Title

Age-dependent aggregation of ribosomal RNA-binding proteins links deterioration in chromatin stability with loss of proteostasis

Author names and affiliations

Julie Paxman¹, Zhen Zhou¹, Richard O’Laughlin², Yang Li¹, Wanying Tian¹, Hetian Su¹, Yanfei Jiang¹, Shayna E. Holness³, Elizabeth Stasiowski², Lev S. Tsimring⁴, Lorraine Pillus^{1,5}, Jeff Hasty^{1,2,4}, and Nan Hao^{1,2,4,6,*}

¹Section of Molecular Biology, Division of Biological Sciences, University of California San Diego, La Jolla, CA 92093, USA

²Department of Bioengineering, University of California San Diego, La Jolla, CA 92093, USA

³Department of Chemistry and Biochemistry, University of California San Diego, La Jolla, CA 92093, USA

⁴BioCircuits Institute, University of California San Diego, La Jolla, CA 92093, USA

⁵UCSD Moores Cancer Center, University of California San Diego, La Jolla, CA 92093, USA

⁶Lead Contact

*Correspondence: nhao@ucsd.edu (N.H.)

Summary

Chromatin instability and loss of protein homeostasis (proteostasis) are two well-established hallmarks of aging, which have been considered largely independent of each other. Using microfluidics and single-cell imaging approaches, we observed that, during the replicative aging of *S.cerevisiae*, proteostasis decline occurred specifically in the fraction of cells with decreased stability at the ribosomal DNA (rDNA) region. A screen of 170 yeast RNA-binding proteins identified ribosomal RNA (rRNA)-binding proteins as the most enriched group that aggregate upon a decrease in rDNA stability. We further found that loss of rDNA stability contributes to age-dependent aggregation of rRNA-binding proteins through aberrant overproduction of rRNAs. These aggregates negatively impact nucleolar integrity and global proteostasis and hence limit cellular lifespan. Our findings reveal a mechanism underlying the interconnection between chromatin instability and proteostasis decline and highlight the importance of cell-to-cell variability in aging processes.

Introduction

Cellular aging is a complex biological phenomenon characterized by damage accumulation leading to loss of homeostatic cellular function and ultimately cell death (Kirkwood, 2005; Ogradnik et al., 2019). As cellular aging has been studied, macromolecular changes have been identified as hallmarks of aging including mitochondrial dysfunction, genomic instability, aberrant protein expression and aggregation, among others (Lopez-Otin et al., 2013). It has been generally believed that these changes occur together during aging, contributing to cellular decline and death. However, from a single-cell perspective, what remains unclear is whether these hallmarks concur in an individual aging cell. And, if so, is there a cascade of molecular events driving the aging process of single cells, in which these hallmark factors interact with and influence one another to induce aging phenotypes, functional deterioration, and ultimately cell death (Crane and Kaerberlein, 2018; Kirkwood and Kowald, 1997).

We have used the replicative aging of the yeast *Saccharomyces cerevisiae* as a genetically tractable model to investigate the dynamic interactions among aging-related processes (O'Laughlin et al., 2020). Yeast replicative aging is characterized as the finite number of cell divisions before cell death occurs (Mortimer and Johnston, 1959). Previous studies have identified many conserved features in yeast aging that also accompany human aging (Janssens and Veenhoff, 2016). Among these, chromatin instability and mitochondrial dysfunction have been considered as major drivers of aging in yeast. In particular, the instability of ribosomal DNA (rDNA), driven by decreased rDNA silencing, was among the first being identified as a causal factor of yeast aging (Defossez et al., 1999; Li et al., 2017; Saka et al., 2013; Sinclair and Guarente, 1997). Furthermore, mitochondrial dysfunction, driven by early-life decreases in vacuolar acidity and intracellular heme level, was also identified as a major contributor to yeast aging (Hughes and Gottschling, 2012; Li et al., 2020; Veatch et al., 2009). Intriguingly, our recent single-cell analyses revealed that rDNA instability and mitochondrial dysfunction are mutually exclusive in individual aging cells. In an isogenic population, about half of aging cells show loss of rDNA stability and nucleolar decline, whereas the other half experiences mitochondrial dysfunction (Li et al., 2020).

Proteostasis decline is another well-recognized hallmark of cellular aging (Hipp et al., 2019; Taylor and Dillin, 2011) and age-related diseases, such as a spectrum of neurodegenerative diseases (Hohn et al., 2020; Kurtishi et al., 2019). During aging, the cell gradually loses the ability to maintain proteostasis,

resulting in damaged protein accumulation, protein aggregation, and eventually cell death. However, how aging causes loss of proteostasis and how this decline interplays with other age-induced processes remain largely elusive. Here we combined microfluidic platforms with advanced imaging techniques to investigate proteostasis decline and protein aggregation during yeast aging at the single-cell level. We observed that proteostasis decline occurred almost exclusively in the aging cells that also experienced loss of rDNA stability. Because many RNA-binding proteins are aggregation-prone and hence sensitive to proteostatic changes, we performed a systematic screen for aggregation of yeast RNA-binding proteins and identified ribosomal RNA (rRNA)-binding proteins as the most enriched group of proteins that aggregate upon decreased rDNA stability. We further found that loss of rDNA stability leads to rRNA-binding protein aggregation through excessive rRNA production and that these age-induced aggregates contribute to overall proteostasis decline and accelerate aging, providing a new mechanistic connection between chromatin instability and proteostasis decline.

Results

Proteostasis decline concurs with ribosomal DNA silencing loss during cell aging

To track the changes in proteostasis in single aging cells, we monitored Hsp104-GFP, a canonical protein stress reporter that forms aggregates (visualized as fluorescent foci) upon loss of proteostasis (Lum et al., 2004; Tkach and Glover, 2004). Consistent with previous studies (Andersson et al., 2013; Erjavec et al., 2007; Saarikangas and Barral, 2015), yeast cells form Hsp104-GFP foci during aging, and the frequency of such appearance increases with age. However, we noticed that Hsp104-GFP foci did not appear universally in all aging cells. Instead, only a fraction of cells showed foci formation during aging (Fig. 1A).

We recently discovered and designated two distinct forms of aging processes in isogenic yeast cells as, “Mode 1” and “Mode 2” (Jin et al., 2019; Li et al., 2020). Mode 1 aging is driven by loss of rDNA silencing, as indicated by an increased fluorescence in a GFP reporter inserted at the rDNA region (rDNA-GFP). Mode 1 cells also produce elongated daughter cells at the late stages of lifespan. In contrast, Mode 2 aging retains rDNA silencing and is associated with production of small round daughters (Fig. 1A, top and middle). We classified a population of aging cells into Mode 1 and Mode 2 (see Fig. 1. legend,

Figure 1. Hsp104-GFP foci form specifically in aging cells that undergo loss of rDNA silencing. (A) Representative time-lapse images of WT Mode 1 and Mode 2 aging processes. Top: Phase, Middle: rDNA-GFP, Bottom: Hsp104-GFP. Replicative age of mother cell is shown at the top left corner of each image. For phase images, aging and dead mothers are marked by yellow and red arrows, respectively. In fluorescence images, aging and dead mother cells are circled in yellow and red, respectively. White arrows point to fluorescence foci of Hsp104-GFP. Mode 1 and Mode 2 cells were classified based on their age-dependent changes in their daughter morphologies (Li et al., 2020). (B) Single-cell color map trajectories of Hsp104-GFP foci formation in WT Mode 1 and Mode 2 cells. Each row represents the time trace of a single cell throughout its lifespan. Color represents the absence (light blue) or presence (dark blue) of foci within a given cell-cycle. Cells are sorted based on their lifespans. Single-cell color map trajectories of iRFP fluorescence and cell cycle length from the same cells are shown in Fig. S1 to confirm the classification of Mode 1 and Mode 2. (C) Representative time-lapse images of *sir2Δ* cells during aging. (D) Single-cell color map trajectories of Hsp104-GFP foci formation in *sir2Δ* cells.

1 Methods, and Fig. S1) and found that Hsp104-GFP form foci almost exclusively in Mode 1 aging cells,
2 but rarely in Mode 2 cells (Fig. 1, A and B; Video 1 and 2), indicating that loss of proteostasis occurs
3 specifically in Mode 1 aging. We independently monitored two additional reporters of proteostatic stress,
4 Δ ssCPY*-GFP, and Sis1-GFP. The Δ ssCPY*-GFP reporter is an unstable carboxypeptidase-GFP fusion
5 protein (Eisele and Wolf, 2008; Medicherla et al., 2004), and Sis1-GFP is an Hsp40 co-chaperone and a
6 reporter for nuclear proteostatic stress (Feder et al., 2021; Klaips et al., 2020). Consistent with the
7 observed pattern of Hsp104-GFP aggregation, both Δ ssCPY*-GFP and Sis1-GFP exhibit responses to
8 proteostatic stress specifically in Mode 1 aging cells (Fig. S2).

9 Because Mode 1 aging is driven by rDNA silencing loss and at the same time exhibits proteostasis
10 decline, we speculated that rDNA silencing might influence the state of proteostasis. To test this

possibility, we deleted Sir2, a conserved lysine deacetylase that mediates chromatin silencing at rDNA, in the Hsp104-GFP reporter strain. We found that, compared to WT, a dramatically larger fraction of cells showed Hsp104 foci formation in the short-lived *sir2Δ* mutant with sustained loss of rDNA silencing (indicated by a constantly high rDNA-GFP signal) (Li et al., 2017) (Fig. 1, C and D). In addition, these foci were formed earlier in life and persisted for a larger portion of the lifespan in *sir2Δ* cells than those in WT cells (Fig. 1D) and we observed a correlation between the time of first foci appearance and the final lifespan, suggesting that proteostasis decline, indicated by Hsp104 foci formation, contributes to cell aging and death (Fig. S3). Taken together, these results suggest that rDNA silencing loss can serve as a contributing factor for proteostasis decline during aging. Sir2, by maintaining rDNA silencing, promotes proteostasis and represses age-dependent protein aggregation, consistent with the role of sirtuins in alleviating protein aggregation-induced cytotoxicity and disorders (e.g. Huntington disease), in yeast and mammalian models (Cohen et al., 2012; Jiang et al., 2011; Kobayashi et al., 2005; Sorolla et al., 2011).

A screen identifies rRNA-binding proteins that aggregate in response to a loss of Sir2 activity

RNA-binding proteins bind to RNAs and form ribonucleoprotein complexes that regulate the localization, processing, modification, translation, storage and degradation of associated RNAs (Buchan, 2014; Lee and Lykke-Andersen, 2013; Mitchell and Parker, 2014; Ramaswami et al., 2013). A disproportionately high number of RNA-binding proteins contain low complexity, prion-like domains and hence are aggregation-prone (Calabretta and Richard, 2015; Kato et al., 2012; Weber and Brangwynne, 2012). Therefore, we reasoned that RNA-binding proteins may be especially sensitive to the intracellular proteostasis environment and considered whether aging or loss of Sir2 activity will lead to RNA-binding protein aggregation as early causal factors for age-induced protein misfolding and proteostasis decline.

To test this, we performed a screen to identify RNA-binding proteins that aggregate in response to a sustained loss of Sir2 activity, which induces rDNA silencing loss and mimics the later phases of Mode 1 aging. We used a recently-developed synthetic genetic sensor for protein aggregation – the yeast transcriptional reporting of aggregating proteins (yTRAP) RNA-binding protein sensor library for the screen (Newby et al., 2017). The yTRAP RNA-binding protein sensor library is composed of 170 unique sensor strains, encompassing every known RNA-binding protein with an experimentally confirmed

physical interaction with RNAs in yeast (Fig. S4A). The aggregation state of an RNA-binding protein can be reflected by the fluorescence signal of its sensor strain. When RNA-binding proteins are in a soluble unaggregated state, the sensor GFP fluorescence is high, however if the RNA-binding protein enters an aggregated state the GFP fluorescence is reduced (Fig. 2A).

Figure 2. A screen identifies 27 RNA-binding proteins (RBPs) that aggregate in response to loss of Sir2 activity. (A) Schematic of the yTRAP synthetic genetic system that functions by coupling aggregation states of proteins to the expression of a fluorescent reporter (diagrams adapted and modified from Newby et al. Cell. 2017). (B) Representative images of yeast cells following 5 mM NAM treatment. Top: Phase images; Bottom: Fluorescence images of rDNA GFP. (C) Representative time-lapse images of Hsp104-GFP cells treated with NAM during aging. (D) Representative time traces of fluorescence changes for a "non-responder" sensor strain (top) and "responder" sensor strains (middle, bottom). NAM induction time shown on graph in grey. Purple shades represent standard deviations of the traces. The time traces show raw fluorescence without normalization. (E) Functional categories of RBPs tested (left), the responders with more than 50% decrease in normalized fluorescence signal upon NAM (middle), and the responders with more than 75% decrease in normalized fluorescence signal upon NAM (right). Complete lists of RBPs tested and responder RBPs are included in Fig. S4.

To conditionally trigger a loss of Sir2 activity, we exposed cells to nicotinamide (NAM), a commonly-used inhibitor of Sir2 (Bitterman et al., 2002; Kato and Lin, 2014; Orlandi et al., 2017). The NAM treatment induced elongated cell morphology, rDNA silencing loss, and increased Hsp104 aggregation (Fig. 2, B and C), recapitulating the aged phenotypes in *sir2Δ* and in the late phases of Mode

1 aging. To track the fluorescence changes of yTRAP RNA-binding protein sensor strains in response to
2 NAM at high-throughput over time, we used a new version of our recently-published large-scale
3 microfluidic platform “DynOMICS” (Graham et al., 2020) that was specifically modified to permit the
4 analysis of libraries of fluorescent yeast strains. This device enables simultaneous quantitative
5 measurements of 48 different fluorescent sensor strains over the course of several days. We observed
6 that some sensor strains exhibited a dramatic decrease in fluorescence, indicating RNA-binding protein
7 aggregation, upon the NAM treatment (Fig. 2D, “Responders”); in contrast, other sensor strains showed
8 modest fluorescence changes, indicating minor changes in the aggregation state (Fig. 2D, “Non-
9 Responder”). We note that because all the RNA-binding protein sensors in the library are under the same
10 constitutive promoter, the fluorescence changes were specifically due to sensor aggregation state
11 changes, not differential expression level changes (Newby et al., 2017). Of the 170 RNA-binding proteins
12 tested in our screen (Fig. 2E, left, and Fig. S4A), we identified 43 Responders, which displayed at least
13 a 50% decrease in the normalized fluorescence signal upon the NAM treatment (Fig. 2E, middle, and
14 Fig. S4B), and 15 Responders with more than 75% decrease in the normalized fluorescence signal (Fig.
15 2E, right, and Fig. S4C). From both Responder groups, we found a consistent and striking enrichment of
16 rRNA-binding proteins involved in rRNA processing. These results indicate that rRNA-binding proteins
17 are a major class of proteins that aggregate upon a loss of Sir2 activity and hence they became the focus
18 of our further study below.

20 **Age-dependent rRNA-binding protein aggregation limits cellular lifespan**

21 To confirm that the identified rRNA-binding proteins indeed aggregate during natural aging, we
22 generated strains with an integrated copy of each rRNA-binding protein candidate C-terminally tagged
23 with mNeon (from the Responder list with more than 75% decrease in signal; Fig. 2E, right, and Fig.
24 S4C). To visualize age-induced rRNA-binding protein aggregation, we tracked single aging cells using
25 microfluidics, and then used confocal microscopy to capture high-resolution images of young cells (1
26 hour after loading) and aged cells (aged for 40 hours, ~80% of the average lifespan), respectively (Fig.
27 3A). We observed that the rRNA-binding proteins in young cells are uniformly localized along one side of
28 the nucleus, forming a single crescent shape, characteristic of the yeast nucleolus. At the late stages of

Figure 3. Age-dependent aggregation of rRNA-binding proteins and their effects on cellular lifespan. (A) Representative confocal images of rRNA-binding proteins in young cells, Mode 1 aged, and Mode 2 aged cells. Young cells and aged mother cells are circled in yellow. White arrows point to the aggregates. (B) The effects of 2-fold overexpression of each rRNA-binding protein on the lifespans of Mode 1 (left) and Mode 2 (right) cells. The percentage changes of the mean replicative lifespan (RLS) relative to that of WT have been shown in the bar graphs. Asterisk indicates the significance of the changes: *** represents $p < 0.001$; * represents $p < 0.05$. The complete RLS curves and p-values are shown in Fig. S6.

aging, most rRNA-binding proteins formed multiple irregular-shaped coalescences or aggregates (visualized as fluorescent patches or foci) in Mode 1 aged cells. In contrast, in Mode 2 aged cells, these rRNA-binding proteins all remained in the uniform crescent shape like that of young cells (Fig. 3A). These data confirmed the yTRAP screen results (Fig. 2) and are in accord with the observation that proteostasis decline occurs specifically in Mode 1 aging (Fig. 1), suggesting a connection between loss of rDNA silencing with age-induced rRNA-binding protein aggregation. We also observed that age-dependent aggregation led to a partial loss of colocalization of rRNA-binding proteins (e.g. Nop13 and Nop15; Fig. S5), which may be indicative of a deterioration of their coordinated functions in rRNA processing and ribosomal biogenesis in the nucleolus.

To determine the effect of rRNA-binding protein aggregation on lifespan, we overexpressed each of the rRNA-binding proteins, which leads to increased aggregation based on the law of mass-action. For each of the rRNA-binding proteins tested, we observed consistently that 2-fold overexpression of each significantly shortened the lifespan of Mode 1 aging cells, but not that of Mode 2 aging cells in the same isogenic population (Fig. 3B and Fig. S6). These results indicate that the aggregated form (in Mode 1 cells) of these rRNA-binding proteins causes cell deterioration and limits cellular lifespan, whereas increasing the non-aggregated form (in Mode 2 cells) shows either no effect or a small extension of the lifespan.

Excessive rRNA production induces rRNA-binding protein aggregation

We next considered the mechanism underlying rRNA-binding protein aggregation during aging and, in particular, how Sir2 and rDNA silencing, which primarily function in maintaining chromatin stability, influence the aggregation process. Since age-dependent aggregation and the effects on cellular lifespan were consistent for all the rRNA-binding proteins tested (Fig. 3), we chose to perform in-depth genetic analysis of one. We selected Nop15, which functions in 60S ribosomal biogenesis, as a representative to investigate the pathways and factors that regulate rRNA-binding protein aggregation. To monitor age-dependent progression of aggregation in single cells, we tracked the aging processes of a large number

Figure 4. Age-dependent aggregation of Nop15 in various mutants. Single-cell color map trajectories indicate the timing and extent of age-dependent Nop15 aggregation in (A) WT, (B, left) *rpn4Δ*, (B, right) *ubr2Δ*, (C, left) *sir2Δ*, (C, right) *fob1Δ*, and (D) *fob1Δ* + *RRN3* overexpression (O/E). Each row tracks the aggregation state of a single aging cell. Confocal images were acquired at indicated time points during the aging experiments. As indicated in the legend on the right, the aggregation state of Nop15 in each aging cell was classified as “no aggregation” – evenly distributed fluorescence with a normal crescent shape (light blue), “moderate aggregation” – unevenly distributed fluorescent patches with irregular shapes (blue), or “severe aggregation” – multiple distinct fluorescent foci (dark blue). Bottom panels: Bar charts show the percentage of cells in each aggregation state as a function of age, quantified from the data in corresponding top panels. For *RRN3* O/E, a TetO-inducible *RRN3* construct was integrated into the *fob1Δ* strain. Upon loading into the device, cells were exposed to 2 μ M doxycycline to induce *RRN3* overexpression throughout the aging experiment. As a control, *fob1Δ* cells without the inducible *RRN3* construct were exposed to doxycycline to exclude the possibility that the drug treatment itself causes enhanced aggregation (Fig. S7).

of individual cells using microfluidics and time-lapse microscopy (phase images acquired every 15 mins), and, in the same experiment, visualized Nop15-mNeon aggregation using confocal microscopy every 13 hours throughout the entire lifespans. We observed that Nop15 formed aggregates during aging of WT cells, with the frequency and severity increasing with age (Fig. 4A).

Recent studies showed that RNA-binding proteins, many of which contain intrinsically disordered domains, are frequent substrates of proteasomal degradation (Myers et al., 2018; Thapa et al., 2020). The proteasome, in cooperation with disaggregases, functions to remove misfolded or aggregated proteins (Pohl and Dikic, 2019). To examine the effects on rRNA-binding protein aggregation, we began with Rpn4, a transcriptional regulator of the 26S proteasome components that is required for normal levels of proteasome activity (Xie and Varshavsky, 2001). In cells lacking Rpn4, which are characterized by a reduced proteasome pool (Ju et al., 2004; Xie and Varshavsky, 2001), we found increased, earlier and more severe Nop15 aggregation during aging (Fig. 4B, left). In contrast, deletion of *UBR2*, which encodes a ubiquitin ligase that mediates Rpn4 degradation, leads to elevated proteasome capacity (Wang et al., 2004) and thereby dramatically alleviated Nop15 aggregation (Fig. 4B, right). These results suggest that the proteasome participates in the process of removing age-induced rRNA-binding protein aggregates, probably through degradation of intrinsically disordered protein monomers as in the case of other misfolded protein aggregates or aberrant RNA-binding protein aggregates (Berke and Paulson, 2003; Brown and Kaganovich, 2016; Hjerpe et al., 2016; Reiss et al., 2020; Thapa et al., 2020).

We next examined the roles of Sir2 and rDNA stability on rRNA-binding protein aggregation. As shown in Fig. 3A, rRNA-binding protein aggregates formed in Mode 1 aged cells, characterized with loss of rDNA silencing. Consistently, we observed much earlier and more frequent appearance of Nop15 aggregation during aging of *sir2Δ* cells (Fig. 4C, left), indicating that deletion of Sir2 or loss of rDNA silencing promotes rRNA-binding protein aggregation.

The yeast rDNA contains 100-200 tandemly arrayed copies of a 9.1 kb rDNA repeat, coding for rRNA subunits of ribosomes. Whereas rDNA is the site of active RNA polymerase (Pol) I-mediated rRNA transcription, it is also one of the three heterochromatin regions in yeast that are subject to distinct forms of transcriptional silencing (Smith and Boeke, 1997). During aging, loss of silencing at the rDNA enhances the rate of DNA double strand breaks and recombination (Lindstrom et al., 2011; Saka et al., 2013), leading to the formation of extrachromosomal rDNA circles (ERCs) excised from this fragile genomic site. ERCs are self-replicating DNA circles, asymmetrically segregated to mother cells during cell division. As a result, ERCs accumulate exponentially in aging mother cells and have been proposed to be a causal factor of aging (Sinclair and Guarente, 1997). To determine whether loss of Sir2 or loss of rDNA silencing

drives rRNA-binding protein aggregation through ERC accumulation, we monitored Nop15 aggregation in the absence of Fob1, a replication fork-barrier protein that, when deleted, prevents rDNA recombination and abolishes ERC formation (Defossez et al., 1999; Johzuka and Horiuchi, 2002). We observed a dramatic reduction in Nop15 aggregation in the *fob1Δ* strain, indicating that ERC accumulation can be a major driver of age-dependent rRNA-binding protein aggregation (Fig. 4C, right).

ERCs can impact various aspects of cellular functions, such as cell cycle progression (Neurohr et al., 2018) and nuclear pore integrity (Denoth-Lippuner et al., 2014). However, how ERCs mechanistically limit cellular lifespan is not clear. A recent study showed that ERC accumulation dramatically increases the number of transcriptionally-active rDNA copies, resulting in a massive increase in pre-rRNA levels in the nucleolus. These pre-rRNAs, however, cannot mature into functional ribosomes (Morlot et al., 2019). Because increasing the level of RNA content generally promotes phase transition and aggregation of ribonucleoprotein complexes (Lin et al., 2015; Zhang et al., 2015), we hypothesized that excessive production of rRNAs could induce age-dependent aggregation of rRNA-binding proteins. To test this, in the *fob1Δ* mutant where ERC formation is abolished, we overexpressed Rrn3, the RNA Pol I-specific transcription factor that promotes rRNA transcription (Moorefield et al., 2000; Philippi et al., 2010; Yamamoto et al., 1996). In support of our hypothesis, we observed that the excessive rRNA production by Rrn3 overexpression is sufficient to induce Nop15 aggregation in the absence of ERCs (Fig. 4D; Fig. S7).

Taken together, these results revealed that age-dependent loss of rDNA silencing promotes rRNA-binding protein aggregation through ERC accumulation and, more specifically, excessively high levels of rRNAs transcribed from ERCs. This aggregation event may impair pre-rRNA processing and maturation in the nucleolus and hinder the assembly of functional ribosomes, accounting for the decoupling between rRNA transcription and ribosomal biogenesis observed in aging cells (Morlot et al., 2019).

Elevated rRNA-binding protein aggregation contributes to global proteostasis decline

Previous studies showed that increased expression and aggregation of intrinsically disordered proteins impose an increased burden on protein folding resources and the proteasome, leading to global proteostasis decline (Andersson et al., 2013; Bence et al., 2001; Outeiro and Lindquist, 2003; Stefani and Dobson, 2003; Verhoef et al., 2002; Yu et al., 2019). We speculated that age-induced elevation in aggregation of rRNA-binding proteins could contribute to the global proteostasis decline observed in aging cells (Fig. 1, A and B). In agreement with this hypothesis, the deletion of *SIR2*, which enhances rRNA-binding protein aggregation (Fig. 4C, left), also promotes proteostasis decline during aging, as reflected by increased Hsp104 foci formation (Fig. 1C and Fig. 5A). In contrast, the deletion of *FOB1*, which reduces rRNA-binding protein aggregation (Fig. 4C, right), showed dramatically decreased Hsp104 foci formation (Fig. 5A). To test whether elevated rRNA-binding protein aggregation can impact global proteostasis independent of ERCs, in the *fob1Δ* mutant where ERC formation is abolished, we overexpressed Rm3 and Nop15, respectively, as both perturbations can enhance rRNA-binding protein aggregation. We observed increased Hsp104 foci formation, indicating that elevated rRNA-binding protein aggregation is sufficient to induce global proteostasis decline (Fig. 5A).

Figure 5. Elevated rRNA-binding protein aggregation promotes Hsp104-GFP foci formation during aging. (A) Boxplots show the distributions of percentage of lifespan with Hsp104-GFP foci appearance in single aging cells for WT (n=87), *sir2Δ* (n=46), *fob1Δ* (n=60), *fob1Δ* + *RRN3* overexpression (n=117), and *fob1Δ* + *NOP15* overexpression (n=130). In the plot, the bottom and top of the box are first (the 25th percentile of the data, q1) and third quartiles (the 75th percentile of the data, q3); the red band inside the box is the median; the whiskers cover the range between $q1 - 1.5 \times (q3 - q1)$ and $q3 + 1.5 \times (q3 - q1)$. The *RRN3* O/E and *NOP15* O/E experiments were conducted in the same way as in Fig. 4D. (B) A schematic depicts the divergent pathways underlying single-cell aging in yeast. (C) Replicative lifespans (RLSs) for WT (n=216), *ubr2Δ* (n=139), *HAP4* O/E (n=134), and *HAP4* O/E, *ubr2Δ* (n=143). RLSs shown indicate the mean lifespans.

Taken together, our results revealed a sequential cascade of interconnected molecular events that underlies the aging process in a fraction (Mode 1) of yeast cells (Fig. 5B): age-dependent loss of

rDNA stability results in ERC accumulation and consequently excessive rRNA production, which promotes aggregation of rRNA-binding proteins. These aggregates contribute to age-induced global proteostasis decline and cell death. The other fraction (Mode 2) of isogenic cells undergo heme depletion and mitochondrial decline during aging (Li et al., 2020), but not rRNA-binding protein aggregation or proteostasis decline. In support of our working model, deletion of *UBR2*, which increases proteasome capacity and alleviates rRNA-binding protein aggregation and proteostasis decline, and overexpression of *HAP4*, which enhances mitochondrial biogenesis, target two divergent aging pathways and hence exhibit an additive effect on lifespan extension (Fig. 5C).

Discussion

Chromatin instability and proteostasis decline are two commonly described hallmarks of aging, which have been previously considered independent of each other. However, an increasing number of recent studies suggest that these two processes may be interconnected. For example, Sir2, a conserved deacetylase encoded by the best-studied longevity gene to date, mediates deacetylation and silencing of heterochromatin regions and serves as a major regulator of chromatin stability and lifespan in yeast. Deletion of Sir2, which causes a loss of chromatin stability, dramatically elevates damaged protein accumulation and aggregation (Cohen et al., 2012; Erjavec et al., 2007), suggesting potential interplays between processes that mediate chromatin stability and proteostasis. In line with this, similar patterns of co-regulation have been observed in mammals upon perturbations that target the sirtuin family of deacetylases (Min et al., 2010; Park et al., 2020; Tomita et al., 2015; Westerheide et al., 2009). However, the molecular basis underlying these connections has remained largely unclear.

In this study, we exploited the power of single-cell imaging technologies, which enabled us to track proteostasis decline throughout the lifespans of a large number of single yeast cells. Interestingly, we observed that proteostasis decline occurs specifically in the fraction of aging cells that undergo loss of rDNA stability (Mode 1), and mutants with decreased rDNA stability also show accelerated proteostasis decline. We further found that loss of rDNA stability causes age-dependent aggregation of rRNA-binding proteins through aberrant overproduction of rRNAs. These aggregates impair nucleolar integrity and contribute to global proteostasis decline, and thereby limit cellular lifespan. Importantly, throughout our

analyses, the fraction of aging cells that undergo mitochondrial dysfunction (Mode 2) did not show proteostasis decline or rRNA-binding protein aggregation and therefore provided a powerful isogenic control for evaluating the regulation and effects of age-induced aggregation.

Our findings establish a mechanistic connection between chromatin stability and proteostasis decline during aging and highlight the importance of cell-to-cell variability when considering aging hallmarks and longevity-modulating perturbations. In addition, our working model can help interpret some intriguing previous observations. For instance, it has been puzzling why overexpression of Hsp104, a disaggregase that clears protein aggregation, can dramatically extend the lifespan of *sir2Δ*, but not WT cells (Erjavec et al., 2007). Based on our results, only a fraction of WT cells (Mode 1) experience chromatin instability and proteostasis decline at late stages of aging and therefore the effect of Hsp104 overexpression on lifespan is rather modest. In contrast, the majority of *sir2Δ* cells age with accelerated and severe rRNA-binding protein aggregation and proteostasis decline due to sustained loss of chromatin stability. As a result, Hsp104 overexpression, which alleviates protein aggregation, exhibits a much more dramatic pro-longevity effect.

It is important to acknowledge that chromatin stability could be linked with proteostasis through other mechanisms, beyond that reported in this study. For example, in *C.elegans*, age-dependent changes in epigenetic landscape and chromatin accessibility lead to a repression of the heat-shock response (HSR) that combats protein misfolding, resulting in proteostasis decline (Labbadia and Morimoto, 2015). During human cell senescence, the losses of silencing and repression on transposon and satellite repeat activation cause disorganized nuclear distribution of HSF1 (the central transcription factor that mediates HSR), contributing to HSR suppression and proteostasis decline (Gaglia et al., 2020; Jolly et al., 2002; Swanson et al., 2013; Van Meter et al., 2014). A very recent report showed that ERC-like DNA circles in yeast cells impair nuclear pore integrity, causing aberrant pre-mRNA nuclear export and translation and consequently contributing to loss of proteostasis (Meinema et al., 2021). We speculate that multiple different mechanisms may operate in parallel to mediate the interplay between chromatin stability and proteostasis during aging. Further investigations will be needed to determine the relative contributions and dynamic coordination among these mechanisms.

We have focused our investigation of protein aggregation specifically on RNA-binding proteins. Recently, substantial interest has focused on the emerging connections between RNA-binding protein aggregation and age-related diseases. For example, aberrant formation and persistence of RNA-binding protein aggregates have been found to contribute to degenerative diseases, including Multi-System Proteinopathy, Paget's disease, Amyotrophic Lateral Sclerosis, Frontotemporal Lobar Degeneration, and Alzheimer's disease, making RNA-binding protein aggregates promising therapeutic targets for treating degenerative diseases (Khalil et al., 2018; Li et al., 2013; Ramaswami et al., 2013). However, a systematic analysis of age-dependent RNA-binding protein aggregation remained missing, in part due to a lack of quantitative and high-throughput cellular reporters. In this study, we took advantage of a recently-developed synthetic genetic system, yTRAP, which couples protein aggregation states to fluorescent reporter output (Newby et al., 2017), and a high-throughput microfluidic platform, DynOMICS, which enables simultaneous time-lapse measurements of a large number of fluorescent strains over an extended period of time (Graham et al., 2020). We screened the yTRAP RNA-binding protein sensor library, which encompasses most of the confirmed yeast RNA-binding proteins, and identified rRNA-binding proteins as the most enriched group of proteins that aggregate in response to loss of Sir2 activity. These rRNA-binding protein candidates have been independently confirmed and further analyzed, uncovering a mechanistic link between chromatin instability and proteostasis decline.

In addition to the rRNA-binding proteins, our screen has also identified a number of mRNA-binding proteins (Fig. 2E and Fig. S4). These proteins bind to mRNAs and form ribonucleoprotein complexes, which play important roles in posttranscriptional control of gene expression and a wide array of physiological functions (Buchan, 2014; Chakravarty et al., 2020; Jiang et al., 2020; Lee and Lykke-Andersen, 2013; Mitchell and Parker, 2014; Ramaswami et al., 2013). Age-dependent aggregation of mRNA-binding proteins may affect their regulatory functions, contributing to aging phenotypes and cellular decline. For example, previous studies showed that the yeast RBP Whi3 forms aggregates during aging, resulting in sterility in aged yeast cells (Schlissel et al., 2017). Future studies will be poised to confirm age-induced aggregation of each mRNA-binding protein identified from our screen and determine the regulation and consequence of their aggregation. In particular, proteins involved in pre-mRNA splicing are highly enriched among the responders from our screen (Fig. 2E) and would be of special interest for

in-depth analyses. These investigations, combined with our current analyses of rRNA-binding proteins, will lead to a comprehensive understanding about the role of RNA-binding protein aggregation in yeast aging.

Research on aging biology has benefited tremendously from the development of genomic sequencing and systematic analyses, which have revealed many conserved genes and hallmarks that change during cell aging and influence cellular lifespan (Hendrickson et al., 2018; Janssens et al., 2015; Lopez-Otin et al., 2013; McCormick et al., 2015; McCormick and Promislow, 2018). An emerging challenge is to understand how these genes and factors interact and operate collectively to drive the aging process. Furthermore, it has been increasingly recognized that cell aging is a highly dynamic and stochastic process in which isogenic cells age with distinct molecular and phenotypic changes, and thereby markedly different lifespans. Such complexity has hindered the progress in mechanistic understanding of aging biology and the rational design of effective intervention strategies to promote longevity. Recent advances in single-cell technologies provide enabling tools for tracking the aging processes of a large number of individual cells and defining the core aging networks that govern the fate and dynamics of deterioration in aging cells (He et al., 2020; O'Laughlin et al., 2020). As an example, the study presented here showcases how these technologies were applied to identify a cascade of events linking chromatin instability with proteostasis decline, two major age-induced processes in single cells, which allowed us to propose a simple molecular network that underlies single-cell aging in yeast populations. Importantly, this network model can be used to guide the design of combinatorial perturbations that target multiple core network nodes, rather than single genes, to dramatically extend cellular lifespan. We envision that systems-level single-cell analyses will become increasingly appreciated and adopted in aging research to unravel comprehensive regulatory networks that determine aging dynamics and to advance a mechanistic understanding of the causes, progression, and consequences of aging.

Acknowledgements

We thank Dr. Ahmad S Khalil (Boston University) for generously providing us the yTRAP RBP sensor library, and Dr. Dieter H Wolf (University of Stuttgart, Germany) for generously providing us the pRS316- Δ ssCPY*-GFP plasmid. This work was supported by National Institutes of Health R01AG056440 (to N.H., J.H., L.P., L.S.T.), GM111458 and AG068112 (to N.H.), and NSF MCB1716841 (LP).

Author Contributions

Conceptualization, J.P., L.S.T., L.P., J.H., and N.H.; Methodology, J.P., Z.Z., R.O., L.S.T., L.P., J.H., and N.H.; Investigation, J.P., Z.Z., R.O., Y.L., W.T., H.S., Y.J., S.E.H., E.S.; Formal Analysis, J.P., Z.Z., R.O., Y.L., W.T., H.S., Y.J., S.E.H., E.S.; Writing - Original Draft, J.P., and N.H.; Writing - Review & Editing, J.P., Z.Z., R.O., Y.L., W.T., H.S., Y.J., S.E.H., E.S., L.S.T., L.P., J.H., and N.H.; Resources, L.S.T., L.P., J.H., and N.H.; Supervision, N.H.; Funding Acquisition, L.P., L.S.T., J.H., and N.H.

Declaration of Interests

The authors declare no competing interests.

Materials and Methods

Strains and plasmid construction: Standard methods for growth, maintenance, and transformation of yeast and bacteria were used throughout. The *S.cerevisiae* yeast strains used in this study were generated from the BY4741 strain background (MATa *his3Δ1 leu20Δ met15Δ0 ura3Δ0*). Yeast integrative transformations were performed using the standard lithium acetate method and confirmed by PCR. To make the Hsp104-GFP reporter, yEGFP-*HIS3* was amplified by PCR and integrated at the C-terminus of *HSP104* at the native locus by homologous recombination. To make the Sis1-mNeon reporter, pKT209 (ref) was subcloned to replace yEGFP with mNeon, and then mNeon-*URA3* was PCR amplified and integrated into the C-terminus of *SIS1* at the native locus by homologous recombination. The plasmid pRS316-ΔssCPY*-GFP was generated in a previous study (Park et al., 2007). We subcloned into pRS306 by digesting both pRS306 vector and ΔssCPY*-GFP using Sall and HindIII and then ligating together. The newly assembled plasmid pRS306-ΔssCPY*-GFP was linearized using StuI and integrated into the *ura3-1* locus by homologous recombination. This strain background was BY4741 in which *ura3Δ0* was replaced by the W303 *ura3-1* locus (Li et al., 2020). The strain library used in the screen for RNA-binding protein aggregation were generated in a previous study (Newby et al., 2017).

To create the strains carrying each of the mNeon tagged rRNA-binding proteins, we used Gibson assembly, or traditional cloning to assemble plasmids containing the coding sequences for rRNA-binding proteins tagged C-terminally with mNeon under their native promoters. These plasmids were linearized using a restriction enzyme that would specifically cut within the promoter sequence, and then integrated into the genome by homologous recombination. Integration was verified using PCR and microscopy, and the copy number of the plasmid integrated was verified using PCR to ensure single-copy insertion. These strains were used to generate the data in Fig. 3A. The rRNA-binding protein overexpression strains were similarly created. The plasmids containing the coding sequences for rRNA-binding proteins under their native promoters were constructed without any fluorescent protein tagging (to avoid potential interference of protein function by tags) were also digested in the promoter region and integrated into the native genomic loci using homologous recombination. Integration and copy number were verified by PCR to ensure single-copy plasmid integration. These strains were used to generate the data in Fig. 3B.

The *sir2*Δ mutant was created by amplifying either *CgURA3* or *CgHIS3* fragment to replace the *SIR2* open reading frame by homologous recombination. Other genetic deletions were similarly created where each open reading frame was replaced with a specific nutrient marker: *fob1*Δ was created using *CgURA3* or *CgHIS3*, *rpn4*Δ was created using *CgHIS3*, *ubr2*Δ was created using *CgHIS3* or *CgLEU2*. The *HAP4* overexpression strain was constructed and verified previously (Li et al., 2020).

Because constitutive overexpression of *RRN3* or *NOP15* adversely affects cellular physiology and condition, we constructed doxycycline-inducible expression plasmids for both genes to conditionally activate their overexpression during aging. For plasmid construction, we used the MoClo toolkit of yeast gene parts and followed the gene assembly strategy as described previously (Lee et al., 2015). Any Tet-On expression system consists of the same basic components: a constitutive promoter driving the transactivator rtTA expression and a promoter with a TetO element driving a gene of interest. More specifically, the ORF of *RRN3* or *NOP15* was PCR amplified and assembled into the entry vector pYTK001 by Golden Gate Assembly. Then it was assembled with the following plasmids by Golden Gate Assembly to create a specific combination of promoter-ORF-fluorescent tag-terminator: pYTK003 (ConL1), NHB1016 (TetO7-*P_{LEU2m}*), pYTK-ORF (*RRN3* or *NOP15*), pYTK034 (mRuby2), pYTK065 (*T_{ENO2}*), pYTK072 (ConRE), and pYTK095 (AmpR-*ColE1*). In the meanwhile, the coding sequence of rtTA3 was put under the *P_{RPL18B}* promoter from the the MoClo yeast toolkit, creating the *P_{RPL18B}-rtTA3* plasmid (NHB1028). The resulting two plasmids and NHB0980, which is a backbone plasmid containing *LEU2* as a nutritional selection marker, were then assembled by Golden Gate Assembly to get the final plasmid, *P_{RPL18B}-rtTA3-T_{ADH1}-TetO7-P_{LEU2m}-RRN3-mRuby2-T_{ENO2}-LEU2* (NHB1148) or *P_{RPL18B}-rtTA3-T_{ADH1}-TetO7-P_{LEU2m}-NOP15-T_{ENO2}-LEU2* (NHB1150). All the pYTK plasmids were from Lee et al., 2015. The inducible overexpression yeast strains were then created through genomic integration of NHB1148 or NHB1150 (linearized using *NotI*) by homologous recombination with the flanking sequences of the *leu2*Δ0 locus.

Microfluidic device fabrication: Design and fabrication of the microfluidic device for yeast replicative aging followed previously published work (Jin et al., 2019; Li et al., 2020; Li et al., 2017). In brief, 4-inch

silicon wafers (University Wafer Inc.) where patterned with SU8 2000 series (Kayakli Advanced Materials, Inc.) photoresists using standard photolithography techniques in general accordance with the guidelines provided by the manufacturer. A polydimethylsiloxane (PDMS) device was made from the silicon wafer mold by mixing 33g of Sylgard 184 (Dow Inc.) and pouring it on the wafer surrounded with aluminum foil. The wafer and PDMS are then degassed in a vacuum chamber and cured on a level surface for at least 1 hr. Design and fabrication of the DynOMICS device was carried out using techniques described previously (Graham et al., 2020). This PDMS device was made from the silicon wafer mold by mixing 77 g of Sylgard 184 and pouring it on the wafer centered on a level 5"x5" glass plate surrounded by an aluminum foil seal. The degassed PDMS is placed on a level surface and allowed to cure at 95C for 1hr.

Single-cell aging microfluidics setup: A PDMS aging device was cleaned and sonicated in 100% ethanol for 15 minutes then followed by a rinse sonication in milliQ water. The device was dried and cleaned with adhesive tape. A glass coverslip was cleaned in a series of washes heptane, followed by methanol, followed by milliQ water, and then dried by air gun. Both the coverslip and the PDMS device were exposed to oxygen plasma to bond and create a fully assembled device. Once assembled, each single-cell aging device was inspected to ensure no defects or dust contamination were present.

To begin the experiment setup, the device was first placed under vacuum for 20 minutes. All media ports covered were then immediately covered by 0.075% Tween 20 for approximately 10 minutes. The device was then placed on an inverted microscope with a 30C incubator system. Media ports were connected to plastic tubing and 60 mL syringes with fresh SCD media (prepared from CSM powder from Sunrise Science, #1001-100, with 2% glucose) medium containing 0.04% Tween-20. Initially the height of the syringes was approximately 2ft above the microscope stage. The waste ports of the device were also connected to plastic tubing, which were attached by tape to stage height. Yeast cells were inoculated into 1.5 mL of SCD and cultured overnight at 30C. This saturated overnight culture was then diluted 1:10,000 and grown at 30C overnight until cells reached approximately OD_{600nm} 0.6. For loading, cells were diluted approximately 2-fold and transferred to a 60mL syringe (Luer-Lok Tip, BD) and connected to plastic tubing (TYGON, ID 0.020 IN, OD 0.060 IN, wall 0.020 IN). Cells were loaded by temporarily replacing the input media port with the syringe filled with yeast culture. The syringe containing the yeast

is also placed approximately 2 ft above the stage. The media and cells flow into the device using gravity driven flow. Cell traps are generally filled with cells in under a minute, at which point the loading tubing is replaced with the media tubing and syringe. Once cells are loaded syringes are raised to be approximately 60 inches above the stage. Waste tubing is lowered to the floor and waste is collected in a 50 mL tube to measure flow rate of about 2.5 mL/day. Note that Tween-20 is a non-ionic surfactant that helps reduce cell friction on the PDMS (Ferry et al., 2011). We have validated previously that this low concentration of Tween-20 has no significant effect on cellular lifespan or physiology. This setup protocol was developed and described in our previous studies (Jin et al., 2019; Li et al., 2020; Li et al., 2017).

Time-lapse microscopy: Time-lapse microscopy experiments were performed using a Nikon Elipse Ti-2 inverted fluorescence microscope with Perfect Focus and a back-illuminated sCMOS camera (Teledyne Photometrics Prime 95B). The light source is a Lumencor SpectraX. Images were taken using a CFI plan Apochromat Lambda DM 60X oil immersion objective (NA 1.40 WD 0.13MM). Microfluidic devices were taped to a custom-built stage adapter and placed on the motorized stage. In all experiments, images were acquired using Nikon Elements software every 15 minutes for the duration of the yeast lifespan, typically 80 hours or longer, unless otherwise stated. The exposure and intensity settings were as follows for each of the fluorescence channels were: GFP 10 ms with 10% light intensity, mCherry 50 ms with 5% light intensity, Cy5 (iRFP) 200 ms with 2% light intensity.

Confocal microscopy: Confocal images were acquired using a CSU-X1 spinning disk confocal module on the Nikon Eclipse Ti2-E scope used for aging experiments. The excitation light is controlled using an Agilent laser box with 405 nm, 488 nm, 561 nm, or 640 nm lasers. Laser light is focused through the microlenses of the spinning excitation disk (Yokogawa CSU-X1). Images were taken using either Plan Apo lambda 60x NA 1.40 oil, or SR HP APO TIRF 100x 1.49 NA objectives. Laser intensity settings were: 30% for 488 nm, 50% for 561 nm, and 50% for 640 nm.

DynOMICS and yTRAP screen setup: Four 48-strain yeast DynOMICS devices were cleaned with 70% ethanol, DI water and Scotch tape (3M) and each was aligned to a custom Singer ROTOR compatible

fixture. Both the fixture and a clean glass slide sonicated with 2% Hellmanex III were exposed to oxygen plasma. Cells were spotted from the previously arrayed agar plate to the aligned PDMS device using the Singer ROTOR spotting robot. The device and glass slide were bonded together and cured for 2 h. Bonded chips were placed in a vacuum for 20 minutes before removal and covering of the inlet and outlet ports with SCD without folic acid or riboflavin media with 0.04% Tween-20. The microfluidic devices were then placed on two Nikon Ti microscopes, with two chips being placed on each Ti microscope. Media ports were then connected, using plastic tubing and 60mL syringes containing SCD media (without folic acid or riboflavin) with 0.04% Tween-20. At this point, we began imaging using a 4X objective on each microscope as yeast cells began to grow to fill the “bulb” regions of each position of the device and eventually fill the downstream “HD biopixels.” Both scopes were equipped with CoolSnap HQ2 cameras (Photometrics). Initial imaging conditions on the first Ti scope containing half of the yTRAP library were the following: Phase contrast 10 ms, GFP 400 ms, RFP 300 ms. For the second Ti microscope Phase contrast was 20 ms, GFP 600 ms and RFP 300 ms. Approximately two days after initial setup of the experiment, yeast had grown to fill the downstream biopixels at each position across the 4 devices. At this point, the GFP exposure time for each scope was increase to 700 ms and we marked this point as the starting point for future data analysis of fluorescent trajectories. Images were acquired every 20 minutes throughout the length of the experiment.

Classification of Mode 1 and Mode 2 aging cells: We classified Mode 1 and Mode 2 aging cells based on the age-dependent changes in their daughter morphologies, as described in our previous study (Li et al., 2020). Mode 1 cells produce elongated daughters at the late stage of lifespan, whereas Mode 2 cells produce small rounded daughters until death. The classification was further confirmed by iRFP fluorescence (indicating the intracellular heme level) and cell cycle lengths during aging. Mode 1 and Mode 2 cells exhibit distinct dynamics of iRFP fluorescence during aging – the iRFP fluorescence increases toward the late stage of Mode 1 aging; in contrast, iRFP signal sharply decreases at the early stage of Mode 2 aging and remains extremely low throughout the entire lifespan. Mode 1 and Mode 2 cells show age-dependent extension of cell cycle length with different timing and extents. Mode 1 cells show a gradual extension of cell cycle length at the late stages of aging; in contrast, Mode 2 cells show

a much earlier and more dramatic extension of cell cycle length during aging (Fig. S1)(Li et al., 2020). These age-dependent iRFP fluorescence and cell cycle length dynamics provide robust and quantitative metrics to further confirm the classification of the two aging modes, independent of the need for specific microfluidic devices or imaging setup.

Experiments with doxycycline-induced protein overexpression: Doxycycline was used as an activator to induce expression of TetO promoter-driven constructs including *RRN3* and *NOP15*. It was introduced into the media syringe to a final concentration of 2 μ M. Doxycycline was delivered immediately after the cells were loaded into the microfluidic device and image acquisition began. Aging cells were exposed to doxycycline throughout the entire aging experiments. To control for the effect of doxycycline itself on Nop15 aggregation, strains lacking the inducible constructs (e.g. *fob1 Δ*) were tested in parallel with and without doxycycline.

Quantification and statistical analysis:

Sample size for each experimental result can be found in the corresponding figure legend. To evaluate the statistical significance of lifespan differences, p-values were calculated using the Gehan-Breslow-Wilcoxon method, as performed in previous publications (Crane et al., 2019; Li et al., 2020), and were included in the corresponding RLS plots or the figure legend.

Tables and their legends

Table S1. Plasmids constructed in this study

Plasmid Description	Source	Identifier
pRS306- <i>P_{NOP15}</i> - <i>NOP15</i> - <i>mNeon</i> - <i>T_{ADH1}</i>	This study	NHB0904
pRS306- <i>P_{NOP15}</i> - <i>NOP15</i> - <i>T_{ADH1}</i>	This study	NHB0902
pRS306- <i>P_{SOF1}</i> - <i>SOF1</i> - <i>mNeon</i> - <i>T_{ADH1}</i>	This study	NHB0892
pRS306- <i>P_{SOF1}</i> - <i>SOF1</i> - <i>T_{ADH1}</i>	This study	NHB0901
pRS306- <i>P_{RPL7}</i> - <i>RPL7</i> - <i>T_{ADH1}</i>	This study	NHB0896
pRS306- <i>P_{NOP13}</i> - <i>NOP13</i> - <i>mNeon</i> - <i>T_{ADH1}</i>	This study	NHB0893
pRS306- <i>P_{NOP13}</i> - <i>NOP13</i> - <i>T_{ADH1}</i>	This study	NHB0903
pRS306- <i>P_{MRD1}</i> - <i>MRD1</i> - <i>mNeon</i> - <i>T_{ADH1}</i>	This study	NHB0927
pRS306- <i>P_{MRD1}</i> - <i>MRD1</i> - <i>mNeon</i> - <i>T_{ADH1}</i>	This study	NHB0895
<i>P_{RPL18B}</i> - <i>rtTA3</i> - <i>T_{ADH1}</i> - <i>TetO7</i> - <i>P_{LEU2m}</i> - <i>RRN3</i> - <i>mRuby2</i> - <i>T_{ENO2}</i> - <i>LEU2</i>	This study	NHB1148

<i>P_{RPL18B}-rtTA3-T_{ADH1}-TetO7-P_{LEU2m}-NOP15-T_{ENO2}-LEU2</i>	This study	NHB1150
--	------------	---------

Table S2. Yeast strains constructed in this study

Strain Description	Source	Identifier
BY4741 MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> , NHP6a-iRFP-kanMX, HSP104-GFP-HIS3	This study	NHGFP0068
BY4741 MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> , NHP6a-iRFP-kanMX, HSP104-GFP-HIS3, <i>sir2::CgURA3</i>	This study	NH0761
BY4741 MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> , NHP6a-iRFP-kanMX, HSP104-GFP-HIS3, <i>fob1::CgURA3</i>	This study	NH0778
BY4741 MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> , NHP6a-iRFP-kanMX, SIS1-mNeon-URA3	This study	NH1324
BY4741 MATa <i>his3Δ1 leu2-1 met15Δ0 ura3-1</i> , NHP6a-iRFP-kanMX, <i>ura3-1::P_{PRC1}-ΔssCPY*-GFP-URA3</i>	This study	NH1036
BY4741 MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> , NHP6a-iRFP-kanMX, NOP15::P _{NOP15} -NOP15-mNeon-T _{ADH1} -URA3	This study	NH1212
BY4741 MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> , NHP6a-iRFP-kanMX, SOF1::P _{SOF1} -SOF1-mNeon-T _{ADH1} -URA3	This study	NH1213
BY4741 MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> , NHP6a-iRFP-kanMX, RLP7::P _{RLP7} -RLP7-mNeon-T _{ADH1} -URA3	This study	NH1187
BY4741 MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> , NHP6a-iRFP-kanMX, NOP13::P _{NOP13} -NOP13-mNeon-T _{ADH1} -URA3	This study	NH1186
BY4741 MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> , NHP6a-iRFP-kanMX, MRD1::P _{MRD1} -MRD1-mNeon-T _{ADH1} -URA3	This study	NH1251
BY4741 MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> , NHP6a-iRFP-kanMX, NOP15::P _{NOP15} -NOP15-T _{ADH1} -URA3	This study	NH1218
BY4741 MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> , NHP6a-iRFP-kanMX, SOF1::P _{SOF1} -SOF1-T _{ADH1} -URA3	This study	NH1216
BY4741 MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> , NHP6a-iRFP-kanMX, RLP7::P _{RLP7} -RLP7-T _{ADH1} -URA3	This study	NH1211
BY4741 MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> , NHP6a-iRFP-kanMX, NOP13::P _{NOP13} -NOP13-T _{ADH1} -URA3	This study	NH1219
BY4741 MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> , NHP6a-iRFP-kanMX, MRD1::P _{MRD1} -MRD1-T _{ADH1} -URA3	This study	NH1215

<i>BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, NHP6a-iRFP-kanMX, NOP15::P_{NOP15}-NOP15-mNeon-T_{ADH1}-URA3, sir2::CgHIS3</i>	This study	NH1477
<i>BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, NHP6a-iRFP-kanMX, NOP15::P_{NOP15}-NOP15-mNeon-T_{ADH1}-URA3, rpn4::CgHIS3</i>	This study	NH1478
<i>BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, NHP6a-iRFP-kanMX, NOP15::P_{NOP15}-NOP15-mNeon-T_{ADH1}-URA3, fob1::CgHIS3</i>	This study	NH1479
<i>BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, NHP6a-iRFP-kanMX, NOP15::P_{NOP15}-NOP15-mNeon-T_{ADH1}-URA3, ubr2::CgHIS3</i>	This study	NH1630
<i>BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, NHP6a-iRFP-kanMX, NOP15::P_{NOP15}-NOP15-mNeon-T_{ADH1}-URA3, leu2Δ0::P_{RPL18B-rtTA3-T_{ADH1}-TetO7-P_{LEU2m}-RRN3-mRuby2-T_{ENO2}-LEU2, fob1::CgHIS3}</i>	This study	NH1507
<i>BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, NHP6a-iRFP-kanMX, RDN1::NTS1-P_{TDH3}-GFP-URA3, ubr2::CgHIS3</i>	This study	NH1408
<i>BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, NHP6a-iRFP-kanMX, RDN1::NTS1-P_{TDH3}-GFP-URA3, HAP4::P_{TDH3}-HAP4-LEU2, ubr2::CgHIS3</i>	This study	NH1642
<i>BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, NHP6a-iRFP-kanMX, HSP104-GFP-HIS3, fob1::CgURA3, leu2Δ0::P_{RPL18B-rtTA3-T_{ADH1}-TetO7-P_{LEU2m}-RRN3-mRuby2-T_{ENO2}-LEU2,}</i>	This study	NH1666
<i>BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, NHP6a-iRFP-kanMX, HSP104-GFP-HIS3, fob1::CgURA3, leu2Δ0::P_{RPL18B-rtTA3-T_{ADH1}-TetO7-P_{LEU2m}-NOP15-T_{ENO2}-LEU2}</i>	This study	NH1685

References:

- Andersson, V., Hanzen, S., Liu, B., Molin, M., and Nystrom, T. (2013). Enhancing protein disaggregation restores proteasome activity in aged cells. *Aging (Albany NY)* 5, 802-812.
- Bence, N.F., Sampat, R.M., and Kopito, R.R. (2001). Impairment of the ubiquitin-proteasome system by protein aggregation. *Science* 292, 1552-1555.
- Berke, S.J., and Paulson, H.L. (2003). Protein aggregation and the ubiquitin proteasome pathway: gaining the UPPer hand on neurodegeneration. *Curr Opin Genet Dev* 13, 253-261.
- Bitterman, K.J., Anderson, R.M., Cohen, H.Y., Latorre-Esteves, M., and Sinclair, D.A. (2002). Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. *J Biol Chem* 277, 45099-45107.
- Brown, R., and Kaganovich, D. (2016). Look Out Autophagy, Ubiquitin UPS Its Game. *Cell* 166, 797-799.

- 1 Buchan, J.R. (2014). mRNP granules. Assembly, function, and connections with disease. *RNA*
2 *Biol* 11, 1019-1030.
- 3
- 4 Calabretta, S., and Richard, S. (2015). Emerging Roles of Disordered Sequences in RNA-
5 Binding Proteins. *Trends Biochem Sci* 40, 662-672.
- 6
- 7 Chakravarty, A.K., Smejkal, T., Itakura, A.K., Garcia, D.M., and Jarosz, D.F. (2020). A Non-
8 amyloid Prion Particle that Activates a Heritable Gene Expression Program. *Mol Cell* 77, 251-
9 265 e259.
- 10
- 11 Cohen, A., Ross, L., Nachman, I., and Bar-Nun, S. (2012). Aggregation of polyQ proteins is
12 increased upon yeast aging and affected by Sir2 and Hsf1: novel quantitative biochemical and
13 microscopic assays. *PLoS One* 7, e44785.
- 14
- 15 Crane, M.M., and Kaeberlein, M. (2018). The paths of mortality: how understanding the biology
16 of aging can help explain systems behavior of single cells. *Curr Opin Syst Biol* 8, 25-31.
- 17
- 18 Crane, M.M., Russell, A.E., Schafer, B.J., Blue, B.W., Whalen, R., Almazan, J., Hong, M.G.,
19 Nguyen, B., Goings, J.E., Chen, K.L., *et al.* (2019). DNA damage checkpoint activation impairs
20 chromatin homeostasis and promotes mitotic catastrophe during aging. *Elife* 8.
- 21
- 22 Defossez, P.A., Prusty, R., Kaeberlein, M., Lin, S.J., Ferrigno, P., Silver, P.A., Keil, R.L., and
23 Guarente, L. (1999). Elimination of replication block protein Fob1 extends the life span of yeast
24 mother cells. *Mol Cell* 3, 447-455.
- 25
- 26 Denoth-Lippuner, A., Krzyzanowski, M.K., Stober, C., and Barral, Y. (2014). Role of SAGA in
27 the asymmetric segregation of DNA circles during yeast ageing. *Elife* 3.
- 28
- 29 Eisele, F., and Wolf, D.H. (2008). Degradation of misfolded protein in the cytoplasm is mediated
30 by the ubiquitin ligase Ubr1. *FEBS Lett* 582, 4143-4146.
- 31
- 32 Erjavec, N., Larsson, L., Grantham, J., and Nystrom, T. (2007). Accelerated aging and failure to
33 segregate damaged proteins in Sir2 mutants can be suppressed by overproducing the protein
34 aggregation-remodeling factor Hsp104p. *Genes Dev* 21, 2410-2421.
- 35
- 36 Feder, Z.A., Ali, A., Singh, A., Krakowiak, J., Zheng, X., Bindokas, V.P., Wolfgeher, D., Kron,
37 S.J., and Pincus, D. (2021). Subcellular localization of the J-protein Sis1 regulates the heat
38 shock response. *J Cell Biol* 220.
- 39
- 40 Ferry, M.S., Razinkov, I.A., and Hasty, J. (2011). Microfluidics for synthetic biology: from design
41 to execution. *Methods Enzymol* 497, 295-372.
- 42
- 43 Gaglia, G., Rashid, R., Yapp, C., Joshi, G.N., Li, C.G., Lindquist, S.L., Sarosiek, K.A., Whitesell,
44 L., Sorger, P.K., and Santagata, S. (2020). HSF1 phase transition mediates stress adaptation
45 and cell fate decisions. *Nat Cell Biol* 22, 151-158.
- 46
- 47 Graham, G., Csicsery, N., Stasiowski, E., Thouvenin, G., Mather, W.H., Ferry, M., Cookson, S.,
48 and Hasty, J. (2020). Genome-scale transcriptional dynamics and environmental biosensing.
49 *Proc Natl Acad Sci U S A*.
- 50

- 1 He, X., Memczak, S., Qu, J., Belmonte, J.C.I., and Liu, G.H. (2020). Single-cell omics in ageing:
2 a young and growing field. *Nat Metab* 2, 293-302.
- 3
- 4 Hendrickson, D.G., Soifer, I., Wranik, B.J., Kim, G., Robles, M., Gibney, P.A., and Mclsaac, R.S.
5 (2018). A new experimental platform facilitates assessment of the transcriptional and chromatin
6 landscapes of aging yeast. *Elife* 7.
- 7
- 8 Hipp, M.S., Kasturi, P., and Hartl, F.U. (2019). The proteostasis network and its decline in
9 ageing. *Nat Rev Mol Cell Biol* 20, 421-435.
- 10
- 11 Hjerpe, R., Bett, J.S., Keuss, M.J., Solovyova, A., McWilliams, T.G., Johnson, C., Sahu, I.,
12 Varghese, J., Wood, N., Wightman, M., *et al.* (2016). UBQLN2 Mediates Autophagy-
13 Independent Protein Aggregate Clearance by the Proteasome. *Cell* 166, 935-949.
- 14
- 15 Hohn, A., Tramutola, A., and Cascella, R. (2020). Proteostasis Failure in Neurodegenerative
16 Diseases: Focus on Oxidative Stress. *Oxid Med Cell Longev* 2020, 5497046.
- 17
- 18 Hughes, A.L., and Gottschling, D.E. (2012). An early age increase in vacuolar pH limits
19 mitochondrial function and lifespan in yeast. *Nature* 492, 261-265.
- 20
- 21 Janssens, G.E., Meinema, A.C., Gonzalez, J., Wolters, J.C., Schmidt, A., Guryev, V., Bischoff,
22 R., Wit, E.C., Veenhoff, L.M., and Heinemann, M. (2015). Protein biogenesis machinery is a
23 driver of replicative aging in yeast. *Elife* 4, e08527.
- 24
- 25 Janssens, G.E., and Veenhoff, L.M. (2016). Evidence for the hallmarks of human aging in
26 replicatively aging yeast. *Microb Cell* 3, 263-274.
- 27
- 28 Jiang, M., Wang, J., Fu, J., Du, L., Jeong, H., West, T., Xiang, L., Peng, Q., Hou, Z., Cai, H., *et*
29 *al.* (2011). Neuroprotective role of Sirt1 in mammalian models of Huntington's disease through
30 activation of multiple Sirt1 targets. *Nat Med* 18, 153-158.
- 31
- 32 Jiang, Y., AkhavanAghdam, Z., Li, Y., Zid, B.M., and Hao, N. (2020). A protein kinase A-
33 regulated network encodes short- and long-lived cellular memories. *Sci Signal* 13.
- 34
- 35 Jin, M., Li, Y., O'Laughlin, R., Bittihn, P., Pillus, L., Tsimring, L.S., Hasty, J., and Hao, N. (2019).
36 Divergent Aging of Isogenic Yeast Cells Revealed through Single-Cell Phenotypic Dynamics.
37 *Cell Syst* 8, 242-253 e243.
- 38
- 39 Johzuka, K., and Horiuchi, T. (2002). Replication fork block protein, Fob1, acts as an rDNA
40 region specific recombinator in *S. cerevisiae*. *Genes Cells* 7, 99-113.
- 41
- 42 Jolly, C., Konecny, L., Grady, D.L., Kutsikova, Y.A., Cotto, J.J., Morimoto, R.I., and Vourc'h, C.
43 (2002). In vivo binding of active heat shock transcription factor 1 to human chromosome 9
44 heterochromatin during stress. *J Cell Biol* 156, 775-781.
- 45
- 46 Ju, D., Wang, L., Mao, X., and Xie, Y. (2004). Homeostatic regulation of the proteasome via an
47 Rpn4-dependent feedback circuit. *Biochem Biophys Res Commun* 321, 51-57.
- 48

- 1 Kato, M., Han, T.W., Xie, S., Shi, K., Du, X., Wu, L.C., Mirzaei, H., Goldsmith, E.J., Longgood,
2 J., Pei, J., *et al.* (2012). Cell-free formation of RNA granules: low complexity sequence domains
3 form dynamic fibers within hydrogels. *Cell* 149, 753-767.
- 4
- 5 Kato, M., and Lin, S.J. (2014). Regulation of NAD⁺ metabolism, signaling and
6 compartmentalization in the yeast *Saccharomyces cerevisiae*. *DNA Repair (Amst)* 23, 49-58.
- 7
- 8 Khalil, B., Morderer, D., Price, P.L., Liu, F., and Rossoll, W. (2018). mRNP assembly, axonal
9 transport, and local translation in neurodegenerative diseases. *Brain Res* 1693, 75-91.
- 10
- 11 Kirkwood, T.B. (2005). Understanding the odd science of aging. *Cell* 120, 437-447.
- 12
- 13 Kirkwood, T.B., and Kowald, A. (1997). Network theory of aging. *Exp Gerontol* 32, 395-399.
- 14
- 15 Klaips, C.L., Gropp, M.H.M., Hipp, M.S., and Hartl, F.U. (2020). Sis1 potentiates the stress
16 response to protein aggregation and elevated temperature. *Nat Commun* 11, 6271.
- 17
- 18 Kobayashi, Y., Furukawa-Hibi, Y., Chen, C., Horio, Y., Isobe, K., Ikeda, K., and Motoyama, N.
19 (2005). SIRT1 is critical regulator of FOXO-mediated transcription in response to oxidative
20 stress. *Int J Mol Med* 16, 237-243.
- 21
- 22 Kurtishi, A., Rosen, B., Patil, K.S., Alves, G.W., and Moller, S.G. (2019). Cellular Proteostasis in
23 Neurodegeneration. *Mol Neurobiol* 56, 3676-3689.
- 24
- 25 Labbadia, J., and Morimoto, R.I. (2015). Repression of the Heat Shock Response Is a
26 Programmed Event at the Onset of Reproduction. *Mol Cell* 59, 639-650.
- 27
- 28 Lee, M.E., DeLoache, W.C., Cervantes, B., and Dueber, J.E. (2015). A Highly Characterized
29 Yeast Toolkit for Modular, Multipart Assembly. *ACS Synth Biol* 4, 975-986.
- 30
- 31 Lee, S.R., and Lykke-Andersen, J. (2013). Emerging roles for ribonucleoprotein modification and
32 remodeling in controlling RNA fate. *Trends Cell Biol* 23, 504-510.
- 33
- 34 Li, Y., Jiang, Y., Paxman, J., O'Laughlin, R., Klepin, S., Zhu, Y., Pillus, L., Tsimring, L.S., Hasty,
35 J., and Hao, N. (2020). A programmable fate decision landscape underlies single-cell aging in
36 yeast. *Science* 369, 325-329.
- 37
- 38 Li, Y., Jin, M., O'Laughlin, R., Bittihn, P., Tsimring, L.S., Pillus, L., Hasty, J., and Hao, N. (2017).
39 Multigenerational silencing dynamics control cell aging. *Proc Natl Acad Sci U S A* 114, 11253-
40 11258.
- 41
- 42 Li, Y.R., King, O.D., Shorter, J., and Gitler, A.D. (2013). Stress granules as crucibles of ALS
43 pathogenesis. *J Cell Biol* 201, 361-372.
- 44
- 45 Lin, Y., Protter, D.S., Rosen, M.K., and Parker, R. (2015). Formation and Maturation of Phase-
46 Separated Liquid Droplets by RNA-Binding Proteins. *Mol Cell* 60, 208-219.
- 47
- 48 Lindstrom, D.L., Leverich, C.K., Henderson, K.A., and Gottschling, D.E. (2011). Replicative age
49 induces mitotic recombination in the ribosomal RNA gene cluster of *Saccharomyces cerevisiae*.
50 *PLoS Genet* 7, e1002015.

- 1
- 2 Lopez-Otin, C., Blasco, M.A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks
- 3 of aging. *Cell* 153, 1194-1217.
- 4
- 5 Lum, R., Tkach, J.M., Vierling, E., and Glover, J.R. (2004). Evidence for an unfolding/threading
- 6 mechanism for protein disaggregation by *Saccharomyces cerevisiae* Hsp104. *J Biol Chem* 279,
- 7 29139-29146.
- 8
- 9 McCormick, M.A., Delaney, J.R., Tsuchiya, M., Tsuchiyama, S., Shemorry, A., Sim, S., Chou,
- 10 A.C., Ahmed, U., Carr, D., Murakami, C.J., *et al.* (2015). A Comprehensive Analysis of
- 11 Replicative Lifespan in 4,698 Single-Gene Deletion Strains Uncovers Conserved Mechanisms
- 12 of Aging. *Cell Metab* 22, 895-906.
- 13
- 14 McCormick, M.A., and Promislow, D.E.L. (2018). Recent Advances in the Systems Biology of
- 15 Aging. *Antioxid Redox Signal* 29, 973-984.
- 16
- 17 Medicherla, B., Kostova, Z., Schaefer, A., and Wolf, D.H. (2004). A genomic screen identifies
- 18 Dsk2p and Rad23p as essential components of ER-associated degradation. *EMBO Rep* 5, 692-
- 19 697.
- 20
- 21 Meinema, A., Al-Bayati, M., Aspert, T., Lee, S., Dörig, C., Charvin, G., and Barral, Y. (2021).
- 22 Increased Intron Retention Propagates Aging from the Nucleus to the Cytoplasm. Available at
- 23 SSRN: <https://ssrncom/abstract=3778332> or <http://dxdoiorg/102139/ssrn3778332>.
- 24
- 25 Min, S.W., Cho, S.H., Zhou, Y., Schroeder, S., Haroutunian, V., Seeley, W.W., Huang, E.J.,
- 26 Shen, Y., Masliah, E., Mukherjee, C., *et al.* (2010). Acetylation of tau inhibits its degradation and
- 27 contributes to tauopathy. *Neuron* 67, 953-966.
- 28
- 29 Mitchell, S.F., and Parker, R. (2014). Principles and properties of eukaryotic mRNPs. *Mol Cell*
- 30 54, 547-558.
- 31
- 32 Moorefield, B., Greene, E.A., and Reeder, R.H. (2000). RNA polymerase I transcription factor
- 33 Rrn3 is functionally conserved between yeast and human. *Proc Natl Acad Sci U S A* 97, 4724-
- 34 4729.
- 35
- 36 Morlot, S., Song, J., Leger-Silvestre, I., Matifas, A., Gadai, O., and Charvin, G. (2019). Excessive
- 37 rDNA Transcription Drives the Disruption in Nuclear Homeostasis during Entry into Senescence
- 38 in Budding Yeast. *Cell Rep* 28, 408-422 e404.
- 39
- 40 Mortimer, R.K., and Johnston, J.R. (1959). Life span of individual yeast cells. *Nature* 183, 1751-
- 41 1752.
- 42
- 43 Myers, N., Olender, T., Savidor, A., Levin, Y., Reuven, N., and Shaul, Y. (2018). The Disordered
- 44 Landscape of the 20S Proteasome Substrates Reveals Tight Association with Phase Separated
- 45 Granules. *Proteomics* 18, e1800076.
- 46
- 47 Neurohr, G.E., Terry, R.L., Sandikci, A., Zou, K., Li, H., and Amon, A. (2018). Deregulation of
- 48 the G1/S-phase transition is the proximal cause of mortality in old yeast mother cells. *Genes*
- 49 *Dev* 32, 1075-1084.
- 50

- 1 Newby, G.A., Kiriakov, S., Hallacli, E., Kayatekin, C., Tsvetkov, P., Mancuso, C.P., Bonner, J.M.,
2 Hesse, W.R., Chakrabortee, S., Manogaran, A.L., *et al.* (2017). A Genetic Tool to Track Protein
3 Aggregates and Control Prion Inheritance. *Cell* 171, 966-979 e918.
4
- 5 O'Laughlin, R., Jin, M., Li, Y., Pillus, L., Tsimring, L.S., Hasty, J., and Hao, N. (2020). Advances
6 in quantitative biology methods for studying replicative aging in *Saccharomyces cerevisiae*.
7 *Transl Med Aging* 4, 151-160.
8
- 9 Ogrodnik, M., Salmonowicz, H., and Gladyshev, V.N. (2019). Integrating cellular senescence
10 with the concept of damage accumulation in aging: Relevance for clearance of senescent cells.
11 *Aging Cell* 18, e12841.
12
- 13 Orlandi, I., Pellegrino Coppola, D., Strippoli, M., Ronzulli, R., and Vai, M. (2017). Nicotinamide
14 supplementation phenocopies SIR2 inactivation by modulating carbon metabolism and
15 respiration during yeast chronological aging. *Mech Ageing Dev* 161, 277-287.
16
- 17 Outeiro, T.F., and Lindquist, S. (2003). Yeast cells provide insight into alpha-synuclein biology
18 and pathobiology. *Science* 302, 1772-1775.
19
- 20 Park, J.H., Burgess, J.D., Farooqi, A.H., DeMeo, N.N., Fiesel, F.C., Springer, W., Delenclos, M.,
21 and McLean, P.J. (2020). Alpha-synuclein-induced mitochondrial dysfunction is mediated via a
22 sirtuin 3-dependent pathway. *Mol Neurodegener* 15, 5.
23
- 24 Park, S.H., Bolender, N., Eisele, F., Kostova, Z., Takeuchi, J., Coffino, P., and Wolf, D.H. (2007).
25 The cytoplasmic Hsp70 chaperone machinery subjects misfolded and endoplasmic reticulum
26 import-incompetent proteins to degradation via the ubiquitin-proteasome system. *Mol Biol Cell*
27 18, 153-165.
28
- 29 Philippi, A., Steinbauer, R., Reiter, A., Fath, S., Leger-Silvestre, I., Milkereit, P., Griesenbeck, J.,
30 and Tschochner, H. (2010). TOR-dependent reduction in the expression level of Rrn3p lowers
31 the activity of the yeast RNA Pol I machinery, but does not account for the strong inhibition of
32 rRNA production. *Nucleic Acids Res* 38, 5315-5326.
33
- 34 Pohl, C., and Dikic, I. (2019). Cellular quality control by the ubiquitin-proteasome system and
35 autophagy. *Science* 366, 818-822.
36
- 37 Ramaswami, M., Taylor, J.P., and Parker, R. (2013). Altered ribostasis: RNA-protein granules
38 in degenerative disorders. *Cell* 154, 727-736.
39
- 40 Reiss, Y., Gur, E., and Ravid, T. (2020). Releasing the Lockdown: An Emerging Role for the
41 Ubiquitin-Proteasome System in the Breakdown of Transient Protein Inclusions. *Biomolecules*
42 10.
43
- 44 Saarikangas, J., and Barral, Y. (2015). Protein aggregates are associated with replicative aging
45 without compromising protein quality control. *Elife* 4.
46
- 47 Saka, K., Ide, S., Ganley, A.R., and Kobayashi, T. (2013). Cellular senescence in yeast is
48 regulated by rDNA noncoding transcription. *Curr Biol* 23, 1794-1798.
49

- 1 Sinclair, D.A., and Guarente, L. (1997). Extrachromosomal rDNA circles--a cause of aging in
2 yeast. *Cell* 91, 1033-1042.
- 3
- 4 Smith, J.S., and Boeke, J.D. (1997). An unusual form of transcriptional silencing in yeast
5 ribosomal DNA. *Genes Dev* 11, 241-254.
- 6
- 7 Sorolla, M.A., Nierga, C., Rodriguez-Colman, M.J., Reverter-Branchat, G., Arenas, A., Tamarit,
8 J., Ros, J., and Cabiscol, E. (2011). Sir2 is induced by oxidative stress in a yeast model of
9 Huntington disease and its activation reduces protein aggregation. *Arch Biochem Biophys* 510,
10 27-34.
- 11
- 12 Stefani, M., and Dobson, C.M. (2003). Protein aggregation and aggregate toxicity: new insights
13 into protein folding, misfolding diseases and biological evolution. *J Mol Med (Berl)* 81, 678-699.
- 14
- 15 Swanson, E.C., Manning, B., Zhang, H., and Lawrence, J.B. (2013). Higher-order unfolding of
16 satellite heterochromatin is a consistent and early event in cell senescence. *J Cell Biol* 203, 929-
17 942.
- 18
- 19 Taylor, R.C., and Dillin, A. (2011). Aging as an event of proteostasis collapse. *Cold Spring Harb*
20 *Perspect Biol* 3.
- 21
- 22 Thapa, P., Shanmugam, N., and Pokrzywa, W. (2020). Ubiquitin Signaling Regulates RNA
23 Biogenesis, Processing, and Metabolism. *Bioessays* 42, e1900171.
- 24
- 25 Tkach, J.M., and Glover, J.R. (2004). Amino acid substitutions in the C-terminal AAA+ module
26 of Hsp104 prevent substrate recognition by disrupting oligomerization and cause high
27 temperature inactivation. *J Biol Chem* 279, 35692-35701.
- 28
- 29 Tomita, T., Hamazaki, J., Hirayama, S., McBurney, M.W., Yashiroda, H., and Murata, S. (2015).
30 Sirt1-deficiency causes defective protein quality control. *Sci Rep* 5, 12613.
- 31
- 32 Van Meter, M., Kashyap, M., Rezazadeh, S., Geneva, A.J., Morello, T.D., Seluanov, A., and
33 Gorbunova, V. (2014). SIRT6 represses LINE1 retrotransposons by ribosylating KAP1 but this
34 repression fails with stress and age. *Nat Commun* 5, 5011.
- 35
- 36 Veatch, J.R., McMurray, M.A., Nelson, Z.W., and Gottschling, D.E. (2009). Mitochondrial
37 dysfunction leads to nuclear genome instability via an iron-sulfur cluster defect. *Cell* 137, 1247-
38 1258.
- 39
- 40 Verhoef, L.G., Lindsten, K., Masucci, M.G., and Dantuma, N.P. (2002). Aggregate formation
41 inhibits proteasomal degradation of polyglutamine proteins. *Hum Mol Genet* 11, 2689-2700.
- 42
- 43 Wang, L., Mao, X., Ju, D., and Xie, Y. (2004). Rpn4 is a physiological substrate of the Ubr2
44 ubiquitin ligase. *J Biol Chem* 279, 55218-55223.
- 45
- 46 Weber, S.C., and Brangwynne, C.P. (2012). Getting RNA and protein in phase. *Cell* 149, 1188-
47 1191.
- 48

- 1 Westerheide, S.D., Anckar, J., Stevens, S.M., Jr., Sistonen, L., and Morimoto, R.I. (2009).
2 Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. *Science* 323, 1063-
3 1066.
4
- 5 Xie, Y., and Varshavsky, A. (2001). RPN4 is a ligand, substrate, and transcriptional regulator of
6 the 26S proteasome: a negative feedback circuit. *Proc Natl Acad Sci U S A* 98, 3056-3061.
7
- 8 Yamamoto, R.T., Nogi, Y., Dodd, J.A., and Nomura, M. (1996). RRN3 gene of *Saccharomyces*
9 *cerevisiae* encodes an essential RNA polymerase I transcription factor which interacts with the
10 polymerase independently of DNA template. *EMBO J* 15, 3964-3973.
11
- 12 Yu, A., Fox, S.G., Cavallini, A., Kerridge, C., O'Neill, M.J., Wolak, J., Bose, S., and Morimoto,
13 R.I. (2019). Tau protein aggregates inhibit the protein-folding and vesicular trafficking arms of
14 the cellular proteostasis network. *J Biol Chem* 294, 7917-7930.
15
- 16 Zhang, H., Elbaum-Garfinkle, S., Langdon, E.M., Taylor, N., Occhipinti, P., Bridges, A.A.,
17 Brangwynne, C.P., and Gladfelter, A.S. (2015). RNA Controls PolyQ Protein Phase Transitions.
18 *Mol Cell* 60, 220-230.
19

Supplemental Figures:

Fig. S1. Age-dependent dynamics of iRFP fluorescence and cell cycle length used to confirm the classification of Mode 1 and Mode 2 cells. (A) Representative time-lapse images of iRFP fluorescence during WT Mode 1 and Mode 2 aging processes. Replicative age of mother cell is shown at the top left corner of each image. Aging and dead mother cells are circled in yellow and red, respectively. Single-cell color map trajectories of (B) iRFP fluorescence and (C) cell cycle length for WT Mode 1 (left) and Mode 2 (right) aging cells. Each row represents the time trace of a single cell throughout its lifespan. Color represents fluorescence intensity as indicated in the color bar. Cells are sorted based on their lifespans. Data in (B) and (C) were from the same cells in Fig. 1B and were shown to confirm the classification of Mode 1 and Mode 2 aging cells (Li et al., 2020).

Fig. S2. $\Delta ssCPY^+$ -GFP and Sis1-GFP exhibit responses to proteostatic stress specifically in Mode 1 aging cells. Representative time-lapse images of (A) $\Delta ssCPY^+$ -GFP and (B) Sis1-GFP during WT Mode 1 and Mode 2 aging processes. Time-lapse images are representative of all Mode 1 and Mode 2 cells measured in this study. Replicative age of mother cell is shown at the top left corner of each image. Aging and dead mother cells are circled in yellow and red, respectively.

1

15 **Fig. S3. Relationship between the age at first Hsp104 foci appearance and final lifespan in single**
 16 **cells.** Each circle represents a single aging cell. Blue circles denote WT cells and red circles denote
 17 *sir2Δ* cells. Single-cell data were from in Fig. 1, B and D. The yellow trend line represents the line of best
 18 fit for all points.

19
 20

A 170 RBPs in yTRAP library tested

mRNA decay	PUF3	mRNA 3' - cleavage and polyadenylation	YTH1	Translation factor	SLF1	rRNA binding and rRNA processing	NUG1	Other	IMD3
	NGR1		SNP1		MRN1		NOC2		IMD4
	CTH1		HRB1		RQC2		NOP15		GLC7
	MPT5		PAB1		TIF4632		NOP8		RNQ1[RNQ-]
	PCF11		HRP1		EAP1		NOP7		NEW1
	SSU72		FIP1		TIF35		EBP2		SUP35
	FIR1		MEX67		TIF3		NOP13		WHI4
	DHH1		GBP2		PRT1		NOP4		PIN4
	GIS2		YRA1		DED1		NSR1		UBP3
	BFR1		RNA15		NIP1		NOP12		HEK2
	SBP1		MPE1		TIF4631		RLP7		SCW4
	PAT1		PAP1		RPG1		RPS3		PUF6
	XRN1		PAP2		TIF2		RPS20		JSN1
	RAT1		NAB2		MSC6		NOP6		TEX1
	NAB3		RNA14		PET54		KRR1		SCP160
	VMA1		CLP1		SGN1		RRP12		MTR2
	DUS3		CFT1		SRO9		MRD1		THP1
	SPT5		SYC1		TIS11		PWP2		ASM4
	REF2		SWD2		YGR054W		NOP58		MIP6
	YGR250C		PFS2		TIF1		NOP56		RPB2
rRNA synthetase	MOT2	RNA helicase	PTA1	mRNA splicing	MER1	Telomere	NOB1		THO2
	NAB6		NRD1		MUD1		SOF1		BRE5
	NPL3		PBP1		MUD2		BMS1		UFD1
	SKI2		RMD9		MSL5		ESF2		PUF4
	UPF3		YSH1		SLU7		NAN1		CYS4
	NRP1				HSH49		NOP6		CPR1
					CWC2		PNO1		TMA46
					NAM8		CBF5		PSP1
					LUC7				KSP1
					MSS116				RRT5
rRNA synthetase	PUS1		PRP43		PRP8		RFA1		RIM4
	STP1		YLR419W		PRP24		PBP2		STE20
	KRS1		DBP9		IST3		CBC2		YLL032C
	TY51		NAM7				MFT1		YME2
	GUS1		MTR4				HPR1		[RNQ+]-RNQ1
	GLN4		DBP1						[RNQ-]-RNQ1
	LHP1		ECM32						
	HTS1		SLH1						
			SUB2						

B 43 RBPs identified (50% decrease in signal)

PUF3	NUG1	IMD3
SBP1	NOP15	RNQ1[RNQ-]
	NOP13	WHI4
YTH1	NSR1	PIN4
HRB1	RLP7	SCP160
GBP2	RPS20	THO2
YRA1	RRP12	CPR1
RNA15	MRD1	
PAP1	PWP2	
PTA1	NOP58	
	NOP56	
DBP1	SOF1	
	ESF2	
MRN1	CBF5	
TIF35	PNO1	
TIF3		
	RFA1	
MUD2	PBP2	
MSL5	CBC2	
SLU7		
HSH49		
NAM8		

C 15 RBPs identified (75% decrease in signal)

PUF3	NOP15
	NOP13
GBP2	RLP7
YRA1	MRD1
	SOF1
TIF35	
	SCP160
HSH49	WHI4
MSL5	
SLU7	
MUD2	

Fig. S4. Lists of RNA-binding proteins (RBPs) in the yTRAP library tested and those identified as “Responders” in the screen. (A) The 137 RBPs in the yTRAP library tested in the screen and their functional categories. The colors of boxes correspond to the colors in the pie chart in Fig. 4E. Note that there were a few strains without detectable signals and hence were not tested in our screen. (B) The 27 “Responders” identified from the screen and their functional categories.

Fig. S5. Partial loss of co-localization of Nop13 and Nop15 upon aggregation in Mode 1 aged cells. Representative confocal images of Nop13-mNeon and Nop15-mCherry in young (top row), Mode 1 aged (middle row), and Mode 2 aged (bottom row) cells. White arrows point to Nop13 and Nop15 aggregates that are not colocalized in Mode 1 aged cells. Nop13 and Nop15, at the non-aggregated form, are colocalized in the nucleolus of young and Mode 2 aged cells.

Fig. S6. Replicative lifespans (RLSs) of Mode 1 and Mode 2 cells upon 2-fold overexpression of rRNA-binding proteins. (A) RLS curves for Mode 1 cells in WT (n=89), *NOP15* 2-fold overexpression (O/E) (n=156), *SOF1* 2-fold O/E (n=163), *RLP7* 2-fold O/E (n=190), *NOP13* 2-fold O/E (n=225), and *MRD1* 2-fold O/E (n=165). The mean RLSs for WT (blue) and each O/E mutant (red) were indicated. P-values were calculated using the Gehan-Breslow-Wilcoxon method. (C) RLS curves for Mode 2 cells in WT (n=127), *NOP15* 2-fold O/E (n=219), *SOF1* 2-fold O/E (n=180), *RLP7* 2-fold O/E (n=224), *NOP13* 2-fold O/E (n=111), and *MRD1* 2-fold O/E (n=230). Note that, in WT, Mode 1 cells have a longer RLS (RLS=26) than that of Mode 2 cells (RLS=17), as reported previously (Li et al., 2020).

Fig. S7. The effect of doxycycline on Nop15 aggregation. Single-cell color map trajectories indicate the timing and extent of age-dependent Nop15 aggregation in *fob1Δ*, (A) without doxycycline, (B) with 2 μ M doxycycline, and (C) *fob1Δ* + TetO-inducible *RRN3* with 2 μ M doxycycline. Each row tracks the aggregation state of a single aging cell. Confocal images were acquired at indicated time points during the aging experiments. The aggregation state in each aging cell was classified as “no aggregation” – evenly distributed fluorescence with a normal crescent shape (light blue), “moderate aggregation” – unevenly distributed fluorescent patches with irregular shapes (blue), or “severe aggregation” – multiple distinct fluorescent foci (dark blue), similar to that in Fig. 4. Panels A and C are the same as Fig. 4C, right, and Fig. 4D, respectively. Panel B is the control in the same set of experiments to exclude the possibility that doxycycline itself, but not induced *RRN3* overexpression, causes enhanced aggregation

Video 1. Tracking Hsp104-GFP during aging of a representative WT Mode 1 cells. White arrow points to the mother cell undergoing Mode 1 aging.

Video 2. Tracking Hsp104-GFP during aging of a representative WT Mode 2 cells. White arrow points to the mother cell undergoing Mode 2 aging.