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# Elevated histone expression promotes lifespan extension

**Jason Feser**[2], **David Truong**[2], **Chandrima Das**[2], **Joshua J. Carson**[2], **Jeffrey Kieft**[2,3], **Troy Harkness**[4], and **Jessica K. Tyler**[1,2,*]

[1]Department of Biochemistry and Molecular Biology, UT MD Anderson Cancer Center, Houston, TX 77030

[2]Department of Biochemistry and Molecular Genetics, University of Colorado School of Medicine, Aurora, CO, 80010

[3]Howard Hughes Medical Institute, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

[4]Department of Anatomy and Cell Biology, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

## Summary

Changes to the chromatin structure accompany aging, but the molecular mechanisms underlying aging and the accompanying changes to the chromatin are unclear. Here we report a mechanism whereby altering chromatin structure regulates lifespan. We show that normal aging is accompanied by a profound loss of histone proteins from the genome. Indeed, yeast lacking the histone chaperone Asf1 or acetylation of histone H3 on lysine 56 are short lived and this appears to be at least partly due to their having decreased histone levels. Conversely, increasing the histone supply by inactivation of the Hir (histone information regulator) complex or overexpression of histones dramatically extends lifespan, via a pathway that is distinct from previously known pathways of lifespan extension. This study indicates that maintenance of the fundamental chromatin structure is critical for slowing down the aging process and reveals that increasing the histone supply extends lifespan.

### Keywords

Aging; lifespan extension; chromatin; histone acetylation; transcription

## Introduction

Mitotic cells divide a finite number of times (replicative lifespan) before they exhibit signs of senescence and cease replicating. The length of time that a post-mitotic or senescent cell exists in a non-dividing state, termed chronological lifespan, also has a profound influence on the physiology and longevity of an organism. Gradual alterations of biological macromolecules characterize normal aging, while adverse alterations to macromolecules are risk factors for age-related conditions and disease states. Notably, aging is the single highest risk factor for the majority of human malignancies. Despite the fundamental importance of



*Contact: jtyler@mdanderson.org.

the aging process, there are still huge gaps in our knowledge as to the biological changes that lead to aging and the molecular causes of these changes.

Several important cellular processes have been implicated in the regulation of yeast replicative lifespan, including glucose sensing, nutrient sensing, stress response, mitochondrial function and transcriptional silencing (Steinkraus et al., 2008). The conservation of the fundamental mechanisms of aging and lifespan determination across eukaryotes (Smith et al., 2007; Smith et al., 2008) enables the study of aging in budding yeast. Determining how replicative lifespan can be extended in yeast will ultimately help facilitate the development of therapeutic regimens to extend lifespan and delay the onset of age-associated disease in humans.

It is clear that the packaging of the eukaryotic genome together with histones to form the chromatin structure plays a critical role in regulating the activities of the genome (Groth et al., 2007; Li et al., 2007). Tightly packaged chromatin structure reduces access to the DNA to limit inappropriate gene expression and genomic instability, while an open chromatin structure promotes unregulated gene expression and genomic instability. Notably, increased genomic instability and inappropriate transcription are associated with increased age (Busuttil et al., 2007; Maslov and Vijg, 2009). A clear example of the influence of chromatin structure on aging is provided by the Sir2/Hst2 NAD-dependent protein and histone deacetylases that have a key role in transcriptional silencing and maintenance of chromatin structure. Inactivation of Sir2 or Hst2 shortens lifespan (Lamming et al., 2005), while introduction of an extra copy of the gene encoding Sir2 extends lifespan (Kaeberlein et al., 1999). The role of Sir2/Hst2 in yeast aging is likely related to its ability to inhibit formation of extrachromosomal rDNA circles (ERC) (Kaeberlein et al., 1999) by repressing rDNA recombination (Gottlieb and Esposito, 1989). A key substrate of the deacetylase activity of Sir2 is lysine 16 of histone H4 (Moazed, 2001). Sir2 protein levels normally decrease during aging, leading to increased levels of acetylated H4 K16 and concomitant loss of histones from specific subtelomeric regions of the genome (Dang et al., 2009). However, it is unclear how altered chromatin structure at the gene-depleted subtelomeric regions of the genome would lead to aging, or if reversed, would extend lifespan.

Chromatin is modified by the cell in a myriad of different ways. The ultimate and most profound chromatin changes are the loss of histones from the DNA, or the deposition of histones onto the DNA. The removal of histones from DNA and the incorporation of histones onto DNA are mediated by a class of proteins termed histone chaperones. Anti silencing function 1 (Asf1) is a highly conserved central chaperone of histones H3 and H4 that is required for proper regulation of gene expression, histone acetylation and the maintenance of genomic integrity (Chen et al., 2008; Ramey et al., 2004; Tyler et al., 1999). We were prompted to investigate whether yeast cells lacking Asf1 have a reduced lifespan, due to the difficulty we encountered in obtaining re-growth of *asf1* mutant cells from old colonies. Our studies have led to our uncovering a causal relationship between chromatin structure and aging, while discovering a means to greatly extend yeast replicative lifespan via manipulating the chromatin structure.

## Results

### Yeast lacking the histone chaperone Asf1 are short-lived

Yeast deleted for *ASF1* have a very short chronological lifespan, shorter than that of yeast lacking the well known anti-aging protein Sir2 and comparable to that of yeast lacking Superoxidase dismutase 1 (Sod1) (Fig. S1). In order to determine whether yeast lacking Asf1 also have a defect in replicative lifespan we took advantage of the unique asymmetric nature of cell division in budding yeast. We found that yeast lacking Asf1 are short-lived,

having a median replicative lifespan of about 7 generations in comparison to the median lifespan of about 27 generations for wild type yeast in multiple yeast backgrounds (Fig. 1A, B). While shortening of lifespan can result from several causes, *asf1* mutants exhibit phenotypes characteristic of aging. Loss of transcriptional silencing occurs in telomeric regions of aged yeast (Kim et al., 1996) and *asf1* mutants have transcriptional silencing defects (Le et al., 1997; Singer et al., 1998; Tyler et al., 1999). Also, yeast lacking the protein Rtt109 that functions in the same pathway as Asf1 for lifespan determination (see later) have an elevated rate of ERC formation (Han et al., 2007b), which correlates with premature aging.

To investigate whether Asf1 functions in previously established pathways of aging we compared the effect of deletion of *ASF1* to deletion of *SIR2* on replicative lifespan. We found that *asf1* mutants have a more drastic decrease in lifespan than *sir2* mutants (which have a median lifespan of 15 generations) (Fig. 1C). Furthermore, yeast lacking both Asf1 and Sir2 were extremely short-lived (median lifespan of 4 to 5 generations) (Fig. 1C). These data demonstrate that Asf1 and Sir2 are functioning in non-identical pathways to promote longevity.

Next, we set out to determine through which molecular pathway Asf1 mediates attainment of a full replicative lifespan. Asf1 promotes replication-dependent chromatin assembly in collaboration with the histone chaperone CAF-1 (Tyler et al., 1999). Inactivation of CAF-1 via deletion of the *CAC1* gene encoding the largest subunit of CAF-1 (Kaufman et al., 1997) shortens yeast replicative lifespan but to a lesser degree than deletion of *ASF1* (Fig. 2A). In agreement, the double *cac1asf1* mutant had a lifespan that was shorter than either single mutant (Fig. 2A), indicating that Asf1 is not solely influencing lifespan via its role in replication-dependent chromatin assembly. Genomic instability contributes to aging and Asf1 has been shown to play a role in the maintenance of genomic stability, apparently due to its role in reducing the amount of DNA damage occurring during DNA replication (Myung et al., 2003; Ramey et al., 2004; Tyler et al., 1999). Notably, yeast lacking the checkpoint protein Mrc1 that normally senses replicational stress (Robert et al., 2006) do not have a reduced lifespan even though they also experience genomic instability occurring during DNA replication (Fig. 2B). These data suggest that the short replicative lifespan of *asf1* mutants is unlikely due to genomic instability during DNA replication.

## The role of Asf1 in longevity is due to Asf1 facilitating acetylation of histone H3 on lysine 56

Asf1 has an additional function in helping several histone acetyl transferases (HATs) acetylate their histone substrates (Adkins et al., 2007; Fillingham et al., 2008; Han et al., 2007a). To examine whether the function of Asf1 in promoting histone acetylation by the HAT Rtt109 was responsible for normal lifespan, we compared the effect of deletion of *ASF1* and *RTT109* on replicative aging. The replicative lifespan was indistinguishable between strains deleted for *ASF1*, *RTT109* or both *ASF1* and *RTT109* together (Fig. 2C), demonstrating that Asf1 and Rtt109 function together in the same pathway to promote achievement of a full lifespan.

Asf1 and Rtt109 together mediate acetylation of histone H3 on lysine 56 and lysine 9 (Fillingham et al., 2008; Han et al., 2007a). To determine which lysine was the critical acetylation target for anti-aging, we measured the replicative lifespan of yeast unable to acetylate K56 using a strain bearing a mutation in the sole copy of the H3 gene that leads to a lysine to arginine (K56R) substitution. Note that although these strains only have one pair of genes expressing H3/H4 (*HHT2/HHF2*), they produce equivalent amounts of histone proteins as a strain with both pairs of genes expressing H3/H4 (Fig. S2). The H3 K56R mutant had a reduced lifespan (Fig. 2D) (Dang et al., 2009), which was not quite as short as

that of yeast with unacetylated K56 (such as the *asf1* or *rtt109* mutants). This is presumably because the arginine is unlikely to fully mimic the lysine for forming the proper histone-DNA contacts mediated by K56 within the nucleosome structure (Luger et al., 1997), and is consistent with the increased chromatin accessibility and reduced transcriptional silencing that occurs in yeast carrying the H3 K56R mutation (Xu et al., 2007). Nevertheless, the drastically reduced lifespan of the H3 K56R mutant clearly shows that failure to acetylate histone H3 on K56 results in a shortened lifespan (Fig. 2D). Furthermore, deletion of *ASF1* did not exacerbate the effect of the H3 K56R mutation on lifespan (Fig. 2D). As such, the role of Asf1 in determining a normal lifespan is mediated via acetylation of histone H3 on K56.

Next, we examined the consequence of having too much H3 K56 acetylation on replicative lifespan. To do this, we first used a strain where the only copy of H3 had been mutated such that lysine 56 is substituted for glutamine (H3 K56Q) to mimic permanent acetylation. The H3 K56Q mutant had a greatly shortened lifespan, which was only slightly longer than that of the *asf1* and *rtt109* mutants (Fig. 2D) (Dang et al., 2009). To rule out the possibility that the shortened lifespan of the H3 K56Q mutant was due to the glutamine not completely mimicking acetylation of H3 K56, we examined the replicative lifespan of yeast deleted for the genes encoding the two partially redundant histone deacetylases that remove the acetyl group from H3 K56Ac; Hst3 and Hst4 (Maas et al., 2006). Deletion of *HST3* and *HST4* leads to a greatly elevated level of H3 K56Ac (Maas et al., 2006) and the *hst3hst4* double mutant had an extremely short replicative lifespan (Fig. 2E) (Tsuchiya et al., 2006). Because it is not yet known whether deletion of *HST3* and *HST4* leads to alterations in the levels of other histone modifications, we took another approach to increase the level of acetylated H3 K56Ac in the yeast cells. We had previously shown that overexpression of Asf1 leads to elevated levels of H3 K56Ac (Adkins et al., 2007). Using a strain in which the endogenous *ASF1* gene was under the control of the galactose inducible *GAL1* promoter (Zabaronick and Tyler, 2005), we found that induction of Asf1 with 0.5% galactose leads to a 19% reduction in the median lifespan, while inducing even more Asf1 with 1% galactose leads to a 28% reduction in the median lifespan (Fig. 2F). These data demonstrate that to achieve normal lifespan it is not only important to be able to acetylate H3 on K56, but it may be equally important to be able to deacetylate H3 K56Ac. Alternatively, there may be a delicate balance or amount of acetylated H3 K56Ac that is required for achieving a normal lifespan.

## Histone protein levels drop during aging

We wondered whether the short lifespan of cells that fail to acetylate histone H3 on K56 might be related to the requirement of H3 K56Ac and Asf1 for accurate expression of the cell cycle regulated histone genes (Sutton et al., 2001; Xu et al., 2005), *HHT1* and *HHT2* encoding H3, *HHF1* and *HHF2* encoding H4, *HTB1* encoding H2B and *HTA1* encoding H2A. Expression of these cell cycle regulated histone genes is repressed outside of S-phase by the **h**istone **i**nformation **r**egulator (Hir) complex (Spector and Osley, 1993; Spector et al., 1997). Consistent with earlier studies, we find histone transcript levels are altered upon deletion of *ASF1, RTT109 or HIR1* (Fig. S3, 3A). To investigate whether histone transcript levels change during the normal aging process, we biochemically separated young and old yeast cells. We observed a significant increase in histone transcripts in the aging population of wild type yeast (Fig. 3A, S3B, S3C). In agreement with the role of H3 K56Ac for accurate expression of the cell cycle regulated histone genes, we found that the short-lived *rtt109*Δ mutant also had significantly lower levels of these transcripts compared to wild type strains when the data were normalized to tubulin levels (Fig. S3A, B). Aging or deletion of *ASF1* or *RTT109* leads to an accumulation of cells with a $G_2$/M DNA content (Fig. S4A). Towards understanding the possible effects of altered histone transcription on nucleosome density we decided to correct for these cell cycle defects by normalizing the transcript levels

to DNA content. Accordingly, when we independently normalized the histone transcript levels to the amount of DNA per cell, histone transcript levels are slightly higher in young *asf1* and *rtt109* mutant cells than young wild type cells (Fig. 3A, S3C). However, although histone transcripts also increased in the *asf1* and *rtt109* mutant cells during the aging process, this was to a much lesser degree than in wild type cells (Fig. 3A).

Given that the levels of histone transcripts increase during aging, we asked whether histone protein levels also increase during aging. We loaded equal amounts of total proteins from young, old or very old wild type yeast and determined the levels of histone H3 by quantitative western analysis. The levels of H3 and H2A protein drastically decrease during aging in wild type yeast (Fig. 3B, S4B). We see a very similar drastic decrease in histone levels during aging when we normalize to DNA content (Fig. S4C). It is possible that the increased abundance of histone transcripts during aging (Fig. 3A) is the cell's attempt to replace the histone proteins that are lost or not properly synthesized during aging. Consistent with the histone transcript analyses above, the short-lived *rtt109* and *asf1* mutants have significantly lower levels of total histone H3 protein than the isogenic wild type strain (Fig. S4D). Furthermore, it is clear that the total histone protein levels also decrease during aging in the short-lived *rtt109* mutant and the *hir1* mutant (Fig. S4E). Moreover, the relative amount of histone H3 protein in the young and old short-lived *rtt109* mutant is less than that seen in the respective young and old wild type cells, while the amount of histone H3 protein in the old *hir1* mutant cells is higher than that in the old wild type cells (Fig. S4E). These data are consistent with the total abundance of histone proteins decreasing during aging, and show that the short-lived *asf1* and *rtt109* mutants have even lower levels of histone proteins than normal.

To determine whether the decrease in histone protein levels in the short-lived *asf1* and *rtt109* mutants and during the normal aging process reflects a loss of free histones or chromatinized histones, we performed chromatin fractionations. We analyzed the same DNA equivalents of proteins for the total, supernatant (free proteins) and pellet (chromatin proteins) fractions to enable direct comparison of the distribution of the histones between the free and chromatinized forms. In order to isolate sufficient numbers of aged cells for this analysis, we utilized a new system developed by Dan Gottschling's lab, termed the “mother enrichment program” (Lindstrom and Gottschling, 2009). Coupled with the traditional biochemical isolation of old cells, this system enabled us to easily isolate much older cells, that had reached approximately 50% of the maximum lifespan for each strain. As before, we observe a greatly reduced total amount of histones during aging (Fig. 3C). Furthermore, we find that in both young and old wild type and *hir1* cells, the vast majority of histone H3 is chromatinized (Fig. 3C). Quite unexpectedly, we find that in the *asf1* and *rtt109* short-lived mutants, a significant amount of the histones are free, even in the mixed age (asynch) population (Fig. 3C). This may reflect a defect in chromatin assembly in the absence of Rtt109 and Asf1 (Li et al., 2008). To provide further evidence for reduced histone occupancy on the DNA during aging, we performed chromatin immunoprecipitation (ChIP) analysis of histone H3. Again, we utilized the mother enrichment program (Lindstrom and Gottschling, 2009) to isolate old cells (median 30 generations) for this analysis. At all areas examined, including the active Actin gene and the silent HML locus, the rDNA and telomere proximal loci, we observed a 50-75% reduction in histone occupancy in the aged population in comparison to the mixed age asynchronous culture (Fig. 3D, S4F).

## Supplying extra histone proteins increases longevity

We hypothesized that the increase in histone transcript levels that normally occurs during aging serves to protect cells from premature aging, while the reduced histone expression in the short-lived mutants is a cause of their shortened lifespan. To test this hypothesis, we

investigated whether ectopic expression of extra histones could extend the lifespan of the short-lived *asf1* mutants. A high copy number plasmid encoding all four core histones extended the median lifespan of *asf1* mutants by 65% (Fig. 3E). Overexpression of the H2A/H2B pair of histones from a galactose inducible promoter also significantly increased the lifespan of *asf1* mutants (Fig. 3F). Overexpression of H3/H4 did not significantly extend the lifespan of the *asf1* mutant (Fig. S5A,B), which is not too surprising given that these cells are lacking the major H3/H4 histone chaperone. These data show that the shortened lifespan of the *asf1* mutant can be partly reversed by supplying additional histones.

Given that the reduced level of histone proteins in the *asf1* mutant is partly responsible for the shortened lifespan of this strain, we predicted that supplying higher levels than normal of histone proteins would extend the lifespan of wild type yeast. To test this prediction, we analyzed the replicative lifespan of the *hir1* mutant that has significantly higher histone protein levels in the old cells as compared to wild type old cell (Fig. 3G). In addition, we examined the other three components of the Hir complex; Hir2, Hir3, Hpc2 (Green et al., 2005). Inactivation of any component of the Hir complex extended the median replicative lifespan by 25-35% (Fig. 3G). This extent of lifespan extension is comparable to that achieved by other known individual manipulations that result in lifespan extension. In summary, mutants with low histone protein levels such as *rtt109* and *asf1* mutants are short-lived, while mutants with high histone protein levels such as *hir1, hir2, hir3* and *hpc2* mutants have extended lifespan.

## Increased histone supply appears to extend replicative lifespan via a pathway that is distinct from other lifespan extension pathways

To determine how the histone supply pathway of longevity relates to other known pathways of lifespan extension, we performed epistasis analyses. Deletion of *HIR1* significantly extends the lifespan of the short-lived *sir2* mutant (Fig. 4A), indicating that the extra histone supply pathway does not require the *Sir2* histone deacetylase to achieve lifespan extension. The short lifespan of *sir2* mutants is due to the accumulation of ERCs because it is suppressed by deletion of *FOB1* (Defossez et al., 1999) (Fig. 4B), where *FOB1* encodes a replication fork protein whose deletion prevents ERC formation (Defossez et al., 1999). Notably, additional deletion of *HIR1* from the *sir2fob1* double mutant led to a significant extension in lifespan beyond that of the *sir2fob1* double mutant (Fig. 4B), indicating that extra histone supply is unlikely to extend lifespan via reducing ERC formation. An extra copy of the gene encoding Sir2 (Sir2 OX) extends lifespan (Kaeberlein et al., 1999) (Fig. 4C), mediated via reduced ERC accumulation because it is blocked by *FOB1* deletion (Imai et al., 2000). Deletion of *HIR1* leads to a greater degree of lifespan extension than overexpression of Sir2 (Fig. 4C), while deletion of *HIR1* and Sir2 overexpression together significantly increased lifespan when compared to Sir2 overexpression alone (Fig. 4C). Collectively, these data indicate that extra histone supply is unlikely to extend lifespan via the identical pathways by which ERC accumulation and Sir2 influence lifespan.

Lifespan extension due to caloric restriction (CR) is likely mediated through the highly conserved nutrient-responsive target of rapamycin (Tor1) kinase (Kaeberlein et al., 2005). To investigate the relationship between the lifespan extension due to increased histone supply and the lifespan extension due to *Tor1* deletion, we combined the *hir1* and *tor1* mutants. The *hir1* mutant extended lifespan more the *tor1* mutant (Fig. 5A). However, the double *hir1tor1* mutant had a lifespan that was intermediate between that of the *tor1* mutant and the *hir1* mutant (Fig. 5A). CR of yeast is achieved by growth in 0.5% glucose in contrast to the 2% glucose that is used for standard yeast growth. Although individually CR or *HIR1* deletion extends lifespan, CR of the *hir1* mutant led to a lifespan extension that was intermediate between that resulting from the individual manipulations, not additive (Fig.

5B). These results suggest two possibilities: either (i) that deletion of *TOR1/*CR and deletion of *HIR1* function through the same pathway to extend lifespan or alternatively, (ii) that *HIR1* deletion cannot extend lifespan in the context of a *tor1* mutant/CR.

Depletion of the 60S ribosomal subunits extends lifespan (Steffen et al., 2008). Genetic evidence places CR, *tor1* inhibition and depletion of the 60S subunits in a longevity pathway that is partially dependent on upregulated translation of the transcription factor Gcn4 that promotes stress resistance (Steffen et al., 2008). To investigate whether the histone supply pathway extends lifespan via the same pathways that are targeted by deletion of *TOR1* and CR, we measured Gcn4 translation and the abundance of 60S ribosomal subunits in the mutants that influence histone supply. Using a dual luciferase reporter construct to measure Gcn4 translation, we observed increased Gcn4 levels upon deletion of the gene encoding the 60S subunit Rpl20b (Fig. 5C) as observed previously (Steffer et al., 2008). However, the isogenic short-lived *rtt109* and *asf1* mutants and the long-lived *hir1* mutant did not have significant alterations in Gcn4 protein synthesis (Fig. 5C), suggesting that 60S ribosomal subunit abundance is not drastically altered in these strains.

To examine directly whether the abundance of the 60S ribosomal subunit was altered in the mutants that effect histone supply, we performed polysome profiling. The *rpl20b* mutant has a lower amount of 60S ribosomal subunit as compared to wild type (Fig. 5D, Fig. S5C) as seen previously (Steffer et al., 2008). We note that the amount and size of the polysomes was lower in the *rpbl20b* mutant compared to wild type indicating reduced amounts of translation in general and each polysome peak was a doublet although the relevance of this is unclear (Fig. 5D). The short-lived *asf1* and *rtt109* mutants had less polysomes than the wild type, indicating that less translation in general is occurring. However, no significant change in the 60S to 40S ratio was observed with the short-lived *asf1* and *rtt109* mutants or the long-lived *hir*1 mutant, as compared to the wild type strain (Fig. 5D). Taken together, these data indicate that extra histone supply is not influencing lifespan via the same pathways of altered protein synthesis that are influenced by inhibition of Tor1 to extend lifespan.

To further investigate the relationship between lifespan extension by extra histone supply and lifespan extension due to deletion of *TOR1* or DR, we examined total histone protein levels during these manipulations. Surprisingly, we found that biochemically isolated old cells from *tor1* mutants or during DR have significantly less histone proteins than old wild type yeast cells (Fig. 5E,F, Fig. S5D). These data indicate that the pathway by which DR and *TOR1* deletion leads to extended lifespan is not a consequence of increased histone supply. These data also raise the possibility that deletion of *HIR1* does not further extend the lifespan of *tor1* mutants or during DR because *HIR1* deletion may not be sufficient to raise the histone levels significantly during these manipulations. Taken together, our analyses suggest that *HIR1* deletion leads to lifespan extension via a pathway that is not shared with other known mechanisms of lifespan extension.

## Ectopic expression of histones H3 and H4 profoundly increases replicative lifespan

To determine which of the core histones impart anti-aging properties, we individually integrated extra copies of the genes encoding H3/H4 or H2A/H2B under the control of the pGAL1/10 promoters. Overexpression of integrated genes encoding H3/H4, but not H2A/H2B extended the median lifespan of wild type cells by 30% (Fig. 6A, Fig. S6A). Higher levels of H3/H4 induction, via addition of more galactose to more strongly induce the promoters controlling H3/H4, resulted in lifespan extension of up to 50% (Fig. 6B). Noteworthy, this degree of yeast replicative lifespan extension is far greater than that usually attained by most other single manipulations in yeast. Expression of H3/H4 in these conditions leads to approximately a 50% increase in the level of total histone proteins (Fig.

S6B,C). Overexpression of histones H3/H4 in these conditions did not lead to significant differences in the polysome profile compared to wild type cells (data not shown) indicating that excess histones are not drastically influencing protein synthesis or ribosomal subunit composition. Also, no significant changes in the cell cycle resulted from overexpression of H3/H4 (Fig. S6D) Importantly, the expression of extra histone proteins does not lead to resistance to the oxidative damage that results from treatment with hydrogen peroxide, replication stress due to treatment with hydroxyurea or DNA damage due to treatment with the alkylating agent methylmethane sulfonate (Figs. S6E,F). As such, the extended lifespan that results from extra histone supply is not due to their protecting the genome from genomic instability.

## Discussion

In this study we show that aging is accompanied by a profound loss of histone proteins. This loss of histones is causal for aging because significant lifespan extension is achieved by ectopically increasing histone expression in wild type yeast, via a pathway that is distinct from other known lifespan extension pathways.

### Age dependent changes in chromatin structure

The drastic decrease in the level of histone proteins on chromatin during the normal aging process in yeast (Fig. 3B-D) is consistent with the loss of transcriptional silencing that occurs in wild type yeast during aging (Kim et al., 1996). More recently, an age-dependent increase in levels of histone H4 K16Ac at the X elements within subtelomeric regions of yeast chromosomes has been shown to occur due to a progressive loss of the Sir2 deacetylase protein during aging (Dang et al., 2009). This particular histone modification is unique in that it prevents higher order packaging between adjacent nucleosomes (Shogren-Knaak et al., 2006), implying that increased H4 K16ac during aging would lead to a more open chromatin structure in aged cells. Indeed, when reporter genes were inserted into the X elements within subtelomeric regions, the age dependent increase in H4 K16Ac levels led to increased expression of these reporter genes during aging (Dang et al., 2009). However the mechanistic relevance of opening up the chromatin structure of the X elements of subtelomeric regions on aging is not clear.

Many other previous studies have also revealed transcriptional dysregulation occurring during the aging process and it is clear that at least some of these transcriptional changes are due to age-dependent alterations in the chromatin structure. For example, DNA methylation, which is known to lead to a tighter packaging of the chromatin structure (Fuks, 2005), diminishes throughout the lifespan of mouse liver cells with consequent expression of previously repressed genes (Mays-Hoopes et al., 1983; Singhal et al., 1987). Age-related mRNA changes have also been documented extensively in yeast (Yiu et al., 2008). Similarly, microarray studies of global messenger RNAs (mRNAs) in aging animals and in mutants considered to affect the aging process have found many transcriptional changes (Zahn and Kim, 2007). Furthermore, the finding that stochastic differences in gene expression between individuals can influence lifespan in *C. elegans* points to a causal role for epigenetic changes during aging (Rea et al., 2005).

### How does extra histone supply extend lifespan?

Our model for how interventions that increase histone supply result in lifespan extension proposes that the resulting enlarged soluble pool of free histones facilitates histone exchange via stimulating the ongoing equilibrium of dynamic chromatin assembly and disassembly (Fig. 6C). The resulting higher rate of histone exchange would accelerate the rate of removal of post-translationally modified and damaged histone proteins from the DNA, and would

additionally enable sporadically occurring nucleosome free regions in the genome to be repackaged into chromatin. This in turn would reduce inappropriate access of proteins to the genome during aging, which would otherwise lead to the increased transcription and genomic instability – each of which are frequent characteristics of increased age and conditions that exhibit accelerated aging phenotypes.

If the chromatin is packaged into to a tighter structure by overexpressing histones, one would predict that the increased histone supply would restore the transcriptional silencing that is normally lost during aging (Kim et al., 1996). Indeed, *hir1* mutants, which have elevated histone levels, have enhanced transcriptional silencing as compared to wild type cells (Kaufman et al., 1998; Smith et al., 1999). By analogy, inactivation of the histone deacetylase Rpd3 also leads to elevated histone levels, extending lifespan in some yeast backgrounds and increasing transcriptional silencing (Bernstein et al., 2000; Kim et al., 1999; Rogina et al., 2002; Smith et al., 1999). Although the increased transcriptional silencing that occurs upon inactivation of Rpd3 and Hir1 provides a marker for altered chromatin structure, it is thought that the influence of Rpd3 on the aging process is most likely mediated through influencing the expression of euchromatin genes (Frankel and Rogina, 2005; Kim et al., 1999). Indeed, inactivation of Rpd3 and the concomitant increase in histone levels results in significant down-regulation of 40% of genes located within 20kb of the telomeres (Bernstein et al., 2000). This is reminiscent of the induction of 40% of genes located within 20kb of the telomeres that occurs upon depletion of histone H4 (Wyrick et al., 1999).

It is clear from the increased silencing in the *hir* mutants and *rpd3* mutants that supplying extra histone proteins results in a tighter chromatin structure, and we anticipate that this will be the case not only at the silent regions but also at other regions of the genome. We propose a model where the expression of euchromatic genes increases inappropriately during aging due to the deterioration of the chromatin structure with age and that the mis-expression of genes contributes to the aging process. By extension, we propose that our manipulations that result in increased histone supply with age result in a tighter chromatin structure, maintaining the regulated state of these genes, leading to lifespan extension (Fig. 6C). The next challenge is to identify the specific transcript(s) that are down regulated by the formation of a tighter chromatin structure that lead to lifespan extension.

## Relevance to lifespan extension in higher eukaryotes and via other pathways

The ultimate goal of this field is to achieve lifespan and healthspan extension in humans. As such, it is critically important to consider the potential relevance of our results in the yeast system to the situation in higher eukaryotes. Consistent with the reduction in histone proteins that we observed in aged yeast, we have found that levels of histone H3 protein decrease in mitotically active mouse tissues during aging (unpublished data), suggesting that histone protein loss may also occur in mammals during aging. This could be the cause of the elevated transcript levels that are characteristic during the aging of renewable tissues in mice (Warren et al., 2007). By examining the published transcriptome analyses, it is apparent that at least one regimen of lifespan extension in *C. elegans* is accompanied by increased histone expression (McColl et al., 2008). Furthermore, calorie restriction in mice is accompanied by increased histone expression (Barger et al., 2008). It will be important to determine whether the increased histone transcript levels during these lifespan regimens in higher eukaryotes are mirrored in a higher histone protein level. Indeed, we find increased histone transcript levels of 4 of the 6 cell cycle regulated histone genes in our long-lived *tor1* yeast mutants (Fig. S3A), while histone protein levels are decreased in the old *tor1* mutants and during CR (Fig. 5E & 5F), suggesting that they are trying to compensate for the depletion of histone proteins by transcribing more histones. Presumably the lower level of histone proteins in the

yeast *tor1* mutants and during CR (Fig. 5E & 5F) is a reflection of the greatly decreased degree of protein synthesis that occurs during nutritional stress and in the absence of Tor1. Our model leads us to ask why deletion of *TOR1* or CR extends lifespan if they have lower histone protein levels than normal in their old cells? Presumably the pathway by which deletion of *TOR1* and CR extends lifespan, which is largely unknown, can function without the need for extra histones to repress the key aging transcript(s) that are inappropriately generated during aging. This may be due to the fact that the reduced protein synthesis of these key aging transcripts during CR or Tor1 inactivation circumvents a requirement for their transcriptional repression in old cells for extended lifespan. Given the conservation of the mechanistic basis of aging, it will be necessary to identify these key aging transcripts to better understand and potentially ameliorate the age-related rise of human disease.

## Experimental Procedures

### Yeast Strains and Media

Yeast strains are described in Supplemental Table I. Replicative lifespan assays were performed on YPD with 2% glucose or YP with 1 % raffinose and 0.5%, 1 % or 2% galactose as indicated. Plasm ids expressing histones were maintained by growth in synthetic complete media lacking the appropriate amino acid and supplemented with raffinose and galactose for strains carrying galactose inducible promoter fusions.

### Lifespan Analysis

Replicative lifespan of virgin mothers cells was determined as described previously (Kennedy et al., 1994). The number of mother cells analyzed for each Figure is itemized in Suppl. Table 2. Yeast cells were kept overnight at 12°C to slow cell division. Yeast were initially grown on YP glycerol plates to eliminate yeast cells lacking mitochondria. For yeast with extended lifespan, the Wilcoxon Rank-Sum Test was used to determine statistical significance. The Wilcoxon.test function was used in the R software version 2.80 and p values less than the significance cut off of 0.05 are given in the figures and in Suppl. Table 2.

### Isolation of Young and Old Yeast Cells

Old yeast cells were isolated as described previously by using EZ Link Sulfo-NHL-LC-LC-Biotin (Pierce) to label cell surface proteins and MagnaBind Streptavidin beads (Thermo Scientific) (Lin et al., 2001). The old cells generated by this protocol had divided an average 8 times, as determined by calcafluor staining. The “very old” cells were isolated by two sequential rounds of this affinity purification, and had divided an average of 15 times, as determined by calcafluor staining.

For isolating older cells, we utilized the MEP as described in (Lindstrom and Gottschling, 2009). For the chromatin fractionations, strains UC5181 and DTY011 were grown for a total of 36 hours in the presence of estradiol, DT009 and DT010 for 16 hours. For ChIP experiments in galactose, UC5181 was grown for 72 hours total.

### Reverse Transcriptase Real-time PCR

RNA was analyzed by real-time RT-PCR analysis using a Roche Light Cycler and SYBR green detection. Primer sequences are given in the supplemental methods.

### Quantitative Western Blotting Analysis, ChIP and Chromatin Fractionations

Total protein extracts were isolated by boiling in Llaemli buffer prior to loading gels. Anti-rat and anti-rabbit secondary antibodies were used that fluoresced at 800nm and 700nm

respectively. Membranes were scanned with a LiCor Odyssey scanning system and their software was used to quantitate the bands, using the average density method. The methods for ChIP were published previously (Williams et al., 2008) and are detailed in the supplemental methods section. The chromatin fractionation was performed using a variation of the method published in (Frei and Gasser, 2000), and is detailed in the supplemental methods section.

### Gcn4 Reporter Assay and Polysome Profiling

The analysis of Gcn4 translation used a dual reporter assay as previously described (Steffen et al., 2008). Briefly, a reporter plasm id pVW31 containing the GCN4 promoter fused to firefly luciferase and Renilla luciferase under the control of a constitutive promoter was used. Cells were grown overnight in synthetic media lacking uracil to maintain the plasmid. Before lysing, cells were grown 2 hrs in YPD to minimize GCN4 expression due to nutrient stress. The Promega Dual-Luciferase Reporter Assay was used along with a Luminoskan Ascent to monitor expression. Firefly luciferase was normalized to Renilla luciferase activity. For polysome profiling, cells were grown in 75 mL of YPD to an OD of 0.8. Cultures were centrifuged and cooled with ice-cold YPD containing 100 μg/ml cycloheximide. After centrifuging again, cells were rinsed with ice-cold dd$H_2O$ containing 100 μg/ml cycloheximide and polysome profiles were measured as previously described (Steffen et al., 2008).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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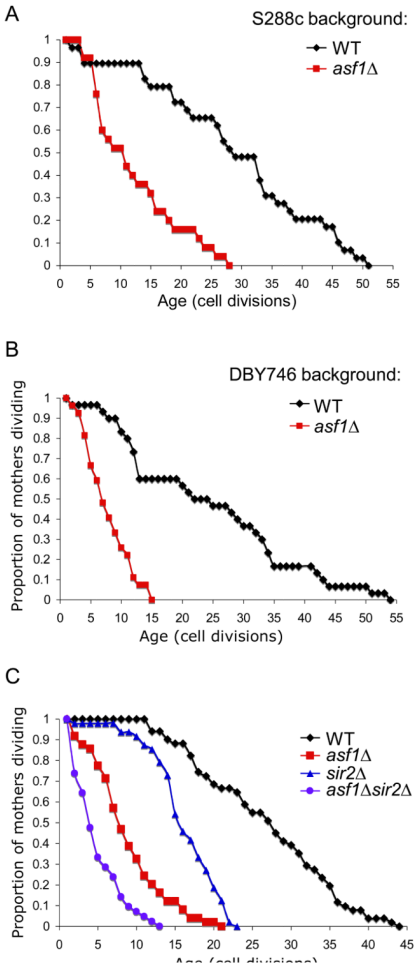


**Figure 1. Asf1 is required for normal lifespan**

**A.** Replicative lifespan of isogenic strains BY4741 (“WT”) or BY4747asf1 (“*asf1*”). **B.** Replicative lifespan of isogenic strains that were wild type (DBY746) or deleted for *ASF1* (JFY022) in the DBY746 background often used for aging studies. **C.** Replicative aging analysis of isogenic yeast strains BY4741 (“WT”), BY4747asf1 (“*asf1*”), BY4747sir2 (“*sir2*”), and BY4747*asf1sir2* (“*asf1sir2*”).

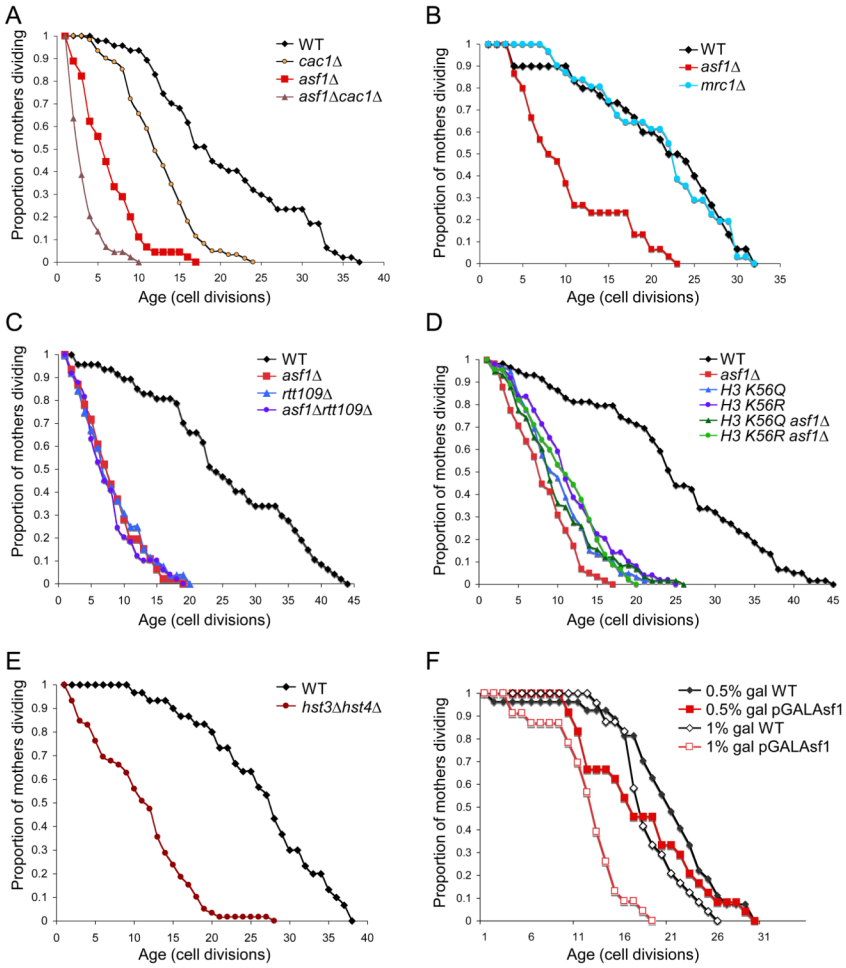


**Figure 2. Genetic identification of the pathway through which Asf1 regulates replicative lifespan**

**A.** Replicative aging analysis of wild type W303 yeast ("WT") or this strain deleted for *ASF1* ("*asf1*"), *CAC1* ("*cac1*") or *both ASF1 and CAC1* ("*asf1cac1*"). **B.** Isogenic strains that were wild type (BY4741) or deleted for *ASF1* or *MRC1* from the genome wide deletion collection in the S288c strain background were analyzed for replicative lifespan. **C.** Replicative aging analysis of yeast strains YB ("WT"), ZGY608 ("*asf1*"), ZGY906 ("*rtt109*"), and ZGY964 ("*asf1rtt109*"). **D.** Replicative aging analysis of yeast strains HMY152 ("WT"), JFY004 ("*asf1*"), HMY139 ("H3 K56Q"), HMY140 ("H3 K56R"), JFY005 ("H3 K56Q *asf1*"), and JFY006 ("H3 K56R *asf1*"). **E.** Replicative aging analysis of isogenic yeast strains BY4741 ("WT") and YNML7 ("*hst3hst4*"). **F.** Replicative aging analysis of isogenic yeast strains SHY0015 ("WT") and SHY0014 ("pGALAsf1"). 0.5% galactose leads to endogenous levels of Asf1, while 1% galactose leads to higher than endogenous levels of Asf1 (Zabaronick and Tyler, 2005).

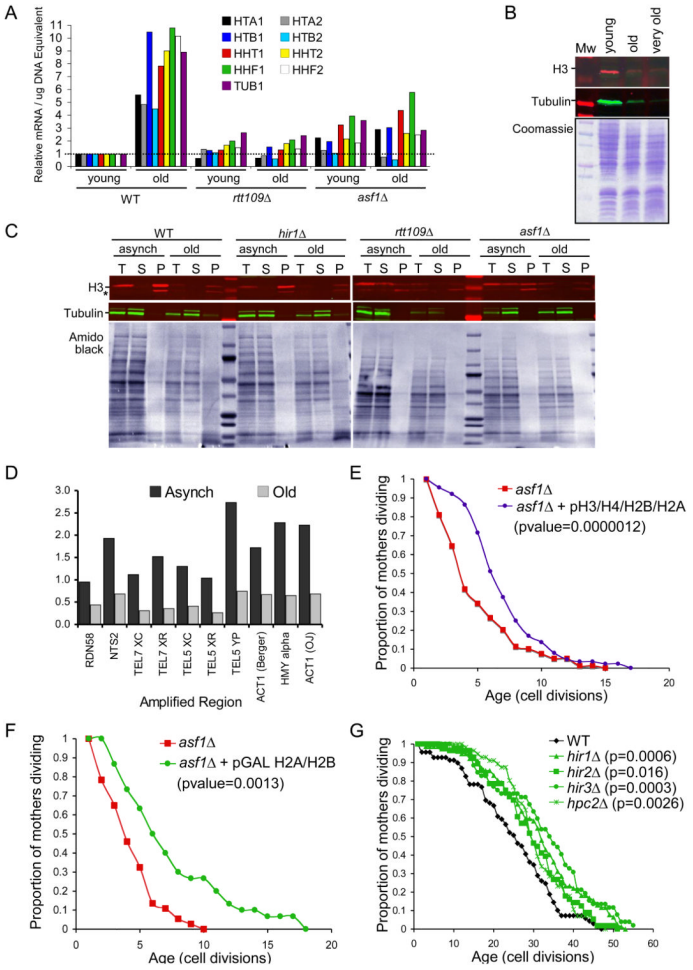


**Figure 3. Aging cells have reduced histone protein levels**

**A.** RNA levels for H3 *(HHT2 and HHT1)*, H4 *(HHF2* and *HHF1)*, H2A *(HTA1*, *HTA2)*, H2B *(HTB1*, *HTB2)* and tubulin (TUB) from strain BY4741 ("WT") or this strain deleted for *ASF1* ("*asf1*Δ"), or *RTT109* ("*rtt109*Δ"). RNA levels were normalized to total DNA content, and the RNA levels were normalized to 1 for young WT RNA (data prior to normalizing to 1 are given in Suppl. Fig. 3C). Representative results are shown. **B.** Histone H3 protein and tubulin levels in wild type strain BY4741 were measured by western analysis of equivalent amounts of total protein extracts from biochemically separated wild type yeast. Below is shown a Coomassie stained gel of the same amounts of the same total yeast protein extracts analyzed in the western analyses. The western analyses used infrared secondary antibodies and the images were taken in the linear range of detection. **C**. Total (T), soluble (S) and pellet (P) proteins were isolated from strain UCC5181 ("WT") or this strain deleted for *HIR1* ("*hir1*Δ"), *ASF1* ("*asf1* Δ"), or *RTT109* ("*rtt109*Δ"), that were growing asynchronously of mixed age or were aged via the "mother enrichment program". The same DNA equivalents of each fraction were analyzed for histone H3 protein levels by western blotting, where non-chromatin bound histones reside in the soluble fraction and chromatinized histones reside in the pellet fraction. "*" denotes a likely proteolytic cleavage product of H3 that arises upon over-handling of the protein. Tubulin was assayed to demonstrate the efficiency of the fractionation. Below are shown amido black staining of the membranes used for the western analyses. The western analyses used infrared secondary antibodies and the images were taken in the linear range of detection. **D.** Cells of mixed age

("Asynch") or following aging via the mother enrichment program ("aged") from strain UCC5181 were subject to ChIP analysis for histone H3 occupancy at the indicated DNA regions. Data shown are the average of two independent experiments, whose results are shown individually in Suppl. Fig. S6A and B. **E.** Replicative lifespan of strain JFY047 and JFY048 ("*asf1*") carrying the empty vector pRS426 or the 2 micron vector pFB1156 carrying the *HHF1/HHT1* and *HTB1/HTA1* genes. The pvalue indicates the significance of the lifespan extension. **F.** Replicative lifespan of strains JFY056 and JFY050 ("*asf1*") carrying the empty vector pRS315 or the vector pRO689 carrying the pGAL driven *HTB1/HTA1* genes. **G**. Replicative lifespan of isogenic strains with the indicated gene encoding subunits of the Hir complex deleted.

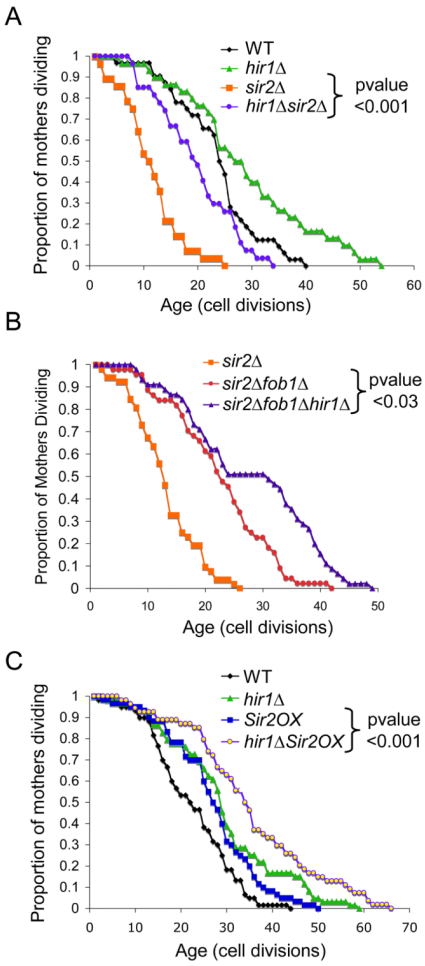


**Figure 4. Increased histone supply does not extend lifespan by the same pathways as Sir2 or ERCs**

**A.** Replicative lifespan of isogenic strains with the indicated genes deleted or containing an extra copy of Sir2 (Sir2OX). **B.** Replicative lifespan of isogenic strains with the indicated genes deleted. **C.** Replicative lifespan of isogenic strains with the indicated genes deleted and/or containing an extra copy of Sir2 (Sir2OX).

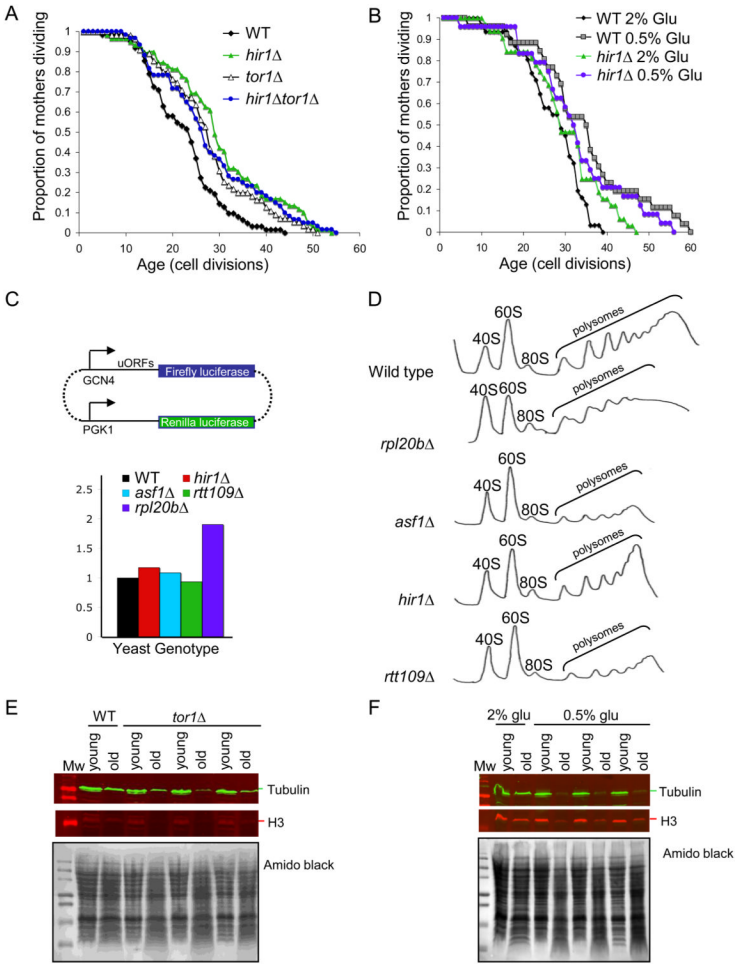


**Figure 5. Epistasis analysis with the Tor1 pathway or CR**

**A.** Replicative lifespan of isogenic strains with the indicated genes deleted. **B.** Replicative lifespan of isogenic strains with or without *HIR1* deleted, grown on either 0.5% glucose to induce CR or on 2% glucose. **C.** Schematic of the dual luciferase reporter plasmid used to measure Gcn4 translation levels. The ratio of the signal from the GCN4-firefly luciferase to renilla luciferase is shown for isogenic strains from the genome wide deletion collection in the S288c strain background. **D.** Polysome profiles of the indicated isogenic strains from the genome wide deletion collection in the S288c strain background. The position of the 60S, 40S, 80S ribosome and the polysomes are indicated. **E.** Histone H3 protein and tubulin levels were measured by western analysis of equivalent amounts of total protein extracts from biochemically separated young and old wild type and *tor1* mutant yeast. Below is shown the amido black stained membrane to show equivalent total protein loading. The western analyses used infrared secondary antibodies and the images were taken in the linear range of detection. **F.** As for E, but with wild type yeast grown in normal conditions (2% glucose) or calorie restricted conditions (0.5% glucose).

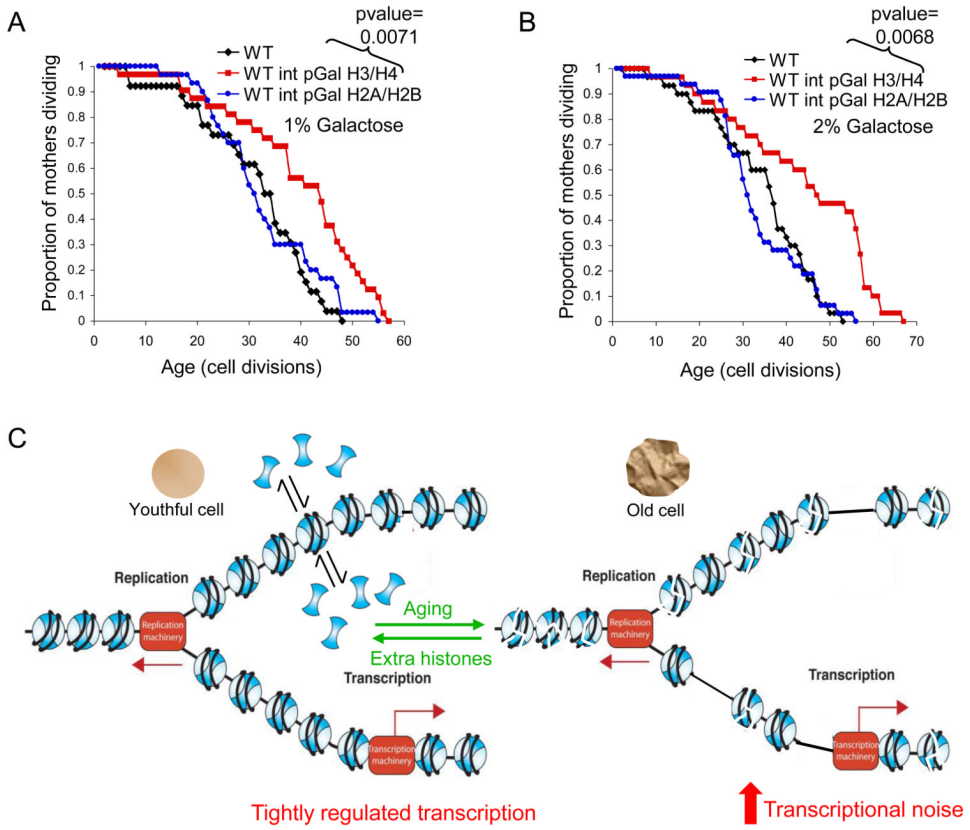


**Figure 6. Overexpression of histones H3/H4 extends lifespan**

**A.** Replicative lifespan of isogenic strains that are wild type (“WT”) or carrying an extra copy of either *HHT2/HHF2* or *HTB1/HTA1* driven from the galactose inducible pGAL1/10 divergent promoter integrated into the genome grown on 1% galactose and **B.** on 2% galactose. **C.** Model for lifespan extension by increasing histone supply.