ROLE OF HETEROCHROMATIN VARIATION IN THE LONGEVITY AND GENE EXPRESSION PROFILES OF HETEROCHROMATIC AND AGING-ASSOCIATED GENES IN D. MELANOGASTER

MOJTABA AHMADI

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ABSTRACT

Aging is a complex biological process that is accompanied by the progressive accumulation of deleterious changes that result in the demise of the cellular and tissues function over time, and the increased susceptibility to disease and death as we age. Among many age-associated chromatin modifications, loss of heterochromatin has been considered as a model of organismal aging. Heterochromatin domains established during embryogenesis are gradually lost with advancing age, causing genetic instability through de-repression of the silenced genes and aberrant changes in the pattern of gene expression in the cell.

Heterochromatin is a highly condensed structure of chromatin that remains compacted throughout the entire cell cycle, and largely consists of highly repetitive satellite DNA sequences and transposable elements. Early genetic studies suggested the lack of genetic loci within heterochromatin, but subsequent gene mapping studies provided evidence for the presence of heterochromatic genes which required the condensed structure of the heterochromatic environment for their normal function and showed variegated expression when they were displaced to the proximity of a euchromatic block. The spreading ability and the silencing effect of heterochromatin on the expression of the nearby genes play an important role in chromatin packaging and segregation, and genome stability.

In this thesis, the process of aging in animals, and its possible causes will be reviewed and then the model of heterochromatin loss in aging will be explored. This is followed by an extensive review of the literature on constitutive heterochromatin. Finally, the value of Drosophila heterochromatin in aging studies will be discussed. This study aimed to explore the effects of changes in heterochromatin levels on the process of aging in *D. melanogaster*. To do this, X/O and

XXY karyotypes which lacked the Y chromosome and contained an extra copy of Y chromosome, respectively were first generated, and then their average lifespan relative to that of the normal XY males and XX females was measured. The entire Y chromosome in *D. melanogaster* is nearly heterochromatic and contains very few protein-coding genes that are only expressed in the male germline cells. My goal was to see if changing the heterochromatin levels would impact the longevity of these karyotypes.

Second, I investigated the expression profiles of the heterochromatic and aging-associated genes in relation to the removal or addition of Y chromosome in different karyotypes of the fruit fly models. Moreover, flies from Df(2R) MS2-10/CyO, S stock missing a portion of the pericentromeric heterochromatin from the right arm of chromosome 2 were generated to see whether the removal of a heterochromatic portion from a chromosome other than the Y chromosome will have the same effects on the relative expression profiles of the same genes as the Y chromosome deletion does. Based on our prediction, the removal of the Y chromosome from the nucleus of the *D. melanogaster* cells will induce an increase in the lifespan measurement and the overall expression levels of the heterochromatic and aging genes. The redistribution of heterochromatin components such as HP1 is predicted to result in the increased expression levels of these heterochromatic and aging genes. However, contrary to our predictions, we found the expression profiles of the heterochromatic and aging genes differentially varied between X/O vs XY males, and XX vs XXY females from 2-days y^1/Dp(1;Y)B^s stock. In addition, the differential gene expression patterns were confirmed between the same karyotypes at 60 days, as well as between the Df(2R)Ms2-10/CyO, S males and females which carried a heterochromatic deletion from the right arm of chromosome 2.

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I. INTRODUCTION

I.1 Definition of aging

Aging is a complex biological phenomenon that is accompanied by the progressive accumulation of deleterious changes that result in the demise of the cellular and tissues function over time, and the increased susceptibility to disease and death as we age (Harman, 2003). Aging is marked by a slow accumulation of damage to DNA and other macromolecules, and the subsequent decline in vital bodily functions such as regeneration and reproduction at various cellular and tissue levels (Harman, 2003). The aging process is also associated with the loss of homeostatic state and the reduced ability to withstand stress, injuries, and diseases, ultimately leading to death (Weinert and Timiras, 2003). Knight (1995) believes aging, regardless of how it is defined, will have the following criteria: "a progressive decrease in the efficiency and vigor of essentially all physiological functions; atrophy of most, if not all, organs and tissues; increased vulnerability to trauma, infections, and various immune system malfunctions; increased susceptibility to most malignant processes; and decreased $V0_2$ max".

The study of human diploid fibroblast cells in vitro allowed Hayflick and Moorhead (1961) to conclude that the limited lifespan of these cells was controlled by certain intrinsic mechanisms. This discovery developed the path for further aging studies at the cellular and molecular levels to uncover the underlying mechanisms that caused or were associated with the aging process (see review by Weinert and Timiras, 2003). These findings demonstrate the aging of an organism is linked to the cellular changes that accompany the aging process, how these changes contribute to the aging process, however, remains unclear. Over the past few decades, aging research studies have dramatically increased our knowledge of the key contributors to the aging process. As put

forth by Gavrilov and Gavrilova (2002), the tremendous number of facts obtained through aging studies requires a general theory of aging to successfully explain these facts in a form of knowledge.

However, findings from aging studies have raised many controversial theories on what factors cause aging and/or whether these factors are the actual causes of aging, or they are simply by-products of the aging process. As quoted by Medvedev (1990), more than 300 theories of aging have been proposed, but none of them are adequate as a single theory that could explain the ultimate biological and evolutionary cause(s) of aging (see minireview by Ashok and Ali, 1999). Gerontologists now agree that a combination of multiple theories should be used in conjunction to explain the underlying mechanisms of the aging process (Knight, 1995; Franceschi et al., 2000). This is because of the complex nature and multiple aspects of aging (Kowald and Kirkwood, 1996; Weinert and Timiras, 2003), the variable rate of progression of aging, and its phenotypic characteristics which are different in different species, different organisms of the same species, different tissues, and different cell types (reviewed by Rattan, 2006)

The theories of aging have been now classified into several categories to account for the mechanisms involved in aging. Generally, these theories can be divided into two broad categories: the programmed theories of aging, and the damage and error theories (Jin, 2010). The programmed theories suggest that aging is the manifestation of a biological clock that regulates the development and growth of organisms. This regulation occurs through specific patterns of gene expression in the nervous, endocrine, and immune systems which are responsible for the maintenance of homeostasis, defense, and repair responses. However, the damage and error theories posit that accumulation of damage induced by extrinsic factors, at different levels are the underlying causes of aging (Jin, 2010). The programmed theory of aging is further divided into sub-categories,

including the programmed longevity, the endocrine, and the immunological theories (Jin, 2010). The damage and error theories include the wear and tear theory, cross-linking theory, free radical theory, and the somatic DNA damage theory, to encompass different sub-categories.

I.2 Heterochromatin loss model of aging

Among many proposed theories of aging, chromatin changes are also associated with the senescent phenotype of old age (reviewed by Benayoun et al., 2015). Among many age-related chromatin modifications, loss of heterochromatin has been considered as a model of organismal aging. This concept was first proposed by Villeponteau (1997) who claimed that heterochromatin domains established during embryogenesis are gradually lost with advancing age. He believed the loss of these heterochromatic domains causes genetic instability through de-repression (activation) of silenced genes, nuclear disorganization, and aberrant changes in the pattern of gene expression in the cell.

The loss of heterochromatin was first noted in patients with premature aging diseases. For instance, mutations in nuclear membrane-associated protein lamin A, seen in Hutchinson-Gilford Progeria and the Atypical Werner Helicase syndromes which mimic premature aging disease, are associated with the loss of the markers involved in heterochromatin formation such as H3K9me3 and HP1 protein (Scaffidi and Misteli, 2005; Schotta et al., 2002). Similarly, loss of heterochromatin markers is also seen in elderly individuals, indicating the association of heterochromatin loss with the normal aging process (Zhang et al., 2015). Moreover, genetic studies of aging in Drosophila and *C. elegans* have also revealed loss of heterochromatin in aging (Brandt et al. 2008; Haithcock et al., 2005). Further studies have shown that the overexpression of the heterochromatin-associated protein HP1 in fruit flies increases lifespan while HP1 knock-out

negatively affects the longevity of flies (Larson et al., 2012). Similarly, Sir4 is a heterochromatic protein that interacts with Sir2 and Sir3 to repress genes near telomeres in yeast in a similar fashion to the position-effect variegation (PEV) in Drosophila. Studies show this silencing effect of Sir4 decreases as the cells age, indicating that the loss of Sir-mediated silencing effect due to the loss of heterochromatin happens with aging (Kim et al., 1996). However, it is unclear how the loss or redistribution of heterochromatin within chromatin structure causes or contributes to age-associated changes at the tissue and organ-system levels.

I.3 Process of heterochromatin loss

As speculated by Villeponteau (1997), euchromatic domains and heterochromatic domains, which are established during embryogenesis, are needed throughout the lifespan because they maintain a stable regulatory pattern of gene expression that gives rise to cell lineage differentiation and tissue development. The heterochromatin loss model of aging assumes that the more compacted structure of heterochromatic domains tends to convert to the more relaxed (less condensed) euchromatic structure. This decay of the heterochromatic domains coupled with their need to be regenerated each time the DNA is repaired or replicated subjects them to the heterochromatin loss (Villeponteau, 1997). In addition, heterochromatin loss can lead to incremental changes in the transcription of a protein or a regulatory element that is located at the periphery of the heterochromatin domain. This change in turn leads to a positive feedback mechanism that generates an incremental gradual loss of heterochromatin. The heterochromatin loss model of aging also predicts that heterochromatin loss leads to shortening of the heterochromatic domains. Every cycle of gradual increase in heterochromatic domain shortening results in incremental changes in the expression of age-related genes that are located at the periphery of the heterochromatic domain. Therefore, DNA damage or cell division can generate

many dramatic changes in the expression of many genes localized at the periphery of the heterochromatin domain (Villeponteau, 1997). Therefore, heterochromatin shortening has been proposed as a key regulator of the aging process.

I.4 Heterochromatin structure

Heterochromatin largely consists of highly repetitive satellite DNA sequences and middle repetitive sequences of transposable elements (Lohe et al., 1993) and accounts for about 1/3 of the entire genome in the male and 1/4 in the female *Drosophila melanogaster* (D. melanogaster) flies (Adams et al., 2000). Heterochromatin is a chromatin structure found in the nucleus of all higher eukaryotic cells. It was first described by the cytological work of Emil Heitz (1928) which was based on the differences in the compaction levels of chromosomes stained with carmine acetic acid during the interphase of the mitosis. Heterochromatin is a highly condensed form of chromatin that remains compacted throughout the entire cell cycle. This contrasts with the euchromatin which forms a condensed structure of chromatin only during cell division (Heitz, 1928). In addition, heterochromatin is transcriptionally less accessible due to its highly condensed and highly organized structure in nucleosomal arrays. This differs from euchromatin which is generally less condensed and more accessible to transcriptional factors (Huisinga et al., 2006). The observation of heterochromatin in the Polytene chromosomes of *Drosophila* led Heitz to conclude that the structure of the chromosome is discontinuous and differs along the length of the chromosome.

Around the same time, Hans Muller noted that chromosome rearrangements placing the euchromatic *white* gene in the proximity of a heterochromatic block led to a variegated expression of the gene, with some patches of red and some patches of white facets in the eye (see review by Eissenberg and Hilliker, 2000). This phenomenon called position effect-variegation was first noted

in *Drosophila* and caused the flies carrying the chromosome rearrangement to show a mosaic pattern of the wild-type and the mutant phenotypes due to the variegated expression of the gene in some cells and its inactivation in the others (reviewed by Eissenberg and Hilliker, 2000). Genetic mapping studies further proved that the genes closer to heterochromatin were silenced first followed by the inactivation of more distal genes (Demerec and Slizynska, 1937; Schultz, 1936). This idea gave rise to the important finding that heterochromatin propagates its silencing effect in a linear fashion along the length of the chromosome.

Early genetic studies by Heitz (1928) established the notion that heterochromatin lacked any active genetic loci and, therefore, was of little interest to geneticists. However, subsequent gene mapping studies proved the notion to be wrong by showing that the *rolled* and *light* genes were located within the heterochromatin (Hilliker and Holm, 1975). In addition, using a series of overlapping deficiencies generated by the detachment of compound autosomal chromosomes to genetically map the loci of some genes in the proximal heterochromatic region of chromosome 2 allowed Hilliker and Holm (1975) to provide evidence for the existence of genes within heterochromatin. Further analysis of the ethyl-methanesulphonate (EMS)-induced recessive lethals of the 2L and 2R proximal deficiencies associated with the detached compound chromosomes led Hilliker (1976) to discover the existence of seven genetic loci in the 2L and six genes in the 2R heterochromatin. Furthermore, analysis of the ribosomal DNA (rDNA), a vital heterochromatic gene on the X chromosome in *Drosophila*, showed that this region is intrinsically heterochromatic and retains its heterochromatic properties in the absence of the flanking heterochromatic region (reviewed by Hilliker and Apples, 1982).

Further studies on position effect variegation of heterochromatic genes demonstrated that heterochromatic genes required the condensed structure of the heterochromatic environment for

their normal function and were transcriptionally silenced in a variegated fashion when they were displaced to the proximity of a euchromatic block (Eissenberg and Hilliker, 2000). For example, the function of the *rolled* (*rl*) gene, which is a heterochromatic gene located in 2R chromosome, was severely compromised when it was relocated to different positions in the euchromatic region through successive chromosomal rearrangements (Eberl et al., 1993). Subsequent studies showed reduced expression of the heterochromatic genes *rolled* and *light* in larvae when the Su(var)2-5 gene encoding HP1 was mutated, demonstrating the essential role of the heterochromatin protein HP1 for the normal expression of the heterochromatic genes (Lu et al., 2000). Further genetic and molecular analysis including RNA interference lines to knock down gene expression provided evidence for the existence of some novel essential genes on the right arm of chromosome 2 (2R) and left arm of chromosome 3 (3L) (Coulthard et al., 2010; Syrzycka et al., 2019).

I.5 Heterochromatin properties

Heterochromatin possesses two important characteristics: the ability to spread across chromatin and the silencing effect on the gene expression of the nearby sequences. The silencing effect is due to the ability of heterochromatin to spread across regions of DNA in a sequence-independent manner and cause the inactivation of the nearby genes. This phenomenon is best observed in the inactivation of X-chromosome in mammalian females, a process in which heterochromatin regulates gene dosage compensation by silencing almost the entire X-chromosome (Boumil and Lee, 2001). The propagation of the silencing effect allows heterochromatin to play an important role in chromatin packaging and segregation, and to be essential for genome stability. As discussed by Larson et al. (2012), it has been shown in Drosophila that heterochromatin is essential for genome stability as it suppresses illegitimate recombination of the repetitive DNA sequences, especially at the rDNA locus. Studies suggest the

evolutionary advantage of heterochromatin silencing at the sites of transposable elements is to regulate these elements (Hall and Grewal, 2003). The other characteristics of heterochromatin as compared to that of euchromatin include low gene density, late replication during S-phase, and decreased frequency of meiotic recombination (review by Hilliker et al., 1980). Heterochromatin also has relative plasticity, a term used to describe the reduced level of heterochromatin in certain somatic tissues when there is an upregulation of heterochromatin in the germline of some organisms (see review by Gatti and Pimpinelli, 1992).

One class of heterochromatin called constitutive heterochromatin includes a highly repetitive sequence "satellite" DNA in long tandem arrays which are found near the centromeres (reviewed by Hilliker and Apples, 1989). The other class of heterochromatin called facultative heterochromatin is a euchromatic region that becomes heterochromatinized during a specific developmental stage, or in certain cell types (Brown, 1966). This study focuses on constitutive heterochromatin and will refer to it as heterochromatin for simplicity from now on. The repeat sequences of pericentric heterochromatin, unlike telomers, could differ between different organisms or among members of the same species. In addition, these pericentric regions need to be tightly regulated because chromosomal rearrangements involving the pericentric regions causing malfunctioning of heterochromatin have been linked to abnormalities such as cancer (reviewed by Saksouk et al., 2015). This implies the importance of the pericentric heterochromatin modifications through epigenetic means.

I.6 Position effect variegation

Position effect variegation (PEV) refers to the silencing ability of heterochromatin when chromosomal rearrangements or transposition place a euchromatic gene into the proximity of a

heterochromatic block. The euchromatic gene is completely repressed in some cells that normally express the gene and is partially inactivated in some other cells, a condition that leads to the variegated expression pattern of the gene (Weiler and Wakimoto, 1995). PEV was first discovered in *Drosophila* when the inversion of the *white*$^{+}$ gene that produces red-eye pigmentation placed the gene near the centromeric heterochromatin. This inversion resulted in the variegated eye bearing some patches of white and some patches of red facets (reviewed by Wallrath, 1998). In addition, PEV is also observed with some heterochromatic genes when they are rearranged in the juxtaposition of a euchromatic block. This was shown by inversion of the *lt*$^{+}$ gene which showed a variegating expression pattern when it was removed from the heterochromatin into distal euchromatin. This observation raised the hypothesis that heterochromatic genes required the heterochromatin environment for their proper expression (Schultz and Dobzhansky, 1934). This observation was supported by further studies which revealed that the rearrangements that placed the *lt*$^{+}$ gene in smaller blocks of the displaced heterochromatin resulted in lower expression of the *lt*$^{+}$, showing that reducing heterochromatin amount reduces the expression of the gene (Hessler, 1958; Wakimoto and Hearn, 1990). A general explanation for the PEV of euchromatic genes considers the spreading of a complex containing the heterochromatin-associated proteins Su(var)3-7, HP1 proteins, and Su(var)3-9 methyltransferases from the euchromatin-heterochromatin border into the adjacent euchromatin that contains the variegated gene, therefore exerting their silencing effect on the euchromatic gene near the border (reviewed by Gatti and Pimpinelli, 1992; Elgin and Reuter, 2013). However, the mechanisms of gene inactivation by PEV and maintenance of the inactive state have not been fully understood yet.

I.6.1 Modifiers of PEV

Genetic screens have identified mutations in about 150 loci that affect gene silencing by centromeric heterochromatin. These mutations are either suppressors (Su(var) or SUV) of PEV which result in the loss of silencing or enhancers (E(var)) of PEV which increase the silencing effect (Weiler and Wakimoto, 1995). These PEV modifiers are usually either chromatin structural proteins or the modifiers of these structural proteins, and the DNA-replication factors which regulate chromatin formation indirectly (Wallrath, 1998). For example, heterochromatin protein 1 (HP1) and Su(var)3-7 (SUV37) are the structural building blocks of heterochromatin. Defects in either protein lead to the suppression of PEV. For the modifiers of structural proteins, the origin of replication complex (ORC) is a multi-subunit factor that recruits silencing factors such as SIR1 to the mating-type loci in *saccharomyces cerevisiae* (*S. cerevisiae*). Mutation in ORC2, a subunit of ORC in Drosophila that is recruited to heterochromatin, leads to suppression of PEV (reviewed by Wallrath, 1998). A few of these modifiers have also a dosage-dependent effect on PEV, meaning that one copy of the gene suppresses PEV whereas three copies enhance suppression. The proteins encoded by such genes impact heterochromatin spreading in a dosage-dependent manner (Locke et al. 1988). HP1 is an example of dosage-dependent modifiers of PEV that is found in pericentric heterochromatin, at telomeres, and the different sites of euchromatic regions (reviewed by Grewal and Elgin, 2002). HP1 is encoded by Su(var)2-5 gene and causes variegation of both euchromatic and heterochromatic genes in a dosage-dependent manner. Mutation of this gene increases expression of a variegating gene such as white gene placed inside a heterochromatic block while three copies of the Su(var)2-5 reduces expression of the gene.

However, studies have demonstrated that in addition to providing a platform for silencing factors, HP1/Swi6 also mediates the recruitment of transcriptional activating factors that promote accessibility of Poll II and transcription of heterochromatic repeats (Zofall and Grewal, 2006). For

example, heterochromatic genes *rolled* and *light* require both HP1 and heterochromatin for their full expression (Eberl et al., 1995). The mechanism of HP1-mediated gene activation is not clear, but it is predicted that the balance between silencing factors and transcriptional activators that are recruited by HP1/Swi6 determine the transcriptional activity of a heterochromatic gene. Heterochromatin proteins recruited to the transcribed genes might also recruit HDACs and nucleosome-remodeling factors that are required to re-establish the heterochromatin structure after transcription to prevent spurious transcription of cryptic start sites (Grewal and Jia, 2007). However, heterochromatin spreading is a complex process that requires the organization of different factors within the silenced region in addition to the adjacent heterochromatin mass. In addition, histone modification also regulates chromatin formation and impacts PEV. Increased levels of histone acetylation are generally predicted to be associated with increased transcriptional activity and therefore suppression of PEV (Grewal and Jia, 2007).

I.6.2 Mechanisms of gene silencing by PEV

Among many models proposed, the two mechanisms of cis-spreading and trans-inactivation are the prominent models of PEV-associated gene silencing in *Drosophila*. The cis-spreading model seems to be the most popular explanation for how heterochromatin induces PEV of the euchromatic genes (see minireview by Wakimoto, 1998). It proposes that the linear spreading of the condensed structure of heterochromatin impacts the chromatin structure of the nearby rearrangement breakpoint and therefore, imposes conformational changes in the chromatin structure of a euchromatic gene. This change in chromatin structure makes the euchromatic loci less accessible to transcriptional factors and results in transcriptional repression (see minireview by Wakimoto, 1998). The prediction that PEV results from changes in chromatin structure is based on the observation that the euchromatic region near the breakpoint changes its banding pattern in

polytene chromosomes (see review by Elgin, 1996). Experiments with transgenes such as hsp26 inserted into the heterochromatin have revealed that the promoter of these transgenes, when placed in euchromatin, is less condensed and more accessible to digestion with nucleases. However, when placed in heterochromatic regions, these transgenes are packaged into regular nucleosomal arrays that become transcriptionally repressed and inaccessible to nuclease digestion (reviewed by Grewal and Elgin, 2002). These findings suggest PEV-associated silencing of a transgene might involve a special chromatin packaging of a given domain.

However, the trans-inactivation model of PEV includes plausible explanations for some of the important features of PEV in *Drosophila* that can not be rationalized with the cis-spreading model. For example, rearrangements that include heterochromatin-euchromatin breakpoints induce transcriptional inactivation of genes that are located long distances away from the breakpoint. This phenomenon is hard to be explained by simple linear propagation of the heterochromatin along chromatin. Moreover, the variegation level is shown to be dependent on the position of the breakpoint along the chromosome and its proximity to other heterochromatic regions, the complexity of the rearrangement, and the interactions between different chromosomes including homolog pairing (reviewed by Wakimoto, 1998). Therefore, based on the trans-inactivation model, PEV is influenced by interactions between different heterochromatic regions and the overall organization of the chromosomes in interphase. Chromosomal rearrangements with heterochromatin-euchromatin breakpoints can disrupt this organization and cause the displaced gene to settle in a specific nuclear compartment and show a variegated expression pattern depending on the accessibility of the transcriptional factors within the nuclear compartment (reviewed by Wakimoto, 1998).

This explanation gives rise to the nuclear compartmentalization theory of PEV which suggests that chromosomes are localized in specific nuclear territories that are exposed to a non-uniform distribution of the silencing factors and transcriptional machinery throughout the nucleus (Sun et al., 2001). This theory implies that changes in chromosome linkage that change the position of a gene within the nucleus dramatically impact the amount of gene silencing (reviewed by Wallrath, 1998). For example, a mini-white + transgene inserted into tandemly repeated arrays that are placed in euchromatin is repressed by PEV. Relocating the arrays containing the transgene by chromosomal rearrangement at a distal location will yield higher expression of the transgene whereas bringing the arrays closer to a heterochromatic block will cause the greater repression of the transgene (Dorer and Henikoff, 1997). Similar changes in gene silencing have been observed for the mini-white + transgene when the position of the transgene in the nucleus changes but its local chromosome region stays the same. Therefore, chromosome linkage changes the position of a gene in the nucleus and results in changes in the availability of silencing factors. The nuclear compartmentalization theory can also explain the trans-inactivation of a wild-type allele on a chromosome when it is paired with the allele of a transgene transcriptionally variegated in a heterochromatic region on the homologous chromosome. It means that the allele placed within a heterochromatic region is localized to a silent compartment and drags the wild-type allele to the same silent compartment by somatic pairing (reviewed by Wallrath, 1998). This trans-inactivation of a homologous wild-type allele by pairing does not involve the spreading of heterochromatin across the homologous chromosome as cytological studies do not support heterochromatinization of the homologous chromosome (Belyaeva et al., 1997).

I.7 Heterochromatin function

Heterochromatin plays diverse cellular functions that might be opposing each other. In fact, contrary to the notion that heterochromatin formation is always associated with gene silencing, recent studies have suggested the activation of gene expression requires heterochromatin formation (Weiler and Wakimoto, 1995; Lu et al., 2000). Similarly, HP1 and histone H3 methylation at lysine 9 (H3K9me), the essential components of heterochromatin formation, are associated with transcriptionally active genes within different regions of chromatin (Greil et al., 2003; Cryderman et al., 2005). In addition, heterochromatic proteins assist in recruiting factors that are involved in the recruitment of RNA polymerase II to heterochromatic loci (Zofall and Grewal, 2006). Heterochromatin also acts as a platform where different regulatory proteins are recruited to perform diverse chromosome functions. The spreading ability of heterochromatin allows these regulatory proteins to be recruited to specific chromatin regions in a sequence-independent manner (review by Grewal and Jia, 2007). This recruitment is in addition to the sequence-specific recruitment of these proteins to specific loci. Therefore, heterochromatin assists in the regulation of genetic loci that are incapable of the recruitment of the regulatory proteins by themselves (review by Grewal and Jia, 2007). The multidimensional function of heterochromatin provides an evolutionary advantage to eukaryotes; different eukaryotes can use the silencing effect of heterochromatin in different ways to allow for heterochromatin regulation based on different chromatin contexts. Therefore, it is not surprising to see histone modifications and proteins involved in heterochromatic silencing in one context are used for gene activation in another context (Grewal and Jia, 2007).

I.7.1 Heterochromatin regulation of gene Silencing at transcriptional and post-transcriptional levels

Gene silencing at the transcriptional level is one of the functions of heterochromatin achieved by chromatin condensation and nucleosome repositioning. Chromosome condensation is achieved through Swi6/HP1 binding to heterochromatic loci. These molecules in turn recruit chromatin-modifying enzymes that reduce the accessibility of the transcriptional machinery to the bound sequences (Grewal and Elgin, 2002). For example, in *S. pombe*, Swi6 and other chromatin-bound proteins recruit HDACs that are spread from the nucleation site to the target loci. Deacetylation of histones at the target loci by HDACs preserves H3K9 trimethylation and prevents Pol II from accessing the target site (reviewed by Grewal and Jia, 2007). Therefore, deacetylation of the histones coupled with nucleosomes repositioning allows the establishment of a condensed chromatin structure. Other modifications like histone methylation which promote the recruitment and spreading of HDACs assist this process. Similar mechanisms of gene silencing by heterochromatin have been observed in studies with mammals but a complex network of interactions between DNA methylation and histone methylation are also present in gene silencing in mammals (reviewed by Grewal and Jia, 2007).

Heterochromatin-induced gene silencing also occurs through post-transcriptional processing of the target transcripts by RNAi machinery. In addition to being recruited to the repetitive DNA sites, the RNAi complex also acts on large chromosomal domains. It identifies and removes inappropriate transcripts that are generated by the repetitive sequences (review by Grewal and Jia, 2007). However, it is yet unclear how heterochromatin takes advantage of the RNAi machinery to induce silencing effects on the transcripts derived from repeat elements.

I.7.2 Heterochromatin and genome stability: protecting proper mitosis

Constitutive heterochromatin plays an important role in genome stability by controlling proper chromosomes segregation during mitosis. Centromeres which are surrounded by

pericentromeric repeats of heterochromatin are the sites of kinetochore formation in mitosis. CENP-A, which is a histone 3 (H3) variant specifically found at centromeres, allows proper chromosome segregation during mitosis (reviewed by Janssen et al., 2018). Studies in fission yeast have revealed the essential roles of the components of the pericentromeric heterochromatin for de novo synthesis of CENP-A at centromeres (Folco et al., 2008) and proper attachment of kinetochore and microtubules, resulting in proper segregation of chromosomes to both daughter cells (Ekwall et al., 1996). Similarly, HP1 regulates the formation and the stability of the cohesion complexes in mammals (Kang et al., 2011) and is required as a component of pericentromeric heterochromatin in fission yeast for sister chromatids cohesion (Bernard et al., 2001). In addition to maintaining cohesion, Hp1α is also involved in recruiting chromosomal complex that corrects improper kinetochore and microtubule attachment in mitosis. Improper chromosome segregation is also shown to results from mutations in HP1 or H3K9 methyltransferases in fission yeast, Drosophila, and mice. It can lead to chromosomal defects including chromosomal breakage during cytokinesis, and aneuploidy (unequal segregation of chromosomes to each daughter cell), and the subsequent increase in the rate of tumor incidence (reviewed by Janssen et al., 2018).

I.7.3 Heterochromatin prevents aberrant repeat recombination

The condensed structure of heterochromatin protects the integrity of the multiple repeats present within heterochromatin. Improper DNA damage repairs, more specifically double-strand break (DSB) repairs, are very dangerous in repetitive sequences. There are different repair mechanisms for DNA double-strand breaks but homologous recombination (HR) is one of the main repair systems. HR relies on end resection which is a biochemical process that includes the removal of a few nucleotides from the 5' end of the double-strand break site to produce a 3' single-stranded sequence that can line up with the matching sequence on the sister chromatids or

homologous chromosome to copy and repair the DSB (reviewed by Janssen et al., 2018). However, the presence of a large number of repetitive homologous sequences from different chromosomes located within heterochromatic domains can result in recombination of the DSB with these repeats and the subsequent generation of dicentric chromosomes, insertions, deletions, and other chromosomal abnormalities (see review by Janssen et al., 2018). Therefore, the compacted structure of heterochromatin maintains the integrity of the repeats and prevents abnormal chromosomal structures.

I.8 Mechanisms of heterochromatin formation

I.8.1 Histone modifications of heterochromatin

Post-translational modifications (PTMs) of the amino-terminal of the core histone tails referred to as the epigenetic modifications regulate gene expression in a DNA sequence-independent manner. Histone PTMs along with the addition or removal of the linker histone protein H1 interact with transcription factors and non-histone chromatin binding proteins to permit the higher-order structure of chromatin by promoting the accessibility of the underlying DNA to transcription, replication, and repair and packaging it into the highly condensed structure of chromosomes during mitosis (reviewed in Allshire and Madhani, 2018).

The mechanism of chromatin silencing was originally identified in the budding yeast *S. cerevisiae.* This mechanism contains the SIR (Silent Information Regulator) protein system in which four proteins, including Sir1, Sir2, Sir3, and Sir4 are recruited in response to the recognition of the silencing factors by sequence-specific DNA-binding proteins. Sir2 is a histone deacetylase (HDAC) that removes the acetyl groups from H4K16 sites, allows Sir3-Sir4 complex to bind nucleosomes, and promotes further recruitment of Sir proteins complex to establish the silent

chromatin state. The SIR system is a unique feature of the budding yeast and is quite distinct from the mechanism of constitutive heterochromatin formation (reviewed in Allshire and Madhani, 2018).

Constitutive heterochromatin formation is regulated by several distinct chromatin-modifying enzymes, which generally promote hypo-acetylation and hyper-methylation of histone 3 lysine 9 (H3K9me) relative to euchromatin (cited in Wang et al, 2016). Studies using antibodies against histones H3 and H4 show that pericentromeric heterochromatin is hypoacetylated compared to euchromatin. Similarly, the heterochromatin of the Barr body (inactivated X chromosome) also shows hypoacetylation of H3 and H4 (see review by Dillon, 2004). This hypoacetylation of histones along with other modifications of the underlying DNA sequence is thought to promote the condensed structure of chromatin.

Similarly, early studies showed that Su(var)3-9 gene, which encodes for a histone methyltransferase (HMT) protein, specifically facilitates methylation of H3K9, a prominent marker of heterochromatin assembly (Rea et al., 2000). The specificity of this HMT for H3K9 proves the role of H3K9 methylation in heterochromatin formation. Also, the malfunctioning of centromere due to the targeted knockdown of Su(var)3-9 in mice is another evidence for such a role (Peters et al., 2001). However, more than one methyl group can bind lysine, leading to mono, di, and trimethylation of H3K9. This makes the studies of heterochromatin formation challenging as different enzymes are involved in each type of methylation. However, recent studies have shown that H3K9 tri-methylation and H3K27 mono-methylation are enriched in pericentric heterochromatin where these forms of methylation require the activity of HMTase produced by the expression of Su(var)3-9h gene (Peters et al., 2003). H3K9 mono- and di-methylations mainly occur at euchromatinic regions and require the activity of G9 HMTase protein. This is evident that

the pericentric heterochromatin marked by H3K9 tri-methylation differs in terms of histone methylation pattern from the epigenetically repressed euchromatin which is marked by H3K9 di-methylation (Plath et al., 2003).

I.8.2 Role of HP1 in heterochromatin assembly

HP1 is a highly conserved chromatin-binding protein originally identified in *Drosophila* as a dosage-dependent modifier of PEV (James and Elgin, 1986). Its homologous forms have been identified in many species from Swi6, Chp1, and Chp2 in fission yeast to HP1α, HP1β, and HP1γ in humans (Eissenberg and Elgin, 2000). Most of HP1, such as HP1α and HP1β, is found at the heterochromatic regions while some forms, such as HP1γ, are localized at different regions of the euchromatin (James et al., 1989). HP1 is produced by the Su(var)2-5 gene which causes changes in the variegation of both euchromatic and heterochromatic genes in a dosage-dependent manner. Knocking down the activity of HP1 causes increased variegated expression of the white gene while the overexpression of HP1 decreases the gene expression (Eissenberg et al., 1992). HP1 proteins mediate heterochromatin modification through three distinct domains: a chromodomain which binds H3K9me sites, a chromoshadow domain which allows for the dimerization of the HP1 and interaction with other proteins, and a flexible (non-conserved) hinge region which is involved in DNA and RNA binding (reviews by Fanti and Pimpinelli, 2008; Maison and Almouzni, 2004). HP1 proteins, through the actions of these domains, bind and maintain the chromatin structure in a highly compacted state. In addition, they recruit a variety of factors that are involved in different aspects of heterochromatin modification (Huisinga et al., 2006).

The three isoforms of HP1 in human cells and Swi6 in fission yeast have a dynamic binding activity to chromatin (Cheutin et al., 2003; Festenstein et al., 2003). They are shown to rapidly change from a chromatin-bound state to a free-floating version or vice versa or rapidly displacing

among different heterochromatic domains. In addition, HP1 proteins are known to change their activity at different stages of cell differentiation. For example, HP1β is shown to be more mobile in human embryonic stem cells and induced pluripotent stem cells as compared to fibroblasts. This dynamic activity of HP1 isoforms can be controlled through regulatory mechanisms or changes in chromatin structure in these embryonic stem cells. However, the dynamic activity of HP1 can promote the recruitment of other factors that can ultimately bind and modify the underlying DNA sequence (Wang et al., 2016). For example, in *D. melanogaster*, methylation of H3K9 sites acts as a binding site for the chromodomain of HP1 to be recruited to the heterochromatic domains (Nakayama et al., 2001; Bannister et al., 2001). HP1 binding facilitates the direct recruitment of the HTMs produced by Su(var)3-9 or indirectly through the recruitment of Su(var)3-7 (Schotta et al., 2002). In this manner, the process of heterochromatin formation continues until Su(var)3-9 stops methylation of the adjacent histone. A similar process occurs in *S. pombe* where H3K9 methylation is initiated through the action of the cryptic regulator 4 (Clr4: SUV39h homolog) in a way that is independent of Swi6. Clr4 recruitment is initiated through the involvement of non-coding RNA (ncRNA) and RNA interference (RNAi). However, Swi6 controls the spreading of methylation across the heterochromatic domain (reviewed by Grewal and Jia, 2007). In addition, Swi/HP1 proteins can bind to other factors such as histone deacetylases (HDACs) that are involved in different aspects of heterochromatin assembly. Therefore, the binding of Swi6/HP1 to methylated H3K9 sites acts as a platform for the recruitment of many chromatin-modifying complexes that maintain and spread the heterochromatic domains (Eskeland et al., 2007). Similarly, in addition to the H3K9 methylation pathway, gene silencing (heterochromatinization) also occurs by H3K27 methylation through polycomb repressive pathway but the underlying

mechanisms of how the two pathways interact with each other at the overlapping regions of heterochromatin are not understood yet (reviewed by Zeng et al., 2010).

I.8.3 Role of transcription in heterochromatin assembly

First discovered in fission yeast but afterward in *C. elegans* and *Drosophila* as well (Pal-Bhadra et al., 2004; Grishok et al., 2005), it was found that the constitutive heterochromatin assembly, which forms at the repetitive DNA sequences and suppresses transcription of these sequences, requires transcription of these repeats. It seems that these repeats are the targets for the formation of constitutive heterochromatin (reviewed by Grewal and Jia, 2007). Studies have shown that heterochromatin assembly is mediated by the active roles of non-coding RNAs and RNAi. Early observation in plants suggested the role of the small double-stranded RNA (dsRNA) in DNA methylation and transcriptional gene silencing (Mette et al., 2000). Further studies with *S. pombe* showed that components of the RNAi machinery which include Dicer (Dcr), Argonaute (Ago), and RNA-dependent RNA polymerase (RdRP) were required for the transcriptional silencing of genes. It was shown that mutation of a gene encoding ago1 caused defects in chromosome segregation, a defective phenotype that is associated with defects in heterochromatin formation (Grewal et al., 1998). Further studies proved the core components (Ago1, Dcr1, Rdp1) of the RNAi machinery are necessary for the silencing effect of heterochromatin and chromatin modifications like H3K9me which are associated with pericentric heterochromatin regions in Drosophila and the mating-type locus in fission yeast (Volpe et al., 2003; Hall et al., 2002).

I.8.4 Mechanism of RNAi-mediated heterochromatin assembly

It is believed that the factors involved in chromatin modifications such as histone deacetylases Clr3 and Clr6 (homologs of HDAC1 and RPD3 in mammals, respectively) and Sir2

(which deacetylases H3K9 and H4K16 sites) initiate the process of heterochromatin assembly in *S. pombe*. This step is followed by histone methylation of H3K9 sites by the action of Clr4 (homolog of SUV39 in mammals). Histone methylation acts as an anchoring site that is recognized by the chromodomain of Swi6, chp1, and chp2 proteins which are the HP1 homologs. Heterochromatin spreading also depends on the deacetylase activity of Sir2 on the neighboring histones to recruit Swi6 and Clr4 proteins. The components of RNAi machinery are further required for Clr4 recruitment and subsequent steps towards heterochromatin formation (reviewed by Saksouk et al., 2015).

The specific mechanisms of how RNAi recruits silencing factors and histone modifications to the repetitive sequences are not fully known but it is predicted that the opening of chromatin structure by DNA Polymerase at the replication fork during DNA replication allows transcription of the repetitive sequences. The RNA-dependent RNA polymerase complex (RDRC) converts these transcribed RNAs to double-stranded sequences which are in turn converted to small interfering RNAs (siRNAs) by the action of ribonuclease Dicer. RNA-induced transcriptional silencing (RITS), which contains the Ago1 protein, forms a complex with siRNAs and binds the CLRC complex. CLRC complex consists of an H3K9 methyltransferase that promotes methylation of H3K9 sites. The other subunit of RITS, Chp1 with a chromodomain, recognizes H3K9me and through its association with heterochromatin forms a heterochromatin loop and allows for the transcription of repeat sequences through RNAi (see Figure 1) (Wang et al., 2016). Therefore, transcription of repetitive DNA sequences both provides small RNAs and allows for the recruitment of other factors that are involved in heterochromatin formation. The heterochromatin and the RNAi machinery then spread across the chromatin from the initial nucleation sites to neighboring regions to exert their heterochromatic effect (Wang et al., 2016).

The same mechanisms of heterochromatin assembly by RNAi machinery in *S. Pombe* and plants exist in *Drosophila* (reviewed by Hennig, 1999). It is suggested that mutations in *aubergine, homeless, and piwi,* which are components of the RNAi machinery in *Drosophila,* cause de-repression of the silencing effect such as the decrease in H3K9me level and the delocalization of HP1 along the entire polytene chromosome (Pal-Bhadra et al., 2004). This observation indicates the role of RNAi machinery in heterochromatin formation in *Drosophila.* Whether this role is direct or indirect in heterochromatin assembly needs to be investigated in the future (Dillon, 2004). Similarly, studies with the mammals suggest factors involved in chromatin modifications such as DNMT, HMTase, and HDACs, transcription factors, and other proteins like NuRD, NoRC, and ATRX, and co-repressors are contributing elements in heterochromatin formation. However, the presence of the RNA-mediated machinery in heterochromatin assembly in mammals remains controversial. As suggested by Saksouk et al. (2015), the components of RNAi machinery that contribute to heterochromatin formation might be important at specific developmental stages where the heterochromatic domains are established.

The spreading of heterochromatin components from the nucleation site allows heterochromatin expansion in a DNA sequence-independent manner. This is best observed with PEV where heterochromatin spreads over a large distance into euchromatin. Similar mechanisms of positive feedback loop seen in heterochromatin assembly are also involved in heterochromatin spreading (reviewed by Allshire and Madhani, 2018). However, the spreading ability of heterochromatin needs to be tightly regulated to prevent errors in gene silencing. Different mechanisms prevent heterochromatin spreading including transcription factors that create nucleosome-free regions, and recruitment of anti-silencing factors by transcription factors and other regulatory elements (reviewed by Allshire and Madhani, 2018).

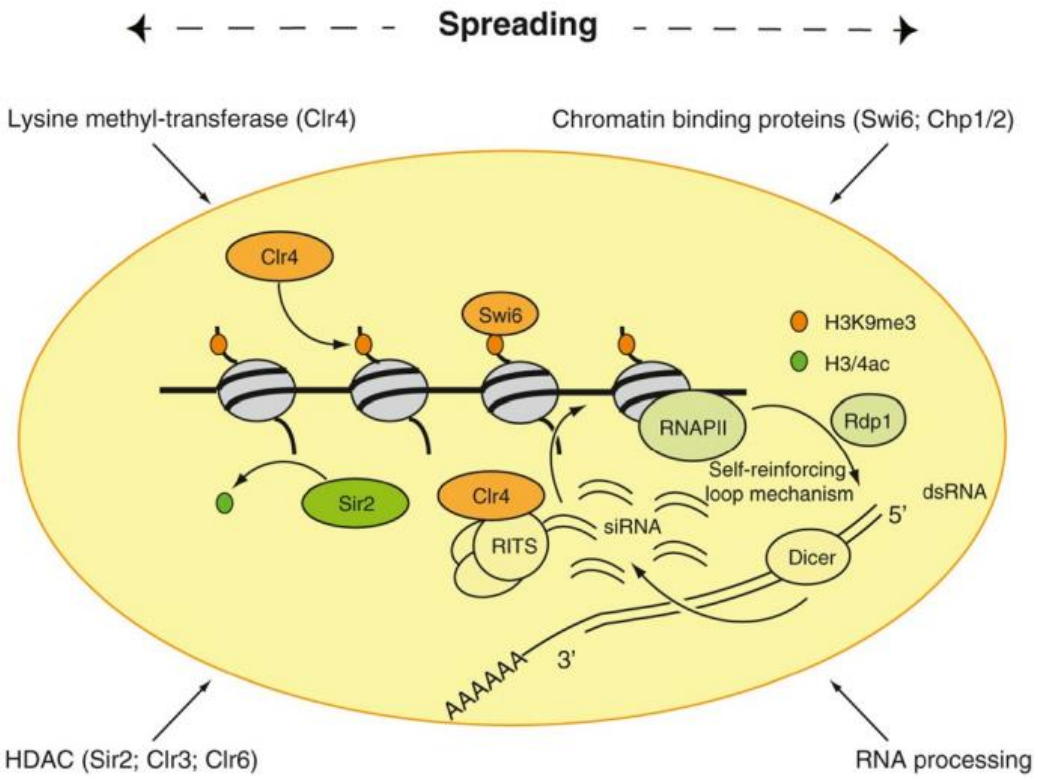

Spreading
Lysine methyl-transferase (Clr4)
Chromatin binding proteins (Swi6; Chp1/2)
Clr4
Swi6
H3K9me3
H3/4ac
RNAPII
Rdp1
Self-reinforcing
loop mechanism
dsRNA
Sir2
Clr4
siRNA
5'
RITS
Dicer
AAAAAA
3'
HDAC (Sir2; Clr3; Clr6)
RNA processing

Figure I.1: Mechanism of heterochromatin formation in fission yeast.

Sir2 initiates heterochromatin assembly in *S. pombe* by deacetylating histone tails at H3K9 and H4K16 sites, followed by histone methylation of H3K9 sites by the action of Clr4 (homolog of SUV39 in mammals). Histone methylation acts as an anchoring site that is recognized by the chromodomain of Swi6, chp1, and chp2 proteins which are the HP1 homologs. RNA Polymerase II (RNAPII) transcribes pericentromeric noncoding repeats in single strand RNA which are converted to double-stranded RNAs by Rdp1, a component of the RNA-dependent RNA polymerase complex (RDRC). Double-strand RNAs are converted to small interfering RNAs (siRNAs) by the ribonuclease Dicer. RNA-induced transcriptional silencing (RITS) forms a complex with siRNAs and binds the CLRC complex, a methyltransferase that promotes further trimethylation of H3K9 by Clr4 and maintains heterochromatin. Reproduced with permission from Saksouk et al., 2015.

I.9 *D. melanogaster* as a Model of Heterochromatin and Aging

D. melanogaster has been a powerful model organism for the study of aging over the last 100 years. Since the discovery that some aspects of aging are genetically controlled, studies trying to modify aging process employing genetic manipulation have extensively experimented the alterations of lifespan by gene mutations in different model organisms. The earliest genetic studies discovered mutations in the components of insulin and IGF-1 like signaling pathway that is associated with the extension of lifespan in worms, fruit flies, and mice (Holzenberger et al., 2003). Thus, knowing that some genetic aspects of aging are conserved among different species paved the path for the study of the underlying molecular mechanisms of aging by utilizing small short-lived invertebrates such as *Drosophila*. Fruit flies become a very useful tool in the study of conserved pathways of aging when findings from flies are combined with those of nematode worms, as well as other short-lived invertebrates such as *S. cerevisiae*. These findings from invertebrates could be further applied to the longer-lived vertebrates like rats to discover the evolutionarily conserved mechanisms to enhance lifespan in more complex organisms (see review by Piper and Partridge, 2018). Some of the properties of *Drosophila* that makes this organism advantageous in aging studies include: the low cost of rearing and maintaining, the ease of generating large numbers of a given sample in a short period, the ease of distinct tissues dissection and genetic manipulation, availability of a large number of genetic tools such as CRISPR/CAS9 system for gene editing, and the ease of generating constructs for gene overexpression or knock-down in a specific tissue and at a specific time-period (Kennedy et al., 2017). In addition, many tissues found in mammals such as the human heart and kidneys can be also found in flies. Moreover, 77 % of genes involved in aging-related diseases in humans are expressed in the equivalent tissues in flies. Similarly, the short lifespan of flies allows experimenters to modify the

experimental conditions to generate stocks with maximum lifespan (reviewed by Piper and Partridge, 2018).

In addition, studies with different model organisms have greatly raised our understanding of the mechanisms of heterochromatin formation. Different pathways of histone modifications and DNA methylation in heterochromatin assembly have been identified (Goll and Bestor, 2005; Maison and Almouzni, 2004). However, due to the increasing number of identified factors linked to heterochromatin assembly and the complex nature of the higher eukaryotic genome, the mechanisms of heterochromatin formation have not been fully understood yet. Similarly, *Drosophila* has been a great tool for the genetic study of heterochromatin properties and understanding the underlying mechanisms of its formation. The small size of *the Drosophila* genome which contains a large amount of heterochromatin provides an advantage for the study of chromatin structure and function. For instance, 30 % of female (XX) and 35 % of male (XY) genome of *D. melanogaster* contains heterochromatin. The pericentric regions (which make 25 %) of chromosomes 2 and 3, the entire Y-chromosome, the proximal end (40 %) of X-chromosome, and most (75 %) of the chromosome 4 are found to be heterochromatic (Lohe et al., 1993). In addition, recent experimental findings have demonstrated the presence of many conserved pathways, which are involved in heterochromatin formation, between fruit flies and other higher eukaryotes including humans.

II. RATIONALE, HYPOTHESES, AND OBJECTIVES

Among many models of aging, the heterochromatin loss model of aging has received little attention. This model was first proposed by Villeponteau (1997) who claimed that heterochromatin domains established during embryogenesis are gradually lost with advancing age, causing genetic instability through de-repression (activation) of silenced genes, nuclear disorganization, and aberrant changes in the pattern of gene expression in the cell. In addition to the loss of heterochromatin markers and heterochromatin proteins seen in HGPS and normal physiological aging (Scaffidi and Misteli, 2005), the shortening of pericentric and centromeric heterochromatin has been observed in many different senescent cells (Swanson et al., 2013). Recent studies suggest the heterochromatin loss model of aging could be possibly termed "the global heterochromatin loss" model of aging (Tsurumi and Li, 2012). Similarly, studies exploring the roles of heterochromatin in aging in *Drosophila* suggest that the loss of heterochromatin marked by HP1 loss of function leads to an increase in the transcription level of rRNA that is involved in ribosomes and protein synthesis (Larson et al., 2012). Although recent advances in the aging field have uncovered a large number of factors and major mechanisms involved in the process of aging due to heterochromatin loss, a key question remains unanswered; whether these mechanisms are simply the by-products of the normal aging, or they are the factors associated with heterochromatin loss which trigger generation of age-related phenotypes.

The objectives of my MSc thesis were to explore the effects of changes in the levels of heterochromatin on the process of aging in *D. melanogaster*. My goal was to see what would happen to the expression levels of some of the known heterochromatic genes as well as some euchromatic genes involved in aging (Table 1) when a large block of heterochromatin was either removed from or added to the nucleus of the fruit fly models. To do this, the principles of the

classic genetics were applied to generate X/O males lacking the Y chromosome, and XXY females with an added copy of the Y chromosome, through meiotic non-disjunction (Bridges, 1916). Almost the entire Y chromosome in *D. melanogaster* is heterochromatic and contains very few protein-coding genes that are only expressed in the male germline cells (cited by Brown et al., 2020). Therefore, the genetic variation due to the removal or addition of the Y chromosome in different X/O, XY, XX, and XXY karyotypes is minimized. In addition, the benefit of utilizing meiotic non-disjunction in producing X/O and XXY flies is that these flies are isogenic to XY and XX flies, except for the presence or absence of the Y chromosome. It was also possible to construct the X/O and XXY males and females from flies carrying double compound or attached X-Y chromosomes (C(1:Y)) (Brown et al., 2020) but there is a high probability of the segregation of the attached chromosomes and the Y chromosome breakage. Moreover, flies from Df(2R) Ms2-10/CyO, S stock missing a portion of the pericentromeric heterochromatin from the right arm of chromosome 2 (Hilliker and Holm, 1975) were generated to see whether the removal of a heterochromatic portion from a chromosome other than the Y chromosome will have similar effects on the expression profiles of the same heterochromatic and aging genes as the Y chromosome deletion does.

It is hypothesized that the removal of the Y chromosome from the nucleus of the *D. melanogaster* cells will induce an increase in the overall expression levels of the heterochromatic loci as well as some aging-related euchromatic genes. We hypothesized that the deletion of a heterochromatin portion in *D. melanogaster* would have similar effects on the expression profiles of the underlying genes as the process of Y chromosome removal does.

It was also of interest to measure the lifespan of these X/O and XXY flies in addition to that of the Df(2R)MS2-10/CyO, S males and females, and compare results with those from the normal wild-

type XY male and XX female counterparts to see if changing the heterochromatin levels will impact the longevity of the flies. Studies have shown that male XY flies live shorter than XX female flies (Tower and Arbeitman, 2009; Yoon et al., 1990) and similar results have been found in some other organisms, including humans (see review by Marias et al., 2018).

III. METHODOLOGY

III.1 Drosophila stock preparation

The isogenic sample of y^1/Dp(1;Y)B^s flies carrying the duplication of the Bar Stone marker on the Y chromosome was ordered from Bloomington Drosophila Stock Center (Stock # 1542). The Df(2R)Ms2-10/CyO, S flies carrying the deletion of a portion of the right arm of chromosome 2 and the CyO, S marker on the homologous chromosome 2 were also used along with the ry^{5+} wild-type flies as control. Flies collected for screening under the light microscope were anesthetized with diethyl ether vapor, and progenies were scored 1-2 days after eclosion.

III.2 Drosophila crosses

Flies were raised at room temperature and normal day/light cycle in small culture vials containing standard sugar and yeast medium with propionic acid to prevent mold formation. They were transferred to fresh food-containing culture vials every 4-5 days until enough progenies were generated. The y^1/Dp(1;Y)B^s males and y^1/y^1 (homozygous recessive yellow) females were raised in large culture bottles and crossed together to generate a large number of flies for screening of X/O and XXY flies (see Figure III.1). XXY females were further crossed to corresponding y^1/Dp(1;Y)B^s males to produce more XXY female progenies.

III.3 Longevity test

A maximum of 10 flies of XX, XXY, XY, or X/O karyotype from y^1/Dp(1;Y)B^s line as well as XX females and XY males from Df(2R)MS2-10/CyO, S stock were kept in separate culture

vials 1-2 days after eclosion and recorded as 1-day old flies. Alive flies were transferred to fresh culture vials every 2 days and the number of dead, strayed, and alive flies were recorded until the complete death of all the flies in the vial. A total of 180-190 flies from each karyotype and each line were used for lifespan measurement.

III.4 Total RNA purification and quantification

To extract total RNA, five healthy flies of the same age from each karyotype and each line were manually homogenized in 500 μL of TRIzol Reagent (Invitrogen Canada Inc., Burlington) according to the manufacturer's instructions. Chloroform was added after incubation to dissociate nucleic acids and proteins, samples were centrifuged at 13000 rpm for 15 minutes, and the aqueous top layer was transferred without disturbing the other layers. After incubation and centrifugation with isopropanol, the RNA pellet was washed with 70 % ethanol, and then air-dried for 5-10 minutes. The pellet was dissolved in 20 ul of DEPC-treated nuclease-free water by incubation at 55 °C for 10 minutes. The RNA concentration was measured using NanoDrop, and then diluted to 50 ng/ul using DEPC-treated nuclease-free water, and the aliquoted samples were maintained at -70 to -80° C.

III.5 Quantitative reverse transcriptase PCR (qRT-PCR)

iTaq Universal SYBR Green One-Step Kit (Bio-Rad Catalog # 172-5150) was used to convert total RNA to cDNA and amplify the sequence of interest through qRT-PCR. The Kit instructions were followed but the PCR tubes were not sealed with optically transparent caps since the Rotor-Gene Q (QIAGEN) used for qRT-PCR analysis reads through the bottom of the tube. Each PCR tube contained 20 ul of the total volume reaction, including 2 ul of 50 ng/ul total RNA.

III.6 Primers

Table III.1 lists the genes and the corresponding forward and reverse primers used for gene expression analysis in this study.

III.7 qRT-PCR analysis and normalization

qRT-PCR analysis was done to quantify the relative expression levels of the selected genes. To do this, the Beta tubulin at 56D (BTub 56D) gene was selected as the reference gene (Lü et al., 2018), its expression level was arbitrarily set to one, and the expression levels of all the other genes were calculated relative to that of BTub 56D gene. In total, five technical replicates for each gene and each karyotype at either 20 days or 60 days of age were used along with the same number of technical replicates for the BTub 56D gene for the same karyotype as the internal control. The cycle threshold (C_T) value of the target gene for each karyotype was subtracted from the corresponding C_T value of the BTub 56D gene and raised to the power of 2 (2ˆ[C_T (reference gene) – C_T (target gene)]. Same calculation was repeated for the other technical replicates and the average of 2ˆ[C_T (reference gene) – C_T (target gene) values for the replicates were used to determine the average relative expression levels of the gene of interest. The standard error of the mean was calculated for each gene and each karyotype and presented as +/- error bar. Student's t.test (two samples, two-tailed and unequal variance) was calculated to determine the statistically significant difference ($P < 0.05$, $P < 0.01$, and $P < 0.001$) in gene expression levels between the two karyotypes.

Table III.1: **List of heterochromatic and aging genes and their corresponding primers**

The table also shows the location of the gene within the genome, the biological function(s), and the corresponding forward (F or L) and reverse primers (R) used in this study. R and L in the "Gene Location" column denote the right and left arms of a chromosome, respectively. Primers sequences are shown in the 5'→3' direction.

Name	Gene	Location	Gene Type	Biological Function	Sequence
βTub56D R	β-Tubulin at 56D	2R	Control	Microtubule cytoskeleton organization (31)	CCAACTGAACGCTGATCTCC
βTub56D R	β-Tubulin at 56D	2R			AACATCTGCTGGGTCAGCTC
Cht3 R	Chitinase 3	2L	Heterochromatin	Chitin catabolic process and carbohydrate metabolic process (1,2)	GCCTGGGAGTTTCCTGTATG
Cht3 L	Chitinase 3	2L			TTACTGGGCGAGACTGCTG
CG17715R	CG17715	2L	Heterochromatin	Not known	CGTTCCTCGTCATCAAGGTT
CG17715L	CG17715	2L			GAGGCAGAGAGCTATTGAGG
lt L	light	2L	Heterochromatin	Essential for normal levels of pigmentation (3)	AGCCCTACGACCTGTATTATGC
lt R	light	2L			GCGATTCTCCTCGATTATGC
Spf45R	Spf45	2L	Heterochromatin	Bifunctional: both DNA splicing and DNA repair protein (4)	GATGGTCAAGGTCTCGGAAA
Spf45L	Spf45	2L			ATAGAAGGTGGCGACAATGC
rlF	rolled	2R	Heterochromatin	Epidermal growth factor receptor signalling pathway(17), adult lifespan determination (18)	GGAAGCTCTTGCACATCCCT
rlR	rolled	2R			AACGACTTCAGGGCATCTCG
YetiF	Yeti	2	Heterochromatin	Chromosome organization, meiotic and mitotic chromosome condensation 20-21)	AGCTGTGTTCCGAGAAATCG
YetiR	Yeti	2			GGTGGCGTCGTCTTTTTCAC
ParpF	Poly-(ADP-ribose) polymerase	3R	Heterochromatin	Nucleolus organization (22)	TCGGATTGCTGTCATGGTTCA
ParpR	Poly-(ADP-ribose) polymerase	3R			TCTCCTACTGAGCTGGGACG

Name	Gene	Location	Gene Type	Biological Function	Sequence
RpL5F	Ribosomal protein L5	2L	Heterochromatin	Assembly of ribosomal large subunit (23)	TTCCGGCGAGGTATGTGTAA
RpL5R	Ribosomal protein L5	2L			ACTGCTTGTTCTTGACTACCTT
SlmapF	Sarcolemma associated protein	2L	Heterochromatin	Negative regulation of hippo signalling (24), positive regulation of Ras signalling (25)	CCAGAGAATGCACTGCGAAAC
SlmapR	Sarcolemma associated protein	2L			GTTCCTGGTAGACCTTCCGC
Maf1F	Maf1	2R	Heterochromatin	Repression of transcription by RNA Polymerase III (26)	ACGAAGAAGGTAGAAGAAGTAACAG
Maf1R	Maf1	2R			GTCTGAGCATCGTTCTTGCG
HaspinF	Haspin	2R	Heterochromatin	Maintenance of sister chromatid cohesion, protein phosphorylation (27)	TGTGCAGCCTTCGAACAAGT
HaspinR	Haspin	2R			CGGATGGTCATTTTCAGATCCC
eIF4BF	eukaryotic translation initiation factor 4B	3L	Heterochromatin	Translation initiation (28)	GCAACAGTTAGTTGGCGCAC
eIF4BR	eukaryotic translation initiation factor 4B	3L			GACACAGAACGGTCATTGCG
CG40178F	CG40178	3L	Heterochromatin	Not known	TCACTTCATCATACTATGCCCAACA
CG40178R	CG40178	3L			TGTACACAGGACCAGAATGGTT
Sod1F	Superoxide dismutase 1	3L	Aging gene	Age-dependent response to oxidative stress (5), determination of adult lifespan (6)	TGCGTAATTAACGGCGATGC
Sod1R	Superoxide dismutase 1	3L			CATGCTCCTTGCCATACGGA

Name	Gene	Location	Gene Type	Biological Function	Sequence
Sod2F	Superoxide dismutase 2 (Mn)	2R	Aging gene	Reactive oxygen species metabolic process (7), determination of adult lifespan (8)	GTGGCCCGTAAAATTTCGCA
Sod2R	Superoxide dismutase 2 (Mn)	2R			CTCTTCGACTTGGCCTCCTC
CatF	Catalase	3L	Aging gene	Reactive oxygen species defense mechanism by neutrilization of hydrogen peroxide to water (9), impacts adult lifespan (10)	ACCGAAGGCGGCTAGAAATC
CatR	Catalase	3L			GGGAGGCATCCTTGATTCCA
foxoF	forkhead box, sub-group O	3R	Aging gene	Transcription factor involved in: determination of adult lifespan (11), oxidative sress (12), insulin receptor signalling pathway (13)	TGAGTCAGATTTACGAGTGG
foxoR	forkhead box, sub-group O	3R			CATAAAGCGGTTGTGCAGCG
myoF	myoglianin	4	Aging gene	Determination of adult lifespan (14)	CTATTAATACCAACGATGAGG
myoR	myoglianin	4			CTTATCCAATCCCAACCACG
Sirt2F	Sirtuin 2	3R	Aging gene	Histone deacetylation (15), determination of adult lifespan (16), neurogeneration	AAGGAGGAACAACCGACGAC
Sirt2R	Sirtuin 2	3R			GAAACCATGCACACGCCAAT
TorF	Target of rapamycin	2L	Aging gene	Determination of adult lifespan (30)	CAAACGCATTTGGGTGAGGG
TorR	Target of rapamycin	2L			AAAGGCAGCCAATCGAGGAA
p38bF	p38b MAP kinase	2L	Aging gene	Determination of adult lifespan (29)	GAAAAGGTGTAGGCGCAGCG
p38bR	p38b MAP kinase	2L			GAATTTGGCCATTTTGCGCG

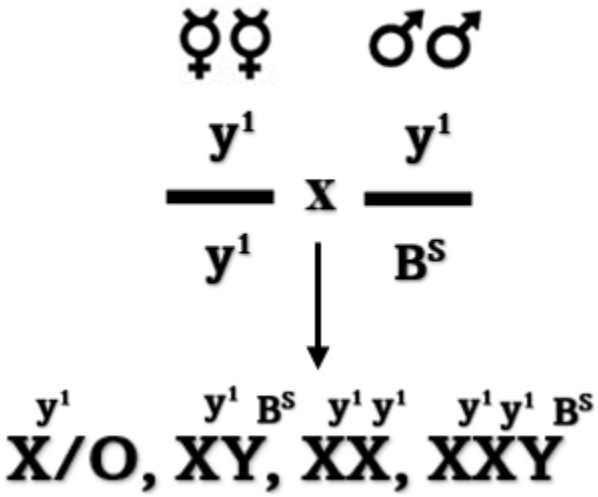

♀♀ ♂♂
y¹ y¹
x
y¹ B^S
y¹ y¹ B^S y¹ y¹ y¹ y¹ B^S
X/O, XY, XX, XXY

Figure III.1: Schematic representation of the *D. melanogaster* cross

The figure depicts the self-cross of yellow Bar Stone-eye males and homozygous recessive yellow female flies from the isogenic $y^1/Dp(1;Y)B^s$ stock to produce X/O, XY, XX, and XXY karyotypes through meiotic nondisjunction. XY males and XXY females carrying the duplication of the Bar Stone (B^s) on the Y chromosome show narrow brown eyes (typical of B^s phenotype) while X/O males and XX females missing Y chromosome and the B^s marker show normal red eye color.

IV. RESULTS

IV.1 Lifespan measurement of different karyotypes

To see if changes in heterochromatin levels affect the lifespan of flies, approximately 180 flies from each XX, XXY, XY, and X/O karyotypes from the $y^1/Dp(1;Y)B^s$ stock were collected and distributed into culture vials, each containing a maximum of 10 flies, for lifespan measurement. The Bar Stone eye phenotype was used to identify flies that either carried an added copy of the Y chromosome (XXY) or lacked it (X/O males). Normal XY males have narrow Bar eyes as they carry the Bar Stone marker on their Y chromosome, but X/O males have normal red eyes due to the absence of the marker on the Y chromosome. Similarly, XXY Bar Stone-eye females are distinguished from the normal red-eye XX females in the same manner.

The average lifespan of the $y^1/Dp(1;Y)B^s$ flies showed X/O males had a significantly longer lifespan (approximately 14 %) relative to XY males (Figure IV.1). This means the removal of the Y chromosome affects the longevity of the flies and is associated with increased average lifespan in X/O males. Similarly, XX females showed a significantly longer average lifespan relative to the XY male flies. The increased longevity of XX females and X/O males relative to XY males is consistent with the findings from the studies which have demonstrated a shorter lifespan of males compared to females in different taxa, including humans (Marais et al., 2018) as well as studies showing increased longevity of X/O males relative to XY flies due to the absence of the Y chromosome in X/O males (Brown et al., 2020). However, contrary to our prediction, XX females did not show increased longevity compared to XXY females. XXY flies had a longer lifespan than XX females albeit not significant (Figure IV.1). Based on the prediction that the Y chromosome removal would be associated with increased longevity, we hypothesized that flies

lacking Y chromosome (X/O) would have the longest average lifespan followed by XX females, and XY males and XXY females having the shortest lifespan. However, it was found that XXY females had the longest average lifespan (about 72.5 days) as they lived significantly longer than X/O and XY males but not significantly longer than XX females (Figure IV.1).

IV.2 Lifespan measurement of Df(2R)MS2-10/CyO, S flies

In addition, Df(2R)MS2-10/CyO, S male and female flies were scored for average lifespan measurement to further explore whether the removal of a heterochromatic region from an autosomal chromosome will induce similar changes in the longevity of the affected flies as the Y-chromosome removal does. Both males and females showed similar average lifespan scores possibly because the impact of the presence or lack of Y chromosome in these flies on the average lifespan measurement was negligible when compared to the significant influence of the deletion of a heterochromatic region from chromosome 2 on the longevity measurement (Figure IV.1). Similarly, maximum lifespan measurement showed no significant difference among different karyotypes of y^1/Dp(1;Y)B^s flies (95-105 days), indicating loss or adding a copy of the Y chromosome does not impact the maximum lifespan measurement (unpublished data). However, both Df(2R)MS2-10/CyO, S males and females appeared to have a similar maximum lifespan but showed a significant reduction in their maximum lifespan (76 and 79, respectively) relative to the normal XY males and XX females from y^1/Dp(1;Y)B^s stock.

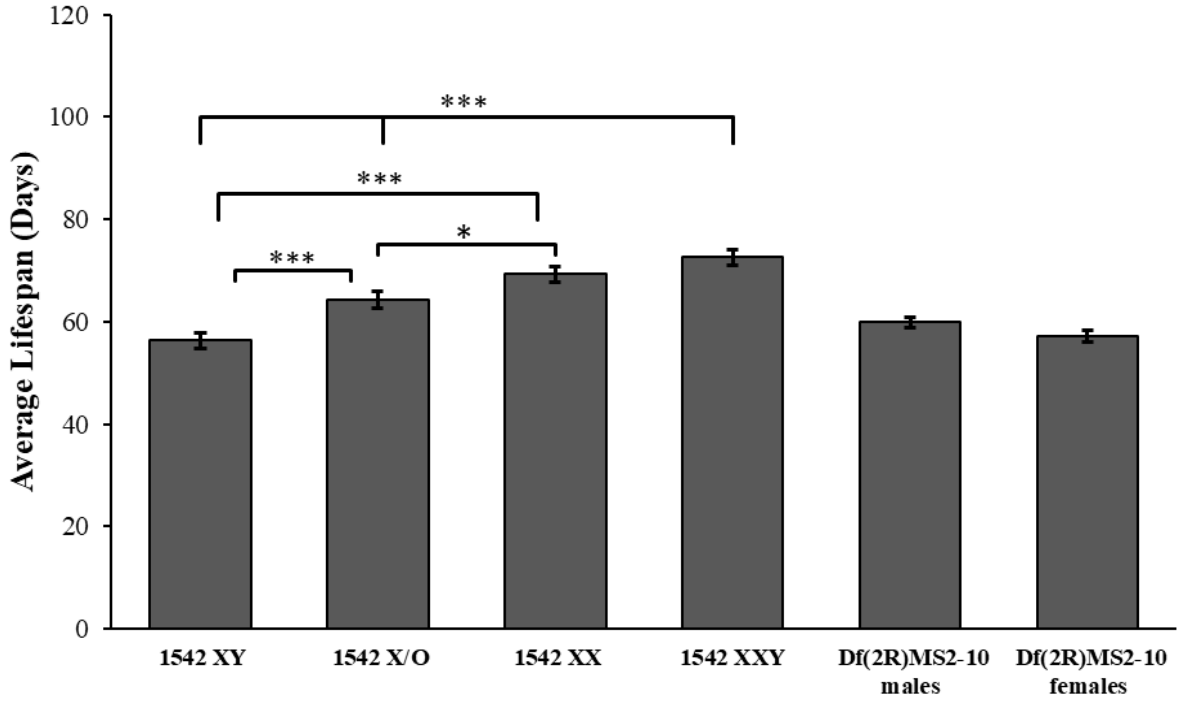

120
100
80
60
40
20
0
Average Lifespan (Days)
***
***
*
***
1542 XY
1542 X/O
1542 XX
1542 XXY
Df(2R)MS2-10 males
Df(2R)MS2-10 females

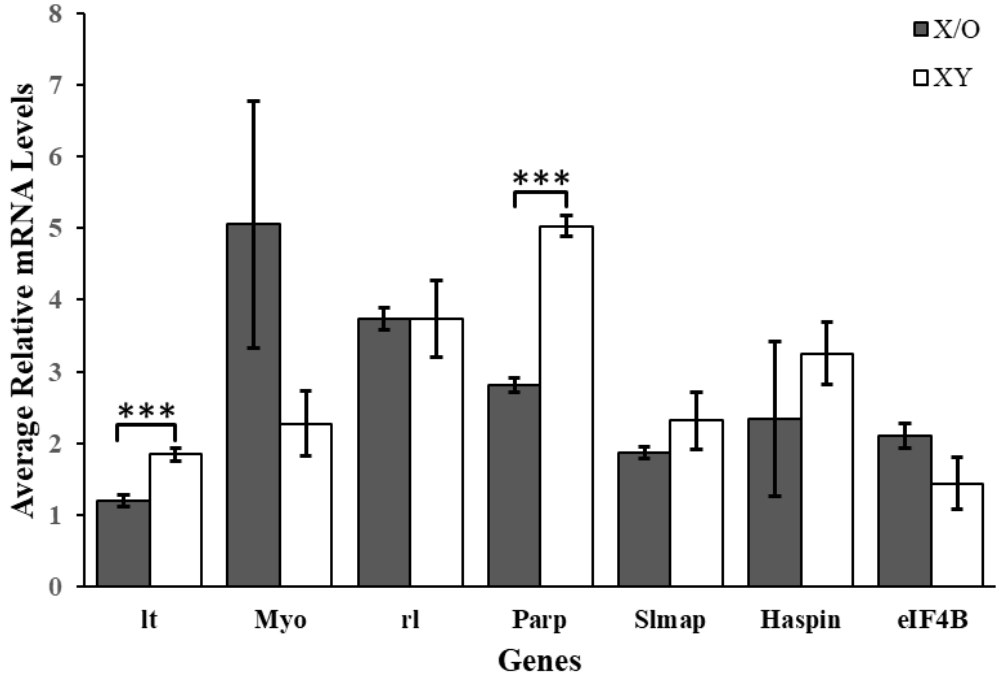
Figure IV.1: Longevity measurement of different karyotypes from y^1/Dp(1;Y)B^s stock and Df(2R)MS2-10/CyO, S male and female flies.

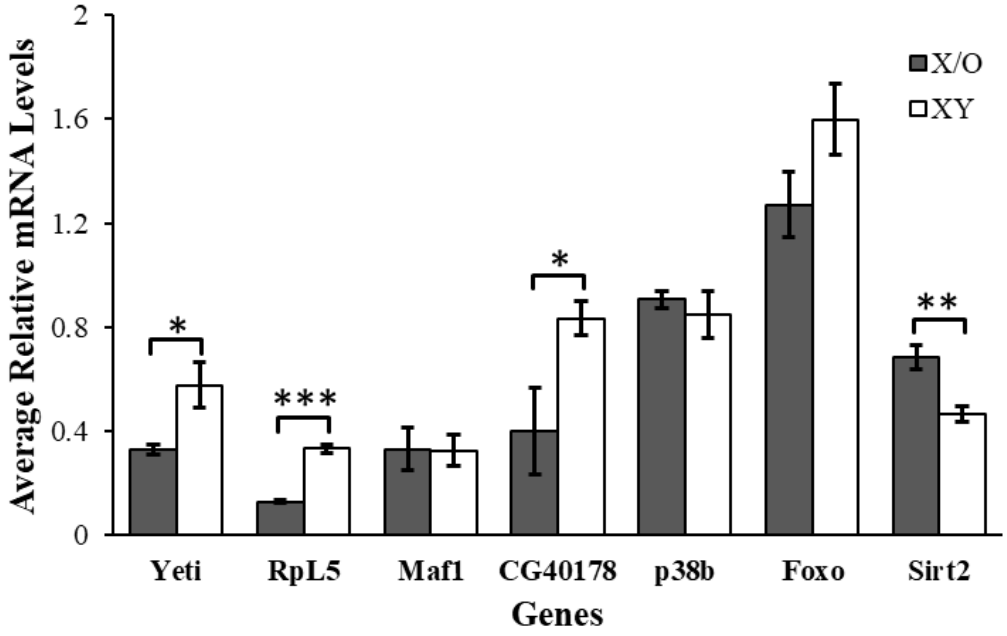
The average lifespan in days is shown on the y-axis and the different karyotypes from y^1/Dp(1;Y)B^s stock as well as Df(2R)MS2-10/CyO, S males and females (listed as Df(2R)MS2-10 in the figure) are listed on the x-axis. The figure shows X/O males lived significantly longer than XY males, and XX and XXY females outlived all the other karyotypes. Df(2R)MS2-10/CyO, S males and females showed comparable lifespan measurement and approximately similar average lifespan relative to XY males from y^1/Dp(1;Y)B^s stock. ∗, ∗∗, and ∗∗∗ on the error bars denote $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Student's t.test (unpaired, unequal variance) was applied to determine the statistical significance of the longevity test results.

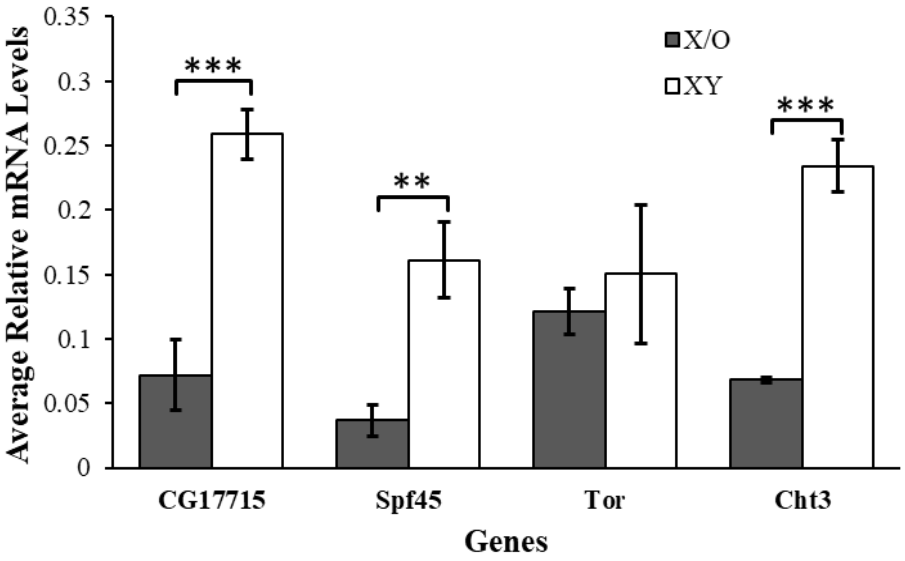
IV.3 Differential gene expression profiles of 2-days karyotypes

To investigate whether changes in heterochromatin levels are associated with changes in transcriptional activity, we sought to study the expression profiles of 21 genes, including genes that reside within heterochromatin as well as euchromatic genes that are associated with aging phenotypes (see Table III.1). Total RNA from X/O males lacking Y chromosome and XXY female flies carrying an extra copy of Y chromosome was used for qRT-PCR analysis. The average relative expression levels of the selected genes in these karyotypes were compared to those of the normal XY male and XX females from the same (y^1/Dp(1;Y)B^s stock. The βTub 56D was used as a reference gene to normalize the relative expression levels of the genes. The qRT-PCR analysis of 2-days flies revealed that six genes, *catalase*, *Sod1*, *Sod2*, *Sirt2*, *Myo*, and *eIF4B*, were overexpressed in X/O males relative to XY males. Four of these genes, *catalase*, *Sod1*, *Sod2*, and *Sirt2*, showed a significant increase in gene expression levels in X/O males, as compared to XY males (Figure IV.2). Only one of these genes, *eIF4B*, overexpressed in X/O males relative to XY males, was heterochromatic while the others were euchromatic aging-associated genes. Except for *rl*, *p38b*, and *Maf1* which had comparable expression levels between XY and X/O males, all the other 12 genes were overexpressed in XY males relative to X/O males while the overexpression of 8 of these genes in XY males was statistically significant (Figure IV.2). All the significantly overexpressed genes, *Yeti*, *RpL5*, *CG40178*, *lt*, *Parp*, *CG17715*, *Spf45*, and *Cht3*, in XY males were found to be heterochromatic in addition to the other two heterochromatic genes, *Haspin* and *Slmap*, being non-significantly overexpressed in XY males in comparison to X/O males (Table IV.1).

Further qRT-PCR analysis of the same genes in 2-days old XX and XXY females was performed to determine the differential gene expression profiles of these flies (Figure IV.3). *Cht3, CG17715,* and *lt* were significantly overexpressed in XX females relative to XXY females. This result is consistent with our prediction that female flies with an added copy of the Y chromosome would have reduced expression levels compared to XX females, lacking the Y chromosome. Except for *Sod1* and *Sod2* which are aging genes, all the other genes overexpressed either significantly or non-significantly in XX flies relative to XXY females turned out to be localized within the heterochromatin (Table IV.1). However, *eIF4B*, *Slmap*, *CG40178*, and *p38b* had significantly increased expression levels in XXY females relative to XX females (significantly reduced expression levels in XX females). In addition, the other six genes, *Sirt2*, *RpL5*, *Tor*, *Foxo*, *Parp*, and *catalase*, had considerably but non-significantly increased transcriptional activity in XXY flies compared to XX females (see Figure IV.3, Table IV.1). All the significantly overexpressed genes in XXY females were found to be heterochromatic as well as nearly half of the non-significantly overexpressed genes. A general trend can be drawn from the expression profiles of 2-day old flies, suggesting the downregulation of heterochromatic genes and the upregulation of aging genes in X/O males relative to XY flies. However, this trend is not observed with XX and XXY females which show upregulation of nearly half of the heterochromatic genes as well as half of the aging-related genes in XX females, as compared to XXY flies (Table IV.1).


X/O
XY
Average Relative mRNA Levels
0
1
2
3
4
5
6
7
8
***
***
lt
Myo
rl
Parp
Slmap
Haspin
eIF4B
Genes


X/O
XY
Average Relative mRNA Levels
0
0.4
0.8
1.2
1.6
2
*
***
*
**
Yeti
RpL5
Maf1
CG40178
p38b
Foxo
Sirt2
Genes


Average Relative mRNA Levels
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
X/O
XY
***
**
***
CG17715
Spf45
Tor
Cht3
Genes

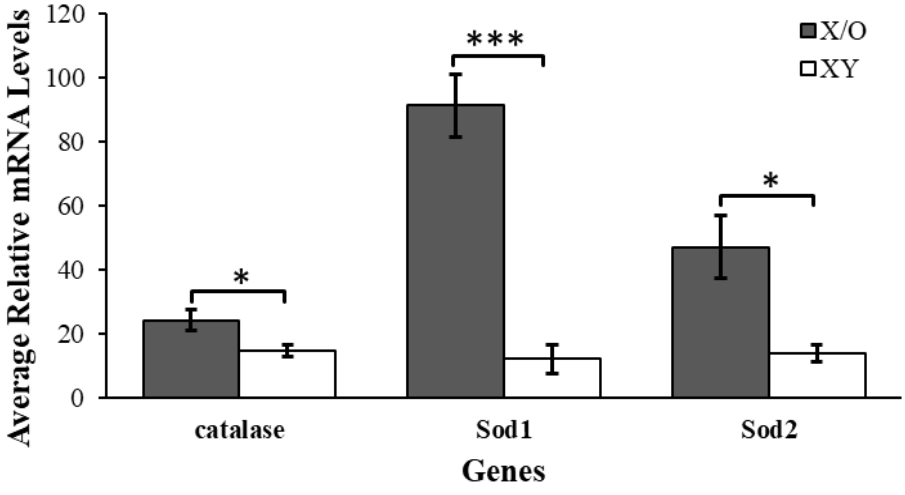
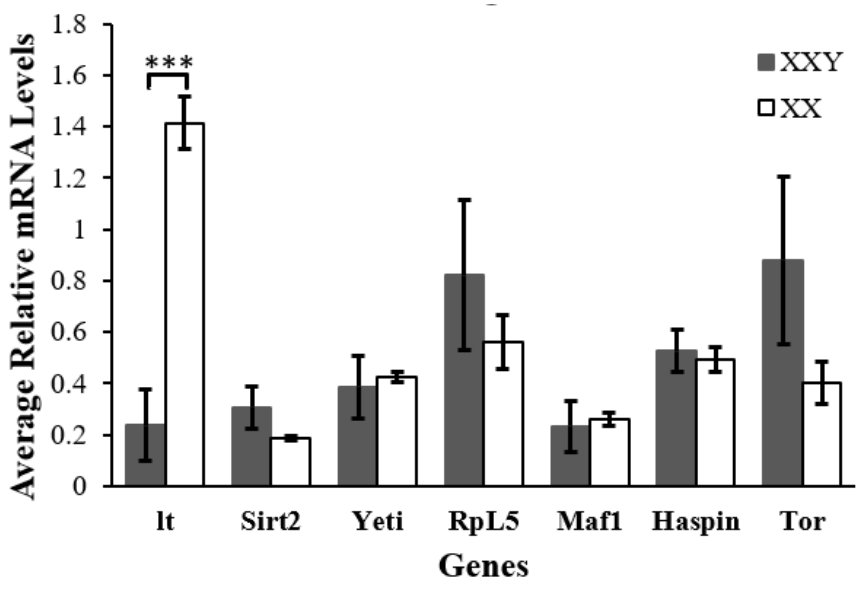
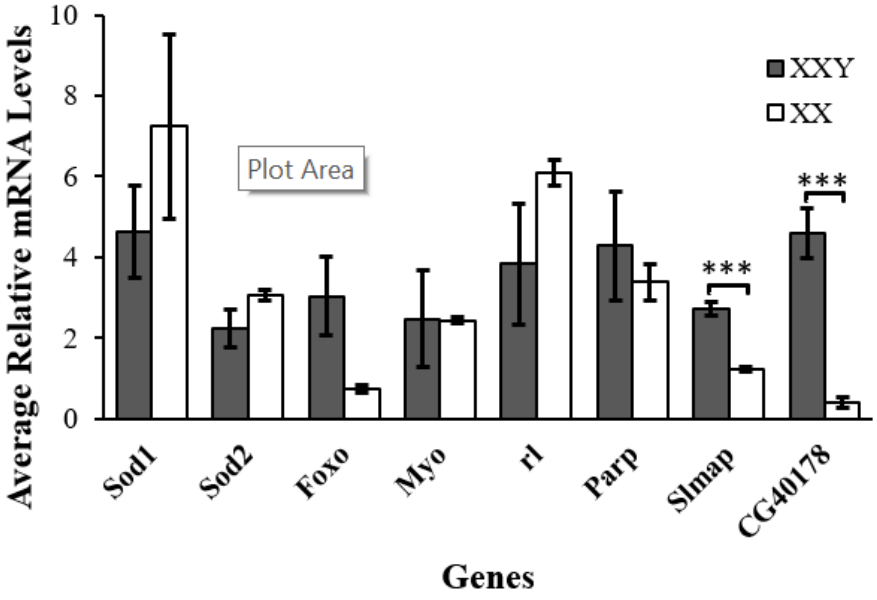
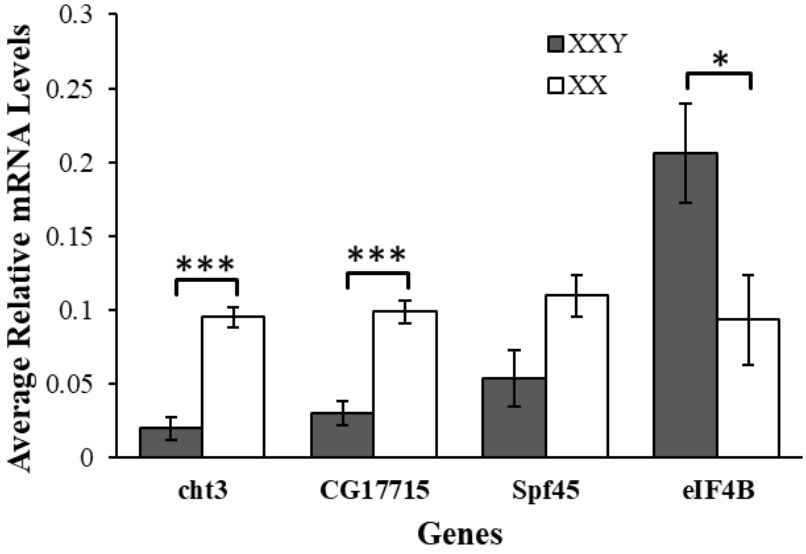

Average Relative mRNA Levels
120
100
80
60
40
20
0
X/O
XY
*
***
*
catalase
Sod1
Sod2
Genes

Figure IV.2: Average relative mRNA levels of heterochromatic and aging genes in 2-days old X/O and XY males.

Quantitative RT-PCR was used to measure the relative expression levels of the heterochromatic and aging-associated genes of 2-days old X/O and XY male karyotypes from y^1/Dp(1;Y)B^s stock. Data represent mean ± SEM of five technical replicates relative to the BTub 56D (internal control) gene. An overall change in the relative expression profiles of the heterochromatic and aging-associated genes was observed when the level of heterochromatin was reduced as the result of Y chromosome removal in X/O males relative to the wild-type XY flies. Student's t.test (two-tailed and unequal variance) was used to determine the statistical significance ($P < 0.05$, $P < 0.01$, and $P < 0.001$ as denoted by $*$, $**$, and $***$ on the error bars, respectively).


Average Relative mRNA Levels
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
XXY
XX
lt
Sirt2
Yeti
RpL5
Maf1
Haspin
Tor
Genes


Average Relative mRNA Levels
10
8
6
4
2
0
XXY
XX
Plot Area
***
***
Sod1
Sod2
Foxo
Myo
rl
Parp
Slmap
CG40178
Genes


Average Relative mRNA Levels
0.3
0.25
0.2
0.15
0.1
0.05
0
XXY
XX
***
***
*
cht3
CG17715
Spf45
eIF4B
Genes

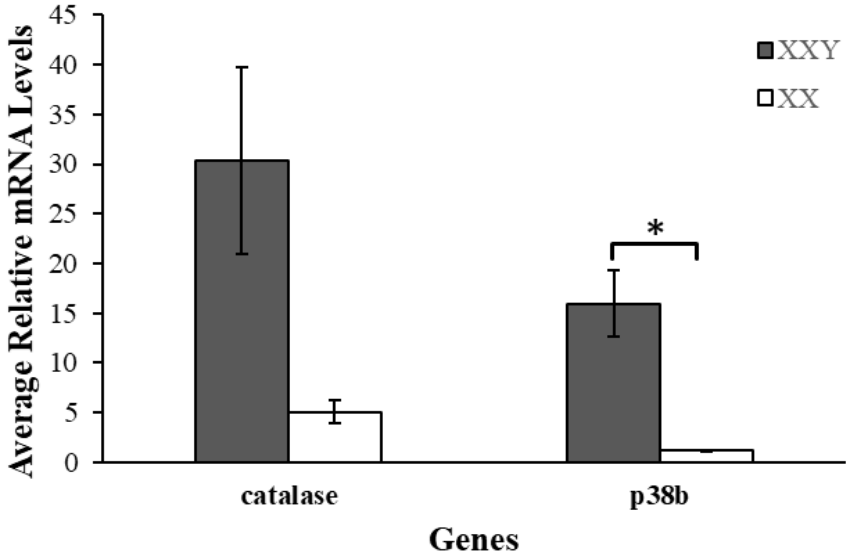

Average Relative mRNA Levels
45
40
35
30
25
20
15
10
5
0
XXY
XX
*
catalase
p38b
Genes

Figure IV.3: Average relative mRNA levels of heterochromatic and aging genes in 2-days old XX and XXY females.

Quantitative RT-PCR was used to measure expression levels of heterochromatic and aging-associated genes from 2-days old XX and XXY karyotypes of y^1/Dp(1;Y)B^s stock. Data represent mean ± SEM of five technical replicates relative to the BTub 56D (control) gene. An overall change in the relative expression profiles of the heterochromatic and aging-associated genes was observed when the level of heterochromatin was increased as the result of adding an extra copy of the Y chromosome in XXY females relative to the normal XX flies. Student's t.test (two-tailed and unequal variance) was used to determine statistical significance ($P < 0.05$, $P < 0.01$, and $P < 0.001$ as denoted by $*$, $**$, and $***$ on the error bars, respectively).

Table IV.1: Comparison of gene expression levels in 2-days old karyotypes.

Data represent the ratio of the relative gene expression levels of 2-days old X/O vs XY males and XX vs XXY females. An increase or a reduction in the relative gene expression levels are shown by values greater than one or less than one, respectively. The result demonstrates an overall change in the relative transcriptional activity of most heterochromatic and aging-related genes due to variation in the amounts of heterochromatin as seen by the removal or addition of a copy of the Y chromosome. Statistical significance of the changes in the relative gene expression levels are demonstrated with $*$, $**$, and $***$ on the error bars which denote $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Gene Name	Type of Gene	Flies Age	X/O vs XY males		XX vs XXY females	
			X/O/XY	Chane in Relative Expression level	XX/XXY	Change in Relative Expression level
Cht3	heterochromatin	2 days	0.29	Decreased***	4.81	Increased ***
lt	Heterochromatin	2 days	0.65	Decreased***	6.00	Increased ***
CG17715	Heterochromatin	2 days	0.28	Decreased***	3.30	Increased ***
Spf45	Heterochromatin	2 days	0.23	Decreased**	2.04	Increased
rl	Heterochromatin	2 days	1.00	No Change	1.59	Increased
Yeti	Heterochromatin	2 days	0.57	Decreased*	1.10	Increased
Parp	Heterochromatin	2 days	0.56	Decreased***	0.79	Decreased
RpL5	Heterochromatin	2 days	0.39	Decreased***	0.68	Decreased
Slmap	Heterochromatin	2 days	0.81	Decreased	0.45	Decreased***
Maf1	Heterochromatin	2 days	1.02	No Change	1.12	Increased
Haspin	Heterochromatin	2 days	0.72	Decreased	0.94	No Change
eIF4B	Heterochromatin	2 days	1.46	Increased	0.45	Decreased*
CG40178	Heterochromatin	2 days	0.48	Decreased*	0.09	Decreased***
Sod1	Aging gene	2 days	7.63	Increased***	1.56	Increased
Sod2	Aging gene	2 days	3.45	Increased*	1.36	Increased
Cat	Aging gene	2 days	1.66	Increased*	0.17	Decreased
foxo	Aging gene	2 days	0.79	Decreased	0.25	Decreased
Myo	Aging gene	2 days	2.22	Increased	0.98	No Change
Sirt2	Aging gene	2 days	1.47	Increased**	0.61	Decreased
p38b	Aging gene	2 days	1.07	No Change	0.07	Decreased*
Tor	Aging gene	2 days	0.81	Decreased	0.46	Decreased

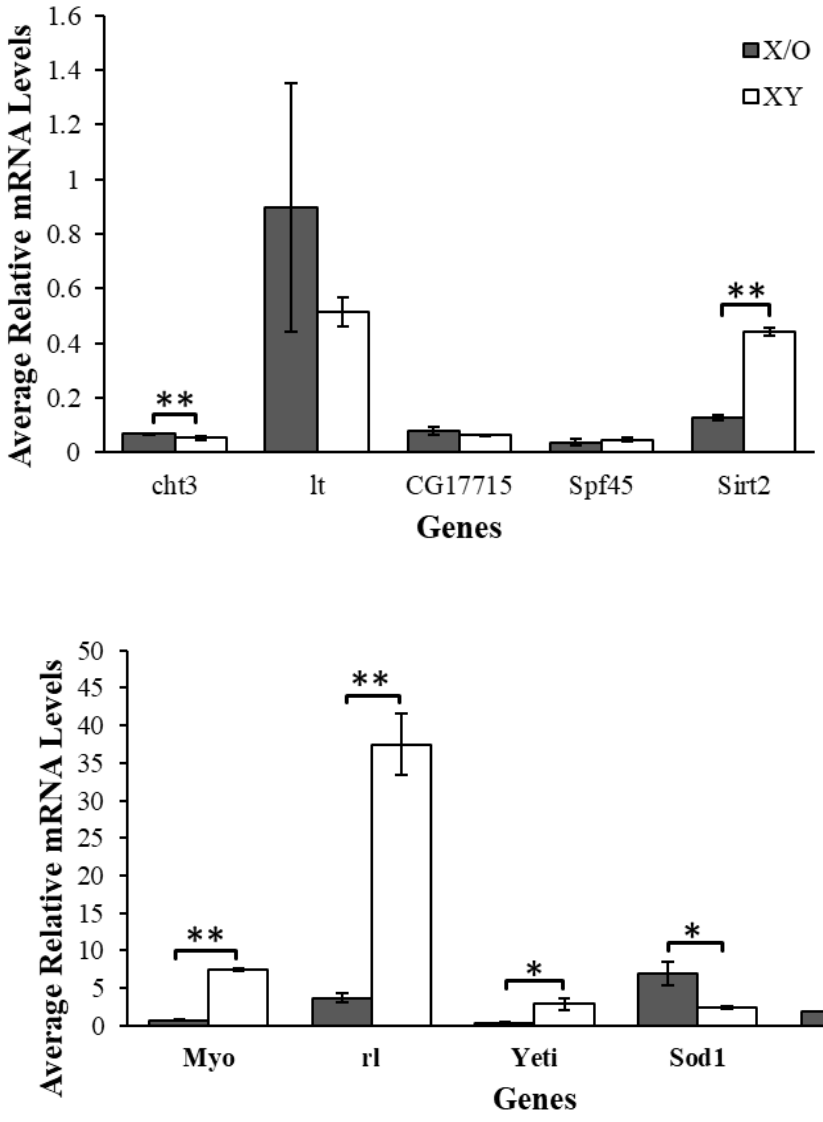
IV.4 Differential gene expression profiles of 60-days karyotypes

We next sought to confirm the differential gene expression patterns seen in 2-days karyotypes as discussed previously by deploying 60-days flies from the same $y^1/Dp(1;Y)B^s$ line for qRT-PCR analysis to determine changes in the gene expression profiles of the old flies. The two aging genes, *Sod1* and *Sod2*, as well as the heterochromatic gene, *Cht3*, were significantly overexpressed in X/O males relative to XY flies. In addition, *lt*, as a heterochromatic gene, also had a considerable but non-significant overexpression level in X/O males relative to XY males. However, the aging genes, *Sirt2* and *Myo*, and the heterochromatic genes, *rl* and *Yeti*, were significantly overexpressed in XY males than X/O males, a result that is inconsistent with our prediction which suggested overexpression of these genes in X/O males. The other two heterochromatic genes, *CG17715* and *Spf45*, experienced nearly no changes in their expression levels between X/O and XY males (Figure IV.4A).

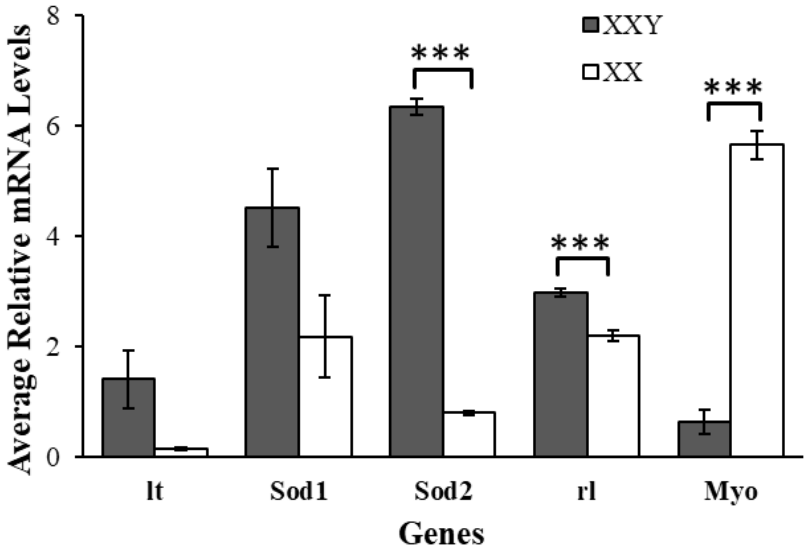
In addition, *Sirt2* and *Myo* showed a statistically significant upregulation while *Parp* had a statistically non-significant but considerable increase in their expression levels in 60-days XX females, as compared to XXY females (Figure IV.4B). However, the heterochromatic genes, *Cht3*, *CG17715*, and *rl*, along with the aging gene, *Sod2*, were significantly overexpressed in XXY females as compared to XX females. In addition, the heterochromatic genes, *Spf45*, *Yeti*, *lt*, and the aging gene, *Sod1*, showed non-significant increased expression levels in XXY females (Figure IV.4B). Moreover, the comparison of the gene expression profiles between X/O and XY males and XX and XXY females at 60 days reveal a general pattern that suggests where the removal of the Y chromosome is associated with an increase in the expression level of a gene, the addition of Y chromosome will reverse the expression pattern for the same gene (see Table IV.2A). This inconclusive result will require further investigation to uncover conclusions about the direction of

changes in the gene expression pattern that might be associated with the changes in the heterochromatin levels in these flies.

A)



B)


Average Relative mRNA Levels
8
6
4
2
0
XXY
XX
***
***
***
lt
Sod1
Sod2
rl
Myo
Genes

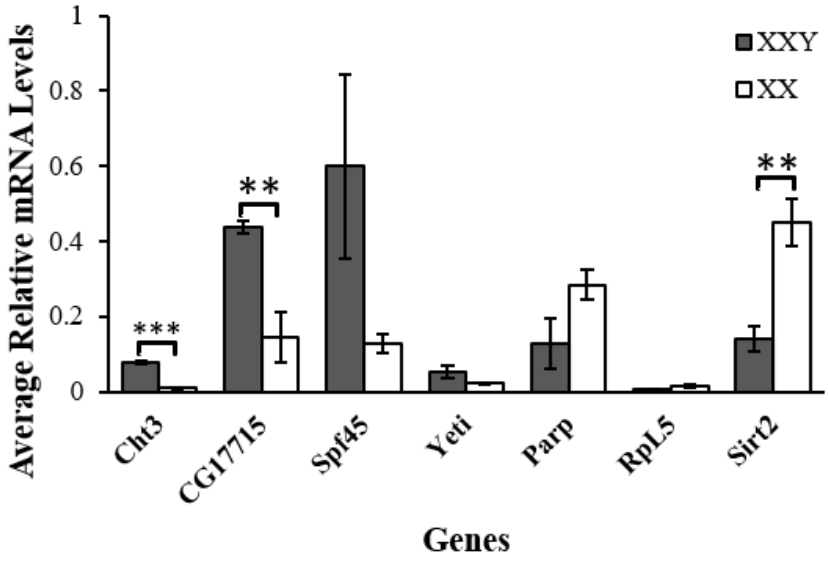

Average Relative mRNA Levels
1
0.8
0.6
0.4
0.2
0
XXY
XX
***
**
**
Cht3
CG17715
Spf45
Yeti
Parp
RpL5
Sirt2
Genes

Figure IV.4: Average relative mRNA levels of heterochromatic and aging genes in 60-days old karyotypes.

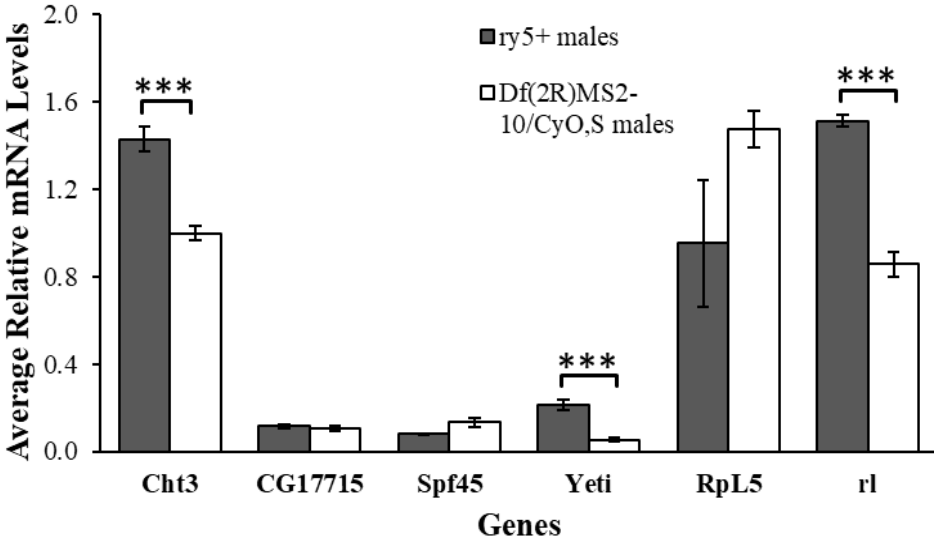
Quantitative RT-PCR was used to measure the expression levels of the heterochromatic and aging-associated genes in 60-days old flies to confirm the differential pattern of gene expression as previously seen in 2-days old different karyotypes. Figures **A)** and **B)** depict the average relative mRNA levels of X/O vs XY males and XX vs XXY females from $y^1/Dp(1;Y)B^s$ stock, respectively. Data represent mean ± SEM of five technical replicates relative to the BTub 56D (control) gene. Student's t.test (two-tailed and unequal variance) was used to determine the statistical significance ($P < 0.05$, $P < 0.01$, and $P < 0.001$) as denoted by $*$, $**$, and $***$ on the error bars, respectively.

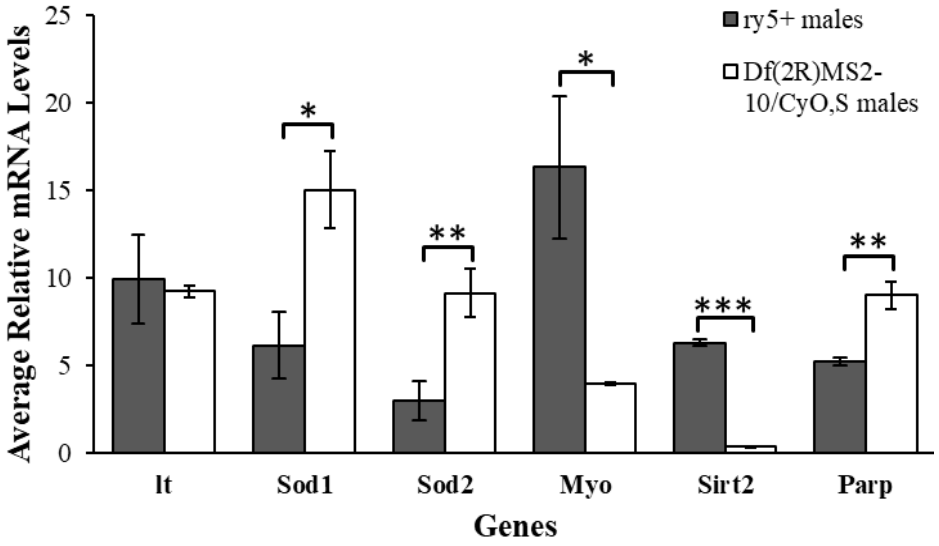
IV.5 Confirmation of differential gene expression profiles upon heterochromatin removal

Next, we deployed Df(2R)MS2-10/CyO, S flies to explore the effects of the deletion of a heterochromatic region from the right arm of chromosome 2 on the gene expression profiles and to see whether the deleted region would induce similar changes in the transcriptional activity of the heterochromatic and aging-associated genes in comparison to the changes in genes expression observed with the Y chromosome removal in X/O males relative to the wild-type XY males of y^1/Dp(1;Y)B^s stock. The gene expression levels of the male and female flies from Df(2R)MS2-10/CyO, S stock were compared to those of the wild-type rosy (ry^{5+}) male and female flies, respectively. QRT-PCR analysis of 20-days old male flies showed significant overexpression of the two aging genes, *Sod1* and *Sod2*, and the heterochromatic gene, *Parp*, in addition to the non-significant increase in expression of *RpL5* and *Spf45* in Df(2R)MS2-10/CyO, S males relative to the wild-type ry^{5+} male flies (Figure IV.5A). However, the heterochromatic genes, *Cht3*, *Yeti*, and *rl* as well as the aging genes, *Myo*, and *Sirt2* were significantly overexpressed in the wildtype ry^{5+} males relative to the deficient males. No noticeable changes in the transcriptional activity of *lt* and *CG17715* genes were detected between the deficient and the wild-type males (Figure IV.5A). Similar findings were obtained from 20-day old females from the same stocks; the heterochromatic genes, *RpL5*, *Parp*, and the aging genes, *Myo* and *Sod2*, had higher average mRNA levels in the deficient females relative to the wild-type females while the heterochromatic genes *CG17715*, and *Spf45* and the aging gene *Sirt2* were overexpressed in the wild-type ry^{5+} female flies relative to Df(2R) MS2-10/CyO, S females (Figure IV.5B). The comparison of the gene expression profiles between the deficient and the wild-type males, and the deficient and the wild-type females did not reveal any general trend of either upregulation or downregulation of the heterochromatic and aging

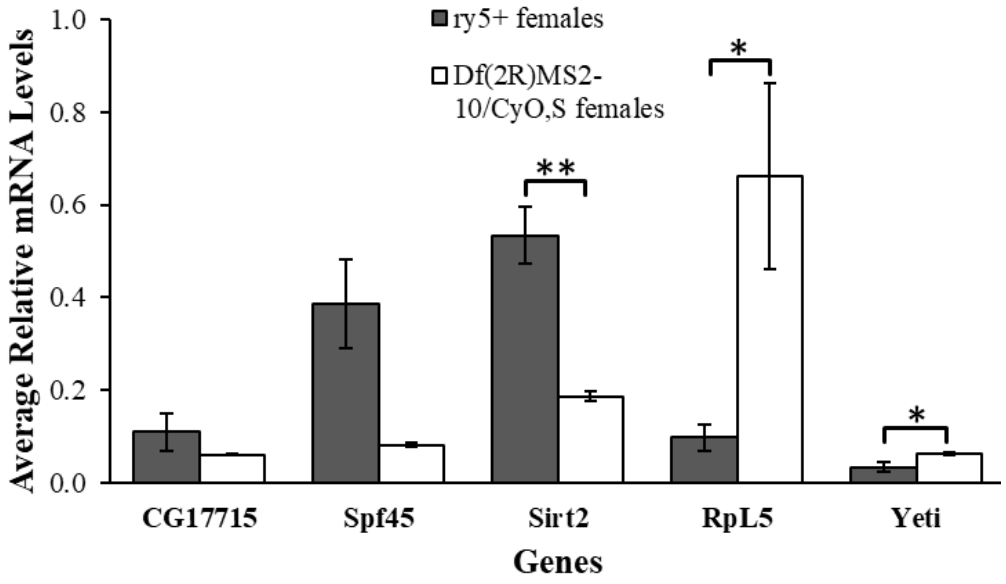
genes when the heterochromatic portion from the 2R chromosome was deleted (see Table IV.2B). Therefore, our data depicted the impact of changes in the heterochromatin levels on the expression pattern of heterochromatic and aging-associated genes. Our study showed the removal or addition of the Y chromosome induces changes in the expression levels of these genes in our experiment. Whether the removal of the Y chromosome upregulates or downregulates gene activity could be studied extensively for a greater number of genes in more detail in the future to uncover the pattern of the changes in the expression levels of a broad range of heterochromatic and aging-associated genes in different X/O, XY, XX, and XXY karyotypes.

A)


Average Relative mRNA Levels
2.0
1.6
1.2
0.8
0.4
0.0
ry5+ males
Df(2R)MS2-10/CyO,S males
***
***
***
Cht3
CG17715
Spf45
Yeti
RpL5
rl
Genes


Average Relative mRNA Levels
25
20
15
10
5
0
ry5+ males
Df(2R)MS2-10/CyO,S males
*
*
**
**
***
lt
Sod1
Sod2
Myo
Sirt2
Parp
Genes

B)


ry5+ females
Df(2R)MS2-10/CyO,S females
Average Relative mRNA Levels
1.0
0.8
0.6
0.4
0.2
0.0
**
*
*
CG17715
Spf45
Sirt2
RpL5
Yeti
Genes

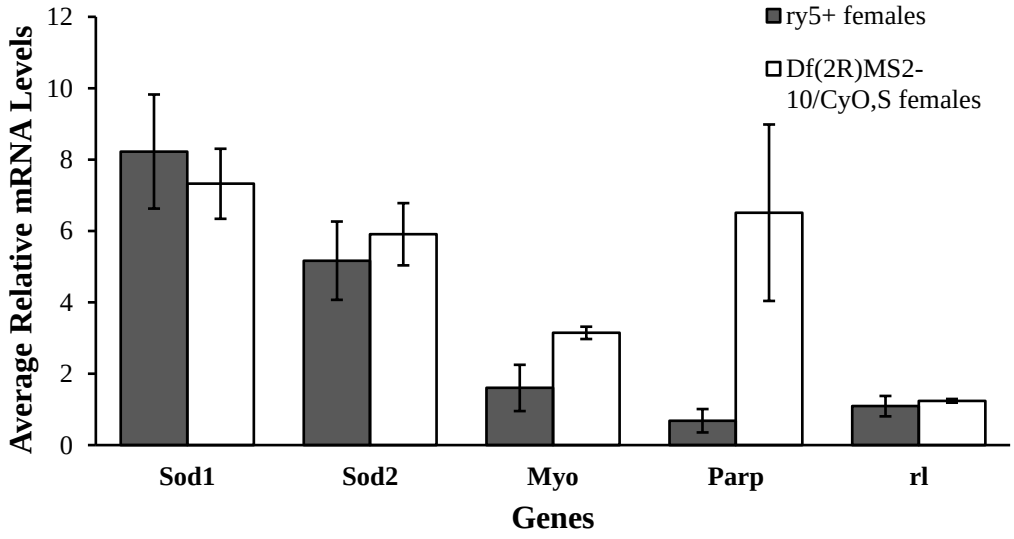

ry5+ females
Df(2R)MS2-10/CyO,S females
Average Relative mRNA Levels
12
10
8
6
4
2
0
Sod1
Sod2
Myo
Parp
rl
Genes

Figure IV.5: Average relative gene expression profiles of heterochromatic and aging genes in Df(2R)MS2-10/CyO, S, and ry^{5+} flies.

These figures depict the quantitative RT-PCR analysis to compare the average mRNA levels of a number of the heterochromatic and aging genes in 20-days old flies between **A)** Df(2R)MS2-10/CyO, S and ry^{5+} males, and **B)** Df(2R)MS2-10/CyO, S and ry^{5+} females. Data represent mean ± SEM of five replicates relative to the BTub 56D (control) gene. ∗**,** ∗∗**,** and ∗∗∗ on the error bars denote statistical significance ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively) of the changes in the relative expression levels as determined by the student's t.test (two-tailed, unequal variance).

Table IV.2: Comparison of gene expression levels among 60-days $y^1/Dp(1;Y)B^s$ karyotypes and 20-days Df(2R)MS2-10/CyO, S vs ry^{5+} flies.

Data represent the ratio of the relative gene expression levels between **A)** 60-days X/O vs XY males, and XX vs XXY females from $y^1/Dp(1;Y)B^s$ stock and **B)** 20-days Df(2R)MS2-10/CyO, S vs ry^{5+} males and Df(2R)MS2-10/CyO, S vs ry^{5+} females. An increase or a reduction in the relative gene expression levels in comparison to BTub 56D gene are shown by values greater than one or less than one, respectively. The result demonstrates overall changes in the relative transcriptional activity of most heterochromatic and aging-related genes due to variation in the amounts of heterochromatin as seen by the removal or addition of a copy of the Y chromosome or deletion of 2R region. $*$, $**$, and $***$ on the error bars denote statistical significance ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively) of the changes in the relative expression levels as determined by the student's t.test (two-tailed, unequal variance.

A)

y1/Dp(1;Y)Bs						
Gene Name	**Type of Gene**	**Flies Age**	**X/O/XY Males**	**Change in Relative Expression Level**	**XX/XXY Females**	**Change in Relative Expression Level**
Cht3	Heterochromatin	60 days	1.26	Increased**	0.14	Decreased***
lt	Heterochromatin	60 days	1.75	Increased	0.16	Decreased
CG17715	Heterochromatin	60 days	1.22	Increased	0.33	Decreased**
Spf45	Heterochromatin	60 days	0.79	Decreased	0.21	Decreased
rl	Heterochromatin	60 days	0.10	Decreased**	0.74	Decreased***
Yeti	Heterochromatin	60 days	0.12	Decreased*	0.43	Decreased
Parp	Heterochromatin	60 days	N/A	N/A	2.22	Increased
RpL5	Heterochromatin	60 days	N/A	N/A	0.11	Decreased
Sod1	Aging gene	60 days	2.88	Increased*	0.48	Decreased
Sod2	Aging gene	60 days	3.01	Increased*	0.13	Decreased***
Myo	Aging gene	60 days	0.10	Decreased**	8.86	Increased***
Sirt2	Aging gene	60 days	0.29	Decreased**	3.23	Increased**

B)

Df(2R)Ms2-10/CyO, S / ry5+						
Gene Name	**Type of Gene**	**Flies Age**	**Males Expression Rtio**	**Change in Relative Expression Level**	**Females Expression Ratio**	**Change in Relative Expression Level**
Cht3	Heterochromatin	20 days	0.70	Decreased***	N/A	N/A
lt	Heterochromatin	20 days	0.93	No Change	N/A	N/A
CG17715	Heterochromatin	20 days	0.89	Decreased	0.56	Decreased
Spf45	Heterochromatin	20 days	1.64	Increased	0.21	Decreased
rl	Heterochromatin	20 days	0.57	Decreased***	1.14	Increased
Yeti	Heterochromatin	20 days	0.26	Decreased***	1.84	Increased*
Parp	Heterochromatin	20 days	1.73	Increased**	9.54	Increased
RpL5	Heterochromatin	20 days	1.55	Increased	6.72	Increased*
Sod1	Aging gene	20 days	2.45	Increased*	0.89	Decreased
Sod2	Aging gene	20 days	3.09	Increased**	1.14	Increased
Myo	Aging gene	20 days	0.24	Decreased*	1.96	Increased
Sirt2	Aging gene	20 days	0.05	Decreased***	0.35	Decreased**

V. DISCUSSION

The Y chromosome in *Drosophila melanogaster* is almost entirely composed of heterochromatin which includes 20 % of the entire haploid genome in males (Brown et al., 2020). It mainly consists of repetitive satellite DNA, transposable elements (TEs), and ribosomal DNA (rDNA) (Bonaccorsi and Lohe, 1991). Although the Drosophila Y chromosome contains a few functional genetic loci that are mainly involved in male reproduction, the main bulk is transcriptionally inactivated through heterochromatin formation (Elgin and Reuter, 2013). It is well known that changes in the Y chromosome are linked to PEV (Gowen and Gay, 1934) but more recent studies have demonstrated that changes in the Y chromosome copy numbers induce significant changes in the regulation of many genes genome-wide (Brown et al., 2020).

Based on the speculation that males live shorter than females in many vertebrates and invertebrates, including the Drosophila flies due to having a Y chromosome (Tower and Arbeitman, 2009; Yoon et al., 1990; Marias et al., 2018), we predicted a longer lifespan for X/O males and XX females than their XY and XXY counterparts. We also hypothesized that X/O flies would have the longest lifespan followed by XX females which have more heterochromatin than X/O males due to the heterochromatic X chromosome, and the XY males and XXY females with the shortest lifespan. To test our prediction, we used flies from y^1/Dp(1;Y)B^s stock which contained females with yellow phenotype and males carrying a duplication of the Bar Stone marker on the Y chromosome. We applied the principles of classic genetics to create the different karyotypes from the above isogenic stock by taking advantage of meiotic non-disjunction. The average longevity test results showed X/O males and XX females lived significantly longer than XY males, supporting consistency with our prediction and the results of other studies which have shown significantly increased longevity of females compared to male flies in 53 strains out of 89 strains

and 68 species of Drosophila (Yoon et al., 1990). However, XXY females surprisingly had the highest longevity score. In addition, the longer lifespan of XX females than X/O males contradicted our predication as well as the findings from another study with Canton S flies which demonstrated the highest longevity score for X/O males due to the sterility of X/O flies and having the least amount of the repetitive DNA (Brown et al., 2020). Since these karyotypes were the progenies from self-cross of the y^1/Dp(1;Y)B^s flies, variation in genetic background is ruled out for this contradiction as these flies are isogenic for autosomal chromosomes, and are only different from each other in terms of either the presence or absence of Y chromosome. Moreover, similar longevity results from three different strains of *Drosophila* suggest that the genetic background is not a determining factor in longevity difference, but the presence or absence of the Y chromosome is responsible for this variation in lifespan measurement (Brown et al., 2020). In addition, the absence of active genetic loci on the Y chromosome, except for a few fertility factors, further reduces the genetic variation that might arise from the addition or removal of the Y chromosome among these different karyotypes. However, we can not completely ignore the possible impacts on the longevity of X/O males that might arise from their sterility and the defects in the process of spermatogenesis. Similarly, a small sample size is another key factor that could be responsible for the deviation of longevity results obtained from this study from our predictions and the results of other studies. It is possible to improve our findings from the average lifespan measurement by utilizing a sufficiently larger sample size for each karyotype to achieve a more reliable data analysis, something that has been done with the other longevity studies (Brown et al., 2020). Therefore, the inconclusive results from average lifespan measurement of the different karyotypes suggested an overall increased longevity of X/O males and XX females, as compared to XY males,

supporting literature findings that the removal of Y chromosome enriched in repeat DNA sequences extends longevity (Brown et al., 2020).

In addition, we performed the qRT-PCR analysis of 2-days and 60-days flies from y1/Dp(1;Y)B^s stock to extrapolate the expression profiles of the heterochromatic and euchromatic aging-associated genes. Contrary to our hypothesis that suggested an overall increase in the relative average gene expression levels upon the Y chromosome removal, we surprisingly found overall changes in the expression levels of most of the studied genes in X/O vs XY male, and XX vs XXY female comparisons, a finding that has been supported by another study that has shown the differential patterns of expression for hundreds of genes between these various karyotypes of *D. melanogaster* (Brown et al., 2020). While some genes showed dramatic increases in gene expression in the X/O karyotype relative to XY males, some others were significantly overexpressed in XY males relative to X/O males. The changes in the relative expression levels were also evident for XX and XXY females. Interestingly, the 2-days old X/O males showed a general downregulation of heterochromatic genes and the upregulation of aging genes relative to XY males, but this trend was not observed with XX and XXY females.

Interestingly, the comparison of the ratio of the relative gene expression levels in X/O and XY males (X/O/XY) with that of the XX and XXY females (XX/XXY) in 2-days and 60-days flies suggests where the removal of the Y chromosome might induce the upregulation of a gene in X/O males relative to XY males, adding a copy of Y chromosome might cause the downregulation of the same gene in XXY females relative to the normal XX females. This inconclusive result can be further extrapolated in the future to determine the increased or reduced transcriptional activity of these genes that might be associated with the variations in the heterochromatic levels. Furthermore, comparing the qRT-PCR data from 20-days old Df(2R)MS2-10/CyO, S males and

females with that of the wild-type ry^{5+} males and females, respectively uncovered changes in the expression profiles of most of the genes investigated in this study; some genes were significantly upregulated in the Df(2R)MS2-10/CyO, S flies while some other genes were significantly upregulated in the wild-type ry^{5+} flies.

Therefore, our results from the gene expression analysis demonstrated that changes in the amount of heterochromatin, as depicted in this study by the addition or removal of the Y chromosome that is enriched in repeat sequences or the deletion of 2R region in Df(2R)MS2-10/CyO, S flies, induce overall changes in the expression levels of the heterochromatic and aging-associated genes. Whether the removal of the Y chromosome causes overexpression of either the heterochromatic genes or the aging genes or both remains unclear and requires transcriptomic analysis, such as RNA-Sequencing, to encompass a much broader range of heterochromatic genes as well as aging-associated genes to elucidate a general trend in the gene expression profiles.

Early studies with the modifiers of PEV in *D. melanogaster* have supported the notion that many of these modifiers might have opposing effects depending on the heterochromatin-euchromatin context. For example, suppressors of the PEV of euchromatic genes that are inserted into heterochromatin are found to enhance PEV of heterochromatic genes since they encode structural components of heterochromatin that affect the chromatin condensation and are required for the normal function of the heterochromatin genes (Hearn et al., 1991). Further studies have revealed that these modifiers such as Su(var) gene products affect the expression of heterochromatic genes (like *lt*) even in its normal heterochromatic location and in the absence of any variegating rearrangement (Clegg et al., 1998). Previous studies have also shown that changing the amount of heterochromatin affects PEV; duplication of heterochromatic materials generally suppresses PEV whereas the deletion of heterochromatic material enhances PEV (Elgin and

Reuter, 2013). Y chromosome, as a large bulk of heterochromatin, poses the same impact on the PEV of the euchromatic genes (Gatti and Pimpinelli, 1992). Considering all these findings, our hypothesis predicted changes in the relative overexpression of the tested heterochromatic genes as well as the age-related genes upon the removal of the Y chromosome. This prediction was based on the findings that Y chromosome polymorphism (changes in the number of repeat sequences within the Y chromosome) affects the global amount of heterochromatin in the genome and, therefore impacts the expression levels of hundreds of genes genome-wide by redistributing heterochromatin proteins such as HP1 and other important chromosomal proteins that are present in the nucleus in limited quantities (Elgin and Reuter, 2013). In other words, the repetitive sequences on the Y chromosome are enriched in heterochromatin proteins and repressive histone modification markers. Y chromosome acts as a "heterochromatin sink" which sequesters the core components of heterochromatin machinery such as the structural proteins or histone-modifying enzymes that play important roles in heterochromatin formation and its maintenance. The result is the depletion of these heterochromatin proteins from their binding sites, and suppression of the other repetitive regions of the genome due to the presence of the Y chromosome (Brown et al., 2020). The protein-coding genes on the Y chromosome are only expressed in male germ cells and are absent from somatic cells of adult tissues. Therefore, the effects of variation in the amount of repetitive DNA on the Y chromosome or changes in the copy number of the Y chromosome on the global expression of a large number of genes genome-wide, as seen with XXY females and XY males can be attributed to the sink effect of the Y chromosome which indirectly redistributes heterochromatin components across the genome (Gatti and Pimpinelli, 1992). The heterochromatin sink model assumes that variation in the amount of repetitive DNA changes the levels of heterochromatin formation genome-wide. This means that increasing the levels of

repetitive DNA causes decreased levels of heterochromatin components at the repetitive regions because the added repeats sequester the heterochromatin factors that are available to other repetitive DNA sequences in a limited amount. The decrease in the amount of repetitive DNA is expected to have the opposite effect (Brown et al., 2020).

Among the heterochromatin proteins, HP1 produced by the Su(var)2-5 gene plays a key role in heterochromatin formation and euchromatic gene silencing. While most of HP1 is found in the heterochromatic regions, some forms, such as HP1γ, are localized at different regions of the euchromatin (James et al., 1989). HP1 causes changes in the variegation of both euchromatic and heterochromatic genes in a dosage-dependent manner (Piacentini et al., 2003). However, contrary to the euchromatic gene silencing role of HP1, recent studies have shown that HP1 positively regulates expression of developmental and heat shock puffs such as Hsp70, pinpointing the multi-complex role of this protein which depends on the chromosomal context (Piacentini et al., 2003). As the genetic studies have shown, the transcriptional activity of heterochromatic genes requires the heterochromatic environment, and HP1 is shown to mediate this transcriptional activity as it is required for heterochromatin formation. In contrast, HP1 is shown to enhance the variegation of euchromatic genes that are placed near the heterochromatin-euchromatin boundary and mediate the silencing of some euchromatic genes in their natural genomic context in the absence of any chromosomal rearrangement (Hwang et al., 2001). It appears that the dynamic activity of HP1 in enhancing PEV, the repression of euchromatic genes, and the induction of heterochromatic genes all depend on the role of HP1 in heterochromatin formation. However, contrary to the repressive role of HP1 in euchromatic gene silencing, recent studies have also linked HP1 activity to the induction of euchromatic gene encoding hsp70 in the larvae of *D. melanogaster*. It is shown that HP1 is positively associated with the expression of the developmental and heat-shock-induced

puffs on polytene chromosomes as these puffs reveal intense gene activity (Piacentini et al., 2003). Therefore, the redistribution of the heterochromatin components, more specifically HP1 protein, is believed to be the main factor that contributes to the differential expression profiles of the heterochromatic and aging-associated genes among different karyotypes of *D. melanogaster* that either lack the Y chromosome or contain an extra copy of it.

VI. CONCLUSION AND PERSPECTIVES

In conclusion, we speculated that variation in the heterochromatin levels as depicted by changes in the copy number of the Y chromosome in this study is not only associated with the differences in the average lifespan measurement of the different karyotypes of y^1/Dp(1;Y)B^s stock but it is also responsible for the relative differential gene expression profiles of these karyotypes as well as the Df(2R)MS2-10/CyO, S males and females as compared to the wild-type ry^{5+} counterparts. This variation in heterochromatin levels is assumed to induce the redistribution of the heterochromatin proteins like HP1 which could be linked to the observed differences in the longevity and the differential expression profiles of many genes genome-wide. Future studies can employ RNA-Seq to consider a genome-wide analysis of the known heterochromatic and aging-associated genes in *D. melanogaster* to reveal more conclusive results with the differential gene expression profiles in the investigated karyotypes.

This study highlights the important role of heterochromatin as a key component of all higher eukaryotic cells in the aging process through the regulation of heterochromatic and aging-associated genes in *D. melanogaster*. It also contributes to the significance of heterochromatin in the literature, a topic that has received little appreciation despite the long history of heterochromatin discovery. Moreover, our study uncovered a possible link between changes in the gene expression pattern and the differences in the longevity of flies as the result of changes in heterochromatin levels. Future studies can explore the molecular mechanisms of this association to reveal the factors that determine the longevity of flies when the transcriptional activity of the genes alters as the result of variation in heterochromatin levels.

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