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A Comprehensive Investigation Of Hp1B, A Chromatin Protein, And Its Role In Aging

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ABSTRACT

Genetic regulation determines which genes are expressed, in which tissues, at which time, and to which level. This regulation is necessary for proper organismal development, and loss of gene regulation can result in phenotypic consequences. As organisms grow older, gene regulation often begins to fail, resulting in negative phenotypic outcomes associated with aging. The exact reason for this failure of gene regulation remains unknown, but current models suggest that chromatin, and its associated proteins, plays a large role. The structure of chromatin impacts gene expression, directly affecting several phenotypes including aging. In this study, we investigate HP1B, a chromatin protein, and how it affects organismal phenotypes including aging by altering chromatin structure. Our research demonstrates that deletion of *HP1b* alters stress resistance, changes longevity, and impacts energy metabolism.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure Page

THE CONNECTION BETWEEN CHROMATIN, METABOLISM, AND AGING

A COMPREHENSIVE INVESTIGATION OF HP1B, A CHROMATIN PROTEIN, AND ITS ROLE IN AGING

THE CONNECTION BETWEEN CHROMATIN, METABOLISM, AND AGING

by
Benjamin B. Mills and Nicole C. Riddle

CHAPTER 1

THE CONNECTION BETWEEN CHROMATIN, METABOLISM, AND AGING

Abstract

The molecular events and pathways that lead to aging are the focus of much recent research. Aging is marked by a progressive deterioration of cellular function and results in many deleterious phenotypes, eventually leading to death. Two important factors implicated in contributing to the aging process are metabolism and chromatin structure. This review will examine the relationship between these two factors and aging, as well as their relationship with each other, to fully explore how these seemingly distinct processes are connected. This review, like several other recent, more comprehensive reviews reveals that investigations into multiple pathways is often necessary to fully understand what causes the physiological and molecular changes associated with aging.

Introduction

Aging is the natural decline in function that affects most organisms over time. It is often characterized by debilitating loss of cellular and bodily functions and can result in degenerative pathologies {3, 4}. In humans, these pathologies include relatively mild conditions, like loss of hair or hearing, but also potentially life-threatening conditions such as heart disease, cerebrovascular disease, and

cancer {1,2, 3, and 4}. For many health conditions, aging is the most important risk factor, and it is estimated that of the roughly 150,000 people who die each day across the globe, approximately two thirds die of age-related causes. In industrialized nations, this fraction rises to 90% {1}, illustrating the impact of degenerative aging processes on human health.

Aging also impacts society through its effect on healthcare cost. Treatment of age-associated health problems in an increasingly elderly population is a major driving force of healthcare costs {1}. For example, US citizens over the age of 65 annually spend nearly $6,000 more on medical expenses than younger individuals {2}. The issue of increased medical spending is projected to grow, because 20.9% of US citizens are expected to be over the age of 65 by the year 2050 compared to only 13% in 2010 {2}. Given these challenges, a better understanding of the causes of aging is urgently needed to mitigate the effects of the aging process, to improve quality of life for elderly people across the globe, and to curb increasing healthcare costs.

Due to this increasing elderly population in many countries, aging – and the prevention of age-associated diseases and disabilities - has become an important research focus. There has been an increase in research in many aging-related fields, including research into cancer, cell loss, mitochondrial mutations, and death-resistant cells {3}. Aging research promises to be highly beneficial because it might lead to early-intervention therapies, offsetting the economic and physiological burdens of aging {3}.

Aging research has uncovered a connection between chromatin structure and metabolism and their role in aging. While at first glance chromatin and metabolism appear to be quite distinct, several lines of evidence, such as dietary restriction, the TOR pathway, and chromatin proteins like Sir2, suggest links between them that may directly impact aging and age-associated phenotypes {22, 34, 38} . In this review, we will examine how both chromatin structure and metabolism affect aging and present a model integrating the chromatin and metabolism impacts on aging. Specifically, this review will highlight the importance of incorporating multiple factors and interacting pathways into models of aging using the example of chromatin and metabolism.

Programmed and Damaged Related Aging

Two distinct types of processes are thought to contribute to aging: programmed processes and damage-related processes {4, 7, 9, 11, and 15}. Programmed aging theories suggest that there is a biological timetable that regulates aging along with growth and development. In other words, organisms are somehow genetically programmed to age over time, leading to degeneration and eventually death {4}. This aging program is envisioned to be species-specific, explaining the differences in lifespan and aging processes between different groups of organisms. Supporting this theory is the fact that organisms of a given species have a species-specific lifespan; e.g. spider monkeys have an average lifespan of 25 years, elephants of 70 years, and humans of 80 years {5}. Programmed aging is thought to be due to gradual changes in macromolecules

that accumulate and eventually negatively impact biological processes, resulting in various age related phenotypes {5}. Thus, the programmed aging theory explains why there is less variation in natural lifespan within a species than between two different species.

Additional support for this theory of programmed aging comes from work in *S. cerevisiae*. Here, transient expression of a single transcription factor, NDT80, involved in the late stages of gametogenesis, can serve to rejuvenate aging mature cells {6}. Expression of this transcription factor is terminated in a developmentally controlled manner suggesting that, even though this factor may prevent aging, the inherent genetic programming of yeast development stops its expression and results in aging. Because NDT80 is only programmed to be produced during development, this finding demonstrates another aspect of programmed aging: organisms that mature and reproduce early typically have shorter lifespans than those with later sexual maturity {7}. Taken together, programmed aging theories can explain many of the characteristics of the aging process and are an important component of aging biology research.

In addition to programmed aging theories, research into damage-related aging theories also has contributed to our understanding of aging biology. The damage-related aging theories suggest that, as organisms live their lives, they experience damage to their DNA, their cellular proteins, and their organelles {4}. Chromatin, specifically, might be damaged during aging due to its constant remodeling in the cell cycle as well as during the course of growth and development {8, 9, and 10}. The age-related damage to chromatin and other

macromolecules can be due to a number of factors, which commonly include stressors such as UV light that can induce DNA damage, or reactive oxygen species (ROS) that can damage mitochondria {8,9, and 10}. Stressful wear and tear during an organism's lifetime and sub-optimally functioning repair or quality control mechanisms also are thought to contribute to age-related damage. The damage-related aging theories predict that organisms that experience less stress, or have an increased ability to mitigate the effects of stress, should have a longer lifespan than their stressed conspecifics. This prediction is supported by work from several model organisms. For example, Drosophila and *C. elegans* strains with increased heat stress resistance have an increased lifespan {113, 114}. Thus, the damage-related aging theories can be used to understand how stressors directly affect the aging process and provide insights into why organisms within a species can show vastly differing lifespans and/or aging phenotypes.

While both the programmed aging and damage-related aging theories successfully explain certain aspects of aging, the processes they describe are not mutually exclusive. Rather, it appears that the processes are functioning in parallel and jointly affect the aging process. This interaction between programmed and damage-related aging processes can be illustrated with chromatin. Chromatin is frequently remodeled during differentiation and development in a programmed fashion {4, 5}. Routine minor chromatin structure changes in the course of programmed development and aging can result in more easily damaged structures, further exacerbating the impacts of stressors such as

UV light exposure on DNA, a process which falls under the domain of damage-related aging {11}. In this example programmed aging changes—in the form of modifications of chromatin structure—are impacting the extent of effects of damage-related aging processes. Given the complex nature of the aging processes, it is generally accepted that an integrative approach, considering both damage-related and programmed aging processes is necessary.

Chromatin and Metabolism as Contributors to Aging

The complexity of aging is illustrated in a recent review by Kennedy and colleagues that discusses the “seven pillars of aging” – inflammation, adaptation to stress, proteostasis, stem cells and regeneration, macromolecular damage, metabolism, and epigenetics, which includes chromatin {12}. While these factors are all important, additional research, such as research examining the mechanisms governing life-extending effects of caloric restriction {33, 40}, has suggested a closer link between chromatin/epigenetics and metabolism which is the focus of this review.

The importance of chromatin structure in aging biology is likely due to its role in the maintenance of gene regulation and genome stability {13}. Changes in chromatin structure, for example via the alteration of post-translational modification of histones, impact which genes are accessed by RNA polymerases and how much transcript is made {13}. Chromatin changes happen throughout development and aging processes, and specific chromatin changes can be correlated with the development of age-associated disorders {14}. In general,

molecular hallmarks of aging include chromatin changes such as global heterochromatin loss {15, 16, 17, and 18} and the development of senescence associated heterochromatin foci that silence proliferation genes {19, 20}. These chromatin changes lead to global changes in gene expression. For example, in human skin cells, 1,672 genes are differentially regulated with increased age {21}. Interestingly, the specific genes differently regulated with age are not conserved between species. For instance, when comparing humans to chimpanzees or mice, it was found that there are many different “aging” genes, or genes differently regulated due to the aging process {14}. While these results do not support a model of conserved “aging” genes, they suggest that changes in chromatin structure that accompany increasing chronological age lead to gene expression changes which ultimately might contribute to age-associated degeneration.

In addition to its role in gene regulation, chromatin also contributes to aging through its role in the maintenance of genome stability. Chromatin structure can help protect the DNA from damage such as mutations and double-stranded breaks {5, 10, and 79}. In addition, chromatin structure has essential functions at centromeres and telomeres that ensure proper segregation of chromosomes during cell division, and it protects the genome against mobilization of transposable elements {5, 10, and 13}. The various protective functions of chromatin begin to decline as an organism ages. For example, older individuals typically exhibit reduced levels of heterochromatin and an increase in transposable element transposition due to a loss of repressive histone marks,

altered composition of nucleosomes, and an increase in nucleosome-free regions {5, 22, 23, 24, and 113}. These age-associated chromatin changes, for example the increased nucleosome-free regions, lead to genome instability because the affected genome regions are more susceptible to UV damage {23, 24}. The importance of genome stability can be observed also in individuals with progeria disorders such as Werner's syndrome (WS) or Hutchinson-Gilford progeria syndrome (HGPS). Progeria syndromes result in accelerated aging due to the disruption of nuclear organization, altered chromatin structure, decreased genome stability (including faster shortening telomeres), and increased DNA damage {29}. The cells of these rapidly aging individuals show characteristics very similar to normally aged cells, suggesting that genome stability provided by chromatin structure is a major factor in the aging process {5}. While the reasons for why organisms undergo chromatin changes as they age are yet to be fully understood, it is clear that these changes in chromatin structure contribute to aging phenotypes.

Chromatin proteins, the proteins required to regulate chromatin structure, are intimately involved in controlling the structural changes of chromatin observed during the aging process {26, 27, 28, and 29}. Chromatin proteins fall into several classes, including structural components of chromatin (histones, HP1, etc), chromatin modifying enzymes (histone modifiers, DNA methyltransferases, etc), and chromatin remodelers (SWI/SNF, etc). Several of these different chromatin protein classes have been linked to aging including Sir2 (a histone deacetylase), RPD3 (also a histone deacetylase), and HP1a (a

member of the Heterochromatin Protein 1 family) {5, 22, 26, 29, and 114}. These chromatin proteins work through a variety of different pathways to affect the aging process, and their effect on aging can be either positive or negative {5, 22, 26}. A comprehensive evaluation of chromatin proteins and their impacts on aging is necessary to fully understand how chromatin structure changes impact aging phenotypes.

A second important factor affecting aging is metabolism, or the chemical processes in a living organism required to maintain life by synthesizing substances and providing energy. Typically, a lower metabolic rate, either within the same species or across species, results in an increased lifespan {4, 30, and 31}. How a lower metabolic rate leads to increased lifespan is not entirely clear, but it is thought to be due to slower development or lower levels of ROS creation {4, 30, and 31}. Dietary restriction {32, 33} and nutrient signaling {34, 35} are factors that have a major impact on both metabolism and aging (Fig. 1). These factors illustrate the wide-ranging impact of metabolism throughout an organism and how metabolism can play a large part in the aging process.

Genes involved in metabolic processes are altered during the aging process resulting in a change in phenotype {4, 5, and 7}. In a recent study comparing gene expression in young vs. old human muscle before and after exercising, it was observed that four metabolic genes had greater expression in young muscle pre-workout: insulin-like growth factor-1 and its binding protein IGFBP5, ciliary neurotrophic factor, and the metallopeptidase MMP2 {36}. In young muscle these genes showed significant changes in expression, but this

change did not occur in older muscle {36}. This finding not only documents differences in gene expression levels between young and old individuals, but it also suggests a potential change in the muscle cells ability to remodel chromatin to change gene expression. There are a number of metabolic pathways that could explain this potential connection between metabolism and gene expression. For instance, a decrease in intake of methionine, the amino acid needed to make *S*-adenosyl-methionine (SAM), the main methyl donor in mammalian DNA methylation, can increase lifespan {37}. We will explore this connection further in the following sections.

Dietary Regimens Link Chromatin to Aging

A major focus of aging research has been on the impact of dietary restriction (DR) on the aging process {38, 39, 40, 41, and 47}. DR is defined as a reduction in food intake, either of total amount or of a particular nutrient, which does not result in malnutrition {38}. Therefore, DR can include caloric restriction, which is a reduction in total calories consumed. It also includes nutrient restriction, which is usually a reduction in a major macromolecule (proteins, carbohydrates, or fats) or specific nutrients like methionine (discussed below). DR has profound effects on the lifespans of many different organisms, including prolonging life in rodents {39}, *C. elegans* {40}, and *S. cerevisiae* {41}. The situation in primates is controversial, as one study has seen positive effects on lifespan and age-related diseases {42 and 43}, while a second independent study, only detected a reduction in age-related diseases, without a change in

median lifespan (maximum lifespan data have not been published yet due to an ongoing study at this time) {44}. Given the wide range of species showing increased lifespan in response to DR, understanding the molecular mechanisms mediating this response has been an important goal of aging biology research.

While it is broadly accepted that DR can positively impact lifespan {38, 39, 40, 41, 47}, the underlying causes of this effect are less understood and appear to be highly complex. Most DR regimes do not simply lower food intake, but often also affect the relative ratio of various nutrients in the diet. Carefully controlled studies in Drosophila models have demonstrated that simply lowering food intake in terms of calories is not sufficient to explain the results seen during DR regimens {45 and 46}. These studies suggest that the more likely cause of the lifespan-extending effect of DR is an altered nutrient balance.

Researchers have begun to test the hypothesis that DR extends lifespan by altering the nutrient balance in a variety of model organisms. For instance, in Drosophila, increased triglyceride accumulation and lifespan is favored by diets that reduce the protein to carbohydrate ratio, but more fecundity is seen with an increased protein to carbohydrate ratio in the diet {47}. Low protein diets increase rodent lifespan {39}, and low protein diets in humans result in decreased insulin-like growth factor 1 (IGF-1) production, which has been shown to protect against chronic age-associated pathologies in a small Ecuadorian population mutant for growth hormone receptor (GHR) and IGF-1 {48,49}. This finding suggests that altered nutrition, and the resulting altered metabolism, impacts the activity of several proteins like GHR and IGF-1. Because these transcription factors impact

gene expression by altering chromatin structure, these findings link metabolism, chromatin and the aging process.

Methionine Restriction Results in Slower Aging

Methionine has been identified as a major factor in DR's effect on lifespan {55, 57, and 59}. The methionine pathway is an evolutionary conserved pathway that produces S-adenosyl-methionine (SAM) from methionine utilizing the enzyme *S*-adenosyl-methionine synthase (Sams) (Fig. 2) {37}. SAM is the methyl donor required for the majority of methyltransferase activity in eukaryotes {37, 52, and 53}. By adding methyl groups, methyltransferases directly affect the structure and function of several important biomolecules including histones, chromatin proteins, and nucleic acids {51}. This connection between methionine and methyltransferase activity suggests that a change in the levels of methionine, for example due to DR, can result in changes in chromatin structure due to altered levels/positioning of DNA methylation and histone methylation.

One classic study that clearly demonstrated the impact of dietary methionine levels on DNA methylation levels—and organism-level phenotypes—involved the *viable yellow agouti* mouse {53}. Normally, the *agouti* gene produces yellow banding on the black hairs of the mouse in a developmentally controlled pattern, resulting in the brown agouti fur color seen in the wild-type mice {53}. In mutant mice carrying the *agouti viable yellow* allele, the *agouti* gene is dysregulated due to a cryptic promoter that drives ectopic *agouti* expression resulting in a yellow hair color {53}. The wild-type version of the *agouti* gene

displays a very predictable and consistent DNA methylation pattern—mostly absence of 5-methyl-cytosine—but the *agouti yellow* mutant has highly variable DNA methylation patterns due to the presence of a repetitive intra-cisternal A-type particle (IAP) element in this allele {53}. Because the DNA methylation at the *agouti yellow* allele is inversely correlated with the intensity of yellow coat color, the coat color can serve as a read-out for DNA methylation levels at the *agouti* IAP {52}. A shift in fur color from the yellow to brown color range can be induced in *agouti yellow* mice by reducing methionine in their diet {53}. This effect was due to a change in methylation levels at the IAP in the *agouti* locus {54}, illustrating how diet can induce a change in local DNA methylation levels, alter local chromatin structure, and ultimately impact an organism-level phenotype.

Chromatin structure changes resulting from methionine restriction have been shown to decrease the speed of aging {55, 56, 57, and 58}. Methionine restriction results in a comparable lifespan increase in several organisms including yeast, in *C. elegans*, Drosophila, and rodents. In *C. elgans,* lifespan increased by 36% under methionine restriction, while in rodents, lifespan is increased by 10-20% depending on the specific study protocols {55, 56, 57, and 58}. Additionally in *C. elegans*, methionine restriction was able to restore a metabolic phenotype more akin to younger organisms by altering gene expression through a change in methylation levels {59}. In 12-month-old mice, methionine restriction was able to reverse age-induced alterations in body weight, adiposity, physical activity, and glucose tolerance to the levels measured

in healthy 2-month-old control mice {59}. These phenotypic changes were accompanied by chromatin structure changes, resulting in decreased hepatic lipogenic gene expression, remodeling of lipid metabolism in white adipose tissue, and increased insulin-induced phosphorylation of the insulin receptor (IR) and Akt in peripheral tissues {59}. Additionally, methionine restriction in these older mice increased circulating levels of FGF21, phosphorylation of eIF2a, and expression of ATF4 {60, 61}. Together, these findings suggest that methionine levels have a strong influence on the aging process by modifying metabolism and changing chromatin structure.

Acetyl-CoA Availability Affects Lifespan

Another metabolite implicated as a causative agent in DR's lifespan-extending effects is acetyl-CoA. Acetyl-CoA is a central metabolite best known for its role in cellular energy generation in the Krebs cycle {62}. It also serves as a donor of acetyl groups in many other biological processes, such as for enzymes generating post-translational modification of proteins {62}. One major group of proteins that undergoes post-translational acetylation is histone proteins. Presence or absence of acetyl-groups on histone tails has profound impacts on chromatin structure, with hyper-acetylation leading to open, transcription-permissible chromatin states, and hypo-acetylation leading to closed, transcription-impermissible chromatin states {62}. Thus, acetyl-CoA provides yet another link between metabolism, chromatin, and aging.

In *S. cerevisiae*, the effects of acetyl-CoA availability has been examined for a) impact on chromatin structure and b) on lifespan {63}. High levels of acetate, obtained via diet, lead to increased production of acetyl-CoA via the enzyme Acs2 {63}. High levels of acetyl-CoA then lead to acetyl-CoA-dependent hyperacetylation of histones H2A, H2B, and H3, which result in reduced lifespan {63}. It has been proposed that this reduced lifespan is due to restriction of autophagy {63}, the breakdown of damaged organelles to recover recyclable macromolecules, because when the mitochondrial acetyl-CoA transferase Ach1 is removed, autophagy is increased due to resulting histone deacetylation {63}. Increased lifespan in response to an increase in autophagy has been observed for many organisms including worms, flies and mammals {64}. Therefore, it is suggested that autophagy regulates aging in three possible ways: via the limitation of nutrient flux by enhancing provisions of substrates for metabolism, via the removal of harmful organelles, or via the clearance of potentially toxic proteins {64}. Together, these findings suggest a large role for acetyl-CoA levels in control of chromatin structure and specifically levels of autophagy that directly impact lifespan and aging.

Nutrient Sensing through the TOR Pathway Impacts Lifespan

Another factor involved in DR that may link chromatin structure and metabolism to aging is the nutrient/amino acid sensing mechanistic target of rapamycin (TOR) pathway (Fig. 3) {34}. A reduction in amino acid intake is sufficient to increase lifespan in yeast {66}, *Drosophila {67}*, and rodents {68},

and it has been suggested that the nutrient signaling and gene expression altering ability of TOR may explain why {34, 35, 66, 67, and 68}. TOR is a conserved serine/threonine protein kinase with at least one orthologue in all eukaryotic genomes examined {69}. TOR regulates protein synthesis and growth in response to nutrient intake in part by inducing ribosomal protein gene expression {34, 69}. Reducing levels of TOR signaling either pharmacologically (using methionine sulfoximine or rapamycin) or through RNAi increases lifespan in *S. cerevisiae* by nearly 50% {66}, *C. elegans* by 40 % {70}, and *Drosophila* by nearly 40% {71}. These results connect the TOR pathway, which responds to nutrient levels, directly to lifespan and aging.

It has been proposed that TOR's effect on lifespan is a result of decreased proteins synthesis {69}. A reduction in protein synthesis can be caused by limited nutrient availability as seen under DR conditions {38}. Reducing protein synthesis might reduce energy requirements allowing for a reduction in respiration and decreased ROS production. A reduction in ROS could lead to decreased DNA damage, explaining the observed increases in lifespan due to less damage-related aging processes {6, 11, 64, and 72}. Alternatively, TOR's effect on lifespan might be due to the gene expression and chromatin structure changes precipitated through the TOR signaling pathway {69, 70, and 71}. Signaling occurs via two distinct multiprotein complexes, TOR complex 1 and 2, which promote, for example, the transcription of genes involved in mitochondrial function {72}. In high nutrient conditions, the TOR signaling pathway would thus promote the generation of the energy and amino acids needed for protein

synthesis, while in low nutrient conditions the opposite would occur {72}. According to this second theory, TOR affects lifespan by linking the availability of metabolites, in this case amino acids, to changes in gene expression that result in an effect on lifespan {72}. The exact mechanism by which nutrient signaling mediated by TOR alters lifespan is still being investigated, but TOR appears to be an important factor in the connection between DR and aging.

Sir2 Partially Mediates the Lifespan Effect of Dietary Restriction

While in the previous sections, we have examined metabolism-focused studies that support a chromatin-metabolism link in aging, we will now review the evidence for such a link from chromatin-centered studies. One of the best known chromatin associated proteins that links chromatin, metabolism, and aging is the NAD+-dependent histone deacetylase (HDAC), Sir2 {32, 73, 74, 80, and 81}. Sir2's histone deacetylase activity was discovered because Sir2 repressed formation of extrachromosomal rDNA circles (ERCs) {73}. This repression requires the removal of acetyl groups from certain lysine near the terminal end of histone H3 and H4 tails {73}. Sir2 was also identified because yeast has been shown to have increased global deacetylation in Sir2 overexpressed lines {74}. Sir2's repression of ERCs also originally linked it to aging because ERCs were once thought to contribute to aging - mutations in the sgs1 gene, the yeast Werner's syndrome gene, caused rapid ERC accumulation {16}. While the role of ERCs in aging has since been refuted {75}, Sir2's role in aging has been confirmed by other studies {80, 81, 82 86, 87, and 88}.

Since linking Sir2 to ERCs, the sirtuin family of proteins, for which Sir2 was the founding member, has been linked to a number of different gene silencing mechanisms {76, 77, 78}. In S*. cerevisiae*, Sir2 functions in silencing at repeated sequences, the mating type loci {76}, telomeres, and ribosomal DNA (rDNA) {77}. While Sir2, Sir3, and Sir4 are all required for silencing at mating type loci and telomeres, only Sir2 is necessary for rDNA silencing {76, 77, and 78}. Silencing caused by these and other chromatin proteins directly results in a more closed and inaccessible local chromatin structure {76, 77, and 78}. Additionally, further linking Sir2 to genome stability, it is proposed that Sir2 is also involved in DNA repair through non-homologous end joining {79}. These examples illustrate the diverse roles Sir2 proteins play in gene regulation.

Sir2 affects aging via several different pathways. In the budding yeast *S. cerevisiae*, Sir2 promotes replicative longevity, or the amount of daughter cells produced by a mother cell before senescence {80}. Interestingly, it does not promote chronological aging, which measures the time yeast cells can survive in stationary phase without dividing {81}. Removal of Sir2 was originally observed to decrease replicative lifespan in budding yeast {80} and maximum lifespan *C. elegans* {82}. Overexpression of the Sir2 protein caused lifespan increases in both organisms {80, 82}. In budding yeast, Sir2 is controlled by PNC1, a gene the activity of which is sensitive to both stress and caloric intake {83}. PNC1 catalyzes the rate-limiting first step in NAD+ synthesis {83}, therefore regulating Sir2 activity, because Sir2 needs NAD+ to carry out its HDAC function {32}. This finding suggests a nutrient/metabolic dependent role of Sir2 linked to increased

replicative longevity. Interestingly, Sir2 appears to have the opposite effect on chronological lifespan than it does on replicative lifespan - deletion of Sir2 in *S. cerevisiae* results in an up to a 60% increase in chronological lifespan {84}. This change in aging is attributed to Sir2's effect on stress resistance as it was discovered that deletion of Sir2 conferred both increased oxidative stress and heat shock resistance {84}. While the results from replicative and chronological lifespan measures in budding yeast are conflicting, they clearly link Sir2 to the aging process and, due to the relationship with PNC1, suggest a link to metabolism.

The link between chromatin, metabolism, and aging is further supported by the finding that Sir2 affects the aging process through the same pathways as DR {83, 85, and 87}. DR does not increase lifespan in yeast strains lacking Sir2, demonstrating that Sir2 is necessary for lifespan extension via DR {85}. Follow-up studies demonstrated that caloric restriction causes an increase in Sir2 silencing activity *in vivo* via chromatin immunoprecipitation using an anti-acetylated histone antibody {85}. This increase in activity potentially happens because, somewhat counterintuitively, DR may cause an increase in respiration in yeast (respiration is a more efficient process than fermentation) {85}. Increased levels of respiration result in more oxidation of NADH, which leads to higher levels of NAD+, and results in more Sir2 activity {85}. This model is further supported by the finding that an activator of Sir2, resveratrol, extended yeast replicative lifespan {86}. In fact, an experiment with 32 different longer-lived yeast strains showed that deletion of Sir2 removed all replicative lifespan phenotypes

{87}. Sir2's essential role in mediating the effects of DR on lifespan demonstrates a clear connection between metabolism, chromatin structure, and aging.

The exact mechanism of how Sir2 prolongs lifespan due to caloric restriction remains unknown. One popular model suggests that the unusual NAD-dependent HDAC activity of Sir2 allows it to sense the metabolic rate of cells due to its NAD+ dependence and modify lifespan accordingly {88}. This hypothesis implies that the rate limiting step in NAD+ synthesis mediated by PNC1 is upstream of the silencing actions of Sir2 {88}. A relationship like this would effectively allow nutrient availability to control the amount of silent chromatin. This theory is strengthened by the finding that Sir2-related genes regulate the formation of specialized survival forms such as spores in yeast {89} and dauer larvae in *C. elegans* {82}. These specialized survival forms are entered into when food is scarce, are marked by chromosomal inactivation, and are typically preceded by an increase in autophagy to recycle macromolecules for usable components {90}. An example of Sir2's impact on survival states can be seen in *C. elegans,* where overexpression of Sir2 promotes longevity and predisposes animals to a dauer state {82}. This predisposition is most likely caused by increased genome silencing, similar to DR conditions, due to increased Sir2 levels {82}. Together, these examples illustrate how the chromatin silencing activity of Sir2 is linked to food intake and possibly the lifespan increasing effects of autophagy.

In budding yeast, there are two separate, known pathways that implicate Sir2 in a caloric restriction response. The first pathway suggests that caloric

restriction causes yeast to shift away from fermentation and instead increase oxygen consumption and respiration {85}. When food is abundant, *S. cerevisiae* performs fermentation to generate ethanol in order to store excess energy {85}. The increase in respiration leads to reduction in NADH {91}, an electron donor in respiration and a competitive inhibitor of Sir2. Reduction of a competitive inhibitor would increase Sir2 activity resulting in increased chromatin silencing {91}. This model is consistent with observations from mammals, where calorically restricted animals shift from using glucose in muscle cells to making lactate and utilizing fatty acids {22}. A second theory suggests that caloric restriction reduces nicotinic acid NA, another competitive inhibitor of Sir2, thus leading to increased Sir2 activity {92}. Specifically, CR upregulates the PNC1 that synthesizes NAD from NA and ADP-ribose {92}. Supporting this idea, deletion of PNC1 diminished the ability of caloric restriction to extend lifespan {83}. Most likely, both pathways contribute to Sir2's effect on caloric restriction.

Sir2 is thought to function in similar ways in multiple organisms in mediating the lifespan effects of caloric restriction. For example, in Drosophila lifespan can be extended by lowering the yeast and glucose levels of standard fly food (typically considered to be food restriction) {93}. This food reduction increases Sir2 mRNA levels, suggesting an increase in Sir2 activity {94}, which might attribute to the lifespan extension seen under these conditions. Further supporting the idea that Sir2 may be responsible for this change in longevity, addition of resveratrol, an activator of Sir2, also increases lifespan in Drosophila (but not under DR conditions) {95}. Additionally, caloric restriction does not affect

Sir2 mutant flies {96}, and overexpression of Sir2 has been shown to increase lifespan {22}. Together, these findings suggest that the chromatin protein Sir2 is an important mediator of caloric restriction's impact on lifespan, and suggest a conserved role in metazoans.

It is suggested that Sirt1, the mammalian Sir2 ortholog, might also have effects on longevity. Sirt1 has the ability to regulate glucose and fat metabolism {84}. Unfortunately, mice that are mutant for Sirt1 die very early in development, and the few animals that reach adulthood appear mostly normal as opposed to showing signs of advanced aging, possibly due to compensatory mechanisms {84}. Murine fibroblast cells lacking Sirt1 are more resistant to senescence caused by oxidative stress {97}, and the animals mutant for Sirt1 that have been screened appear to have phenotypes consistent with longer lived animals, such as decreased body weight and fat mass, smaller pituitary size, and lower levels of free IGF-1 {98}. While many questions about the function of Sir2 and its orthologs remain, it is clear that this family of proteins has an impact on the aging process. This family of proteins demonstrates a strong connection between chromatin modification, nutrient availability, metabolism, and longevity.

RPD3, an HDAC Linked to the Lifespan Extending Effects of Dietary Restriction

Like Sir2, RPD3 is an HDAC that impacts lifespan in a number of organisms {94, 100, 101, and 102}. RPD3 is a class 1 HDAC that has specificity for lysine residues 5 and 12 of histone H4 {99}. RPD3 is involved in many cellular pathways including cell proliferation, development, and metabolism {94, 100,

101, 102, and 103}. Similar to deletion of Sir2, deletion of RPD3 in yeast results in histone hyperacetylation {100}, but at different target sites. Interestingly, a shared molecular function as HDACs is where the similarity between Sir2 and RPD3 ends. While deletion of Sir2 reduces silencing at mating loci, telomeres, and rDNA, deletion of RPD3 increases silencing at all three of these loci {23, 99, 101}. While, both Sir2 and RPD3 affect replicative timing in yeast, the effect is in the opposite direction: while presence of RPD3 delays initiation at late origins, Sir2 is required for the timely activation of early origins {102}. Similar to Sir2, RPD3 is an HDAC that changes chromatin structure and alters lifespan, but the actions of RPD3 appear to be generally antagonistic of Sir2.

In terms of their impact on lifespan, RPD3 and Sir2 also show opposite effects, despite their shared molecular function as HDACs. Removal of RPD3 in yeast significantly increases replicative lifespan by 41% {23}; the complete opposite of what is observed with Sir2 {80, 87}. RPD3 partial loss of function mutants in Drosophila also show increased lifespan, with the increase being 41% above the wildtype level {94}. This effect occurs in both male and female flies, but the impact of RPD3 levels on lifespan appears to be dosage sensitive, as full removal of RPD3 is lethal {94}. The molecular pathways to increased lifespan impacted by decreased RPD3 levels are distinct from those impacted by Sir2, because increases in lifespan due to DR are not dependent on RPD3 {103}. In fact, the lifespan impacts of RPD3 and DR are partially additive, suggesting distinct but interacting pathways {103}. However, RPD3 does play part in the caloric restriction pathway in flies: levels of RDP3 are decreased under calorically

restricted conditions {94, 104}. The relationship between decreased calories and resulting decreased levels of RPD3 protein has also been observed in yeast {100}. These examples demonstrate that RPD3 could potentially have a role in DR and aging.

The genome-wide pattern of acetylation left by RPD3 and Sir2 helps to regulate and govern the expression at loci that appear to be associated with growth and longevity {102}. Sir2 and RPD3 operate in two independent pathways that converge under DR conditions creating levels of silencing based on nutrient availability {102}. This relationship causes increased or decreased silencing depending on nutrient availability, connecting metabolism to chromatin structure. The effects of these two pathways ultimately results in differential gene expression based on nutrient availably, causing in a potential effect on lifespan.

Interactions between RPD3 and the TOR pathway have also been documented {103}. When RPD3 is heterozygous, male Drosophila have mean longevity extension of around 40%, but when male Drosophila are heterozygous for TOR they have a 19% decrease in mean lifespans at normal food concentrations {103}. Interestingly, in Drosophila under nutrient limiting conditions, the mRNA levels of 4E-BP, coding for TOR, is decreased {103}. Researchers have also tested how the results interacted with each other. Flies mutant for both *TOR* and RPD3 have slight mean lifespan decreasing effects, 4-14% {103}. These findings imply that multiple discrete, but interacting pathways take place within an organism to affect longevity {103}. This result, as well as other results detailed above, suggests that RPD3 is an important protein in the

aging process, potentially due to its impact on chromatin structure. More research into this protein should result in much better understanding of DR and how chromatin proteins affect the aging process.

HP1a Might Affect Aging through a Metabolic Pathway

While Sir2 and RPD3 are well known chromatin proteins that affect aging through metabolic pathways, another chromatin protein that has been linked to aging is Heterochromatin Protein 1a (HP1a). The Heterochromatin Protein 1 (HP1) family is highly conserved across eukaryotes, performing several roles including gene repression via heterochromatin formation, maintenance of heterochromatin integrity, and transcriptional activation {105, 106}. As demonstrated in previous sections, maintenance of genome stability is an important part of preventing aging, and HP1a is essential for genome stability {105}. Due to its function in heterochromatin formation, HP1a also carries out gene silencing functions that reduce gene expression {105, 106}.

The impact of chromatin structure and integrity on aging can be observed by looking at an aged organism within a number of different species. For example, in older Drosophila, differential enrichment of HP1a between euchromatin and heterochromatin is reduced, leaving levels nearly even throughout the genome {105, 106}. Also seen in older Drosophila more transposable elements (TEs) become active, potentially due to decreased loss of heterochromatin {113}. This increases in TEs can further disrupt chromatin structure furthering the aging process. Additionally, genome instability in older

individuals can be seen in many organisms including yeast, *C. elegans*, and humans {28, 107, and 108}. For example, patients with the progeria Werner's syndrome have decreased levels of overall chromatin integrity, decreased levels of nuclear proteins including HP1a, and are marked by both shortening telomeres and increased DNA damage {25}. In *Drosophila melanogaster,* upregulation of HP1a has been shown to increase median lifespan by 23% and maximum lifespan by 12% due to an increase in heterochromatin formation that can preserve genome stability {107}, and loss of function mutants have been shown to have decreased lifespans {107}. These findings suggest a model of age-associated global heterochromatin loss: as organisms age, heterochromatin levels decrease, allowing for an increase in genome damage that manifests as deleterious age-associated phenotypes.

However, heterochromatin levels are not decreased everywhere in the genome with age, suggesting that a model of global heterochromatin loss with aging is over-simplified {19}. For example, in senescent cells, there are often localized increases in facultative heterochromatin called Senescence-Associated Heterochromatin Foci (SAHF) {19}. These SAHFs were identified as DAPI-bright puncta in immunofluorescence studies of tissue culture cells {109}. SAHF occur at specific sites in the genome, often associated with proliferation-promoting genes {110}. SAHF are a hallmark of the aging process and can lead to irreversible cell cycle arrest {19}. While SAHF are often studied in senescent cell cultures, they occur *in vivo* as well, for example, in skin cells of older organisms including primates and humans {111}. Thus, depending on the proliferative status

of the cell, there are at least two types of events occurring with age with regard to heterochromatin: in senescent cells, SAHF sequester proliferation genes, while in other cells heterochromatin marks decrease globally across the genome.

Some of the effect of HP1a on aging may be linked to DR mechanisms {113}. As mentioned previously, Sir2 has the ability to increase silencing of rDNA in response to low nutrient availability in yeast {112}. In other organisms, regions of heterochromatin created by HP1 proteins also have this ability {105, 106}. Nutrient sensing through the TOR pathway might be affected by the silencing ability of HP1a. Flies heterozygous for an HP1a mutation (decreased ability to create heterochromatin) are larger in size {107} potentially because silencing of the TOR pathway is less effective. When the TOR pathway is not properly repressed, it leads to the upregulation of many genes resulting in increased proteins production and increased growth rate {69}. Furthermore, overexpression of HP1a, resulting in more silencing, causes flies to be smaller {107}. Here, increased TOR silencing might lead to decreased gene expression, potentially resulting in decreased protein production and reduced growth. These results suggest that more heterochromatin may lead to slower growth because of decreased protein synthesis {15}. More research is required to determine if the lifespan and body size altering effects of HP1a are linked to TOR signaling. HP1a provides a good example of a protein that has a role in chromatin structuring and an effect on aging that may be linked to metabolism when investigated further.

Conclusion

This review has provided detailed analyses of a few mechanisms linking chromatin structure and metabolism to the aging process. These mechanisms connect seemingly district phenotypes resulting in a combined effect on aging. This inter-connectedness is illustrated well by DR where nutrient intake has a direct effect on chromatin modifying proteins that in turn modify chromatin structure affecting organismal levels phenotypes. Proteins that have been identified in this interaction include Sir2, RPD3, and potentially HP1a, but likely additional unidentified proteins contribute as well. Nutrients such as methionine and acetyl-CoA have been linked to this pathway due to their regulation of chromatin proteins suggesting a direct effect of metabolism and diet. Finally, nutrient signaling through the TOR pathway has been linked to the aging process by its regulation of chromatin proteins. To date many of the mechanism linking metabolism and chromatin structure to aging remain undiscovered but provided an exciting challenge for future research.

The identified connection between chromatin structure, metabolism, and aging is clearly an important concept in the field of aging research. Much remains to be discovered, and the continued research effort into this field of study will develop a more integrated understanding of the aging process and possibly lead the way to new therapeutic opportunities. Hopefully future research will be able to combine these areas or research to allow a complete and detailed understanding of how chromatin structure and metabolism affect the aging process.

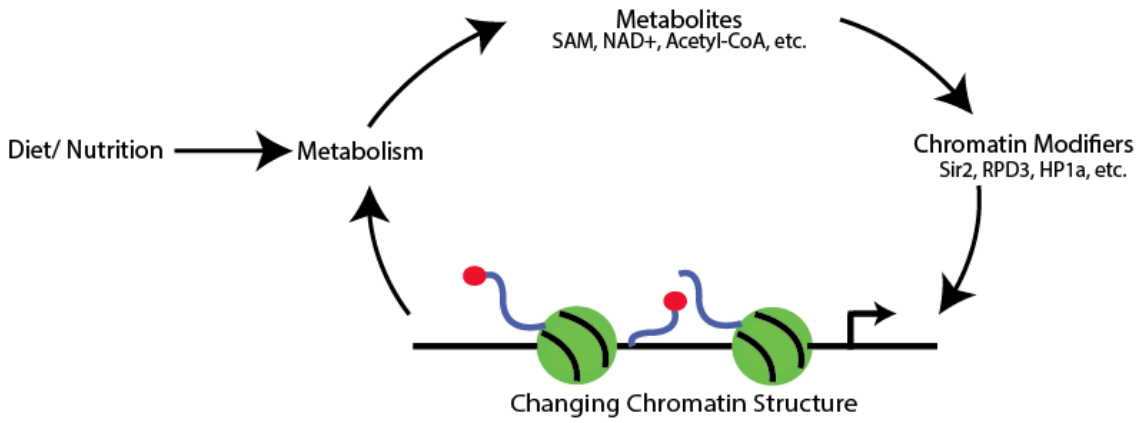

Figure 1. Chromatin modifiers link diet and transcription changes. The availability of intermediary metabolites is dictated by diet. Dietary restriction conditions result in altered metabolite levels, directly affecting chromatin modifier activity, resulting in a change in chromatin structure and transcription. Effects on chromatin modifiers by metabolites are dose dependent.

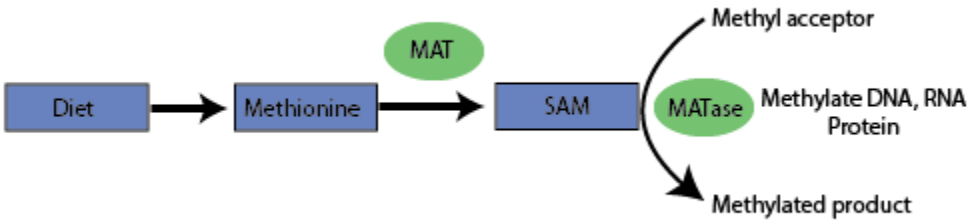

Figure 2. Diet directly affects levels of SAM production and methylation. Methionine required for methylation via SAM comes from diet in a dose-dependent manner. Limited or restricted methionine leads to decreased SAM production and therefore decreased methylation of DNA, RNA, and proteins, potentially altering phenotypes.

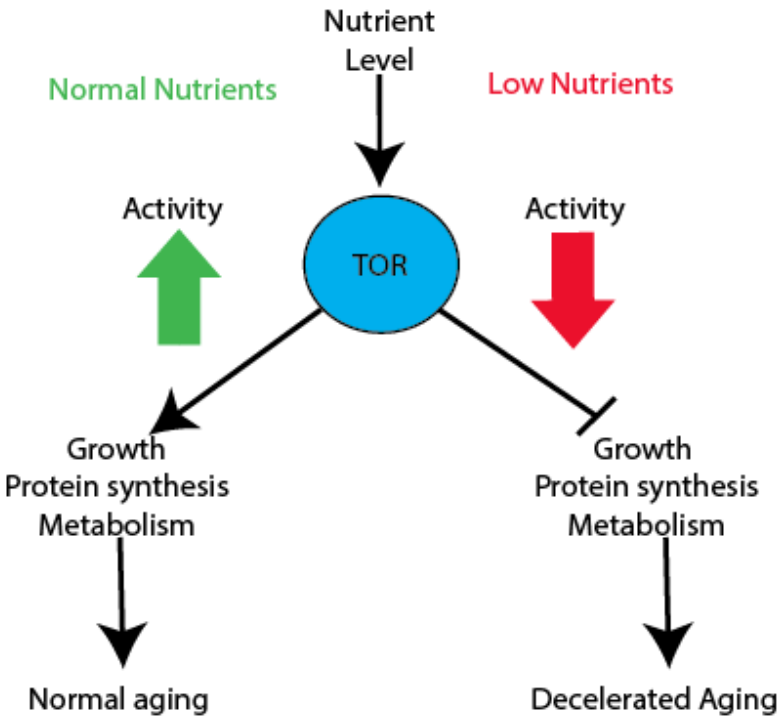

Figure 3. Nutrient sensing capabilities of the TOR pathway helps regulate growth and protein production. Nutrient and growth factor signaling regulates TOR dependent downstream signaling. Decreased TOR signaling affects chromatin structure leading to a decrease in rRNA production, decreasing overall growth and potentially slowing the aging process.

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A COMPREHENSIVE INVESTIGATION OF HP1B, A CHROMATIN PROTEIN, AND ITS ROLE IN AGING

by
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CHAPTER 2

A COMPREHENSIVE INVESTIGATION OF HP1B, A CHROMATIN PROTEIN, AND ITS ROLE IN AGING

Gene regulation plays a vital role in the overall health and development of an organism by ensuring that genes are activated or silenced at the proper time as well as in the proper tissues. Chromatin, the composite structure of DNA and its associated proteins, contributes to gene regulation, undergoing a variety of structural modifications to ensure proper gene expression {1}. This regulation of chromatin composition is essential to maintaining the balance of transcriptionally active and silent genes necessary for development {1, 2}. Euchromatin, the decondensed form of chromatin, consists of coding and regulatory segments of the genome that are accessible for transcription {3, 4}. In contrast, heterochromatin is highly compacted and less accessible to the transcriptional machinery {3, 4}. Histone and DNA modifications such as methylation, phosphorylation, and acetylation play a key role in the regulation of these basic chromatin types {5, 6, 7, and 8}. Misregulation of histone-modifying enzymes can lead to chromosomal instability and the disruption of important biological processes {5, 6, 7 and 8}.

Chromosomal proteins, also called chromatin proteins, play an important role in the formation of different chromatin types or chromatin states, directly altering gene expression, thereby affecting pathways such as aging and

metabolism {9, 10, 11, and 12}. Members of the HP1 (Heterochromatin Protein 1) family are non-histone chromosomal proteins that are highly conserved across eukaryotic organisms {13, 14}. HP1 family proteins have a chromo domain (CD), a chromo-shadow domain (CSD), and a variable hinge domain separating the two. The CSD facilitates HP1 dimerization, allowing for formation of a new surface that facilitates interaction with a variety of other proteins {13, 14}. The CD is responsible for recognizing di- and trimethylated H3K9 (histone 3 lysine 9) residues typically found in heterochromatic regions of the genome {13, 14}. The hinge domain found between the CSD and CD is the least conserved region, but commonly contains the nuclear localization sequence {13, 14}. Through the CD's ability to bind directly to methylated histones, as well as the CSD's ability to factilitate binding to other chromatin modifying proteins, HP1 proteins serve as "hubs" for other proteins including other chromatin modifying proteins to interact at histone marks throughout the genome {15}. In turn, this hub protein function allows HP1 proteins to be involved in diverse roles such as gene regulation, chromatin integrity maintenance, DNA replication, and DNA repair {15}.

HP1 proteins are found in most studied eukaryotic genomes as small gene families {13, 14}. HP1a, HP1B, and HP1C are the three somatically expressed HP1 homologs in Drosophila {13, 14, and 15}. HP1B is found at both euchromatic and heterochromatic regions of the genome {16}. Studies using RNAi knockdown suggest that loss of HP1B leads to decreased euchromatic and heterochromatic gene expression, while overexpression leads to specific decondensation of centromeric heterochromatin and is accompanied by disassociation of HP1a and

loss of H3K9 methylation {17}. However, to date, no detailed characterization of an HP1B null mutant has been published.

Here, we provide a detailed characterization of an *HP1b* null mutant, focusing on organism-level phenotypes, including longevity, stress resistance, and metabolism. We utilize two *HP1b* null alleles to demonstrate that *HP1b* null mutants have increased stress resistance, increased average lifespan, but no increase in maximum lifespan. *HP1b* null mutants exhibit decreased activity levels, decreased food intake, and altered body composition (increased body fat). Metabolite analysis and mitochondrial function assays indicate that these phenotypes might be due to altered energy metabolism. Thus, our results suggest that HP1B might be another example of a chromatin protein that is linked to longevity and metabolism.

Results

Loss of HP1B is Associated with Increased Average Lifespan

In an effort to further characterize the roles of HP1B, we focused on lifespan because previous studies have documented a role of Drosophila HP1a – as well as other chromatin proteins – in longevity {17, 18, 19, 20, and 21}. Survival curves were generated for males and females from two strains lacking HP1B ($HP1b^{86}$ and $HP1b^{16}$) as well as a control strain (*yw*). We find that the survival curves of the three strains trend towards being significantly different from each other (Fig. 1, Kaplan-Meier survival function, $HP1b^{86}$ and *yw* p=.106, $HP1b^{16}$ and *yw* p= .0685). The two mutant strains show increased average

survival, with female HP1b mutants living 46.73 +/- 1.44 days and the control females living on average 43.46 +/- 2.06 days. Similarly, for males the two mutant strains show increased average lifespan, with male HP1b mutants living 35.50 +/- 0.79 days and the control males living on average 34.76 +/- 1.2 days. In addition, the shape of the survival curve is altered, with the mutants experiencing a sharp decrease in survival after day 40, leading to a lack of increased maximum lifespan. Due to these shifts in the survival curve, the two *HP1b* mutant strains differ from the *yw* control strain when comparing them with the Kaplan-Meier survival function in an analysis combining both sexes although not significantly for males. This result is mainly driven by the shift in the females, which is stronger than the shift in males (Fig. 1, Kaplan-Meier survival function, (A) females, $HP1b^{86}$ and *yw* p=.0056, $HP1b^{16}$ and *yw* p= .0256, (B) males, $HP1b^{86}$ and *yw* p=.119, $HP1b^{16}$ and *yw* p= .278). These results show that loss of *HP1b* has a complex effect on survival, affecting the two sexes differently, and mainly impacting survival in early/mid-life.

HP1b Mutants Have Increased Starvation Resistance

Mutations that affect lifespan typically also affect stress resistance {19, 20, 21, 22, and 23}. Given the positive results in the lifespan assays described above, three separate stress resistance assays were performed investigating starvation stress, oxidative stress, and heat stress in *HP1b* mutants. To assay resistance to starvation stress, flies were grown under standard conditions and transferred to 1% agar vials providing hydration but no nutritive value. Deaths

were recorded every 8hr, and survival curves were generated. Results from this starvation assay show that the survival curves of the *HP1b* mutants are shifted to the right compared to the *yw* control, indicating increased stress resistance (Fig. 2). Due to this shift, the survival curves are significantly different between *HP1b* mutants and control flies (Fig. 2, Kaplan-Meier survival function, (A) female, $HP1b^{86}$ and *yw* p=4.67e-8, $HP1b^{16}$ and *yw* p=2.36e-10, (B) males, $HP1b^{86}$ and *yw* p=.011, $HP1b^{16}$ and *yw* p= 1.86e-9). *HP1b* mutants have significantly increased average lifespan under the starvation conditions (Confidence intervals p=.05, average *HP1b*=50.52 +/- .84 hours, average *yw*=45.6+/- 1.06 hours). These data demonstrate that loss of HP1B allows flies to survive longer under starvation conditions.

HP1b Mutants Show Increased Resistance to Oxidative Stress

Increased resistance to oxidative stress has been linked to increased lifespan as well. To examine this relationship in *HP1b* mutants, we utilized a paraquat feeding assay. Paraquat is an herbicide that induces oxidative stress when ingested, and it is widely used to measure oxidative stress resistance in flies {22}. Flies were provided a paraquat-laced sucrose solution as food source to induce oxidative stress. They were monitored, and time to death was recorded for each fly to generate survival curves. Results from this assay reveal that the survival curves of *HP1b* mutants differ significantly from control flies (Fig. 2, Kaplan-Meier survival function, (C) females, $HP1b^{86}$ and *yw* p= 0, $HP1b^{16}$ and *yw* p= 8.13e-13, (D) males, $HP1b^{86}$ and *yw* p=.1.29e-14, $HP1b^{16}$ and *yw* p= 6.96e-

9). The survival curve of the *HP1b* mutants is shifted to the right, and the control flies (red) show a much steeper drop-off in survival rate than the two *HP1b* mutant strains (blue and green). *HP1b* mutants have significantly increased average lifespan in this assay compared to the *yw* control strain (Confidence intervals p=.05, average *HP1b*=59.62 +/- 1.43 hours, average *yw*=46.04+/- 1.57 hours). These data demonstrate that loss of HP1B increases oxidative stress resistance as measured by paraquat feeding assays.

HP1b Mutants do not have Increased Heat Stress Resistance

Heat stress was chosen for a final investigation of stress resistance in the *HP1b* null mutants. Increased resistance to heat stress is typically associated with an increased lifespan {23}. To examine this association in *HP1b* mutants, flies were raised under standard conditions and then moved to 37°C. The flies' time of death was recorded hourly to generate survival curves. We did not find an increased stress resistance to heat stress in the *HP1b* mutants, neither in males nor in females. In contrast, one of the lines, $HP1b^{86}$, actually showed decrease resistance to heat compared to the control (Fig. 2**,** Kaplan-Meier survival function, (E) females $HP1b^{86}$ and *yw* p=.0002, (F) males $HP1b^{86}$ and *yw* p=7.02e-7). These results demonstrate that *HP1b* mutant flies do not have increased heat stress resistance. This result is unexpected, because typically, in long-lived mutants, stress resistance in general is increased, irrespective of assay. Together with the fact that *HP1b* mutants increase average but not maximum

lifespan, this finding suggests that HP1B affects lifespan differently from other long-lived mutants.

HP1b Mutants Consume Less Food

Dietary restrictions often result in altered survival curves and can increase lifespan (24), but might not positively impact all types of stress resistance (25). To investigate if a change in feeding might contribute to the altered survival curves of *HP1b* mutants, we measured food intake using a food consumption (CAFE) assay (26). Flies were fed a nutrient solution via capillaries, and consumption was recorded over an 8hr time period. In this assay, *HP1b* mutants on average consumed ~33% less food than control flies. This change in consumption is mainly driven by the greater decrease of food consumption in the males than in the females. While decreased food consumption is seen in both sexes, the change is more pronounced in males. (Fig. 3, ANOVA, (A) females $HP1b^{86}$ and *yw* p=.19, $HP1b^{16}$ and *yw* p=.046, (B) males $HP1b^{86}$ and *yw* p=.015, $HP1b^{16}$ and *yw* p=.0007) The results from the CAFÉ assay show that loss of *HP1b* impacts food consumption, suggesting that the physiology of these flies might have been altered.

Decreased Food Consumption is the Cause of Increased Oxidative Stress Resistance Observed in the Paraquat Feeding Assay

As illustrated above, flies lacking HP1B exhibit increased resistance to oxidative stress in a paraquat feeding assay. However, mutants lacking HP1B tended to consume less food, thus, raising the possibility that this lower feeding

rate might explain the increased resistance to paraquat-induced oxidative stress – because the *HP1b* mutant flies ingest less paraquat through their food, it will take longer for them to consume a lethal dose. To examine this hypothesis, the paraquat concentration used in the assay was adjusted based on feeding rate to equalize the total amount of paraquat consumed between all groups. In this modified assay, control flies lived significantly longer than *HP1b* mutant flies (Fig. 3, ANOVA, $HP1b^{16}$ and *yw* p=.7.59e-9, $HP1b^{86}$ *and yw* p=2.03e-11). This finding is opposite of what was seen in the initial oxidative stress assay, where the mutants lacking HP1B lived significantly longer. This result suggests that loss of the HP1B protein does not actually confer increased oxidative stress resistance *per se*, but that increased lifespan in the paraquat oxidative stress assay was due to the decreased food consumption by the *HP1b* mutant flies, thus limiting paraquat intake and exposure.

HP1b Mutants Have Decreased Activity Levels

The results from the food consumption assay suggest that there might be additional physiological changes occurring in *HP1b* mutants. We hypothesized that due to the lower level of energy intake, *HP1b* mutant flies might have less energy to expend on physical activity and thus exhibit decreased levels of physical activity. To test this hypothesis, activity levels *HP1b* mutants and control flies were assayed utilizing an activity monitor. This activity monitor measures fly activity levels by recording every time a fly crosses a laser beam dissecting the middle of the monitor chamber. In this assay, *HP1b* mutants have significantly

decreased levels of activity compared to the controls with *HP1b*^{16} mutants having an average of 15,394 recorded crossings, *HP1b*^{86} mutants having 10,204 recorded crossings, and control flies having 17,588 recorded crossings (Fig. 4, A. ANOVA, *HP1b*^{86} and *yw* p=.0000004, *HP1b*^{16} and *yw* p=.00026; n=5). This significant decrease is observed in both males and females, with males in general being more active for all genotypes (total male activity= 26,079 recorded movements, total female activity= 21,890 recorded movements). Thus, loss of *HP1b* decreases activity in addition to decreasing food intake of the animals.

HP1b Mutants Have Increased Fat Percentage

Decreased food consumption and lower activity levels suggest *HP1b* mutants may have altered body composition as well. Body composition has been shown to be important for lifespan, but the exact effect of body size on fly lifespan remains unclear {27, 28}. To examine body composition, we used quantitative magnetic resonance (QMR), which provides an estimate of fat and lean body mass. Weights do not differ between mutants and wildtype significantly (ANOVA, *HP1b*^{16} and *yw* p=.997, *HP1b*^{86} and *yw* p=.960), but *HP1b* mutants have significantly increased body fat content. Combining data from males and females, the average *HP1b* mutant has 15.6% body fat, while the body fat level of *yw* is 10.0% (Fig. 4 B. *HP1b*^{16} and *yw* p=.003, *HP1b*^{86} and *yw* p=.002). These results demonstrate that *HP1b* mutations have an effect on body composition, shifting overall composition towards increased fat levels.

HP1b Mutants Exhibit Decreased Levels of Citrate and Malate

Given the alterations to food intake, activity levels, and body composition, we wanted to investigate if metabolite levels in the *HP1b* mutants might be affected as well. Metabolite levels have been shown to affect the aging process {29}. We chose to focus specifically on metabolites in the Krebs cycle (pyruvate, lactate, citrate, glutarate, succinate, fumarate, malate, glutamate, glutamine, and aspartate) and performed mass spectrometry analysis on 3-5 day old flies. While most Krebs cycle intermediates were unaffected, *HP1b* mutants trend towards having decreased levels of citrate (ANOVA, $HP1b^{16}$ and *yw* p=.0327, $HP1b^{86}$ and *yw:* p =.0559, average citrate for *HP1b*= 4.56e-2 ug/mL*,* Average citrate for yw= 7.54e-2 ug/mL). There was also a trend towards decreased levels of malate (ANOVA, $HP1b^{16}$ and *yw* p=.0343, $HP1b^{86}$ and *yw:* p =.119, average citrate for *HP1b*= 4.30e-2 ug/mL*,* Average citrate for yw= 5.80e-2 ug/mL). These results suggest that loss of HP1B alters select metabolite levels in the Krebs cycle and might impact energy metabolism.

HP1B Mutants Have Decreased Complex III Activity

Citrate synthase activity has been used extensively as a measure of overall metabolic rate {30,31}, and mutations in genes associated with complex III typically result in increased body weight, exercise resistance, and decreased activity through metabolic decompensation {32, 33}. Given the phenotypes we observed in the *HP1b* mutants, we measured the activity levels of these enzymes in *HP1b* mutants and compared them to the *yw* control. The results show that

citrate synthase activity was unchanged among all three lines (ANOVA, *HP1b*^{16} and *yw* p=.97, *HP1b*^{86} and *yw:* p =.99), but that *HP1b* mutants had decreased levels of complex III activity (Fig. 5, ANOVA, *HP1b*^{16} and *yw* p=.048, *HP1b*^{86} and *yw:* p =.006, average activity yw= 5.4 nmol/mg/min, average activity *HP1b*= 2.91 nmol/mg/min). These results show that deletion of *HP1b* results in a measurable change in enzymes involved in energy metabolism, which might explain the altered activity levels and food intake.

Discussion

Results from a variety of assays demonstrate that loss of HP1B has far-reaching impacts on organismal health. Overall, our data show that HP1B has an impact on several different organism-level phenotypes, most likely by affecting components of overall metabolism. Our findings provide an additional link between HP1 proteins and longevity, which is supported also by findings from HP1a, a protein family member previously shown to increase longevity when overexpressed {34}. HP1B seems to affect longevity through different means than HP1a – loss of HP1B increases average lifespan, while it is overexpression of HP1a that results in longevity. HP1B loss also leads to an unusual change of shape in the survival curve. We also demonstrated that removal of HP1B has a direct impact on stress resistance. This finding initially seemed consistent with how chromatin proteins such as SWI/SNF remodel chromatin to better tolerate stress events {35}, but further investigation revealed a different mechanism for the observed increase in stress resistance in *HP1b* mutants. Typically, increased

stress resistance is associated with an increased maximum lifespan {19, 20, 21, and 22}, a result that has been observed in Drosophila, *C. elegans,* and mice {36, 37}. Interestingly, unlike other Drosophila mutants, *HP1b* mutants showed increases in some but not all forms of stress resistance, and actually performed worse under heat stress conditions. In addition, unlike other increased stress resistance mutants, *HP1b* mutants actually show a decrease in maximum lifespan. This phenotype stands in stark contrast with other stress resistance studies, suggesting that deletion of *HP1b* affects stress resistances via an important alternative pathway.

Our studies of metabolic changes in an *HP1b* mutation revealed that HP1B affects stress resistance through a change in metabolic demand. Mutants lacking HP1B have decreased food intake, decreased activity levels, and higher body fat content and are thus able to withstand starvation conditions longer than control flies. HP1b mutants have decreased food intake and are thus able to avoid oxidative stress as measured in a paraquat feeding assay. These observed changes in physiology and metabolism could explain the impacts longevity we detect in our study. Metabolites can directly impact chromatin and can alter the function of various proteins such as DNMTs, HMTs, and HDACs {2, 3, 17, 29, 38, 39, 40, and 41}, illustrating how metabolism is linked to chromatin and chromatin proteins such as HP1B. Other examples include both Sir 2, a histone deacetylase whose increased expression has been shown to increase lifespan through its role in caloric restriction assays {40}, as well as Rpd3, another histone deacetylase that has been shown to have roles in metabolism and aging {41}. Both of these

chromatin proteins alter metabolism to affect longevity, further suggesting that HP1B might alter longevity and stress resistance pathways via a change in metabolic activity. For example, increased starvation and oxidative stress resistances are usually attributed to overall increased stress resistance, implying better chromatin maintenance and therefore a longer lifespan {19, 20, 21}. However, we have determined that other factors should be examined to determine the true cause of stress resistance. In *HP1b* mutants there are several other factors at play that affect the results of stress resistance assays including: decreased activity, increased body fat, and decreased food consumption under normal conditions. Knowledge of these differences have allowed better understanding of why *HP1b* mutants appear to have stress resistance increases that, contrary to other stress resistance mutants, do not result in increased longevity.

Mechanistically, it appears as through the differential metabolism caused by deletion of HP1B stems from changes in multiple parts of the metabolic process. Our results have shown the removal of HP1B results in decreased levels of two identified Krebs metabolites (citrate and malate). Interestingly, these metabolites have both been linked to longevity pathways in past studies {38, 39}. For citrate, it has been shown that decreasing the expression of the *Indy* gene, the fly homolog of a mammalian SLC13A5 plasma membrane transporter, increases longevity {38}. INDY is a transporter of Krebs cycle intermediates and has the highest affinity for citrate, potentially connecting citrate levels to the observed changes in longevity {38}. Malate levels have been previously linked to longevity in yeast due to an associated overexpression of Mdh1, malate

dehydrogenase resulting in increased longevity {39}. Both of these results provide a possible explanation for the observed metabolic phenotypes and could further explain the stress resistance and longevity phenotypes displayed by *HP1b* mutants.

A potential mechanism that might explain how removal of HP1B alters several phenotypes through a change in metabolism is the result seen in complex III analysis. The results of these assays show that *HP1b* mutants have decreased activity levels in this major component of the electron transport chain, suggesting that removal of HP1B leads to metabolic decompensation in a similar manner to previous studies {32, 33}. This metabolic difference between HP1B mutants and the wildtype control could be a differentially regulated process leading to other metabolic abnormalities found as a part of this research, which then may lead to the associated changes seen in stress resistance and longevity. More research is required to fully understand how decreased complex III activity might result in these phenotypes, but these results suggest that reduced complex III activity might lead to a negative impact in the overall metabolic demand of the organism.

Maintaining proper chromatin structure is paramount for healthy and consistent gene expression, and chromatin proteins like HP1B are integral in this process. This concept is supported by the various changes in phenotypic outcomes that were observed over the course of these studies. In this research we demonstrated that removal of HP1B has major phenotypic effects in several different areas including longevity, stress resistance, and metabolism. We also

demonstrated that in order to gain a full understanding of the effects of chromatin proteins several different underlying mechanisms of action must be considered. These results suggest that the differences in stress resistance and longevity observed between test groups are actually a result of a change in overall metabolic demand. We propose that future research into the effects of chromatin proteins will reveal more interactions between pathways similar to these results with HP1B.

Materials and Methods

The *HP1b* mutant alleles were backcrossed to the control strain (*yw*) six times and were screened by PCR to confirm the *HP1b* deletion. In all assays, *HP1b* mutants were compared to *yw* control flies. Stock bottles were set up with 15 females and 10 males and kept at 25°C and 60% humidity on a 12 hour light-dark cycle. Both male and female virgin flies were collected and used at 3-5 days of age unless otherwise stated. All flies were raised on Jazzmix medium (Fisher Scientific).

Starvation Stress

Flies were placed onto starvation media (1.5ml of 1% agar to provide hydration), returned to the incubator (25°C, 60% humidity, 12 hr light-dark cycle), and monitored every 8h to record deaths until all flies had died (n= 100 for each sex and genotype; 20 flies per vial) {42}. We performed three independent

replicates of this assay. Data analysis was performed in R using the R package “Survival,” specifically, its survival difference formula (Kaplan-Meier) {43, 44}.

Oxidative Stress

Filter paper disks saturated with paraquat (an herbicide that serves as an oxidizer) solution (20mM paraquat, 5% sucrose) were placed into vials containing starvation media (1.5ml of 1% agar). Flies were placed inside vials (n= 100 for each sex and genotype; 20 flies per vial), returned to the incubator (25°C, 60% humidity, 12 hr light-dark cycle), and monitored every 4hr until all flies had died {22}. We performed three independent replicates of this assay. Data analysis was performed in R using the R package “Survival,” specifically, its survival difference formula (Kaplan-Meier) {43, 44}.

Oxidative stress was measured in a concentration dependent manner by scaling paraquat concentration based on food consumption. This was done in order to accurately determine the effect of an HP1b mutation on oxidative stress resistance. Paraquat concentration given to the wildtype was decreased by 33% (13.2mM paraquat, 5% sucrose). Other than scaled paraquat concentration, this assay was kept consistent with previous oxidative stress assays. We performed one trial of this assay (n= 100 for each sex and genotype; 20 flies per vial). Data analysis was performed in R using the R package “Survival,” specifically, its survival difference formula (Kaplan-Meier) {43, 44}.

Heat Stress

Flies were transferred to fresh Jazzmix medium (Fisher Scientific) and placed in a warm room maintained at 37°C. Flies were monitored every 1hr until all flies had died (n= 100 for each sex and genotype; 20 flies per vial) {45}. We performed three independent replicates of this assay. Data analysis was performed in R using the R package “Survival,” specifically, its survival difference formula (Kaplan-Meier) {43, 44}.

Lifespan Assay

Flies were maintained on Jazzmix (Fisher Scientific) under standard conditions (25°C, 60% humidity, 12 hr light-dark cycle). Flies were moved to new food every 3 days without the use of CO_2 and monitored daily until all flies had died (starting population: n= 100 for each sex and genotype; 20 flies per vial) {45, 46}. We performed three independent replicates of this assay. Data analysis was performed in R using the R package “Survival,” specifically, its survival difference formula (Kaplan-Meier) {43, 44}.

Food Consumption

Vials and food were prepared as described previously for Capillary Feeder (CAFE) assays {28}. In brief, standard fly vials were cut in half, four equally spaced holes were made around the perimeter using a 22-gauge needle (for evaporation consistency), and the vials were filled with starvation media (1.5ml of 1% agar to provide water). Two capillaries, held in place by pipette tips, were

then positioned inside each foam vial top to provide a nutrient solution (5% sucrose, 5% yeast solution). At 4PM of the day prior to the start of the CAFÉ assay, flies were placed into the vials (n= 8 for each sex and genotype; five flies per vial), capillaries were filled with the nutrient solution, and returned to the incubator (25°C, 60% humidity, 12 hr light-dark cycle). The following day at 8AM, the capillaries were refilled with the nutrient solution and recorded for an initial time point. Eight hours later, liquid level in the capillaries was measured again for a final time point. We assayed the same flies twice over two eight hour time periods (8AM to 4PM) on two consecutive days. Evaporation control vials without flies were set up to average food evaporation within the capillary tubes across vials and replicates. We performed three independent replicates of this assay. Data analysis was performed in R using analysis of variance (ANOVA) with a Tukey's honest significant difference (HSD) *post hoc* test {44}.

Activity

Basal activity of flies was monitored using a locomotion activity monitor (LAM25) from TriKinetics. Flies were placed into vials (n= 5 for each sex and genotype; ten flies per vial), vials were then placed into the monitor, and the monitor was placed in the incubator (25°C, 60% humidity, 12 hr light-dark cycle). Flies were allowed to recover for 1h prior to the start of the measuring period. Activity was recorded every 5 minutes from 8AM to 10AM. We performed six independent replicates of this assay. Data analysis was performed in R using ANOVA with a Tukey's HSD *post hoc* test {44}.

Body Composition Analysis

Body fat content was obtained using quantitative magnetic resonance (QMR) for flies at different ages. For this measure, flies were sorted by sex and genotype, aged for the desired time (3-5, 20, 30, 40 or 50 days old), placed 10 to a microcentrifuge tube, and stored on ice. Flies were then processed using an EchoMRI 3-in-1 QMR machine (Echo Medical Systems, Houston, TX) by the UAB Small Animal Phenotyping core. Flies were placed in the biopsy tube and scanned using the biopsy setting with 9 primary accumulations. We performed four biological repetitions at each time point. Data were analyzed in R using ANOVA with a Tukey's HSD *post hoc* test {44}.

Metabolite Analysis

Metabolite concentrations were measured by mass spectrometry for flies aged 3-5 days. Flies were sorted by sex and genotype, aged 3-5 days, flash frozen in liquid nitrogen, homogenized in cold methanol, and delivered to the UAB Targeted Metabolomics and Proteomics Laboratory (TMPL) for analysis via mass spectrometry.

TMPL protocol was as follows: Supernatants were transferred to new glass tubes and stored at -80°C until further processing. Standards were generated as a master mix of all compounds at 100 □g/mL in H_2O and serial diluted to 10x of the final concentrations (0.05-10 □g/ml, 9 standards). Standards were further diluted to 1x in methanol to a total volume of 1 mL, and dried by a gentle stream of N_2.

For cell extracts, 1 mL of each were transferred to a glass tube and dried under a gentle stream of N_2. Standards and samples was resuspended in 50 □l of 5% acetic acid and vortexed for 15 seconds. Amplifex™ Keto Reagent (SCIEX, Concord, Ontario, Canada) (50 □L) was added to each sample and allowed to react for 1 h at room temperature. Standards and samples were then dried under a gentle stream of N_2 and resuspended in 1 ml of 0.1% formic acid.

Samples were analyzed by LC-multiple reaction ion monitoring-mass spectrometry. Liquid chromatography was performed by LC20AC HPLC system (Shimadzu, Columbia, MD) with a Synergi Hydro-RP 4 □m 80A 250 x 2 mm ID column (Phenomenex, Torrance, CA). Mobile phases were: A) 0.1% formic acid and B) methanol/0.1% formic acid. Compounds were eluted using a 5-40% linear gradient of B from 1 to 7 min, followed by a column wash of 40-100% B from 7 to 10 min, and re-equilibrated at 5% B from 10.5 - 15 min. Column eluant was passed into an electrospray ionization interface of an API 4000 triple-quadrupole mass spectrometer (SCIEX). The following mass transitions were monitored in the positive ion mode: *m/z* 261/118 for □-ketoglutarate, *m/z* 247/144 for oxaloacetate and *m/z* 204/144, 204/118 and 204/60 for pyruvate. In the negative mode, the following transitions are monitored: *m/z* 115/71 for fumarate, *m/z* 89/43 for lactate, *m/z* 117/73 for succinate, *m/z* 133/115 for malate, *m/z* 173/85 for cis-aconitate, *m/z* 191/87 for citrate, *m/z* 191/73 for isocitrate, *m/z* 147/129 for 2-hydroxyglutarate, *m/z* 146/102 for glutamate, *m/z* 145/42 for glutamine and *m/z* 132/88 for aspartate. The 16 transitions were each monitored for 35 ms, with a

total cycle time of 560 ms. MS parameters were CAD 4, CUR 15, GS1 60, GS2 30, TEM 600, IS -3500 volts for negative polarity mode and IS 4500 for positive polarity mode. Peak areas of metabolites in the sample extracts are compared in MultiQuant software (SCIEX) to the those of the known standards to calculate metabolite concentrations.

We performed four biological replicates for each group. Data analysis was performed in R using ANOVA with a Tukey's HSD *post hoc* test.

Mitochondrial Complex Analysis

Mitochondrial complex 3 and citrate synthase activity were measured in male flies using a spectrometer with the help of UAB's Bio-analytical Redox Biology (BARB) core. For these measures all flies were aged 3-5 days. Thoraces were dissected the day of mitochondrial isolation and placed in MIWA buffer on ice {47}. The thoraces were then homogenized, filtered, and protein concentration was measured with a Lowry assay {48}. We performed five biological replicates of both assays. Data analysis was performed in R using ANOVA with a Tukey's HSD *post hoc* test.

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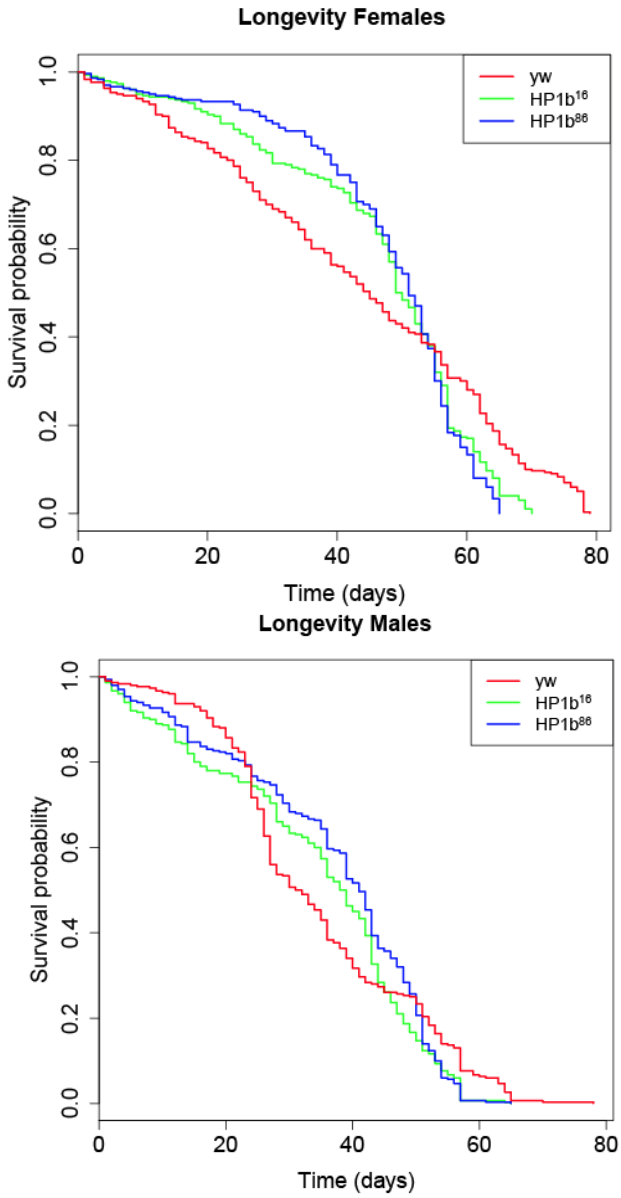

Figure 1. Loss of *HP1b* is associated with increased average lifespan but decreased maximum lifespan. *HP1b* longevity was compared to wild type using a longevity assay. Over three trials *HP1b* mutants showed increased average lifespan but decreased maximum lifespan. Survival curves were compared using a Kaplan-Meier survival function. The y-axis is percent surviving. The x-axis is time in days. For females (top) n= 300, $HP1b^{86}$ and *yw* p=.0056, $HP1b^{16}$ and *yw* p= .0256. For males (bottom) n= 300 $HP1b^{86}$ and *yw* p=.119, $HP1b^{16}$ and *yw* p= .278

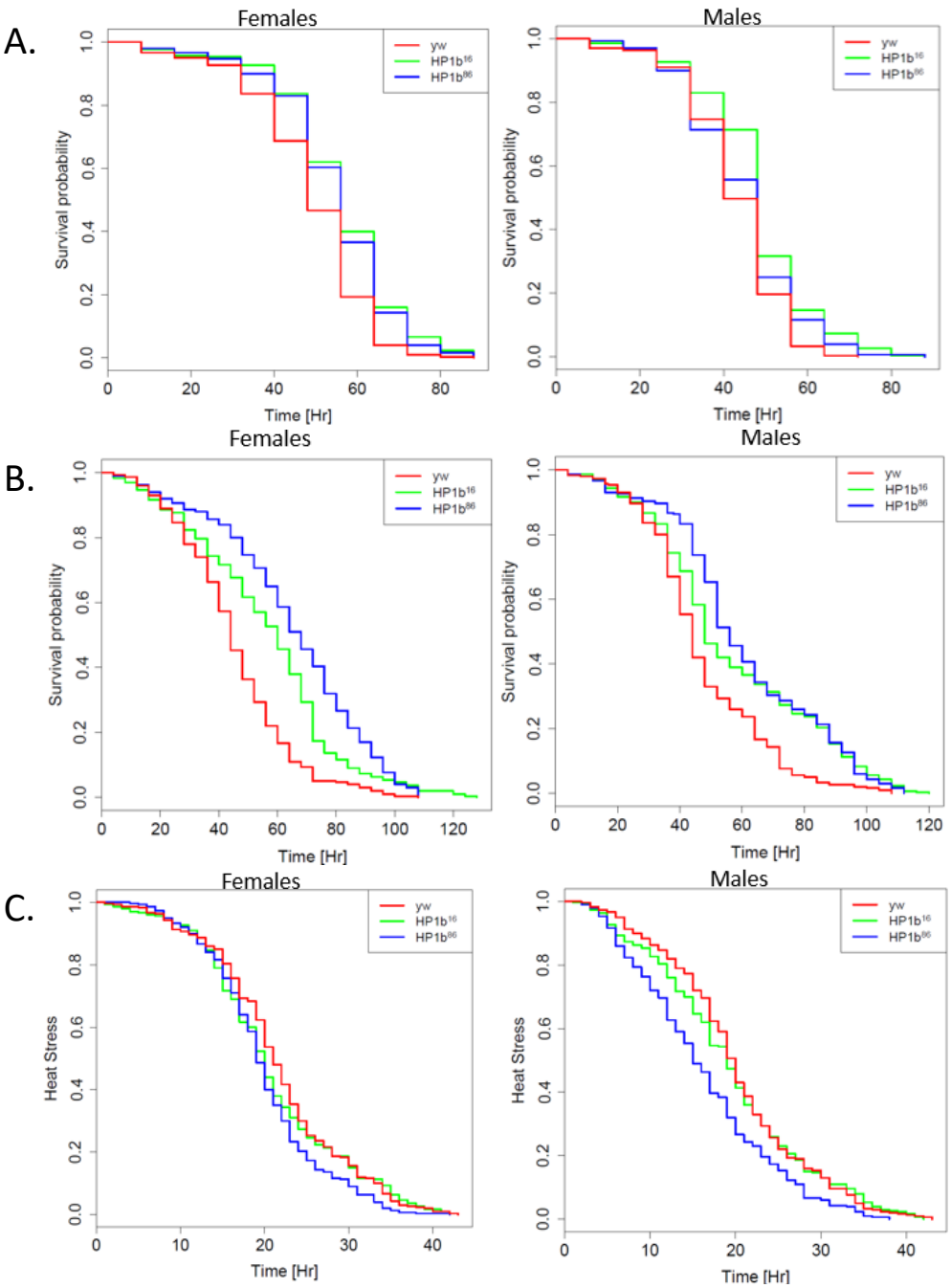

Figure 2. Flies were tested for starvation (A) Oxidative stress (B) and heat stress resistance (C). All survival curves were compared using Kaplan-Meier survival functions. The y-axis is percent survival. The x-axis is time in hours. (A) In three trials *HP1b* mutants exhibited increased starvation resistance, n= 600, $HP1b^{86}$ and *yw* p=9.83e-9, $HP1b^{16}$ and *yw* p=1.11e-16. (B) In three trials *HP1b* mutants exhibited increased oxidative stress resistance, n=600, $HP1b^{86}$ and *yw* p= 0, $HP1b^{16}$ and *yw* p= 0. (C) In three trials *HP1b* mutants exhibited either no change in heat stress resistance or performed worse, n=600, $HP1b^{16}$ and yw p= .419, $HP1b^{86}$ and yw p=2.39e-9

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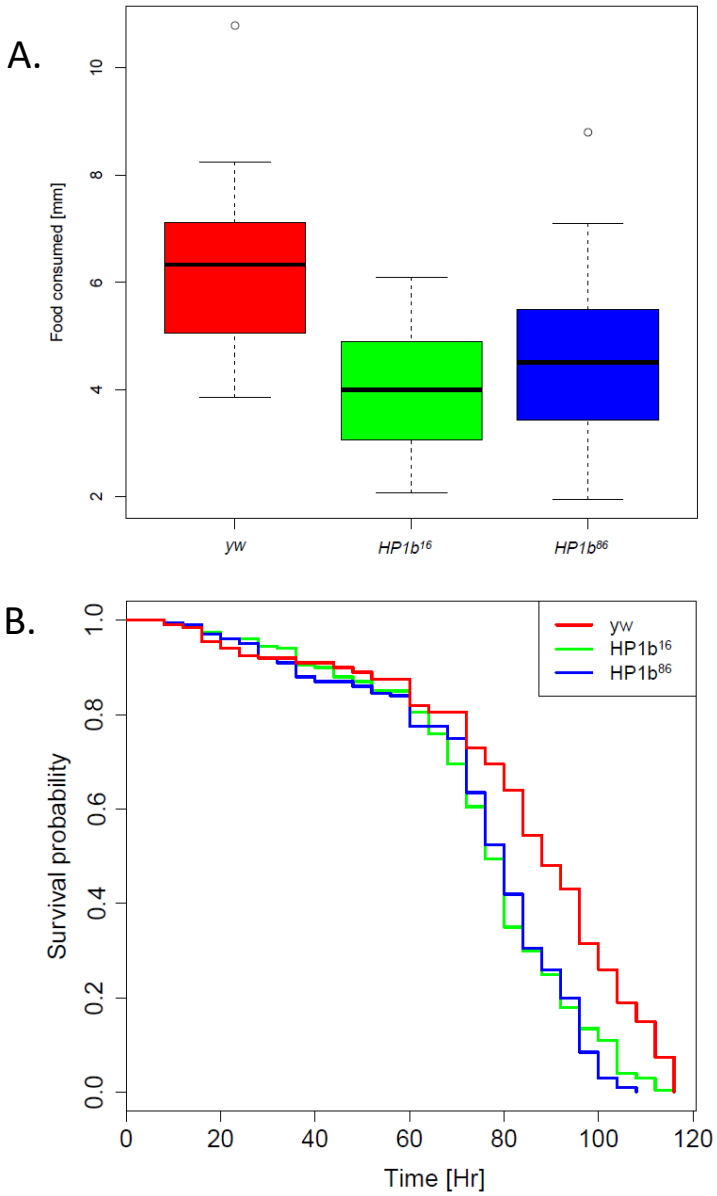

Figure 3. *HP1b* mutant flies have decreased levels of food consumption. *HP1b* mutants were compared to wild type to investigate an effect on food consumption. (A.) Over three separated individual trails *HP1b* mutants consumed less food than the wild type. Results were compared using an ANOVA and a Tukey's hsd *post hoc* test, n=24, $HP1b^{86}$ and *yw* p=.0098, $HP1b^{16}$ and *yw* p=.0003. (B.) HP1b mutants were again tested for oxidative stress resistance but with paraquat concentration scaled for food consumption. Results were compared using a Kaplan-Meier survival function. *HP1b* mutant flies had decreased oxidative stress resistance compared to wild type, n=200, $HP1b^{16}$ and *yw* p=.7.59e-9, $HP1b^{86}$ *and yw* p=2.03e-11

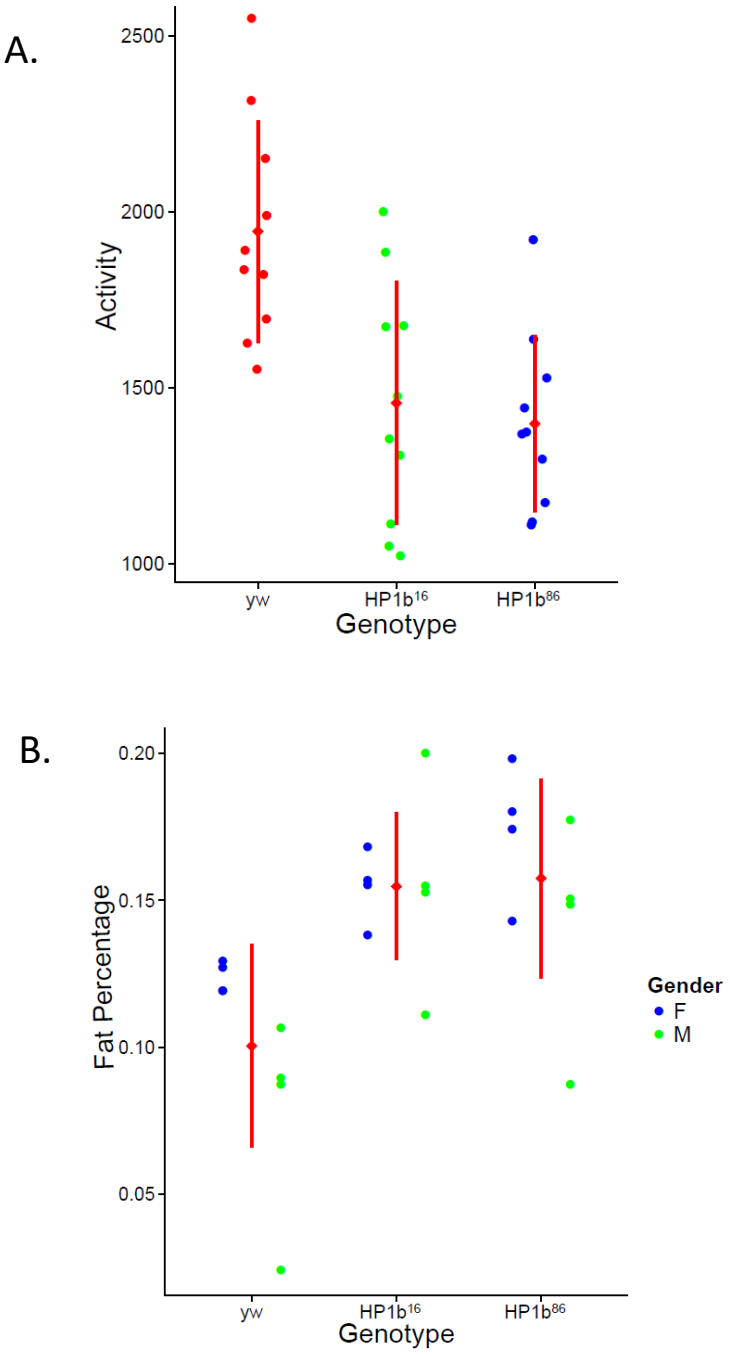

Figure 4. *HP1b* mutants have decreased activity levels and increased fat percentage. *HP1b* mutants were compared to wild type to test for (A) basal activity levels and (B) fat percentage. Both assays were analyzed using an ANOVA and a Tukey's hsd *post hoc* test. (A) In three independent trials *HP1b* mutants had decreased overall basal activity levels, n= 30, $HP1b^{86}$ and *yw* p=.0000004, $HP1b^{16}$ and *yw* p=.00026. (B) With eight biological replicates, 3-5 day old *HP1b* mutants had increased fat percentage, n=8, $HP1b^{16}$ and *yw* p=.003, $HP1b^{86}$ and *yw* p=.002.

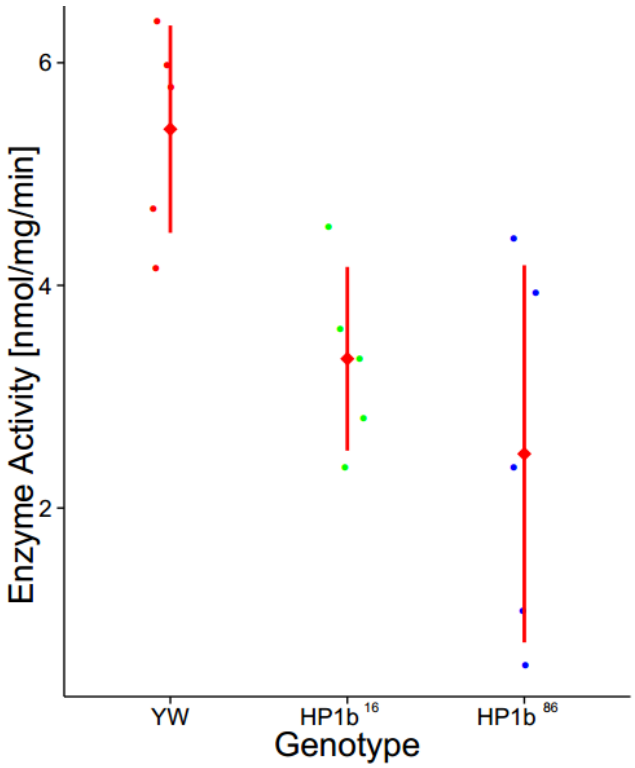

Figure 5. Loss of *HP1b* causes decreased complex III activity. HP1b mutants were compared to wild type to determine the effect of an HP1B deletion on the enzymatic activity of mitochondrial complex iii. Results were analyzed using an ANOVA and a Tukey's hsd *post hoc* test. With 5 biological replicates *HP1b* mutant flies had significantly decreased overall complex iii activity, n=5, $HP1b^{16}$ and *yw* p=.048, $HP1b^{86}$ and *yw* p =.006

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CONCLUSION

by
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CHAPTER 3

CONCLUSION

In researching the heterochromatin protein HP1B, and its effects on metabolism and aging, we discovered that deletion of *HP1b* in *Drosophila melanogaster* affects a range of phenotypes including stress resistance, lifespan, and metabolism. Our research focused on the connections between these seemingly disparate phenotypes to gain a better understanding of the effects of removing HP1B. While the findings presented in this thesis help build a better understanding of HP1 proteins, more research is needed to fully understand the complex functions of this protein family. Here, I present o findings in the context of the current literature and illustrate how they can serve as a starting point for further investigations into HP1 biology and the link of these proteins to aging and metabolism.

A number of studies have linked chromatin structure to the aging process, and have proven that several chromatin proteins are involved in the control of lifespan. These proteins include Sir2, a histone deacetylase implicated in the mechanism through which dietary restriction increases lifespan; RPD3, another histone deacetylase that is potentially involved in dietary restriction; and HP1a, a member of the heterochromatin protein family linked to lifespan control {1, 2, 3, 4}. Given the precedence provided by these three proteins, research into other

chromatin proteins and their effect on aging may aid in our understanding of the mechanism controlling aging.

Metabolism is the set of chemical processes employed in a living organism to synthesize substances and provide the energy required to maintain life. Metabolic processes can impact gene expression through a number of different pathways and interventions including dietary restriction (e.g. methionine or acetyl CoA restriction), nutrient sensing/signaling pathways via the TOR protein, or through endocrine pathways governed by growth hormone and/or insulin {5, 6, 7, and 8}. Gene expression changes via these pathways are mediated by changes in chromatin structure and potentially affect the aging process. Thus, metabolism-mediated pathways dictate some of actions of chromatin proteins, connecting seemingly distinct mechanisms to an effect on aging.

The action of Sir2 under dietary restriction conditions illustrates a direct link connecting chromatin structure and metabolism to the aging process. Organisms, such as yeast, *C. elegans*, Drosophila, and primates, subjected to dietary restriction conditions have an increased lifespan, however removing the histone deacetylase Sir2 nearly eliminates this effect {9,10,11,12}. According to the currently accepted model, Sir2 responds to current metabolic levels in cells, potentially due to TOR signaling, and the increase or decrease of expression levels based on available nutrients {13, 14}. Altered gene expression resulting from decreased nutrient levels could be the mechanism that leads to increased lifespan {15}. The findings from Sir2 suggest a potential connection between metabolism

and chromatin proteins and a role for this interaction in the aging process, demonstrating the need for further research into this connection.

Proteins of the HP1 family might also be a component of the connection between chromatin proteins, metabolism, and aging. The HP1 protein family is highly conserved, and members are found across all studied eukaryotes {16, 17}. This family of proteins performs several roles, including gene repression, via heterochromatin formation, maintenance of heterochromatin integrity, and transcriptional activation, making this family of proteins of significant interest {16, 17}. In *Drosophila melanogaster*, researchers have discovered that HP1a protein concentration, one of five HP1 family members in this species, might affect the aging process through its role in heterochromatin formation {18, 19}. Overexpressing HP1a in Drosophila results in increased levels of heterochromatin, and increases median lifespan by 23% and maximum lifespan by 12% {18}. Conversely, reducing expression of HP1a reduces overall heterochromatin levels and decreases overall lifespan {18}. Also it has been found that in older Drosophila more transposable elements (TEs) become active, potentially due to decreased loss of heterochromatin {44}. This increases in TEs can further disrupt chromatin structure furthering and accelerating the aging process. This result clearly links HP1a to the aging process through a change in chromatin structure.

Interestingly, the connection between HP1a and lifespan may be partially mediated by metabolism. Similar to Sir2, HP1a has a role in gene silencing, and this function may be partially mediated through availability of nutrients. Nutrient signaling through the TOR pathways could be governed by the silencing effects of

HP1a. This is supported by the fact that fruit flies heterozygous for HP1a (decreased ability to create heterochromatin) are larger in size, because silencing of the TOR pathway is less effective {18}. When the TOR pathway is not properly repressed, it leads to the upregulation of many genes, resulting in increased protein production and increased growth rate. Furthermore, overexpression of HP1a, resulting in more silencing, causes fruit flies to be smaller {18}. This is due to increased TOR silencing, causing decreased gene expression and leading to decreased protein production and reduced growth. These results demonstrate that increased heterochromatin levels lead to slower growth due to decreased protein synthesis {20}. This implies that chromatin structure is important for determining how nutrients are sensed, resulting in an overall effect on body size. Heterochromatin levels, modified by proteins like Sir2 and HP1a, therefore affect overall protein synthesis and growth. It is also known that smaller and slower developing organisms within a species typically have increased lifespans. This would suggest a relationship between chromatin structure that affects gene expression, nutrient sensing that is regulated by chromatin structure, and body size that is regulated by nutrient sensing. These findings suggest that additional studies into chromatin proteins and how they are interconnected with metabolism could provide valuable insights for aging biology.

The goal of this research was to characterize the connection between metabolism, chromatin structure, and aging for a less-studied member of the HP1 protein family member in *Drosophila melanogaster*, HP1B. This research tests the hypothesis that, like HP1a, HP1B contributes to the control of lifespan and aging,

possibly by impacting metabolism. This research is the first to examine the effects of HP1B loss on the aging process and to investigate how removal of HP1B affects metabolism. This research uniquely combines aging protocols with methods from metabolism research to create a comprehensive model demonstrating how removal of HP1B affects the organism-level phenotypes in Drosophila.

The first major finding of my research is that removal of HP1B provides increased resistance to some, but not all, stress types. Increased stress resistance is often associated with increased lifespan, so these results suggested that removal of HP1B might increase lifespan. However, HP1B knockdown flies did not exhibit increased stress resistance under all conditions as is typically observed with many long-lived stress-resistant organisms. These conflicting results agree with other stress resistance studies carried out on HP1 proteins. In a few cases HP1 proteins inhibit stress resistance. For example, in *C. elegans,* it appears as through downregulation of heterochromatin protein-like 2 (HPL-2), the homolog of HP1a, provides increased resistance to endoplasmic reticulum stress {30}, in human cells, it was shown that reducing levels of all three HP1 isoforms increased DNA repair {31}. However, there are other examples of decreasing levels of HP1s inhibiting stress response. For example, in human cells, HP1 proteins are shown to be recruited to UV-induced lesions, oxidative lesions, and DNA breaks {31}, and decreased levels of HP1s in *C. elegans* and mice cause genome instability {31}. These seemingly contradictory results, are most likely due to the diverse roles of HP1 proteins.

A second major finding of my work is that HP1B has an effect on longevity (See Fig. 1, Chapter 2). This effect is complex; *HP1b* mutants have decreased maximum lifespan and an increased average lifespan compared a control strain. Similar to our stress resistance findings, these results are unusual. An increased average lifespan implies a period of increased overall health, but the decreased maximum lifespan suggests a sharp and sudden decline in organismal/cellular function. These results suggest several interacting factors operate together to create this complex phenotype. Again, these findings are consistent with the fact that HP1 proteins have many varied roles within an organism.

The third major finding of this research is that removal of HP1B has a major effect on metabolism and might be able to explain other phenotypes observed in these mutants. Due to observations that HP1B null mutants had decreased levels of overall movement, it was hypothesized that the mutants would have a decreased overall metabolic rate, which could help in explaining stress resistance and longevity results. Metabolism has been linked to aging several times through pathways such as dietary restriction, nutrient signaling, or body composition, and several of these pathways involve chromatin proteins {36, 37, 38, and 39}. In order to fully investigate the role of metabolism on the phenotypes of HP1B null mutants, several assays were performed. We discovered that HP1B null mutants consume less food, move significantly less, have increased body fat percent, have decreased levels of citrate and malate, and decreased complex three activity. Coupling these results together with stress resistance and longevity results, this research examines the phenotypes of an HP1B null mutation in a unique manner

that helps gain a more thorough understanding of the underlying pathways. Our research shows that chromatin structure, metabolism, and aging are all connected, and that examining multiple pathways to explain results strengthens research.

Future Experiments for this Research

Going forward, there are several important questions that remain unanswered. More research is needed to investigate the effects of HP1B loss on mitochondrial complexes. To date, we know that complex 3 has decreased activity in HP1B mutants, other complexes could be affected as well. More complex assay trials may reveal other connections to further elucidate the effect of HP1B deletion on each step of metabolism. In addition, measuring respiration directly would explicitly show an altered metabolism instead of utilizing proxy measurements. Unfortunately, measuring respiration in Drosophila requires costly equipment that is not available at UAB, and thus, likely, collaborations will have to be established to achieve this goal.

Having examined the impact of HP1B loss, experiments using HP1B overexpression lines would be an important next step. As previous studies of HP1B overexpression suggest that a second HP1 paralog, HP1a, is impacted by this manipulation, additional experiments with these lines are important not only to understand HP1B, but to understand HP1 proteins in general. Additionally, preforming assays on flies carrying mutations for several HP1 homologs, would provide an insight into how these proteins interact. While homozygous HP1a and

HP1C mutants are not viable, results revealed from examining heterozygotes would still provide better insight into the relationships between the proteins.

Conclusion

Understanding the multiple relationships and pathways that ultimately culminate in an aging phenotype is important for maintaining quality research going forward. While it is important to individually study and each factor involved in aging to allow for development of ideas, it is also necessary to combine these factors to fully understand the aging process. A large number of researchers have identified this idea and have begun to view aging through multiple connected pathways instead of as isolated components {1, 4, 5, and 45}. This approach should result in a more unified theory of aging, in which pieces of information from several different pathways are used to explain a single result.

In my research, multiple components of aging were investigated individually and then combined to gain additional insight into how removal of the heterochromatin protein HP1B affects an organism. We repeatedly showed that chromatin proteins have an effect on stress resistance pathways, providing a proxy for lifespan estimation. Removal of HP1B did have an effect on stress resistance, but not the expected resulting effect on lifespan. In order to understand the observed phenotypes, metabolic effects of removal of HP1B needed to be understood. When combined together, results from metabolic screenings assisted in explaining results from stress resistance assays, ultimately helping to explain effects on longevity.

HP1B clearly is an important chromatin protein that, when removed, affects phenotypes in many different areas including stress resistance, longevity, and metabolism. My research emphasizes the importance of considering many various pathways to understand a complex set of phenotypes such as the ones induced by loss of HP1B.

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