 mM Na Phosphate, 0.5 mM EDTA, pH = 6.8 buffer. Protein identification was checked by mass spectrometry and SDS-PAGE gel. Protein was stored at -80C.

UV/Vis Spectrophotometric Absorbance Assays: UBQLN2 samples were prepared to achieve 50 μM concentration of protein, 200 mM concentration of NaCl, and 20 mM Na Phosphate buffer with 0.5 mM EDTA pH 6.8, and 0.02% Sodium Azide. Buffer served as a blank and was subtracted from the absorbance value. Absorbance, at OD_{600} nm, was measured as a function of temperature, and recorded automatically on a Beckman DU-640 UV/Vis Spectrophotometer, temperature changing every 2 minutes on 1 ˚C intervals, increasing from 20 ˚C to 44 ˚C and then to measure reversibility, brought back down to 20 ˚C. Sample trials and measurements were recorded in duplicates with n = 4, n = 6, n = 8, and n = 10 depending on the construct. Constructs were prepped with varying levels of Ubiquitin, all represented as ratios relative the protein constructs of UBQLN2 450C being measured. Ratios include 1:0.25, 1:1, 1:3, 1:4, 1:5 and 1:6 depending on varying levels of mutant constructs interaction strength with Ubiquitin.

Results

Here, results from the UV/Vis Spectroscopy assays are represented in graphical representations and also through "phase" plots, which are visual displays of liquid-liquid phase separation represented by varying levels of darkness indicating more or less phase separation.

UBQLN2 450C LLPS is Reduced When Regions Participating in Multivalent Interactions are Removed or Substituted

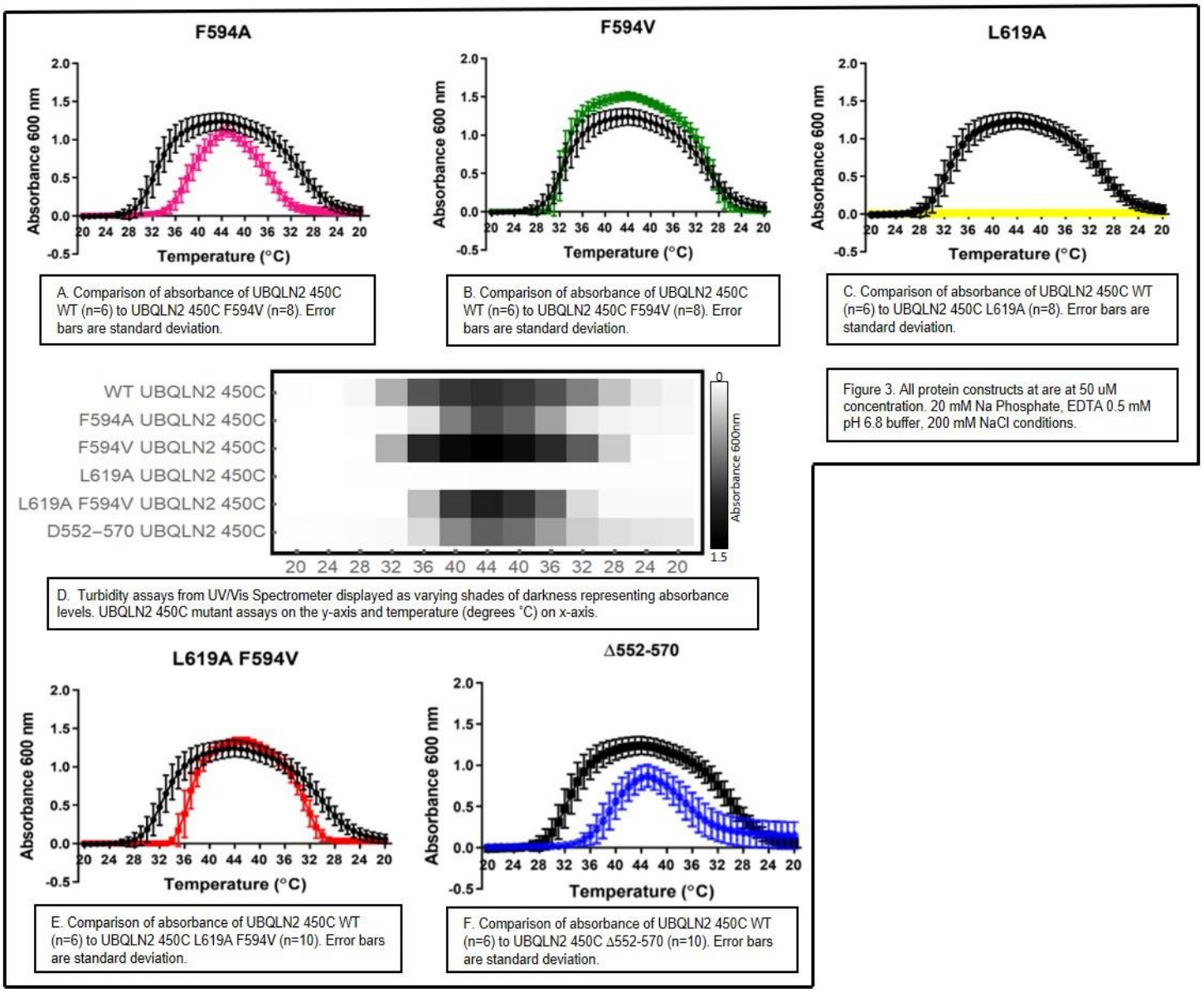


Figure 3. All protein constructs at are at 50 uM concentration. 20 mM Na Phosphate, EDTA 0.5 mM pH 6.8 buffer, 200 mM NaCl conditions.

Our lab recently characterized many interaction sites on UBQLN2 450C that drive UBQLN2 LLPS, including residues 470-487, 505-508, 554-571, 592-594, and 616-620 (Figure 4). These residues were determined to contribute to phase separation behavior of UBQLN2 by analyzing a residue-by-residue NMR map, which measured chemical shift perturbations, meaning the change in microenvironment of different amino acids, representing oligomerization

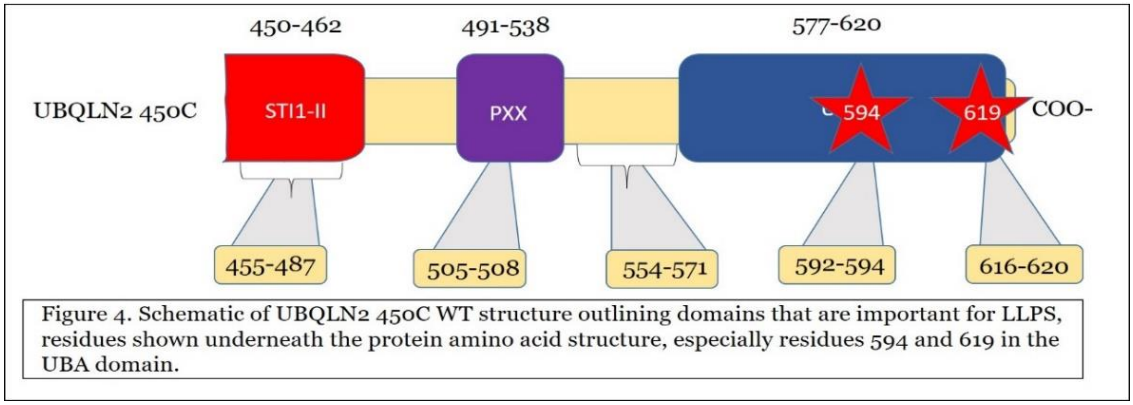


Figure 4. Schematic of UBQLN2 450C WT structure outlining domains that are important for LLPS, residues shown underneath the protein amino acid structure, especially residues 594 and 619 in the UBA domain.

of LLPS behavior of UBQLN2 (Dao et al. 2018). This suggested that these regions mentioned earlier are involved in multivalent interactions between UBQLN2. We investigated the effect of a deletion of one of these domains (residues 552-570) on UBQLN2 450C LLPS. We used UV/Vis Spectrophotometry to measure absorbance as a proxy of for LLPS. We measured the protein solution in 20 mM Na Phosphate, 0.5 mM EDTA, pH 6.8 buffer with 200 mM NaCl. All UBQLN2 450C constructs were at 50 μM concentration. As seen in Figure 3F, the absorbance of UBQLN2 450C Δ552-570 began to increase at a lower temperature than UBQLN2 450C WT and the peak absorbance was significantly lower than the peak of the UBQLN2 450C WT peak. This suggests that the deleted sequence of amino acids residues, 552-570, participates in multivalent interactions, suggesting that reducing the number of sites involved in multivalent interactions in UBQLN2 450C reduces liquid-liquid phase separation.

UBQLN2 450C LLPS is Reduced When Hydrophobicity is Decreased

There is evidence that UBQLN2 LLPS is promoted by hydrophobic and polar interactions. The addition of NaCl increases UBQLN2 LLPS behavior as it is theorized that salt strengthens the interactions between hydrophobic and polar amino acids. Increased electronic strength occurs as a result. Another reason is that UBQLN2 LLPS is driven by lower critical solution temperature (LCST) transitions, meaning LLPS in UBQNL2 is induced by higher temperatures and hydrophobic amino acids (Martin & Mittag, 2018).

We wanted to investigate the effect of different levels of amino acid hydrophobicity at particular regions on UBQLN2 LLPS. To quantify these effects, we choose residues that are located in regions that participate in multivalent interactions and that are also hydrophobic

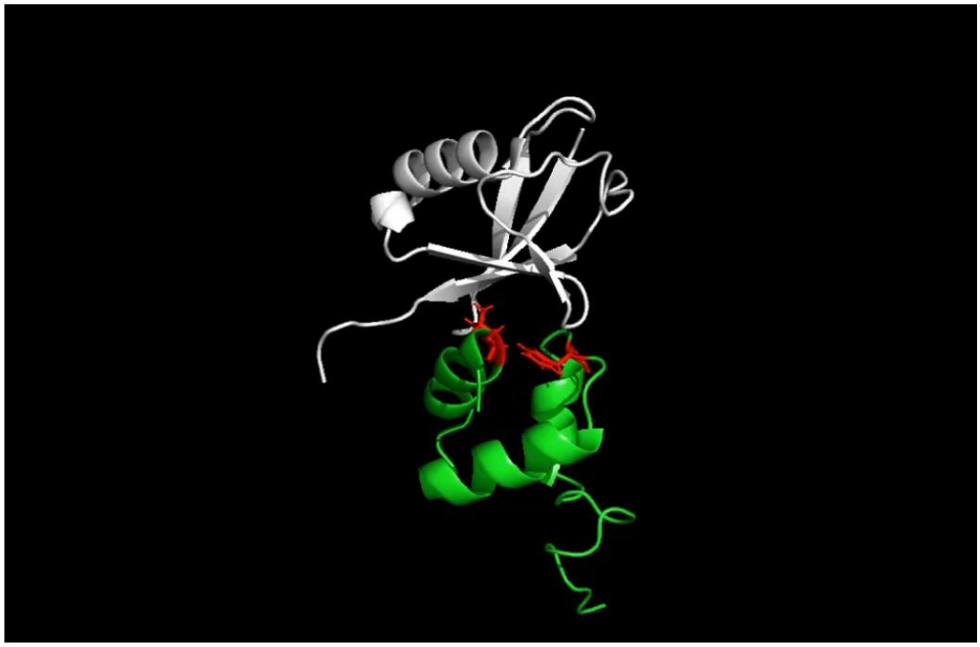


Figure 5. Pymol image of ubiquitin and with UBQLN1 UBA domain interaction highlighting amino acids at 594 and 619

residues (residues F594 and L619). We created four UBQLN2 450C constructs with amino acid substitutions including F594V, F594A, L619A and a double substitution construct L619A

F594V. The amino acid sequence of the UBQLN2 UBA is 99% similar to the UBQLN1 UBA domain and the residues 594 and 619 of UBQLN1 UBA domain is displayed in Figure 5.

Prior structural data reveals that ubiquitin (Ub), which interacts with UBQLN2 via the UBQLN2 UBA domain, binds to the surface of the UBA domain. The residues at this interface

Table 1. Compilation of AA Hydrophobicity. Comparing Previously Published Hydrophobicity Scales for V, L, F, & A. Original values presented with rankings followed.

AA	(Kyte & Doolittle, 1982)	(Hopp & Woods, 1981)	(Eisenberg, D., Schwarz E., Komaromy M., 1984)	(Radzicka & Wolfenden, 1988)
Valine	4.2; 1	-1.5; 3	1.08; 2	4.04; 2
Leucine	3.8; 2	-1.8; 2	1.06; 3	4.92; 1
Phenylalanine	2.8; 3	-2.5; 1	1.19; 1	2.98; 3
Alanine	1.8; 4	-0.5; 4	0.62; 4	1.81; 4

include residues 592-594 and 610-620. These regions of amino acids are also the same residues that exhibit high CSPs values dependent on concentration (Dao et al. 2018). Therefore, the motivation underlying amino acid substitutions at these sites is two-fold. First, the substitutions allow for the investigation of the effect of hydrophobic amino acids on LLPS. Second, we study the effect of these substitutions on how Ub binding modulates UBQLN2 LLPS.

Definition of the order of hydrophobicity of amino acids is not agreed upon in the field, although there have been around 98 studies that have created their own scales of hydrophobicity using varying methods of measurement (Simm, Einloft, Mirus, & Schleiff, 2016). Some of the most referenced hydrophobicity scales in the field are presented for the amino acids V, L, F, and A (Table 1). Based on these past studies, it seems to be the consensus that A is the least hydrophobic of the amino acids (Table 1). However, there is little agreement on the order of the other three amino acids.

The UBQLN2 450C F594V construct is a substitution of Phenylalanine to Valine and may be an increase in hydrophobicity (Kyte & Doolittle, 1982), however some consider it a decrease in hydrophobicity (Hopp & Woods, 1981). As seen in Figure 3B, the absorbance of UBQLN2 450C F594V began to increase at a similar temperature to UBQLN2 450C WT, but the peak absorbance was higher than the peak of the UBQLN2 450C WT. This suggests that the substituted Valine causes an increase in phase separation. Valine is sometimes considered hydrophobic, suggesting that increasing hydrophobicity may increase phase separation. The absorbance of UBQLN2 450C F594A began to increase at a higher temperature than UBQLN2 450C WT and the peak absorbance was lower than the peak of the UBQLN2 450C WT. This suggests that the substituted Alanine decreases UBQLN2 450C, suggesting that decreasing hydrophobicity in UBQLN2 450C at residue 594 may decrease LLPS.

Although the order of hydrophobicity of Leucine compared to all amino acids, is not completely agreed upon, it is the consensus that Leucine is more hydrophobic than Alanine. This suggests that the substituted Alanine decreases hydrophobicity at residue 619. The absorbance of UBQLN2 450C L619A began to increase at a higher temperature than UBQLN2 450C WT and the peak absorbance was lower than the peak of the UBQLN2 450C WT (Figure 3C), suggesting that decreasing hydrophobicity at residue 619 completely eliminates LLPS behavior in UBQLN2 450C.

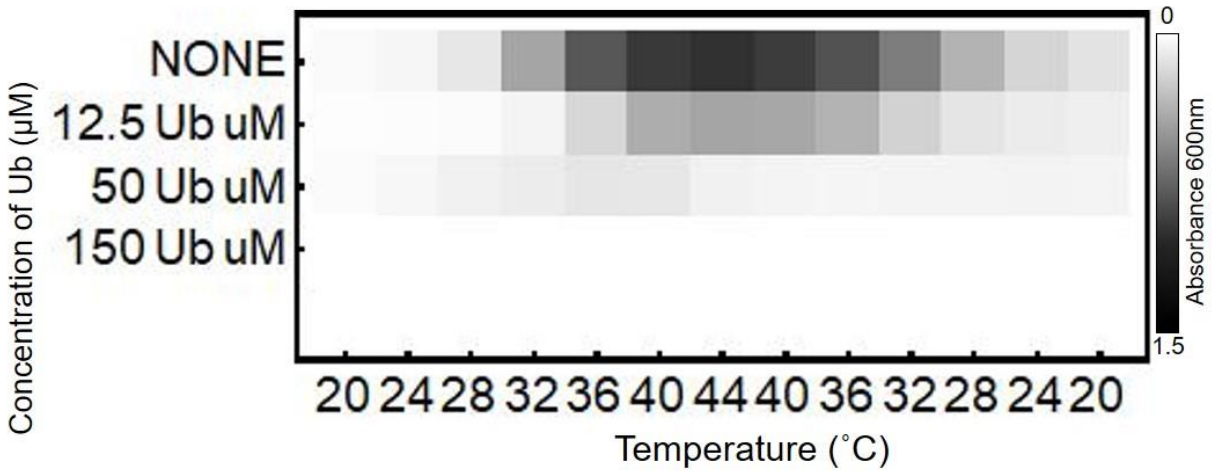
The UBQLN2 450C double substitution construct L619A F594V is a combination of a substitution of Leucine to Alanine at residue 619 and a substitution of Phenylalanine to a Valine at residue 594, and a combination of a reduction in hydrophobicity at residue 619 and an increase in hydrophobicity at residue 594 (Kyte & Doolittle, 1982). As seen in Figure 3E, the absorbance of UBQLN2 450C L619A F594V began to increase at a similar temperature to UBQLN2 450C

WT and the peak absorbance was also similar to the peak of UBQLN2 450C WT absorbance. This suggests that the double substitution of amino acids, which simultaneously increases and decreases hydrophobicity in UBQLN2 450C L619A F594V, is creating a similar chemical environment and level of hydrophobicity at residues 594 and 619 to UBQLN2 450C WT.

UBQLN2 450C WT LLPS is Disrupted and Modulated by Ubiquitin

The UBA domain located near the carboxyl end of UBQLN2 interacts directly with the Ub molecule, then shuttles Ub and its ubiquitinated substrate to the proteasome (Walters et al. 2002). It was previously shown the Ub binding affects UBQLN2 LLPS, through DIC, fluorescent microscopy, and turbidity assays as a function of temperature (Dao et al. 2018). Here, we confirm the same results using the turbidity assay again with increasing ratio of ubiquitin in solution. UBQLN2 450C WT LLPS is eliminated completely as presence of Ubiquitin increases in solution as seen in Figure 6. For all turbidity assays involving ubiquitin addition, we used a UBQLN2 concentration of 50 μM. At stoichiometric ratios of 1:1 Ub:UBQLN2, phase

Figure 6. UBQLN2 450C WT phase separation in the presence of varying concentrations of ubiquitin. UBQLN2 450C WT at 50 μM. 20 mM Na Phosphate, EDTA 0.5 mM pH 6.8 buffer, 200 mM NaCl conditions.



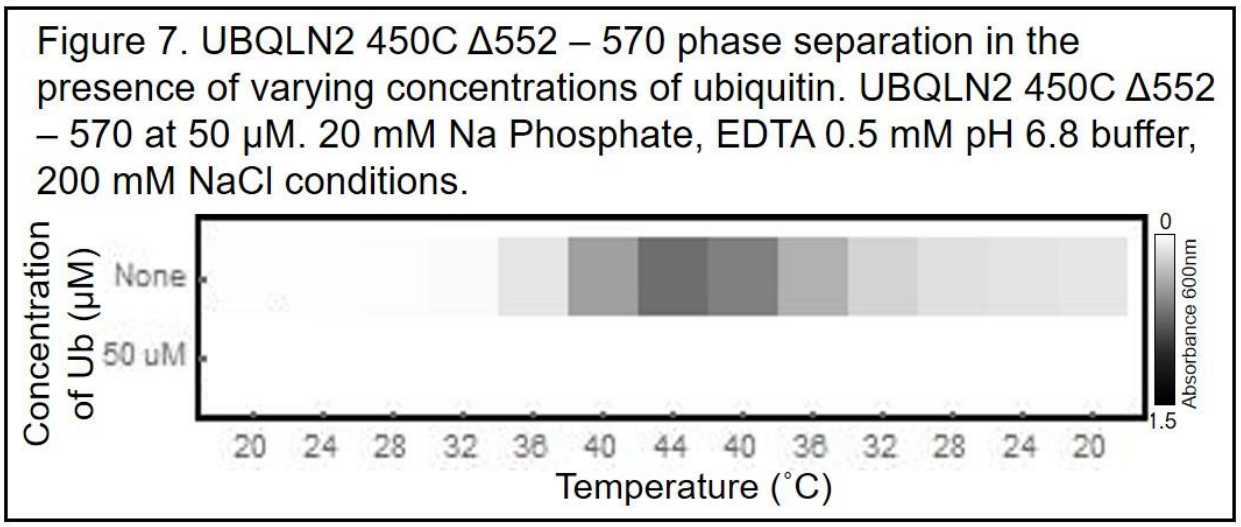
separation of UBQLN2 is markedly decreased. Trials with Ub concentration above 150 μM were not completed because the LLPS behavior was entirely eliminated.

UBQLN2 450C Δ552-570 LLPS is Disrupted by the Presence of 50 μM Ubiquitin

Many interaction sites on UBQLN2 450C that drive LLPS have been recently characterized by our laboratory, including residue regions 470-487, 505-508, 554-571, 592-594, and 616-620. Comparing NMR chemical shift perturbation data, defining UBQLN2 450C residue by residue at both high and low concentrations showed that many amino acids are participating in interactions that increase with the concentration of protein (Dao et a. 2018). This data suggests that these residue regions mentioned may be involved in multivalent interactions between UBQLN2 molecules, as described in the introduction. As seen in Figure 3F, the effect of deletion of one of these domains participating in multivalent interactions (residues 552-570) on UBQLN2 450C LLPS was investigated. The effect was that deletion of residues 552-570 showed a decrease in UBQLN2 LLPS propensity. However, it is important to note that this reduction in multivalency elements in UBQLN2 450C does not fully eliminate the protein's LLPS behavior.

As mentioned earlier, there are specific residue sites that are predicted to participate in UBQLN2 LLPS Ub binding, by NMR titrations of Ub into UBQLN2 450-624, demonstrating that residues 450-580 are not involved in binding to Ub (Dao et al. 2018). The regions of predicted binding of Ub include residues 592-594 and residues 610-620, excluding the region of residues 552-570. We confirmed this data by performing turbidity assays with the 552-570 deletion construct and Ub at 50 μM concentration. Ub presence completing eliminated UBQLN2 450C D552-570 LLPS at these conditions as seen in Figure 7. Elimination of LLPS behavior in

mutant D552-570 is similar to the marked decrease in WT.



F594V, F594A, & L619A F594V Mutations Disrupt Ubiquitin's Ability to Modulate UBQLN2 450C LLPS

To probe the effect of UBQLN2 mutations in the UBA domain (residues 594 and/or 619) on how UBQLN2 LLPS is modulated by Ub, we performed turbidity assays of the UBQLN2 mutants using varying amounts of Ub protein. We examined the effects of four UBQLN2 mutants: F594A, F594V, and L619A, and the double mutant F594V L619A.

We hypothesized that an alteration in the amino acid at the binding interface between UBQLN2 and Ub may affect how Ub modulates UBQLN2 LLPS. In Figure 8B, the Ub presence at a concentration of 50 µM did not have any significant effect on UBQLN2 F594A LLPS behavior suggesting that Ub is not binding to UBQLN2 F594A as well as wild-type UBQLN2. Therefore, Ub is not able to modulate UBQLN2 450C LLPS at this concentration. The mutant F594A does start to have decreased LLPS behavior at around 150 µM Ub, but the decrease in the LLPS behavior is not as marked as the decrease in WT LLPS behavior (Figure 8A). UBQLN2

450C F594A LLPS is still present at 300 μM Ub or six times the amount of Ub compared to the UBQLN2 mutant.

The LLPS behavior of UBQLN2 F594V and L619A F594V double mutant increase in comparison to the WT protein. Here, we investigate the effect of a substitution of any amino acid that is more hydrophobic than alanine. The mutant constructs F594V and L619A F594V have LLPS behavior that is even more persistent in the presence of Ub than the F594A mutant (Figure

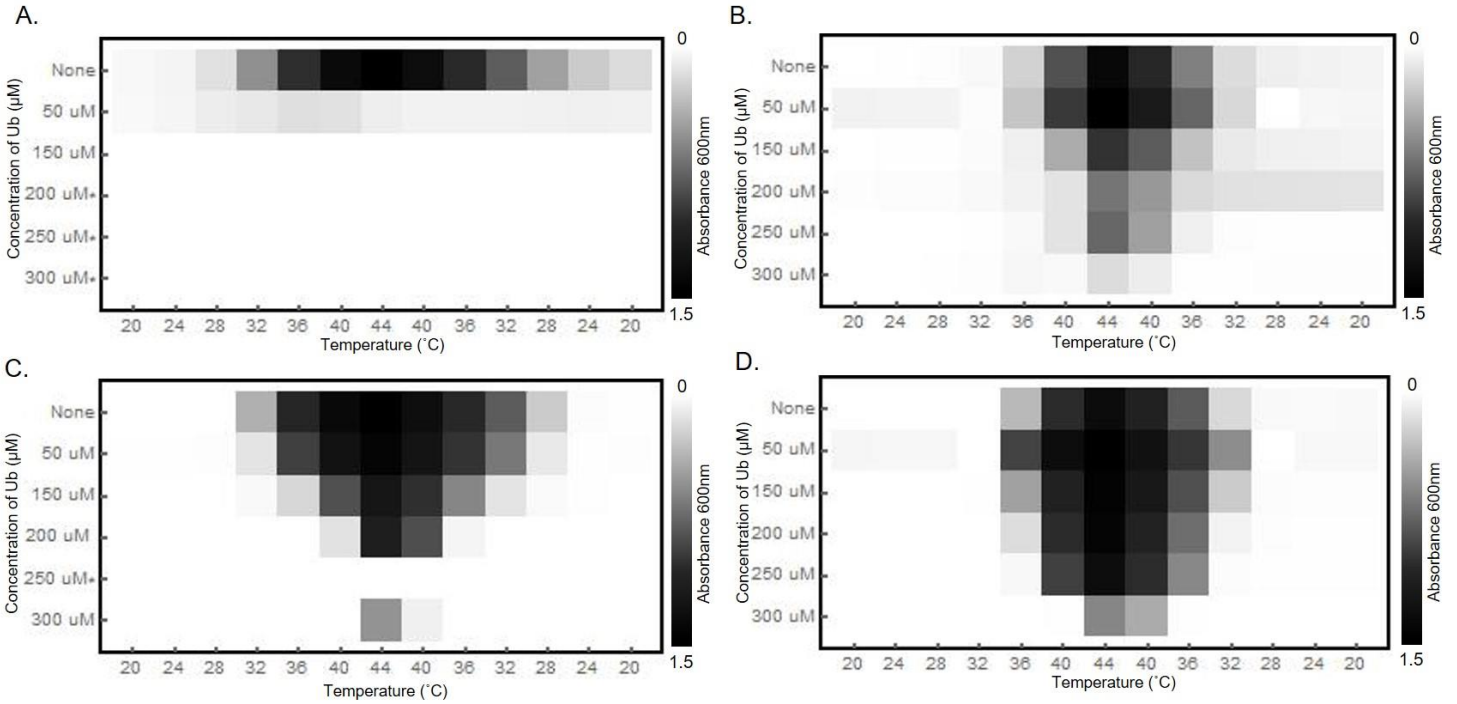


Figure 8. UBQLN2 450C constructs at 50 μM comparison of phase separation in the presence of varying concentrations of ubiquitin. (A) UBQLN2 450C WT. (B) UBQLN2 450C F594A. (C) UBQLN2 450C F594V. (D) UBQLN2 450C L619A F594V. 20 mM Na Phosphate, EDTA 0.5 mM pH 6.8 buffer, 200 mM NaCl conditions. Note * next to concentration of ubiquitin on left y-axis indicates no data for that trial. There is no data for WT after 150 μM ubiquitin because phase separation ceased at 150 μM.

8B, 8C & 8D). Mutants F594V and L619A F594V have very strong LLPS behavior and persist at the highest of Ub concentrations. This means Ub must be in excess of 6x the amount of UBQLN2 mutants F594V and L619A F594V in order for any modulation of LLPS to occur.

In order to visualize my results to compare how different constructs of UBQLN2 450C respond to Ub, I compared the data from all the UBA domain mutants represented in Figure 9.

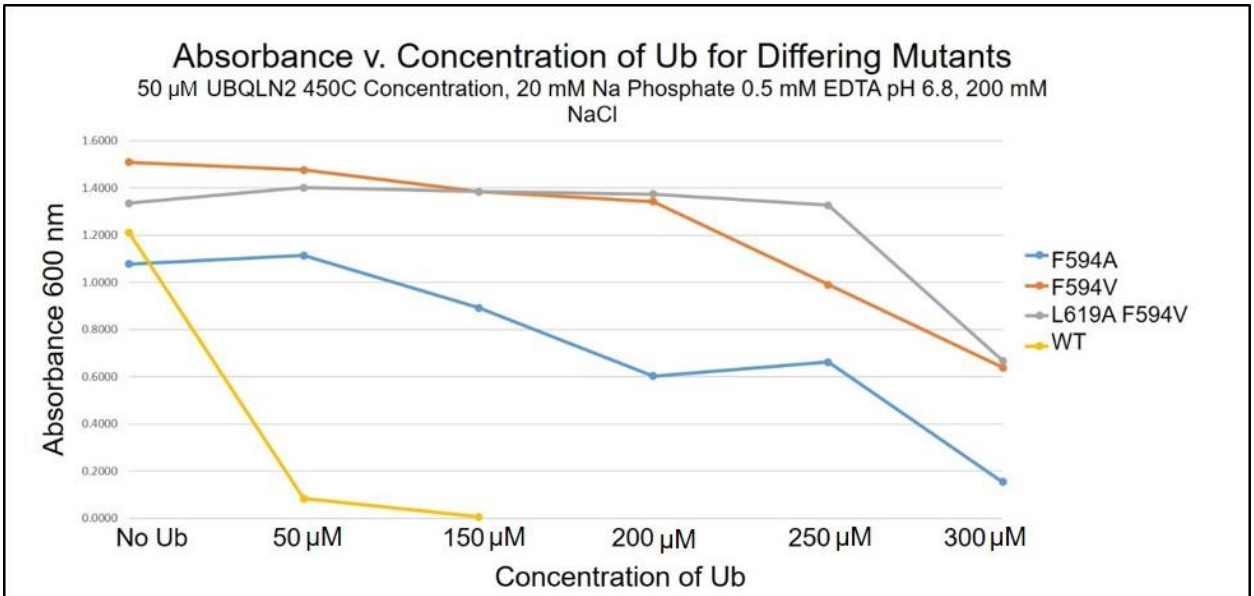


Figure 9. Comparison of UBQLN2 450C constructs at varying concentrations of ubiquitin. All UBQLN2 450C constructs at 50 µM. Absorbance is the average maximum absorbance value for all trials. Note data for F594V at 250 µM was not collected, so 200 µM and 300 µM were average to get this value.

In Figure 9, the maximum absorbance of turbidity profile was plotted as a function of Ub protein concentrations. For example, at 50 µM Ub, the maximum absorbance for the UBQLN2 450C WT construct is less than 0.1, while for the F594V mutant LLPS persists and the maximum absorbance remains at around 1.5 (Figure 9). This plot demonstrates the differences in how the LLPS of mutant UBQLN2 proteins respond to Ub as compared to UBQLN2 450C WT. The data suggest that LLPS persists for mutant UBQLN2 even at high concentrations of Ub, in other words, Ub's ability to eliminate UBQLN2 LLPS is significantly attenuated by mutations to the UBA domain of UBQLN2.

Discussion

Recent studies have shown the connection between the development of neurodegenerative diseases and the process of LLPS and pathological inclusion formation (Kim et al., 2013; Molliex et al., 2015). Persistence of stress granules in particular may lead to ALS or other neurodegenerative diseases (Li, King, Shorter, & Gitler, 2013). UBQLN2, a human protein involved with protein quality control mechanisms and shown to be present in pathological inclusions of both familial and sporadic ALS patients. UBQLN2 localizes into stress granules under stressful conditions in the cell and is shown to undergo LLPS under physiological concentration, temperature, and ionic strength (Dao et al. 2018). UBQLN2 is a well-suited protein to serve as a model system for studying the molecular grammar behind LLPS.

The "molecular grammar" of LLPS for proteins such as NICD and FUS, have been recently studied (Pak et al., 2016; Wang et al., 2018). The amino acid sequence and composition of these proteins differ, and the properties and composition of their amino acid sequences affect their LLPS behavior. For example, the protein NICD requires interaction with positively charged partners to phase separate while the protein also contains aromatic and hydrophobic residues (Pak et al., 2016). The proteins in the FUS family require a different composition and primary sequence. FUS proteins typically contain a N-terminal PLD domain and C-terminal RBD domain, which are prerequisites for its LLPS behavior. The FUS proteins also contain tyrosine and arginine residues which govern the concentration at which the solution becomes saturated and phase separates, and glycine residues which maintain liquidity of the protein. FUS family proteins follow a system called the associative polymer model, which can predict the phase separation behavior of the protein (Wang et al., 2018). With more data on the phase separation of

UBQLN2, models that can predict the WT protein and mutant protein's LLPS behavior may be elucidated, with this thesis progressing towards that goal.

In this work, we show that decreasing the number of regions UBQLN2 decreases its ability to phase separate and the level of amino acid hydrophobicity also effects LLPS of UBQLN2. This supports the theory that multivalent interactions between multiple participating domains between many molecules are the driving force for liquid-liquid phase separation in the cell and there is growing proof that many proteins have sites for multivalent interactions (Pak et al., 2016; Wang et al., 2018). These findings also show that reduction in some of the multivalent elements or sticker regions in the UBQLN2 450C protein, reduces LLPS behavior. However, maintenance of some multivalent regions in the protein protect UBQLN2 450C from completely losing its ability to phase separate. Importantly, these findings provide further evidence that UBQLN2 LLPS is promoted by numerous multivalent interaction elements, especially hydrophobic interaction sites, which was hypothesized through our earlier work (Dao et al. 2018). This further investigation to the molecular sequences responsible for UBQLN2 LLPS gives insight into these mechanisms by identifying amino acids that increase or decrease phase separation. Further studies will address the molecular determinants of LLPS for UBQLN2.

This *in vitro* observation confirmed by Dao et al. 2018 shows that Ub disrupts and may even eliminate UBQLN2 LLPS is important for understanding how phase separation behavior is modulated by protein-protein interactions. In the cell, it is important to know how ligands and other molecules modulate phase boundaries, including the conditions for membraneless organelle assembly and disassembly. The mechanism of how ligands affect phase separation boundaries is generally called polyphasic linkage. An example of this is how profilin binds to regions of the huntingtin protein, Htt-NTFs (Posey et al., 2018). Dao et al suggests a mechanistic

scheme, similar to that of a polyphasic linkage mechanism, where UBQLN2 can shuttle Ub-tagged proteins out of membraneless organelles such as stress granules to the proteasome. These results show that the amino acid mutations demonstrated here, within the regions 592-594 and 610-620, would disrupt this behavior and UBQLN2 could not perform its hypothesized role to shuttle Ub-tagged proteins out of membraneless organelles.

Future directions are to perform NMR titrations of Ub into UBQLN2 450C mutants to determine the exact binding affinity of Ub to UBQLN2 at these mutation sites and to discover the effect of certain amino acids on Ub binding. Creation of a complete phase diagram for differing mutation constructs of UBQLN2 would provide greater insight into the ultimate and complete effects of mutations on the protein and the effect of UBA mutations on how UBQLN2 interacts with ubiquitin. Different conditions such as higher or lower temperatures, increased UBQLN2 construct concentrations, increased or decreased salt (NaCl) concentrations would greatly increase the amount of information that a phase diagram could provide. The threshold concentration at which UBQLN2 phase separates may be elucidated.

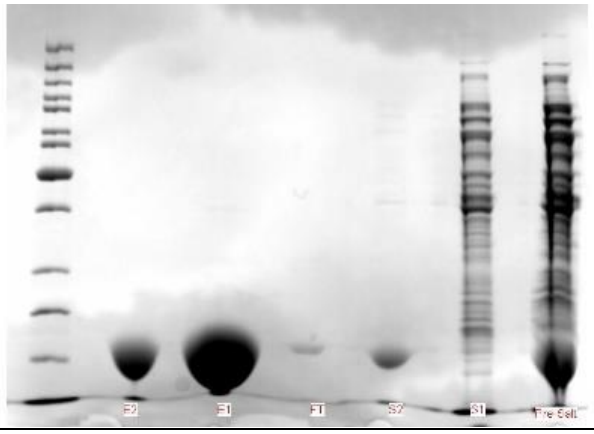
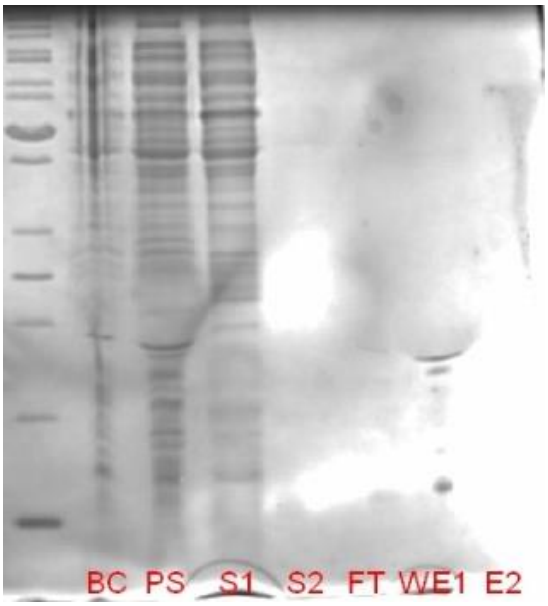
Further studies that investigate the mechanism of the free energy of the UBQLN2 system by contribute to comprehending the thermodynamics and molecular driving forces for phase separation (Banani, Lee, Hyman & Rosen, 2017). It would also be extremely interesting to determine the effects of substituting amino acids near the Ub binding domains on UBQLN2 450C UBA, but at the interaction sites. This may demonstrate the different roles of amino acid categories on LLPS by controlling for the interaction of ubiquitin with the UBA domain of UBQLN2.

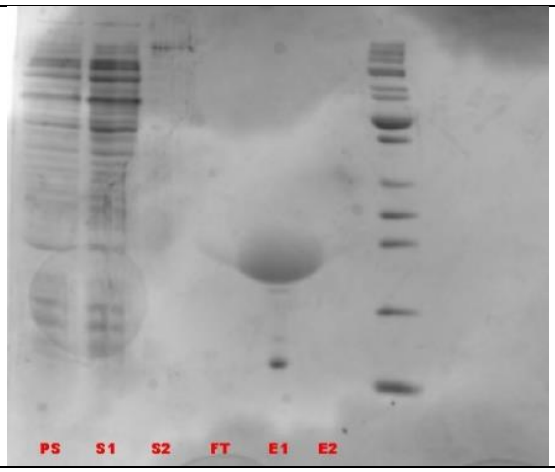
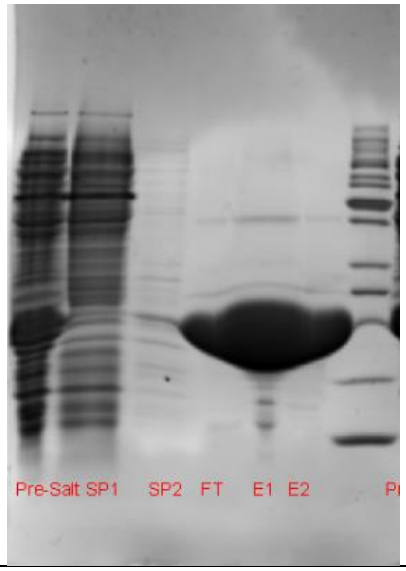
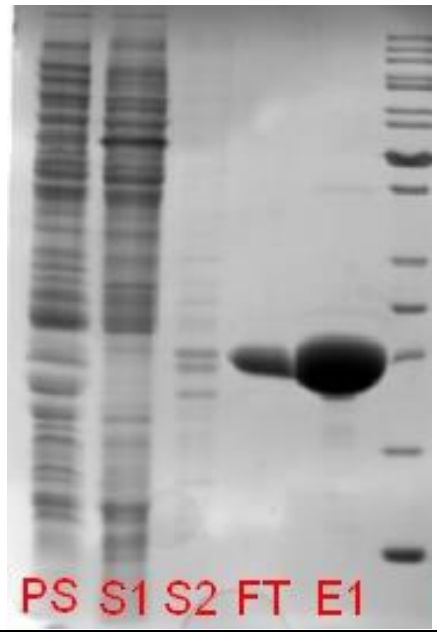
Conclusions

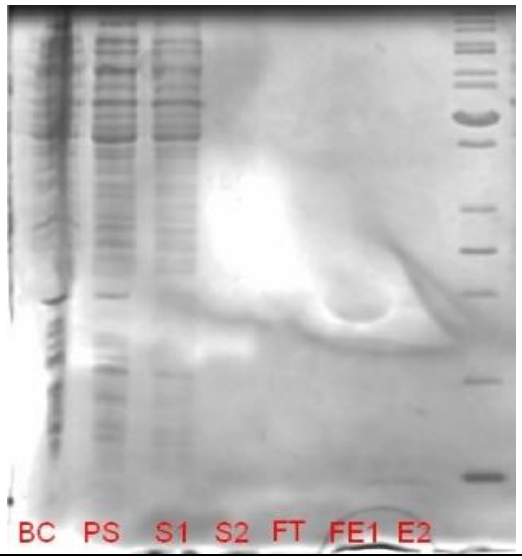
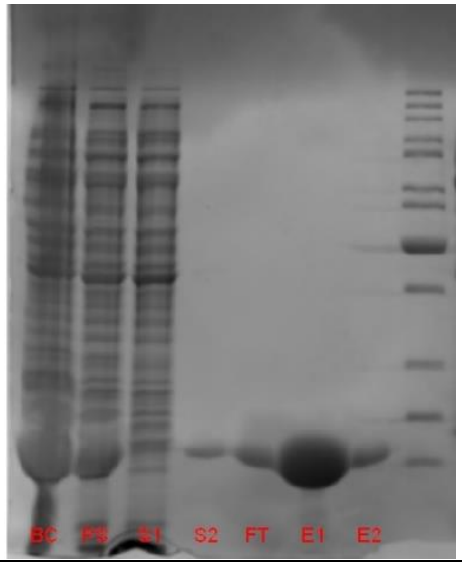
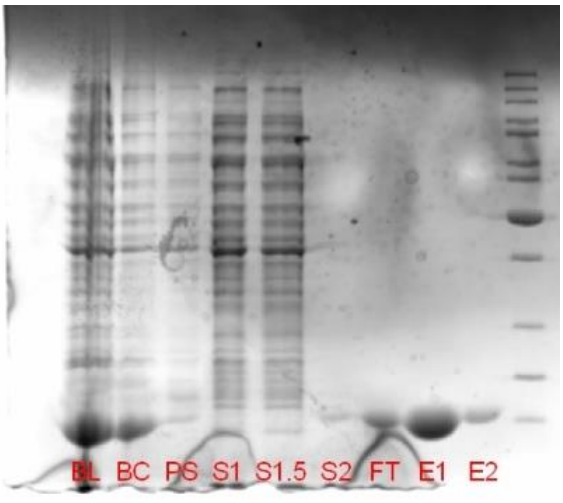
This report shows that UBQLN2 LLPS is decreased by (a) loss of one of the regions that participates in multivalent interactions and/or (b) the reduction in amino acid hydrophobicity at residues 594 and 619. This report also shows that UBQLN2 LLPS is decreased by the presence of Ub at certain concentrations. However, certain single amino acid substitutions and double amino acid substitutions disrupt Ub's ability to eliminate or modulate UBQLN2 LLPS behavior. In a broader view, these conclusions give more support for the increasing evidence that the driver of membraneless organelle formation through LLPS are weak, multivalent interactions between molecules. These data also give insight into the differing compositions involved in driving LLPS, whether (in the case of UBQLN2) the amino acids are hydrophobic and polar or involving alternating electrostatic charges. These data will contribute further to identifying the "molecular grammar" behind LLPS and may give further information regarding the biochemical and biophysical binding affinity of Ub related to the structure and character of amino acids at these certain regions.

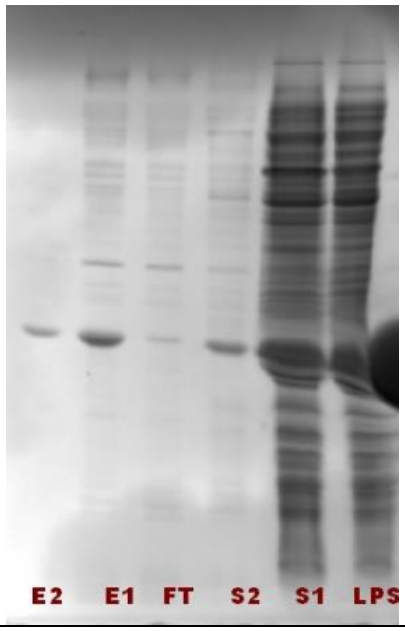
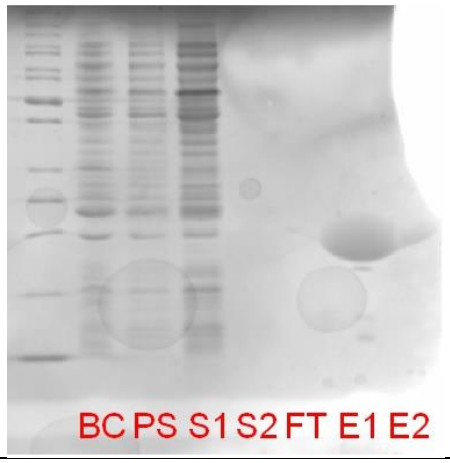
Supplemental Data

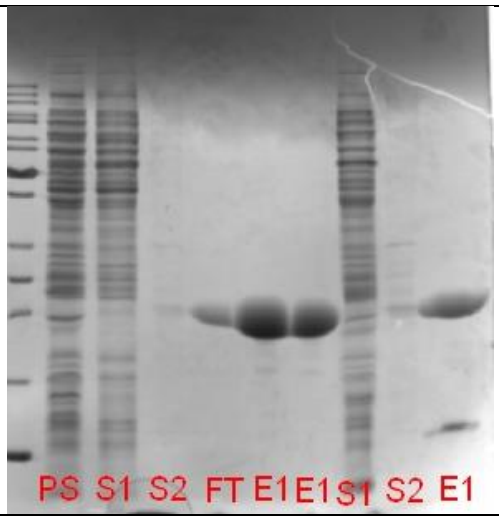
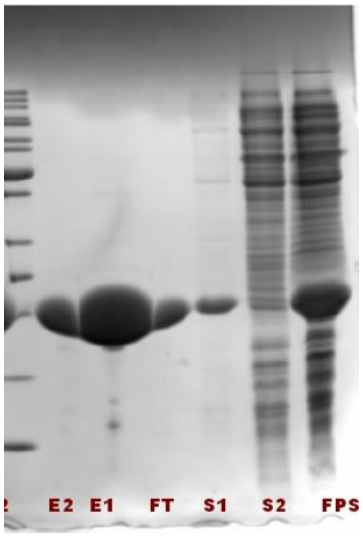
Appendix 1: Organization of Growths, Gels, and Protein Concentration/Purification. If Mass Spectrometry deconvolutions are available, growths labeled Y or N and have hyperlink on the date of the growth.

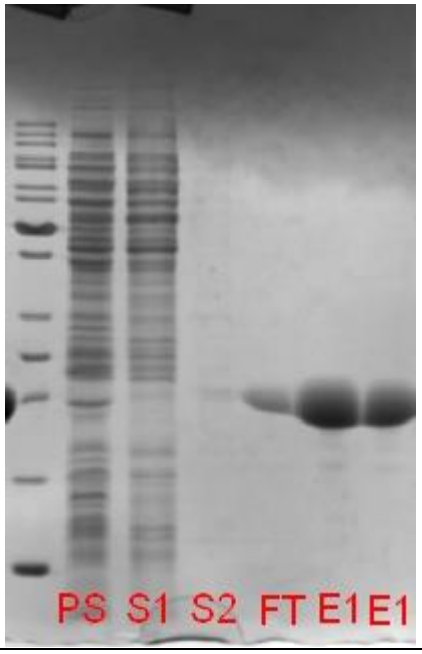
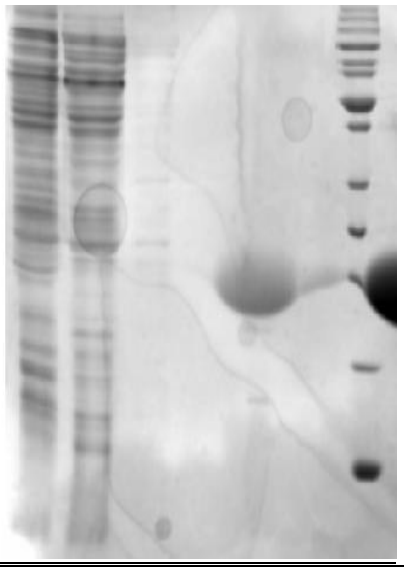
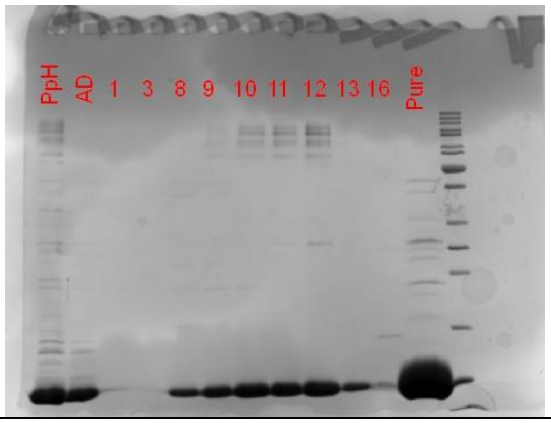
Type of Protein	Date of Purification	Concentration (Conc.), Purity (260/280), and Mass Spec (Y or N)	Gel (Protein is ~ 17700 kDa) BL= Before Lyse; BC= Before Centrifuge; PS= Pre-Salt; S1= Supernatant 1; S2= Supernatant 2; FT= Flow through; E1= Eluent 1; E2= Eluent 2
UBQLN2 450C WT	9.7.17	Conc: 1900 uM 260/280: 0.77 MS: Y	
UBQLN2 450C WT	6.25.18	Conc.: 1584 uM and 260/280: 0.78 MS: N	 Gel not stained enough but WT is there

UBQLN2 450C WT	10.18.18	Conc.: 1530 uM 260/280: 0.77 MS: N	
Δ552-570	2.21.18	Conc.: 1600 uM 280/260: 0.83 MS: N	
Δ552-570	7.3.18	Conc.: 823 uM 280/260: 0.81 MS: N	

450C F594V	6.25.18	Conc.: 290.9 uM 280/260: 0.83 MS: N	
450C F594V	6.7.18	Conc.: 527 uM 280/260: 0.76 MS: Y (6.6.18)	
450C F594V	6.13.18	Concentration: 367 uM 280/260: 0.78 MS: Y	

450C F594V	2.4.18	Conc.: 260/280: MS: N	
450C L619A	6.22.18	Basically zero protein	
450C L619A	6.27.18	Conc.: 1228 uM 260/280: 0.79 MS: Y	
450C L619A	7.3.18	Conc.: 1034 uM 260/280: 0.81 MS: N	L619A on the right (S1, S2, and E1)

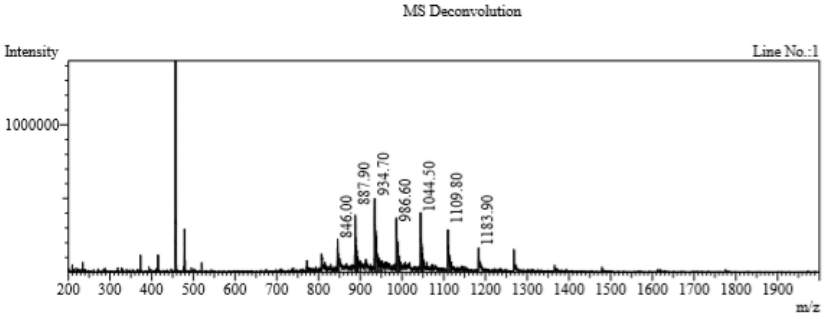
			
450C F594A	6.20.18	Conc.: 1020 uM 260/280: 0.77 MS: Y	
450C F594A	7.2.18	Conc.: 860 uM 260/280: 0.76 MS: Y (7.5.18)	

450C L619A F594V	7.6.18	Conc.: 454 uM 260/280: 0.78 MS: Y	
450C L619A F594V	7.13.18	Conc: 940 uM 260/280: 0.77 MS: N	
Ubiquitin	7.31.18	Conc.: 4000 uM 260/280: 0.69 MS: Y	

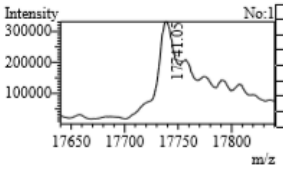
			PpH= pre-acidic pH; AD= after dialysis; 1-16= HPLC collections; Pure= final pure ub

Appendix 2: Mass Spectrometry

9.7.17: UBQLN2 450C WT protein from Completed on 6/22/18. Predicted MW: 17742.1621 Actual MW: 17741.05

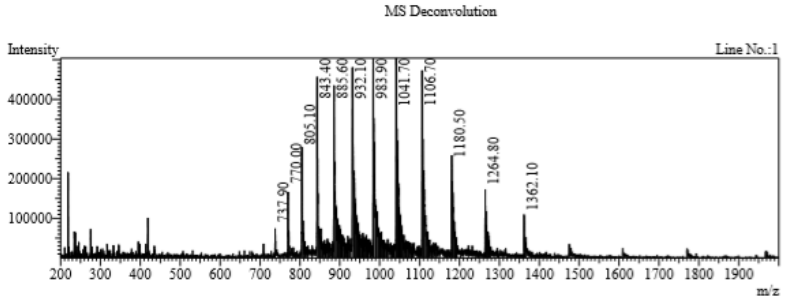


No.	Name	# Peaks	A.Mass	SD	R.Mass
1	17741.0493	7	1.00782	2.203	17741.05

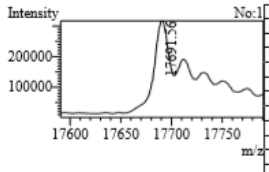


No.	Int.	Charge	Real Charge	m/z	Weight	Mass
1	179909	15	14.998	1183.90	0.20	17743.38
2	330151	16	16.000	1109.80	0.17	17740.67
3	407866	17	17.002	1044.50	0.15	17739.37
4	342773	18	18.000	986.60	0.14	17740.66
5	519513	19	19.001	934.70	0.12	17740.15
6	454029	20	20.004	887.90	0.11	17737.84
7	229869	21	20.996	846.00	0.10	17744.84

6.6.18: F594V protein from Completed on 6/22/18. Predicted MW: 17694.1191 Actual MW: 17691.56

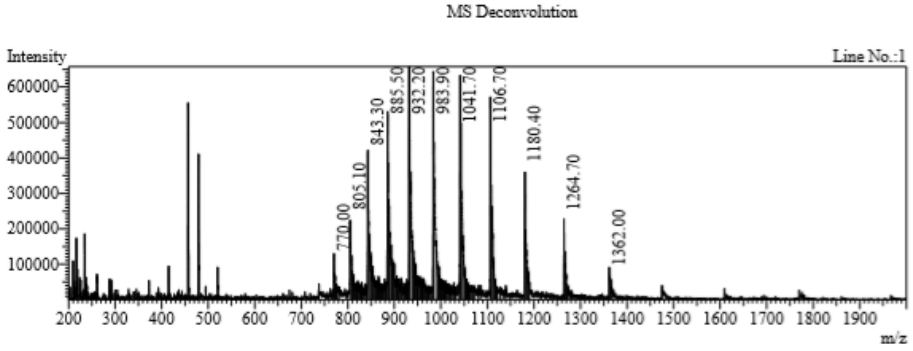


No.	Name	# Peaks	A.Mass	SD	R.Mass
1	17691.5649	12	1.00782	2.512	17691.56



No.	Int	Charge	Real Charge	m/z	Weight	Mass
1	119046	13	12.998	1362.10	0.15	17694.20
2	184325	14	13.999	1264.80	0.13	17693.09
3	253548	15	14.999	1180.50	0.11	17692.38
4	477010	16	16.000	1106.70	0.10	17691.07
5	492384	17	17.000	1041.70	0.09	17691.77
6	518080	18	17.999	983.90	0.08	17692.06
7	505690	19	19.001	932.10	0.07	17690.75
8	447913	20	20.000	885.60	0.06	17691.84
9	480923	21	21.002	843.40	0.06	17690.24
10	285103	22	22.002	805.10	0.05	17690.03
11	187204	23	23.006	770.00	0.05	17686.82
12	89541	24	24.008	737.90	0.04	17685.41

6.13.18:
F594V measured on 6/20/18 after being in fridge for a week: Completed on 6/22/18. Predicted MW: 17694.1191 Actual MW: 17691.1191



No.	Name	# Peaks	A.Mass	SD	R.Mass
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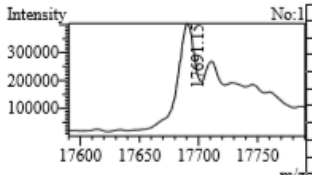
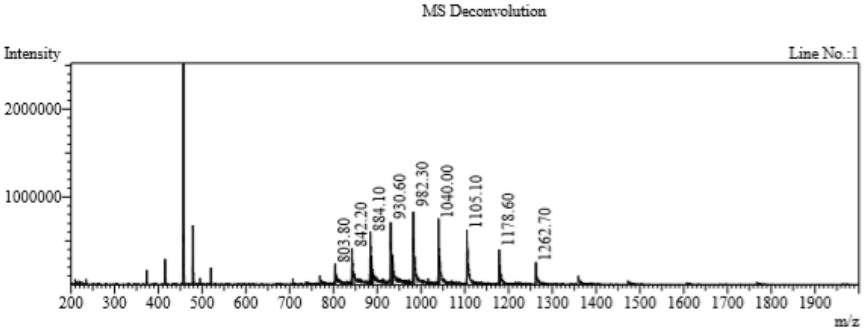


No.	Int.	Charge	Real Charge	m/z	Weight	Mass
1	96188	13	12.999	1362.00	0.16	17692.90
2	251891	14	14.000	1264.70	0.14	17691.69
3	389068	15	15.000	1180.40	0.12	17690.88
4	599914	16	16.000	1106.70	0.10	17691.07
5	650891	17	16.999	1041.70	0.09	17691.77
6	648544	18	17.999	983.90	0.08	17692.06
7	676492	19	18.998	932.20	0.07	17692.65
8	566361	20	20.001	885.50	0.07	17689.84
9	435133	21	21.004	843.30	0.06	17688.14
10	253336	22	22.001	805.10	0.06	17690.03
11	136760	23	23.006	770.00	0.05	17686.82

6.20.18:
F594A protein Completed on 6/22/18. Predicted MW: 17666.0645 Actual MW: 17663.47



No.	Name	# Peaks	A.Mass	SD	R.Mass
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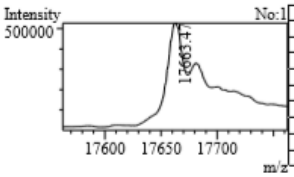
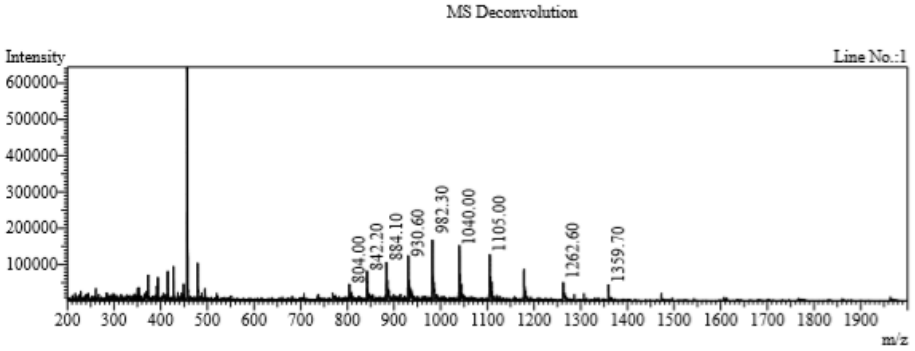
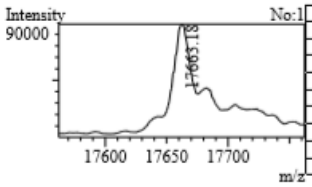


No.	Int.	Charge	Real Charge	m/z	Weight	Mass
1	270857	14	14.000	1262.70	0.17	17663.69
2	435776	15	15.000	1178.60	0.15	17663.88
3	641351	16	15.998	1105.10	0.13	17665.47
4	757077	17	17.001	1040.00	0.12	17662.87
5	890780	18	18.000	982.30	0.10	17663.26
6	747230	19	19.001	930.60	0.09	17662.25
7	605025	20	20.002	884.10	0.08	17661.84
8	429048	21	20.998	842.20	0.08	17665.04
9	246259	22	22.003	803.80	0.07	17661.43

7.5.18: Completed on 7/10/18. F594A from 7/5/18: Predicted MW: 17666.0645 Actual MW: 17663.18

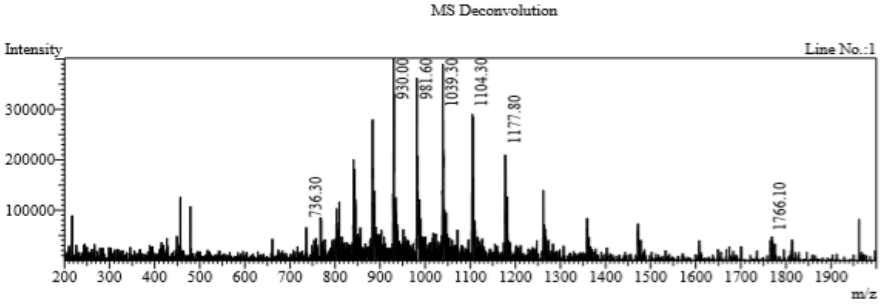


No.	Name	# Peaks	A.Mass	SD	R.Mass
1	17663.1809	9	1.00782	1.270	17663.18

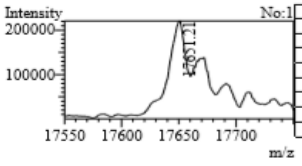


No.	Int.	Charge	Real Charge	m/z	Weight	Mass
1	50481	13	13.000	1359.70	0.19	17663.00
2	60941	14	14.001	1262.60	0.16	17662.29
3	134679	16	15.999	1105.00	0.13	17663.87
4	153711	17	17.000	1040.00	0.11	17662.87
5	186297	18	18.000	982.30	0.10	17663.26
6	121936	19	19.001	930.60	0.09	17662.25
7	109808	20	20.002	884.10	0.08	17661.84
8	83709	21	20.998	842.20	0.07	17665.04
9	48187	22	21.997	804.00	0.07	17665.83

7.6.18: Completed on 7/10/18. L619A F594V protein from 7/6/18: Predicted MW: 17652.0391 Actual MW: 17651.21

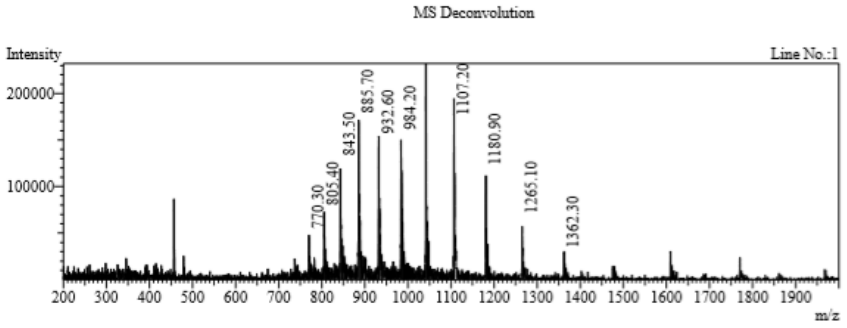


No.	Name	# Peaks	A.Mass	SD	R.Mass
1	17651.2131	8	1.00782	1.620	17651.21

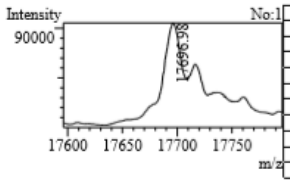


No.	Int.	Charge	Real Charge	m/z	Weight	Mass
1	97680	9	9.000	1962.30	0.30	17651.63
2	59065	10	10.000	1766.10	0.24	17650.92
3	231272	15	14.999	1177.80	0.11	17651.88
4	311308	16	15.999	1104.30	0.09	17652.67
5	437115	17	17.000	1039.30	0.08	17650.97
6	372366	18	18.001	981.60	0.07	17650.66
7	473478	19	19.000	930.00	0.07	17650.85
8	85123	24	24.006	736.30	0.04	17647.01

6.27.18: Completed on 7/10/18. L619A from 6/27/18: Predicted MW: 17700.0820 Actual MW: 17696.98

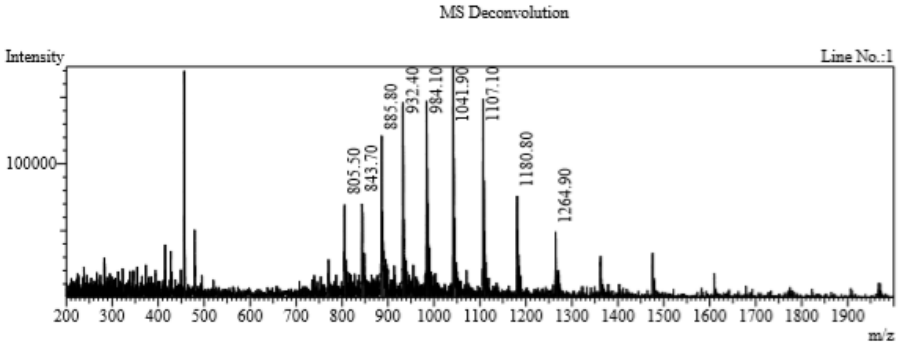


No.	Name	# Peaks	A.Mass	SD	R.Mass
1	17696.9841	10	1.00782	2.441	17696.98

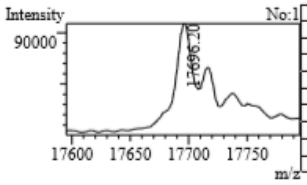


No.	Int.	Charge	Real Charge	m/z	Weight	Mass
1	34049	13	13.000	1362.30	0.17	17696.80
2	71112	14	14.000	1265.10	0.15	17697.29
3	122983	15	14.999	1180.90	0.13	17698.38
4	218090	16	15.998	1107.20	0.12	17699.07
5	160837	18	18.000	984.20	0.09	17697.46
6	156863	19	18.996	932.60	0.08	17700.25
7	178784	20	20.004	885.70	0.07	17693.84
8	132952	21	21.006	843.50	0.07	17692.34
9	79404	22	22.000	805.40	0.06	17696.63
10	54601	23	23.004	770.30	0.06	17693.72

7.3.18: Completed on 7/10/18. L619A from 7/3/18: Predicted MW: 17700.0820 Actual MW: 17696.20

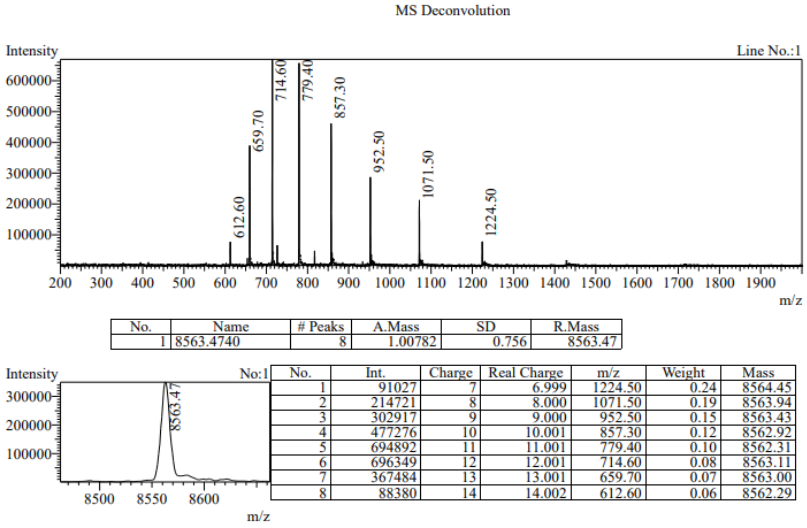


No.	Name	# Peaks	A.Mass	SD	R.Mass
1	17696.2030	9	1.00782	1.228	17696.20



No.	Int.	Charge	Real Charge	m/z	Weight	Mass
1	55201	14	14.001	1264.90	0.17	17694.49
2	82765	15	14.999	1180.80	0.15	17696.88
3	161151	16	15.999	1107.10	0.13	17697.47
4	202002	17	17.001	1041.90	0.12	17695.17
5	165609	18	18.001	984.10	0.10	17695.66
6	158082	19	19.000	932.40	0.09	17696.45
7	125257	20	20.000	885.80	0.08	17695.84
8	79182	21	21.000	843.70	0.08	17696.54
9	76827	22	21.997	805.50	0.07	17698.83

7.31.18: Ub Mass Spec completed Tuesday, 9/4/18. Ub from 7.31.18: Predicted MW: 8564.8203 Actual MW: 8563.47



No.	Name	# Peaks	A.Mass	SD	R.Mass
1	8563.4740	8	1.00782	0.756	8563.47

No.	Int.	Charge	Real Charge	m/z	Weight	Mass
1	91027	7	6.999	1224.50	0.24	8564.45
2	214721	8	8.000	1071.50	0.19	8563.94
3	302917	9	9.000	952.50	0.15	8563.43
4	477276	10	10.001	857.30	0.12	8562.92
5	694892	11	11.001	779.40	0.10	8562.31
6	696349	12	12.001	714.60	0.08	8563.11
7	367484	13	13.001	659.70	0.07	8563.00
8	88380	14	14.002	612.60	0.06	8562.29

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