THE RETROTRANSPOSON TY1 EXTENDS CHRONOLOGICAL LIFESPAN IN *SACCHAROMYCES PARADOXUS* BY INFLUENCING CELLULAR RESPONSES TO STRESS

by

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A Thesis Submitted to the Graduate
Faculty of Rensselaer Polytechnic Institute
in Partial Fulfillment of the
Requirements for the degree of
DOCTOR OF PHILOSOPHY
Major Subject: BIOLOGY

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April 2015
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ACKNOWLEDGEMENTS

This work could not have been conducted or completed without the incredible amount of support, guidance, and assistance that I received from countless individuals. My first acknowledgement has to be to my doctoral advisor, Dr. Patrick Maxwell, whose time and patience was greatly appreciated for someone who had been away from the lab bench for quite some time when I began this journey. Your support and guidance allowed this dissertation to be completed and it has been an honor working under you.

Secondly I would like to thank the additional members of my doctoral committee members: Dr. Cynthia Collins, Dr. Douglas Conklin, and Dr. Susan Gilbert. The encouragement and support I received allowed me to push through the rigors and struggles encountered with scientific research. With the support of my committee, I took ownership of my project and was able to do research that I found exciting and added meaningful knowledge to the scientific community.

I would be remiss not to acknowledge the other members of the Maxwell lab group. First I need to acknowledge Melissa Paterson who always offered insightful suggestions to this work, adeptly observing events that may have been overlooked. Your advice with experimental design and keen knowledge of laboratory protocols enabled us to overcome challenges we were troubleshooting. Secondly I wish to acknowledge Alison (Kenny) Scannapieco for keeping the lab afloat and the necessary relief from study. I had many students that worked alongside me during this study that I would like to acknowledge. Most important was my then-undergrad Meredith Giblin, who helped with some of the published data. I wish you luck as you continue your education and quest to become your own scientist. Lastly, Drew Pfeifer and Emily Ross for being an integral part of the Maxwell lab.

I would also like to acknowledge friends and past members of the CBIS community. I need to especially than Clay Albrecht, Brandon Bensel, Teri Hreha, Chris Connors, and Kristien Van Vlasselaer. You all are my RPI “Band of Brothers” and I am glad to have gone through this journey with you all. They have understood my frustrations, celebrated my successes, and supported my family and interests.

Ultimately I need to thank my family and friends. Mom and Dad, thanks for always pushing me to achieve my best and to encourage me on my crazy, worldwide adventures. I am the person I am today because of the way you raised me. Most importantly I need to thank my wonderful wife and partner Hiromi Kunisada-VanHoute. She has been there from the start, from our move from Brooklyn up till now. I look forward to having you by my side again as we begin our next chapter. Your love and support guided me through this process and I appreciate the sacrifices you had to make. In the last 5 years two big changes occurred: we got married in Japan and we had our first son, Kai. With that I need to dedicate this dissertation to the both of them. I can’t imagine my life without you two. Every morning Kai you brighten my day and your continued curiosity helps to drive my curiosity in my scientific career. I am so thankful to have you both by my side.

ABSTRACT

Advancing age of the global population is resulting in an increase in the accumulation of age-related pathologies. Many potential molecular and cellular causes of aging are still being debated. Endogenous retroelements are a class of mobile DNA elements that have coexisted in the genomes of virtually every eukaryotic organism for much of evolution. Recent work indicates that retrotransposons are active in advanced age but their impact on aging has not been explored in detail. Evidence is accumulating to suggest that retrotransposon-mediated events are a potential source of genomic change that can influence aging. In this study I took advantage of a unique yeast model system to characterize aging in cells with or without Ty1 retroelements. I identify here a novel role of retroelements in extending yeast chronological lifespan in certain media conditions, rather than observing an anticipated pro-aging effect. This effect was correlated with changes in factors known to influence aging such as mitochondrial function, reactive oxygen species, and sensitivity to a chemical inhibitor of nutrient sensing pathways. These results demonstrate that the presence of these retroelements have unforeseen direct or indirect roles in influencing cellular processes relevant to lifespan. The ability of retrotransposons to regulate cellular functions has not been well investigated, in contrast to the ability of cellular pathways to regulate retrotransposons. The work here broadens our perspective on the role retrotransposons have in host cells to influence certain aspects of eukaryotic aging.

Chapter 1

1. INTRODUCTION

1.1. Significance

1.1.1. Aging and disease

The functional decline of tissue and cellular function is considered to be the phenotypic outcome of the aging process. The conventional wisdom is that cells and tissues accumulate damage to important macromolecules necessary to perform their respective functions, and that this results in aging. While experimental models have elucidated changes that can slow down the aging process, there is still much we do not understand about aging. Specifically it is still unclear whether a single factor or mechanism, such as DNA damage and genomic instability, is primarily responsible for the aging process. Recently, a variety of molecular and cellular factors associated with aging have been reviewed to broaden our perspective on aging. For example, is cellular dysfunction at the level of organelles, chromosomes as a whole, interactions with the extracellular matrix, cell-cell signaling, etc., more critical to aging than the total accumulation of damage to macromolecules [1]? The variety of cellular processes thought to contribute to aging increases the complexity of investigating this topic.

By 2050, the U.S. population over 65 years of age is expected to represent 19%

of the total U.S. population, or just over 81 million persons [2]. While modern medicine and health practices have substantially improved life expectancy worldwide, the incidence of age-associated diseases has also increased. Specifically the incidence of cancer, neurodegenerative diseases such as Alzheimer's, and cardiac-related deaths in the U.S. are expected to represent 22.2%, 4.2%, and 28.2% of all deaths, respectively [3]. Thus approximately half of all deaths will be influenced by age-associated changes. Studying the molecular and cellular factors that contribute to aging may lead to improved treatments of age-associated diseases and interventions that promote healthy aging in the near future.

Multiple models have been used to explore the nature of organismal aging, and a recent review of studies focused on mammals led to nine proposed hallmarks of aging: genomic instability, telomere attrition, epigenetic alterations, unregulated nutrient sensing, stem cell depletion, senescence, loss of proteostasis, impaired mitochondrial function, and altered cell-to-cell communication [4]. All of the hallmarks are associated with aging but it remains to be established whether these changes are primary drivers of aging or merely correlated with changes to lifespan. Generally more than one of these factors needs to be changed prior to observing a change to lifespan. While there is ample evidence to support these factors as drivers of aging, there is insufficient data to make a definitive conclusion. The complex nature of organismal aging is generally a result of an accumulation of one or more of these factors.

One of the well-established theories in aging is the “Somatic Mutation Theory of Aging” [5,6]. This theory suggests that aging results from the accumulation of damage to DNA or larger chromosomal aberrations. Associated with this theory is the “Free Radical Theory of Aging” proposed by Harlan in 1956 based on the idea that reactive oxygen species (ROS) generation leads to intracellular damage of important macromolecules and that this accumulation could lead to aging phenotypes [7–10]. Three of the nine hallmarks form the basis of theory: genomic instability, telomere attrition, and stem cell dysfunction. An alternative viewpoint is that malfunction due to hyperstimulation of cellular pathways is a main driver of organismal aging [1,11], which was first proposed by Blagosklonny et al. [12]. “The Hyperfunction Theory of Aging” suggests that overstimulation of pathways that were once beneficial early in life are detrimental later in life and lead to late-life pathologies. For example, rapid growth and reproduction early in life can be beneficial, but hyperstimulation of growth pathways late in life may lead to diseases associated with aging. Many of the examples to support this theory involve the activity of conserved nutrient-signaling pathways, and perturbations to these pathways have age-related consequences. Hence, many of the other hallmarks of aging (6/9) perhaps fit better under the umbrella of hyperfunction theory of aging. Caloric restriction (CR) can extend lifespan in multiple organismal and cellular models suggesting that inhibition of nutrient signaling can have beneficial effects for aging and supporting the idea that hyperfunction of certain cellular pathways does affect the aging process. From

the perspective of the damage-based theories, reduced nutrients would limit growth and stimulate somatic maintenance through decreased damage and elevated cellular repair/maintenance activities to promote lifespan. It might be thought that an excess of calories would provide ample energy for energy-intensive maintenance functions, but growth signaling might shift cellular resources away from maintenance functions. From the hyperfunction theory perspective, limited nutrients would reduce inappropriate biosynthesis and cellular signaling with age to reduce cellular dysfunction, regardless of the cellular investment in maintenance functions [1]. It is important to note that neither of these theories alone will necessarily account for all aspects of aging, and many points of the hyperfunction and somatic mutation theories of aging converge on similar hallmarks. Hyperfunction of critical pathways can lead to more cellular damage while certain mutations may lead to hyperfunction of critical age-associated pathways, and thus there is still a debate as to the true driver of aging in higher organisms.

1.1.1.1. Primary sources of cellular damage and genomic instability

Molecular damage associated with aging includes oxidative damage, genomic instability, chromatin changes that alter epigenetic regulation, and loss of proteostasis. While there is much controversy regarding the exact role ROS may have on the aging process, excessive damage to all macromolecules can occur due to ROS. ROS are generated as by-products of cellular respiration and other cellular processes. Many studies demonstrate increases in ROS and oxidative

damage with age in multiple models and that decreasing ROS can increase lifespan [13,14]. However, genetic manipulations to increase ROS production or overexpression of antioxidant defenses doesn't always produce expected changes in lifespan, so the role ROS play in the aging process has been questioned [15–18]. For example, certain studies have shown that deactivation of protective catalases, which break down hydrogen peroxide, can have lifespan extending effects in yeast although there is an increase in oxidative damage [19]. Paradoxically as well, the shorter lifespans of mutant yeast deficient for mitochondrial superoxide dismutase is not the result of increases in oxidative damage[19]. Furthermore, long-lived naked mole rats have 10 times the lifespan of mice and rats yet have consistently higher levels of oxidative damage [20,21]. In fact, while there is ample evidence that ROS can contribute to aging; a study involving *C. elegans* showed that elevated ROS levels actually extended lifespan, despite elevated oxidative damage levels were elevated, even with all 5 *sod* genes removed [22,23]. Similar results were also found in mice when care was taken in the genetic backgrounds, elevating antioxidant defenses (catalase, cytosolic Cu/ZnSOD, mt-MnSOD) still led to normal lifespans [24,25]. This leads to the idea that mitochondrial ROS may not be a strong driver of aging under the "Somatic Mutation Theory of Aging". If the appropriate ROS levels are maintained, where damage is limited, ROS may have a more important role in stress signaling pathways that promote lifespan. Coincidently, cells should be capable of dealing with the occasional rampant increase in ROS levels through antioxidant defense mechanisms. Thus the longstanding view that oxidative

damage promotes the aging process is in need of re-evaluation, and it appears that ROS levels and mitochondrial health and biogenesis have a much more complex role in the aging process of cells.

Genomic instability encompasses changes to nuclear and mitochondrial DNA, nuclear architecture, chromosomal rearrangements and translocations, telomere attrition, and genetic change due to incorporation of genetic material from viruses and mobile DNA elements [4]. Many reports have documented that aging cells and tissues have increased genomic damage and genomic instability. Cells are under constant attack from endogenous and exogenous sources of DNA damage, so genomes are in a constant flux between damage and repair. Unrepaired or incorrectly repaired DNA damage can have severe downstream effects for the health of the cell and surrounding tissue. These downstream effects include phenotypic changes, apoptosis, cell-cycle arrest, senescence, malignancy, and the depletion of stem cell pools [26]. Large scale chromosomal rearrangements or the simplest of point mutations have the propensity to cause abnormalities that left unrepaired can result in phenotypic changes or lead to apoptosis or senescence, further depleting the regenerative capacity for tissue repair [27,28]. The amount of damage accrued per day is astonishing. For example, it is estimated that 50,000-200,000 apurinic/apyrimidic (AP) sites caused naturally or through repair mechanisms after damage by ROS and 20,000-40,000 spontaneous DNA single-strand breaks (SSBs) occur daily in human cells, based on factors such as diet, age, and lifestyle [29]. Accelerated-aging

disorders known as progerias support genomic instability as a driver of aging. While these disorders, such as Werner Syndrome and Hutchinson's-Gilford Progeroid Syndrome (HGPS), are classified as "accelerated aging diseases" it is important to note that only some characteristics of aging are accelerated in progerias. These disorders generally result from mutations in one of a small set of genes that play roles in DNA replication, DNA repair, and nuclear architecture, which lead to large-scale genomic instability and shortened lifespan in affected individuals. The *LMNA* gene codes for the lamin A protein that is part of the nuclear lamina, which has roles in tethering chromatin and genome maintenance, and mutations in *LMNA* that lead to a dominant negative form of the lamin A protein (progerin) result in HGPS. Progerin disrupts nuclear architecture and DNA repair, and general decline in genome maintenance by the nuclear lamina has also been implicated in the normal aging process [30,31]. Cao et al. showed that telomere dysfunction during aging can cause an increase in progerin in aging fibroblasts, and lamin B1 levels, another lamin protein, have been shown to decrease in aging cells approaching senescence [32–35]. Conversely, inhibiting progerin levels can help prevent age-onset disorders in progeroid mouse models [36,37]. As the respiratory control centers, mitochondrial harbor extreme oxidative environments that can result in damage to mtDNA through the increased production of ROS. Additionally, mtDNA lack the protective properties of histones afforded to nuclear DNA. As a result mutations and damage to mitochondrial DNA is thought to contribute to the aging process [38].

The Hayflick limit is the maximum proliferative capacity of replicative cells, and results from the attrition of the protective caps on chromosomes known as telomeres [39]. Human somatic cells lack the regenerative polymerase telomerase that is responsible for telomere maintenance and thus after each successive round of DNA replication, telomere length decreases in these cells. Telomere maintenance is known to influence the aging process in humans and mice, and ectopic expression of telomerase is enough to allow some cell types to replicate indefinitely [40]. Additionally, there is evidence that telomere shortening is accelerated by mitochondrial dysfunction, potentially linking the damaging effects of ROS to telomere erosion [41,42]. Telomeres are more prone to oxidative damage due to the high frequency of guanine triplets [42] and persistent single-stranded or double-stranded breaks due to ROS may lead to premature senescence. Animal studies have elucidated a causal link between telomere loss and cellular senescence, and thus to a reduction in replicative regeneration during aging [43–46]. More interestingly and quite recently, Boonekamp and colleagues demonstrated a strong relation between telomere length and mortality that further supports the role of telomeres in the aging process [47].

Proper function and folding of proteins and degradation of old or misfolded proteins are essential for a cell to perform its intended duties. The accumulation of misfolded or non-functioning proteins has been linked to age-associated changes within cells [48]. Late-onset degenerative diseases such as Alzheimer's

and Parkinson's result from the aggregation of protein products. Chaperone proteins have important roles in protein folding, and studies have shown that there are declines in chaperone proteins during aging that can contribute to an imbalance in proteostasis. Conversely, overexpression of chaperone proteins in worms and flies has been shown to extend the longevity in these organisms [49,50]. The activation of heat shock transcription factor HSF-1 in nematodes increases longevity and thermotolerance [51]. Proper proteolytic recycling is the responsibility of two protein degradation systems, the autophagy-lysosome system and the ubiquitin-proteasome system, and both decline with age [52,53]. Autophagy occurs within lysosomes to degrade old, damaged, defective, aggregate proteins and defective autophagy mechanisms are implicated to reduce lifespan [54]. Work in aged rat livers showed a decline in autophagy and a subsequent increase in damage within these tissues [55]. Rapamycin treatment increases lifespan in yeast, worms, and flies at least in part due to stimulation of autophagy, while stimulation of the mTOR signaling pathway represses it, but as of yet, this specific example of rapamycin inducing autophagy however has not been demonstrated in mammal models [52,56]. The effects of CR also have implications on autophagy systems. CR-mediated longevity involves an upregulation of autophagy pathways in yeast, worms, and rat models [57,58]. Other studies suggest important links between chaperone mediated autophagy and aging. In human fibroblast, instability of the lysosome autophagy mediated protein LAMP2a, a protein in the lysosome membrane, results in failure to transport tagged cargo to the lysosome due to instability of LAMP2a and not a

reduction in transcription [54,59]. In mice, overexpression of LAMP2a retards age-associated declines in autophagy [52,53]. Taken together, not only are properly functioning macromolecules necessary for normal lifespan but also their proper maintenance, recycling, and degradation in determining healthy lifespan.

Chromatin and chromatin-associated proteins contribute to epigenetic gene regulation, and chromatin changes may influence the aging process. The basic structure of chromatin is the organization of DNA into nucleosomes. This is achieved by wrapping DNA around highly conserved histone octamer core proteins: H2A, H2B, H3, H4. These can be packaged further into highly complex and dense structures, all the way up to fully condensed chromosomes present during cell division. There are two types of chromatin: euchromatin and heterochromatin. Euchromatin is associated with transcriptionally active gene regions and has “open” epigenetic marks that make it accessible to the transcriptional machinery. Heterochromatin is typically associated with gene-poor regions and “repressive” epigenetic marks [4]. Silent regions of the genome typically are hypermethylated. Changes in methylation/acetylation patterns of histones or other chromatin-associated proteins have shown to be strong influencers of aging in mammalian cells [60]. Many of these marks consist of posttranslational modifications of lysine residues in the N-terminal tails of core histone proteins. Specifically, increased histone H4K16 acetylation, H2K20 and H3K4 trimethylation, or decreased H3K9 methylation or H3K27 trimethylation are known age-related epigenetic marks, normally activating or repressing

respectively [61,62]. These chromatin-remodeling processes are controlled by a multitude of histone methylases, acetylases, demethylases, and DNA methyltransferase proteins. In mice, histone acetylation decreased while histone methylation increased in cochlea during aging [63]. Mouse brains were shown to have high levels of deacetylation of H4K12 in the hippocampus in old tissues compared with younger tissue and this was correlated to a decline in cognitive abilities [64]. Conversely, treatment with deacetylase inhibitors reversed the effect in aged mice.

Sir2, a well-studied NAD-dependent protein deacetylase for lysine residues on the amino-terminal tails of histones H3 and H4, has strong lifespan extending effects in yeast, worms, and flies. This established a role for the sirtuin family of deacetylases and ADP ribosyltransferases in the aging process [65,66]. *SIRT1*, the mammalian homolog to the invertebrate *Sir2*, has not shown the same lifespan extending effects, but can improve genomic integrity and enhance metabolic activity in embryonic stem cells in mice [67,68]. Mutant mice deficient in another mammalian sirtuin, *SIRT6*, did show signs of accelerated aging [69]. In another study, it was shown that *SIRT6* had a role in repressing heterochromatin regions, and that overexpression of *SIRT6* could extend lifespan *in vitro* [70]. Furthermore *SIRT3* can improve replicative capacity in aged stem cells in mice [71]. So while the lifespan enhancing effects of the sirtuin family of proteins is more evident in invertebrates, at least three sirtuin proteins in mammals have age-related influences.

Additional chromatin changes besides changes in posttranslational modifications of histones are associated with aging. Aged cells or pathological cells from primary dermal fibroblasts show diminished capacity for chromatin remodeling [72]. Also, heterochromatin loss or redistribution occurs with age. Overexpression of heterochromatin remodeling complexes in fruit flies resulted in enhanced longevity and a delay in certain age-associated dystrophies [73–75]. A loss of histones in heterochromatic regions or decreases in histone proteins are known to occur in aging yeast, as well as some important histone chaperone proteins like Asf1 [66,76]. A loss of histone proteins has also been observed in senescent cells in human fibroblasts [77]. Interestingly, heterochromatic regions of chromosomes associate with lamin proteins of the nuclear lamina. Association of heterochromatin with the nuclear lamina is altered with age [78]. Silent heterochromatin regions become more transcriptionally active as they disassociate from the nuclear lamina during the aging process [79]. Greater transcriptional activity in heterochromatin could result in elevated expression of mobile DNA elements, which are frequently present in heterochromatin. Activation of mobile DNA elements could then result in genomic instability. From a therapeutic perspective, epigenetic modifications are in theory reversible, and therefore might prove easier to correct than problems resulting from accumulation of mutations. Treatments with deacetylase inhibitors prevented memory impairment in mice and inhibition of histone acetyltransferases helped to promote lifespan in progeroid mice models. While the influences of chromatin

remodeling is most likely much more complex in humans, age-related changes to chromatin in multiple invertebrate and mammalian systems supports the idea that chromatin structure has a strong influence on the aging process.

Both the “Free Radical Theory and Somatic Mutation Theory of Aging” propose that accumulation of macromolecular damage drives aging. In contrast, the hyperfunction theory of aging proposes that inappropriate activities of cellular signaling pathways drive aging, and that macromolecular damage does not reach levels necessary to directly limit lifespan. In support of the hyperfunction theory, it has been argued that frequency of changes to the nuclear genome may be too low in somatic cells to cause aging [80]. Some of the longevity promoting effects of CR do not correlate with decreased accumulation of genomic damage [81–83]. It has already been noted that levels of oxidative damage are not always correlated with changes in lifespan. Furthermore, epigenetic and transcription profiles are known to change in aging populations. It could be argued that changes to one single gene in DNA maintenance or oxidative protection would be inefficient to rescue an aging phenotype alone [84,85]. Instead it is the coordinated effort of multiple pathways and transcriptional changes that are needed to rescue an aging phenotype. Decreasing protein synthesis by reducing activity of growth signaling pathways has been shown to increase lifespan, and decreased protein translation could result in improved proteostasis and higher levels of recycling of cellular components via autophagy mechanisms. From the perspective of the “hyperfunction theory of aging”,

damage to macromolecules is correlated with aging but not the primary driver of aging.

1.1.1.2. Cellular level dysfunction during aging

De-regulated nutrient signaling, mitochondrial dysfunction, increased cellular senescence, stem cell depletion, and altered cell-to-cell communication are hallmarks of aging that could be considered consequences of damage to macromolecules or that could be considered to be primary drivers of aging independent of accumulated damage. Intercellular nutrient signaling pathways involved in the somatotrophic axis are the most evolutionarily conserved age-controlling pathway and affect multiple downstream targets that have conserved roles in aging [4]. Insulin and insulin-like growth factor (IGF/IGF-1) enable mammalian cells to sense and respond to nutrient levels by activating transcription of genes responsible for promoting growth and development at the expense of potential increases in genomic damage. Inhibiting expression of these

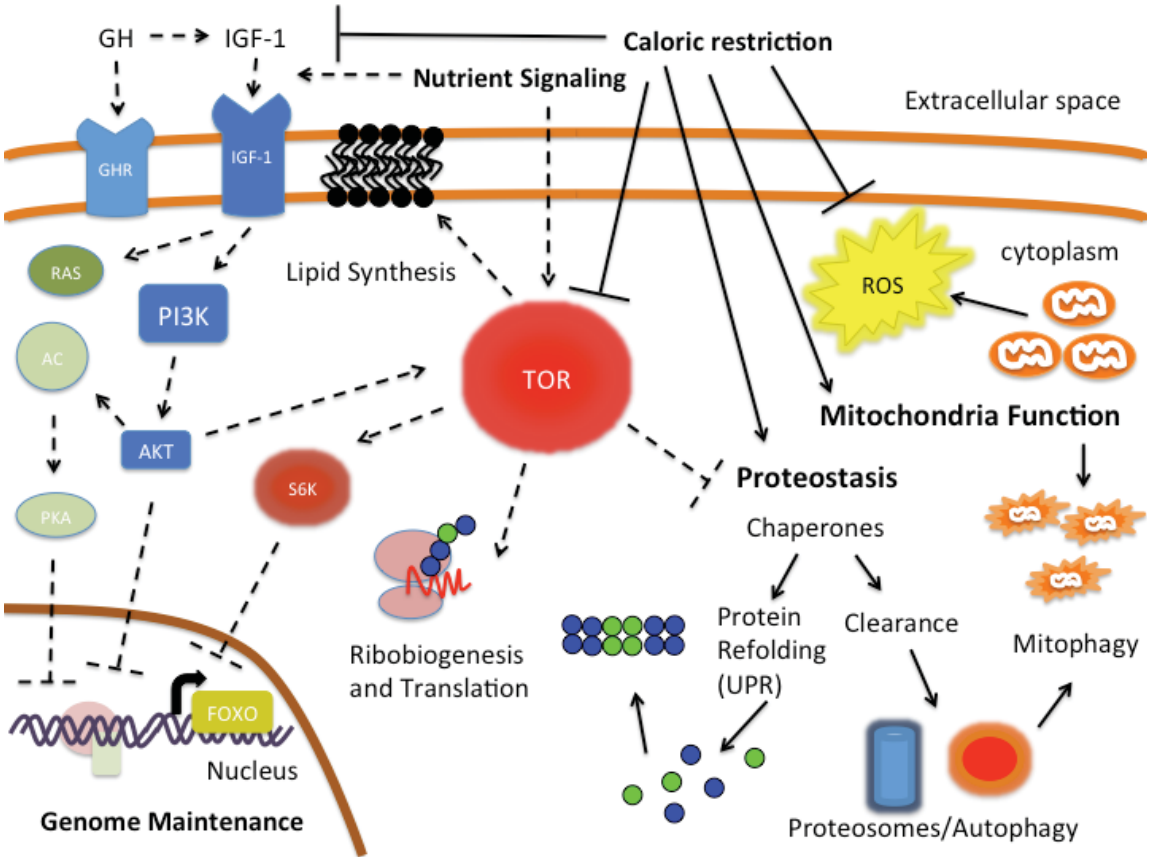

Figure 1.1. Unregulated nutrient signaling leads to cellular changes that influence aging

Caloric restriction is known to promote lifespan (solid lines and "T" bars) by inducing proper mitochondrial function by limiting excessive ROS generation and stimulating the activation of mitochondria-specific autophagy pathways (mitophagy). Inhibition of nutrient signaling pathways also induces proper proteostasis. The upregulation of the unfolded protein response (UPR) to maintain protein folding, stimulating the recycling of cellular components via autophagy, or simple clearance of worn-out, aged, or aggregated cellular components in proteosomes promotes healthspan under CR. Nutrient signaling inhibition influences aging that is highly conserved among eukaryotes. In mammals, this is mediated mainly by inhibition (solid "T" bar) of target of rapamycin (mTOR) and growth hormone (GH)/insulin and insulin-like growth factor (IGF-1). Inhibition of these nutrient-signaling pathways results in upregulation of stress response transcription factors; including the forkhead family of transcription factors (FOXO), to induce transcription of stress response genes. In this manner the upregulation of stress response genes facilitates to protect the genome from age-dependent instability. In non-CR conditions (dashed lines and "T" bars), nutrient signaling is upregulated to promote rapid growth and development. This "hyperfunction" condition is believed to inhibit stress protective measures to proteostasis, genome maintenance, proper mitochondrial function and ROS generation to limit lifespan.

pathways has been shown to retard aging at multiple levels. Downstream effectors that influence lifespan include the FOXO group of transcription factors in worms and flies, but the results in mice models are still unclear, although

mouse *FOXO1* is required for the tumor-suppressor effect of CR [86–88]. However, mice knockouts to growth hormone receptor are longer-lived [89]. Three other nutrient signaling pathways known to influence lifespan sense amino acid concentrations (mTOR), low energy status detected through high AMP levels (AMPK) or low NAD^+ levels (sirtuins) [4]. mTOR is present in mTORC1 and mTORC2 complexes, and its inhibition through mutations, CR, or treatment with rapamycin can extend longevity in yeast, worms, and flies. mTORC1 has multiple downstream effectors that increase synthesis of ribosomes, proteins, lipids, and nucleotides to promote growth (Figure 1.1)[4,11]. Low ATP conditions stimulate AMPK to inhibit mTORC1, while low NAD+ levels can stimulate *SIRT1* to deacetylate multiple targets in response to metabolic and exogenous and endogenous stresses. A few examples such as PPARα coactivator 1α(PGC-1α), NF-κB, and Ku70 acetylation are involved in responses to increase mitochondrial gluconeogenesis, decrease inflammation, and stimulate DNA repair after *SIRT1* mediated acetylation respectively [11,90]. *SIRT1* deficient mice develop insulin resistance and isolated mitochondria from these samples have reduced respiratory function and increase levels of ROS [91]. The main role sirtuins play in nutrient signaling in mammalian aging remains incomplete as overexpression of *SIRT1* in mice does not extend lifespan under normal diet conditions, but treatment with resveratrol in high-fat diet fed mice may increase lifespan due to upregulation of *SIRT1* [92,93]. Thus, it appears sirtuins can provide protective roles during aging only under stressed conditions in mammals. Overall, factors that increase nutrient signaling accelerate aging, while factors

that work to limit nutrient signaling promote healthy aging potentially by activating stress-response pathways to prevent excessive damage (Figure 1.1).

Mitochondrial dysfunction can have profound impacts on the redox potential of cells, energy status, and cellular signaling, so it could contribute to aging in multiple ways. Evidence suggests that impaired mitochondrial function without increasing ROS levels in mice is more detrimental to lifespan than simply an increase in ROS [4]. Accumulating mutations to mtDNA, membrane potential deficiencies, oxidative damage to the respiratory chain, and defective mitochondrial autophagy are thought to have a role in mitochondrial decline. Severe mitochondrial dysfunction has a negative influence on aging, but recent evidence suggests that mild mitochondrial stress may actually be beneficial to the cell [11]. Mild stress is thought to trigger a hormetic response in mitochondria, known as mitohormesis, that is believed to help contribute to healthy aging through stimulation of mitochondrial stress responses as seen in worms [94,95]. Also, it is becoming apparent that stress-induced increases in ROS can activate signaling pathways to promote cell survival and proliferation [96]. Increases in ROS beyond a certain threshold are still thought to be detrimental to cells, though [97]. *SIRT1* in mammals can promote healthy aging by regulating mitochondrial biogenesis through the transcriptional coactivator 1α(PGC-1α) and autophagy to remove defective mitochondria [4,98,99]. These examples illustrate some of the complexities that mitochondria may contribute to aging.

The ability to replace damaged or old cells is essential for tissues to remain functional, and the proliferative nature of stem cell pools is responsible for this activity. Therefore, impaired renewal of stem cell pools can contribute to aging. For example, studies in mice have demonstrated that the replicative capacity of hematopoietic stem cells (HSCs) is reduced with age [100]. There is also evidence that telomere shortening is responsible for stem cell decline [101,102]. Interestingly, excessive stem cell proliferation is detrimental to the aging process and can lead to premature exhaustion of stem cell pools contributing to premature aging. Thus it appears that stem cell pools require specific signals that prevent excessive proliferation but retain proper regenerative properties to maintain normal aging.

Cellular senescence was initially described as permanent exit from the cell cycle due to erosion of telomeres, but we now know that additional factors can cause cells to become senescent. While senescence is thought to be a protective measure against tumorigenesis and the proliferation of cancerous cells, the premature and or continuous exit of many cells into a senescent state is thought to lead to a progressive decline in tissue function that can contribute to aging [103]. While the anti-cancer protective effect from the removal of damaged or potentially oncogenic cells is beneficial to the overall health of tissues, senescent cells are now known to still be actively secreting proinflammation cytokines and matrix metalloproteinases [4]. This acquired phenotype increases inflammation of the surrounding tissues and can recruit neighboring cells to enter a senescent state. Thus, increased senescence in cellular tissues can

contribute to aging phenotypes. The best-studied inflammatory response pathway to influence aging involves nuclear factor kappa beta (NF-κB). Overexpression of NF-κB is known to promote aging and its inhibition is shown to rejuvenate aged skin tissue in mice, and can reverse some signs of premature aging in progeroid mouse models [104–106]. Interestingly it appears that sirtuins can also help to regulate the inflammatory pathway involved with NF-κB. Deacetylation of NF-κB histones and subunits by *SIRT1, SIRT2*, and *SIRT6* can down-regulate the inflammatory response [107–109]. Thus, inflammation appears to influence the aging process in such a way that is not only intrinsic to one cell, but potentially can alter the fate of neighboring cell and tissue types.

The main focus in current research is still determining what the main drivers of aging are. “The free radical and somatic theories of aging” postulate that macromolecular damage drives cellular dysfunction by altering cell signaling, inflammation, stem cell renewal, and other cellular processes to limit lifespan. “The hyperfunction theory of aging” postulates that macromolecular damage never reaches a critical point to directly limit lifespan. Rather the activities of signaling pathways beneficial for development and reproduction early in life directly lead to cellular dysfunction later in life. There are numerous challenges still awaiting our discovery but it is hopeful that new technologies will aid in the development of potential therapeutics to combat pathologies of advancing age, or at a minimum increase our knowledge of the complex biological mechanisms that can influence the aging process.

1.2. Do mobile DNA elements contribute to aging?

While the above information serves to identify factors that are known to influence the aging process, other aspects of cellular life are proving to have an influence on the aging process. Interestingly, known factors of genetic change like mobile DNA elements have been implicated in the aging process and could have "hyperfunction-type" impacts on the aging process of cells (Maxwell, Burhans, & Curcio, 2011; St. Laurent, Hammell, & McCaffrey, 2010; Wood & Helfand, 2013). While much is known of the genotoxic nature of mobile DNA elements, the complex relationship of mobile DNA to its host is still not well understood. For example, are there other factors into the dynamic nature of mobile DNA elements that contribute to the aging process? Does hyperfunction of these elements or the increasing numbers of elements have a role in the longevity of a cell? The role mobile DNA elements have in interacting with some of the above-mentioned hallmarks of aging is an interesting prospect that has not been explored in detail.

Endogenous mobile DNA elements (transposons) have been shown to cause DNA damage, mutations and chromosomal rearrangements to destabilize genomes [110]. Found in the genomes of many organisms, the specific manner by which mobile elements destabilize genomes is not well understood. Mobile DNA elements alter genomes by excising from one location and inserting into a new location, or some types of mobile DNA elements copy themselves out of the genome and insert into new locations elsewhere. Mobile elements that copy out

of the genome increase their copy number in that genome. Two different perspectives on the genetic variation caused by mobile DNA are that it can be beneficial to evolution of populations and that it is primarily detrimental to genomic instability. Activation of many mobile DNA elements in response to stress could result in random genetic variants within populations that could promote survival of those populations during times of stress. Mobile DNA transposition in the germ-line results in inherited mutations, but transposition can also occur in somatic tissues. Found in all kingdoms of life, the exact beneficial (evolutionary) or detrimental (genotoxic) roles these elements play in cell function and survival is gaining attention as more is being learned about their regulation and impact on host genomes.

1.2.1. The diverse nature of mobile DNA elements

Many types of mobile elements exist with diverse replication strategies. These fall into two different classes: DNA transposons and retrotransposons, and retrotransposons can be divided into long terminal repeat (LTR) or non-LTR elements. Examples of DNA transposons include the bacterial Tn5 and Tn7 transposons, *Drosophila melanogaster* P elements, and *Caenorhabditis elegans* Tc1 transposons. Examples of LTR retrotransposons include Tnt1 in tobacco plants, the Ty family of retroelements in *Saccharomyces cerevisiae*, Tf1 and Tf2 in *Schizosaccharomyces pombe*, and the *gypsy* element in *Drosophila*. The human genome is composed of approximately 45% mobile DNA elements [111]. DNA transposons, LTR retrotransposons, and non-LTR retrotransposons

represent approximately 3%, 8%, and 34% of the human genome, respectively. The most common mammalian non-LTR retrotransposons are the long interspersed nuclear element-1 (LINE-1 or L1) and short interspersed nuclear elements (SINE)/*Alu* elements. The L1 element is the only autonomously active mobile DNA element in the human genome. L1 constitutes approximately 17% of the human genome, and while most of these thousands of copies are defective, there are about 100 active copies per genome [111–113]. The mouse genome also contains autonomously active MusD and intracisternal A-particle (IAP) elements, which are LTR retrotransposons. Non-autonomous SINE/*Alu* elements can be mobilized by L1-encoded proteins, and human *Alu* and SINE-R-VNTR-*Alu* (SVA) SINE elements are the only other mobile DNA elements known to be actively mobilized in the human genome.

DNA transposons like bacterial Tn5 and Tn7 transpose in a “cut and paste” fashion. They are excised out of their initial genomic locations and insert into new regions of the genome, without duplication of the elements. This occurs via a coded transposase enzyme that will bind and excise the inverted terminal repeats of the element and then will cleave a target site in a new location of the genome to integrate the transposon. Transposase generates staggered ends at the cleavage site that when repaired lead to target site duplications (TSDs) of about 4-8 base pairs flanking the transposon [114]. While DNA transposons are active in a variety of species, they are not active within human genomes and have not been associated with aging.

LTR retrotransposons contain two long-terminal repeats that flank the coding region. The 5'-LTR contains a promoter region that is recognized by host RNA polymerase II [114]. LTR retrotransposons replicate via a "copy out, paste in" mechanism that involves reverse transcription of an mRNA transcript into a complementary DNA (cDNA) molecule. LTR retrotransposons code for Gag and Gag-Pol or Gag-Pro-Pol fusion proteins that carry out retrotransposition. Gag forms a cytoplasmic virus-like particle (VLP) within which the element mRNA, a tRNA primer for reverse transcription, and fusion protein are packaged. Proteolytic processing of Gag and the fusion protein by Pro or a domain of Pol (for Gag-Pol fusion proteins) matures the VLP and allows the reverse transcriptase activity of Pol to generate a cDNA copy of the mRNA. Upon completion of reverse transcription, the cDNA is transported into the nucleus in a complex with the Pol integrase domain through a mechanism that is not fully defined. In the nucleus, integrase inserts the cDNA into a genomic site or the cDNA can replace existing retrotransposon copies through homologous recombination. LTR retrotransposition differs little from retrovirus replication, except that LTR retroelements lack the envelope (*env*) gene required for retroviruses to bud from cells and be infectious [115]. Unlike DNA transposons, retrotransposons are capable of increasing their copy number within the genome.

Non-LTR retrotransposons also increase their copy number within genomes by a similar “copy out, paste in” mechanism. However, non-LTR retrotransposition does not include a VLP intermediate step and instead involves a target-primed reverse transcription (TPRT) mechanism. These elements frequently code for two proteins, one with nucleic acid binding and chaperone activities and a second with endonuclease and reverse transcriptase activities. The proteins form a complex with the element RNA and the complex then associates with a genomic target site. The endonuclease domain of the second protein cleaves one DNA strand at a target site in the genome, with different elements showing different sequence preferences, resulting in a 3’-OH overhang that can serve to initiate reverse transcription of the element RNA. The endonuclease then cleaves at a staggered site on the second DNA strand, providing a 3’-OH to prime second-strand DNA synthesis and resulting in target site duplications. Second-strand synthesis and the final steps of retrotransposition are still not well understood.

Mobile DNA elements belong to a broad range of different classes and exhibit diverse mechanisms to remain mobile in host genomes. The consequences of such mobility events have attracted the attention of researchers in many fields. By attempting to understand when, why, and how mobile DNA elements integrate into new genomic regions, we can better understand their role in genomes.

1.2.2. Levels of mobility, regulation, and consequences of retrotransposon genomic change

1.2.2.1. Retrotransposons are mobile in eukaryotic genomes

It has been proposed that most transposition is absent in eukaryotic genomes but that view has been recently challenged as more studies continue to find on-going retromobility [116]. LTR retrotransposons are quite prolific in yeast, *Drosophila*, and mouse genomes, and it is stated that approximately 10-12% of sporadic mutations in the mouse are due to LTR retrotransposition events [117]. While LTR retrotransposons are mobile in many eukaryotes, they are not mobile in human genomes although they account for ~8-9% of the genome [111]. Human genomes contain LTR retrotransposons known as endogenous retroviruses (ERVs), but they are all defective due to deletions and mutations [118]. On the contrary, non-LTR retrotransposons are active and more abundant in the human genome. Human L1 non-LTR elements are autonomously mobile, and their protein products also mobilize the non-autonomous human SINE elements *Alu* and SVA. Although it is difficult to calculate the relative rate of retrotransposition with current technologies, it is thought that *Alu* elements have a new insertion in about every 20 births, making it the most active element in humans, with 1 in a 100 and 1 in 900 for L1 and SVA respectively [112,119,120]. It has been shown that the protein products from the two coding regions of L1 (ORF1 & ORF2) are capable of processing cellular mRNAs. Processed genes lacking a promoter generated by L1 proteins are known as pseudogenes, and those that have functional capabilities are termed retrogenes [121].

1.2.2.2. Retrotransposition can result in changes that lead to disease

The consequences of mammalian L1 retrotransposition are profound and the 100 active copies still account for approximately 1/1000 of spontaneous mutations to cause disease in human populations [122,123]. The most straightforward effect occurs when L1 elements insert into the coding region of genes, disrupting their function directly. Exon skipping or mis-splicing of genes can occur due to insertions into intronic regions of genes [124]. Gene expression can be altered by premature polyadenylation of gene transcripts when intronic L1 insertions are present, due to adenosine rich regions of L1. Gene expression can also be altered due to the presence of nearby L1 transcriptional control sequences [125]. L1 can mobilize genomic 3'-flanking sequences during a retrotransposition event, via L1-mediated transduction. The ability of L1 to mediate such events could have profound impacts on genome arrangements such as exon shuffling and or the creation of new genes. A retrotransposition event in owl monkeys resulted in insertion of a cyclophilin A cDNA into the TRIM5-α locus that led to production of a TRIM5- α-cyclophilin A fusion protein that confers HIV resistance [126]. L1 can contribute to gross chromosomal rearrangements when homologous recombination occurs between L1 sequences at non-allelic sites. L1 sequences have also been shown to insert via an endonuclease-independent mechanism, filling in gaps in DNA present because of genomic damage or DNA repair activities. The two protein products of L1 (ORF1 & ORF2) are capable of reverse transcribing cellular mRNAs to integrate cDNA copies of genes lacking their transcriptional control sequences,

to produce pseudogenes [121]. Occasionally these sequences are expressed from promoters at the insertion site and produce proteins, forming mammalian retrogenes. There are approximately 8,000-15,000 processed pseudogenes in the human genome [127–129]. To date, however, there are no human diseases attributed to processed pseudogenes, although there is a disease-causing pseudogene in 19 specific dog breeds [130]. Replication of L1 is initiated and completed at site, potentially having adverse effects to genome integrity at the site of retrotransposition. For example the endonuclease activity of ORF2 protein initially results in multiple cuts in the genomic DNA before the actual site for TPRT is determined [114,131]. These nicks, including double-strand breaks (DSBs) are highly mutagenic and if left unrepaired can result in increases in senescence, apoptosis, or cancer [131,132]. These additional cut sites must be repaired and can occasionally result in transversion or transition mutations, or in extreme cases simply repaired via non-homologous end joining (NHEJ) resulting in loss of DNA nucleotides [116].

Reports of non-LTR retrotransposon insertion polymorphisms in different human genomes and over 100 disease-causing insertions offer evidence of retrotransposition in the human germline [112]. L1 is expressed in rodent and human germline cells, germline mobility have been observed in transgenic mice with engineered L1 elements, and human oocytes are permissive for retrotransposition of an engineered L1 element [133]. It is estimated that 0.3% of all disease-causing mutations are caused by mobile DNA elements in

humans[122,134]. Recent evidence has also shown that retrotransposition can occur in mammalian somatic tissues. Retromobility in somatic tissue won't harm further generations but may contribute to genomic instability and be detrimental to the host organism. Somatic cell retrotransposition is shown to occur quite frequently in neurons and tumors, but can be in induced in vitro in multiple tissues through ectopic expression of elements on plasmid-based vectors. Occasionally new insertions are found within coding regions, although this is quite rare. A few examples of somatic retrotransposition of L1 have been found in cancers. One such case was a mutagenic L1 insertion into the *APC* gene that led to colon cancer in a particular individual [135]. The surrounding tissue lacked the same mutagenesis, and this served as a basis for classifying the insertion as a somatic event. Similarly, Iskow et al., found nine de novo somatic L1 insertions in six of 20 non small-cell lung cancers that were not found in the surrounding healthy tissue [136]. For unknown reasons, human and mouse neurological tissue exhibit high levels of L1 retrotransposition compared with other tissues. Quantitative PCR experiments showed increased insertions in post-mortem brain tissue over heart and liver tissue from the same individual [137]. Engineered L1 elements retrotranspose in rat neuronal progenitor cells, human embryonic stem cell derived neural progenitor cells, and in human fetal derived neural progenitor cells [137,138]. It is thought that retrotransposition may contribute to the plasticity inherent in neuronal development in the brain, but direct evidence for this has not been reported. Regardless, it is evident that retrotransposition is not

restricted to solely the germline but in fact there can be mobility in somatic cells as well.

1.2.2.3. Regulation and changes in expression of retrotransposons

Host cells have many mechanisms that help to limit retrotransposition and its genotoxic effects. Epigenetic changes via DNA methylation, histone modifications, and the activity of chromatin modeling complexes are known to regulate retrotransposition [114,139–141]. Genome-wide searches have documented that most mobile DNA elements are found within non-coding regions of the genome, namely within heterochromatin and are usually heavily silenced [78]. Regulation through DNA methylation is supported by increased retrotransposition in *MeCP2* (methyl-CpG-binding protein 2) knockout mice neuronal cells, as well as increased L1 genomic copy number in Rett syndrome patients that have *MeCP2* mutations [142]. Additionally, many species have RNAi machinery to block rampant retrotransposition [143–146]. The resulting mechanism of RNAi on retrotransposons is an exciting area of research. Two mechanisms work to limit retrotransposition under RNAi but function in a similar manner: small-interfering RNA (siRNA) and PIWI-interacting RNA (piRNA). siRNA interferes blocks retrotransposition post-transcritionally after double-stranded RNA species coded from the retroelement are detected. Dicer processing protein degrades dsRNA into short single stranded 21-24 nucleotide sequences that can be loaded onto Argonaute processing proteins to form RNA-inducing silencing complexes (RISC) that cleave retroelement mRNA sequences [143].

RISC is then free to bind to newly transcribed retroelement mRNA, leading to degradation and effectively blocking retromobility post-transcriptionally. piRNA processing happens in a similar manner and is reserved for the germ-line [144]. Long genomic sequences that may contain retroelement sequences are processed into short 24-35 nucleotide anti-sense piRNAs by Argonaute subfamily of proteins (PIWI clade of proteins) to bind to retroelement mRNA sequences. The endonuclease activity of PIWI proteins produces sense-strand sequences that associate with Argonaute 3 protein, and the cycle can repeat after these sequences can bind to piRNA transcripts to be processed once more to generate complementary piRNA complexes [114]. With age however the efficiency of these complexes is known to decrease, leading to changes in the silencing profile and increases in expression of retrotransposons [114].

While many mobile elements reside in transcriptionally silenced regions of the genome, recent evidence points to important links between chromatin and mobile DNA elements. Since replicative senescence is now considered a major driver of mammalian aging [103], changes to chromatin and therefore the “opening” of once repressed mobile DNA elements could have profound impacts on cellular processes. Four main mechanisms have been considered as to how age-dependent changes in chromatin structure could alter retroelement expression and mobility. First, chromatin silencing factors can be relocated to other parts of the genome, opening up transcriptionally repressed regions normally silent in young cells. An example of this is the redistribution of SIR

complexes in yeast away from telomeres and towards rDNA sequences as cells age [147]. The mammalian longevity protein SIRT6 was shown to be a powerful suppressor of L1 mobility by regulating repressive chromatin marks at L1 5' untranslated regions, and loss of SIRT6 binding with age led to increased L1 expression in human cells and mouse tissues [70]. Second, an overall decrease in histone acetylation and methylation, and a decrease in DNA methylation at promoters with age in mammals at some transposable elements has been observed [148–150]. Third, the spatial organization of chromatin within the nucleus can change with age. As the majority of heterochromatin is located and tethered to the nuclear lamina, changes to this architecture with age may induce these regions to become transcriptionally active [78]. Since many retrotransposons are located within heterochromatin, this is another potential avenue to induce mobility. Interestingly, the efficiency of homologous recombination (HR) increases in yeast when repetitive regions are in close proximity to each other, and localization of repetitive regions (i.e. telomeres, centromeres) to the periphery of the nucleus can prevent genomic instability due to non-allelic HR between repetitive sequences [151]. It is then interesting to speculate that repetitive elements such as retrotransposons could pose the same problem for cells if the spatial organization of heterochromatin to the nuclear lamina changes. With age however the efficiency of these chromatin-regulating complexes is known to decrease, leading to changes in the silencing profile and increases in expression of retrotransposons. Lastly, changes to RNAi mechanisms decline in function resulting in derepression of retroelements. As an

increase in retroelement transcripts are no longer being degraded by RNAi machinery, this can lead to a global increase in the retroelements available for insertion.

1.2.2.4. Retrotransposons are mobilized by endogenous and exogenous sources of stress

With many mechanisms in place to help restrict or regulate retroelements, certain factors are known to increase the frequency of mobility of these elements. Both DNA damage and oxidative stresses like ROS increase the frequency of retroelements [152–154]. While it is not well understood as to why activation of retroelements occurs under these conditions, these stresses are associated with aging and increased retromobility could help to fuel greater genomic instability (Figure 1.2). L1 can be activated in human genomes by a variety of stresses including gamma-radiation and metabolic oxidative stress [152,155,156]. As oxidative stress has been associated with aging and can drive certain pathologies such as tumor progression and neurodegenerative disorders, understanding how L1 is activated in humans is of particular interest. The activation of L1 by certain stressors is context-dependent and it has been suggested that changes to the regulatory mechanisms that inhibit mobility must be altered [157]. Stress activation of retroelements may have developed in response for the need to adapt during environmental changes.

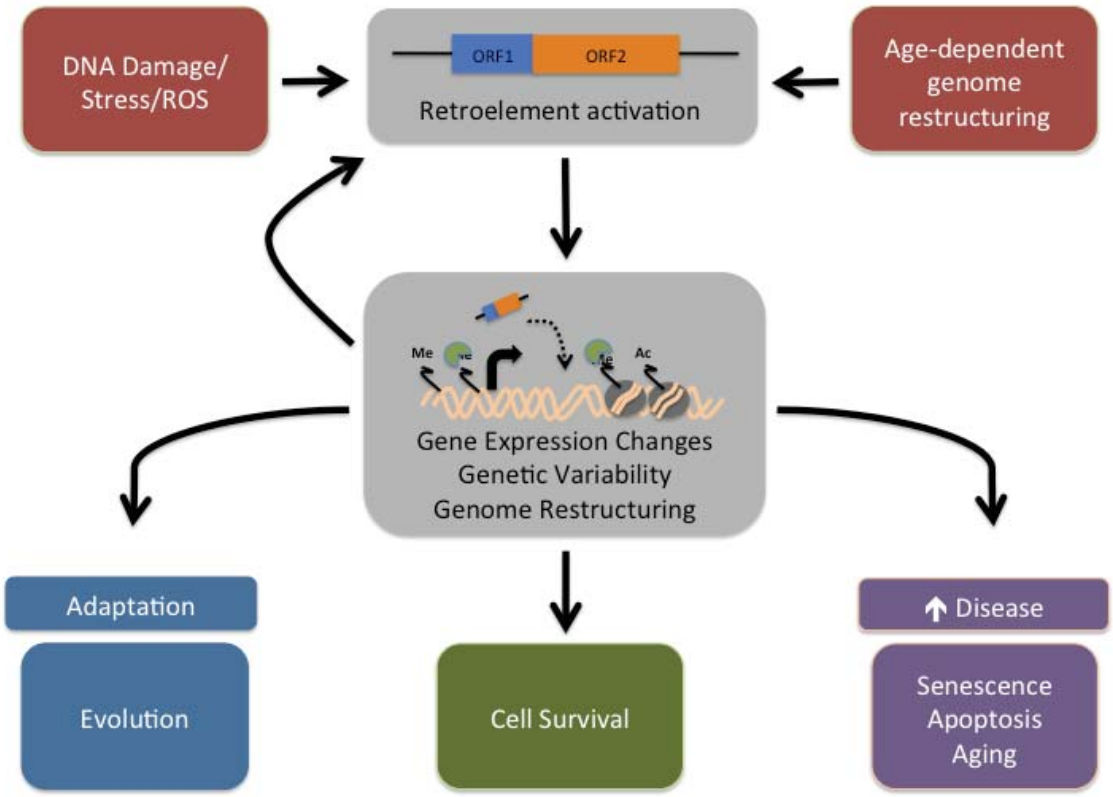

Figure 1.2. Genome changes associated with retromobility and the potential outcomes

Activation of retroelements can be stimulated by stress, damage to cells, and age-dependent changes to the genome structure that can lead to potential retrotransposon-dependent genome altering consequences (top). Retroelement mobility and insertions can destabilize the genome to promote irreparable DNA damage and decreases in tissue regenerative capacity, leading to aging and disease (bottom right). Normal age-mediated genomic changes can stimulate retrotransposon activation initiating a "vicious cycle" of retromobility (middle). The same mechanisms that promote instability can also accelerate the evolutionary process by influencing gene expression changes and genetic variability, promoting adaptation during times of stress (bottom left).

Activation of retroelements in response to stress may have beneficial outcomes. Changes in gene expression or stimulation of pathways that normally restrict retrotransposons might sometimes have beneficial changes in cells (Figure 1.2). In *S. cerevisiae*, Ty5 was shown to change its preference for insertion from regions of heterochromatin to coding regions under low nitrogen conditions [158]. Survival promoting advantages to changes in retromobility are also evident in *S.*

pombe retroelements Tf1 where 95% of insertions are near ORFs [159]. Activation of this element by stress and the frequency of insertion near coding regions, suggests a mechanism in which mobility can help to improve survival under environmental stress [114,160]. While it is easy to see how simpler organisms growing in a population could benefit from this adaptation, it is more difficult to rationalize the added benefit in somatic tissues of a higher organism. In fact there are not many reports of an adaptive benefit to retromobility in mammals. Retromobility in development is context and stage specific, and is in part regulated by changes to heterochromatin where repetitive elements reside. Fadloun et al. demonstrated using mice L1 models that different classes of retroelements are more or less active at different times post-fertilization prior to implantation of the embryo. This corresponded to changes in histone modifications resulting in atypical heterochromatin configurations [161]. While changes to chromatin structure are known to allow increase in retromobility, increased retroelement activity early in development may be a remnant artifact to increase genome plasticity that has evolved into mammalian genomes. Another context in which retroelements can lead to greater plasticity in genomes is in neuronal tissues. Neuronal somatic tissues are permissive for retrotransposition [137]. It is hypothesized that somatic retroelement insertions lead to greater diversity in the genomes of neurons that have implications on gene expression and function in these tissues [162]. While this thought is still only speculative and independent of stress activation, it does suggest that L1 retromobility can have a positive and functional value during development in

mammalian tissues (Figure 1.2). Understanding the consequences of stress-induced retromobility will aid in the understanding of how retroelements can lead to genetic diversity.

Some studies have found relationships between retrotransposon mobility and aging. While the activation of retrotransposons can be stimulated by ROS and DNA damage, the direct role for retroelement-mediated aging remains unknown although there is evidence to support such a role. Recent studies have shown that retromobility is increased during aging in yeast, *Drosophila*, and there are correlations between retromobility and aging in mammals [116,153,163]. A functional decline in neuronal processes in fruit fly brains correlated with increased transposition in these tissues. Additionally, this group showed that compromising RNAi pathways, through use of *Drosophila* with Argonaute 2 (*Ago2*) mutations, increased transposition frequency and led to a correlative decline in brain function [164]. There also appears to be a relationship between cellular senescence, an aging phenotype, and the increased expression of retroelements. Expression of *Alu* elements increased in human adult stem cells as they became senescent, and suppression of *Alu* elements in the senescent stem cells allowed them to return to a self-renewing state [165]. Chromatin containing retrotransposon sequences (and other repetitive sequences) was observed to become more open in human senescent cells, leading to elevated L1 RNA levels and genomic L1 copy number increases, supporting mobilization in senescent cells. Inhibiting degradation of *Alu* transcripts by a Dicer1 knockout

led to increase expression of *Alu* in retinal-pigmented epithelial cells, resulting in further disease progression of Geographical atrophy in these tissues [166]. Defects to miRNA processing have been shown to occur in aged mice tissues and this deregulation of miRNA is implicated in allowing increased retromobility in cancer tissues [167]. These examples, in multiple experimental models, provide a link between changes in retroelement regulation and aging. This is consistent with the notion that uncontrolled expression of retroelements may contribute to aging.

1.3. *Saccharomyces cerevisiae* as a model for investigating the influence of retrotransposons on aging

1.3.1. *Saccharomyces cerevisiae* has been instrumental for aging research

The experimental advantage of working with yeast and conservation of cellular pathways and processes influencing aging have made yeast an important organism for studying the aging process. There are two commonly used aging models in *S. cerevisiae*: chronological aging and replicative aging. Chronological aging (chronological lifespan-CLS) is characterized by the loss of viability of non-dividing cells after exhausting a carbon source in stationary phase, while replicative aging (replicative lifespan-RLS) is monitored by the number of times a mother cell can continue to divide, representative of aging of post-mitotic and mitotically active cells, respectively [168]. Mother cells do not divide continuously, much like human stem cells, and generally cease replication after about 20-25 replication cycles during RLS. During division damaged macromolecules are asymmetrically retained in mother cells that reset lifespan in daughter cells and

mutations affecting this phenomenon such as *bud6Δ* mutations abrogate the reset in daughter cells, suggesting that the accumulation of damaged molecules is in some part responsible for aging in yeast.

Changes in epigenetic marks, nutrient availability and signaling, mitochondrial homeostasis, and genomic instability influence the aging process in yeast. Deletions of *SIR2*, which encodes a protein deacetylase that is the single yeast sirtuin, decrease RLS and overexpression increases RLS [66]. *SIR2* does not influence yeast CLS in the same manner. Although yeast cells are fungi, they share many homologous pathways with humans and other mammals. Caloric restriction (CR) has been shown to increase lifespan in many well-studied eukaryotic organisms and even in non-human primates, making it one of the most conserved mechanisms involved in the aging process. In yeast RLS and CLS, nutrient signaling is modulated by multiple cellular factors in both aging models and decreases in caloric intake promote lifespan. The main influencers of nutrient signaling regulation in yeast are *TOR*/Sch9 and Ras/*AC*/*PKA* [169]. Yeast *tor1Δ*, *sch9Δ*, and *ras2Δ* mutants with inhibited growth signaling all have extended lifespan, and decreases in *TOR* and ribosomal S6 kinase (homologue of yeast Sch9) signaling can increase lifespan in nematodes, fruit flies, and mice, suggesting parallels in longevity pathways in these organisms [168]. *TOR* signaling during times of ample nutrients leads cells through the cell cycle, increases translation of mRNA and biogenesis, while inhibiting autophagy and stress response pathways, all modulators of yeast aging. On the other hand,

during times of nutrient unavailability or by inactivation of TOR, growth and translational biogenesis is inhibited while there is an increased activation of stress responses and autophagy [170]. Interestingly this applies to yeast RLS and CLS. More specifically, the *TOR*/Sch9 pathway can promote aging in all major model organisms while inhibition of Ras, *AC*, and *PKA* are shown to extend the lifespan in mice [171,172]. Elevated levels of mRNA translation are thought to affect RLS in yeast. This in part is due to *TOR/PKA*/Sch9 signaling, as mRNA translation decreases when these pathways are inhibited. While poorly understood, influences on the mitochondrial retrograde response can alter Sir2 responses in the formation of rDNA circles that results in instability [169]. In the CLS model in yeast, *TOR*/Sch9/Ras converge on Rim15 during times of ample nutrients. In times of nutrient depletion, these pathways are inhibited which elevates Rim15 response to activate stress-responsive transcription factors to promote CLS. This response is coupled with an increase in mitochondrial respiration and membrane potential leading to a differential response in mitochondria function implicated in adaptive ROS signaling and growth response. While these examples are reserved to the yeast aging model, Sir2 overexpression, *TOR*-inhibition, Sch9/Akt, and *PKA* have been conserved longevity mechanisms in worms and flies and have helped elucidate mechanisms of nutrient signaling in mammals and humans [168].

Genomic instability is also associated with yeast aging. Much of what is known about the DNA damage response to double-stranded breaks (DSB) and

replication fork stalling has been determined using yeast, since proteins involved in the DNA damage response are highly conserved from yeast to humans. Examples include yeast Mec1 and Tel1 kinases that activate signaling pathways to mediate DSB repair and restart of replication forks, which are homologous to mammalian *ATR* and *ATM*, respectively.

1.3.2. *Saccharomyces cerevisiae* has active LTR-retrotransposons

Saccharomyces cerevisiae harbors five different families of LTR retrotransposons Ty1, Ty2, Ty3, Ty4, and Ty5 with Ty1 being the most abundant and well studied in *S. cerevisiae* [173–175]. As with other LTR retroelements, Ty1 replicates via a similar "copy out, paste in" mechanism (Figure 1.3). Full-length Ty1 elements are 5.9 kilobase pairs in length and include 334 base pair (bp) LTR sequences (delta sequences) at their flanks that begin and end in dinucleotide inverted repeats that are well conserved among other retroelements (5'-TG…CA-3'). Solo LTR sequences can be found throughout the genome, remnants of LTR-LTR recombination events that lead to the loss of the main element sequence. The internal region of Ty1 contains open-reading frames (ORFs) required for successful replication: *GAG*, coding for the protein that forms the VLP, and *POL*, coding for conserved enzymatic domains: protease (PR), reverse transcriptase (RT), RNase (RH),

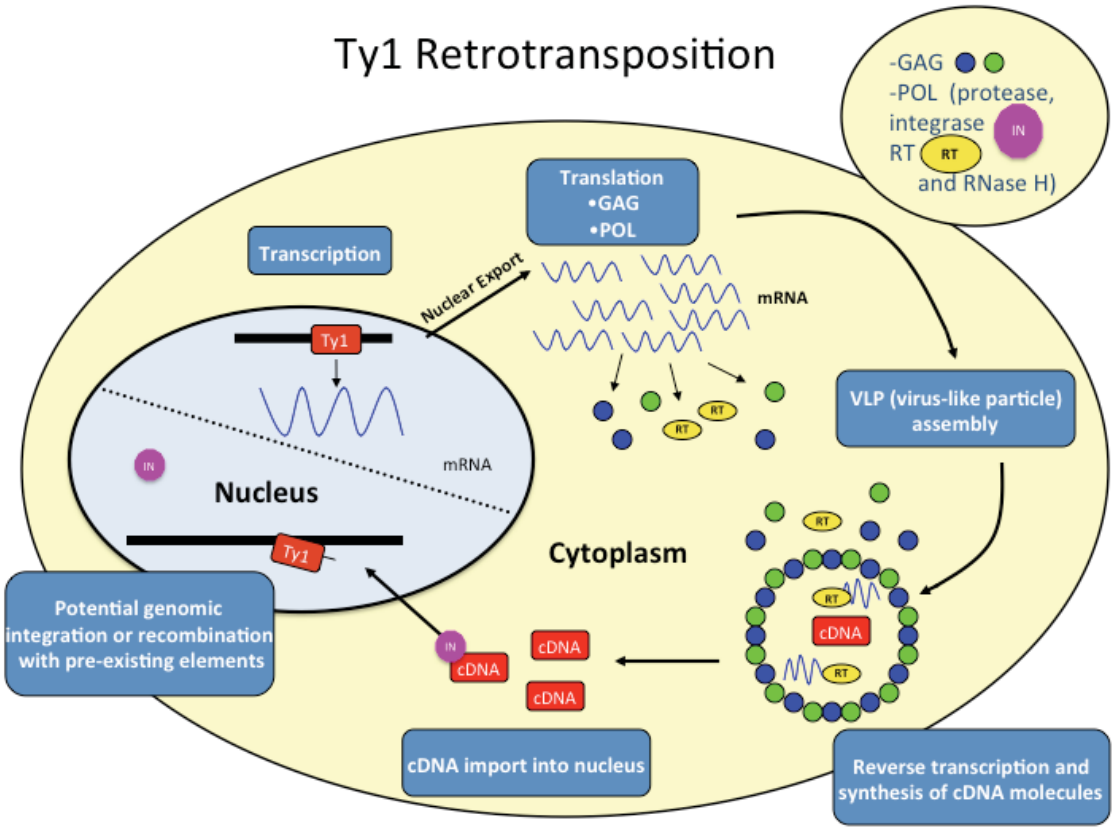

Figure 1.3. Ty1 retrotransposition in *Saccharomyces cerevisiae*

The LTR retrotransposon Ty1 in yeast is the most active of the five Ty retroelements. As with all LTR retroelements, translation of retroelement transcripts produces a structural proteins (*GAG*) resulting in the formation of a VLP (virus-like particle) where reverse transcription of retroelement mRNA into cDNA occurs using a retroelement-coded reverse transcriptase (RT) enzyme. cDNA products are then carried across the nuclear membrane by integrase (IN) for integration into new genomic sites or recombination with pre-existing elements.

and integrase (IN). Following transcription of Ty1, which begins in the 5' LTR and ends in the 3' LTR, the newly synthesized mRNA is exported out of the nucleus where translation and assembly of VLPs occurs. Translation of the proper ratio of Gag to Pol is regulated by a +1 frameshifting mechanism that results in translation of only Gag or a Gag-Pol fusion protein. This frameshift is important for Ty1 retrotransposition to occur as experimental evidence shows that *hsx1* mutants that don't produce a rare tRNA-Arg (CCU) or *spe1/spe2*

mutants with polyamine deficiency alter frameshifting and greatly reduce Ty1 retrotransposition [175]. Posttranslational processing of Gag (p49) and Gag-Pol (p199) proteins occurs after their translation by the encoded PR. A 45kD protein is formed from the cleavage of Gag-p49 and constitutes the majority of the incorporated Gag protein in VLPs. Similarly, Gag-Pol-p199 cleavage also occurs to activate the enzymatic domains of Pol (protease, integrase, and reverse transcriptase/RNase H). Mature VLPs house Gag and Pol proteins, Ty1 mRNA, methionine tRNA to initiate reverse transcription, and probably other unknown factors related to Ty1 mobility [175]. It is within mature VLPs that reverse transcription can take place.

Ty1 cDNA synthesis is a complex process that involves two strand exchanges and the coordinated catalytic processing of RNA/DNA hybrids and full-length cDNA elements via RT and RH. cDNA synthesis begins when the methionine tRNA anneals to a primer binding site (PBS) adjacent to 5'-LTR sequences on the mRNA, allowing minus strand synthesis of cDNA in the 5' direction. Once synthesis reaches the end of the mRNA, RH recognizes and degrades the resultant RNA/DNA hybrid, which allows the cDNA strand to anneal to redundant sequences in the 3' end of Ty1 mRNA to complete minus-strand synthesis in the 5' direction. This long RNA/DNA hybrid product is once again the target of RNA degradation by RH, except for two polypurine residue tracts (GGGTGGTA) [175]. These purine rich regions prime plus-strand synthesis of the 3' end of the element, after which the second strand exchange occurs, resulting in the plus-

strand product annealing to redundant sequences at the 5'-end of the cDNA molecule. Plus-strand synthesis is completed along the full-length cDNA molecule generating a new sequence ready for integration [175]. Newly synthesized Ty1 cDNA can be directly integrated by IN into new sites within the yeast genome or can replace pre-existing elements through homologous recombination. IN binds the free LTR ends of a cDNA molecule to form a pre-integration complex (PIC). Formation of the PIC results in nuclear localization through a mechanism that is not yet defined. IN cleaves a target site, generating 5' overhangs that are joined to 3'-OH groups at each end of the cDNA. The cellular repair machinery must then repair one strand at each end of the insertion, generating TSDs, and completing insertion of the Ty1 element. Occasionally, insertions occur within pre-existing elements resulting in multiple tandem repeats. These most often result in the disruption of the coding sequences of the existing element. Integration deficient Ty1 elements demonstrate a 70% increase in homologous recombination (HR)-mediated events that result in tandem Ty1 repeats, indicating that HR involving Ty1 elements could involve multiple copies of Ty1 and potentially contribute to genomic instability [175].

1.3.3. *Saccharomyces cerevisiae* has mechanisms to inhibit rampant Ty1 mobility

Interestingly, Ty1 mRNA can account for 0.1-8% of the total number of transcripts in haploid strains of yeast cells, yet rates of retrotransposition remain

quite low at 10^{-3}-10^{-7} per element per cell generation [176]. This indicates that there is strong posttranscriptional or posttranslational inhibition of retrotransposition. It has been shown in yeast that many Ty1 regulators have conserved roles in genome maintenance. Mutations to *RTT* genes (regulators of Ty1 transposition genes), several of which are orthologs to human retroviral restriction factors, increase Ty1 mobility up to 111-fold [115,177,178]. Such genes include members of the *RAD52* epistasis group, normally involved in DNA recombination and repair functions in both yeast and humans, and yeast Ku70/Ku80, involved in DSB repair and telomere maintenance in yeast and humans. The ability of such genes to inhibit retrotransposition at posttranslational steps suggests that genomes have evolved mechanisms to decrease the damaging effects retrotransposition can pose to overall genomic integrity.

Higher organisms regulate retroelements through chromatin modifications, DNA methylation, RNAi, and other mechanisms. DNA methylation and RNAi are absent in *S. cerevisiae* [114]. However, *S. cerevisiae* do demonstrate transcriptional control in the form of copy number dependent silencing, and posttranscriptional control through antisense RNA (asRNA) transcripts that inhibit Ty1 protein levels, processing, and activity in VLPs during replication [114,179,180]. Previous studies have demonstrated that *S. cerevisiae* cells harboring fewer elements have far higher retrotransposition rates, and mobility is reduced as the number of retroelements increases, indicating that Ty1 is operating under

a ‘copy number control’ (CNC) mechanism [181]. CNC is thought to have developed to limit damage due to Ty1 retrotransposition as cells acquired new Ty1 elements after rounds of retromobility. Cosuppression at the DNA level has been shown to inhibit Ty1 mobility through a mechanism that is distinct from cosuppression found in plants, but that is not fully defined [179,181]. What is apparent is that cosuppression at the transcriptional level begins as copy number increases, requires the native Ty1 promoter, and exists within either an “on” or “off” state [179]. Cosuppression can also occur posttranscriptionally since cDNA levels have been shown to decrease as copy number increased, even though mRNA levels remained high [175]. Ty1 asRNA transcripts produced from an antisense Ty1 promoter increase in number as copy number increases to induce CNC [180,182]. These asRNA species are approximately 0.5-1kbp in length and hybridize to the 5’-end of *GAG* but not *POL* regions of Ty1 [180]. The expression of these asRNAs has been shown to inhibit Ty1 mobility posttranslationally by associating with VLPs and in a mechanism independent of Ty1 mRNA interaction [183].

Ty1 frequently inserts at genomic sites where retrotransposition is thought to result in minimal consequences, which may reflect selection for Ty1 preferences or cellular regulation that reduce consequences of retrotransposition on cellular fitness. Ty1 and other yeast retroelements have preferred insertion sites upstream of tRNA (RNA Pol III-transcribed) genes, within rDNA regions, and within heterochromatic regions at telomeres and hidden mating type (HM) loci

[173]. Over 90% of all Ty1 insertions occur upstream of tRNA genes, and only approximately 2-5% into ORFs [184,185]. This preference for non-coding regions may have allowed Ty1 to persist and remain mobile within the genome. However, when Ty1 insertions occur outside the preferred sites of targeting, integration has the propensity to alter expression of neighboring genes. Inactivating mutations can occur when Ty1 integrates between the coding region of a gene and its natural promoter or directly into the coding region of a gene. Activating mutations occur when upstream insertions cause genes to be placed under transcriptional control of Ty1. Ty1 has an insertional bias for regularly spaced nucleosomes flanking tRNA genes or ORFs, so insertions near genes can frequently alter normal promoter activity [186].

1.3.4. Yeast as a model for retrotransposition in aging

While L1 elements are non-LTR type retrotransposons and yeast Ty1 elements are LTR retrotransposons, both are associated with the same types of genomic changes. Ty1, like L1, can act as an insertional mutagen [173]. Multiple Ty1 copies and remnant LTRs in the genome can facilitate chromosomal rearrangements to destabilize the genome, as do non-allelic copies of mammalian retrotransposons [187]. Both Ty1 and L1 cDNA can also be captured at sites of DNA repair, and both Ty1 and L1 retroelements can produce pseudogenes and retrogenes via reverse transcription of cellular mRNA [121,188–190]. Ty1 insertions can alter patterns of gene expression, and L1 and mouse IAP element insertions upstream of genes can have a profound impact on gene expression [112]. Some of these gene

expression changes result in truncated transcripts that can alter gene function and create oncogenic responses [191,192]. These shared characteristics of Ty1 and L1 retrotransposons make Ty1 a useful model for investigating the influence of retrotransposons on cell function and survival during aging.

Therefore it is conceivable that yeast would serve as an appropriate models for investigating the influence retrotransposons have on genomic integrity and aging. Retrotransposition is much easier to quantify and investigate in yeast than in humans or mammalian models, such as mice. Many studies in human cells or mice have relied on quantitative PCR to analyze retrotransposon copy number increases to infer increased mobility. In yeast, a *his3AI* reporter system has been used extensively to quantify Ty1 mobility frequencies from a single chromosomal element. Activation of the reporter gene after completion of retrotransposition provides a direct assay for new insertions. Yeast also enables study of endogenous retrotransposons at normal genomic positions, whereas many studies directly testing retrotransposition in mammalian models make use of plasmid-based elements. Results from such studies may be confounded by the fact that these elements are not at normal locations of retrotransposons in the genome. In addition, the relative ease to quantify retromobility events and create various mutations within strains to observe the consequences of such mutations during experimentation enable yeast to be an attractive model in aging investigations. Usefully, it is also possible to work with a strain of *Saccharomyces paradoxus* that lacks endogenous retroelements, specifically

yeast Ty1. Since it is impossible to work with retroelement-less human cell lines, this species is experimentally advantageous for retrotransposition copy number-based studies. A previous study showed that high Ty1 copy number strains can selectively out compete cellular strains with fewer elements [193]. This hints at the possibility of a selective advantage of multiple copies in the genome, but how this mechanism operates specifically is still unclear. It is possible that CNC may limit Ty1 mobility and further destructive genomic instability in a copy number dependent manner, yet be activating during times of stress to convey selective advantage in particular variants within a population. This type of study would be impossible to perform in most other models.

While it is clear that human retroelements like L1 and yeast Ty1 retrotransposons can influence genomes in a similar ways, some questions related to retrotransposon mobility and regulation during aging are better suited for study using Ty1 as a model. Two comprehensive aging models (RLS and CLS) that represent actively dividing and non-dividing cells in human tissues, respectively, facilitate the ease by which aging research can be conducted. The many similarities that yeast share with humans, the ease to genetically manipulate and quantify retromobility events makes yeast an even more attractive model. Since many eukaryotic genomes are composed of mobile DNA elements, using this strong model we wished to investigate the role multiple endogenous copies have on chronological aging. Taking advantage of a *S. paradoxus* lab strain devoid of Ty1 elements, we were able to successfully

introduce Ty1 elements back into the genome to create strains with zero, low, and high Ty1 copy strains. Using these genetic backgrounds we could directly observe how retroelement copy number could influence CLS and how that potentially could be interpreted from a human aging perspective. From this starting point we wanted to ask the questions: Do differences in copy number result in greater genomic instability from de novo mobility events or other genomic changes? Is it possible that Ty1 expression, or overexpression of Ty1 components has similar consequences for L1 in human genomes? It is interesting to speculate that using yeast Ty1 model systems could potentially uncover some novel host factors that regulate retromobility not yet discovered in mammals. Potential mechanisms that limit L1 mobility in a similar fashion like CNC in yeast, or other unknown contributions of L1-based biology come to mind. Therefore, yeast models systems may prove to be beneficial in elucidating some of the unknown mechanisms retroelements may have on aging genomes that cannot be conducted in human tissues. This may result in the discovery of new, novel and complex relationships between host and retroelements that have not yet been considered.

Chapter 2

2. EXTENSION OF *SACCHAROMYCES PARADOXUS* CHRONOLOGICAL LIFESPAN BY RETROTRANSPOSONS IN CERTAIN MEDIA CONDITIONS IS ASSOCIATED WITH CHANGES IN REACTIVE OXYGEN SPECIES

2.1. Introduction

Retrotransposons are mobile DNA elements that replicate through reverse transcription of an RNA intermediate and are known to be capable of promoting genome instability [194]. Retrotransposon sequences can comprise up to 30-80% of eukaryotic genomes, and cells commonly inhibit retrotransposon expression and mobility through use of repressive chromatin marks and/or posttranscriptional silencing mechanisms [146]. Changes in chromatin and genome instability are observed with aging, and recent work is demonstrating that the regulation and mobility of retrotransposons also changes during aging [78,195]. Increased expression of retrotransposons with age has been observed in gonads of *C. elegans*, brains of *Drosophila melanogaster*, somatic tissues in mice, normal human cells grown *ex vivo*, and in yeast mother cells [165,196,163,197,198]. Increased mobility of retrotransposons has been detected at late stages of *Saccharomyces cerevisiae* chronological lifespan and in brains of

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This chapter previously appeared as: VanHoute, D. & Maxwell, P. H. Extension of *Saccharomyces paradoxus* chronological lifespan by retrotransposons in certain media conditions is associated with changes in reactive oxygen species. *Genetics* **198,** 531-545 (2014).

aged *D. melanogaster* [163,153]. Inhibition of elevated *Alu* retrotransposon expression during senescence of normal human cells grown *ex vivo* reversed senescence phenotypes [165]. Exploring the relationship between retrotransposons and aging might provide additional insights into the aging process.

Retrotransposons have been hypothesized to provide an evolutionary advantage through increased genetic variation at the cost of reduced lifespan due to genome instability [116]. Complementary DNA (cDNA) generated by reverse transcription of retrotransposon RNA is integrated into the genome during retrotransposition, producing genetic variation and potentially mutating genes [199]. Nearly 100 disease-causing alleles resulting from retrotransposon insertions in humans have been characterized [200]. Retrotransposons can produce DNA double-strand breaks, even in the absence of successful retrotransposition [201]. Some retrotransposons, such as the mammalian L1 element and yeast Ty1 element, are frequently present at sites of chromosome rearrangements and can produce retrotransposed copies of gene transcripts [115,121,187,202–206]. Furthermore, mammalian L1 elements and yeast Ty1 elements are both activated by increased reactive oxygen species (ROS) and DNA damage [199,155,154,156], which are stresses associated with aging [13,207]. However, the potential role of retrotransposons and the genome instability they cause in aging has not yet been well investigated.

The Saccharomyces research model offers a unique opportunity to address the potential contribution of retrotransposons to aging. *S. cerevisiae* has five families of long terminal repeat (LTR) retrotransposons, Ty1-Ty5, and Ty1 is an abundant and active retrotransposon in this yeast [199]. LTR retrotransposons are also referred to as extrachromosomally primed (EP) retrotransposons, since they code for a protein that forms a cytoplasmic virus-like particle in which the retrotransposon RNA is packaged and reverse transcribed by an element-encoded reverse transcriptase prior to integration into a genomic site [199]. *Saccharomyces paradoxus* is a very closely related species to *S. cerevisiae*, and a strain of *S. paradoxus* has been reported to completely lack sequences from the coding regions of Ty retrotransposons [208]. This is an exceptional system, since the multi-copy nature of retrotransposons typically prevents the generation of populations of a species that do not have retrotransposons. Gains and losses of Ty1 elements and regulation by a copy-number-control mechanism have been examined following insertion of defined numbers of genomic Ty1 elements into this Ty-less *S. paradoxus* strain [174]. Copy-number-control results in reduced Ty1 mobility when many copies of Ty1 are present in the genome, but high Ty1 mobility when only one or a few Ty1 elements are present in the genome. Similarities in regulation by cellular stresses and influences on genome instability between Ty1 and mammalian retrotransposons make Ty1 a good model element for investigating the contribution of retrotransposons to a complex phenotype, such as aging [199,154,156].

Two aging models have been used in yeast to obtain information relevant to aging in diverse species. Yeast replicative lifespan is the number of times a mother cell can divide to produce a daughter cell, while chronological lifespan is the length of time that cells remain viable in a non-dividing state in nutrient-depleted medium [169]. Yeast lifespan can be altered by the activity of evolutionarily conserved growth signaling pathways, such as the TOR kinase pathway, mitochondrial function, proteasome function, sirtuin gene function, levels of autophagy, dietary restriction, and oxidative stress, among other processes and gene functions [168,169]. Many of these processes and homologous pathways also regulate aging in other eukaryotes [168,169]. Facile genetic approaches and short lifespan have made yeast an efficient system for identifying and characterizing these aging-associated gene/pathway functions.

As associations between retrotransposons and aging continue to be identified, it becomes important to find approaches to test for any direct influence of retrotransposons on aging. We used the Ty-less *S. paradoxus* strain to determine whether the presence and mobility of Ty1 elements in the genome would reduce yeast chronological lifespan by comparing strains with zero, one to three, or approximately 20 genomic copies of Ty1. Lifespan data did not support this hypothesis, but surprisingly, we found that strains with high Ty1 copy number had extended lifespan when cells were grown either in synthetic medium or in rich medium in the presence of a low dose of the ribonucleotide reductase inhibitor hydroxyurea. Hydroxyurea can stimulate Ty1 mobility and

causes DNA replication stress, and replication stress has been correlated with reduced yeast chronological lifespan [177,209,210]. However, lifespan extension in high Ty1 copy number strains did not appear to be due to high retrotransposition levels or substantial differences in DNA replication stress. Rather, lifespan extension was correlated with changes in ROS accumulation. These results identify a novel role for retrotransposons in promoting lifespan that may uncover additional associations between retrotransposons and ROS production or signaling.

2.2. Results

We used a plasmid to introduce copies of a Ty1 retrotransposon into the genome of a Ty-less lab strain of *S. paradoxus* [181,208] to address the impact of retrotransposons on yeast chronological lifespan. Conditions to induce Ty1 mobility were varied to obtain strains with different Ty1 copy numbers, and the approximate Ty1 copy number in clonal populations was determined through quantitative PCR. We grouped the strains with one to three genomic Ty1 insertions as low copy strains and the strains with approximately 20 genomic Ty1 insertions as high copy strains, since Ty1 mobility is restricted through a copy-number-control mechanism [181] and the initial expectation was that Ty1 mobility would be correlated with any observed influence on chronological lifespan. Four different low copy strains and four different high copy strains were used for this work to reduce the possibility that lifespan changes would simply be due to a particular Ty1 insertion or pattern of Ty1 insertions that directly altered

expression or function of a gene regulating lifespan. To confirm the presence of independent insertions, genomic DNA from these strains was digested separately with two restriction enzymes to produce fragments containing sequences from the 3' end of Ty1 and flanking genomic DNA. A Southern blot of these samples hybridized with a probe to the 3' end of Ty1 demonstrated that different fragment sizes were present in different low or high copy strains with no distinct fragments common to all low or all high Ty1 copy strains (Figure 2.1). Low copy strains had one to three fragments, and high copy strains had over ten distinct fragments. This indicates that each low or high Ty1 copy strain harbors insertions at different genomic sites, so a consistent change in lifespan for all low Ty1 copy strains or for all high Ty1 copy strains would likely be a general phenotype of a low or high copy strain.

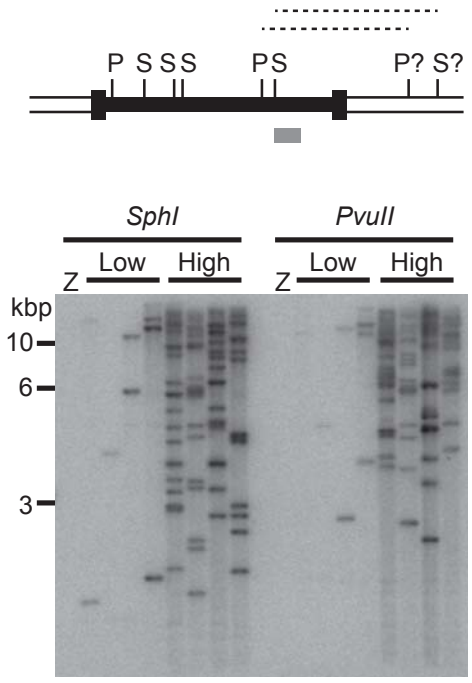

Figure 2.1. Individual low and high Ty1 copy strains have distinct Ty1 insertions

The diagram at the top depicts a Ty1 element as a black box bounded by narrow taller boxes (long terminal repeats) and flanked by genomic DNA sequences (double lines). Relative positions of sites for the restriction enzymes *PvuII* (P) and *SphI* (S) are indicated and the question marks indicate that the distance to the nearest flanking site is variable for each insertion. The light gray box shows the relative position of the probe and dotted lines indicate the restriction fragments detected by the probe. Below the diagram is a Southern blot of genomic DNA digested with *PvuII* or *SphI* from the zero copy, "Z", four low copy, "Low", and four high copy, "High", Ty1 strains probed for 3' Ty1-flanking DNA restriction fragments. Positions of selected size standards in kilobase pairs, "kbp", are indicated to the left of the blot image.

Strains were grown and aged in rich medium (YPD) at 20˚ to determine chronological lifespan (CLS) so that Ty1 would be able to actively retrotranspose [211,212]. Note that growth at 20˚ can substantially lengthen CLS compared to standard growth at 30˚ [153]. The number of days required for populations to reach 50% and 10% viability were calculated as representations of median (50%) and maximum (10%) lifespans to facilitate comparisons between multiple strains and

replicate trials. The mean days to 50% and 10% viability for the zero Ty1 copy parent strain were 15.1 and 18.6, respectively, and there was no significant difference in lifespan between the zero, low, or high Ty1 copy number strains in YPD (Figures 2.2A and 2.2B).

We considered that it might be necessary to stress cells to detect an influence of Ty1 on lifespan, since various stresses induce retromobility and a number of stresses can also influence lifespan [11,146,199]. Increased DNA replication stress has been correlated with decreased yeast CLS [209,210], so we repeated CLS experiments in YPD medium with a low concentration (30 mM) of the ribonucleotide reductase inhibitor hydroxyurea (HU). This concentration of HU led to a 30-40% increase in the doubling time of all strains (Table 1), but did not decrease viability when cells first reached stationary phase. Unexpectedly, chronic exposure to HU significantly increased the median lifespan of the zero Ty1 copy strain by 28% to 19.3 days ($p<0.05$) and the maximum lifespan by 37% to 25.4 days ($p<0.01$, Figures 2.2C and 2.2D). Significant increases were also observed for the median lifespan of the low Ty1 copy strains ($p<0.05$) and both the median and maximum lifespans of the high Ty1 copy strains ($p<0.01$, Figure 2.2D). Since growth signaling is known to influence yeast lifespan [169], the slower growth of these strains in HU could be at least partly

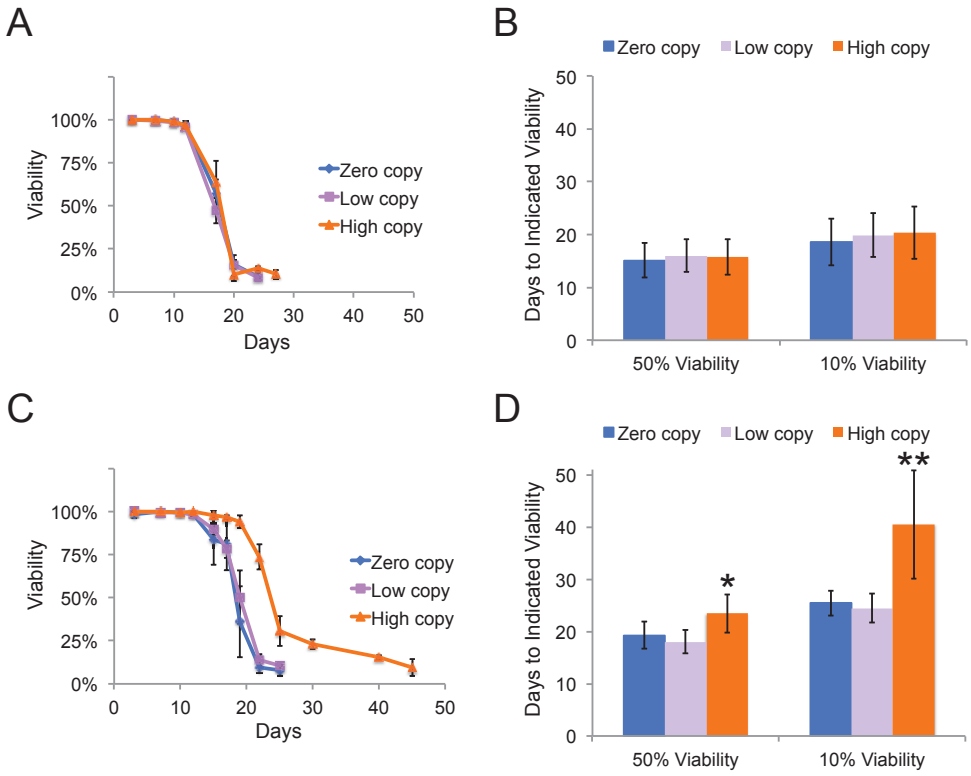

Figure 2.2. High Ty1 copy strains have extended lifespan in the presence of hydroxyurea

(A) Representative chronological lifespan experiment in YPD medium using triplicate cultures of the zero Ty1 copy parent strain, one low Ty1 copy strain, and one high Ty1 copy strain. (B) Mean ± standard deviation of median chronological lifespan (days to 50% viability) and maximum chronological lifespan (days to 10% viability) in YPD medium for the zero Ty1 copy strain (blue columns), four low Ty1 copy strains (light purple columns), and four high Ty1 copy strains (orange columns) from five to nine independent trials. (C) Representative chronological lifespan experiment in YPD + 30 mM hydroxyurea using triplicate cultures of the zero Ty1 copy strain, one low Ty1 copy strain, and one high Ty1 copy strain. (D) Mean ± standard deviation of median chronological lifespan (days to 50% viability) and maximum chronological lifespan (days to 10% viability) in YPD + 30 mM hydroxyurea medium for the zero Ty1 copy parent strain (blue columns), four low Ty1 copy strains (light purple columns), and four high Ty1 copy strains (orange columns) from nine to twelve independent trials. Significant differences are indicated with asterisks: "*" = $p<0.05$, "**" = $p<0.01$.

responsible for this lifespan extension. There was no significant difference in CLS between zero Ty1 copy and low Ty1 copy strains in HU. Surprisingly, median and maximum lifespans of high Ty1 copy strains were significantly extended by 22% and 59%, respectively, in YPD medium with HU compared to

the zero copy strain (Figure 2.2D). Cell doubling times of the high Ty1 copy strains were not significantly different from the parent zero copy strain in YPD medium with HU (Table 1), indicating that a difference in growth rate is unlikely to be the reason for the lifespan extension.

Table 1. High Ty1 copy strains have a slightly slower growth rate in rich medium than the zero Ty1 copy strain.

Strain (Ty1 copy number)	Trials	Doubling time in YPD[a]	Trials	Doubling time in YPD + 30 mM Hydroxyurea[a]
Zero	6	3.8 ± 0.2	7	5.2 ± 0.3
Low	6	3.9 ± 0.2	6	5.0 ± 0.3
High	8	4.0 ± 0.2*	8	5.5 ± 0.4

Triplicate cultures were grown at 20˚C for each trial, and a total of four low Ty1 copy and four high Ty1 copy strains were tested.
[a] Mean ± standard deviation in hours.
* $p<0.05$ versus zero Ty1 copy strain in same medium.

2.2.1. Increased lifespan of high Ty1 copy strains grown in medium with HU is not correlated with Ty1 retrotransposition

Since Ty1 mobility did not appear to negatively influence CLS, relative Ty1 retrotransposition levels were examined to confirm that low Ty1 copy strains had higher levels of retrotransposition and high Ty1 copy strains had lower levels of retrotransposition, due to restriction of retrotransposition by a copy-number-control mechanism [181]. We assayed for Ty1 integration events upstream of 5S rRNA genes by PCR (Figure 2.3A), since Ty1 elements are known to frequently integrate upstream of genes transcribed by RNA polymerase III [213]. The expected PCR result is a ladder of products representing different sites of Ty1 insertions relative to a 5S rRNA gene in different subpopulations of cells in a given culture. The yield of PCR products was greatly reduced using DNA

templates prepared from cells grown in the presence of phosphonoformic acid, a reverse transcriptase inhibitor, demonstrating that the products resulted from new retrotransposition events (Figure 2.3A). Relative Ty1 integration levels were compared by normalizing the total signal intensities of all integration products for each template to the signal intensity of a PCR product for a single-copy gene, and then using that normalized value for one high Ty1 copy strain to normalize all other integration PCR values. Low Ty1 copy strains had approximately 10- to 40-fold higher Ty1 integration levels using this assay than the reference high Ty1 copy strain, and integration levels in other high copy strains ranged from 0.5- to 4-fold the value of the reference strain (Figure 2.3B). These results indicate that copy number control is occurring in these strains.

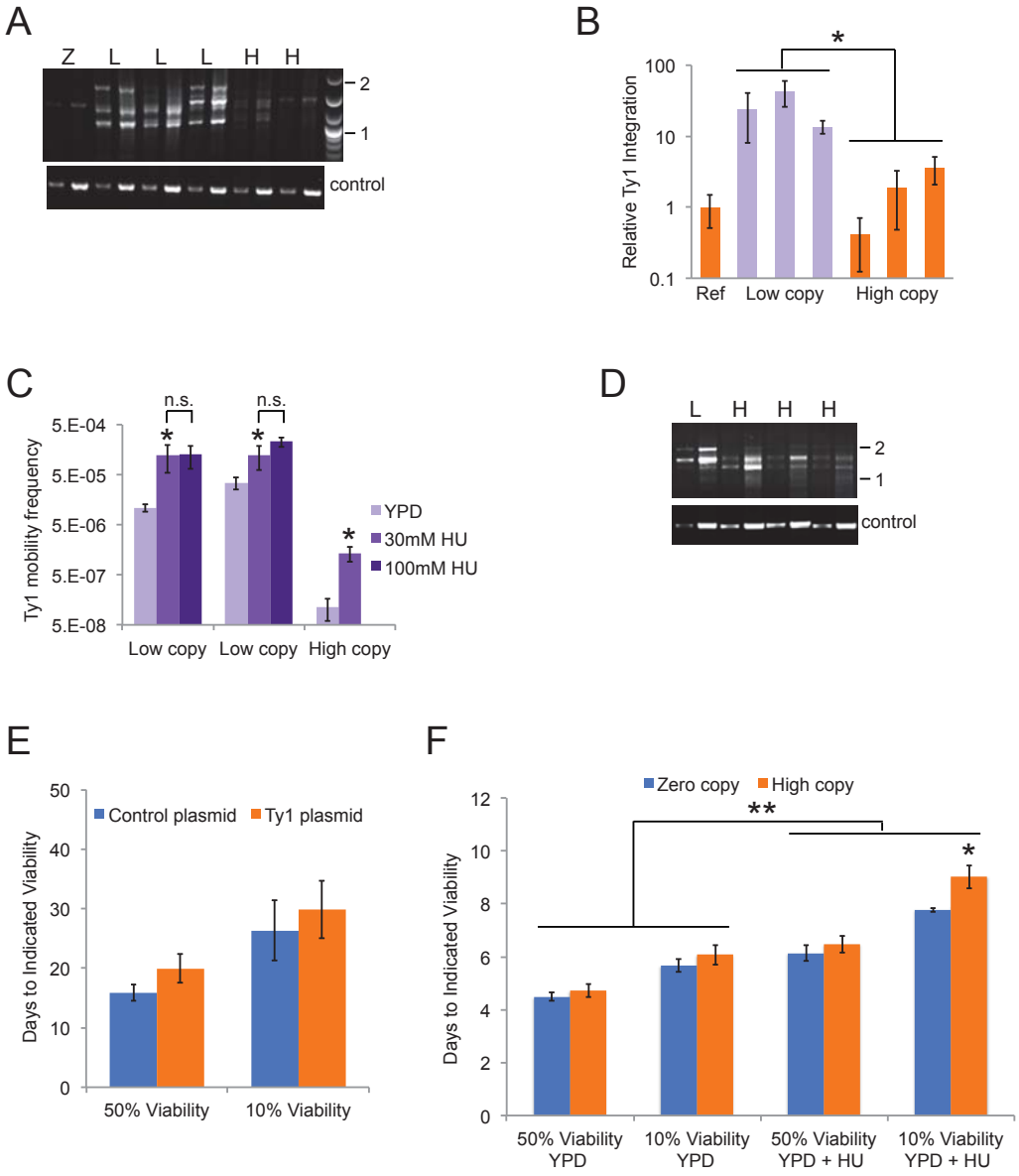

Figure 2.3. Ty1 mobility is elevated in low Ty1 copy strains and in the presence of hydroxyurea.

(A) Representative pairs of ethidium bromide-stained agarose gel images showing Ty1 integration PCR products (upper panel of each pair) obtained after 31 and 34 cycles from the same template or single copy gene control product obtained after 17 and 19 or 20 and 22 cycles from the same template (upper "control" and lower "control" images, respectively). Results in the upper pair of images are for the zero Ty1 copy parent strain, "Z", three low Ty1 copy strains, "L", and two high Ty1 copy strains, "H". Results in the lower pair of images are for two low Ty1 copy strains grown in the absence, "no PFA", or presence, "+ PFA", of 200 µg/ml phosphonoformic acid to inhibit Ty1 reverse transcriptase. The positions of one kilobase pair and two kilobase pair

markers are indicated (“1” and “2”). (B) A comparison of the relative yield of Ty1 integration PCR products from two to three independent trials using a reference high Ty1 copy strain, “Ref”, three low Ty1 copy strains, “Low copy”, and three additional high Ty1 copy strains, “High copy”. Columns indicate mean ± standard deviation. The symbol “*” indicates $p<0.05$ for the means of low versus high Ty1 copy strains. (C) Frequencies of forming His^+ prototrophs as a measure of Ty1 mobility in two low Ty1 copy, “Low copy”, and one high Ty1 copy, “High copy”, strains harboring a chromosomal Ty1*his3AI* element grown in the absence, “YPD”, or presence of two different concentrations of hydroxyurea, “30mM HU” and “100mM HU”. Columns indicate mean ± standard deviation for three trials per strain per condition, the symbol “*” indicates $p<0.05$, and the symbol “n.s.” indicates no significant difference. (D) Representative results of Ty1 integration PCR following growth of cells in YPD medium with 30 mM HU, as described for (A), and control product was obtained after 20 and 22 cycles of PCR. (E) The median and maximum lifespans for zero copy Ty1 strains harboring a control plasmid (blue columns) or a Ty1 plasmid (orange columns) grown in YPD + 30 mM hydroxyurea + 200 µg/ml G418 are shown as the mean ± standard deviation of three trials. (F) Median, “50% viability”, and maximum, “10% viability”, lifespans for zero Ty1 copy (blue columns) and high Ty1 copy (orange columns) strains grown at 30˚ in YPD medium without or with 30 mM hydroxyurea, “YPD” or “YPD + HU”, respectively. Data are mean ± standard deviation from three trials for the zero copy strain and four trials for the high copy strains. The symbol “*” indicates $p<0.05$ for maximum lifespans of zero compared to high Ty1 copy strains in YPD + HU, and the symbol “**” indicates $p<0.01$ for lifespans in YPD compared to those in YPD + HU.

The influence of HU on Ty1 retromobility in low and high copy strains was compared using the *his3AI* retromobility indicator gene [176]. Ty1 retromobility frequencies in two low copy strains harboring only a single genomic Ty1*his3AI* element and a high Ty1 copy strain with a single genomic Ty1*his3AI* were measured as the frequencies with which cells became His^+ prototrophs. HU was reported to activate Ty1 retromobility in a dose-dependent manner in an *S. cerevisiae* lab strain harboring Ty1*his3AI* [177], which would be comparable to the high Ty1 copy strain with Ty1*his3AI* in the current study. Treatment with 30 mM HU increased Ty1*his3AI* retromobility approximately 4- or 11-fold in the two low copy strains and approximately 12-fold in the high Ty1 copy strain (Figure 2.3C). Increasing the HU concentration to 100 mM led to a marginal, but not significant, further increase in mobility for the low copy strains. The substantially higher mobility in the single-copy strains compared to the high Ty1 copy strain again verified Ty1 regulation through copy number control (Figure 2.3C). Furthermore,

integration upstream of 5S rRNA genes was still observed with HU treatment, indicating that Ty1 insertion preferences were not grossly altered in response to HU (Figure 2.3D). These results indicate that lifespan extension in high Ty1 copy strains in the presence of HU occurs in the context of moderate levels of Ty1 mobility, and higher levels of retromobility in low Ty1 copy strains do not negatively influence CLS in rich medium with or without HU.

A high-copy plasmid with a Ty1 element under the control of a galactose-inducible promoter was introduced into the zero Ty1 copy strain to test whether the presence of many Ty1 DNA sequences in the nucleus of cells would somehow extend lifespan in the presence of HU. Strains with the Ty1 plasmid or a control plasmid were grown and aged in rich medium with HU and an antibiotic, G418, to maintain the plasmids. Glucose medium was used to repress the galactose-inducible promoter and thereby minimize expression of the Ty1 element. PCR with DNA extracts from early and late time points during aging confirmed the presence of the plasmid throughout aging (data not shown). No significant differences in median or maximum lifespan were identified between strains with the control plasmid or the Ty1 plasmid (Figure 2.3E)**.** Therefore, the presence of Ty1 sequences on a high-copy plasmid is not enough to reproduce the phenotype of high Ty1 copy strains grown in YPD with HU.

Ty1 mobility is inhibited at 30˚, as previously noted, which is largely due to reduced Ty1 protein levels and reduced proteolytic processing of Ty1 Gag-Pol fusion protein by Ty1 protease [212]. The lack of Gag-Pol processing inhibits

reverse transcription, but Ty1 transcription is not altered at high temperature [212]. Earlier work also demonstrated that Ty1 transcripts are abundant in cells grown at 30˚ [214]. We therefore aged zero and high Ty1 copy strains at 30˚ in the presence and absence of HU to determine whether Ty1 protein expression and processing were required for lifespan extension. Incubation at 30˚ significantly decreased lifespan for all strains compared to incubation at 20˚ (p<0.005), as expected (Figures 2.2B, 2.2D, and 2.3F). No significant differences in lifespan between strains were noted during aging in YPD medium alone, and lifespans of all strains were significantly extended when HU was added to the medium (p<0.001, Figure 2.3F). High Ty1 copy strains no longer had extended median lifespan relative to the zero copy strain when incubated at 30˚ in YPD with HU (Figure 2.3F). Maximum lifespan was still significantly extended, but the relative extension was significantly reduced from 59% at 20˚ to 16% at 30˚ (p<0.01, Figures 2.2D and 2.3F). These results indicate that the presence and/or functions of Ty1 proteins may contribute to lifespan extension in HU.

2.2.2. Extension of lifespan in high Ty1 copy strains does not appear to result from substantial differences in DNA replication stress between zero and high Ty1 copy strains

A negative correlation has previously been reported between treatments and mutations that alter yeast CLS and levels of DNA replication stress [209]. This negative correlation was established in part by examining the ability of yeast cells to correctly arrest in G1 stage of the cell cycle when they reach stationary

phase through DNA content analysis [209]. The possibility that high Ty1 copy strains lived longer in HU medium due to improved arrest in G1 during the transition to stationary phase was examined through use of a fluorescent DNA stain and flow cytometry. Nearly all cells for both zero and high Ty1 copy strains had G1 DNA content when grown in YPD medium for three days, and there was little change in this profile at day seven **(**Figure 2.4A). Growth in YPD with 30 mM HU caused a pronounced increase in the fluorescence intensities of cell populations at day three, indicative of many cells in S and G2 stages, but this effect was diminished by day seven as cells began to return to a G1 DNA content (Figure 2.4A). DNA content profiles for the zero and high Ty1 copy strains were very similar in the presence of HU at both time points (Figure 2.4A), and we did not observe consistent or substantial differences in profiles in multiple experiments. Therefore, lifespan extension in high Ty1 copy strains does not seem to be due to substantially improved arrest in G1 during stationary phase in the presence of HU.

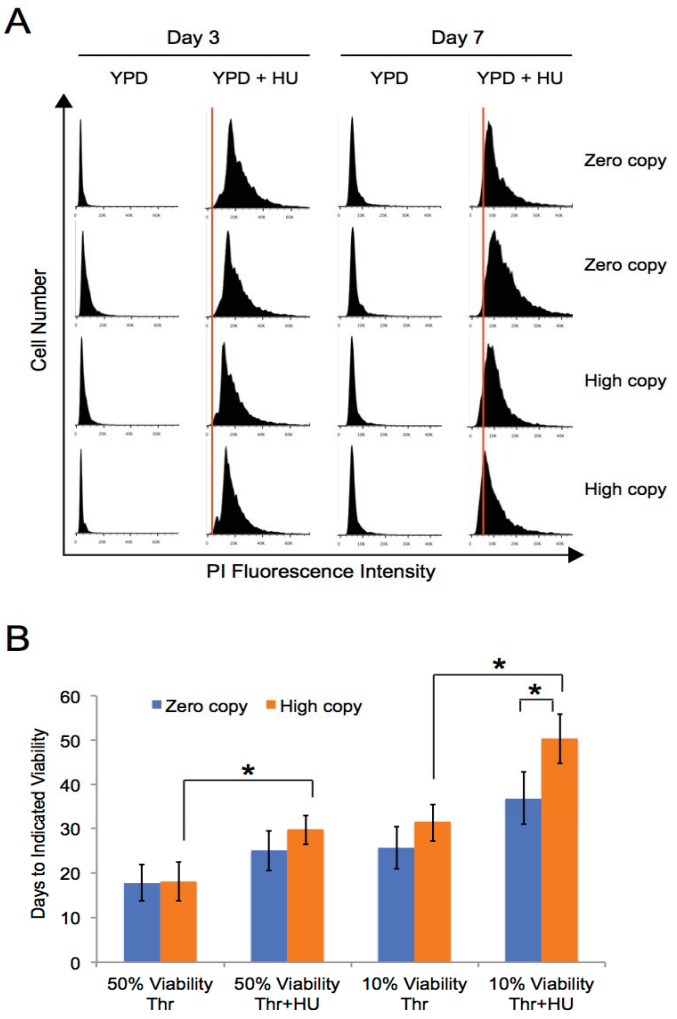

Figure 2.4. Differences in replication stress are unlikely to fully explain the lifespan extension in high Ty1 copy strains.

(A) Representative histograms from flow cytometry using propidium iodide (PI) to determine DNA content of two replicates of the parent zero Ty1 copy strain, “Zero copy”, and two different high Ty1 copy strains, “High copy”. Cells were analyzed after three, “Day 3”, or seven, “Day 7”, days of growth in rich medium without or with 30 mM hydroxyurea, “YPD” or “YPD + HU”, respectively. Each panel for Day 3 has the same x-axis scale, and each panel for Day 7 has the same x-axis scale. An orange vertical line on the “YPD + HU” panels indicates the average position of the peak signal from the corresponding set of “YPD” graphs to facilitate comparisons. (B) Mean ± standard deviation of median, “50% viability”, and maximum, “10% viability”, lifespans for the zero Ty1 copy strain (blue columns) and three high Ty1 copy strains (orange columns) grown in rich medium with an excess of threonine and without or with 30 mM hydroxyurea, “Thr” or “Thr + HU”, respectively. Data are from three trials, and “*” indicates $p<0.05$ comparing high Ty1 copy strains grown without or with HU and maximum lifespans of zero and high Ty1 copy strains in Thr + HU.

We further investigated the potential relationship between HU-induced DNA replication stress and lifespan extension by high Ty1 copy number by growing and aging cells in the presence of excess threonine. *S. cerevisiae* cells deficient for threonine biosynthesis genes, such as *THR1*, show increased sensitivity to HU, which can be suppressed by growing cells with an excess of threonine [215]. A metabolic pathway involving threonine has been proposed to buffer dNTP levels in the presence of HU or when other means are used to inhibit ribonucleotide reductase activity [215]. Also, *thr1*Δ mutants have been reported to have decreased CLS, which can be suppressed by overexpression of the ribonucleotide reductase subunit gene *RNR1*, and excess threonine can suppress the shortened CLS of cells experiencing replication stress due to high glucose [210]. Strains were grown and aged in rich medium with four times the normal concentration of threonine to determine whether buffering dNTP levels would result in more similar lifespans for zero and high Ty1 copy strains in the presence of HU. The maximum lifespan of high Ty1 copy strains was still significantly longer by approximately 37% compared to the parent strain in the presence of both excess threonine and HU, which was not significantly different from the lifespan extension originally observed in rich medium and HU without threonine (Figures 2.2D and 2.4B). The difference in median lifespan between zero and high Ty1 copy strains in medium with threonine and HU was not significant, though. Two additional aspects of these data are noteworthy. First, HU still extended lifespan of all strains in the presence of excess threonine (compare Thr vs. Thr + HU in Figure 2.4B), but the increase was not quite

significant for the zero copy strain. Second, the median and maximum CLS of both zero and high Ty1 copy strains were significantly longer in YPD with HU and excess threonine than they were in YPD with only HU ($p<0.05$ for zero copy and $p<0.01$ for high copy, compare Figures 2.2D and 2.4B). This indicates that depletion of dNTPs may limit CLS of these strains in HU, even though HU has an overall positive influence on CLS. Since the maximum CLS of high Ty1 copy strains was still longer than the parent strain despite threonine supplementation, differential response to DNA replication stress may not be the primary reason for the increased longevity.

2.2.3. High Ty1 copy strains have a very moderate reduction in mutation frequency when grown in medium with HU

Mutations and chromosome rearrangements accumulate during yeast chronological aging [216], so we examined young cell populations to determine whether reduced mutation rates early in lifespan could potentially contribute to lifespan extension in high Ty1 copy strains. *CAN1* encodes a permease that transports arginine and the toxic arginine-analog canavanine into yeast cells, and loss-of-function mutations in *CAN1* provide canavanine resistance [217]. Modest increases or decreases in *CAN1* mutation rates per cell generation were observed for individual low Ty1 copy and high Ty1 copy strains compared to the zero copy

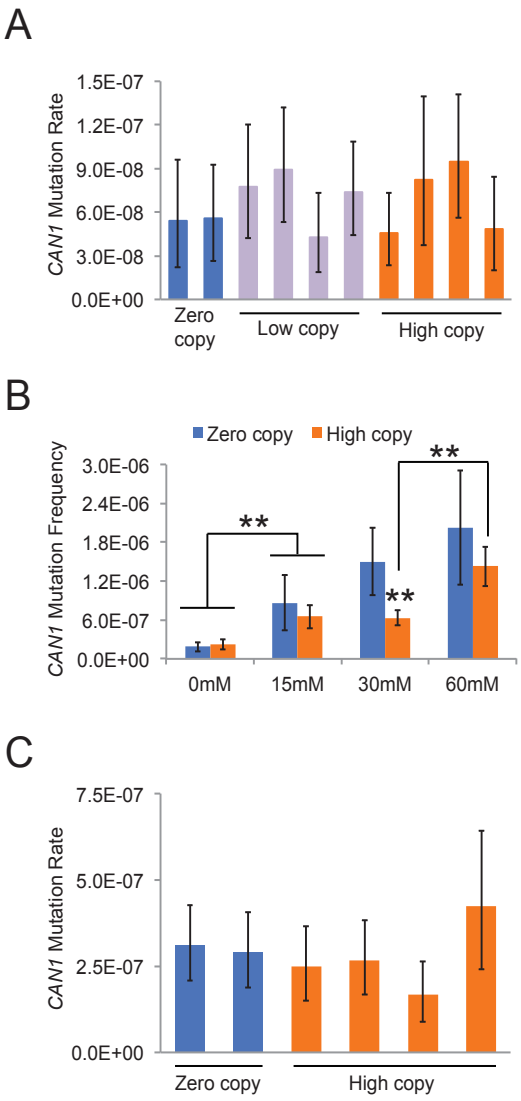

Figure 2.5. High Ty1 copy strains have a modest reduction in mutation frequency in the presence of hydroxyurea.

(A) Rate per cell generation of forming canavanine-resistant mutants for two trials of the zero copy Ty1 strain (blue columns), four low Ty1 copy strains (light purple columns), and four high Ty1 copy strains (orange columns) in YPD medium determined using the FALCOR calculator [218]. Error bars indicate 95% confidence intervals. (B) Frequency of forming canavanine-resistant mutants following growth in rich medium without or with the indicated concentrations of hydroxyurea for zero (blue columns) or four high Ty1 copy (orange columns) strains. Data represent the mean ± standard deviation from four or five trials, and the symbol "**" indicates $p<0.01$ comparing zero to high Ty1 copy strains in 30 mM HU or for indicated comparisons. (C) Mutation rates as for (A) for two trials of the zero copy strain and four high Ty1 copy strains using YPD medium with 30 mM HU.

strain when grown in YPD medium, but the 95% confidence intervals were largely overlapping **(**Figure 2.5A**)**. *CAN1* mutation frequencies (fraction of cells in the population with a mutation) were significantly increased by growth in YPD with 15 mM HU compared to YPD only for both zero and high Ty1 copy strains, though the additional increases in mutation frequency with each additional increase in HU concentration were not quite significant in most cases (Figure 2.5B**).** High copy Ty1 strains showed a moderately diminished mutation frequency response to HU compared to the zero copy strain, particularly in 30 mM HU (Figure 2.5B). However, substantial differences in mutation rates for the zero copy and four high Ty1 copy strains in YPD with 30 mM HU were not observed (Figure 2.5C). There were marginal decreases in rate for three high copy strains and a modest increase in mutation rate for the fourth compared to the zero copy strains, but the 95% confidence intervals were again largely overlapping. Differences in mutation rates therefore do not appear to be responsible for the lifespan extension in the high Ty1 copy strains.

2.2.4. Differences in ROS accumulation play a role in lifespan extension of high Ty1 copy strains

Yeast CLS is substantially reduced in synthetic medium compared to rich medium, which is at least partly due to acetic acid accumulation and is associated with increased replication stress [209,219]. We observed that these *S. paradoxus* strains had very short lifespans in synthetic medium with 2% glucose

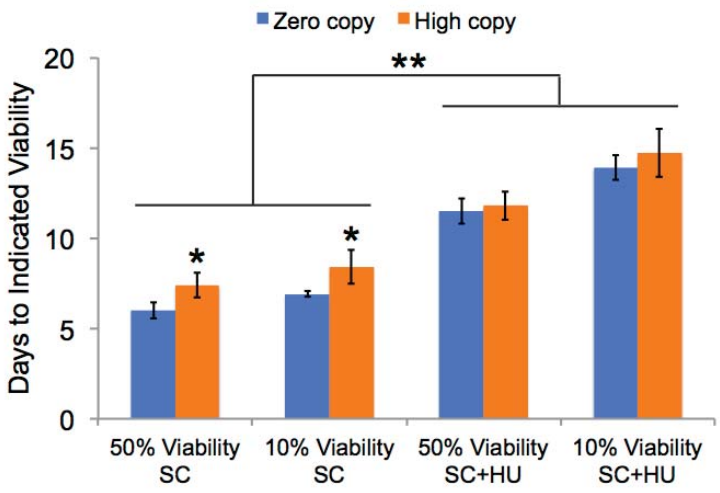

Figure 2.6. High Ty1 copy strains have moderately extended lifespan in synthetic medium without hydroxyurea.

Median, "50% viability", and maximum, "10% viability", lifespans for zero Ty1 copy (blue columns) and high Ty1 copy (orange columns) strains grown in synthetic medium without or with 30 mM hydroxyurea, "SC" or "SC + HU", respectively. Data are the mean ± standard deviation from three to five trials. The symbol "*" indicates $p<0.05$ for lifespans of zero compared to high Ty1 copy strains in SC, and the symbol "**" indicates $p<0.01$ for lifespans in SC compared to those in SC + HU.

(SC), especially considering that their growth was at 20˚, rather than the more typical 30˚ used for most published CLS experiments. In SC medium, high Ty1 copy strains had significantly increased median and maximum lifespans compared to the zero copy strain (7.4 versus 6.0 days and 8.4 versus 6.9 days, respectively, Figure 2.6). All strains had a significantly longer lifespan when 30 mM HU was added to the SC medium, but there was no difference between zero and high Ty1 copy strains in this medium (Figure 2.6). The difference in results using YPD with HU and SC with HU could indicate that acetic acid accumulation,

increased DNA replication stress, or other stresses/cellular changes associated with aging in SC medium may mask the ability of HU treatment to further extend lifespan of the high Ty1 copy strains.

Since changes in ROS have been associated with yeast CLS [169,220,221], we measured ROS levels in the zero and high Ty1 copy strains. No significant differences in levels of peroxides and other ROS detected by the fluorescent reagent dihydrorhodamine, DHR, and flow cytometry were noted between zero and high Ty1 copy strains grown in YPD during the transition to stationary phase and early time points during aging. In contrast, high Ty1 copy strains had a significant reduction in ROS detected by DHR compared to the zero copy strains at day three when 30 mM HU was present in the YPD medium (Figure 2.7A). This difference diminished and was no longer significant at later time points. Cells grown in SC medium were sampled at comparable points during early stationary phase and early aging, but sampling was on different days to compensate for

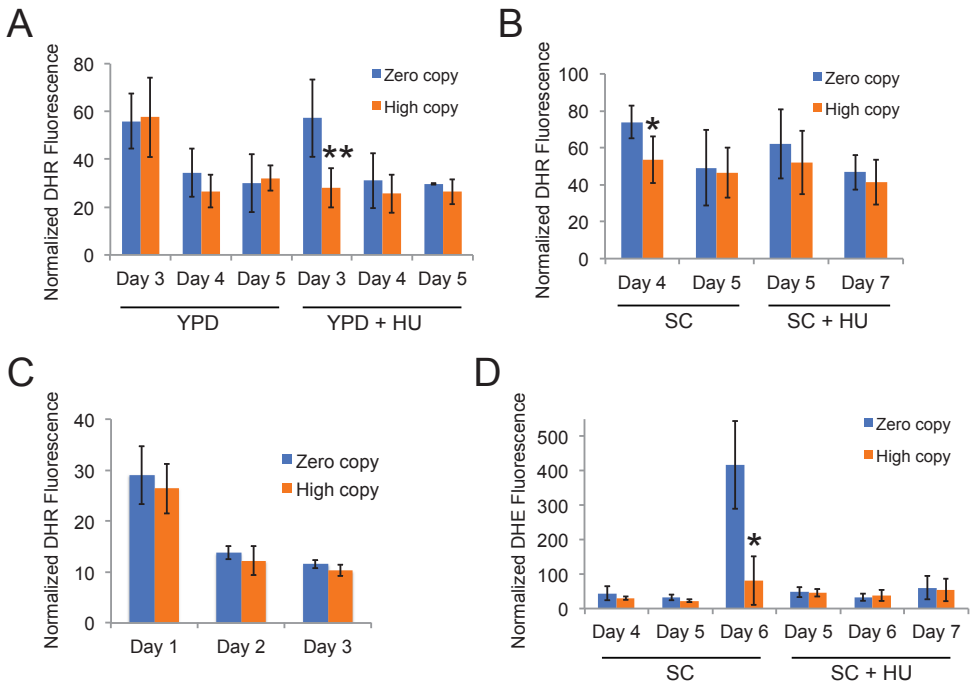

Figure 2.7. Reduced ROS levels in high Ty1 copy strains at early time points may contribute to lifespan extension.

(A) Mean of normalized dihydrorhodamine, "DHR", fluorescence obtained from at least four trials of zero Ty1 copy (blue columns) and high Ty1 copy (orange columns) strains after the indicated number of days in rich medium without or with 30 mM hydroxyurea, "YPD" or "YPD + HU", respectively. Error bars indicate standard deviation, and the symbol "**" indicates $p<0.01$ comparing zero and high Ty1 copy strains at the indicated day. (B) As for (A) for three trials using synthetic medium without or with 30 mM hydroxyurea, "SC" or "SC + HU", respectively. The symbol "*" indicates $p<0.05$ comparing zero and high Ty1 copy strains. (C) As for (A) for five trials using YPD with 30 mM hydroxyurea and incubation at 30˚. (D) Mean of normalized dihydroethidium, "DHE", fluorescence obtained from three trials of zero Ty1 copy (blue columns) and high Ty1 copy (orange columns) strains in synthetic medium without or with 30 mM hydroxyurea, "SC" or "SC + HU", respectively. The symbol "*" indicates $p<0.05$ comparing zero and high Ty1 copy strains.

slightly slower growth in SC medium compared to YPD and a further reduction in growth rate in SC medium with HU (data not shown). High copy Ty1 strains again had a significant reduction in ROS detected by DHR compared to the zero copy strain on the initial day of sampling in SC medium alone (Figure 2.7B), but no significant differences were seen for either day of sampling of cells grown in

SC with HU. No differences in ROS levels measured with DHR were noted between zero and high Ty1 copy strains grown at 30˚ in YPD medium with HU, consistent with the diminished lifespan extension observed with incubation at 30˚ (Figure 2.7C). Sampling was done on different days in the latter case to account for faster growth at 30˚. We also observed a significant increase in fluorescence with dihydroethidium (DHE), which detects superoxide anions, in the zero Ty1 copy strain as populations neared their median lifespan in SC medium alone that was significantly diminished in the high Ty1 copy strains at the same time point (Figure 2.7D). The results of these ROS experiments show a correlation between media conditions in which high Ty1 copy strains have extended longevity and significant differences in ROS levels compared to the zero Ty1 copy strain.

Zero and high Ty1 copy strains were grown and aged in the presence of reduced glutathione as an antioxidant to further explore the connection between ROS levels and extension of lifespan in high Ty1 copy strains. The median and maximum CLS of the zero Ty1 copy strain were virtually the same as for the high Ty1 copy strains in YPD medium with 30 mM HU and either 5 or 10 mM glutathione (Figure 2.8A). The moderate reduction in CLS of all strains when 10 mM glutathione was used indicates that excessive levels of glutathione may have negative effects on cells. The median and maximum CLS of the zero copy strain in YPD with HU and 5 mM glutathione were not significantly different from

the median and maximum CLS of the high Ty1 copy strains in YPD with HU only (Figures 2.2D and 2.8A). This demonstrates that addition of glutathione to the

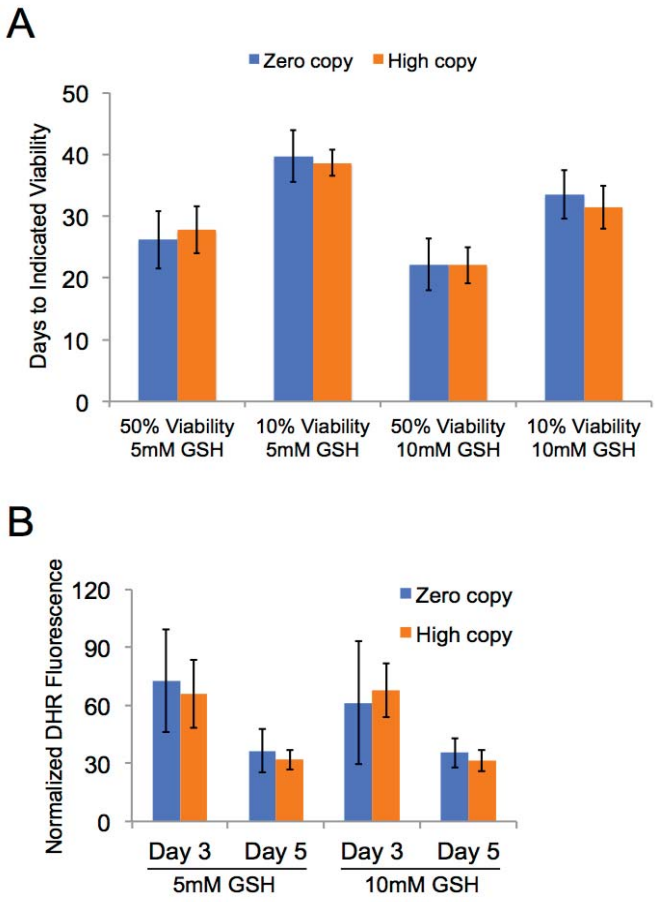

Figure 2.8. Lifespan of the zero Ty1 copy strain increases to that of high Ty1 copy strains in hydroxyurea with antioxidant treatment.

(A) Median, "50% viability", and maximum, "10% viability", lifespans for zero Ty1 copy and high Ty1 copy strains (blue and orange columns, respectively) grown in YPD medium with 30 mM hydroxyurea and the indicated concentration of glutathione, "5mM GSH" or "10mM GSH". Data represent means ± standard deviations of three to five trials. (B) Mean of normalized dihydrorhodamine, "DHR", fluorescence obtained from at least three trials of zero Ty1 copy and high Ty1 copy (blue and orange columns, respectively) strains after the indicated number of days in rich medium with 30 mM hydroxyurea and the indicated concentration of glutathione, "5mM GSH" or "10mM GSH". Error bars indicate standard deviation.

medium increased the lifespan of the zero copy strain so that it was equivalent to that of the high Ty1 copy strains in HU. Treatment with 5 mM glutathione led

to a modest increase in median CLS of the high Ty1 copy strains (p<0.05), but did not increase their maximum CLS in YPD with HU (Figures 2.2D and 2.8A). This limited influence of glutathione is consistent with longevity-promoting changes in ROS accumulation occurring in the high copy strains without exposure to an antioxidant. These data from glutathione exposure are consistent with changes in ROS contributing to the lifespan extension of high copy Ty1 strains grown in YPD with HU. An overall comparison of the relative lifespan of high Ty1 copy strains compared to the zero copy parent strain in glutathione-containing medium and all other media conditions tested is shown in Table 2.

DHR fluorescence was compared in zero and high Ty1 copy strains exposed to both HU and reduced glutathione to test whether ROS levels would be similar, since these strains had similar CLS. No significant differences were noted in ROS levels between these strains when grown and aged in YPD with HU and either 5 or 10 mM glutathione for three or five days (Figure 2.8B). This is in contrast to the results for YPD with HU and no glutathione (Figure 2.7A**).** These results further support the observation that conditions that increase CLS of the high Ty1 copy strains relative to the zero copy strain are associated with changes in ROS between these strains, while conditions that do not increase CLS of the high Ty1 copy strains are associated with similar ROS levels between high and zero Ty1 copy strains.

Table 2. Mean lifespan of high Ty1 copy strains as a percentage of mean lifespan of the zero Ty1 copy strain for each medium or growth condition.

MEDIUM/CONDITION	MEDIAN LIFESPAN[a]	MAXIMUM LIFESPAN[b]
YPD 20˚C	104	109
YPD 20˚C + HU	122*	159**
YPD 30˚C	106	107
YPD 30˚C + HU	106	116**
YPD 20˚C + Threonine	101	119
YPD 20˚C + Threonine + HU	122	137*
SC 20˚C	123*	122*
SC 20˚C + HU	103	106
YPD 20˚C + 5mM Glutathione + HU	106	97.2
YPD 20˚C + 10mM Glutathione + HU	99.5	93.7

[a] Days to 50% viability.

[b] Days to 10% viability.

* $p<0.05$ versus zero Ty1 copy strain.

** $p<0.01$ versus zero Ty1 copy strain.

2.3. Discussion

This work identifies an unanticipated positive contribution of yeast Ty1 elements to chronological lifespan through use of an exceptional yeast model system for studying retrotransposons. Much of the current focus on the possible contribution of retrotransposons to aging concerns the potential for these elements to negatively influence lifespan by promoting genome instability [116,141]. We did not observe a negative influence of retrotransposition on lifespan of non-dividing yeast cells, but rather, strains with many Ty1 copies and low retrotransposition levels survived longer in stationary phase when grown in rich medium with a low dose of the ribonucleotide reductase inhibitor HU or in synthetic medium. Lifespan extension was correlated with reduced ROS during early stationary phase, and antioxidant treatment allowed the strain with no Ty1 elements to live as long as the high Ty1 copy strains in rich medium with HU. The presence of many chromosomal Ty1 retrotransposons may indirectly or directly lead to cellular changes that affect ROS production and/or scavenging and promote chronological lifespan. The potential contribution of retrotransposons to aging could therefore be complex and should be investigated from a broad perspective.

The increased lifespan of all the *S. paradoxus* strains due to treatment with a low dose of HU is in contrast to a recent report of decreased CLS in *S. cerevisiae* strains treated with the same dose of HU [210]. This discrepancy could be due to differences in methods, but we note that there are certain

consistencies between our results and those of the previous report. First, *S. cerevisiae* cells exposed to 30 mM HU have substantially decreased reproductive potential (ability to form colonies on fresh medium) in stationary phase but no reduction in terminal cell density [210]. We also observed no decrease in terminal cell density and a moderately lower density of colony forming units (lower reproductive potential) at the start of stationary phase with HU treatment (data not shown). Second, many cells exposed to HU in the previous study and in our work were in S or G2 stage of the cell cycle during stationary phase [210]. Third, both our study and the previous study found that addition of excess threonine to buffer dNTP levels could extend lifespan of cells aging in the presence of HU [210,215]. However, we found no loss of cell viability due to HU by direct staining of cells and longer maintenance of cell viability and reproductive potential in HU medium. An important difference between the studies is that we grew cells from a low initial density (5×10^3 cells/ml) to stationary phase, while the previous study used cultures inoculated at a higher initial density (5×10^7 cells/ml), so cells in our experiments spent more time actively growing in the presence of HU [210]. We propose that a longevity-promoting effect resulting from exposure to a low dose of HU during exponential growth in our experiments overshadows a negative effect of HU on CLS due to replication stress during stationary phase.

Extended lifespan of high Ty1 copy strains was correlated with decreased ROS levels during early stationary phase. Changes in mitochondrial function and ROS

levels during active growth that lead to decreased ROS levels during early stationary phase underlie yeast CLS extension due to deletion of the *TOR1* gene [222,223]. *TOR* genes in diverse organisms encode kinases responsive to nutrient conditions and growth signaling that regulate processes such as translation, ribosome biogenesis, and autophagy to influence cell growth, metabolism, and aging [56]. Functional mitochondria are required for increased CLS of yeast *tor1*Δ mutants [222]. These mutants exhibit elevated mitochondrial membrane potential and mitochondrial superoxide during active growth, but reduced mitochondrial membrane potential, superoxide, and ROS detected by DHR in early stationary phase [223]. Overexpression of the mitochondrial superoxide dismutase, *SOD2*, prevents lifespan extension in *tor1*Δ mutants. Exposure to a low dose of the superoxide-generating reagent menadione during exponential growth of wild type cells causes lifespan, ROS, and mitochondrial changes similar to those of *tor1*Δ mutants [223]. These observations indicate that ROS changes during active growth and early stationary phase are important for mediating lifespan extension. The ROS changes we observed in high Ty1 copy strains could reflect similar changes in mitochondrial function or ROS production/scavenging that contribute to lifespan extension in these *S. paradoxus* strains. How the presence, expression, and/or mobility of Ty1 elements could indirectly or directly produce such changes is a topic that we are actively investigating.

Our findings do not rule out potential negative effects of retrotransposons on lifespan in other contexts or aging models, including replicative aging of actively

dividing yeast mother cells [168]. Retrotransposon expression increases with age in gonads of *C. elegans*, brain tissue of *Drosophila melanogaster*, normal human cells maintained *ex vivo*, multiple mouse tissues, and yeast mother cells [165,196,163,197,198]. Increased mobility of retrotransposons occurs at late time points during yeast chronological lifespan and in brains of aged *D. melanogaster* [163,153]. Increased copy numbers of retrotransposons in human cells aged *ex vivo*, somatic tissues from aged mice, and during yeast replicative aging have also been interpreted as signs of increased retrotransposition [196–198]. These correlations have led to proposals that retrotransposons might promote the aging process and/or genome instability during aging. The *S. paradoxus* Ty-less strain background has allowed us to carefully address such a potential role during chronological aging of haploid yeast strains in certain media conditions. Whether Ty1 elements could negatively influence CLS in the context of other lifespan altering mutations or treatments, and whether they have a negative influence on the lifespan of dividing cells will require further study.

Additionally, mutation rate of a representative gene was not increased due to elevated retrotransposition in our haploid strains in young populations, in contrast with previous work showing that retrotransposition was correlated with loss of heterozygosity and chromosome loss during chronological aging of diploid *S. cerevisiae* strains [153]. The difference in these results could be due to the use of different cell types (haploids vs. diploids), cells of different ages, or to the different measures of genome instability. While high levels of

retrotransposition did not negatively influence CLS, it remains possible that some forms of genome instability are elevated in haploid cells during chronological aging due to retrotransposition. Furthermore, while mutations and chromosome rearrangements accumulate during yeast chronological aging [224], additional research is needed to understand how this relates to the progressive loss of viability during stationary phase.

This work also provides a new perspective on the relationship between ROS and retrotransposons. Prior work identified increased mobility of yeast Ty1 and mammalian L1 elements in response to oxidative stress and that activation of Ty1 elements by the DNA-damaging agent MMS requires mitochondrial function and ROS production [154,156]. Regulation of retrotransposon expression and mobility by numerous stresses could have consequences for cell function and survival in stressful conditions [146,225]. However, our findings show that retrotransposons can indirectly or directly alter ROS levels in certain contexts, indicating that these elements might influence cellular responses to stress. Dispersed copies of retrotransposons that are subject to chromatin modifications and that initiate transcripts that can read through into neighboring genes may alter gene expression patterns [146], which could affect cellular stress responses. Chimeric transcripts containing retrotransposon sequences and sequences of other genes are observed in human cells, including tumor cells, and can regulate gene expression [226,227]. Chromatin marks on mammalian SINE retrotransposons can change in response to stress [228], and RNA from these elements can directly

interact with RNA polymerase to influence gene expression in response to heat shock [229]. Yeast Ty1 elements can alter transcription of neighboring genes and establish stress-responsive patterns of expression on genes [173,230]. However, the results from growth at 30˚ are consistent with the expression and activity of Ty1 proteins directly or indirectly influencing lifespan and ROS, since substantial Ty1 transcription occurs at 30˚ [212,214]. Also, the different patterns of Ty1 insertions in the high Ty1 copy strains indicate that it is unlikely that the same gene or genes would have altered expression due to neighboring Ty1 sequences in all the high copy strains. Ty1 Gag protein forms the Ty1 virus-like particle and Ty1 Pol protein has protease, reverse transcriptase, and integrase activities [199]. One or more of these activities might indirectly alter stress-response pathways or, in the case of reverse transcriptase or integrase, influence DNA metabolism. An alternative possibility is that Ty1 protein expression/function (or moderate levels of Ty1 mobility) is needed in combination with transcriptional effects on gene expression for lifespan extension.

Overall, a longevity-promoting role for retrotransposons in yeast chronological lifespan in certain contexts provides a new point of view for investigating the relationship between these elements and aging. The advantages of the *S. paradoxus* Ty-less model offer a unique opportunity to directly test for additional roles of retrotransposons during aging. Continued work in this system will likely produce findings relevant to aging and transposable elements in many species,

considering common aspects of regulation and impacts of retrotransposons in diverse species, as well as fundamental similarities between aging in budding yeast and other eukaryotes.

ACKNOWLEDGEMENTS

The authors would like to thank David Garfinkel for generously providing the Ty-less *S. paradoxus* parent strain and members of the Maxwell lab for helpful comments. We also thank Meredith Giblin, Alison Kenny, and Andrew Peifer for technical assistance. This work was supported in part by grant R00AG031911 from the National Institute on Aging to P. H. M. Content of this article does not necessarily represent official views of the National Institutes of Health.

2.4. Materials and methods

2.4.1. Yeast Strains, Media, and Plasmids

Standard rich (YPD) and synthetic (SC) media were used for growing yeast strains [231]. All strains were derivatives of a Ty-less strain of *S. paradoxus*, DG1768 (*MAT*α, *his3-*Δ*200hisG*, *ura3,* kindly provided by David Garfinkel) [181]. A galactose-inducible plasmid copy of Ty1 containing a *his3AI* retrotransposition indicator gene [176] was used to obtain derivatives of DG1768 with a single genomic copy of a Ty1*his3AI* element, as described previously [153]. The high copy *URA3*-marked plasmid pGTy1H3CLA containing an unmarked Ty1 element under the control of a galactose-inducible promoter [232] was used to introduce unmarked copies of Ty1 into the genome of strain DG1768. Ura^+ transformants

harboring pGTy1H3CLA were grown on SC + 2% glucose medium lacking uracil, and selected Ura^+ transformants were then grown in liquid SC + 2% glucose medium lacking uracil at 30˚ overnight. Aliquots of these cultures were diluted and spread onto solid SC medium lacking uracil + 2% galactose to induce Ty1 retrotransposition, and were then grown at 20˚ or 30˚ to vary the level of retrotransposition (Ty1 mobility is restricted at 30˚, [211,212]). Single colonies obtained after induction were grown on YPD medium to allow for loss of pGTy1H3CLA and analyzed for Ty1 copy number by qPCR using a Roche LightCycler® 480. Genomic DNA was extracted by glass bead disruption in phenol/chloroform and ethanol precipitation, followed by further purification using a Wizard® SV Gel and PCR Clean-up System (Promega). Equal concentrations of DNA from these strains were amplified with primers to the *TYA1* region of Ty1 and primers to a single copy gene, *ACT1*. The cycle threshold (Ct) values for the *TYA1* PCR were normalized to the *ACT1* Ct values for each strain, and approximate copy number was then determined by comparing these normalized values to the normalized *TYA1* Ct for a single copy Ty1*his3AI* control strain. Strains were grouped as low copy (1-3 genomic Ty1 elements) or high copy (~20 genomic Ty1 elements). A pGTy1H3*kanMX* plasmid was constructed by first amplifying the *kanMX* marker present in the *S. cerevisiae MAT*α deletion collection (Thermo Scientific Open Biosystems) with PCR primers that added sequences corresponding to Ty1-H3 positions 5516-5566 and 5567-5616 to either end of *kanMX* (Ty1-H3 GenBank accession M18706.1). This PCR product was then co-transformed into strain DG1768 with pGTy1H3CLA linearized at the

*Cla*I site present between the 3' end of *TYB1* and the 3' LTR. Cells in which the Ty1-*kanMX* PCR product was used to repair the plasmid were selected on YPD medium containing 200 µg/ml G418 sulfate and verified by PCR. A high-copy *URA3*-marked control plasmid lacking Ty1 sequences was generated by cloning a *BamH*I-*EcoR*I fragment of the PCR-amplified *kanMX* gene into pRS426 digested with *BamH*I and *EcoR*I. Strains with pGTy1H3*kanMX* or pRS426-*kanMX* were grown using YPD medium containing 200 µg/ml G418 sulfate to maintain each plasmid.

2.4.2. Southern Blotting to Detect Ty1 Insertions

Approximately 1-2 µg of genomic DNA prepared from cells grown at 30˚ by glass bead disruption in phenol/chloroform and ethanol precipitation was digested with *PvuII* or *SphI* restriction enzymes and used for Southern blotting as described previously [188]. A radioactive probe was prepared from a PCR product corresponding to positions 4221-4842 of Ty1-H3 (GenBank accession M18706.1) by random priming using the Ambion DECAprime II kit (Life Technologies), and signal was visualized using a Typhoon Trio+ imager (GE Healthcare).

2.4.3. Growth Rate Experiments

Cells from fresh streaks were inoculated into 5 ml YPD broth with or without 30 mM hydroxyurea at an initial density of 1 x 10^5 cells/ml to determine cell-doubling times. Triplicate cultures were grown for each strain for each trial on a

culture tube rotator at 20˚. Cell densities were determined at several time points between seven and 33 hours of growth by counting cells on a hemocytometer to measure growth rate from late lag phase to mid-exponential phase. Exponential function trend lines fit to plots of cell densities compared to hours of growth were used to calculate doubling times in hours.

2.4.4. Chronological Lifespan Determination

Cells from fresh streaks were inoculated at an initial density of 5 x 10^3 cells/ml in triplicate 25 ml cultures in 125-ml Erlenmeyer flasks and aged at 20˚ (or 30˚ for some trials) with shaking. For SC medium experiments, the same initial density of cells was inoculated into triplicate four ml cultures in culture tubes and aged at 20˚ on a tube rotator. Media used for lifespan experiments included YPD with or without 30 mM hydroxyurea, 0.8 mg/ml threonine, or 5 mM or 10 mM reduced glutathione, as well as SC + 2% glucose medium with or without 30 mM hydroxyurea. At early stationary phase and at regular time points thereafter, aliquots of cells were removed to assess viability and colony-forming units/ml (cfu/ml). Viability was measured by mixing diluted cells with two volumes of 0.4% trypan blue in phosphate-buffered saline (PBS), incubating for 45 minutes at room temperature, and determining the fraction of unstained cells to total cells for populations of approximately 200 cells by microscopy. Cfu/ml were determined by spreading aliquots of diluted cells onto YPD agar. Trend lines were fit to graphs of viability versus days of incubation and used to calculate

days to 50% and 10% viability as measures of median and maximal lifespan, respectively.

2.4.5. Ty1 Integration and Retromobility Frequency

A previously described semi-quantitative assay to measure Ty1 integration upstream of 5S rRNA genes was modified in two ways to compare Ty1 retrotransposition levels in different yeast strains [153]. First, primers to the *S. paradoxus LEU2* gene were used to control for PCR efficiency. Second, the total signal intensity of Ty1 integration PCR products for each template minus the background intensity obtained from a zero Ty1 copy template was determined using a ChemiDoc XRS+ imager and Image Lab version 4.0 (Bio-Rad) and normalized to the signal intensity of the control *LEU2* PCR product to obtain a semi-quantitative relative measure of Ty integration. In some trials, retrotransposition was inhibited by growing cells in the presence of 200 µg/ml phosphonoformic acid to strongly inhibit Ty1 reverse transcriptase without noticeably altering cell growth [233]. Ty1 retromobility was measured in strains with a chromosomal Ty1*his3AI* element by determining the frequency of His^+ prototroph formation [176]. Seven replicate cultures initiated at a density of 5×10^3 cells/ml were grown to near saturation at 20˚ for each strain/trial, aliquots of diluted cells were spread onto YPD medium to determine cfu/ml, and appropriate volumes of cells were spread onto SC + 2% glucose medium lacking histidine. Mobility frequencies were calculated as the number of His^+ prototrophs

divided by the number of cfu in the same volume of culture. Median values for three independent trials were averaged.

2.4.6. Mutation Rate and Frequency Measurements

Fluctuation tests were performed to obtain rates of loss-of-function mutations in the *CAN1* gene that produce resistance to canavanine, and rates were calculated using the online calculator FALCOR [218,234]. Ten replicate one ml cultures in YPD or YPD with 30 mM hydroxyurea inoculated at initial densities of 1 x 10^3 cells/ml were grown to late log/early stationary phase at 20˚. Aliquots of culture were diluted in water and spread onto YPD agar to determine colony-forming-units/ml and the remainder of each culture was pelleted and resuspended in water to spread onto SC + 2% glucose medium lacking arginine and containing 60 µg/ml canavanine. Plates were incubated for up to four days at 30˚. Mutation frequencies were measured in a similar manner using three or seven replicate cultures inoculated at initial densities of 5 x 10^3 cells/ml grown in YPD or YPD with varying concentrations of hydroxyurea. Mutation frequencies were calculated as the number of mutant colonies obtained divided by the number of cfu spread on selective medium, and median values from independent trials were averaged.

2.4.7. Analysis of Reactive Oxygen Species Levels and DNA Content

Strains were grown in flasks at 20˚ (or 30˚ for some trials) as described for chronological lifespan experiments and sampled after three to seven days (one

to three days at 30˚) of growth to determine levels of reactive oxygen species (ROS). Methods to measure ROS using dihydroethidium (DHE) or dihydrorhodamine 123 (DHR) were based on published protocols [19,221]. Briefly, approximately 1 x 10^7 cells were washed in 1X PBS buffer twice, resuspended in 1X PBS, and incubated with a 50 μM final concentration of either DHR or DHE for 2 hours at room temperature (in the dark with rocking). After incubation, cells were washed twice in 1X PBS buffer before analysis on a BD LSR II™ flow cytometer using excitation at 488 nm and a 530/30 nm (long pass 505) emission filter for DHR or excitation at 515 nm and a 610/20 nm (long pass 600) emission filter for DHE. The median fluorescence intensity for each sample was determined using FlowJo version X.0.7 software (TreeStar, Inc.) and normalized based on the median fluorescence intensity of the corresponding unstained sample. Similar results were obtained analyzing geometric means. Reported values are averages of median values for three or more trials. Analysis of DNA content in cells was performed as described previously [209], except that Propidium Iodide ReadyProbes Reagent (PI, Life Technologies) was used instead of SYBR Green. Cells were incubated with two drops per ml of the PI reagent for 15 minutes prior to analysis by flow cytometry using excitation at 515 nm and a 610/20 nm (long pass 600) emission filter.

2.4.8. Statistical Analysis

Mean values were compared for significant differences using unpaired, two-tailed t-tests assuming equal or unequal variance, depending on the variance in particular data sets. Levels of significance are indicated in figures and the text.

Chapter 3

3. EXTENSION OF CHRONOLOGICAL LIFESPAN IN HIGH TY1 COPY NUMBER STRAINS OF *SACCHAROMYCES PARADOXUS* IS ASSOCIATED WITH CELLULAR CHANGES

3.1. Introduction

Retroelements have the potential to alter and shape genomes through their mutagenic effect and are hypothesized to contribute to the aging process [116,194]. Retroelements have been shown to increase their frequency of mobility as they lose repressive chromatin marks during aging [78]. This could potentially contribute to aging due to the detrimental effects of new retrotransposition events. However, in this project we discovered a positive role the retroelement Ty1 can have under certain media conditions, by extending lifespan when present in high copy number. This positive influence of multiple Ty1 elements on CLS appears independent of genomic instability, as we did not observe substantial changes in mutation rates and frequencies of a representative gene due to the presence or mobility of these elements. Thus, using this model to study the impact of Ty1 copy number on yeast chronological aging provides an opportunity to study novel ways by which retroelements influence aging that may also have implications for aging in other organisms.

Several factors have been discovered to directly impact CLS in the yeast model and could be relevant for characterizing the positive influence of Ty1 on lifespan.

Accumulation of acetic acid as yeast age in synthetic complete media can limit CLS and this could in turn result in drastic transcriptional changes within the nuclear genome and mitochondrial changes that are associated with aging [209,219,235]. Manipulations that alter nutrient signaling pathways and mitochondrial function can have profound implications for yeast lifespan [222,236], and these factors also influence aging in other models [4]. Deleting or inhibiting TOR or Ras/AC/PKA nutrient-signaling pathways greatly increases CLS in yeast. Inhibiting these pathways extends CLS in part by inducing stress responses. One such example is stimulation of autophagy pathways upon nutrient depletion. Clearance of damaged macromolecules and intracellular recycling of cell components are important to extend CLS in yeast. Depleting TOR treatment with rapamycin is known to stimulate autophagy pathways [237]. TOR signaling is intimately linked to the energy status of the cell and affects mitochondrial function and ROS levels [222,238]. Specifically the deletion of *TOR1* leads to the immediate upregulation of mitochondrial translation, proteins involved in oxidative phosphorylation (OXPHOS), and respiration leading to increases in mitochondrial ROS (mtROS) [169,222,238]. Additionally the lifespan extending effects of CR on yeast CLS are in part due to increases in mitochondrial respiration [239].

While it is evident that mitochondrial function is important for aging in yeast, the role ROS has is slightly more complicated. ROS is a known oxidative agent that can damage macromolecules including DNA. However it is becoming clear that ROS is also involved in intracellular signaling and in adaptive stress responses,

depending on the level of ROS [19]. How and at what levels ROS signaling are transduced to influence aging throughout the cell remains to be discovered. Interestingly, the strong link between mitochondrial function, ROS, and nutrient signaling suggests that these three factors of yeast longevity may have other implications on aging. For example, mitochondria dysfunction may impact other processes involved in cellular signaling in a ROS independent manner. Specifically induction of mitochondrial-specific autophagy pathways and reduced efficiency in mitochondrial bioenergetics involving *SIRT3* to limit lifespan [240–243].

Multiple stresses can stimulate Ty1 retromobility, but the possibility that Ty1 expression and activity can also influence stress pathways and cellular processes has not been directly investigated. It may be that the presence of multiple copies of Ty1 represses these pro-aging pathways and functions. Addressing this topic could identify novel cellular roles for Ty1 and retrotransposons in general and could alter the way we view the contribution of retrotransposons to aging in higher eukaryotes. We followed up on the ability of Ty1 to promote CLS that was identified in Chapter 2 by addressing the potential role Ty1 might have in altering pathways known to influence CLS in yeast. Specifically we investigated whether the effect of Ty1 on CLS in SC medium depended on medium acidification, and whether the presence of Ty1 altered mitochondrial membrane potential or sensitivity to rapamycin, an inhibitor of TOR, using our zero and high Ty1 copy strains. We observed that strains with and without Ty1 had similar lifespans in buffered SC medium. Furthermore, high

Ty1 copy strains were more resistant to rapamycin treatment and had lower mitochondrial membrane potentials when grown in rich medium with HU than the zero Ty1 copy strain.

3.2. Results

3.2.1. Lifespans of zero and high Ty1 copy strains are similar in buffered SC medium

We chronologically aged strains of *S. paradoxus* with zero or high copies of Ty1 in SC medium with or without 30mM HU to which a final concentration of 1X PBS was added to test the possibility that acidification of SC medium was required for high Ty1 copy strains to exhibit an extended lifespan. As expected, median and maximal CLS increased in buffered SC medium for both zero and high Ty1 copy strains compared to unbuffered SC medium, by approximately two-fold (compare Figure 3.1 and Figure 2.6). High Ty1 copy strains had significantly extended lifespans in buffered SC medium containing 30mM HU compared to the zero copy strain for median lifespan (25.0 versus 21.8 days) and maximum lifespan (31.8 versus 27.3 days), respectively. No significant differences in CLS in buffered SC medium lacking HU were observed for median (14.3 versus 12.7 days) or maximum lifespan (18.5 versus 16.5 days) for high and zero copies, respectively. In HU, this represents extensions of 114% and 118% for median and maximum lifespan for high Ty1 copy strains over the zero copy strain. Strikingly, the zero copy strain had similar maximal CLS in buffered SC medium with HU compared to rich medium with HU (27.3 and 25.4 days,

respectively). However, high copy strains had a significantly lower maximal CLS in SC medium with HU compared to rich medium with HU (31.8 and 40.4 days, respectively). These results indicate that mitigating the effects of medium acidification in SC medium prevents high Ty1 copy number from extending lifespan, but also “unmasks” the ability of HU treatment to increase lifespan in high Ty1 copy strains relative to the zero copy strain.

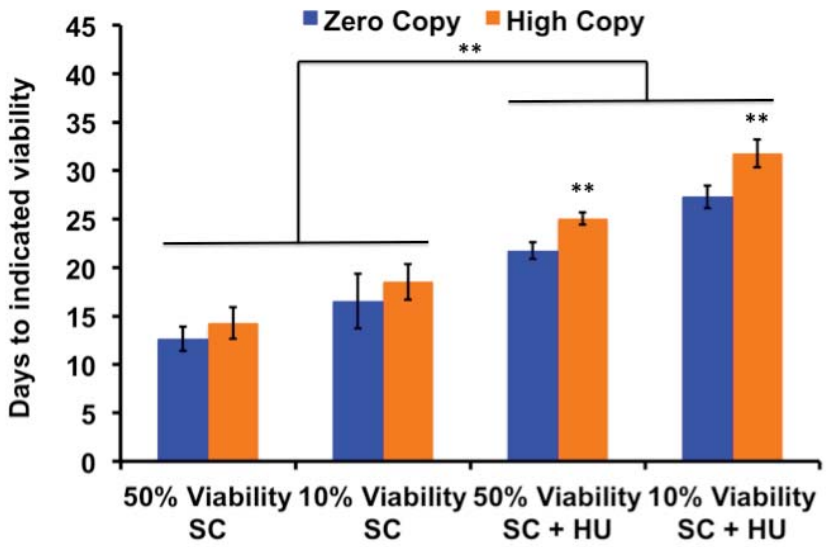

Figure 3.1. High Ty1 copy strains have extended lifespan in 1X PBS buffered synthetic complete medium with hydroxyurea.

Median, “50% viability”, and maximum, “10% viability”, lifespans for zero Ty1 copy (blue columns) and high Ty1 copy (orange columns) strains grown in synthetic medium buffered with 1X PBS and without or with 30 mM hydroxyurea, “SC” or “SC + HU”, respectively. Data are the mean ± standard deviation from three to five trials. The symbol “**” indicates $p<0.01$ for lifespans in SC compared to those in SC + HU and between high Ty1 and zero copy strains.

3.2.2. High Ty1 copy strains have reduced mitochondrial membrane potential in rich medium with HU

Mitochondria structure and function are important during aging in many organisms [4], and lifespan extension in high Ty1 copy strains was associated with reduced ROS levels (Figure 2.7). To test whether mitochondrial function is altered in the high Ty1 copy strains, we treated cells with the fluorescent reagent *3,3'*-dihexyloxacarbocyanine and performed flow cytometry to measure mitochondrial (mt) membrane potential. Mitochondrial membrane potential has been shown to decrease with RLS in yeast [244]. We observed similar mitochondrial membrane potentials in both zero and high copy strains when grown in YPD medium, and the approximately 20% reduction in high copy strains relative to the zero copy strain on day one was not a significant decrease. However, the ~35% reduction in mitochondrial membrane potential in YPD medium with 30mM HU in high copy strains relative to the zero copy strain was a significant decrease. This reduction in mitochondrial membrane potential between zero and high Ty1 copy strains was not observed at later time points. This result indicates that high Ty1 copy strains have altered mitochondrial function early during growth in the presence of HU. Unfortunately, some further experiments to explore the role of mitochondrial function in Ty1-dependent lifespan extension could not be carried out, due to the observation that none of the *S. paradoxus* strains grew to stationary phase in media conditions that are commonly used to force yeast cells to respire (such as glycerol or ethanol as the sole carbon source). Further work will be required to determine the reason for

this inability to grow well on non-fermentable carbon sources. The mitochondrial membrane potential results show a further correlation between conditions that extend CLS and alter cellular functions in high Ty1 copy strains compared with zero copy strains.

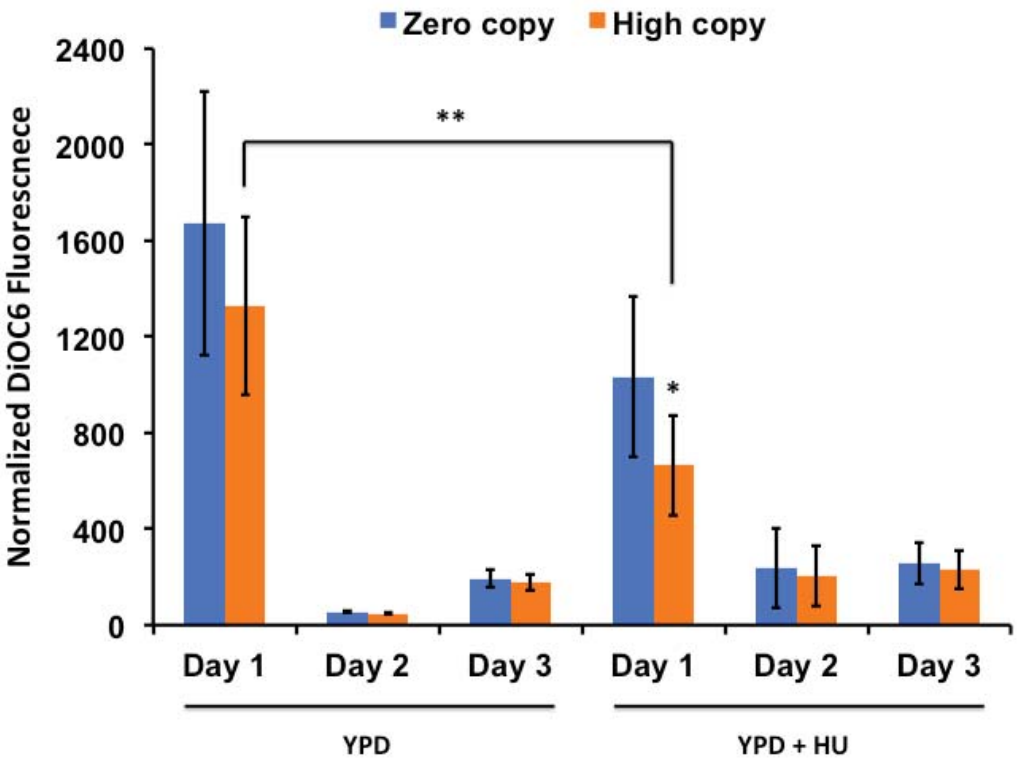

Figure 3.2. High Ty1 copy strains have moderately reduced mitochondrial membrane potential in early log phase in high Ty1 copy strains grown in rich medium and hydroxyurea.

Median of normalized *3,3'*-dihexyloacarbocyanine (DiOC6) fluorescence obtained from at least four trials of zero Ty1 copy (blue columns) and high Ty1 copy (orange columns) strains after the indicated number of days in rich medium without or with 30 mM hydroxyurea, "YPD" or "YPD + HU", respectively. Error bars indicate standard deviation, and the symbol "**" indicates $p<0.01$ comparing zero and high Ty1 copy strains at the indicated day. The symbol "*" indicates $p<0.05$

3.2.3. High Ty1 copy strains are more resistant to rapamycin in rich medium with HU

Rapamycin is an inhibitor of the TOR nutrient-sensing kinase, and TOR activity is a well-known pro-aging signal. We assayed zero and high Ty1 copy strains for sensitivity to rapamycin over the course of three days of growth in rich medium with HU. We observed no significant effect in relative colony-forming units (CFUs/ml) in zero and high copy strains grown in the presence of 25nM or 100nM rapamycin for 24 hours in rich medium YPD with 30mM HU. These results indicate that treatment with rapamycin does not affect growth in these strains prior to stationary phase, and the presence of Ty1 did not alter rapamycin sensitivity during initial growth of the populations. At early stationary phase (Day 3), we observed a significant difference in relative CFU/ml between zero and high copy strains on day three in YPD with 30mM HU and 25nM rapamycin. Under these conditions, high Ty1 copy strains had relative CFU/ml values similar to population growth prior to stationary phase (Day 1) in YPD with 25nM rapamycin and HU, whereas the relative CFU/ml of zero copy strains was reduced by approximately 80% compared to growth in YPD with 25nM rapamycin and HU on day 1. When the rapamycin concentration was increased to 100nM, both zero and high copy strains had a similar reduction in relative CFU/ml in YPD with HU medium. These results indicate that high Ty1 copy strains may have altered TOR signaling or changes in other cellular activities that increase resistance to rapamycin when cells are stressed by exposure to HU.

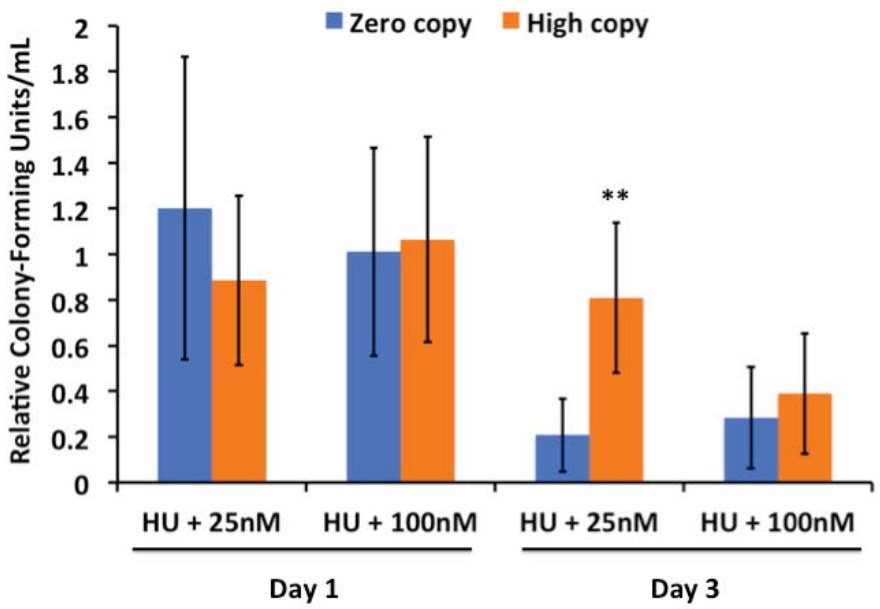

Figure 3.3. Zero copy strains are significantly more sensitive to 25nM rapamycin treatment when grown in 30mM HU rich media compared with high Ty1 copy strains.

Relative colony forming units were determined numerically by dividing values obtained from rapamycin treated cultures by values obtained from control cultures for zero (blue columns) and high (orange columns) Ty1 copy strains and the symbol "**" indicates $p<0.01$.

3.3. Discussion

This work further characterizes the pro-longevity effects of high Ty1 copy number on yeast grown under mild chronic replication stress of HU or in SC medium, but the mechanisms responsible for the effects have yet to be fully determined. Previous experiments (Chapter 2) had correlated the positive influence of Ty1 on longevity with low levels of ROS early during growth to stationary phase. The current experiments have extended this analysis by

showing that the influence of Ty1 on lifespan is affected by medium acidification and high Ty1 copy number is associated with reduced mitochondrial membrane potential and increased resistance to rapamycin when cells are exposed to HU. Overall, these results indicate that changes in growth signaling, mitochondrial activity, or both, may at least partly account for the effect of Ty1 on CLS. After cells grow and begin to deplete nutrients as they approach stationary phase, they divert resources from growth and reproduction to stress resistance and maintenance of lower metabolic rates. Changes that influence this shift from rapid growth into stationary phase in yeast are known to have dramatic effects on CLS. Therefore, changes identified during initial growth or during early stationary phase have the potential to account for changes in longevity.

The moderate extension of CLS in high Ty1 copy strains grown in SC medium originally observed (Chapter 2) was no longer observed when the SC medium was buffered against acidification. While yeast in fermentative growth excrete numerous organic acids in addition to acetic acid, Burtner et al. determined that accumulation of physiological levels of acetic acid in media was sufficient to induce cellular damage and limit CLS [219]. Increases in acetic acid concentration or a decrease in the pH of the media, may make cells more sensitive to oxidative stress. This same report also highlighted that buffering SC medium can reverse the negative effects of acetic acid accumulation. Therefore, we were curious if the CLS results we showed in Chapter 2 could be mitigated by treatment with a buffer to raise the pH of SC medium. Our results presented here in experiments

with CLS in buffered SC media highlight the how varying environmental factors can impact CLS of our zero and high Ty1 copy strains in different ways. For example, decreases in pH of the media by acetic acid accumulation may increase sensitivity to oxidative stress. Additionally, high concentrations of acetic acid are known to induce programmed cell death in yeast [245]. Like apoptotic pathways, programmed cell death in yeast requires changes to mitochondrial function, decreases in membrane potential and release of cytochrome *c* [246]. Furthermore, hyperactivation of Ras-cAMP-*PKA* signaling pathways can also lead to dysfunctional mitochondria [247] The extended lifespan of high Ty1 copy strains in unbuffered SC medium is not further extended relative to zero copy strains by HU treatment. However, in buffered SC medium, high Ty1 copy strains do not have extended lifespan unless they are treated with HU. There may be some interaction between these two stresses (acidification and HU) such that acidification of the medium prevents HU from causing a greater extension of lifespan in high copy strains relative to the zero copy strain. Determining what effects of acidification are necessary for the differential CLS of zero and high copy strains could address the nature of this interaction to help clarify how Ty1 may be promoting lifespan.

Mitochondrial function has important influences on aging, and high Ty1 copy number led to reduced mitochondrial membrane potential during exposure to HU. It remains to be established whether this is a direct or indirect effect of Ty1, but this could represent an unprecedented role for retrotransposons in the

regulation of cellular functions. Interpreting this effect on mitochondrial membrane potential is complicated, though, because other studies have correlated reduced membrane potential with mitochondrial dysfunction and functional decline during aging. Since the reduced mitochondrial potential observed here was in the context of stress (HU treatment), then perhaps the reduction represents an adaptive change in growth and metabolism, rather than mitochondrial dysfunction. The reduced mitochondrial membrane potential early in growth is consistent with identification of lower ROS levels early in stationary phase in high Ty1 copy strains (Chapter 2). Fully understanding how Ty1 might influence mitochondria and how this relates to extension of CLS will require additional work, including a better characterization of the *S. paradoxus* strain background. We found that these strains grow very poorly when forced to respire on non-fermentable carbon sources, which indicates that the strain background could have a mitochondrial deficiency or change in mitochondrial activity. Determining the basis for this poor growth may provide insight into the effects of Ty1 in these strains. Regardless, our data here support recent literature highlighting the potential "mitohormetic" stress responses to promote CLS, which suggests that cellular fitness is improved by mild stress treatments to stimulate compensatory cellular responses [1,4,248,249]

Diversion from anabolic activities to pathways that promote stress resistance occurs during aging and is a common feature of inhibiting the TOR nutrient-sensing pathway and its downstream effectors. Since TOR signaling influences

lifespan, pursuing the reason for the increased resistance of high copy strains to rapamycin may help identify the basis for Ty1-dependent lifespan extension in HU. TOR kinase is the direct target of rapamycin, so increased resistance to rapamycin in high Ty1 copy strains could result from greater TOR activity. Elevated TOR activity is associated with accelerated aging, though, so it does not seem likely that greater TOR activity would account for extended lifespan of high Ty1 copy strains in HU. Alternatively, a number of gene functions have been found to be necessary for sensitivity to rapamycin in yeast. Included in this set were genes with roles in mitochondria, and loss of these gene functions provided resistance to rapamycin [250]. The reduced mitochondrial activity in high Ty1 copy strains exposed to HU could contribute to rapamycin resistance in these strains when exposed to HU.

Inhibiting TOR signaling is known to upregulate autophagy pathways to extend lifespan [237], and treatment with rapamycin is sufficient to induce autophagy. The recycling of cellular components during autophagy to extend CLS raises the possibility that autophagy pathways are regulated differently between zero and high Ty1 copy strains during HU stress. Exactly how changes in mitochondrial activity or potential changes in autophagy in high copy strains could be extending lifespan will require continued investigation.

Multiple studies also highlight changes in mitochondrial respiration, ROS output, and adaptive signaling that correlate well with CLS extension in yeast

[222,223,236,238,251]. Deletion or inhibition of TOR signaling in yeast increases the metabolic output in mitochondria and increased membrane potential, and this coincides with adaptive ROS signaling by the mitochondria to extend CLS [238]. This same study observed increases in mt membrane potential during logarithmic growth in *tor1Δ* over wt, but then was lower during stationary phase and this promoted lifespan. Since TOR signaling has strong pro-aging effect in yeast, these results suggest a strong link between increasing mitochondrial activity with decreases in nutrient signaling and the role ROS signaling may have in promoting lifespan.

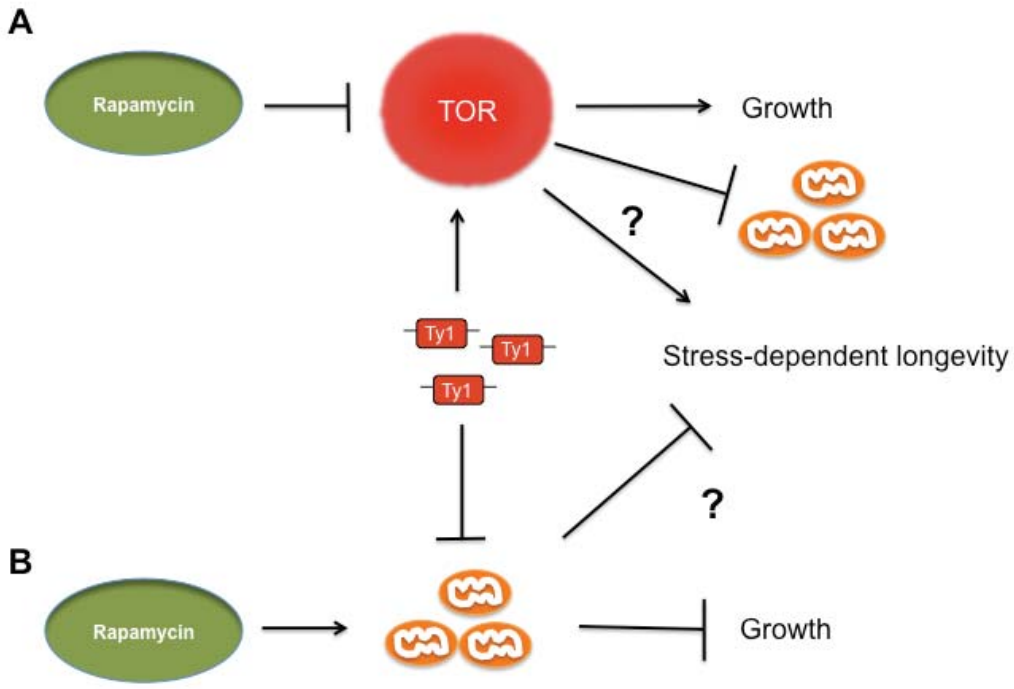

Figure 3.4. Two potential models to account for rapamycin resistance in high Ty1 copy number strains to extend chronological lifespan in hydroxyurea media conditions

Rapamycin functions to inhibit TOR function to alter nutrient signaling and growth leading to decreased growth. The presence of Ty1 elements could lead to overstimulation of TOR resulting

in rapamycin resistance, leading to lower mitochondrial function and membrane potential, promoting growth and longevity under stress conditions (A). Alternatively the presence of Ty1 inhibits mitochondria function leading to rapamycin resistance to promote survival by inducing stress responsive longevity promoting effects (B).

Overall we demonstrate here that differences in lifespan of zero and high Ty1 copy strains are correlated with changes in cellular physiology. While the results are still preliminary, they raise the possibility that retrotransposon expression influences specific cellular activities to alter aging (Figure 3.4). One scenario in this model would suppose that TOR positively influences cellular aging in hydroxyurea. The presence of Ty1 elements or cellular components of Ty1 leads to resistance to rapamycin under low doses (Figure 3.3 and Figure 3.4A). Overactivated signaling would stimulate growth and under this model induce stress-dependent longevity. The connection between increased TOR activity and longevity is unexpected and may not seem intuitive. Alternatively, model B suggests that heightened mitochondrial activity negatively influences CLS, and Ty1 biology may inhibit mitochondrial function in unknown ways, resulting in resistance to rapamycin (Figure 3.4B). Inhibition of growth by rapamycin is known to require a certain level of mitochondrial activity. In this scenario a small, but appropriate amount of stress is generated that encourages mitochondrial to function at the correct level perhaps through a “mitohormetic” effect based on our ROS and mitochondrial membrane potential data (see Figure 2.7 and Figure 3.2). This level of mitochondrial function could induce stress response genes and autophagy to promote lifespan. These connections to longevity are unexpected and the first thing to investigate is TOR and mitochondrial function directly in the context of aging. The contribution of retrotransposons to cellular

functions has not been well investigated, in contrast to regulation of retrotransposons by various cellular pathways. Continued work on this topic may identify novel mechanisms through which retrotransposons alter cell function and survival, providing a broader perspective on how they can contribute to aging.

3.4. Materials and methods

3.4.1. Determination of chronological lifespan in buffered medium

Cells from fresh streaks were inoculated at an initial density of 5 X 10^3 cells/ml in triplicate four ml cultures of SC medium (with or without 30mM HU) with a final concentration of 1X phosphate-buffered saline (PBS) pH 7.4 and aged on a culture tube rotator at 20˚C. Viability was measured by mixing diluted cells with two volumes of 0.4% Trypan blue in PBS, incubating for 45 min at room temperature, and determining the fraction of unstained cells to total cells for populations of ~200 cells by microscopy. CFU/ml were determined by spreading aliquots of diluted cells onto YPD agar. Trend lines were fit to graphs of viability versus days of incubation and used to calculate days to 50 and 10% viability as measures of median and maximal lifespan, respectively.

3.4.2. Determination of mitochondrial membrane potential

Strains were inoculated at 1 X 10^5 cells/ml in YPD with or without 30mM HU and grown in 125ml flasks at 20˚C. Cells were sampled after one to three days of

growth to determine mitochondrial membrane potential using (*3,3'*-dihexyloacarbocyanine (DiOC6)) based on a published protocol [244]. Briefly, 1 X 10^7 cells were washed twice in 1X PBS and then incubated in 0.5ml of PBS with a final concentration of 200nM DiOC6 dye for 30 minutes rotating at 30˚C. Cells were pelleted, washed twice with 1X PBS, and then analyzed by a BD LSR II flow cytometry using excitation 488 nm and a 530/30 nm (long pass 505) emission filter. The median fluorescence intensity for each sample was determined using FlowJo version X.0.7 software (Treestar) and normalized based on the median fluorescence intensity of the unstained sample. Similar results were obtained analyzing geometric means. Reported values are averages of normalized median values for three or more trials.

3.4.3. Characterization of rapamycin sensitivity

Strains were inoculated at 1 X 10^5 cells/ml in culture tubes with four ml of either rich medium + 30mM HU only, or this same medium supplemented with 0nM (mock), 25nM, or 100nM final concentration of rapamycin (Alfa Aesar) and grown rotating at 20˚C until stationary phase. At time of sampling, cells were diluted and plated onto YPD to assay for CFU/ml. Plates were incubated up to four days at 30˚C. Relative CFU/ml for each strain and trial was calculated by dividing the CFU/ml of the treated sample by the product of CFU/ml of the untreated sample and mock treatment (ethanol only) control average.

Chapter 4

4. CONCLUSIONS

4.1. General discussion

The work presented here offers a new perspective on the role mobile DNA elements, specifically retrotransposons, can play in the aging process. The negative impact of retromobility on host genomes is well documented [136,153,226,252,152]. Even so, there to date are less than a hundred documented cases in which retromobility is known to have resulted in a human disease phenotype [253]. This suggests that host genomes are quite adept at preventing retromobility from impacting the host in a severe manner, and this also suggests that the continued presence of retroelements within higher complex eukaryotes still serves a potential, continued function. George Williams in 1957 coined the term “antagonistic pleiotropy” to define genetic traits that can influence the lifespan in organisms by observing that certain gene functions that are beneficial during reproductive age become detrimental later in the aging process after reproduction and growth are no longer needed [254]. This overlaps with the hyperfunction theory of aging to suggest that stimulation of cellular processes/pathways when they are not needed will influence the aging process. Good examples that fit with antagonistic pleiotropy and hyperfunction are nutrient excess and senescence. Availability of excess nutrients will permit rapid growth and reproduction, which are initially beneficial for a population, but has been shown to contribute to age-related pathologies that are then detrimental to

longevity. The same can be said for cellular senescence. While cellular senescence is thought to have evolved to inhibit tumorigenesis, overactivation of senescence pathways will ultimately reduce the rejuvenative capacity of tissues and lead to pro-aging effects later in life.

St. Laurent et al. reviewed the concept of antagonistic pleiotropy in the context of L1 (LINE-1) elements in human genomes and developed the “LINEage theory of aging” [116]. This idea posits that known stressors activate L1 and the resulting retromobility either benefits a population of cells by causing genetic variation to fuel evolution or stimulates enough DNA damage and DNA damage responses to cause cell cycle arrest, senescence, or apoptosis that contributes to aging. This work here provides a testable hypothesis related to this idea of antagonistic pleiotropy as it relates to retroelements, highlighting the unexpected extension in yeast CLS with high Ty1 copy number under certain medium conditions. We observed a novel function in the ability of multiple copies of Ty1 in yeast to confer pro-lifespan stimulating effects over strains with zero copy in certain media contexts.

Although continued study is necessary to characterize the specific mechanism of action through which Ty1 can extend CLS, we observed correlations between some factors known to regulate yeast CLS and high Ty1 copy number. Our work presented here suggests that there are beneficial CLS promoting effects of having multiple Ty1 elements under stressed media conditions early in growth.

These results support certain aspects of St. Laurent et al. hypothesis of an antagonistic pleiotropic role for retroelements under certain contexts. Prolifespan extending effects in cellular populations of high Ty1 copy strains under certain conditions may be the result of an adaptive response within the population. While it is hard to determine if there were detrimental pro-aging effects to individual cells to support the LINEage theory of aging, as a whole population the extension of CLS in Ty1 strains could be due to evolutionary benefitting adaptive changes in cellular responses. Lower ROS levels and mitochondrial membrane potential differences and intrinsic changes in nutrient signaling capabilities leading up to stationary phase in our strains is evidence that the presence of multiple Ty1 copies can influence these cellular activities. This was shown to be independent of Ty1 mobility and genomic instability. Thus this work is in accordance to the positive role retroelements play in driving beneficial changes of host genomes, and in this case, promoting CLS in *S. paradoxus*.

4.1.1. Certain forms of genomic instability did not impact aging in high Ty1 copy strains.

The initial premise for this project was that increased genomic instability would lead to accelerated aging in strains of *S. paradoxus* with few Ty1 elements, since they would have elevated levels of Ty1 mobility and experience higher levels of genomic damage. However, this was not what we observed in these strains during chronological aging, although we did show that Ty1 mobility levels were elevated in low Ty1 copy strains and inhibited in high Ty1 copy strains

(Figure 2.3 A-D). As a surprise we observed a novel CLS-extending effect of high Ty1 copy number when strains were grown in certain media conditions. Differences in mutation rate at the *CAN1* locus were not correlated the lifespan extension in the high Ty1 copy strains (Figure 2.5). It is possible that changes in other forms of genomic instability are correlated with this lifespan extension, though, since we did not assay for diverse types of genetic damage. Hydroxyurea acts as a replication stress, inhibiting ribonucleotide reductase function by interacting with the subunit Rnr2, and thereby lowering levels of dNTPs available for replication. We did not observe substantial changes in cell cycle arrest during stationary phase or differential responses to buffering dNTP levels through threonine supplementation during aging in zero and high Ty1 copy strains exposed to HU. These experiments did not identify differential responses to replication stress in zero and high Ty1 copy strains. We performed preliminary experiments in strains lacking *RAD52*, a gene required for most forms of homologous recombination in yeast, including double strand break repair by homologous recombination. No extension of CLS in high Ty1 copy *rad52*Δ strains relative to zero copy *rad52*Δ strains was observed in rich medium (data not shown). These results support the interpretation that CLS extension in high Ty1 copy strains does not directly depend on differential responses to decreased dNTP levels or to defects in DNA repair activities.

Acknowledging that we didn't look at all forms of genomic instability or DNA damage responses, it would be interesting to more thoroughly investigate

whether zero and high Ty1 copy strains do show differences in some forms of genomic instability. For example, it may be important to investigate if there are differences in stability of the mitochondrial genome which has fewer mechanisms for protection, considering that lifespan extension in high Ty1 copy strains is correlated with reduced ROS and mitochondrial activity [4]. An erythromycin-resistance assay could be used to measure frequencies of mutations in the 21S rRNA gene in the mitochondrial genome. Alternatively, marker genes could be inserted between direct repeats in mitochondrial DNA to test for direct repeat recombination, or mitochondrial genomes from zero and high Ty1 copy strains could be isolated and sequenced to identify the frequency and spectrum of mutations. The frequency with which strains spontaneously produce petite mutant cells with defective mitochondrial genomes could also be measured. Such experiments could identify a link between CLS and mitochondrial damage that is relevant to Ty1-dependent lifespan extension.

Retroelements, like LINE-1 and Ty1, are capable of causing large-scale chromosomal rearrangements, and this increases with age [153,255–257]. It has been demonstrated that Ty1 cDNA can mediate repair of DSBs when homologous recombination pathways are inhibited, and this cDNA-mediated repair of DSBs may serve as a secondary approach to promote longevity and stability when DNA repair mechanisms become faulty, serving as “DNA band-aids” [258]. A specific assay for retrotransposon-mediated gross chromosomal rearrangements would provide more direct evidence that retroelements can

drive such events and provide a means to quantify their occurrence in different aging contexts. Repair of DSBs by retroelement cDNA can promote translocations leading to greater genomic instability when recombination occurs with pre-existing elements [173,153]. Testing the idea that retroelement repair of DNA breaks leads to large-scale chromosomal rearrangements during aging could be accomplished by designing a selectable system to detect when a newly integrating Ty1 cDNA recombines with a pre-existing homologous sequence elsewhere in the genome. More specifically, a system could involve a genomic Ty1 element in which the 3' untranslated region contains half of an intron followed by a truncated gene. The second truncated half of the gene and the intron could be inserted into a known "hotspot" for Ty1 insertions (ex: upstream of tRNA genes). A Ty1 cDNA targeted to a site upstream of the second truncated allele could then recombine with the engineered Ty1 element with the first truncated allele. This could result in a translocation that would bring together the two halves of the reporter gene, which would be separated by an intron, leading to expression of the reporter dependent on splicing of the intron. Thus, Ty1-mediated translocation frequencies could be quantified through selection on appropriate medium. This system could potentially demonstrate whether Ty1-mediated chromosome rearrangements are more or less frequent during aging to more specifically show whether Ty1 retrotransposition events increase genomic instability with age.

4.1.2. Ty1 and the genomic landscape

Mammalian retroelements typically reside in areas of heterochromatin that are heavily silenced regions of the genome. It has been shown that repressive chromatin marks decrease with age, transforming inaccessible regions of the genome into accessible regions for transcription [78]. Similarly it has been shown that increases in retrotransposition occur during aging, partly due to the opening of heterochromatin to transcription and the removal of repressive epigenetic marks that inhibit retrotransposons [70]. The presence of retroelements in particular genomic regions could also regulate the expression of neighboring sequences. “Heterochromatic spreading” induced by retroelements can occur when new insertions of retroelements cause flanking DNA sequences to undergo epigenetic changes, possibly inducing nearby genes/promoters to become silenced. This has been shown to occur for *gypsy* elements in *Drosophila* and also for the mouse LTR retroelement IAP, though these events are rare [259–261]. Rebollo et al. reported increases of H3K9me3-H4K20me3-chromatin spreading into regions nearby IAP elements in mouse embryonic cells. This same study also demonstrated that such insertions can have strong influence on the regulation of a nearby gene, beta-1,3-galactosyltransferase-like gene (*B3galtl*) in this case. *S. cerevisiae* has relatively few regions of heterochromatin, and Ty1 elements are not preferentially found in heterochromatin. However, there are reports that Ty1 integration can mediate gene transcriptional changes under stressed conditions [230,262]. It is reasonable to consider that the initial Ty1 insertions leading to the production of our high Ty1

copy strains could alter basal or stress-responsive transcription of nearby genes through chromatin alterations to promote survival in certain contexts. One step in addressing this hypothesis would be to determine the Ty1 integration sites in each of the high Ty1 copy strains by genome sequencing. While we have shown that many of the Ty1 insertions in our high Ty1 copy strains appear to have occurred at independent genomic sites (Figure 2.1), the addition of multiple Ty1 elements to the genome might globally change chromatin structure in similar ways in each strain. Experiments to test this hypothesis could use assays to detect nucleosome-free and nucleosome-rich regions of genomes, such as FAIRE (formaldehyde-assisted isolation of regulatory elements) or micrococcal nuclease digestion, to compare zero copy and high Ty1 copy strains for genome-wide differences in nucleosome occupancy and distribution.

4.1.3. Lower mitochondrial membrane potentials and ROS levels suggests a Ty1-mediated mechanism to extend chronological lifespan in hydroxyurea media

Mitochondrial function is key to extending longevity in yeast and mitochondrial dysfunction causes multiple diseases in humans [236]. The CLS promoting effects of CR in yeast depend on multiple factors influencing the upregulation of mitochondrial responses [251,263]. In this study we correlated extended CLS in high Ty1 copy strains with reduced ROS levels early in stationary phase. We also observed lower mitochondrial membrane potentials in these strains compared with zero copy strains early in growth. By manipulating growth conditions or with

antioxidant treatment, we could remove the Ty1-dependent CLS extension. We therefore suggest that our high Ty1 copy strains have altered mitochondrial function or regulation, however the mechanism at this point is unclear. Higher respiratory activity can decrease mitochondrial ROS levels and extend CLS in yeast [264]. It is possible that our high Ty1 copy strains have respiratory changes associated with growth under media conditions that were shown to promote CLS due to changes in ROS levels.

Experiments to further explore differences in mitochondrial function could include directly measuring O_2 consumption/CO_2 generation rates to determine the respiratory status of our strains and indirect evaluation of mitochondrial function by deleting the *ADE2* gene from our strains (which is in progress at the time of this writing). *ADE2* is part of the biosynthetic pathway necessary for the production of purine nucleotides, and strains lacking this gene accumulate pink bioprecursors within the vacuole that turn yeast colonies pink/red [265]. These colonies become white if there are dysfunctions within the mitochondria, so evaluation of colony color can be used to measure the ratio of respiratory-competent/incompetent cells. This common auxotrophic screen for mitochondrial function would allow us to observe if populations of our zero or high Ty1 copy strains have different proportions of cells with dysfunctional mitochondria. Mitochondrial fragmentation is also characteristic of aging yeast cells [266]. By generating a GFP-reporter system tagging mitochondrial outer membrane proteins to examine mitochondrial fragmentation, or staining with a mitochondrial

fluorescent dye like MitoTracker® DeepRed, we would be able to use fluorescence microscopy to visualize any differences in mitochondrial morphology between zero and high Ty1 copy strains as a function of age.

Adaptive ROS signaling due to increased mitochondrial activity resulting from decreases in TOR signaling are sufficient to extend CLS in yeast [238]. Changes in mitochondrial function early in growth of high Ty1 copy yeast strains may allow cells to adapt for survival during stationary phase. Our results from treatment with rapamycin and mitochondrial membrane potential experiments would support these findings. Additionally, our results also support work in which CR led to lower ROS levels to help promote CLS in yeast via increased ability to correctly arrest growth in stationary phase [221].

Reduced mitochondrial membrane potential, increases in ROS, and fragmentation of the mitochondrial network are examples of factors that would upregulate autophagic machinery to eliminate dysfunctional mitochondria in yeast [267–270]. Upregulation of autophagy pathways is required for normal CLS in yeast [237,271]. In our study, growth with chronic exposure to HU might upregulate autophagy pathways to promote survival. Alternatively, high Ty1 copy number could alter mitochondrial function, which is supported by observations of reduced ROS and lower mitochondrial membrane potential, and indirectly upregulate autophagy to enhance survival. Selective autophagy can limit Ty1 retrotransposition in yeast under CR by selectively removing protein components of VLPs [272]. It is plausible that stimulation of Ty1 replication by HU could

upregulate autophagy pathways to degrade VLP components, and that this stimulation of autophagy could help to extend lifespan in high Ty1 copy strains. This effect would be absent during growth in rich media lacking HU as there is a lack of HU-stimulated stress. Testing this hypothesis could involve expressing an N-terminally GFP tagged Ty1 Gag p45 or p49 protein (a component of mature VLPs) and monitoring associations with vacuolar proteins like vacuolar aminopeptidase I (ApeI) via microscopy or autophagy-related gene products like Atg19. If Ty1 did stimulate autophagy during stress, then this future work could represent an example of a hormetic response involving Ty1 retroelements to promote CLS in yeast.

4.1.4. Ty1 copy number leads to partial resistance to rapamycin to influence certain aspects of nutrient signaling

We discovered that chronic exposure to a low dose of rapamycin (25nM) in the presence of HU (30mM) more strongly inhibited growth of zero Ty1 copy strains compared to high copy strains (Figure 3.3). This effect could indicate that there is a difference either in activity of the TOR nutrient-sensing pathway between zero and high Ty1 copy strains or in other gene functions required for sensitivity to rapamycin. Nutrient signaling is a main driver of aging and these nutrient signaling pathways are highly conserved among species. However, potential relationships between nutrient-signaling pathways and retroelements have not been widely addressed. Our work could reflect a novel mechanism through which the presence of retrotransposons influences the activities of certain

nutrient-sensing pathways, but no firm conclusion can be made until any potential mechanism is better characterized.

TOR signaling is at the crossroads for regulating metabolism, stress and aging. TOR signaling is repressed during nutrient depletion and stimulated under ample nutrient conditions [11]. Since TOR is the target of rapamycin, examining TOR signaling in zero and high Ty1 copy strains may provide an explanation for the difference in rapamycin sensitivity. TOR has many downstream targets and one option for future experiments is to look at transcription levels of these targets by qRT-PCR. Inhibition of TOR by rapamycin treatment greatly increases transcription of general amino acid permeases such as Gap1 and the allantoate permease Dal5 [273,274]. Nitrogen permease reactivator kinase (Npr1) is a Ser/Thr kinase responsible for activating Gap1 under nitrogen-starvation, but is negatively regulated by TOR-dependent phosphorylation under plentiful nitrogen sources [274]. We are currently examining transcript levels of these downstream TOR targets to test for differences in expression between zero copy and high Ty1 copy strains in different media conditions that might explain differences in CLS and rapamycin sensitivity. The extra hydroxyl group on hydroxyurea is the only difference distinguishing it from the nitrogen compound urea, which in *S. cerevisiae* constitutes a poor nitrogen source and physiologically upregulates *GAP1* expression [274]. Growth in YPD with 30mM urea did not extend lifespan in our high Ty1 copy strains (data not shown), so lifespan extension in HU is not likely to result from a direct role of HU as a nitrogen source.

The influence of rapamycin in TOR signaling may be more complicated than previously thought. In rodents, different cell lines have experimentally exhibited differences in resistance to rapamycin treatment, and rapamycin can lead to insulin resistance and inhibition of mitochondrial biogenesis and function [275–281]. In yeast the insensitivity of high Ty1 copy strains at low doses of rapamycin in HU raises some new interesting questions about TOR signaling on the aging process. While it is well established in yeast that CLS is extended in *tor1Δ*, the molecular mechanism governing ribosome biogenesis and translation have not all been identified and some gaps remain in TOR regulation of these processes [250]. Importantly, yeast TOR consists of two complexes: TORC1 and TORC2, and TORC2 is naturally insensitive to rapamycin whereas it can bind readily with TORC1 [282]. Thus the clear inhibition by rapamycin on TOR is not straightforward. Our rapamycin results would suggest that high Ty1 copy strains are resistant to low doses of rapamycin as they enter stationary phase. It is hard to reconcile how Ty1 elements may contribute to this process. Ty1 cellular components may interfere indirectly with the mechanistic action of rapamycin at low doses leading to insensitivity in HU. Ty1 regulatory functions may “hijack” normal cellular responses to cellular signaling, rendering cells insensitive to certain aspects of nutrient sensing and signaling. Recent work points to a role of Ty1 coded proteins in inhibiting certain aspects of its own replication during CNC [182]. It may be possible that certain aspects of the complex CNC regulatory

function can interfere with other aspects of cellular metabolism, and in this case the binding of rapamycin to TORC1.

The presence or absence of gene functions not directly related to TOR function may influence rapamycin sensitivity. There is some evidence that suggests rapamycin resistance in yeast results in changes to mitochondria function. Fournier et al. discovered that deletion of the nuclear gene *GGC1* which is critical for mitochondrial genome maintenance as a GTP/GDP transporter of iron, led to increased resistance to rapamycin treatment and enhanced growth in yeast [250]. This effect resulted in derepression of ribosomal and ribosome biogenesis and changes in cell cycle gene expression patterns. They suggest that *GGC1* is important for mitochondrial protein synthesis and DNA metabolism [250]. It is interesting to speculate then that high Ty1 copies or Ty1 gene products may influence mitochondrial function (Figure 3.4), but this remains unknown. This is mere speculation at this point and requires further testing, but for the single-cell organism *S. paradoxus*, Ty1 may exert more pressure on adaptive internal cellular processes for survival then is currently believed.

4.2. Final conclusions

To conclude, this work has identified a positive role for retroelements in promoting CLS under conditions of stress in strains of *S. paradoxus.* Elucidating the precise mechanism behind Ty1-dependent extension of CLS requires further investigation. We have shown that there is a correlation between CLS extension

in HU and internal cellular changes, directly or indirectly affecting mitochondria function and nutrient signaling pathways, known cellular factors that contribute to aging under certain contexts. We did not find extensive genomic instability as a result of retroelement mobility which is currently hypothesized to contribute to aging in yeast and higher organisms [78,116,153]. However, we did not completely rule out the possibility that some forms of genomic instability are relevant for the observed lifespan differences. Our observations point more towards the evolutionary aspect of the host/retroelement relationship and provide evidence that the presence of retroelements may alter how cells adapt during times of stress. We propose that these results reflect novel interactions between retroelements and cellular pathways that influence stress responses, which can be beneficial for cell survival or function in a context-dependent manner. Wilke and Adams reported that when yeast strains with and without Ty1 elements were co-cultured and subjected to serial subculturing in liquid medium, all cells in the final populations were cells that had Ty1 elements [193]. This work raised the possibility that the presence of Ty1 may provide some beneficial effect that allowed cells with Ty1 to outcompete cells lacking Ty1. Our data is consistent with this earlier observation by providing examples of Ty1 positively influencing survival during chronological aging. This supports the possibility that Ty1 can help cells adapt to stress and new environments.

Several groups have published reports of increased retrotransposon activity during aging, and retrotransposons are considered as agents of genetic damage

that could promote aging in these studies. The positive role retroelements can play during aging that we have shown represents a new viewpoint for investigating retrotransposons and aging. We expect that this work will lead to follow up studies characterizing specific mechanisms that allow retrotransposons to alter cellular activities, rather than simply respond to cellular regulation. This story adds a new chapter to the already complex nature of organismal aging and the role that retroelements may have on influencing longevity.

REFERENCES

1. Gems, D. & Partridge, L. Genetics of longevity in model organisms: Debates and paradigm shifts. *Annu. Rev. Physiol.* **75,** 621–44 (2013).

2. United States Census Bureau, *National Population Projections Data* Available at: http://www.census.gov/population/projections. (Accessed: 4th January 2015).

3. Larson, K., Centers for Disease Control and Prevention (CDC). Available at: http:// www.cdc.gov. (Accessed: 24th February 2012).

4. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. X. The hallmarks of aging. *Cell* **153,** 1194-1217 (2013).

5. Failla, G. The aging process and cancerogenesis. *Ann. N. Y. Acad. Sci.* **71,** 1124–1140 (1958).

6. Szilard, L. On the nature of aging process. *Proc. Nati. Acad. Sci. USA* **45,** 30-35 (1959).

7. Garinis, G. A., van der Horst, G. T. J., Vijg, J. & Hoeijmakers, J. H. J. DNA damage and ageing: New-age ideas for an age-old problem. *Nat. Cell Biol.* **10,** 1241–1247 (2008).

8. Gensler, H. L. & Bernstein, H. DNA damage as the primary cause of aging. *Q. Rev. Biol.* **56,** 279–303 (1981).

9. Harmon, D. Aging: A theory based on free radical and radiation chemistry. *J. Gerontol.* **11,** 298–300 (1956).

10. Hoeijmakers, J. H. J. DNA damage, aging, and cancer. *N. Engl. J. Med.* **361,** 1475–1485 (2009).

11. Haigis, M. C. & Yankner, B. A. The aging stress response. *Mol. Cell* **40,** 333–344 (2010).

12. Blagosklonny, M. V. Paradoxes of aging. *Cell Cycle* **6,** 2997–3003 (2007).

13. Kirkwood, T. B. L. & Kowald, A. The free-radical theory of ageing-older, wiser and still alive: Modelling positional effects of the primary targets of ROS reveals new support. *BioEssays* **34,** 692–700 (2012).

14. Stadtman, E. R. Serial review: Oxidative stress and aging. *Free Radic. Biol. Med.* **33,** 597– 604 (2002).

15. Ristow, M. & Schmeisser, S. Extending life span by increasing oxidative stress. *Free Radic. Bio. Med.* **51,** 327–336 (2011).

16. Schmeisser, S. *et al.* Neuronal ROS signaling rather than AMPK/sirtuin-mediated energy sensing links dietary restriction to lifespan extension. *Mol. Metab.* **2,** 92–102 (2013).

17. Van Remmen, H. *et al.* Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. *Physiol. Genomics* **16,** 29–37 (2003).

18. Zhang, D.-Y. *et al.* Wnt/β-catenin signaling induces the aging of mesenchymal stem cells through promoting the ROS production. *Mol. Cell. Biochem.* **374,** 13–20 (2013).

19. Mesquita, A. *et al.* Caloric restriction or catalase inactivation extends yeast chronological lifespan by inducing H2O2 and superoxide dismutase activity. *Proc. Natl. Acad. Sci. USA* **107,** 15123–15128 (2010).

20. Yu, C. *et al.* RNA sequencing reveals differential expression of mitochondrial and oxidation reduction genes in the long-lived naked mole-rat when compared to mice. *PLoS One* **6,** e26729 (2011).

21. Andziak, B. *et al.* High oxidative damage levels in the longest-living rodent, the naked mole-rat. *Aging Cell* **5,** 463–471 (2006).

22. Raamsdonk, J. M. Van & Hekimi, S. Superoxide dismutase is dispensable for normal animal lifespan. *Proc. Natl. Acad. Sci. USA* **109,** 5785–5790 (2012).

23. Van Raamsdonk, J. M. & Hekimi, S. Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in Caenorhabditis elegans. *PLoS Genet.* **5,** e1000361 (2009).

24. Pérez, V. I. *et al.* Is the oxidative stress theory of aging dead? *Biochim. Biophys. Acta* **1790,** 1005–1014 (2009).

25. Pérez, V. I. *et al.* Protein stability and resistance to oxidative stress are determinants of longevity in the longest-living rodent, the naked mole-rat. *Proc. Natl. Acad. Sci. USA* **106,** 3059–3064 (2009).

26. Erol, A. The functions of PPARs in aging and longevity. *PPAR Research.* 39654 (2007).

27. Jones, D. L. & Rando, T. A. Emerging models and paradigms for stem cell ageing. *Nat. Cell Biol.* **13,** 506–512 (2011).

28. Rossi, D. J., Jamieson, C. H. M. & Weissman, I. L. Stems cells and the pathways to aging and cancer. *Cell* **132,** 681–696 (2008).

29. Martin, L. J. DNA damage and repair: relevance to mechanisms of neurodegeneration. *J. Neuropathol. Exp. Neurol.* **67,** 377–387 (2008).

30. Ragnauth, C. D. *et al.* Prelamin a acts to accelerate smooth muscle cell senescence and is a novel biomarker of human vascular aging. *Circulation* **121,** 2200–2210 (2010).

31. Scaffidi, P. & Misteli, T. Lamin A-dependent nuclear defects in human aging. *Science* **312,** 1059–1063 (2006).

32. Cao, K. *et al.* Progerin and telomere dysfunction collaborate to trigger cellular senescence in normal human fibroblasts. *J. Clin. Invest.* **121,** 2833–2844 (2011).

33. Shah, P. P. *et al.* Lamin B1 depletion in senescent cells triggers large-scale changes in gene expression and the chromatin landscape. *Genes Dev.* **27,** 1787–1799 (2013).

34. Shimi, T. *et al.* The role of nuclear lamin B1 in cell proliferation and senescence. *Genes Dev.* **25,** 2579–2593 (2011).

35. Freund, A., Laberge, R.-M., Demaria, M. & Campisi, J. Lamin B1 loss is a senescence-associated biomarker. *Mol. Biol. Cell* **23,** 2066–2075 (2012).

36. Osorio, F. G. *et al.* Splicing-directed therapy in a new mouse model of human accelerated aging. *Sci. Transl. Med.* **3,** 1-11 (2011).

37. Varela, I. *et al.* Combined treatment with statins and aminobisphosphonates extends longevity in a mouse model of human premature aging. *Nat. Med.* **14,** 767–772 (2008).

38. Park, C. B. & Larsson, N.-G. Mitochondrial DNA mutations in disease and aging. *J. Cell Biol.* **193,** 809–818 (2011).

39. Hayflick, L. & Moorhead, P. S. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25,** 585–621 (1961).

40. Blasco, M. A. The epigenetic regulation of mammalian telomeres. *Nat. Rev. Genet.* **8,** 299–309 (2007).

41. Passos, J. F., Von Zglinicki, T. & Kirkwood, T. B. L. Mitochondria and ageing: Winning and losing in the numbers game. *BioEssays* **29,** 908–917 (2007).

42. Passos, J. F., Saretzki, G. & Von Zglinicki, T. DNA damage in telomeres and mitochondria during cellular senescence: Is there a connection? *Nucleic Acids Res.* **35,** 7505–7513 (2007).

43. Armanios, M. *et al.* Short telomeres are sufficient to cause the degenerative defects associated with aging. *Am. J. Hum. Genet.* **85,** 823–832 (2009).

44. Samper, E., Flores, J. M., Lee, H. & Blasco, A. Disease states associated with telomerase deficiency appear earlier in mice with short telomeres. *Embo. J.* **18,** 2950–2960 (1999).

45. Rudolph, K. L. *et al.* Longevity, stress response, and cancer in aging telomerase-deficient mice. *Cell* **96,** 701–712 (1999).

46. Tomás-Loba, A. *et al.* Telomerase reverse transcriptase delays aging in cancer-resistant mice. *Cell* **135,** 609–622 (2008).

47. Boonekamp, J., Mulder, G., Salomons, H., Dijkstra, C. & Verhulst, S. Nestling telomere shortening, but not telomere length, reflects developmental stress and predicts survival in wild birds. *Proc. R. Soc. B Biol. Sci.* **281,** 1-8 (2014).

48. Koga, H., Kaushik, S. & Cuervo, A. M. Protein homeostasis and aging: The importance of exquisite quality control. *Ageing Res. Rev.* **10,** 205–215 (2012).

49. Morrow, G. & Tanguay, R. M. Heat shock proteins and aging in *Drosophila melanogaster*. *Semin. Cell Dev. Biol.* **14,** 291–299 (2003).

50. Walker, G. A. & Lithgow, G. J. Lifespan extension in *C. elegans* by a molecular chaperone dependent upon insulin-like signals. *Aging Cell* **2,** 131–139 (2003).

51. Chiang, W. C., Ching, T. T., Lee, H. C., Mousigian, C. & Hsu, A. L. HSF-1 regulators DDL-1/2 link insulin-like signaling to heat-shock responses and modulation of longevity. *Cell* **148,** 322–334 (2012).

52. Rubinsztein, D. C., Mariño, G. & Kroemer, G. Autophagy and aging. *Cell* **146,** 682–695 (2011).

53. Tomaru, U. *et al.* Decreased proteasomal activity causes age-related phenotypes and promotes the development of metabolic abnormalities. *Am. J. Pathol.* **180,** 963–972 (2012).

54. Cuervo, A. M. Autophagy and aging: Keeping that old broom working. *Trends Genet.* **24,** 604–612 (2008).

55. Vellai, T., Takács-Vellai, K., Sass, M. & Klionsky, D. J. The regulation of aging: Does autophagy underlie longevity? *Trends Cell Biol.* **19,** 487–494 (2009).

56. Bjedov, I. & Partridge, L. A longer and healthier life with TOR down-regulation: Genetics and drugs. *Biochem. Soc. Trans.* **39,** 460–465 (2011).

57. Aris, J. P. *et al.* Autophagy and leucine promote chronological longevity and respiration proficiency during calorie restriction in yeast. *Exp. Gerontol.* **48,** 1107–1119 (2013).

58. Cavallini, G., Donati, A., Gori, Z. & Bergamini, E. Towards an understanding of the anti-aging mechanism of caloric restriction. *Curr. Aging Sci.* **1,** 4–9 (2008).

59. Kiffin, R. *et al.* Altered dynamics of the lysosomal receptor for chaperone-mediated autophagy with age. *J. Cell Sci.* **120,** 782–791 (2007).

60. Talens, R. P. *et al.* Epigenetic variation during the adult lifespan: Cross-sectional and longitudinal data on monozygotic twin pairs. *Aging Cell* **11,** 694–703 (2012).

61. Fraga, M. F. & Esteller, M. Epigenetics and aging: The targets and the marks. *Trends Genet.* **23,** 413–418 (2007).

62. Han, S. & Brunet, A. Histone methylation makes its mark on longevity. *Trends Cell Biol.* **22,** 42–49 (2012).

63. Watanabe, K. I. & Bloch, W. Histone methylation and acetylation indicates epigenetic change in the aged cochlea of mice. *Eur. Arch. Oto-Rhino-Laryngol.* **270,** 1823–1830 (2013).

64. Peleg, S. *et al.* Altered histone acetylation is associated with age-dependent memory impairment in mice. *Science* **328,** 753–756 (2010).

65. Guarente, L. Sirtuins, aging, and metabolism. *Cold Spring Harb. Symp. Quant. Biol.* **76,** 81–90 (2011).

66. Kaeberlein, M., McVey, M. & Guarente, L. The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. *Genes Dev.* **13,** 2570–2580 (1999).

67. Wang, R. H. *et al.* Impaired DNA damage response, genome instability, and tumorigenesis in SIRT1 mutant mice. *Cancer Cell* **14,** 312–323 (2008).

68. Oberdoerffer, P. *et al.* SIRT1 redistribution on chromatin promotes genomic stability but alters gene expression during aging. *Cell* **135,** 907–918 (2008).

69. Mostoslavsky, R. *et al.* Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. *Cell* **124,** 315–329 (2006).

70. Van Meter, M. *et al.* SIRT6 represses LINE1 retrotransposons by ribosylating KAP1 but this repression fails with stress and age. *Nat. Commun.* **5,** 1-10 (2014).

71. Brown, K. *et al.* SIRT3 reverses aging-associated degeneration. *Cell Rep.* **3,** 319–327 (2013).

72. Pegoraro, G. & Misteli, T. The central role of chromatin maintenance in aging. *Aging (Albany NY).* **1,** 1017–1022 (2009).

73. Oberdoerffer, P. & Sinclair, D. A. The role of nuclear architecture in genomic instability and ageing. *Nat Rev Mol Cell Biol* **8,** 692–702 (2007).

74. Larson, K. *et al.* Heterochromatin formation promotes longevity and represses ribosomal RNA synthesis. *PLoS Genet.* **8,** e1002473 (2012).

75. Tsurumi, A. & Li, W. X. Global heterochromatin loss: A unifying theory of aging? *Epigenetics* **7,** 680–688 (2012).

76. Feser, J. *et al.* Elevated histone expression promotes lifespan extension. *Mol. Cell* **39,** 724–735 (2010).

77. Adams, P. D. *et al.* Lysosome-mediated processing of chromatin in senescence. *J. Cell Biol.* **202,** 129–143 (2013).

78. Wood, J. G. & Helfand, S. L. Chromatin structure and transposable elements in organismal aging. *Front. Genet.* **4,** 1–12 (2013).

79. Peric-Hupkes, D. & van Steensel, B. Role of the nuclear lamina in genome organization and gene expression. *Cold Spring Harb. Symp. Quant. Biol.* **75,** 517–524 (2010).

80. Stuart, G. R., Oda, Y., De Boer, J. G. & Glickman, B. W. Mutation frequency and specificity with age in liver, bladder and brain of lacI transgenic mice. *Genetics* **154,** 1291–1300 (2000).

81. Edman, U. *et al.* Lifespan extension by dietary restriction is not linked to protection against somatic DNA damage in Drosophila melanogaster. *Aging Cell* **8,** 331–338 (2009).

82. Page, M. M., Withers, D. J. & Selman, C. Longevity of insulin receptor substrate1 null mice is not associated with increased basal antioxidant protection or reduced oxidative damage. *Age.* **35,** 647–658 (2013).

83. Jacobson, J. *et al.* Biomarkers of aging in *Drosophila*. *Aging Cell* **9,** 466–477 (2010).

84. McElwee, J. J. *et al.* Evolutionary conservation of regulated longevity assurance mechanisms. *Genome Biol.* **8,** R132 (2007).

85. Amador-Noguez, D., Yagi, K., Venable, S. & Darlington, G. Gene expression profile of long-lived Ames dwarf mice and Little mice. *Aging Cell* **3,** 423–441 (2004).

86. Kenyon, C. J. The genetics of ageing. *Nature* **464,** 504–512 (2010).

87. Slack, C., Giannakou, M. E., Foley, A., Goss, M. & Partridge, L. dFOXO-independent effects of reduced insulin-like signaling in *Drosophila*. *Aging Cell* **10,** 735–748 (2011).

88. Yamaza, H. *et al.* FoxO1 is involved in the antineoplastic effect of calorie restriction. *Aging Cell* **9,** 372–382 (2010).

89. Bonkowski, M. S. *et al.* Disruption of growth hormone receptor prevents calorie restriction from improving insulin action and longevity. *PLoS One* **4,** e4567 (2009).

90. Kahn, B. B., Alquier, T., Carling, D. & Hardie, D. G. AMP-activated protein kinase: Ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab.* **1,** 15–25 (2005).

91. Boily, G. *et al.* SirT1 regulates energy metabolism and response to caloric restriction in mice. *PLoS One* **3,** e1759 (2008).

92. Herranz, D. & Serrano, M. SIRT1: Recent lessons from mouse models. *Nat. Rev. Cancer* **10,** 819–823 (2010).

93. Baur, J. A. *et al.* Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* **444,** 337–342 (2006).

94. Tapia, P. C. Sublethal mitochondrial stress with an attendant stoichiometric augmentation of reactive oxygen species may precipitate

many of the beneficial alterations in cellular physiology produced by caloric restriction, intermittent fasting, exercise and dietary phytonutrients: "mitohormesis" for health and vitality. *Med. Hypotheses* **66,** 832–843 (2006).

95. Durieux, J., Wolff, S. & Dillin, A. The cell-non-autonomous nature of electron transport chain-mediated longevity. *Cell* **144,** 79–91 (2011).

96. Sena, L. A. & Chandel, N. S. Physiological roles of mitochondrial reactive oxygen species. *Mol. Cell* **48,** 158–166 (2012).

97. Hekimi, S., Lapointe, J. & Wen, Y. Taking a 'good' look at free radicals in the aging process. *Trends Cell Biol.* **21,** 569–576 (2011).

98. Rodgers, J. T. *et al.* Nutrient control of glucose homeostasis through a complex of PGC-1α and SIRT1. *Nature* **434,** 113–118 (2005).

99. Lee, I. H. *et al.* A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy. *Proc. Natl. Acad. Sci. USA* **105,** 3374–3379 (2008).

100. Rossi, D. J. *et al.* Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature* **447,** 725–729 (2007).

101. Flores, I., Cayuela, M. L. & Blasco, M. A. Effects of telomerase and telomere length on epidermal stem cell behavior. *Science* **309,** 1253–1256 (2005).

102. Sharpless, N. E. & DePinho, R. A. How stem cells age and why this makes us grow old. *Nat. Rev. Mol. Cell Biol.* **8,** 703–713 (2007).

103. Campisi, J. Cellular senescence : When bad things happen to good cells. *Nature* **8,** 729–740 (2007).

104. Adler, A. S. *et al.* Motif module map reveals enforcement of aging by continual NF-κB activity. *Genes Dev.* **21,** 3244–3257 (2007).

105. Osorio, F. G. *et al.* Nuclear lamina defects cause ATM-dependent NF-κB activation and link accelerated aging to a systemic inflammatory response. *Genes Dev.* **26,** 2311–2324 (2012).

106. Tilstra, J. S. *et al.* NF-κB inhibition delays DNA damage-induced senescence and aging in mice. *J. Clin. Invest.* **122,** 2601–2612 (2012).

107. Xie, J., Zhang, X. & Zhang, L. Negative regulation of inflammation by SIRT1. *Pharmacol. Res,* **67,** 60–67 (2013).

108. Kawahara, T. L. A. *et al.* SIRT6 links histone H3 lysine 9 deacetylation to NF-κB-dependent gene expression and organismal life span. *Cell* **136,** 62–74 (2009).

109. Rothgiesser, K. M., Erener, S., Waibel, S., Lüscher, B. & Hottiger, M. O. SIRT2 regulates NF-κB dependent gene expression through deacetylation of p65 Lys310. *J. Cell Sci.* **123,** 4251–4258 (2010).

110. Belgnaoui, S. M., Gosden, R. G., Semmes, O. J. & Haoudi, A. Human LINE-1 retrotransposon induces DNA damage and apoptosis in cancer cells. *Cancer Cell Int* **6,** 13 (2006).

111. Lander, E. S. *et al.* Initial sequencing and analysis of the human genome. *Nature* **409,** 860–921 (2001).

112. Maxwell, P. H. Consequences of ongoing retrotransposition in mammalian genomes. *Adv. Genomics Genet.* **4,** 129–142 (2014).

113. Brouha, B. *et al.* Hot L1s account for the bulk of retrotransposition in the human population. *Proc. Natl. Acad. Sci. USA* **100,** 5280–5285 (2003).

114. Levin, H. L. & Moran, J. V. Dynamic interactions between transposable elements and their hosts. *Nat. Rev. Genet.* **12,** 615–627 (2011).

115. Maxwell, P. H. & Curcio, M. J. Host factors that control long terminal repeat retrotransposons in *Saccharomyces cerevisiae*: Implications for regulation of mammalian retroviruses. *Eukaryot. Cell* **6,** 1069–1080 (2007).

116. St. Laurent, G., Hammell, N. & McCaffrey, T. A. A LINE-1 component to human aging: Do LINE elements exact a longevity cost for evolutionary advantage? *Mech. Ageing Dev.* **131,** 299–305 (2010).

117. Maksakova, I. a. *et al.* Retroviral elements and their hosts: Insertional mutagenesis in the mouse germ line. *PLoS Genet.* **2,** 1–10 (2006).

118. Belshaw, R. *et al.* Genomewide screening reveals high levels of insertional polymorphism in the human endogenous retrovirus family HERV-K(HML2): implications for present-day activity. *J. Virol.* **79,** 12507–12514 (2005).

119. Cordaux, R. & Batzer, M. A. The impact of retrotransposons on human genome evolution. *Nat. Rev. Genet.* **10,** 691–703 (2009).

120. Xing, J. *et al.* Mobile elements create structural variation: analysis of a complete human genome. *Genome Res.* **19,** 1516–1526 (2009).

121. Esnault, C., Maestre, J. & Heidmann, T. Human LINE retrotransposons generate processed pseudogenes. *Nat. Genet.* **24,** 363–367 (2000).

122. Kazazian, H. H. & Moran, J. V. The impact of L1 retrotransposons on the human genome. *Nat. Genet.* **19,** 19–24 (1998).

123. Chen, J. M., Férec, C. & Cooper, D. N. LINE-1 endonuclease-dependent retrotranspositional events causing human genetic disease: Mutation detection bias and multiple mechanisms of target gene disruption. *J. Biomed. Biotechnol.* **2006,** 1-9 (2006).

124. Beck, C. R., Garcia-Perez, J. L., Badge, R. M. & Moran, J. V. LINE-1 elements in structural variation and disease. *Annu. Rev. Genomics Hum. Genet.* **12,** 187–215 (2011).

125. Han, J. S., Szak, S. T. & Boeke, J. D. Transcriptional disruption by the L1 retrotransposon and implications for mammalian transcriptomes. *Nature* **429,** 268–274 (2004).

126. Sayah, D. M., Sokolskaja, E., Berthoux, L. & Luban, J. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. *Nature* **430,** 569–573 (2004).

127. Torrents, D., Suyama, M., Zdobnov, E. & Bork, P. A genome-wide survey of human pseudogenes. *Genome Res.* **13,** 2559–2567 (2003).

128. Zhang, Z., Carriero, N. & Gerstein, M. Comparative analysis of processed pseudogenes in the mouse and human genomes. *Trends in Genetics* **20,** 62–67 (2004).

129. Zhang, Z., Harrison, P. M., Liu, Y. & Gerstein, M. Millions of years of evolution preserved: A comprehensive catalog of the processed pseudogenes in the human genome. *Genome Res.* **13,** 2541–2558 (2003).

130. Parker, H. G. *et al.* An expressed fgf4 retrogene is associated with breed-defining chondrodysplasia in domestic dogs. *Science* **325,** 995–998 (2009).

131. Wallace, N. A., Belancio, V. P. & Deininger, P. L. L1 mobile element expression causes multiple types of toxicity. *Gene* **419,** 75–81 (2008).

132. Belancio, V. P., Hedges, D. J. & Deninger, P. Mammalian non-LTR retrotransposon for better or worse, in sickness and health. *Genome Res* **18,** 343–358 (2008).

133. Georgiou, I. *et al.* Retrotransposon RNA expression and evidence for retrotransposition events in human oocytes. *Hum. Mol. Genet.* **18,** 1221–1228 (2009).

134. Deininger, P. L. & Batzer, M. A. *Alu* repeats and human disease. *Mol. Genet. Metab.* **67,** 183–193 (1999).

135. Miki, Y. *et al.* Disruption of the APC gene by a retrotransposal insertion of L1 sequence in a colon cancer. *Cancer Res.* **52,** 643–645 (1992).

136. Iskow, R. C. *et al.* Natural mutagenesis of human genomes by endogenous retrotransposons. *Cell* **141,** 1253–1261 (2010).

137. Coufal, N. G. *et al.* L1 retrotransposition in human neural progenitor cells. *Nature* **460,** 1127–1131 (2009).

138. Muotri, A. R. *et al.* Somatic mosaicism in neuronal precursor cells mediated by L1 retrotransposition. *Nature* **435,** 903–910 (2005).

139. Garcia-Perez, J. L. *et al.* Epigenetic silencing of engineered L1 retrotransposition events in human embryonic carcinoma cells. *Nature* **466,** 769–773 (2010).

140. Tsukahara, S. *et al.* Bursts of retrotransposition reproduced in *Arabidopsis*. *Nature* **461,** 423–426 (2009).

141. Sedivy, J. M. *et al.* Death by transposition-the enemy within? *BioEssays* **35,** 1035–1043 (2013).

142. Muotri, A. R. *et al.* L1 retrotransposition in neurons is modulated by MeCP2. *Nature* **468,** 443–446 (2010).

143. Van Rij, R. P. & Berezikov, E. Small RNAs and the control of transposons and viruses in *Drosophila*. *Trends in Microbiology* **17,** 163–171 (2009).

144. Aravin, A. A., Hannon, G. J. & Brennecke, J. The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. *Science* **318,** 761–764 (2007).

145. Czech, M. P., Aouadi, M. & Tesz, G. J. RNAi-based therapeutic strategies for metabolic disease. *Nat. Rev. Endocrinol.* **7,** 473–484 (2011).

146. Slotkin, R. K. & Martienssen, R. Transposable elements and the epigenetic regulation of the genome. *Nat. Rev. Genet.* **8,** 272–285 (2007).

147. Kennedy, B. K. *et al.* Redistribution of silencing proteins from telomeres to the nucleolus is associated with extension of life span in *S. cerevisiae*. *Cell* **89,** 381–391 (1997).

148. Faulkner, G. J. *et al.* The regulated retrotransposon transcriptome of mammalian cells. *Nat. Genet.* **41,** 563–571 (2009).

149. Cruickshanks, H. a. & Tufarelli, C. Isolation of cancer-specific chimeric transcripts induced by hypomethylation of the LINE-1 antisense promoter. *Genomics* **94,** 397–406 (2009).

150. Ryu, S. H., Kang, K., Yoo, T., Joe, C. O. & Chung, J. H. Transcriptional repression of repeat-derived transcripts correlates with histone hypoacetylation at repetitive DNA elements in aged mice brain. *Exp. Gerontol.* **46,** 811–818 (2011).

151. Agmon, N., Liefshitz, B., Zimmer, C., Fabre, E. & Kupiec, M. Effect of nuclear architecture on the efficiency of double-strand break repair. *Nat. Cell Biol.* **15,** 694–699 (2013).

152. Farkash, E. a. & Prak, E. T. L. DNA damage and L1 retrotransposition. *J. Biomed. Biotechnol.* **2006,** 1–8 (2006).

153. Maxwell, P. H., Burhans, W. C. & Curcio, M. J. Retrotransposition is associated with genome instability during chronological aging. *Proc. Natl. Acad. Sci. USA* **108,** 20376–20381 (2011).

154. Stoycheva, T., Pesheva, M. & Venkov, P. The role of reactive oxygen species in the induction of Ty1 retrotransposition in *Saccharomyces cerevisiae*. *Yeast* **27,** 259–267 (2010).

155. Rockwood, L. D., Felix, K. & Janz, S. Elevated presence of retrotransposons at sites of DNA double strand break repair in mouse models of metabolic oxidative stress and MYC-induced lymphoma. *Mutat. Res. - Fundam. Mol. Mech. Mutagen.* **548,** 117–125 (2004).

156. Giorgi, G., Marcantonio, P. & Del Re, B. LINE-1 retrotransposition in human neuroblastoma cells is affected by oxidative stress. *Cell Tissue Res.* **346,** 383–391 (2011).

157. Teneng, I., Stribinskis, V. & Ramos, K. S. Context-specific regulation of LINE-1. *Genes to Cells* **12,** 1101–1110 (2007).

158. Dai, J., Xie, W., Brady, T. L., Gao, J. & Voytas, D. F. Phosphorylation Regulates Integration of the Yeast Ty5 Retrotransposon into Heterochromatin. *Mol. Cell* **27,** 289–299 (2007).

159. Guo, Y. & Levin, H. L. High-throughput sequencing of retrotransposon integration provides a saturated profile of target activity in *Schizosaccharomyces pombe*. *Genome Res.* **2,** 239–248 (2010).

160. Feng, G., Leem, Y. E. & Levin, H. L. Transposon integration enhances expression of stress response genes. *Nucleic Acids Res.* **41,** 775–789 (2013).

161. Fadloun, A. *et al.* Chromatin signatures and retrotransposon profiling in mouse embryos reveal regulation of LINE-1 by RNA. *Nat Struct Mol Biol* **20,** 332–338 (2013).

162. Singer, T., McConnell, M. J., Marchetto, M. C. N., Coufal, N. G. & Gage, F. H. LINE-1 retrotransposons: Mediators of somatic variation in neuronal genomes? *Trends Neurosci.* **33,** 345–354 (2010).

163. Li, W. *et al.* Activation of transposable elements during aging and neuronal decline in *Drosophila*. *Nat. Neurosci.* **16,** 529-532 (2013).

164. Li, W., Jin, Y., Prazak, L., Hammell, M. & Dubnau, J. Transposable elements in TDP-43-mediated neurodegenerative disorders. *PLoS One* **7,** e44099 (2012).

165. Wang, J. *et al.* Inhibition of activated pericentromeric SINE/*Alu* repeat transcription in senescent human adult stem cells reinstates self-renewal. *Cell Cycle* **10,** 3016–3030 (2011).

166. Kaneko, H. *et al.* DICER1 deficit induces *Alu* RNA toxicity in age-related macular degeneration. *Nature* **471,** 325–330 (2011).

167. Shalgi, R., Pilpel, Y. & Oren, M. Repression of transposable-elements - a microRNA anti-cancer defense mechanism? *Trends Genet.* **26,** 253–259 (2010).

168. Kaeberlein, M. Lessons on longevity from budding yeast. *Nature* **464,** 513–519 (2010).

169. Longo, V. D., Shadel, G. S., Kaeberlein, M. & Kennedy, B. Replicative and chronological aging in *Saccharomyces cerevisiae. Cell Metab.* **16,** 18–31 (2012).

170. Kaeberlein, M., Burtner, C. R. & Kennedy, B. K. Recent developments in yeast aging. *PLoS Genet.* **3,** 655–660 (2007).

171. Borrás, C. *et al.* RasGrf1 deficiency delays aging in mice. *Aging (Albany NY).* **3,** 262–276 (2011).

172. Enns, L. C. *et al.* Disruption of protein kinase A in mice enhances healthy aging. *PLoS One* **4,** e 5963 (2009).

173. Lesage, P. & Todeschini, A. L. Happy together: The life and times of Ty retrotransposons and their hosts. *Cytogenet. Genome Res.* **110,** 70–90 (2005).

174. Garfinkel, D. J., Nyswaner, K. M., Stefanisko, K. M., Chang, C. & Moore, S. P. Ty1 copy number dynamics in *Saccharomyces*. *Genetics* **169,** 1845–1857 (2005).

175. Voytas, D. Ty1 and Ty5 in *Saccharomyces cerevisiae* in *Mobile DNA II*. (ed. Craig, N. l.) Ch. 26, 631–662 (ASM Press, 2002).

176. Curcio, M. J. & Garfinkel, D. J. Single-step selection for Ty1 element retrotransposition. *Proc. Natl. Acad. Sci. USA* **88,** 936–940 (1991).

177. Curcio, M. J. *et al.* S-phase checkpoint pathways stimulate the mobility of the retrovirus-like transposon Ty1. *Mol. Cell. Biol.* **27,** 8874–8885 (2007).

178. Scholes, D. T., Banerjee, M., Bowen, B. & Curcio, M. J. Multiple regulators of Ty1 transposition in *Saccharomyces cerevisiae* have conserved roles in genome maintenance. *Genetics* **159,** 1449–1465 (2001).

179. Jiang, Y. W. Transcriptional cosuppression of yeast Ty1 retrotransposons. *Genes Dev.* **16,** 467–478 (2002).

180. Matsuda, E. & Garfinkel, D. J. Posttranslational interference of Ty1 retrotransposition by antisense RNAs. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 15657–15662 (2009).

181. Garfinkel, D. J., Nyswaner, K., Wang, J. & Cho, J. Y. Post-transcriptional cosuppression of Ty1 retrotransposition. *Genetics* **165,** 83–99 (2003).

182. Saha, A. *et al.* A *trans*-dominant form of Gag restricts Ty1 retrotransposition and mediates copy number control. *J. Virol.* **89,** 3922-3938 (2015).

183. Purzycka, K. J. *et al.* Exploring Ty1 retrotransposon RNA structure within virus-like particles. *Nucleic Acids Res.* **41,** 463–473 (2013).

184. Hani, J. & Feldmann, H. tRNA genes and retroelements in the yeast genome. *Nucleic Acids Res.* **26,** 689–696 (1998).

185. Kim, J. M., Vanguri, S., Boeke, J. D., Gabriel, A. & Voytas, D. F. Transposable elements and genome organization: A comprehensive

survey of retrotransposons revealed by the complete *Saccharomyces cerevisiae* genome sequence. *Genome Res.* **8,** 464–478 (1998).

186. Mularoni, L. *et al.* Retrotransposon Ty1 integration targets specifically positioned asymmetric nucleosomal DNA segments in tRNA hotspots. *Genome Res.* **22,** 693–703 (2012).

187. Umezu, K., Hiraoka, M., Mori, M. & Maki, H. Structural analysis of aberrant chromosomes that occur spontaneously in diploid Saccharomyces cerevisiae: Retrotransposon Ty1 plays a crucial role in chromosomal rearrangements. *Genetics* **160,** 97–110 (2002).

188. Maxwell, P. H. *et al.* Ty1 mobilizes subtelomeric Y' elements in telomerase-negative *Saccharomyces cerevisiae* survivors. *Mol. Cell. Biol.* **24,** 9887–9898 (2004).

189. Derr, L. K. The involvement of cellular recombination and repair genes in RNA-mediated recombination in *Saccharomyces cerevisiae*. *Genetics* **148,** 937–945 (1998).

190. Moore, J. K. & Haber, J. E. Capture of retrotransposon DNA at the sites of chromosomal double-strand breaks. *Nature* **383,** 644–646 (1996).

191. Druker, R. & Whitelaw, E. Retrotransposon-derived elements in the mammalian genome: A potential source of disease. *J. Inherit. Metab. Dis.* **27,** 319–330 (2004).

192. Shukla, R. *et al.* Endogenous retrotransposition activates oncogenic pathways in hepatocellular carcinoma. *Cell* **153,** 101–111 (2013).

193. Wilke, C. M. & Adams, J. Fitness effects of Ty transposition in *Saccharomyces cerevisiae*. *Genetics* **131,** 31–42 (1992).

194. Belancio, V. P., Deininger, P. L. & Roy-Engel, A. M. LINE dancing in the human genome: Transposable elements and disease. *Genome Med.* **1,** 97 (2009).

195. Moskalev, A. a. *et al.* The role of DNA damage and repair in aging through the prism of Koch-like criteria. *Ageing Res. Rev.* **12,** 661–684 (2013).

196. De Cecco, M. *et al.* Transposable elements become active and mobile in the genomes of aging mammalian somatic tissues. *Aging (Albany NY).* **5,** 867–883 (2013).

197. Hu, Z. *et al.* Nucleosome loss leads to global transcriptional up-regulation and genomic instability during yeast aging. *Genes Dev.* **28,** 396–408 (2014).

198. De Cecco, M. *et al.* Genomes of replicatively senescent cells undergo global epigenetic changes leading to gene silencing and activation of transposable elements. *Aging Cell* **12,** 247–256 (2013).

199. Beauregard, A., Curcio, M. J. & Belfort, M. The take and give between retrotransposable elements and their hosts. *Annu. Rev. Genet.* **42,** 587–617 (2008).

200. Hancks, D. C. & Kazazian, H. H. Active human retrotransposons: Variation and disease. *Curr. Opin. Genet. Dev.* **22,** 191–203 (2012).

201. Gasior, S. L., Wakeman, T. P., Xu, B. & Deininger, P. L. The human LINE-1 retrotransposon creates DNA double-strand breaks. *J. Mol. Biol.* **357,** 1383–1393 (2006).

202. Abeysinghe, S. S., Chuzhanova, N., Krawczak, M., Ball, E. V. & Cooper, D. N. Translocation and gross deletion breakpoints in human inherited disease and cancer I: Nucleotide composition and recombination-associated motifs. *Hum. Mutat.* **22,** 229–244 (2003).

203. Robberecht, C., Voet, T., Esteki, M. Z., Nowakowska, B. A. & Vermeesch, J. R. Nonallelic homologous recombination between retrotransposable elements is a driver of *de novo* unbalanced translocations. *Genome Res.* **23,** 411–418 (2013).

204. Dunham, M. J. *et al.* Characteristic genome rearrangements in experimental evolution of *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA* **99,** 16144–16149 (2002).

205. Derr, L. K., Strathern, J. N. & Garfinkel, D. J. RNA-mediated recombination in *S. cerevisiae. Cell* **67,** 355–364 (1991).

206. Gilbert, N., Lutz-Prigge, S. & Moran, J. V. Genomic deletions created upon LINE-1 retrotransposition. *Cell* **110,** 315–325 (2002).

207. Burhans, W. C. & Weinberger, M. DNA damage and DNA replication stress in yeast models of aging. *Subcell. Biochem.* **57,** 187–206 (2012).

208. Moore, S. P. *et al.* Analysis of a Ty1-less variant of *Saccharomyces paradoxus*: the gain and loss of Ty1 elements. *Yeast* **21,** 649–660 (2004).

209. Weinberger, M. *et al.* DNA replication stress is a determinant of chronological lifespan in budding yeast. *PLoS One* **2,** e748 (2007).

210. Weinberger, M., Sampaio-Marques, B., Ludovico, P. & Burhans, W. C. DNA replication stress-induced loss of reproductive capacity in *S. cerevisiae* and its inhibition by caloric restriction. *Cell Cycle* **12,** 1189–1200 (2013).

211. Paquin, C. E. & Williamson, V. M. Temperature effects on the rate of Ty transposition. *Science* **226,** 53–55 (1984).

212. Lawler, J. F., Haeusser, D. P., Dull, A., Boeke, J. D. & Keeney, J. B. Ty1 defect in proteolysis at high temperature. *J. Virol.* **76,** 4233–4240 (2002).

213. Devine, S. E. & Boeke, J. D. Integration of the yeast retrotransposon Ty1 is targeted to regions upstream of genes transcribed by RNA polymerase III. *Genes Dev.* **10,** 620–633 (1996).

214. Curcio, M. J., Hedge, A. M., Boeke, J. D. & Garfinkel, D. J. Ty RNA levels determine the spectrum of retrotransposition events that activate gene expression in *Saccharomyces cerevisiae*. *MGG Mol. Gen. Genet.* **220,** 213–221 (1990).

215. Hartman, J. L. Buffering of deoxyribonucleotide pool homeostasis by threonine metabolism. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 11700–11705 (2007).

216. Madia, F. *et al.* Oncogene homologue Sch9 promotes age-dependent mutations by a superoxide and Rev1/Polζ-dependent mechanism. *J. Cell Biol.* **186,** 509–523 (2009).

217. Whelan, W. L., Gocke, E. & Manney, T. R. The *CAN1* locus of *Saccharomyces cerevisiae*: Fine-structure analysis and forward mutation rates. *Genetics* **91,** 35–51 (1979).

218. Hall, B. M., Ma, C.-X., Liang, P. & Singh, K. K. Fluctuation analysis CalculatOR: A web tool for the determination of mutation rate using Luria-Delbrück fluctuation analysis. *Bioinformatics* **25,** 1564–1565 (2009).

219. Burtner, C. R., Murakami, C. J., Kennedy, B. K. & Kaeberlein, M. A molecular mechanism of chronological aging in yeast. *Cell Cycle* **8,** 1256–1270 (2009).

220. Fabrizio, P., Pletcher, S. D., Minois, N., Vaupel, J. W. & Longo, V. D. Chronological aging-independent replicative life span regulation by

Msn2/Msn4 and Sod2 in *Saccharomyces cerevisiae*. *FEBS Lett.* **557,** 136–142 (2004).

221. Weinberger, M. *et al.* Growth signaling promotes chronological aging in budding yeast by inducing superoxide anions that inhibit quiescence. *Aging (Albany NY).* **2,** 709–726 (2010).

222. Bonawitz, N. D., Chatenay-Lapointe, M., Pan, Y. & Shadel, G. S. Reduced TOR signaling extends chronological life span via increased respiration and upregulation of mitochondrial gene expression. *Cell Metab.* **5,** 265–277 (2007).

223. Pan, Y., Schroeder, E. a., Ocampo, A., Barrientos, A. & Shadel, G. S. Regulation of yeast chronological life span by TORC1 via adaptive mitochondrial ROS signaling. *Cell Metab.* **13,** 668–678 (2011).

224. Madia, F., Gattazzo, C., Fabrizio, P. & Longo, V. D. A simple model system for age-dependent DNA damage and cancer. *Mech. Ageing Dev.* **128,** 45–49 (2007).

225. Capy, P., Gasperi, G., Biémont, C. & Bazin, C. Stress and transposable elements: Co-evolution or useful parasites? *Heredity* **85,** 101–106 (2000).

226. Belancio, V. P., Roy-Engel, A. M., Pochampally, R. R. & Deininger, P. Somatic expression of LINE-1 elements in human tissues. *Nucleic Acids Res.* **38,** 3909–3922 (2010).

227. Cruickshanks, H. a. *et al.* Expression of a large LINE-1-driven antisense RNA is linked to epigenetic silencing of the metastasis suppressor gene TFPI-2 in cancer. *Nucleic Acids Res.* **41,** 6857–6869 (2013).

228. Hunter, R. G. *et al.* Acute stress and hippocampal histone H3 lysine 9 trimethylation, a retrotransposon silencing response. *Proc. Natl. Acad. Sci. USA* **109,** 17657–17662 (2012).

229. Ponicsan, S. L., Kugel, J. F. & Goodrich, J. A. Genomic gems: SINE RNAs regulate mRNA production. *Curr. Opin. Genet. Dev.* **20,** 149–155 (2010).

230. Servant, G., Pennetier, C. & Lesage, P. Remodeling yeast gene transcription by activating the Ty1 long terminal repeat retrotransposon under severe adenine deficiency. *Mol. Cell. Biol.* **28,** 5543–5554 (2008).

231. Amberg, D. C. in *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual 1st edn,* (eds Inglis, J. *et al.*) 1-230 (Cold Spring Harbor Press, 2005).

232. Garfinkel, D. J., Mastrangelo, M. F., Sanders, N. J., Shafer, B. K. & Strathern, J. N. Transposon tagging using Ty elements in yeast. *Genetics* **120,** 95–108 (1988).

233. Lee, B. S., Bi, L., Garfinkel, D. J. & Bailis, a M. Nucleotide excision repair/TFIIH helicases RAD3 and SSL2 inhibit short-sequence recombination and Ty1 retrotransposition by similar mechanisms. *Mol. Cell. Biol.* **20,** 2436–2445 (2000).

234. Foster, P. L. Methods for determining spontaneous mutation rates. *Method. Enzymol.* **409,** 195–213 (2006).

235. Fabrizio, P. & Wei, M. Conserved role of medium acidification in chronological senescence of yeast and mammalian cells. *Aging* **3,** 1127–1129 (2011).

236. Bonawitz, N. D., Rodeheffer, M. S. & Shadel, G. S. Defective mitochondrial gene expression results in reactive oxygen species-mediated inhibition of respiration and reduction of yeast life span. *Mol. Cell. Biol.* **26,** 4818–4829 (2006).

237. Alvers, A. L. *et al.* Autophagy is required for extension of yeast chronological life span by rapamycin. *Autophagy* **5,** 847–849 (2009).

238. Pan, Y. & Shadel, G. S. Extension of chronological life span by reduced TOR signaling requires down-regulation of Sch9p and involves increased mitochondrial OXPHOS complex density. *Aging (Albany NY).* **1,** 131–145 (2009).

239. Lin, S.-J. *et al.* Calorie restriction extends *Saccharomyces cerevisiae* lifespan by increasing respiration. *Nature* **418,** 344–348 (2002).

240. Giralt, A. & Villarroya, F. SIRT3, a pivotal actor in mitochondrial functions: metabolism, cell death and aging. *Biochem. J.* **444,** 1–10 (2012).

241. Rizzuto, R., De Stefani, D., Raffaello, A. & Mammucari, C. Mitochondria as sensors and regulators of calcium signalling. *Nat. Rev. Mol. Cell Bio.* **13,** 566–578 (2012).

242. Wang, K. & Klionsky, D. J. Mitochondria removal by autophagy. *Autophagy* **7,** 297–300 (2011).

243. Graef, M. & Nunnari, J. Mitochondria regulate autophagy by conserved signalling pathways. *EMBO J.* **30,** 2101–2114 (2011).

244. Hughes, A. L. & Gottschling, D. E. An early age increase in vacuolar pH limits mitochondrial function and lifespan in yeast. *Nature* **492,** 261–265 (2012).

245. Ludovico, P., Sousa, M. J., Silva, M. T., Leão, C. & Côrte-Real, M. *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. *Microbiology* **147,** 2409–2415 (2001).

246. Ludovico, P. *et al.* Cytochrome c release and mitochondria involvement in programmed cell death induced by acetic acid in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **13,** 2598–2606 (2002).

247. Giannattasio, S., Guaragnella, N., Zdralević, M. & Marra, E. Molecular mechanisms of *Saccharomyces cerevisiae* stress adaptation and programmed cell death in response to acetic acid. *Front. Microbiol.* **4,** 1-7 (2013).

248. Zarse, K. *et al.* Impaired insulin/IGF1 signaling extends life span by promoting mitochondrial L-proline catabolism to induce a transient ROS signal. *Cell Metab.* **15,** 451–465 (2012).

249. Yang, W. & Hekimi, S. Two modes of mitochondrial dysfunction lead independently to lifespan extension in *Caenorhabditis elegans*. *Aging Cell* **9,** 433–447 (2010).

250. Fournier, M. L. *et al.* Delayed correlation of mRNA and protein expression in rapamycin-treated cells and a role for Ggc1 in cellular sensitivity to rapamycin. *Mol. Cell. Proteomics* **9,** 271–284 (2010).

251. Ocampo, A., Liu, J., Schroeder, E. A., Shadel, G. S. & Barrientos, A. Mitochondrial respiratory thresholds regulate yeast chronological life span and its extension by caloric restriction. *Cell Metab.* **16,** 55–67 (2012).

252. Rodić, N. & Burns, K. H. Long interspersed element-1 (LINE-1): Passenger or driver in human neoplasms? *PLoS Genet.* **9,** 1–5 (2013).

253. Kaer, K. & Speek, M. Retroelements in human disease. *Gene* **518,** 231–241 (2013).

254. Williams, G. C. Pleiotropy, natural selection, and the evolution of senescence. *Evolution.* **11,** 398–411 (1957).

255. Han, K. *et al.* Genomic rearrangements by LINE-1 insertion-mediated deletion in the human and chimpanzee lineages. *Nucleic Acids Res.* **33,** 4040–4052 (2005).

256. Han, K. *et al. Alu* recombination-mediated structural deletions in the chimpanzee genome. *PLoS Genet.* **3,** 1939–1949 (2007).

257. Lee, J., Han, K., Meyer, T. J., Kim, H. S. & Batzer, M. A. Chromosomal inversions between human and chimpanzee lineages caused by retrotransposons. *PLoS One* **3,** e4047 (2008).

258. Yu, X. & Gabriel, A. Patching broken chromosomes with extranuclear cellular DNA. *Mol. Cell* **4,** 873–881 (1999).

259. Gdula, D. A., Gerasimova, T. I. & Corces, V. G. Genetic and molecular analysis of the gypsy chromatin insulator of *Drosophila. Proc. Natl. Acad. Sci. USA* **93,** 9378–9383 (1996).

260. Rebollo, R. *et al.* Retrotransposon-induced heterochromatin spreading in the mouse revealed by insertional polymorphisms. *PLoS Genet.* **7,** e1002301 (2011).

261. Eichten, S. R. *et al.* Spreading of heterochromatin is limited to specific families of maize retrotransposons. *PLoS Genet.* **8,** e1003127 (2012).

262. Servant, G. *et al.* Tye7 regulates yeast Ty1 retrotransposon sense and antisense transcription in response to adenylic nucleotides stress. *Nucleic Acids Res.* **40,** 5271–5282 (2012).

263. Fehrmann, S. *et al.* Aging yeast cells undergo a sharp entry into senescence unrelated to the loss of mitochondrial membrane potential. *Cell Rep.* **5,** 1589–1599 (2013).

264. Barros, M. H., Bandy, B., Tahara, E. B. & Kowaltowski, A. J. Higher respiratory activity decreases mitochondrial reactive oxygen release and increases life span in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **279,** 49883–49888 (2004).

265. Cherry, J. M. *et al.* SGD: *Saccharomyces* genome database. *Nucleic Acids Res.* **26,** 73–79 (1998).

266. Lam, Y. T., Aung-Htut, M. T., Lim, Y. L., Yang, H. & Dawes, I. W. Changes in reactive oxygen species begin early during replicative aging of *Saccharomyces cerevisiae* cells. *Free Radic. Biol. Med.* **50,** 963–970 (2011).

267. Suzuki, K. Selective autophagy in budding yeast. *Cell Death Differ.* **20,** 43-48 (2013).

268. Schiavi, A. & Ventura, N. The interplay between mitochondria and autophagy and its role in the aging process. *Exp. Gerontol.* **56,** 147–153 (2014).

269. Kanki, T., Klionsky, D. J. & Okamoto, K. Mitochondria autophagy in yeast. *Antioxid. Redox Signal.* **14,** 1989–2001 (2011).

270. Okamoto, K., Kondo-Okamoto, N. & Ohsumi, Y. Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. *Dev. Cell* **17,** 87–97 (2009).

271. Fabrizio, P. *et al.* Genome-wide screen in *Saccharomyces cerevisiae* identifies vacuolar protein sorting, autophagy, biosynthetic, and tRNA methylation genes involved in life span regulation. *PLoS Genet.* **6,** 1–14 (2010).

272. Suzuki, K., Morimoto, M., Kondo, C. & Ohsumi, Y. Selective autophagy regulates insertional mutagenesis by the Ty1 retrotransposon in *Saccharomyces cerevisiae*. *Dev. Cell* **21,** 358–365 (2011).

273. Georis, I., Feller, A., Tate, J. J., Cooper, T. G. & Dubois, E. Nitrogen catabolite repression-sensitive transcription as a readout of Tor pathway regulation: The genetic background, reporter gene and GATA factor assayed determine the outcomes. *Genetics* **181,** 861–874 (2009).

274. Crespo, J. L. & Hall, M. N. Elucidating TOR signaling and rapamycin action: Lessons from *Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev.* **66,** 579–591, table of contents (2002).

275. Cunningham, J. T. *et al.* mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. *Nature* **450,** 736–740 (2007).

276. Schieke, S. M. *et al.* The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. *J. Biol. Chem.* **281,** 27643–27652 (2006).

277. Lamming, D. W. *et al.* Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. *Science* **335,** 1638–1643 (2012).

278. Houde, V. P. *et al.* Chronic rapamycin treatment causes glucose intolerance and hyperlipidemia by upregulating hepatic gluconeogenesis and impairing lipid deposition in adipose tissue. *Diabetes* **59,** 1338–1348 (2010).

279. McKee Alderman, J. *et al.* Calorie restriction and dwarf mice in gerontological research. *Gerontology* **56,** 404–409 (2010).

280. Nisoli, E. *et al.* Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. *Science* **310,** 314–317 (2005).

281. Chen, Y., Zheng, Y. & Foster, D. A. Phospholipase D confers rapamycin resistance in human breast cancer cells. *Oncogene* **22,** 3937–3942 (2003).

282. Loewith, R. *et al.* Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol. Cell* **10,** 457–468 (2002).

283. VanHoute, D. & Maxwell, P. H. Extension of *Saccharomyces paradoxus* chronological lifespan by retrotransposons in certain media conditions is associated with changes in reactive oxygen species. *Genetics* **198,** 531-545 (2014).