UNIVERSITY OF CALIFORNIA SAN DIEGO

Emerging Links Between Chromatin Stability and Protein Homeostasis in Aging

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by

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The dissertation of Julie Paxman is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

I dedicate this thesis to my family. To my mom for her passion for higher education and my dad for his inspirational work ethic. As a first-generation college student, I would have never imagined myself in this spot, but I am grateful for their examples that pushed me to achieve these things. I dedicate my work also to my young family, my husband for always believing in me and pushing me through challenging times, and to my children in hopes my work will lead to better lives and better futures for them.

EPIGRAPH

The longer I live, the more beautiful life becomes.

Frank Lloyd Wright

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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ABSTRACT OF THE DISSERTATION

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Aging, an inevitable path of life defined by gradual deterioration of function and ultimately death. While young cells and organisms are generally able to maintain health and homeostasis how cells lose this ability over time remains a great biological question. A common theme throughout this thesis will be the networked interaction of cellular processes and how an aging landscape can shape these interactions and drive cellular aging fates.

In Chapter 1, we take a deep analysis of the molecular interactions that govern aging with a specific focus on what drives changes cellular proteostasis as cells age. We assess the knowledge regarding what governs the cellular proteostasis network and what factors change with age that drive a loss of protein homeostasis. We take particular interest in RNA binding proteins as these proteins are highly prone to aggregation and they have been widely implicated in many age-related and neurodegenerative disorders. We further analyze data about specific types of RBPs and the implications that their aggregation has on cellular aging and aging pathologies. We highlight the role of the nucleolus and rRNA binding proteins in regulating cellular and protein homeostasis, as we hypothesize that increase rRNA transcription is a major driver of loss of nucleolar stability, and loss of cellular protein homeostasis. One of the ways rRNA transcription is regulated is by chromatin silencing and we posit that increased chromatin stability via Sirtuins and other chromatin modifiers may increase nucleolar stability and protein homeostasis thereby increasing cellular longevity.

In Chapter 2, we perform a systematic study of ribosomal RNA-binding proteins and uncover an interesting link between chromatin stability and loss of protein homeostasis. Using time-lapse microscopy and microfluidics we discover differences in cellular protein homeostasis within populations of isogenic cells and discover that this divergence is consistent with two distinct aging modes. The two distinct aging modes are characterized by changes in daughter cell morphology and by changes in Sir2 activity and hence rDNA silencing activity. “Mode 1” aging cells are characterized by elongated daughter aging morphology and dramatic loss of rDNA silencing whereas “Mode 2” cells are characterized by small, rounded daughter aging morphology and maintained rDNA

silencing. We provide evidence that not only is there a correlation between cells that lose rDNA silencing and cells that have proteostatic stress, but also that accompanying ERC formation and elevated rDNA transcription drives loss of proteostasis. Next, we systematically investigate RNA binding proteins, which are among the most aggregation prone cellular proteins, to see if and which of these proteins aggregate in response to loss of Sir2 activity. We perform a high-throughput screen of nearly 150 RBPs and identify 27 RBPs that aggregate in response to Sir2 inhibition. We find the most enriched sub-category within the hits are ribosomal RNA binding proteins. Given the link between Sir2, rDNA, and rRNA binding proteins we chose to systematically assess the aggregation of each of these genes during aging. Here we find that, consistent with general proteostasis decline, that Mode 1 cells exhibit aggregation of these rRNA binding proteins whereas Mode 2 cells do not. We hypothesize that elevated rRNA transcription that occurs in Mode 1 cells helps drive the nucleolar dysfunction and aggregation of these rRNA binding proteins. We provide evidence that elevated rRNA transcription does indeed increase rRNA binding protein aggregation and cellular protein aggregation more broadly. Finally, we demonstrate the lifespan impacts that these rRNA binding proteins have, illustrating the negative impacts that rRNA binding protein aggregation has on cellular lifespan.

INTRODUCTION

Aging seems the inevitable fate of all living things, however, understanding this process can improve understanding of the mechanisms that drive cellular dysfunction and can elucidate how interconnected systems affect one another within a dynamically changing cellular environment (Crane and Kaeberlein, 2018; Dillin et al., 2014; Fontana et al., 2010; Kirkwood and Kowald, 1997). In addition to providing in-depth insights into dynamic molecular and cellular interactions, the study of cellular aging also provides therapeutic targets whereby cellular function can be optimized for improved cell and organismal healthspan (Figure I.1.B) (Fontana et al., 2010; Gouveia et al., 2017; Janssens et al., 2015; Martin-Montalvo et al., 2013; Zaman et al., 2011). This work becomes increasingly important as the world population is becoming more and more aged. Most of the leading causes of death in the U.S. are now are age related (Table I.1), and by 2050 there will be several parts throughout the world where more than 30% of the population will be 65 and older (He et al.; Kochanek et al., 2019).

Cellular aging is a complex biological phenomenon characterized by damage accumulation leading to loss of homeostatic cellular function and ultimately cell death (Ogrodnik et al., 2019). Even though biological organisms vary greatly in size, structure, and lifespan, the genes and molecular and cellular processes that control cellular aging are conserved across species (Fontana et al., 2010; Steinkraus et al., 2008). 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 (Janssens and Veenhoff, 2016; López-Otín

et al., 2013). The co-occurrence of many aging hallmarks suggests that many aging mechanisms may operate in parallel or experience some degree of crosstalk, thereby functioning as an integrated network that affects the rate and mechanism of cell aging and decline (Figure I.1.C) (Li et al., 2020). While young cells are generally able to maintain homeostasis, even in the presence of mild environmental stress, it is unknown how the molecular landscape of the cell dynamically changes during aging to prevent the cells from maintaining proper order and function (Figure I.1.A). Additionally, though genes may dictate differences between populations it remains to be understood why genetically identical cells can age with such different lifespans. Systems-level and single-cell research is needed to investigate the dynamic aging landscape, and single-cell context may more clearly elucidate how specific molecular and cellular changes directly affect cell health and lifespan(O'Laughlin et al., 2020).

In our study we use *Saccharomyces cerevisiae* as a model to investigate cellular aging. Despite being single-celled, these organisms have a finite lifespan and the genes and mechanisms that govern this lifespan are conserved across biological species (Fontana et al., 2010; Steinkraus et al., 2008). Yeast's asymmetric division enables us to track single-cells over time and count the number of divisions they undergo before they ultimately senesce and die—this count being their replicative lifespan (Figure I.2.A) (Mortimer and Johnston, 1959). Asymmetric division is also key to how yeast segregate damage and damaging factors (Saarikangas et al., 2017). As yeast replicate and divide the mother cell retains damage or damaging factors while producing a fully rejuvenated daughter cell with full replicative lifespan potential (Crane and Kaeberlein, 2018; McMurray and Gottschling, 2004). This asymmetric division has fascinated scientists for

decades as they've worked to identify what factors are retained and accumulating that are ultimately driving the mother cell's demise (Fehrmann et al., 2013; Lesur and Campbell, 2004; Moreno et al., 2019; Mortimer and Johnston, 1959; Sinclair and Guarente, 1997). Yeast has been a pioneering model for proteomic and genomic analysis providing us with enabling tools to systematically study genes, molecular mechanisms, and environmental factors that control replicative lifespan(Botstein and Fink, 2011; Cherry et al., 2012; Forsburg, 2001; Ghaemmaghami et al., 2003; Giaever and Nislow, 2014; Huh et al., 2003). In early yeast aging work, and even today, lifespan experiments are conducted using microdissection—literally placing single mother cells on an agar pad and allowing them to grow, the budding daughter cells are removed at every cell division and counted until each of the mothers die (I.2.B) (Steffen et al., 2009). Work done using microdissection has contributed greatly to our understanding of how certain genes affect lifespan, but this work is limited in dimensionality to single genes or genetic combinations and their effects on lifespan (McCormick et al., 2015). Recently more high-throughput methods have been developed and used including the yeast mother enrichment program, the mini-chemostat-aging device, and microfluidics (Figure I.2.C) (Crane et al., 2014; Hendrickson et al., 2018; Lindstrom and Gottschling, 2009). Each of these methods allow for some other measurement beyond just lifespan.

Microfluidics have served as a key advancement to quantitatively investigate cellular aging (O'Laughlin et al., 2020). The use of microfluidics to study cellular aging not only decreases labor and improves throughput, but also allows for multidimensional data collection. When microfluidics is combined with and microscopy, high-throughput accurate replicative lifespan counts can be made and changes in cellular morphology

during aging can be observed and quantified. Fluorescence microscopy enables observation and quantification of dynamic changes in gene expression at single-cell resolution for the duration of the cell's lifespan, and in some instances, changes in protein localization or aggregation state can also be observed (Jo et al., 2015; Li et al., 2017; Moreno et al., 2019; Morlot et al., 2019). Additionally, use of multiple fluorescence reporters in the same cells or strain helps elucidate cause and effect changes in the molecular aging network. Microfluidics have enabled study of the dynamic cellular changes that drive divergent aging trajectories, uncovering relationships between chromatin silencing, mitochondrial decline, and nucleolar morphologies (Jin et al., 2019; Li et al., 2020). This technology has also allowed investigation of stochasticity and age-related gene expression noise and has demonstrated how chromatin silencing can influence noisy gene expression and variability among isogenic cells (Crane et al., 2014; Elowitz et al., 2002).

In this work we used time-lapse fluorescence microscopy with a custom-built microfluidic technology (Figure I.2.C) to investigate protein aggregation during cellular aging (Li et al., 2017). We were particularly interested in the relationship we identified between Sir2 activity and cellular proteostasis. While Sir2 is a chromatin factor its activity appears essential for maintenance of lifespan and cellular proteostasis.

Sir2 is an NAD+ dependent histone deacetylase and one of the best-studied pro-longevity genes to date (Figure I.3.A) (Landry et al., 2000; Rine and Herskowitz, 1987). Sir2 is conserved across species and, in general, increased Sir2 activity is associated with increased lifespan (Anderson et al., 2003; Kaeberlein et al., 1999; Lindstrom et al., 2011). In yeast, one of the major regions of DNA that Sir2 is responsible for maintaining

chromatin silencing and genomic stability is the ribosomal DNA region (Saka et al., 2013). This region is composed of 100-200 tandem repeats of ribosomal DNA and, without silencing is susceptible to high levels of mutation and recombination(Kaeberlein et al., 1999; Lindstrom et al., 2011). In fact, one of the major hallmarks and believed causes of yeast aging is the recombination and formation of extrachromosomal rDNA circles. Beyond protecting particularly fragile regions of the genome from DNA damage and recombination, Sir2 activity also functions to regulate the expression of hundreds of genes (Ellahi et al., 2015). Recent work highlights specifically how dynamic changes in Sir2 activity drive cells towards two distinct aging trajectories (Figure I.3.B) (Jin et al., 2019; Li et al., 2017, 2020). While each of these aging phenotypes have defining phenotypic characteristics, the major driving mechanism diverging these two paths appears to be Sir2 activity. Cells that age according to what we have designated as the Mode 1 Aging phenotype exhibit a dramatic loss in Sir2 activity that immediately precedes death. While loss of Sir2 can promote genomic instability, this change alone could not be enough to explain the rapid descent to cellular death. Each of these aging modes have been investigated using various reporters to determine what other cellular and molecular changes are occurring distinctively in each aging mode. See table I.2 for a summary of each aging mode and their distinctive molecular and phenotypic differences.

Protein aggregation is a commonly described aging hallmark (Janssens and Veenhoff, 2016; López-Otín et al., 2013; Moreno et al., 2019) however, it is unknown what dynamic cellular changes occur during aging, including among other aging hallmarks, that make an aged cell unable to maintain protein homeostasis (proteostasis). Loss of proteostasis is a readily observed aging hallmark across biological species, however, it

becomes increasingly difficult to pinpoint what initiates the progressive, age-related loss of cellular proteostasis (Klaips et al., 2018; Shrestha and Megeney, 2015; Taylor and Dillin, 2011; Walther et al., 2015). In addition to working to identify the causes of cellular protein aggregation, time-lapse studies are needed to understand how changes in cellular protein homeostasis affect other cellular pathways. One piece of this puzzle includes identifying what proteins are aggregate in the cell during aging. While certain genes and factors have been identified as aggregation prone, there remains missing a systematic study of aggregating proteins during aging. RNA binding proteins (RBPs) are enriched among aggregation prone proteins and have recently been implicated in neurodegenerative diseases and cancer, however, it is unknown how the dynamically changing cellular landscape affects their aggregation and how RBP aggregation affects cellular lifespan (Calabretta and Richard, 2015; Conlon and Manley, 2017; Harrison and Shorter, 2017). Use of microfluidics to perform systematic study of protein aggregation and other aging hallmarks can provide molecular context to what is driving protein aggregation(Newby et al., 2017). Divergent yeast phenotypes and the factors that drive these changes may enable better understanding of how aging hallmarks affect or are affected by one another.

In chapter 1, we asses current literature regarding age-related loss of proteostasis. While a great body of research has demonstrated loss of proteostasis as an age-related hallmark, and a driver of age-related disease we wanted to find sources that illustrate how this decline happens and what other factors and hallmarks interact and affect the process of proteostatic decline. Emergent research has identified many aggregation-prone genes that appear to be key drivers of aging pathologies. We examine similarities among these

aggregation prone genes and assess what common factors may be at work in driving their aggregation, and how aggregation of these types and classes of proteins may specifically affect cellular function. Among the most enriched aggregation-prone genes across species are RNA binding proteins (RPBs)(Agrawal et al., 2019; Conlon and Manley, 2017; Harrison and Shorter, 2017; Newby et al., 2017). These genes are characterized by low-complexity domains and disordered regions, and, in part, this common feature among RPBs is also what enables their function (Calabretta and Richard, 2015; Harrison and Shorter, 2017; Kato et al., 2012). Of further interest is the liquid-droplet condensates that naturally form from RBPs and nucleic acids. It is believed that these phase-separated ribonucleoprotein particles (RNPs) are necessary structural formations that enable RBP function and necessary RNA processing(Hubstenberger et al., 2017; Kroschwald et al., 2015; McSwiggen et al., 2019). While these condensed structural bodies enable function, they must also be tightly regulated as volume and density may be drivers of phase change pushing these RNP bodies from a functional liquid phase to a dysfunctional solid phase.

While a majority of age-related RBP research is focused on mRNA binding proteins (Johnson et al., 2008; Khalil et al., 2018; Lukong et al., 2008; Mitchell and Parker, 2014; Ramaswami et al., 2013)we found it of importance to highlight data about the nucleolus and rRNA binding proteins. The nucleolus is a particularly large liquid phase RNP body where rRNA transcription and ribosome biogenesis take place (Lafontaine et al., 2020). Evidence across biological species shows that nucleolar irregularities are associated with advanced age or shortened lifespans, however little investigation has occurred to discover what cellular changes are occurring to drive formation of nucleolar aggregates

or nucleolar irregularities (Duncan et al., 2017; Montanaro et al., 2008; Morlot et al., 2019; Tiku et al., 2016; Yang et al., 2018). One conserved trend however is elevated rRNA transcription which may alter the stoichiometric balance between rRNA and rRBPs in the nucleolus and drive these irregularities (Morlot et al., 2019; Sharifi and Bierhoff, 2018; Tiku et al., 2016).

In chapter 2 we investigated loss of cellular proteostasis during aging. While loss of proteostasis is a well-established aging hallmark, we wanted to leverage our single-cell aging device to investigate what other molecular and cellular changes were co-occurring with loss of proteostasis. We used a canonical reporter of protein stress Hsp104-GFP(Saarikangas and Barral, 2015), which forms single juxtanuclear foci upon proteins tress, and found increased protein stress with advanced age. However, we identified and interesting pattern. Only about half of the cells exhibit loss of protein homeostasis during aging and it happened to be that these differences also corresponded to the two divergent aging modes. In Mode 1 aging, cells that die with elongated daughter cells and a dramatic loss of Sir2 activity exhibit loss of proteostasis, whereas in Mode 2 aging, cells that die with small-rounded daughters and maintain Sir2 activity maintain proteostasis (Li et al., 2017). We validated the relationship between loss of proteostasis and Sir2 activity and proceeded to identify specific proteins that aggregate in response to loss of Sir2 activity. In a screen of 137 unique RNA binding proteins we identified 27 RBS that readily aggregate in response to Sir2 inhibition and found a significant enrichment for ribosomal RNA binding genes.

We thought it would be of interest to systematically investigate the rRBPs and the mechanism driving their aggregation because one of the major genomic regions Sir2

regulates is the rDNA region. We hypothesized that the molecular mechanism that initiates the aggregation of these rRBPs may be elevated rRNA transcription resulting from Sir2 activity within the rDNA region. To test this, we first wanted to visually confirm that each of these hits from our screen aggregate in response to loss of Sir2 activity. We separately tagged each rRBP with mNeon and using our single-cell aging device we imaged each rRBP at late stage lifespan. Consistent with our hypothesis we find in Mode 1 cells, those with loss of Sir2 activity, nucleolar irregularities, and aggregation of the rRBPs whereas the nucleoli of Mode 2 cells are comparable to young cells with no aggregation. We next wanted to specifically confirm that elevated rRNA transcription drives increased levels of rRBP aggregation. Using an inducible RRN3 (Yamamoto et al., 1996) construct, we artificially increased rRNA transcription and found, consistent with our hypothesis, that rRBP aggregation also increased. We found that elevated rRNA transcription and aggregation of rRBPs globally affects cellular proteostasis, providing a potential mechanism for age-related loss of cellular proteostasis. Finally, we assess the lifespan impacts of rRBPs and find that increased expression can separately impact cells depending on their cellular landscape, where elevated rRBP expression and aggregation causes a significant shortening of replicative lifespan.

This thesis dissertation presents studies aimed at understanding how the cellular landscape changes dynamically during aging to prevent cells from maintaining protein homeostasis at late-stage lifespan. Determining the networked interactions between cellular aging hallmarks will help in identifying factors that initiate cellular decline and loss of homeostasis. The tools and systematic approaches used in this study will be helpful in expanding our understanding of these age-related networked interactions and will aide in

identifying essential nodes and therapeutic targets for increasing lifespan and improving healthspan.

Table I-1 Top 10 Leading Causes of Death in the United States in 2019

Cause of Death	Age-adjusted death rate per 100,000
Heart disease	**163.6**
Cancer	**149.1**
Unintentional Injury	48.0
Chronic lower respiratory disease	**39.7**
Stroke	**37.1**
Alzheimer's disease	**30.5**
Diabetes	**21.4**
Kidney Disease	**12.9**
Influenza and pneumonia	14.9
Suicide	14.2

Age-related diseases are in bolded in table. Note that 7/10 of the leading causes of death are driven by aging. Even influenza and pneumonia are more deadly in aged individuals as age brings decreased immune activity. Data sourced from CDC.

Table I-2 Phenotypic comparison of yeast aging modes

Aging Mode 1	Aging Mode 2
rDNA silencing lost at late-stage lifespan	rDNA silencing maintained at late-stage lifespan
Sir2 becomes inactive	Sir2 maintains activity
Elongated daughter aging morphology	Rounded daughter aging morphology
High heme levels	Low heme levels
High HAP activity	Low HAP activity
Mitochondrial homeostasis	Mitochondrial dysfunction
Enlarged irregular nucleus and nucleolus	Normal or small nucleus and nucleolus
Loss of proteostasis	Maintain proteostasis
Fast cell cycle	Irregular and slowed cell cycles
Replicative lifespan ~26	Replicative lifespan ~17

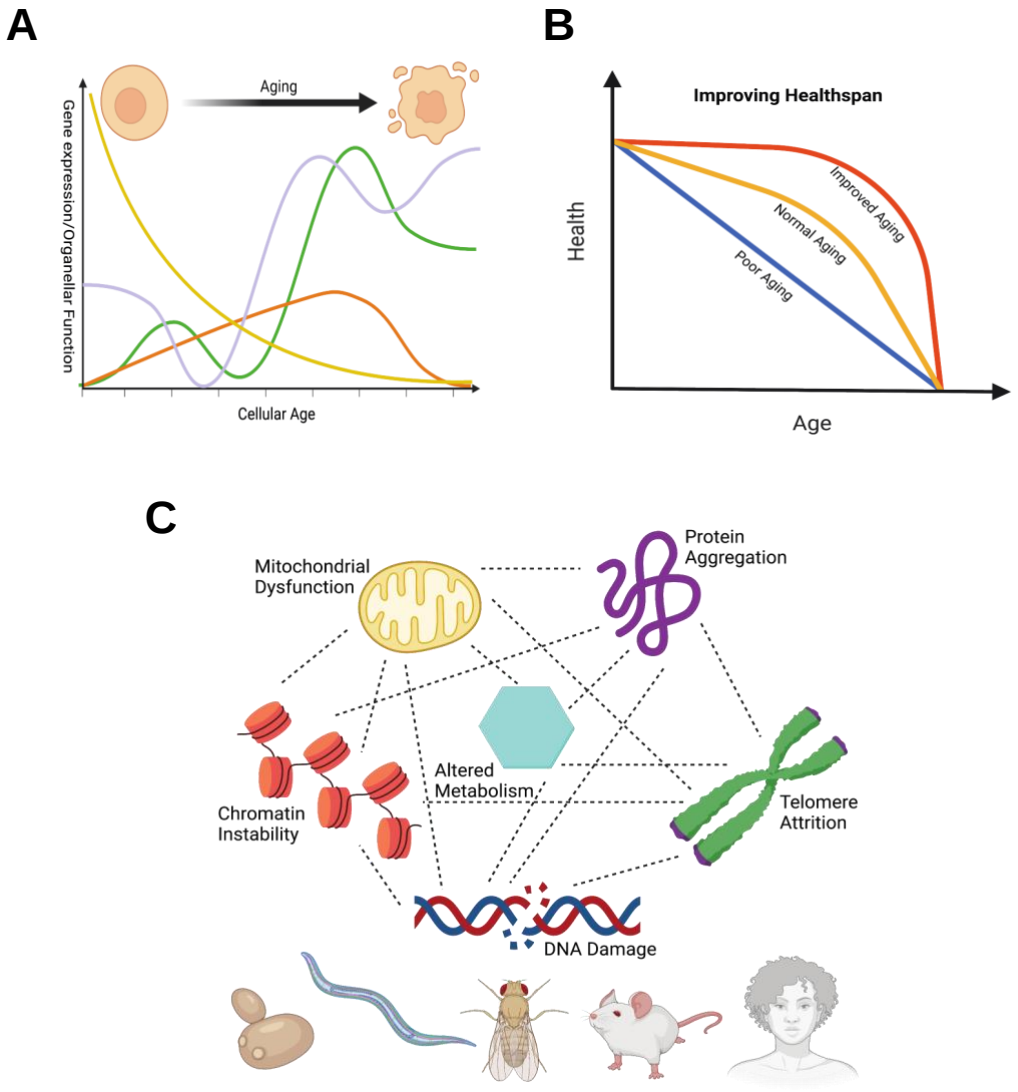

Figure I.1 Conserved aging hallmarks are interconnected in a dynamic cellular environment.
(A). Gene expression, chromatin silencing, and organellar function all dynamically change during cellular aging. (B). Aging therapies can improve healthspan providing improved health and function while increasing in age. (C). Conserved aging hallmarks include mitochondrial dysfunction, protein aggregation, altered metabolism, chromatin instability, telomere attrition, and DNA damage. These hallmarks are highly interconnected and are conserved across biological species.

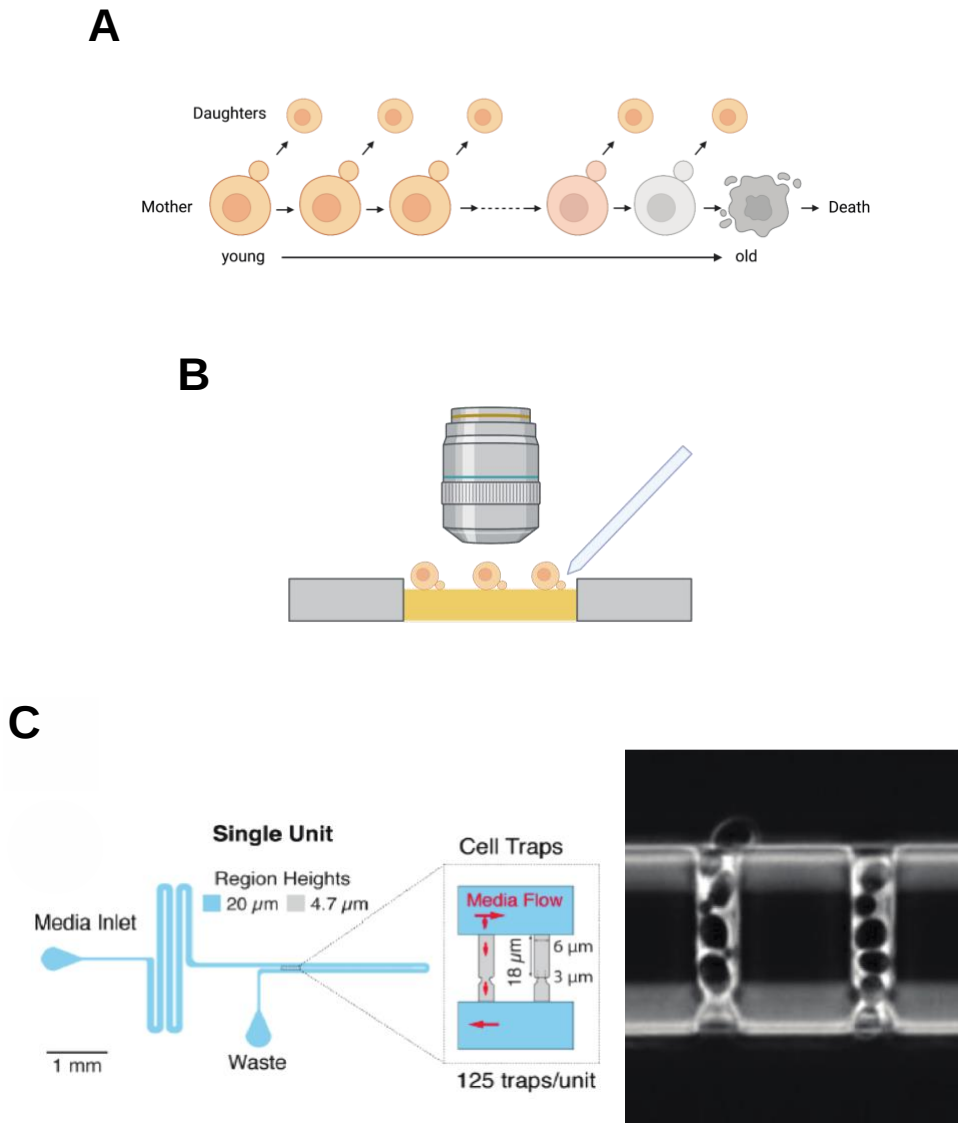

Figure I.2 Methods of studying replicative aging in *Saccharomyces Cerevisiae.*
(A). Yeast replicate and divide asymmetrically. The mother cell buds and produces fully rejuvenated daughter cells until the mother cell ultimately senesces and dies. (B). Yeast microdissection, a classical method for isolating yeast mother cells and counting the number of cellular divisions before death. Yeast mother cells are spotted on an agar pad and a glass-pulled needle is used to remove budding daughter cells. (C). Diagram depicting microfluidic device used in our study (left), phase image of yeast cells in trapping chambers during aging experiment (right).

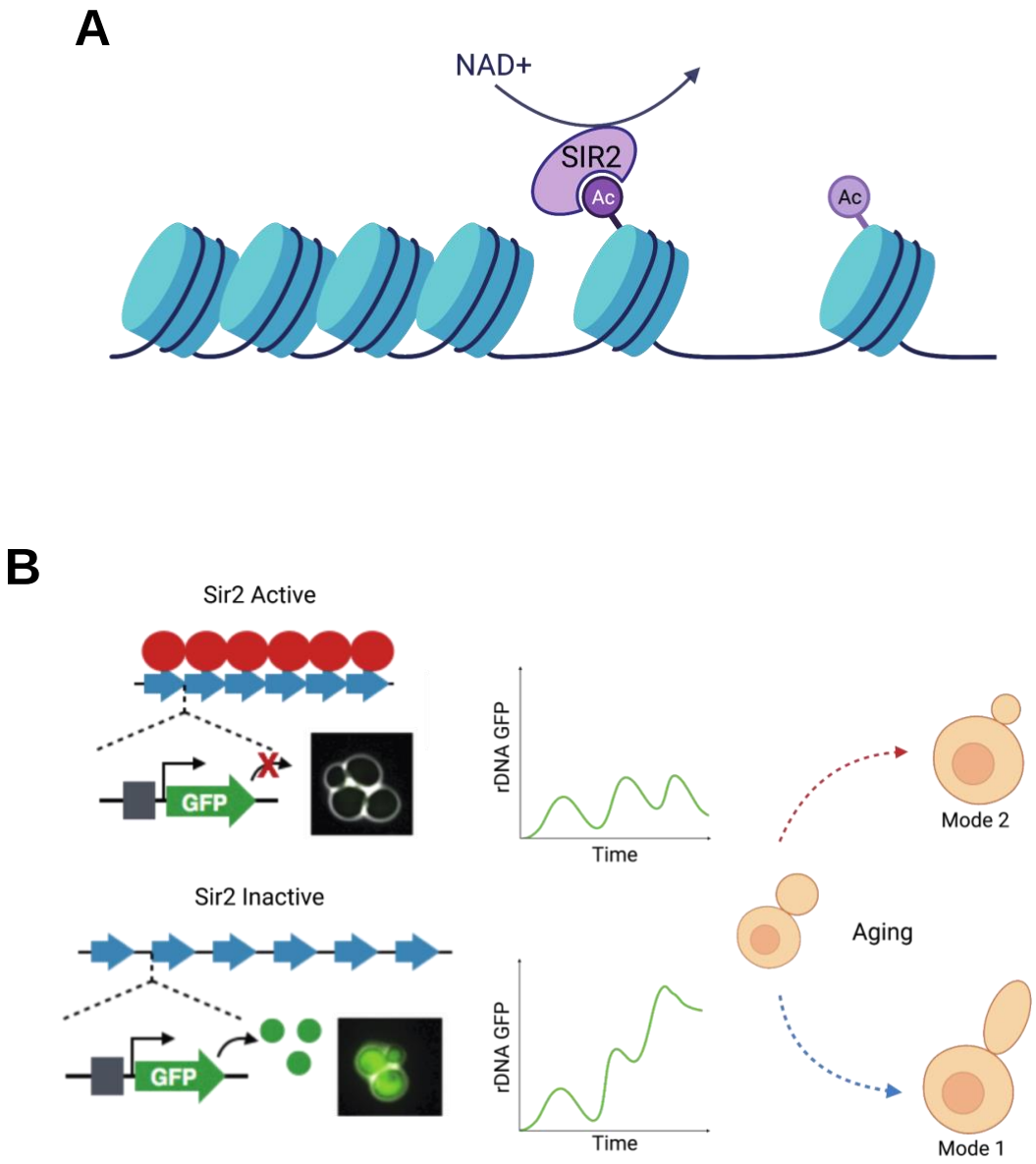

Figure I.3 Sir2 activity drives cellular fate decision during aging.
(A). Sir2 is an NAD+ dependent lysine deacetylase whose activity regulates chromatin silencing across the yeast genome. (B). Chromatin silencing in the ribosomal DNA region of the genome is tightly regulated by Sir2. To investigate silencing dynamics within the rDNA region we integrated a GFP reporter driven by a constitutive promoter. Sir2 activity exhibited two distinct patterns during aging, one with a general maintenance of activity and one with a significant loss of Sir2 activity at late-stage lifespan. These two patterns of Sir2 activity dictated two distinct aging modes characterized by daughter morphology.

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Chapter 1

Cause And Consequence of Protein Aggregation During Aging

Introduction

Cellular aging is a complex biological phenomenon that has fascinated scientists for decades (Klass, 1983; Mortimer and Johnston, 1959; Szilard, 1959; Tissenbaum and Guarente, 2001). The study of cellular aging has led to the discovery of genes, environmental factors, and even drugs that can help slow the aging process or improve lifespan (Haigis and Guarente, 2006; Heilbronn and Ravussin, 2003; Kaeberlein et al., 1999; López-Otín et al., 2013; Martin-Montalvo et al., 2013; McCormick et al., 2015). While major discoveries have been made, the current understanding of hallmark genes and phenotypes associated with aging are disconnected relative to the dynamic network changes that undergird the cellular aging process. Systems-level studies with time-series data and single-cell studies are enabling new insights into the interactions between aging hallmarks and helping to identify more upstream factors that initiate the cellular aging process (Crane et al., 2019; Jin et al., 2019; Mathys et al., 2019; McCormick et al., 2015; Xu et al., 2006). In this review we discuss interactions and dynamic changes among aging hallmarks that explain age-related loss of proteostasis, we characterize proteins that are prone to aggregation, and explain the implications of protein aggregation with respect to cellular function and disease pathology.

Age related changes in cellular landscape affect protein homeostasis

Loss of protein homeostasis is a commonly described hallmark of cellular aging and age-related disease (Janssens and Veenhoff, 2016; Klaips et al., 2018; López-Otín

et al., 2013; Shrestha and Megeney, 2015; Walther et al., 2015). Loss of proteostasis can both be a result of cellular aging, where a cell is no longer able to maintain stable checkpoints within the proteostasis network, and it can act as a driver of cellular aging as misfolded proteins accumulate and prevent proper cellular function. The proteostasis network is a system of proteins that work together to prevent protein aggregation and involves protein synthesis, folding and maintenance, disaggregation, and degradation (Klaips et al., 2018; Labbadia and Morimoto, 2015). Protein stress can be induced in many ways—DNA mutation, mistranslation (Gonskikh and Polacek, 2017; Taylor and Dillin, 2011), oxidative damage (Hanzén et al., 2016; Harley et al., 2021), improper protein modifications (Calabretta and Richard, 2015), failure to fold properly, heat-shock, and environmental stress . In young cells, protein stress can be readily resolved, in aging cells however, cellular and molecular changes have altered the cell landscape such that aged cells are unable to resolve the protein aggregation (Klaips et al., 2018; Saarikangas and Barral, 2015; Taylor and Dillin, 2011; Walther et al., 2015).

Cellular metabolism and altered nutrient sensing are one of the many ways in which the cellular landscape changes with age (Kamei et al., 2014; Kato and Lin, 2014; Yao et al., 2015). Cellular metabolic function serves to provide the necessary energy and building blocks for cellular activity, replication, and life itself. The cell's ability to rapidly respond to changes in nutrient availability allows it to maintain proper energy and cellular homeostasis. A shift in metabolic rate, function, or even metabolic substrates may cause short-term changes resolved through metabolic shifts or stress responses. Prolonged changes in nutrient availability and cell metabolism, however, can drive shifts in epigenetic structure, chromatin stability, and consequently, gene expression. In general,

metabolic activity is negatively correlated with lifespan. This suggests that metabolism naturally produces byproducts that cause cellular damage. For example, early and constitutive inhibition of the IGF-1 and insulin signaling (IIS) pathway will extend lifespan by lowering rates of cell growth and metabolism and improving resistance to oxidative damage (Lechler et al., 2017). Dietary restriction is another means of lowering cell growth and metabolism and can activate and inhibit various enzymes and pathways that promote longevity(Fontana et al., 2010). Some modulators of the effects of Dietary Restriction and IIS pathway include Sirtuins, TOR, stress-responsive transcription factors (TFs), and others (Allard et al., 2009; Fontana et al., 2010). Sirtuin activity increases genomic stability, TOR inhibition decreases ribosomal production protein translation, and stress-responsive TFs drive expression of Heat Shock proteins. Overall, these changes in metabolism and nutrient sensing have major effects on cellular function and homeostasis which, in part, may explain the aged cell's inability to maintain protein homeostasis. Metabolically induced changes in chromatin structure, and damage to nucleic acids can indirectly impact protein stability whereas, metabolic byproducts can directly damage cellular proteins.

Epigenetic modifications and chromatin instability are other hallmarks of aging cells that are inhibitory to aged cell's maintenance of proteostasis (Dang et al., 2009; Gonskikh and Polacek, 2017; Janssens and Veenhoff, 2016; Jin et al., 2011; O'Sullivan et al., 2010). Epigenetic modifications and changes in chromatin stability can affect all three branches of the proteostasis network including protein synthesis, protein maintenance, and protein degradation (Figure 1.1A) (Lardenoije et al., 2015). Maintenance of chromatin structure protects DNA from mutation and recombination

preserving the integrity of genes and gene expression. Aged cells loss of nucleosomes and heterochromatin has been shown to result in aberrant transcription with elevated mutations (Hu et al., 2014), improper RNA processing (Sawyer et al., 2019), and excess or inappropriate protein production (Lardenoije et al., 2015). Histone modifying enzymes are responsible for not only modifying histones but also a broad array of proteins (Jedrusik-Bode et al., 2013). Thus, changes in activity of histone modifying enzymes could have more broad implications on protein folding and maintenance. Finally, aberrant protein expression due to changes in epigenetic structure can lead to oversaturation of the proteasome driving accumulation of damaged proteins and loss of proteostasis.

Loss of proteostasis in aging cells

Mutation, damage, and cellular stress can drive changes in protein conformations. Generally, this damage can be readily addressed via chaperones and the cell's degradation machinery, but if the damage exceeds the cell's capacity for refolding and degradation, these aggregates begin to build up and form assemblies. Though low-level expression of some misfolded proteins seems harmless, some misfolded proteins act as seeding factors that initiate and accelerate protein aggregation within the cell (Speldewinde and Grant, 2017). Furthermore, some protein modifications may be directly toxic to the cell where exposed hydrophobic regions interact with and disrupt cellular and organellar membranes or proteins within the cell (Moreno et al., 2019). With age misfolded proteins accumulate and the availability of chaperones in late-stage lifespan becomes very limited. Additionally, chaperones may be among proteins that are compromised by damage and misfolding with further reducing the cell's capacity to deal with the growing aggregation problem (Soti and Csermely, 2003). One of the important

responses cells mount to cellular protein stress is a heat-shock or unfolded protein response, however, with age this pathway becomes impaired resulting in lower levels of stress-responsive heat shock proteins to address the growing aggregation problem (Stroo et al., 2017; Taylor and Dillin, 2011).

Protein aggregates with highly ordered insoluble structures are known as amyloids (Stroo et al., 2017). These fibrils or amyloids can be found extracellularly or within the nucleus or cytoplasm. Regardless of location, the formation of these aggregates is associated with impaired cellular function and the pathology of various age-related neurodegenerative diseases. (Aguzzi and O'Connor, 2010). In yeast, aging mother cells retain protein aggregates in a small juxtanuclear deposits to promote protein quality control within the cytosol (Hill et al., 2014; Saarikangas et al., 2017). In each case, formation of organized amyloids or sequestered deposits indicates some level of protein stress, however the ordered and sequestered form of these aggregates is generally perceived to be protective. In neurodegenerative disease there is some debate regarding the role of amyloid formation in the disease pathology. The amyloid formation may serve as a protective sequestration of the amyloid-forming proteins, where the individual protein intermediates may carry the true cytotoxic effects (Stroo et al., 2017). In yeast, the prevalence of singular deposits increases with failures in the proteostasis network, however, severe defects within the proteasome result in scattered foci and aggregate formation throughout the cytosol (Saarikangas and Barral, 2015).

Effects of protein aggregation at a cellular and organismal level

Intermediate misfolded protein species that form aggregates have frequently been shown to be cytotoxic (Klaips et al., 2018; Stroo et al., 2017). Many mechanisms are

proposed to explain their toxicity, but one of the most common is that these intermediate species can drive damages to plasma membranes, chaperones, and other cellular components when hydrophobic regions in misfolded proteins become accessible (Figure 1.1B) (Campioni et al., 2010; Chimon et al., 2007; Fändrich, 2012). Because proteins are essential to every structure and function of the cell, protein aggregation and the accumulation of such aggregation can have vast and diverse cytotoxic consequences to the cell. Formation of aggregates could drive the sequestration of proteins involved in regulating genomic stability, proteins within the cytoskeleton, proteins needed for cellular respiration and metabolism, and proteins required for translation with loss of such proteins causing decline or damage within their respective functions and pathways (Figure 1.1B) (Harley et al., 2021; Olzscha et al., 2011; Stroo et al., 2017). Disruption of lipid membranes by misfolded proteins can result in aberrant membrane permeability allowing for unintended diffusion of various molecules into and out of the cell (Campioni et al., 2010). Protein aggregation can also drive cell-cycle delay and ultimately cellular senescence (Moreno et al., 2019). These cell specific damages can result in diverse outcomes for cell-cell communication and more broadly, for the whole organism.

Just as diverse as the cellular effects of protein aggregation are the pathogenic effects of protein aggregation in the context of human disease. Most age-related diseases, including neurodegenerative disease, cardiovascular disease, type II diabetes, and cancer, appear to have some underlying dysfunction in protein homeostasis (Campioni et al., 2010; Dugger and Dickson, 2017; Gouveia et al., 2017; López-Otín et al., 2013; Wang and Fersht, 2015). Neurodegenerative diseases are among the best-studied age-related diseases that are driven by protein aggregation (Aguzzi and

O'Connor, 2010; Cohen et al., 2012; Hetman and Pietrzak, 2012; Johnson et al., 2008; Ramaswami et al., 2013; Shrestha and Megeney, 2015). In neurodegenerative disease, populations of neurons progressively deteriorate and die resulting in devastating corporeal dysfunction including cognitive decline, behavioral disorders, and motor decline (Dugger and Dickson, 2017). Cardiovascular disease frequency increases with age and is closely tied to protein aggregate formation. In conserved form, proteostasis becomes impaired and aggregates become toxic causing cellular damage and loss of function within cells and tissues (Gouveia et al., 2017). Type II diabetes is another example of age-associated disease driven by aggregation of the islet amyloid peptide. This disease is characterized by compromised insulin release, hyperglycemia, insulin resistance, and loss of ß-cell function damage(Campioni et al., 2010). In cancer, aggregation of tumor-suppressor gene p53 is believed to drive the chemoresistance and tumor growth that underlies the disease pathology (Wang and Fersht, 2015).

RNA binding proteins are associated with age-related protein decline

All proteins are susceptible to mutation, misexpression, and misfolding however, certain motifs and structures make some proteins less stable and more prone to misfolding. Some structures even promote or seed cellular protein aggregation. Proteins that are enriched with glutamine and asparagine residues or proteins that have low complexity domains (LCDs) are more prone to aggregation (Calabretta and Richard, 2015; Harrison and Shorter, 2017; Kato et al., 2012; Lechler et al., 2017; Maziuk et al., 2017). These residues often serve as prion domains, and as such, will self-propagate their misfolding and aggregation within the cell. Prions or proteins with prion-like domains

have been identified as some of the causative agents of age-related neurodegenerative disorders.

Yeast prions [PSI+] and [RNQ1+] have served as models to understand prion formation and their role in protein aggregation(Harrison and Shorter, 2017; Speldewinde and Grant, 2017), cellular decline, and applications to neurodegenerative disease. Each of these proteins contain low complexity domains that are enriched for asparagine and glutamine, increasing their propensity for misfolding and conformational changes. [RNQ1+] prion and native protein are of unknown function, though [RNQ1+] colocalizes with [PSI+] suggesting it may act as a seeding factor for their polymerization and accumulation. The native form of [PSI+] is Sup35, an essential RNA binding protein (RBP) required for translational termination.

Yeast RNA binding proteins (RBPs) are the most enriched categories of aggregation prone genes that contain prion-like or low complexity sequence domains (Newby et al., 2017). Whi3 is one example RBP that has been shown to form aggregates in aged cells, and whose aggregation results in sterility in aging yeast (Mizunuma et al., 2013; Schlissel et al., 2017). These aggregation prone genes have been primarily identified using bioinformatics approaches and until recently have only started to be systematically studied. In a screen of all yeast RBPs with confirmed RNA binding activity, Hrp1, a gene homologous to the human hnRNPA1 associated with MSP and ALS, was identified to have the highest aggregation propensity. In addition to its own aggregation propensity Hrp1 could induce the aggregation of other cellular proteins including several proteins involved in ribosome biogenesis. This systematic study not only unveiled

aggregation propensity among a large class of proteins, but also identified factors that co-aggregate and likely have functional downstream effects (Newby et al., 2017).

In worms the proteome becomes increasingly altered with age, not only do transcripts and proteins change in abundance during aging, but the correlation between these changes drops significantly with age illustrating post-transcriptional and translational dysregulation. One of the most abundant increases in protein abundance included extracellular transthyretin (TTR)-like factors (Walther et al., 2015). TTR proteins are among the most abundant proteins found within amyloid deposits suggesting the elevated expression of TTR-like factors may have severe detrimental effects to the proteostasis environment. Proteome-wide analysis of *C. elegans* protein aggregation revealed that genes with the highest aggregation propensity were enriched in the nucleus, and another study found accumulation of stress-granule related RPBs in aged C. *elegans*. Specifically, increased aggregation or puncta formation for two mRNA binding proteins with low complexity domains, PAB-1 and TIAR-2 occurs with age (Lechler et al., 2017). These C. elegans-based models of aging illustrate how RBPs, post-transcriptional and translational processes all impact cellular proteostasis.

In humans, there are 240 genes that have been identified with prion-like motifs and these genes are enriched for genes with RNA binding and RNA processing activity (March et al., 2016). Several specific aggregate forming proteins have been identified that cause cellular decline, neurodegenerative disease, and other age-related pathologies. These proteins include TDP-43, TAF15, EWSR1, hnRNPA2B1, hnRNPA1, hnRNPA3, p53, and IAPP whose aggregation and dysfunction cause: ALS, Chondrosarcoma, Ewing Sarcoma, Paget disease, ALS, Multisystem proteinopathy, cancer, and diabetes,

respectively (Harrison and Shorter, 2017). Most of these genes are among the 240 human genes that harbor low-complexity domains, and all except IAPP have DNA or RNA binding activity. Given the pathological capacity of these few mutated or misfolded RBPs in age-related disease, systematic study of RNA binding proteins could reveal previously missed causal factors and provide new insights into mechanisms of aging and proteostasis decline.

RBP form and function makes them prone to aggregation

Across species RNA binding proteins are among the most aggregation prone genes because of the low-complexity domains (LCDs) and intrinsically disordered regions (IDRs) common among this class of proteins. The LCDs and IDRs enable the RBP the flexibility necessary to bind various substrates and fulfill the multiple functions required of these proteins (Hofmann et al., 2021; Hubstenberger et al., 2017). This flexibility allows for these proteins to bind nucleic acids, interact with RNA pol II and other proteins. Furthermore, the LCDs and IDRs allow for protein interactions that drive compartmentalization of functional proteins and liquid-liquid phase separated condensates necessary to fulfill various cellular functions (Figure 1.2).

RNA and proteins associate together forming ribonucleoprotein complexes which further co-assemble into a diversity of large RNP bodies. These liquid-liquid phase separated bodies or membraneless organelles are found in both the nucleus and cytoplasm. Nuclear bodies include the nucleolus, paraspeckles, Polycomb, and Cajal bodies. In the cytosol processing bodies (P-bodies), stress granules (SGs), and other RNP bodies can be found (Figure 1.2) (March et al., 2016; Sawyer et al., 2019). While liquid-liquid phase separation is necessary for various RBP functions, the size number

and composition of these bodies must be highly regulated to prevent aberrant interactions and formation of solid aggregates.

RNP bodies: P-body and stress-granule formation during aging

P-bodies and stress granules are cytoplasmic RNP bodies accumulate different types of RNAs and proteins in response to various cellular changes and stresses (Rieckher and Tavernarakis, 2017). Stress-granules primarily contain translation initiation machinery whereas P-bodies contain factors that assist in RNA stabilization, mRNA silencing, and mRNA degradation and have 2-fold enrichment of RNA-binding proteins relative to stress-granules (Anderson and Kedersha, 2009). P-bodies interact with a large array of RNA transcripts with more than one-fifth of all cytoplasmic transcripts selectively accumulating within P-bodies (Hubstenberger et al., 2017). The ability to interact with such a wide array of transcripts and proteins is largely enabled by the LCDs and the IDRs which also promote P-body condensation. The interaction network between the RBPs and the RNAs within P-bodies both complex and dense.

Cytoplasmic RNA granules increase in size and number with age, and age-induced P-bodies may have both cytoprotective and pathological consequences (Choi et al., 2021; Schisa, 2014). P-bodies contain mRNA silencing and mRNA degradation machinery, and elevated P-body accumulation at late-stage lifespan may explain the uncoupling between the transcriptome and the proteome (Hubstenberger et al., 2017). While stress-granules and P-bodies are generally separate and contain unique RNP compositions, they have been found physically interact during cellular stress which could be of importance in an aging context (Rieckher and Tavernarakis, 2017). One of the pathological changes associated with stress-granules and P-bodies is their shift from liquid-phase condensates

to solid-phase condensates. These changes affect the RNP function by impairing proper assembly and disassembly. For example, SIRT6 colocalizes with and is important for regulation of C. elegans and mammalian stress granules. Inhibition SIRT6 activity results in impaired stress-granule formation and delays disassembly during recovery (Jedrusik-Bode et al., 2013). Delayed or impaired stress-granule disassembly results in persistent aggregate formation and loss of cellular viability. Such problems with stress granule disassembly are associated with several neurodegenerative diseases including Alzheimer's, ALS, and Parkinson's disease (Hofmann et al., 2021). Overall, the aggregation and impairment of RBPs in stress granules, P-bodies, or other RNP bodies will impact RNA processing and protein expression and alter the cellular proteostasis environment such that age and lifespan are negatively impacted.

RNP bodies: Nucleolar decline in aging

The nucleolus is a small membraneless nuclear subcompartment, but one of the largest and most specialized of the cell's RNP bodies. This region of the cell is among them most active as it is the site of the most significant RNA transcription within the cell, and it is where the most abundant protein particles are assembled into functional ribosomal subunits(Boisvert et al., 2007; Lafontaine et al., 2020). The ribosome synthesis and associated activities including transcription, rRNA processing, and ribosomal RNP assembly are highly regulated to ensure proper cellular replication and growth (Boulon et al., 2010). Across biological species including yeast, worms, flies, mice, and even human cell lines longevity is associated with small nucleoli, whereas large nucleoli are associated with decreased lifespans and cellular decline (Tiku et al., 2016).

In *S. cerevisiae* the nucleolus becomes enlarged and fragmented in late-stage lifespan and select genetic mutants accelerate nucleolar enlargement and fragmentation which coincides with a significant shortening of the replicative lifespan. (Sinclair and Guarente, 1997). One of the major mechanisms by which the nucleolus is believed to become enlarged in yeast is by Extrachromosomal rDNA Circle formation (ERC) due to loss of Sir2 activity and loss of chromatin silencing within the rDNA region of the genome. Morlot et al. show that ERC formation is one of the initial cellular changes that occurs before any physiological decline is detected, they further show that ERC formation drives significant increases in rRNA transcription but that increases in functional ribosome synthesis don't correlate with the elevated rRNA transcription levels. The elevated rRNA transcription affects the delicate balance within the nucleolus leading to loss of homeostasis, cellular senescence and death. (Morlot et al., 2019)

Nucleolar size in *C. elegans* is inversely correlated with worm longevity. The gene, NCL-1 inhibits rRNA transcription and protein synthesis thus mutant *ncl-1* worms demonstrate larger nucleoli and reduced lifespans (Tiku et al., 2016). Expression of the FIB-1/fibrillarin protein, a conserved nucleolar methyltransferase necessary for rRNA processing and maturation, is upregulated in *ncl-1* mutants (Yi et al., 2015), and *fib-1* RNAi knockdown was shown to decrease nucleolar size and increase worm lifespan. In mutants with decreased nucleolar size and increased lifespan rRNA levels and ribosomal proteins were also reduced.

In Drosophila, young males exhibit single round "normal" nucleolar morphology in their GSCs while aged males exhibit increasing percentages of abnormal fragmented or deformed GSC nucleoli. While rDNA copy number appears to decrease during aging, Lu

et al. find striking activation of the normally transcriptionally silent X rDNA correlated with the abnormal nucleolar morphology. Flies have a genetic program to maintain and restore normal rDNA copy number, however, if this system is mutated or perturbed than nucleolar morphology cannot be recovered in subsequent generations. (Lu et al., 2018)

As with the aforementioned models of aging, mice also exhibit major differences in nucleolar structure in young versus aged mice. Duncan et al. performed in-depth analysis to characterize observable differences between young and aged mouse oocytes. One of the major initial differences was the observable change in nucleolar architecture between young and old—young oocytes generally contained multiple nucleoli whereas aged oocytes displayed only one nucleolus. They analyzed various nucleolar proteins involved in the stages of ribosome biogenesis and found major differences in rRNA transcription as indicated by more frequent and larger giant fibrillar centers (GFCs), where high levels of rDNA transcription occur, in aged oocytes. Like C. elegans, fibrillarin expression increases in nucleoli of oocytes. In mice, increased fibrillarin expression is implicated in aberrant rRNA methylation resulting in ribosomal production with compromised translational fidelity. Nucleolin, a protein involved in late-stage ribosome biogenesis showed no difference with age suggesting disruption in the coordination of rDNA transcription and processing and ribosome biogenesis (Duncan et al., 2017).

In humans, muscle biopsies from elderly individuals revealed that a calorie restriction diet, or longevity inducing conditions was associated with smaller nucleolar size (Tiku et al., 2016). Donor fibroblasts from patients with Hutchinson-Gilford progeria syndrome (HGPS), a genetic condition that causes premature aging, display enlarged nucleoli comparable to that of elderly donors. Comparison between HGPS donor cells

and normal donor cells reveal increased rRNA production, nucleolar size, and ribosome biogenesis with age.

Each of these models provide corroborative evidence of conserved nature of nucleolar decline with cellular age. In all cases the major driving factor for decline in nucleolar structure appears to be dysregulation of rRNA transcription through loss of silencing in the rDNA region(s) of the genome. While ERCs are commonly cited as a reason for cellular decline and aging in yeast (Sinclair and Guarente, 1997), the elevated rRNA transcription seen across species suggests that elevated rDNA transcription and potentially altered rDNA chromatin structure are sufficient to initiate the cellular decline. The conservation of these events suggests that one potential intervention to slow or prevent nucleolar decline could be via activation or upregulation of factors that can increase rDNA genomic silencing and thereby decrease rDNA transcription. Though increased rDNA transcription and altered or enlarged nucleolar morphology appear to be universal markers of cellular age and decline, it is necessary to understand how these changes affect the intracellular environment and cellular function more broadly and how they interact with and influence other age-associated hallmarks.

RNP bodies: Effects of nucleolar decline

Defects in nucleolar structure and function have consequential effects on ribosome biogenesis. Changes in rRNA production, rRNA processing, altered ribosome biogenesis will have dramatic affects in cellular protein production and turnover, cellular growth rates, cellular protein homeostasis, and on energy metabolism and nutrient sensing (Montanaro et al., 2008; Morlot et al., 2019; Turi et al., 2019). These intertwined cause and effect mechanisms illustrate the highly networked structure of genetic factors and aging

hallmarks within the cellular aging process. In yeast, rDNA transcription and ribosome biogenesis are normally tightly coordinated, however on the path of cellular decline rRNA transcription and ribosome biogenesis becomes uncorrelated, and despite dramatic increases in rDNA production with age, ribosome levels remain relatively constant (Morlot et al., 2019). Comparatively, in *C. elegans*, *Drosophila*, mice, and humans, ribosome biogenesis increases with elevated rRNA levels. Furthermore, mutations which extend lifespan in *C. elegans* and yeast involve some reduction of ribosome biogenesis or a deletion of a ribosomal protein (Macinnes, 2016). Overall, it appears that cells and organisms are very sensitive to perturbations in homeostasis of ribosome biogenesis. While decreases in ribosome biogenesis could have damaging growth effects, excessive ribosome biogenesis appears to drive early senescence and cell death. Elevated ribosome biogenesis is believed to affect cellular homeostasis in two major ways. The elevated rRNA transcription and ribosome assembly that takes place in these aged and deregulated cells is likely to result in assembly of ribosomes with rRNA that has not been fully or properly processed (Marcel et al., 2013), this has downstream consequences for translation fidelity and cellular proteostasis. Under normal cellular conditions ribosome biogenesis already constitutes over half of the cells transcriptional and translational activity, thus dramatically increasing transcription of rRNA and ribosomal proteins, and translation of ribosomal proteins will have dramatic energetic and metabolic costs for the cell (Macinnes, 2016) potentially depleting or affecting resources needed for other cellular organelles and processes.

Excess protein biogenesis machinery and elevated protein production are likely to drive many downstream cellular dysfunctions through several different mechanisms.

Elevated levels of protein biogenesis machinery may affect protein stoichiometry throughout the cell affecting the procession of essential cellular processes; too much protein production may occur exceeding the capacity of the proteasome; elevated protein production may increase the cell size resulting in metabolic challenges for the cell.

Many analyses have shown that lower translation rates and protein synthesis is associated with longevity, i.e., calorie restriction and rapamycin treatment both extend lifespan and both treatments cause decreased translation rates. Systems-level research has revealed that, as cells age, not only is there a significant trend towards protein overabundance, but also there is a decoupling that occurs between the cellular transcriptome and the cellular proteome. The most significant uncoupling occurs for genes related to translational machinery—proteins for ribosome and protein biogenesis machinery increase and accumulate with age inconsistent with transcript changes for these proteins. Janssens et al. reveal through their network analysis that uncoupling of protein biogenesis genes appears to be one of the most upstream drivers of cellular age and decline with metabolic shifts, mitochondrial changes, and stress responses all falling downstream in this process of decline (Janssens et al., 2015).

Discussion and Conclusion

Loss of proteostasis during aging is associated with many negative cellular and pathological outcomes. Systems-level research is helping to specifically uncover what proteins are forming aggregates, and what dynamic age-related cellular changes occur to drive aggregation of such proteins. As we begin to understand cause and effect we can help treat and prevent age-related disease caused by loss of proteostasis. These studies can also elucidate how epigenetic modifications, transcription and translation, and repair

mechanisms operate together to maintain proper cellular function. Single-cell research may provide insights on how to best optimize the cellular aging process. In some instances, it appears cells make a fate decision, basically taking a bet hedging strategy for survival with age. Depending on environmental circumstances certain fate choices are better than others, but ideally if the optimal characteristics of each aging strategy could be combined this could provide for significant improvements to cellular aging and lifespan.

Changes in the cell's epigenetic landscape, including changes in nutrient sensing and altered chromatin structure, can impact cellular protein homeostasis with broad implications for cellular viability and disease. LCDs and IDRs are some of the canonical characteristics that make proteins prone to aggregation and these motifs are common among RNA Binding Proteins. While some specific RNA binding proteins have been implicated in age-related and neurodegenerative disease, the aggregation-prone nature of this class of proteins suggests need for their systematic study. Recently RNA binding proteins have begun to be systematically studied to better understand their function, localization, and their role in aging and loss of cellular proteostasis. RNA binding proteins commonly function in liquid-liquid condensates of varying size, composition, and function within the cell. Dysfunction of any of these RNP bodies negatively affects cellular proteostasis and cellular viability. RBPs in part, explain interconnected relationship between gene expression regulation and protein aggregation and how proper RBP and proteasome function are crucial for cell viability.

Recent work using quantitative single-cell approaches has unveiled the significant ways in which an aging landscape affects cell fate-decisions and damage outcomes. Microfluidics combined with time-lapse fluorescent imaging has enabled scientists to

capture changes in chromatin structure and gene expression concomitant with cell morphological changes, and cellular proteostasis changes revealing new correlative and causative relationships. Li et al. find that during aging Sir2 dynamics not only shape cell lifespan, but also determine cellular fate decision including how damage accumulates and affects cell organelles—Sustained loss of Sir2 activity impacts nuclear homeostasis, whereas maintenance of Sir2 activity results in dysfunctional mitochondria (Figure 1.3) (Li et al., 2020). Chromatin instability has long been implicated as an aging factor, but recent work shows just how chromatin stability may undermine other cellular processes and affect global cellular proteostasis. Loss of Sir2 activity in yeast has been shown to be a driver of cellular aging because of the production of ERCs, but why these ERCs had such negative cellular impacts was not well understood. Recently, it was found that loss of Sir2 activity, ERC production, and consequent increases in rDNA transcription, drive aggregation of rRNA binding proteins triggering loss of cellular proteostasis and a shortening of replicative lifespan (Paxman et al., 2021). As we continue to advance our understanding of the networks and cellular landscapes that shape the aging process, we can begin to pinpoint the factors that initiate cellular decline and identify important targets that can be modulated to optimize cellular aging and improve heathspan and longevity.

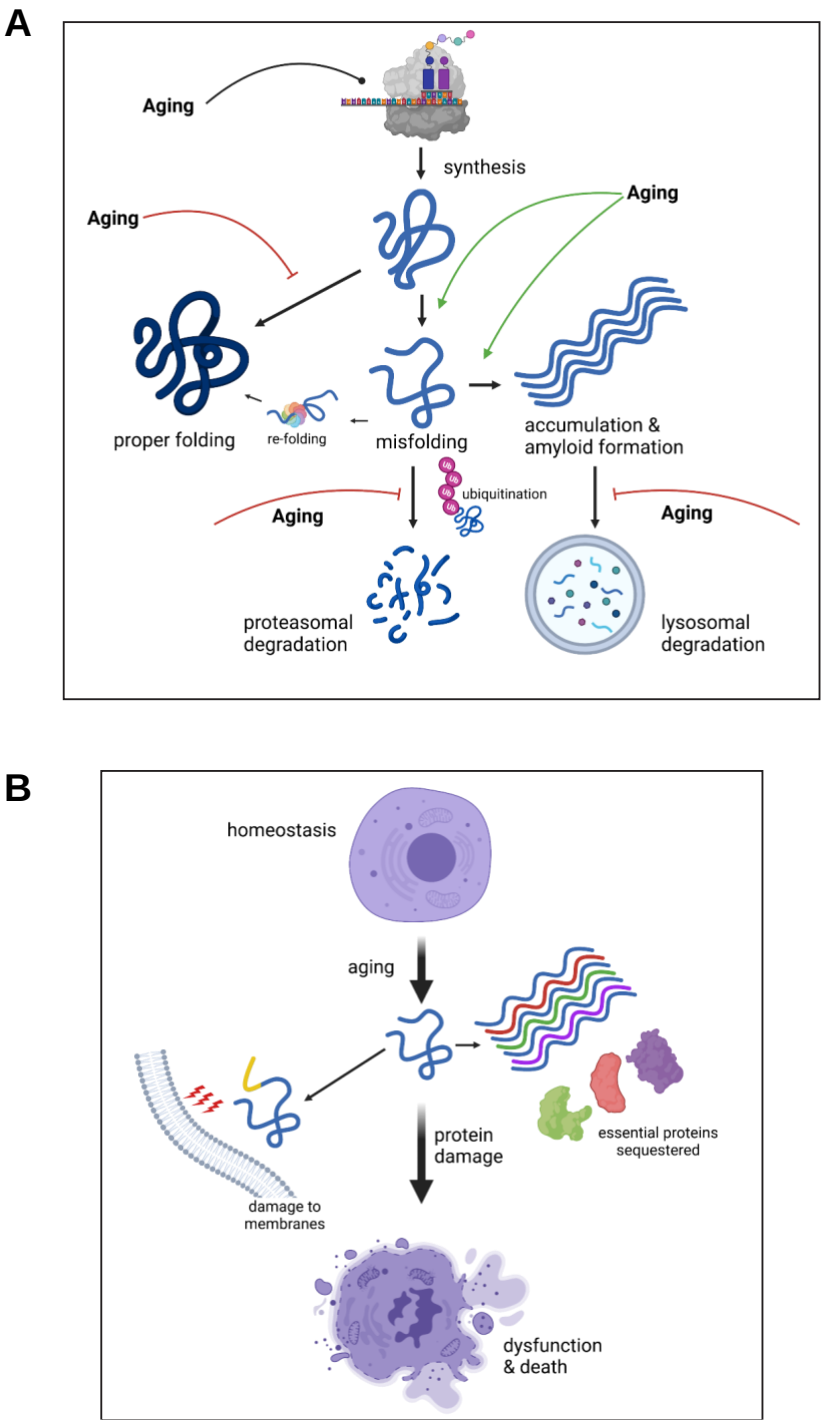

Figure 1.1 Proteostasis Network and Proteotoxicity
(A). Aging drives changes in protein synthesis, protein folding, and protein degradation. Red inhibition lines indicate where aging inhibits or impairs processes, green arrows indicate where aging accelerates or upregulates processes, and black arrows indicate bidirectional changes including upregulation and downregulation. (B) Protein misfolding can be directly toxic to the cell shown here is sequestration of essential proteins in amyloid formations and toxic interactions of hydrophobic protein regions with cell membranes and structures.

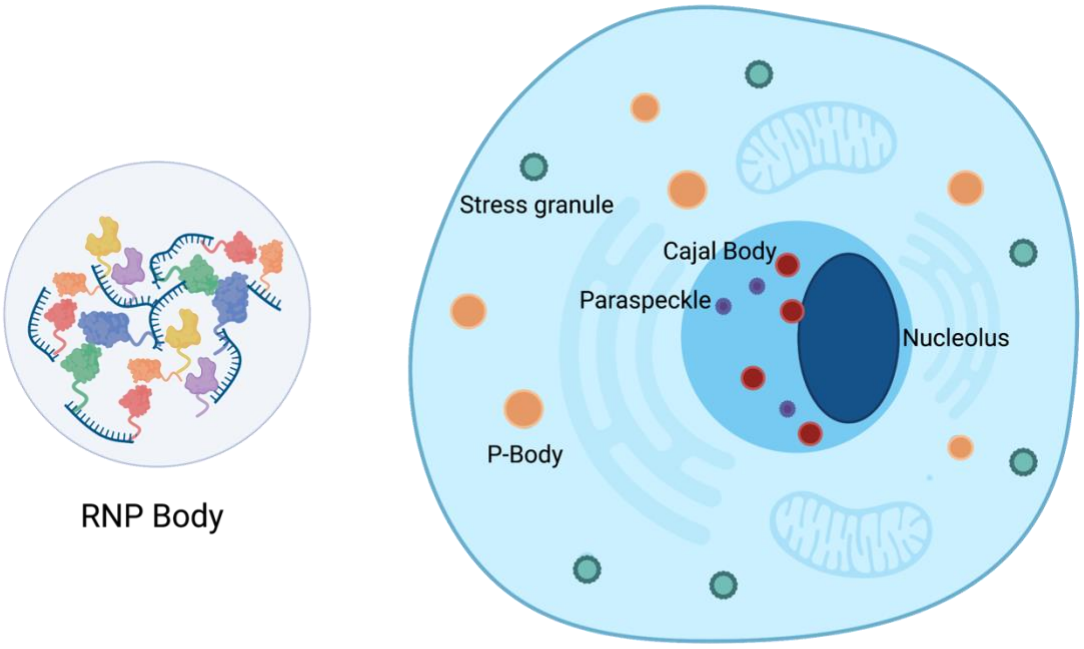

Figure 1.2 RNA Binding Proteins Form Condensates in the Cytoplasm and Nucleus
RNA binding proteins have low-complexity domains which allows for their diverse interactions. RNA-RNA, RNA-RBP, and RBP-RBP interactions allow for formation liquid-liquid phase condensates known as RNP bodies. RNP bodies can be found throughout the cell, in the cytoplasm stress-granules and p-bodies form, in the nucleus can be found the nucleolus, Cajal bodies, and paraspeckles.

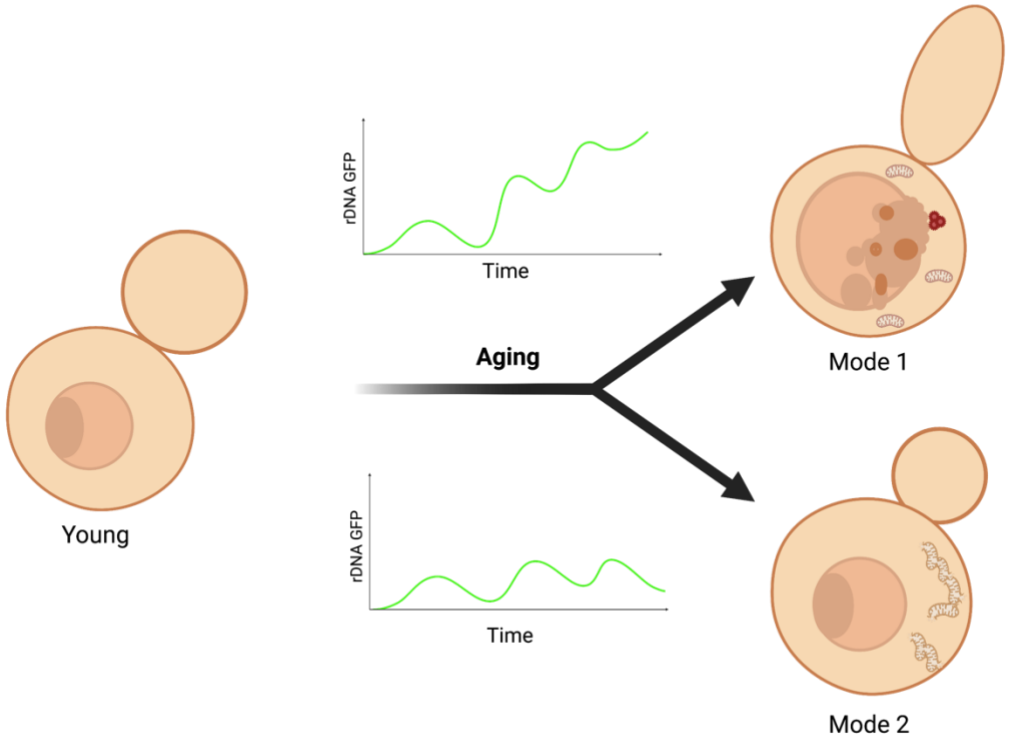

Figure 1.3 Epigenetic landscape drives two distinct aging trajectories and damage fates.

In yeast Sir2 regulates silencing of rDNA during aging, and increased rDNA GFP levels indicates loss of Sir2 activity. Sir2 activity is oscillatory, but Mode 1 aging cells exhibit a dramatic change in their epigenetic landscape with a significant loss of Sir2 activity at late-stage lifespan. The epigenetic divergence between these two aging modes shapes how damage accumulates in the cell. Mode 1 cells exhibit nucleolar decline, rRNA binding protein aggregation, and loss of cellular proteostasis. Mode 2 cells maintain Sir2 activity and nucleolar stability but exhibit mitochondrial aggregation and dysfunction.

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Chapter 2

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

Summary

Loss of protein homeostasis (proteostasis) is a well-established hallmark of aging; however, we discover that proteostatic stress indicated by Hsp104-GFP foci, is not universal and instead is only observed in in approximately half of aging isogenic yeast cells. The two distinct populations of cells, those with protein aggregation and those without, parallel two divergent aging populations characterized by differences in both cellular morphology and Sir2 enzymatic activity. We propose that Sir2 activity affects cellular proteostasis via its maintenance of rDNA silencing and nucleolar stability. In a screen of 137 RNA binding proteins (RPBs) we find that rRNA binding proteins are the most enriched subtype of RBPs that aggregate in response to loss of Sir2 activity. Finally, we characterize the Sir2-dependent aggregation patterns and lifespan effects of rRNA binding proteins aggregation with single-cell resolution and reveal the crosstalk between genomic instability and protein aggregation.

Introduction

Cellular aging is a complex biological phenomenon characterized by damage accumulation leading to loss of homeostatic cellular function and ultimately cell death(Ogrodnik et al., 2019; Vijg and Suh, 2013). 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(Janssens and Veenhoff, 2016; López-Otín et al., 2013). The

co-occurrence of many aging hallmarks suggests that many aging mechanisms may operate in parallel or experience some degree of crosstalk, thereby functioning as an integrated network that affects the rate and mechanism of cell aging and decline (Crane and Kaeberlein, 2018; Kirkwood and Kowald, 1997).

Genomic instability is a commonly described hallmark of aging (Gravel and Jackson, 2003; Hu et al., 2014; Lombard et al., 2005; McMurray and Gottschling, 2004; Vijg and Suh, 2013). While experiments and analyses have been done to characterize differences between young and old cells, only recently have the dynamic changes in chromatin structure and genomic stability been investigated. In yeast, the ribosomal DNA region with 100-200 copies of a 9.1kb rDNA repeat, is particularly fragile and subject to recombination (Sinclair and Guarente, 1997). Chromatin silencing of the rDNA region is regulated by Sir2, one of the best-studied pro-longevity genes, and its activity is key to the protection and stability of this genomic region (Brachmann et al., 1995; Haigis and Guarente, 2006; Kaeberlein et al., 1999; Tissenbaum and Guarente, 2001). Li et al. recently found that the chromatin silencing in this region is dynamic during aging, and subgroups of cells exhibit different Sir2 dynamics during aging. One aging subgroup exhibits a dramatic loss of rDNA silencing, or Sir2 activity at late-stage lifespan which immediately precedes cell death (Li et al., 2017).

Protein aggregation is another commonly described aging hallmark(Janssens and Veenhoff, 2016; Labbadia and Morimoto, 2015; López-Otín et al., 2013; Saarikangas and Barral, 2015; Shrestha and Megeney, 2015), however, it is unknown what dynamic cellular changes occur during aging, including other aging hallmarks, that make an aged cell unable to maintain protein homeostasis (proteostasis). While certain proteins have

been identified as aggregation-prone, there remains missing a systematic study of aggregating proteins during aging. RNA-binding proteins (RBPs) are enriched among aggregation prone proteins and have recently been implicated in neurodegenerative diseases and cancer (Harrison and Shorter, 2017; Maziuk et al., 2017; Ramaswami et al., 2013), however, it is unknown how the dynamically changing cellular landscape affects their aggregation and how RBP aggregation affects cellular lifespan.

In our study, we use *Saccharomyces cerevisiae* as a model to investigate the interactions between the aging cellular landscape and protein aggregation formation during the aging process. Yeast has been a pioneering model for proteomic and genomic analysis (Botstein and Fink, 2011; Forsburg, 2001; Giaever and Nislow, 2014; Huh et al., 2003; McCormick et al., 2015) providing us with enabling tools (Newby et al., 2017) to systematically study age-related protein aggregation specifically with RBPs. Here we combine high-throughput and single-cell microfluidics platforms with advanced imaging techniques to quantitatively investigate how molecular and cellular changes affect cellular protein aggregation and how protein aggregation influences cellular lifespan.

Results

Proteostasis decline coincides 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 (Andersson et al., 2013; Hanzén et al., 2016; Saarikangas and Barral, 2015; Saarikangas et al., 2017). Consistent with previous studies, yeast cells form Hsp104-GFP foci during aging, and the frequency of such appearance increases

with age (Andersson et al., 2013; Saarikangas and Barral, 2015; Saarikangas et al., 2017). However, we noticed that Hsp104-GFP foci did not appear universally in all aging cells. Instead, only a fraction (~50%) of cells showed foci formation during aging (Fig. 2.1A).

We recently discovered and designated two distinct forms of aging processes in isogenic yeast cells as, “Mode 1” and “Mode 2.” Mode 1 aging is driven by loss of ribosomal DNA (rDNA) silencing, as indicated by an increased fluorescence in a GFP reporter inserted at the non-transcribed space region of rDNA (rDNA-GFP), and is associated with elongated daughter cell morphology (Fig. 2.1A, top and middle). In contrast, Mode 2 aging retains rDNA silencing and is associated with production of small round daughters. We classified isogenic aging cells with Hsp104-GFP into Mode 1 and Mode 2 and found that Hsp104-GFP form foci almost exclusively in Mode 1 aging cells (95.4% of Mode 1 cells show Hsp104 foci during aging), but not in Mode 2 cells (Fig. 2.1, A and B; Fig. 2.2), indicating that loss of proteostasis occurs specifically in Mode 1 aging. We independently monitored two additional reporters of proteostatic stress, ΔssCPY*-GFP, and Sis1-GFP. The ΔssCPY*-GFP reporter is an unstable carboxypeptidase-GFP fusion protein (Andersson et al., 2013; Hanzén et al., 2016; Park et al., 2007), and Sis1-GFP is an Hsp40 co-chaperone and a reporter for nuclear proteostatic stress (Klaips et al., 2020). Consistent with the observed pattern of Hsp104-GFP aggregation, both ΔssCPY*-GFP and Sis1-GFP exhibit responses to proteostatic stress specifically in Mode 1 aging cells (Fig. 2.3).

Because Mode 1 aging is driven by rDNA silencing loss and at the same time exhibits proteostasis 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 a dramatically larger proportion (67%) of cells showed Hsp104 foci formation in the short-lived *sir2*Δ mutant with sustained loss of rDNA silencing (indicated by a constantly high rDNA-GFP signal)(Li et al., 2017) (Fig. 2.1, C and D). In addition, these foci were formed earlier in life and persisted for a larger portion of the lifespan in *sir2*Δ cells than those in WT cells (Fig. 2.1D and 2.4A, B) and we observed a correlation between the time of first foci appearance and the final lifespan, suggesting that proteostasis decline, as indicated by Hsp104 foci, contributes to cell aging and death (Fig. 2.4A). Taken together, these results suggest that rDNA silencing loss can serve as a driving factor for proteostasis decline during aging. Sir2, by maintaining rDNA silencing, functions to promote proteostasis and repress 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 (Alba Sorolla et al., 2011; Cohen et al., 2012; Jiang et al., 2012; Kobayashi et al., 2005).

A screen identifies ribosomal RBPs that aggregate in response to a loss of Sir2 activity

Because RBPs are aggregation-prone and play important roles in cellular functions, we next considered whether aging and Sir2-mediated loss of proteostasis will cause RBP aggregation. To test this, we performed a screen to identify RBPs that aggregate in response to a loss of Sir2 activity, which mimics the later phases of Mode 1 aging using a recently-developed synthetic genetic sensor for protein aggregation--the

yeast transcriptional reporting of aggregating proteins (yTRAP) RBP sensor library. The yTRAP RBP sensor library is composed of 137 unique sensor strains, encompassing every known RBP with an experimentally confirmed physical interaction with RNAs in yeast. The aggregation state of an RBP can be reflected by the fluorescence signal of its sensor strain. When RBPs are in a soluble unaggregated state, the sensor GFP fluorescence is high, however if the RBP enters an aggregated state the GFP fluorescence is reduced (Newby et al., 2017) (Fig. 2.5A).

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. 2.5, B and C), recapitulating the aged phenotypes in *sir2*Δ and in the late phases of Mode 1 aging. To track the fluorescence changes of yTRAP RBP sensor strains in response to NAM in a high-throughput manner, we used a 48-strain version of our recently-developed large-scale microfluidic platform “DynOMICS” (Graham et al., 2020) that enables simultaneous quantitative measurements of 48 fluorescent sensor strains over a long period of time. We observed that some sensor strains exhibited a dramatic decrease in fluorescence, indicating RBP aggregation, upon the NAM treatment (Fig. 2.5D, “Responders”); in contrast, other sensor strains showed modest fluorescence changes, indicating minor changes in the aggregation state (Fig. 2.5D, “Non-Responder”) (Fig. 2.6). Of the 137 RBPs tested in our screen, we identified 27 positive Responders, among which we found a striking enrichment of ribosomal RBPs involved in ribosomal RNA processing and biogenesis: 10 out of the 22 ribosomal RBPs tested in the library showed aggregation

responses to a loss of Sir2 activity (Fig. 2.5E). Therefore, we focused our investigation on these ribosomal RBPs.

Age-dependent ribosomal RBP aggregation affects cellular lifespan

To confirm that the identified ribosomal RBPs indeed aggregate during natural aging, we generated 8 yeast strains, each with an integrated copy of a ribosomal RBP candidate C-terminally tagged with mNeon (out of the 10 candidates). To visualize age-induced ribosomal RBP aggregation, we cultured single aging cells using microfluidics, and then used confocal microscopy to capture high-resolution images of young cells (~1 hour after loading) and aged cells (aged for 48 hours, ~60% of the longest lifespans), respectively (Fig. 2.7A). We observed that the ribosomal RBPs in young cells are uniformly localized along one side of the nucleus, forming a characteristic crescent shape. At the late stages of aging, most ribosomal RBPs formed multiple irregular aggregates (visualized as fluorescent foci or patches) in Mode 1 aged cells. In contrast, in Mode 2 aged cells, these RBPs all remained in the uniform crescent shape like that of young cells (Fig. 2.7A). These data confirmed the yTRAP screen results (Fig. 2.5) and are in accord with Hsp104 aggregation observed specifically in Mode 1 aging (Fig. 2.1), suggesting a connection between losses of Sir2 activity and rDNA silencing with age-induced RBP aggregation and proteostasis decline.

To determine the effect of ribosomal RBP aggregation on lifespan, we overexpressed each of the ribosomal RBPs, which, based on the law of mass-action (Newby et al., 2017), leads to increased aggregation (Bolognesi et al., 2016; Patel et al., 2015). For each of the ribosomal RBPs 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 populations (Fig. 2.7, B and C). These results indicate that the aggregated form (in Mode 1 cells) of these ribosomal RBPs causes cell deterioration and accelerates aging, whereas increasing the non-aggregated form (in Mode 2 cells) does not affect longevity. In agreement with the negative effect of ribosomal RBP aggregation on lifespan, we observed that Nop13 and Nop15, two representative ribosomal RBPs, partially lost the colocalization in the nucleolus when they form aggregates, suggesting a deterioration in their coordinated functions in rRNA processing and ribosomal biogenesis (Fig. 2.8).

Excessive rRNA production induces ribosomal RBP aggregation

We next considered the mechanisms underlying ribosomal RBP aggregation during aging and, in particular, how Sir2 and rDNA silencing, involved in maintaining chromatin stability, contribute to the aggregation process. Since the age-dependent aggregation and the effects on cellular lifespan were consistent across all the ribosomal RBPs tested (Fig. 2.7), we chose to perform in-depth genetic analysis using Nop15 as a representative to investigate the pathways and factors that regulate ribosomal RBP aggregation. To monitor age-dependent progression of aggregation in single cells, we tracked the aging processes of a large number 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.

We first examined the role of the ubiquitin/proteasome system, which functions to remove damaged or aggregated proteins, maintain proteostasis and hence promote longevity (Ju et al., 2004). Rpn4 is a transcriptional regulator of the 26S proteasome components and is required for normal levels of intracellular proteasome activity (Xie and Varshavsky, 2001). In cells lacking Rpn4, which are characterized by a reduced proteasome pool (Ju et al., 2004), we found increased, earlier and more severe Nop15 aggregation during aging (Fig. 2.9B, left). In contrast, deletion of Ubr2, a ubiquitin ligase that mediates Rpn4 degradation, leads to elevated proteasome capacity (Kruegel et al., 2011; Nillegoda et al., 2010)and thereby dramatically alleviated Nop15 aggregation (Fig. 2.9B, right). These results indicate that the proteasome system is responsible for clearing age-induced ribosomal RBP aggregates, like in the cases of many other toxic protein aggregates (Andersson et al., 2013; Kruegel et al., 2011).

We showed above that ribosomal RBP aggregates formed in Mode 1 aged cells (Fig. 2.7A), which are characterized by rDNA silencing loss (Li et al., 2017, 2020). Consistently, we observed much earlier and more frequent appearance of Nop15 aggregation during aging of *sir2*Δ cells (Fig. 2.9C, left), suggesting that deletion of Sir2 or loss of rDNA silencing mediated by Sir2 promotes ribosomal RBP aggregation. Previous studies showed that loss of Sir2 or rDNA silencing results in an increased rate of rDNA recombination and consequently accumulation of extrachromosomal rDNA circles (ERCs) that drives yeast aging (Morlot et al., 2019; Sinclair and Guarente, 1997).To determine whether loss of Sir2 or rDNA silencing drives ribosomal RBP 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. We observed a dramatic reduction in Nop15 aggregation in the *fob1*Δ strain, indicating that ERC accumulation is a major driver of age-dependent ribosomal RBP aggregation (Fig. 2.9C, right). ERCs impact multiple aspects of cellular functions, including cell cycle progression, nuclear pore integrity, and ribosomal RNA (rRNA) production. Among these, we hypothesized that excessive rRNA production from ERC accumulation (Morlot et al., 2019)causes aggregation of ribosomal RBPs (e.g. Nop15), as increasing the level of RNA content generally promotes phase separation and aggregation of ribonucleoprotein complexes (Lin et al., 2015). To test this, we overexpressed Rrn3, the RNA polymerase I-specific transcription factor that promotes rRNA transcription (Moorefield et al., 2000; Philippi et al., 2010; Yamamoto et al., 1996), in the *fob1*Δ mutant, in which ERC formation is abolished. 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. 2.9D). Taken together, these results showed that age-dependent loss of rDNA silencing loss promotes ribosomal RBP aggregation through ERC accumulation and, more specifically, excessive rRNA production caused by ERCs, providing the mechanistic link between rDNA stability and ribosomal RBP aggregation.

Ribosomal RBP aggregation contributes to global proteostasis decline during aging

Because cells use the ubiquitin/proteasome system to remove ribosomal RBP aggregates (Fig. 2.9B), we speculate that age-induced ribosomal RBP aggregation may impose an increased burden on the proteasome system, which contributes to the global proteostasis decline observed in aged cells. In agreement with this, we showed that

deletion of Sir2, which enhances ribosomal RBP aggregation (Fig. 2.9C), also promotes proteostasis decline during aging, as reported by increased Hsp104 foci formation (Fig. 2.1C). In contrast, *fob1*Δ, with reduced ERC accumulation and ribosomal RBP aggregation, showed dramatically decreased Hsp104 foci formation (Fig 2.4B), further supporting our hypothesis. It is possible that ERCs may affect proteostasis through processes other than ribosomal RBP aggregation. To test whether ribosomal RBP aggregation can impact global proteostasis, we overexpressed Rrn3 in the *fob1*Δ strain . We observed that Rrn3 overexpression increased Hsp104 foci formation, indicating that ribosomal RBP aggregation, caused by excessive rRNA production in the absence of ERCs (Fig. 2.9D), is sufficient to trigger proteostasis decline. In summary, our results revealed a cascade of interconnected molecular events that leads to cell deterioration during aging, in which age-dependent loss of rDNA silencing results in ERC accumulation and consequently excessive rRNA production, causing aggregation of ribosomal RBPs. The aggregation impairs the normal functions of rRBPs as well as global proteostasis, accelerating the aging process.

Mitigating ribosomal RBP aggregation effectively extends cellular lifespan

As a further test of our working model, we considered how we can design perturbations, based on our discoveries in this study, to effectively promote longevity. Our previous work revealed divergent aging processes (Mode 1 and Mode 2) in an isogenic yeast population, with different molecular causes and phenotypic changes (Jin et al., 2019; Li et al., 2017, 2020). Our findings here showed that ribosomal RBP aggregation and the associated proteostasis decline are major drivers of Mode 1 aging, but not Mode 2 aging. As a result, perturbations that alleviate ribosomal RBP aggregation or

proteostasis decline will be more effective on extending lifespan under the conditions where the majority of cells in a population undergo Mode 1 aging. To test that, we first overexpressed *HAP4*, which enhances mitochondrial biogenesis (the defect of which drives Mode 2 aging) and hence pushes cells to Mode 1 aging (Li et al., 2020). In the same strain, we then deleted *UBR2* to increase proteasome capacity and thus alleviate ribosomal RBP aggregation. We found that the deletion of *UBR2*, in combination with *HAP4* overexpression, significantly extended the average lifespan of the cell population, much more dramatic than either perturbation alone, supporting our model from this study. In contrast, deleting *UBR2* in the strain with *SIR2* 2-fold overexpression, a long-lived mutant with increased rDNA stability (Li et al., 2020), only modestly extended the lifespan, again confirming our model where these two perturbations converge on alleviating ribosomal RBP aggregation and thereby cannot promote longevity additively.

Discussion

Recent work showed that overexpression of the protein disaggregase Hsp104 in cells where Sir2 has been deleted prevents the accelerated cellular aging that occurs in *sir*2Δ mutant cells (Erjavec et al., 2007), illustrating the cross-talk between factors that promote chromatin stability and factors that promote proteostasis. High-throughput single-cell analysis of cells during aging has enabled us to uncover a novel relationship between Sir2 activity and cellular protein homeostasis. Here we identify that Sir2 is important for maintaining proteostasis, we specifically identify rRNA binding proteins that aggregate in response to loss of Sir2 activity, and we show that increased rDNA transcription resulting from loss of Sir2 activity is the major driver of rRNA Binding Protein aggregation and, more generally, loss of cellular proteostasis.

In previous work, we found that the temporal maintenance of oscillatory Sir2 activity correlated with the length of lifespan in Mode 1 aging cells. In other words, if Sir2 activity was continuously fluctuating within a dynamic range, the cells remained happy and continued a healthy lifespan. However, in late-stage lifespan, Mode 1 aging cells consistently exhibited a dramatic loss of Sir2 activity that immediately preceded cell death. Additionally, if Sir2 was inhibited or deleted, cellular lifespan would be dramatically reduced. While Sir2 is important for maintaining chromatin stability, we believed DNA recombination and damage resulting from loss of Sir2 activity alone couldn't explain such rapid cellular deterioration(Li et al., 2017). The results shown here help identify downstream cellular changes including rRNA binding protein aggregation and general cellular protein stress, providing reason for the rapid cellular deterioration following loss of Sir2 activity.

Sir2 presents a double-edge sword paradox--loss of Sir2 activity drives rapid cellular deterioration and death, however, consistent high-level Sir2 activity results in similar reduction in cellular lifespan. This study may provide explanation. Elevated rDNA transcription from temporary loss of Sir2 activity may be necessary for adequate ribosome biogenesis. However, excessive rDNA transcription levels from sustained loss of Sir2 activity creates imbalance in the rRNA and rDNA binding proteins stoichiometry thereby promoting the rRBP aggregation. HAP4 overexpression combined with ubr2Δ helps to simultaneously tune two parameters in this process to optimize lifespan extension. HAP4 overexpression improves mitochondrial outcomes while ubr2Δ resolves rRBP aggregation and promotes cellular proteostasis thereby providing significant lifespan extension.

The crosstalk between two aging hallmarks—loss of proteostasis and chromatin instability highlight the complexities of the aging process and suggest that there may exist other crosstalk and interplay among additional aging hallmarks. We hope to continue systematic, dynamic, single-celled analysis to uncover these complex relationships. Noting that essential genes for rRNA processing and thus ribosome biogenesis are among the genes that aggregate in Mode 1 aging, it will also be of interest to investigate how these changes impact cellular ribosome biogenesis and determine whether they are responsible for dysregulation of ribosome biogenesis relative to the elevated rRNA production that occurs in late-stage lifespan. Another question we hope to address with further investigation is whether rRNA binding protein aggregate formation contributes to nuclear and nucleolar aggregation in such a way that nuclear localized genes such as Sir2 are impeded in their normal function or activity. Coupling our dynamic, single-cell view with improved image resolution techniques, super-resolution imaging, or using genetic applications such as optogenetics we believe we can uncover the intricate interplay between aging hallmarks and additionally identify novel feedback mechanisms.

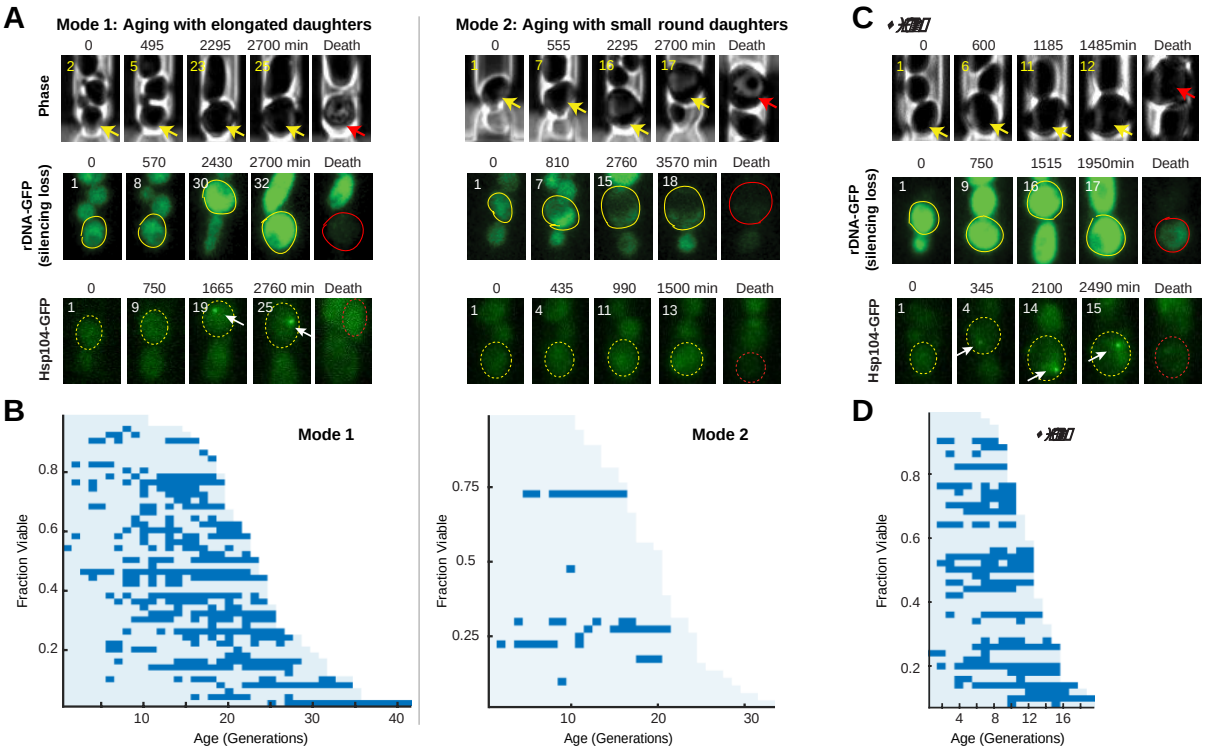


Figure 2.1 Protein aggregation occurs specifically in cells that lose Sir2 activity.

Time lapse images are representatives of all Mode 1, Mode 2, and *sir2*Δ cells in this study. 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. How to classify mode 1 vs mode 2 (iRFP; Fig. 2.2) (A) Representative time-lapse images of WT Mode 1 and mode 2 aging processes. Top: Phase, Middle: rDNA-GFP, Bottom: Hsp104-GFP (B) Single-cell color map trajectories of Hsp104-GFP aggregation during aging. Each row represents the time trace of a single cell throughout its lifespan. Color represents the prescence (red) or absence (blue) of aggregation within a given cell-cycle, as reflected by a visible focus point within the cell. Cells are sorted top to bottom according to their lifespans. (C) Representative time-lapse images of sir2Δ cells during aging. Top: Phase, Middle: rDNA-GFP, Bottom: Hsp104-GFP. (C) Single-cell color map trajectories of Hsp104-GFP aggregation during aging for *sir2*Δ cells.

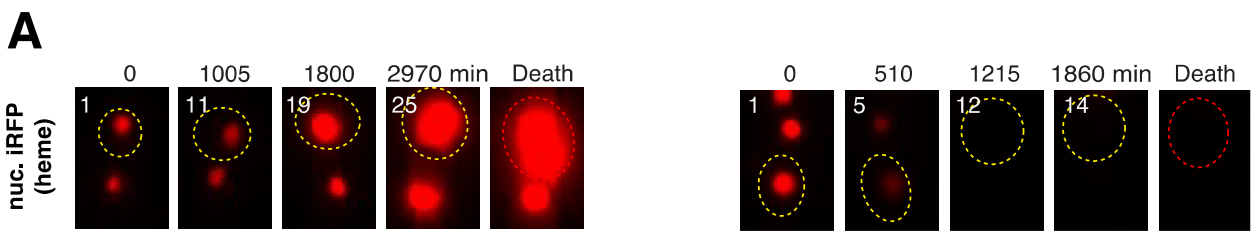


Figure 2.2 Aging mode classification.

Aging modes were classified according to iRFP intensity. Mode 1 aging cells exhibit increased iRFP intensity at late-stage lifespan whereas Mode 2 aging cells exhibit very low or no iRFP fluorescence at late-stage lifespan.

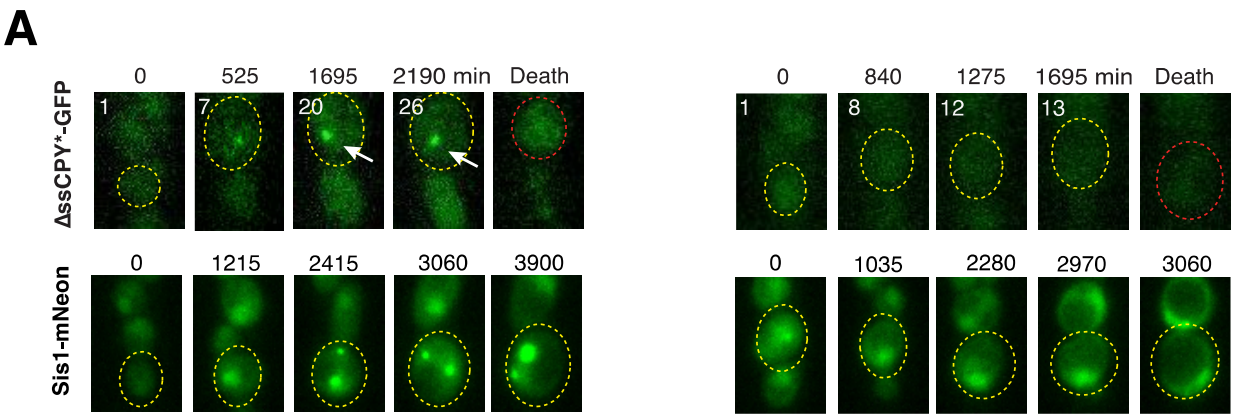
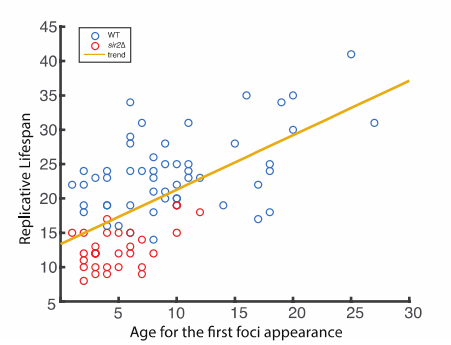


Figure 2.3 Protein stress occurs in Mode 1 aging cells but not Mode 2 aging cells.

Two separate reporters of cellular protein stress, ΔssCPY*-GFP and Sis1-mNeon each show foci or aggregate formation in Mode 1 cells but not Mode 2 aging cells. Representative Mode 1 cells are shown left panel and representative Mode 2 cells are shown in the right half of the panel for ΔssCPY*-GFP and Sis1-mNeon.

A



B

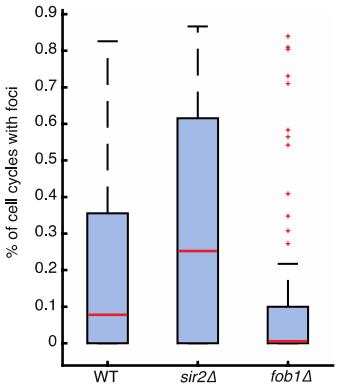


Figure 2.4 Aggregate formation as a function of age and as a percentage of cell cycles.

(A) A plot of cellular lifespan as a function of cellular age at first foci appearance, blue circles denote WT cells and red circles denote *sir2*Δ cells. The yellow trend line represents the line of best fit for all points. (B) Quantification showing the distribution each of the percentage of cell cycles with foci for each cell respective to genotype. Red line indicates median, bottom and top of each box represents the 25th and 75th percentiles, respectively.

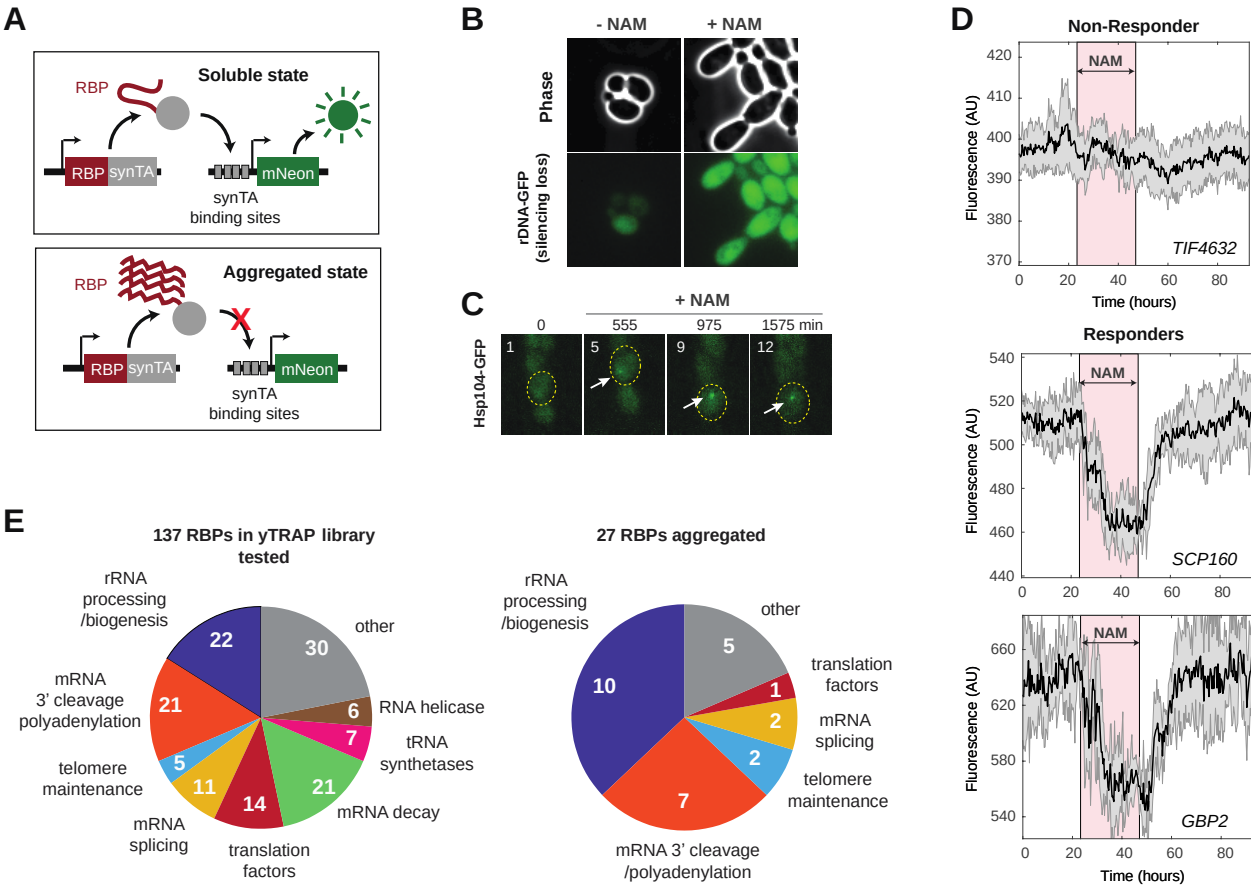


Figure 2.5 A screen identifies 27 RBPs that aggregate in response to loss of Sir2 activity.

(A)Schematics 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 5mM NAM treatement. 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 pink. (E) Functional categories of RBPs tested (left) and responders (right).

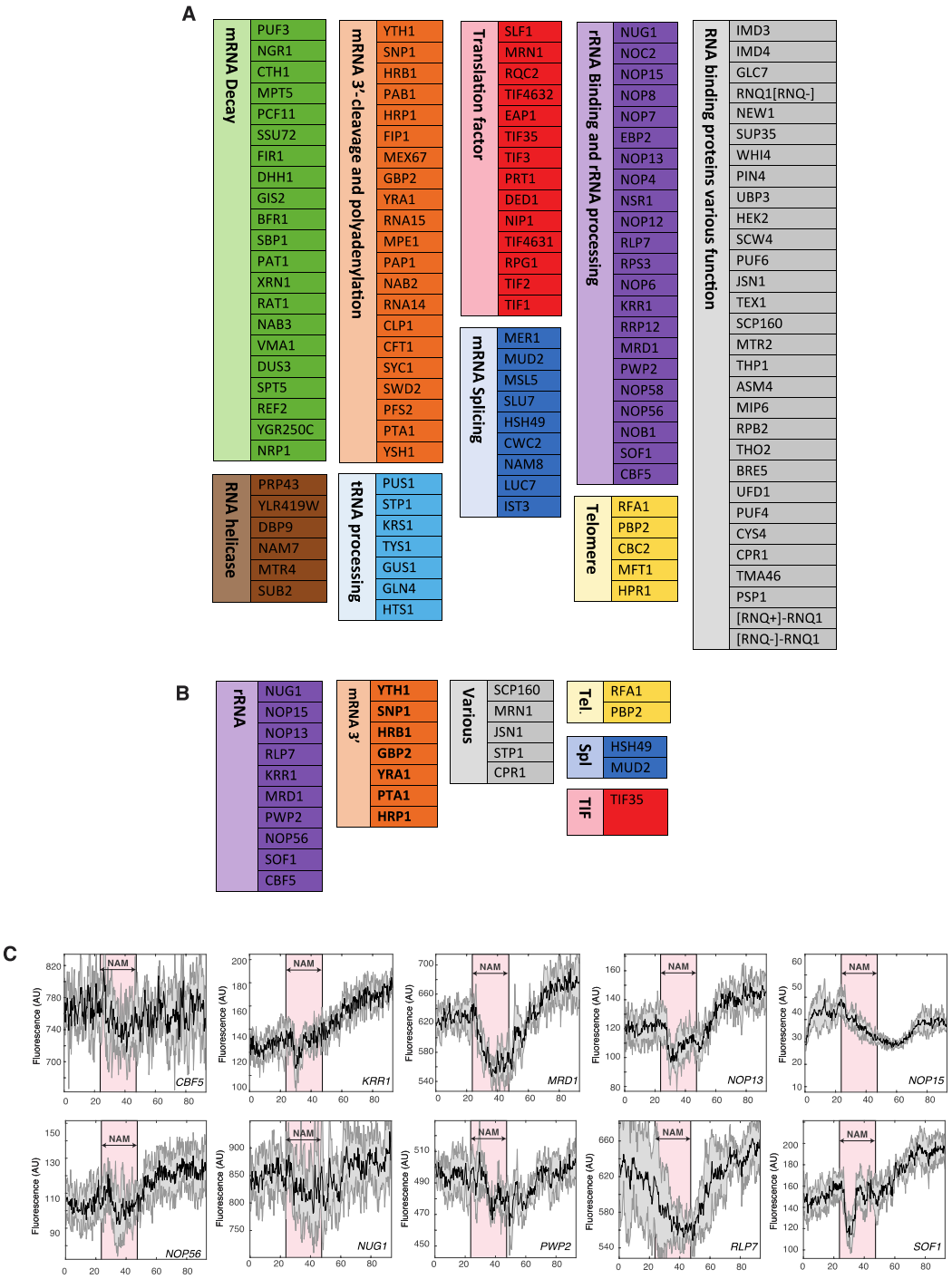


Figure 2.6 Sir2 responsive RBPs.

(A) Complete list of RBPs tested during screen labeled and separated according to functional group. (B) Complete list of Sir2 responsive RBPs labeled and separated as in (A). (C) Representative time traces of fluorescence changes for each responsive rRNA sensor strain, NAM induction time shown on graph in pink.

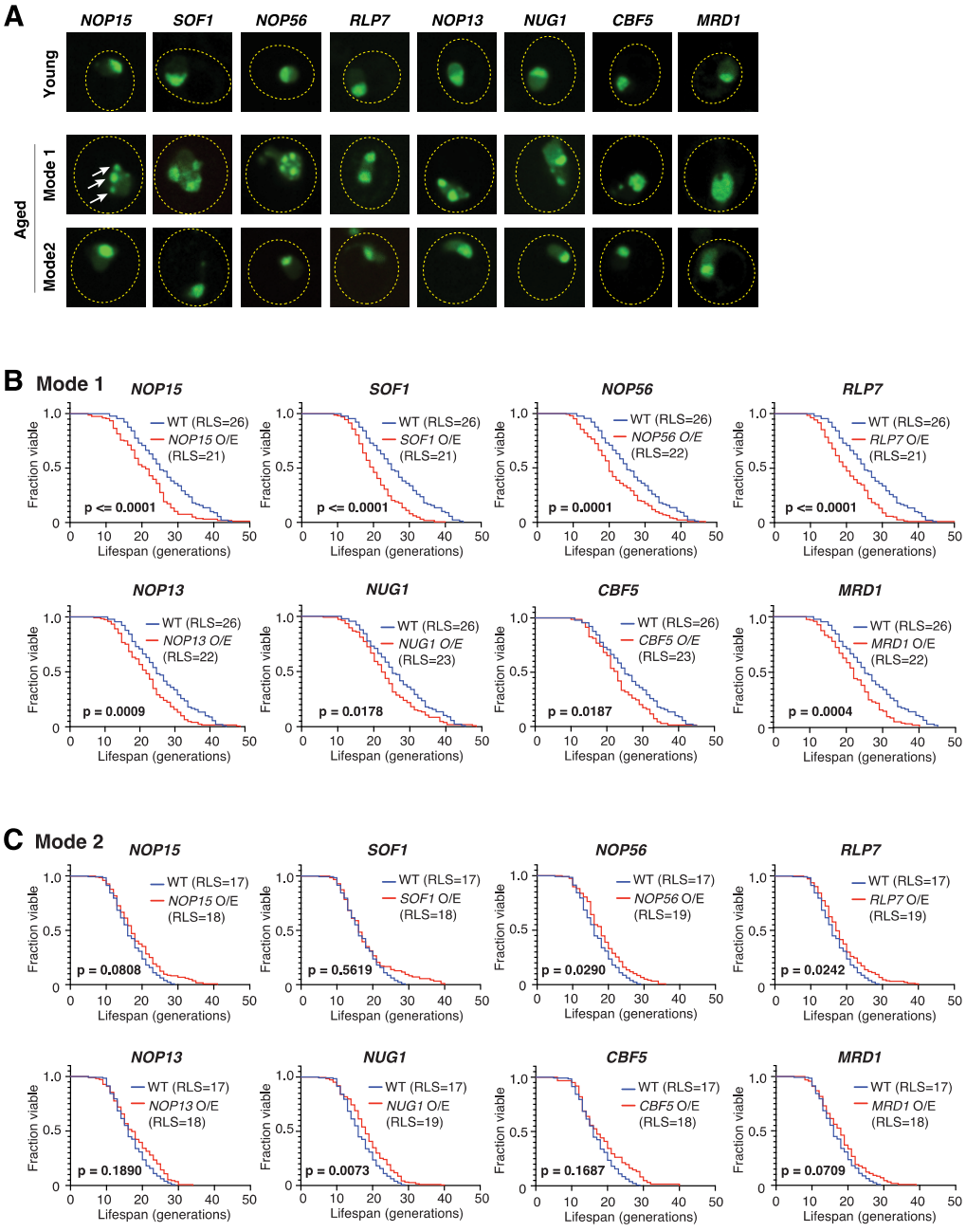


Figure 2.7 Aggregation of responder ribosomal RBPs is universal in Mode 1 aging cells but not in young or Mode 2 aging cells. 2-Fold overexpression of responder ribosomal RBPs shortened the lifespan of Mode 1 aging cells, but not that of Mode 2 aging cells.

(A) Representative images of yeast cells with each responder rRNA binding protein tagged with mNeon. Young cells and aged mother cells are circled in yellow. White arrows indicate representative aggregation spots identified in Mode 1 aging cells. (B) RLS curves for Mode 1 cells in WT (n=89), *NOP15* 2-fold overexpression (O/E) (n=156), *SOF1* O/E (n=163), *NOP56* O/E (n=145), *RLP7* O/E (n=190), *NOP13* O/E (n=225), *NUG1* O/E (n=105), *CBF5* O/E (n=131), and *MRD1* O/E (n=165). (C) RLS curves for Mode 2 cells in WT (n=127), *NOP15* O/E (n=219), *SOF1* O/E (n=180), *NOP56* O/E (n=118), *RLP7* O/E (n=224), *NOP13* O/E (n=111), *NUG1* O/E (n=187), *CBF5* O/E (n=138), and *MRD1* O/E (n=230). P-values determined using Gehan-Brreslow-Wilcoxon test.

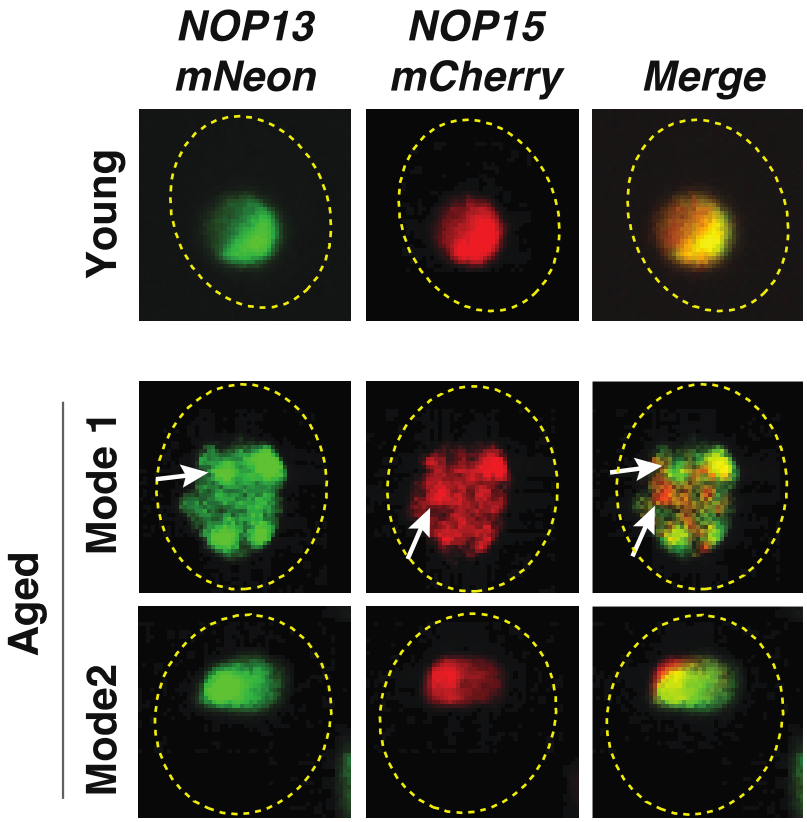


Figure 2.8 Colocalization of RBPs during aging.

Representative images showing NOP13-mNeon and NOP15-mCherry co-localization in young cells (Top), Mode 1 aging cells (Middle), and Mode 2 aging cells (bottom). Mother cells are outlined in yellow, arrows indicate foci or aggregate formations that no longer colocalize in Mode 1 aging cells.

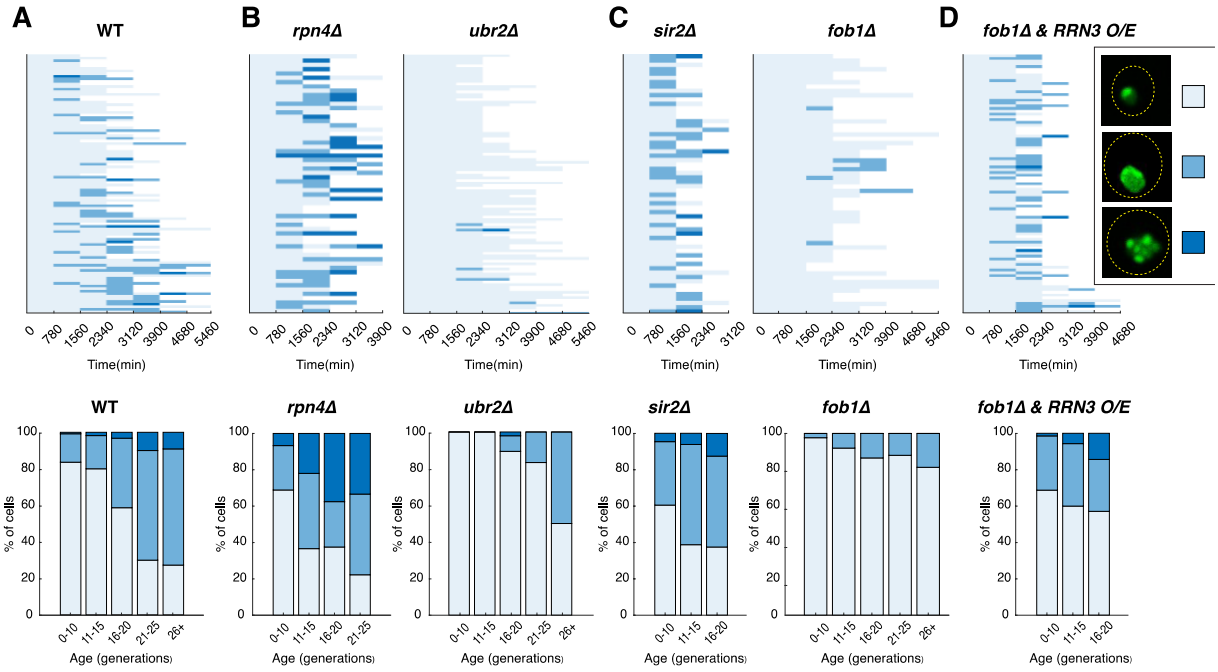


Figure 2.9 Elevated rDNA transcriptions drives aggregation of rRNA binding proteins.

Characterization of Nop15-mNeon aggregation in genetic mutants. Top: Heatmaps represent quantified Nop15-mNeon aggregation according to aggregation severity, see legend (right) for representative cellular examples of each aggregation state: no aggregation, medium aggregation, severe aggregation. Each bar represents the lifespan of single cell. Bottom: Bar charts quantify percentage of cells in each aggregation state relative as a function of specific age range. (A) Quantification of Nop15-mNeon aggregation in WT strain background. (B) Quantification of Nop15-mNeon aggregation with decreased (*rpn4*Δ*)* and elevated (*ubr2*Δ*)* proteasome activity. (C) Quantification of Nop15-mNeon aggregation with elevated (*sir2*Δ*)* and decreased (*fob1*Δ*)* rDNA recombination. (D) Quantification of Nop15-mNeon aggregation in strain with elevated rDNA transcription.

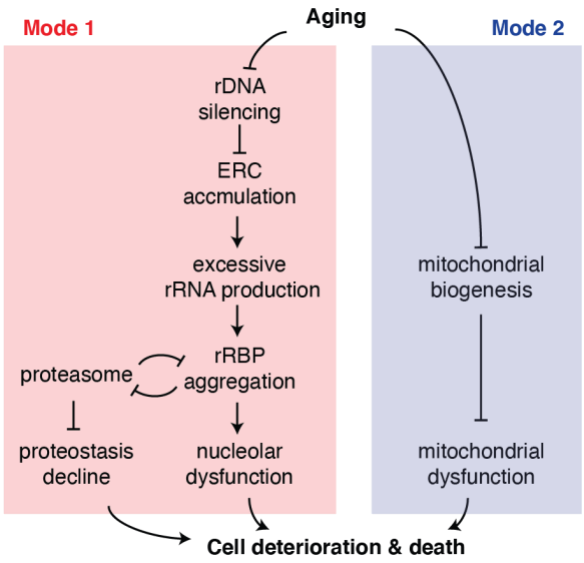


Figure 2.10 Proposed model linking chromatin instability RBP aggregation and proteostasis decline in aging.

As cells age, they diverge along two distinct trajectories characterized by changes in rDNA silencing and mitochondrial biogenesis in Mode 1 and Mode 2, respectively. In Mode 1 aging rDNA silencing is lost with age allowing for ERC accumulation and excess rRNA production. These changes promote rRPB aggregation which overwhelms or impairs the proteasome system resulting in nucleolar dysfunction, loss of proteostasis and ultimately cell deterioration and cell death.

Materials and Methods

Strains and Plasmid Construction

Standard methods for growth, maintenance, and transformation of yeast and bacteria and standard methods of DNA manipulation were used throughout. The yeast strains used in this study were generated from the BY4741 strain background (MATa *his3∆1 leu20∆ met15∆0 ura3∆0).* Tables detailing strains and plasmids used in this study are provided in tables below.

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 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-∆ssCPY*-GFP was obtained from(Park et al., 2007), we subcloned into pRS306 by digesting both pRS306 vector and ∆ssCPY*-GFP using SalI and HindIII and then ligating together. The newly assembled plasmid pRS306-∆ssCPY*-GFP was linearized using StuI and integrated into the *ura3-1* locus by homologous recombination. This strain background was BY4741 but had the *ura3∆0* locus replaced by the W303 *ura3-1* locus.

The strains used in the screen for RNA binding protein aggregation were from(Newby et al., 2017).

To create each of the mNeon tagged rRNA binding protein strains we used Gibson assembly, or traditional restriction cloning to assemble plasmids containing RBP promoter-RPB coding sequence-linker sequence-mNeon- tADH1. These plasmids were linearized using a restriction enzyme that would specifically cut within the promoter

sequence of the RBP, and then using homologous recombination integrated into the RBP promoter region. Integration was verified using PCR or microscopy, and copy number was verified using PCR to ensure single-copy insertion. The rRNA binding protein strains were similarly created. The plasmids were assembled using Gibson assembly or restriction cloning to create plasmids containing the RBP promoter-RBP coding sequence-tADH1. The plasmids were also digested in the promoter region and integrated into the native RBP promoter locus using homologous recombination. Integration and copy number were verified by PCR to ensure single-copy plasmid integration.

The sir2Δ 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 deletion strains were created using CgURA3 and and CgHIS3, *rpn4*Δ was created using CgHIS3, ubr2Δ strains were created using CgHIS3 and CgLeu2.

The Hap4 overexpression and Sir2-2x expression strains were originally constructed and verified in (Li et al., 2020) and UBR2 was deleted from each of these strains as described above.

The TetO-inducible Rrn3 overexpression strains were created by transformation with NHB1147 inducible RRN3, and NHB1148, inducible RRN3 with mCherry tag digested with *NotI* for integration at the *leu2Δ0* locus.

Yeast transformations were performed using the standard lithium acetate method and confirmed by PCR.

Microfluidic Device Fabrication

Design and fabrication of the microfluidic device for yeast replicative aging followed previously published work (Li et al., 2017). In brief, SU8 2000 series photoresists (MicroChem), chrome glass masks (HTA Photomask) and an EVG620 contact mask aligner (EV Group) were used to construct and pattern the desired features for each layer onto silicon wafers (University Wafer Inc.). Feature heights were validated using a Dektak 150 surface profiler (Veeco). A polydimethylsiloxane (PDMS) device was made from the silicon wafer mold by mixing 33g of Slygard 184 and pouring it on the wafer surrounded with aluminum foil. The wafer and PDMS asre then degassed and cured on a flat surface for 1h at 80C.

Design and fabrication of the 48-strain 2k dynomics device was carried out using techniques described in(Ferry et al., 2011) and 2000-strain design detailed in(Graham et al., 2020) .

A PDMS device was made from the silicon wafer mold by mixing 77 g of Slygard 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.

Microfluidic Experiment Setup

Aging Experiments

PDMS is cleaned and sonicated in 100% ethanol for 15 minutes then followed by a rinse sonication in milliQH20, the device is dried and cleaned with adhesive tape. A glass coverslip is cleaned in a series of washes heptane, followed by methanol, followed by milliQH20, and then dried by air gun, both glass and PDMS are exposed to oxygen

plasma to bond and create fully assembled device. Each single-cell aging device contains four separate experimental chambers, once assembled inspected to ensure no defects dust contamination present.

To begin experiment setup, device must be placed under vaccum for 20 minutes. Once removed, the device must immediately have all media ports covered by 0.075% Tween 20 for approximately 10 minutes. Once prepared the device is 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 are approximately 2ft above the microscope stage, the waste ports of the device are also connected to plastic tubing which are 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 gown at 30C overnight until cells reached approximately OD600nm 0.6. When loading cells were diluted approximately 2-fold and transferred to a 60mL syringe (Leur-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 2ft 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. We have validated previously that this low concentration of Tween-20 has no significant effect on cellular lifespan or physiology (6).

48-strain 2K Dynomics Experiments

A PDMS device cleaned with 70% ethanol and adhesive tape was aligned to a custom fixture compatible with the Singer ROTOR. 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 chip was placed in vacuum for 20 minutes before removal and covering with SCD media with 0.04% Tween-20. Media ports are then connected to plastic tubing and 60mL syringes containing SCD media with 0.04% Tween-20 and yeast are allowed to grow until traps were filled to confluence at which point imaging would begin.

Aging Microfluidics

Time-lapse microscopy experiments were performed using a Nikon Elipse Ti2-E large field of view inverted fluorescence microscope with perfect focus and an 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. The exposure and intensity settings were as follows for each of the

fluorescence channels: GFP 10ms with 10% light intensity, mCherry 50 ms with 5% light intensity, Cy5 200 ms with 2% light intensity.

Confocal Microscopy

Confocal images were obtained using the Nikon Eclipse Ti2-E scope with automated stage and perfect focus and an sCMOS camera (Teledyne Photometrics Prime 95B or PrimeBSI). The excitation light is controlled using an Agilent laser box with 405nm, 488nm, 561nm, or 640nm 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, orSR HP APO TIRF 100x 1.49 NA objectives. Laser intensity settings were: 30% for 488nm, 50% for 561nm, and 50% for 640nm.

Drug Treatments

2uM doxycycline was used to activate TetO inducible products. It was introduced into the microfluidic experiment by direct addition and mixing into the syringe. Time from drug addition to induction was approximately 5hrs as determined by induction and expression of fluorescent reporters.

Image Analysis

Image background was subtracted using ImageJ “rolling ball” algorithm with 50-pixel radius. Cellular divisions of each mother cell were manually identified and counted using nuclear marker separation as the time point of cell division. Cell aging mode was categorized based on their aging phenotypes. Mode 1 cells are characterized by late-stage elongated daughter morphology whereas Mode 2 cells are characterized by small-rounded daughter morphology. Cells that displayed abnormal morphologies from the

beginning of experiment and have a lifespan less than 5 generations were excluded from analysis.

Hsp104-GFP foci appearance was identified and quantified by eye. During each frame that a foci was visible, the frame number was noted. Heat maps were generated based on when foci were visible within a given cell-cycle. RBP aggregation was also quantified by eye. Severity of aggregation was characterized on a 1-3 scale (1 no aggregation-3 severe aggregation).

Classification of Mode 1 and Mode 2 aging cells

Mode 1 and Mode 2 cells were classified according to iRFP intensity. Any cell with x-fold increase in iRFP fluorescence during aging was classified as Mode 1 aging type. Mode 2 aging type is characterized by a loss of iRFP fluorescence, any cell which never showed increase in iRFP signal or exhibited a dramatic decrease in iRFP signal was classified as Mode 2.

Table 2-1 Yeast strains used or created for this study

Strain Name	Description
NH0270	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, Nhp6a-iRFP-kanMX RDN1::NTS1-pTDH3-GFP-URA3*
NHGFP0068	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX HSP104-GFP-HIS*
NH0761	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX HSP104-GFP-HIS sir2::CgURA3*
NH0778	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX HSP104-GFP-HIS fob1::CgURA3*
NH1324	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX Sis1:mNeon-URA*
NH1036	*BY4741 MATa his3Δ1 leu2-1 met15Δ0 ura3-1, Nhp6a-iRFP-kanMX ura3-1::ΔssCPY*-GFP-pRS306*
NH1212	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, Nop15:pNop15-Nop15-mNeon-tADH1-pRS306*
NH1213	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, Sof1:pSof1-Sof1-mNeon-tADH1-pRS306*
NH1217	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, Nop56:pNop56-Nop56-mNeon-tADH1-pRS306*
NH1187	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, RLP7:pRLP7-RLP7-mNeon-tADH1-pRS306*
NH1186	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, Nop13:pNop13-Nop13-mNeon-tADH1-pRS306*
NH1185	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, Nug1:pNug1-NNug1-mNeon-tADH1-pRS306*
NH1223	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, CBF5:pCBF5-CBF5-mNeon-tADH1-pRS306*
NH1251	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, MRD1:pMRD1-MRD1-mNeon-tADH1-pRS306*
NH1218	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, Nop15:pNop15-Nop15-tADH1-pRS306*
NH1216	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, Sof1:pSof1-Sof1-tADH1-pRS306*
NH1220	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, Nop56:pNop56-Nop56-tADH1-pRS306*
NH1211	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, RLP7:pRLP7-RLP7-tADH1-pRS306*
NH1219	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, Nop13:pNop13-Nop13-tADH1-pRS306*
NH1221	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, Nug1:pNug1-Nug1-tADH1-pRS306*

Table 2-1 continued. Yeast strains used or created for this study

Strain Name	Description
NH1222	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, CBF5:pCBF5-CBF5-tADH1-pRS306*
NH1215	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, MRD1:pMRD1-MRD1-tADH1-pRS306*
NH1477	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, Nop15:pNop15-Nop15-mNeon-tADH1 sir2::CgHIS*
NH1478	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, Nop15:pNop15-Nop15-mNeon-tADH1 rpn4::CgHIS*
NH1479	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, Nop15:pNop15-Nop15-mNeon-tADH1 fob1::CgHIS*
NH1630	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, Nop15:pNop15-Nop15-mNeon-tADH1 ubr2::CgHIS*
NH1506	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, Nop15:pNop15-Nop15-mNeon-tADH1, pRPL18B_rtTA3_tADH1, TetO7pLeu2_RRN3_mRuby2*
NH1507	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX, Nop15:pNop15-Nop15-mNeon-tADH1 fob1Δ::HIS, pRPL18B_rtTA3_tADH1, TetO7pLeu2_RRN3_mRuby2*
NH1408	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX RDN1::NTS1-pTDH3-GFP-URA3, ubr2::CgHIS3*
NH1642	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX RDN1::NTS1-pTDH3-GFP-URA3 HAP4::pTDH3-HAP4-LEU2 colony 1,single copy ubr2::CgHIS*
NH1645	*BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nhp6a-iRFP-kanMX pSIR2::pSIR2-SIR2-pRS303 extra single copy of SIR2, ubr2::CgLEU2*

Table 2-2 Plasmids used or created for this study.

Plasmid Name	Description
NHB0904	pNOP15-NOP15-mNeon-tADH1-pRS306
NHB0902	pNOP15-NOP15-tADH1-pRS306
NHB0892	pSOF1-SOF1-mNeon-tADH1-pRS306
NHB0901	pSOF1-SOF1 -tADH1-pRS306
NHB0891	pNOP56-NOP56-mNeon-tADH1-pRS306
NHB0897	pNOP56-NOP56-tADH1-pRS306
	pRLP7-RLP7-mNeon-tADH1-pRS306
NHB0896	pRLP7-RLP7-tADH1-pRS306
NHB0893	pNOP13-NOP13-mNeon-tADH1-pRS306
NHB0903	pNOP13-NOP13-tADH1-pRS306
NHB0894	pNUG1-NUG1-mNEON-tADH1-pRS306
NHB0908	pNUG1-NUG1-tADH1-pRS306
NHB0911	pCBF5-CBF5-mNeon-tADH1-pRS306
NHB0909	pCBF5-CBF5-tADH1-pRS306
NHB0927	pMRD1-MRD1-mNeon-tADH1-pRS306
NHB0895	pMRD1-MRD1-mNeon-tADH1-pRS306
NHB1147	Inducible RRN3
NHB1148	Inducible RRN3 mRuby
NHB0658	pRS305_SalI_pTDH3_HAP4_SacI
NHB0638	pSir2-SIR2_pRS303

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