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Proteasome condensate formation is driven by multivalent interactions with shuttle factors and ubiquitin chains

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Stress conditions can cause the relocalization of proteasomes to condensates in yeast and mammalian cells. The interactions that facilitate the formation of proteasome condensates, however, are unclear. Here, we show that the formation of proteasome condensates in yeast depends on ubiquitin chains together with the proteasome shuttle factors Rad23 and Dsk2. These shuttle factors colocalize to these condensates. Strains deleted for the third shuttle factor gene, *DDI1*, show proteasome condensates in the absence of cellular stress, consistent with the accumulation of substrates with long K48-linked ubiquitin chains that accumulate in this mutant. We propose a model where the long K48-linked ubiquitin chains function as a scaffold for the ubiquitin-binding domains of the shuttle factors and the proteasome, allowing for the multivalent interactions that further drive condensate formation. Indeed, we determined different intrinsic ubiquitin receptors of the proteasome—Rpn1, Rpn10, and Rpn13—and the Ubl domains of Rad23 and Dsk2 are critical under different condensate-inducing conditions. In all, our data support a model where the cellular accumulation of substrates with long ubiquitin chains, potentially due to reduced cellular energy, allows for proteasome condensate formation. This suggests that proteasome condensates are not simply for proteasome storage, but function to sequester soluble ubiquitinated substrates together with inactive proteasomes.

proteasome | liquid–liquid phase separation (LLPS) | proteasome storage granule (PSG) | shuttle factors | yeast

Protein degradation mediated by the proteasome plays important roles in many cellular processes. Proteasomes have traditionally been considered as machines that are available whenever ubiquitinated substrates present themselves. They are also transcriptionally up-regulated under conditions of proteolytic stress. However, more recently this view has been challenged because proteasome activity can also be modulated by post-translational modifications (1). Furthermore, superfluous or defective proteasomes can be targeted for autophagic degradation (2–5). Finally, proteasome localization is regulated in response to cellular changes, reflecting a localized need, e.g., in ER-associated degradation or surrounding aggregates. Interestingly, in yeast, proteasomes have been shown to be exported from the nucleus and to relocalize into distinct biomolecular condensates known as proteasome storage granules (PSGs) upon glucose starvation (6–8). Consistent with the liquid–liquid phase separation (LLPS) nature of these condensates, PSGs have liquid-like behavior and rapidly dissolve (within 10 min) upon re-addition of glucose to cells. While glucose utilization by fermenting single-celled organisms, like yeast, is a rather specialized response, recent work has shown that osmotic stress or amino acid starvation can induce proteasome LLPS in mammalian cells (9–11), suggesting there is a general cellular benefit to depositing proteasomes into condensates. Currently, it is unclear whether the fundamental mechanism used by yeast and mammals to induce LLPS of proteasomes is conserved.

The yeast PSGs were proposed to be storage granules for proteasomes, as proteasomes rapidly returned to cell nuclei from cytoplasmic condensates upon glucose replenishment. Another model suggested proteasomes are sequestered into PSGs to protect them from incorporation into autophagosomes and subsequent degradation (8, 12). However, it remains unclear why nuclear proteasomes would be prompted to leave the nucleus, as their nuclear localization also protects them from autophagy (2, 4). Regardless, both models propose that proteasomes are dissociated into regulatory particles (RP) and core particles (CP) before translocation to PSGs, together with monoubiquitin and the deubiquitinating enzyme Ubp6 (6–8, 12–14). However, these models do not provide a rationale for the multivalent forces required for the formation of proteasome condensates (15). Interestingly, in the mammalian system, proteasome condensates appear to actively degrade specific substrates (9, 11), providing an alternative function for proteasome relocalization into condensates. Formation of these condensates depended on one specific proteasome shuttle factor, RAD23B.

Significance

Stress conditions can cause relocalization of proteasomes to condensates in yeast as well as mammalian cells. Our work shows that the formation of proteasome condensates in yeast depends on ubiquitin chains, the proteasome binding shuttle factors Rad23 and Dsk2 and proteasome intrinsic ubiquitin receptors. Here, different receptors are critical for different condensate inducers. These results indicate distinct condensates can form with specific functionality. Our identification of key factors involved in the process is crucial for understanding the function of proteasome relocalization to condensates. We propose that cellular accumulation of substrates with long ubiquitin chains results in the formation of condensates comprising those ubiquitinated substrates, proteasomes, and proteasome shuttle factors, where ubiquitin chains serve as the scaffold for condensate formation.

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Proteasomes can bind ubiquitinated substrates either directly via three intrinsic receptors—Rpn1, Rpn10, and Rpn13—that have affinity for polyubiquitin chains (16), or indirectly using extrinsic receptors that can bind both ubiquitinated substrates and the proteasome (17, 18). The extrinsic receptors are known as shuttle factors and consist of three members in yeast, Dsk2, Rad23, and Ddi1, each with additional orthologs in humans (Fig. 1*A*). The shuttle factors are characterized by the presence of a ubiquitin-like (Ubl) domain that binds proteasomal ubiquitin receptors and at least one ubiquitin-associated (UBA) domain that interacts with ubiquitinated substrates. While shuttle factors exhibit redundancy (particularly Rad23 and Dsk2), instances of substrate selectivity for each factor have been reported (19–22). Phenotypic evidence suggests shuttle factors are involved in a variety of cellular processes, including cell cycle progression, spindle pole body duplication, and DNA damage responses (20, 23–25). Recent work shows ~90% of proteasomal substrates are delivered to proteasomes by Dsk2 or Rad23 suggesting shuttle factors are prominent contributors to overall ubiquitin proteasome system (UPS) function (22). Although generally considered substrate transporters (18, 26–28), alternative models suggest shuttle factors protect ubiquitin signal integrity or function in direct opposition of proteolysis (29, 30). The latter could be achieved by stabilizing substrates through obstruction of ubiquitin chain elongation or sequestration of substrates away from degradation machinery (31–33). Thus, how shuttle factors influence proteasome dynamics is an emerging and important question.

In this report, we show that shuttle factors play an important role in proteasome condensate formation in yeast, like they do in mammals. While these shuttle factors do not appear to be essential for condensate formation following glucose starvation, proteasome condensates induced by mitochondrial stress depend on Rad23 and Dsk2 for their formation. Like proteasomes, Rad23 and Dsk2 are enriched in these granules and their ability to bind proteasomes is important for proteasome condensate formation, indicating a direct role for Rad23 and Dsk2. Furthermore, we observed a reduction in K48-linked ubiquitin chains resulted in reduced condensate formation, while K63- or K11-linked chains were not required. Consistent with a role for ubiquitin chains as a scaffold in these condensates, mutations of intrinsic proteasome ubiquitin receptors or the Dsk2 UBA domain disrupted condensate formation. In all, we propose a model where the accumulation of substrates with long K48-linked ubiquitin chains is a prerequisite for proteasome condensate formation. Here, multivalent interactions between proteasomes, Rad23, Dsk2, and K48-linked ubiquitinated substrates trigger LLPS.

Results

Shuttle Factors Are Required for Proteasome Condensate Formation. While the shuttle factors are generally considered to deliver substrates to the proteasome, we were interested in testing whether these factors play a role in relocalization of proteasomes. To explore this, we evaluated their role in proteaphagy as well as proteasome condensate formation. In mammalian cells, p62 has been identified as a proteaphagy adaptor linking proteasomes to autophagosome components (34). A p62 homolog does not exist in yeast, however, yeast shuttle factors and p62 share structural and functional similarities [namely, the ability to bind polyubiquitinated proteins and interact with proteasomes (35)]. Furthermore, ubiquilins, the mammalian orthologs of yeast shuttle factor Dsk2, are important for general autophagy (36). To determine whether shuttle factors were required for proteaphagy in yeast, we starved shuttle factor deletion strains for nitrogen or treated them with proteasome inhibitor (the latter only shows modest proteaphagy in our hands). These strains have a GFP-tag at the endogenous locus of RPN1, a regulatory particle subunit, or α1, a core particle subunit, such that every copy of each subunit is tagged and both subcomplexes can be monitored. Both Rpn1 and α1 are essential, and the tagged subunit is efficiently incorporated into the proteasome (37). After 24 h of nitrogen starvation or proteasome inhibitor treatment, the deletion mutants showed proteaphagy similar to the wild type (WT), as was apparent from the accumulation of "free" GFP on immunoblots and a vacuolar GFP localization comparable to WT by fluorescence microscopy (*SI Appendix*, Fig. S1). This indicates that, in yeast, shuttle factors are not required for efficient proteaphagy following nitrogen starvation or proteasome inhibitor treatment.

Next, we evaluated whether the yeast shuttle factors were required for proteasome condensate formation. This was based on the observations that human ubiquilin-2 (UBQLN2) is capable of liquid–liquid phase separation (LLPS) and localizes to foci reminiscent of proteasome condensates (38) and that human RAD23B colocalizes with proteasome condensates (9, 11). We monitored the localization of GFP-tagged proteasomes in WT and shuttle factor mutant strains following growth in rich media (YPD) for 48 or 72 h, here onward referred to as prolonged growth. Consistent with previous reports, approximately 10% of WT cells exhibited cytosolic foci with proteasomes at 24 h of growth in YPD (6). This percentage increased to ~40% at 48 h (Fig. 1*B*). The deletion of either *DSK2* or *RAD23* reduced the fraction of cells with condensates by ~65 to 75% compared to WT cells at both time points (Fig. 1*B*). A deletion of both genes resulted in the complete absence of condensates, indicating a redundant role for Rad23 and Dsk2 in this process. Such a redundancy is not surprising and has been observed for other phenotypes as well (18, 22).

Some labs monitor proteasome condensates formation at stationary phase (quiescence), which involves growth for more than 5 d in YPD media (7). To determine whether the absence of these shuttle factors caused a delay in condensate formation or whether the shuttle factors are essential for the process, we grew cells in YPD up to 6 d. While the RAD23 and DSK2 single deletion strains showed some increase in the amount and intensity of condensates when comparing 3 and 6 d with 1 and 2 d, the lack of condensate formation in the *rad23*Δ *dsk2*Δ strain shows these two shuttle factors are required for this process and do not simply delay or decrease the efficiency of condensate formation (*SI Appendix*, Fig. S2). In sum, our data indicate that both Dsk2 and Rad23 contribute to the localization of proteasomes in condensates under conditions of gradual nutrient depletion.

We recently reported that the treatment of cells with inhibitors of mitochondrial oxidative phosphorylation, like sodium azide, induced the formation of proteasome condensates (39). In this condition, the deletion of both RAD23 and DSK2 also resulted in an almost complete loss of condensate formation, indicating that the shuttle factors play a critical role in proteasome localization following mitochondrial inhibition/stress (Fig. 1*C*). Surprisingly, acute glucose starvation, another condition that results in PSG formation, did not show any measurable deficiencies in the formation of proteasome condensates in *rad23*Δ *dsk2*Δ cells (Fig. 1*D*). This contrasts with a recent study observing that *rad23*Δ cells were defective in the formation of glucose starvation–induced condensates (11). This study, however, was mainly focused on mammalian cells and did not study the role of the yeast shuttle factors in detail. In addition to the differential requirement for shuttle factors in proteasome condensates formed by prolonged growth, sodium azide treatment, or glucose starvation, we also

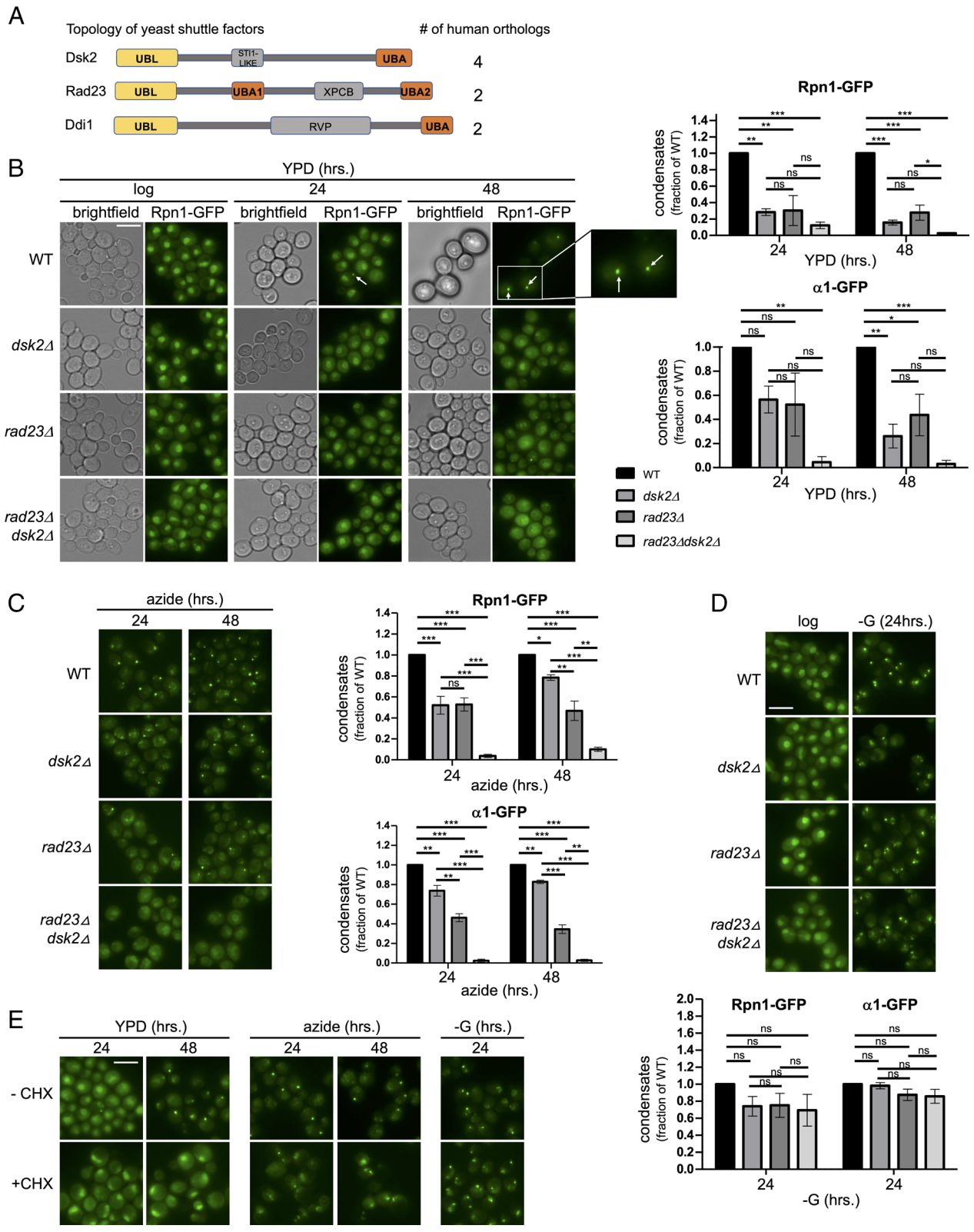


Fig. 1. Rad23 and Dsk2 are important for the formation of proteasome condensates. (*A*) Topology of yeast shuttle factors and the number of human orthologues. UBL (ubiquitin-like domain), UBA (ubiquitin associated domain), STI1 (stress inducible 1 domain), XPCB (XPC binding domain), and RVP (retroviral protease domain). (*B*) Wild-type (WT), *dsk2*Δ, *rad23*Δ, and *rad23*Δ *dsk2*Δ yeast expressing Rpn1-GFP (microscopy images and quantification) or α1-GFP (quantification), tagged at their endogenous locus, were grown in yeast extract peptone dextrose (YPD) media and proteasome localization in live cells was visualized at indicated times using fluorescence microscopy. (*C*) Strains as in (*B*) were grown logarithmically and then treated with sodium azide for 24 or 48 h before microscopic analysis of proteasome localization. (*D*) The indicated yeast strains were starved for glucose (-G) for 24 h. Quantification shows the fraction of cells that formed condensates relative to wild-type cells. (*E*) Rpn1-GFP-tagged cells were exposed to condensate-inducing conditions in the presence or absence of cycloheximide. Proteasome localization in live cells was visualized using fluorescence microscopy at indicated time points. Quantification in (*B–D*) was carried out using FIJI. The average of at least three biological repeats are shown and error bars represent SD. One-way ANOVA and Tukey's multiple comparison test was used to determine significance (ns = not significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001). The scale bars represent 5 μm.

observed a difference in the need for protein synthesis. Condensates that were dependent on Rad23 and Dsk2 for their formation, i.e., those induced by prolonged growth and azide treatment, also required protein synthesis, as the addition of the translation inhibitor cycloheximide disrupted their formation (Fig. 1*E*). In mammalian cells, cycloheximide treatment was shown to inhibit the formation of proteasome condensates known as starvation-induced proteasome assemblies in the nucleus (SIPANs) because it prevented depletion of amino acid pools (11). However, this is unlikely to play a role here because yeast, unlike the mammalian cells, do not form proteasome condensates upon amino acid starvation (3). In all, we observed a striking difference in the requirements for condensates formed upon acute glucose starvation compared to other condensate-inducing conditions, and Rad23 and Dsk2 are not a priori essential for the formation of proteasome condensates.

Proteasomes, Rad23, and Dsk2 Co-Localize in Condensates. Several factors have been shown to be important for proteasome condensate formation, such as the N-terminal acetylase NatB and the kinase Pak1. However, these factors do not colocalize with proteasomes in condensates, suggesting an indirect or regulatory role (7, 40). The requirement for Rad23 and Dsk2 in proteasome condensate formation, and their ability to bind proteasomes suggests a direct role for these factors. To test this, we C-terminally tagged the shuttle factors with mCherry in our GFP-tagged proteasome strains. It should be noted that tagging Rad23 or Dsk2 reduced the number of condensates that formed, presumably because the tag compromises the function of these shuttle factors to some extent. Most condensates that were induced with azide or prolonged growth, which depend on Rad23 and Dsk2 for formation, showed colocalization of proteasomes with Rad23 or Dsk2 (Fig. 2*A*). This is consistent with a direct role of Dsk2 and Rad23 in the formation of these proteasome condensates. Interestingly, while the condensates that formed under glucose starvation did not depend on Rad23 or Dsk2 (Fig. 1), we observed that these shuttle factors still colocalized with proteasomes under this condition (Fig. 2*A*).

To test whether the direct interaction between Rad23 or Dsk2 and proteasomes contributed to condensate formation, we used CRISPR/Cas9 to generate strains expressing Rad23^{I45A} or Dsk2^{I45A} mutations. These mutations, located in the Ubl domains, are predicted to disrupt interactions with proteasomes but not the protein structure (27). Strains harboring these mutations, in a background where the redundant shuttle factor was deleted, showed a strong reduction in the ability to form proteasome condensates (Fig. 2*B*). This reduction was also observed for glucose starvation, a condition where *rad23*Δ *dsk2*Δ cells still form condensates (Fig. 1*D*). We speculate this reflects a dominant effect of these mutations, as they would occupy binding sites that otherwise could contribute to multivalent interactions needed for condensate formation. It should be noted that any reduction in condensates was not due to the absence of the mutated proteins, as we could readily detect Dsk2 in a fluorescently tagged background (*SI Appendix*, Fig. S3). In sum, the shuttle factors Rad23 and Dsk2 co-localize with proteasome condensates, and their ability to bind proteasomes is critical for the formation of proteasome condensates.

Ddi1 Limits Proteasome Condensate Formation. In addition to the shuttle factors Rad23 and Dsk2, there is a third shuttle factor, Ddi1. Similar to Rad23 and Dsk2, Ddi1 contains Ubl and UBA domains for proteasome and substrate interactions, respectively. Like Rad23 and Dsk2, we did not observe a role for Ddi1 in proteaphagy (*SI Appendix*, Fig. S1). However, unlike Rad23 and Dsk2, we did not observe a strong dependence on Ddi1 for condensate formation during prolonged growth or sodium azide treatment, and none of the shuttle factors were required for glucose starvation–induced condensates (Fig. 3 *A* and *B*). Instead, a deletion of DDI1 substantially increased the fraction of cells with GFP-positive foci during prolonged growth. Furthermore, Ddi1 did not consistently co-localize with proteasome foci, although some co-localization was observed (Fig. 3*C*). Interestingly, we observed some Ddi1 foci where proteasomes were absent (see the gray arrow in Fig. 3*C*). The nature of these foci was not further investigated in this study.

While Ddi1 was not required for, nor consistently localized with, proteasome condensates, we did observe a striking phenotype in the DDI1 knockout cells growing logarithmically. That is, without any inducer or stressor, *ddi1Δ* cells formed proteasome condensates in log phase (Fig. 3*D*). These condensates were weaker in fluorescence intensity compared to the condensates observed with prolonged YPD growth and were still dependent on Rad23 and Dsk2 for their formation as they were absent in the *rad23Δ dsk2Δ ddi1Δ* cells (Fig. 3*D*). Thus, Ddi1 prevents the formation of cytosolic proteasome condensates in logarithmically growing yeast.

Long K48-Ubiquitin Chains Trigger Proteasome Condensate Formation. Both human and yeast Ddi1 contain a viral protease domain that was recently shown to cleave substrates tagged with long polyubiquitin chains (41). This function of Ddi1 potentially

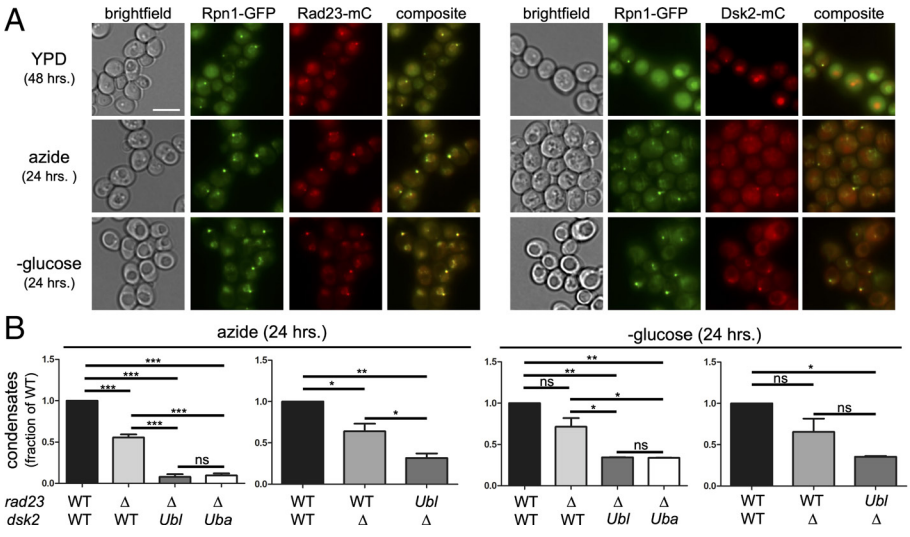


Fig. 2. Rad23 and Dsk2 co-localize with proteasomes in condensates. (*A*) Rad23-mCherry and Dsk2-mCherry were introduced into an Rpn1-GFP tagged strain to monitor their localization during prolonged growth, sodium azide treatment, and glucose starvation. The scale bars represent 5 μm. (*B*) Strains with mutations in the Ubl domain of Rad23 or Dsk2, predicted to disrupt their binding to the proteasome, were analyzed for their ability to form condensates as described previously. Similarly, a strain with mutations in the Dsk2 UBA domain, predicted to disrupt the binding to ubiquitin chains, was analyzed. One-way ANOVA and Tukey's multiple comparison test was used to determine significance (ns = not significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001).

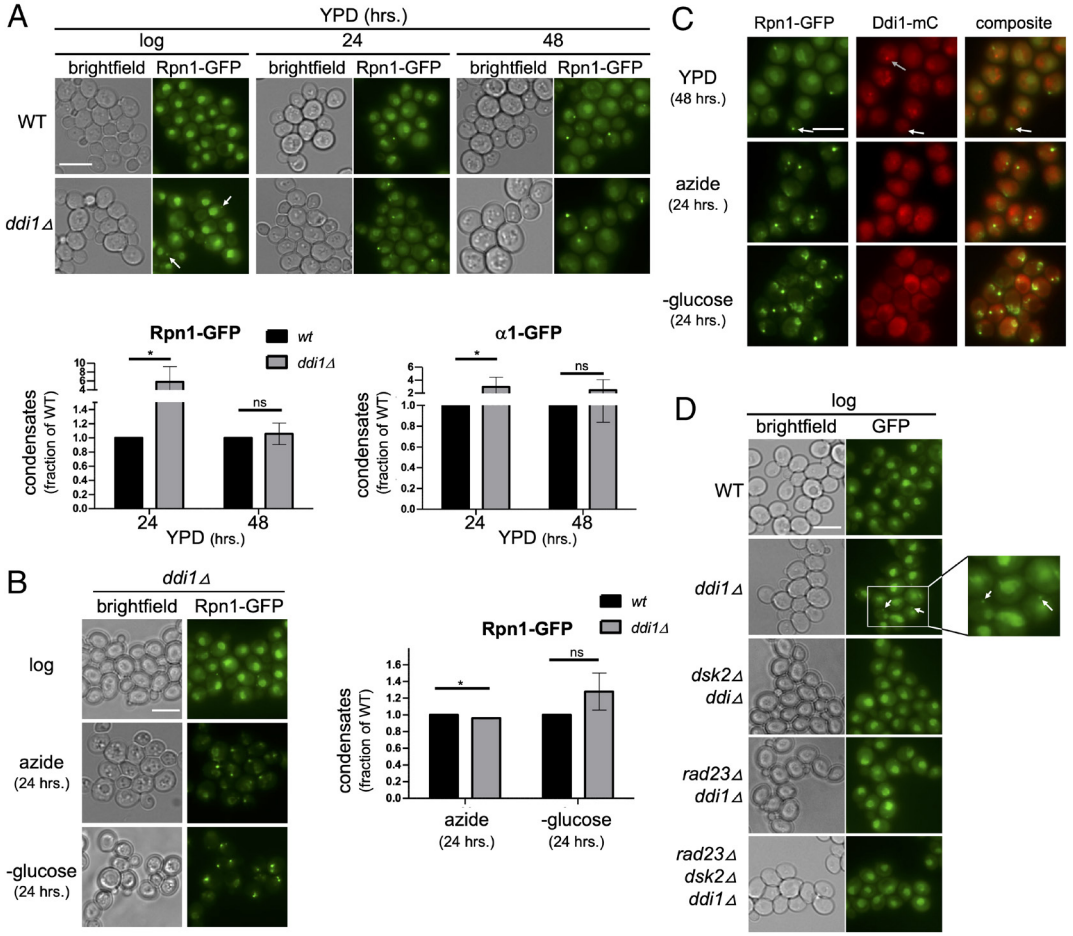


Fig. 3. Ddi1 counteracts proteasome condensate formation. (*A*) Wild-type and *ddi1Δ* cells expressing Rpn1-GFP were grown in YPD media, and proteasome localization was monitored at the indicated times. Quantifications were carried out as in Fig. 1 and error bars represent SD. *t* tests were used to determine significance. (*B*) *ddi1 Δ* cells were monitored for a role of Ddi1 in proteasome condensate formation following sodium azide treatment or glucose starvation. Quantifications show this mutant relative to the wild-type strain shown in Fig. 1 *C* and *D*. SD is presented, and *t* tests were used to determine significance. (*C*) Ddi1 was tagged with mCherry in an Rpn1-GFP tagged strain and its localization monitored at the indicated times and following sodium azide or glucose starvation for 24 h. White arrows provide example of co-localization of proteasomes and Ddi1; gray arrow shows a Ddi1 condensate without proteasomes. (*D*) Indicated strains, all Rpn1-GFP tagged, were grown logarithmically prior to fluorescent imaging. ns = not significant, $*P < 0.05$. The scale bars represent 5 μm.

reflects a role in the cleavage and degradation of substrates that are difficult to degrade, e.g., due to the lack of an initiation site for proteasome engagement. In all, the presence of Ddi1 is critical to prevent the accumulation of substrates with long ubiquitin chains during logarithmic growth. Our observation that *ddi1*Δ strains show proteasome condensates in logarithmically growing cells led us to hypothesize that the lack of Ddi1 protease activity, and consequently the accumulation of proteasome substrates in this mutant, trigger proteasome condensate formation. To test this, we overexpressed either wild-type Ddi1 or Ddi1^{D220N}, a protease-dead mutant, in a DDI1 deletion background and monitored condensate formation. While expression of WT Ddi1 rescued the phenotype of proteasome condensates in logarithmically growing cells, expression of the protease-dead mutant did not (Fig. 4*A*). This shows that the protease activity of Ddi1 is required to prevent proteasome condensate formation during logarithmic growth. Thus, in *ddi1*Δ cells, condensate formation is not due to the loss of a competitor among the shuttle factors for proteasome binding, but instead likely reflects a role for the accumulation of proteasome substrates with long ubiquitin chains in condensate formation.

Condensates generally rely on numerous low-affinity multivalent interactions and a scaffold to trigger their formation (15, 42). Based on the observed condensates in the *ddi1*Δ strain, we reasoned that it is likely that substrate-attached polyubiquitin chains form a scaffold for proteasome condensates to nucleate. We hypothesized that to function as a scaffold, a critical length needs to be reached to allow the ubiquitin chain to interact with the UBA domains of Rad23 or Dsk2 and/or multiple intrinsic ubiquitin receptors (i.e., Rpn1, Rpn10, or Rpn13). To test this, we integrated wild-type ubiquitin, ubiquitin^{K48R}, or ubiquitin^{K63R} into the yeast genome at the URA3-Tim9 locus driven by the strong GPD promoter. Ubiquitin^{K48R} overexpression specifically reduces the length of K48-linked ubiquitin chains, the type of chain typically found on substrates targeted for proteasomal degradation (43, 44). Consistent with this we saw a strong reduction in K48-linked ubiquitin chains as well as ubiquitin chains in general (Fig. 4*B*). Overexpressing ubiquitin^{K63R} is expected to reduce the length of K63-linked ubiquitin chains, a modification that more commonly regulates signaling pathways or endocytosis of substrates (43). Crucially, reducing the length of K48-linked chains, but not K63-linked chains, interfered with the formation of proteasome condensates following azide treatment (Fig. 4*B*). Ideally, we would test whether K48-linked chains are essential for condensate formation to distinguish between a specific role for K48-linked ubiquitin chains versus a general reduction in ubiquitinated material. However, a strain that exclusively expresses ubiquitin^{K48R} is lethal. Instead, we tested if the second or third most abundant type of ubiquitin linkage (45) is required for condensate formation, namely K63- or K11-linked chains. In

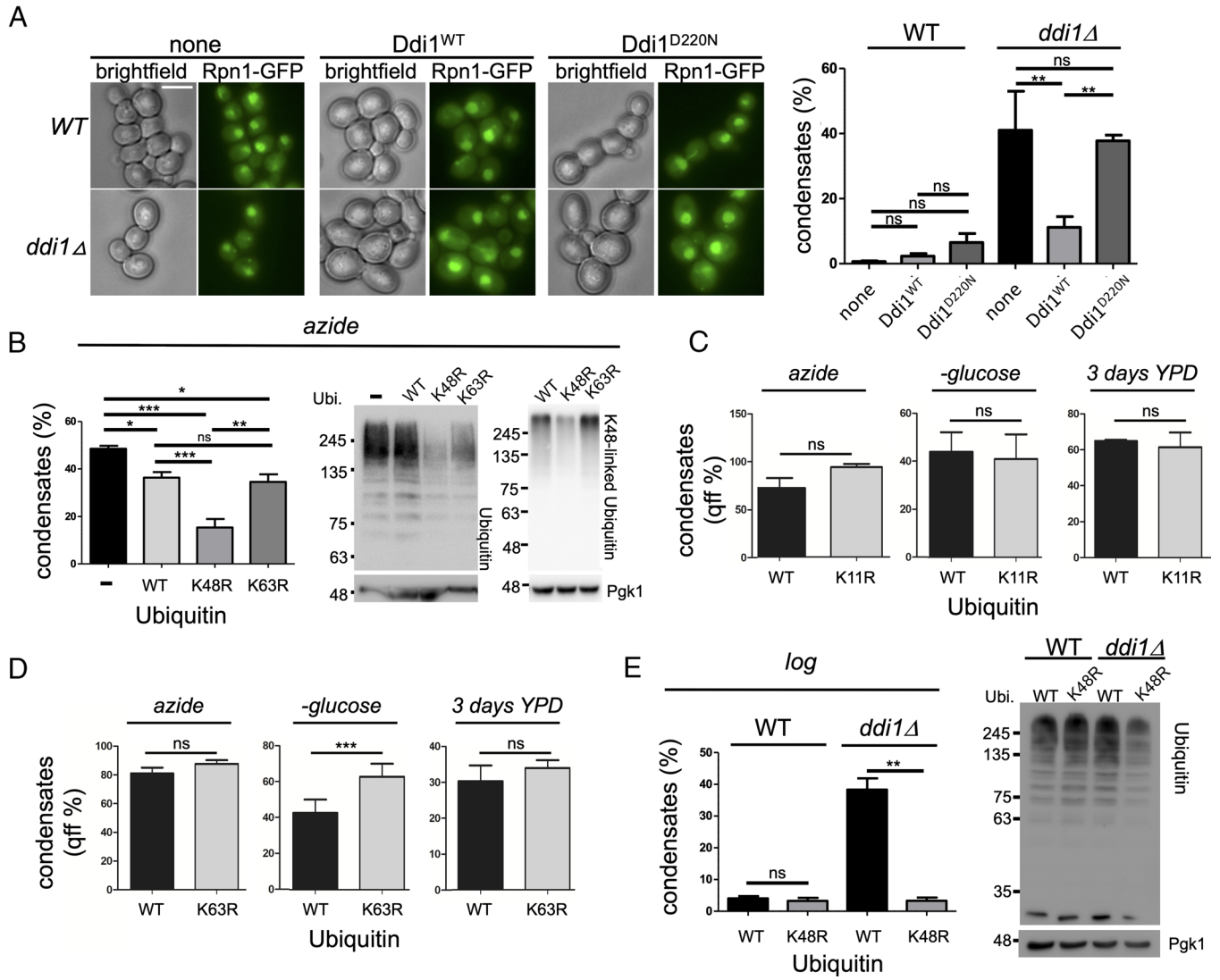


Fig. 4. Long K48-linked ubiquitin chains are a critical component of proteasome condensates. (*A*) Logarithmically growing WT and *ddi1*Δ yeast containing a plasmid without DDI open reading frame (none), expressing Ddi1 ($Ddi1^{WT}$), or a protease-dead Ddi1 ($Ddi1^{D220N}$), were monitored by fluorescence microscopy for proteasome localization. Quantification shows the percentage of cells that formed proteasome condensates. One-way ANOVA and Tukey's multiple comparison test was used to determine significance. (*B*) Wild-type cells expressing Rpn1-mCherry (–) were compared with strains overexpressing ubiquitin (WT), ubiquitin with lysine 48 mutated to arginine (K48R), and ubiquitin with lysine 63 mutated to arginine (K63R) following treatment with sodium azide. Ubiquitin variants were introduced at the URA-TIM9 locus to avoid issues with plasmid loss and expression was driven by a GPD promoter. The average of four independent experiments is shown with SEM. One-way ANOVA and Tukey's multiple comparison test was used to determine significance. Samples were collected after azide treatment and cell lysates were immunoblotted for ubiquitin and Pgk1. (*C*) Strains expressing Rpn1-GFP and a single, integrated, GPD promoter-driven ubiquitin gene, either wild type or $ubiquitin^{K11R}$, were analyzed for their ability to form proteasome condensates under indicated stress conditions. The average of three independent experiments are presented and error bars show SD. *t* tests were used to analyze significance. (*D*) Strains expressing Rpn1-GFP and a single, plasmid-derived, CUP promoter-driven ubiquitin gene, either wild type or $ubiquitin^{K63R}$, were analyzed for their ability to form proteasome condensates under indicated stress conditions. The average of three independent experiments are presented and error bars show SD. *t* tests were used to analyze significance. (*E*) Rpn1-GFP WT and *ddi1*Δ yeast expressing ubiquitin (WT) or K48R ubiquitin were grown logarithmically and monitored for proteasome condensates (*Left*). The average of four independent experiments is presented with SEM. The paired *t* test was used to determine statistical significance. Cells were also collected and lysed, followed by immunoblotting for ubiquitin and loading control Pgk1 (*Right*). ns = not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. The scale bar represents 5 μm.

particular, K63 is of interest as it has been associated with phase separation (46–48). We used the SUB280 strain background, where all endogenous ubiquitin genes have been deleted and only a single ubiquitin gene is present that is either wild-type ubiquitin, $ubiquitin^{K11R}$, or $ubiquitin^{K63R}$ (44). Immunoblots showed no reduction in K48-linked ubiquitin in these strains (*SI Appendix*, Fig. S4) and there was no reduction in the number of condensates formed (Fig. 4 C and *D*), indicating K11- and K63-linked ubiquitin chains are not required for proteasome condensate formation.

Overexpression of $ubiquitin^{K48R}$ in the *ddi1*Δ cells prevented the formation of proteasome condensates during logarithmic growth (Fig. 4*E*). A similar trend of reduced condensate formation upon overexpression of $ubiquitin^{K48R}$ was observed in the wild-type background for glucose starvation and prolonged growth (*SI Appendix*, Fig. S4), however the manipulation of ubiquitin under those conditions might not be as effective (7) and manipulating the endogenous ubiquitin genes might be required to determine whether K48-linked ubiquitin chains are involved under those conditions. Nevertheless, the notion that K48-linked ubiquitin chains are required for some proteasome condensates is supported by our observation that $ubiquitin^{K48R}$ expression prevented proteasome condensates induction by azide as well as following antimycin A treatment, another inducer that we previously characterized (*SI Appendix*, Fig. S4) (39). In all, these data indicate

that proteasome condensates in yeast consist of proteasomes, the shuttle factors Rad23 and Dsk2, and substrates with K48-linked ubiquitin chains. While the contribution of other types of ubiquitin chains cannot be excluded based on our data, we did see that K11 and K63-linked chains are not essential for proteasome condensate formation.

Different Requirements for Proteasome Intrinsic Receptors in Condensate Formation. Considering polyubiquitin chains serve as scaffolds holding proteasome condensates together, we would predict a critical role for the proteasome intrinsic ubiquitin receptors via binding to the ubiquitin chains and/or the shuttle factors' Ubl domains. However, a previous model proposed that proteasome storage granules consist of proteasome CP and RP in a dissociated state, together with the de-ubiquitinating proteasome-associated factor Ubp6, and monoubiquitin (7). It is unclear in this model what forces induce condensate formation, but it presumably involves unidentified intrinsically disordered regions within some of the proteasome subunits and cellular pH as a potential trigger (7, 8). To distinguish between this and our model, we next analyzed the contribution of the proteasome ubiquitin receptors Rpn13, Rpn10, and Rpn1. To determine the role of the peripheral subunit Rpn13, we deleted the RPN13 gene, which does not impact overall proteasome stability or structure (49). We also deleted RPN10; however, this subunit plays a critical role in the stability of the RP, and its absence can result in some dissociation of the lid subcomplex. Therefore, we also introduced mutations, via CRISPR/Cas9 mutagenesis, that have previously been shown to strongly reduce the affinity of Rpn10 for ubiquitin, while not affecting proteasome structure (26). The Rpn1 subunit is essential, playing a structural role through its interaction with several other RP subunits. Therefore, we introduced a set of mutations that have been shown to disrupt ubiquitin binding (16). Analyzing these mutant strains, we observed surprising differences among the ubiquitin receptors for their role in proteasome condensate formation. The ability to efficiently form condensates following glucose starvation was clearly dependent on Rpn10, and perhaps modestly on Rpn13, while mutating Rpn1 had little to no impact on the ability of cells to form glucose starvation–induced condensates (Fig. 5*A*). However, when we analyzed azide-induced condensates, we noticed a strong dependence on Rpn13 and Rpn1 and little to no role for Rpn10 (Fig. 5*B*). All three ubiquitin receptors appear to play a role in the formation of condensates following prolonged growth in YPD (Fig. 5*C*). As the levels of ubiquitinated proteins did not correlate with the number of condensates observed in these mutant strains (*SI Appendix*, Fig. S5), our observations reflect a direct role of these three receptors in condensate formation and not an indirect effect due to less efficient degradation of substrates. These data indicate a striking physiological and likely functional difference among these proteasome ubiquitin receptors. It remains to be determined whether the mutations of proteasome ubiquitin receptors in these different conditions mainly impact condensate formation via disruption of the binding of ubiquitin chains, the Ubl's of Rad23 and Dsk2, or both. However, when we mutated the UBA domain of Dsk2 [disrupting Dsk2's ability to bind ubiquitin chains (50), $Dsk2^{G343A,\ F344A}$] we also observed a disruption of condensate formation (Fig. 2*B*). This further supports a role of ubiquitin chains in condensate formation. Interestingly, this is different from the human ortholog UBQLN2, where in vitro the absence of Ubl and UBA domain increased its condensate formation (51), and the UBA domain contributed to the recruitment of E6AP to condensates (52). In all, our data show proteasome condensates are formed and maintained through a network of critical interactions among (K48-linked) ubiquitin chains, the shuttle factors Rad23 and Dsk2, and proteasomes (via the intrinsic proteasome ubiquitin receptors).

Discussion

Proteasome condensates were first reported in yeast more than 15 y ago (described as PSGs). However, it has remained unclear what triggers their formation and what key interactions facilitate their liquid–liquid phase separation into condensates. Here, we report the presence of the proteasome shuttle factors Rad23 and Dsk2 in yeast proteasome condensates. We also found that long K48-linked ubiquitin chains are a key component of proteasome condensates in yeast, particularly upon inhibition of mitochondria and in *ddi1*Δ cells (see summary Fig. 6). The disruption of condensate formation by the overexpression of $ubiquitin^{K48R}$, which reduces the length of K48-linked ubiquitin chains, provides a strong indication these condensates contain long ubiquitin chains. Long chains would provide the capacity to serve as a scaffold, analogous to how mRNA works as a scaffold in stress granules, by allowing multiple binding partners to interact with the chain. The shuttle factors Rad23 and Dsk2 can interact with these chains utilizing their UBA domains, and we showed the ability of the Dsk2 UBA domain to bind ubiquitin is important for proteasome condensate formation. As the shuttle factors can also bind to the proteasome intrinsic ubiquitin receptors (via their Ubl domains) and ubiquitin chains, all components needed for multivalent interactions that allow condensate formation are present (Fig. 6).

Indeed, mutating the intrinsic ubiquitin receptors of the proteasome disrupts proteasome condensate formation, indicating intrinsic receptors are critical for proteasome localization to these condensates. However, p62 and the human shuttle factors RAD23B and UBQLN2 have been shown to form condensates without proteasomes in vitro and/or in vivo (9, 38, 47, 53). For RAD23B, K48-linked ubiquitin chains were more efficient than K63-linked in inducing LLPS in vitro (9). In contrast, UBQLN2 condensate formation is more efficient with K63-linked ubiquitin chains as compared to K48-linked (48). However, neither of these in vitro studies included proteasomes in their assay. Shuttle factors could be the scaffolds or "stickers" that form the essence of the condensates. Proteasomes might thus be "clients" that enter in these condensates but are not critical for their formation. However, we do not believe this to be the case because mutations of the intrinsic ubiquitin receptors do not cause an appearance of condensates that contain shuttle factors but lack proteasomes; instead, it causes a general drop in condensates observed with either GFP-tagged proteasomes or mCherry-tagged Dsk2 (*SI Appendix*, Fig. S5). Thus, the intrinsic receptors of the proteasome contribute critical multivalent interactions required for condensate formation.

Our data show that specific protein–protein interactions between these components are critical for condensate formation. This contrasts with a previous model that postulated an increase in monoubiquitin in cells triggers condensate formation in the cytosol, while polyubiquitinated proteins associate with and retain proteasomes in the nucleus (7). Our data suggest a more conserved general mechanism of the sequestration of proteasomes in condensates, as our data shows parallels to proteasome condensates in human cells (9–11). While the location and number of human proteasome condensates differ from yeast, their formation also involves a shuttle factor, RAD23B or p62, and polyubiquitin chains. In yeast, generally 1 or 2 larger condensates are observed in the cytosol, which is different from the many small condensates that have been observed in the nucleus of mammalian cells following specific stressors. Mammalian proteasome condensates

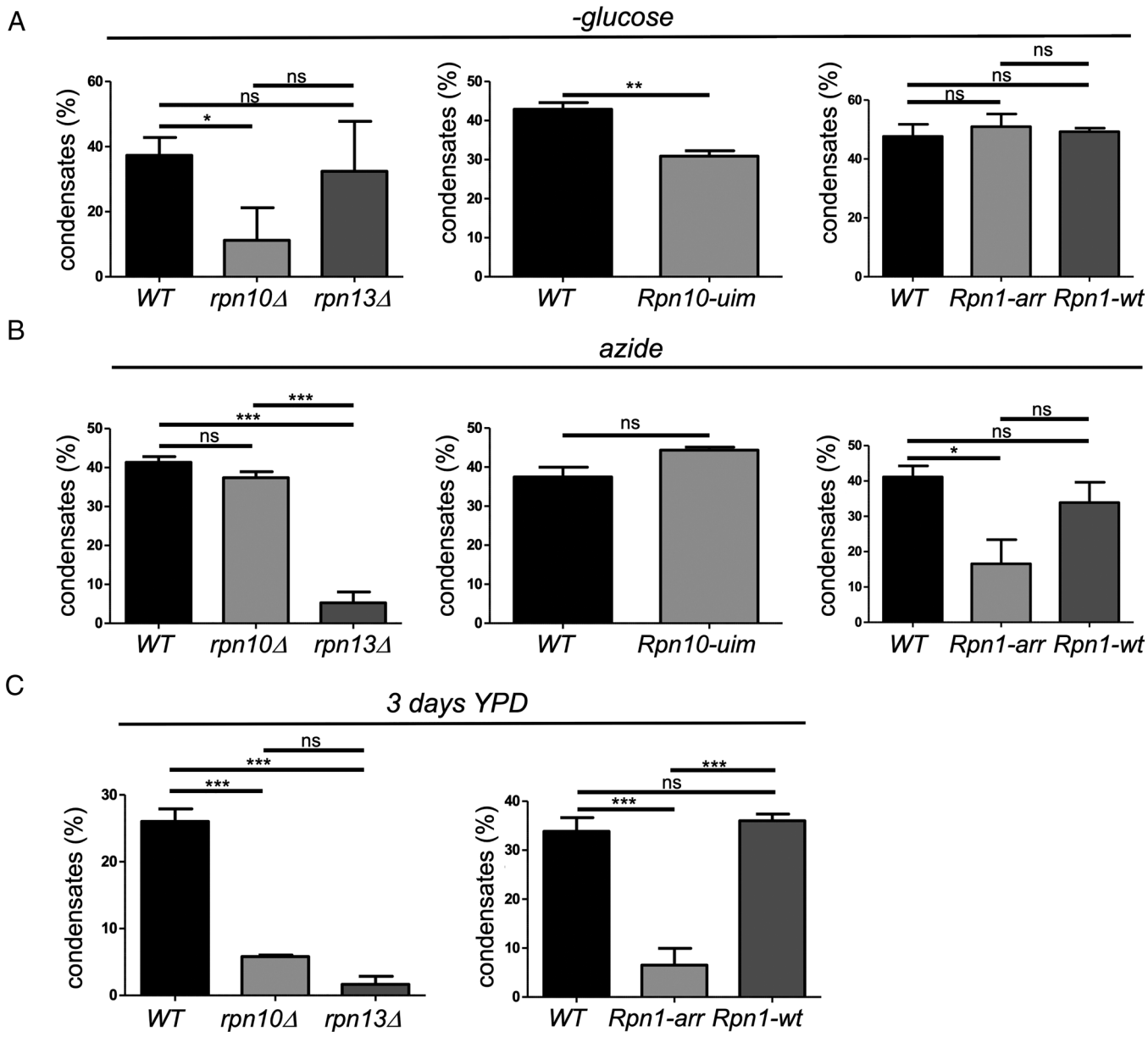
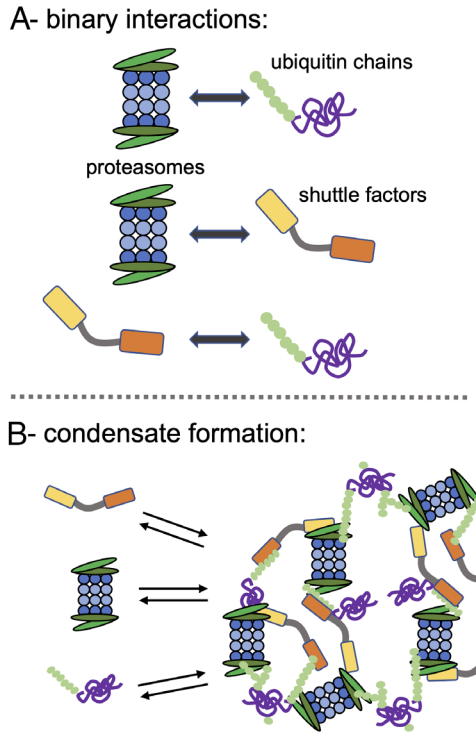


Fig. 5. Intrinsic proteasome ubiquitin receptors have unique roles in proteasome condensate formation. (*A*) *Left* panel, Rpn1-GFP expressing yeast deleted for Rpn10 or Rpn13 were starved for glucose, and proteasome localization was monitored. The average of four independent experiments are presented with one-way ANOVA, and Tukey's multiple comparison test was used to determine significance. *Center* panel, the ubiquitin interacting motif (UIM) of Rpn10 was mutated (Rpn10-uim). Cells were starved for glucose, and proteasome localization was monitored. Quantification from four independent experiments is presented with SEM. The paired *t* test was used to determine significance. *Right* panel, one of the ubiquitin interacting motifs of Rpn1 was mutated (Rpn1-ARR) and compared to wild-type cells and cells with the same genetic modification, only lacking the ARR mutation (Rpn1-WT). Quantifications show the average of three independent experiments. One-way ANOVA and Tukey's multiple comparison test was used to determine significance. (*B*) The strains presented in (*A*) were treated with sodium azide to monitor the effects on proteasome condensates induced in this condition. The first two panels show the results of four independent experiments with one-way ANOVA, and Tukey's multiple comparison test was used to determine significance for Rpn10 and Rpn13 mutants. SEM is presented for the Rpn10-uim mutant, and the *t* test was used to determine significance. The *Right* panel shows the results of three independent experiments and one-way ANOVA and Tukey's multiple comparison test was used to determine significance. (*C*) (*Left*) Rpn1-GFP yeast deleted for RPN13 or RPN10 were grown for 3 d in YPD and then imaged microscopically. One-way ANOVA and Tukey's multiple comparison test was used to determine significance. (*Right*) Yeast as in (*A*) and (*B*) harboring WT Rpn1, Rpn1-arr, or Rpn1-wt were grown to stationary phase and monitored microscopically for condensate formation. One-way ANOVA with Tukey's multiple comparison test was used to determine significance. ns = not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

have been proposed to be actively degrading substrates, suggesting there is an equilibrium between accumulating ubiquitinated substrates that enable LLPS versus the degradation of these substrates that counteracts this. In yeast, on the other hand, the condensates have been proposed to mature and store proteasomes, hence their name proteasome storage granules (PSGs) (6, 7, 54, 55). The difference in dynamics between human and yeast proteasome condensates could also explain why in general yeast cells end up with only one or two large, stable proteasome condensates while human cells have many small nuclear condensates. It remains unclear why proteasomes would need to be stored, as substrate selection and degradation fate are determined at the ubiquitination step. A proposed function of their storage has been to protect proteasomes from autophagy (8, 12). While attractive as a model, it should be noted that the majority of proteasomes are normally localized in the nucleus where they are already protected from autophagy (2, 4). Thus, it is unclear why proteasomes would be exported to the cytosol to protect them from autophagy. Either



C- summary

Factor	Mitochondrial inhibition	Acute carbon starvation	Gradual carbon starvation
induced by	24 hrs. azide	- glucose	YPD > 24 hrs.
localization	cytosolic condensate	cytosolic condensate	cytosolic condensate
reversible?	Yes, within ~ 10 min.	Yes, within ~ 10 min.	Yes, within ~ 10 min.
colocalization Dsk2 & Rad23	Yes	Yes	Yes
in dsk2Δ *rad23*Δ	absent	present	absent
translation dependent?	Yes	No	Yes
OE ubi^{K48R}	reduced	reduced*	reduced*
OE ubi^{K63R}	no change	reduced*	no change
26S receptor	Rpn13 and Rpn1	Rpn10	Rpn1, Rpn10, and Rpn13

Fig. 6. Model showing the multivalent interactions that drive proteasome condensate formation. (*A*) pairwise interactions that contribute to proteasome condensate formation. Proteasomes have three intrinsic ubiquitin receptors (Rpn1, Rpn10, Rpn13) that can interact with ubiquitin chains as well as Ubl domains of proteasome shuttle factors (yellow). Shuttle factors also have 1 or 2 UBA domains that can interact with ubiquitin chains (orange). (*B*) Our data show, at least for condensates induced by prolonged growth or inhibition of mitochondria, that shuttle factors, K48-linked ubiquitin chains, and intrinsic ubiquitin receptors are all critical for condensate formation, suggesting that condensation occurs via a network of interactions between folded domains. (*C*) Summary of properties identified in this study for condensates formed under different stress conditions. Common features as well as distinguishing factors among these condensates are indicated. *shows trend, but is not statistically significant.

nuclear localization of proteasomes is unfavorable under those conditions, or the proteasome condensates have a function beyond protecting proteasomes. Interesting to note here is that we previously reported the involvement of a MAP kinase pathway in proteasome autophagy and the formation of proteasome condensates (3). We proposed that this pathway was involved in regulating the nuclear export of proteasomes, suggesting that in addition to ubiquitinated substrates and shuttle factors, proteasomes need to be present at sufficient concentration in the cytosol to facilitate condensate formation.

The specific involvement of long K48-linked ubiquitin chains suggests that proteasome condensates include polyubiquitinated substrates. This is supported by our observation that a strain deleted for DDI1 shows condensates under conditions of logarithmic growth, i.e., without additional stress. In the absence of Ddi1, cells accumulate substrates with long ubiquitin chains (41), suggesting that proteasome condensate formation can be triggered by the accumulation of substrates with long K48-linked ubiquitin chains. That said, our data cannot distinguish between a critical need for K48-linked ubiquitin chains or a more general need for a certain level of ubiquitinated material (irrespective of chain linkage), especially since K48-linked ubiquitin chains are generally also the most abundant form of linkage in the cell (45). As the condensate-inducing conditions we tested in yeast all involve a drop in cellular energy levels (due to lack of a carbon source or inhibition of mitochondrial oxidative phosphorylation), we hypothesize that such conditions lead to the accumulation of substrates in the cytosol, likely due to reduced proteolytic activity of the proteasome under those conditions, potentially combined with changes in activities of E3 ligases and deubiquitinating enzymes, something that remains to be explored. With the ubiquitin chain length and concentration as key triggers for condensate formation, we propose that the role of the condensates is to sequester ubiquitinated substrates, similar to how accumulated misfolded proteins are sequestered in membrane-less structures like IPODs or JUNQs. While these structures are less dynamic and contain aggregated material (55–57), many short-lived proteins targeted for degradation by ubiquitination are not misfolded but soluble and functional. Such proteins are unlikely to engage with the protein quality control machinery that would otherwise transport them to IPODs or JUNQs. Proteasome condensates could function to sequester these soluble ubiquitinated proteins and prevent them from remaining biologically active under conditions of low proteolytic activity. This would be similar to how stress granules sequester certain mRNAs to prevent translation under stress, thereby not spending energy producing superfluous proteins (58).

An interesting observation that remains to be further explored is why different condensate-triggering conditions required different proteasome intrinsic ubiquitin receptors: with condensates induced by glucose starvation depending on Rpn10 and those induced by azide depending on Rpn1 and Rpn13. These condensates also differ in their dependence on Rad23, Dsk2, and protein synthesis. Thus, there are clear distinguishing features between condensates induced by glucose starvation and those induced with mitochondrial stress. Interestingly, the degradation of substrates presented to the proteasome by shuttle factors or their Ubl domain appears to be primarily mediated through Rpn1 and Rpn13 (59, 60). We hypothesize that different substrates accumulate based on the stressor, and those substrates interact differently with proteasome receptors. As the role of the different intrinsic ubiquitin receptors on the proteasome is still being debated, the dependence on different intrinsic receptors for specific conditions provides an

exciting observation with significance beyond their role in proteasome condensate formation.

Material and Methods

Yeast Strains and Gene Manipulations. Strains used in this work are reported in *SI Appendix*, Table S1. Strains harboring different mutations or with C-terminal fluorescent fusions on proteasome subunits or shuttle factors were generated using standard PCR-based procedures (61, 62). Plasmids and primers used in this study are presented in *SI Appendix*, Table S2. All newly made plasmids were confirmed by sequencing. The DDI1 ORF was amplified from the yeast genome using primers pRL1106 and pRL1107 and cloned using NEBuilder® HIFI DNA assembly into pRS415 (CEN/LEU2), which had been amplified with pRL1108 and pRL1109, creating plasmid pJR980. Subsequent PCR-based mutagenesis was performed to generate the protease-dead D220N mutant plasmid (primers pRL1110 and pRL1111, plasmid pJR985). Yeast expressing these plasmids were grown overnight in selection media to maintain the plasmid, then diluted in YPD for microscopic analysis after logarithmic growth. The URA-TIM9 genomic region was used to integrate wild-type and mutant ubiquitin. First, ubiquitin driven by the strong GPD promoter and followed by a ADH1 terminator and HIS3 MX6 selection cassette was cloned into a pNC1124 [Addgene #41560, (63)] derived plasmid such that it was flanked by a region from the URA3 and Tim9 genes that serve for cross-over. The resulting plasmid, pJR1040, was mutated to create ubiquitin^{K48R} (pJR1048) or ubiquitin^{K63R} (pJR1050). For integration of this cassette into strain backgrounds with a mutated URA3, the plasmids were digested with SalI and SacI to create a linear fragment that was utilized in yeast transformation. For backgrounds with a clean deletion of the URA3 gene (such as the yeast knock-out collection), which lack the URA3 flanking region for cross-over, PCR with primers pRL995 and pRL559 was used to create a linear fragment for transformation. CRISPR-Cas9 mutagenesis was used to generate yeast harboring mutation in the Rpn10 ubiquitin interacting motif (rpn10-uim, replacing LAMAL with NNNNN), Dsk2^{I45A}, Dsk2^{G343AF344A}, and Rad23^{I45A}. The sequence coding for the guide RNA was cloned into CRISPR vector pML107 [Addgene plasmid # 67639 (64)], creating the plasmids reported in *SI Appendix*, Table S3. The sgRNA/CRISPR plasmid and the repair duplex (*SI Appendix*, Table S1) were transformed into yeast and mutations were confirmed by sequencing the genomic region. To introduce the mutation of the T1 ubiquitin-binding site in Rpn1 at the genomic RPN1 locus, we digested plasmid YSp97 (for WT control) or pEL356c (encoding *rpn1-arr* mutation) with XhoI and NotI (16). Linear fragments were transformed into yeast and successful integration of the mutant RPN1 was confirmed by sequencing the genomic region.

Yeast Growth Conditions. Yeast strains were inoculated in yeast extract peptone media containing dextrose as a carbon source (YPD) and grown overnight. For starvation, sodium azide treatment or antimycin A treatment, cultures were diluted to OD_{600} = 0.5 in fresh YPD media and grown for 4 h. Sodium azide was added to a final concentration of 0.5 mM and antimycin A at a concentration of 0.1 mM. For starvation, logarithmically growing cultures were harvested, washed, and inoculated in either SD media lacking carbon or nitrogen as previously described (2). The proteasome inhibitor PS-341 (also known as Bortezomib or Velcade) was added at a final concentration 100 μM. For cycloheximide treatment, overnight cultures were diluted in fresh YPD as above, grown for 4 h, and cycloheximide was added to a final concentration of 50 μg/mL for a 20-min pretreatment. Then cells were washed with respective media, inoculated at an OD_{600} of 1.5 in respective media containing 50 μg/mL cycloheximide, and allowed to grow to indicated time points.

Fluorescence Microscopy. Live yeast cells were collected by centrifugation (2,000 g, 2 min.) and resuspended in PBS buffer, or a small volume of supernatant. Cells were immobilized on a microscopy slide using a 1% agarose pad supplemented with PBS buffer (modified from https://www.youtube.com/watch?v = ZrZVbFg9NE8, 2019). Images were acquired at room temperature on a Nikon Eclipse E800 microscope using a Nikon Plan Apo ×60/1.40 objective and R3 Retiga camera. For GFP images, Sedat Quad filter set (Chroma 86000v2, Bellows Falls, VT) was set to an excitation wavelength of 490/20 nm and emission wavelength of 528/38 nm. mCherry images were obtained using excitation and emission wavelengths of 555/28 nm and 685/40 nm, respectively. Images of one focal plane were collected using Metamorph (Molecular Devices) within 10 min of cell immobilization on microscopy slides. For proteasome condensate quantification, over 100 cells per experimental replicate were manually counted to determine the percent of cells that have at least one condensate. GFP images used for quantifications in Fig. 4 *C* and *D* ("qff%) were collected on a Keyence BZ-X810 microscope using a Nikon Plan Apo ×60/1.40 objective and the "quick full focus" (extended depth of focus) acquisition setting. This setting captures in-focus light in whole cells in Z steps of about 0.8 μm, allowing us to capture all focal planes in one two-dimensional image. Images were collected using Metamorph (Molecular Devices) within 10 min of cell immobilization on microscopy slides and processed using FIJI. Scale bars represent 5 μm.

Cell Lysis and Immunoblotting Analysis. At indicated time points, for each strain, an OD_{600} of 2 was harvested by centrifugation and immediately lysed or stored at −80 °C. Alkaline lysis was carried out as previously reported (65). In short, pellets were resuspended in 100 μL distilled water to which 100 μL 200 mM NaOH was added, and samples were incubated at room temperature for 5 min. Cell suspensions were pelleted, resuspended in 50 μL SDS-PAGE sample buffer (0.06 M Tris–HCl, pH 6.8, 5% glycerol, 2% SDS, 4% β-mercaptoethanol, 0.0025% bromophenol blue), boiled at 98 °C for 5 min, and supernatant was collected. SDS-PAGE of these samples was followed by western blotting. Membranes were analyzed by immunoblotting using antibodies against GFP (Roche Applied Science, catalog no.11814460001), Pgk1 (Invitrogen, catalog no.459250), ubiquitin (Vu1, LifeSensors, catalog no. VU101), and K48-linked polyubiquitin (Cell Signaling Technology, catalog no. 8081). Images were acquired using a Gbox imaging system (Syngene) and captured with GeneSys software.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*. All protocols are described in the Experimental Procedures and *SI Appendix* or in the references therein. Plasmids and yeast strains used in the study are freely available upon request to the corresponding author.

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