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Review

Protein quality control machinery: regulators of condensate architecture and functionality

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Protein quality control (PQC) mechanisms including the ubiquitin (Ub)-proteasome system (UPS), autophagy, and chaperone-mediated refolding are essential to maintain protein homeostasis in cells. Recent studies show that these PQC mechanisms are further modulated by biomolecular condensates that sequester PQC components and compartmentalize reactions. Accumulating evidence points towards the PQC machinery playing a pivotal role in regulating the assembly, disassembly, and viscoelastic properties of several condensates. Here, we discuss how the PQC machinery can form their own condensates and also be recruited to known condensates under physiological or stress-induced conditions. We present molecular insights into how the multivalent architecture of polyUb chains, Ub-binding adaptor proteins, and other PQC machinery contribute to condensate assembly, leading to the regulation of downstream PQC outcomes and therapeutic potential.

Highlights

Recruitment of protein quality control (PQC) machinery to biomolecular condensates is an additional spatiotemporal regulatory mechanism for PQC in cells.

The multivalency and oligomerization stemming from the architecture and interactions among ubiquitin(Ub)-binding adaptor proteins, polyUb chains, chaperone proteins, and proteasomes make these PQC components ideal candidates to be recruited into condensates or to form their own.

Substrate ubiquitination drives the assembly of condensates that are precursors for immune system activation, autophagosome formation, DNA repair, and endocytosis.

Dysregulation of condensates is one hypothesized mechanism leading to the formation of protein-containing inclusions found in neurodegenerative disorders.

PQC machineries and biomolecular condensates

Protein homeostasis in cells relies on PQC machineries, which enable protein degradation via the **UPS** (see Glossary) or **autophagy**, and, by contrast, **chaperone**-mediated protein refolding [1–3]. Perturbations to PQC can cause protein mislocalization and/or accumulation of misfolded or damaged proteins in cells. These misfolded protein accumulations, known as inclusions or **aggregates**, are characteristic of many proteinopathies (protein aggregation diseases) and neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), Alzheimer's, Huntington's, and Parkinson's diseases [1,4,5]. Relevant to our story here is that such inclusions trap PQC components, such as Ub, ubiquitinated proteins, Ub-binding adaptor proteins, chaperones, and UPS/autophagy components, among many others [6,7].

Recently, studies have highlighted that PQC components can accumulate in several types of **biomolecular condensates**, membraneless compartments that regulate various cellular processes by sequestering macromolecular components and/or compartmentalizing biological reactions (Figure 1A and Box 1) [8]. These condensates are hypothesized to form via **phase separation**, a process whereby distinct phases (e.g., liquids, solids) coexist in solution (Box 1). Further, evidence suggests that accumulation of PQC components in some biomolecular condensates offers spatiotemporal control to mediate PQC outcomes differentially throughout the cell under physiological conditions (Figure 1B) [9–11]. As biomolecular condensates are implicated in both physiological and disease-associated processes [8,12], it has been proposed that one mechanism of aggregate formation may stem from the dysregulation of biomolecular condensates (Figure 1A).

Here, we review how PQC machineries contribute to the scaffolding and regulation of biomolecular condensates with downstream biological consequences. While extensive biochemistry and advanced structural biology techniques have uncovered the key molecular steps in the UPS

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and autophagy, recent work has revealed that many of these steps can be further manipulated by the interplay with and recruitment of PQC components into condensates (Figure 1C). We must emphasize that PQC is also occurring outside condensates, as previously demonstrated and reviewed in [5,6,13,14]. However, within PQC-containing condensates, interactions among varied combinations of polyubiquitinated substrates, Ub-binding adaptor proteins, and other PQC components lead to **emergent properties** that control functions such as activation of immune signaling, initiation of autophagy, and regulation of protein levels in a spatiotemporal manner. Deciphering the physicochemical determinants of these behaviors offers therapeutic avenues to regulate the functions of condensates and the ability to dissect disease-linked mechanisms in neurodegeneration associated with dysregulated PQC.

PQC components localize to condensates

PQC often operates via Ub, a small but highly conserved protein that is a common post-translational modification of nearly every eukaryotic protein [14,15]. Ub is covalently attached to substrate proteins primarily at lysine residues via the E1, E2, and E3 **ubiquitination** enzyme cascade. While PQC occurs throughout the cell, PQC components can localize to condensates. These components include Ub (in the form of ubiquitinated substrates), ubiquitination enzymes, **proteasomes**, chaperones [e.g., heat shock proteins (HSPs)], and Ub-binding adaptor proteins for the UPS and autophagy (Figure 2A). As presented later, some PQC components can drive the assembly of condensates and be recruited to known condensates in the nuclei and/or cytoplasm of many eukaryotic organisms and cell types. Recruitment of PQC components to condensates is often linked to proteotoxic stress conditions [11,16]. Examples of well-studied condensates include cytoplasmic stress granules (SGs), nuclear promyelocytic leukemia (PML) bodies, and nucleoli (Figure 1B). Current data suggest that PQC components participate in processes that regulate condensate assembly/disassembly and tune condensate material properties.

PQC components form their own distinct condensates

Certain PQC components [e.g., Ub-binding adaptor proteins Rad23, p62, and ubiquilins (UBQLNs)] are important for the assembly of UPS-associated condensates (Figures 1A,C and 2A). For example, proteasomes accumulate into nuclear condensates under various stress conditions, including nutrient starvation and hyperosmotic stress [17–19] (Table 1); the formation of proteasome puncta is regulated by Rad23B and VCP/p97 **segregase**, with the latter mediating the interaction between ubiquitinated proteins and Rad23B [17]. Under amino acid starvation conditions, proteasome condensates contain Rad23B and polyUb chains [18]. Additionally, a population of p62 was shown to form nuclear proteasome-active condensates, although p62 was engineered to be nucleus restricted in this case [20]. In yeast, proteasomes cluster into stress-induced cytoplasmic condensates with Rad23 and UBQLN-homolog Dsk2 [19], but also into so-called cytoplasmic proteasome storage granules (PSGs) with as-yet-undetermined function [21,22]. Proteasomes also cluster at the endoplasmic reticulum (ER) in *Chlamydomonas reinhardtii* for directed ER-associated degradation (ERAD) in a manner consistent with condensate assembly [23]. These proteasomes may mediate the degradation of ER-specific protein substrates while also serving as degradation centers for cytoplasmic proteins.

Besides UPS-associated condensates, substantial evidence points towards PQC-containing condensates mediating autophagy (see recent reviews for in-depth coverage [24–26]). Mammalian p62/SQSTM1 condenses into 'pre-autophagosomes' with polyubiquitinated cargo that recruit other autophagy drivers [27,28]. Co-condensation of p62 with the Ub-binding adaptor NEMO is important for aggregate clearance [29]. Other p62-like bodies include ones formed by the autophagic adaptor NBR1 that condense with ubiquitinated cargo; these data suggest that condensates facilitate cargo-sorting and potentially have other functionalities prior to autophagosome formation

Glossary

Aggregates: the clumping of proteins into a large accumulation, where the proteins adopt non-native conformations and no longer function correctly. Protein aggregation is a physiological process managed by PQC, but dysregulation can be symptomatic of a disease state or be pathological to a cell's health.
Autophagy: a PQC pathway important for the degradation of large protein aggregates and organelles using a double-membrane-bound vesicle that fuses with lysosomes.
Biomolecular condensates: membraneless bodies with a range of material properties (e.g., liquid, gel, solid) that are hypothesized to form via phase separation of biological macromolecules (e.g., proteins, RNA).
Chaperones: family of multidomain proteins that assist in the *de novo* folding of nascent proteins or refolding of unfolded or partially folded polypeptides to maintain proteostasis. Chaperones can prevent protein aggregation and/or facilitate protein degradation.
Emergent properties: properties that emerge from the totality of interactions among components (e.g., in condensates). Emergent phenomena stem from the collective actions of many molecules to produce properties/behaviors that are not predicted from properties of just a few molecules.
Multivalent interactions: noncovalent interactions that involve multiple binding sites on a macromolecule. Multivalent interactions are important on both the intramolecular (changes in macromolecule conformation) and intermolecular (self-association, oligomerization) level.
Oligomerization: self-association of proteins into multimeric assemblies via noncovalent interactions.
Phase separation: a density-driven transition whereby a solution of macromolecules separates into at least two distinct phases. In a single-component system, the dense phase is macromolecule rich and the dilute phase is macromolecule poor. Many macromolecules that phase separate also undergo percolation transitions driven by crosslinks involving multivalent interaction sites on macromolecules (see Box 1 in the main text).
Proteasome: a large, barrel-shaped megadalton complex with multiple catalytic subunits that performs

[30]. These mechanisms appear conserved, as pre-autophagosomal condensates also exist in yeast [31]. Importantly, several of these condensate-driving factors (e.g., p62, UBQLN) are involved in both the UPS and autophagy, while NEMO has roles in NF-κB activation and autophagy [20,28,29,32–34]. These PQC-containing condensates may direct cargo and substrates to different pathways.

PQC component recruitment leads to regulation of well-known condensates

In the cytoplasm, SGs are dynamic, reversible, stress-responsive (e.g., oxidative stress, osmotic stress, heat shock) condensates comprising mRNA, translation components, and core protein **scaffolds**, such as G3BP and other RNA-binding proteins [35,36]. SGs are centers of arrested translation and mRNA sequestration; on dissipation of the stress condition, SGs disassemble and translation resumes [36,37]. Via recruitment to SGs, PQC components regulate SG disassembly and prevent aberrant SG phase transitions [38–40]. Depending on the stress condition, PQC components in SGs include Ub, VCP/p97 segregase, proteasomes, HSP chaperones, and Ub-binding adaptor proteins (e.g., ZFAND1, UBQLN2) [39,41–47]. Inhibition of PQC components such as E1, the proteasome, and/or HSP70 delays SG disassembly [44]. HSP70 also prevents aberrant liquid-to-solid transitions of SGs triggered by accumulation of misfolded proteins [38]. PQC mechanisms underlying SG clearance include autophagy, G3BP ubiquitination, and chaperone-mediated disassembly [38,39,44,48]. Different PQC pathways are likely to be invoked as different stress conditions lead to differences in SG composition, longevity, and biophysical properties.

The nucleolus, a multiphase condensate responsible for ribosome biogenesis and PQC in the nucleus, sequesters and protects unfolded/misfolded proteins during heat stress partly by preventing their structural transitions into irreversible, pathological aggregates [11,49]. On removal of stress, epigenetic modifiers (i.e., Polycomb proteins) refold via recruitment of PQC component HSP70 and subsequently redistribute to the nucleoplasm to resume their normal functions [50]. Misfolded proteins and defective ribosomal products (DRiPs) can be sequestered into PML bodies. Here, the accumulation of DRiPs leads to a transition into a solid amyloid-like state that can immobilize 20S proteasomes and deplete the nuclear Ub pool [51]. However, the transition is reversible, as chaperones (HSP70 and VCP/p97) can be recruited to deliver DRiPs/nascent proteins for degradation [51]. Ubiquitination machinery, including deubiquitinases such as USP42 and E3 ligase adaptors such as SPOP, can be recruited into nuclear speckles, key regulators of splicing and transcription [52,53]. These PQC components impart dynamic control of condensates, as SPOP can use its phase-separating ability with target substrates to form additional ubiquitination-active condensates, while the deubiquitinase USP42 uses its enzymatic activity to be recruited into nuclear speckles and putatively regulates their fluidity. In summary, PQC components are found throughout many cytoplasmic and nuclear condensates with implications for the sequestration of misfolded proteins and regulation of condensate assembly, disassembly, and dynamics.

PQC uses condensates to communicate between nucleus and cytoplasm

An emerging paradigm is that cytoplasmic condensates sense conditions that are communicated to condensates in the nucleus and vice versa, and PQC can be spatiotemporally managed across intracellular compartments by using such mechanisms [10,54]. In yeast, soluble misfolded proteins in the cytoplasm sequester into ER-associated liquid-like structures called Q bodies, which coalesce to form the juxtanuclear quality control compartment (JUNQ) (Figure 1B) [9,55]. In the nucleus, misfolded proteins accumulate into an analogous compartment called the intranuclear quality control compartment (INQ) [56]. These membraneless, dynamic compartments exhibit properties reminiscent of condensates, but this remains to be rigorously examined. After formation, JUNQ and INQ appear to move towards each other and end up adjacent to each

degradation of misfolded proteins or proteins no longer needed in an ATP-dependent manner. The 26S proteasome comprises a 19S regulatory particle and a 20S core particle. The 19S regulatory particle contains Ub-binding receptors Rpn1, Rpn10, and Rpn13.

Scaffold: a macromolecule (e.g., protein, RNA) with multivalent architecture that has multiple binding sites for other macromolecule components.

Segregase: macromolecular assembly (e.g., p97/VCP) that removes protein components from macromolecular complexes or compartments to mark these proteins for degradation via subsequent association with the proteasome.

Ubiquitination: Ub molecules are covalently attached to proteins to target them for degradation, autophagy, DNA repair, or many other signaling processes. Ubiquitination uses a cascade of enzymes, including Ub-activating E1, Ub-conjugating E2, and E3 ligase enzymes.

Ubiquitin-proteasome system (UPS): major proteolytic pathway conserved across eukaryotes that processes Ub-modified protein targets for degradation. The UPS machinery comprises proteasomes, chaperones (e.g., VCP/p97), and Ub-binding adaptor proteins, among others.

Viscoelasticity: biomolecular condensates possess a range of material properties that represent their flow behavior of being both viscous as fluid and elastic as solid.

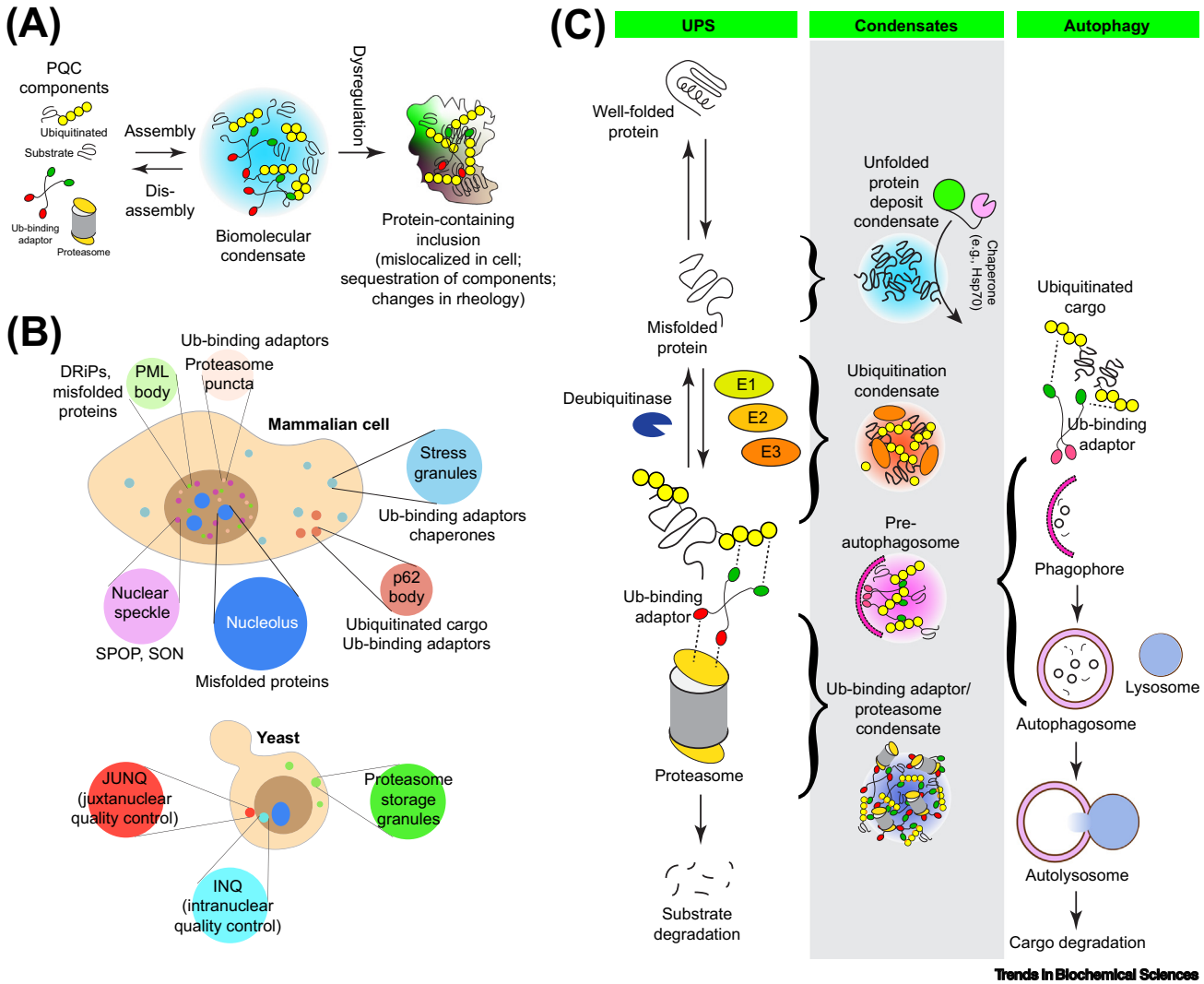

Figure 1. Protein quality control (PQC) components localize to condensates. (A) PQC components can reversibly assemble into condensates. Dysregulation can lead to protein-containing inclusions that are characteristic of neurodegenerative disorders and other diseases. (B) PQC components can be recruited into condensates in eukaryotes. Note that PQC functions are occurring both inside and outside condensates. Labeled adjacent to condensates are components that are found inside condensates and discussed in the review. Ubiquitin (Ub)-binding adaptor proteins are critical for the formation of proteasome puncta in the nucleus and p62 bodies in the cytoplasm. Note that not all condensates simultaneously exist in the cell, as some are stress induced (e.g., stress granules). (C) Evidence from the literature on different condensates at distinct stages in PQC pathways. Unfolded proteins can expose hydrophobic regions that favor condensate assembly with chaperone recruitment [107]. Ubiquitination can also stimulate condensate assembly, including E3 ligase autoubiquitination [71,81]. p62 and p62-like proteins condense with ubiquitinated cargo to form pre-autophagosomes [27,28,30]. Ub-binding proteins form proteasome condensates under stress conditions [17–20]. Abbreviations: DRiPs, defective ribosomal products; INQ, intranuclear quality control compartment; JUNQ, juxtanuclear quality control compartment; PML, promyelocytic leukemia.

other separated only by the nuclear envelope. These data suggest communication between the JUNQ and INQ compartments, perhaps via the nuclear pore [9]; much remains to be resolved regarding whether these compartments operate synergistically to manage PQC.

Additional examples of how cytoplasmic and nuclear condensates affect each other include cytoplasmic SGs cooperating with nuclear condensates to maintain nuclear PQC [57] and hindered

Box 1. Molecular driving forces of biomolecular condensate assembly

Biomolecular condensates (formerly called membraneless organelles) are hypothesized to form via phase separation, a thermodynamically driven process that results in demixing to form coexisting phases (e.g., macromolecule-dense droplets in solution). Biomolecular condensates are heterogeneous in composition, potentially containing hundreds of different protein, RNA, or DNA molecules [8]. Many condensates exhibit liquid-like properties stemming from dynamic interactions that rapidly form or break among macromolecular components (akin to the dynamic hydrogen-bond network in liquid water). These condensates tend to be reversible (e.g., SGs that rapidly assemble on stress and disassemble once the stress condition is dissipated). However, condensates exhibit diverse material properties ranging from liquid to solid characteristics that can change with time in response to altered environment (temperature, pH, etc.) or composition (macromolecules, ligands, metabolites). As condensates locally increase the concentration of macromolecules, condensates may serve as a precursor step leading to protein aggregation (the clumping together of proteins resulting in loss of protein function). Importantly, condensates are not always required for aggregation [118].

From a technical perspective, in phase separation macromolecules undergo a density transition characterized by a saturation concentration (c_{sat}) above which the system phase separates. The c_{sat} threshold is determined by both environmental conditions and composition. Furthermore, there can be a percolation threshold (c_{perc}), above which the **multivalent interactions** among macromolecules create a dynamic network of physical crosslinks that can span the condensate. These fundamental concepts are well reviewed in [118,119]. Multivalent interactions can be distributed among individual amino acids, short linear motifs (e.g., within intrinsically disordered regions in proteins), and patches on folded domains, among others. Differences in the molecular grammar underlying these multivalent interactions drive condensate assembly under different conditions and the recruitment of different macromolecular components [107,118]. The sticker–spacer framework can be used as a conceptual model to describe macromolecules as associative polymers [120]. Following the sticker–spacer framework, interactions among stickers on a multivalent macromolecule drive the assembly of condensates, while spacers link the sticker regions on the same macromolecule and can further modulate the material (rheological) properties of condensates. The language of scaffolds and clients has been used to describe the composition of condensates, whereby scaffolds are macromolecules without which the condensate could not assemble. Clients are those macromolecules that are recruited into condensates. Care must be taken with these definitions as the same macromolecule can be a scaffold in one scenario but a client in another.

PML body formation in the nucleus leading to dysregulated cytoplasmic SGs [58]. Condensates also buffer protein levels between the cytoplasm and nucleus, as exemplified by LEAFY, a regulator of flower development in *Arabidopsis* [59]. LEAFY ubiquitination modulates cytoplasmic LEAFY condensates such that nuclear LEAFY levels remain unaffected and effectively buffered; if cytoplasmic LEAFY condensates dissolve, nuclear LEAFY levels increase. While mechanisms underlying intracellular communication between condensates remain to be resolved, data implicate that PQC-containing condensates have a substantial role in cellular processes. These observations necessitate the determination of how condensates affect PQC pathways and vice versa.

Multivalent architecture of PQC components enable exquisite tuning of condensates

To address the question of how PQC components regulate condensation at a molecular level requires a physicochemical understanding of the molecular driving forces that underpin the recruitment of macromolecules to and scaffolding of condensates (Box 1). The multivalent architecture of macromolecules is critical to condensate assembly by enabling multiple interactions among a large array of components (e.g., SG formation is driven by interactions among RNA-binding proteins and RNA [60], PML bodies assemble via SUMO/SIM interactions [61,62]). Interacting patches on the macromolecules contribute to the overall valency, and these patches include disordered and ordered domains. As discussed in Box 1, once the concentrations of the scaffolding macromolecules reach a saturation threshold, it becomes thermodynamically favorable for the system to phase separate and form condensates [63].

An inherent property of nearly all PQC components is their multivalency (Figure 2A), enabling them to both form their own condensates and to be recruited into condensates under the right

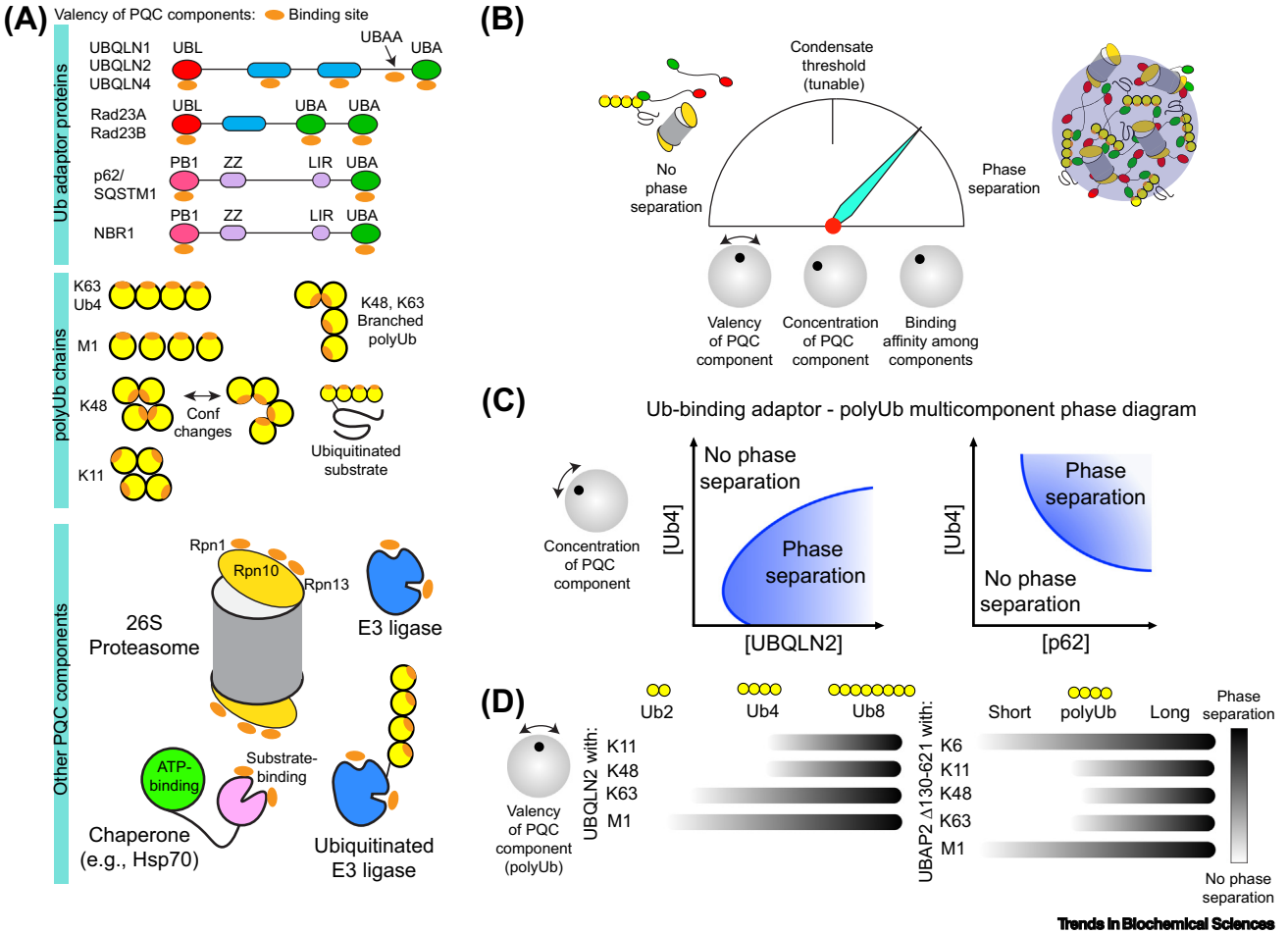

Figure 2. Molecular driving forces (valency, concentration, and binding affinity) enable protein quality control (PQC) components to be recruited into condensates and form their own. (A) PQC components are multivalent in architecture, enabling binding to multiple interaction partners at once (orange ovals indicate binding sites to other components). Polyubiquitin (polyUb) chains adopt both compact (K11, K48) and extended (K63, M1) conformations, while also exhibiting the ability to form mixed, branched chains. Chaperones have multiple binding sites for substrates, while E3 ligases can also engage Ub and substrate. E3 ligases can also be ubiquitinated, providing additional multivalency. Only a subset of PQC components is highlighted here for brevity. (B) Control of the condensation threshold is provided through at least three knobs, including valency, concentration, and binding affinity. Condensates assemble above the condensate threshold and disassemble if below the threshold. Real-time changes in component concentration alter the condensate threshold (e.g., stress-induced ubiquitination can stimulate condensate assembly). Note that there is not a single preset saturation concentration threshold given the multicomponent nature of PQC-containing condensates. (C) Phase diagrams illustrating how polyUb chains drive phase separation of Ub-binding adaptor proteins in different ways [27,28,71], as discussed in the main text. (D) Through differences in chain length and linkage type, polyUb chains can tune conditions favoring phase separation of Ub-binding adaptor proteins. Ubiquilin 2 (UBQLN2) favors K63 and M1 linkages for phase separation, but increased length of K11 and K48 chains could also promote phase separation [71]. UBAP2 preferentially interacts with K6 and M1 linkages for phase separation [84]. Abbreviations: UBA, Ub-associating; UBAA, UBA-adjacent

conditions. In addition to multivalency, two other parameters contribute to the tuning of PQC-containing condensates: binding affinity among PQC components and the concentration of the component (Figure 2B). The tuning of these three dials determines the condensation threshold conditions above which condensates form and below which condensates disassemble. Alterations in expression levels of PQC components (up- or downregulation) and environmental conditions (e.g., temperature, pH) further control when condensates assemble/disassemble in real time. Enzymatic activity [through recruitment of ubiquitination enzymes (e.g., E3 ligases), proteasomes, deubiquitinases] tunes condensates through modulation of multivalency (e.g., changing the length of polyUb chains).

Table 1. Proteasome-containing puncta in cells

Organism/cell line[a]	Condition for observation	Proteasome component identified in puncta	Note[b]	Subcellular location	Refs
Yeast	ATP deficiency	19S and 20S particles of 26S proteasome	Puncta are PSGs	Cytoplasm	[21]
Yeast	Glucose starvation	Rpn1, α1 (core)	Puncta contain Rad23, Dsk2	Cytoplasm	[19]
Yeast	Mitochondrial inhibition (sodium azide)	Rpn1	Puncta contain Rad23, Dsk2	Cytoplasm	[19]
HCT116	Hyperosmotic stress	PSMB2, PSMD4, PSMA3 (26S proteasome)	Puncta contain K48-linked polyUb	Nucleus, nuclear membrane	[124]
HCT116, hTERT, RPE-1	Hyperosmotic stress	PSMB2, 26S proteasome	Puncta contain Rad23B, p97, Ube3A, Ub, K48-linked polyUb	Nucleus	[17]
IMR90, HCT116, COS7, MCF7, etc.)	Nutrient starvation	Various subunits of 26S proteasome	Puncta called SIPAN and contain Rad23B, Ub, K48-linked polyUb	Nucleus	[18]
HeLa, HEK293T	Arsenite-induced oxidative stress	19S and 20S particles of 26S proteasome	SGs also containing ZFAND1, p97	Cytoplasm	[47]
HeLa, A549, MCF7	Leptomycin (nuclear export inhibitor) or p62ΔNES, or heat stress (43C)	Various subunits (α6, Rpn1) of 19S and 20S particles of 26S proteasome	p62 puncta contain UBA1 E1, UbcH5 E2, K48- and K63-linked polyUb, HSP70/90	Nucleus	[20]
WI-38, IMR-90, etc.	Senescence, ETP-induced senescence	Various subunits (Rpt6, Rpn13) of 26S proteasome	Puncta called SANP and contain Rad23B, p97, UBE3A, and K48-linked polyUb	Nucleus	[96]
SH-SY5Y, COS7, H4	Hyperosmotic stress	20S particle α5 subunit	Ub-independent BAG2 puncta containing PA28, HSP70	Cytoplasm	[125]

[a]Mammalian cell line unless otherwise noted.
[b]Abbreviations: SANP, senescence-associated nuclear proteasome puncta; SIPAN, starvation-induced proteasome assemblies in the nucleus.

Ub-binding adaptor proteins can be scaffolds for condensates to drive proteasomal degradation and/or autophagy

The major classes of Ub-binding adaptors discussed earlier include UBQLN, Rad23, and p62 (Figure 2A); each is either recruited to biomolecular condensates or required for condensate formation [17,20,28,32,43,46,64–66]. These proteins are conserved in higher eukaryotes; recent reviews cover their physiological roles [33,67,68]. These proteins adopt a common domain architecture with N-terminal Ub-like fold (UBL) and C-terminal Ub-associating (UBA) domains that make these proteins ideal for bridging interactions between substrates and PQC machineries. More specifically, the UBA domain binds polyUb or ubiquitinated substrates, while the UBL domain directly engages the proteasome and autophagy adaptors [32]. These adaptor proteins also broadly interact among themselves. For example, the UBL and UBA domains of Rad23 interact with the UBA and UBL domains of UBQLNs, respectively [69]. Further, UBQLNs are known to form hetero-oligomers with each other [33,69]. To further promote phase-separating conditions, these proteins also contain **oligomerization** domains (e.g., STI1 in UBQLNs, the PB1 domain in p62) that enable self-association. UBQLNs can independently undergo condensation and p62 can form both condensates and filaments [28,46,70].

A distinguishing feature among these proteins is that Rad23 and p62 require polyUb chains or ubiquitinated substrates to efficiently form condensates *in vitro* and in cells, while this is not necessary for UBQLNs *in vitro*. Such behavior for Rad23 and p62 suggests that they phase separate via heterotypic interactions with polyUb, while UBQLNs can phase separate solely by homotypic interactions. However, polyUb affects UBQLN phase separation such that polyUb can either

inhibit or promote phase separation in a concentration-dependent manner [46,71]. We quantify the distinctions for these different systems via concentration–concentration phase diagrams, highlighting the importance of how the addition of just one component reorganizes the landscape for the phase separation of Ub-binding adaptor proteins (Figure 2C).

Multivalency of polyubiquitinated substrates enables them to act as scaffolds to recruit PQC machinery

PolyUb chains are very heterogeneous in architecture, specifically in length (number of Ub units) and linkage type (how Ub units are connected to each other) (reviewed in [13,72]) (Figure 2A). Ub-Ub linkages are generally isopeptide bonds between the side chain amine (K6, K11, K27, K29, K33, K48, K63) of one Ub and the C terminus of another Ub; these result in K-linked polyUb chains. M1-linked polyUb chains are also formed between the N-terminal methionine of one Ub and the C terminus of another Ub. Each of these polyUb chain types signals for many PQC processes including protein degradation (K48), cell cycle regulation (K11), and autophagy (K63, M1) with links to neurodegenerative disease states [5,6,13,73]. However, polyUb chains do not exclusively signal specific processes and can also contain multiple linkages, adding to the complexity of the Ub code [13,15,65,72]. Further, substrates can be ubiquitinated at multiple positions, resulting in multimonoubiquitination, multipolyubiquitination, or a combination thereof. Therefore, polyUb chains and ubiquitinated substrates offer diverse multivalent scaffolds. Emerging evidence highlights that polyUb chains dynamically regulate the assembly/disassembly of condensates through polyphasic linkage, the principle by which ligand binding is coupled to modulation of phase equilibria (Box 2). Inhibiting ubiquitination abrogates the formation of proteasome puncta under hyperosmotic stress conditions [17], and ubiquitination stimulates phase separation of E3 ligases to drive further ubiquitination in a feed-forward mechanism [74].

Increased polyUb chain length enhances multivalency and promotes phase separation via Ub-mediated interactions. Longer polyUb chains favor condensation with UBQLN2, Rad23B, and p62 [17,28,71]. Ubiquitinated substrates with long polyUb chains promote UBQLN2 phase separation, even driving condensate formation at physiological concentrations of UBQLN2 *in vitro* [75]. Long polyUb chains also promote Rap80 phase separation at DNA lesion sites that need to be repaired [76]. In yeast, knockdown of the Ub-binding protein DDI1 or expression of a protease-dead DDI1 variant leads to accumulation of substrates with long polyUb chains [77]. Under these conditions, the long polyUb chains on substrates contribute positively to the formation of proteasome/Dsk2/Rad23 condensates [19].

Box 2. Polyphasic linkage framework describes how ligands modulate condensates

The assembly and disassembly of condensates are heavily regulated by ligands, noncovalent binding partners of condensate components. Ligand effects on condensate assembly and disassembly can be described by the thermodynamic principle of polyphasic linkage, where the equilibria of ligand binding and phase transitions are inexorably linked. Initially illustrated by Wyman and Gill on how oxygen binding regulates the phase transition of sickle cell hemoglobin between soluble and solid phases [121], the thermodynamic principles of polyphasic linkage are identical for how ligands regulate the phase transitions of scaffold macromolecules that lead to assembly or disassembly of condensates. The valency, interaction strength (binding affinity), and concentration of ligands are all important modulators of phase transitions (see Figure 2B in the main text). Monovalent ligands lead to disassembly of condensates [122], as demonstrated experimentally by how monoUb disassembles UBQLN2 condensates (K_d in the low-micromolar range) [46]. By contrast, multivalent ligands can either stabilize (promote assembly of) or disrupt (promote disassembly of) condensates. These effects depend on where multivalent ligands interact with scaffolds, specifically stickers or spacers. Such principles are illustrated experimentally in at least two systems: inhibitory synapse components on gephyrin condensates [123] and polyubiquitin modulation of UBQLN2 condensates [71,78]. In both systems, the valency of the ligand and the binding affinity between the ligand and scaffold are critical parameters in controlling the conditions for condensate assembly. Polyphasic linkage principles can be used to determine the effects of multiple types of ligands (e.g., proteasome, polyubiquitinated substrate, Ub-binding adaptor proteins) on condensates.

PolyUb linkage type controls Ub-Ub spacing, modulating the ability to interact with Ub-binding partners [78]. Chains that adopt extended conformations in solution (e.g., K63-linked, M1-linked) readily promote phase separation as their Ub-binding sites are far apart [28,71,79]. The canonical K48-linked polyUb chain, which largely mediates protein degradation signals, adopts compact conformations in solution stemming from Ub-Ub noncovalent interactions, thus requiring a conformational change to engage binding partners [71,80]. Comparison between K48- and K63-linked polyUb chains of similar lengths showed that K63-linked chains favored condensation with p62 and UBQLN2 (Figure 2D) [28,71]. K63-linked ubiquitination is a common driver of condensate formation, as recently illustrated with Dishevelled-2, a protein critical to cell fate decisions during development [81], and with Eps15, a protein involved in forming precursor condensates for clathrin-mediated endocytosis [82]. K63- and M1-linked polyUb chains also form condensates efficiently with NEMO, a protein critical to immune system regulation via NF-κB activation. A pathogenic NEMO mutant, incapable of binding M1-linked polyUb, does not form condensates, leading to both the impairment of cellular processes (NF-κB activation) and downstream accumulation of protein aggregates in the brain [29,34,79]. Compact polyUb chains (e.g., K6, K11, K48 linkages) also promote condensate assembly, but often require longer chain length to drive phase separation (compared with extended polyUb conformations) or enhanced binding affinity with Ub-binding partners over other polyUb linkages. Such examples include Rad23A/B's interaction with K48-linked polyUb to form nuclear proteasome condensates, where one Rad23 UBA domain preferentially interacts with K48-linked polyUb [83], and the protein UBAP2 that preferentially interacts with K6-linked polyUb to form condensates critical for purinosomes [84].

Last, the requirements for polyUb-coupled condensation depend on Ub concentration. Estimates from HEK293 cells suggest that the total Ub concentration is around 85 μM with roughly 10% of this pool in polyUb chains [85]. Of these chains, K48-linked polyUb is seven times more represented than K63-linked polyUb. As polyUb concentrations change in response to stress conditions and other linkages are more prevalent during different stages of the cell cycle (e.g., K11 during mitosis), up- and downregulation of polyUb chains can dynamically control condensate assembly and disassembly.

Emergent properties of PQC-containing condensates lead to distinct functional outcomes

The ability of condensates to selectively sequester and enrich macromolecular components results in their dynamic composition and internal organization that differ from those encountered in the surrounding cytoplasm or nucleus. The condensate's unique environment leads to emergent properties. Condensates can deliver distinct functional outcomes, such as either promoting a ubiquitinated substrate's degradation or protecting the substrate from degradation (Figure 3A). Activity of condensates can be manipulated over a wide range of material properties ranging from liquid-like to solid-like behavior.

Modulation of Ub-binding adaptor and proteasome functionality in condensates

Evidence has accrued that Ub-binding adaptor proteins (e.g., UBQLN, Rad23, p62) can either inhibit or promote degradation of substrates [86–90]; recent results implicate condensates in this decision-making process. For UBQLNs, some substrates interact with the oligomerization STI1 domains that are also known to drive phase separation [89,91]. Similarly, E3 ligases such as UBE3A engage UBA-adjacent (UBAA) sequences that contribute to phase separation of UBQLN1/2 [70,89]. Using designed substrates and reconstitution *in vitro* experiments, we demonstrated that K63-polyubiquitinated substrates form condensates with UBQLN2 such that their proteasomal degradation is inhibited, while K48-polyubiquitinated substrates (that do not

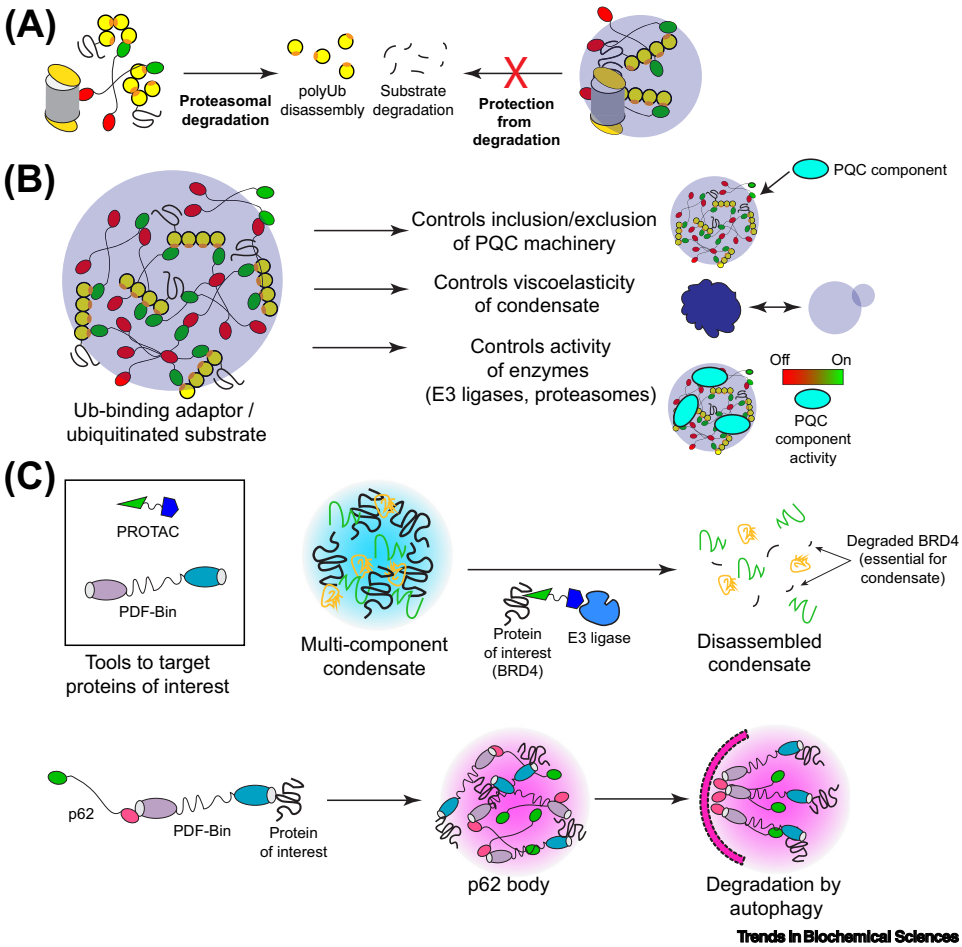

Figure 3. Emergent properties of protein quality control (PQC)-containing condensates that can be used for therapeutic potential. (A) Condensates can regulate PQC outcomes such as substrate degradation or protection from degradation. One recent example shows that condensates can protect substrates from degradation. (B) Condensates containing PQC machinery can modulate the inclusion/exclusion of components, enzymatic activity, and viscoelastic properties. The viscoelastic properties of condensates can be controlled by chaperone proteins thus affecting the aggregation potential of substrates. (C) At least two approaches have recently been developed that use PQC machinery to either dynamically control condensates or selectively degrade components via condensates. Proteolysis-targeting chimeras (PROTACs) target a protein of interest for E3-mediated ubiquitination and subsequent degradation [116]. PDF-Bin uses p62's ability to form condensates to direct a protein of interest to degradation via autophagy [117].

condense as well with UBQLN2) are efficiently degraded [75]. Besides differences in propensity to drive condensate assembly, different polyUb chains on substrates could further alter the internal network architecture of condensates with consequences for the modulation of internal dynamics and condensate **viscoelasticity** [71]. Via increased conformational entropy, intrinsically disordered regions in substrates or Ub-binding adaptor proteins may reorganize the internal network of condensates such that certain components are excluded [92,93]. Similarly, the conformational ensemble heterogeneity of polyUb chains could drive the inclusion/exclusion of certain components from condensates, such as proteasomes or deubiquitinases (Figure 3B). Interestingly, proteasome localization in condensates varies, as proteasomes are located at the periphery of nuclear p62 condensates while they are located internally in other PQC-containing condensates (e.g., Rad23B) [17,20]. Enzymatic activity within these condensates can also be manipulated via a

tradeoff between increased concentrations of PQC components (speeding up reactions) and increased crosslinking among components (slowing reactions) [94,95].

Evidence exists for both active and inactive states of the proteasome in condensates. In mammalian cells, nuclear p62 puncta contain Ub conjugates, ubiquitination machinery, and active proteasomal subunits [20]. Functionally, these p62 condensates protect the cells from apoptotic cell death by constant degradation of ubiquitinated substrates after heat stress. Proteasomal degradation machinery is recruited to nuclear p62 condensates and substrate degradation is enhanced as demonstrated with c-Myc [20]. Cells entering senescence also accumulate active 26S proteasomes into nuclear puncta with Rad23; these puncta regulate mitochondrial activity by preventing reactive oxygen species production [96]. In yeast, cytoplasmic PSGs form when cells enter quiescence as a result of glucose deprivation [21]. Proteasomes are considered inactive in PSGs, which presumably protect proteasomes from degradation. Together, these data suggest that the composition of condensates and internal network architecture of condensates (dictated by PQC component valency, polyUb linkage, binding affinity, and substrate) constitute a code that dictates their biological function and specific PQC outcomes. The rules underlying the control of proteasomes and other PQC components in these condensates remain to be determined.

PQC machinery modulates condensate viscoelasticity

Changes in condensate material properties can result in: (i) altered enzymatic activity inside condensates; (ii) differential recruitment of macromolecular components; and (iii) different condensate clearance mechanisms [48,94,95,97]. Chaperones are the best-known PQC component to manipulate liquid–solid phase transitions affecting downstream functionality [45,98]. Generally, chaperones bind damaged or unfolded proteins to prevent their aggregation and/or guide their refolding. Given the diversity of condensates and their variable composition due to different physiological or stress conditions, multiple chaperones, including HSP70, HSP40, and small HSPs (sHSPs), are implicated in regulating condensate viscoelasticity [99–104]. Chaperones also regulate condensate assembly and disassembly and disperse endogenous condensates during stress response [45] as well reviewed in [98,105].

HSP70 is a family of key chaperones that interact with unfolded protein deposits, misfolded protein substrates, Ub-binding adaptor proteins, and other PQC machinery [106,107]. HSP70 prevents liquid-to-solid phase transitions of SGs and other condensates [99–101]; the liquidity of arsenite-induced SGs is decreased in HSP70 knockdown cells [108]. HSP70 prevents the accumulation of misfolded proteins in SGs to enable the clearance of aberrant SGs [38]. HSP70 recruitment to condensates also prevents the maturation of aggregation-prone ALS-linked RNA-binding protein FUS or TDP-43 into the amyloid fibril state or solid-like state, respectively [99,100]. Conversely, overexpression of HSP70s (HSPA1A and HSPA8 together) increases the liquidity of the large nuclear inclusions formed by TDP-43 [100].

sHSPs also maintain liquid-like properties of condensates and/or prevent substrate aggregation [103,104]. sHSPs lack an ATPase domain and reduce the load on ATP-dependent chaperones by acting as holdases to stabilize proteins and prevent further aggregation [109]. Recruitment of HSP27 (HSPB8) to SGs maintains their liquid-like properties and prevents the aggregation of FUS into amyloid fibrils [104]. Large sHSP complexes distinctly accumulate at the interface of *in vitro* FUS droplets, which are also initiation sites for FUS fibril aggregation [110,111]. Two other sHSPs, HSPB2 and HSPB3, when overexpressed form nuclear condensates in a concentration- and stoichiometry-dependent manner that lead to either liquid-like or solid-like aggregates [112].

Besides chaperones, other PQC machineries can manipulate and recognize different viscoelastic properties of condensates. For example, tau has an increased propensity to aggregate via condensation, but tau ubiquitination prevents this *in vitro* [113]. p62 cooperates with HSP27 to regulate the liquidity of p62 condensates that are recruited to damaged lysosomes for autophagic clearance [114]. Also, cargo-containing condensates (as demonstrated for Ape1) may maintain an optimal liquidity that controls whether these condensates are cleared by selective autophagy [97]. Continued work on building reconstituted systems and bridging *in vitro* experiments to cell-based systems will further accelerate our understanding of PQC-mediated effects on the regulation of condensate material properties.

Therapeutic potential of PQC-containing condensates

As condensates can selectively enrich components and potentially control PQC outcomes, efforts are being made to engineer PQC condensates with therapeutic potential. These applications range from transcriptional control to selective degradation of proteins and/or protein aggregates (Figure 3C). One approach uses proteolysis-targeting chimeras (PROTACs), which are designed heterobifunctional molecules to target a protein of interest for degradation by the UPS via interaction with a ubiquitinating E3 ligase [115]. Recently, a PROTAC was designed to target BRD4, a protein essential to the regulation of superenhancer transcriptional condensates in cells [116]. By degrading BRD4, superenhancer condensates are disassembled, resulting in downstream consequences on gene transcription. PROTAC-induced degradation is recoverable or reversible; thus, PROTACs could be used to dynamically regulate select condensates after further development.

Another recent effort employs p62 condensates as a tool to direct the degradation of specific targets using a bispecific nanobody-targeting strategy [117]. Nanobodies specific to p62 are coupled with another nanobody that specifically targets a protein of interest. Through this approach (termed PDF-Bin, for p62 degradation factory), a targeted protein is recruited into p62 condensates. As p62 condensates are linked to pre-autophagosomes (Figure 1B), these proteins can be subsequently degraded via autophagy. The advantage of employing p62 is that, as a Ub-binding adaptor protein, it can direct substrates towards both proteasome-mediated degradation and autophagy. Such a strategy could be employed for UBQLNs, as they are involved in both the UPS and autophagy.

Concluding remarks

Condensates are implicated in every stage of PQC pathways (Figure 1), providing the cell with an additional spatiotemporal control of PQC mechanisms under normal physiological and stress-induced conditions. Evidence showcases the importance of condensates in selectively including/excluding components, providing change-able viscoelastic environments, and controlling enzymatic activity. These effects imply that condensates, through unique composition of components and control of dynamics, can offer distinct environments that modulate PQC functions such as deciding if/when substrates are degraded, recruiting chaperones to substrates for refolding or degradation, or directing specific PQC outcomes involving the UPS or autophagy. Condensates offer a new layer of control atop the previously identified molecular steps of PQC pathways. Consequently, PQC-containing condensates also present new therapeutic potential to selectively degrade substrates/aggregates or to manipulate protein functions through spatiotemporally controlled degradation.

In this review, we highlighted that many PQC components (including common polyUb chains on substrates) dynamically control condensation leading to emergent properties such as differential PQC functional outcomes and changes in viscoelasticity. Determining the rules that lead to these emergent functions is a part of the critical future directions in this field (see Outstanding questions). The answers

Outstanding questions

Protein-containing inclusions, characteristic of neurodegenerative disorders, contain PQC components. Do PQC-containing condensates mature into these inclusions, and if so, how?

What controls how different PQC elements are involved in condensates under physiological or stress-induced conditions?

How are decisions made inside PQC-containing condensates to selectively protect certain substrates from degradation, refold substrates, and/or promote the degradation of other substrates?

What is the relationship between the network architecture within PQC-containing condensates and their emergent properties? What experimental strategies can be devised to probe internal structures of PQC-containing condensates?

What molecular features of chaperones enable their dynamic control of condensate material properties?

Several Ub-binding adaptors (including p62 and UBQLNs that localize to condensates) interface with multiple PQC pathways. Do condensates play a role in directing substrates towards the UPS, autophagy, or other PQC pathways?

What are the determining factors that control the kinetics of enzymatic processes inside PQC-containing condensates?

To what extent can reconstituted PQC-containing condensates be used to understand how PQC processes are manipulated by condensates inside cells?

Can PQC-containing condensates be designed for therapeutic intervention and drive substrates to specific PQC outcomes?

to these questions will require continued insights from experimental, theoretical, and computational approaches that examine the composition and network architecture of PQC-containing condensates. Given the underlying common physical chemistry principles that describe condensate assembly and disassembly, we can draw on parallels from other systems. For example, the multivalency intrinsic to polyUb chains is found in other post-translational modifications such as SUMOylation and polyPARylation that are linked to condensate-mediated physiological and stress-induced processes. While challenges exist in deciphering the functions of PQC-containing condensates in cells (stemming from the essential nature of PQC to cell viability), the use of reconstituted systems *in vitro* can reduce the experimental complexities and offer a controlled environment. Insights across these diverse systems will be crucial to delineate how PQC-containing condensates function and how they contribute to human health and disease.

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Declaration of interests

The authors declare no competing interests.

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