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UNDERSTANDING THE MECHANISM OF GENOMIC INSTABILITY DURING REPLICATIVE AGING IN BUDDING YEAST

Sangita Pal

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UNDERSTANDING THE MECHANISM OF GENOMIC INSTABILITY
DURING REPLICATIVE AGING IN BUDDING YEAST

by

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DURING REPLICATIVE AGING IN BUDDING YEAST**

A
DISSERTATION

Presented to the Faculty of
The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

By
Sangita Pal, M.S.
Houston, Texas
May, 2017

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Dedication

*This dissertation is dedicated to my parents and my sister,
for their unconditional love and support to make this journey possible
and for making me who I am today*

Acknowledgements

I would like to express my sincere gratitude to my doctoral advisor, Dr. Jessica Tyler, for providing me the opportunity to work in the lab that I could call my second home for all these years, for having faith in my abilities, for having tremendous patience during all the difficult times and for guiding me through this entire process. Joining her lab helped me in many ways to grow as a scientist. With her exceptional and unmatched professional skills, she serves as the perfect role model I could look up to and learn from. I also have a great respect for her for being an amazing human being, and I feel very fortunate to have the opportunity to know her so closely.

I would also like to express my sincere appreciation to all my committee members, Drs. Pierre D. McCrea, Xiaobing Shi, Kevin A. Morano, Alison A. Bertuch and Weiwei Dang for providing time for me out of their busy schedules and for guiding my research for all these years with their valuable insights, and supporting me whenever needed. A special note of gratitude goes to Dr. Pierre D. McCrea for serving as my on-site advisor for these last couple of years and for all his support. It meant a lot to me. I would also like to thank Drs. Michelle Barton, Elsa Flores and William Mattox, for serving in my former committees, either advisory or exam committees.

As part of the great Tyler lab for all these years, I do not know where to start acknowledging. With some uncanny ability, Jess picks the best people in the world for her lab. Without all my lab members, past and present, my graduate

school experience would be very different. They have a special corner reserved in my heart, and some of them I consider my family. Special thanks to Pingping for being my greatest support in the lab and listening to all my complaints. Myrriah and Sarita, for the special connection we shared. Xuan, Richard, JaHwan, Jim, Brandee, Hillary, Briana, Varija, Candice, Reva, Zhihong, Zhi-Jie, Li-Ting, Faith, Rhiannon, for being great friends over the years, for supporting me in my tough times and for spending lots of fun time with me; Chandrima and Siddhartha, for all your guidance and love; and to all others for making the lab a favorable and friendly place to work. I also want to thank Sleckman lab members at Weill Cornell, especially Putzer, for sharing a great camaraderie.

I want to express my heartfelt gratitude to my parents, Dr. Ranjan Kumar Pal and Mrs. Puspa Pal, and my elder sister Silpi for always loving, caring, encouraging and supporting me. They are the pillars of strength in my life. Everything I am today is because of them. I would also like to acknowledge my dearest niece and my brother-in-law for their continuous support. A special note of thanks goes to my parents-in-law and my husband's family as well, for loving me the way I am and supporting me through everything. Without all these people in my life, I would not be able to cross the hurdles in life so easily.

I am fortunate that my graduate school provided me the chance to meet some special people, who constantly supported me through all the ups and downs I faced in these years. I am looking forward to sharing a lifelong relationship with them. Especially I would like to mention my friends Kaushik Thakkar, Pingping Wang, Niza Nemkul, Amanda Haltom, Aundrietta Duncan and

William Munoz who made my stay in graduate school lovely and enjoyable. I would also like to express my sincere thanks to all my friends and acquaintances those are not listed here, for their friendship and support over the years and to provide me some of the best memories I would cherish my whole life.

Words will not be enough to express my sincere gratitude to G&D program for providing a very supportive learning environment and especially Elisabeth Lindheim, who is a pillar of support for students enrolled in G&D program, with the solutions to almost any problem with a smiling face. I also want to thank some of the most friendly and considerate people in GSBS, who loved and supported me throughout this time. Especially I would like to mention Lily, Joy, Brenda, Bunny and Tracey among others for sharing such a special relationship with me. I value all these people a lot.

Last, but the definitely not the least, very special thanks go to the most important person in my life, my husband, Atanu, without whom I doubt I would make it through. I consider myself so blessed to have my best friend in my husband. I can truly be myself in front of him and I appreciate it so much for him always taking so good care of me. He really means the world to me. I cannot express in words how fortunate I am to have somebody in my life who has the tolerance to bear with me, for always understanding me in good or bad times, for supporting me through any situation and for being there with me no matter what. We already shared a good portion of our life together and I am looking forward to sharing the next chapters of my life with him.

UNDERSTANDING THE MECHANISM OF GENOMIC INSTABILITY DURING REPLICATIVE AGING IN BUDDING YEAST

Sangita Pal, M.S.

Advisory Professor: Jessica K. Tyler, Ph.D.

Aging brings a gradual decline in molecular fidelity and biological functionality, resulting in age related phenotypes and diseases. Despite continued efforts to uncover the conserved aging pathways among eukaryotes, exact molecular causes of aging are still poorly understood. One of the most important hallmarks of aging is increased genomic instability. However, there remains much ambiguity as to the cause. I am studying the replicative life span (RLS) of the genetically tractable model organism *Saccharomyces cerevisiae*, or budding yeast using the innovative “mother enrichment program” as the method to isolate unparalleled numbers of aged yeast cells to investigate the molecular changes associated with aging. My goal is to determine the possible causes of loss of genomic integrity during replicative aging in budding yeast to gain potential insight into this vastly complex process.

In my work presented here, I uncovered a global loss of cohesion in mitotically aged yeast cells and this most likely serves as the cause of increased rDNA instability and/or ERC accumulation as observed during aging. These events, in turn, influence the global genomic integrity in replicatively aged cells.

Furthermore, I discovered a profound defect in double strand break (DSB) repair with aging due to limiting levels of key components of the homologous recombination machinery. This DSB repair defect in old cells limited the replicative lifespan, because restoration of DSB repair by overexpressing key HR proteins ameliorated age-associated changes, to extend lifespan. We propose that the limiting levels of repair factors and cohesin proteins impair the ability of the aged cells to counteract the increased burden of genomic damage accumulation coupled with chromosomal rearrangements and potentially chromosome loss, eventually to cross a threshold of genomic damage that is sensed by the cell to cause cessation of cell division marking the end of the replicative lifespan.

Table of Contents

Approvals	i
Title	ii
Copyright	iii
Dedication	iv
Acknowledgements	v
Abstract	viii
Table of Contents	x
List of Figures.....	xv
List of Tables	xvii
List of Appendix.....	xviii
CHAPTER 1: INTRODUCTION.....	1
1.1. General overview of the aging process.....	2
1.2. Hallmarks of aging.....	4
1.2.1. Epigenetic changes during aging.....	7
1.2.2. Genomic instability during aging.....	13
1.3. Human aging disorders and genomic instability.....	19
1.4. Use of model organisms for aging studies.....	20
1.4.1. Chronological aging in budding yeast.....	22
1.4.2. Replicative aging in budding yeast.....	23
1.5. Structure of yeast rDNA locus and connection with aging.....	28

1.5.1. Structure of yeast rDNA.....	28
1.5.2. ERC (extrachromosomal rDNA circle) theory of aging.....	31
1.5.3. rDNA theory of aging.....	32
1.6. Double strand break (DSB) repair in yeast and connection with aging.....	33
1.6.1. Double strand break (DSB) repair in yeast.....	33
1.6.2. Connection of impairment of DNA repair with aging.....	37
 CHAPTER 2. MATERIALS AND METHODS.....	39
2.1. Yeast strains.....	40
2.2. Yeast media and plates.....	41
2.3. Yeast genomic DNA isolation.....	42
2.4. Yeast transformation.....	42
2.5. Purification of old cells by mother enrichment program.....	43
2.6. Purification of old cells by serial biotin purification.....	44
2.7. RNA isolation, cDNA synthesis and quantitative PCR (qPCR).....	45
2.8. Pulse field gel electrophoresis (PFGE).....	48
2.9. Southern blot analysis.....	49
2.10. Analysis of branched recombination intermediates (Stuck DNA analysis).....	49
2.11. DSB analysis.....	50
2.12. Chromatin immuno-precipitation (ChIP) and qPCR.....	50
2.13. Western blot analysis.....	52
2.14. Cohesion assay (GFP two spt analysis).....	53

2.15. Repair assay using MMS.....	54
2.16. Immunofluorescence (IF) analysis.....	54
2.17. PCR analysis of HO cutting and repair efficiency.....	55

CHAPTER 3: ROLE OF RIBOSOMAL DNA (rDNA) INSTABILITY IN

REGULATION OF LIFESPAN IN BUDDING YEAST.....57

3.1. Introduction and Rationale.....	58
--------------------------------------	----

3.2. Results	60
--------------------	----

3.2.1. Non-coding RNA transcription is highly induced from <i>NTS1</i> and <i>NTS2</i> in the intergenic spacer region of the rDNA locus in old cells.....	60
--	----

3.2.2. Cohesin occupancy is reduced from the rDNA region during aging.....	63
--	----

3.2.3. Cohesion is lost not only from the rDNA locus, but also globally during aging.....	66
---	----

3.2.4. Cohesin displacement from the rDNA does not correlate with non-coding RNA transcription.....	68
---	----

3.2.5. The majority of the non-coding RNA in old cells arises from ERCs.....	71
--	----

3.2.6. Expression of Mcd1, the limiting sub-unit of cohesin complex, is reduced during aging.....	71
---	----

3.2.7. The rDNA locus undergoes unequal recombination and repeat number variation during aging.....	73
---	----

3.2.8. Formation of branched recombination intermediates increases from rDNA during aging	76
3.2.9. DNA double-strand break formation at the rDNA locus does not increase during aging <i>per se</i>	78
3.3. Conclusions.....	78

CHAPTER 4: MECHANISTIC INSIGHTS INTO UNDERSTANDING INCREASED DNA LESIONS FORMATION DURING REPLICATIVE

AGING	84
4.1. Introduction and Rationale.....	85
4.2. Results	86
4.2.1. Increased chromosomal instability proportional to chromosome lengths is observed during aging.....	86
4.2.2. Global chromosomal instability in old cells is proportional to rDNA instability.....	88
4.2.3. DSB repair is significantly impaired in old cells.....	93
4.2.4. Levels of several DNA repair proteins are reduced in old cells.....	99
4.2.5. Over-expression of key DNA repair proteins (Rad51 and Mre11) partially reverses the accumulation of DNA damage with age	101
4.3. Conclusions.....	101

CHAPTER 5: DISCUSSION & FUTURE DIRECTIONS.....105

APPENDIX 1: The commercial antibodies against histone H3 lysine 56	
acetylation (H3K56Ac) are non-specific <i>in vitro</i> and in mammalian	
cells.....	121
Bibliography.....	134
Vita.....	167

List of Figures

Figure 1. Decline of cellular functions and accumulation of cellular damage with aging.....	3
Figure 2. Hallmarks of aging.....	5
Figure 3. Epigenetic alterations and genomic instability during aging.....	6
Figure 4. Replicative aging model in budding yeast.....	24
Figure 5. Model of the “mother enrichment program (MEP)”.....	27
Figure 6. Structure of the yeast rDNA locus.....	29
Figure 7. Key steps of homologous recombination (HR) in budding yeast.....	35
Figure 8: Transcriptional up-regulation from non-transcribed regions of rDNA during aging.....	61
Figure 9. No significant difference in RNA Polymerase I (Pol I) transcriptional activity is observed during aging.....	64
Figure 10. Mcd1 occupancy at different genomic regions is reduced during aging.....	65
Figure 11. Loss of cohesion is observed during aging.....	67
Figure 12: <i>NTS1</i> or <i>NTS2</i> transcription does not directly correlate with cohesin association.....	69
Figure 13. <i>NTS1</i> and <i>NTS2</i> intergenic transcripts in old cells are largely contributed by extra-chromosomal rDNA circles (ERCs).....	72
Figure 14. Cohesin sub-unit, Mcd1, level is lower in old cells.....	74
Figure 15. The rDNA locus undergoes unequal recombination with age.....	75

Figure 16. Increased rDNA recombination products is observed in old cells.....	77
Figure 17. rDNA locus does not accumulate more DSBs <i>per se</i> during aging...	79
Figure 18. The aging genome accumulates random damage.....	87
Figure 19: Increased chromosomal instability proportional to chromosome length is observed during aging in WT cells.....	89
Figure 20. rDNA instability appears to promote global genomic instability.....	91
Figure 21. The HO endonuclease is ineffective in inducing DSBs in old cells....	94
Figure 22: Old cells have impaired DSB repair.....	96
Figure 23. Old cells are predominantly at G ₂ /M phases of the cell cycle.....	98
Figure 24. Reduced expression level is observed for key DNA repair proteins in old cells.....	100
Figure 25: Over-expression of key repair proteins reduces genomic instability.....	102
Figure 26. Working model for rDNA instability and reduced homologous recombination (HR) causing global genomic instability to limit lifespan.....	115
Figure 27. The commercial antibodies against histone H3 lysine 56 acetylation (H3K56Ac) are non-specific <i>in vitro</i>	127
Figure 28. The commercial antibodies against histone H3 lysine 56 acetylation (H3K56Ac) are non-specific when tested in mammalian cells.....	129
Figure 29. A commercial antibody against histone H3 lysine 56 acetylation (H3K56Ac) showing specificity <i>in vitro</i> is non-specific when tested in breast cancer tissue.....	132

List of Tables

Table 1: A list of yeast strains used in this study.....	40
Table 2: A list of primers used in this study.....	46
Table 3: A list of antibodies used in this study.....	55

List of Appendix

Appendix 1: The commercial antibodies against histone H3 lysine 56 acetylation (H3K56Ac) are non-specific <i>in vitro</i> and in mammalian cells.....	121
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CHAPTER 1: INTRODUCTION

Some parts of this chapter are based upon our published review: Pal, S., and Tyler, J.K. (2016). "Epigenetics and aging". *Science Advances* 2, e1600584. Copyright permission is not required as the copyright policy for this article published in the journal of *Science Advances* states that "This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial license, which permits use, distribution, and reproduction in any medium, so long as the resultant use is not for commercial advantage and provided the original work is properly cited."

1.1. General overview of the aging process

In general, propagation of any given organism primarily depends on either forever maintaining its cellular homeostasis or effectively renewing the population with healthy young individuals. Nature primarily selected for the latter option throughout evolution and potentially selected against any organism living forever. Aging is a complex multi-factorial biological process that is an inevitable outcome of life and is universally shared by all organisms. Generally, organismal health is directly proportional to the overall fitness of its organs, tissues, cells and finally biological macromolecules. It is characterized by a progressive decline of molecular, cellular, physiological and organismal function in a time-dependent manner. As an organism ages, its ability to retain normal cellular functions starts to decline, and susceptibility to diseases and other environmental stresses rises strikingly. How this functional decline initiates during organismal aging remains elusive till now. Understanding the molecular details of organismal aging thus

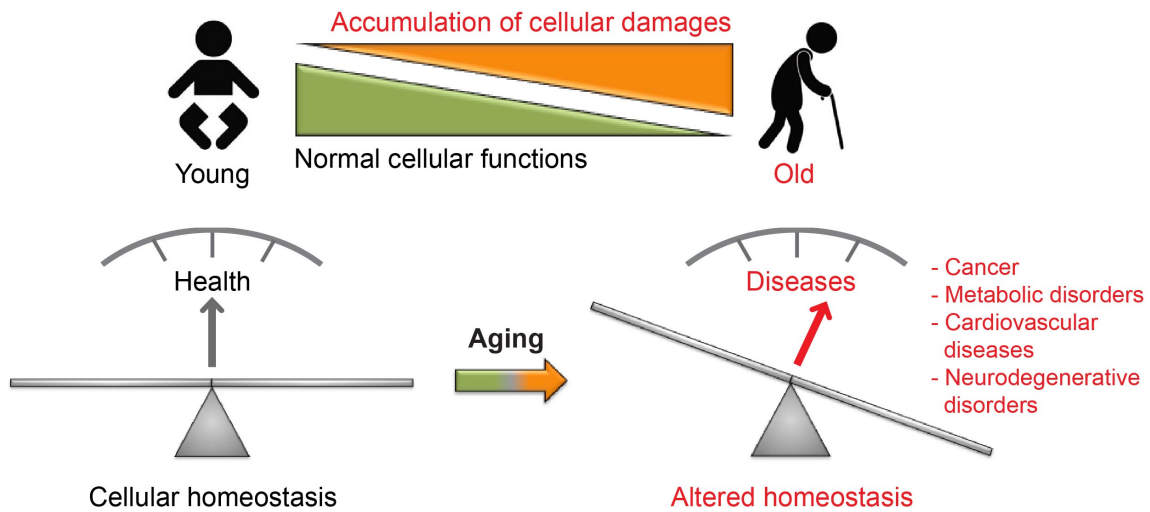


Figure 1. Decline of cellular functions and accumulation of cellular damage

with aging. A simplified model illustrates that maintenance of proper cellular homeostasis in young cells is imperative to maintain proper health. However, with aging functional deteriorations of different cellular functions together with increased accumulation of cellular damage leads to altered cellular homeostasis resulting in increased susceptibility to different age-associated conditions, including cancer, metabolic disorders, cardiovascular diseases and neurodegenerative disorders.

holds significant importance for human health because aging serves as the greatest risk factor for development of many diseases including cancer, metabolic disorders such as diabetes, cardiovascular disorders, neurodegenerative diseases and many others (**Fig. 1**) (1-8). Although the causes of aging are poorly understood, there are continued efforts to delineate longevity pathways conserved among all eukaryotes effectively linking physiology with signal transduction and genetic regulations to mediate extension of health and lifespan.

1.2. Hallmarks of aging

Over the past years, great strides have been made by numerous groups in the aging research field that efficiently categorized the cellular and molecular hallmarks of aging (9). These nine hallmarks are generally considered to be the pillars greatly contributing to and determining the aging process (**Fig. 2**). In order to be considered as a hallmark of aging, these candidates needed to be fulfilling the following criteria: that this event happens during normal aging, experimentally worsening this event would accelerate the natural aging process and experimental improvement of this event delays onset of aging to extend natural lifespan (9). Although some of these hallmarks are specific for metazoans, the majority of them are conserved in all eukaryotic organisms from yeast to humans, including epigenetic alterations and genomic instability (**Fig. 3**), which will be further elaborated here.

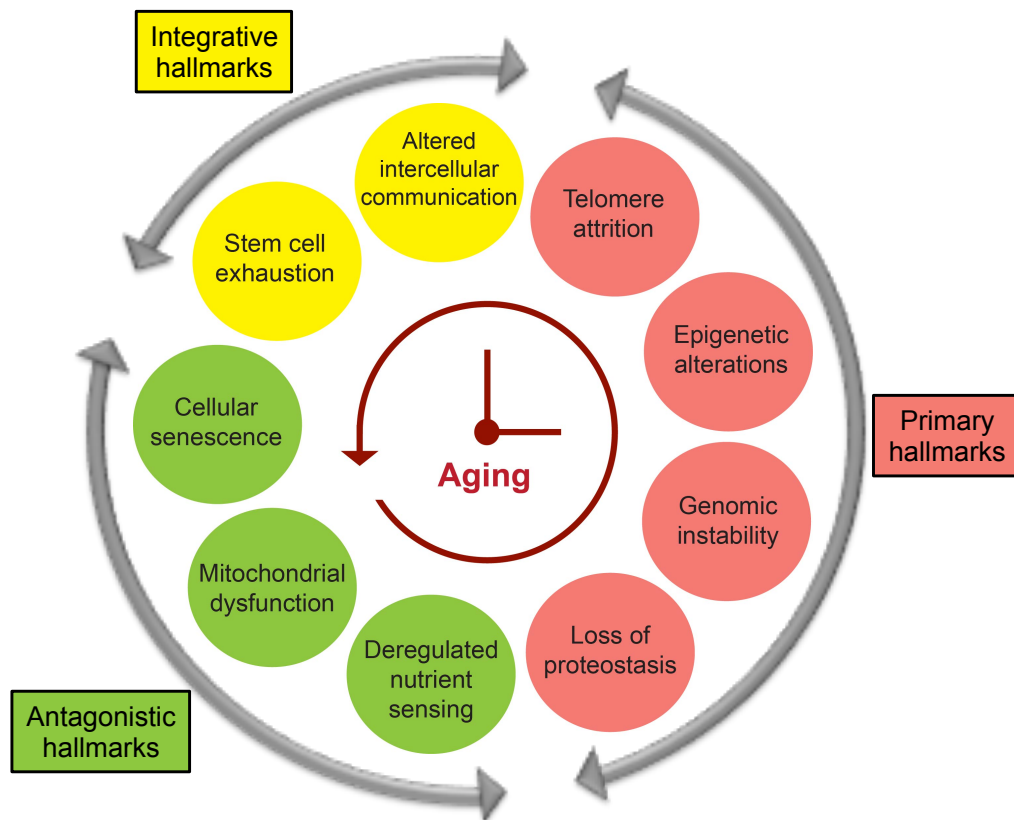


Figure 2. Hallmarks of aging. Schematic representation of nine known hallmarks of aging in multicellular eukaryotes, while some of them are conserved across species to unicellular eukaryotes also. The nine hallmarks are further grouped into three categories of primary, antagonistic and integrative hallmarks of aging. Epigenetic alterations and genomic instability are conserved aging hallmarks in multiple organisms and here are categorized as primary hallmarks of aging, i.e. those that serve as the drivers of aging. (Adapted from: Lopez-Otin, C., M. A. Blasco, L. Partridge, M. Serrano, and G. Kroemer. 2013. The hallmarks of aging. *Cell* 153: 1194-1217. The figure is used with permission from Elsevier under the license number 4091620498639)

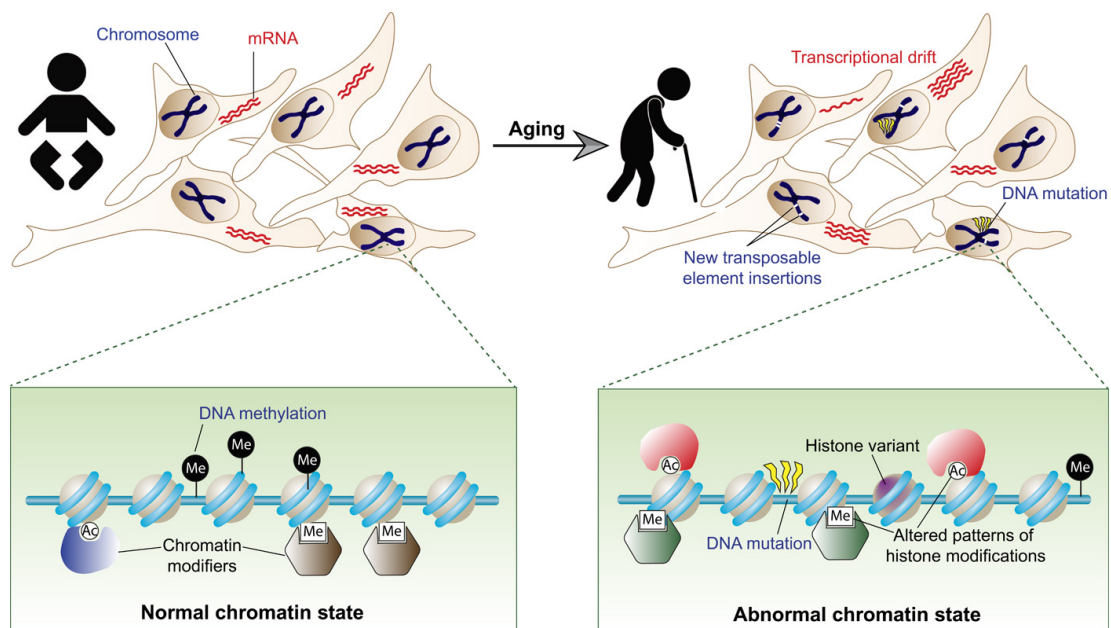


Figure 3. Epigenetic alterations and genomic instability during aging. While in young individuals cells maintain uniformity in their epigenetic information and also a stable genome, during aging exposures to different exogenous and endogenous factors result in abnormal chromatin states coupled with increased genomic instability. (Adapted from: Pal, S., and J. K. Tyler. 2016. Epigenetics and aging. *Science advances* 2: e1600584)

1.2.1. Epigenetic changes during aging

Among these hallmarks, epigenetic alterations represent one crucial mechanism behind the deteriorated cellular functions observed during aging and in age-related disorders. By definition, epigenetics represents the reversible heritable mechanisms that occur without any alteration of the underlying DNA sequence. Although the chromosomes in our genome carry the genetic information, the epigenome is responsible for the functional use and stability of that valuable information. In other words, it connects the genotype with the phenotype (10-13). These epigenetic changes can either be spontaneous or driven by external or internal influences. Epigenetics potentially serves as the missing link to explain why the pattern of aging is different between two genetically identical individuals, such as identical twins or in the animal kingdom between animals with identical genetic make-up, such as queen bees and worker bees (2, 14, 15). Though longevity studies in the human population have shown that genetic factors could explain a fraction (20-30%) of the differences observed in the lifespans of monozygotic twins, the majority of the remainder of variation is thought to have arisen through epigenetic drift during their lifetime (11, 12, 14, 16, 17). Similarly, different environmental stimuli including diet cause differential alterations of stored epigenetic information to create a striking contrast in physical appearance, reproductive behavior, and lifespan in queen and worker honeybees, despite their identical DNA content (18). The resulting variability in the pattern of epigenetic information within individual cells in the population during aging, in turn, leads to transcriptional drift and genomic instability. Being

established by enzymes, epigenetic information is reversible. As such, epigenetics holds great prospects for targeting by therapeutic interventions, which may potentially lead the way to the development of novel therapeutic approaches to delay aging and age-related diseases.

There are different types of epigenetic information encoded within our epigenome, including but not limited to the presence or absence of histones on any particular DNA sequence, DNA methylation, chromatin remodeling, post-translational modifications of the histone proteins, structural and functional variants of histones, and transcription of non-coding RNAs (ncRNAs) (2, 4, 12). These different types of epigenetic information together comprise our epigenome and are important determining factors behind the function and fate of all cells and tissues, in uni- and multi-cellular organisms. Invariably, each of these different types of epigenetic information is functionally significant for the process of aging. Growing evidence in recent years also clearly implicates chromatin structure, which carries much of the epigenetic information, as a major player during the aging process. The basic unit of chromatin structure is the nucleosome, which consists of 147 bp of DNA wrapped around a histone octamer that comprises two copies of each core histone protein, H2A, H2B, H3 and H4 (19, 20). The addition of linker histones, such as histone H1, and other non-histone proteins, such as heterochromatin protein 1 (HP1), facilitate the formation of higher-order repressive chromatin structures, such as heterochromatin (21). The packaging of the genomic DNA into the highly organized chromatin structure regulates all

genomic processes in the nucleus including DNA replication, transcription, recombination and DNA repair, by controlling access to the DNA (4).

Studies in humans and the powerful models of aging are revealing that, similar to all other biological structures inside the cells, the epigenome suffers from a progressive loss in its configuration during aging. This results in a profound change in the chromosomal architecture, genomic integrity and gene expression patterns (2, 12). When examined, these effects are mostly conserved all the way from single-celled organisms such as budding yeast to complex multicellular eukaryotes. These conserved mechanisms help us gain a clearer picture of the aging process.

The heterochromatin loss model of aging

One of the earlier proposed models of aging was “the heterochromatin loss model of aging” (22, 23). This model suggests that the loss of heterochromatin that accompanies aging leads to changes in global nuclear architecture and expression of genes residing in those regions, directly or indirectly causing aging and cellular senescence. As with any other model of aging, the heterochromatin loss model is supported by experimental data, but there are also confounding observations. Loss of transcriptional silencing, due to decay of the heterochromatin, occurs during aging in all eukaryotes examined from yeast to humans (22-26), and there is evidence that accelerating or reversing this process can either shorten or lengthen lifespan, respectively (Fig. 2). Gene silencing requires the absence of histone acetylation within

heterochromatin regions. Accordingly, treatment with histone deacetylase (HDAC) inhibitors or deletion of genes encoding HDACs, such as yeast SIR2 or its sirtuin counterparts in metazoan species, shortens lifespan while chemical activation or overexpression of SIR2 / sirtuins extends lifespan (27, 28). Yeast Sir2 was first recognized as an H4 K16Ac deacetylase, while mammalian SIRT1 is an H3 K9Ac / H4 K16Ac deacetylase (27). However, it is now appreciated that sirtuins do not just deacetylate histones, but also many other transcriptional regulators and indeed their roles in aging extend far beyond heterochromatin, including genome maintenance (29). It has been observed previously in budding yeast that redistribution of Sir proteins from the silent mating type loci to sites of increased genomic instability during aging causes loss of silencing from heterochromatic mating type loci to cause sterility and to promote aging (30). Similarly, the mammalian homolog of Sir2, SIRT1, has been shown to repress repetitive elements and other regions across the mouse genome. However, in response to DNA damage, SIRT1 is redistributed from these loci to DNA breaks, resulting in alterations in gene expression that is comparable to those in the aging mice brain (29). Therefore, repression of heterochromatic repeat elements seems to be evolutionarily conserved and extremely critical in lifespan maintenance (29, 31). Another example where it is clear that loss of heterochromatin promotes aging is at the rDNA locus, where loss of silencing of the rDNA promotes genomic instability and aging in budding yeast (32, 33). Further evidence supporting the heterochromatin loss theory comes from the analysis of chromatin structures from Hutchinson-Gilford Progeria Syndrome

(HGPS) patients and most recently from a Werner syndrome stem cell model of premature aging (12, 34-37). These disorders exemplify how the deregulation of heterochromatin, illustrated by global loss of heterochromatin marks (discussed more below) and altered heterochromatin structure, accelerates aging. Although some of the defects observed in premature aging patients have also been observed in aged individuals (35), it is still debatable whether the findings are equally applicable to physiological aging.

Global histone protein reduction during aging

Recently, the aging research field has experienced a leap from the earlier paradigm of the heterochromatin loss model. Not only is the heterochromatin reorganized during aging, but in addition, a global loss of core histone proteins from the genome during aging has been observed in multiple scenarios, and this has been shown to be a cause of aging in yeast. In budding yeast, replicative aging is accompanied by loss of approximately half of the core histone proteins (38, 39). The extensive nucleosome loss from the entire yeast genome during replicative aging results in globally increased transcription in aged cells (40). These global changes in transcription and chromatin structure were only detectable due to the development of novel normalization approaches for genomic datasets (41). Notably, during yeast replicative aging, not only transcriptionally silent regions showed dramatic de-repression, but all genomic regions showed transcriptional up-regulation, presumably due to increased access of the transcription machinery to the DNA sequences (40). The drastic

decline of the core histone proteins in *S. cerevisiae* is due to reduced protein synthesis of the histones (38). Clearly, the fact that the levels of histone transcripts, like all other transcripts, actually increase during replicative aging in yeast (38, 39, 42) has no functional consequence because of the reduced histone protein synthesis in old yeast. Other cells may indeed use transcriptional regulation to reduce histone levels during aging because quiescent muscle stem cell aging is accompanied by reduced histone transcript levels (43). Supply of extra histone H3 and H4 proteins, either from an inducible promoter, by deleting the genes encoding the Hir repressor of histone gene transcription, or deleting the gene encoding the protein Tom1 that is involved in degradation of excess histone proteins, results in lifespan extension of yeast, identifying loss of histone proteins as a cause of aging (38, 44). The pathway of lifespan extension utilized by supplying extra histones appears to be independent from the pathway of calorie restriction (CR) in budding yeast (38), which is a widely-accepted way to extend lifespan in seemingly all species (45). Interestingly, the lifespan extension that results from lithium exposure in worms is accompanied by increased histone gene expression (46), although it is not known if increased histone gene expression plays a causative role in lifespan extension. The reduction of bulk core histone protein levels observed during replicative aging is not restricted to budding yeast, but has also been observed during aging in worms (47), during replicative aging of human diploid primary fibroblasts (48) and in senescent human cells (49). It is noteworthy that global histone loss has not yet been reported in mitotically dividing mammalian tissues in vivo. In human primary

fibroblasts, the reduced synthesis of new histones during replicative senescence was a consequence of the shortened telomeres activating the DNA damage response (48), potentially explaining the mechanism by which telomere shortening limits the number of cell divisions. As such, loss of core histones may be a more generalized phenomenon observed with aging in many organisms.

Decreased levels of core histone proteins in aged cells would likely cause a dramatic effect on the chromatin landscape by providing inappropriate access to the genetic material. The consequences of having limiting amounts of histone proteins on DNA during age progression has been elaborately examined in a recent global analysis of chromatin from aged cells in budding yeast in comparison to the young rejuvenated population (40). Most of the nucleosomal positions are maintained in aged cells, while nucleosome occupancy is reduced to half the normal proportion, and nucleosome locations on particular DNA sequences become less stringent, or fuzzier. Additionally, moderate over-expression of histone proteins H3/H4 partially reversed the transcriptional defects observed during aging. It will be intriguing to investigate in the future whether the longevity effect conferred by supplying extra histones is equally pertinent in multicellular organisms.

1.2.2. Genomic instability during aging

Genomic instability represents another major hallmark that has long been implicated as a major causal factor for aging (9, 50). It refers to alterations in the genome under physiological conditions in response to endogenous or exogenous

stresses. The stability and integrity of the genome are under continuous challenge from these endogenous or exogenous threats. Lesions in the genome can arise either as chemical damage to the DNA, mutations of actual base pairs or epimutations of DNA that refers to heritable changes in DNA modifications affecting gene expression without changing the actual sequence of DNA (9, 50). The resulting effects of these genomic lesions accumulated in DNA are in general highly diverse and include point mutations, deletions/insertions, inversions, transpositions, translocations, chromosomal gains/losses, telomere shortening etc. While the ability to endure a certain level of changes in the genomic content of any species is important for its evolvability, too much change also drives a species towards its extinction. Therefore, very complex yet refined systems of genome maintenance exist to guard the genome, which is the primary template for structural and functional information in any organism (50, 51). Although distinct from the nuclear DNA, changes in mitochondrial DNA and nuclear architecture can give rise to genomic instability (9). The focus of this study will be geared towards the genetic instability of nuclear DNA during aging.

As mentioned earlier, the eukaryotic genome is packaged into chromatin structures. Therefore, a more relaxed chromatin structure observed during aging, due to heterochromatin loss or histone loss, is predictive of not only transcriptional deregulation but also genome instability. Indeed, the reduced nucleosome occupancy in old yeast led to increased genomic perturbations, including higher levels of DNA breaks, damage foci formation, inter- and intra-chromosomal translocations, insertion of mitochondrial DNA into the nuclear

genome and high levels of retrotransposition (40). Noteworthy, partial over-expression of histone proteins H3 and H4 partially reversed the transcriptional defects observed during aging and reduced retrotransposition, indicating that the increased retrotransposition in old yeast was a consequence of histone loss during aging (40). Increased levels of DNA breaks or unrepaired DNA damage, as illustrated by formation of γ -H2AX foci (a hallmark of DNA damage) have been observed in cells from multiple species including aged mice, senescent human cells and cells derived from patients with premature aging disorders (52-55). In addition, other forms of DNA damage including copy number variations and chromosomal aneuploidy have also been shown to be associated with increased age (56, 57). This evidence suggest that DNA damage may be a driving force behind organismal aging, although the full extent of the role of DNA damage during age progression still needs to be explored.

One form of genomic instability observed during aging that results from heterochromatin decay is increased mobility of endogenous genetic elements, called retrotransposable elements. Retrotransposable elements have been implicated in the aging process by several lines of evidence across organisms including budding yeast, flies, worms, mice and even during cellular senescence in adult human stem cells (40, 58-63). Retrotransposable elements are tightly silenced by heterochromatin in young cells/organisms. However, the loss of heterochromatin with aging leads to increased expression of otherwise silent retrotransposons, essentially causing transposition of these elements, because the transcripts are reverse transcribed to make a genomic cDNA copy that gets

integrated elsewhere into the genome. The end result is increased mobility of retrotransposable elements within genomes, resulting in disruption of cellular homeostasis during aging (64). Proof that the loss of chromatin-mediated silencing of retrotransposable elements during the course of aging directly leads to their increased transposition during aging came from the ability to repress Ty transcription and retrotransposition in old yeast by overexpressing histones (40). Lifespan-extending interventions, such as calorie restriction, have also been shown to counteract the increased expression of retrotransposons in aged mice (63).

As mentioned previously, sirtuins play important roles not only in deacetylating histones but also in their roles in genome maintenance (29). In addition to redistribution of Sir proteins from the silent mating type loci to sites of increased genomic instability during aging to cause loss of silencing from mating type loci and to promote aging in budding yeast (30), in mice SIRT1 has also been shown to repress repetitive elements and other regions across the genome. In response to DNA damage, SIRT1 is redistributed from these loci to DNA breaks, resulting in alterations in gene expression comparable to those in the aging mice brain (29). Another example where it is clear that loss of heterochromatin promotes aging is at the rDNA locus, where loss of silencing of the rDNA promotes genomic instability and aging in budding yeast (32, 33). The most recent evidence for sirtuin proteins acting in genome maintenance come from studies focusing on SIRT6. While the lifespan of transgenic male mice overexpressing Sirt6 is significantly longer than WT mice (65), SIRT6 deficient mice

are smaller in size, have acute early-aging degenerative phenotypes and early death (66). In the absence of SIRT6, increased genomic abnormalities and increased sensitivity to DNA damaging agents were observed, implying roles of SIRT6 in genome maintenance (66). Another study of SIRT6 in mouse cells showed that it has ADP-ribosylase activity and SIRT6-mediated mono ADP-ribosylation of KAP1 promotes its interaction with HP1 and the packaging of L1 LINE elements into repressive heterochromatin (67). During aging, SIRT6 is sequestered away from the L1 elements, presumably to DNA breaks elsewhere in the genome, resulting in activation of the retrotransposons (67). Recently, it has also been shown that SIRT6 is responsible for rescuing the declining efficiency of homologous recombination *in vitro* during senescence of human fibroblasts (68). All these evidence further emphasize the critical roles of sirtuin proteins in genome maintenance, and lifespan extension (29, 31).

It is a long-standing belief that accumulation of DNA mutations over a period of time impacts organismal aging, as mutation accumulation happens as a function of age in different organisms such as flies or mice, and in some cases also correlates with lifespan (69, 70). However, a recent study indicated that accumulation of somatic DNA mutations at least in budding yeast does not have a causal role in aging (71). Therefore, it is yet to be conclusively determined if the persistent DNA damage observed in aging cells is the result of increased susceptibility of the accessible genome to accumulate DNA damage with time or the inability of aged cells to efficiently repair the damage or a combination of both. It has been hypothesized for a long time that a declining DNA repair

response is a possible contributing factor for the increased DNA damage observed during aging, thus contributing to the aging process itself (51, 72). Notably, another recent analysis of the DNA repair transcriptome of liver in species with significant lifespan differences revealed that the longer-lived species express certain DNA repair genes at higher levels compared to species with shorter lifespan, suggesting a superior genome maintenance mechanism in the longer-lived species, which could potentially be responsible for their longer lifespan (73).

Importantly, cancer is considered a disease of aging as cancer development exponentially increases in the aging human population, but the mechanism by which aging predisposes cells to cancer initiation still remains elusive (74, 75). Aging and cancer, in the majority of instances, share common etiologies (76). The increased genomic instability that is a prominent hallmark for aging also serves as one of the primary hallmarks of cancer (9, 77). Thus, understanding the events associated with an increase in genomic instability during aging will also enhance our knowledge about carcinogenesis, and therefore help in the accurate prediction, effective treatment, and prevention of cancer.

Noteworthy, the processes described in this section are unlikely to function independently from each other, and one event may lead to another during the aging process. For example, heterochromatin decay and histone loss may lead to increased genomic instability and changes in gene expression. In consideration of the increase in genome instability during aging, strong lines of

evidence are there to demonstrate genomic alterations in aging cells but how it is causally related to aging needs further exploration. We are currently limited in our knowledge of the sequence of the causal events during aging in healthy individuals. Given the complexity of the genome maintenance mechanisms in eukaryotic cells and the redundant roles played by different factors, it is difficult to model physiological aging in any given model organisms. Attempts should be made in the near future to define the cascade of events during age progression to attain a comprehensive view of the aging process.

1.3. Human aging disorders and genomic instability

The importance of genome maintenance mechanisms on the aging process is further exemplified by different premature aging disorders, the majority of which result from defects in genes important for DNA repair pathways (78, 79). Hutchinson-Gilford Progeria Syndrome (HGPS), Werner syndrome (WS), Cockayne syndrome (CS) and trichothiodystrophy (TTD) are rare human genetic disorders characterized by premature aging phenotypes with a shortened lifespan (79). This group of diseases resembles physiological aging to a certain extent, serving as excellent models to gain insight into the biology of aging in humans (80, 81). These diseases are either due to mutations in genes involved in the DNA repair pathways or the A-type lamin, leading to defective DNA damage response and/or disorganized chromatin structures (78, 79, 82). The causative mutations behind these progeria syndromes indicate that genomic instability and chromatin deterioration are causes of human aging. Furthermore,

the knowledge we gain from understanding the molecular pathology of these human premature aging diseases provides us with useful information to understand the complex aging process. Individuals with progeric syndromes do not always recapitulate all aging phenotypes, as they usually show segmental progeria affecting multiple tissues (79). Furthermore, the accelerated aging phenotypes observed in these disorders may not be solely due to the chromatin/repair defects, but a considerable contribution of ineffective genome maintenance to these disease etiologies has to be acknowledged. By recapitulating some molecular and cellular changes characteristic of the natural aging process, these models provide us with a unique opportunity to understand the aging process in a human model (80, 81).

1.4. Use of model organisms for aging studies

Use of model organisms in studying aging is invaluable for laying the groundwork in this field of study. Much of our knowledge of aging today comes from the studies in different model organisms (83, 84). Studies on the aging of mammals are rather limited by the long lifespan of the commonly used model organisms. Both non-vertebrate and invertebrate organisms, with their shorter lifespan and ease of genetic and environmental manipulations, are popular among researchers in the aging field as experimental models for aging studies. Among them, budding yeast or *Saccharomyces cerevisiae* is a highly informative organismal model for aging studies with its genetic tools, short lifespan and fully sequenced genome (85, 86). It is also possible to investigate mitotic and post-

mitotic aging in this model organism. Though unicellular, budding yeast has been an excellent model to identify and characterize conserved basic biological processes, including aging (5, 6). It has been extensively used for identifying genes and interventions responsible for lifespan extension, to gain insights relevant to the aging processes of all eukaryotic organisms. In parallel, over the years studies in invertebrate organisms such as *Drosophila melanogaster* (flies) and *Caenorhabditis elegans* (worms) and in certain vertebrate models such as mice, zebrafish, naked mole rats and most recently African turquoise killifish have also provided invaluable information to help us understand the complexity of the process of aging and the effect of overlapping pathways influencing longevity (5, 8, 84). Both flies and worms have a relatively short lifespan and are extensively used to characterize the involvement of different genetic pathways in longevity, but both of these model organisms primarily focus on post-mitotic aging (5). African killifish rather recently provided the aging field with a short-lived vertebrate model that recapitulates age-associated human pathologies, but this model system is the newest in the field and yet to be developed fully (84). On the other hand, mice and zebrafish represent well-established vertebrate models of aging those are evolutionarily closer to humans. In particular, the available resources and different models of aging including premature aging and long-lived aging mice were instrumental in our current understanding of this complex process (5). However, the relatively longer lifespans of mice and zebrafish models (3-4 years and 5 years, respectively) make the experimental aging studies in these models more complicated (5, 84). In addition to these model

organisms, accelerated aging disorders in humans or progeria syndromes also provide some insightful details of the aging process (5, 6). Having taken all these variable factors into consideration, we utilized *Saccharomyces cerevisiae* or the budding yeast as our experimental model in our study. In this unicellular model organism, we can distinguish and characterize mitotic aging (Replicative aging) from post-mitotic aging (Chronological aging) very easily. In this study, our entire focus will be on the replicative aging or mitotic aging in budding yeast. Since the replicative age curve of budding yeast follows the same shape as the lifespan distribution in multicellular eukaryotes and the majority of the cellular processes are conserved among all eukaryotes, the knowledge I gain from my studies in budding yeast has the potential to be directly relevant to understanding the aging of multicellular eukaryotes, including humans.

1.4.1. Chronological aging in budding yeast

Chronological lifespan (CLS) is the aspect of longevity in budding yeast that measures the amount of time a cell is viable in a quiescent or non-dividing state (87, 88). It is a model of aging for non-dividing cells in multicellular eukaryotes. During CLS, following the initial growth phase, the cells are normally maintained in a postdiauxic phase that is a high metabolism phase, followed by a low metabolism stationary phase until death (88). Experimentally, CLS is commonly achieved by initially growing the yeast cell population in rich medium until it reaches a saturation level and then maintaining the cells in the same media without further replenishing any components. In the rich medium, yeast cells grow very rapidly, but then they go through first the diauxic shift and stop

growing. Then there is another switch from the fermentative stage to respiratory metabolism, following which the cells resume growth at a very slow rate, followed by cell cycle arrest and entry into the low metabolism stationary phase (88).

While many different pathways have been implicated so far in regulation of chronological lifespan in yeast, a consensus mechanism for its regulation is yet to be developed, but nutrient sensing pathways, mitochondrial respiratory capacity and oxidative stress responses seem to play vital roles in regulating CLS (87).

1.4.2. Replicative aging in budding yeast

The second aspect of longevity that is extensively studied in budding yeast is Replicative lifespan (RLS), which is the measurement of the reproductive potential of a yeast mother cell achieved by measuring the number of times a mother cell produces a daughter cell before entering senescence. This is a model of aging for mitotically dividing cells in multicellular eukaryotes (**Fig. 4**) (87, 89-91). Budding yeast divides a finite number of times, and in general, the median lifespan for a yeast mother cell is around 25 generations (89). RLS assays utilize the asymmetric division of budding yeast and the size difference between the mother and daughter cells to its advantage. This means after each cell division the daughter cell produced is rejuvenated, i.e. its age clock is set back to zero, and the aging factors are asymmetrically segregated to the mother cell. Conceptually the assay is simple and relies on the fact that when a mother cell produces a daughter cell by budding, the size of the daughter cell is smaller

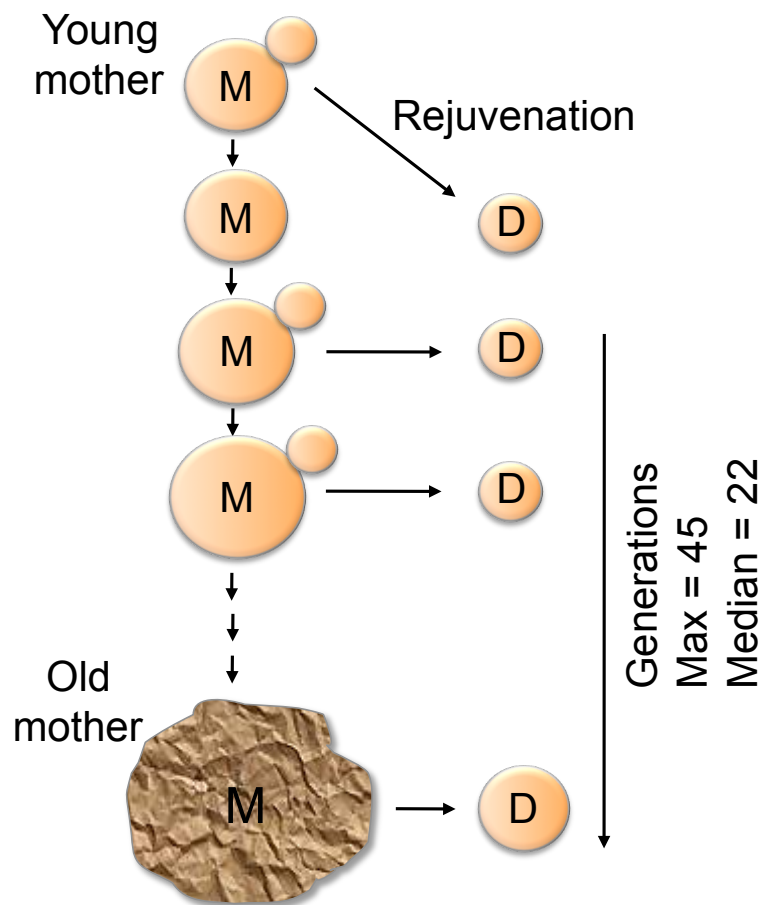


Figure 4. Replicative aging model in budding yeast. Replicative aging of budding yeast as the model of mitotic aging of dividing cells in multicellular eukaryotes. It represents the reproductive potential of an individual yeast mother cell, measuring the number of times a mother cell divides before entering senescence. M indicates mother cell, while D indicates daughters.

and distinguishable from the mother cell. Towards the end of its life cycle, the mother cell increases in its size (89, 92-94) and develop a loose and wrinkled appearance (89, 95, 96), and starts to produce daughter cells that are larger in size, with apparent breakdown in the asymmetric division (94). With age, old mother cells also start to produce daughters with heterogenous morphologies that include both spherical and ellipsoidal shapes (92).

Budding of the daughter cell from the mother cell leaves behind a new bud scar, which is mainly composed of chitin, on the surface of the mother cell (97). Replicative age of a mother cell can be determined via staining of the yeast cell wall by calcofluor and counting the number of bud scars on the mother cell surface that represents how many daughter cells the mother cell has produced. However, the more precise method of measuring replicative lifespan, namely micromanipulation, involves manual separation of daughter cells from a mother cell using a standard tetrad dissection microscope (87). Although enabling accurate determination of the RLS, the process is labor-intensive as well as low throughput. More recently, high-throughput methods have been developed to study budding yeast during the RLS using microfluidic systems coupled with high-resolution microscopy at the single cell level (92, 98), that can efficiently measure cell morphology and other aging-associated characteristics. Attempts to isolate old mother cells for molecular analyses previously have used either the elutriation method (42) or affinity purification of mother cells following cell wall labeling with biotin (24, 99). However, isolation of a large amount of old mother cells is difficult because the daughter cells quickly outpopulate the old cells. The

end result is that these methods are only able to isolate aged cells that have undergone less than 15 divisions. Recently, the development of another powerful genetic system called “mother enrichment program (MEP)” improved the ability to obtain a larger amount of older aged mother cells (100).

The “Mother Enrichment Program (MEP)”

The more recently developed genetic method of isolating old mother cells, called mother enrichment program (MEP), utilizes an inducible selection against daughter yeast cells coupled with a biotin purification system of the mothers (100). Selection against daughter cells is achieved by disruption of two essential genes, *CDC20* and *UBC9*, only in daughter cells using Cre-*lox* recombination. The expression of Cre is regulated by the transcription factor Ace2, which is specifically expressed in daughter cells owing to the asymmetric division of budding yeast. Cre activity is also inducible in nature as it is a fusion protein between Cre and estradiol-binding domain of the estrogen receptor (Cre-EBD78). Induction of Cre activity is achieved by the addition of estradiol which mediates translocation of the fusion protein Cre-EBD78 to the nucleus, where Cre functions to disrupt the two essential genes (*CDC20*, *UBC9*) via its recombinase activity, resulting in permanent mitotic arrest of daughter cells selectively (**Fig. 5**) (100). For the purification step, a biotin pulse is used to label the initial mother cell population so that the mother cell wall is coated with biotin. This biotin labeling is only restricted to the cell surface of mothers as the cell wall synthesis in budding yeast is generated *de novo* (24), meaning that the biotin is

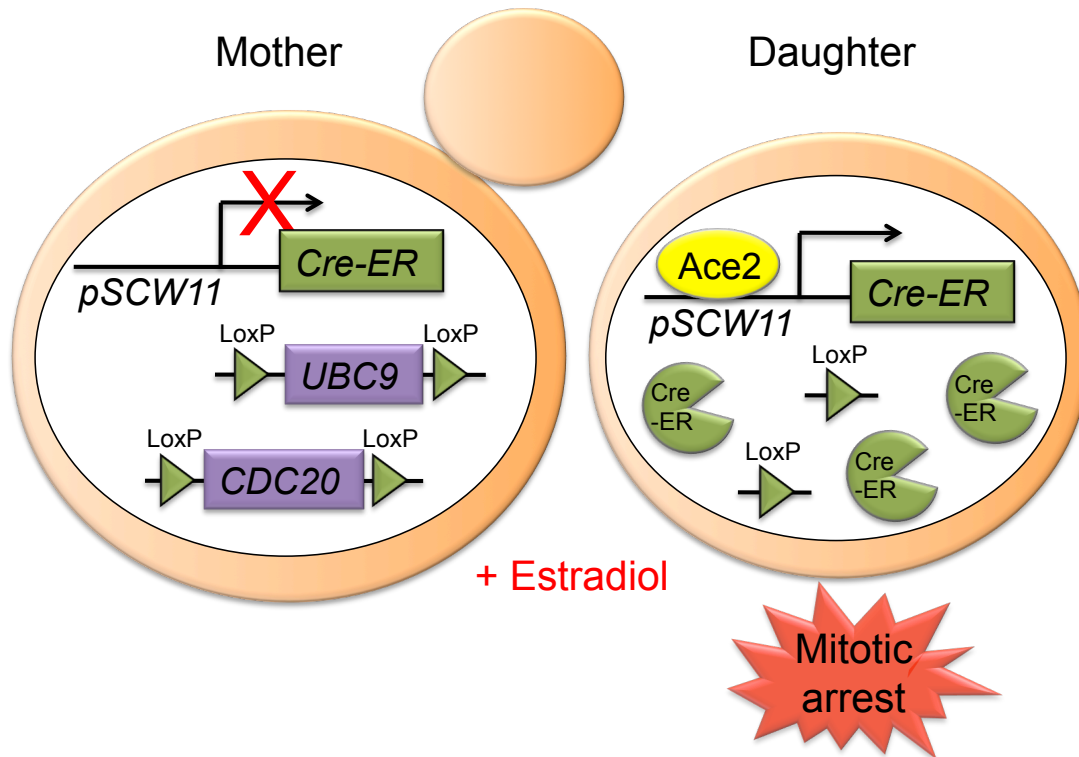


Figure 5. Model of the “mother enrichment program (MEP)”. Illustration of the genetic system called the MEP, to selectively induce mitotic arrest of daughter cells via estradiol induced Cre-loxP mediated deletion of two essential genes, and to allow yeast mother cells to continue replicating and age. This genetic method coupled with a biochemical purification approach provides a powerful system to isolate replicatively aged mother cells. (Adapted from: Lindstrom, D. L., and D. E. Gottschling. 2009. The mother enrichment program: a genetic system for facile replicative life span analysis in *Saccharomyces cerevisiae*. *Genetics* 183: 413-422, 411SI-413SI)

non-transferrable to newborn daughter cells. After aging the cells in liquid culture in the presence of estradiol to ensure the mitotic arrest of daughter cells, it is possible to magnetically sort the starting population of biotin-labeled old mother cells utilizing the high-affinity interaction of biotin and streptavidin (100).

Throughout my studies presented here, I extensively utilized this highly efficient genetic system to isolate sufficient numbers of aged mother cells at a much older age than previously possible to analyze the events determining yeast replicative lifespan as a model for aging of mitotically dividing cells of multicellular eukaryotes. These analyses enabled me to gain potential insight into the natural aging processes and to broaden our overall knowledge in the aging research.

1.5. Structure of the yeast rDNA locus and connection with aging

1.5.1. Structure of yeast rDNA

The ribosomal RNA gene cluster, also known as the rDNA locus is a unique locus in the eukaryotic genome. It has a large number of tandemly repeated sequences, comprising both genes and intergenic regions with non-coding elements (**Fig. 6**). In budding yeast, the rDNA locus resides within the nucleolus in a single cluster on chromosome XII and contains approximately 150 tandemly repeated copies in the haploid genome. The rDNA locus occupies approximately 60% of the chromosome and almost 10% of the budding yeast genome. The rDNA cluster is critical as it produces the structural RNA components of ribosomes, serving a crucial housekeeping function in the cell (33, 101, 102). The rRNA genes are the most abundant genes and are highly

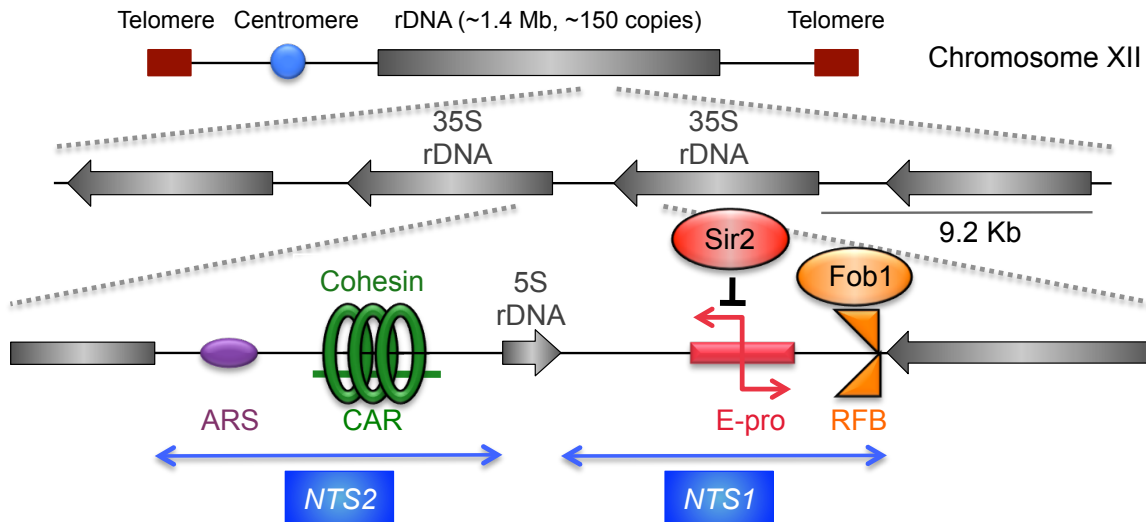


Figure 6. Structure of the yeast rDNA locus. The budding yeast rDNA locus is a part of chromosome XII right arm, comprising of ~150 tandem repeating arrays. Each repeat (~9.2 Kb) contains 35S rDNA and 5S rDNA genes, and intergenic spacer regions NTS1 and NTS2. The NTS1 region contains a bidirectional promoter E-pro, which is normally silenced via recruitment of Sir2, and contains a replication fork barrier (RFB) site, which is bound by Fob1. The NTS2 region contains a replication origin (ARS) and a cohesin associating region (CAR). (Adapted from: Ganley, A. R., and T. Kobayashi. 2014. Ribosomal DNA and cellular senescence: new evidence supporting the connection between rDNA and aging. FEMS yeast research 14: 49-59.)

conserved across species (33). In budding yeast, rRNAs (26S, 18S, and 5.8S) are transcribed from 35S rDNA repeats by RNA polymerase I and then mature into three individual rRNAs via splicing. 5S rRNAs are independently transcribed from 5S rDNA repeats located in between two 35S repeats (**Fig. 6**). All these rRNAs together form the ribosome structure along with ribosomal proteins (102). Repetitive in nature and actively transcribing, the rDNA is one of the most fragile sites in the genome and highly recombinogenic resulting in frequent fluctuations in rDNA copy numbers (103). Though earlier studies primarily emphasized its roles in ribosome biogenesis, later evidence underscores its extra-coding functions including crucial roles in determining lifespan (33, 102).

An individual rDNA repeat is approximately 9.2 Kb in size, consisting of two rRNA genes (35S rRNA and 5S rRNA) and two non-transcribing spacer regions (*NTS1* and *NTS2*). The *NTS2* region contains a replication origin (ARS) and cohesin associating region (CAR). The *NTS1* region contains a replication fork barrier (RFB) site, which is responsible for inhibiting rightward movement of the replication fork, through the action of a specific protein, Fob1. The RFB prevents the replication fork from colliding with RNA polymerase I, and results in a DNA double-strand break during replication that can be used to maintain the proper number of rDNA repeats via sister chromatid recombination. The *NTS1* region further contains a bidirectional promoter, E-pro, which is normally silenced via recruitment of a silencing complex that includes Sir2 (**Fig. 6**). The rDNA cluster is unique in this way that it contains all these diverse genomic elements (33, 101-103).

1.5.2. The ERC (extrachromosomal rDNA circle) theory of aging

The rDNA locus has long been implicated in genome maintenance, having critical effects on lifespan in yeast and *Drosophila* (25, 33, 102, 104). The rDNA is located within the nucleolus of the cell and aging yeast cells typically display the phenotype of nucleolar enlargement and fragmentation, which serves as an indicator of rDNA changes with aging (105, 106). An initial aging theory was proposed that emphasized the roles of extrachromosomal rDNA circles (ERCs) as causative agents for yeast aging (32). According to this theory, ERCs are excised from the rDNA locus mostly due to unequal sister chromatid recombination between the rDNA repeats. When excised, they form circular loops with the ability to replicate because of the presence of autonomous replicating sequence (*ARS*) elements in them. These ERCs are asymmetrically segregated to yeast mother cells, causing aging mother cells to accumulate ERCs exponentially (32, 107). This inherent instability of the rDNA locus because of its RFB and its tandem repeats, together with the asymmetric segregation of ERCs serving as aging factors in the yeast mother cells may provide an explanation for why there is an enlarged and fragmented nucleolus during aging. Although it is unknown exactly how ERCs form during aging or how ERCs cause aging, it has been proposed that exponential accumulation of ERCs in aged yeast mother cells may possibly titrate away factors important to maintain diverse nuclear functions including replication, transcription or genome maintenance (32). Validation of this model of aging comes from lifespan analysis of certain mutants, such as *sir2* and *fob1* mutants, where increased accumulation of ERCs

lead to shortening of lifespan (in the *sir2* mutant) and decreased accumulation of ERCs lead to lifespan extension (in the *fob1* mutant) (108-110).

1.5.3. rDNA theory of aging

While ERC accumulation had been proposed earlier as a cause of aging in yeast (32), in several other instances it has been observed that there is an inverse correlation between accumulation of ERC levels with lifespan and lifespan cannot always be explained by the ERC theory of aging (104, 111, 112). Thus, later studies suggested ERCs were merely a byproduct of rDNA instability and that the rDNA instability itself was the regulator of aging (102, 104, 109, 112). Notably, the rDNA locus is one of the most unstable regions in the budding yeast genome (112). In the rDNA theory of aging, instead of the accumulation of ERCs, rDNA instability itself has been proposed to be the major driving force behind yeast aging (112). At the molecular level, transcription from the intergenic regions of the rDNA locus that occurs in *sir2* mutants has been proposed to specifically displace cohesin from the rDNA leading to unequal sister chromatid recombination, rDNA instability and aging (112, 113). The rDNA theory of aging proposes that being already unstable, the rDNA locus shows more sensitivity to the age-associated accumulation of DNA damage compared to other parts of the genome, and that the unstable rDNA locus serves as a DNA damage sensor that eventually induces senescence in aging cells (112). Evidence supporting this theory comes from a study partially decoupling ERC levels with rDNA instability by using strains with different strengths of rDNA replication origins (rARS) (104).

Here two strains with differing rARS activities, one with increased and the other with decreased ERC levels, lived shorter than a wild-type (WT) strain, but both displayed increased rDNA instability in terms of marker loss assays and a less intact chromosome XII (104). Although several other studies suggested the rDNA theory of aging would better explain the connection of rDNA to aging than the ERC theory of aging (112), the causal relationship of this theory has not yet been tested in the context of physiological aging in budding yeast.

1.6. DNA double-strand break (DSB) repair in yeast and connections with aging

1.6.1. DNA double-strand break (DSB) repair in yeast

The integrity of the genome is under constant threat due to exposure to different exogenous and endogenous damaging agents. Exposure to damage generates a variety of DNA lesions including, but not limited to, DNA single-strand break (SSB) and DNA double-strand break (DSB) formation. Among all different DNA lesions, the most deleterious kinds are DSBs because they can lead to loss of chromosome arms. In order to faithfully maintain chromosomal integrity, the DSB need to be accurately repaired. Thus, cells possess several complex and intricate but related signal transduction and repair pathways that efficiently repair the DNA (114-116). The elaborate system of repairing the DSBs within the cells is called the DNA damage response that includes sensing the damage, transducing the signals and efficiently repairing the DNA (115). Along with the numerous proteins involved in these signal transduction pathways, there

is involvement of different post-translational modifications as well. The major pathways to repair DSBs are homologous recombination (HR) and non-homologous end joining (NHEJ). HR is considered as an error-free repair pathway while NHEJ is widely regarded as a more error-prone repair pathway (117-119).

HR is the primary DNA repair pathway utilized by budding yeast in response to DSBs (117). The crucial step for HR is the invasion of single-stranded DNA (ssDNA) generated following resection into a homologous DNA duplex (119). In budding yeast, following DSB formation, repair by the HR pathway involves DNA end processing/resection by Mre11-Rad50-Xrs2, known as MRX (MRN in mammals) and the Sae2 protein, followed by Replication Protein A/RPA loading onto ssDNA. The RPA is removed and replaced by the ssDNA providing a substrate for Rad51 nucleoprotein filament assembly. There are a few critical mediators, such as Rad52, in budding yeast that promote Rad51 binding. Rad51 assembly onto the ssDNA, assisted by Rad52 and other proteins, is needed for strand invasion into the duplex DNA of the sister chromatid. The 3' end of the invading strand provides a primer for repair synthesis while the intact sister chromatid serves as the template. The strand invasion intermediate (D-loop) can then be resolved in a number of ways, leading to either crossover (CO) or non-crossover (NCO). But in either scenario, precise information is copied from the sister chromatid template to drive error-free repair and to restore the genomic information (**Fig. 7**) (116, 117, 119, 120).

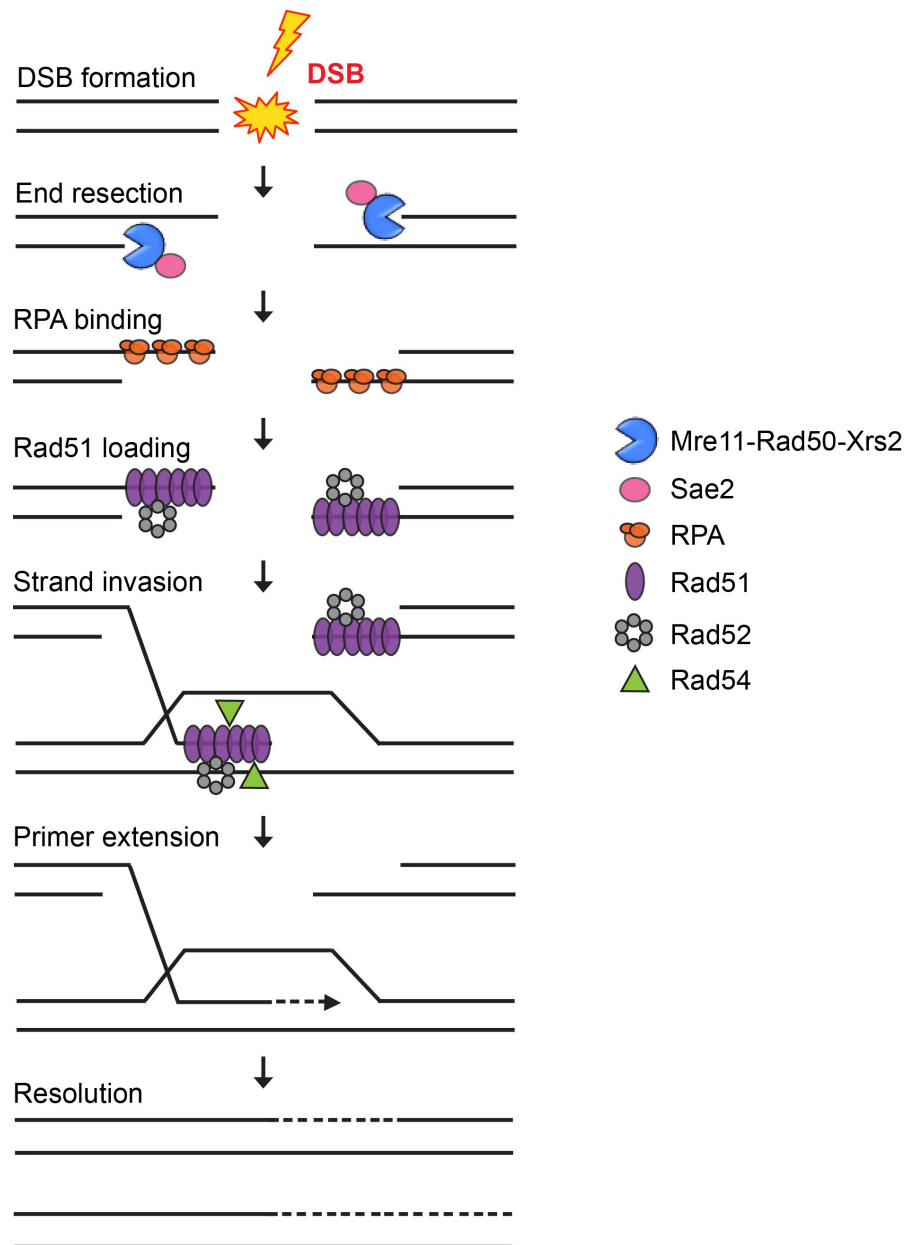


Figure 7. Key steps of homologous recombination (HR) in budding yeast.

Upon induction of a DSB, the Mre11-Rad50-Xrs2 (MRX) complex is recruited to DSB sites to drive end resection of DSB ends generating single-stranded DNA (ssDNA). RPA hetero-trimers then bind to the ssDNA ends and is subsequently

replaced with RAD51 nucleoprotein filament formation. The RAD51/ssDNA complex invades the complementary sister chromatid strand in search of homology, and then, DNA synthesis occurs. HR repair is completed following the resolution of resulting Holliday junction. (Adapted from: Jasin, M., and R. Rothstein. 2013. Repair of strand breaks by homologous recombination. Cold Spring Harbor perspectives in biology 5: a012740., and Mehta, A., and J. E. Haber. 2014. Sources of DNA double-strand breaks and models of recombinational DNA repair. Cold Spring Harbor perspectives in biology 6: a016428.)

1.6.2. Connection of impairment of DNA repair with aging

As mentioned earlier, one important hallmark of aging is increased genomic instability due to constant insults from numerous DNA damaging agents, and it has long been implicated as a causal factor behind aging (9, 50). Of note, premature aging syndromes in humans are often characterized by defects in genes involved in DSB repair, such as *WRN*, which is involved in both HR and NHEJ repair pathways, is mutated in Werner syndrome (121-123). Maintenance of genomic integrity thus remains a major mechanism to retain viability and promote longevity (79). Our earlier studies showed that replicative aging in budding yeast is associated with significant increases in DNA breaks, DNA amplifications and chromosomal translocations (40). As HR is the major pathway of DNA repair in budding yeast, defects in the HR pathway could potentially account for the defective DNA repair that occurs in old yeast cells leading to these chromosomal rearrangements and more DNA damage. It is intriguing to observe that DNA lesions accumulate to such a great extent during aging, given that the DNA repair system is highly proficient in nature and abundant in cells (50). This observation leads to the prediction that the DNA repair activity would decline with aging (123, 124). Declining DNA repair efficiency during aging has so far been reported only in *C. elegans* at the organismal level, although the exact mechanism is yet to be characterized (125). There are additional instances of reduced DSB repair activity in aging rat neurons and cultured primary human cells (68, 126-128), as well as in peripheral lymphocytes from aged humans (129-131), indicating the generalized nature of

an impaired DNA repair system during aging among all eukaryotes. Using the replicatively aged yeast cells, it is possible to determine whether this decline in repair efficiency occurs during aging and whether this contributes to genomic instability and aging.

CHAPTER 2: MATERIALS AND METHODS

2.1. Yeast strains

All the yeast strains used in this study are haploid strains and are listed in Table 1. Yeast deletion mutants were made by homologous recombination-mediated gene replacement, that is the replacement of the open reading frame of a specific gene with a selection marker gene. Tagging yeast strains with an HA epitope tag at the C-terminus of a specific gene was achieved by PCR-mediated DNA fragment integration followed by selection for the appropriate marker. The pFA6a-3XHA-KANMX6 Longline vector was used as the PCR template to generate the DNA fragment for making C-terminal 3XHA tagged strains (132). Over-expression of a single copy gene was achieved by integrating yeast integrative vectors with a *HIS3* marker containing the specific genes with the flanking sequence. Standard YEP media and SC media were used to grow yeast cells. YSI129 and YSI130 strains were kindly gifted to us by T. Kobayashi (133).

Strain	Genotype	Source
ZHY2	<i>MATalpha ade2::hisG his3 leu2 met15Δ::ADE2 trp1Δ63 ura3Δ0:: URA3 hoΔ::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-HPMX</i>	Hu et al, Genes & Dev, 2014
<i>rad52Δ</i>	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rad52Δ::KANMX</i>	Giaever et al, Nature, 2002
SPY006	<i>MATalpha ade2::hisG his3 leu2 met15D::ADE2 trp1D63 ura3D0:: URA3 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-HPMX MCD1-6HA:his5+</i>	This study
SPY021	<i>MATalpha ade2::hisG his3 leu2 met15D::ADE2 trp1D63 ura3D0:: URA3 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-HPMX fob1Δ::KANMX4</i>	This study
YSI129	<i>MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 fob1Δ::LEU2 his3-11::GFP-LacI-HIS3 RDN1::LacO(50)-ADE2 (110 rDNA copies)</i>	Ide et al, Science, 2010

YSI130	<i>MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 fob1Δ::LEU2 his3-11::GFP-LacI-HIS3 LacO(50)-ADE2:445kb ChXII (110 rDNA copies)</i>	Ide et al, Science, 2010
SPY056	<i>MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 MCD1-6HA:his5+</i>	This study
SPY065	<i>MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 E-proΔ::GAL1/10-URA3 MCD1-6HA:his5+</i>	This study
SPY048	<i>MATalpha ade2::hisG his3 leu2 met15D::ADE2 trp1D63 ura3D0:: URA3 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-HPMX RAD54-3HA::KANMX6</i>	This study
SPY054	<i>MATalpha ade2::hisG his3 leu2 met15D::ADE2 trp1D63 ura3D0:: URA3 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-HPMX SAE2-3HA::KANMX6</i>	This study
SPY031	<i>MATalpha ade2::hisG his3 leu2 met15D::ADE2 trp1D63 ura3D0:: URA3 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-HPMX RAD51-HIS3</i>	This study
SPY074	<i>MATalpha ade2::hisG his3 leu2 met15D::ADE2 trp1D63 ura3D0:: URA3 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-HPMX MRE11-HIS3</i>	This study
SPY025	<i>MATalpha ade2::HisG his3 leu2 met15Δ::ADE2 trp1Δ63 hoΔ::SCW11pr-CRE-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX ade3::GAL HO</i>	This study

Table 1: A list of yeast strains used in this study

2.2. Yeast media and plates

YEP (yeast extract peptone) media: To make 1L YEP, 10 g of yeast extract and 20 g of peptone were dissolved in 900 ml distilled water. The yeast media was sterilized by autoclaving at 120°C for 20-30 min. For YEP, either glucose (20% w/v stock, sterilized by autoclaving), galactose (20% w/v stock, sterilized by filtration) or raffinose (20% w/v stock, sterilized by filtration) was added to make YEPD or YEPR respectively to make a final concentration of 2% of the

appropriate sugar. To make solid media plates, 1.5% agar A was added to the liquid media prior to autoclaving.

2.3. Yeast genomic DNA isolation

Yeast genomic DNA was isolated as previously described (134). Cell pellets from overnight cultures were resuspended in 200 μ l genome preparation buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0), 250 μ l glass beads, and 250 μ l phenol:chloroform:isoamyl alcohol (25:24:1) and then vortexed in the cold room for 5 minutes. The sample was centrifuged for 5 minutes at maximum speed at room temperature and the top aqueous layer was transferred to a fresh microfuge tube. 1 mL 100% ethanol was added to the sample and mixed well before centrifugation for several minutes until a small pellet of DNA was obtained on the side of the tube. Finally, the pellet was washed twice with 70% ethanol, the excess ethanol was removed, and the pelleted DNA was resuspended in 200 μ l millipore water for further application.

2.4. Yeast transformation

Yeast transformation was performed using the lithium acetate method and single-stranded salmon sperm DNA as the carrier DNA (135). In brief, cultures were inoculated into 5 ml of YEPD for growing overnight at 30⁰C. The following morning, overnight cultures were diluted to an optical density (OD₆₀₀) of 0.3-0.4 in 5 ml fresh YEPD media and allowed to double in density at 30⁰C to reach OD₆₀₀ of 0.8. Cells were then spun to get a pellet and the pellet was washed in 1X TE /

1X LiAc (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM LiAc) and vortexed vigorously for 30 seconds before finally resuspending in 50 μ l of 1X TE / 1X LiAc solution. In a separate tube, 10 μ l of denatured salmon sperm DNA and 3-5 μ l of transforming DNA (either a plasmid or PCR amplified product) were mixed and then added to the resuspended cell pellet. 300 μ l of polyethylene glycol (PEG) solution (40% PEG, 100 mM LiAc, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) was then added to the cell suspension followed by addition of 50 μ l DMSO. The resuspension was mixed thoroughly by vortexing followed by incubation at 30°C for 10 min and heat shock at 42°C for 20 min. Following the incubations, cells were spun and washed in YEPD media before resuspending in 100 μ l distilled water and plating in appropriate selection agar media plates to grow yeast colonies.

2.5. Purification of old cells by the mother enrichment program method

Isolation of aged cells was performed using the “mother enrichment program” (MEP), as described previously (40, 100). Briefly, 2×10^7 cells from YEPD (1% yeast extract, 2% peptone, 2% dextrose) mid-log phase culture were collected and washed twice in 1X PBS, followed by resuspension in PBS containing 3 mg/ml Ez-link Sulfo-NHS-LC-LC biotin (Thermo-scientific, P121338). Cells were incubated at room temperature for 30 min and washed twice using YEPD. These cells were used to seed a culture at a density of 2×10^4 biotinylated cells per ml in YEPD. 17 β -Estradiol (Sigma, E8875) was added to a final concentration of 1 μ M to initiate the MEP and cells were cultured at 30°C for 30 hours to obtain cells of

desired age (confirmed by calcofluor staining). Typically, cultures were 2L in volume. At the collection time, cells were collected via centrifugation and washed twice in PBS, resuspended at a density of 5×10^8 cells/ml in 1 ml of PBS, and incubated for 30 min at room temperature with 50 μ l streptavidin-coated magnetic beads (Miltenyi Biotec, NC9821945). After streptavidin incubation, cells were washed in PBS, resuspended in 8 ml of PBS, and loaded onto an LS MACS column (Miltenyi Biotec, NC9777034) equilibrated with 5 ml of PBS. After gravity flow-through of unlabeled cells and debris, columns were washed thrice with 8 ml of PBS. The columns were then removed from the magnetic field and aged cells were eluted by gravity flow with 8 ml of PBS, and processed accordingly for the subsequent experimental purpose.

2.6. Purification of old cells by serial biotin purification

Yeast old mother cells were isolated using 2–3 rounds of purification; the initial population of cells was labeled with 3 mg/ml biotin (Thermo-scientific, P121338) in 10 ml cultures for 30 minutes, then the cells were washed with 1X PBS, 3 times to remove the biotin, cultured overnight, followed by affinity purification using 50 μ l streptavidin-coated magnetic beads (Miltenyi Biotec, NC9821945) in LS MACS column (Miltenyi Biotec, NC9777034) in the cold room following published method (136). Mean ages of isolated cells were estimated by counting Calcofluor-stained bud scars. Briefly, cells were grown in YEPD media to OD_{600} of 0.7. Then the cells were spun, washed twice with in 1X PBS, and resuspended in cold 1X PBS. 3 mg/ml Ez-link Sulfo-NHS–LC-LC biotin was

added to the cells following resuspension in PBS. The mixture was incubated for 30 min at room temperature followed by spinning and washing three times in 1X PBS. Cells were then diluted and grown in YEPD for 6-8 generations and spun down, washed with 1X PBS, and resuspended in 10 ml of cold 1X PBS. 50 μ l Streptavidin coated magnetic beads were added to the resuspension and incubated for 45 min at room temperature. The suspension was washed three times using 1X cold PBS and purified using LS MACS columns. Isolated cells were used again for another 1-2 rounds of growth and purification using similar procedure maintaining cell density within logarithmic growth phase ($OD_{600} < 1.0$).

2.7. RNA isolation, cDNA synthesis and quantitative PCR (qPCR)

Total RNA isolation was performed using the Master Pure Yeast RNA Isolation Kit (Epicentre Biotechnologies, MPY03100) following the manufacturer's protocols using the same number of cells for each sample. The DNase I digestion steps were included in our RNA isolation to obtain a purer sample for subsequent analyses. The quality of the RNA was confirmed by running RNA samples on formaldehyde gels. cDNA synthesis was performed using Transcriptor first strand cDNA synthesis kit (Roche, 04379012001) using strand specific primers, to be analyzed by subsequent qPCR analyses using LightCycler 480 SYBR green I master mix (Roche, 04887352001) using manufacturer's instructions in a LightCycler 480 II PCR machine (Roche). Plotted are the average and standard error of the mean of three independent experiments.

P values were determined using Student *t*-test using Prism 6 software (GraphPad Software, Inc.). The primers used are listed in Table 2.

Primers	Purpose	Sequence
NTS2-1_A	cDNA synthesis of NTS2 (+) strand	5'- GGT AGG TCG AAA CAG AAC ATG AAA GTT GG -3'
NTS2-1_B	cDNA synthesis of NTS2 (-) strand	5'- GCT ACT CTC ATG GTC TCA ATA CTG CC -3'
NTS1-2_A	cDNA synthesis of NTS1 (+) strand	5'- CAG AGC GGC AAA CAT GAG TGC TTG TAT AAG -3'
NTS1-2_B	cDNA synthesis of NTS1 (-) strand	5'- CGT AGT ACA TCT TAC AAC TCC GCA TAC C -3'
ACT1_RV	cDNA synthesis of ACT1 (Crick strand)	5'- CGG ACA ATT TCT CTT TCA GCA GTG GTG G -3'
qNTS2_1_FW	qPCR analysis of NTS2 region	5'- GAG GTA GTT TCA AGG TGA CAG G -3'
qNTS2_1_RV		5'- CTC TCA TGG TCT CAA TAC TGC C -3'
qNTS1_2_FW	qPCR analysis of NTS1 region	5'- GAG GCT ACT GGG AAG AAG AAA GAG -3'
qNTS1_2_RV		5'- CAT CTT ACA ACT CCG CAT ACC G -3'
qACT1_1_FW	qPCR analysis of ACT1 control	5'- GTG ATG GTG TTA CTC ACG TC -3'
qACT1_1_RV		5'- GTA GTC AGT CAA ATC TCT ACC GGC -3'
rDNA_probe2_FW	Primers to design probe for Southern blot against rDNA (Chr XII)	5'- CAG GTT ATG AAG ATA TGG TGC AA -3'
rDNA_probe2_RV		5'- AAA ATG GCC TAT CGG AAT ACA -3'
Chr2_1.FW	Primers to design probe for Southern blot against Chr II	5'- GTC TAT ATC GAC GGT AGC CCA CAC TTG C -3'
Chr2_1.RV		5'- CTT GTC GCC CTG TTT CAC CAC ATC G -3'
qCARL2_.FW	ChIP primers to check Mcd1 occupancy at different regions of Chr XII	5'- CCT GCA TTG TCC TCA TTT AGT GCT CAG G -3'
qCARL2_.RV		5'- TTT GGC AAC GAG ATA GTT GTG CCC -3'
qCARL3_.FW		5'- ATG CCA CCT ACC GAC CAA CTT TCA -3'
qCARL3_.RV		5'- AGA GGT GTT ATG GGT GGA GGA CAA -3'

qCARL3-N_.FW		5'- TCC ACT TTC AAC CGT CCC TCC AAA -3'
qCARL3-N_.RV		5'- AGA ATG TCG GCG GCA GTA TTG AGA -3'
qCARC1_positive. FW	ChIP primers to check Mcd1 occupancy at centromeric regions of Chr III (+ve control)	5'- CAG ACG ATA AGT TGA GTA GCG G -3'
qCARC1_positive. RV		5'- GTC CAC TAG GAG ACT CTT GAA C -3'
qCARC_negative. FW	ChIP primers to check Mcd1 occupancy at genomic regions of Chr III lacking Mcd1 binding (-ve control)	5'-CAG TGC TAT CGG ATC TAG GAA G -3'
qCARC_negative. RV		5'- GTA TTT GAC GTC TCC GCT TTC C -3'
DSB probe.FW	Primers to design probe for Southern blot against DSB probe	5'- GCC ATT TAC AAA AAC ATA ACG -3'
DSB probe.RV		5'- GGG CCT AGT TTA GAG AGA AGT-3'
Probe C.FW	Primers to design probe for Southern blot against Control probe	5'- ACA GAT GTG CCG CCC CAG CCA AAC TCC -3'
Probe C.RV		5'- CCT GGA TAT GGA TTC TTC ACG GTA ACG -3'
Chr1_left.FW	Primers to design probe for Southern blot against Chr I	5'- GAT TGC CTC TTT TGG GAG GTC TGG -3'
Chr1_left.RV		5'- CCA TCA GGC TCA GAT GAA TCA TGG GCC -3'
ChrX_right.FW	Primers to design probe for Southern blot against Chr X	5'- CAC CAC ACC CTG CTT ATT AAA GC -3'
ChrX_right.RV		5'- GAC AGA GGA CTT GTG TGA CG -3'
ChrIV_left.FW	Primers to design probe for Southern blot against Chr IV	5'- CAT TGG GAC AGG TAC TAG ATG G -3'
ChrIV_left.RV		5'- CTA TCT GTC TCT GCT CAG TGT GG -3'
HOMAT.FW	Primers used for multiplex PCR to analyze HO cutting and repair efficiency	5'- AGG TAA ATT ACA GCA AAT AG -3'
HOMAT.RV		5'- AAC AAC AAC CTA GAG TAA TG -3'
RAD27.FW		5'- ACA TCG CGC AAA TGA AGG TT -3'
RAD27.RV		5'- TCA AGT TCC CAG AAA AAC TG -3'

Table 2: A list of primers used in this study

2.8. Pulse field gel electrophoresis (PFGE)

Analyses of intact yeast chromosomes were performed using a CHEF DR II system (Bio-Rad), which is followed by Southern hybridization using probes against different chromosomes. Yeast cells grown in YEPD or YEPR were isolated at different time points using the MEP as indicated in the figure or figure legends. In general, 2×10^7 cells were used in each well to prepare agarose gel molds following the manufacturer's instructions (Bio-Rad, Cat#1703593). In brief, the cell pellet was resuspended in cell suspension buffer and equilibrated at 50°C . Plug mold was created using 2% CleanCut agarose in cell suspension buffer (10 mM Tris, pH 7.2, 20 mM NaCl, 50 mM EDTA), to make a final concentration of 1% agarose in agarose plug molds. Just before mixing the cells with agarose, 10 mg/mL Zymolyase (100T) was added, followed by solidification of the agarose plugs. The agarose plugs were further enzymatically treated with 10 mg/ml Zymolyase in buffer A (10 mM Tris, pH 7.2, 50 mM EDTA) and incubated at 35°C for 2 hours followed by Proteinase K digestion with 1mg/ml Proteinase K in Proteinase K reaction buffer (100 mM EDTA, pH 8.0, 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine) at 50°C overnight. After enzymatic digestions, the plugs were washed in wash buffer (20 mM Tris, pH 8.0, 50 mM EDTA) at room temperature and stored at 4°C for future use. The electrophoretic conditions used for my analysis: 3V/cm for 72 h at 14°C with a 300 to 900 sec pulse time and a 1% agarose gel with 0.5X Tris-borate-EDTA (TBE). The gel was stained with SYBR safe dye (Invitrogen, Cat#S33102) and visualized using a ProteinSimple Imager (FluorChem E system).

2.9. Southern blot analysis

Intact chromosomes were resolved in by PFGE on a CHEF agarose gel using 0.5% TBE or digested DNA was resolved in 1% TBE agarose gels. In both cases, the gels were partially depurinated using 0.25N HCl for 20 minutes at room temperature and were denatured using denaturing solution (0.5M NaOH/0.6M NaCl) for 20 minutes at room temperature. The DNA was then transferred to a nitrocellulose membrane via the capillary method using 2X SSCPE buffer (to make 1L of 20X concentrate of SSCPE buffer, we combined 140g NaCl, 88g Na-citrate, 35g KH₂PO₄, 7.4g EDTA, and pH adjusted to 7.2 using NaOH) and UV crosslinked to the membrane. The membrane was then pre-hybridized using Ultra-hyb buffer (Applied Biosystems, Cat# AM8669) at 42⁰C for 1 hour, followed by hybridization using specific probes against particular chromosomes or specific DNA sequences at 42⁰C overnight. The probes were labeled by random prime labeling with ³²P (Rediprime II Random Prime Labelling system, GE, RPN1633) following manufacturer's protocol. The primer sequences used for amplifying the probes are listed in the tables for Primers (Table 2). Specific signal was detected after washing off unbound probe, using 10-15 ml wash buffer (2X SSPCE, 0.2% SDS), 2 times for 30 minutes each at 65-70⁰C, using autoradiography.

2.10. Analysis of branched recombination intermediates (stuck DNA analysis)

Branched recombination intermediates do not enter the gels during PFGE and remain in the plug well (stuck DNA). The analysis of branched recombination intermediate formation has been done for two chromosomes, chr II and chr XII (rDNA), in both young and old samples. Relative proportions of stuck DNA in the well during aging was measured by quantifying the signal from Southern blot using probes against the respective chromosomes. Plotted are the average and standard error of the mean of three independent experiments. *P* values were determined using Student *t*-test using Prism 6 software (GraphPad Software, Inc.).

2.11. DSB analysis

Measurement of DSB formation at the rDNA (using restriction digestion by BglII) was performed according to the following procedure with certain modifications (137). The procedure of embedding the cells in agarose plugs followed by all different enzymatic digestions was followed as described and the gel plugs were loaded into 1% agarose gels, ran in 1XTBE at 30V for 14 hours. The gel was stained with SYBR safe dye to visualize DNA and used for subsequent Southern blot using specific probes as mentioned in the figure.

2.12. Chromatin immunoprecipitation (ChIP) and qPCR

Samples for ChIP were collected at different time points of the MEP as indicated in the figure legends. In brief, cells were cross-linked with 1% formaldehyde for 20 minutes and quenched by addition of 1M glycine to the final concentration of

125 mM. Cells were lysed using lysis buffer (140 mM NaCl, 50 HEPES pH 7.5, 1 mM EDTA, 0.1% deoxycholic acid, 1% Triton X-100) in the presence of a protease inhibitor cocktail (protease inhibitor cocktail set IV, Calbiochem, cat# 539136) using glass beads at 4⁰C for 10 minutes in a bead beater and then kept on ice for 15 minutes. The extract was removed, transferred to a fresh tube, followed by shearing of the chromatin by sonication using a Branson 350 Sonifier (Output: 2, Timer: Hold, Duty cycle: Constant, 100% power). The samples were centrifuged at maximum speed at 4⁰C and for 5 minutes, 2 times to separate supernatant from the cell pellet. A small portion of supernatant (50 µl) was mixed with 30 µl ChIP elution buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% SDS) to be used as input controls and immunoprecipitation was performed with the rest of the supernatants using specific antibody. For immunoprecipitation for each sample, 10 µl Dynabeads Protein A (Invitrogen, 100-02D) pre-mixed with 2 µl antibody was added to the cell lysate to incubate at 4⁰C overnight. The following day beads were washed using magnetic stand in the following buffers: two times washing in cold Lysis buffer (0.1% deoxycholic acid, 1mM EDTA, 50mM HEPES pH 7.5, 140mM NaCl, 1% Triton X-100), two times washing in cold Lysis buffer-500 (0.1% deoxycholic acid, 1mM EDTA, 50mM HEPES pH 7.5, 500mM NaCl, 1% Triton X-100), two times washing in cold LiCl detergent (0.5% deoxycholic acid, 1mM EDTA, 250mM LiCl, 0.5% NP-40, 10mM Tris pH 7.9) and once with cold 1X TE buffer. Following the washes, for each IP sample 80 µl of ChIP elution buffer was added to the beads. After elution step, 20 µl 20 mg/ml Pronase was added to both 80 µl of Input and IP samples to incubate in a PCR cycler for

the following program (42°C for 2 hours; 65°C for 8 hours; 4°C, hold) to reverse crosslink and to digest proteins. The DNA was then purified using MinElute DNA purification kit (Qiagen, 28004). Quantitation of the DNA sequences in the input samples and immune-precipitates was performed via real-time qPCR analyses using LightCycler 480 SYBR green I master mix (Roche, 04887352001) using manufacturer's instructions in a LightCycler 480 II PCR machine (Roche). A polyclonal antibody against rabbit hemagglutinin (HA) (Abcam, ab91110) was used to measure the occupancy of Mcd1, a cohesion subunit, at different regions around the rDNA locus and control regions. Plotted are the average and standard error of the mean of three independent cultures for each experiment. *P* values were determined using Student *t*-test using Prism 6 software (GraphPad Software, Inc.). The primers used are listed in Table 2.

2.13. Western blot analysis

Total protein extracts were isolated by boiling samples (same cell number for each sample) in 2X sample buffer (0.06M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.0025% bromophenol blue) prior to loading gels. Samples were resolved using 4-20% Criterion TGX precast gels (Biorad, 567-8095) using 80 volt for 2 hours, and then transferred to nitrocellulose membrane using 450 mAmps for 45 minutes at cold room. The membrane was blocked using 5% non-fat milk diluted in 1X TBS with Tween 20 (TBST) for 30 minutes, followed by incubation in presence of primary antibody in TBST for 1 hour at room temperature, and washing the membrane with 1X TBST three times, 10

minutes each. After the washes incubation with secondary antibody in TBST was done, 1 hour at room temperature, followed by three washes with 1X TBST, 10 minutes each. Membranes were then developed using either Amersham ECL western blotting detection reagent (GE) or Immobilon Western HRP substrate (Millipore). Dilutions for primary and secondary antibodies used are listed in Table 3.

2.14. Cohesion assay (GFP two spot analysis)

To investigate sister-chromatid cohesion, strains where the LacO array is inserted into rDNA (RDN) or telomere (TEL) expressing LacI-GFP fusion protein were used (133). Aged cells were collected following two rounds of biotin affinity sorting. The exponential growth of the culture was maintained by limiting the cells to going through ~6 doublings before each round of sorting. Cell surface was labeled with Ez-link Sulfo-NHS–LC-LC biotin (Thermo-scientific) with a 30 minute long pulse of 3 mg/ml concentration biotin in YPD, were washed 3 times in 1X PBS, followed by 10-12 hrs of exponential growth in YPD media and affinity purified following incubation with streptavidin-coated magnetic beads (Miltenyi Biotec, NC9821945) using LS MACS column (Miltenyi Biotec) using the procedure as previously described (138). Cells at G₂/M phase (large budded cells) were scored for a single fluorescent GFP dot (maintenance of cohesion) versus two separated fluorescent GFP dots (loss of cohesion) as visualized by fluorescence microscopy. More than 100 cells were counted for each sample. Plotted are the average and standard error of the mean of three independent

experiments. *P* values were determined using Student *t*-test using Prism 6 software (GraphPad Software, Inc.).

2.15. Repair assay using MMS

For the repair assay, biotinylated cells were grown in YEPD media according to the MEP method and samples were collected before inducing any damage. For damage induction, 0.07% Methyl Methanesulfonate (MMS) (Sigma-Aldrich, 129925-5G) was used for 30 minutes followed by washing the cells in fresh YPD media thrice. For analyzing post-damage repair kinetics samples were collected 3 and 6 hours after washing out the MMS. All the samples collected at different time points were subject to magnetic biotin-streptavidin purification as described previously in the cold room. For old samples, damage induction with MMS was started at 22 hours into growing the cells following MEP procedure so that the 6 hours repair time course was finished within 30 hours of the start of the MEP. For each time point, 2×10^7 cells were collected and processed for making DNA plugs in agarose for subsequent PFGE analysis.

2.16. Immunofluorescence (IF) analysis

Samples for IF analysis were collected following the MEP procedure in 2L cultures. Cells were fixed with 4% formaldehyde followed by spheroplasting using 10 mg/mL Zymolyase (100T) (USBiological, Z1004) for 40 to 60 minutes. Spheroplasting was confirmed visually under the microscope. Spheroplasts were harvested and applied to a poly-lysine coated microscope slide. The cells were

permeabilized using methanol and acetone followed by blocking with 3% PBS-BSA and incubation with primary and appropriate fluorescent secondary antibodies. The antibodies used are listed in Table 3.

Antibody (animal source)	Source (Catalog #)	Dilution
HA (Rabbit)	Abcam (ab9110)	1:4000 (WB); 2 μ l (per ChIP)
H3-C (Rabbit)	Abcam (ab1791)	1:5,000 (WB)
Rfa1 (Rabbit)	Brush et al., NAR, 2000	1:2,500 (WB)
Rfa2 (Rabbit)	Brush et al., NAR, 2000	1:2,500 (WB)
Rad51 (Rabbit)	Abcam (ab63798)	1:5,000 (WB)
Rad52 (Rabbit)	Santa Cruz (sc-50445)	1:500 (WB)
Rad53 (Mouse)	Fiorani et al., Cell cycle, 2008	1:100 (WB)
Mre11 (Rabbit)	LSBio (LS-C155765)	1:1,000 (WB)
α -Tubulin (Rat)	AbD Serotec (MCA78G)	1:1,000 (WB)
phospho-H2A S129 (Rabbit)	Abcam (ab15083)	1:10,000 (IF)
Anti-mouse, HRP	Promega (W4021)	1:10,000 (WB)
Anti-rabbit, HRP	Promega (W4011)	1:10,000 (WB)
Anti-rat, HRP	Sigma (A5795)	1:4,000 (WB)
Anti-rabbit IgG (H+L) Alexa flour 594	Invitrogen (A-11037)	1:1,000 (IF)

Table 3: A list of antibodies used in this study

2.17. PCR analysis of HO cutting and repair efficiency

Following collection of samples at each time point of the repair time-course experiment, yeast cells were collected by centrifugation and genomic DNA was isolated as described earlier. A multiplex PCR assay was done following published protocol (139) using HOMAT and RAD27 (as a control) primers (140). PCR products were resolved by 1.5% agarose gel electrophoresis. The gel was stained with SYBR safe dye and visualized using a ProteinSimple Imager

(FluorChem E system). While the PCR product generated by *RAD27* primers is expected to be ~1.4 Kb, HOMAT primer pairs generated a *MATa* product with the size of ~1.1 Kb, and a *MATα* product with the size of ~1.2 Kb. Quantification of the relative amount of *MATa* or *MATα* products (normalized to control) during HO cutting and repair assay was performed using AlphaView software available with the ProteinSimple Imager.

**CHAPTER 3: ROLE OF RIBOSOMAL DNA (rDNA)
INSTABILITY IN REGULATION OF LIFESPAN IN
BUDDING YEAST**

3.1. Introduction and Rationale

As described in Chapter 1, the ribosomal DNA (rDNA) locus is a unique locus in the eukaryotic genome. It is located within the nucleolus and is crucial to maintain critical housekeeping functions for the cells, as well as other extra-coding functions that have been emphasized more recently (33, 102). It is also one of the highly unstable regions of the genome (141). rDNA instability has long been implicated in lifespan determination in yeast and *Drosophila* (25, 104), but most of the evidence was circumstantial. For example, characteristic aging phenotypes in budding yeast are nucleolar enlargement and fragmentation that implicated that there are changes to the rDNA locus during aging (105, 112). At the same time, even after it was unclear whether ERCs are the cause of aging or simply the by-products of rDNA instability (112), increased accumulation of ERCs during aging only underscores the inherent instability of this locus that worsens with age. Furthermore, increased LOH at the rDNA locus of the daughters of aged yeast cells also serve as an indirect indicator of increased rDNA instability with aging (105, 142). Interestingly, the age-induced hyper-recombinational state of the rDNA is not solely restricted to budding yeast but has also been found in adult solid tumors (143). Of note, aging is the highest risk factor for cancer. All these evidence correlate and potentially implicate changes in rDNA instability to aging. However, it is yet to be addressed directly if there is indeed increased rDNA instability in aged cells and whether rDNA instability affects lifespan as predicted by the “rDNA theory of aging”. As the use of the MEP now enables us to uncover specific events in a truly aged population of yeast cells, which are at

or beyond their median lifespan, it is possible to address what happens at the rDNA locus during the aging process.

Previously our lab revealed chromatin structure as a major modulator of aging in yeast and provided evidence of drastic histone protein loss from different regions of the genome including the rDNA locus during aging (38). A more open chromatin structure with aging is likely to provide inappropriate access to the genome, leading to loss of genomic integrity affecting all the genomic regions, but may be affecting the fragile genomic regions like the rDNA even more. Our earlier study, which showed massive nucleosome loss during replicative aging coupled with drastic transcriptional upregulation and increase in genomic instability, also provided evidence of the involvement of the rDNA in increased – intra and –interchromosomal translocation and amplifications of the chromosome segment distal to the rDNA during aging (40). Thus, the objective of the first part of my project is to test the ‘rDNA theory of aging’ to determine whether there is increased rDNA instability during yeast replicative aging and if so, to characterize the mechanism behind it. As rDNA is universally present in all eukaryotes and most of the fundamental biological pathways are well conserved among eukaryotes, it has the potential to serve as a more common lifespan determinant (102). Altogether, this study has the potential to be applicable to the replicative aging of stem cells, which are mitotically dividing cells, unlike most of the adult tissues in multicellular eukaryotes where cells are maintained in a non-dividing state.

3.2. Results

3.2.1. Non-coding RNA transcription is highly induced from *NTS1* and *NTS2* in the intergenic spacer region of the rDNA locus in old cells

The rDNA locus in budding yeast is located in a single cluster at the right arm of chromosome (chr) XII, consisting of approximately 150 tandem repeats of rDNA units, as described earlier (**Fig. 6**). Within each individual rDNA repeat there are non-transcribed spacer regions 1 and 2 (*NTS1* and *NTS2*). These regions are silenced via the recruitment of the Sir2 containing complex known as RENT (regulator of nucleolar silencing and telophase exit) (144). During the course of our previous RNA-seq analyses during yeast replicative aging (40), we noted that transcription from the *NTS1* and *NTS2* regions was the most induced out of all the yeast genes during aging. Given that transcription of the *NTS1* and *NTS2* non-coding RNAs has never been observed in wild type yeast previously, I validated the RNA-seq results using strand specific reverse transcription PCR. Using aged cells isolated using the MEP that are 25 or more generations old on average, I found 21-87 fold transcriptional induction from *NTS1* when normalized to *ACT1* transcription and 18-34 fold induction from *NTS2* depending on which strand was being examined in old cells in comparison to their young counterparts (**Fig. 8A, B**). Strand-specific transcript analysis revealed induction of non-coding RNA from both Watson (+) and Crick (-) strands at *NTS1* and *NTS2* consistent with their having bidirectional promoters (113, 145). Transcriptional upregulation has been observed from all genes in the yeast genome during replicative aging

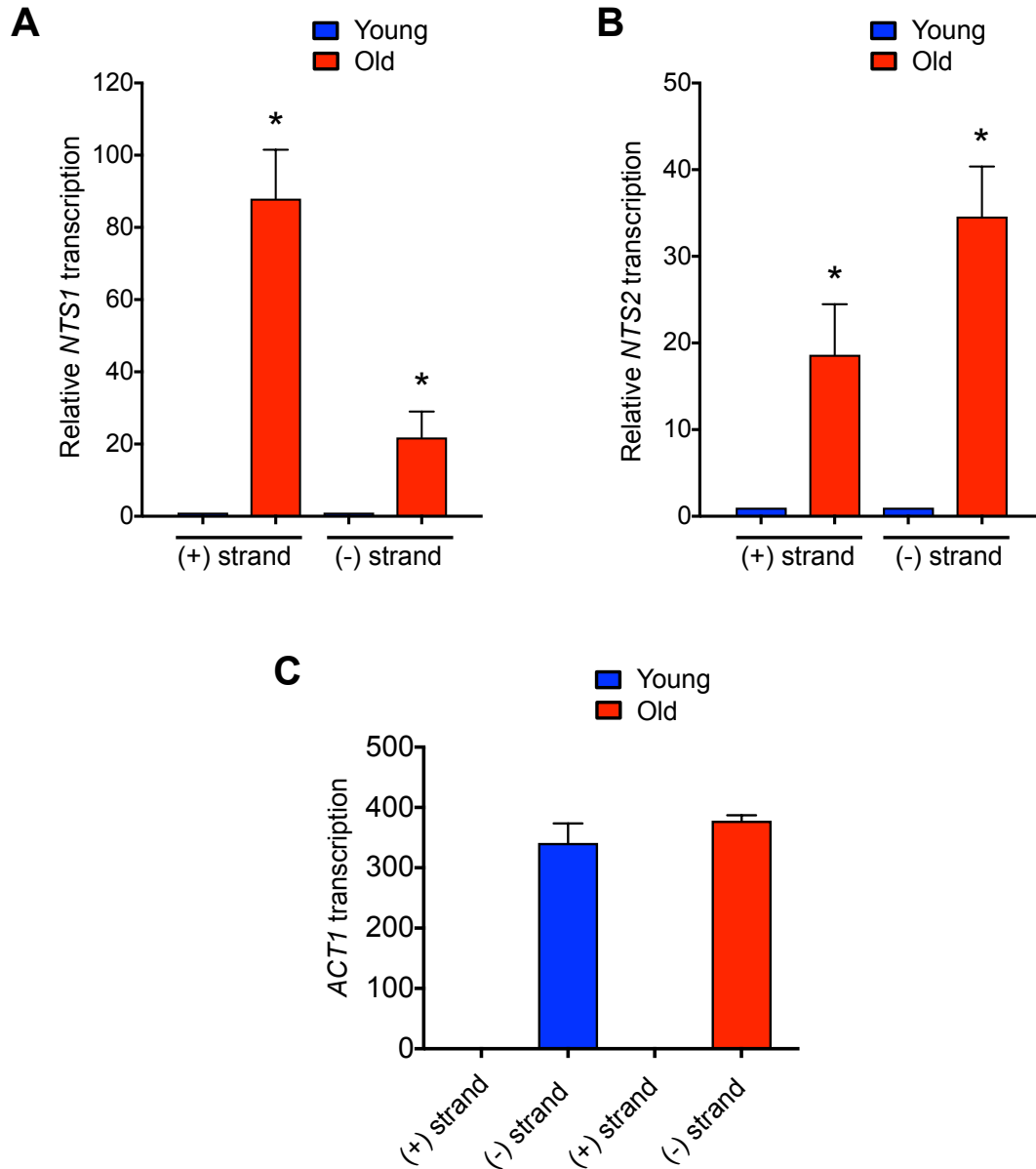


Figure 8: Transcriptional up-regulation from non-transcribed regions of rDNA during aging. (A, B) Strand-specific qRT-PCR analysis to examine the fold increase in non-coding RNA levels in old cells from both Watson (+) and Crick (-) strands of (A) *NTS1* and (B) *NTS2* regions of rDNA in comparison to young cells. RNA levels were normalized to *ACT1* transcript levels in both young and old cells before normalizing to 1 for young cells in each case. Average and

standard error of the mean of three replicates are plotted. Asterisk (*) indicates significant changes in old from young ($p < 0.05$), as determined by the Student's t-test. (C) Levels of *ACT1* strand-specific transcription from same number of young and old cells. Average of two independent replicates are plotted here.

(40). Therefore, before using the *ACT1* transcript as our normalization control, I determined the level of *ACT1* transcription from same number of cells in both young and old cells, and found that the *ACT1* transcript levels were slightly higher in aged cells (**Fig. 8C**). These results validate that transcriptional silencing is lost from both *NTS1* and *NTS2* during aging. As mentioned before, the rDNA locus contains diverse genomic elements and activities of all three RNA polymerases (RNA Pol I, II, and III) are observed at this locus (146). An earlier study found RNA polymerase I transcription is inversely proportional to ncRNA production at the rDNA locus, suggesting that RNA polymerase I activity may act to silence ncRNA production at this locus (146). However, when I compared the levels of 25S and 18S rRNA level as an indication of RNA polymerase I activity in young and old cells, I did not find any significant difference between them (**Fig. 9A, B**), indicating that the increased *NTS1* and *NTS2* transcription observed during aging is not a reflection of loss of RNA polymerase I-mediated regulation.

3.2.2. Cohesin occupancy is reduced from the rDNA region during aging

Transcription from the bidirectional E-pro promoter of *NTS1* in yeast deleted for *SIR2* has been reported to promote dissociation of cohesin from the rDNA locus to regulate recombination (113). Since I observed increased non-coding RNA produced from the rDNA region during aging as a consequence of histone loss during aging (40), I asked whether this transcription displaces cohesin from the rDNA during aging. I epitope tagged Mcd1, the limiting subunit of the cohesin complex (147) with HA and measured cohesin occupancy by

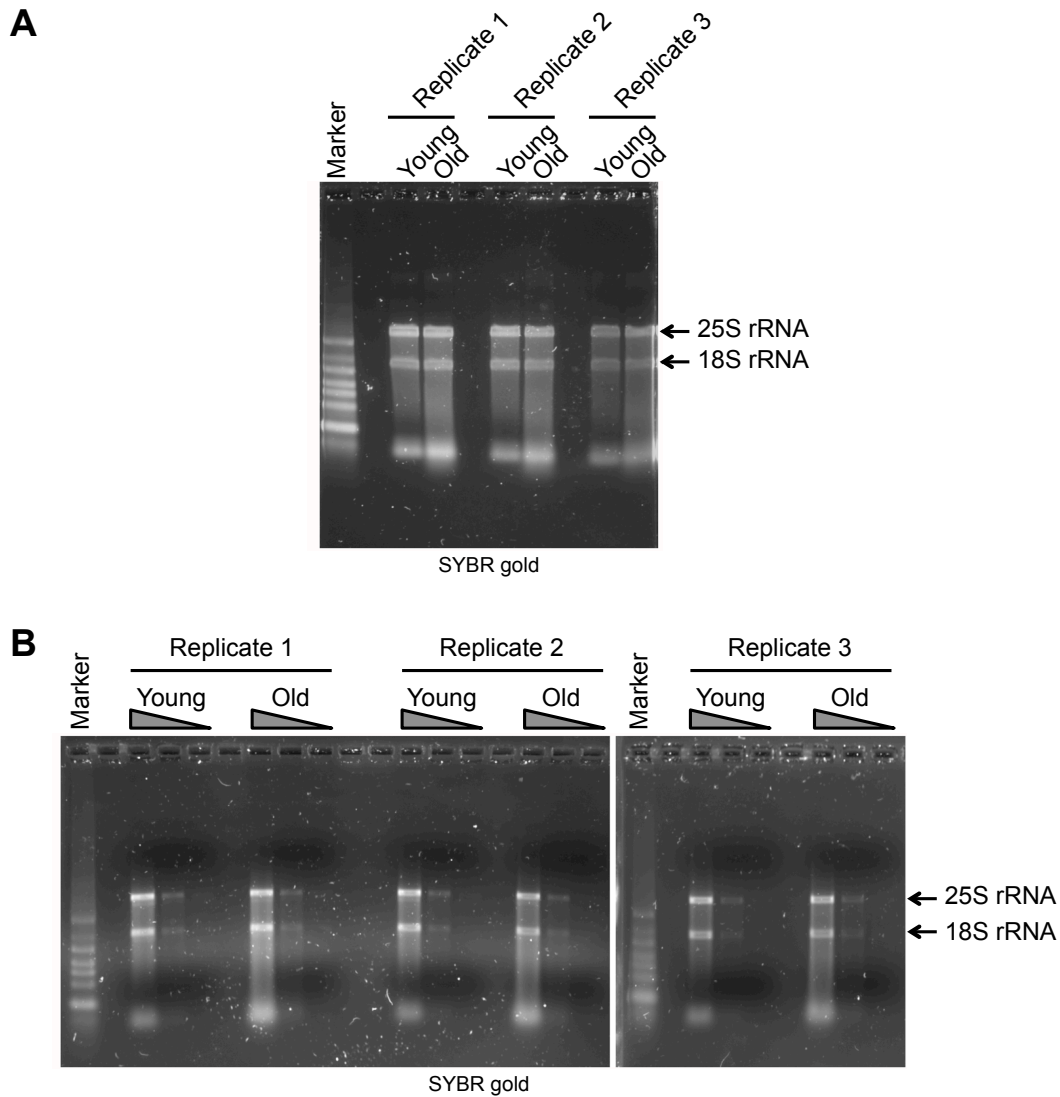


Figure 9. No significant difference in RNA Polymerase I (Pol I)

transcriptional activity is observed during aging. (A, B). Pol I transcriptional activity as observed from the levels of 25S and 18S rRNA levels. (A) Results from three independent replicates are shown. (B) Ten fold serial dilutions of each samples to illustrate the differences between young and old samples from three independent replicates. The same number of cells are analyzed for young and old cells.

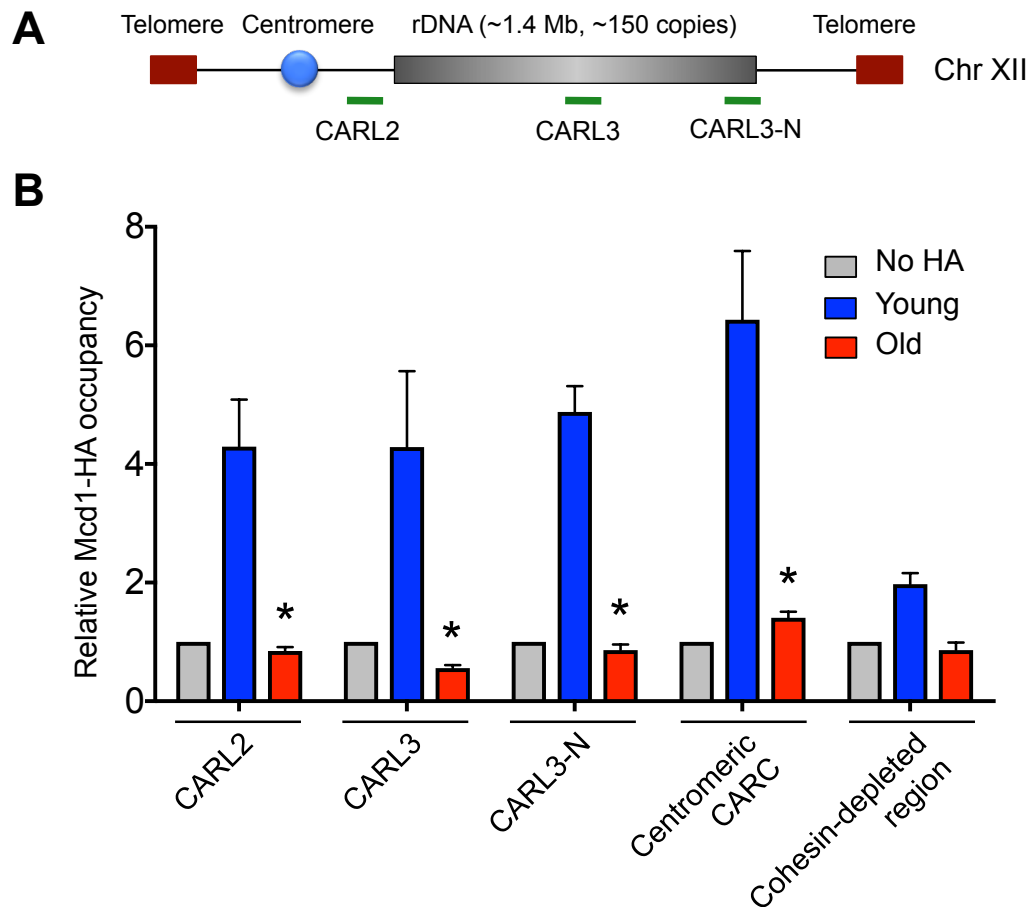


Figure 10. Mcd1 occupancy at different genomic regions is reduced during aging. (A) Location of the ChIP primers around rDNA locus in chromosome XII to check Mcd1 occupancy. (B) Young and old cells isolated via the MEP were subjected to chromatin immuno-precipitation (ChIP) Young cells from untagged strain were used as a negative control. Data shown here are the average of four independent replicates. Error bars represent standard error of the mean. Asterisk (*) indicates significant changes in old from young ($p < 0.05$ as determined by the Student's t-test).

chromatin immunoprecipitation (ChIP) during aging. I examined two known cohesion associated regions (CAR) within different rDNA repeats during aging (CARL3, CARL3-N) and one region outside rDNA locus but still located at the right arm of chromosome XII (CARL2) (**Fig. 10A**). As a positive control, I used the centromeric region of chromosome III and a cohesin-depleted region of chromosome III (Chr III: 309955 to 310355) as a negative control (148). Mcd1 occupancy was significantly lower in aged cells within the rDNA in comparison to young cells (**Fig. 10B**). However, there was also a similar degree of cohesin occupancy reduction at a different cohesin binding site of chromosome XII outside of rDNA locus and also at the centromeric region of chromosome III in old cells (**Fig. 10B**), indicating that reduced cohesin occupancy during aging extends beyond the rDNA locus.

3.2.3. Cohesion is lost not only from the rDNA locus, but also globally during aging

In order to determine whether the reduction of cohesin occupancy from different genomic locations that I observed during aging (**Fig. 10**) is functionally relevant, I measured cohesion during aging using a functional assay. I visualized cohesion at the rDNA and telomere by observing Lac repressor-GFP localization in G₂/M phase cells in strains where the lacO array was inserted into either the rDNA or telomere (33). When the sister chromatids are cohesed, there is a single GFP signal per cell, while cohesion loss gives rise to two GFP spots in each cell (33) (**Fig. 11A**). I found that the percentage of separated sister chromatids was

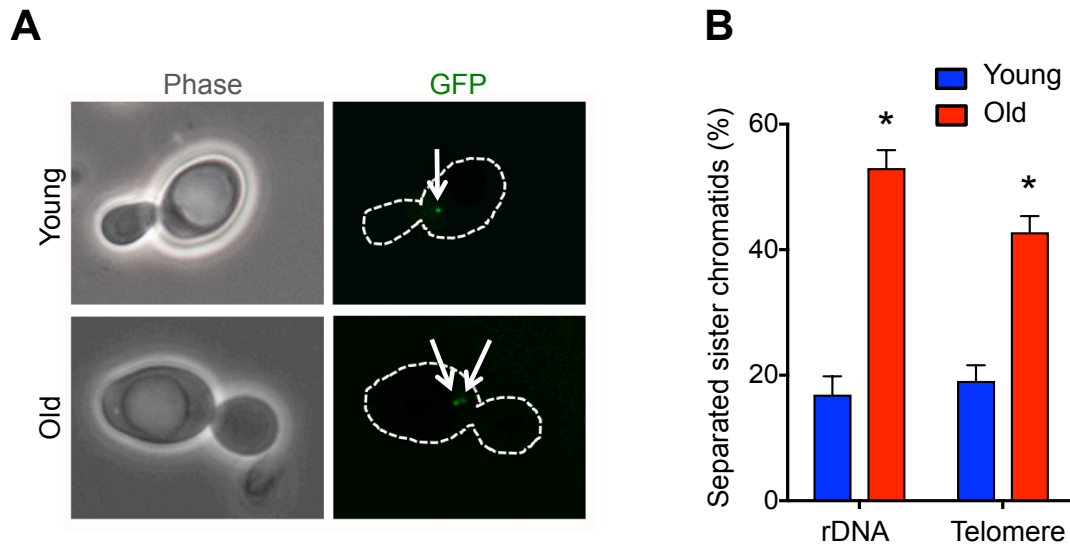


Figure 11. Loss of cohesion is observed during aging. (A) Representative images of the cohesion assay are shown on the left and quantitation is shown on the right. (B) Quantification of loss of cohesion at rDNA and telomeric regions with aging as indicated by separated sister-chromatid percentage, from visualizing GFP lac repressor in G2/M cells. Average and standard error of mean of three replicates are plotted. Arrows indicate one spot (young cell) or two spots (old cell) of GFP lac repressor. Asterisk (*) indicates significant changes in old from young ($p < 0.05$), as determined by the Student's t-test.

significantly higher at both the rDNA and telomere in older cells (**Fig. 11B**). As such, there is a significant loss of cohesion with aging at the rDNA and other regions of the genome.

3.2.4. Cohesin displacement from the rDNA does not correlate with non-coding RNA transcription

I further examined the relationship between non-coding RNA expression from the rDNA and cohesion, as it relates to longevity in the rDNA theory of aging (112). Previously, when transcription was induced from the galactose-inducible bidirectional *Gal1/10* promoter (Gal-pro) that was inserted in place of the native E-pro promoter in the *NTS1* locus (**Fig. 12A**), it increased rDNA instability and reduced lifespan (149). On the contrary, transcription inhibition from Gal-pro by addition of glucose reduced rDNA instability and increased lifespan (149). Using the same strains but with HA-tagged Mcd1, I asked whether induction of non-coding transcription from *NTS1* is sufficient to displace cohesin. I found that neither repression nor induction of *NTS1* transcription by Gal-pro had a significant effect on cohesin occupancy at CARL2 (**Fig. 12**). Meanwhile, both repression and induction of *NTS1* transcription by Gal-pro caused a significant reduction of cohesin occupancy at CARL3 and CARL3-N, two intergenic non-coding regions of the rDNA within other copies of the rDNA repeat (**Fig. 12**). Noteworthy, repression of *NTS1* transcription led to the greatest loss of cohesin occupancy at these sites (**Fig. 12**). As such, while *NTS1* transcription may limit replicative lifespan (149), this is independent of its effect on cohesin occupancy

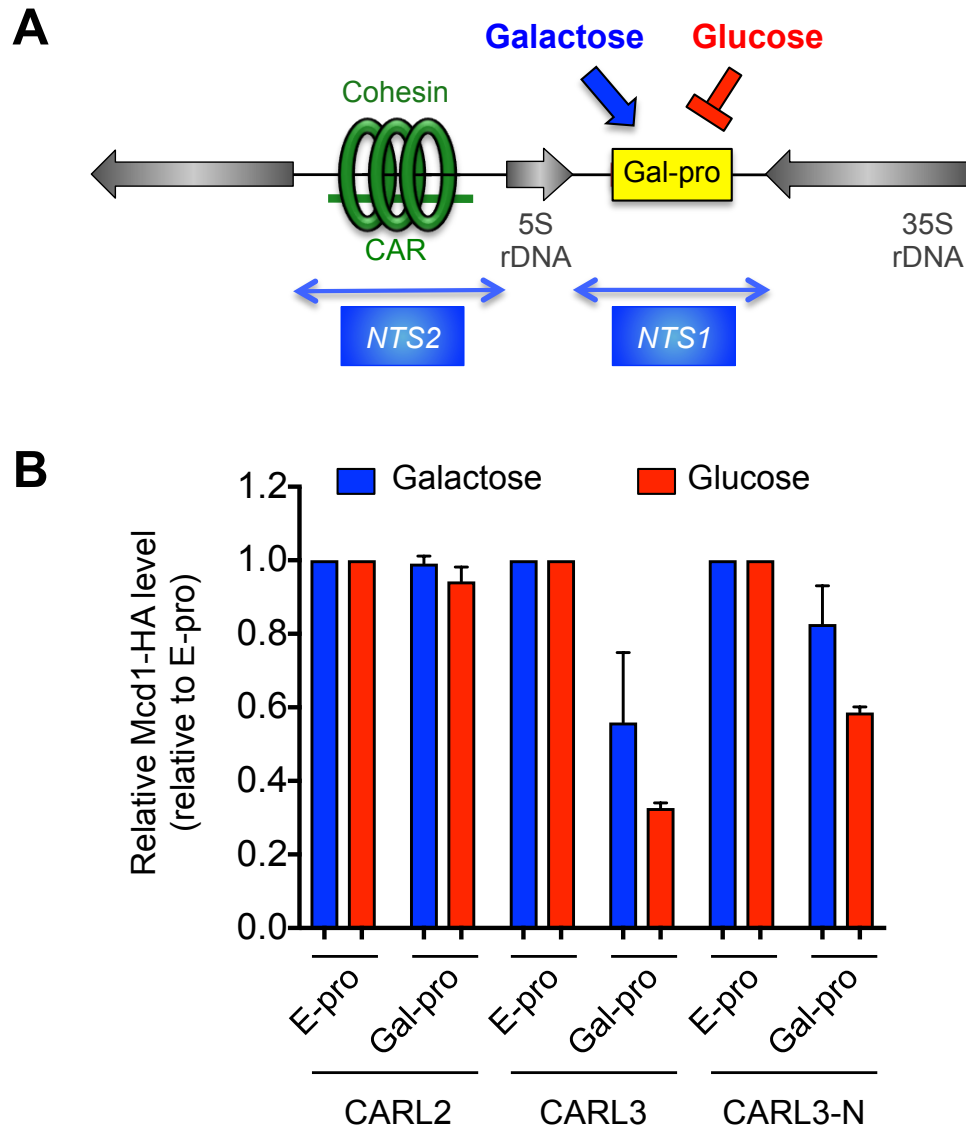


Figure 12: *NTS1* or *NTS2* transcription does not directly correlate with cohesin association. (A) Schematic representation of the experimental system, where native E-pro promoter at *NTS1* locus of all the rDNA repeat units has been replaced with galactose-inducible and glucose-repressible Gal-pro promoter. (B) ChIP analysis to measure Mcd1 occupancy at the indicated regions of rDNA from strains with E-pro or Gal-pro driving expression at *NTS1* following transcriptional induction of Gal-pro by galactose or repression by glucose. Data shown here was

normalized to a positive control region and then to E-pro. Average of two independent experiments are plotted here. Error bars indicate experimental variations.

at the rDNA. Our findings disagree with the previously made conclusion that *NTS1* transcription leads to displacement of cohesin while *NTS1* repression leads to maintenance of cohesion (113).

3.2.5. The majority of the non-coding RNA in old cells arises from ERCs

Since I found no correlation between *NTS1* transcription and cohesin occupancy (**Fig. 12**), I asked whether the non-coding RNAs produced during aging come from the rDNA on chromosome XII or whether they are expressed from the ERCs. I measured the non-coding rDNA transcript levels in wild type yeast and a *fob1* Δ mutant, where ERCs accumulate to a much lower degree during aging (110). Because the *fob1* Δ mutant cells live longer, we isolated cells with a mean age of ~30 replicative divisions and found that deletion of *FOB1* caused an 8-fold reduction in *NTS1* transcription, and a 30 fold reduction in *NTS2* transcription, in old cells (**Fig. 13A, B**). As such, I conclude that the majority of *NTS1/2* transcription in old cells comes from the ERCs and not from chromosome XII.

3.2.6. Expression of Mcd1, the limiting sub-unit of cohesin complex, is reduced during aging

All of our evidence so far suggested that the loss of cohesin and cohesion during aging was not related to non-coding transcription from the rDNA. Rather I found the cohesion loss during aging extends in other genomic regions as well, indicating a more global change. Therefore, I asked whether the protein level of

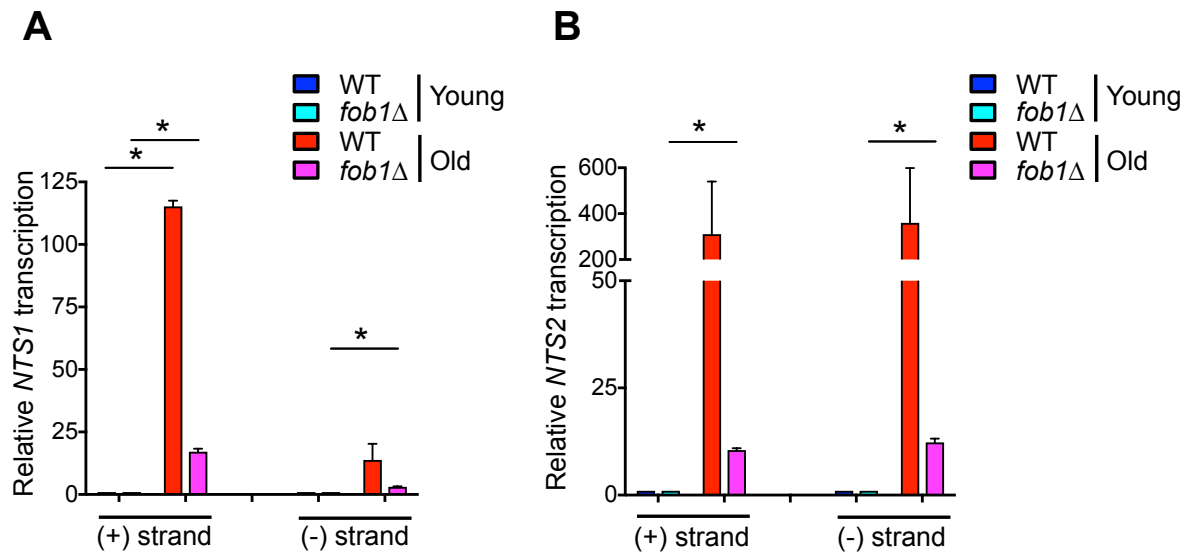


Figure 13. *NTS1* and *NTS2* intergenic transcripts in old cells are largely contributed by extra-chromosomal rDNA circles (ERCs). Strand-specific qRT-PCR analysis to examine the fold increase in non-coding RNA levels in old cells from both strands of (A) *NTS1* and (B) *NTS2* in comparison to young cells in both WT and *fob1*Δ strains. RNA levels were normalized to *ACT1* transcript levels before normalizing to 1 for young cells in each case. Average and standard error of mean of two replicates are plotted. Asterisk (*) indicates significant changes in between groups as indicated ($p < 0.05$), as determined by the Student's t-test.

cohesin components is reduced during aging. Loading total protein samples from equal numbers of cells, I found that the level of the rate limiting Mcd1 subunit of cohesin was drastically reduced during aging (**Fig. 14A**). I also observed a very similar decrease when I analyzed Mcd1 levels during aging when I normalized sample loading to equal DNA content (**Fig. 14B**). Therefore, I conclude that a lower protein level of Mcd1 is the likely explanation for the global cohesion defect in old cells.

3.2.7. The rDNA locus undergoes unequal recombination and repeat number variation during aging

Increased *NTS1* and *NTS2* transcription (**Fig. 8**) together with loss of cohesin occupancy (**Fig. 10**) at the rDNA region during aging, and the already existing correlation of rDNA with aging prompted us to ask if there is unequal recombination at rDNA in old cells resulting in rDNA repeat expansion/contraction. I compared the length of chromosome XII in young and old cells via pulse-field gel electrophoresis (PFGE) followed by Southern blot with an rDNA specific probe (**Fig. 15A**). Chromosome XII in aged cells showed variability in its size with two main populations, one having gained rDNA repeats and one having lost rDNA repeats. Meanwhile young cells showed only one band that corresponds to chromosome XII, indicating uniform rDNA locus length in the majority of young cells. I asked at what age the rDNA locus started showing variability, and found that the rDNA repeat number variability starts arising from middle age (~14-15 divisions) and progressed with the age (**Fig. 15B**). Of note,

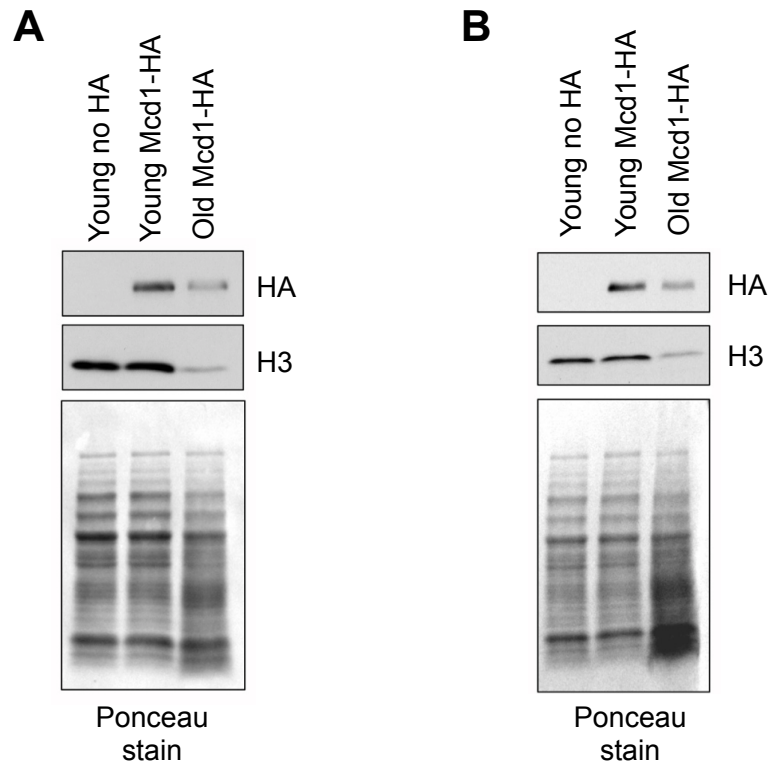


Figure 14. Cohesin sub-unit, Mcd1, level is lower in old cells. (A) Western blot analysis to measure protein levels of HA-tagged Mcd1 in young and old cells, with untagged strain as a control. Samples are loaded according to equal cell numbers. Histone H3 protein level is used as a control, which is known to be decreased in old cells (Feser et al., 2010). (B) Western blot analysis similar to A, but samples are loaded here according to same DNA equivalent.

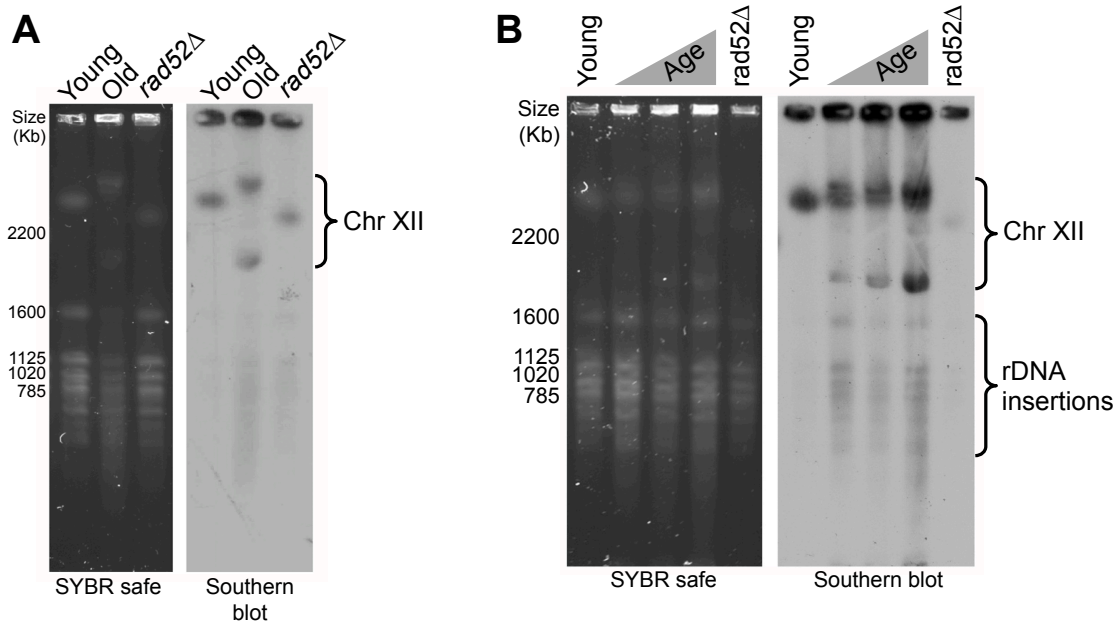


Figure 15. The rDNA locus undergoes unequal recombination with age. (A)

Analysis of intact yeast chromosomes by PFGE from young and old yeast cells.

Left panel shows staining pattern of chromosomes by SYBR safe. Right panel

shows Southern hybridization using a Chr XII (rDNA) specific probe. The *rad52Δ*

mutant is used as a control that shows a different rDNA copy number. (B) Similar

analysis as Figure 15A, but in this case analysis was done from intact yeast

chromosomes collected at indicated time points during aging. Left panel indicates

SYBR safe staining, while right panel indicates Southern hybridization using a

Chr XII (rDNA) specific probe. Fragmentation of DNA apparently increases with

age progression, which may also be indicative of rDNA integration at other

genomic sites.

while the rDNA probe displayed chromosome XII specificity in young cells, it hybridized to seemingly all other chromosomes in old cells, in addition to chromosome XII (**Fig. 15A, B**). Taken together with our previous observation of rDNA sequences fused to other chromosomal sequences apparent from deep sequencing of the old genome (40), these southern blots suggest that small rDNA repeat fragments insert into other yeast chromosomes during aging.

3.2.8. Formation of branched recombination intermediates increases from rDNA during aging

While insertions of rDNA repeats into other chromosomes, chromosomal amplifications having break points at the rDNA (40) and altered chromosome XII sizes (**Fig. 15A**) in old cells are likely to be the product of inaccurate repair of DSBs within the rDNA, I asked whether I could find direct evidence of rDNA recombination actively occurring in old cells. Recombination intermediates are unable to enter the PFGE gel, leading to their being stuck in the wells. An analysis of 'stuck DNA' in the wells has been used to show that there are more rDNA recombination intermediates in mother cells (~3-4 generations) compared to daughter cells (~0-1 generations) (104). By comparing daughters to much older mothers (25 or more generations), I found that chromosome XII from old mothers was 4 times more likely to be stuck in the well compared to chromosome II from old mothers (**Fig. 16A, B**), indicating that old cells have more rDNA recombination intermediates.

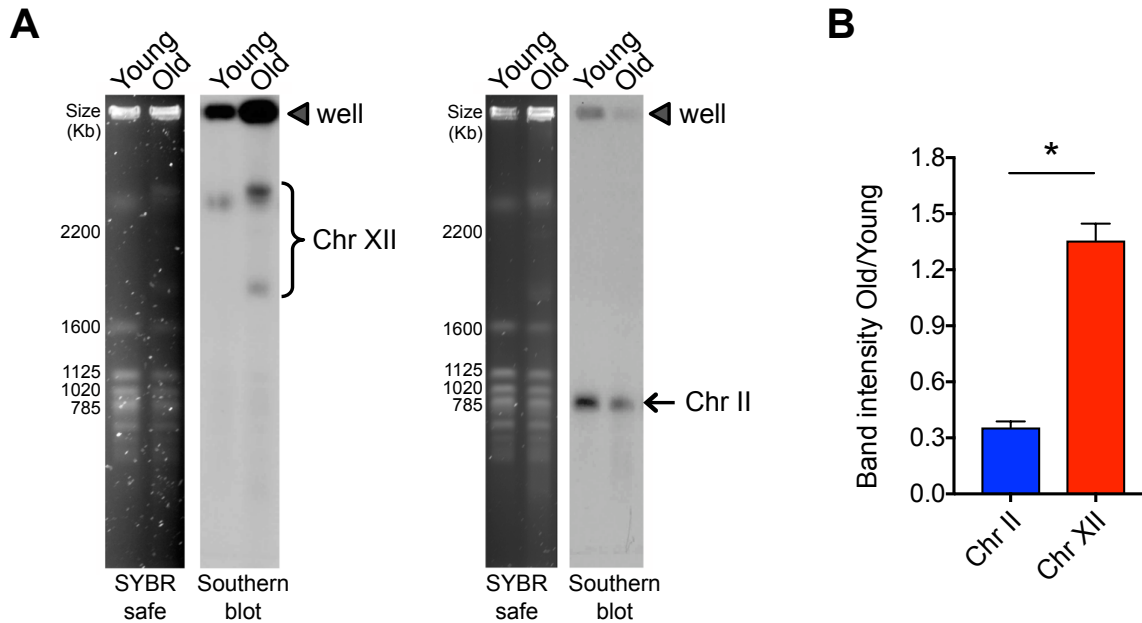


Figure 16. Increased rDNA recombination products is observed in old cells.

(A) Stuck DNA analysis in the gel wells for Chr XII (rDNA) and Chr II. Intact yeast chromosomes were analyzed by PFGE. For each chromosome (XII and II), the left panel indicates the staining pattern of chromosomes by SYBR safe, the right panel shows Southern hybridization using the indicated probe. Closed triangles indicates stuck DNA in the well. The same number of cells were analyzed in each lane of the gel. (B) Quantification of A is shown. The signal of the chromosomes stuck in the well for respective chromosomes was quantified and plotted as the relative intensity of stuck DNA in old cells compared to young cells. Three independent replicates are plotted here and error bars represent standard error of the mean. Asterisk (*) indicates significant changes in band intensity from chr II to chr XII ($p < 0.05$), as determined by the Student's t-test.

3.2.9. DNA double-strand break formation at the rDNA locus does not increase during aging *per se*

Next, I asked directly whether old yeast cells have more DSBs within the rDNA locus. To do this, I performed southern analysis with the BglII restriction enzyme that cuts proximal to the rDNA and used southern blotting to detect the intact DNA fragment and smaller chromosomal fragments due to DSBs at the replication fork block (RFB) site (137) (**Fig. 17A**). While I could detect DNA fragments that are due to DSBs around the RFB in young cells, their abundance was not significantly increased in aged cells (**Fig. 17B**). The evidence taken together suggests that DSBs at the RFB within the rDNA accumulate with the same frequency in old and young cells, but that old cells are more likely to repair these DSBs using unequal sister recombination or inaccurate fusion to other chromosomes.

3.3. Conclusions

According to the 'rDNA theory of aging', the rDNA locus plays a central role in regulating lifespan, where this already fragile region becomes even more unstable with the progression of age and acts as an internal sensor to gradually lengthen the cell cycle and finally induce senescence (112). This theory further emphasizes that ERCs are the by-products of rDNA instability (112), as opposed to the idea of ERCs being the cause of aging in budding yeast, as proposed earlier (32). Increased transcription from the intergenic regions of the rDNA locus due to loss of Sir2-mediated silencing has been proposed to specifically displace

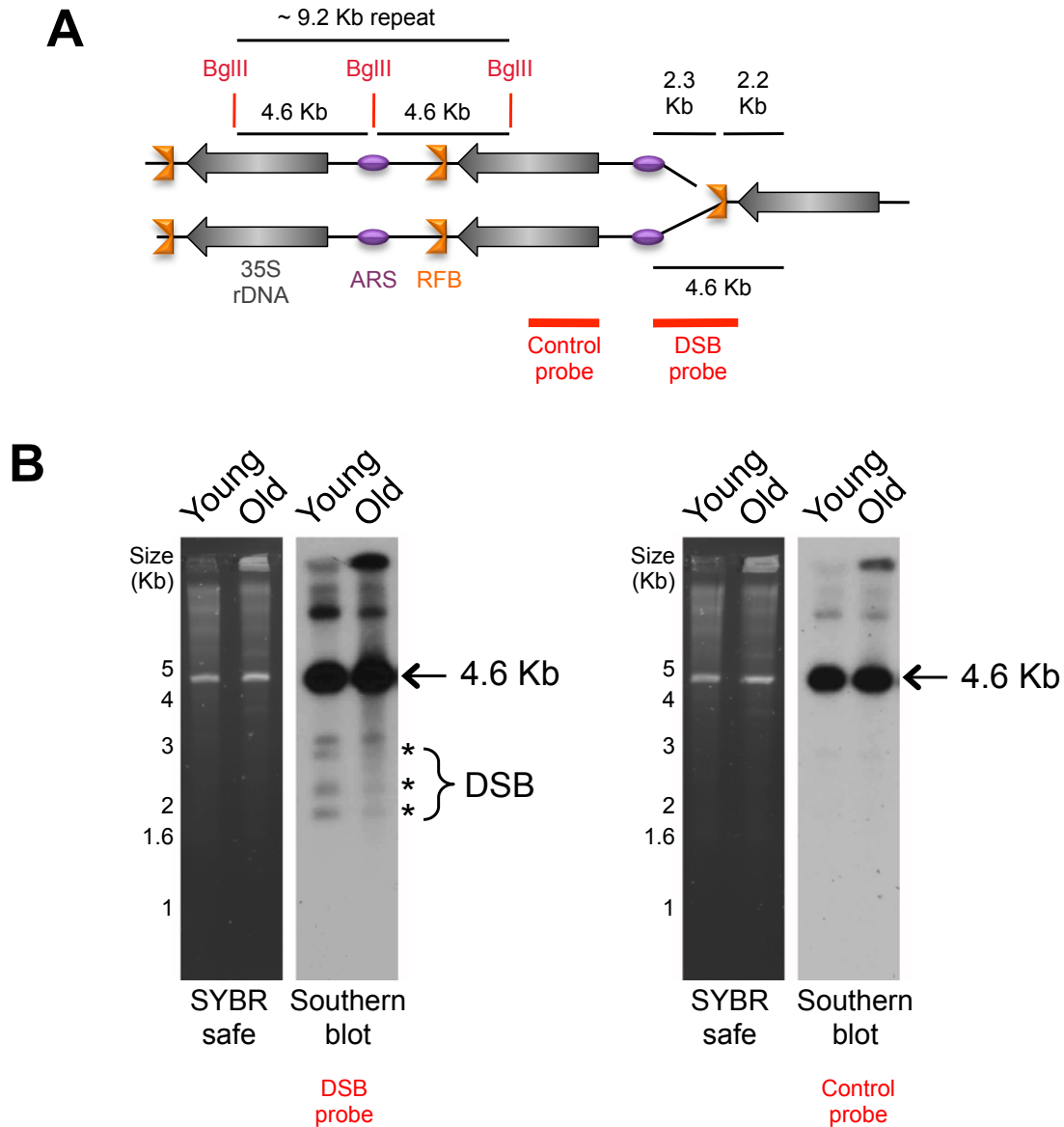


Figure 17. rDNA locus does not accumulate more DSBs *per se* during aging. (A) Schematic representation of the location of the probes for Southern blot used in this assay within a single rDNA repeat unit. (B) Agarose gel electrophoresis measuring DSBs. Genomic DNAs from equal numbers of young and old cells are extracted in an agarose plug by restriction digestion by BglIII and resolved in 1% agarose gel followed by Southern hybridization using DSB probe

or control probe, as indicated. Asterisks (*) indicate DNA fragments generated potentially from DSBs near the RFB, while arrows indicates control fragments resulting from restriction digestion by BglII and no DSB at RFB.

cohesin from the rDNA (113) leading to loss or gain of rDNA repeats, rDNA instability and aging. In my findings described in this chapter by analyzing the events in the true population of aged cells, I have uncovered that rDNA indeed becomes more unstable with age but is acting through a different mechanism than previously assumed. I observed massive increases in non-coding RNA (ncRNA) transcription from the intergenic spacer regions of the rDNA locus of aged cells (**Fig. 8**). However, I found the majority of this ncRNA transcription actually was contributed by ERCs by comparing WT cells with a mutant (*fob1Δ*) that is known to accumulate fewer ERCs during aging (110) and has a longer lifespan (**Fig. 13**). As such, the idea that enhanced transcription from the non-coding regions of the rDNA in old cells is what displaces cohesin to lead to rDNA instability and aging is likely to be inaccurate.

Furthermore, I did not find any correlation between induction of non-coding RNA transcription from the rDNA locus and cohesin occupancy, as even forced transcriptional induction of non-coding RNAs from the rDNA locus in young cells did not displace cohesin from the rDNA (**Fig. 12**). This further indicates that Sir2-mediated silencing of the non-coding spacer regions of the chromosomal rDNA locus is irrelevant to influencing replicative aging. However, my results indicate that the rDNA locus undergoes unequal sister-chromatid recombination, which starts around middle age and progresses with age (**Fig. 15**). I also find that more rDNA recombination intermediates accumulate with aging (**Fig. 16**), which undeniably confirms the proposed increase in rDNA instability during aging. While trying to find the reason behind this repeat number

change of rDNA copies in old cells, I did not observe any increase in DSB accumulation at the rDNA locus in replicatively old cells *per se* compared to young cells (**Fig. 17**), which probably implies that old cells still repair these DSBs at the rDNA but in an inaccurate manner.

Noteworthy, I observed a significant reduction in cohesin occupancy at the rDNA locus during aging (**Fig. 10**). However, I also found this loss of cohesin occupancy and loss of cohesion extended beyond the rDNA locus to other genomic sites such as, telomeres and centromeres during aging (**Fig. 10, 11**). This defect is most likely attributed to the reduced levels of the limiting cohesin protein Mcd1, which is due in turn to reduced protein synthesis of Mcd1 during aging (Tyler lab, unpublished observation) (**Fig. 14**). Taken together, I conclude from this part of my study that the rDNA locus becomes unstable with age, potentially contributing to limiting the replicative lifespan. However, this study does not rule out the contribution of the increased accumulation of ERCs to aging, which may be responsible for possible titration and sequestration of different repair factors away from the genome (see next chapter). The loss of cohesion observed with aging would explain finally the reason behind the accumulation of ERCs during aging, as cohesion is essential for equal sister chromatid recombination between the rDNA repeats. However, the effect cohesion loss supposedly should have larger implications during aging than only affecting the rDNA locus. Given the role of cohesin in promoting DSB repair, loss of cohesion may affect global genomic instability to limit replicative lifespan in yeast. Furthermore, loss of cohesion during aging is also likely to lead to

chromosomal loss and aneuploidy, which would also have a negative contribution to replicative lifespan.

**CHAPTER 4: MECHANISTIC INSIGHTS INTO
UNDERSTANDING INCREASED DNA LESION
FORMATION DURING REPLICATIVE AGING**

4.1. Introduction and Rationale

As discussed in chapter 1, genomic instability serves as a primary hallmark of aging (9). It is a well-conserved aging factor across many different organisms (150, 151). As mentioned earlier, the implication of genomic instability during aging is further strengthened by the evidence that certain human premature aging diseases are characterized by a defect in DNA repair genes (121-123, 152). Also, mutations of several repair proteins in yeast result in shortening of replicative lifespan (153). However, it is yet to be demonstrated whether enhanced DNA repair causes extension of lifespan. Of note, a very interesting correlation analysis comparing species with extreme differences in lifespan suggested that the longer-living species have higher expression of DNA repair genes and superior genome maintenance versus the short-lived species (73).

Our group previously demonstrated drastic loss of histone proteins during replicative aging in yeast, which also serves as a cause of aging in yeast (38). This loss of histones is a more generalized phenomenon and conserved also in multicellular eukaryotes as this has been observed during *in vitro* replicative aging of human fibroblasts (48). As mentioned previously, loss of histones during aging implies a more open chromatin conformation with less tight regulation for all the biological processes that require access to DNA. Given that chromatin structure is extremely critical to maintain genomic integrity, it is possible that an open chromatin structure observed during aging would give rise to increased genomic instability. We found that this is indeed the case during aging. We

showed significant increases in DNA breaks and associated changes resulting in increased genomic instability during yeast replicative aging (40). Building on these observations, an interesting and challenging goal is to determine the molecular mechanism behind the increase in DNA lesions observed during yeast replicative aging. Utilizing the budding yeast RLS model system, I sought to analyze the possible causes of loss of genomic integrity with aging. Using the replicatively aged yeast cells, it is possible to determine whether there is a defect in DNA repair *per se* during yeast replicative aging and whether a decline in DNA repair efficiency contributes to the high level of genomic instability and DNA lesions observed during aging.

4.2. Results

4.2.1. Increased chromosomal instability proportional to chromosome lengths is observed during aging

My results showed clear evidence of chromosomal fragmentation in old cells, apparent from the smearing of the chromosomal signals for all chromosomes from old cells upon total DNA staining in all our PFGE analyses. If the aging genome experiences random DNA breakage, I predict that the loss of intactness of the chromosomes would be inversely proportional to chromosome length, with the longest chromosomes becoming least intact with age. From quantitation of total DNA staining, I did indeed observe that the intactness of chromosomes in old cells was inversely proportional to their length (because we

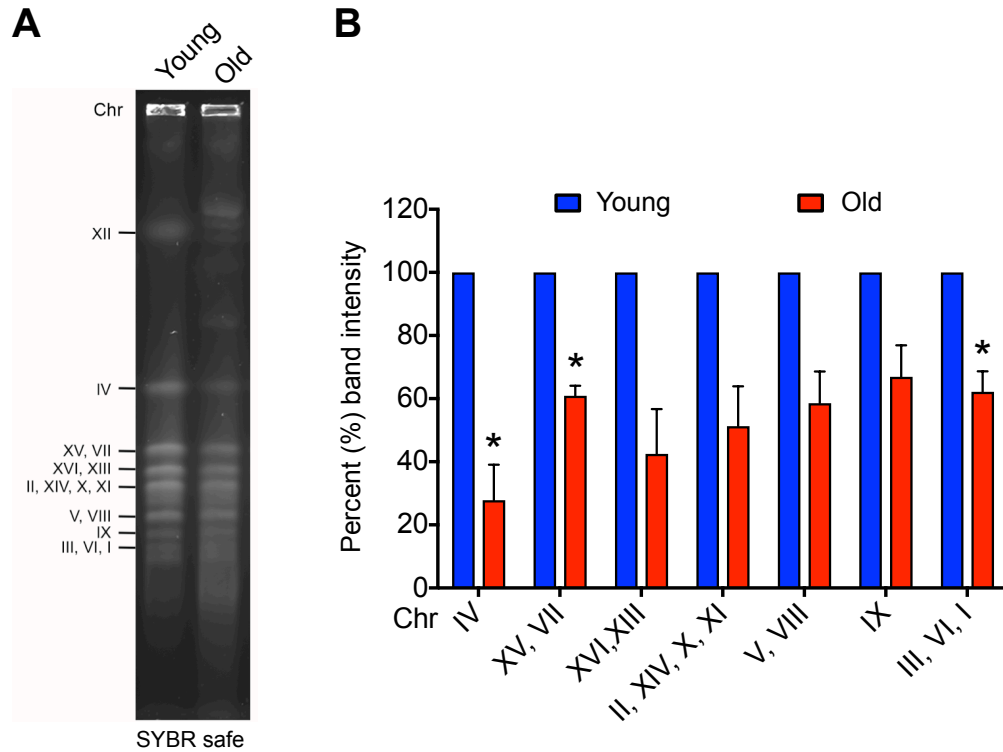


Figure 18. The aging genome accumulates random damage. (A) Analysis of intact yeast chromosomes by PFGE from young and old yeast cells. Left panel shows staining pattern of chromosomes by SYBR safe. (B) Right panel indicates the quantification of the chromosomal band intensities as measured from the SYBR safe stained gel, following normalization of signal intensity of young cells to 100% for each chromosome or group of chromosomes (both for single chromosomal band or band corresponding to multiple chromosomes as indicated). Average and standard error of mean of three replicates are plotted. Asterisk (*) indicates significant changes in old from young ($p < 0.05$), as determined by the Student's t-test.

see multiple bands of chromosome XII indicating increased rDNA instability, we excluded chromosome XII from this analysis) (**Fig. 18A, B**). To more accurately examine this relationship, I performed PFGE followed by southern blot analysis of a long chromosome (chromosome 4, 2nd longest in the yeast genome), a middle sized chromosome (chromosome 10) and the smallest chromosome (chromosome 1). While the loss of intactness of chromosomes was least for the smallest chromosome in old cells, the longer the chromosome was, the greater was the loss of intactness observed during aging (**Fig. 19A, B**). These data indicate that there is DNA double-strand break accumulation along the length of all chromosomes in old cells.

4.2.2. Global chromosomal instability in old cells is proportional to rDNA instability

To determine whether the global chromosomal instability was somehow related to the rDNA instability, we compared the intactness of chromosomes during aging between wild type yeast and a *fob1* Δ mutant which has much less DSBs at the RFB within the rDNA (110). With the *fob1* Δ mutant, we observed a similar trend of chromosomal intactness inversely proportional to chromosome length in old cells by SYBR safe staining, but the general chromosomal intactness was greater in similarly aged *fob1* Δ mutants in comparison to wild type cells (**Fig. 20**). In order to confirm this finding, we performed southern analysis for three chromosomes of different lengths in wild type and *fob1* Δ mutant yeast, comparing the same number of same age cells. In agreement with our previous

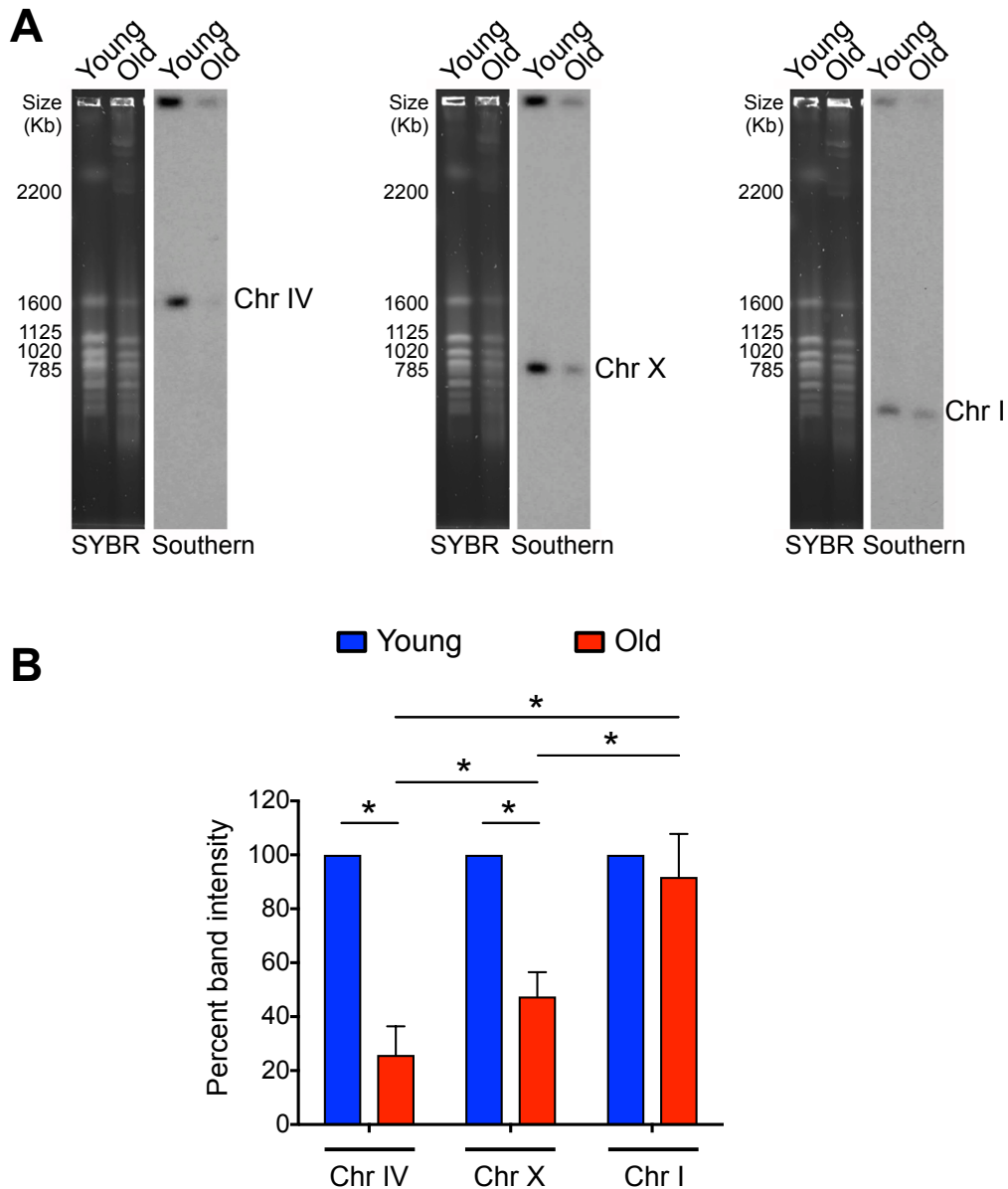


Figure 19: Increased chromosomal instability proportional to chromosome length is observed during aging in WT cells. (A) Analysis of intact yeast chromosomes by PFGE from same numbers of young and old WT yeast cells followed by Southern hybridization using specific probes against three chromosomes of different sizes: long (Chr IV), middle-sized (Chr X) and short (Chr I). In each case, left panel shows staining pattern of chromosomes by SYBR

safe and right panel shows Southern hybridization using the indicated probe. (B) Quantification of A is shown. The graph shows percent loss of band intensity in aged cells measured from Southern blots of respective chromosomes, following normalization signal intensity of young cells to 100% for each chromosome. Average and standard error of mean of three replicates are plotted. Asterisk (*) indicates significant changes in old from young ($p < 0.05$), and also significant changes in chromosomal band intensities between chromosomes of different lengths in old cells as determined by the Student's t-test.

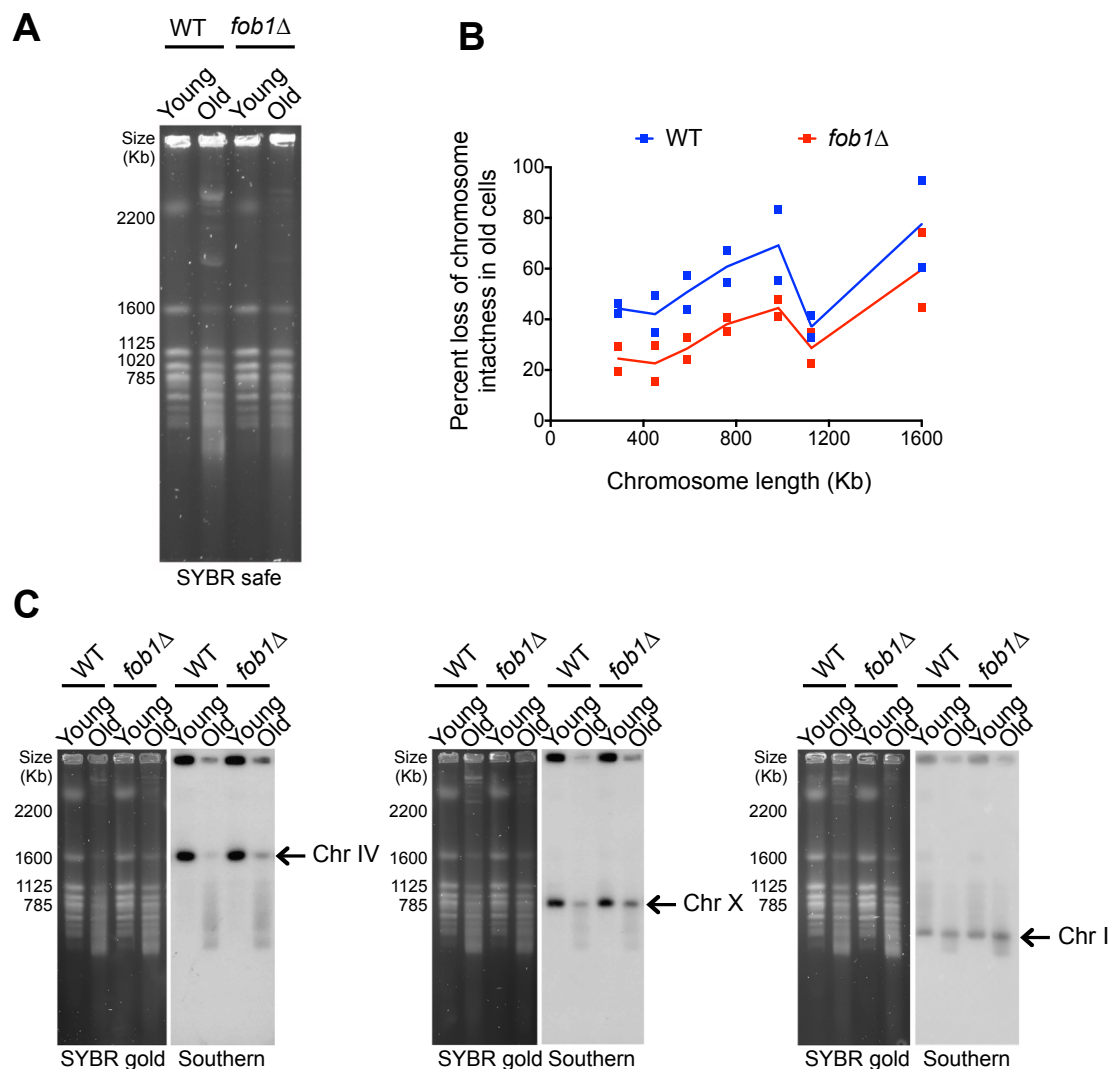


Figure 20. rDNA instability appears to promote global genomic instability.

(A) Analysis of intact yeast chromosomes by PFGE from same numbers of young and old yeast cells from both WT and *fob1Δ* strains. The panel shows staining pattern of chromosomes by SYBR safe. (B) Quantification of A is shown. The net percent loss of chromosomal band intensities during aging measured from quantifying band intensities of all chromosomes from SYBR safe stained agarose gels using AlphaView software following background correction. Average of two

replicates are plotted here. (C) Analysis of intact yeast chromosomes by PFGE from same numbers of young and old yeast cells from both WT and *fob1* Δ strains followed by Southern hybridization using specific probes against three chromosomes, Chr IV, Chr X and Chr I. In each case, left panel shows staining pattern of chromosomes by SYBR gold and right panel shows Southern hybridization using the indicated probe.

finding, we found the chromosomal intactness was better maintained in the *fob1Δ* mutant when compared to wild type for every chromosome, and that the loss of chromosome intactness in old cells was again more pronounced in longer chromosomes than smaller ones (**Fig. 20**). These data indicate that rDNA instability is proportional to, and at least in part likely responsible for, loss of global chromosomal stability in old cells.

4.2.3. DSB repair is significantly impaired in old cells

We observed previously that replicatively aged cells accumulate DNA breaks (40) and above we found that during the course of aging, DNA damage randomly accumulated across the genome (**Fig. 18, 19**). This raises the question of whether there are more DSBs in old cells because there is less repair and / or more DNA damage. In order to determine directly if there is a defect in DSB repair efficiency in aged cells, I attempted to take advantage of an inducible system of DSB induction at a specific site, that is HO endonuclease-inducible DSB formation at the *MAT* locus of budding yeast (139). The expression of HO endonuclease is under the control of galactose-inducible and glucose-repressible promoter. Therefore, it is possible to specifically induce a DSB at the *MAT* locus at any given time by the addition of galactose and follow the repair kinetics after the addition of glucose to repress endonuclease expression. Using semi-quantitative multiplex PCR analysis, it is possible to measure the degree of HO cutting and repair efficiency over the time course, which measures DSB repair kinetics. Utilizing the MEP strain carrying inducible HO endonuclease, I

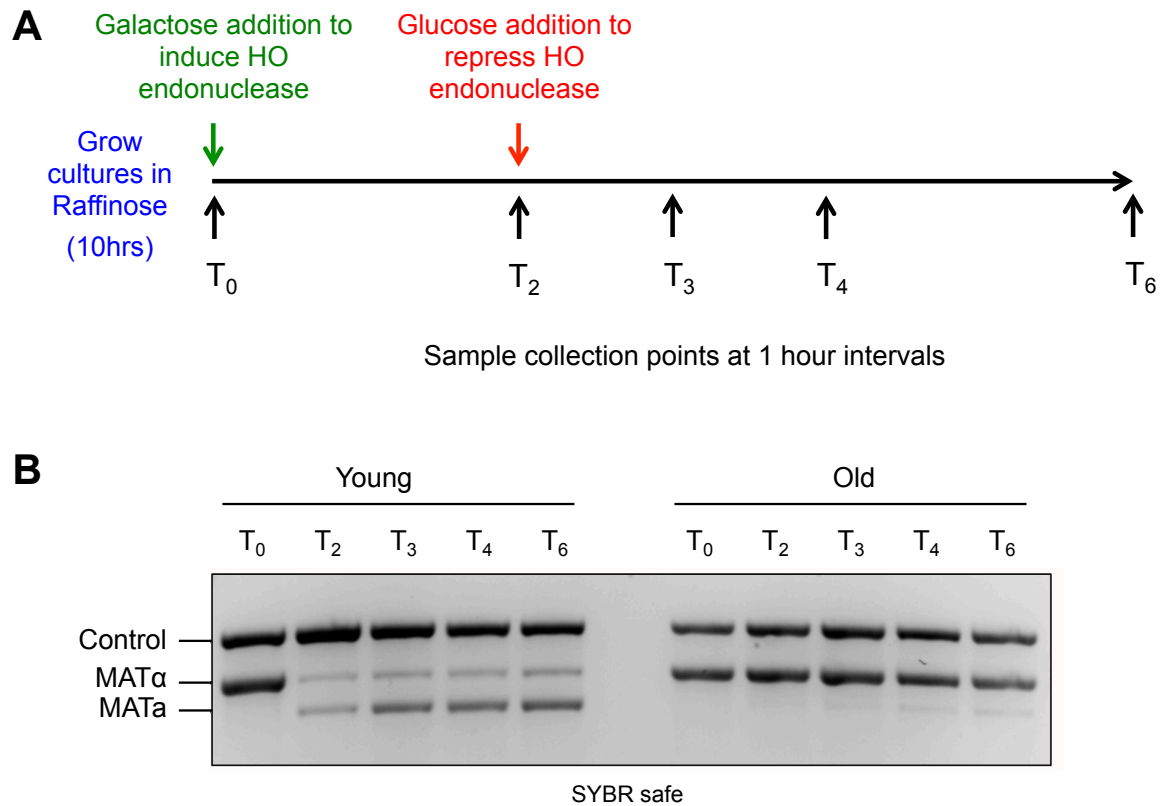


Figure 21. The HO endonuclease is ineffective in inducing DSBs in old cells. (A) Schematic diagram of the experiment using HO endonuclease for damage induction to follow repair dynamics in young and old cells. (B) PCR analyses of HO cutting and repair dynamics in young and old cells. While young cells show HO sensitivity and efficient cutting and repair dynamics, HO endonuclease activity is undetectable in old cells.

measured the HO cutting and repair dynamics in young and old cells following a repair time-course (**Fig. 21A**). While the young cells showed efficient cutting and repair dynamics as seen by loss of the MAT alpha PCR product and appearance of the MATa PCR product which is a consequence of DSB induction and DSB repair at the MAT locus, respectively. However, I could not achieve detectable DSB induction at the MAT locus in aged cells, reflected in the constant levels of the MAT alpha PCR product (**Fig. 21**). The most likely reason for this is that there is a defect in HO endonuclease protein synthesis, as we have recently observed a global defect in protein synthesis during aging (Tyler lab, unpublished observation).

Therefore, in order to examine if DSB repair is defective in old yeast cells, I utilized a different approach and induced global DSBs in young and old yeast cells using the alkylating agent methyl methane sulfonate (MMS), and compared their ability to repair the DSBs by resolving intact yeast chromosomes using PFGE. In young cells, MMS treatment induced smearing of the otherwise intact chromosome bands that is indicative of DSBs (**Fig. 22**). However, following removal of MMS from the media, within 6 hours young cells repaired most of the DNA breaks and the intactness of the chromosomes was restored to the level observed before damage induction (**Fig. 22**). In contrast, there was a clear defect in old cells in the ability to re-establish intact chromosomes following MMS removal (**Fig. 22**). The presence of DNA breaks causes cells to accumulate in G₂/M phase (154, 155). By examining the cell cycle stage of the cell populations during induction of MMS and washing out the DNA damaging agent, it was clear

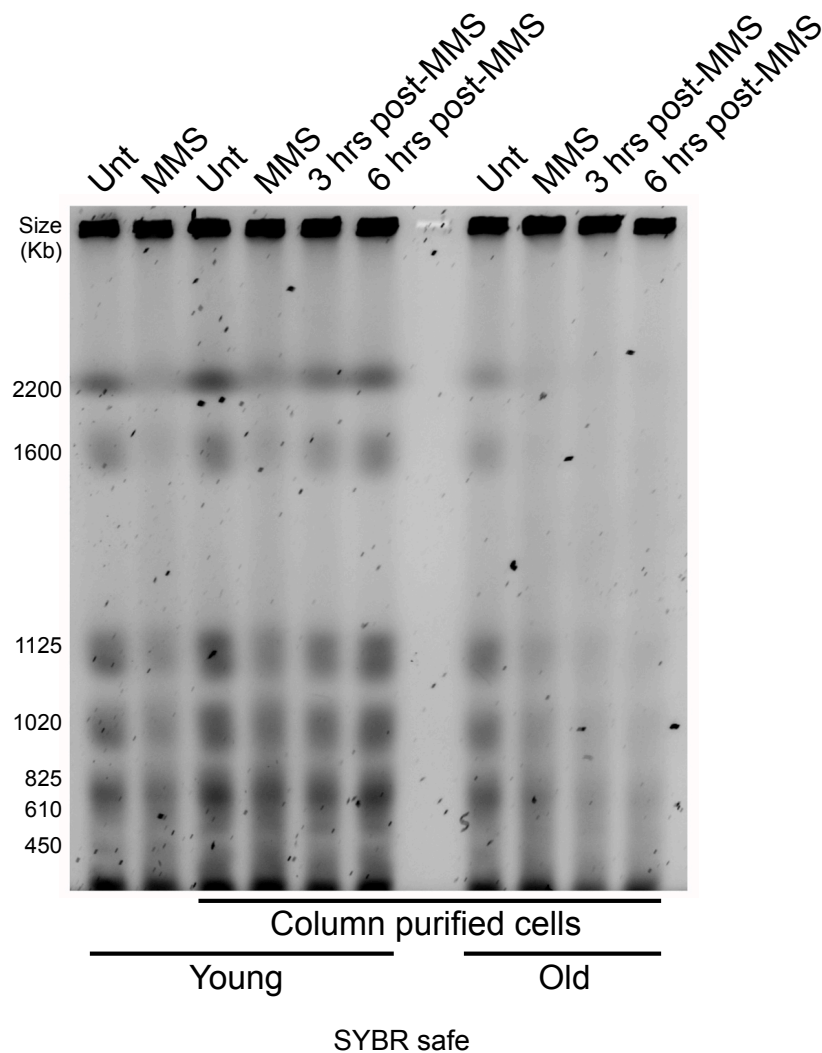
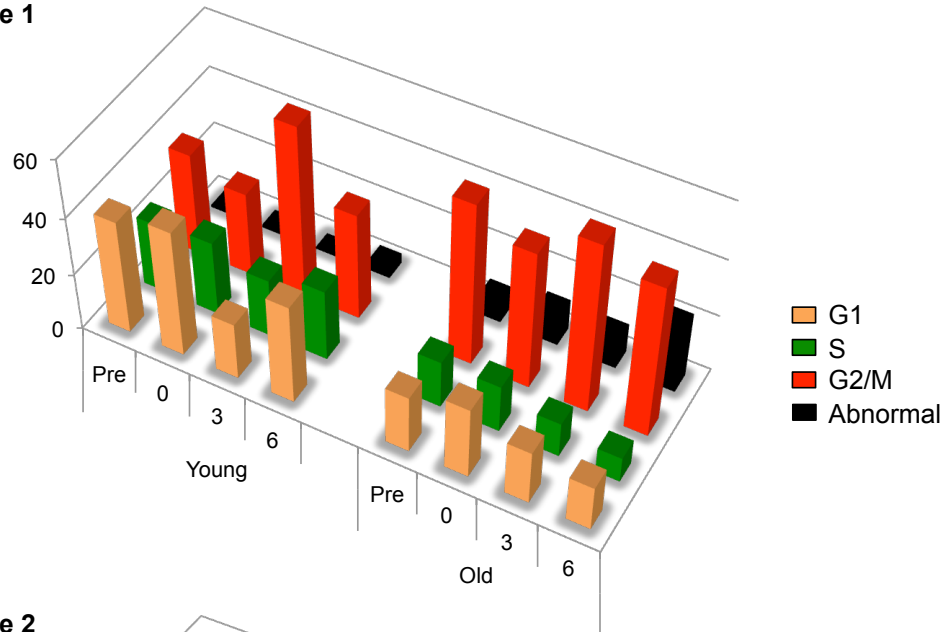


Figure 22: Old cells have impaired DSB repair. Analysis of intact yeast chromosomes by PFGE from same numbers of young and old WT yeast cells for each time point during repair a time-course as indicated in the figure. Samples were collected before damage induction (Unt), addition of MMS to a final concentration of 0.07% for 30 mins (MMS), and post-MMS removal and recovery up to 6 hours (3 and 6, respectively). Samples in the first two lanes (Unt, MMS in Young) are not purified through columns as required for collection of samples

using MEP, as controls for the method. The panel shows staining pattern of chromosomes by SYBR safe.

Replicate 1



Replicate 2

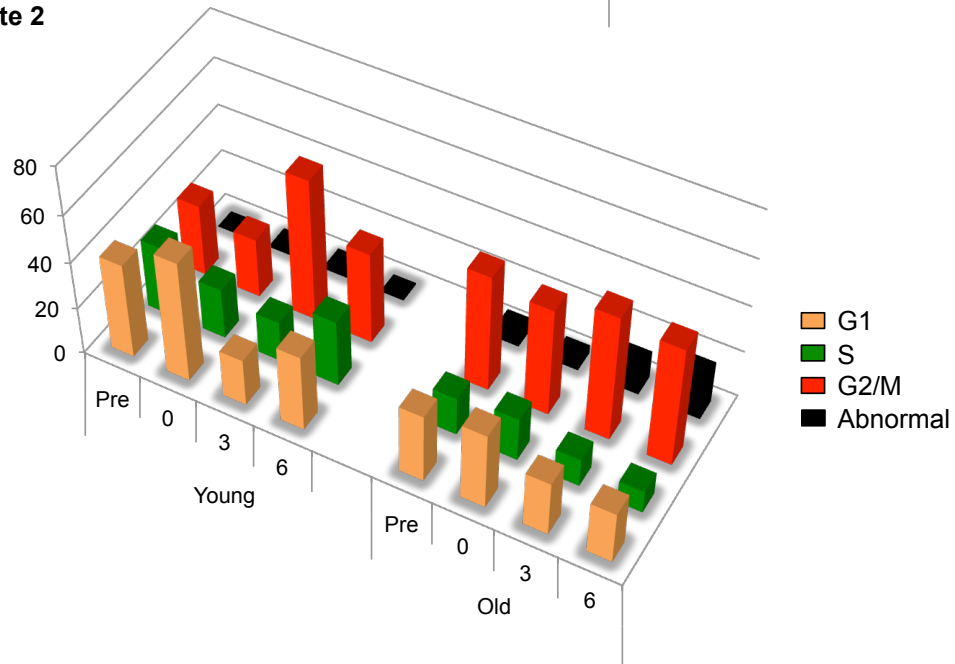


Figure 23. Old cells are predominantly at the G₂/M phase of the cell cycle.

Budding index of young and old samples collected during DNA repair time-course. Result from two independent replicates are shown here. Over a 100 cells were examined before (pre) or at various hours after washing out MMS, as indicated by the numbers.

that the young cells accumulate in G₂/M phase consistent with activation of the DNA damage checkpoint, following DNA damage and subsequently reenter the cell cycle following DNA repair (**Fig. 23**). By contrast, the old cells were already predominantly in G₂/M phase even prior to inducing damage, indicating that they may already be experiencing an activated cell cycle checkpoint due to DNA damage (**Fig. 23**). This is consistent with old cells having more DNA damage perhaps due to the inability of old cells to repair DSBs. Taken together, these data clearly show that DSB repair is defective in old yeast cells.

4.2.4. Levels of several DNA repair proteins are reduced in old cells

Most DSB repair in yeast is performed by the homologous recombination (HR) pathway (118). Therefore, I asked whether there are reduced levels of key HR proteins in old cells. Indeed, the levels of several key proteins involved in different stages of HR were severely reduced during aging when I compared the protein levels in young and old cells (**Fig. 24**), including the resection proteins Mre11 and Sae2, single strand DNA binding proteins RPA (Rfa1 and Rfa2), the strand exchange protein Rad51, the DNA damage response kinase Rad53 and Rad54, the protein involved in a late postsynaptic step of HR to complete the repair process (117, 119, 156). The only HR protein that did not appear to have significantly reduced levels during yeast aging was Rad52. Because the protein levels of most key HR proteins were significantly reduced in old cells, it is likely that this is the reason why DSB repair efficiency is compromised in aged yeast cells.

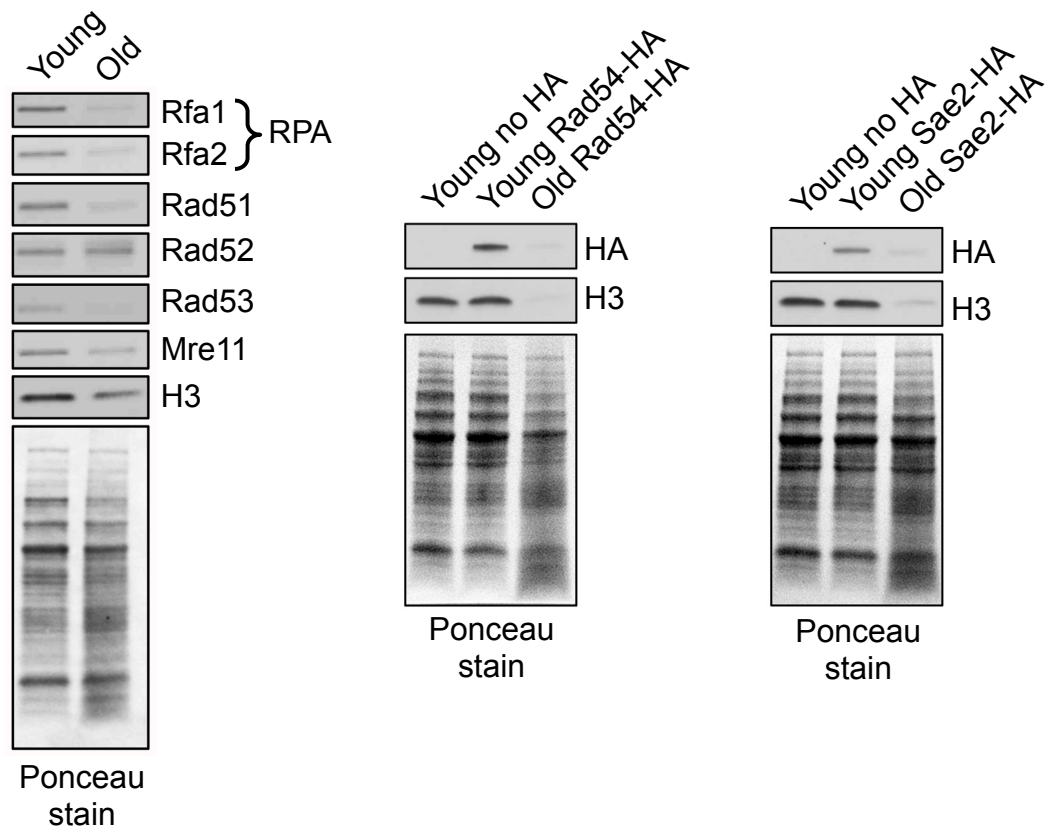


Figure 24. Reduced expression level is observed for key DNA repair proteins in old cells. Western blot analysis to measure levels of key DNA repair proteins in young and old cells. Samples are loaded according to equal cell numbers. Left panel indicates proteins tested using available antibodies, while panel in right shows HA-tagged proteins with untagged strain as additional control. Histone H3 protein level has been shown in each case as a control, which is decreased in old cells. Below is shown the Ponceau stained membrane to show equal protein loading.

4.2.5. Over-expression of key DNA repair proteins (Rad51 and Mre11) partially reverses the accumulation of DNA damage with age

To investigate whether the reduced levels of HR proteins in old cells is responsible for the profound defect in DSB repair in old cells (**Fig. 24**), I overexpressed an extra copy of several of the key HR proteins and asked if it could restore DSB repair. We have shown previously that aged cells show increased accumulation of γ H2A foci, which is indicative of increased break accumulation during aging (40). When I overexpressed Rad51, I found a significant reduction of γ H2A foci formation in old cells (**Fig. 25A**). Similarly, overexpression of Mre11 also led to a significant reduction of γ H2A foci formation in old cells (**Fig. 25B**). It has been shown previously that an extra copy of the *RAD51* gene extends replicative lifespan in yeast (136). Taken together these data indicate that the reduced DSB repair that occurs in old cells is possibly a cause of aging, because partially fixing the DSB repair defect in old cells extends replicative lifespan.

4.3. Conclusions

There are several lines of evidence that illustrates the necessity for proper genome maintenance to rightfully execute different cellular functions (115, 116). Given the gradual decline in all molecular and cellular functions during aging, loss of genomic integrity has long been implicated as a causative factor for aging (9). Significant increase in the accumulation of DNA breaks has already been observed in replicatively aged yeast cells by our laboratory (40). In this chapter, I

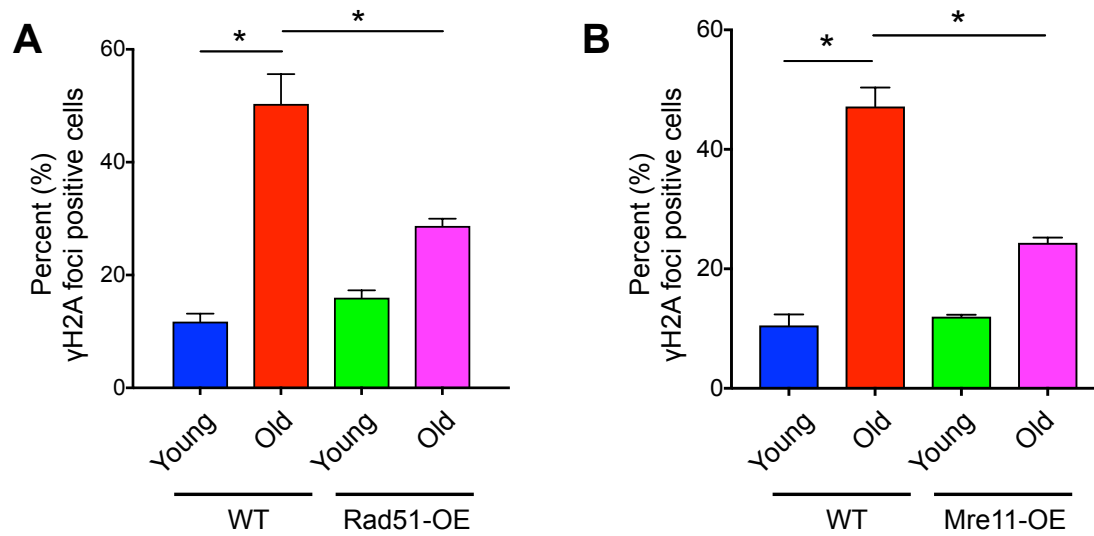


Figure 25: Over-expression of key repair proteins reduces genomic instability. (A) Quantification of percentage of young and old cells with detectable γ H2A foci as measured by immunofluorescence, in both WT and single copy Rad51-overexpression (OE) strains. Three independent replicates are analyzed here. Error bars represent standard error of mean. Asterisk (*) indicates significant changes in between groups as indicated ($p < 0.05$), as determined by the Student's t-test. (B) As in A, but for Mre11-overexpression.

sought to characterize the potential mechanism behind the reason for this increased genomic instability observed during aging.

My findings suggest that an aging genome accumulates random damage as a function of time (**Fig. 18, 19**). I also found a direct correlation between rDNA instability / ERC levels and accumulation of global chromosomal damage during aging (**Fig. 20**). Specifically, global DSB damage accumulation was less in a mutant (*fob1* Δ) where the rDNA is less recombinogenic and has less ERCs (110). It is believed for a long time that accumulation of DNA mutations over a period of time impacts organismal aging (69, 70). However, a recent study indicated that mere accumulation of somatic DNA mutations, at least in budding yeast, does not have a causal role in aging by measuring de novo mutations in daughters from mothers of different ages (71). Therefore, it needed to be conclusively determined whether a declining DNA repair response was responsible for the increased accumulation of damage as a function of age. Here, using the replicatively aged yeast cells, I found an impaired DNA repair response in aging cells that cannot resolve increasing accumulation of DNA lesions efficiently (**Fig. 22**). Although I could not rule out the possibility that this decline in DNA repair response is due to failure of specific DNA repair proteins to be recruited at the DSB sites during aging, I uncovered that the levels of most of the DNA repair proteins are lower in aged cells (**Fig. 24**). This indicates that while the demand for the repair proteins is higher in aged cells due to an increased burden of damage accumulation, the supply of repair proteins is lower, limiting the ability of the aging cell to maintain its genomic integrity. I confirmed this by

over-expressing specific proteins involved in the DNA repair response. Following over-expression of specific repair proteins, I found that similar age-matched cells to WT cells show lower abundance of DNA damage as indicated by decrease in γ H2A foci formation (**Fig. 25**), indicating an improved DNA repair response following over-expression of these proteins. Over-expression of one of these proteins, Rad51, has already been shown to extend budding yeast replicative lifespan (136). Although the reason for this was unknown, this result together with ours indicates that enhancement of the DSB repair response leads to lifespan extension. Furthermore, I found evidence that maintaining rDNA stability protects global genomic integrity during aging, as a strain with higher rDNA stability and longer lifespan showed better chromosomal integrity in comparison to WT age-matched yeast cells. Taken together, I conclude from this part of my study that the combination of rDNA instability and lower levels of DNA repair proteins possibly due to translation defects are accountable for the functional decline of DNA repair in aged cells contributing to the loss of genomic integrity. Maintaining or enhancing the repair response may possibly serve as a potential way for the extension of replicative lifespan in budding yeast.

CHAPTER 5: DISCUSSION & FUTURE DIRECTIONS

DISCUSSION

The inherent complexity of aging biology is an ongoing area of interest, as there are continued efforts to find out therapeutic ways to delay the onset of aging or age-associated diseases. Understanding the molecular details during the aging process is thus imperative to achieve that goal. During an organisms' lifetime, aging cells experience diverse changes and the aging phenotypes are the manifestation of the cumulative effect of all these alterations. Even after all these efforts by multiple groups to understand aging biology, there are still large gaps in our knowledge of what changes happen during aging. In this thesis work, I have presented an unparalleled analysis of the changes an aging cell experiences from a genomic integrity point of view. The goal of this work was to attain a comprehensive understanding of the loss of function experienced by an aging genome. To my advantage, I used the single-celled eukaryote budding yeast RLS, which provides a simplified model of the organismal aging process. Given the high similarity of signaling pathways among eukaryotic organisms, the knowledge I gained from these analyses may apply to the aging process of multicellular eukaryotes as well.

Here, in the work presented in chapters 3 and 4, I have uncovered a global loss of cohesion occurring in replicatively aged cells and this is the likely cause of increased rDNA instability and ERC accumulation in old cells, which we find, in turn, leads to global genomic instability in replicatively old cells. Furthermore, I have discovered a profound defect in DSB repair in replicatively old yeast due to limiting levels of key components of the homologous

recombination machinery. This DSB repair defect in old cells limits the replicative lifespan, because restoration of DSB repair by overexpressing HR proteins ameliorates age-associated changes, extending lifespan.

The vast majority of yeast aging studies to date have focused on identifying different mutants or conditions that shorten or lengthen lifespan. This was because budding yeast enables the accurate measurement of replicative lifespan. Conversely, the experimental difficulty in collecting sufficient numbers of old yeast is responsible for the very sparse knowledge of the phenotype, physiology and molecular health of replicatively old yeast cells. The transformative MEP genetic system has enabled the molecular study of replicatively old yeast cells (100) really for the first time. I used the MEP in this study to uncover novel events occurring in yeast that are at, or beyond, their median replicative lifespan, providing extensive novel insight into genomic integrity in these cells and its role in determining lifespan.

Loss of cohesion during replicative aging

The likely reason why I observed an increase in rDNA instability during aging was the decreased cohesin occupancy at the rDNA locus of aged cells. Loss of cohesion may serve as the reason behind unequal recombination in between tandem repeats resulting in changes in repeat numbers. In addition to the rDNA locus, cohesion defects observed during aging extended also to other genomic sites including telomeric and centromeric regions in aged cells. This defect is likely caused by the reduced levels of the limiting subunit of cohesin

complex, Mcd1, due to reduced protein synthesis of Mcd1 during aging as found from the work done in other studies in our lab (unpublished observations). Given the reduced levels of cohesin in old cells, it is probable that there is a global loss of cohesion in mitotically aged cells beyond the regions that we have examined. The potential implications of this loss of cohesion for human disease and lifespan are multifold. First, loss of cohesion at centromeres would lead to chromosome loss and gain during mitotic aging. Indeed, cohesion loss has previously been observed during meiotic aging mostly at centromeres, and to some degree at chromosome arms. This loss of cohesion in meiosis has been demonstrated to be the leading cause for mis-segregation of chromosomes associated with advanced maternal age, giving rise to birth defects and developmental abnormalities (157, 158). Loss of cohesion during mitotic aging would also contribute towards increased genomic instability given that cohesin is recruited to DSBs to promote accurate DSB repair (159, 160). Loss of cohesion would also lead to unequal sister chromatid recombination as I observed during aging between repeats and ERC accumulation.

The accumulation of ERCs has long been known to occur during yeast aging and has even been proposed to be a causative reason for yeast aging (32). However, the molecular reason for ERC accumulation during aging has never been known. ERCs accumulate due to unequal sister chromatid recombination between the rDNA repeats (107). The loss of cohesion during aging that I have uncovered here is undoubtedly the cause for the accumulation

of ERCs during aging, as cohesion is essential for equal sister chromatid recombination between the rDNA repeats.

Formation of extrachromosomal circular DNA similar to ERCs has also been reported during *Drosophila* aging (25). Importantly, extrachromosomal circular DNAs also appear during both in vitro and in vivo aging of mammalian cells (161, 162). It is possible that cohesion is also lost during aging of metazoans including mammals, and is responsible for the circular DNA accumulation seen in these systems. We know very little about the roles played by these circular nucleic acids in complex model organisms during aging. Interestingly, rDNA instability has been shown to occur during some adult solid tumors as well (143), where cancer is a disease of aging. Given that previous studies (149) have correlated the levels of non coding RNA from the rDNA with lifespan in budding yeast, it is relevant that I have found that the vast majority of the non-coding RNA transcription occurring during aging is coming from the ERCs. This places ERCs center stage again for contributing to perturbing cellular homeostasis, potentially by sequestering factors that are required for efficient DSB repair. This brings us to the causal role that we have observed for rDNA instability/ERCs in global chromosomal instability.

Instability of the rDNA and its influence on global chromosomal stability during aging

My findings reveal inability of aging cells to maintain genomic integrity as demonstrated by the accumulation of random damage along chromosome arms

compromising chromosomal stability. This is most likely due to a declining DNA repair efficiency in aging cells. But a potential influence of rDNA instability over global genomic integrity cannot be ignored as well. It is probable that an unstable rDNA locus and / or accumulated ERCs in the nucleolus that accumulates with increased mitotic age, titrates key limiting factors, which would otherwise be available to maintain genomic integrity, as suggested previously (29, 32). Future studies should aim to determine whether key repair factors translocate to fragile sites like rDNA in the genome during age progression.

Another study using the MEP, albeit analyzing much younger old cells that had undergone approximately 9 divisions, compared to our analysis of cells that have undergone 25 or more divisions, found reduced pausing of the replication machinery at the RFB within the rDNA in older cells (163). The molecular reason for this is currently unknown. Regardless, this result is consistent with the fact that we find the level of DSBs close to the RFB to be similar in old and young cells, while the efficiency of DSB repair is greatly reduced in old cells. As such, it is possible that old cells have reduced generation of DSBs near the RFB because of reduced replication fork pausing, but in old cells these DSBs are not effectively repaired, leading to equivalent steady state levels of DSBs at the RFB in young and old cells. This would be in agreement with the fact that the length of chromosome XII in old cells adopts two primarily distinct lengths, as opposed to a distribution of chromosome XII fragments that would result if there were multiple unequal sister chromatid recombination events due to the loss of cohesion in old cells. The fact that old cells only show two main lengths of chromosome XII

indicates that either somehow all old cells undergo unequal sister chromatid cohesion in order to yield similarly reduced and increased numbers of rDNA repeats. Alternatively, there may be one event of unequal sister chromatid recombination in middle age and subsequent cells maintain these numbers of rDNA repeats, perhaps because old cells are unable to efficiently repair the DSB at the RFB by recombination.

Reduced homologous recombination in yeast limits the replicative lifespan

My studies reveal a defect in DSB repair, presumably via homologous recombination, in replicatively aged yeast cells that may function to limit replicative lifespan. The importance of the role of DSB repair in determining lifespan and normal cellular health is consistent with the fact that yeast deleted for key HR proteins have a very short lifespan (153). I uncovered the molecular reason for the HR repair defect in replicatively old yeast cells as reduced levels of key HR proteins in old cells, possibly due to a defect in their protein synthesis (Tyler lab, unpublished observation). In agreement with our observations in yeast, a decline in HR repair efficiency has been reported during replicative senescence of human fibroblast cells *in vitro* and also during meiotic aging in germ cells of old *Drosophila* using reporter assays (68, 164). It is interesting to note that replicative senescence of human fibroblasts was accompanied by reduced levels of key HR proteins, including Rad51, Rad52, NBS1 and SIRT6 (68), demonstrating that reduced levels of key HR proteins is a common feature of replicative aging of yeast and humans. In human fibroblasts, however, the HR

repair defect during replicative senescence is likely due to reduced protein levels of the histone deacetylase SIRT6, as SIRT6 overexpression reestablished DSB repair (68).

Interestingly, maintaining the optimal levels of the HR protein Rad51 seems extremely important in maintaining genomic integrity. While deficiency of Rad51 renders increased sensitivity for DNA damaging agents in both budding and fission yeast (165, 166), targeted Rad51 disruption results into accumulations of chromosomal aberrations leading to cell death in vertebrate cells and cell lethality in mouse ES cells (167, 168). Rad51-overexpression also has certain adverse effects on both yeast influencing DSB-induced HR and mammalian cells promoting chromosomal instability (169-171). However, over-expression of a single copy of Rad51 in yeast seems to be better tolerated and is in fact beneficial for longevity, as illustrated by moderate lifespan extension in yeast (136). Our analyses further indicate that over-expression of a single copy of Rad51 could partially fix the DSB repair defect in aged yeast cells, which may serve as a potential reason behind the extension of replicative lifespan observed following Rad51 over-expression. Importantly, a single extra copy over-expression of another protein implicated during DNA repair, Mre11, shows very similar effect in aged cells in partially fixing the DSB repair defect. Although very limited information is available about the effect of Mre11 over-expression, previously deficiency for this protein has been shown to cause increased damage sensitivity and a profound deficiency in DSB repair in budding yeast (172). Taken together, these results indicate that decline in the level of key proteins during

aging results into a compromised genomic integrity and modulating the level of repair proteins in a balanced way to compensate the repair defect observed during aging may serve as a potential way to extend lifespan.

As mentioned earlier, a declining HR response with replicative age seems to be a more generalized phenomenon than previously assumed. Aging also serves as the greatest carcinogen, and in certain instances aging and cancer share common etiologies (76). An impaired HR response has long been associated with increased genomic instability to initiate carcinogenesis as well (173). While hypoxic microenvironment characteristic to cancer cells seems to accelerate genomic instability by reducing Rad51 expression, therefore also causing a reduction in HR efficiency (174), wide ranges of tumor tissues also show elevated Rad51 levels (171, 175), which seems somewhat counter-intuitive. In general genomic instability has a negative impact on cell growth. Therefore, over-expression of Rad51 may be assumed as a plausible counter-measure taken by tumor cells with HR defects to selectively overcome that defect, in order to re-gain genome stabilization as well as to experience a survival advantage as proposed by one earlier model (173). Additionally, Rad51 over-expression is also associated with a number of consequences including increased damage resistance. Over-expression of Rad51 possibly also contributes for the resistance to radiation therapy or DNA damage therapeutics for these tumors (171). Interestingly, in a similar fashion over-expression of Mre11 has also previously been shown to promote radioresistance via an enhanced DNA repair mechanism in breast cancer (176). How and where the

mechanisms behind these two outcomes, a declining HR response during aging and selective pressure of tumor cells to survive by overcoming that response diverge remains subject to further investigation, but all these evidence further emphasize that tightly controlled and balanced regulation of repair proteins is crucial for proper functioning of the cells.

With mitotic age progression, the available cellular resources become limiting, and problems start arising, including defective cohesion, defective DSB repair and inaccurate repair of DSB lesions within the rDNA, as we have uncovered here (**Fig. 26**). Eventually the limiting levels of repair factors and cohesin in old cells causes the accumulation of genomic damage, chromosomal rearrangements and potentially chromosome loss. We propose that eventually a threshold of genomic damage is crossed that is sensed by the cell to cause cell division to halt marking the end of the replicative lifecycle.

FUTURE DIRECTIONS

The major limitation in the field of aging research is that most of the time it depends on correlational analysis. This is because of the difficulty in performing functional analyses at the molecular level, the long length of the aging experiments especially when performed in larger eukaryotes and the complexity of aging biology with the potential influence of other environmental factors. There is still a great deal to learn about this highly complex biological process.

Development of new vertebrate and invertebrate model systems suitable for aging research such as African killifish and naked mole rats, and transformative

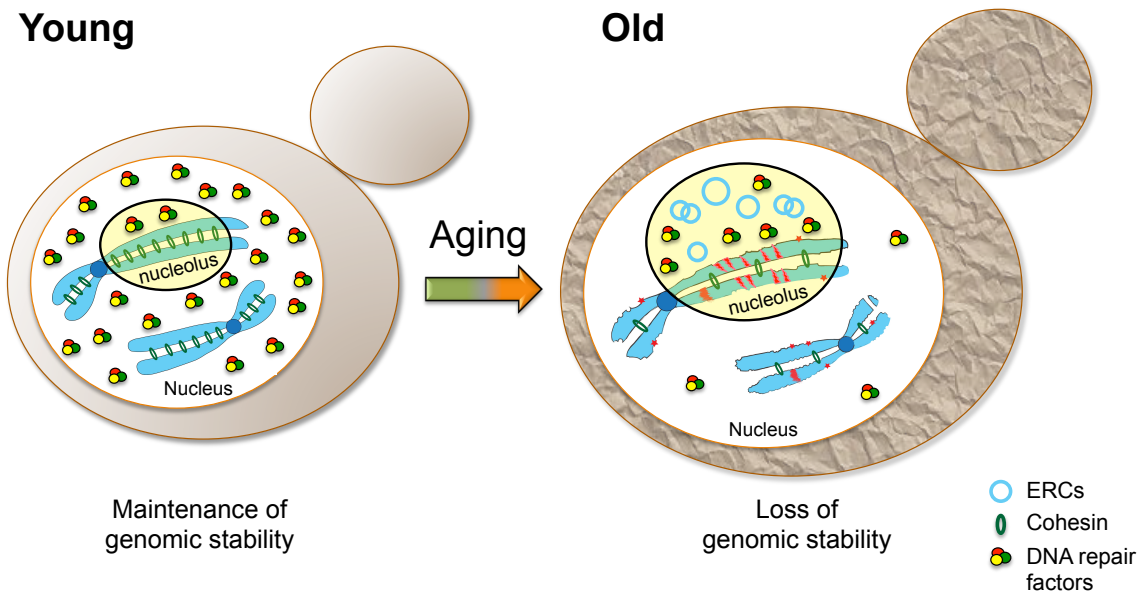


Figure 26. Working model for rDNA instability and reduced homologous recombination (HR) causing global genomic instability to limit lifespan.

genetic systems such as the MEP provide invaluable tools in this complex field of research. Similarly, development of microfluidic systems also enables researchers to perform broad genetic screens in a high throughput manner (98). Our current progress in knowledge and extensive utilization of next-generation sequencing techniques are further empowering us to analyze age-associated changes at ever increasing resolution. These developments, together with simultaneous gain-of-function or loss-of-function studies performed in different organisms, will enable the field eventually to comprehend the causal effects of certain pathways during aging, to move beyond correlation analyses.

The major body of research illustrated in this thesis work demonstrates how genomic stability is perturbed during aging. However, we are only starting to learn details about the molecular changes happening during aging, not relying solely on the correlative analyses. But there is still a lot to learn about the process and there are plenty of unanswered questions. I have observed altered levels of key repair proteins in aged yeast cells, but my analyses were restricted to only a few proteins. An unbiased approach may have been useful in understanding the full detailed picture, such as quantitative mass spectrometry. However, it needs to be kept in mind that aging cells undergo profound changes in all macromolecular levels. Therefore, use of proper normalization controls and cautious interpretation of results are extremely important to derive any logical conclusion. It will be also interesting to find out whether there are certain changes in cellular localization pattern of these repair proteins and if in aging cells whether these proteins are sequestered to regions with high fragility. As

mentioned earlier, a possible hypothesis suggests ERCs perturb cellular homeostasis during aging, potentially by sequestering factors that are required for efficient DSB repair. The use of a microfluidic platform coupled with high-resolution microscopy will be helpful in addressing this question by tracking large number of mother cells and following the events during aging. Specifically, it would be of interest to use a marker of induced DNA damage response, to visualize whether the DNA damage checkpoint is activated during the final replicative cell cycle.

Since extra-chromosomal circular DNAs similar to ERCs have been found in other organisms, and also in aged mammalian cells, another interesting aspect of study will be to illustrate the roles of these circular nucleic acids in aging, and also in other aspects, as we have very little knowledge about them. It will be also of interest to learn if loss of cohesion serves as a causal factor to the generation of these circular DNAs. As genomes of larger eukaryotes carry more repeat sequences than budding yeast, it is highly likely to happen beyond yeast also. In particular, it will be important to investigate the broader impact of loss of cohesion during aging and to determine if this can serve as a cue for the cells to end replicative lifespan. Analysis of cohesion using the GFP assay that I utilized here, coupled with microfluidic analyses of the RLS would enable it to be determined whether loss of cohesion was consistently apparent in the final cell division. To determine whether the loss of centromeric cohesion leads to aneuploidy during aging, chromosome loss assays should be performed during the yeast RLS to determine if there is a threshold of maternal age above which

chromosome loss and gain (aneuploidy) occurs, much like the threshold of age for loss of heterozygosity (LOH) observed by Dan Gottschling's laboratory previously. To further analyze whether loss of cohesion is the cause of aging, attempts should be made to overexpress cohesins, to see if they extend lifespan. I attempted to do this with Mcd1, but the cells did not tolerate the overexpression of Mcd1. Perhaps lower level expression could be attempted, to see if it will extend the yeast RLS. I also used a strain where cohesin was artificially driven to the rDNA via LacO arrays. Unfortunately, this shortened lifespan, presumably due to persistent chromosome cohesion throughout the cell cycle (data not shown). To determine the relevance of these studies beyond yeast, it will be of interest to determine whether there is a loss of cohesion during mitotic aging in other organisms, such as replicative senescence of human fibroblasts in culture, or hematopoietic stem cells.

In addition, as we have observed over-expression of certain DNA repair proteins could partially ameliorate certain aging phenotype, another interesting area of study will be to address what specific changes are associated with the compromised genome in aged cells and how over-expression of particular repair proteins helps to overcome those defects to possibly extend lifespan. It will also be interesting to know if it is a generalized response or only certain proteins involved in the DNA repair pathway may serve as the aging factors. In this context, it will be important to remember that many of the DNA repair factors are involved in interconnected pathways. Therefore, caution should be taken when

interpreting the results, to separate the effects of one pathway from the other and in certain contexts it may be very difficult to address.

As genome instability serves as a general hallmark of aging and there is evidence suggesting its conserved nature across different organisms, it will be also interesting to test whether the molecular reasons for genome instability uncovered in my analyses, such as cohesion loss, rDNA instability, and an impaired repair response are responsible for aging pathways in other eukaryotes as well. Besides the questions above, from a different approach, it will be interesting to find out whether specific lifespan-extending regimens influence this particular hallmark of aging to cause an extension of lifespan. For example, does calorie restriction or rapamycin treatment promote genomic stability? Calorie restriction is known to reduce ERC accumulation, and therefore it would be of interest to look at the integrity of chromosomes during aging upon calorie restriction, to provide further support for our hypotheses. However, any lifespan-extending regimen will likely have an impact on multiple pathways, making it difficult again to separate specific effects of individual pathways.

While some of the molecular changes observed during aging are categorized as causal factors of aging, there are other changes that simply accompany the aging process. While attempting to characterize the causes or consequences of aging, extreme cautions need to be taken, because most of the relevant pathways are interconnected with each other. It is thus necessary to establish the hierarchical relationship among the relevant pathways to

understand the causal network of aging in order to derive effective therapies to counteract the aging process and to impede age-induced complications.

APPENDIX 1: The commercial antibodies against histone H3 lysine 56 acetylation (H3K56Ac) are non-specific *in vitro* and in mammalian cells

This section is based upon our published work: **Pal, S.**, Graves, H., Ohsawa, R., Huang, T.H., Wang, P., Harmacek, L., and Tyler, J. (2016). The Commercial Antibodies Widely Used to Measure H3 K56 Acetylation Are Non-Specific in Human and Drosophila Cells. PLoS One 11, e0155409.

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INTRODUCTION:

Chromatin structure is the template for all different nuclear processes in all eukaryotes. The basic repeating unit of chromatin is called the nucleosome. It consists of approximately 147 base pairs of DNA wrapping two molecules of each of the histone proteins, H2A, H2B, H3, and H4 around 1.75 times (177). Nucleosomes are responsible for efficient packaging and protecting our entire

genome. Earlier studies carried out in budding yeast showed that the histone needs to be disassembled from chromatin structure for different processes such as transcription, DNA repair, and replication to occur (178, 179). Chromatin disassembly allows different other machineries mediating these genomic processes to gain proper access to the DNA to perform their function efficiently. The process of removal of histones from the DNA during these genomic events is, in part, understood from the work done in budding yeast. But this process requires the involvement of specific proteins called histone chaperones that bind to the histones in a stoichiometric manner to remove them from the DNA (180). Histone removal from the DNA is further facilitated by different post-translational modifications (PTMs) of histone proteins that potentially weaken histone-DNA interactions. One of such PTMs is acetylation of histone H3 lysine 56 (H3 K56Ac) (181-183). Although the process of histone removal has been described in budding yeast, it is yet to be determined whether similar mechanisms exist during these genomic processes in metazoans as well.

The functional study of histone PTMs is rather simplified in budding yeast, as there are only two copies for each histone genes and it is relatively easy to study the functional effect of any histone PTM by mutating the histone encoding genes. In sharp contrast, in metazoan cells, there are hundreds of genes encoding each of the canonical histones (184). Therefore, our current knowledge about histone PTMs in metazoan cells is mostly restricted to the correlation analysis those examine the genome-wide distribution of histone PTMs (185). However, these genome-wide analyses do not provide any functional studies of

the histone PTMs, and more importantly, such correlative analyses are largely dependent on the specificity of the antibodies used against a particular histone PTM.

Histone H3 K56Ac has been initially identified by mass spectrometry analysis in mammalian cells (186, 187). Acetylation at this site of histone H3 is catalyzed by the histone acetyl transferases (HATs) CBP and p300 in coordination with the histone chaperone Asf1 in humans. Chromatin immunoprecipitation (ChIP) analyses demonstrating the localization pattern of H3 K56Ac in stem cells showed this modification to be associated with the regulatory regions of different pluripotency genes (186), indicating a potential role of this PTM in transcriptional regulation. Since then, large number of studies implicated this PTM in transcriptional regulation in metazoan cells, mostly using commercial antibodies against H3 K56Ac, and showing H3 K56Ac to be localized at active promoters or enhancers in mammalian cells (186, 188-197). Of note, all newly synthesized histone H3 proteins in budding yeast are acetylated on H3 K56 residue. In contrast, in human cells the abundance of this PTM is exceptionally low. Mass spectrometry analysis suggests that only 0.03% of all histones carry this particular PTM (198). Because of this difference, it is not possible to directly apply the knowledge we gained from studies performed in budding yeast on humans, as it may be inaccurate considering the physiological difference. All these abovementioned studies imply possible roles H3 K56Ac play in transcriptional regulation in metazoan systems mostly based on the of its localization and/or distribution patterns. However, it is yet to be tested at the

functional level. Earlier, mutational analysis in budding yeast showed that H3 K56Ac promotes transcriptional activation in budding yeast by promoting chromatin disassembly by allowing histone proteins to be dissociated from promoter regions more readily (199). Based on all these evidence, we were interested to examine whether H3 K56Ac plays a similar role during transcription in mammalian cells. As there are certain concerns raised about the specificity of commercially available antibodies against H3 K56Ac (198), I rigorously tested the specificity of the available commercial antibodies against H3 K56Ac. During our analyses, I found that all of the commercially available H3 K56Ac antibodies showed non-specificity when tested *in vitro*, and also in mammalian cells, recognizing other acetylated lysine residues than lysine 56 on histone H3. Therefore, our study provides the proof that the studies being performed in mammalian cells using H3 K56Ac antibodies available commercially should be interpreted with caution.

MATERIALS AND METHODS

Dot blot

Lyophilized peptides were rehydrated in 1X PBS at a 300 μ M concentration and then diluted 10 fold, prior to spotting 10 μ l onto an activated PVDF membrane. The membrane was air-dried and then stained with amido black to verify the presence of the peptides. The membranes were washed in TBST and then blocked in 3% non-fat milk in TBST. The blots were incubated in primary

antibodies overnight at 4°C. The blots were washed and probed with specific HRP conjugated secondary antibodies at room temperature for 1 hr.

Western blot

Acid extracted histones were separated by SDS-PAGE, probed with the indicated antibodies in each figure. Where necessary, the secondary antibody IR dye 680RD Goat anti-Rabbit IgG (H + L) multiplexed with the IRDye® 800CW Goat anti-Mouse IgG (H + L) was used for the 2-color detection method by the Odyssey LI-COR imaging system. The H3 K56Ac antibodies used in this and other experiments are: Epitomics monoclonal Ab (2134–1), Epigentek polyclonal Ab (A-4026-050), Cell Signaling polyclonal Ab (4243S), Upstate polyclonal Ab (07–677), Active Motif polyclonal Ab (39281), Active Motif monoclonal Ab (61061). The other antibodies used are: N-terminal histone H3 mAb (Active Motif 39763), C-terminal histone H3 pAb (Abcam ab1791), IRDye 680 Goat anti-rabbit IgG (LICOR 926–32221) and IRDye 800CW Goat anti-mouse IgG (LICOR 926–32210).

Immunohistochemistry (IHC)

Slides containing paraffin embedded breast cancer tissue samples fixed in 10% neutral buffered formalin were obtained from the MD Anderson Pathology core and IHC method was optimized in assistance with the Pathology core. In short, the sections of the tissues were deparaffinized with xylene and rehydrated in a graded series of ethanol (two 100% ethanol wash, followed by 95% ethanol, 70%

ethanol, 50% ethanol, 30% ethanol, followed by water). Antigen retrieval was performed using heat-induced method following standard procedure. After blocking non-specific binding, the sections were incubated with primary antibody at indicated dilution (overnight, 4°C, humidified chamber) followed by incubation with HRP-conjugated secondary antibody (1 hr, room temperature). For signal detection, DAB substrate kit (Vector Laboratories) was used followed by counterstaining with Mayer's Hematoxylin (Sigma).

RESULTS AND DISCUSSION:

To ensure the specificity of the H3 K56Ac antibodies commercially available, I used multiple approaches and tested the specificity of this PTM rigorously both *in vitro* and in mammalian cells within the context of other cellular proteins, which is more similar to physiological setting. I first performed a dot blot analysis using purified peptides carrying specifically histone H3 K56Ac modification, but I simultaneously also used other purified acetylated peptides on other sites of histone H3, to ensure the specific binding of the antibody being tested. On the dot blot, several of the commercial antibodies (Epitomics, Epigentek, and Upstate) failed to recognize H3 K56Ac specifically and recognized one or more other acetylated lysine residues from N-terminus of H3 in addition to recognizing the peptide carrying H3 K56Ac (**Fig. 27**). From the dot blot analysis, it seemed that the more specific antibodies for H3 K56Ac were the antibodies from Cell Signaling and Active Motif (two antibodies from Active Motif,

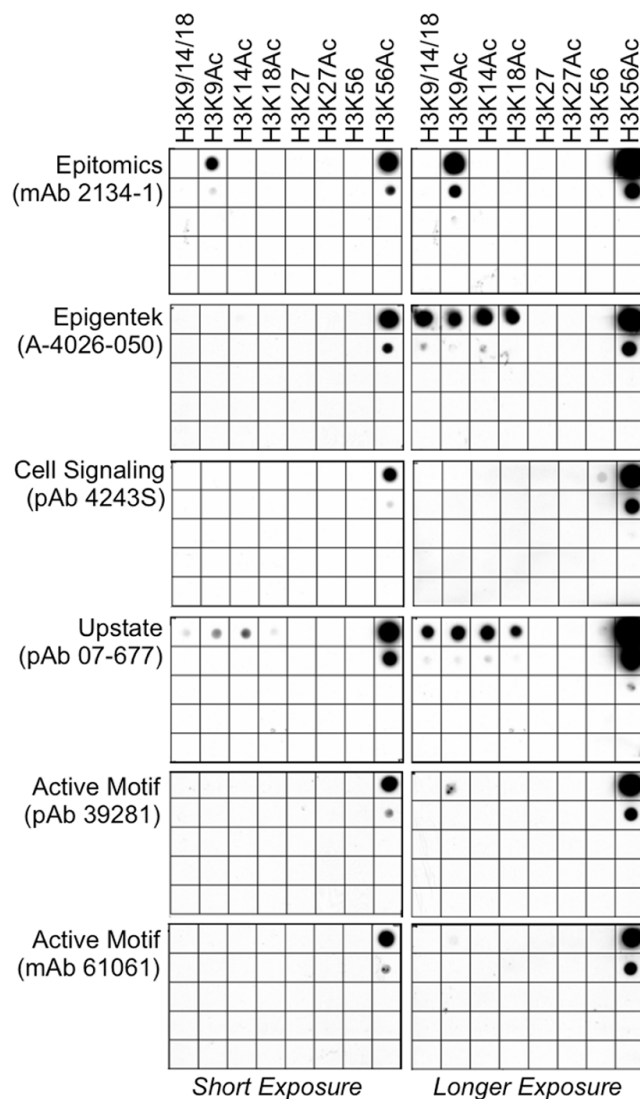


Figure 27. The commercial antibodies against histone H3 lysine 56 acetylation (H3K56Ac) are non-specific *in vitro*. Dot blot analysis of the indicated commercial H3 K56Ac antibodies, tested against the peptides indicated above. 10 μ l of each peptide at 300 μ M concentration was spotted on the top row, followed by 10 fold serial dilutions below. A short and longer exposure are shown.

one monoclonal, and one polyclonal). Therefore, we continued using these antibodies for our next level of specificity test in mammalian cells.

Next, I analyzed the specificity of the antibodies in more physiologically relevant condition that is in a crude protein extract. In a crude protein extract, all the acetylated lysine residues would exist in physiologically relevant ratios as similar to the level normally found inside the cell. To test the specificity of the H3 K56Ac antibodies mentioned above, I used mammalian H1299 and 293T cell lines carrying stably integrated FLAG tagged H3.1 (200), carrying either a wild type lysine (K) or lysine 56 (K56) mutated to arginine (R) to prevent acetylation at this specific site. As the FLAG-tagged H3 expression level is only about 5% of the level of endogenous histones, for this analysis I used acid extracted histones to isolate enough amount of protein and to enrich for histone proteins to achieve the level of detection for the FLAG-tagged H3 by western blot. Here in this analysis, I included wild type 293T cells not expressing any tagged histones also as a control, to ensure that the H3 K56Ac antibody was recognizing the right band, and not just a background band. Both the histone H3 antibody and the H3 K56Ac antibodies being tested could recognize full length FLAG-tagged H3 (**Fig. 28**). Interestingly, I observed that all of the commercial H3 K56Ac antibodies being tested here (Active Motif polyclonal, Active Motif monoclonal, Cell Signaling, and Epitomics) could recognize the mutated residue (H3 K56R-FLAG tagged protein) almost to the same efficiency as the wild type H3-FLAG (**Fig. 28**). As K56R mutant is deficient in its ability to be acetylated on this residue, this result indicates that all of these H3 K56Ac antibodies could recognize either

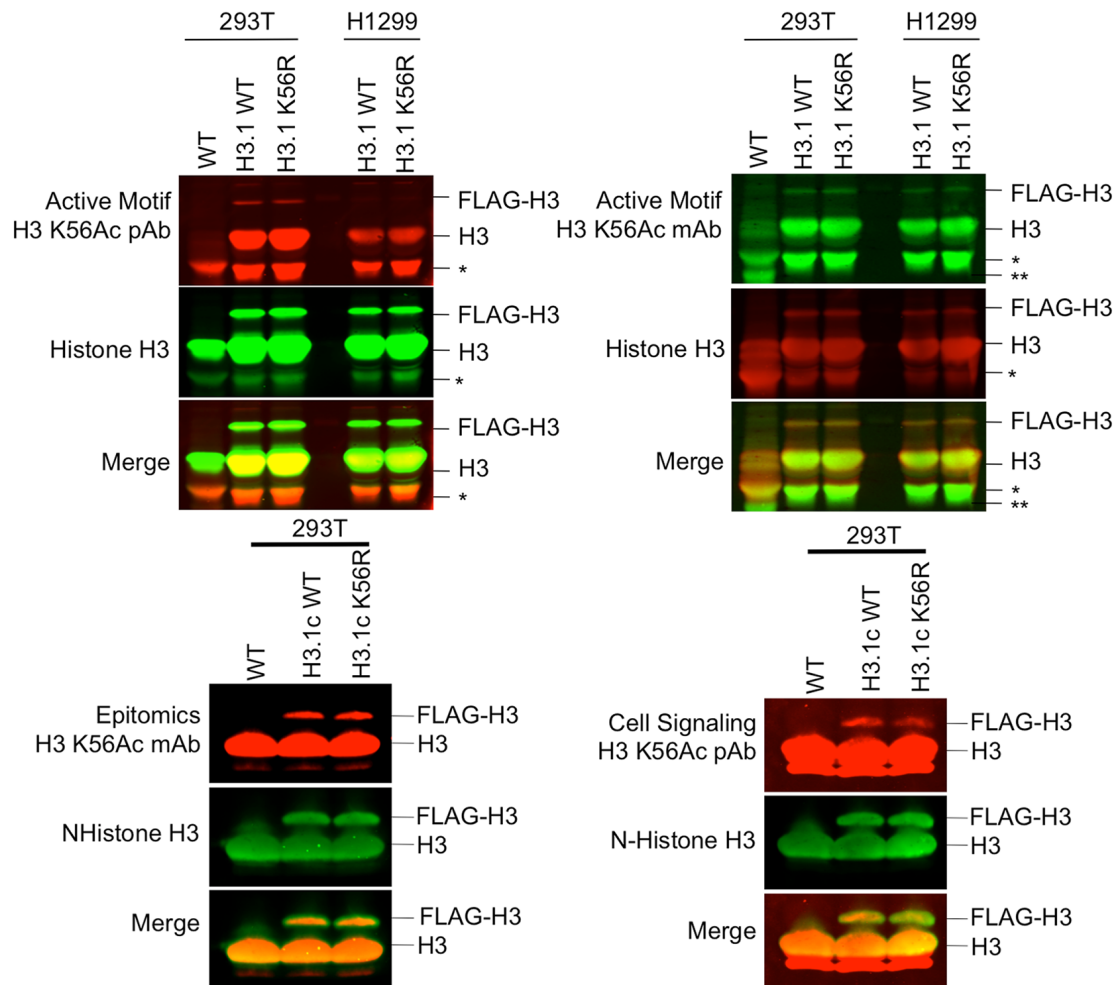


Figure 28. The commercial antibodies against histone H3 lysine 56 acetylation (H3K56Ac) are non-specific when tested in mammalian cells.

Western blot analysis of acid extracted histones from 293 cells that were wild type (WT) or stably expressed H3.1c-FLAG or H3.1c K56R-FLAG, or from H1299 cells that stably expressed H3.1c-FLAG or H3.1c K56R-FLAG. * indicates a proteolytic degradation product of H3 generated by cleavage of the N-terminus that occurs upon generating the total protein extracts. ** indicates a smaller H3 degradation product seen in the wild type sample only, due to possible over

handling of the extract. Data is shown for the Active Motif, the Epitomics and the Cell signalling H3 K56Ac antibodies. 75 micrograms of total protein extract was loaded for each lane, and western blotted with the indicated antibodies, followed by detection with infrared antibodies on a Licor Odyssey machine.

histone H3 or other acetylated residues on histone H3 in the absence of H3 K56Ac. In order to eliminate the possibility that this non-specificity shown by these antibodies was not due to excess antibody concentration being used, I tried different dilutions of these antibodies to find an effective dilution at which these antibodies would show specificity for H3 K56Ac residue. But for all the dilutions I tested, all of these antibodies recognized both wild type H3-FLAG and H3 K56R-FLAG almost to an equal level.

Because the Active Motif monoclonal antibody was the most recently developed commercial antibody and it was supposed to be a specific antibody for the application of ChIP, for which the success of the application depends on the ability of the antibody to recognize the native epitopes, I wanted to test the specificity of this antibody for recognizing native epitopes but for the application of immunohistochemistry (IHC), which utilizes a similar mode of recognition following similar fixation approach (**Fig. 29A, B**). Interestingly, I found this antibody to recognize significant amounts of cytoplasmic proteins, even at high dilutions (**Fig. 29B**), further confirming its non-specificity as occurrence of H3 K56Ac is presumably mostly restricted to the nucleus given that this event is mediated by p300 and CBP (187). We also further confirmed the non-specific nature of these antibodies in an *in vivo* model using flies (201).

Taken together, all these data indicate that at least in my analyses, I could not achieve enough specificity for any of the commercially available H3 K56Ac antibodies, via dot blot, western analyses, or in certain instances IHC application. The non-specificity conferred by these available H3 K56Ac antibodies would

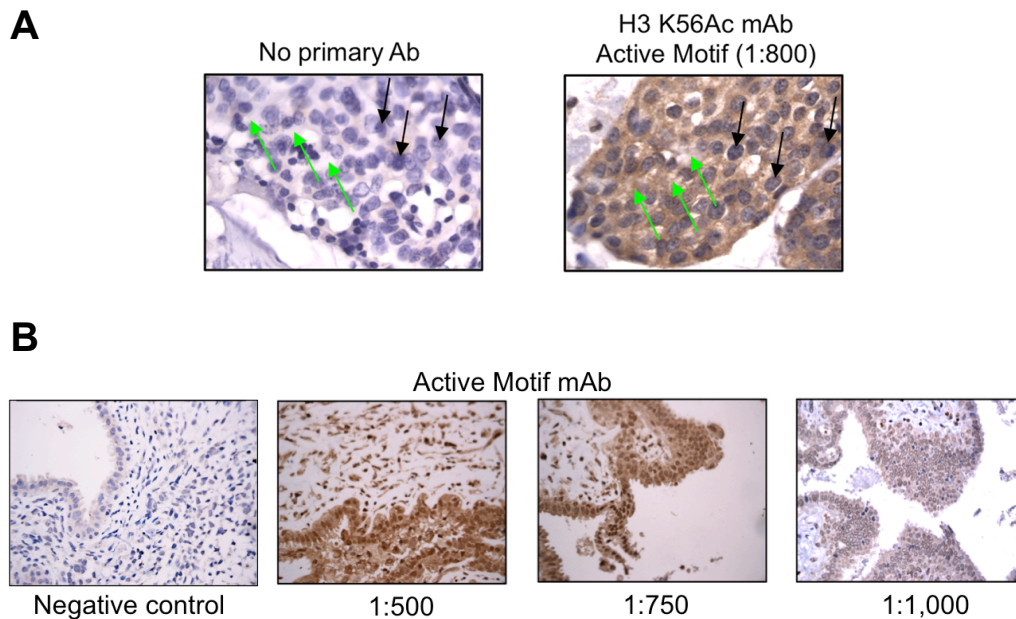


Figure 29. A commercial antibody against histone H3 lysine 56 acetylation (H3K56Ac) showing specificity *in vitro* is non-specific when tested in breast cancer tissue. (A) Immunohistochemistry (IHC) analysis of the Active Motif monoclonal antibody, on breast cancer tissue samples shows non-specific cytoplasmic staining instead of distinct nuclear staining. Green arrows point to cytoplasm and black arrows point to nuclei. Both slides are also stained with haematoxylin and eosin (H&E) stain, which stains nuclei blue and eosinophilic structures pink. (B) IHC analysis of breast cancer tissue as (A), using either no primary antibody or other indicated dilutions of the antibody being tested here.

possibly explain the reason for the confusion for identifying the responsible histone deacetylase (HDAC) in mammalian cells, because SIRT1, SIRT2, SIRT6, HDAC1, and HDAC2 each have been reported as the potential H3 K56Ac HDAC (187, 202-210). Furthermore, the non-specificity of these antibodies could also explain the contradictory findings of the occurrence of H3 K56Ac during different cellular events such as during the cell cycle and DNA repair (187, 200, 202, 211-218). Therefore, data obtained with commercially available H3 K56Ac antibodies in metazoan cells, is likely due to its ability to recognize additional acetylated lysine residues on histone H3, and should be interpreted with utmost caution.

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Vita

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