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Intrinsically Disorder Proteins and Liquid-Liquid Phase Transitions in Neurodegenerative Diseases

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Intrinsically Disorder Proteins and Liquid-Liquid Phase Transitions in Neurodegenerative Diseases

by

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DEDICATION

To my parents for providing unconditional support. To Beau for always supporting me and believing in me no matter what, without you none of this would be possible.
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Many of the discoveries here are from the work and ideas of Dr. Chad Dickey who tragically passed away too young but was one of the most inspirational scientists I have ever had the privilege of meeting. Also, I want to thank my mentor Dr. Vladimir Uversky who has always unconditionally supported me and my best interests in both science and in life. The life lessons I have learned from him are invaluable. And finally, I would like to thank Dr. Dali Zheng for teaching me almost everything I know about conducting experiments in the lab, always providing me with helpful well-thought out advice and teaching me that integrity in science is the most important aspect of being a scientist.
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ABSTRACT

Neurodegenerative diseases have a negative impact on health and economics, effecting more than 20 million people in the United States and costing around $800 billion in 2018. Additionally, most neurodegenerative diseases such as Alzheimer’s Disease (AD), Amyotrophic Lateral Sclerosis (ALS), Frontotemporal Dementia (FTD), and several others are fatal and have no cure or effective treatment. Many of these diseases have a common theme of protein aggregates being present in disease tissue. These aggregates are thought to be toxic to the cell and prevention of pathological aggregation and elimination of these cellular aggregates may serve as a potential therapeutic approach to treat disease. Furthermore, identifying a common mechanism shared in all diseases that seeds the toxic aggregation of proteins would enable therapeutic approaches that prevent protein aggregation to be more broadly applicable.

Here we demonstrate the ability of intrinsically disordered proteins found in neurodegenerative diseases to undergo liquid-liquid phase separation and show that this can seed their aggregation and lead to detrimental effects on the cell. We also show that this behavior can be modulated by exploited ratios of expressed pathological proteins as well as increasing the expression of chaperone proteins. This work offers some key insight to a potential mechanism shared across several neurodegenerative disease related proteins that could be targeted in order to alleviate disease progression.
Chapter One:
Protein Liquid-Liquid Phase Separation in Neurodegenerative Disease

1.1 Abstract
Protein misfolding and misassembly are key molecular themes in many neurological disorders. This aberrant protein behavior is found in disorders such as Amyotrophic Lateral Sclerosis (ALS), Frontotemporal Dementia (FTD), Alzheimer’s Disease (AD), and Huntington’s Disease [1] and leads to neuronal loss and cognitive and motor impairments. Although protein aggregation is a well-accepted aberration in these diseases, the challenge is to determine what is driving the process and what is just simply a consequence of it. Many of the proteins found in pathological aggregates in neurological diseases have the unique ability to undergo liquid-liquid phase separation and are found in cellular membraneless organelles. Here we will discuss the physiological role of liquid-liquid phase separation and membraneless organelle formation. We will also discuss how sustained phase separation and membraneless organelle formation can lead to protein aggregation and disease pathology.

1 Portions of this work were previously published (Darling, A.D. et al., 2018) and are used with permission of the publisher.
1.2 Introduction

Although pathological misfolding of proteins has been a key observation in several neurodegenerative diseases, the exact mechanism that initially seeds the aggregation has not been fully realized. While clues have been provided for why particular proteins aggregate, there is not a general mechanism that explains most cases. However, it has been shown that increasing the local concentration of protein increases the propensity of the protein to aggregate [2]. Additionally, increasing protein local concentration is the key driving factor for proteins to undergo liquid-liquid phase separation (LLPS) with leads to the formation of membraneless organelles (MLOs) inside the cell [3]. LLPS can result in a more confined space for the proteins contained in the phase separated droplet and therefore increases their rate of aggregation if droplet formation is sustained. Because of the connection of LLPS and protein aggregation, it is conceivable that it may be an early step that seeds the aggregation of proteins involved in the pathology of neurodegenerative diseases. Here we will discuss the physiological role of LLPS and MLOs in cells and how they can become pathological and lead to detriments in various neurodegenerative diseases.

1.3 Discussion

1.3.1 Aging and Disease

The primary risk factor for most neurodegenerative diseases is aging [4]. All cells age due to a time dependent accumulation of cellular damage which leads to a progressive decline in physiological functioning [5, 6]. In younger cells, the mechanisms to repair the damage are viable therefore allowing the cell to overcome stress. However, in aged cells, homeostasis mechanisms are weakened to the point where the
cell is no longer able to repair itself when faced with insults. Therefore at its core, the process of aging seems to result from a loss of homeostatic capacity but is associated with several hallmarks including loss of proteostasis, genomic instability, mitochondrial dysfunction, stem cell exhaustion, cellular senescence, and defective or deregulated signaling [4]. The major problem currently faced is to discern the connection of these aging hallmarks and figure out how they individually contribute to the overall process.

The cause for the deterioration of homeostatic mechanisms in aged cells is not well understood. There are several concepts which have been proposed to explain this phenomenon including a decline in metabolic activity [7-10], damage to biomolecules such as protein and nucleic acids [11], and deregulated gene expression [12, 13]. However, the challenge is to determine what is driving the process and what is just simply a consequence of it. Whichever the case, aging results in a universal decline in cell health and it inevitably leads to the emergence of age-related diseases and death of the organism.

During the process of aging, there are major fluctuations in the physical-chemical parameters of the cell. This is driven by two factors. The first is a decline in metabolic activity due to damage associated with the mitochondria [7, 8, 10] and the other is the failure of homeostatic systems such as proteostasis [11]. Aberrations in these factors lead to rapid changes in the cellular environment which can cause detrimental effects on the ability of the cell to maintain intracellular organization and spatiotemporal control. In fact, this concept may explain the multifaceted hallmarks of aging and the increase of disease that accompanies it.
1.3.2 Membrane-less Organelles

Compartmentalization of biomolecules is essential for a cell to carry out its biological functions and maintain intracellular organization. Physical separation is achieved using compartments in the cell which are known as organelles. Some organelles achieve separation using a membrane while others are known as membraneless organelles [14]. Some examples of membrane bound organelles include the nucleus, mitochondria, and chloroplast. MLOs form by spontaneous liquid-liquid phase separation (LLPS) into multi-component viscous liquid structures [42]. The phase-separation occurs due to changes in the cell environment that trigger molecular supersaturation such as alterations in salt concentration, pH, and changes to protein charge due to post-translational modifications [42]. The selective partitioning produces a specialized chemical microenvironment that enables specific reactions to occur such as the remodeling of nucleic acids [43] and ensures spatiotemporal control over biochemical reactions that are diffusion-limited [15].

There are many kinds of MLOs in cells that perform a variety of functions, the vast variety of organelles are shown in Figure 1.1. The localization and functioning of some of the main organelles is shown in Table 1.1. The nucleus is a membrane bound compartment but is further partitioned into MLOs such as the nucleolus and cajal bodies, just to name a few [46]. The cytoplasm, mitochondria, and chloroplasts also are sites of membrane-less organelles. The cytoplasm contains organelles that form because of cellular stress such as stress granules. Many of these membraneless compartments have been shown to have an association with age-related diseases which showcases the vulnerabilities of these components during the aging process. For
example, mutations associated with ALS are mainly in proteins that are prominent members of membrane-less organelles such as TDP-43 and FUS [16-20]. Additionally, there is increasing evidence showing that there are defects in membrane-less organelles which are associated with protein misfolding diseases and cancer [21]. Some of the most abundantly studies MLO’s will be discussed below.

**Nuclear Pore Complex**

In eukaryotic cells, the nuclear pore complex (NPC) is embedded in the nuclear membrane and are the largest protein complexes in the cell, possessing a mass of ~125 megaDaltons (MDa) in vertebrates [22] and 66 MDa in yeast [23]. It functions to enable the selective transport of molecular across the nuclear membrane. NPCs are large molecular machines operated by proteins rich is phenylalanine and glycine domains (FG domains) that are intrinsically disordered [24, 25]. Proteins with these domains can form dense hydrogel-like phases allowed for the privileged passage of molecules through the nuclear membrane [24, 25]. The FG domain proteins undergo spontaneous phase separation into dense hydrogels that are permeable to the cargo of nuclear transport receptors but act as effective barriers for macromolecules [25].

**Nucleolus**

In the nucleus the largest, and arguably most important, MLO is the nucleolus. It is responsible for the assembly of ribosomal subunits, and perturbations in it led to defects in ribosome assembly and translation [48]. The protein nucleophosmin (NPM1) is the main component of the nucleosome and is required for its formation via phase-separation [49]. Once formed, the nucleosome is dynamic, dissipating once ribosomes are assembled to allow for their export [48]. Aberrations in the formation and dynamics
of the nucleoli can lead to an overall decrease in global protein translation, something that has been observed in several neurodegenerative diseases [25].

Cajal Bodies

Cajal bodies or coiled bodies, called so due to the presence of the protein coilin which appears as coiled threads, are in the nucleus [26]. They have prominent appearance in cells that are highly active such as neurons and cancer cells [27]. They are functionally involved in assembling the U small nuclear ribonucleoproteins that are required for spliceosome formation and are therefore assumed to increase the efficiency of the complexes formation [26].

Paraspeckles

Paraspeckles are another nuclear membraneless organelles. They are involved in the control of gene expression. The formation of paraspeckles occurs through phase separation and is driven by the long non-coding RNA NEAT1, whose knockdown leads to their rapid disintegration [28, 29]. Paraspeckles are the sight of several proteins that are mutated in ALS such as TDP-43 and FUS [30], and therefore may have important implications in the pathophysiology of the disease. For example, TDP-43 downregulation leads to the formation of paraspeckles by inducing the accumulation of NEAT1 [31].

P-Granules

One of the early observations of membrane-less organelles was the discovery of processing bodies (or P-granules), which are germ granules specific to Caenorhabditis elegans. P-granules contain RNA granules and RNA binding proteins, mainly PGL1 and PGL3 as well as DEAD-box proteins [1, 32]. These granules have been shown to
segregate during the development of *Caenorhabditis elegans* germline and exhibit liquid-like behavior [33]. The liquid-like behavior observed involves minimizing surface area, droplet fusions, and flow-like features such as dripping in response to shear stress, which can be explained by the fast-internal molecular rearrangements that occur within P granules. Such rapid rearrangements are enabled by multiple weak interactions between RNA molecules and the RNA-binding proteins present in P granules and constitute a general principle of liquid compartments that partition the intracellular space [33].

**Stress Granules**

When the cell is exposed to specific stressful events stress granules (SGs), which contain untranslated messenger ribonucleoprotein (mRNP), form in the cytoplasm [50]. The stressor halts translation and the aborted translation initiation complex can either be routed to translation initiation or degraded [51]. The assembly of SGs is mediated by the prion-like aggregation of TIA1, which causes it to be recruited into SGs in all cell types [50]. Another SG protein component is a phosphorylation dependent endoribonuclease known as RasGAP SH3-binding protein (G3BP) [51]. G3BP interacts with RasGAP in its central domain, where it is dephosphorylated at serine 149, thus recruiting it to SGs [51].

SG assembly is a dynamic process that resolves once the stress inducing insult has been terminated, causing the SGs themselves to absolve [52]. However, in cases where the insult is too large to overcome, SGs do not clear, and instead the cell switches from a rescue path to a self-induced death pathway [50, 52]. Additionally, if the
stress is sustained, but the cell finds a way to overcome death, it can lead to deleterious effects on the cell later [20, 34]

‘Aging’ of SGs can lead to an increase in protein aggregation. In fact, data shows that sustained SG formation leads to an increase in TDP-43 aggregation and cytoplasmic mislocalization due to loss of mobility, both of which are hallmarks in ALS [53]. Additionally, there is increasing evidence showing that aberrant formation of RNP granules takes a central role in ALS and FTD. Many of the genetic mutations associated with these diseases are in resident SG proteins, such as TIA-1, hNRNPA1, FUS, and TDP-43 [1, 54]. In Alzheimer’s disease, stress granules are associated with tau pathology and tau expression leads to poorly dynamic stress granules that are unable to be cleared [35, 36].

1.3.3 Intrinsically disordered proteins and liquid-liquid phase separation

MLOs frequently contain protein molecules and RNA. The proteins that drive phase transitions are referred to as intrinsically disordered proteins/regions (IDP/IDRP) that contain low complexity domains (LCDs) consisting of repeat amino acids or short amino acid motifs with an intrinsic preference for conformational disorder [42, 44, 45]. The amino acid composition of these proteins is often biased with low amino acid diversity, having polar and charged groups favored [45]. The lack-of structure of IDP/IDPRs is important for MLOs for many reasons. IDP/IDPRs have a conformational flexibility that allows for the fluidity of MLOs and can form a multitude of transient contacts allowing for the stability of the MLO once formed [46]. Once phase separation occurs, the newly-formed MLOs exhibit hallmark behaviors of liquid such as classical
wetting and dripping behaviors, sufficient surface tension to maintain a spherical shape, fusion upon contact, and flow in response to shear stresses [47].

Since protein LLPS is extremely sensitive to the physical-chemical environment, small fluctuations in the cell can lead to large changes in the formation and function of MLOs. *In-vitro* studies have shown that changes to parameters such as protein concentration, pH, ion and small molecule concentration, temperature, affinity of macromolecules, and energy levels can have an effect of LLPS [37].

**1.3.4 Prevalence of intrinsic disorder in proteins in membraneless organelles**

All proteinaceous MLO’s contain specific sets of resident proteins. Several studies have indicated that the presence of significant levels of intrinsic disorder represents a characteristic feature of some of the proteins associated with proteinaceous MLO’s 35-46 However, no systematic analysis of the intrinsic disorder predisposition was conducted so far for the MLOs in the human proteome. To fill this gap, we discuss below results of a systematic bioinformatics analysis of the disorder status of 4796 human proteins from 20 PMLOs. These proteins were retrieved mostly using the outputs of the QuikGO tool (https://www.ebi.ac.uk/QuickGO) complemented with some literature search. The analyzed proteins were distributed among the human PMLOs as follows: Nucleolus (1626) > Chromatin (1350) > Nuclear speckles (650) > Centrosome (530) > Mitochondrial RNA granules (229) > PML bodies (104) > SGs (57) > Perinuclear compartment (55) > CBs (54) > PcG bodies (48) > P-granules (19) > Nuage (18) > Cleavage bodies (14) > Gemini (10) > SAM68 bodies (8) > Paraspeckles (6) > Nuclear SGs (5) = OPT domain (5) > HLB (4) = Neuronal RNP granules (4). This list is not an exhaustive one nor does it contain all human MLO related proteins. In fact,
a recently designed Cell Atlas representing a comprehensive image-based map of the subcellular protein distribution identified localization of 12003 human proteins to 30 subcellular structures assembled into 13 major organelles, such as nucleus (1922 proteins, together with nucleoplasm, nuclear speckles, and nuclear bodies containing 3739, 444 and 482 proteins, respectively), nucleoli (1016 proteins, together with fibrillar center (254 proteins) and rim of nucleoli), nuclear membrane (272 proteins), Golgi apparatus (959 proteins), endoplasmic reticulum (430 proteins), vesicles (1806 proteins, together with lipid droplets containing 35 proteins), plasma membrane (1466 proteins, together with cell junctions containing 285 proteins), mitochondria (1070 proteins), cytosol (4279 proteins, together with cytoplasmic bodies (48 protein), aggresomes (17 proteins), and rods and rings (18 proteins), intermediate filaments (179 protein), microtubules (263 proteins, together with microtubule ends (four proteins), cytokinetic bridge (88 proteins), mitotic spindle (17 proteins), midbody (36 proteins), and midbody ring (12 proteins), centrosome (336 proteins, together with the microtubule organizing center containing 132 proteins), and actin filaments (223 proteins, together with focal adhesions containing 133 proteins) [38].

Figures 1.2, 1.3, 1.4, and 1.5 represent the result of a global analysis of the intrinsic disorder predisposition of 4796 human proteins associated with different MLOs. To this end, first, we looked at their overall disorder levels (protein-average disorder scores, PADS) evaluated by three members of the PONDR family of disorder predictors, PONDR® FIT,[39] PONDR® VLXT, [40] and PONDR® VSL2 [41-43]. Results of this analysis are summarized in Figure 1.2, where correlation between the outputs of these three predictors are shown as 3D plot, and which illustrate that
significant fractions of proteins in most human MLOs are noticeably disordered. This conclusion is based on the consideration of the outputs of this analysis using the criteria of accepted classification, where two arbitrary cutoffs for the levels of intrinsic disorder are used to classify proteins as highly ordered (PADS\textsubscript{ho} < 0.25), moderately disordered (0.25 \leq PADS\textsubscript{md} < 0.5), and highly disordered (PADS\textsubscript{hd} \geq 0.5\%) \[44\]. Figure 1.2 shows that according to this classification, many proteins in almost all PMLOs are highly or moderately disordered. By their disorderedness degree (PADS\textsubscript{md} + PADS\textsubscript{hd}) evaluated by PONDR\textsuperscript{®} FIT, human MLOs can be ranked as follows: nuage (33.4\%) < P-granules (57.9\%) < mitochondrial RNA granules (62.0\%) < perinuclear compartment (67.3\%) < neuronal RNP granules (75.0\%) < centrosome (81.5\%) < nucleolus (81.9\%) < paraspeckles (83.3\%) < nuclear speckles (87.8\%) < gemini (90.0\%) < PML bodies (90.3\%) < CBs (90.7\%) < SGs (93.0\%) < PcG bodies (93.7\%) < chromatin (95.5\%) < cleavage bodies (100.0\%) = SAM68 bodies (100.0\%) = nuclear SGs (100.0\%) = OPT domain (100.0\%) = HLB (100.0\%). Analogous analysis conducted for the entire human proteome (20228 proteins retrieved from the Consensus Coding Sequence database \[45\text{-}47\] revealed that this set contains 3969 (19.6\%), 5164 (25.5\%), and 11095 (54.9\%) highly disordered (PADS\textsubscript{hd} \geq 0.5\%), moderately disordered (0.25 \leq PADS\textsubscript{md} < 0.5), and highly ordered (PADS\textsubscript{ho} < 0.25) proteins, respectively. In other words, according to their overall disorderedness degree (PADS\textsubscript{md} + PADS\textsubscript{hd}) evaluated by PONDR\textsuperscript{®} FIT (45.1\%), proteins of human proteome are noticeably less disordered than proteins in all human MLOs (except for nuages). These data clearly show that the proteome of MLOs is, in general, highly intrinsically disordered.
1.3.5 Physiological consequences of protein phase separation

Protein phase separation is a physiological process used by the cell to reach several goals. The formation of membraneless organelles due to phase separation can tune reactions, organize the intracellular environment, and has a role in cellular fitness. Each of these functions enabled by protein phase separation are discussed below.

Reaction tuning

Microdroplet chemistry has been used to accelerate reaction rates by several orders of magnitude by providing a specific microenvironment to tune reaction rates [48]. The same concept is true of membraneless organelles. Their phase-separation behavior allows them to maintain a partitioned environment that can obtain conditions that vary from the outside aqueous environment [37]. Specifically, phase separation leads to an increase in the concentration of certain molecules. Because the reaction concentrations of molecules have an impact on the reaction rate, higher concentrations may lead to increases in rates of reaction within the enclosed space [49]. For example, concentrating RNA into liquid droplets led to a significant increase in ribozyme’s substrate cleavage activity [50]. Additionally, behavior like this has been observed during the formation of cytoskeleton components, such as microtubules [51].

Additionally, the presence of absence of certain molecules can enable or disable reactions to occur. Liquid droplets formed by phase separation in the cell can act as filters to regulate which molecules can enter and exit, similar to membranes found on more traditionally known organelles such as the nucleus. A common molecule that is selectively allowed to enter MLO’s is RNA. The structure, length, and interactions of RNA effect its ability to partition into phase separated droplets in the cell [52]. In fact, it
was demonstrated that longer RNA species more effectively partition into phase separated liquid droplets [50]. The ability of protein phase separation to tune reactions could lead to the ability to artificially increase or decrease reactions in the cell which are desirable or pathological. Therefore, finding out more about the mechanisms by which membraneless organelles selectively partition molecules and the microenvironments they are in is an important avenue that should be explored.

**Intracellular Organization**

One of the most important roles of phase separation in a cell is it enables the cell to organize its intracellular environment. It does this through compartmentalization that allows for specific biochemical reactions to occur, internal rearrangements, and the selective diffusion of molecules in and out of the organelle [53].

Due to the large surface area of neurons, spatially confining molecules in certain locations is important and they often utilize LLPS to aid in this process. For example, neurons utilize LLPS in synaptic vesicles located at synapses as a method of intracellular organization. It is used for the functional purpose of clustering replenishable pools of synaptic vesicles during period of heightened synaptic activity [54]. Synapsin, which is the mediator of synaptic vesicle clustering, undergoes LLPS which recruits the vesicles into the clusters where they are more easily accessible [54].

Another example of neurons exploiting LLPS to achieve intracellular organization lies in protein-rich compartments on the post-synaptic membrane which remodel protein composition during long-term potentiation called postsynaptic densities [55]. These protein-rich compartments are what underlie learning and memory. It was recently proposed that the formation of post-synaptic densities occurs through LLPS of two
protein components, SynGAP and PSD-95, allowing the neuron to locally concentrate proteins without influencing global translation [56]. Because of the importance of cellular organization, phase separation has a large physiological role and it can easily be hypothesized that aberrations in the ability to maintain this intracellular organization would have determinantal effects on the viability of the cell.

**Cellular fitness**

Strategies to increase cellular fitness are explored as potential treatments for most diseases. Since phase separation is highly responsive to environmental changes, it has the potential to be exploited to allow the cell to sense, respond to, and recover from stress [53]. In other words, phase separation and the subsequent dissipation of droplets may increase cellular fitness if it occurs at advantageous times. One challenge that cells often incur is protein misfolding which leads to stress. This process can trigger phase separation of membraneless organelles that can store and attempt to refold proteins or push them for degradation to relieve the stressor [57]. For example, disturbances in proteostasis can lead to the stalling of translation and the subsequent formation of stress granules which contain protein and RNA. Misfolded proteins in stress granules can then be cleared by chaperone protein complexes [58], and eventually the entire stress granule is dissipated by the Hsp70, Hsp40, and Hsp110 complex [59]. Therefore, the ability of a cell to respond to stress without pathological consequences can be altered by the formation of membraneless organelles.

**1.3.6 Pathological phase transitions in neurodegenerative diseases**

Although protein phase transitions have several roles in the cell, changes in the environment can cause physiological phase transitions to become pathological and lead
to disease phenotypes. Here we discuss the pathological changes that can occur such as changes in the internal dynamics, aberrant post translational modifications (PTMs), and mutations.

*Changes in internal dynamics of MLOs*

Different phase-separated proteinaceous entities are characterized by physical, dynamic, and mechanical properties that can vary in a broad range. Some of these entities are highly dynamic liquid-like droplets [60-67], whereas others, such as albinian bodies, centrosomes, nuclear pores, and amyloid bodies, are much less dynamic "bioreactive gels" with properties ranging from viscous liquids to gels and even to solid-like functional amyloids [68]. These non-dynamic "bioreactive gels" or "biomolecular condensates" are not formed instantaneously. Instead, the very first step of their biogenesis is the formation of dynamic, liquid-like droplets that quickly mature into much less dynamic structures [68]. Furthermore, although many MLOs (e.g., SGs) are liquid-like in the norm, they can mature or age into much less dynamic state, typically coinciding with the formation of fibrous structures [69]. Such maturation leads to changes in the mechanical and physical properties of cellular bodies that can be of biological importance [69]. Of great importance are recent observations that SGs are characterized by the heterogeneous (or biphasic) structures containing a core, where the proteins are more densely packed, and a more diffused shell favoring exchanges of constituents between SGs and the surrounding cytoplasm [70]. Kinetically, these different SG phases are formed at distinct stages of the SG biogenesis, with dense core being assembled at an early event in granule assembly [70]. It was also indicated that the SG maturation and
time-dependent changes in the dense core of this MLO can serve as a potential source of insoluble protein aggregates [71].

Therefore, at least for some MLOs there is a specific time of existence before pathology ensues, since aberrant biogenesis of PMLOs and their abnormal aging can be accompanied by the misfolding and pathological aggregation of MLO-residing IDPs/IDPRs, being related to the pathogenesis of various human diseases [20, 72, 73]. In other words, if the stress is sustained, but the cell finds a way to overcome death, this can generate some deleterious effects on the cell later. In fact, ‘aging’ of SGs can lead to an increase in protein aggregation, and data show that sustained SG formation results in the increased aggregation and cytoplasmic mislocalization of Tar DNA Binding Protein-43 (TDP-43) due to the loss of mobility, both of which are hallmarks in ALS [74]. Recently, it was directly shown that pathological aggregation and fibrillation of low-complexity domain (LCD) of TDP-43 was dramatically accelerated under phase separated conditions, suggesting that aberrations in the process may contribute to pathogenesis in neurodegenerative disease by promoting pathological TDP-43 aggregation [75].

Liquid droplets formed by the positively charged microtubule-binding domain of intrinsically disordered microtubule associated protein tau were shown to undergo coacervation with negatively charged molecules and this coacervation promoted amyloid fibril formation [76]. In another study, soluble tau was shown to undergo LLPS under cellular conditions, with resulting phase-separated tau droplets rapidly undergoing transition to the gel-like species that eventually matured to amyloid-like fibrils, suggesting that these droplets served as an intermediate toward tau aggregate formation [77]. Finally, many intrinsically disordered RNA-binding proteins (RBPs) possessing LCD
domains that are aggregated in patients with different neurodegenerative diseases were found in SGs, suggesting that the dynamics of SGs can be altered by inclusion of such pathology-related proteins [78].

*Aberrant PTMs and pathological phase separation*

The activity of many proteins is regulated by various PTMs. Since PTMs represent one of the sides of “biological dark matter” (which is composed of biologically important protein species that are not amendable to structural characterization by traditional tools developed to investigate ordered proteins [79]), and since many PTMs occur in IDPRs (that themselves represent another component of the ‘dark matter of biology’), it was indicated that such disorder-centered PTMs constitute the darker side of the biological dark matter [80]. Altogether, by extending the range of structures and physico-chemical properties of amino acids, PTMs play important roles in the increase in the variability and diversity of protein structures and functions [81]. In fact, due to the variability of PTMs, the actual number of chemically different amino acids typically utilized in protein biosynthesis increases from 20 to more than 140, and as many as 300 different PTMs can be found in proteins [82].

PTMs changes protein structure at many different levels by covalently adding various chemical groups (such as different small molecules, carbohydrates, lipids, and even entire proteins or nucleic acids) to amino acid side chains, or removing various chemical groups, or via enzymatic cleavage of peptide bonds. Since different PTMs can differently affect physicochemical properties of a protein [83], different modifications can graft different functions to the same protein [84]. Although natural variability of PTMs is very broad, these modifications are typically very specific. Many PTMs are catalyzed by
special enzymes that recognize motifs in target sequences of specific proteins. Some PTMs (e.g., phosphorylation, acetylation, glycosylation, lipidation, methylation, and nitration) are readily reversible due to the concert action of modifying and demodifying enzymes. Such and interplay between the conjugating and deconjugating enzymes represents an economical and rapid way of the controlling the protein function. Furthermore, although mutations (which represent another means of changing the chemical properties of a polypeptide chain) can only occur once per position, different forms of PTMs may happen in tandem [85]. Since PTMs represents crucial means for the regulation of protein structure and function, deregulation of PTMs is commonly associated with the development of various pathological conditions [80, 86, 87]. Therefore, it is not surprising that aberrant PTMs can affect disorder-based LLPSs and MLOs.

An illustrative example of this concept is given by poly(ADP-ribosylation) (PARylation), one of the PTMs associated with neurodegeneration [88]. PARylation is a reversible enzymatic attachment of multiple NAD-derived ADP-ribose (ADPr) units to target proteins. PARylation is catalyzed by a family of PARP enzymes [89, 90], whereas dePARylation is conducted by the hydrolyzing enzyme poly(ADP-ribose) (PAR) glycohydrolase (PARG) [91, 92]. Furthermore, some proteins are capable of non-covalent PAR-binding [93]. In addition to numerous physiological roles of PARylation that range from gene expression to DNA repair, mitochondrial biogenesis, neuroinflammation, and regulation of a variety of signaling pathways inducing different forms of cell death, alterations in this PTM were associated with aberrant phase transitions and pathological aggregation of several proteins associated with Alzheimer’s disease (AD), Parkinson’s disease [1], Huntington disease [38], and amyotrophic lateral sclerosis (ALS), such as α-
synuclein, TDP-43, and heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) [88]. Since PAR is characterized by a multivalent anionic polymeric structure resembling nucleic acids, and since many neurodegeneration-related proteins are RBPs that also contain the PAR-binding motifs (PBM), increased levels of PAR can directly influence amyloid aggregation of some PBM-containing proteins or modulate phase transitions in other pathology-associated RBPs, such as TDP-43 and hnRNPA1 [94], or stimulate association of some of these RBPs with SGs [88]. The picture is further complicated by the fact that hnRNPA1 can be PARyated, and this PTM facilitates the phase transition of hnRNPA1 alone and stimulates the co-phase-transitions of TDP-43 and hnRNPA1 [95]. Additionally, the LCD of RNA-binding protein hnRNPA2B1 (heterogeneous nuclear ribonucleoprotein A2B1) can undergo LLPS and promotes hnRNPA2B1-TDP-43 co-phase separation, the arginine methylation of this domain reduces the efficiency of hnRNPA2 phase separation and inhibited co-phase separation of this protein with TDP-43 [96].

The AD related intrinsically disordered microtubule-associated protein tau is known to undergo liquid-liquid phase transitions [76], efficiency of which can be affected by various mutations and PTMs, such as truncation, hyperphosphorylation, and hyperacetylation [76, 97]. Importantly, these different factors differently affected the phase transition behavior of tau, with truncation, mutation, and hyperphosphorylation enhancing tau phase transitions and aggregation [76], and with hyperacetylation disfavoring phase transitions and inhibiting the heparin-induced aggregation of this protein [97].

**Mutations and pathological phase separation**

Biogenesis of MLOs can be affected by pathological mutations in proteins either
undergoing LLPS or proteins involved in MLO regulation. For example, different properties of SGs, such as their number, mean size, lifespan and internal dynamics, as well as the SG capability to control stress suppression are all affected by TDP-43 with ALS-related point mutations [98, 99]. Similarly, biogenesis of SGs (namely, kinetics of their assembly and disassembly) is affected by ALS-related point mutations in FUS, which becomes incorporated into SGs [100]. Similarly, heterogeneous nuclear ribonucleoproteins (hnRNPs) A2B1 and A1 (hnRNPA2B1 and hnRNPA1) with ALS-related point mutations in their prion-like domains noticeably alter SG biogenesis and dynamics, being excessively incorporated into this MLO [101]. On the other hand, ALS-related point mutations in T-cell-restricted intracellular antigen-1 (TIA1) were not only impact the SG dynamics but also promoted the accumulation of stable SGs that contained TDP-43 [74]. Systematic analysis of several familial ALS-related point mutations in the proteasomal shuttle factor UBQLN2 mostly affecting the proline-rich (Pxx) region of this protein revealed that the UBQLN2 LLPS was differently affected by these ALS-linked Pxx mutations [102]. This differential effect was dependent on the type and sequence position of a given amino acid substitution, suggesting that ALS-linked Pxx mutations altered physical properties of UBQLN2, modified the in vivo behavior of this protein, and contributed to the aberrant morphology and dynamics of SGs, eventually resulting in the appearance of ALS specific inclusions [102].

It was shown that the unnatural dipeptide repeat (DPR) proteins (poly(glycine-alanine), polyGA; poly(glycine-arginine), polyGR; poly(proline-alanine), polyPA; poly(proline-arginine), polyPR; and poly(glycine-proline), polyGP) generated as a result of the hexanucleotide (GGGGCC) repeat expansion in the gene chromosome 9 open
reading frame 72 (C9ORF72), which is considered now as the most common genetic cause of ALS and frontotemporal dementia (FTD), were able to alter the liquid-like state of PMLOs [103]. Furthermore, arginine-rich DPRs (polyGR and polyPR) were shown to undergo LLPS themselves and were able to efficiently induce phase separation of a large set of proteins related to the RNA metabolism and SG biogenesis [104].

1.4 Conclusions

LLPS and the formation of MLOs is extremely important to maintain cellular fitness. Since the phenomenon is so sensitive to the conditions of the environment, the physiological functions can easily be affected by changes that naturally occur due to aging and disease. These changes can lead to pathological LLPS and membraneless organelles that lose the ability to dissipate. Loss of dynamics has been shown to lead to the pathological aggregation of proteins, something that is a common phenomenon seen in neurodegenerative disease. Therefore, maintaining or restoring the dynamics of pathological MLO’s may be an enticing avenue for therapeutic endeavors.
Figure 1.1 Diversity of membraneless organelles found in eukaryotic cells. Schematic representation of the multitude of cytoplasmic, nuclear, mitochondrial, and chloroplast membraneless organelles.
Figure 1.2 Abundance of intrinsic disorder in human PMLO-related proteins A) and in human proteome B). In both plots, consensus 3D PONDR® FIT vs. PONDR® VSL2 vs. PONDR® VLXT plot representing the correlation between the disorder content in human PMLO-related proteins A) and in human proteome B) evaluated by PONDR® VLXT (X-axis), PONDR® VSL2 (Y-axis), and PONDR® FIT (Z-axis). Following the accepted practice, two arbitrary cutoffs for the levels of intrinsic disorder are used to classify proteins as highly ordered (PADSho < 0.25), moderately disordered (0.25 ≤ PADSmd < 0.5) and highly disordered (PADShd ≥ 0.5%). The values in brackets show the content of highly disordered, moderately disordered, and highly ordered proteins in each PMLO A) or in human proteome B). Data for the human proteome (20,228 proteins) were retrieved from the Consensus Coding Sequence database.
Figure 1.3 Evaluation of the overall disorder levels and peculiarities of disorder distribution in human proteins associated with PMLOs. A) Spread of the protein-average disorder scores in individual PMLOs evaluated by PONDR® VSL2 (black bars), PONDR® VLXT (red bars), and PONDR® FIT (green bars). Bars show mean protein-average disorder scores in corresponding PMLOs, whereas error bars reflect the corresponding standard deviations calculated by SigmaPlot software. B) Box-and-whisker plot representing statistical analysis of the commonness of long IDPRs in human proteins associated with various PMLOs. In this plot, the top of each box indicates the third quartile, a horizontal line near the middle of the box indicates the median, and the bottom of the box indicates the first quartile. A vertical line extending from the top of the box indicates the maximum value, whereas a vertical line extending from the bottom of the box indicates the minimum value. Black circles represent outliers. Boxes without whiskers correspond to the PMLOs with small number of proteins (4–8).
Figure 1.4 Finding potential disorder-based protein–protein interactions sites in human MLO-related proteins. Analysis performed using the α-MoRF-Pred I (A and B), α-MoRF-Pred II (C and D), and ANCHOR algorithms (E and F). For all three tools, data are aggregated to show predicted binding regions per protein (plots A, C, and E) and predicted binding residues per residue (plots B, D, and F). In these plots, horizontal bars correspond to median values, whereas error bars show 2.5 percentile and 97.5 percentile, all estimated by 100,000 bootstrap iterations. Between 2.5 and 97.5 is the 95% confidence interval on the median.
Figure 1.5 Evaluating global intrinsic disorder predisposition of all human proteins A) and human proteins associated with various PMLOs B) by combining the outputs of binary disorder classifiers, CH-plot and CDF. Here, the coordinates of each point were calculated as a distance of the corresponding protein in the CH-plot from the boundary (Y-coordinate) and an average distance of the respective CDF curve from the CDF boundary (X-coordinate). The four quadrants correspond to the following predictions: Q1, proteins predicted to be disordered by CH-plots, but ordered by CDFs; Q2, ordered proteins; Q3, proteins predicted to be disordered by CDFs, but compact by CH-plots (i.e., putative molten globules or hybrid proteins); and Q4, proteins predicted to be disordered by both methods.
Table 1.1 Examples of some membraneless organelles and their location, size, and function.

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Location</th>
<th>Size (µm)</th>
<th>Known Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear pore</td>
<td>Nucleus</td>
<td>0.1-0.2</td>
<td>Transport of molecules across nuclear envelope</td>
</tr>
<tr>
<td>Nucleolus</td>
<td>Nucleus</td>
<td>0.2-3.5</td>
<td>Ribosome biogenesis</td>
</tr>
<tr>
<td>Cajal bodies</td>
<td>Nucleus</td>
<td>0.1-2</td>
<td>Regulation of snRNP maturation</td>
</tr>
<tr>
<td>Paraspeckles</td>
<td>Nucleus</td>
<td>0.2-1</td>
<td>Regulation of gene expression</td>
</tr>
<tr>
<td>P-bodies</td>
<td>Cytoplasm</td>
<td>0.1-0.3</td>
<td>Decay and processing of mRNA</td>
</tr>
<tr>
<td>Stress Granules</td>
<td>Cytoplasm</td>
<td>0.1-0.3</td>
<td>Store translationally stalled ribonucleoproteins</td>
</tr>
</tbody>
</table>
Chapter Two:
Protein Liquid-Liquid Phase Separation in ALS/FTD

2.1 Abstract

A microsatellite expansion mutation in C9orf72 is the most common genetic cause of Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD). The expansion mutation leads to C9orf72 loss of function, RNA foci, and five species of non-AUG RAN translated dipeptide repeat proteins (GA, GP, GR, PA, and PR). More than one peptide species can be present in the same cell in a patient with the expansion, however the interplay between the different species has not been established. Here we determined that PR causes cytotoxicity, induces the spontaneous formation of poorly dynamic stress granules, and phase separates with bound RNA. However, co-transfection of PR and GA ablates the cytotoxicity of PR alone and absolves the appearance of spontaneous stress granules. In addition, we showed that the interaction between synthetic GA and PR polypeptides leads to the formation of a protein complex which is structurally and biochemically distinct from the individual comprising components. Thus, the combined expression of distinct C9orf72-derived dipeptide repeat species produces cellular outcomes and structural differences that are unique compared to the expression of a single species.

Portions of this work were previously published (Darling, A.D. et al., 2018) and are used with permission of the publisher.
2.2 Introduction

An increasing number of neuromuscular and neurodegenerative disorders are being classified as microsatellite expansion disorders due to the pathological link to genetic mutations in specific microsatellite regions. These regions consist of tandem DNA repeats that are prone to strand slippage during replication, which leads to the addition or deletion of repetitive segments and a high level of length variation in repeat sequences among individuals [105]. Strand slippage can lead to the accumulation of large repetitive elements in the genome which can cause pathology via three separate mechanisms. Expansions can lead to a loss of function in the gene that it is located within, production and accumulation of toxic RNA species that sequester RNA-binding proteins, and a gain of novel toxic function from the translation of aberrant polypeptide species [106].

Two diseases with pathology driven by microsatellite expansions include amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). A link connecting these two disorders was discovered when two groups identified a hexanucleotide repeat expansion [107] in the 5’ non-coding region of the gene C9 open reading frame 72 (C9orf72) gene [108, 109]. The discovery of the link between the two diseases has led to the intensive investigation to determine how this aberration leads to pathology and the possibilities are shown in Figure 2.1. After the genetic link was discovered, two groups identified aggregation-prone dipeptide repeat (DPR) proteins GA, GP, and GR in CNS tissue from patients with the C9orf72 expansion mutation [110, 111]. Later, it was determined that the antisense species can also be translated leading to the discovery of PA, PR, and PG polypeptides [112]. The absence of a start codon
indicates these dipeptide species are most likely by repeat-associated non-ATG (RAN) translation [112].

Although six DPR species are translated because of C9orf72’s HRE, the biochemical differences between these peptide species causes them to behave differently in cells and produce diverse cytotoxic outputs. The arginine-rich polypeptides GR and PR, which are highly charged, have been shown to be the most toxic species in Drosophila, yeast, and mammalian primary neurons [113-117], whereas GA is able to form toxic amyloid species that can be spread between cells and cause toxicity [118, 119]. However, the exact mechanism by which DPRs cause cell toxicity remains unclear. Studies conducted to examine the cellular effects of DPR expression primarily focus on the effects driven by the expression of a single DPR. However, both sense and anti-sense RNA foci for C9orf72 expansions have been identified in the same cell; suggesting that multiple DPRs can be translated simultaneously [120, 121]. The phenomena of multiple DPR expression was recently explored in a report which identified GA as the most abundant DPR expressed in the frontal cortex of patients with expanded C9orf72 and that GA could sequester other DPRs [122]. However, additional studies are necessary to more thoroughly evaluate the cellular effects of DPR co-expression.

Here we examined the toxicity produced by individual DPR expression. We identified PR as the most toxic DPR species in cell models capable of modulating the behavior of membraneless organelles. Since GA was suggested to be the most prominent DPR species, we then investigated potential interactions between PR and GA. We showed that expression of GA can ablate PR-driven toxicity. We determined
that the combination of GA and PR reduced markers of ER stress and lowered the number of stress granules present. We then demonstrate that the altered toxicity is likely due to significant structural changes which sequester PR and GA. Our data illustrates the complex biological interactions that occur when multiple DPR variants are simultaneously expressed and highlights the need for further investigative studies into how DPR expression affects the disease state.

2.3 Materials and methods

2.3.1 Cell Culture and Transfection

NSC34, HEK293T, and HT22 cells were cultured in DMEM with 10% FBS and kept at 37°C with 5% CO₂. NSC34 and HT22 transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. PEI transfection was used for the HEK293T cells. The DPR-containing plasmids used to transfect cells included pEGFP-GA(50), pEGFP-GP(47), pEGFP-GR(50), pEGFP-PA(50), and pEGFP-PR(50) and were all generous gifts from Dr. Leonard Petrucelli (Mayo Clinic, Jacksonville, FL). Other plasmids used included pCDNA3.1-p62-HA and pEGFP-C-1 (Clontech, Mountainview, CA).

2.3.2 Primary Neuron Preparation and Transfection

All procedures involving the experimentation on animals were done in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of the University of South Florida. Cortico-hippocampal neurons were prepared using a previously developed protocol [123]. Briefly, P0 mouse pups were extracted from the womb, their brains were removed, cortices dissected in cold isotonic buffer, washed, digested in trypsin, triturated, and resuspended in DMEM supplemented
with 10% FBS and Antibiotic-Antimycotic Solution (Fisher Scientific, Waltham, MA). The cells were then plated on poly-l-lysine (1:5; Sigma, St. Louis, MO) coated coverslips. 24 hours after plating, the media was changed to Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B27 (Life Technologies, Carlsbad, CA) and Glutamax (Life Technologies, Carlsbad, CA). Neurons were transfected using Lipofectamine LTX (Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions.

2.3.3 Antibodies

The following primary antibodies were used in this study: p-PERK (Thr 981) (1:500 Western blot, 1:200 immunofluorescence, Cat. sc-32577, Santa Cruz, Dallas, TX), PERK (C33E10) (1:1000, Cat, 3192S Cell Signaling, Danvers, MA), Anti-C9ORF72/C9RANT (poly-GA) (1:1000 Western blot, 1:200 immunofluorescence, Cat. MABN889, EMB Millipore, Billerica, MA), Anti-C9ORF72/C9RANT (poly-PR) (1:1000 Western blot, 1:200 immunofluorescence, Cat. ABN1354, EMB Millipore, Billerica, MA), TIA-1 (1:200 immunofluorescence, Cat. Sc-1751, Santa Cruz, Dallas, TX), SQSTM1/p62 (cat. 5113, Cell Signaling, Danvers, MA), GFP Tag Antibody (1:1000, Cat. G10362, Invitrogen, Carlsbad, CA), and Actin (1:1000, Cat. A2066, Sigma, St. Louis, MO). Secondary antibodies used include Alexa-Fluor fluorescently labelled secondary antibodies (1:500, Invitrogen, Carlsbad, CA) and horseradish-peroxidase linked secondary antibodies (1:1000, Southern Biotech, Birmingham, AL).

2.3.4 Cell Staining and Imaging

NSC34, HT22, and primary neurons were prepared for staining 24 hours post transfection. Cells were washed with 1× PBS, fixed in 4% paraformaldehyde (w/v) at room temperature for 20 minutes, and then permeabilized for 10 minutes with 1× PBS
containing 0.1% of Triton X-100. Cells were then blocked with 1× PBS containing 10% goat or donkey serum (Lampire Biological Laboratories Inc. Pipersville, PA). Primary antibody incubations were performed overnight at 4°C, and secondary antibodies were incubated for 1 hour at room temperature. DAPI was added at a concentration of 1:5000 in 1× PBS for 5 minutes at room temperature. The cover-slips were mounted onto glass slides using Prolong Gold mounting reagent (Life Technologies, Carlsbad, CA). An Olympus FluoView FV10i confocal microscope equipped with a 60× UIS2 SAPO objective was used for imaging. Quantitative analysis was performed using ImageJ software (National Institutes of Health).

2.3.5 Peptide Staining and Imaging

0.5 µg of the peptides were added to cover-slips coated with 0.2% electron microscopy grade glutaraldehyde (Electron Microscopy Services, Hatfield, PA) for 2-hours at room temperature. They were then washed with 1x TBS, blocked with 1% BSA in TBS overnight at 4°C, incubated with the primary antibody for 1-hour at room temperature, washed with TBS, and then incubated with the secondary antibody for 1-hour at room temperature. The coverslips were then mounted and slides were imaged as described above.

2.3.6 Western Blotting

Cells were lysed using RIPA protein extraction reagent (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 1% NP-40, 0.5% sodium dodecyl sulfate) containing 1mM of PMSF as well as a protease inhibitor mixture (Sigma, St. Louis, MO). 40 µg of the total extracted protein was run on 5-15% gradient SDS-PAGE gels (Bio-Rad, Hercules, CA). The protein was then transferred to a PVDF
membrane (Amersham Biosciences, Little Chalfont, United Kingdom), blocked with 7% nonfat milk for 1 hour, incubated with the primary antibody overnight at 4°C, and then incubated with the secondary antibody for 1 hour at room temperature. ECL (Fisher Scientific, Waltham, MA) was used to develop blots imaged using a LAS-4000 mini imager (GE Healthcare, Little Chalfont, United Kingdom). ImageJ software (National Institutes of Health) was used to perform densitometry and samples were normalized to the loading control.

2.3.7 Recombinant Protein Production and Purification

A 50-repeat PR plasmid was synthesized by Genscript (Piscataway, NJ) in a pet22b vector with a C-terminal GB1 solubility tag. The 50-repeat GA plasmid was in the pGEX6P.1 vector and was a generous gift from Dr. Leonard Petrucelli (Mayo Clinic, Jacksonville, FL). E. coli BL21 cells were transformed with the plasmids above then grown at 37°C in LB media containing 100 μg/mL carbenicillin. Once their OD600 reached 0.8 the cells were induced with 1mM of IPTG (Gold Biotec hnology, Olivette, MO) for 2 hours. Centrifugation at 5,000 g for 15 min was used to harvest the cells, which were then resuspended with either 1× PBS (GA50) or Nickel chromatography running buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 mM Imidazole) (PR50) containing protease inhibitors. The cells were then lysed using a freeze-thaw cycle followed by sonication. The lysed cells were centrifuged at 50,000 g for 1 hour at 4°C. The GA50 supernatant was affinity purified using a standard gravity column packed with Glutathione Agarose Resin (Fisher Scientific, Waltham, MA), while PR50 affinity purification was performed using a HisPur™ Ni-NTA Resin (Fisher Scientific, Waltham, MA). The eluted fractions were dialyzed into 1× PBS overnight and concentrated using
an EMD Millipore Amicon™ Ultra-15 Centrifugal Filter Unit (Fisher Scientific, Waltham, MA).

2.3.8 Peptide Synthesis

The GA$_{20}$ and PR$_{20}$ polypeptides were synthesized by DgPeptidesCo., LTD (Hangzhou, China) using the following amino acid sequences:

GA(20):N’GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA GW
DYKDDDDK
PR(20):N’PRPRPRPRPRPRPRPRPRPRPRPRPRPRPRPRPRPRPRPRPRPRPRPRPRRWYP
YDVPDYA

Aromatic residue (tryptophan) was added at the C-terminal regions of the synthesized peptides to simplify their spectroscopic analyses. Distinct C-terminal tags, FLAG (DYKDDDDK; GA$_{20}$) and hemagglutinin (YPYDVPDYA; PR$_{20}$), were added to each DPR.

2.3.9 Isothermal titration calorimetry (ITC)

Solution of the poly-GA polypeptide (10 µM) in PBS (10 mM phosphate, 150 mM NaCl, pH 7.5, 1.7 ml) was placed in the sample cell of the VP-ITC calorimeter (Malvern Instruments) and incubated at 25 °C. A solution of the poly-PR polypeptide (20 µM) in the same buffer was added (19 injections, 15 µl each) and ITC measurements performed. Data was analyzed with Origin software.

2.3.10 Circular dichroism (CD)

Far-UV CD (190–260 nm) spectra of synthesized polypeptides were measured using a JASCO J-815 spectropolarimeter at 25 °C. A solution of polypeptide (250 µl, 0.1 mg/ml) in the appropriate buffer (10 mM) was incubated for 1 h at 25 °C, placed into a 1
mm pathlength cell, and the CD spectra were acquired with 10 nm/min scan speed at 0.2 nm step size and 1.0 nm bandwidth under constant purging with nitrogen. Two spectra were accumulated and averaged for each sample. Spectra in the presence of 0.15 M NaCl were measured in the 0.2 mm path-length cell at 0.5 mg/ml protein concentration (60 µl sample volume).

Buffers used were citrate (pH 2.0-3.0), acetate (pH 4.0-5.0), phosphate (pH 6.0-7.5), Tris (pH 8.0) and borate (pH 9.0-10.0). The same buffers were used for CD, fluorescence and DLS measurements.

2.3.11 Fluorescence spectroscopy

The solution of synthesized polypeptide (13.3 µl, 1 mg/ml stock solution, 5 µg/ml final concentration) was mixed with buffer (final volume 400 µl, final buffer concentration 10 mM). The solution was incubated for 1 h at 25 °C, and the intrinsic protein fluorescence was measured. Excitation wavelength was 280 nm and emission spectrum was recorded in the 295-380 nm range. Excitation and emission slits were at either 2.5 or 5 nm. The measurements were performed in duplicate for each sample.

2.3.12 Dynamic light scattering (DLS)

100 µl of the synthesized polypeptide solution (0.1 mg/ml) in the appropriate buffer (10 mM) was placed in the well of the 96-well clear bottom plate (Corning, Corning, NY), and dynamic light scattering was measured. DynaPro plate reader (Wyatt Technology, Goleta, CA) equipped with an 830 nm laser and a temperature control module. Ten 30-s measurements were taken for each well, and the measurements were performed in duplicate. The Dynamics software (Version 7.0.1, Wyatt Technology Corporation) was used for scheduled data acquisition and analysis.
2.3.13 Nanoparticle tracking analysis

Samples were diluted 500-fold into 1 mL of 0.02 μm filtered de-ionized water. Approximately 300 μl of sample was loaded onto the Malvern Nanosight LM10 equipped with a 633 nm red laser. Protein particle data were captured with a Marlin CCD camera in duplicate. Graphs were generated by Nanosight software.

2.3.14 Transmission electron microscopy

100 μM of each recombinant GA$_{50}$, PR$_{50}$, and GA$_{50}$+PR$_{50}$ was adsorbed onto prewashed 200 mesh formvar/carbon-coated copper grids for 5 min. The grids were washed with water (10 μl), stained with filtered 2% uranyl acetate (10 μl) for 1 min and washed with water again. The samples were analyzed with a JEOL 1400 Digital Transmission Electron Microscope, and images were captured with a Gatan Orius wide-field camera at the Electron Microscopy Core Facility in the College of Medicine at the University of South Florida.

2.3.15 Image Analysis and Statistics

Quantitative analysis was performed using ImageJ software (National Institutes of Health). The statistical significance performed for each analysis was done using ANOVA with Tukey post tests for group comparison as well as Student’s t-tests.

2.4 Results

2.4.1 Co-Expression of PR with GA Alters the Localization, Cytotoxic Output, and Morphology of PR

We began by characterizing the cytotoxicity of each individual DPR in a cell culture system. Expression of PR$_{50}$ was the most potent reducer of cell viability, though each DPR reduced cell viability relative to the control transfection (Figure 2.1A). Since
PR$_{50}$ showed the highest toxicity, we examined if the co-expression of the most abundantly expressed DPR, GA$_{50}$, altered the effects of PR$_{50}$, similar to the sequestration of other DPRs by GA, as previously reported [122]. To examine this, we expressed GA$_{50}$ alone or in tandem with an increasing amount of PR$_{50}$. GA$_{50}$ ablated PR$_{50}$ toxicity when expressed at ratios of 10:1 and 5:1 (GA$_{50}$:PR$_{50}$) (Figure 2.1B). We found this result surprising due to precedent in the literature for GA presenting cellular toxicities [118, 119, 122].

Next, we noticed that PR$_{50}$ was always expressed in the nucleus, but GA$_{50}$ was cytoplasmic (Figure 2.1C). We wanted to see if the localization of PR$_{50}$ was altered because of co-expression with GA$_{50}$. We found that when PR$_{50}$ and GA$_{50}$ are co-expressed, it results in a significant change to the localization of PR$_{50}$ with a strong reduction in nuclear species (Figure 2.1C and 2.1D).

2.4.2 PR expression leads to PERK phosphorylation and the spontaneous formation of stress granules, GA rescues effect

Since the co-expression of GA$_{50}$ with PR$_{50}$ rescued PR$_{50}$-driven cytotoxicity, we examined other PR-associated cellular stresses to identify the cell stress mechanism being ablated by GA co-expression. Specifically, we were interested in looking at the PERK pathway of the unfolded protein response due to the fact that other disease-related repeat peptides induce cell death in this manner [124]. We found that expression of PR$_{50}$ in HEK293T cells (Figure 2.2A) and primary neurons (Figure 2.2C) increased PERK phosphorylation; a cellular event associated with a response to cellular stress, which can trigger apoptosis [125]. The expression of the other DPR species did not lead to any significant differences in the levels of phosphorylated PERK as compared to the
control, apart from PA$_{50}$. We then wanted to see if the increase in PERK phosphorylation produced by PR$_{50}$ expression could be altered by co-expression with GA$_{50}$ in primary neurons. We co-transfected primary neurons with GA$_{50}$ and PR$_{50}$, then immunostained these cells for p-PERK. We found that co-expression of GA with PR significantly decreased phosphorylated PERK levels compared to PR$_{50}$ alone (Fig. 2.2C and 2.2 D).

The phosphorylation of PERK is an event that occurs and can trigger stress granule formation. Since literature has shown that expression of PR$_{50}$ can lead to aberrations in stress granules, we wanted to explore this. To do that, we expressed PR$_{50}$ alone or in combination with GA$_{50}$ then used immunostaining of TIA-1, a resident stress granule protein, to quantify the number and size of stress granules. We found that expression of PR$_{50}$ was able to significantly increase the spontaneous formation of stress granules but did not change the size (Figure 2.3A and 2.3B). Additionally, we found that co-expression with GA$_{50}$ resulted in a rescue of this effect (Figure 2.3A and 2.3B).

### 2.4.3 GA and PR Interact and Co-Localize in a Cell-Free Environment

We then speculated that the ablation of PR$_{50}$ toxicity was due to DPR sequestration driven by structural changes. To examine this, PR$_{50}$ and GA$_{50}$ were incubated separately or together in the 5:1 ratio observed to reduce PR$_{50}$ toxicity. Using transmission electron microscopy (TEM), we observed that the combination of PR$_{50}$ and GA$_{50}$ presented a structure distinct from either individual DPR (Figure 2.4A).

The observed structural/morphological uniqueness of the PR and GA combination prompted us to characterize this interaction further. To more efficiently
address structural issues, 20-repeat GA and PR peptides were synthesized (GA\textsubscript{20} and PR\textsubscript{20}). A tryptophan residue was added near each C-terminus and distinct C-terminal tags, FLAG (DYKDDDDK; GA\textsubscript{20}) and hemagglutinin (YPYDVPDYA; PR\textsubscript{20}), were added to each DPR. We first utilized isothermal titration calorimetry (ITC) to monitor binding of GA\textsubscript{20} and PR\textsubscript{20}. After optimization of assay conditions, we found that these polypeptides interacted at \(\sim 5:1\) GA to PR ratio at pH 7.5 in the presence of 150 mM NaCl (Figure 2.4B); the same ratio at which GA negated PR toxicity in our cellular experiments and we observed a dramatic morphological change. The binding between GA and PR had a high affinity with a binding constant \(\sim 100\) nM and was favored both enthalpically and entropically. This binding affinity is similar to that observed for binding of proteins to other highly charged biopolymers, such as heparin [126]. We then performed peptide-immunostaining to confirm peptide interaction following co-incubation. Peptide immunostaining demonstrated that the GA\textsubscript{20} and PR\textsubscript{20} peptides do co-localize in a cell free environment (Figure 2.4C). (Rcoloc=0.830).

2.4.4 Co-Incubation of PR with GA Leads to Structural Changes in Synthetic Polypeptides

To further characterize the structures formed by the interaction of GA and PR, we used dynamic light scattering (DLS) to examine the particle sizes of GA, PR, or the combination of GA and PR. Particle size analysis revealed that co-incubation of GA\textsubscript{20} and PR\textsubscript{20} produced particles larger than either individual DPR (Figure 2.5A). As a supplement to these DLS results we used nanoparticle tracking analysis to follow protein aggregation by directly observing protein aggregates with light microscopy and tracking their Brownian motion [127]. Similar to our DLS observations, we recorded
different populations of aggregates of various sizes for individual polypeptides.

However, the nanoparticle tracking analysis of co-incubated GA$_{20}$ and PR$_{20}$ reported that the resulting aggregates were larger in size and occurred at a higher frequency than either individual peptide (Figure 2.5B). These results supported the presence of an interaction between GA$_{20}$ and PR$_{20}$ in a cell-free environment.

Next, we examined the secondary structure of these peptides using far-UV circular dichroism (CD) spectroscopy. We measured far-UV CD spectra of GA$_{20}$ and PR$_{20}$ individually and in combination at physiological pH and salt concentrations to determine whether interaction results in structural changes. We found that a spectrum of GA$_{20}$ has a minimum at 215-217 nm, which is typical for the proteins with β-sheet rich structures (Figure 2.5C). Spectra of PR contained a minimum at 198-200 nm typical for highly disordered polypeptides (Figure 2.5C). Spectra of the mixtures of these polypeptides contained both spectroscopic bands as expected. To determine whether structural changes occurred upon mixing of the polypeptides, the spectra of individual polypeptides were subtracted from the spectrum of their mixture (Figure 2.5C; dashed line). The results indicated that there is a loss of β-sheet structure and appearance of disordered structure upon mixing (Figure 2.5C), which is an additional indication of interaction between these polypeptides.

We then analyzed the dependence of the GA$_{20}$ and PR$_{20}$ complex on chemical factors such as pH. First, we monitored aggregation of both polypeptides individually and as a mixture by dynamic light scattering over the 2-10 pH range. Dynamic light scattering data showed that GA$_{20}$ is highly aggregation prone at pH 3 while PR$_{20}$ does not aggregate (Figure 2.5D; red and blue lines, respectively). Co-incubation of these
polypeptides resulted in the shift of the aggregation optimum from pH 3 to physiological pH (pH 6-8) (Figure 2.5D; purple line), an indication of direct interaction between the polypeptides. We then examined if secondary structure formation was altered across the same pH range. We recorded the far-UV CD spectra of either the individual polypeptides or the combined GA_{20} and PR_{20} in solutions with a pH range of 2-10. Similar to our observations at physiological pH, we found that the spectrum of GA_{20} has a negative band at 215-217 nm typical for β-sheet-rich structures with minimal alterations occurring across the pH range (Figure S2.1A) PR_{20} maintained a minimum around 198-200 nm across pH range (Figure S2.1B). The mixture of GA_{20} and PR_{20} demonstrated no structural changes at low pH values (pH 2-4). However, at higher pH values (pH 6-10), we observed a loss of β-sheet structure and the appearance of disordered structure as indicated by characteristic changes in the far-UV CD spectra (Figure 2.5E).

An additional measure of protein structure is intrinsic fluorescence of aromatic residues [128]. The addition of tryptophan (Trp) residues near the C-terminus allowed us to examine intrinsic fluorescence as a measure of the hydrophobicity of the polypeptide at different pH values (schematically represented in Figure 2.6). We found that the Trp environment in PR_{20} was hydrophilic at all pH values (λ_{em} 349 nm), whereas the Trp environment in GA_{20} was moderately hydrophobic (λ_{em} 339 nm) at pH below 4 and became less hydrophobic (λ_{em} ~343 nm) at higher pH values (Figure 2.5F). We generated a calculated fluorescent value for the Trp environment of a mix of GA and PR (λ_{em} ~345 nm; Figure 2.5F, dashed line). Interestingly, the measured Trp environment from the 1:1 mix of GA_{20} and PR_{20} became steadily more hydrophobic with increasing
pH; distinct from the mathematically averaged $\lambda_{em}$ values (Figure 2.5F). These data suggest the interaction between GA$_{20}$ and PR$_{20}$ produces a distinct secondary or tertiary structure, not observed with either GA$_{20}$ or PR$_{20}$ independently.

We also assessed if the structures formed by GA$_{20}$ and PR$_{20}$ are dependent on physiological salt concentrations. We again monitored the Trp environment in the polypeptides over a 2-10 pH, now in the absence of salt. Similar to conditions with physiological salt concentrations (see: Figure 2.5F), we found that PR$_{20}$ was fairly hydrophilic over the pH range ($\lambda_{em}$ 349 nm). GA$_{20}$ was hydrophobic ($\lambda_{em}$ 339 nm) below pH 4, but loses hydrophobicity ($\lambda_{em}$ ~343 nm) at higher pH values (Figure 2.5G). We found that the co-incubation of GA$_{20}$ and PR$_{20}$ produced a Trp environment intermediate to those observed in GA$_{20}$ and PR$_{20}$ individually ($\lambda_{em}$ ~345 nm), and the environment became more hydrophobic when pH values increased (Figure 2.5G). We again compared the calculated average of $\lambda_{em}$ values of each polypeptide and saw that it diverged from the values measured for the GA$_{20}$-PR$_{20}$ mixture, indicating the formation of a distinct structure when the polypeptides are present at physiological pH values (Figure 2.5G).

Since both GA$_{20}$ and PR$_{20}$ contain Trp residues, the extent of contribution of each of these polypeptides to the observed change in Trp environment hydrophobicity is not immediately obvious. To investigate it further and validate our method, we incubated GA$_{20}$ with high molecular weight poly-lysine (poly-K). Though poly-K is not a perfect mimic of PR$_{20}$, we anticipate the poly-K/GA$_{20}$ interaction to present similarly to GA$_{20}$ and PR$_{20}$ due to the highly positively charged nature and polarity of poly-K. Additionally, poly-K doesn’t contain aromatic residues; thus, any changes in the fluorescence
emission wavelength are specific to GA\textsubscript{20}. We observed no significant change in Trp emission wavelength upon addition of poly-K to GA\textsubscript{20} (Figure 2.5G). This indicates that the secondary and tertiary structures adopted by GA and PR following incubation are unique to these peptides and are not a generic structural state which occurs when GA is present with positively charged peptides. Together, these results suggested that the interaction between GA\textsubscript{20} and PR\textsubscript{20} results in the formation of a structure that is unique to the complex between these two DPR species and is dependent on salt and pH conditions.

\textbf{2.5 Discussion}

In this study we identified a unique interaction between GA and PR which presents dramatic effects on cell health and stress pathways. Though PR\textsubscript{50} presented as the most cytotoxic DPR species, co-expression with GA\textsubscript{50} abrogated this toxicity. An examination of the interaction occurring between recombinant and synthesized PR and GA polypeptides demonstrated a clear interaction presenting distinct and discrete structural, biophysical, and morphological properties, as well as an increased aggregation propensity. Taken together, these results indicate that the simultaneous expression of multiple DPR species influence the cytotoxic output of cells in ALS/FTD patients with the C9 expansion mutation.

In previous studies it has been shown that PR has a strong proclivity for interacting with proteins that contain low complexity domains (LCD) [129, 130]. Low complexity domains are extremely abundant in eukaryotes and are generally composed of amino acid repeats [131]. The promiscuity of binding exhibited by PR to LCD-containing proteins has been hypothesized to interfere with several aspects of cellular
functioning causing alterations in homeostasis and cytotoxicity [130]. Proteomic analysis of PR showed that it was able to interact with ALS associated proteins FUS, hnRNPA1, and hnRNPA2B1 [115, 129], all of which contain LCDs thought to mediate assembly into higher ordered structures such as liquid droplets [132]. Furthermore, it has been shown that the binding of PR to LCDs stabilizes these structures [115, 129]. Interestingly, ALS associated mutations in these proteins have also been shown to lead to the stability of higher ordered structures and an increase in membrane-less organelles such as stress granules [20, 73, 133]. The main mutations associated with ALS/FTD have convergence based on ability to modify properties of membrane-less organelles such as stress granules. The ratio of expressed DPRs can modify this.

GA, similar to other ALS pathogenic proteins, can be considered as a low complexity sequence due to its repeated glycine and alanine residues. The chemical nature of the amino acid repeats in GA is somewhat like that contained in the FG repeats in the LCD domain of Nups. Our studies demonstrated that PR can interact with GA. When GA and PR interact, there is a morphological change in the peptides that present as spherical liquid-like or highly aggregated higher ordered structures. Since PR binding to LCDs of ALS related proteins promotes the stabilization of higher ordered structures such as liquid droplets and dense aggregates, it is conceivable that the interaction between PR and GA promotes a stabilized higher-order structure that sequesters the toxic PR, and GA, species; preventing the toxicities associated with either individual DPR. These stabilized higher-order structures containing GA and PR may have an increased ability to be recognized and cleared by the cell, possibly through autophagy; however, additional studies are necessary to confirm this hypothesis.
Collectively, our results indicate that the specific DPR species being expressed in a cell, as well as the ratio between different species expressed simultaneously may influence the cytotoxic output of cells in ALS/FTD patients with the C9 expansion mutation. Therefore, further work needs to be done to explore which specific ratios may be exploited to induce or attenuate DPR mediated cytotoxicity and through what mechanism that is occurring by. By elucidating this fact, there will be a clearer pathway for therapeutic developments since the phenomenon of simultaneous DPR expression has been shown to occur in patient tissue.
Figure 2.1 Schematic showing how the expansion mutation in C9orf72 can lead to toxicity. The potential mechanisms for pathology are shown in red.
Figure 2.2 DPR Localization and Toxicity  (A) NSC34 cells were transfected with 50-repeat GFP-tagged DPR plasmids for 72 hours, with Alamar Blue being added for the last 4 hours. Absorbance readings taken at 570nm showed that PR$_{50}$ significantly reduced cell viability and/or cell growth (mean +/- SEM, ANOVA with Tukey tests, *P ≤ 0.05, ***P ≤ 0.001; n=3). (B) Co-transfection of varying amount of PR$_{50}$ with 250ug of GA$_{50}$ in the same Alamar Blue assay as that listed above, resulted in a rescue of cell viability and/or growth compared to PR$_{50}$ expression alone at specific ratios (mean +/- SEM, ANOVA with Tukey tests, *P ≤ 0.05; n=3). (C) NSC34 cells were transfected with 50-repeat GFP-tagged GA or 50-repeat mCherry-tagged PR for 48 hours then fixed, stained, and imaged. Scale bar 10µm. (D) Quantification of the nuclear/cytoplasmic ratio of PR$_{50}$ in the NSC34 cells showed that localization changes to be less nuclear when co-expressed with GA$_{50}$ (mean +/- SEM, t-test, P=0.0048; n=12).
Figure 2.3 GA Rescues PR Induced Increases in pPERK (A) NSC34 cells were transfected with GFP-tagged 50-repeat DPR plasmids for 48 hours and the levels of PERK phosphorylation were measured by immunoblotting. (B) Quantitation showed that PR_{50} significantly increases PERK phosphorylation, while the others did not have an effect (mean +/- SEM, ANOVA with Tukey tests, *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; n=2). (C) Primary murine neurons were transfected with the same plasmids listed above individually and co-transfected with GA_{50} and PR_{50}. They were then immunostained for phosphorylated PERK. Scale bar 10nm. (D) The quantitative analysis of PERK levels showed PR_{50} significantly increased levels, but this was rescued by co-transfection with GA_{50} (mean +/- SEM, ANOVA with Tukey tests, **P ≤ 0.01; ***P ≤ 0.001; n=11).
Figure 2.4 GA Rescues PR Induced Increases in Stress Granule Formation (A) NSC34 cells were transfected with GFP-tagged 50-repeat DPR plasmids for 48 hours and the levels of TIA-1 were measured by immunofluorescence. (B) Quantitation showed that PR significantly increases the number but not size of TIA-1 positive stress granules, and co-transfection of GA rescues this effect (mean +/- SEM, ANOVA with Tukey tests, ***P ≤ 0.001; n=2).
Figure 2.5 GA and PR Interaction Characterization (A) Transmission electron microscopy was used to visualize recombinant 50-repeat GA, PR, and the combination. Images revealed that upon co-incubation of recombinant GA<sub>50</sub> with PR<sub>50</sub> there was a dramatic structural change. Scale bar 100nm. (B) Isothermal titration calorimetry was utilized to evaluate direct binding of GA with PR. 10µM of GA<sub>20</sub> in PBS was placed in a sample cell of the VP-ITC calorimeter and a 20µM solution of PR<sub>20</sub> in PBS was injected (19 injections, 15µl each) while ITC measurements were performed. After the run was complete the data was analyzed using Origin software. It revealed that the polypeptides interacted at a GA to PR ratio of 5:1 in the presence of 150mM NaCl at pH 7.4. (C) 20-repeat GA-Flag, PR-HA, or a combination were fixed, immunostained with HA and Flag, and imaged. Imaging showed that upon co-incubation the polypeptides are more aggregation prone and colocalize in-vitro (Rcoloc=0.830). Scale bar 10µm.
Figure 2.6 Physical Properties of GA and PR Interaction. (A) Light scattering intensity of GA\textsubscript{20} and PR\textsubscript{20} incubated either individually or in combination (0.1 mg/ml) at 25°C at pH 7.0 in the presence of 0.15 M NaCl. (B) Nanoparticle tracking analysis of GA\textsubscript{20} and PR\textsubscript{20} polypeptides incubated either individually (0.1 mg/ml) or in combination at 25°C at pH 7.5. Red–GA\textsubscript{20}; blue–PR\textsubscript{20}; purple–1:1 mixture of GA\textsubscript{20} and PR\textsubscript{20}. (C) Far UV CD spectra of GA\textsubscript{20} (red line), PR\textsubscript{20} (blue line), and their mixture (purple line) at pH 7.5 in the presence of 0.15 M NaCl. (D) Light scattering intensity of GA\textsubscript{20} and PR\textsubscript{20} incubated either individually or in combination (0.1 mg/ml) at 25°C at different pH values. Red–GA\textsubscript{20}; blue–PR\textsubscript{20}; purple–1:1 mixture of GA\textsubscript{20} and PR\textsubscript{20}. (E) Far UV CD spectra of combined GA and PR peptides at different pH values. (F) Intrinsic fluorescence emission wavelength of GA\textsubscript{20} and PR\textsubscript{20} incubated individually or in combination (0.025 mg/ml) at 25°C at different pH values in the presence of 0.15 M NaCl. Red–GA\textsubscript{20}; blue–PR\textsubscript{20}; purple–1:1 mixture of GA\textsubscript{20} and PR\textsubscript{20}; black–an average of intrinsic fluorescence emission wavelengths of GA\textsubscript{20} and PR\textsubscript{20}. (G) Intrinsic fluorescence emission wavelength of GA\textsubscript{20} and PR\textsubscript{20} polypeptides incubated either individually or in combination (0.025 mg/ml) at 25°C at different pH values. Red–GA\textsubscript{20}; blue–PR\textsubscript{20}; purple–1:1 mixture of GA\textsubscript{20} and PR\textsubscript{20}; black–an average of intrinsic fluorescence emission wavelengths of GA\textsubscript{20} and PR\textsubscript{20}, green–1:1 mixture (by weight) of GA\textsubscript{20} and poly-K.
Figure 2.7 Schematic of the proposed tryptophan (W) environment in the different conditions. With GA_{20} only the tryptophan is buried in a hydrophobic environment while in PR_{20} only it is freely interacting with the aqueous environment. Upon interaction of the two peptides, the tryptophan in PR_{20} becomes more buried and the environment more hydrophobic.
Figure S2.1 CD spectra of GA and PR at different pHs (A) Far UV CD spectra of GA\textsubscript{20} at a 2-10 pH range. (B) Far UV CD spectra of PR\textsubscript{20} at the same pH range.
Chapter Three:
Protein Aggregation and Liquid-Liquid Phase Separation in Alzheimer’s Disease

3.1 Abstract

Alzheimer’s disease (AD) is a progressive fatal neurodegenerative disease with no cure. The hallmarks of disease include extracellular protein plaques and intracellular tangles of aggregated protein. The intracellular tangles consist of the microtubule associated protein tau (MAPT, tau). Preventing the pathological aggregation of tau may be an important therapeutic approach to treat disease. In this study we show that small heat shock proteins, specifically heat shock protein 22kDa (Hsp22), can prevent the aggregation of tau in vitro. Additionally, we show that tau can undergo liquid-liquid phase separation which causes it to have an increased aggregation rate and reduced dynamics. We demonstrate that Hsp22 can impact the aggregation and behavior of phase separated tau.

3.2 Introduction

Tau aggregation is a hallmark in AD and promotes neuronal loss and cognitive decline [134-138]. Additionally, there are several other neurodegenerative disorders that are characterized by the aberrant aggregation of tau referred to as tauopathies such as progressive supramolecular palsy and frontotemporal dementia [139, 140]. When tau aggregates it forms a structure distinguished by β-sheets that can form long fibers that promote misfolding of other protein species known as amyloid [141]. While it has long been recognized that hyperphosphorylation of tau can cause it to dissociate from
microtubules and facilitate self-interaction which can lead to aggregation [142], the exact mechanism that causes tau to become pathological has remained enigmatic. However, strategies to reduce tau aggregation and increase degradation of aberrant species have been shown to be effective in restoring cognitive decline in AD mouse models and cellular functioning [143, 144].

One strategy that has been used is to increase the expression of chaperone proteins which are involved in maintaining cellular proteostasis [145-150]. Depending on the chaperone protein, they can lead to an increase or decrease in tau loads [145, 146, 148-151]. Additionally, modifications of chaperones can cause them to have an increased ability to chaperone tau or conversely lose the ability altogether [151].

Studies using the small heat shock chaperone protein (sHsps) heat shock protein 27kDa (Hsp27) have demonstrated the dramatic effect that chaperone modifications can have on functioning by comparing the activity of WT Hsp27 to a perpetually pseudophosphorylated mutant 3xS/D Hsp27 [151]. Data showed that while both versions were able to prevent tau aggregation in-vitro, WT Hsp27 was able to reduce tau levels and restore loss in long term potentiation in a tau mouse model while 3xS/D Hsp27 increased tau levels and did not restore long term potentiation [151]. This study demonstrated that the dynamic ability of sHSPs like Hsp27 to be phosphorylated is important for their chaperone activity towards client proteins such as tau. In this study, we wanted to test a similar sHSP, heat shock protein 22kDa (also referred to as HSPB8), to see if it could also prevent tau aggregation in-vitro.

In addition to tau hyperphosphorylation driving aggregation, it was recently discovered that tau can undergo LLPS which can initiate its aggregation and cause it to
seed the aggregation of other proteins [77]. In this study we wanted to see if 4R0N WT tau could undergo LLPS as well as two disease related mutants P301L and ΔK280 tau. Additionally, we looked at how tau LLPS effects its aggregation behavior and dynamics. Lastly, since chaperone proteins have an influence on the behavior and structure of tau, we wanted to see if chaperone proteins, namely Hsp22, could affect the LLPS behavior of tau.

3.3 Materials and methods

3.3.1 Molecular Cloning

WT tau, P301L tau, ΔK280 tau, Hsp22 WT, Hsp22 3x S/D, and Hsp22 3x S/A were generated in lab by insertion into a pet28a vector backbone using restriction enzyme digests followed by ligation.

3.3.2 Protein expression and purification

E. coli BL21 cells were transformed with 4R0N WT tau, 4R0N P301L tau, 4R0N ΔK280 tau, WT Hsp22, S/A Hsp22, and S/D Hsp22 plasmids in a pet28a vector with a 6x histidine tag and TEV protease site. The cells were then grown at 37°C in LB media containing 100 μg/mL kanamycin. Once their OD600 reached 0.8 the cells were induced with 1mM of IPTG (Gold Biotechnology, Olivette, MO) for 3 hours. Centrifugation at 5,000 g for 15 min was used to harvest the cells, which were then resuspended with nickel chromatography running buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 mM Imidazole) containing protease inhibitors. The cells were then lysed using a freeze-thaw cycle followed by sonication. The lysed cells were centrifuged at 50,000 g for 1 hour at 4°C. The supernatant was affinity purified using a standard gravity column packed with HisPur™ Ni-NTA Resin (Fisher Scientific, Waltham, MA). The eluted fractions were
treated with TEV protease for 4 hours at room temperature then dialyzed back into nickel chromatography running buffer overnight. A second nickel purification column was run and the efficiency of the TEV cleavage was assessed by SDS-PAGE followed by Coomassie staining. For the tau constructs, size exclusion chromatography was performed using a HiLoad 16/600 Superdex 200pg column. Fractions containing >95% pure tau were pooled and concentrated. The concentrated protein was then aliquoted, flash frozen with liquid nitrogen, and frozen at -80°C until use.

3.3.3 Fluorescently labeling recombinant proteins

An Alexa Fluor® 488 protein labeling kit (Fisher, Cat# A10235/A10239) was used to label recombinant proteins. Briefly, recombinant proteins were concentrated into 1mL at a concentration of 10mg/ml. They were then dialyzed into 100mM sodium bicarbonate buffer pH 8 overnight. Following dialysis, 1 vial of reactive dye was added to 1mL of protein and allowed to react at 37 degrees for 1 hour. After 1 hour, the reaction was quenched by adding 100uL of 100mM glycine buffer pH 8. The proteins were then subjected to 10 rounds of buffer exchange into 50mM sodium phosphate pH 8 to get rid of residual dye. The degree of labeling was determined by measuring the absorbance of the conjugate solution at 280nm and 494nm in a cuvette with a 1cm pathlength.

3.3.4 Thioflavin T fluorescent assays

Recombinant tau and Hsp22 proteins were dialyzed into 100mM sodium acetate buffer pH 7 overnight. 20uM of P301L or ΔK280 tau or 10uM of WT tau was mixed with varying amounts of WT Hsp22, S/A Hsp22, or S/D Hsp22 as well as 10uM heparin and 10uM thioflavin T. 100uL of each condition was loaded onto 96-well black clear-bottom
plates (Fisher, Cat#07-200-525) in triplicate. Fluorescence was then read at 440nm excitation and 482 nm emission every 10 minutes over a 72-hour period using a BioTek Synergy H1 plate reader.

3.3.5 Transmission electron microscopy

10uL of the end products of the thioflavin T assay were adsorbed onto prewashed 200 mesh formvar/carbon-coated copper grids for 5 min. The grids were washed with water (10 μl) two times, stained with filtered 2% uranyl acetate (10 μl) for 1 minute, then dried. The samples were analyzed with a JEOL 1400 Digital Transmission Electron Microscope, and images were captured with a Gatan Orius wide-field camera at the Electron Microscopy Core Facility in the College of Medicine at the University of South Florida.

3.3.6 Liquid droplet formation and visualization

Recombinant tau protein was dialyzed into 50mM sodium phosphate buffer pH 8 overnight. Droplet formation was induced by using either 10% polyethylene glycol, 12.5% ficoll, or 12.5% dextran in the presence of 1mM DTT. Tau droplets were observed with an EVOS fluorescent microscope using the visible light and fluorescence channels.

3.3.7 Turbidity assays

Tau turbidity was measured using absorbance at 350nm and was performed using a BioTek Synergy H1 plate reader.
3.3.8 Fluorescent recovery after photobleaching

A 488nm laser line of a confocal ZEISS LSM 880 (Confocal Laser Scanning Microscope) with Airyscan was used on a 1µm diameter spot on tau droplets at 100% transmission for 5 seconds. Pre-bleached and post-bleached images were captured.

3.3.9 Image analysis and statistics

Quantitative analysis of the tau droplet number and size was performed using ImageJ software (National Institutes of Health). The statistical significance performed for each analysis was done using unpaired t-tests. Statistical analysis was performed using the GraphPad Prism version 5.02 software.

3.4 Results

3.4.1 Hsp22 prevents tau aggregation in-vitro

To investigate the role that Hsp22 has on tau aggregation, we first purified WT Hsp22 and two genetically altered versions, 3X S/D which is perpetually pseudo-phosphorylated and 3X S/A which is unable to be phosphorylated. Additionally, we purified three different versions of tau, WT tau, P301L tau, and ΔK280 tau. In order to investigate whether tau aggregation could be altered by Hsp22, we conducted a thioflavin T assay and subsequently imaged the end products using transmission electron microscopy (TEM). Tau was induced to aggregate with heparin and aggregation was followed over a 72-hour period taking readings every 10 minutes. Results showed that all 3 versions of Hsp22 were able to reduce aggregation of all versions of tau tested (Figure 3.1A).

We then further characterized the aggregation of the final ThT products by visualizing them using TEM. Results showed that all 3 version of tau formed large fibril
structures, and all versions of Hsp22 were able to dramatically reduce the size of those fibrils. It should be noted that in the Hsp22 conditions, tau fibrils were still present but the size, specifically the width of fibrils, was reduced (Figure 3.1B). Taken together, these data indicate that Hsp22 can prevent the aggregation of WT tau and disease related mutants P301L and ΔK280.

3.4.2 Tau phase separates in-vitro

To determine if tau was able to undergo LLPS, we used crowding agents to induce the process. Fluorescently labeled (488nm) WT tau, P301L tau, and ΔK280 tau were mixed with 10% polyethylene glycol, the crowding reagent, and were then visualized via microscopy both in the visible light spectra as well as by fluorescent visualization. Results showed that all 3 versions of tau were able to efficiently undergo phase transition into droplet like structures (Figure 3.2A). Additionally, to ensure phase separation had occurred, we took absorbance readings at 350nm which should increase if a protein undergoes a phase transition. Results demonstrated that all 3 versions of tau had a dramatic increase in turbidity upon formation of droplets in solution (Figure 3.2B). These results indicate that WT tau and disease related mutants can undergo LLPS in-vitro in the presence of crowding agents.

3.4.3 Tau phase separation causes an increase in aggregation rate

The aggregation behavior of tau was assessed via thioflavin T fluorescence. Tau with and without 10% PEG was induced to aggregate with 10uM of heparin and aggregation was monitored via 10uM of thioflavin T over a period of 72 hours with readings taken every 10 minutes. Results showed that phase separated P301L tau has an increased rate of aggregation and produces more thioflavin T reactive species as
compared to tau that is not phase separated (Figure 3.3). These results are not surprising since phased separated proteins are forced into a more confined area where they have a higher probability to self-interact.

3.4.4 **Phase separated tau loses dynamics**

In order to measure the dynamics of *in-vitro* phase separated tau we used turbidity readings over time as well as fluorescent recovery after photobleaching (FRAP). Initial turbidity readings of P301L tau showed a dramatic increase in turbidity, but that was steady over a time of 72 hours (Figure 3.4B). Because there was no change in turbidity, it can be assumed that tau phase separation occurs immediately, and the behavior does not increase over time.

To look more carefully at the dynamics of tau droplets, we used FRAP. After photobleaching, if molecules are dynamic, fluorescence should return to the bleached area due to diffusion. We bleached an area on P301L tau droplets and monitored it for 24 hours. In that time, we saw no recovery of fluorescence, indicating a loss of dynamics in the tau droplets (Figure 3.4A). Additionally, we noticed that the droplets that were formed on the coverslip used to visualize the protein did not move over time. This indicated to us that they were no longer dynamic and did not possess liquid like structures which are able to freely flow and fuse. Taken together, these data indicate that phase transitioned tau quickly converts to a less dynamic structure. We hypothesize this is due to tau undergoing a transition from a liquid like state into a gel.

3.4.5 **Hsp22 has an impact on phase separated tau**

Next, we wanted to see if Hsp22 was able to alter the behavior of tau LLPS since phase separated tau has an increased aggregation rate. To do that we mixed P301L tau
with WT Hsp22 at a 20:1 tau:hsp22 ratio and then induced tau phase transition. Turbidity at 350nm was measured and showed that P301L tau was able to phase separate in the presence of Hsp22 (Figure 3.5A). The tau droplets were then visualized by fluorescent microscopy. Results showed that Hsp22 causes a significant increase in the number of tau droplets (Figure 3.5B). Additionally, tau droplets formed in the presence of Hsp22 had a trend of being smaller, but it was not a significant result. We also wanted to see if Hsp22 could alter the increased rate of phase separated tau aggregation. To do this, we used thioflavin T to measure tau aggregation induced with heparin over time. We did this in the presence and absence of WT Hsp22. Results showed that Hsp22 can prevent the increased rate of tau droplet aggregation and smaller species overall are formed (Figure3.5C). These results show that Hsp22 has an impact on the LLPS behavior of tau in-vitro.

3.5 Discussion

There has been a large level of research aimed at trying to determine tau’s aggregation kinetics, the structure of tau aggregates, and pathological consequences of tau in the brain [152, 153]. However, the question of what the early trigger of tau aggregation remains elusive. Here we show that WT tau and disease relative mutants (P301L and ΔK280) can undergo LLPS and aggregation can be triggered by this process. Additionally, we show that tau LLPS leads to the formation and droplets that lose dynamics. Physiologically, tau can undergo LLPS to enhance the polymerization of microtubules by pulling tubulin dimers into the confined space of a droplet and once polymerization has ensued, tau droplets can dissipate [51]. However, it is possible that
disease related mutations or pathological phosphorylation can alter the dynamics of tau droplets and disable them from dissipating. Since that would trap tau in the confined space of the droplet, it is conceivable that this would seed its aggregation. Therefore, the mechanisms of physiological tau LLPS should be studied further to see what aberrations can lead to loss of dynamics and pathological aggregation.

Additionally, since chaperone proteins have been shown to have an effect on tau aggregation [145, 147, 150], we decided to test the ability of Hsp22 to prevent tau aggregation in-vitro. Our studies showed that Hsp22 can prevent the aggregation of tau, both phase-separated and non-phase-separated versions. Since Hsp22 can access proteins that are in phase separated droplets such as those in stress granules due to its intrinsically discorded N-terminal domain [58], it is not surprising that it would be able to access LLPS tau and have a role in its aggregation. Future studies should be aimed at determining how chaperone proteins such as Hsp22 can prevent the aberrant aggregation of phase separated tau. This could present as a potential mechanism to deal with tau aggregation and therefore could be posed as a possible therapeutic option to treat tauopathies.
Figure 3.1 Hsp22 WT and mutants prevent tau aggregation in-vitro. (A) Heparin induced tau aggregation was measured over 72 hours for thioflavin T (ThT) fluorescence (ex/em 448/482nm) and the endpoint ThT RFU values were normalized to tau by itself and plotted (bars represent SEM, unpaired t-test, p-values shown, n=3). Representative 20,000x transmission electron microscopy images of the ThT endpoint tau fibrils alone and incubated with Hsp22 at a 20:1 tau:Hsp22 ratio (scale bar=1uM).
Figure 3.2 WT tau and disease relevant mutants undergo LLPS. Fluorescently labeled tau was induced to undergo LLPS with 10% polyethylene glycol. (A) Fluorescent images were captured to show the tau droplets that were formed. (B) The turbidity (absorbance at 350nm) was measured for tau with and without 10% PEG. (bars represent SEM, unpaired t-test, n=3).
Figure 3.3 Phase separated P301L tau has an increased rate of aggregation. Heparin induced P301L tau aggregation was measured in the absence and presence of a crowding agent, which induced tau droplet formation over 72 hours. Thioflavin T (ThT) fluorescence (ex/em 448/482nm) was measured and the endpoint ThT RFU values were plotted (bars represent SEM, unpaired t-test, p-values shown, n=2) as well as the aggregation rate over time.
Figure 3.4 Phase separated P301L tau loses dynamics. (A) P301L tau droplets labeled with Alexa fluor-488 were subjected to bleaching in a spot with a diameter of 1µm. From 30 seconds after bleaching to 24 hours later, there was no fluorescent recovery in the area that was photobleached and the droplets did not change location. (B) The turbidity (absorbance at 350nm) of phase-separated tau was compared to that of non-phase-separated tau over a 96-hour period. When tau phase separated, the turbidity had a drastic immediate increase. However, over time the turbidity remained constant.
Figure 3.5 Hsp22 WT changes the characteristics of tau droplets. (A) The turbidity (absorbance at 350nm) was measured for phase separated tau with and without Hsp22 (error bars represent SEM). (B) 488-labeled P301L tau droplets were visualized with fluorescent microscopy in the presence of absence of Hsp22 WT. Quantification of droplets size and number showed that Hsp22 significantly increase the number of phase separated tau droplets and had a trend (non-significant) of decreasing their size (bars represent SEM, unpaired t-test, p-values shown, n=3). (C) Thioflavin T fluorescence of phase separated tau with and without Hsp22 was measured over 72 hours and the endpoint ThT values were plotted (bars represent SEM, unpaired t-test, n=3).
Chapter Four:
Overall Conclusions and Future Studies

4.1 Overall Conclusions

Within the work of this manuscript we provide evidence that protein LLPS is a key element in neurodegenerative diseases. We showed that in AD and ALS/FTD, there are intrinsically disordered proteins that undergo phase separation, and this causes problems in cellular functioning and pushes proteins towards pathological aggregation. Additionally, we demonstrated that there are ways to modulate this behavior.

To modulate LLPS behavior in ALS/FTD, the ratio of dipeptide repeat proteins derived from the C9orf72 hexanucleotide repeat expansion can be altered. We showed that if you express GA and PR at a 5:1 ratio of GA:PR it can have a protective role in the cell and reduce aberrant LLPS seen in the form of stress granule assembly. Therefore, this finding could be exploited to alter the aberrant phase separation behavior in the cell, something that is a hallmark of ALS/FTD [30].

Similarly, there are ways to modulate the behavior of protein LLPS in AD. Here, we showed that Hsp22 can prevent tau aggregation of both LLPS tau and tau that is not phase separated. Additionally, we show that Hsp22 can influence the number of tau droplets formed as well as their size. Because of this, chaperone proteins should be
further explored for activity involved in modulating the LLPS behavior of proteins involved in neurodegenerative diseases.

4.2 Future Studies

To examine how protein LLPS can lead to pathological effects on the cells, methods should be used to induce LLPS in a more physiological setting. One proposed method is using optogenetics. By using cytochrome2 (cry2), a protein derived from *Arabidopsis Thaliana*, it is now possible to study this. Cry2 oligomerizes upon exposure to blue light [154]. Therefore, plasmid constructs could be engineered that contain the protein of interest with Cry2 [154]. By doing that, blue light exposure could be used to induce LLPS in a cell, either transfected with the plasmid or engineered to stably express it, at specific times and specific locations [154]. This would enable one to study the dynamics of protein LLPS and how disease relevant mutations could alter those dynamics in a more physiological setting without using crowding reagents.

Additionally, since there is a way to modulate protein LLPS in ALS/FTD by exploiting the ratio of DPRs expressed, more studies should be conducted to further examine how GA may perturb the phase transition of arginine rich DPRs in-vitro and more in-depth analysis should be done on how this impacts the physiological functioning in the cell. Also, other combinations and ratios of DPRs should be tested to see if there are similar effects with other combinations.

Furthermore, since chaperone proteins have long been used to alter the protein folding activity of proteins as well as their degradation [147], they would be an interesting target to test to see how they could affect protein LLPS and subsequent aggregation. Because chaperone proteins have been shown to have opposing effects
on tau aggregation, it would be interesting to test these same chaperones with LLPS tau to see if they could alter the LLPS behavior or aggregation. Also, chaperone proteins could be tested with other proteins involved in neurodegenerative diseases that are known to undergo LLPS such as the arginine rich DPRs.

Determining the very initial steps that seed the aggregation of pathological proteins found in neurodegeneration will be key to prevent downstream aggregation. However, once the initial steps are determined, there must be a strategy to modulate this behavior. Exploiting protein ratios and adding in chaperone proteins may serve as a therapeutic route to modulate LLPS and potentially prevent subsequent pathological aggregation in neurodegenerative diseases.
References


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