

**Search for genes that can modify heterochromatic  
gene expression in *Drosophila melanogaster***

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## Abstract

Transcription of genes is sometimes believed to be physically blocked by a chromatin state known as heterochromatin. This is not totally true because there are genes in *Drosophila melanogaster* that are found in constitutive heterochromatin and do get transcribed. The study of these heterochromatic genes could lead to new knowledge about gene expression.

Heterochromatin is also interesting because of its potential connection to aging. Chromatin gets perturbed with age, and there are some cases where heterochromatin manipulations extend an organism's lifespan. This project identifies some mutants that can modify the expression of the *Drosophila melanogaster* heterochromatic gene light (*lt*), and a new method for extending the lifespan of *D. melanogaster* by removing some constitutive heterochromatin from chromosome 2.

## Contents

Abstract.....	ii
Contents.....	iii
List of Abbreviations.....	iv
Introduction.....	1
Methods.....	24
Results.....	32
Discussion.....	41
References.....	44
Appendix.....	56

### List of abbreviations

ACD = ACD shelterin complex subunit and telomerase recruitment factor; ADP = adenosine diphosphate; AGE = advanced glycation end product; AGO3 = Argonaute 3; AKT = AKT kinase; AKT1 = AKT serine/threonine kinase 1; AKT3 = AKT serine/threonine kinase 3; AMP = adenosine monophosphate; AMPK = AMP-activated protein kinase; APOE = apolipoprotein E; AT = the nitrogenous bases adenine and thymine; ATM = ATM serine/threonine kinase; bp = base pairs; CETP = cholesteryl ester transfer protein; CDK4/6 = cyclin dependent kinase 4 or cyclin dependent kinase 6; da = daughterless; DNA = deoxyribonucleic acid; FANCE = Fanconi anemia complementation group E; foxo = forkhead box, sub-group O;  $\gamma$ H2AX = H2A histone family member X that has received a post-translational modification of a phosphate group to a specific serine residue; H1 = histone 1; H2A = histone 2A; H2B = histone 2B; H3 = histone 3; H3K9 = the lysine amino acid in histone 3 that is modified in H3K9ac, H3K9me2 and H3K9me3; H3K9ac = a post-translational modification of histone 3 that has an acetyl group attached to a specific lysine residue; H3K9me2 = a post-translational modification of histone 3 that has 2 methyl groups attached to a specific lysine residue; H3K9me3 = a post-translational modification of histone 3 that has 3 methyl groups attached to a specific lysine residue (the same residue as H3K9me2); H3K23ac = a post-translational modification of histone 3 that has an acetyl group attached to a specific lysine residue; H3K27me3 = a post-translational modification of histone 3 that has 3 methyl groups attached to a specific lysine residue; H3K36me3 = a post-translational modification of histone 3 that has 3 methyl groups attached to a specific lysine residue; H3S10ph = a post-translational modification of histone 3 that has a phosphate group attached to a specific serine residue; H4 = histone 4; H4K20me3 = a post-translational modification of histone 4 that has 3 methyl groups attached to a specific lysine residue; HGPS =

Hutchinson-Gilford progeria syndrome; HP1 = Heterochromatin protein 1; HP1a = Heterochromatin protein 1a; INK4/ARF = p16; JIL-1 = JIL-1 kinase; kuk = kugelkern; Lam = *Drosophila melanogaster* gene called Lamin; LDL = low-density lipoprotein; LIPL-4 = lipase like 4; LKB1 = liver kinase B1; LMNA = lamin A/C; lt = *Drosophila melanogaster* gene called light;  $\mu\text{m}$  = micrometre; mof = males absent on the first; mRNA = messenger RNA; msl-1 = male-specific lethal 1; MTOR = mechanistic target of rapamycin;  $\text{NAD}^+$  = nicotinamide adenine dinucleotide; nm = nanometre; p16 = cyclin dependent kinase inhibitor 2A; p21 = cyclin dependent kinase inhibitor 1A; p53 = tumor protein p53; PARP = Poly-(ADP-ribose) polymerase; PDPK1 = 3-phosphoinositide-dependent protein kinase-1; PEV = position-effect variegation; PIK3CA = phosphatidylinositol 3-kinase, catalytic, alpha; piRNAs = Piwi-interacting RNAs; PI3K = phosphoinositide 3-kinase; POT1 = protection of telomeres 1; PTEN = phosphatase and tensin homolog; RAGE = Receptor for advanced glycation end-products; Rb = RB transcriptional corepressor 1; RISC = RNA-induced silencing complex; RNA = ribonucleic acid; RNAi = RNA interference; roX1 = long non-coding RNA on the X 1; RT-PCR = reverse transcriptase – polymerase chain reaction; SASP = senescence-associated secretory phenotype; Sirt1 = Sirtuin 1; Sod1 = Superoxide dismutase 1; SOD2 = superoxide dismutase 2; tau = tubulin associated unit; TERC = telomerase RNA component; TERF1 = telomeric repeat binding factor 1; TERF2 = telomeric repeat binding factor 2; TERF2IP = TERF2 interacting protein; TERT = telomerase reverse transcriptase; TFEB = transcription factor EB; TINF2 = TERF1 interacting nuclear factor 2; tj = traffic jam; TORC1 = target of rapamycin complex I; Trp53bp1 = transformation related protein 53 binding protein 1; ULK1 = UNC-51-like kinase 1; woc = without children; WRN = WRN RecQ like helicase; X0 = an organism with one X chromosome

as the only sex chromosome per diploid cell; XPA = XPA, DNA damage recognition and repair factor;

## Introduction

### Heterochromatin

In eukaryotes, nuclear DNA is bound by many proteins. The complex of DNA and proteins is called chromatin. Chromatin is divided into 2 types: heterochromatin and euchromatin. This model is simplistic. More complicated and possibly more accurate models of chromatin architecture are refinements of this basic model.<sup>1</sup> The simple euchromatin and heterochromatin model remains as the model verified in many eukaryotic species.

Before describing heterochromatin in detail, chromatin in general will be described.

Nucleosomes can be found in eukaryotes.<sup>2</sup> There are 5 classes of histones: H1, H2A, H2B, H3, and H4. H1 is the linker histone, and the other four are the core histones. One of the functions of histones is to pack DNA.<sup>2</sup> The nucleosome is the repeating unit of chromatin and is composed of an octamer of proteins and about 200 bp of DNA wrapped around it.<sup>2</sup> About 146 bp of DNA is in contact with the octamer and the rest forms a link to the next octamer.<sup>2</sup> The protein octamer is made of 2 copies each of the 4 core histones.<sup>2</sup> The histone octamer and the DNA in contact with the octamer is called the nucleosome core particle. H1, the linker histone, binds to nucleosomes and promotes formation of the 30-nm filament.<sup>2</sup> H1 can bind to the DNA between histone octamers, and it can lower mobility of nucleosomes.<sup>2</sup> Nucleosomes at promoters can help regulate transcription.<sup>2</sup> Transcription elongation can occur on DNA that has nucleosome core particles.<sup>2</sup> Nucleosomes are found in both euchromatin and heterochromatin.<sup>3</sup>

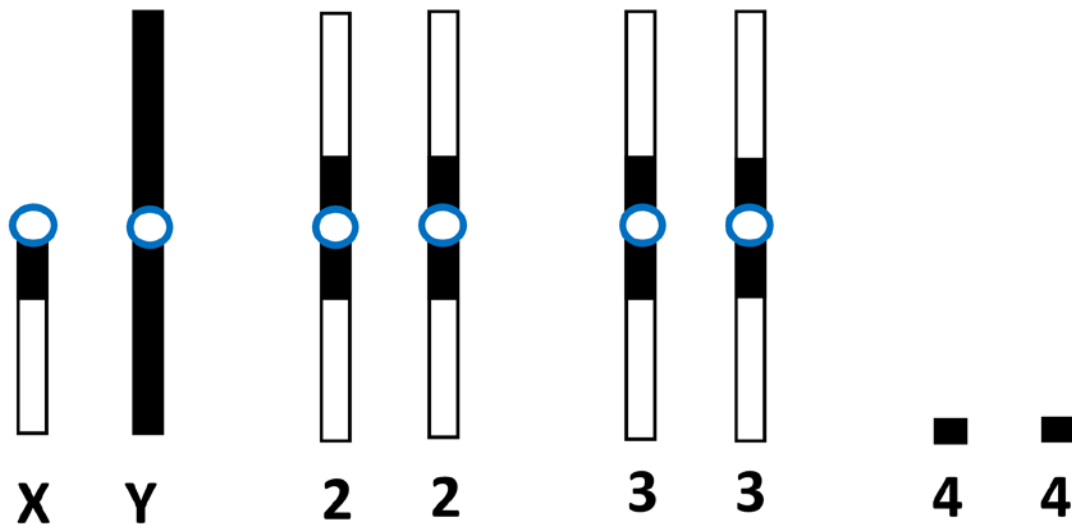
Heterochromatin is distinguished from euchromatin because heterochromatin has these properties. Heterochromatin is denser than euchromatin (density in this case is base pairs /  $\mu\text{m}^3$ ).<sup>4</sup>

Heterochromatin replicates after euchromatin.<sup>1,5</sup>

One of the functions of heterochromatin is maintaining chromosome alignment prior to segregation during mitosis or meiosis.<sup>3</sup> Centromeres require heterochromatin.<sup>3</sup>

One of the functions of heterochromatin may be to trap transposable elements.<sup>3</sup> Transposable elements are DNA sequences that can change their location within a genome. Transposable elements are widespread within the eukaryotes.<sup>6</sup> There are 2 classes of transposable elements, retrotransposons and DNA transposons.<sup>6</sup> Retrotransposons get transcribed, then reverse transcribed, then the new DNA gets integrated into the genome.<sup>6</sup> DNA transposons get cut out, then integrated into the genome.<sup>6</sup>

Heterochromatin can be subdivided in various ways. One of the ways of describing a type of heterochromatin is as constitutive or facultative heterochromatin. Constitutive heterochromatin is a region of a chromosome that is always in the heterochromatic chromatin state.<sup>7</sup> Constitutive heterochromatin prevents double-strand breaks, and homologous recombination between 2 sites that have a similar DNA sequence but are not alleles.<sup>7</sup> Constitutive heterochromatin tends to be found near centromeres and telomeres.<sup>7</sup> The constitutive heterochromatin of *Drosophila melanogaster* can be seen in metaphase chromosomes as preferentially stained regions after applying a particular staining procedure.<sup>8</sup> *D. melanogaster* constitutive heterochromatin covers the Y chromosome, the 4<sup>th</sup> chromosome, one end of the X chromosome, and around the centromeres of chromosomes 2 and 3 (**Figure 1**).<sup>8</sup> Pericentric heterochromatin is the constitutive heterochromatin found next to centromeres. The DNA of constitutive heterochromatin is enriched with repetitive sequences.<sup>3,9</sup>



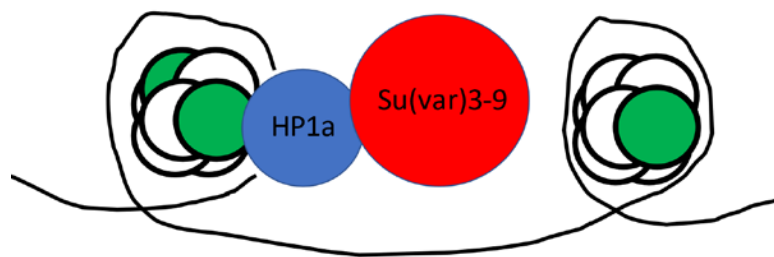
**Figure 1.** The approximate distribution of constitutive heterochromatin on the *Drosophila melanogaster* chromosomes. The symbols at the bottom indicate the names of chromosomes. White circles with blue borders represent centromeres and they are omitted from chromosome 4 in this figure. The black areas are constitutive heterochromatin. The white areas are considered euchromatic regions of the genome.

A type of heterochromatin other than constitutive heterochromatin is facultative

heterochromatin. Facultative heterochromatin contains genes that can be regulated.<sup>7</sup> Facultative heterochromatin is dense, transcriptionally silent, and capable of converting to euchromatin.<sup>10</sup> At a molecular level, facultative heterochromatin is often distinct from constitutive heterochromatin. Facultative heterochromatin can be a chromatin state that is identified by enrichment of H3K27me3 and Polycomb.<sup>10</sup>

Histones can be post-translationally modified, which often occurs on their N-terminal tails.<sup>2,3</sup> H3K9me3 is found in constitutive heterochromatin.<sup>1</sup> H3K9me3 is histone 3 that has been post-translationally modified. The modification of H3K9me3 is trimethylation at a lysine residue near the protein's N-terminus. The methylation of histone 3 at this position is catalyzed by a histone methyltransferase, the histone methyltransferase is encoded by the *Drosophila* gene called

Su(var)3-9.<sup>11</sup> Another protein that is found in constitutive heterochromatin is Heterochromatin protein 1 (HP1a).<sup>12,13</sup> HP1a binds to H3K9me3.<sup>14</sup> Su(var)3-9 mutants have less H3K9me2 and H3K9me3 at pericentric heterochromatin, and also less HP1a at pericentric heterochromatin.<sup>13</sup> HP1a is a structural part of heterochromatin.<sup>3</sup> HP1a can also bind to Su(var)3-9.<sup>3,15</sup> Su(var)3-9 localizes to heterochromatin, and HP1a is required for this.<sup>3,15</sup> The combination of HP1a, Su(var)3-9, and H3K9me3 may lead to the phenomenon of heterochromatin spreading.<sup>3</sup> These components lead to a cycle when Su(var)3-9 binds to HP1a and creates new H3K9me3, then more HP1a binds to the new H3K9me3, and Su(var)3-9 can shift to the new heterochromatin/euchromatin boundary (**Figure 2**). Histone H1 binds to Su(var)3-9 and HP1.<sup>16</sup> Histone H1 promotes the enzymatic activity of Su(var)3-9.<sup>16</sup> *Drosophila melanogaster* pericentric heterochromatin is enriched with H3K9me3, H3K9me2, Su(var)3-9, and HP1a.<sup>1,13</sup> Compared to the rest of the genome, pericentric heterochromatin has less H3K23ac, and H3.3.<sup>1</sup> H3.3 is a histone variant of histone 3 that is created by a different gene.



**Figure 2.** Model of fundamental heterochromatic components. The DNA, represented by a black line, wraps around histone octamers (clump of small circles) to form nucleosomes. Nucleosomes are found in all types of chromatin. Heterochromatin has the following extra features. H3 is post-translationally modified with the mark H3K9me3 (green circles), HP1a is a non-histone protein that binds to H3K9me3, and Su(var)3-9 is an enzyme that attaches methyl groups to H3 to create the mark H3K9me3. Su(var)3-9 physically binds to HP1a, which puts it in a position to modify nearby histones. You can imagine Su(var)3-9 in the figure is in the process of modifying H3 in the right nucleosome; once that is complete the nucleosome on the right will have affinity for HP1a. Components are not drawn to scale.

Heterochromatin might help maintain genome stability.<sup>17</sup> Su(var)3-9 mutants have more genome instability.<sup>18</sup> Su(var)205 mutants have more DNA damage.<sup>17</sup> Su(var)205 is the fly gene that produces HP1a.<sup>17</sup> Overexpressing HP1 reduces lethality caused by irradiation.<sup>17</sup>

Heterochromatin helps create the condensed chromosomes that occur during mitosis. Su(var)205 mutants have abnormally large chromosomes during mitosis.<sup>17</sup> Mice that are null for both Suv39h1 and Suv39h2 have more missegregation of chromosomes during meiosis and nonhomologous chromosomes interact during meiosis.<sup>19</sup> Suv39h1 and Suv39h2 are mammalian versions of Su(var)3-9.

Position-effect variegation (PEV) is an important phenomenon related to heterochromatin. A mutation that moves a euchromatic gene near constitutive heterochromatin can lead to the variegated expression of that euchromatic gene.<sup>20</sup> Variegated expression means that some cells in the organism will seem to be expressing the gene, and other cells in the same organism will seem to not be expressing the gene. This phenomenon is called position-effect variegation. This phenomenon is caused by the euchromatic DNA segment taking on the heterochromatic state and becoming transcriptionally inactive.<sup>3,20</sup> Heterochromatin can spread from the natural block of heterochromatin and cover the euchromatic gene.<sup>3,20</sup> The distance of heterochromatin spreading is random, and some cells will retain euchromatic gene expression at that locus; this is the reason for variegated expression.<sup>20</sup> DNA sequences found naturally in heterochromatin may be essential for position-effect variegation.<sup>20</sup> Variegating genes are less accessible to restriction enzyme digestion, which implies that they really do become heterochromatic.<sup>21</sup> Position-effect variegation can be modified by temperature, amount of heterochromatin in the genome, inhibitors of histone metabolism, and mutations of individual genes.<sup>20</sup> The set of genes that can modify position-effect variegation tend to all have something to do with chromatin, many of

these genes are essential.<sup>20</sup> These genes tend to code for structural proteins or enzymes that modify histones.<sup>3</sup> Checking if a gene mutation modifies PEV is a way to determine if that gene has something to do with heterochromatin. Proteins that are important for constitutive heterochromatin were mentioned previously. All the genes that code for those proteins are modifiers of PEV. Su(var)3-9 is a modifier of PEV.<sup>11</sup> Su(var)205, the gene coding for HP1a, is a modifier of PEV.<sup>22,23</sup> Histone H1 is a modifier of PEV.<sup>24</sup>

In *Drosophila melanogaster*, constitutive heterochromatin contains genes, some of which are essential.<sup>25-27</sup> This is interesting because heterochromatin is well-known for suppressing gene expression (see position-effect variegation discussed above). The genes within constitutive heterochromatin are called heterochromatic genes, and they produce RNA transcripts, which can code for proteins. Heterochromatic genes are not duplications of euchromatic genes. Functions of proteins produced by heterochromatic genes do not seem to be biased to any particular job. *D. melanogaster* heterochromatic genes tend to be long, much of the gene made of introns, found in regions of low gene density, and are AT-rich.<sup>1,28</sup> The long introns of heterochromatic genes are filled with transposable element-like sequences.<sup>28</sup>

Heterochromatic genes may have special expression conditions as compared to normal euchromatic genes. HP1a and Heterochromatin protein 2 (HP2) are found at promoters of heterochromatic genes.<sup>13</sup> However, the 5' regions of expressed heterochromatic genes are relatively depleted of HP1a, H3K9me2, H3K9me3, and Su(var)3-9 compared to the rest of the gene.<sup>29</sup> There is a heterochromatic gene called rolled, that when moved to a euchromatic location will have reduced function.<sup>30</sup> There is a heterochromatic gene called light, that has variegated expression when moved to a euchromatic environment.<sup>31</sup> HP1a or Su(var)3-7 mutants enhance light variegation.<sup>32,33</sup> Mutations that suppress position-effect variegation will enhance the

variegation of heterochromatic genes put near euchromatin.<sup>31</sup> This is similar to PEV except heterochromatic gene instead of euchromatic gene, euchromatic environment instead of heterochromatic environment, mutation enhances instead of suppressing. Mutating HP1a can reduce expression of some heterochromatic genes on the 4<sup>th</sup> chromosome.<sup>23</sup> Mutating HP1a can reduce expression of some pericentric heterochromatic genes on the 2<sup>nd</sup> and 3<sup>rd</sup> chromosomes.<sup>34,35</sup> This is important because it implies that the heterochromatin environment is required for proper expression of heterochromatic genes. Heterochromatic genes share some chromatic features with euchromatic genes. Heterochromatic genes that are expressed and euchromatic genes that are expressed have H3K36me3.<sup>29</sup>

How is it possible that heterochromatin can positively influence the expression of heterochromatic genes? Heterochromatin might promote the folding of DNA to bring distant enhancers close to heterochromatic gene promoters.<sup>36</sup> Heterochromatin components might act as transcription factors that boost heterochromatic gene expression.<sup>36</sup> Heterochromatin might silence transposable elements. Active transposable elements could theoretically impair the expression of heterochromatic genes by competing for transcription machinery or by producing transcripts that would lead to silencing of heterochromatic genes by RNA interference.<sup>36</sup> HP1a can bind to and improve stability of mRNAs of some euchromatic genes.<sup>37</sup> HP1a can positively and negatively influence expression of euchromatic genes.<sup>38</sup>

Heterochromatin can spread to cover euchromatic regions as discussed with PEV, but what stops heterochromatin spreading? Heterochromatin spreading might stop when heterochromatin components get used up.<sup>3</sup> A DNA sequence might stop heterochromatin spreading.<sup>3</sup> There is a gene in the H3 class called H3.3. The presence of this gene product in nucleosomes might prevent heterochromatin spreading.<sup>3</sup> H3S10ph might inhibit heterochromatin spreading.<sup>3</sup> JIL-1

makes H3S10ph.<sup>39</sup> JIL-1 prevents heterochromatin spreading and is a modifier of PEV.<sup>39</sup>

H3S10ph inhibits Su(var)3-9.<sup>39</sup> JIL-1 phosphorylates Su(var)3-9.<sup>39</sup>

How does heterochromatin get established? There is probably more than one way. One of the models explaining this question goes as follows.<sup>3</sup> Double-stranded RNA present in the cell gets cut by Dicer-2. Dicer-2's activity creates double-stranded RNA pieces that can be incorporated into RNA-induced silencing complex (RISC). RISC is known to be able to alter H3K9 methylation, Su(var)3-9 and HP1a localization.<sup>3</sup> A different model of establishment of heterochromatin goes as follows.<sup>3</sup> Piwi-interacting RNAs (piRNAs) bind to Piwi. Since piRNAs are complementary to satellite DNA and transposable elements, Piwi will localize to these sequences. Piwi then recruits HP1a. Both of these models suggest that the parts of chromosomes that are heterochromatic are the ones that have DNA sequences that are similar to particular RNA molecules, or are near these sequences. Note that this is only for the establishment of heterochromatin, after heterochromatin is established, the cell does not require these RNAs anymore.

When studying heterochromatin, it might be useful to restrict your investigation to a subcategory of heterochromatin. In this report, I focused on the heterochromatin near the centromere of chromosomes 2 and 3. I did this because I believe heterochromatin is somewhat diverse. For instance, telomeric heterochromatin is probably different from centromeric heterochromatin because Su(var)205 is a modifier of position-effect variegation unless the variegating gene is near the telomeres.<sup>21</sup>

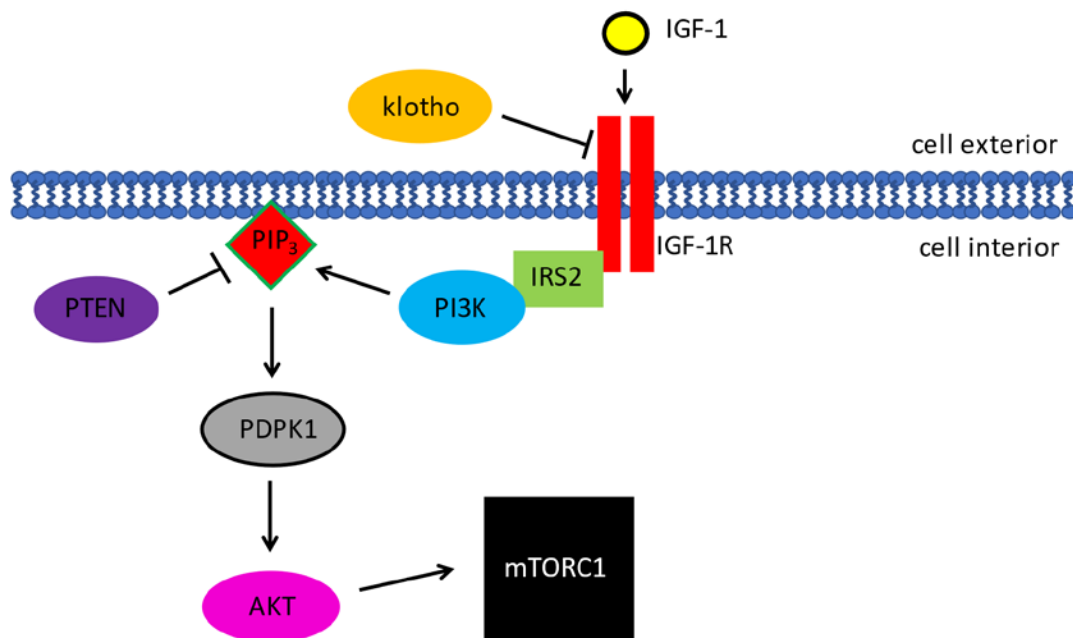
## Aging

Aging is important because age is a risk factor for many diseases.<sup>7,40</sup> Most human deaths are of age-related causes.<sup>41</sup> Aging can be defined as an increase in mortality rate with time, or as progressive loss of function of an organism. This section of the introduction will cover various topics believed to be relevant for explaining the causes of aging.

### *PI3K/AKT pathway*

The PI3K/AKT pathway is a pathway that is believed to be associated with aging. A figure showing the main parts of this pathway is shown below (**Figure 3**). Klotho, an inhibitor of this pathway,<sup>42-44</sup> extends mouse lifespan.<sup>43</sup> Klotho alleles are also associated with long human lifespan.<sup>45</sup> Insulin, a hormone that leads to activation of the PI3K/AKT pathway,<sup>46</sup> is associated with human longevity.<sup>45,47</sup> Insulin like growth factor 1 is a hormone that is similar to insulin and is associated with human longevity.<sup>48</sup> Insulin like growth factor 1 receptor is a receptor that is upstream of PI3K in the PI3K/AKT pathway.<sup>49</sup> Alleles of insulin like growth factor 1 receptor gene are associated with long lifespan in humans.<sup>50,51</sup> Insulin receptor substrate 2 physically interacts with PI3K<sup>52</sup> and alleles of that gene are associated with human longevity.<sup>51</sup> PIK3CA is the gene that makes the catalytic subunit of PI3K, it is a lipid kinase. PIK3CA gene variants are associated with human longevity.<sup>47</sup> Currently, the gene that produces the record in lifespan extension is the *Caenorhabditis elegans* gene called age-1. age-1 is the homolog of PIK3CA. age-1 mutants have an average lifespan 10x longer than wildtype.<sup>53</sup> PTEN is a lipid phosphatase that opposes the function of PIK3CA. PTEN is also associated with human longevity.<sup>50</sup> PTEN overexpression in *Drosophila* fat body extends lifespan.<sup>54</sup> PDPK1 is one of the activators of

AKT in the PI3K/AKT pathway.<sup>55</sup> PDK1 is associated with human longevity.<sup>48</sup> AKT1 is a gene whose protein is a component of the PI3K/AKT pathway. Alleles of AKT1 are associated with long human lifespan.<sup>47,50</sup> AKT3 is very similar to AKT1 and is also a component of the PI3K/AKT pathway. AKT3 gene variants are associated with human longevity.<sup>47</sup> MTOR, a component of PI3K/AKT pathway, is associated with human longevity.<sup>48</sup> Mutations in Tor, the *Drosophila* homolog of MTOR, extends fly lifespan.<sup>56</sup> mTORC1 is a protein complex that has MTOR as one of its components. Reducing mTORC1 function increases lifespan.<sup>57</sup>



**Figure 3.** PI3K/AKT pathway. PIP<sub>3</sub> is a lipid. IGF-1 = Insulin like growth factor 1. IGF-1R = Insulin like growth factor 1 receptor. IRS2 = insulin receptor substrate 2. PI3K = Phosphoinositide 3-kinase. PIP<sub>3</sub> = phosphatidylinositol (3,4,5)-trisphosphate. PTEN = phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase. PDK1 = 3-phosphoinositide-dependent protein kinase-1. mTORC1 = mammalian target of rapamycin complex 1. The roles of IGF-1 and IGF-1R in the pathway can be substituted with insulin and insulin receptor respectively.

After AKT and mTORC1, the PI3K/AKT pathway branches in many directions. One of the downstream targets that may be important for promoting long life is foxo. foxo is a transcription factor.<sup>58</sup> The longevity record of age-1 mutation requires FOXO for the lifespan extension effect.<sup>53</sup> foxo overexpression in *Drosophila* fat body extends lifespan.<sup>54</sup> In *D. melanogaster*, the number of genes that foxo binds to decreases with age.<sup>58</sup> FOXO3 is one of the human homologs of foxo. Variants of FOXO3 are associated with long human lifespan.<sup>50,59-63</sup>

### *Reactive oxygen species*

Reactive oxygen species are very reactive chemicals that contain the element oxygen. Reactive oxygen species cause damage to genomes.<sup>7,64</sup> 8-oxoguanine is a chemical produced by oxidative damage to DNA.<sup>64</sup> 8-oxoguanine accumulates with age.<sup>64</sup> Klotho, an inhibitor of the PI3K/AKT pathway, induces expression of SOD2.<sup>42</sup> SOD2 is an enzyme that reduces damage caused by reactive oxygen species by neutralizing reactive oxygen species within the mitochondria.<sup>65</sup>

Overexpression of Sod2 increases lifespan.<sup>66</sup> Overexpressing Sod1, an enzyme that is similar to Sod2, increases lifespan.<sup>67</sup> Some mutations that promote longevity also boost oxidative stress resistance.<sup>64</sup>

Chemicals can act on proteins, lipids or DNA to produce advanced glycation end products (AGE).<sup>68</sup> AGE formation causes irreversible damage.<sup>68</sup> High amounts of AGEs are associated with mortality.<sup>68</sup> Glyoxalases are enzymes that can help neutralize some of the chemicals that create AGEs.<sup>68</sup> Glyoxalase genes are associated with age-related diseases.<sup>68</sup> Overexpression of a glyoxalase in the nematode extends lifespan.<sup>68</sup> Receptor for advanced glycation end-products

(RAGE) is a membrane receptor that can recognize some AGEs.<sup>68</sup> Binding of an AGE to RAGE can lead to inflammation, and reactive oxygen species.<sup>68</sup>

### *Caloric restriction*

Caloric restriction is eating fewer calories than normal. Caloric restriction slows aging.<sup>40,57</sup>

Dietary restriction is any reduction in diet that does not necessarily lead to a reduction in calories. Dietary restriction prevents many age-related diseases.<sup>69</sup>

One of the possible mechanisms to explain this phenomenon focuses on AMPK. AMPK is a complex that is activated when cellular energy is low.<sup>69</sup> Sometimes, dietary restriction causes AMPK activation.<sup>69</sup> Sometimes, lifespan extension from dietary restriction requires AMPK.<sup>69</sup>

AMPK activation by itself increases lifespan.<sup>69</sup> LKB1 is an activator of AMPK.<sup>69</sup>

Overexpression of LKB1 also increases lifespan.<sup>69</sup>

How does AMPK increase lifespan? AMPK is an inhibitor of the PI3K/AKT pathway mentioned previously. AMPK inhibits the TORC1 complex.<sup>69</sup> AMPK might influence the health of an organism through other mechanisms as well. AMPK phosphorylates FOXO.<sup>69</sup> FOXO members are transcription factors that are associated with long life in several organisms.<sup>69</sup> AMPK promotes autophagy due to AMPK activating ULK1.<sup>69</sup> ULK1 is a component of the autophagy initiation complex.<sup>69</sup> The connection between autophagy and aging is described next.

### *Autophagy*

Autophagy is a cellular process that captures cellular components and delivers it to a lysosome for degradation. Autophagy is known to have connections to other pathways or treatments that are associated with aging. Caloric restriction promotes autophagy.<sup>57</sup> Modification of mTOR and AMPK pathways influence aging partially because they influence autophagy.<sup>57</sup> There is evidence that autophagy may have a connection to normal aging. Lysosomal function and autophagic function declines with age.<sup>57</sup> Lysosomal storage diseases show some phenotypes similar to aging.<sup>57</sup> There are methods related to autophagy that can extend the lifespan of organisms. TFEB is a transcription factor that regulates autophagy.<sup>57</sup> TFEB nuclear localization extends lifespan.<sup>57</sup> Overexpression of LIPL-4, a lipase, in *C. elegans* extends lifespan because it enhances autophagy.<sup>69</sup>

### *Premature aging diseases*

Progeroid syndromes are a set of human diseases. The symptoms of progeroid syndromes mimic some of the characteristics of natural human aging.<sup>70</sup> At a cellular level, progeroid cells and aged normal cells both have increased DNA damage, shorter telomeres, epigenetic changes, nuclear lamina alterations, cell cycle defects, and higher rates of senescence.<sup>70,71</sup> The genes that cause progeroid syndromes seem to fall into 4 function groups. These genes seem to be involved in double-strand break repair, interstrand cross-link repair, nucleotide excision repair, and telomeres.<sup>70</sup> For example, WRN is a gene involved with double-strand break repair and is mutated in Werner syndrome.<sup>70</sup> FANCE is involved with interstrand cross-link repair and is mutated in Fanconi anaemia.<sup>70</sup> XPA is involved with nucleotide excision repair and is mutated in xeroderma pigmentosum.<sup>70</sup> TERT is involved with telomeres and is mutated in dyskeratosis

congenita.<sup>70</sup> These 4 groups all seem to regulate genome stability. The study of progeroid syndromes shows that genome instability may be one of the causes of aging.

The best characterized progeroid syndrome is Hutchinson-Gilford progeria syndrome (HGPS). Hutchinson-Gilford progeria syndrome is caused by a mutation in the LMNA gene.<sup>70,71</sup> Lmna-null cells have more genomic instability than wildtype cells.<sup>72</sup> However, LMNA is known to produce a structural component of the nuclear lamina.<sup>73</sup> This conflicts with the idea that progeroid syndromes are caused by genome instability. LMNA has functions other than structural. One of the functions of the LMNA gene is to prevent proteasomal degradation of the Trp53bp1 protein.<sup>72</sup> Trp53bp1 is involved with double-strand break repair, specifically it is involved with nonhomologous end joining.<sup>74</sup>

Evidence from model organisms can also contribute to our understanding of these human diseases. *Drosophila melanogaster* nuclei increase in size with age.<sup>71</sup> Old nuclei also sometimes show separation of inner nuclear membrane from outer nuclear membrane.<sup>71</sup> Overexpressing Lam or kuk increases nucleus size and alters nucleus shape, similar to effects of age.<sup>71</sup> Lam is a lamin gene from *D. melanogaster*, and kuk is not a lamin but is a similar gene. Overexpressing Lam or kuk shortens fly lifespan.<sup>71</sup> Introducing fly versions of Lam or kuk into mouse cells leads to more DNA damage, less H3K9me3, and less HP1.<sup>71</sup>

Here is some evidence that DNA damage has something to do with aging. DNA damage goes up with age in humans.<sup>75</sup> DNA damage goes up with age in *Drosophila* fat body.<sup>76</sup> The efficiency of the repair of double-strand breaks declines with age.<sup>64</sup>

DNA damage leads to activation of ataxia-telangiectasia mutated kinase (ATM).<sup>64</sup> ATM can then activate p53.<sup>64</sup> p53 activation can lead to apoptosis, senescence, or cell cycle arrest.<sup>64</sup> ATM

mutations can cause a progeroid syndrome.<sup>64,70</sup> ATM-deficient cells have slow growth, genome instability, and early senescence.<sup>64</sup>

### *Telomeres*

Telomeres are special structures found at the ends of linear chromosomes. Like other parts of chromosomes, telomeres are made of DNA and proteins. Telomeric DNA is DNA at telomeres. Telomeric DNA tends to be a simple sequence that is repeated many times. In humans, telomeric DNA has the sequence TTAGGG repeated on one of the strands.<sup>77</sup> Subtelomere is adjacent to the part of the telomere that has telomeric DNA. Subtelomeric DNA tends to be repetitive and to have low gene density.<sup>78</sup> There is a complex of six different proteins called shelterin or telosome that binds to telomeres.<sup>79</sup> In humans, the members of shelterin complex are TERF1, TERF2, TERF2IP, TIN2, ACD, and POT1.<sup>80</sup> The shelterin complex influences telomere length and protection.<sup>78</sup> Proper telomere function requires both shelterin and sufficient telomeric DNA.<sup>78</sup>

One of the functions of telomeres is to prevent chromosomal end-to-end fusions. Shelterin is required for this function.<sup>79</sup> Telomeres are also responsible for buffering DNA loss that occurs at the ends of linear chromosomes after DNA replication.<sup>79</sup>

Telomere shortening does occur in adult cells and this might contribute to aging.<sup>78,79</sup> A somatic cell will divide a finite number of times before cell division stops and the cell enters a senescent state. This is known as the Hayflick limit. Telomere shortening explains the Hayflick limit.<sup>79</sup> Shelterin component mutations can lead to accelerated aging.<sup>79</sup> Shelterin depletion can cause senescence.<sup>40</sup>

Short telomeres can lead to apoptosis.<sup>81,82</sup> Telomere length is maintained by telomerase.<sup>78</sup>

Telomerase is made of a reverse transcriptase called TERT and an RNA called TERC.<sup>77,78</sup>

Telomeric DNA has a single-stranded 3' overhang, and telomerase acts upon the overhang.<sup>78</sup> Mutations in either the RNA or protein component of telomerase leads to shorter telomeres and health problems.<sup>77,79</sup> The reverse transcriptase subunit of telomerase can lengthen telomeres and prevent senescence.<sup>83</sup> Telomere length is variable between species and between individuals within a species.<sup>78</sup> Many human cancers are capable of maintaining telomere length and this preserves the cell's ability to proliferate.<sup>78</sup> Some cancers have active telomerase.<sup>77</sup> This means that inhibiting telomerase might limit the growth of cancer.<sup>77</sup> Double-stranded telomeric DNA can form a DNA structure called a t-loop. In a t-loop, the end of the 3' overhang is tucked between the strands of double-stranded telomeric DNA.<sup>78,81</sup> Not all telomeres follow this standard formula. An example of non-standard telomeres comes from the fruit fly, *Drosophila melanogaster*. Flies do not have telomerase and telomeric DNA is made of retrotransposons.<sup>6,78</sup> Telomere lengthening in flies is caused by transposition.<sup>82</sup>

### *Cellular senescence*

Senescence is a cellular state where the cell will not divide. Senescent cells are not dead, they are functional. Senescent cells have characteristics in addition to the lack of mitosis. Signs of senescence include large and flat cell shape, multiple nuclei, diploid or tetraploid, and  $\beta$ -galactosidase activity.<sup>81</sup> Senescent cells have more autophagy, and secrete hundreds of factors.<sup>40</sup> The secretion is known as senescence-associated secretory phenotype (SASP).

The molecules secreted from a senescent cell are not always the same. A different senescent cell might secrete a different set of molecules.<sup>40</sup> One of the functions of the secreted molecules is to

target the senescent cell to be destroyed by immune cells.<sup>40</sup> Another function is to maintain the senescent state of the original cell and to induce senescence in neighbouring cells.<sup>40</sup>

Senescence is a useful cellular program. Senescence can prevent mutated genomes from propagating.<sup>40</sup> Senescence can prevent tumours.<sup>40</sup> Senescence is used during normal development,<sup>40</sup> analogously to apoptosis being a part of normal development.<sup>84</sup>

The Rb protein causes senescence.<sup>40</sup> In non-senescent cells, CDK4/6 inhibits Rb, which prevents senescence.<sup>40</sup> For activation of senescence, CDK4/6 is inhibited.<sup>40</sup> There is more than one pathway leading to CDK4/6 inhibition. One of the senescent pathways is as follows.<sup>40</sup>  $\gamma$ H2AX and Trp53bp1 are found at sites of DNA damage. Those proteins activate a kinase cascade that eventually leads to p53 activation. p53 induces transcription of p21. p21 inhibits CDK4/6 and causes senescence. Extra copies of p53 delay aging.<sup>40</sup>

One of the other pathways that cause senescence via inhibition of CDK4/6 is as follows.<sup>40</sup> The INK4/ARF locus is usually transcriptionally silent and has H3K27me3. Disrupting those histone modifications permits transcription of the genes at the INK4/ARF locus. One of the genes turned on is p16. p16, like p21, is a CDK4/6 inhibitor. p16 expression increases with age.<sup>40</sup>

The Hayflick limit is the number of times a cell will divide before cell division stops. Cells taken from old people have a lower Hayflick limit than cells taken from young people.<sup>85</sup> Senescent cells accumulate with age.<sup>64</sup> Stem cell populations from old people have a larger percentage of senescent cells compared to young stem cell populations.<sup>40</sup> As a result, stem cell populations in old people have reduced ability to self-renew.<sup>40</sup> This might be the cause of some age-associated diseases.<sup>40</sup> These observations align with the stem cell theory of aging, which is aging is partially

caused by inability of pluripotent stem cells to replenish tissues.<sup>41</sup> Stem cell function declines with age.<sup>41</sup>

More evidence linking aging with stem cells involves epigenetic age. DNA methylation changes with age.<sup>86</sup> Epigenetic age estimates the actual age from analysis of DNA methylation data.<sup>86</sup>

Epigenetic age is associated with mortality and age-related diseases in humans.<sup>86</sup> Epigenetic age might have something to do with stem cells, because Yamanaka factors reduce epigenetic age.<sup>86</sup>

Yamanaka factors are transcription factors that can artificially induce pluripotency.<sup>87</sup>

Pluripotency is the ability of a cell to differentiate into any cell of the body, a property found in embryonic stem cells.<sup>87</sup>

Of the mutations that cause premature aging, many of them also cause senescence.<sup>64</sup> Senescence can cause chronic inflammation.<sup>40</sup> Chronic inflammation occurs during old age.<sup>88</sup> Chronic inflammation is associated with bad outcomes for some diseases.<sup>40</sup> The factors secreted during SASP lead to inflammation.<sup>88</sup> Senescence is associated with some age-related diseases, and killing senescent cells can improve health.<sup>40</sup> However, senescence-based treatments are sometimes associated with higher cancer risk.<sup>40</sup>

### *Hints from centenarian studies*

In humans, variants of the gene cholesteryl ester transfer protein (CETP) have been associated with long life.<sup>89,90</sup> Similarly, variants of the gene apolipoprotein E (APOE) have been associated with long human life.<sup>89-96</sup> Both of these genes have a function connected to lipoproteins.

Lipoproteins are spheres of lipids surrounded by a phospholipid layer. Apolipoproteins are embedded in the phospholipid layer. Lipoproteins are packages stored with lipids that can be

transported through aqueous environments, such as the blood. The APOE gene makes one of the apolipoproteins.<sup>97</sup> The CETP gene creates a protein that transfers cholesteryl ester and triglycerides from one lipoprotein to another.<sup>98</sup> Cholesteryl ester is a lipid that can be found within lipoproteins.<sup>98</sup> These gene variants may influence the development of atherosclerosis and this may be the reason for association with long life. Low-density lipoproteins (LDL), one of many types of lipoprotein, cause atherosclerosis.<sup>99</sup> ApoE, the protein generated by APOE gene, can be found within LDL and can contribute to LDL remaining in the artery wall, which is one of the steps in the development of atherosclerosis.<sup>99</sup> CETP might be important for the creation of some LDL.<sup>99</sup> APOE gene variants are not only associated with length of life, they have also been associated with LDL-cholesterol levels.<sup>99</sup> LDL-cholesterol levels are important because treatments that reduce LDL-cholesterol lead to fewer atherosclerotic cardiovascular disease events.<sup>99</sup>

### Heterochromatin and aging

There is an idea called the heterochromatin loss model of aging. Heterochromatic regions are shrunk/perturbed with age, this then leads to changes in nuclear architecture, and abnormal gene expression.<sup>7</sup> This idea has some support because loss of heterochromatin leads to altered gene expression.<sup>7</sup>

Here are some points that hint at heterochromatin being connected to aging. In mammals, heterochromatin is associated with DNA methylation,<sup>7</sup> and DNA methylation is associated with aging.<sup>86</sup> Heterochromatin loss occurs during senescence.<sup>7</sup> Heterochromatin prevents DNA damage.<sup>7</sup> Knockdown of Su(var)3-9 leads to DNA damage.<sup>100</sup> SUV39H1 (mammalian homolog

of Su(var)3-9) expression goes down with age.<sup>7</sup> tau is associated with several neurodegenerative disorders.<sup>101</sup> tau decreases H3K9me2 and HP1a, which are important components of heterochromatin.<sup>101,102</sup> Also, adding tau suppresses position-effect variegation,<sup>101</sup> further implicating heterochromatin as possibly being important for the neurodegenerative disorders that are associated with tau.

In normal human aging, the abundance of H3K9me3 (a marker of constitutive heterochromatin) goes down with age.<sup>103</sup> The explanation of the cause is as follows. Hutchinson-Gilford progeria syndrome (HGPS) also causes H3K9me3 to go down.<sup>73</sup> HGPS is caused by a mutation at a cryptic splice site in lamin A gene which makes the cryptic splice site more likely to be used for splicing, resulting in an altered lamin A protein.<sup>104</sup> The cryptic splice site is sometimes used for splicing in normal humans, but less frequently than in HGPS patients.<sup>103</sup> Inhibition of the cryptic splice site causes H3K9me3 to go back up.<sup>103</sup> These results suggest that buildup of altered lamin A proteins causes the decline of H3K9me3 with age.

Similarly, in *Drosophila melanogaster*, abundance of H3K9me2 is known to decline with age.<sup>105</sup> Also in *D. melanogaster*, enrichment of H3K9me3 at pericentric heterochromatin compared to the rest of genome goes down with age.<sup>106</sup> However, total H3K9me3 goes up with age in *D. melanogaster*, probably due to an increase in H3K9me3 at euchromatic regions.<sup>106</sup>

In *Drosophila*, X0 males live longer than both wildtype males and wildtype females.<sup>107</sup> X0 males are flies with 1 X chromosome, and no Y chromosome. The Y chromosome is made of constitutive heterochromatin.<sup>8</sup> The Y chromosome is a modifier of position-effect variegation, it is a suppressor.<sup>108</sup> The lifespan extension seen in X0 males may be caused by the effects of losing Y chromosome heterochromatin. X0 males have higher H3K9me2 and H3K9me3 compared to wildtype,<sup>109</sup> this could have some impact on the lifespan effect.

Heterochromatin is known to contain transposable elements and repetitive DNA.<sup>3</sup> Both of these sequence types are associated with genome instability. Transposable elements may cause exponential damage over time.<sup>110</sup> Since transposable elements can change location in the genome, this movement is one of the ways that transposable elements can cause deleterious mutations.<sup>6</sup> Rhino, a *D. melanogaster* gene, promotes integration of transposable elements into heterochromatin.<sup>3</sup> Depleting H1 by RNAi leads to activation of heterochromatic transposable elements.<sup>16</sup> Su(var)3-9 null mutation also seems to activate transposable elements.<sup>16</sup> Reduced DNA methylation can promote transposable element expression.<sup>6</sup> Repetitive DNA can promote mutations that alter the number of repeats. This can be caused by double-strand break repair at the repetitive region.<sup>111</sup> Chromatin states at the repetitive region might influence the likelihood of these mutations.<sup>111</sup> Mammalian heterochromatin is rich in DNA methylation, DNA methylation might prevent homologous recombination at repetitive sequences.<sup>78</sup> One of the functions of heterochromatin may be to prevent genomic instability that would have been caused by both transposable elements and repetitive DNA. Genomic instability may be a contributor to aging so this is another possible connection between heterochromatin and aging.

The piRNA pathway is a mechanism of inhibiting transposable elements by cutting transposable element mRNA.<sup>110</sup> piwi, an important component of the piRNA pathway, is a modifier of position-effect variegation.<sup>112</sup> The piRNA pathway is active predominantly in the germ line.<sup>110</sup> This is noteworthy because the germ line is biologically immortal.

As mentioned previously, telomeres might have something to do with aging. This leads to another possible connection between heterochromatin and aging. Yeast, fly, and mammalian telomeres seem to be heterochromatic.<sup>78</sup> Heterochromatin at telomeres can influence expression of nearby genes.<sup>78</sup> This is called telomere position effect. Telomere length can influence

telomere position effect.<sup>78</sup> Enzymes that methylate H3K9 work at both pericentric heterochromatin and telomeres.<sup>78</sup> In mammals, pericentric heterochromatin and telomeres both have H3K9me3 and H4K20me3.<sup>78</sup> Mammalian telomeres have HP1.<sup>78</sup> The mammalian telomere repeat sequence can't be methylated, but mammalian subtelomeres are methylated.<sup>78</sup> Human telomeres are not late replicating, but yeast telomeres are.<sup>78</sup> Decreased DNA methylation leads to increased telomere length and homologous recombination at telomeres.<sup>78</sup> Telomeres are able to adopt a euchromatic state when telomeres are short and should be lengthened.<sup>78</sup> This might cause telomerase to act preferentially on the shortest telomeres.<sup>78</sup>

A possible explanation for the connection between heterochromatin and aging is heterochromatic genes have an influence on aging. One of the heterochromatic genes in *Drosophila melanogaster*, Poly-(ADP-ribose) polymerase (PARP), is already suspected of having a connection to aging.<sup>113</sup> Aging leads to DNA damage, which leads to PARP activation. Since PARP consumes NAD<sup>+</sup>, the NAD<sup>+</sup> concentration in the cell will go down. Other enzymes in the cell that also require NAD<sup>+</sup>, such as sirtuins, will have decreased activity due to the low NAD<sup>+</sup> concentration.<sup>113</sup> This idea has some support because NAD<sup>+</sup> gets depleted with age.<sup>75</sup> PARP activity is correlated with maximum lifespan in mammals.<sup>114</sup> PARP activity declines with age in humans.<sup>114</sup>

Another heterochromatic gene that could be involved with aging is Argonaute 3 (AGO3). AGO3 is a part of the piRNA pathway.<sup>16</sup> Therefore, it may prevent genomic instability by inhibiting transposable elements. There already exists some evidence that aging has an effect on heterochromatic gene expression. Some heterochromatic genes have altered expression in old ovaries.<sup>115</sup>

There are sometimes coincidences of a mutation that both modifies position-effect variegation, and modifies the lifespan of the organism. In *Drosophila melanogaster*, overexpression of HP1 extends lifespan.<sup>105</sup> Similarly, in *Drosophila melanogaster*, knockdown of HP1a shortens lifespan and leads to DNA damage.<sup>100,116</sup> HP1a is a modifier of position-effect variegation.<sup>22,23</sup> kuk is a modifier of position-effect variegation and there are kuk mutants that shorten lifespan.<sup>71</sup> Overexpression of Lam shortens fly lifespan.<sup>71</sup> Lam is a modifier of position-effect variegation.<sup>117</sup> Sirt1 is a modifier of position-effect variegation.<sup>118</sup> Mammalian SIRT1 deacetylates histones, which might promote heterochromatinization.<sup>7</sup> Sirt1 is an anti-aging gene.<sup>119</sup> These genes imply that heterochromatin is involved in aging in *Drosophila melanogaster*.

## Objectives

For the long-term objective of determining if heterochromatic gene expression has an influence on aging, heterochromatic gene expression needs to be well-understood so it can be precisely manipulated in an experiment. Are there genes that modify heterochromatic gene expression? I studied several candidate genes for their ability to modify the expression of the heterochromatic gene light (lt). light is one of the heterochromatic genes that is quite highly expressed in *Drosophila melanogaster*, so a difference should be quite noticeable. Expression of light was measured with reverse transcriptase polymerase chain reaction (RT-PCR).

I also investigated whether heterochromatin has an influence on aging. I measured the lifespan of flies missing pericentric constitutive heterochromatin from chromosome 2R. Is there a statistically significant difference in the lifespan of these flies compared to normal flies?

## Methods

### *Organism*

All experiments are done with *Drosophila melanogaster*.

### *RT-PCR*

TRIzol reagent (Invitrogen) was used and the manufacturer's protocol was followed for purifying fly RNA. 3-5 whole adult flies were homogenized per collection in 500  $\mu$ L of TRIzol Reagent. I did not use glycogen. RNA was stored between -80 and -70  $^{\circ}$ C.

Reverse transcriptase – polymerase chain reactions were done using and following the instructions of iTaq Universal SYBR Green One-Step Kit except centrifugation was not done at end. Rotor-Gene Q machine was used as the thermocycler and spectrophotometer (this machine also centrifuges, which is why centrifugation was not required prior to loading into machine). 20  $\mu$ L reaction volume was used. Concentration of tube  $i = 2^{\text{Ct of tube 1} - \text{Ct of tube } i}$ . Concentrations of  $\beta$ -Tubulin replicates were averaged. Relative concentration of tube  $i = \text{concentration of tube } i / \text{average of } \beta\text{-Tubulin replicates from the same RNA sample}$ . Reported values are expression of the gene of interest relative to expression of  $\beta$ -Tubulin. **Table 1** lists the primers used.

**Table 1.** Primer sequences used. Sequences are written 5' to 3'.

Gene	Primer Sequences
$\beta$ -Tubulin at 56D	CCAAGTGAACGCTGATCTCC AACATCTGCTGGGTCAGCTC
light	AGCCCTACGACCTGTATTATGC GCGATTCTCCTCGATTATGC

### *Lifespan assay*

The lifespan of flies is determined by counting dead flies every 2 days until the entire population has died. The earliest day that any fly in the vial could have emerged from a pupa is treated as day 0. Live flies are moved to a new vial with food after each count; if dead flies are transferred to the new vial then the number of dead flies in the new vial is recorded.

### *Fly stocks*

Fly stocks were from Bloomington Drosophila Stock Center or from the lab of Dr. Hilliker.

1.  $ry^{+5}$  wildtype flies, also used for some crosses.
2. Df(2R)MS2-10/CyO, S
3.  $y^1 w^*$ ; PBac{ $y^{+mDint2} w^{+mC}=Su(var)3-7-GFP.FPTB$ }VK00037 flies that have 4 copies of Su(var)3-7.
4. In(1) $w^{m4}$ ; Su(var)3-9<sup>1</sup>/TM3, Sb<sup>1</sup> Ser<sup>1</sup> flies used for creating Su(var)3-9 mutants.
5.  $w^{1118}$ ; PBac{ $y^{+mDint2} w^{+mC}=tj-GFP.FPTB$ }VK00033 flies that have 4 copies of tj.
6.  $w^{1118}$ ; PBac{ $y^{+mDint2} w^{+mC}=woc-GFP.FPTB$ }VK00037 flies that have 4 copies of woc.
7.  $w$ ; G32[w+] G32 refers to daughterless-Gal4. Flies that were used to create flies that overexpress foxo.
8.  $w^{1118}$ ; P{ $w^{+mC}=UASp-foxo.S$ }3 flies used for creating flies that overexpress foxo.
9.  $w^{1118}$ ; P{ $w^{+mC}=EP$ }Sirt1<sup>EP2300</sup>/CyO
10.  $y^1 w^*$ ; P{ $w^{+mC}=tubP-GAL4$ }LL7/TM3, Sb<sup>1</sup> Ser<sup>1</sup>

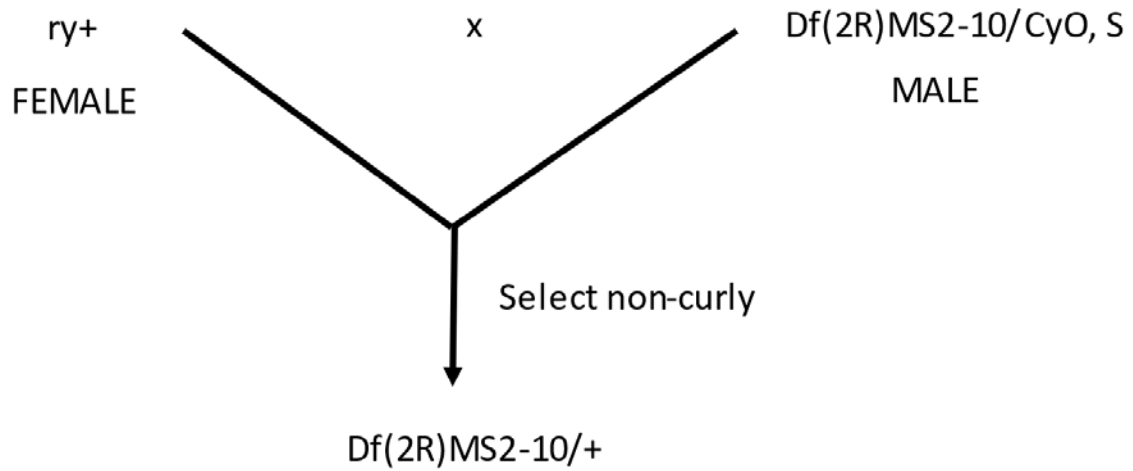
11. w P{w<sup>+mC</sup>=EP}lncRNA:roX1<sup>G958</sup>
12. y<sup>1</sup> w<sup>67c23</sup>; P{y<sup>+mDint2</sup> w<sup>+mC</sup>=EPgy2}msl-1<sup>EY09390</sup>
13. w<sup>\*</sup> P{w<sup>+mC</sup>=EP}mof<sup>G894</sup>

### *Collecting virgins*

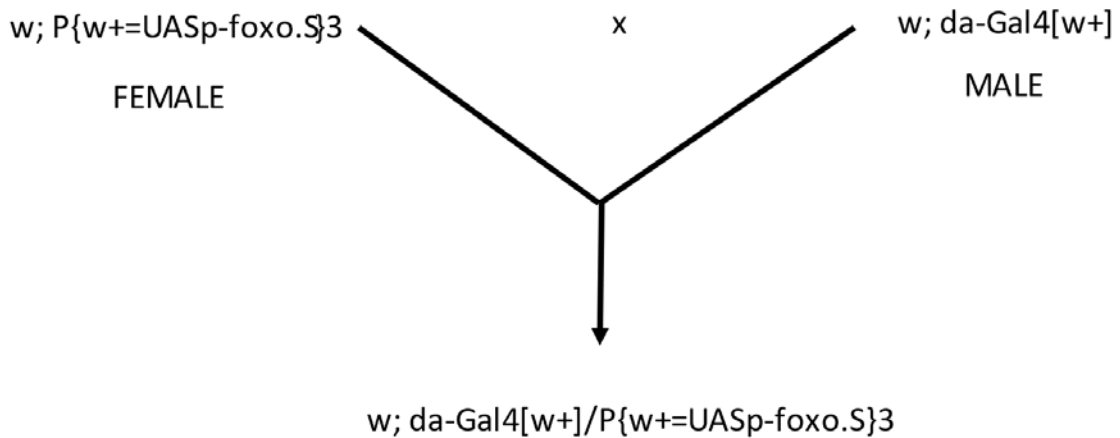
Crosses require virgin females. 2 methods were used for collecting virgins. A dark spot in the abdomen of a female is a mark of a virgin. If a female fly is gathered within 8 hours of eclosing, then it is a virgin. Virginity was double-checked by keeping vials that held virgins to make sure that no larvae come from them.

### *Crosses*

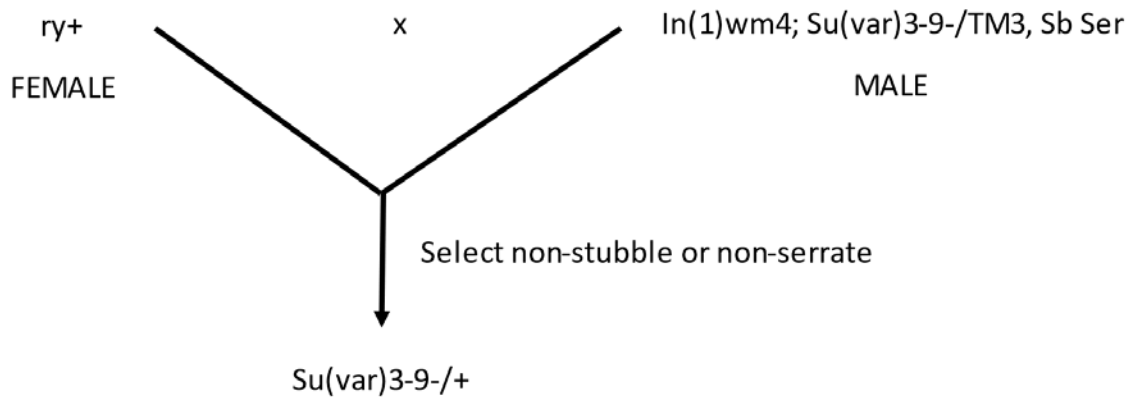
The following figures describe all crosses conducted.



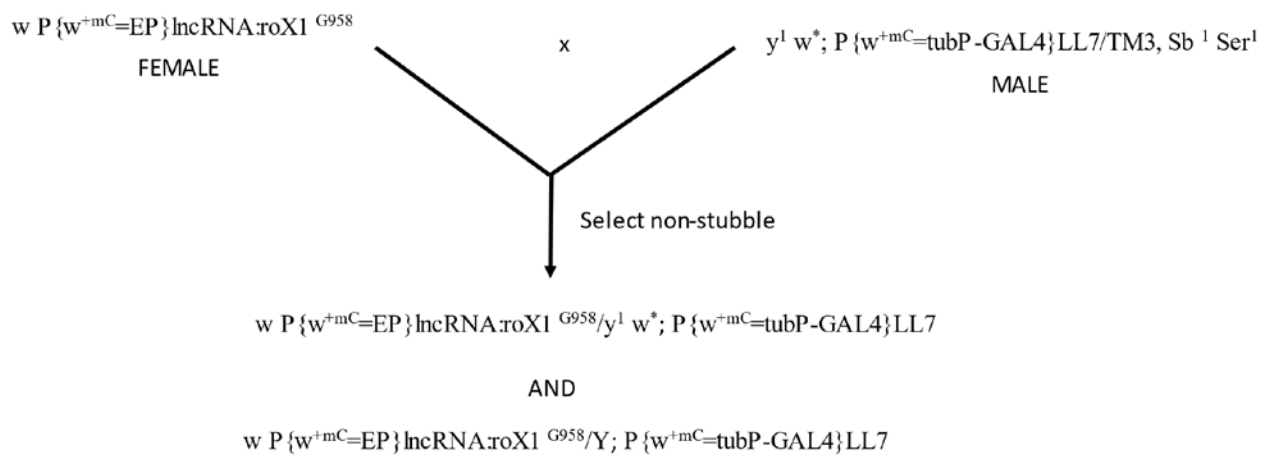
**Figure 4.** Method of creating flies that are heterozygous for a deletion of pericentric constitutive heterochromatin on chromosome 2. The flies produced are called 2Rh-.



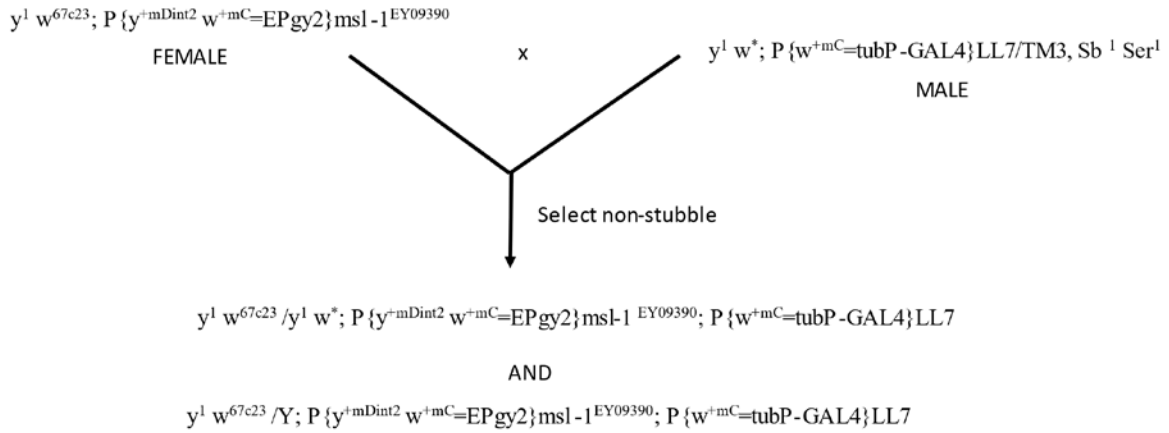
**Figure 5.** Method for creating flies that ubiquitously overexpress foxo. The flies produced are called da>foxo.



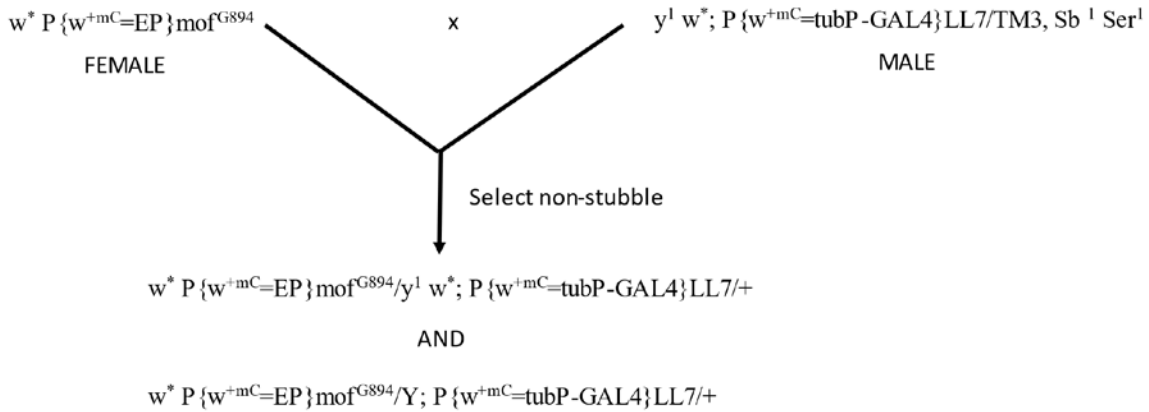
**Figure 6.** Method for creating flies that are heterozygous for a loss-of-function allele of Su(var)3-9. The flies produced are referred to as Su(var)3-9.



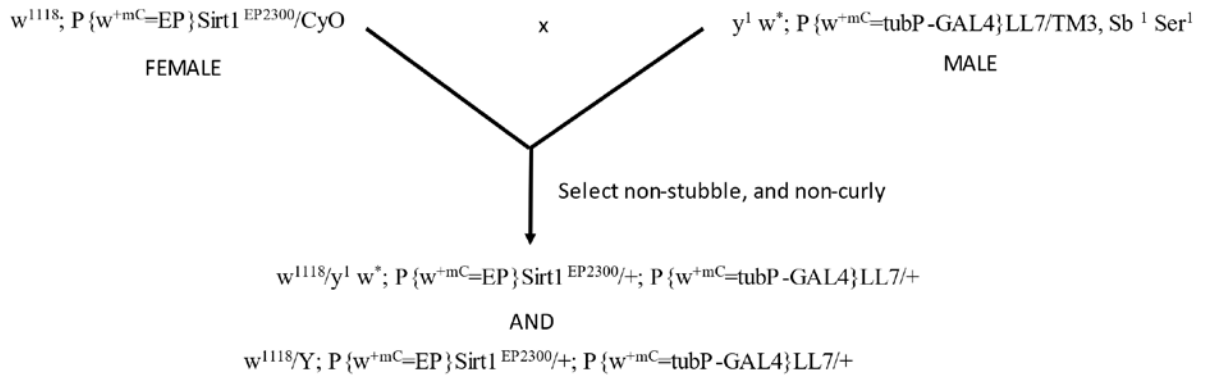
**Figure 7.** Method for creating flies that ubiquitously overexpress roX1. The flies produced are referred to as roX1.



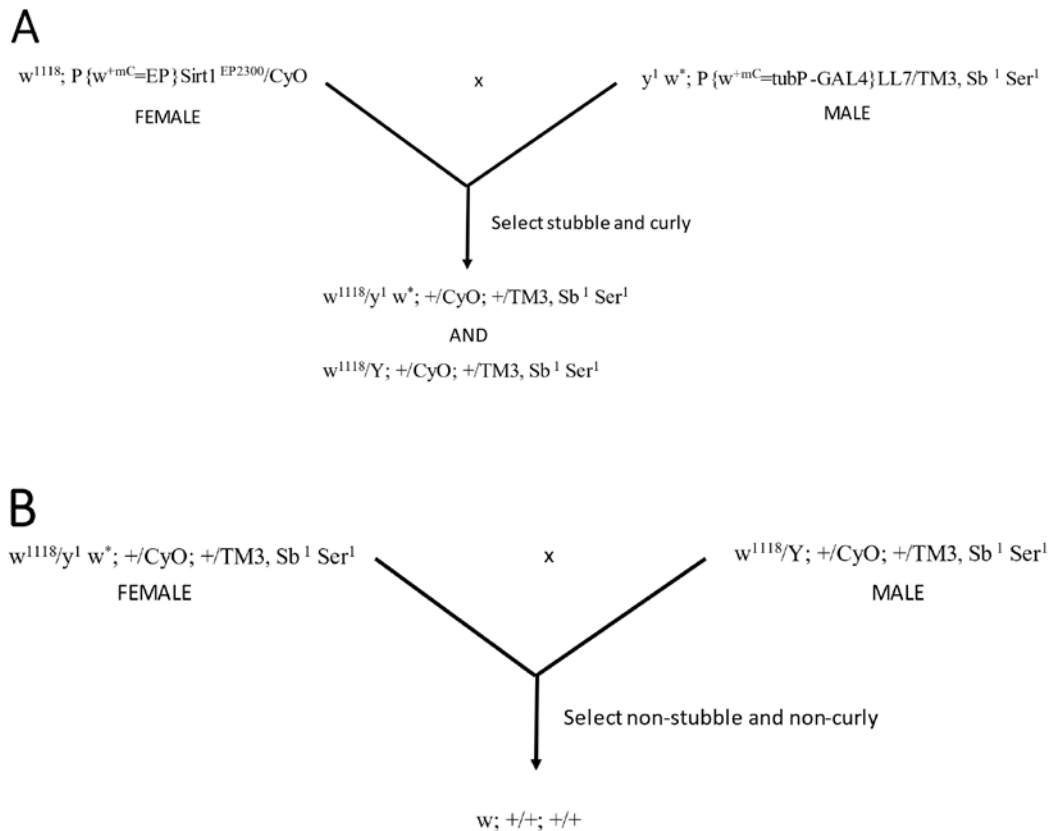
**Figure 8.** Method for creating flies that ubiquitously overexpress *msl-1*. The flies produced are referred to as *msl-1*.



**Figure 9.** Method for creating flies that ubiquitously overexpress *mof*. The flies produced are referred to as *mof*.



**Figure 10.** Method for creating flies that ubiquitously overexpress Sirt1. The flies produced are referred to as sirtuin.



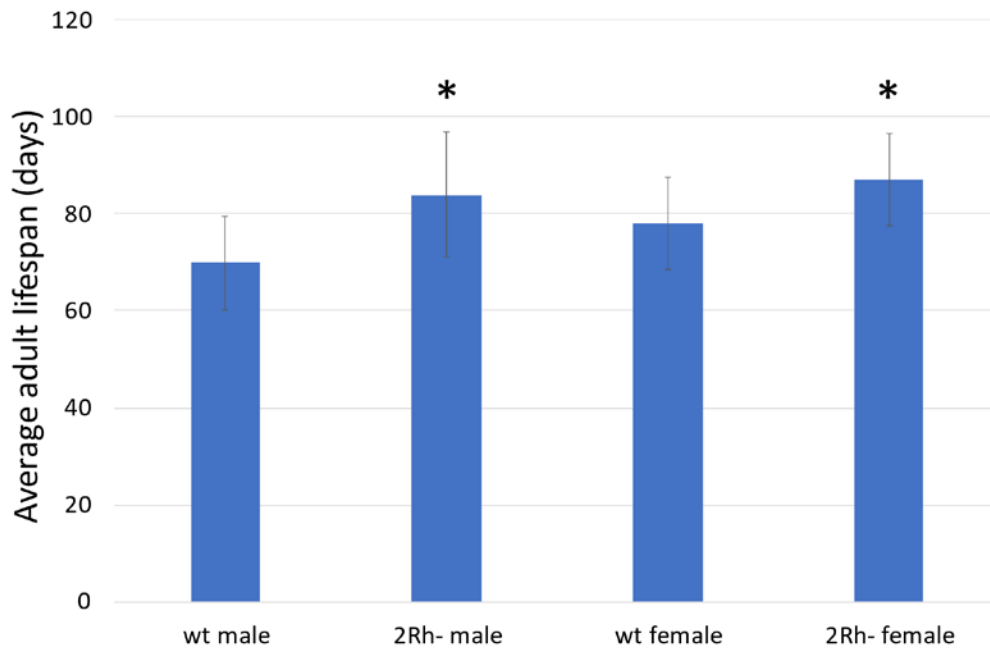
**Figure 11.** Method for creating flies that are used as the controls for sirtuin flies. A is first part of the cross which is identical to the previous figure except different flies are being selected. B is a cross using the flies from part A for generating flies that are related to the sirtuin flies but are lacking balancer chromosomes and relevant mutations.

### *Statistics*

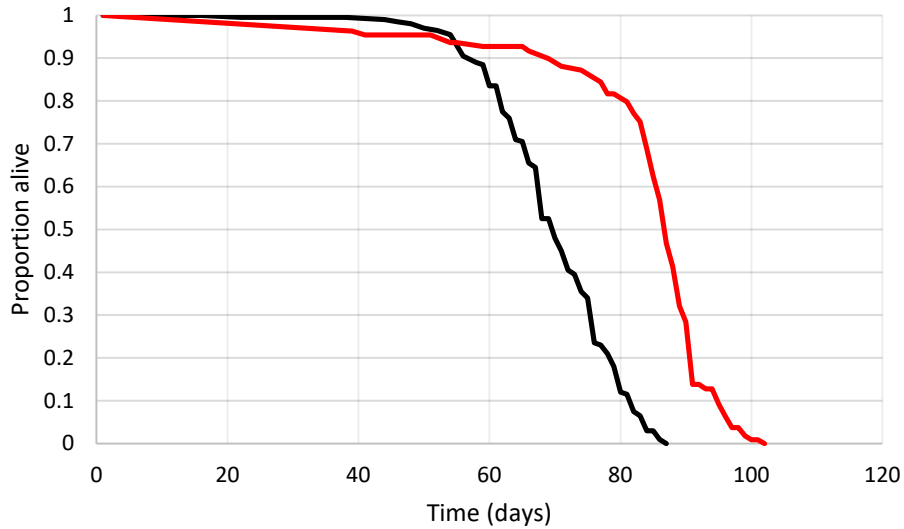
Statistical significance was determined with Excel. The tests were two-tailed t-tests with unequal variance.  $\alpha = 0.05$ .

## Results

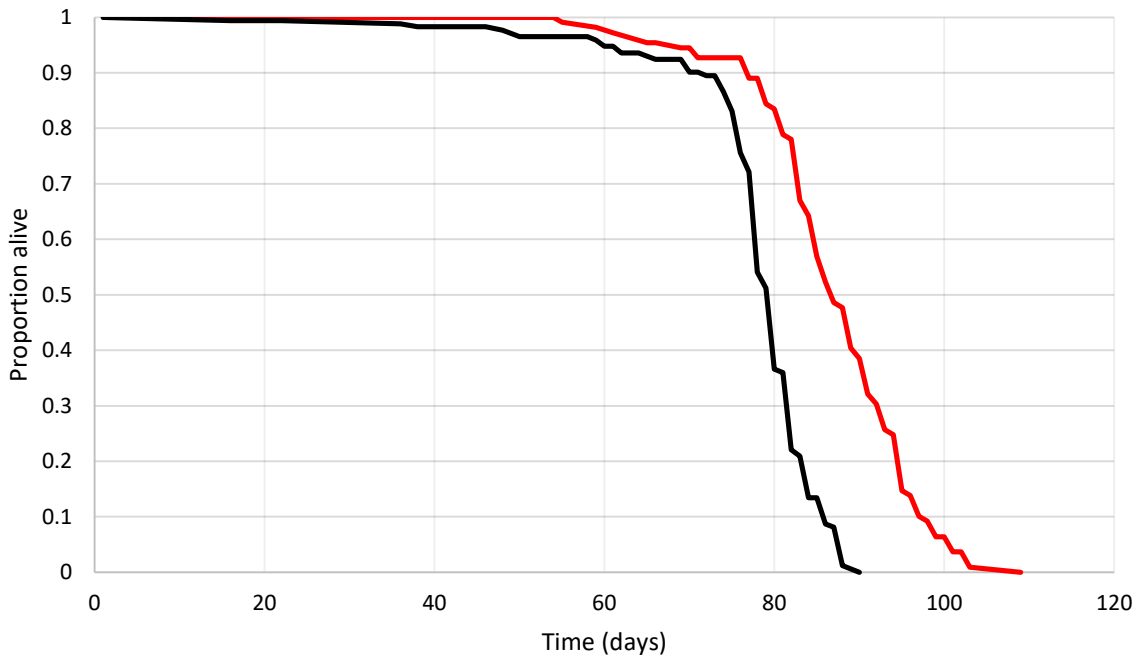
X0 males live longer than normal XY males.<sup>107</sup> Since the Y chromosome is heterochromatic in *Drosophila melanogaster*,<sup>8</sup> the lifespan extension of X0 males may be caused by loss of some of the heterochromatic genome. To test if loss of heterochromatin results in increased lifespan, flies were generated that were missing a large section of pericentric constitutive heterochromatin from chromosome number 2 (see **Figure 4 in Methods section**). The lifespan of these heterozygous flies and flies with normal genomes were measured. Removal of 2R heterochromatin increases the lifespan of both male and female flies (**Figures 12-14**). There was a 20% and 12% increase in average lifespan for males and females, respectively. The results were statistically significant with  $P < 0.05$ .



**Figure 12.** Adult lifespan of wildtype and heterochromatin-deficient *Drosophila melanogaster*. Flies of indicated gender and genotype were collected after emerging from pupae. They were transferred to vials with fresh food every 2 days and deaths were recorded. Error bars represent standard deviation. \* =  $P < 0.05$  compared to wildtype of same gender. Sample sizes from left to right: 200, 109, 172, 109. wt = wildtype. 2Rh- = flies with a heterozygous deletion of a large section of pericentric constitutive heterochromatin on chromosome arm 2R.

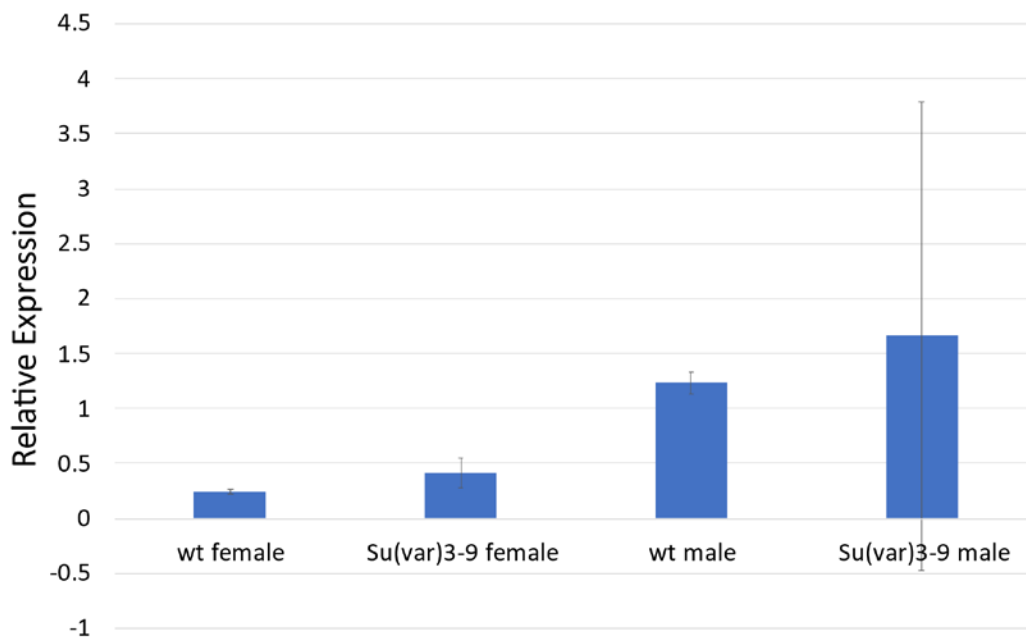


**Figure 13.** Survivorship curves for wild-type and heterochromatin-deficient males. The black curve displays wildtype male fly adult lifespans. The red curve displays adult lifespans from male flies with a heterozygous deletion of a large section of pericentric constitutive heterochromatin on chromosome arm 2R. The data came from the same source as Figure 12.



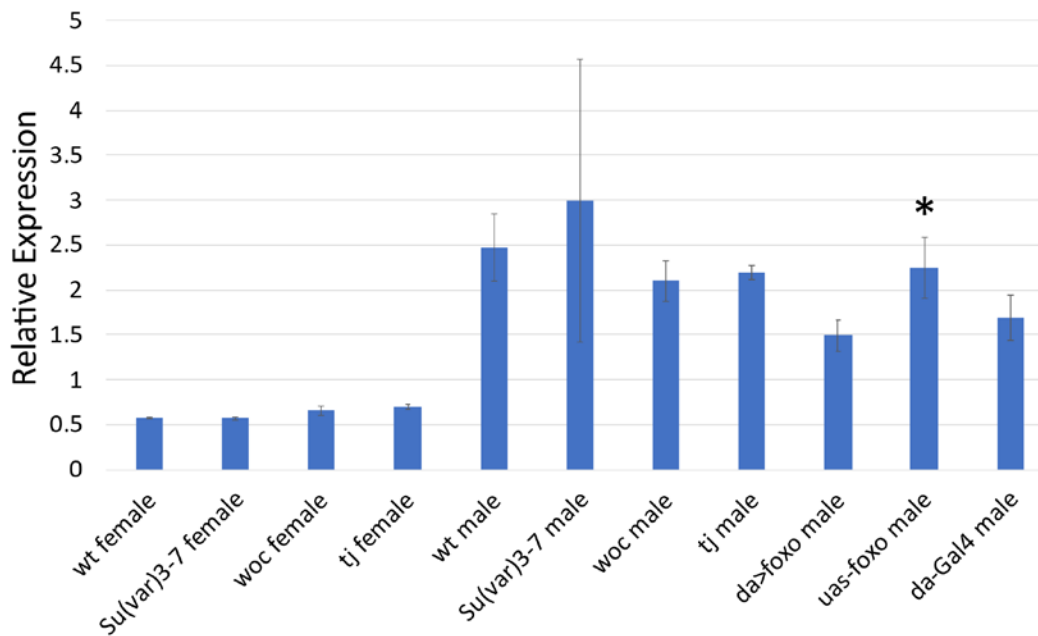
**Figure 14.** Survivorship curves for wild-type and heterochromatin-deficient females. The black curve displays wildtype female fly adult lifespans. The red curve displays adult lifespans from female flies with a heterozygous deletion of a large section of pericentric constitutive heterochromatin on chromosome arm 2R. The data came from the same source as Figure 12.

X0 males live longer than normal XY males.<sup>107</sup> I found in my undergraduate thesis that X0 males also have higher expression of some heterochromatic genes. To investigate whether the expression of heterochromatic genes has an influence on lifespan, the first step was to find mutants of individual genes that are modifiers of heterochromatic gene expression. RT-PCR of the light (lt) gene, a heterochromatic gene, was done on RNA samples from different flies for the purpose of finding mutants that altered heterochromatic gene expression. Su(var)3-9 was one of the genes tested because it is an important component of constitutive heterochromatin. Since heterochromatic gene expression requires the heterochromatin environment,<sup>35</sup> Su(var)3-9 seems like a gene that could modify heterochromatic gene expression. Mutants of Su(var)3-9 did not significantly alter heterochromatic gene expression (**Figure 15**).



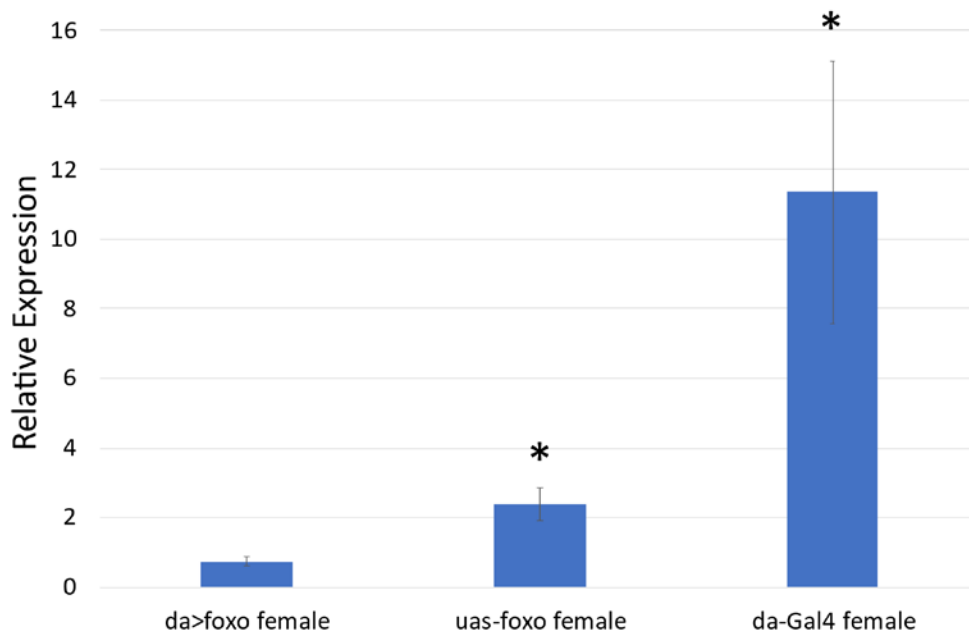
**Figure 15.** Su(var)3-9 underexpressor mutation does not alter expression of the heterochromatic gene light. RT-PCR was conducted on RNA collected from 6-7 day old flies of the indicated gender and genotype to quantify amount of *lt* mRNA. Error bars signify standard deviation. n = 4. wt = wildtype.

Other candidate genes were tested for their ability to modify the expression of the heterochromatic gene light. Su(var)3-7 is a modifier of position-effect variegation, and it physically binds to Su(var)3-9 and HP1a.<sup>15,33</sup> woc and traffic jam (tj) are transcription factors that bind to HP1a.<sup>120,121</sup> Flies bearing extra copies of woc, traffic jam, or Su(var)3-7 do not alter expression of light, a heterochromatic gene (**Figure 16**). foxo is an anti-aging gene<sup>54</sup> and it is a transcription factor.<sup>58</sup> In males, foxo overexpression does not significantly alter the expression of light (**Figure 16**). Expression of light was significantly different for only one of the controls.



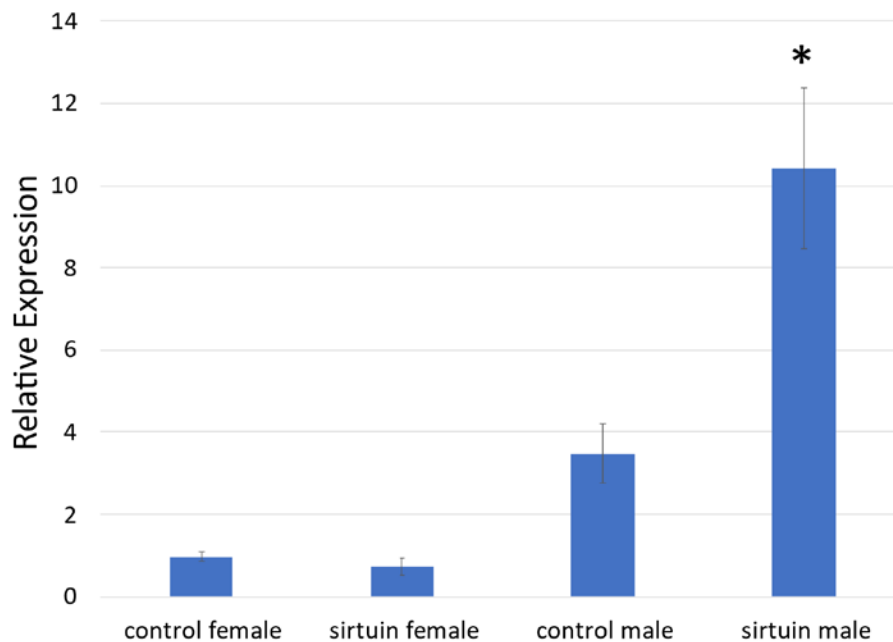
**Figure 16.** Expression of the heterochromatic gene *light* is not influenced by some mutations. RT-PCR was conducted on RNA collected from 5-7 day old flies of indicated gender and genotype to quantify amount of *lt* mRNA. Y-axis indicates relative expression compared to  $\beta$ -Tubulin. Error bars signify standard deviation. \* = statistically significant compared to *da>foxo* male, which is from male flies that ubiquitously overexpress *foxo*. n = 4 for last 3 bars, n = 2 for the rest. wt = wildtype. *uas-foxo* and *da-Gal4* are flies that are related to *da>foxo* but do not overexpress *foxo*.

*foxo*-overexpressing females have significantly lower heterochromatic gene expression compared to both controls (**Figure 17**). *foxo* lowers the expression of the heterochromatic gene *light* in females by at least 2-fold.



**Figure 17.** Overexpressing foxo reduces expression of the heterochromatic gene light in female flies. RT-PCR was conducted on RNA gathered from 5-day old flies to quantify amount of *lt* mRNA. Samples from female flies that overexpressed foxo (*da>foxo*) were compared to samples from the 2 fly lines used to generate the foxo overexpressors. Y-axis is relative expression compared to  $\beta$ -Tubulin. Error bars represent standard deviation. \* =  $P < 0.05$  compared to *da>foxo* female.  $n = 4$ .

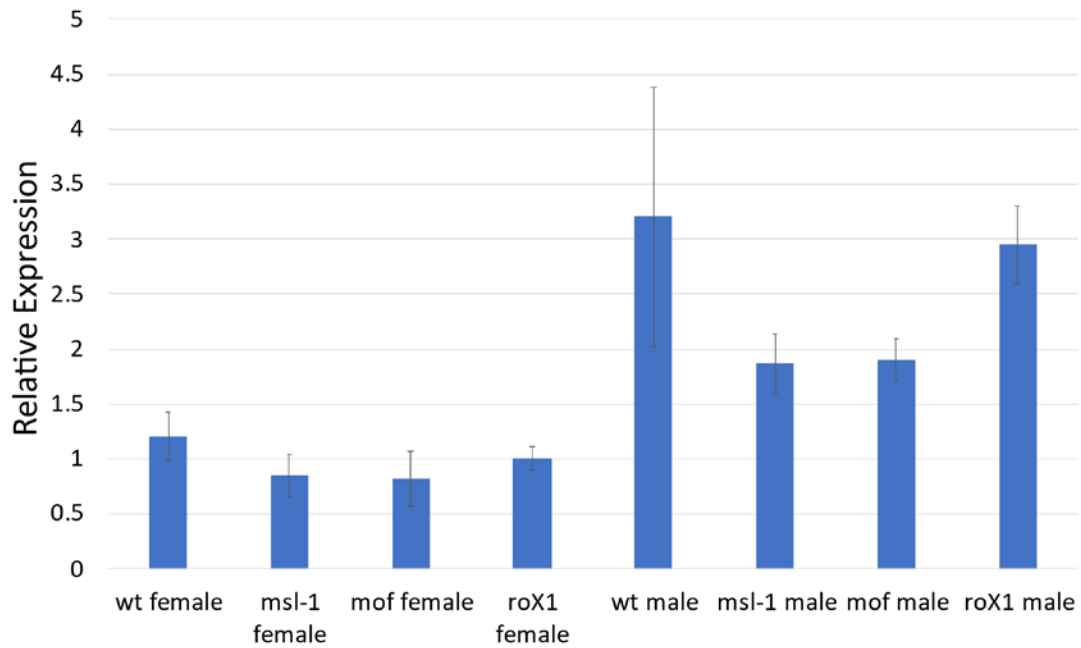
Another candidate gene was Sirt1. Sirt1 is homologous to mammalian SIRT1, a deacetylase that can modify histones including removing H3K9ac (ac is short for acetylated).<sup>122</sup> Removal of H3K9ac permits formation of H3K9me2 or H3K9me3. Mammalian SIRT1 can modify chromatin and silence transcription.<sup>64</sup> Sirt1 is an anti-aging gene.<sup>119</sup> Sirt1 is a modifier of position-effect variegation.<sup>118</sup> The combination of these features on one gene, Sirt1, implies that Sirt1 may be important for understanding the connection between heterochromatin and aging. Overexpressing Sirt1 increases *lt* expression in males, but not females (**Figure 18**).



**Figure 18.** Overexpressing Sirt1 increases expression of the heterochromatic gene light in male flies. RT-PCR was conducted on RNA gathered from 5-7 day old flies to quantify the amount of lt mRNA. Y-axis is relative expression compared to  $\beta$ -Tubulin. Error bars represent standard deviation. \* =  $P < 0.05$  compared to control male (see **Figure 11** in Methods for description of how controls were made). n = 3 for sirtuin male, n = 4 for the rest.

Mutations in members of the male-specific lethal complex reduce heterochromatic gene expression.<sup>123</sup> The function of male-specific lethal complex genes is for dosage compensation in males.<sup>123</sup> The presence of the Y chromosome in males will attract heterochromatic components and could reduce expression of heterochromatic genes. The male-specific lethal complex might counteract this by promoting expression of heterochromatic genes in male flies.<sup>123</sup> I sought to overexpress these same genes to see if heterochromatic gene expression would be increased. Overexpressing members of the male-specific lethal complex does not significantly alter light

expression (**Figure 19**).



**Figure 19.** Overexpression of some genes of the male-specific lethal complex do not influence the expression of the heterochromatic gene light. RT-PCR was conducted on RNA gathered from 4-6 day old flies to quantify the amount of *lt* mRNA. Y-axis is expression of light relative to  $\beta$ -Tubulin. Error bars represent standard deviation. Female samples were not significantly different from each other, same is true for males.  $n = 4$ .

## Discussion

The longevity results support the conclusion that removing some constitutive heterochromatin from *Drosophila melanogaster* increases lifespan. In Brown *et al.*,<sup>107</sup> removal of the Y chromosome generated an increase in lifespan up to 38%. Here, removal of pericentric constitutive heterochromatin from 2R increased lifespan of males by 20% (**Figure 12**). The smaller effect of 2R heterochromatin may be due to its smaller size compared to the Y chromosome. If this is the case, then the effect on lifespan is influenced by the amount of heterochromatin removed. It should be noted that removal of all constitutive heterochromatin from the *Drosophila melanogaster* genome will be lethal because constitutive heterochromatin contains vital genes in this species.<sup>25-27</sup>

Another possible explanation of these results is removing DNA increases lifespan. There are no large deletions of euchromatic DNA because of the relatively high density of vital genes in euchromatin. That would make it seem like removing heterochromatin increases lifespan because there are no large deletions of euchromatin that you can compare it to. Why would removing DNA increase lifespan? One possibility is the cost of DNA replication goes down, and therefore more resources can be used for maintenance. To test if these results are caused by DNA removal, one could generate flies that are missing heterochromatin and have a duplication of a section of euchromatic DNA, then measure lifespan. These flies should have roughly the same amount of DNA as wildtype flies, so if they live longer it is not because of having less total DNA. The idea that removing DNA increases lifespan can be argued against because adding DNA in *Drosophila melanogaster* does not modify lifespan. Normal females and triploids live for the same length of time.<sup>124</sup> In *D. melanogaster*, normal females have a genome with 2 copies of chromosomes X, 2, 3, and 4. Triploids have 3 copies of X, 2, 3, and 4.

The results reported here show that Su(var)3-9, Su(var)3-7, woc, tj, msl-1, mof, and roX1 do not influence the expression of heterochromatic genes. However, in this study only the heterochromatic gene light was tested. A more comprehensive search might find that some of these genes have an effect on some heterochromatic genes.

foxo, an anti-aging gene, suppresses expression of heterochromatic genes in females only. foxo binds to the light gene and other heterochromatic genes.<sup>58</sup> foxo can upregulate and downregulate gene expression.<sup>58</sup> foxo might not systematically influence heterochromatic gene expression. Since foxo is an anti-aging gene,<sup>54</sup> it might selectively target heterochromatic genes that can influence lifespan. foxo binds to some histone genes, and may be a chromatin regulator.<sup>58</sup>

Sirtuins are NAD<sup>+</sup>-dependent deacylases.<sup>122</sup> Overexpressing Sirt1 increases lifespan in both male and female flies.<sup>119</sup> Overexpressing Sirt1 increases heterochromatic gene expression in males, but not females (**Figure 18**). These results can be interpreted more than one way. Firstly, the link between heterochromatin and aging might not be caused by heterochromatic gene expression. If it was, then you would expect increased heterochromatic gene expression in females and males, not just males. There are other proposed mechanisms by which sirtuins can influence lifespan. SIRT6, a mammalian sirtuin, represses activity of retrotransposons.<sup>41</sup> Sirtuin activity inhibits senescence.<sup>40</sup> Another way this can be interpreted is that expression of heterochromatic genes is influenced by the gender of the fly. This might be correct because the influence of foxo on light expression was gender-specific (**Figures 16 and 17**). Since Sirt1 is an anti-aging gene, it might not modify heterochromatic gene expression in bulk. It might optimize the expression of particular heterochromatic genes. The wildtype expression of light in females might already be fairly optimized in that system. If I would have looked into more heterochromatic genes, maybe some of them would have been altered in females that overexpress Sirt1. Sirt1 may be worth

taking a closer look at in the future. At least in males, it seems to be involved with aging, heterochromatin, and heterochromatic gene expression.

The lack of an effect of Su(var)3-9 on heterochromatic gene expression was interesting (**Figure 15**). I suspected the heterozygous mutants tested would have had an effect since the mutant I used is a dominant suppressor of position-effect variegation and Su(var)3-9 is well-known to be important for heterochromatin. Su(var)3-9 is a histone methyltransferase that creates H3K9me2/3.<sup>11</sup> Heterochromatic gene promoters have H3K9me2 and H3K9me3,<sup>13</sup> and this may be important for heterochromatic gene expression. Su(var)3-9 is not the only histone methyltransferase in the *Drosophila melanogaster* genome that can act on H3K9. There may be a different histone methyltransferase that functions to promote expression of heterochromatic genes. eggless (egg) is an enzyme similar to Su(var)3-9 that places H3K9me2/3 at pericentric heterochromatic gene promoters.<sup>13</sup> This is a gene worth investigating in the future for a role in modifying heterochromatic gene expression.

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## Appendix

In an effort to discover DNA sequences unique to heterochromatic genes, the DNA sequences of some *Drosophila melanogaster* heterochromatic genes were compared to their homologous genes from closely related *Drosophila* species. The following procedure was repeated several times. A *Drosophila melanogaster* heterochromatic gene was picked. Its gene sequence, and the homologous sequences from other *Drosophila* species was downloaded (other species were *D. yakuba*, *D. ananassae*, *D. persimilis*, and *D. virilis*). The homologous sequences were assigned a chromatin status of either heterochromatic or euchromatic. Repetitiveness, low gene density, or large gene size were factors that would lead to heterochromatic assignment. Homologous sequences were aligned. Transcription start site locations were determined from available RNA sequence information.

Some of the homologs were also heterochromatic genes, and some of them were euchromatic genes. When comparing homologous heterochromatic genes to the *D. melanogaster* heterochromatic genes, it seems like the transcription start site is fairly well conserved (**Table 2**, row labelled heterochromatin). However, when comparing homologous euchromatic genes to the *D. melanogaster* heterochromatic genes, it seems like the alignment program had difficulty aligning the euchromatic transcription start site region to the *D. melanogaster* gene sequence (**Table 2**, row labelled euchromatin). This implies that conversion of a euchromatic gene to a heterochromatic gene requires mutations that will alter the 5' end of the gene.

**Table 2.** Summary of alignments of transcription start sites between homologous genes. Heterochromatic genes from *Drosophila melanogaster* were compared to their heterochromatic and euchromatic homologs from other *Drosophila* species. The distance between *D. melanogaster* and the other transcription start site, and whether or not the first 10 nucleotides of the other transcript sequence had an insertion or deletion was looked at. The *D. melanogaster* heterochromatic genes were  $\alpha$ -Cat, l(3)80Fj, lt, rl, and scro. bp = distance in base pairs between pair of transcription start sites.

	<100 bp + no insertion + no deletion	>100 bp or insertion or deletion
Heterochromatin	7	3
Euchromatin	1	7