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Aggregation of RNA-binding proteins during yeast aging

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Chemistry

by

Shuhao Wang

Committee in charge:

Professor Brian M. Zid, Chair

Professor Nan Hao

Professor Navtej Singh Toor

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University Of California, San Diego

2017

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ABSTRACT OF THE THESIS

Aggregation of RNA-binding proteins during yeast aging

by

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Master of Science in Chemistry

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Protein aggregation is a hallmark of aging and age-related diseases. Meanwhile, physiological aggregation of proteins and other bio-molecules is used by cells as a strategy to maintain cellular fitness under various conditions. RNA-binding proteins, possibly due to their intrinsically disordered and aggregation-prone nature, are associated with many pathological and physiological aggregates. Here we examine the subcellular localization of a group of proteins from two mRNP structures during yeast replicative aging by fluorescence microscopy. We find that several P-body components form an aggregate during aging in yeast. This P-body-like aggregate differs from normal yeast P-bodies in its insensitivity to glucose starvation and the absence of certain mRNA species. Similar to yeast P-bodies, it has a

relatively dynamic structure, which can be disrupted by hexanediol treatment and upon the inhibition of protein translation. Furthermore, the aggregate does not contain protein disaggregase Hsp104, a marker for misfolded protein inclusions, under different conditions and thus unlikely to be a physiological deposition site of misfolded proteins. Combining several lines of evidence, we speculate that the age-related P-body-like aggregate represents an aberrant state of P-bodies with altered composition and function. We propose that fully understanding its formation and function in yeast will provide insights into mechanisms that underlie human aging and age-related diseases.

INTRODUCTION

Protein aggregation has been studied for decades, initially in diseases and later in other biological models. It includes both physiological and pathological processes in which proteins accumulate to form ultrastructures such as liquid droplets, hydrogels, oligomers, and amyloid fibrils. Protein aggregates first came to light due to their appearance in many age-associated diseases. One of the most well-known instances is amyloid plaques resulting from β -amyloid peptides in Alzheimer's disease, which are often accompanied by intracellular neurofibrillary tangles resulting from hyperphosphorylated tau proteins^{1,2}. Similar aggregates were also found in Parkinson's diseases and termed Lewy bodies, which mainly consist of alpha-synucleins³. In other neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), most of the mutated genes such as *SOD1*, *FUS*, *TDP43*, and more recently identified *C9orf72* encode abnormal proteins that form cytoplasmic inclusions, which might contribute to the disease pathology through both gain-of-function and/or lost-of-function mechanisms^{4,5,6,7}.

Despite being involved in diseases, protein aggregation is commonly employed by cells as a coordinated event to maintain cellular fitness and function. Numerous intracellular membrane-less compartments have been described, which are typically assembled through phase transition and execute a variety of biological functions. For example, germ-cell specification in *Caenorhabditis elegans* requires the migration of germ-line-specific P-granules to the posterior side of the embryo⁸. The function of germ-line granules are likely to be conserved across metazoan. A number of membrane-less bodies have also been identified in the nucleus, including Cajal bodies, nucleoli, and nuclear speckles, which are postulated to be involved in a series of biological events such as the assembly of snRNPs, ribosome biogenesis, and pre-mRNA splicing^{9,10,11,12}. Recently, a research group reported that

the formation of heterochromatin domains is also mediated by phase separation¹³.

Yeast and mammalian cells undergo organized protein aggregation and form messenger ribonucleoprotein (mRNP) granules under stressful conditions. Two well-studied examples are processing bodies (P-bodies) and stress granules, both of which are responsible for the maintenance of RNA homeostasis during stress, but have different functions. P-bodies consist of many enzymes involved in mRNA degradation and mainly serve as intracellular sites for mRNA degradation¹⁴. Stress granules, on the other hand, are enriched in stalled translational machinery and mainly serve as mRNA storage and triage sites¹⁵.

Multiple sites for sorting and degradation of misfolded proteins have also been identified in yeast and mammalian cells. JUNQ and IPOD are two distinct inclusion bodies induced by prolonged proteotoxic stress, which contain misfolded proteins in different states and several molecular chaperones¹⁶. Q-bodies are relatively small foci of misfolded proteins which can be rapidly induced upon heat shock in a cytoskeleton-independent manner and eventually coalesce to larger foci, possibly JUNQ as the end of this pathway, upon the impairment of proteasome activity¹⁷.

The efforts to maintain protein and RNA homeostasis during stress are not necessarily independent from each other. Several studies have described the co-localization of RNA-containing stress granules, exogenous misfolded proteins, and molecular chaperones during heat shock^{18,19,20}. The idea that these two processes are coupled is also supported by the evidence that stress granules are dissolved by molecular chaperone system and autophagy upon recovery from stress^{21,22}. Recently, a research group suggested that endogenous proteins in heat shock granules (HSGs) were not misfolded but aggregated through stress-specific quinary interactions²⁰. Taken together, these findings suggest that the process of stress-induced protein aggregation could be complicated and involve multiple steps.

Although many efforts have been put into understanding both normal and abnormal

protein aggregation, the connection between them and how a well-controlled protein aggregate can transform into a pathological state in diseases remains elusive. Stress granules (SGs), a physiological RNP granule, have been linked to the pathogenesis of ALS and FTD since multiple disease-related proteins such as FUS, TDP43, and profilin 1 have been reported to co-localize with stress granule markers in both stressed cells and patient tissues^{23,24,25,26}. Recently, a research group showed that SGs maintain its normal function by the constant degradation of defective ribosomal products (DRiPs) through molecular chaperone complex and/or autophagy pathway, and the impairment of these protein quality control pathways results in the transformation of SGs into an aberrant state²¹. Later they proposed a model that the accumulation of misfolded proteins in SGs can alter its behavior and lead to aberrant SGs, which may have relevance for ALS and related diseases²⁷.

All organisms experience aging, and aging largely increases the risk of occurrence of many diseases. As protein aggregation is implicated in many age-related diseases, it is considered as a hallmark of aging²⁸. Previous studies have shown a global increase in protein aggregation with age in *C. elegans*²⁹. In yeast, protein aggregates have been viewed as an ‘aging factor’ that accumulates in mother cells through asymmetrical inheritance. For example, carbonylated proteins are asymmetrically inherited in mother cells, which leads to an increase of oxidative damage in yeast with replicative age³⁰. Hsp104-associated protein aggregates induced by stress, such as JUNQ and IPOD, are asymmetrically partitioned in yeast mother cells through controversial mechanisms including limited diffusion, actin cable-mediated retrograde transport, and tethering to cellular organelles^{31,32,33}. In 2014, a research group identified five long-lived asymmetrically retained proteins (LARPS) in yeast mother cells, three of which are plasma membrane proteins and the other two are associated with large cytoplasmic structures³⁴. A similar study later identified another group of mother-enriched proteins in yeast and showed that, compared to other proteins, they are more likely to form

large complexes and limit yeast lifespan at high concentrations³⁵. Both results suggest that ‘aging factors’ accumulate in mother cells through protein aggregation and/or organelle-based diffusion constraints. However, the role of protein aggregation in aging has yet to be elucidated. Yeast has been used as a powerful model organism to study aging, due to its short lifespan, well studied genetic background, and relatively easy genetic manipulation.

Recently, the research group of Barral Yves reported a single, asymmetrically inherited protein aggregate during yeast replicative aging³⁶. Similar to JUNQ, IPOD, and Q-bodies induced by varied stressful conditions, this aggregate contains multiple molecular chaperones and is thought to promote the clearance of misfolded proteins and restore proteostasis³⁶. The difference is that it is even present under normal conditions in yeast, suggesting that protein aggregation is a common strategy for yeast to maintain cellular fitness. Further investigations are required to identify and characterize more age-related protein aggregates in yeast and reveal the mechanisms underlying the transition between beneficial and harmful aggregates as cells age.

RNA-binding proteins (RBPs) are generally considered as promising candidates to study protein aggregation, since they are relatively disordered and aggregation-prone. A previous RBPome study showed that nearly 30% of RBPs contain disordered motifs³⁷. These motifs are termed intrinsically disordered regions (IDRs)³⁸. Many of them are also termed low-complexity or prion-like domains due to their low-complexity and prion-like features. IDRs in proteins are able to execute diverse biological functions, such as RNA binding, DNA binding, and interaction with other proteins³⁸. Furthermore, they play critical roles in the assembly of RNP granules and other ultrastructures like hydrogels, probably through driving membrane curvature and promoting dynamic phase separation³⁸.

In addition to their aggregation-prone nature, RBPs are widely associated with diseases. For example, many mutations linked with ALS are located in genes encoding RBPs,

such as *FUS*, *TDP43*, *hnRNPA1* and *hnRNPA2*. The expansion of CAG repeats in the RBP gene *ATXN2* can cause spinocerebellar ataxia type 2 (SCA2)³⁸. Additional instances include mutant *SMN1* and *Wilms' tumor 1 (WT1)*, which are associated with spinal muscular atrophy and renal cancer, respectively³⁸.

In yeast, RBPs cooperate to regulate gene expression during stress by assembly to cytoplasmic mRNP granules such as P-bodies and stress granules. The formation of these granules is highly organized and requires mRNA molecules as scaffolds and the process of phase separation mediated by IDRs^{39,40}. By increasing the local concentration of enzymes involved in RNA metabolism, these granules change mRNA turnover and translational landscape in yeast cells to accommodate stressful conditions. Moreover, many nutrient stresses can trigger the down-regulation of nutrient signaling pathways and mis-localization of their key components. For example, upon glucose starvation, Kog1 in TOR pathway localizes to a single body near vacuolar membrane and Tpk2, Tpk3 in cAMP/PKA pathway form granules that are partially co-localized with P-bodies and stress granules^{41,42}. Given that aging is also a stressful process, components of mRNP granules and aggregation-prone proteins involved in nutrient signaling pathways might provide strong candidates to study age-related protein aggregation.

In this study, we examine the subcellular localization of a group of proteins mainly associated with two mRNP structures, P-bodies and stress granules, in budding yeast during replicative aging by epifluorescence microscopy. We report a protein aggregate containing multiple P-body components formed during middle aging in yeast. This age-related P-body-like aggregate differs from physiological P-bodies in its insensitivity to glucose starvation and the absence of certain mRNA species. Although the specific role of this aggregate in aging is still unknown, our data suggest that its formation might be associated

with the collapse of protein homeostasis. We propose that a complete understanding of this aggregate will shed light on mechanisms of yeast aging.

RESULTS

1. P-body components, but not stress granule components, form aggregates during yeast aging

To streamline the process of investigating protein aggregation in yeast replicative aging, we took advantage of the mother enrichment program (MEP), a genetic modified strain developed by the research group of Daniel Gottschling in which the division of daughter cells is inhibited upon the addition of estradiol, and thus mother cells can be enriched for the characterization of age-related phenotypes and measurement of replicative lifespan (RLS)⁴³. The protein candidates we used in this study were mainly from two mRNP granules: stress granules and P-bodies, including several major components and three disease-related proteins, and two aggregation-prone proteins involved in nutrient signaling pathways. A brief description of each protein is included in the manuscript (Table. 1.1). Target proteins were endogenously tagged with mNeonGreen, a yellow-green fluorescent protein, in the MEP and visualized under fluorescence microscope at different time points during yeast aging. We found that most of the stress granule proteins did not form foci and remained diffusely localized in cells throughout aging (Fig. 1.3). Tpk2 and Hrp1 both accumulated in the nucleus throughout aging, while Kog1 preferentially localized to the periphery of the vacuole, which is consistent with previous studies^{41,42} (Fig. 1.1,1.3). Interestingly, multiple P-body proteins formed at least one bright focus in a proportion of cells at 24h, which is equivalent to 16 generations and considered as ‘middle aging’ (given that the median lifespan of a haploid yeast is around 26 generations) (Fig. 1.2). Due to the increasing heterogeneity of yeast cells with aging, we classified cells into three categories to maintain intact information: fluorescence diffusely localized, with foci, and low fluorescence (Fig. 1.1~1.3, right panel). In each experiment, a given cell was selected for ‘low fluorescence’ when its mean fluorescence

intensity (Integrated density/Area) fell below 20% of the average level. Consistent with the idea that the number of living cells would decrease over time, we found an increasing trend of percentages of cells with ‘low fluorescence’ at 24h and 48h compared to 3h for most proteins (Fig. 1.1~1.3, right panel). Generally, P-body proteins were more aggregation-prone than other candidate proteins during aging (Fig. 1.2). Two yeast homologs of human disease proteins, Pbp1 and Hrp1, were diffusely localized in the cytoplasm and concentrated in the nucleus, respectively, throughout aging (Fig. 1.3, right panel). Tpk2 and Kog1, two proteins involved in nutrient signaling, showed certain degree of abnormal localization at 24h and 48h compared to 3h, indicating an altered pattern of nutrient signaling in aged yeast cells (Fig. 1.1, right panel).

Further quantification of cells with foci which excluded cells with ‘low fluorescence’ and only focused on presumably living cells showed that the visible foci of major P-body components such as Dcp2, Edc3, and Dhh1 showed up in nearly half of the cells at 24h (Fig. 1.4). The other P-body proteins formed foci in 20-40% of the cells except for Ccr4 (Fig. 1.4). Most of stress granule proteins remained diffusely localized in aging. Interestingly, Pub1, a yeast homolog of human TIA protein, formed foci in around 20% of the cells at 24h (Fig. 1.4). However, subsequent experiments repeated with mRuby2 tagging and in the microfluidic device did not support this result (data not shown). Pab1 also formed foci in aged cells occasionally but, similarly, the result was not reproducible when fused with mRuby2 (Data not shown). To address whether the prion-like properties of individual proteins accounts for the variance of their propensities to aggregate during aging, we assigned a LLR value calculated by PLAAC, an online tool to predict the prion-like property of a given amino acid sequence, to each target protein. We found no strong correlation between the LLR values and the percentages of cells with foci at 24h (Fig. 1.5). Instead, P-body proteins comprised a

distinguishable group of proteins that are more aggregation-prone than the other proteins (Fig. 1.5).

A research group previously reported that a protein aggregate formed during early aging in yeast marked by Hsp104 and Hsp42³⁶. Thus we checked the localization of Hsp104 in the MEP during aging. Similarly, we observed Hsp104 foci in early yeast aging (Fig. 1.1). However, inconsistent with the previous study, we also observed a decrease of Hsp104 foci number at 24h (Fig. 1.1). The percentage of cells with Hsp104 foci dropped from 80% at 3h to 16.7% at 24h (Fig. 1.4). Considering that age-associated Hsp104 aggregates were thought to promote the clearance of misfolded proteins and maintain protein homeostasis, we speculate that the loss of age-related Hsp104 aggregates during aging might be associated with the collapse of protein homeostasis.

To ascertain whether those aggregated P-body proteins are co-localized with each other during aging, we constructed multiple yeast strains containing Dcp2 tagged with mRuby2 and a second P-body protein tagged with mNeonGreen. We found that the foci of P-body proteins strongly overlapped with Dcp2 foci at 24h, suggesting that they formed a P-body-like structure (Fig. 1.6, 1.7). In Dcp2-mNeonGreen/Hsp104-mRuby2 double-tagged strain, we observed two events: the emergence of Dcp2 foci and the disappearance of Hsp104 foci with aging. Whether the two events are linked to each other is still unknown. In individual cells with both Dcp2 and Hsp104 foci, two foci were not co-localized, suggesting they formed distinct aggregates during aging (Fig. 1.6, 1.7). In Dcp2-mNeonGreen/Pab1-mRuby2 strain, we observed no Pab1 foci, as mentioned above, but Dcp2 foci in mother cells at 24h, suggesting that the age-related Dcp2 aggregate is generally devoid of stress granule components (Fig. 1.6, 1.7). Quantification of the Dcp2 foci co-localized with different proteins further confirmed that multiple P-body components were partitioned into a single

P-body-like aggregate during aging (Fig. 1.8). Hereafter we focused on the characterization of this aggregate.

2. The P-body-like aggregate in aging has a relatively dynamic structure and is distinct from multiple misfolded protein inclusions.

Previous studies have shown that P-bodies and stress granules are in different physical states. P-bodies in yeast are more dynamic than stress granules and could be dissolved by hexanediol (HXD), a chemical that can disrupt weak hydrophobic interactions between molecules⁴⁰. In addition, multiple physiological aggregates, such as P-bodies, stress granules, and heat-induced Hsp104 aggregates, are sensitive to cycloheximide (CHX), a translation elongation inhibitor, possibly because the formation of these granules requires the normal production of certain proteins^{40,44,45}. To determine the physical state of the P-body-like aggregate, we treated Dcp2-mNeonGreen/Hsp104-mRuby2 double-tagged yeast cells at 24h with CHX and HXD, respectively. We found that Dcp2 foci were no longer present in cells after 1h treatment of both chemicals (Fig 2.1, 2.2). Similar results were observed in microfluidic experiments where we tracked the dynamics of Lsm4/Dcp2 co-localized foci at different time points after adding chemicals to the microfluidic device (data not shown). We also found that Lsm4/Dcp2 co-localized foci at 24h were less sensitive to CHX than to HXD, as it took much longer for CHX to dissolve these foci (data not shown). Combined, these data suggest that the P-body-like aggregate has a relatively dynamic structure and requires normal protein translation.

In 2008, a research group reported two distinct inclusions within yeast cells that sequester misfolded proteins during prolonged proteotoxic stress, which were termed JUNQ and IPOD¹⁶. Later they reported small bodies called Q-bodies formed during early heat shock in yeast and eventually fused to a larger inclusion, which, they suggest, was JUNQ¹⁷. The

microscopy data demonstrated that all these misfolded protein aggregates recruited Hsp104, a protein disaggregase. Therefore, we used Hsp104 as a marker to indicate the location of Q-bodies, JUNQ, and IPOD in yeast during stress and examined if age-related Dcp2 foci co-localized with these bodies. We treated Dcp2-mNeonGreen/Hsp104-mRuby2 MEP cells at 24h with AZC, a chemical that induces protein misfolding in yeast. As expected, the AZC treatment increased the number of Hsp104 foci after 1h (Fig. 2.3, 2.4). Most of the newly-formed Hsp104 foci, however, were not co-localized with age-related Dcp2 foci (Fig. 2.6). Interestingly, we also observed a slight increase of the number of Dcp2 foci after the treatment compared to the normal aged cells at 24h, suggesting that Dcp2 localization might be somehow affected by the change of protein homeostasis upon stress (Fig. 2.3, 2.4). Similar results were observed in the aged cells after 24h followed by 30min or 1h heat shock at 37°C. Those Q-bodies arising from heat shock were not co-localized with Dcp2 foci in aging (Fig. 2.3, 2.6). Finally, we treated 24-hour-old yeast cells with a combination of MG132, a proteasome inhibitor, and 37°C heat shock, which induces the formation of JUNQ and IPOD in yeast¹⁶. In contrast to young yeast cells, a proportion of aged yeast cells escaped the formation of JUNQ and IPOD upon stress, suggesting an altered mode of stress response during yeast aging (Fig. 2.5). However, we observed no co-localization between Dcp2 foci and those JUNQ and IPOD formed in the aged yeast cells, although some Dcp2 foci were formed in proximity to Hsp104 inclusions (Fig. 2.3, 2.6). Combined, these data show that the age-related P-body-like aggregate is distinguishable from many previously identified Hsp104 aggregates associated with misfolded proteins including Q-bodies, JUNQ, and IPOD. Hence we propose that this P-body-like aggregate is not a normal storage or triage site for misfolded proteins during aging.

3. The P-body-like aggregate forms during middle aging in yeast and is distinct from physiological P-bodies.

We next wanted to track the dynamics of these P-body-like aggregates during aging in yeast cells. To this end, we employed a microfluidic device to fix yeast mother cells while washing away newborn daughter cells by a continuous flow of yeast media. First we confirmed that the microfluidic device exerted no stress on the MEP, which otherwise induced the formation of P-bodies, unless we deprived the glucose in the media (data not shown). Additionally, we observed an interesting phenomenon that MEP cells appeared much larger in estradiol than those without estradiol at 24h and resulted in mother cells being pushed out of the trap (data not shown). Considering the possible negative effect of estradiol on MEP cells, we conducted the rest of microfluidic experiments without the addition of estradiol.

The dynamics of both Dcp2 and Hsp104 foci were tracked by microfluidics for two days in a Dcp2-mNeonGreen/Hsp104-mRuby2 MEP strain. Consistent with the results from batch culture experiments, we observed an increase of the number of Dcp2 foci and a decrease of the number of Hsp104 foci starting 12h (Fig. 3.1, 3.2). At 36h, a proportion of mother cells exhibited abnormally strong red fluorescence (Fig. 3.1, 3.2). Given that this phenomenon was not conserved in other strains expressing mRuby2 fusion proteins, we assumed the strong fluorescence was from the high expression of Hsp104. Interestingly, there was a huge increase of Dcp2 foci number from 12h to 24h, suggesting that most of the P-body-like aggregates were formed in yeast during this period of lifespan. The altered localization and expression of Hsp104 were accompanied by the formation of P-body aggregates during yeast aging, suggesting that there might be a correlation between these two events. Since researchers have shown that Hsp104 aggregates formed in early aging can help to maintain protein homeostasis, the abnormal molecular behavior of Hsp104 protein at later time points together with the

aggregation of P-body components might reflect an ‘unhealthy state’ in aged yeast cells and/or their efforts to restore protein homeostasis.

Since P-bodies are induced by glucose starvation where translation initiation is generally inhibited, we next wanted to understand how the age-related P-body-like aggregate responds to glucose starvation. A series of experiments were conducted in the microfluidic device which enables the real-time quantification of P-body-like aggregates upon stress. In Lsm4-mNeonGreen/Dcp2-mRuby2 double tagged MEP cells, both Lsm4 and Dcp2 foci appeared and co-localized with each other at 24h. Interestingly, the majority of Lsm4/Dcp2 co-localized foci remained at the same size in the mother cells throughout 2h glucose starvation, which suggested that P-body-like aggregates were generally insensitive to glucose starvation (Fig. 3.3, 3.4). As expected, we observed the *de novo* formation of P-bodies in most of the daughter cells and occasionally in the mother cells at distinct sites from the pre-existing aggregates (Fig. 3.3). It should be noted that a small proportion of Lsm4/Dcp2 co-localized foci were gradually dissolved during glucose starvation followed by the formation of new foci, indicating a certain degree of heterogeneity among those P-body-like aggregates (Fig. 3.3). Based on these data we conclude that the age-related P-body-like aggregate is different from normal P-bodies and is not responsive to glucose starvation.

In yeast, P-bodies serve as mRNA degradation sites and its formation requires the presence of mRNA molecules³⁹. A previous study showed that pre-existing mRNAs were generally sequestered into P-bodies during stress, while newly produced mRNAs depending on their categories were diffusely localized or partially directed to stress granules⁴⁶. Therefore, we selected four representative mRNAs and examined if they localized to P-body-like aggregates during yeast aging by the MS2 tagging system⁴⁷ (Table 3.1). PGK1 is an enzyme involved in glycolysis and is constitutively expressed under normal conditions. Its mRNAs are sequestered in P-bodies upon glucose starvation. GLC3 is involved in glycogen accumulation.

Its mRNAs are induced during glucose starvation and localize to both P-bodies and stress granules. HSC82 and HSP30 are both heat shock proteins and involved in protein quality control. The difference is that HSC82 is constitutively expressed under normal conditions, while HSP30 is mainly produced during stress. Our data showed that *HSP30* mRNAs remained diffusely localized while *HSC82* mRNAs were sequestered in P-bodies during glucose starvation (data not shown). To enable the visualization of mRNA localization during aging, plasmids expressing mRNAs fused with MS2 stem loops were transformed into a yeast strain co-expressing MS2 coating protein fused with GFP and Dcp2 endogenously tagged with RFP. Four recombinant strains were grown in the microfluidic device for imaging at different time points. As expected, stress-induced *GLC3* and *HSP30* mRNAs form very few foci throughout aging, although a certain number of *HSP30* foci were observed at 12h (Fig. 3.5). *HSC82* and *PGK1*, two constitutively expressed mRNAs, were able to form small foci even under normal conditions (Fig. 3.5, 3.6). Considering that a potential problem with the MS2 system has been reported that MS2 decay products might be accumulated in yeast over time, we proposed that those mRNA foci under normal conditions might partially arise from the extra MS2 decay products in yeast⁴⁸. Similar to the previous results, Dcp2 foci appeared at 12h, and most of the aged cells at 24h contained at least one Dcp2 focus. Intriguingly, in all four strains we tracked, mRNA foci were no longer co-localized with Dcp2 foci starting 24h, while they did co-localize with some concomitant Dcp2 foci in daughter cells or in mother cells at early aging (Fig. 3.5, 3.7). In a batch culture experiment, we confirmed that *PGK1*, *HSC82* mRNAs and occasionally *HSP30* and *GLC3* mRNAs were sequestered into P-bodies during glucose starvation (data not shown). In the microfluidic device, a small proportion of aged yeast cells exhibited two distinct foci in a single cell: one containing both Dcp2 and mRNAs and the other one only containing Dcp2. These data suggest that another major

difference between the age-related P-body-like aggregates and normal P-bodies is that these aggregates lack certain mRNA species.

DISCUSSION

In this study we report a protein aggregate during yeast replicative aging which resembles physiological P-bodies but shows different properties. After screening fifteen proteins associated with yeast mRNP structures for their ability to aggregate during aging, we found that several major components of P-bodies comprise a unique group of proteins that are more aggregation-prone than the other proteins. This propensity to aggregate during aging has little correlation with the inherent prion-like properties, suggesting that being intrinsically disordered itself is not sufficient for proteins to form visible aggregates in yeast, even under suboptimal conditions such as aging in which proteostasis is impaired. Specific interactions between bio-molecules might be required in this process.

Further co-localization analyses showed that these P-body components, including Dcp2, Lsm4, Dhh1, Edc3, and Xrn1, are present in the same aggregate during aging. We thus speculate that this aggregate is comprised of similar components, if not the same, to physiological P-bodies involved in mRNA turnover. However, our results did not support that what we observed is actual P-bodies: first, the formation of this aggregate was neither dependent on nor affected by glucose starvation, a stressful condition that strongly induces P-bodies; second, this aggregate did not contain certain mRNA species including *PGK1* and *HSC82* observed in normal P-bodies. Therefore, we term it the age-related P-body-like aggregate.

Furthermore, our results showed that, similar to P-bodies and unlike amyloid structures, the P-body-like aggregate can be dissolved by both hexanediol and cycloheximide treatment, suggesting that it has a dynamic structure and requires constant protein translation. This aggregate is distinct from the Hsp104 aggregate formed during aging and many other Hsp104 inclusions formed during proteotoxic stresses in yeast. The absence of Hsp104 and

perhaps other molecular chaperones suggests that it is unlikely to be a well-regulated deposition site for misfolded proteins. However, we cannot exclude the possibility that misfolded proteins are still accumulated in this aggregate with aging but inaccessible to the protein quality control system for some reasons.

A major question to be solved in the future is the composition of this P-body-like aggregate. The presence of P-body components and the absence of Hsp104 suggest that it might be associated with mRNA metabolism rather than protein quality control. Surprisingly, we did not find any mRNA species in this aggregate. One unlikely but still possible model (here we call Model 1) is that these age-related P-body-like aggregates are actually functional P-bodies but contain only a specific group of mRNAs species over-expressed during aging. The other model (here we call Model 2) is that this aggregate represents an aberrant version of P-bodies with altered composition, reminiscent of recently reported aberrant stress granules in mammalian cells caused by the accumulation of misfolded proteins²⁷. Further biochemical analyses such as mass spectrometry and RNA-Seq are required to identify other components and ascertain whether any other mRNA species or misfolded proteins are sequestered in this aggregate.

We found that the P-body-like aggregates showed up in a small proportion of cells at 12h and in more than half of the mother cells at 24h. The proportion of cells with these aggregates stayed at the level around 50% and did not further increase after 24h. Data from both batch culture and microfluidics experiments at 48h showed that some P-body-like aggregates even remained in dying yeast cells (data not shown). Combining these data, we propose that rather than a well-regulated or functional physiological inclusion, this P-body-like aggregate is more like a pathological inclusion which matures during middle aging and remains through late aging. In other words, the aggregate might be indicative of an ‘unhealthy state’ in yeast cells. In order to understand the dynamics of this aggregate during

aging, we need to perform a closer examination of protein aggregation by monitoring individual proteins under a time-lapse microscope. Based on the previous studies on P-bodies and the data from *Lsm4/Dcp2* co-localization experiments, there is unlikely to a huge time variance regarding the appearance of individual P-body components within the P-body-like aggregate. However, we are still interested in whether the abundance of RNA and misfolded proteins will change and whether some P-body components will be lost in the aggregate with aging. As both hexanediol and cycloheximide treatment in this study were performed at 24h, it is also unclear whether this aggregate will transition from a dynamic liquid-like structure to a more solid-like structure during late aging in yeast. In addition, revealing the relative location of this P-body-like aggregate to other organelles or cytoskeletons might help to understand the mechanism of its formation. To address all these questions, we need to further characterize the spatio-temporal behaviors of this aggregate.

A question of keen interest is the biological function of the age-related P-body-like aggregate. While our preliminary data favor the Model 2 mentioned above, we are still uncertain whether the aggregate is truly devoid of mRNAs or contains other mRNA species that were not chosen for this study. Interestingly, we occasionally observed two kinds of *Dcp2* foci in a single cell expressing both *Dcp2-mRuby2* and *mRNA-MS2* during aging, one containing *mRNA-MS2* and one lacking it. Assuming those *Dcp2* foci with *mRNA-MS2* are normal P-bodies, the observation strongly suggests two points: first, the P-body-like aggregate should have a completely different structure from normal P-bodies, otherwise they would fuse together due to their liquid-like properties; secondly, the presence of P-body-like aggregate does not necessarily prevent the formation of new P-bodies, although it might sequester a certain number of P-body proteins. Another piece of evidence that supports this conclusion is that we occasionally observed the *de novo* formation of P-bodies in the mother cells with P-body-like aggregates at 24h upon glucose starvation. Taken together, these data support the

Model 2 and argue against the Model 1. We thus speculate that the P-body-like aggregates are aberrant P-bodies which have lost their normal function in RNA turnover, probably due to the accumulation of misfolded proteins and/or abnormal RNA species.

In order to fully understand the function of this aggregate, we can compare several biochemical properties, such as cellular fitness, RNA homeostasis and protein homeostasis, of those mother cells with and without the P-body-like aggregates. Another method is disrupting the formation of this aggregate by genetic manipulation and monitoring the changes of these properties. To this end, we can perform a genetic screen to identify genetic modifiers of this aggregate. Potential candidates include key P-body components, proteins involved in P-body-related RNA metabolism, molecular chaperones, and proteins involved in autophagy. We can then manipulate the P-body-like aggregate through genetic modifiers to understand its biological function.

Finally, we wish to explore the connection between this P-body-like aggregate and yeast aging. Our results showed that aging did not necessarily trigger the aggregation of P-body components, but it did increase the risk of this event. Interestingly, the aggregation of P-body components with aging was accompanied by a huge increase of Hsp104 expression and a gradual loss of Hsp104 foci. In some aged cells with Dcp2 foci, we observed abnormally high Hsp104 fluorescence intensities. Although there is no direct evidence that these two events are functionally linked, these data suggest that the formation of P-body-like aggregates might be associated with the loss of cellular fitness in yeast. This reminds us of those pathological protein aggregates in human diseases. It is of significance to reveal the upstream mechanisms that underlie the formation of this aggregate during yeast aging.

Another research focus in the future would be the potential effect of this aggregate on yeast aging. Many longevity genes have been identified in yeast, including *Sir2* and *Fob1* involved in epigenetic gene silencing and rDNA recombination, *Hsp104* and *Hsp42* involved

in the maintenance of protein homeostasis, etc. It is not hard to imagine that genes involved in RNA homeostasis like P-body components could be potential modifiers of yeast replicative lifespan. We can perform a genetic screen of P-body components on the MEP to quickly identify novel longevity genes. This will provide more insights into the connection between RNA homeostasis and aging, an area that has been little studied.

In previously studies, researchers reported the accumulation of P-bodies and a universal increase of protein aggregation level during aging in *C. elegans*^{29,49}. In yeast, we observed the formation of a P-body-like aggregate, but not exactly P-bodies during aging. Interestingly, P-bodies are mostly visible under the microscope in mammalian cells even under normal conditions, suggesting that mechanisms of mRNA turnover vary across different species. The long-term goal of this study is to expand these findings in yeast to other biological models. In the future, we hope to explore whether the abnormal aggregation of P-body components and relevant events are associated with human age-related diseases or aging itself.

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APPENDIX

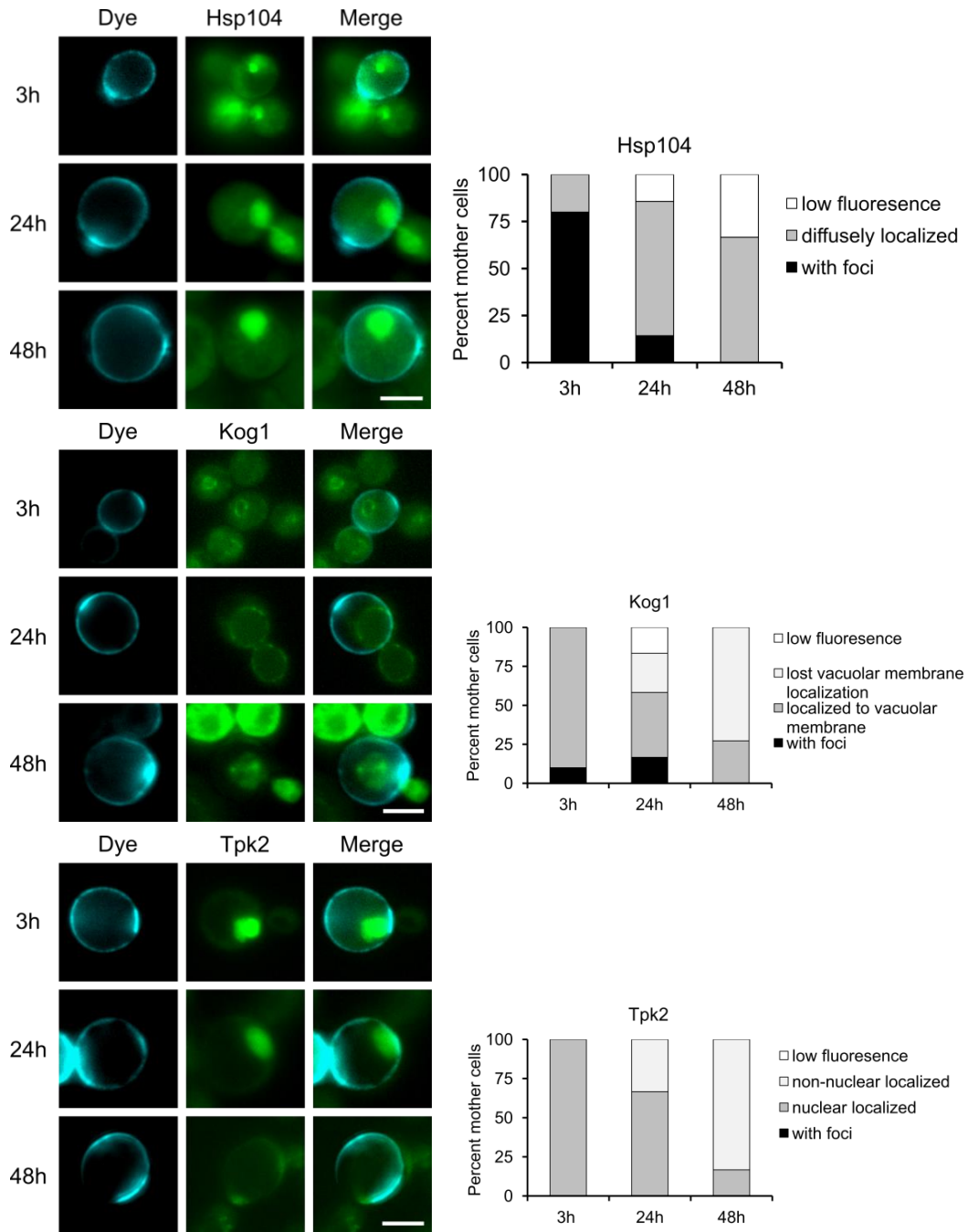


Figure 1.1: Localization of Hsp104, Kog1, and Tpk2 during aging in yeast

(Notes: Dye: DyLight 633 (hereafter), a far-red fluorescent dye to label mother cell walls. All proteins were endogenously tagged with mNeonGreen in the MEP. Cells

were harvested from batch culture and analyzed under fluorescence microscope. Scale bars: 5 μ m. n=3~14 for each experiment.)

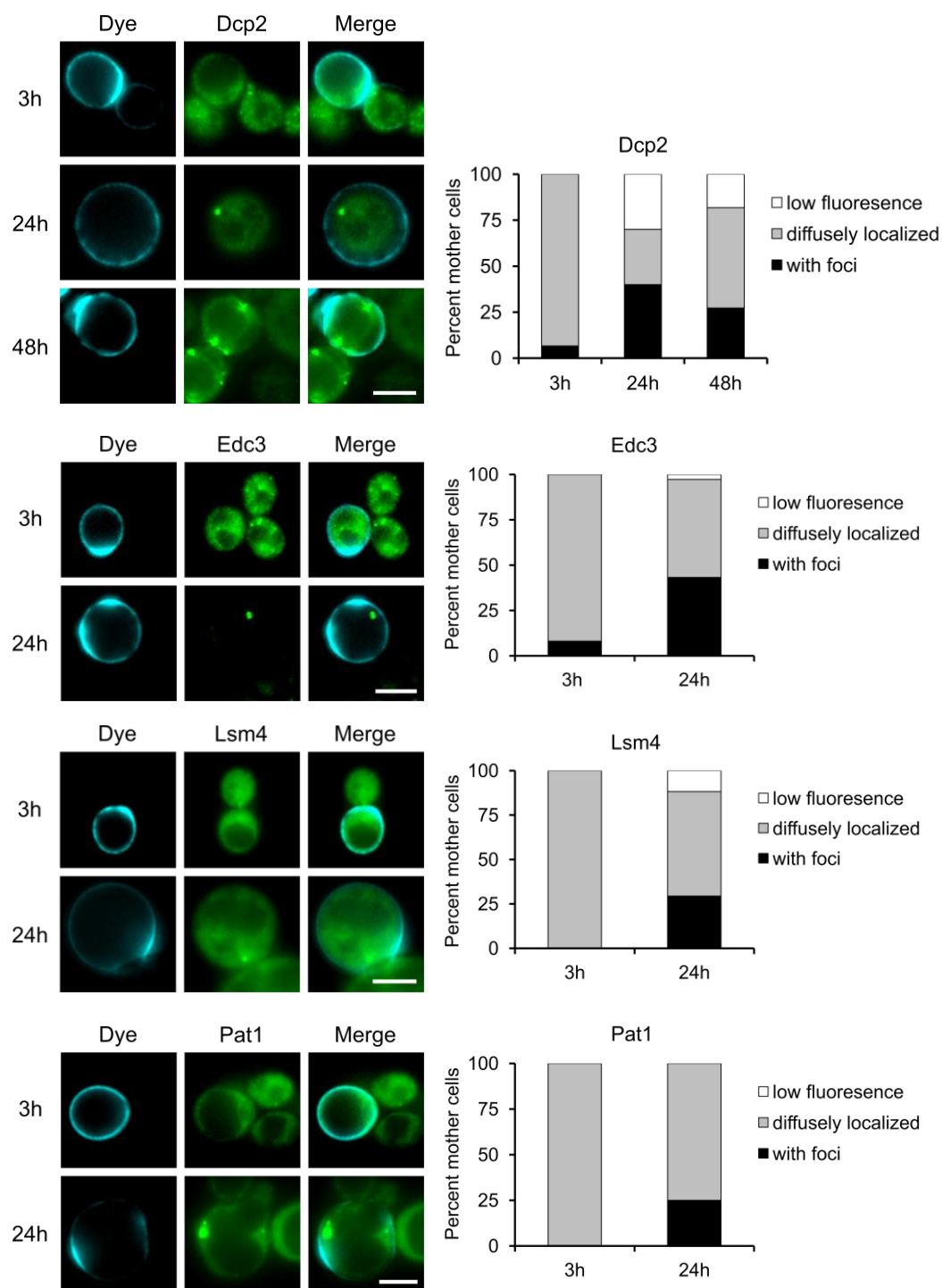


Figure 1.2: Localization of P-body components during aging in yeast

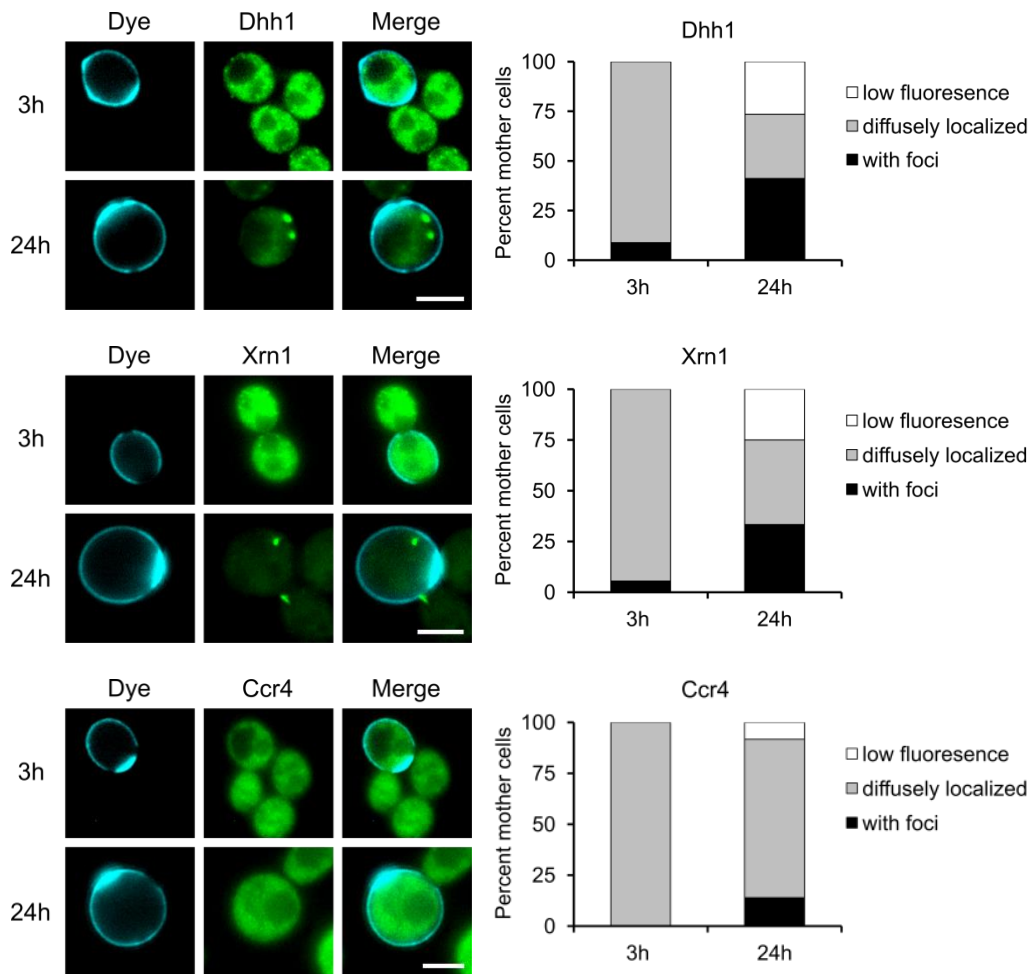


Figure 1.2: Localization of P-body components during aging in yeast, Continued

(Notes: All proteins were endogenously tagged with mNeonGreen in the MEP. Cells were harvested from batch culture and analyzed under fluorescence microscope. Scale bars: 5 μ m. n=10~37 for each experiment.)

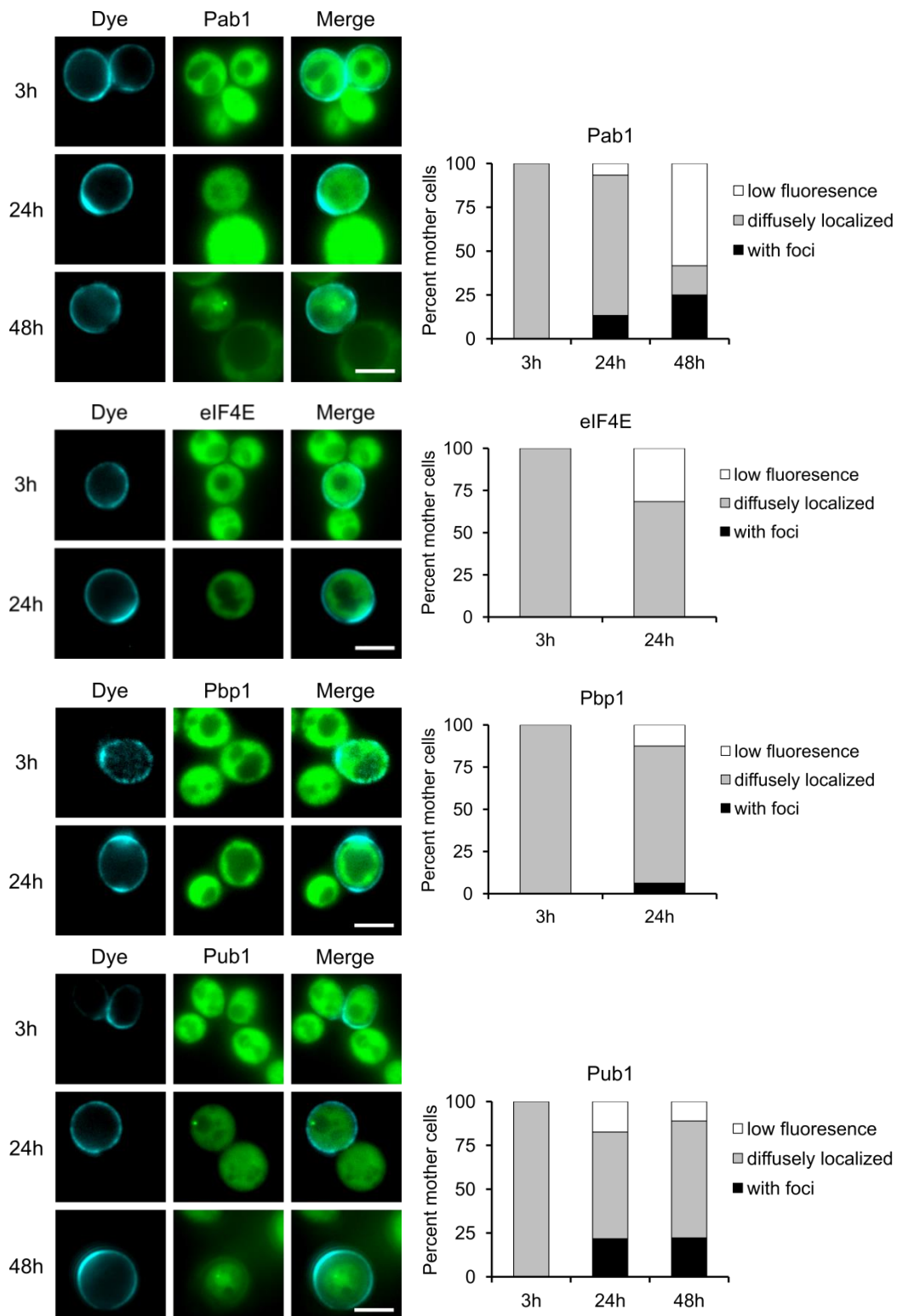


Figure 1.3: Localization of stress granule components during aging in yeast

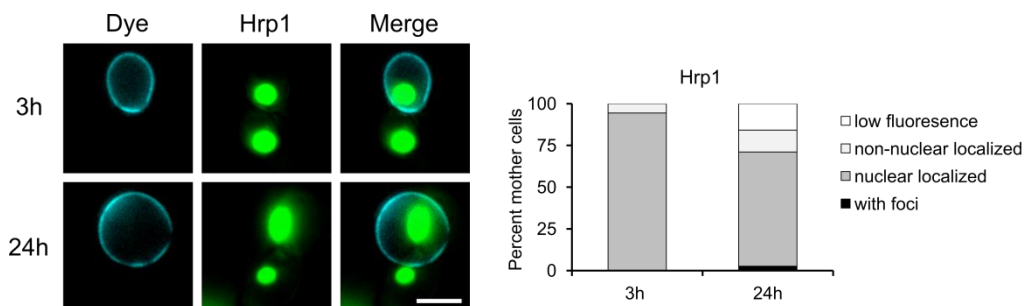


Figure 1.3: Localization of stress granule components during aging in yeast, Continued

(Notes: All proteins were endogenously tagged with mNeonGreen in the MEP. Cells were harvested from batch culture and analyzed under fluorescence microscope. Scale bars: 5 μ m. n=8~38 for each experiment.)

Table 1.1: Candidate proteins in this study

Name	Category	Description
Hsp104	Protein aggregate marker	Disaggregase; heat shock protein that helps to refold and reactivate previously denatured, aggregated proteins
Kog1	Nutrient signaling	Subunit of TORC1; involved in TOR pathway
Tpk2	Nutrient signaling	cAMP-dependent protein kinase catalytic subunit; involved in cAMP-PKA pathway
Dcp2	P-body	Catalytic subunit of Dcp1p-Dcp2p decapping enzyme complex;
Edc3	P-body	Non-essential conserved protein with a role in mRNA decapping;
Lsm4	P-body	Lsm (Like Sm) protein; part of heteroheptameric complexes (Lsm2p-7p and either Lsm1p or 8p)
Pat1	P-body	Deadenylation-dependent mRNA-decapping factor; binds to mRNAs under glucose starvation
Dhh1	P-body	Cytoplasmic DEAD-box helicase, stimulates mRNA decapping; coordinates distinct steps in mRNA function and decay
Xm1	P-body	Evolutionarily-conserved 5'-3' exonuclease
Ccr4	P-body	Component of the CCR4-NOT transcriptional complex; component of the major cytoplasmic deadenylase
Pab1	Stress granule	Poly(A) binding protein; part of the 3'-end RNA-processing complex
eIF4E	Stress granule	mRNA cap binding protein and translation initiation factor eIF4E
Pbp1	Stress granule	Interacts with Pab1p to regulate mRNA polyadenylation; yeast homolog of human disease protein ataxin-2
Hrp1	Stress granule	Subunit of cleavage factor I; yeast homolog of human disease protein TDP43
Pub1	Stress granule	Poly (A)+ RNA-binding protein; yeast homolog of human disease protein TIA1

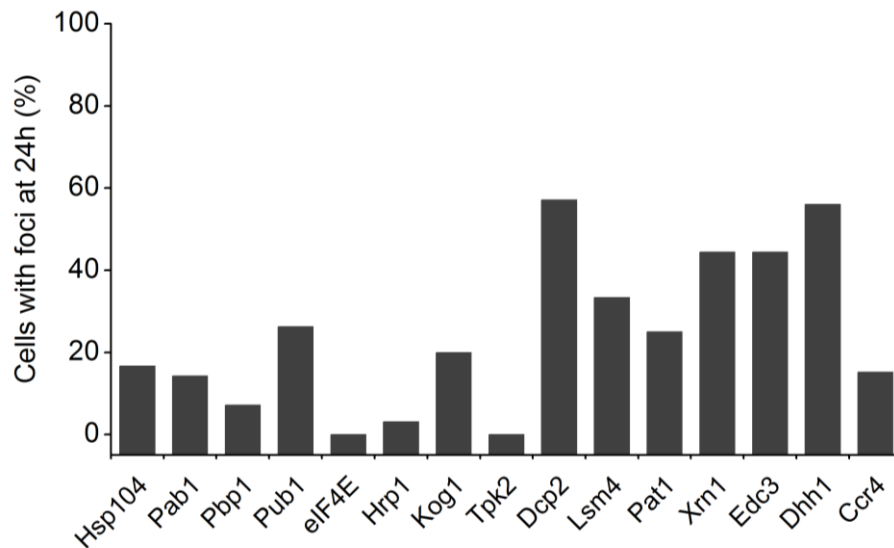


Figure 1.4: P-body components specifically form aggregates in yeast at 24h

(Notes: Proportions of cells with foci were calculated from cell populations excluding 'low-fluorescence' cells which were considered dying.)

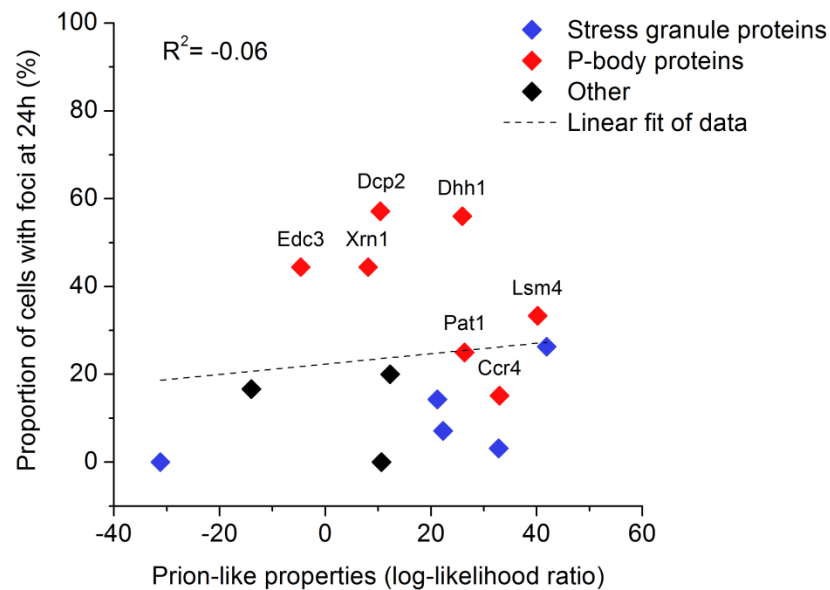


Figure 1.5: Correlation between the propensities to aggregate at 24h and the inherent prion-like properties of individual proteins

(Notes: Linear fit line of two groups of data was obtained by image analysis program, R^2 falls below zero, which indicates poor correlation.)

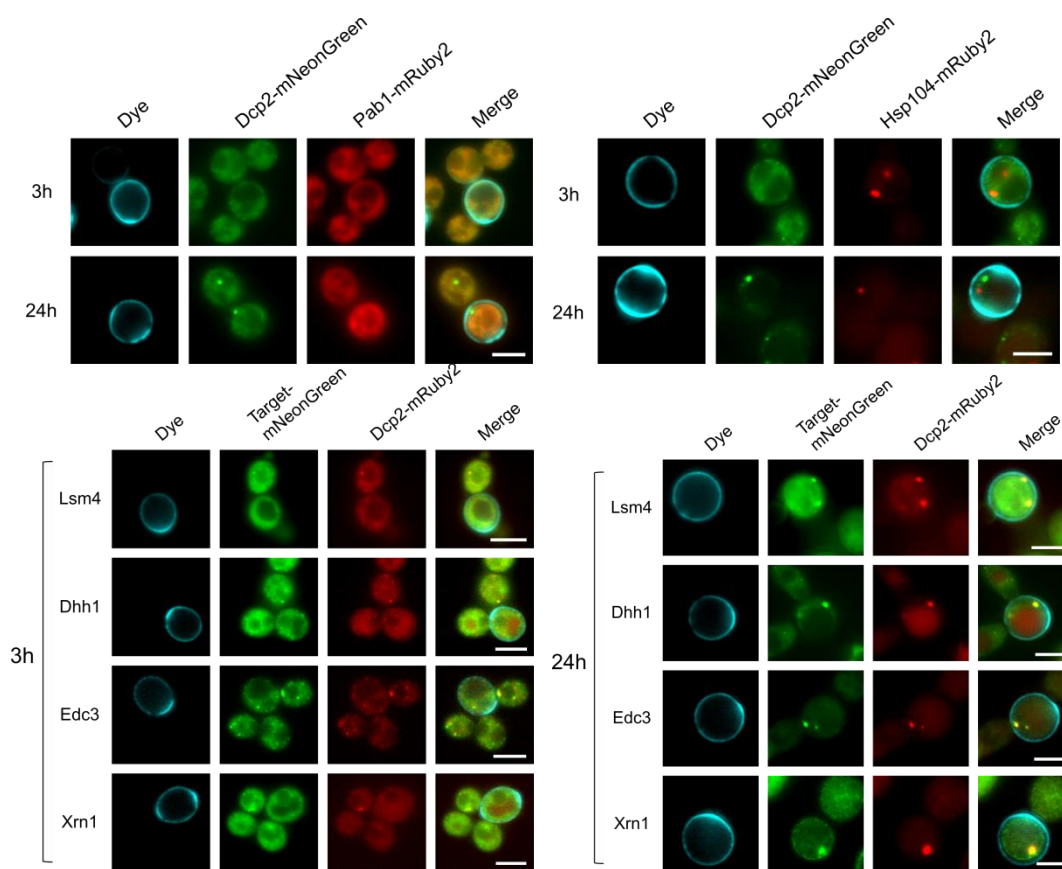


Figure 1.6: Co-localization analyses of Dcp2 with Pab1, Hsp104, and other P-body components during aging in yeast

(Notes: Dcp2 and other target proteins were endogenously tagged with different fluorescent proteins in the MEP, as shown in the figure. Scale bars: 5 μ m.)

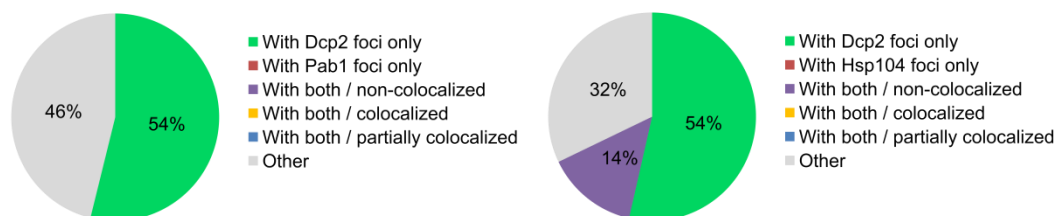


Figure 1.7: Classification of double-tagged yeast cells at 24h

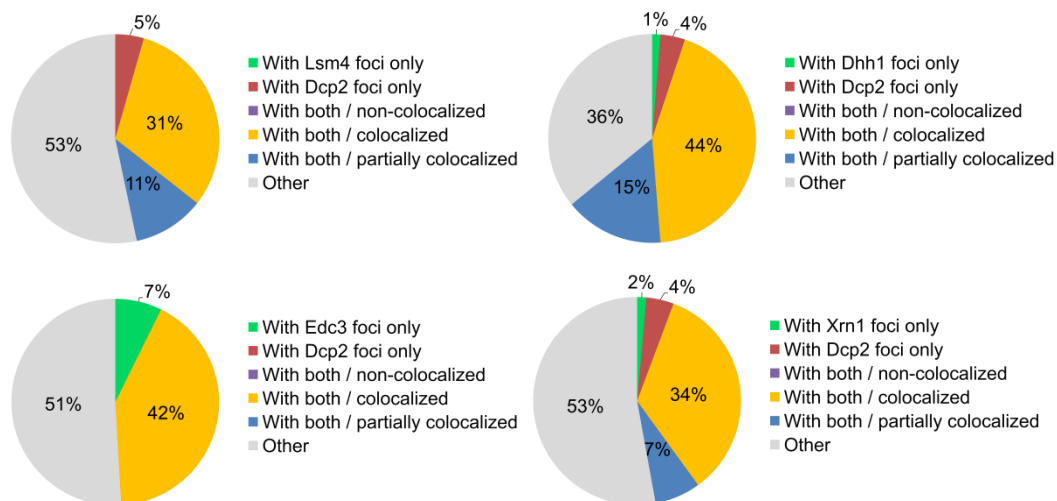


Figure 1.7: Classification of double-tagged yeast cells at 24h, Continued

(Notes: Cells were classified according to foci overlapping status. n=12~70 for each experiment.)

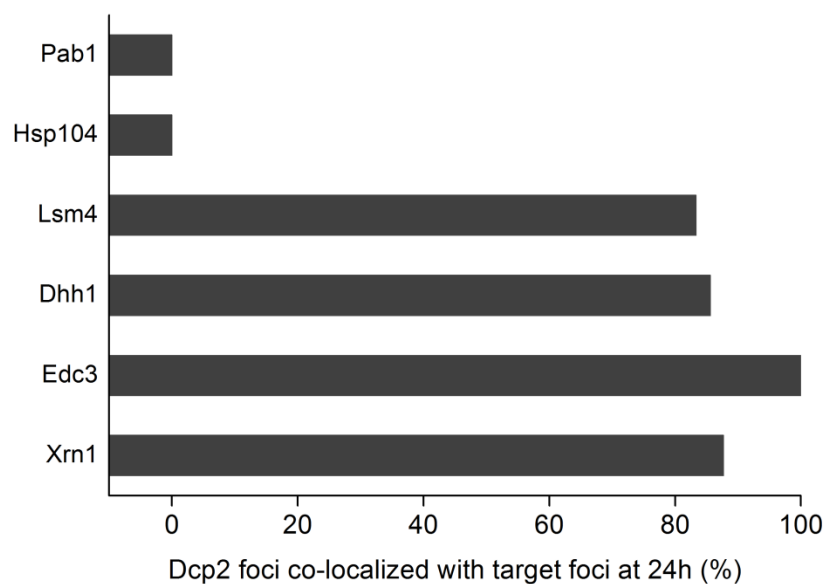


Figure 1.8: Dcp2 co-localizes with other P-body components, but not Pab1 and Hsp104 at 24h

(Notes: The same cell population used in Fig. 1.7 were used for the quantification of Dcp2 foci.)

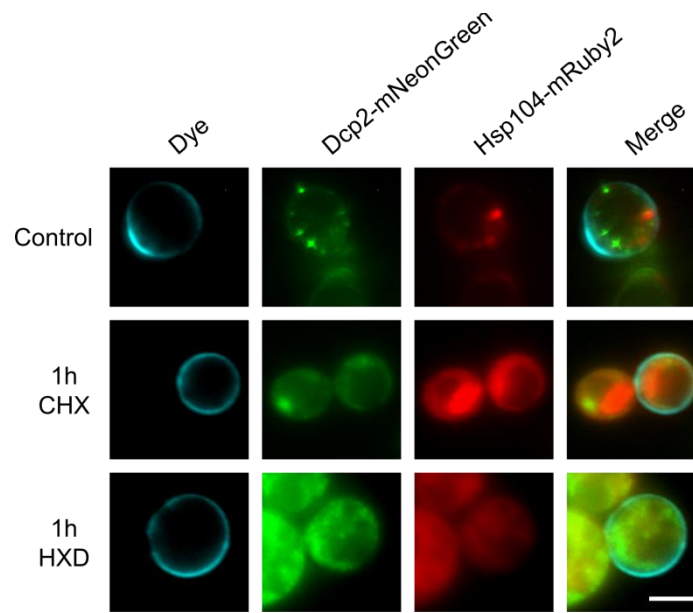


Figure 2.1: Localization of Dcp2 and Hsp104 after cycloheximide and hexanediol treatment at 24h

(Notes: CHX: cycloheximide; HXD: hexanediol. Cycloheximide was directly added to media to a final concentration of 100 $\mu\text{g}/\text{mL}$. Hexanediol was premixed with liquid media at 10% (g/mL) and added to cells after removing original media. Scale bars: 5 μm .)

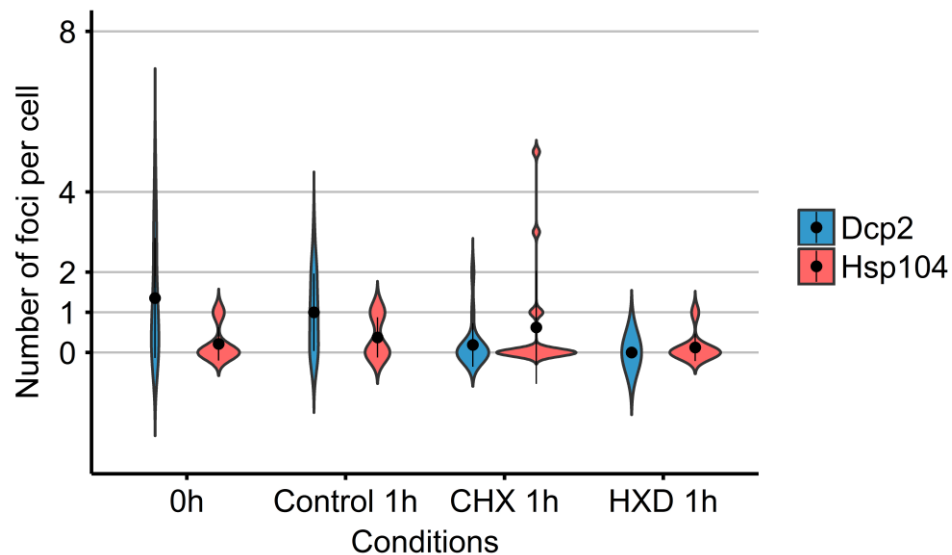


Figure 2.2: The formation of Dcp2 aggregates at 24h was disrupted by cycloheximide and hexanediol treatment to different extents

(Notes: CHX: cycloheximide; HXD: hexanediol; Control: normal media with nothing added. Central points and lines in violin plots indicate mean values and standard deviations, respectively. n=16~28 for each experiment.)

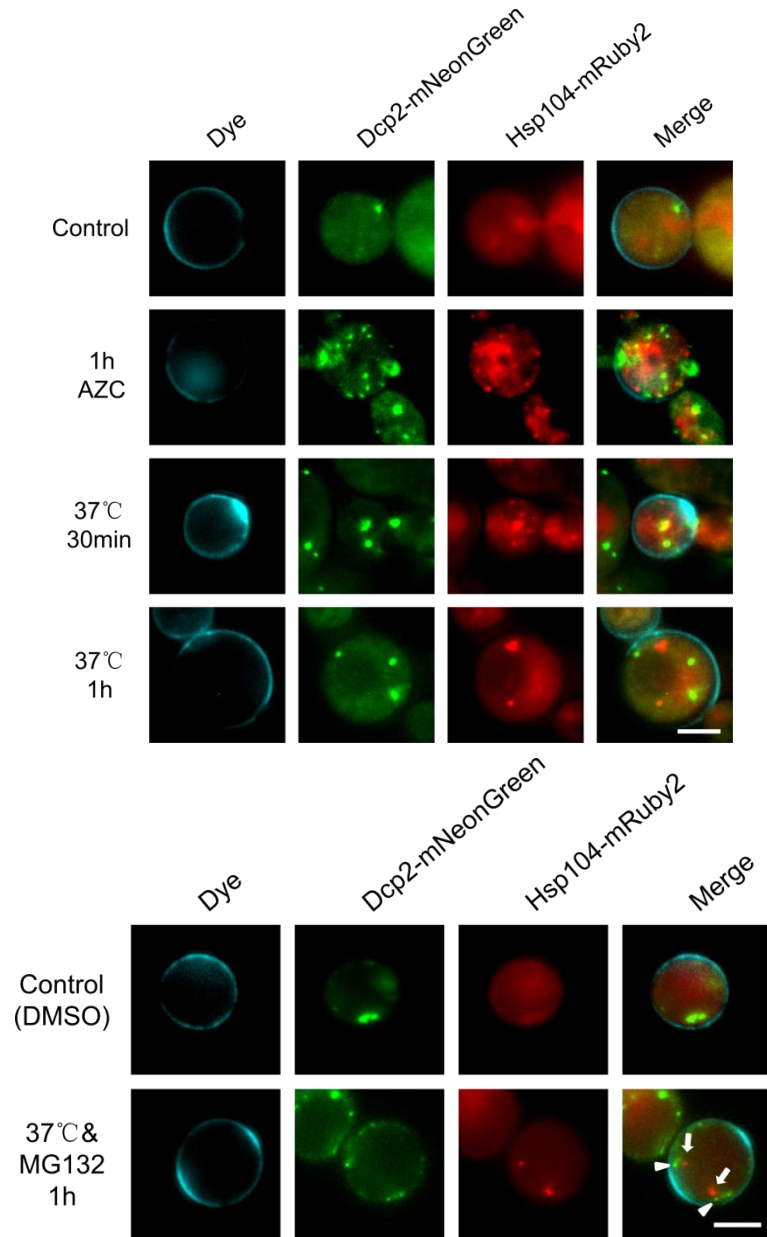


Figure 2.3: Co-localization analyses of Dcp2 and Hsp104 aggregates upon multiple proteotoxic stresses at 24h

(Notes: AZC: L-azetidine-2-carboxylic acid, a chemical that induces protein misfolding; MG132: proteasome inhibitor; Control: normal media; Control (DMSO):

normal media with 1/1000 DMSO (v/v). AZC was directly added to media to a final concentration of 10mM. MG132 was dissolved in DMSO and then diluted 1000 fold in normal media to a final concentration of 100 μ M. White arrowhead: Dcp2 foci at 24h; white arrow: JUNQ and IPOD; scale bars: 5 μ m.)

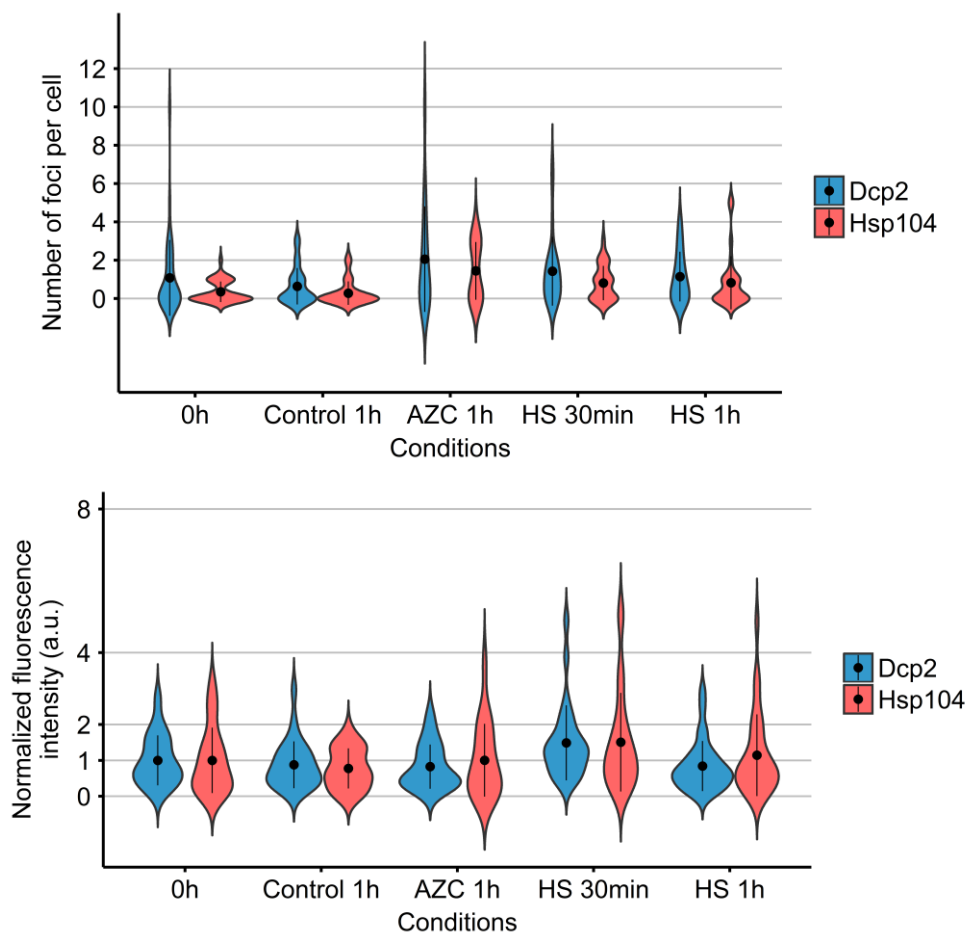


Figure 2.4: Effects of AZC treatment and heat shock on the foci number and expression level of Dcp2 and Hsp104 at 24h

(Notes: AZC: L-azetidine-2-carboxylic acid; HS: heat shock at 37°C; Control: normal media with nothing added. Central points and lines in violin plots indicate mean values and standard deviations, respectively. Fluorescence intensity in each cell was obtained from single plane images and normalized to the mean value at 0h to demonstrate relative protein expression levels. n=18~37 for each experiment.)

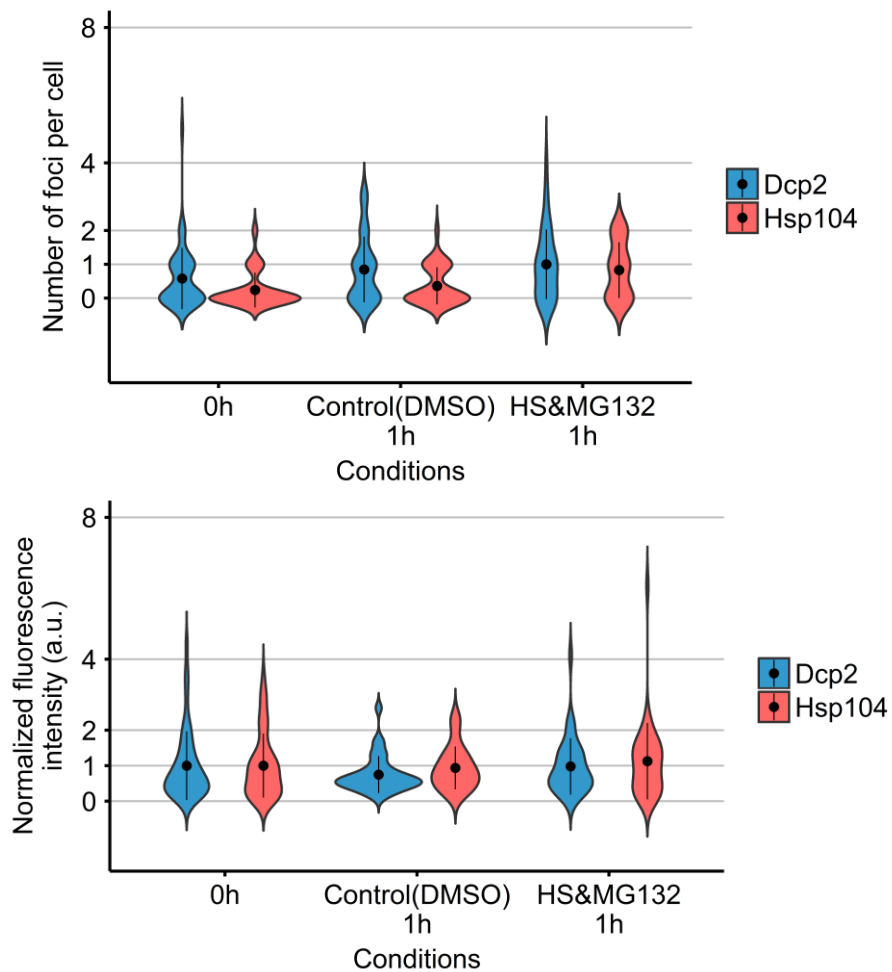


Figure 2.5: Effects of heat shock combined with MG132 treatment on the foci number and expression level of Dcp2 and Hsp104 at 24h

(Notes: HS: heat shock at 37°C; Control (DMSO): normal media with 1/1000 DMSO (v/v). Central points and lines in violin plots indicate mean values and standard deviations, respectively. Fluorescence intensity in each cell was obtained from single plane images and normalized to the mean value at 0h to demonstrate relative protein expression levels. n=33~50 for each experiment.)

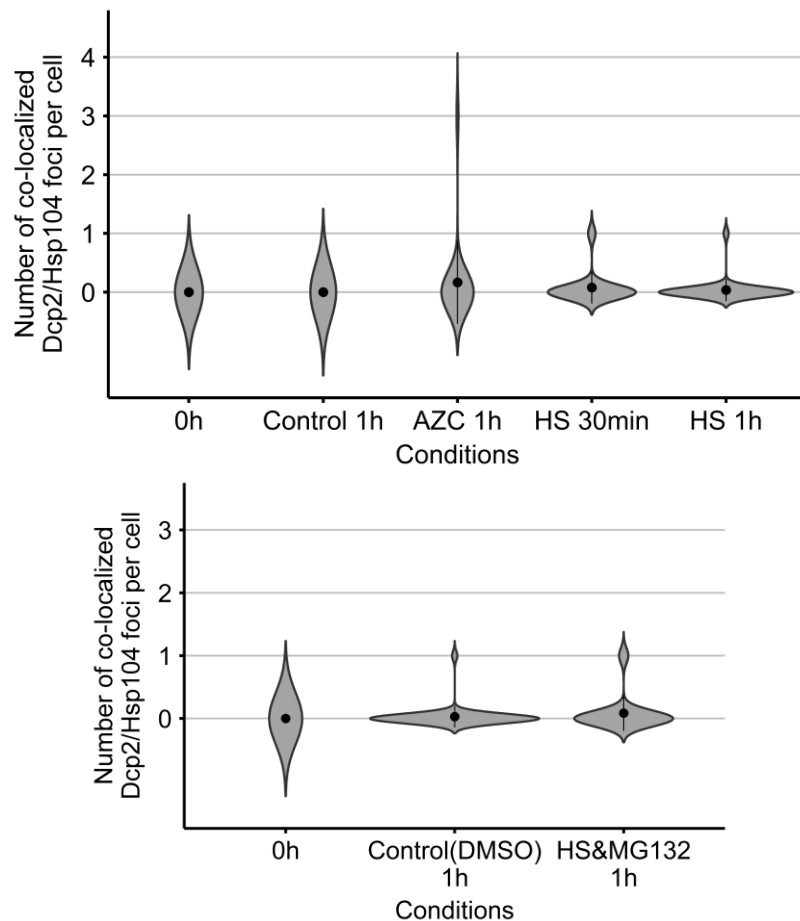


Figure 2.6: The age-related Dcp2 aggregate is distinct from Hsp104 aggregates induced by proteotoxic stresses, including Q-bodies, JUNQ and IPOD

(Notes: The same cell population from Fig. 2.4 and Fig. 2.5 was used for the quantification of foci co-localization. AZC: L-azetidine-2-carboxylic acid; HS: heat shock at 37°C; Control: normal media with nothing added; Control (DMSO): normal media with 1/1000 DMSO (v/v). Central points and lines in violin plots indicate mean values and standard deviations, respectively. n=18~50 for each experiment.)

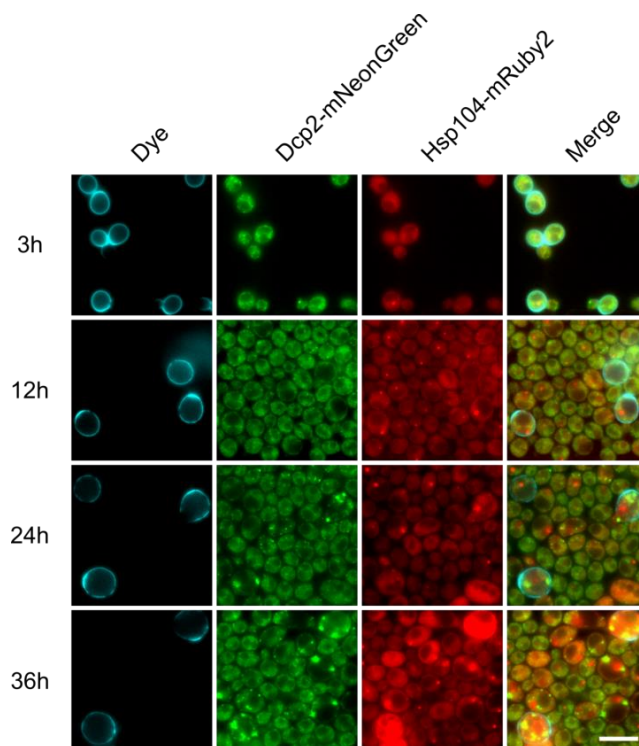


Figure 3.1: Dynamics of Dcp2 and Hsp104 aggregates during aging in yeast

(Notes: Double-tagged MEP cells were trapped in a microfluidic device and analyzed under fluorescence microscope. Scale bars: 10 μ m.)

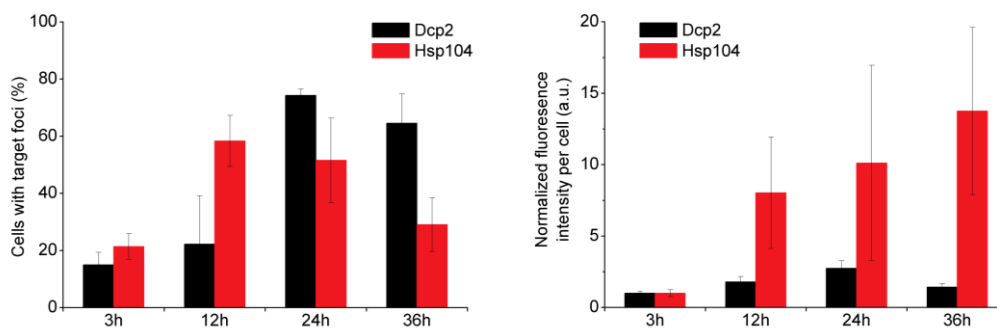


Figure 3.2: The aggregation pattern and expression level of Dcp2 and Hsp104 change with aging in yeast

(Notes: For each time point, data were obtained from three independent experiments with a total $n=25\sim47$. Fluorescence intensity in each cell was obtained from single plane images to demonstrate relative protein expression levels. For each group of data,

mean fluorescence intensity per cell was calculated and normalized to the total mean level at 3h. Both graphs show Mean \pm SEM.)

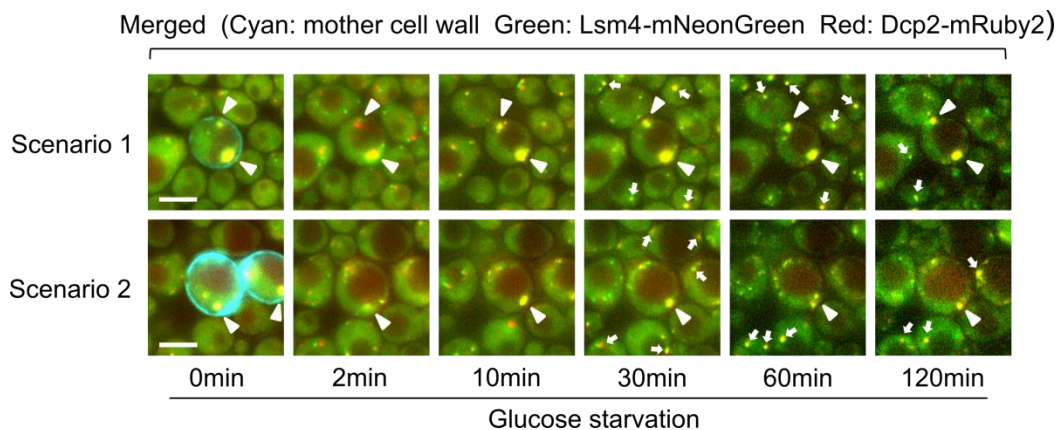


Figure 3.3: Dynamics of P-body-like aggregates in 24-hour-old yeast cells upon glucose starvation

(Notes: Scenario 1: most of the P-body-like aggregates remained in the field during glucose starvation; Scenario 2: some of the P-body-like aggregates were gradually degraded during glucose starvation. White arrowhead: P-body-like aggregates; white arrow: normal P-bodies in daughter cells induced by glucose starvation. Double-tagged MEP cells were trapped in a microfluidic device and analyzed under fluorescence microscope. Scale bars: 5 μ m.)

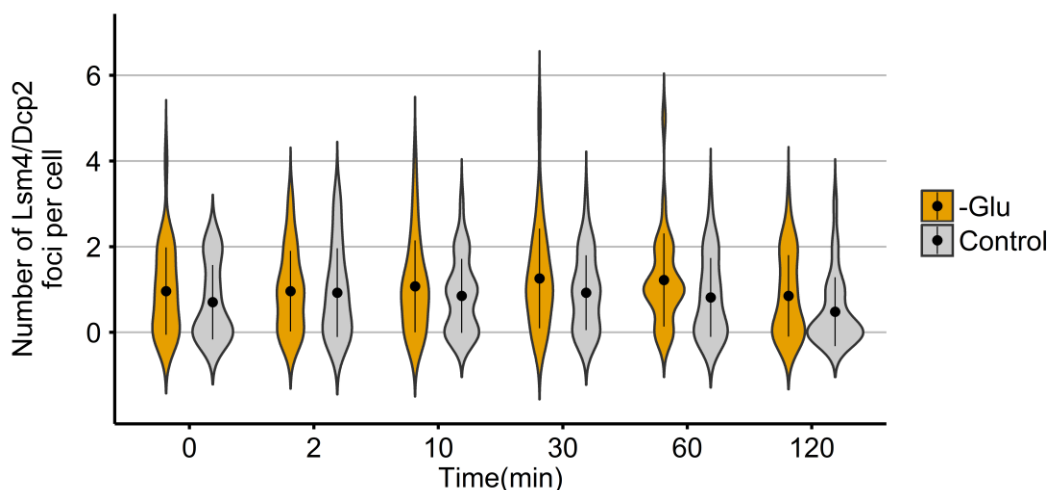


Figure 3.4: P-body-like aggregates at 24h are not sensitive to glucose starvation

(Notes: -Glu: glucose starvation; Control: normal yeast media with 2% (g/mL) glucose. Central points and lines in violin plots indicate mean values and standard deviations, respectively. n=27 for both experiments.)

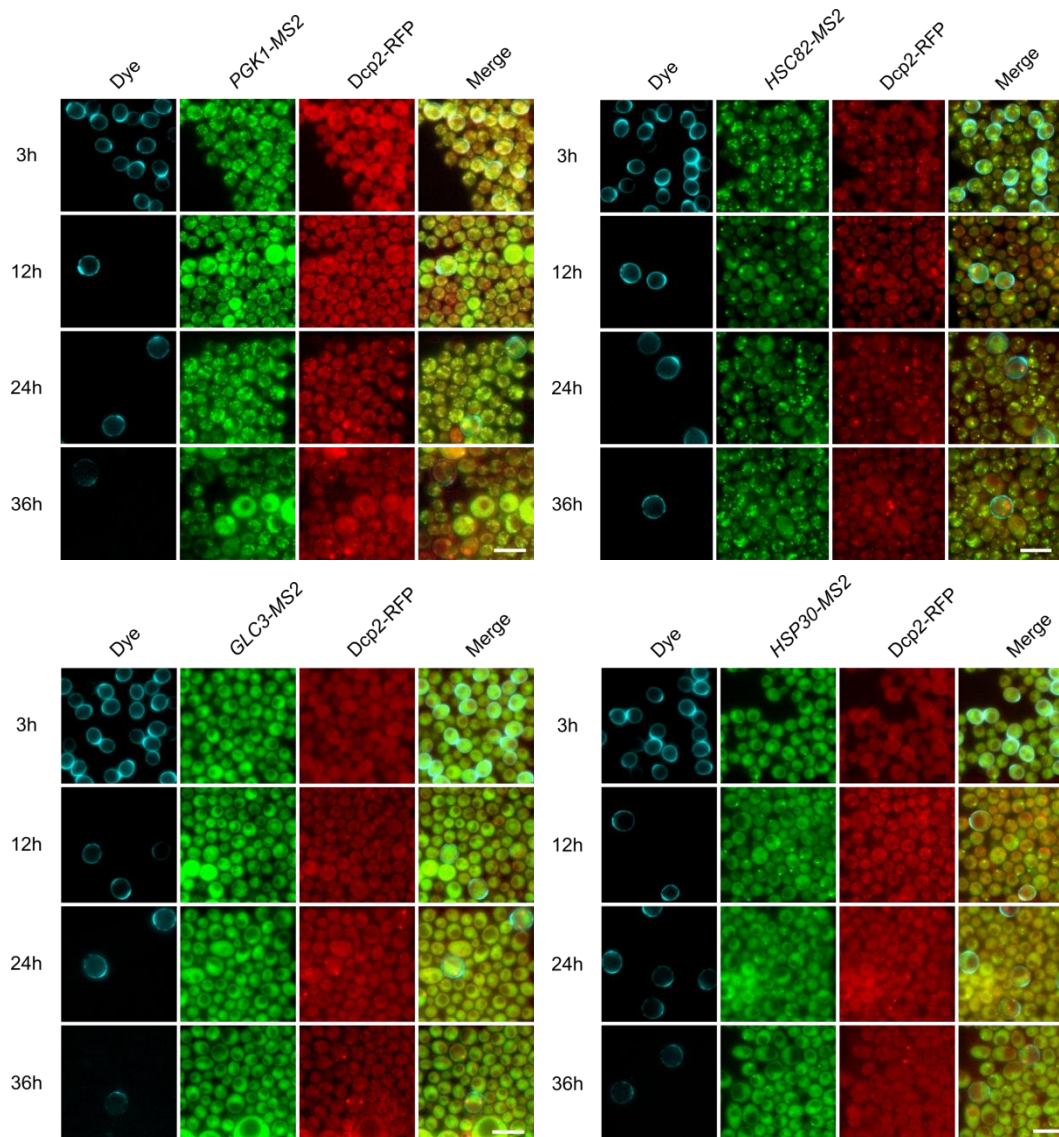


Figure 3.5: Co-localization analyses of different mRNA species and Dcp2 aggregates during aging in yeast

(Notes: For mRNA visualization, plasmids expressing different mRNAs fused with MS2 stem loops were transformed into a yeast strain with W303 background expressing MS2 coat proteins tagged with GFP and Dcp2 endogenously tagged with RFP. Cells were trapped in a microfluidic device and analyzed under fluorescence microscope. Scale bars: 10 μ m.)

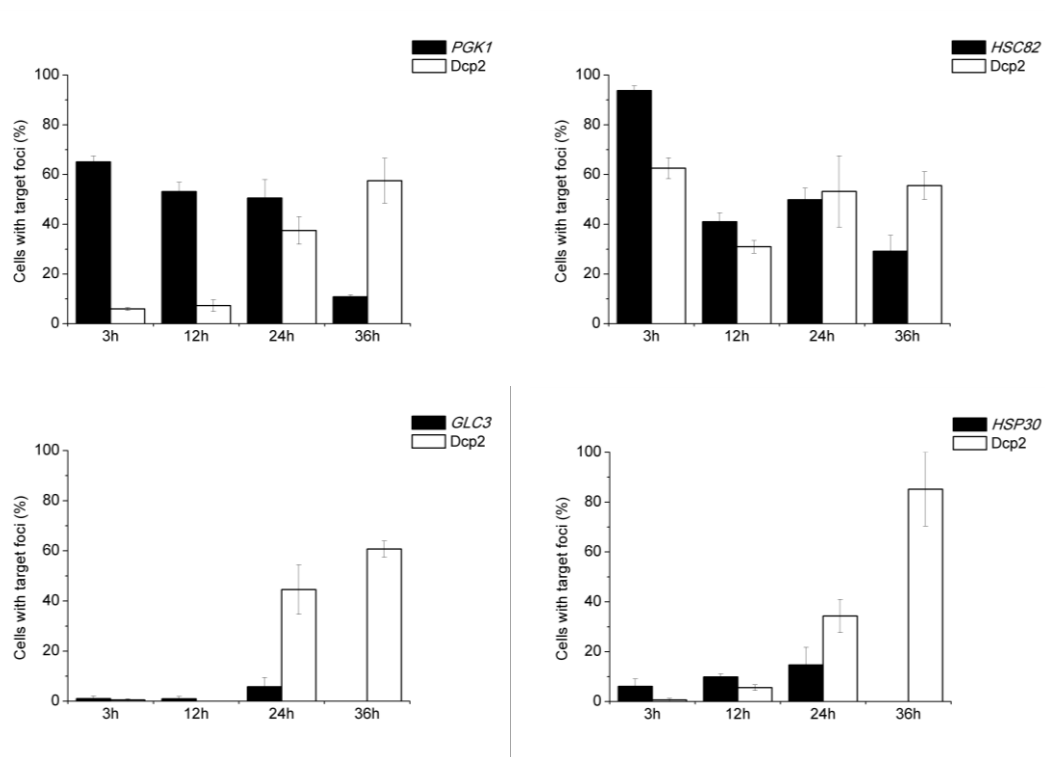


Figure 3.6: The aggregation pattern of Dcp2 and target mRNAs change with aging in yeast

(Notes: For each time point, data were obtained from three independent experiments with a total $n=20\sim 186$. Error bars: \pm SEM.)

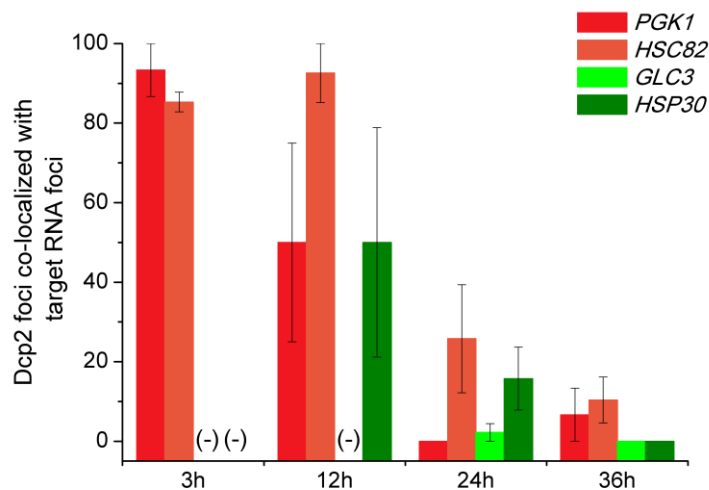


Figure 3.7: The age-related Dcp2 aggregate does not contain target mRNA species, including those observed in normal P-bodies

(Notes: The same dataset in Fig. 3.6 was used for the quantification of Dcp2 foci during aging. (-): lack of data; error bars: \pm SEM.)

Table 3.1: mRNA species selected for Dcp2 co-localization analyses

Name	Description
<i>PGK1</i>	3-phosphoglycerate kinase; key enzyme in glycolysis and gluconeogenesis; constitutively expressed; localized to P-bodies during glucose starvation
<i>HSC82</i>	Cytoplasmic chaperone of the Hsp90 family; constitutively expressed; localized to P-bodies during glucose starvation
<i>GLC3</i>	Glycogen branching enzyme, involved in glycogen accumulation; induced by glucose starvation; localized to P-bodies and stress granules during glucose starvation
<i>HSP30</i>	Stress-responsive protein; induced by multiple stresses; diffusely localized during glucose starvation