The Y chromosome may contribute to sex-specific ageing in Drosophila

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Heterochromatin suppresses repetitive DNA, and a loss of heterochromatin has been observed in aged cells of several species, including humans and Drosophila. Males often contain substantially more heterochromatin DNA than females, due to the presence of a large, repeat-rich Y chromosome, and male flies generally have a shorter average lifespan than females. Here we show that repetitive DNA becomes de-repressed more rapidly in old male flies relative to females, and repeats on the Y chromosome are disproportionately mis-expressed during ageing. This is associated with a loss of heterochromatin at repetitive elements during ageing in male flies, and a general loss of repressive chromatin in aged males away from pericentromeric regions and the Y. By generating flies with different sex chromosome karyotypes (XXY females and XO and XYY males), we show that repeat de-repression and average lifespan is correlated with the number of Y chromosomes. This suggests that sex-specific chromatin differences may contribute to sex-specific ageing in flies.

The chronic deterioration of chromatin structure has been implicated as one of the molecular signatures of ageing, and an overall loss of heterochromatin and repressive histone marks is observed in many old animals. Heterochromatin is enriched at repetitive DNA, and its loss can result in de-repression and mobilization of silenced transposable elements (TEs) while the pericentromeric heterochromatin on the X amounts to only ~10–20 Mb (depending on the strain). This implies, therefore, that a substantially larger fraction of the male genome is heterochromatic compared to that of the female. Males have a shorter average lifespan in many taxa, including humans and most Drosophila species. Indeed, the genetic sex determination system predicts adult sex ratios in tetrapods, with the heterogametic sex being less frequent. Lower survivorship of the sex with the repetitive Y or W chromosome may suggest a link between sex-specific mortality, chromatin and sex chromosomes.

Here, we test for an association between sex-specific heterochromatin loss and a de-repression of repetitive DNA during ageing, by assaying chromatin and gene expression profiles in young and aged individuals of D. melanogaster. We further create flies with different sex chromosome karyotypes (that is, XO and XXY males and XXY females), to assess the influence of the Y chromosome on longevity and sex-specific changes in repeat expression.

Results

Drosophila males and females differ in repeat content and longevity. We chose D. melanogaster to investigate the contribution of the Y chromosome to sex-specific ageing, since a substantial fraction of its heterochromatin, including its Y chromosome, has been assembled. In addition, the availability of mutant strains allows us to generate flies that differ in their sex chromosome configuration. Figure 1a shows a schematic overview of the karyotype for D. melanogaster males and females, and the approximate size and position of large heterochromatic segments. Previous studies have shown that males have approximately 20 Mb more heterochromatin per cell than females (Fig. 1a), and flow cytometry estimates verify that the Canton-S strain used here shows similar differences in repeat content between the sexes (Table 1). This confirms, therefore, that male Canton-S flies contain substantially more repetitive DNA than females.

Previous work in Drosophila showed that, for the vast majority of species, male flies live for a significantly shorter time than females. In particular, a study surveying longevity in 68 species (and 89 strains) of Drosophila found statistically significant differences in longevity between the sexes for 55 strains. Females had higher longevity in 53 strains, and males lived longer in only two. We determined longevity for males and females of two standard laboratory strains of D. melanogaster (Canton-S and Oregon-R), and the 2549 strain from the Bloomington Stock Center, which has a compound metacentric X chromosome (that is, two X chromosomes fused at the centromere) and a hetero-compound X-Y chromosome (that is, an X chromosome inserted between the two arms of the Y chromosome). Lifespan assays confirm that males live significantly shorter than females, for both wild-type (WT) strains and the 2549 strain (Fig. 1b, and see Extended Data Fig. 1 for lifespan assays in Oregon-R and 2549). Increased longevity of females is consistent with multiple studies on sex-specific lifespan in Drosophila.

Heterochromatin loss differs between sexes. We gathered replicate chromatin immunoprecipitation sequencing (ChIP-seq) data for a repressive histone modification typical of heterochromatin (H3K9me2) from young (8-day) and old (64–68-day) D. melanogaster males and females (Canton-S), to test for sex-specific heterochromatin loss during ageing. We used a ‘spike-in’ normalization method to compare the genomic distribution of chromatin marks across samples. Specifically, we spiked in a fixed amount of chromatin from Drosophila miranda to each D. melanogaster chromatin sample before ChIP and sequencing. We chose the former species to serve as an internal standard for our D. melanogaster ChIPs, since they are sufficiently diverged from each other that there is very little ambiguity in the assignment of reads to the correct species.
We employed a previously described normalization strategy\(^2\), where the relative recovery of \textit{D. melanogaster} ChIP signal versus \textit{D. miranda} ChIP signal, normalized by their respective input counts, was used to quantify the relative abundance of the chromatin mark in \textit{D. melanogaster}. We also used a linear regression model to estimate the relative recovery of \textit{D. melanogaster} ChIP signal versus that of \textit{D. miranda}, normalized by their respective input counts\(^2\). Overall enrichment patterns and differences between sexes and ages are quantitatively similar between the two methods, showing that our inferences are robust to our normalization strategy (Supplementary Fig. 1a).

Repetitive regions pose a challenge for mapping with short reads, since one cannot be sure that a particular locus is generating the reads in question if these map to multiple positions. Our study is concerned with the overall behaviour of repetitive regions in the genome during ageing, and is not focused on any particular locus. Thus, analysis of all reads (including those mapping to multiple locations) is most appropriate for our purpose. However, we repeated our analysis using only uniquely mapping reads, which confirms that our inferences are robust when considering only those reads (Supplementary Fig. 1b).

Figure 1c shows the genomic distribution of the repressive histone modification H3K9me2 for young and old male and female flies. As expected, heterochromatin is enriched at repetitive regions, including pericentromeres, the small dot chromosome and the repeat-rich \(Y\). While the genomic distribution of H3K9me2 appears similar between young males and females\(^2\), heterochromatin enrichment changes dramatically in old male flies but less so in old females. In particular, we see a general loss of heterochromatin at repetitive regions in aged males (Fig. 1c; see Extended Data Fig. 2 for biological replicate), and males show significantly more regions that lose H3K9me2 signal (1.5-fold or more) during ageing compared to females (232 versus 73, \(p<2.2\times10^{-16}\), Wilcoxon test) for males (blue) and females (red), with pericentromere boundaries defined by the Release 6 version of the \textit{D. melanogaster} genome. Boxes represent 25th and 75th percentile, and whiskers show the most extreme values within 1.5\times interquartile range.
Mis-expression of heterochromatic and lamina-associated genes in old flies. Sex-specific chromatin changes during ageing are associated with sex-specific expression changes. To study gene expression during ageing, we gathered replicated stranded RNA sequencing (RNA-seq) data from young and old flies (Supplementary Fig. 4 and Supplementary Tables 3 and 4). We found that genes located in pericentromeric regions change their expression more during ageing compared to those in chromosomal arms, in both sexes (Supplementary Fig. 5). While heterochromatin typically has a repressive effect on gene expression, genes located in normally heterochromatic regions (such as the pericentromere) are known to be dependent on this repressive chromatin environment for proper transcription\(^2\). Indeed, the global loss of heterochromatin in pericentromeric regions is associated with reduced expression levels of pericentromeric genes in aged males and females (Supplementary Fig. 5). Genes that gain the H3K9me2 mark during ageing tend to be located close to the pericentromeric boundary (\(P=0.04\), Fisher’s exact test; Supplementary Figs. 2 and 3 and Extended Data Fig. 3). This suggests that heterochromatin–euchromatin boundaries are less efficiently maintained in old flies, resulting in spreading of heterochromatin from the repeat-rich pericentromere into neighbouring regions. Thus, our results show that male \textit{D. melanogaster} flies lose heterochromatin marks more rapidly than female flies, and our results are reproducible using different mapping and normalization strategies and independent biological replicates.

Repeat de-repression in old male flies. In addition, we found sex-specific differences in repeat reactivation during ageing. We mapped our transcriptome data to the consensus repeat library of \textit{D. melanogaster} and detected low levels of expression of repetitive elements in young male and female flies (Fig. 2a). Aged females maintained efficient repression of TEs, while expression for the major classes of annotated TEs increased during ageing for males (Fig. 2a,c). De-repression of TEs is more pronounced in males, both in terms of the number of individual elements showing a significant increase in expression during ageing, as well as the fraction of the transcriptome that consists of repetitive transcripts across all...
Fig. 2 | Sex-specific silencing and expression of repeats during ageing. 

**a** Expression of all repeats from FlyBase consensus library from Release 6 of the *D. melanogaster* genome in young (8-day) and old (64- or 68-day) male and female Canton-S, averaged across replicates, with significance values calculated using the Wilcoxon test (**P < 0.01, ***P < 1x10⁻⁵**). Heatmaps are visualized globally according to the scale, with dark red corresponding to the top 5% of all values across all samples and dark blue corresponding to the bottom 5% of all values across all samples. **b** H3K9me2 enrichment in repeats from FlyBase consensus library in young (8-day) and old (64- or 68-day) male and female Canton-S, averaged across replicates, with significance values calculated using the Wilcoxon test (**P < 0.01, ***P < 1x10⁻⁵**). The heatmap is scaled in the same manner as in **a**. **c** Expression for repeat families for a old and young males and females, with lines indicating s.d. for each estimate of expression across replicates, and colours indicating the class of repetitive element. **d** Expression and H3K9me2 signal in putatively Y-linked repeats in young (8-day) and old (64- or 68-day) Canton-S males, with significance values calculated using the Wilcoxon test (**P < 0.01, ***P < 1x10⁻⁵**). Heatmaps are scaled in the same manner as in **a**, **c** Expression of putatively Y-linked repeats for old and young Canton-S males, with lines indicating s.d. for each estimate of expression across replicates. Boxes represent 25th and 75th percentile, and whiskers show the most extreme values within 1.5x interquartile range. RPKM, reads per kilobase million.

repetitive elements. Overall, we found that in females, six repetitive elements showed a significant increase in expression during ageing and 14 a significant decrease (Fig. 2c), but the total fraction of transcripts derived from repeats increased during ageing (the fraction of repetitive reads in all RNA-seq reads was 2.0% at 8 days versus 4.6% at 68 days (Supplementary Table 1 and Supplementary Fig. 12). The increase in repeat expression was much more pronounced in males, with 32 repetitive elements showing a significant increase in expression during ageing and four showing a significant decrease (Fig. 2c and Supplementary Fig. 12), and the total fraction of repetitive reads increased from 1.6 to 5.8% (Supplementary Table 1). The TE showing the highest level of de-repression in both sexes was *copia*, which was expressed 28-fold more in old versus young males and 15-fold more in old versus young females (Fig. 2c). H3K9me2 profiles at TE families show that there is a general enrichment of this repressive mark in young male and female flies (Fig. 2b). Consistent with genome-wide expression profiles showing overall efficient silencing of repeats in old females, there was no global loss of the repressive chromatin mark at repetitive elements in 68-day-old females (in fact, there was a slight increase; Fig. 2b). However, aged *D. melanogaster* males underwent a general loss of H3K9me2 histone modification at repetitive elements (Fig. 2b). Thus, chromatin and gene expression profiles show that TEs lose their epigenetic silencing and become mis-expressed in old male flies.
Males have approximately 20% more repetitive sequence than females, due to the repeat-rich Y chromosome. The sex-specific increase in repeat expression may be triggered by the presence of the heterochromatic Y chromosome in males, and the Y indeed showed a dramatic loss of heterochromatin during ageing (Fig. 1c). To determine whether Y-linked repeats are especially prone to mis-regulation during ageing, we used de novo assembled male-specific and male-biased (putatively Y-linked) repetitive sequences (Supplementary Fig. 13). Indeed, we found that in males, putatively Y-linked repeats were upregulated more strongly during ageing relative to repeats present in both sexes \( (P = 5.9 \times 10^{-12}, \text{Wilcoxon test}; \text{Fig. 2d,e}) \). Overall, we found that 42 Y-linked repeats showed a significant increase in expression during ageing (and only one a significant decrease; Fig. 2d,e and Supplementary Fig. 14), and the total fraction of transcripts derived from Y-linked repeats increased more than ninefold in old males (Supplementary Table 2). Additionally, putatively Y-linked repeats disproportionately lost the repressive histone modification H3K9me2 during ageing compared to other repeats \( (P = 3.4 \times 10^{-11}, \text{Wilcoxon test}; \text{Fig. 2d}) \). Thus, male-biased and male-specific repeats—that is, repeats located on the Y chromosome—are especially prone to de-repression during ageing in males.

**Flies with additional Y chromosomes have decreased lifespan.** In *Drosophila*, sex is determined by the ratio of X chromosomes to autosomes independently of the presence of the Y chromosome\(^{31}\). This allowed us to test whether the Y chromosome contributes to sex-specific TE de-repression and ageing, by generating *D. melanogaster* females containing a Y chromosome (XXXY flies) and males with either zero or two Y chromosomes (X0 and XYY flies, respectively). We crossed Canton-S flies with different strains having attached-X and attached-X-Y chromosomes to generate XXY females and X0 and XYY males (Fig. 3a; see Methods for strain information and Table 1 for genome size estimates). Note that X0 males and XYY females of a given cross have the same autosomal background but differ in their genomic background from other crosses and from Canton-S, which could contribute to the observed lifespan variation among strains (Fig. 3b,c). Utilizing different strains to generate flies with aberrant sex chromosomes, however, should allow us to control for genomic background effects to some extent, by randomizing across different backgrounds. Indeed, we found qualitatively identical results using three independent strains to generate XO/XX/XXX/XXXY flies, suggesting that differences in longevity are not due to genomic background but are caused by the presence or absence of the Y. Note, however, that we cannot formally exclude background effects using these crosses.

In particular, we compared sex-specific lifespans of WT Canton-S *D. melanogaster* flies and XXX females and X0 and XYY males resulting from (back-)crosses to the three different attached-X and attached-X-Y strains (Fig. 3b,c). Cumulative survival probabilities showed that the lifespan of females containing a Y chromosome (XXX females) is reduced relative to WT females or males lacking a Y chromosome (X0 males) for all crosses assayed (Fig. 3b,c). Indeed, X0 males showed a dramatic increase in lifespan relative to WT males and even outlived WT females (Fig. 3b,c). X0 males are sterile and have the least amount of repetitive DNA of all karyotypes investigated (\(-10 \text{ and } -40 \text{ Mb less than Canton-S females and males, respectively; Table 1}\); both of these factors may contribute to increased lifespan. Males with two Y chromosomes (XYY), in contrast, lived the shortest (Fig. 3b,c) and their lifespan was reduced considerably relative to WT males despite both karyotypes being fertile. Thus, survivorship data are consistent with the hypothesis that the number of Y chromosomes influences organismal survival in *Drosophila*.

**Mis-expression of Y genes and repeats in flies with additional Y chromosomes.** Gene expression changes during ageing in aberrant karyotypes show many of the same patterns as in WT flies, with similar networks of GO terms enriched in both X0 and XYY males, and XXY females, including ‘reproduction’ or ‘immune response’ (Supplementary Fig. 9). Overall, we found that 101 of the top 10% of genes mis-expressed during ageing were shared among all five karyotypes \( (P = 1 \times 10^{-13}, \text{permutation test}) \). These genes do not show any enrichment for a particular GO term, and only six of them are located inside or within 1 Mb of the pericentromere (expect 4.8 genes).

Genomic location also influences gene expression changes during ageing in flies with aberrant karyotypes. As in WT males and females, genes located in the pericentromere showed a decrease in expression during ageing in XXY females and X0 males (Supplementary Fig. 15; XYY flies showed no significant expression change at pericentromeric genes). Y-linked genes in WT males are expressed almost exclusively in male reproductive tissues\(^{29}\), and we did not detect any expression of Y-linked genes even in very old XY male heads (Supplementary Fig. 15). In contrast, we found that Y-linked genes were inefficiently silenced in heads of XXY females and XYY males and were becoming de-repressed even more during ageing (six Y genes were expressed in old XXY females and 14 in old XYY males; Supplementary Fig. 15).

Thus, WT males maintain efficient silencing at their Y-linked genes during ageing, despite global heterochromatin loss on the Y chromosome and mis-expression of Y-linked repeats. Silencing mechanisms on the Y chromosome of XXY and XYY flies, on the other hand, appear to be generally compromised, even in young individuals. Indeed, we previously showed that the Y chromosome affects global heterochromatin integrity\(^{29}\). Young flies with additional Y chromosomes (XXXY females or XYY males) showed lower levels of H3K9me2 enrichment at their TEs and a de-repression of Y-linked repeats relative to WT flies, while X0 flies showed increased levels of H3K9me2 at repeats\(^{29}\). Expression profiles in XXY females and XYY/X0 males suggest that the absence or presence of the Y chromosome modulates expression of TEs during ageing (Fig. 4). Expression profiles from aged flies with aberrant sex chromosome karyotypes confirm our expectation that X0 males show less de-repression of TEs during ageing relative to WT males (Fig. 4a). XYY males, on the other hand, show more mis-expression of repeats during ageing compared to WT females. In XXY females, seven repetitive elements showed a significant increase in expression during ageing while three repetitive elements showed a significant decrease (compared to 6/14 TEs that increased/decreased expression in WT females; Fig. 4a), and the fraction of repetitive transcripts increased more during ageing for XXY females (3.1-fold increase in XXY females versus 2.2-fold in WT females; Supplementary Table 1). XYY males showed the greatest number of repeats with significantly increased expression during ageing (33 repetitive elements, Fig. 4a), but not the highest fold change in total fraction of repetitive reads (Supplementary Table 1), partly because young XXY males already showed the highest expression of repeats of any of the five karyotypes (Supplementary Table 1) and partly because old XXY males were approximately 30 days younger than the other karyotypes (Fig. 3b).

Mis-expression of repetitive elements in XXY females and XYY males is especially pronounced for repeats found on the Y chromosome. Y-linked repeats showed reduced silencing, even in young XXY females and XYY males relative to WT males\(^{29}\) (Fig. 4b), and became de-repressed even more during ageing in XXY and XYY individuals (Fig. 4b). Overall, WT males expressed 64 putatively Y-linked repeats during their lifespan while XXY females and males expressed 86 and 102, respectively. In XXY females, 13 repeats significantly increased in expression during ageing (5 decrease) and 71 repeats significantly increased in expression in XYY males (and 8 decreased; Supplementary Fig. 14). Indeed, even at just 37 days of age, XXY males already showed higher, presumably...
harmful, expression of Y-linked repeats compared to 68-day-old WT males (Fig. 4b).

**Discussion**

Heterochromatin loss during ageing has been observed in a wide variety of species, ranging from yeasts to worms, flies and mammals⁴⁻⁶. Here, we show that sex-specific heterochromatin loss may contribute to sex-specific cellular ageing in *Drosophila*. In particular we found that increased heterochromatin loss in old male flies is associated with increased expression of repeats, and especially of repeats located on the Y chromosome. TE activation in old flies can lead to mutagenic insertions and genomic instability⁸⁻¹¹.

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**Fig. 3 | Survivorship of XXY females and X0 and XYY males.**

*a*, Schematic crossing scheme used to generate flies with aberrant sex chromosomes, with Canton-S used as WT males and females for all crosses, and various lines with C(1)RM and C(1;Y) indicated by the attached-X and attached-XY karyotypes. Crosses between WT females and attached-XY males resulted in X0 flies (left), crosses between attached-X females and WT males resulted in XXY females (middle) and crosses between XXY females and attached-XY males resulted in XYY males (right). **b**, Kaplan–Meier survivorship curves for flies with aberrant sex chromosome karyotype, generated with various C(1)RM and C(1;Y) lines as indicated at the top of each survivorship curve (stock 2549 and 4248 were obtained from the Bloomington Stock Center, and stock 100950 was obtained from the Kyoto Stock Center). The crosses used to obtain the XO, XXY and XYY flies are shown in a. Shaded areas indicate the upper and lower 95% confidence intervals calculated from Kaplan–Meier curves.

**c**, Median lifespan for each of the different karyotypes measured, with error bars indicating the upper and lower 95% confidence intervals (estimated from the Kaplan–Meier curves). Significance is compared to the WT Canton-S of the same sex for each aberrant karyotype, and was calculated using the survdiff package in R (**P < 1 × 10⁻⁶, ***P < 1 × 10⁻¹²).
Fig. 4 | Expression of repetitive elements in XXY females and X0 and XYY males during ageing. a. Expression of all repeats from FlyBase consensus library from Release 6 of the D. melanogaster genome. The heatmap shows averaged expression across replicates, with significance values calculated using a Wilcoxon test (*P < 0.05; **P < 0.01; ***P < 0.001). The scatterplots show expression of all repeats from the FlyBase consensus library in young and old X0 males, XXY females and XYY males, with lines indicating the s.d. of each expression value calculated from replicates, and colours indicating the class of repetitive element. b. Expression for putatively Y-linked (male-specific) repeats in karyotypes with a Y chromosome, averaged across replicates, with significance calculated using the Wilcoxon test (*P < 0.05; **P < 0.001; ***P < 1 × 10⁻⁵). Scatterplots as in a for putatively Y-linked repeats. Boxes represent 25th and 75th percentile, and whiskers show the most extreme values within 1.5x interquartile range.

We further show that the absence or presence of a Y chromosome correlates with increased/reduced lifespan in flies.

The molecular mechanisms underlying heterochromatin loss during ageing are poorly understood, and our study does not allow us to address the causative cellular mechanisms underlying sex-specific heterochromatin loss and ageing. One prominent heterochromatic structure that has been directly implicated in ageing is the nucleolus. The nucleolus, the site of ribosome assembly, is formed at the tandemly repeated rDNA and is embedded in heterochromatin in most eukaryotes. In Drosophila, the rDNA cluster is located on the X and Y chromosomes, each containing a few hundred rDNA transcriptional units in tandem repeats. Nucleolar size and activity have been mechanistically implicated in ageing and longevity. In yeast, RNA instability (that is, reduction of rDNA copy number and associated accumulation of extra-chromosomal rDNA circles) is a major cause of replicative ageing. Destabilization and loss of rDNA has been shown during ageing of male germline stem cells in Drosophila, which manifests cytologically as atypical morphology of the nucleolus. In contrast, no such defects of nucleolar morphology were found in young and old female germline stem cells. The rDNA locus is highly transcribed, and potential collisions between the replication and transcription machinery are thought to contribute to genomic instability of rDNA. In male Drosophila, transcription of rDNA is normally restricted to the Y chromosome (nucleolar dominance), and transcriptionally active Y-linked rDNA copies are preferentially lost in ageing germline stem cells. Flies with only a single rDNA cluster (that is, X0 flies) live the longest, while those with extra Y chromosomes (and thus more rDNA copies; see Supplementary Fig. 15) live shorter (Fig. 3). This suggests a more complex picture on how the nucleolus may contribute to sex-specific ageing, and future detailed functional experimentation is necessary to understand the molecular basis of the sex-specific differences in heterochromatin loss in Drosophila.

Ageing is modulated by both genetic and environmental factors, including diet, temperature and mating status of flies, some of which show sex-biased effects on longevity. Dietary restriction, an intervention known to extend lifespan, has been shown to prevent
TE activation in old flies\(^1\), and it will be of great interest to study how manipulations that are sex-biased in their effects on ageing will influence sex-specific heterochromatin loss and repeat activation in aged flies.

To conclude, our data support the hypothesis that the repeat-rich Y chromosome may decrease lifespan in *Drosophila*. Loss of heterochromatin in repetitive regions during ageing is more pronounced in male flies, and is accompanied by a de-repression of TE\(s\). Y-linked repeats disproportionately lose their repressive marks and become reactivated, and analysis of flies with aberrant sex chromosome configurations is consistent with the notion that the Y has a direct influence on organismal survival. Age-related heterochromatin loss on the repetitive, sex-limited Y or W chromosome and repeat reactivation could contribute to lower survivorship of the heterogametic sex across taxa\(^2\), including humans. The Y chromosomes of *Drosophila* and humans are known to harbour structural polymorphism in heterochromatic sequences and copy number variation in repeats\(^{3,4}\), and polymorphism on the *D. melanogaster* Y has been shown to affect lifespan\(^5\), the formation of heterochromatin\(^6\) and the regulation of TEs and hundreds of genes genome-wide\(^{7,8}\). More generally, individual humans and flies show extensive variation in their repeat content\(^{9,10}\), and our results raise the question of whether natural variation in repetitive sequences can contribute to genetic variation in longevity among individuals.

Methods

**Drosophila strains.** Fly strains were obtained from the Bloomington Stock Center and the Kyoto Stock Center. The following strains were used: Canton-S; Oregon-R: 2549 (C1;Y; y; cv/cv; v/B & C(1)RM; v/v); 4248 (C1;RM, y’; pm’; v & C(1)Y, y’ B’/B & ss+/ss+); from the Bloomington Stock Center and 100950 (C1;1(RM, y’w’, C(tz1);1, y’; y’ ac’; sc’; w’)) from the Kyoto Stock Center. The screening scheme used to obtain X0 and XY males and XXY females is depicted in Fig. 3a. For chromatin and gene expression analyses, flies were grown in incubators at 25 °C, 60% relative humidity and 12 h light for the indicated number of days following eclosion, and were then flash-frozen in liquid nitrogen and stored at −80 °C. See Supplementary Fig. 17 for exact crossing scheme.

**Genome size estimation.** We estimated genome size of the five karyotypes of interest using flow cytometry methods similar to those described in ref. \(^1\). Briefly, samples were prepared using a 2-ml Dounce to homogenize one head each from an internal control (*Drosophila virilis* female, IC = 328 Mb) and one of the five karyotypes in Galbraith buffer (44 mM magnesium chloride, 34 mM sodium citrate, 0.1% (v/v) Triton X-100, 20 mM MOPS, 1 mg/ml RNaseA, pH 7.2). After homogenization of samples with 15–20 strokes, samples were filtered using a nylon mesh filter and incubated on ice for 45 min in 25 µg ml\(^{-1}\) propidium iodide. Using a BD Biosciences LSR II flow cytometer, we measured 10,000 cells for each unknown fluorescent peak interval. We calculated the genome size of the five karyotypes by conditions described above. Lifespan assays were conducted as described in ref. \(^52\).

**Lifespan assays.** Lifespan data were collected for all karyotypes under the rearing conditions described above. Lifespan assays were conducted as described in ref. \(^1\). Briefly, synchronized embryos were collected on agar plates, mobilized with a cotton swab, washed three times with PBS pH 7.4 and 10 µl of embryos was pipetted into a fresh vial of standard molasses fly medium (0.68% agar, 2.7% yeast, 6.07% cornmeal, 0.45% propionic acid, 1.6% sucrose, 0.76% of 95% ethanol, 0.09% Tigespoet, 8.2% molasses, 0.0625% CaCl\(_2\), 0.75% Na tartrate). Adult flies were collected over 2 d and were allowed to mate for a further 2 d. Flies were then sexed quickly in batches under light CO\(_2\) to minimize exposure, and 30 flies were counted into each vial. Vials were then flipped, without using CO\(_2\), every 2–3 d, and fly deaths were recorded. Throughout the experiments, flies were grown in incubators at 25 °C, 60% relative humidity and 12 h light. Flies that were observed escaping the vial were censored. To collect samples for the RNA-seq and ChIP-seq experiment, we censored the entire lifespan once it reached 50% survivorship and flash-froze the remaining flies in liquid nitrogen. In total, 6,829 flies in 297 vials were counted for the lifespan assays reported here.

**ChIP-seq.** We performed ChIP-seq experiments using a standard protocol adapted from ref. \(^1\). Briefly, approximately 2 ml of adult flash-frozen flies were dissected on dry ice, and heads and thoraces were used to fix and isolate chromatin. Following chromatin isolation, we spiked in 60 µl of chromatin prepared from female *D. miranda* larvae (approximately 1 µg of chromatin). We then performed immunoprecipitation using 4 µl of the H3K9me2 antibody (Abcam, no. ab1220). After reversal of cross-links and isolation of DNA, we conducted sequencing library preparation using the BQO Nextera kit. Sequencing was performed at the Vincent J. Coates Genomic Sequencing Laboratory at UC Berkeley, supported by NIH S10 Instrumentation Grants nos. S10RR029668 and S10RR027303. We performed 50–bp single-read sequencing for our input libraries, and 100–bp paired-end sequencing for H3K9me2 libraries, due to their higher repeat content.

We collected replicate datasets for H3K9me2 enrichment in aged males and females to confirm differences seen between the sexes and between young and old samples (Extended Data Fig. 2 and Supplementary Fig. 18). Replicate H3K9me2 data for young flies are derived from ref. \(^1\).

RNA extraction and RNA-seq. We collected replicate RNA samples for aged individuals of all five karyotypes of interest; replicate RNA data for young flies are derived from ref. \(^1\). Additionally, we collected three replicate samples for aged male Canton-S, aged XXY females and aged XYY males, and four replicate samples for aged female Canton-S and aged X0 flies. After flash-freezing in liquid nitrogen, we dissected and pooled five heads from each sample. Extracted RNA and prepared stranded total RNA-seq libraries using Illumina’s TrueSeq Stranded Total RNA Library Prep kit with Ribo-Zero ribosomal RNA reduction chemistry, which depletes the highly abundant RNA transcripts (Illumina, no. RS-122-2201). We performed single-read sequencing for all total RNA libraries at the Vincent J. Coates Genomic Sequencing Laboratory at UC Berkeley.

Mapping of sequencing reads and data normalization. For all *D. melanogaster* alignments, we used Release 6 of the genome assembly and annotation\(^1\). For all ChIP-seq datasets we used Bowtie2 (ref. \(^1\)) to map reads to the genome, using the parameters ‘-D 15 –R 2 –N 0 –L 22 –S 1,0,50 –no-1-mm-upfront’, which allowed us to reduce cross-mapping to the *D. miranda* genome to approximately 2.5% of 50-bp reads and 1% of 100-bp paired-end reads. We also mapped all ChIP-seq datasets to the *D. miranda* genome assembly\(^1\) to calculate the proportion of each library originating from the spiked-in *D. miranda* chromatin versus the *D. melanogaster* sample.

To calculate the ChiP signal, we first calculated the coverage across 5-kb windows for both the ChiP and input, and then normalized by the total library size, including reads that mapped to both *D. melanogaster* and *D. miranda* spike. We then calculated the ratio of ChiP coverage to input coverage for each of the 5-kb windows, normalized by the ratio of *D. melanogaster* reads to *D. miranda* reads in the ChiP library, and then by the ratio of *D. melanogaster* reads to *D. miranda* reads in the input, to account for differences in the ratio of sample to spike present before immunoprecipitation. We describe the validation of this normalization method in ref. \(^1\). Note that this normalization strategy accounts for differences in ploidy levels of sex chromosomes (see Supplementary Fig. 7 in ref. \(^1\)).

**Gene expression analysis.** For each replicate of RNA-seq data, we first mapped RNA-seq reads to the RNAseq scaffold in the Release 6 version of the *D. melanogaster* genome, and removed all reads that mapped to this scaffold because differences in rRNA transcript abundance are likely to be technical artefacts from the total RNA library preparation, which aims to remove the bulk of rRNA transcripts. We filtered out the remaining reads in the Release 6 version of the *D. melanogaster* genome using STAR\(^\(_{55}\)_\), with default parameters. We then counted reads mapping to each transcript using the FeatureCounts module of Subread\(^\(_{57}\)_\). Gene counts were imported into DESeq2 for differential expression analysis\(^\(_{58}\)_\), using the two replicates for each karyotype to calculate log fold change Pvalue estimates. GO analysis was performed using GOrilla, using ranked lists of differentially expressed genes\(^\(_{59}\)_\). GO terms that were enriched, and with FDR < 0.1, were visualized using the software Revigo\(^\(_{60}\)_\). Gene expression changes during ageing for all five karyotypes are given in Supplementary Table 3.

Repeat libraries. We used two approaches to quantify expression of repeats. The first was based on consensus sequences of known repetitive elements that are inserted in the Release 6 version of the *D. melanogaster* genome and are available on FlyBase. These included consensus sequences for 125 TEs and the three largest satellites (359, dodeca and responder).

Our second approach aimed to specifically assess the repeat content of the Y chromosome. Since the Y chromosome is poorly assembled and repetitive elements on the Y are relative nietassembled, we previously assembled repetitive elements de novo from male and female genomic DNA reads using RePair and identified 101 male-specific repeats comprising 13.7 kb of sequence, based on male-specific coverage analysis\(^\(_{61,62}\)_\).

To assess expression of repetitive elements, we mapped RNA-seq reads to each of the repeat libraries (consensus TEs and putatively Y-linked repetitive elements) using Bowtie2 (ref. \(^1\)) and the parameters ‘-D 15 –R 2 –N 0 –L 22 –S 1,0,50 –no-1-mm-upfront’. We then calculated mean coverage across each repetitive element using Bedtools\(^\(_{63}\)_\) and normalized the coverage by the number of uniquely mapping reads in the sequencing library. We made this calculation independently
for each replicate for each time sample and karyotype, and then calculated both the average expression value and s.d. to assess statistical significance and reproducibility (Supplementary Fig. 19).

To assess H3K9me2 signal in repetitive elements, we took a similar approach to that used to calculate ChIP enrichment profiles across the genome. First, we mapped both ChIP and input sequencing reads to each of the repeat libraries using Bowtie2 and the parameters `-D 15 –R 2 –N 0 –l S,1,0.50 –no-1-fragmenton'. We then calculated mean coverage across each repetitive element using Bedtools56, and normalized coverage by the total library size, including reads that mapped to both the D. melanogaster and D. miranda genome. We then calculated the ratio of ChIP coverage to input coverage for each repetitive element, then normalized by the ratio of D. melanogaster reads to D. miranda reads in the ChIP library and then by the ratio of D. melanogaster reads to D. miranda reads in the input, as described above and in ref. 57. This method accounts for differences in copy number of the repetitive elements, through division of ChIP coverage by that of each repeat in the input.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All RNA-seq and ChIP-seq reads are deposited at NCBI under BioProject ID PRJNA594556.

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**Author contributions**

D.B. and E.J.B. conceived the study and wrote the paper. E.J.B. and A.H.N. collected and analysed the data.

**Competing interests**

The authors declare no competing interests.

**Additional information**

Extended data is available for this paper at https://doi.org/10.1038/s41559-020-1179-5.

**Supplementary information** is available for this paper at https://doi.org/10.1038/s41559-020-1179-5.

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Extended Data Fig. 1 | Survivorship curves of additional *D. melanogaster* strains. Shown are Kaplan-Meier survivorship curves for line 2549 males and females ((C(1;Y);y;cv;B/0 & C(1)RM;y;v;v/0) and Oregon-R wild-type males and females.
Extended Data Fig. 2 | Genome-wide enrichment of H3K9me2 for replicate young and old *D. melanogaster* males and females along the different chromosome arms. Pearson correlation coefficients for replicate H3K9me2 datasets for old males and females, and boxplots of normalized enrichment values for the replicates. Genome-wide plots were generated using biological replicate data as in Fig. 1b,d.
Extended Data Fig. 3 | Loss and gain of heterochromatin during ageing. Shown are chromosomal locations of 50 kb windows that gain (red) or lose (blue) at least 1.5-fold H3K9me2 signal during ageing for males and females. Pericentromeric regions are indicated by the red portion of the line beneath each chromosome.
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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- n/a
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☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted Give P values as exact values whenever suitable.

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☐ Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

RNA-seq and ChIP-seq data were sequenced on an Illumina HiSeq 2500 machine. Bases were called using bcl2fastq

Data analysis

RNA-seq reads were aligned to BDGPv6 using STAR v 2.6, and reads mapping to each transcript were counted using Subread v 1.6.3. Differential expression analysis was performed using DESeq v 1.24. GO analysis was performed using the GOrilla online tool (http://cbl-gorilla.cs.technion.ac.il). ChIP-seq data were aligned to BDGPv6 using bowtie2 v2.2.6. Coverage across windows was calculated using bedtools v2.25.0. De novo repeat libraries were constructed using RepARK v 1.2.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Study description**
Drosophila melanogaster individuals of 5 different karyotypes (XX, XY, XO, XXY, and XYY) were assayed for lifespan. Young (5-8 days) and old (50% survivorship) were sampled for RNA-seq analysis for all 5 karyotypes, and ChIP-seq data were collected for young and old XX and XY individuals.

**Research sample**
The Canton-S and Oregon-R lines were used as the wildtype D. melanogaster sample, and was used to generate XO, XXY, and XYY individuals by crossing with multiple attached-X lines; these are among the most widely-used wildtype D. melanogaster lines. The attached-X lines used were 2549 (C(1;Y),y1 cv1v1 B/0 & C(1;RM,y1v1/0) and 4248 [C(1;RM, y1 p1 v1 & C(1;Y)1, y1 B1/0; svspa-pol] from Bloomington Stock Center, and 100950 (0/ C(1;RM, y1w1/1; y1 B1/0) from the Kyoto stock center. These lines were chosen because of their independently-derived attached-X/ attached-X-Y chromosomes, presence of visible markers, and absence of other lesions that may contribute to lifespan differences.

**Sampling strategy**
Both RNA-seq and ChIP-seq data were pooled across multiple individuals, ranging from . This was done with the aim of minimizing individual-to-individual noise in favor of capturing variation between karyotypes. For RNA-seq, 5 heads from each sample were dissected and used for RNA extraction. For ChIP-seq, approximately 2 ml of adult flies were collected, and the abdomens were removed to avoid including gonads.

**Data collection**
Lifespan data were collected by Emily Brown and undergraduate research assistants under her supervision. Data were stored in excel spreadsheets that calculated percent survivorship as data was entered; this was critical in collecting old samples at the correct time, as they passed the 50% survivorship mark.

**Timing and spatial scale**
Data were collected from September 2014- July 2016.

**Data exclusions**
No data were excluded

**Reproducibility**
Lifespan data were collected in separate replicates for each karyotype, with replicates resulting from independent crosses. Young and old samples for RNA-sequencing were also produced from independent crosses.

**Randomization**
Not relevant

**Blinding**
Not relevant

**Did the study involve field work?**
- Yes
- No

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| | Antibodies |
| | Eukaryotic cell lines |
| | Palaeontology |
| | Animals and other organisms |
| | Human research participants |
| | Clinical data |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| | ChIP-seq |
| | Flow cytometry |
| | MRI-based neuroimaging |

### Antibodies

**Antibodies used**
Abcam ab1220 (anti-H3K9me2)
Validation

All batches of antibody are tested by Abcam using ChIP-qPCR against known active and inactive genes as well as heterochromatin regions.

Animals and other organisms

**Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research**

| Laboratory animals | D. melanogaster strains used: Canton-S, Oregon-R, 2549 (C(1);y); y1c1v18B/0 & C(1)RM, y1v1/0), 4248 (C(1)RM, y1 pn1 v1 & C(1);v1), y1 B1/0; svxpa-pol), and 100950 (Y(0); C(1)RM, y1wstr/C(v1); y1 y+ ac1 sc1 w1) |
|--------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Wild animals       | N/A                                                                                                                                                                                              |
| Field-collected samples | N/A                                                                                                                                                                                                 |
| Ethics oversight   | N/A, no vertebrates used                                                                                                                                                                        |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

ChIP-seq

**Data deposition**

☒ Confirm that both raw and final processed data have been deposited in a public database such as GEO.

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Provide a list of all files available in the database submission.

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Provide a link to an anonymized genome browser session for "Initial submission" and "Revised version" documents only, to enable peer review. Write "no longer applicable" for "Final submission" documents.

Methodology

| Replicates | 2 independent replicates were collected for each sample |
| Sequencing depth | Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end. |
| Antibodies | Abcam ab1220 against H3K9me2 |
| Peak calling parameters | Bowtie2 was used for read mapping. Peaks were not called. |
| Data quality | Pearson correlation coefficients were calculated across replicates between ChIP signals calculated in 5kb windows |
| Software | Bowtie2 and bedtools were used to analyze ChIP-seq data |

Flow Cytometry

**Plots**

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

**Sample preparation**

Flies were flash frozen in dry ice. Heads were collected by vortexing frozen flies. A single D.melanogaster head was dounced in a 2ml dounce homogenizer with a single female D.virilis standard in 1mL Galbraith buffer. Samples were stained for 30 minutes with propidium iodide at 25 μg/mL concentration and then run through the flow cytometer.

**Instrument**

BD LSR II
| Software          | BD FACSDiva 6.2                      |
|-------------------|-------------------------------------|
| Cell population abundance | Cell populations of *D. melanogaster* and *D. virilis* contained at least 1,000 cells. |
| Gating strategy   | Gated boundaries set by identifying distinct cell population clusters from low-fluorescence and debris in the FSC-A vs. PE-A scatter. |

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.