Transposable element landscape in *Drosophila* populations selected for longevity

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ABSTRACT

Transposable elements (TEs) inflict numerous negative effects on health and fitness as they replicate by integrating into new regions of the host genome. Even though organisms utilize powerful mechanisms to demobilize TEs, transposons become increasingly derepressed during aging. The rising TE activity causes genomic instability and was suggested to be involved in age-dependent neurodegenerative diseases, inflammation and the determination of lifespan. It is therefore conceivable that long-lived individuals have improved TE silencing mechanisms and consequently fewer genomic insertions and a reduced TE expression relative to their shorter-lived counterparts. Here, we test this hypothesis by performing the first analysis of genome-wide insertions and expression of TEs in populations of *Drosophila melanogaster* selected for longevity through late-life reproduction for 50-170 generations from four independent studies. Surprisingly, we found that TE families were generally more abundant in long-lived populations compared to non-selected controls. Despite simulations showed that this was not expected under neutrality, we found little evidence for selection driving TE abundance differences. Additional RNA-seq analysis revealed only few differentially expressed TEs whereas reducing TE expression might be more important than regulating genomic insertions. We further find limited evidence of parallel selection on genes related to TE regulation and transposition. However, telomeric TEs were genomically and transcriptionally more abundant in long-lived flies, suggesting improved telomere maintenance as a promising TE-mediated mechanism prolonging lifespan. Our results provide a novel viewpoint proposing that reproduction at old age increases the opportunity of TEs to be passed on to the next generation with little impact on longevity.

INTRODUCTION

Aging, also known as senescence, is an evolutionary conserved process described as the progressive loss of physiological homeostasis starting from maturity with disease promotion, decline in phenotypic function, and increased chance of mortality over time as a consequence (Fabian and Flatt 2011; Flatt and Heyland 2011; López-Otín et al. 2013). At the molecular level, studies of loss-of-function mutations in model organisms such as yeast, *Caenorhabditis elegans*, *Drosophila melanogaster*, and mice have successfully identified key pathways underlying aging and longevity including the conserved insulin/insulin-like growth factor signaling (IIS) and target of rapamycin (TOR) nutrient-sensing network (Piper et al. 2008; Fontana et al. 2010; Gems and Partridge 2013; Pan and Finkel 2017). More recently, sequencing of whole genomes, transcriptomes, and epigenomes corroborated that aging has a complex genetic basis involving many genes and is accompanied by changes across a broad range of interconnected molecular functions (López-Otín et al. 2013).

While there has been a predominant focus on identifying the links between genes and phenotypes correlated with aging, the role of transposable elements (TEs) in senescence and longevity has received less attention even though their discovery by Barbara McClintock goes back to more than half a century ago (McClintock 1950). TEs, or transposons, are selfish genetic elements that replicate and move within genomes of their hosts. In eukaryotes they typically constitute a considerable portion of the genome with estimates around ~3% in yeast, ~20% in *D. melanogaster*, ~70% in humans and ~85% in maize (Quesneville et al. 2005; Schnable et al. 2009; de Koning et al. 2011; Carr et al. 2012). To date, several thousand TE families broadly classified into DNA-transposons and retrotransposons multiplying via RNA intermediates have been identified and are known to vary hugely in their transpositional mobility (Jurka et al. 2011; Deniz et al. 2019). For instance, only a small fraction of L1 retrotransposons are responsible for most of the transposition events in the human genome, while the vast majority of L1s and other TE families have been inactivated through the accumulation of structural and point mutations over evolutionary time scales (Brouha et al. 2003).

For a long time, transposons were unfairly deemed as 'junk DNA' that do not have any significant impact on organismal function, but a series of studies proofing TE-mediated adaptive evolution and their role in diseases initiated a paradigm shift in thinking. Nevertheless, the extent to which active transposition and immobile TEs residing in the genome contribute to host fitness are still controversial. TE mobility causes genomic instability through insertional mutagenesis, which can directly affect coding sequences of genes or modify their transcription. Typically, TE insertions into or close to genes impose negative consequences on health and have been associated with ~100 diseases in humans, including cystic fibrosis, haemophilia and cancer (Hancks and Kazazian 2012). Moreover, TE expression and translation allow the formation of toxic TE products that, for example, contribute to autoimmune diseases, whereas TE activity and replication of an increased genomic TE content might also impose metabolic costs to the host (Kaneko et al. 2011; Barrón et al. 2014; Volkman and Stetson 2014; Bogu et al. 2019).

On the other hand, there is mounting experimental evidence for positive selection on segregating TE insertions from multiple taxa confirming beneficial phenotypic properties including insecticide and virus resistance in *Drosophila* (Daborn et al. 2002; Magwire et al. 2011; Kuhn et al. 2014; Li et al. 2018; Rech et al. 2019). Still, recent arguments have emphasized the notion that most TE insertions are likely to be slightly deleterious or neutral with little effects on fitness, and that adaptive TEs are negligibly rare (Arkhipova 2018).

An evolutionary conserved characteristic of TEs is that their rate of transposition and expression is not constant throughout life, but rather changes during aging as demonstrated in various organisms including yeast, *D. melanogaster*, *C. elegans*, mice, and humans (Maxwell et al. 2011; Dennis et al. 2012; Solyom et al. 2012; De Cecco et al. 2013; Li et al. 2013; Chen et al. 2016; Bogu et al. 2019; De Cecco et al. 2019). TEs have further been implicated in age-related neurodegenerative diseases (e.g. Krug et al. 2017; Prudencio et al. 2017; Guo et al. 2018) and might promote chronic inflammation observed during aging (Chen et al. 2014; De Cecco et al. 2019) further supporting the involvement of TEs in senescence and longevity as proposed by the emerging 'transposable element theory of aging'. The age-related change in TE activity detected in many tissues has mainly been attributed to chromatin remodeling and the decline in repressive heterochromatin structure which is

126 commonly rich in transposable elements (Dimitri and Junakovic 1999; Wood and
127 Helfand 2013; Chen et al. 2016; Wood et al. 2016). TEs that are not suppressed by
128 chromatin structure are target of post-transcriptional silencing by the host RNA-
129 interference (RNAi) machinery, mostly the piwi-interacting RNA (piRNA) pathway, that
130 is in turn also necessary for heterochromatin formation and stability (Lippman and
131 Martienssen 2004; Martienssen and Moazed 2015). Indeed, research has identified
132 longevity-promoting effects of several genes involved in the RNAi machinery and
133 heterochromatin formation (Mori et al. 2012; Wood and Helfand 2013; Wood et al.
134 2016). Interestingly, it is possible that age-related misexpression of TEs is exclusive
135 to the soma due to efficient post-transcriptional TE silencing mediated by the piRNA
136 machinery in the germline (Sturm et al. 2015; Elsner et al. 2018; Erwin and Blumenstiel
137 2019). Considering current evidence, it seems natural that longevity can be achieved
138 through impeding TE activity and controlling the genomic content of TEs. However,
139 whether variation in aging and lifespan within species is mediated by transposons and
140 their role in the evolution of senescence is largely unknown.
141
142 Here, we analyze genomes of *D. melanogaster* populations experimentally selected
143 for increased lifespan through postponed reproduction from four independent studies
144 to understand the role of TEs in the evolution and genomic basis of late-life
145 performance and aging. The invertebrate *D. melanogaster* is an excellent model in
146 this respect as it exhibits abundant genetic and phenotypic variation in fecundity and
147 traits related to aging that can be selected for. In the present experiments, replicate
148 populations derived from nature were subjected to a late-life breeding scheme in which
149 only flies surviving and fertile at old age contributed to the subsequent generations,
150 while control individuals reproduced earlier in life. At the point of whole genome
151 sequencing, this process had continued for over 30 years with ~170 and ~150
152 generations of selection for Carnes et al. 2015 (Carnes2015) and Fabian et al. 2018
153 (Fabian2018), and for 58 and 50 generations for Hoedjes et al. 2019 (Hoedjes2019)
154 and Remolina et al. 2012 (Remolina2012) enabling us to quantify differences in TE
155 content of long- and short-term evolutionary responses. Selection for postponed
156 senescence has resulted in phenotypic divergence of multiple fitness traits, most
157 notably an ~8% to ~74% increase in lifespan and improved old age fecundity at the
158 cost of reduced early reproduction (Luckinbill et al. 1984; Rose 1984; Remolina et al.
159 2012; Carnes et al. 2015; Fabian et al. 2018; Hoedjes et al. 2019; May et al. 2019). At

the genome level, analysis of genetic differentiation has revealed a significant sharing in candidate genes across the four studies indicating parallel evolution (Hoedjes et al. 2019), but at the same time exposed multiple novel targets of selection. For instance, three of the studies report genetic and/or transcriptomic divergence in immunity genes, and it has recently been confirmed that these molecular changes reflect differences in traits related to pathogen resistance (Fabian et al. 2018). Thus, despite variations in the experimental designs, numerous evolutionary repeatable phenotypic and genetic adaptations have been observed, but the importance of TEs in these studies has remained unexplored. Our main objective therefore was to investigate for the first time whether TE abundance in the genome, and host genes related to TE regulation, had undergone similar parallel changes. Using RNA-seq data from Carnes et al. (2015), we further test if males and females of selected populations evolved to suppress TE transcription to mitigate potentially negative effects on longevity.

RESULTS

Genomic TE abundance

Our initial goal was to test if transposable elements differ in the number of genomic insertions between late-breeding, long-lived selection (S) and early-breeding control (C) populations of four studies performing experimental evolution of postponed senescence (**Table S1**). As a first step, we excluded all TEs that did not pass our coverage and mapping quality filters and therefore included between 110 to 115 TEs dependent on the study in our downstream analysis. We then used three different approaches to test differences in TE abundance across control and selection regimes (see overview in **Fig. S1**).

As sequencing depth varies throughout the positions within a TE, resulting in a distribution of insertion counts rather than a single point-estimate, we first analyzed the differences between regimes using all insertion value estimates within individual TEs separately for each study (**Fig. 1** and **Table 1**). We found that between 46% and 77% of all TEs had a significantly larger number of genomic insertions in the selected populations relative to controls after Bonferroni correction for multiple testing (from here on referred to as S>C TEs). In contrast, only 12% - 31% of TEs showed the opposite pattern and had more insertions in the controls (from here on referred to as C>S TEs). As expected, the magnitude of difference in TE insertions between regimes

was correlated with the genomic copy number of TE families (Pearson's r, Carnes2015: 0.81, Fabian2018: 0.52, Hoedjes2019: 0.77, Remolina2012: 0.6, all $P < 0.0001$). To correct for this, we also obtained $\log_2$ fold change (FC) values by dividing average insertions of selected by control populations for each TE (lower panels in **Fig. 1**). S>C TEs had significantly larger $\log_2$ FC values in the two short-term evolution studies of Hoedjes2019 and Remolina2012, while the opposite pattern and no difference was observed for Fabian2018 and Carnes2015, respectively (**Fig. S2A**; t-tests between absolute $\log_2$ FC values of S>C and C>S; Carnes2015: $P \sim 0.47$; Fabian2018: $P \sim 0.01$; Hoedjes2019: $P \sim 0.001$, Remolina2012: $P \sim 0.005$).

This demonstrates that the dynamics of TE change between control and selected populations varies among studies. Indeed, we find that studies also differed significantly in the size of $\log_2$ FC values in the order of Carnes2015 > Fabian2018 > Hoedjes2019 = Remolina2012 (**Fig. S2A**; ANOVA with Study term, Tukey post hoc test; $P < 0.001$ for all pairwise comparisons except Hoedjes2019-Remolina2012, which was not significant), seemingly scaling with the length of selection (Carnes2015: 170; Fabian2018: ~146; Hoedjes2019: 58, Remolina2012: 50 generations).

As a second, complementary approach to test for TE abundance differences between selection and control regimes, we also fitted linear models using average number of insertions per TE and population and analyzed the four studies together. Because the total number of TEs was significantly different between studies (ANOVA $P < 0.0001$ of Study factor; **Fig. S3**), we normalized the insertion counts of each individual TE by the total insertions in the corresponding population and analyzed arcsine square root transformed proportions (**Fig. S1B** and **Fig. S1C**). At an FDR of <0.05, we identified 41 TEs with a significant regime factor of which 34 were more and 7 less abundant in selected populations, confirming our findings of individual studies (**Table 1**). Comparable tendencies were obtained when we analyzed the raw insertion counts or standardized z-scores of insertions and changing the significance threshold did not alter the fact that S>C TEs were more frequent than C>S (not shown).

In our third - and most conservative - analysis, we identified TEs with consistent differences in genomic TE insertions between all control and all selected populations (**Table S2**). Interestingly, the number of TEs were generally smaller compared to our statistical approach indicating that TEs within control or selected populations do not always behave in a parallel way. The insertion tendency in favor of S>C was again apparent in three studies. In Hoedjes2019, however, we observed the same pattern

for populations selected on larval diet with low and medium sugar/protein concentration, but the opposite for the high sugar/protein diet. A qualitatively similar results was obtained applying the statistical approach from above (**Fig. S4** and **Table S3**). Yet, the overall increase in TE insertions in late-breeding populations was apparent in our model which corrects for the effect of diet (**Fig. 1** and **Table 1**).

We further tested if our results are influenced by the chosen mapping quality cut-off of 20 and redid the analyses without applying a threshold. The number of genomic insertions per TE were highly correlated between both mapping approaches (Pearson's r range = 0.94 – 1 in all populations and studies, $P < 0.0001$), and changing the cut-off resulted in similar proportions of S>C and C>S TEs (not shown).

To analyze if changes in TE abundance are driven by certain TE subclasses (Long Terminal Repeat, LTR; Non-Long Terminal Repeat, non-LTR; Terminal Inverted Repeat, TIR) or class (RNA, DNA) we tested S>C and C>S TEs for enrichment of these types using two-sided Fisher's exact tests. We only detected a significant underrepresentation of TIRs and DNA-class TEs (i.e. overrepresentation of RNA-class) in the C>S group of TEs of Carnes2015 and Hoedjes2019 (Carnes2015, TIRs: $P \sim 0.044$; DNA/RNA class: $P \sim 0.024$; and Hoedjes2019, TIRs: $P \sim 0.013$; DNA/RNA class: $P \sim 0.008$), while significant TEs of Fabian2018 and Remolina2015 had no enrichment in class or subclass.

Despite the bulk of individual TEs had a higher genomic abundance in the selected populations, the whole genomic TE content was not significantly different between the regimes (ANOVA Regime factor: $P \sim 0.086$; **Fig. S3**). This was at least partly driven by the fact that relative to S>C TEs, the fewer C>S TEs showed a significantly higher magnitude of insertion differences in two studies (**Fig. S2B,** t-test on S>C vs C>S TEs δInsertion values; Carnes2015 $P \sim 0.04$; Fabian2018 $P \sim 0.005$). The non-significant difference in overall genomic TE load could therefore be a result of a large number of S>C TEs with small differences that are balanced by fewer C>S TEs with large differences. We further analyzed the whole genomic content of individual subclasses of TEs and identified that selected populations had on average a significantly higher number of TIRs than controls (**Fig. S3**, ANOVA, both Regime and Regime x Study factors, FDR < 0.001). Tukey tests analyzing differences between selected and control populations indicated that this effect is strongly influenced by Carnes2015 (Tukey

HSD, Regime x Study factor testing for C vs S within studies, Carnes2015: $P < 0.0001$; Hoedjes2019: $P \sim 0.85$; Fabian2018 and Remolina2012: $P \sim 1$). We also detected that selected populations had a larger LTR retrotransposon load than controls (ANOVA, Regime factor, $P \sim 0.026$), whereas non-LTR content did not differ significantly. Finally, we note that studies in general varied significantly in total TE content and subclass-specific loads (ANOVA, Study factor, $P < 0.0001$ in all models).

In summary, our results demonstrate that selection for postponed reproduction leads to evolutionary repeatable increases in copy number of many TEs relative to early bred controls, without affecting the overall genomic TE load.

Approximate TE mobility

We next tested if the change in abundance of TEs between control and selected populations can be explained by differences in average population frequency of TE insertions which serves as a proxy for recent mobility and age of TE invasion (Kofler et al. 2015).

We first determined the exact genomic location and frequency of TE insertions and calculated average population frequency across all insertion sites for each TE family. Importantly, while TE abundance (as above) is quantified by the total number of reads mapping to a TE relative to single-copy genes (Weilguny and Kofler 2019), identifying the exact genomic location of insertions requires mates of a read-pair to map discordantly to the chromosome and TE sequence, and therefore strongly depends on the sequencing depth and number of populations (Cridland et al. 2013; Kofler et al. 2016; Lerat et al. 2019). The genome-wide average coverage ~162, ~101, ~41, and ~23 for Fabian2018, Hoedjes2019, Remolina2012, and Carnes2015, respectively. As expected, the number of detected TE insertions partially scaled with coverage: across all populations within a study, we found 13,018 TE insertions in Hoedjes2019, 8,402 in Fabian2018, and 4,502 in Remolina2012, which is in the range recently identified in natural populations (i.e. 4,277 - 11,649 TE insertions in Lerat et al. 2019). The least number of insertions was found for Carnes2015 for which we identified an unusually small number of 567 TE insertions.

For each TE family, we then averaged frequencies across all of its detected genomic positions to estimate the mean frequency at which a TE is segregating in a population (Kofler et al. 2015). Studies varied in the minimum average TE frequency in the order of Carnes2015 > Remolina2012 > Fabian2018 > Hoedjes2019, which is likely a further

effect of dissimilar sequencing depths and other experimental factors (average frequency ranges of Hoedjes2019: 0.01 - 0.9; Fabian2018: 0.02 - 1; Remolina2012: 0.04 - 0.84; Carnes2015: 0.19 – 0.9). The TE frequencies of Carnes2015 therefore need wo be interpreted with care, considering the likely insufficient amount of data.

To get unbiased average TE frequency estimates independent of coverage fluctuations across studies, we also obtained average frequencies from a single natural South African (SA) population (Kofler et al. 2015; Kofler 2019). The SA population had a higher sequencing depth than all studies here (Fabian2018: ~2x, Hoedjes2019: 3.2x; Remolina2012: ~7.8x, Carnes2015: ~13.9x times more) and thus presumably a more accurate estimate of TE frequencies. Notably, this population was not subjected to any selection or control treatment and was only maintained 8 generations in the lab before sequencing. Average genome-wide TE frequencies of control and selected populations of Fabian2018, Hoedjes2019 and Remolina2012, but not Carnes2015, were significantly correlated with the South African TE frequencies (**Fig. S5**; Spearman's r, Fabian2018: 0.65; Hoedjes2019: 0.61; Remolina2012: 0.58, all three $P < 0.0001$; Carnes2015: 0.1, $P \sim 0.4$), demonstrating that the SA population can function as an appropriate reference here.

Previous studies reported that low frequency TEs are more abundant in genomes of *D. melanogaster* (Petrov et al. 2011; Kofler et al. 2015). We confirmed that for all populations, TE abundance was similarly negatively correlated with average TE frequency of the SA population (Spearman's r range, Carnes2015: -0.49 to -0.54; Fabian2018: -0.4 to -0.5; Hoedjes2019: -0.42 to -0.45; Remolina2012: -0.5 to -0.51; all $P < 0.0001$). Moreover, we obtained comparable results when we performed this analysis using the average TE frequencies of Fabian2018, Hoedjes2019 and Remolina2012 (Spearman's r averaged across all populations, Fabian2018: -0.33; Hoedjes2019: -0.37; Remolina2012: -0.4, all populations $P < 0.001$; Carnes2015: -0.29 to 0.28, two populations significant at $P < 0.05$). The transposable element content of all populations can therefore be characterized by a large number of TEs with low frequency, whereas TEs with a high population frequency are less abundant in the genomes.

We then employed the data of the SA population and asked if C>S and S>C TEs vary in average frequency to identify TEs that have presumably been mobile in the two regimes (**Fig. 2**). C>S TEs had a significantly lower frequency than S>C TEs in all four

328 studies (t-tests between C>S and S>C frequencies, $P < 0.05$ for all three studies). As
329 there were more S>C than C>S TEs, we also contrasted the average frequencies of
330 the top 10 C>S and S>C TEs with the biggest changes in genomic abundance defined
331 by $\log_2$ FC values (lower panel of **Fig. 1**). We only detected a significantly higher
332 frequency in top 10 S>C relative to C>S TEs for Carnes2015 (t-test, $P \sim 0.03$), but not
333 in the other two studies. Except from the marginal difference in Carnes2015, this
334 suggests that TEs with the biggest abundance changes between regimes have similar
335 levels of mobility.
336 In summary, our analysis proposes that C>S TEs are potentially mobile, low frequency
337 TEs expanding to new sites in the genome, whereas S>C TEs span from low to high
338 frequency, possibly reflecting TEs with a broad range of transposition rates.
339
340 **Selection on TE abundance and insertions**
341 A major challenge in experimental evolution studies is to differentiate selection from
342 the confounding genomic signals of genetic drift, which might be amplified by small
343 effective population sizes (N_e) or varying generation times spent in the lab between
344 control and selected populations. We therefore calculated genome-wide nucleotide
345 diversity π and Watterson's θ across 100kb windows as a proxy for N_e. With the
346 exception of Fabian2018, where π was equal between regimes (ANOVA, Regime
347 factor, $P \sim 0.18$), we found that both estimators were significantly higher in selected
348 relative to control populations (**Table S4**; ANOVA, Regime factor, all $P < 0.0001$). Even
349 though a generally reduced N_e in controls should lead to the loss of low and fixation of
350 high frequency TEs under neutrality, we observed the opposite pattern in our analysis
351 above (**Fig. 2**).
352 To further formally test if the increased abundance of many TEs is driven by genetic
353 drift alone, we performed population genetic simulations using the correlated average
354 TE frequencies from the natural South African population (Kofler et al. 2015) as a
355 starting point (see **Fig. S5** and results above). We simulated TE frequency change in
356 selected and control populations 5,000 times given the reported consensus population
357 sizes as N_e, generation times and number of replicates. We then asked how often the
358 same or a higher relative proportion of S>C to C>S TEs as in our observations is
359 obtained (**Table 1**). While the results from Carnes2015, Hoedjes2019, and
360 Remolina2012 were significantly different from the expected proportions, the TE

abundance differences of Fabian2018 could be caused by genetic drift alone (**Fig. S6**). Testing different ranges of the reported population sizes and assuming that only 50% and 25% of flies in the selected populations were able to breed at old age resulted in qualitatively similar results (not shown). We also quantified expected proportions of TEs consistently varying in frequency across simulated replicates: while there were generally more TEs consistently higher in abundance in selected populations (**Table S2**), all our simulations resulted in more TEs with a consistently higher frequency in controls. The increased genomic abundance of many TEs in selected populations is therefore unlikely to be solely caused by genetic drift.

Considering the deviation from neutrality, we next asked if the parallel patterns in TE abundance are caused by the same or different TEs, which could indicate selection acting on genomic copy number of certain TEs. We considered the TEs significantly varying in abundance from the first linear model in which we analyzed each study separately (**Fig. 1** and **Table 1**), and created study-overlaps of S>C and C>S TEs. Among the 103 common TEs, we identified a sharing of 14 S>C and 2 C>S TEs across all four studies (**Fig. 3A**). Even though this seemingly large number of shared S>C TEs, only the overlap between Remolina2012 and Hoedjes2019 was significant ($P < 0.05$). Yet, we found that the most common telomeric TE *HeT-A* (Casacuberta 2017) was significantly more abundant in selected populations in all four studies and in our combined model (**Fig. S1**), suggesting that long-lived populations might have evolved longer telomeres to avoid attrition, which is considered to be a key conserved mechanism of aging (López-Otín et al. 2013). In contrast to S>C TEs, we detected several significant overlaps for the C>S group of TEs. Potentially, a high genomic abundance of several TEs, most importantly *G-element* and *G2* found in all four studies, is detrimental for longevity and late-reproduction (**Fig. 3B**). Despite some significant overlaps, we did not observe any significant Spearman's correlation coefficients in pairwise comparisons of $\log_2$ FC values between studies except for Hoedjes2019-Remolina2012 ($r = 0.28$, $P \sim 0.004$).

Genomic TE abundance in selected populations might also be increased because selection acted on a large number of TEs segregating in the base populations resulting in frequency divergence between control and selected populations. We therefore screened all identified TE insertion sites across the genome for significant frequency

differences between regimes in each study by performing ANOVAs on arcsine square root transformed frequencies and Bonferroni correction. We did not find any TE insertions significantly varying in frequency in Carnes2015 and Remolina2012, and using a less stringent cut-off at FDR < 0.05 did not change this result. However, we detected 38 significant TE insertions out of 8,402 in Fabian2018 and 100 out of 13,018 in Hoedjes2019 (**Fig. 3C** and **Fig. 3D, Table S5**). While there were more TEs with a significantly higher frequency in control populations in Fabian2018 (8 higher vs 30 lower frequency in S), the opposite pattern was observed in Hoedjes2019 (56 higher vs 44 lower frequency in S).

At the gene level, the significant TEs defined 29 and 98 genes in Fabian2018 and Hoedjes2019, respectively, and none were shared between the two studies. In accordance with the notion that most TEs are neutral (Arkhipova 2018), only a negligible fraction of insertions might be beneficial for longevity and postponed reproduction.

To further investigate if frequency changes explain variation in abundance, we tested if each TE family varying in abundance also differs significantly in frequency between the regimes, rather than analyzing individual insertion sites (**Table S6**). We fit the same model as for the abundance analysis but using arcsine square root transformed TE frequencies (**Figure S1A**). At an FDR < 0.05, we found that in Carnes2015, 27 TE families had a higher frequency and abundance in selected populations, while 7 showed a mismatch in direction of frequency and abundance change. In Fabian2018 and Hoedjes2019, we only detected 1 and 3 TE families with significant frequency variation all of which matched the differences in abundance, whereas none were significant in Remolina2012.

Thus, despite differences in TE abundance are likely not driven by neutral evolution alone, we only found limited evidence for parallel evolution of TE copy numbers and sparse TE frequency differentiation.

Differential TE expression

To test whether the increased genomic abundance of TEs in selected flies is explained by a higher transcriptional activity we analyzed RNA-seq data from whole flies of Carnes2015 (**Fig. 4** and **Table S7**). We first fit a model with *Sex*, *Age*, *Regime* and all interactions to every TE and each gene on the major chromosomal arms. In line with abundant sexual dimorphism of gene expression observed by Carnes et al. (2015),

we identified that ~93% of TEs had a significant main factor of sex or interaction including it, whereby all 109 TEs significant for the sex term had a higher expression in males than females **(Fig. 4A** and **Fig. S7)**. To exclude that this is caused by a technical artifact, we asked if a similar male-bias is observed for the genes on the major chromosomal arms. We found that 53% of all 13,255 included genes had a significantly higher expression in males while 37% were biased towards females. Moreover, the proportions of genes with male-, female- or no bias was significantly different from the proportions observed for TEs (χ^2 test on counts of TEs vs genes, P < 0.0001). Because we do not find a similarly strong male-biased expression for genes or a higher number of read counts in males (not shown), we conclude that TEs are generally higher expressed in males than females in the present populations.

We therefore decided to test the effects of *Regime*, *Age*, and the *Regime x Age* interaction in the sexes separately (**Fig. 4**, **Table S7**). We detected several TEs differentially expressed between selected and control regimes. In males, 19 TEs (~16% of total) had a significant regime term of which a majority of 17 TEs were upregulated in controls (**Fig. 4B**). Among these, there were 10 LTRs, 6 non-LTRs, and 1 DNA-class foldback TE, whereas the 2 upregulated in selected populations were both non-LTR TEs (*TART-A*, and *TART-B*). Less TEs changed across regimes in females (10 TEs, ~8%) compared to males. However, females showed a similar tendency towards higher expression in controls: 6 TEs had a higher and 4 TEs a lower expression in controls relative to selected flies. The 6 TEs with a higher expression in controls consist of 1 non-LTR (*R1A1-element*) and 5 LTR TEs (*copia, flea, blood, mdg1,* and *rooA*), while the 4 more in selected were 3 non-LTR (*TART-A*, *TART-B, TAHRE*) and 1 LTR (*1731*). We also observed that the 6 TEs significant in both sexes also had the same directionality of expression change: 4 LTR-class TEs (*copia, flea, blood,* and *mdg1*) were higher expressed in controls, whereas 2 non-LTR TEs (*TART-A*, and *TART-B*) were upregulated in selected populations. Interestingly, *TART-A*, *TART-B,* and *TAHRE* provide the enzymatic machinery for telomeric maintenance (Casacuberta 2017), again suggesting that reduced telomere attrition evolved in response to selection paralleling the genome-based analysis. In general, regime affected TE expression in males and females similarly as indicated by a significant correlation of $\log_2$ fold change values between sexes (**Fig. 4B**, Pearson's r = 0.7, P <

462 0.0001). We further asked if the magnitude of $\log_2$ fold change varies between TEs
463 more expressed in controls or selected populations, and did not find any significant
464 difference (**Fig. S8**, t-test, females: P ~ 0.12; males: P ~ 0.49).
465
466 To investigate if derepression of TEs occurs with age, we compared the proportion of
467 TEs up- or downregulated or unchanged with age to those of genes. For both male
468 and females, we found a significant difference in the distributions of TEs and genes
469 with age (χ^2 test on counts of TEs vs genes, for males: P < 0.0001; for females: P ~
470 0.01), demonstrating that TEs show generally different age-related expression
471 changes than genes. Supporting the notion that TEs become derepressed during
472 aging, the effect of age on TE expression in males was general as all 89 significant
473 TEs (i.e. ~73% of all included TEs) had a higher expression in older flies (**Fig. 4A** and
474 **Fig. 4C**), whereas ~26% of all genes increased and also ~26% decreased expression
475 with age. In contrast to this, the effect of age was less pronounced in females in which
476 only 35 TEs (~28%) had a significant Age factor. Surprisingly, less TEs than genes
477 were upregulated with age in females (7% TEs and 15% genes), while 21% of TEs
478 and 14% of genes decreased expression with age. Still, the TEs upregulated in older
479 females had on average a significantly higher $\log_2$ fold change of 1.3, relative to 0.53
480 for the downregulated TEs (**Fig. S8**, t-test: P < 0.01), consistent with the results of a
481 recent study (Chen et al. 2016).
482 Moreover, 27 TEs had a significant age term in both male and female models, but only
483 9 of them had the same sign and increased in expression with age (**Fig. 4A** and **Fig.**
484 **4C**)**.** Among these were 6 LTR (*copia, gypsy, mdg1, Burdock, rooA, springer*) and 3
485 non-LTR transposons (*jockey, R1A1- and R2-elements*). This confirms previous
486 findings that showed *copia, gypsy, Burdock, R1* and *R2* upregulation with increasing
487 age (Li et al. 2013; Chen et al. 2016).
488 The 16 remaining shared TEs showed downregulation with age in females, but
489 upregulation in males, further highlighting that age has generally different effects on
490 TE expression in both sexes. In females, the 35 age-related TEs were also signficantly
491 enriched for retrotransposons whereas DNA-class TEs were underrepresented
492 (Fisher's exact test, P ~ 0.02). Male flies in contrast, did not show any enrichment.
493 Thus, males exhibit a general upregulation of TEs regardless of their class during

aging, whereas females show a more complicated pattern with mainly RNA-class TEs affected by age.

No TE families showed a significant *Regime x Age* term in males, but the interaction was significant for 28 TEs (~23%) in females (**Fig. 4A**). These consisted of 13 non-LTR, 13 LTR, and 2 TIRs. Interestingly, most of these TEs were defined by a higher expression in young controls compared with selected flies of the same age (see **Fig. S9** for example). Selected populations then increased while controls decreased expression, meeting at a similar expression level at old age. This is comparable with recent studies which suggested that age-dependent changes in TE expression differ between genotypes (Erwin and Blumenstiel 2019; Everett et al. 2019).

To examine if the selected populations might have evolved to maintain a young TE expression profile, we compared differences between regimes to those that occurred with age (**Fig. 4D and 4E**). In both males and females $\log_2$ FC values of all TEs were significantly correlated between regime and age (Pearson's correlation, females: $r = 0.55$, $P < 0.0001$; males: $r = 0.19$, $P < 0.05$). Performing the same analysis only considering genes also resulted in significant positive correlations (Pearson's correlation, females: $r = 0.37$, males: $r = 0.41$, both $P < 0.0001$), and the coefficients between males and females were more similar. Thus, variation in expression of genes and TEs between selected and control populations mirrors the changes between young and old flies.

In summary, our results propose that selected populations of Carnes2015 evolved to reduce TE expression particularly in males and to a smaller magnitude in females, but expression of most TEs was at the levels observed in controls. In agreement with sexual dimorphism (Brown and Bachtrog 2017) and age-related deregulation of TEs (Li et al. 2013; Chen et al. 2016), the effects of sex and age on TE expression were more dominant compared to regime.

Link between genomic and expression differentiation

We next asked if the change in genomic TE abundance reflects the expression differences between selected and control populations. On average, TE expression was positively correlated to the number of genomic insertions in the genome (**Fig. 4F**,

Spearman's r ~ 0.74; P < 0.0001). To analyze if this association depends on the treatment, we separated our samples into different levels of sex, age, and regime. The number of genomic TE insertions predicted TE expression significantly across all conditions (**Table S8**). Interestingly, the correlation coefficients were similar across different sample groups, demonstrating that the relationship between expression and genomic abundance of insertions is not dependent on regime, sex or age. Thus, in Carnes2015, TE copy number is a robust predictor of TE expression regardless of treatment.

Next, we investigated if there are parallel changes in expression and genomic abundance of TEs significantly varying between regimes (**Table S9**). In males, 14 TEs were significant for regime in the genomic and expression analysis all of which were higher expressed in controls. Of these, 9 TEs were upregulated and had significantly more genomic insertions in controls (*blood, copia*, *F-element*, *Doc2-element, Doc3-element, G2, Porto1, ZAM, 17.6*), while 5 TEs (*Circe, G5, mdg1, Tom1, Quasimodo*) had more copy numbers in selected populations. In females, 3 TEs (*blood*, *copia*, *R1A1-element*) were higher expressed and had a higher genomic abundance in controls, while 2 TEs (*1731*, *TAHRE*) were significantly more expressed and more abundant in selected populations. The remaining two TEs signficant in the expression and insertion analysis (*mdg1, rooA*) showed different signs and had despite a larger number of genomic insertions in selected populations a higher expression in controls. This suggests that the absence of a strong link between expression and number of genomic insertions at the individual TE level. Indeed, $\log_2$ FC expression and $\log_2$ FC insertions between regimes was only correlated in males (Pearson's r = 0.31, P < 0.001), and marginally not significant in females (r = 0.18, P ~ 0.05) (**Fig. S10**).
Because our results suggests some positive relationship between TE expression and genomic abundance, we re-did the differential expression analysis from above using RNA-seq read counts corrected for the number of TE insertions in each sample (**Table S10**). The tendency of TEs to be higher expressed in controls was substantially larger compared to the analysis where we did not correct for insertion numbers (males: 55 TEs more, 2 TEs less expressed in controls; females: 16 TEs more, 3 TEs less expressed in controls).
Our results further emphasize that selection for late-reproduction leads to a reduction in TE expression in whole flies. Notably, we assumed that males have the same copy

number as females for the analyses here. As the genomic TE abundance measures came from DNA pools of female flies, copy number could differ from males due to the repetitive nature of the *Y* chromosome.

Genes involved in TE regulation

We next hypothesized that if the effects of TEs on lifespan and aging are predominantly negative, as proposed by many studies, experimental selection for longevity would have likely resulted in clear-cut genetic and expression differentiation in 96 known chromatin-structure, piRNA, and transposition-associated genes likely involved in TE regulation and silencing (**Table 2**, a complete list of genes can be found in **Table S11**). Of these, 10 genes were implicated under selection in Carnes2015, 3 in Fabian2018, 7 in Hoedjes2019, and 6 in Remolina2012. *E2f1* (FBgn0011766) and *Hsp83* (FBgn0001233) were the only genes occurring in more than one study. Thus, the significant candidate gene sharing reported by Fabian et al. (2018) and Hoedjes et al. (2019) does generally not include genes regulating TEs. In line with this, the four studies did not report any significant enrichment of GO terms related to transposon silencing and chromatin structure.

Using the available RNA-seq data from whole flies of both sexes in Carnes2015 and microarray data from female heads and abdomens in Remolina2012, we then asked if TE regulation genes are differentially expressed (**Table S11**). In Carnes2015, we found that the 30 TE regulation genes with a significant regime term tended to be upregulated in controls relative to selected populations (males: 3 more and 8 less expressed; females: 6 more and 14 genes less expressed relative to selected populations). In Remolina2012, we detected generally little differences in transcript abundance between regimes at FDR < 0.05 (only 5 out of 13,995 genes significant in head tissue, none significant for abdomen). We therefore analyzed genes significant at P < 0.01 (491 genes for heads, 8 for abdomen) for the regime term, and only detected 2 TE regulation genes of which one was more and the other lower expressed in selected flies.

Similar to the TE expression patterns in Carnes2015 (**Fig. 4A**), the effect of age was stronger than selection regime in both studies and significant TE regulation genes showed a clear tendency for upregulation with age. In males of Carnes2015, 41 TE regulation genes increased and 6 decreased expression with age. The effect was less pronounced in females in which 19 genes increased transcription and 8 decreased

with age. Likewise, in Remolina2012, all 5 TE regulation genes significant for age in heads ramped up transcription with age. The age effect was even stronger in abdomens, where 24 genes increased with age, while only one showed the opposite pattern.

Taken together, the small number of genetically differentiated TE regulation genes, lack of TE-associated GO enrichment, and overall missing sharing between studies suggests that improving TE repression was either specific to studies and/or not a prime target of selection. Indeed, experimental selection has mainly affected expression of TE regulation genes in the Carnes2015 long-term evolution study but not Remolina2012, suggesting that altering expression of these genes is not a general necessity for increasing lifespan. The boosted expression of TE regulation genes at older ages appears to be common and might be a response to increased TE transcription in old flies.

DISCUSSION

Are transposable elements conferring an adaptive advantage as shown for many traits (Daborn et al. 2002; Magwire et al. 2011; Kuhn et al. 2014; Li et al. 2018; Rech et al. 2019) or should they be purged and repressed during the evolution of longevity due to their widespread negative effects on fitness (Chen et al. 2014; Krug et al. 2017; Prudencio et al. 2017; Guo et al. 2018; De Cecco et al. 2019)? In this report, we attempt to answer this controversial question by employing four independent data sets to present the first characterization of the genome-wide TE content and expression in *D. melanogaster* populations experimentally selected for late-life reproduction and longevity.

Does longevity-selection lead to changes in TE abundance?

Variation in TE copy number has been associated with some geographic and climatic factors (Kalendar et al. 2000; Kreiner and Wright 2018; Lerat et al. 2019) in natural populations of plants and *Drosophila* and was shown to change during experimental evolution in different temperatures (Kofler et al. 2018). Our analysis revealed repeatable predominance of TE families with increased genomic insertions in late-breeding, long-lived populations and indicates that reproductive age, with some dependency on developmental diet, is another factor influencing divergence in TE abundance **(Fig. 1** and **Table 1)**. Interestingly, we find that the degree of TE

abundance change was significantly different between studies and roughly scaled with the number of generations under selection (**Fig. S2A**). While parallel changes in TE characteristics within populations of the same selection regime have been reported by similar experiments (Graves et al. 2017; Kofler et al. 2018), it is striking that we observed this pattern in data created by four independent studies. Despite most TE families are more abundant in long-lived populations, our analysis shows no significant difference in the total genomic TE content between control and selected populations (**Fig. S3**), which was partly driven by a few TEs with large increases in copy number in controls that oppose many TEs with small increases in abundance in selected populations (**Fig. S2B**). Changes in the overall genomic TE load are therefore likely not essential to evolve longevity or fecundity at old age in *Drosophila*. These findings are in contrast to recent work in several killifish species, which reported that TE expansion can cause an increased genome size with possible negative effects on lifespan (Cui et al. 2019). However, our analyses focused exclusively on the genomic TE load, so that we cannot exclude a difference in genome size between control and selected populations, as might be caused by other factors such as non-repetitive InDels or repetitive DNA unrelated to TEs.

Are TEs adaptive during the evolution of aging?

The genomic content of TEs evolves through various factors including replicative transposition, selection, genetic drift, and the TE defense machineries of the host (Charlesworth and Charlesworth 1983; Kofler 2019). By performing population genetic simulations considering just genetic drift, we were able to exclude that population size and generations spent in the lab *per se* cause an increased abundance of TE families in selected populations (**Fig. S6**). Even though it is known that the majority of TE insertions are neutral to fitness (Arkhipova 2018), this proposes that factors other than genetic drift influenced TEs in our experiments.

From a selective point of view, increasing many TE families might be beneficial for longevity, while the fewer TEs less abundant in selected flies could have a negative effect. Under this scenario, selection would lead to parallel increases or decreases of the same TE families across studies. However, when we screened for parallel patterns in abundance change, we found only two TEs (*G-element* and *G2*) that had a decreased copy number in selected flies and were significantly shared across all studies. Both elements are *jockey*-like non-LTR TEs, of which *G2* is highly enriched in

664 centromeric regions of the genome (Chang et al. 2019). Thus, changing centromeric
665 structure by altering its TE content could be one mechanism modulating aging, but
666 experimental evidence for this is still missing. In contrast to this, we did not find any
667 significant overlap between all four studies among TEs with an increased abundance
668 in the late-breeding populations. Unless many TE families had non-repeatable effects
669 on longevity, the small amount of significant sharing suggests that abundance of most
670 TEs is neutral.
671 Another possibility to alter TE abundance is through selection affecting TE insertions
672 that were already present in the base population at a genome-wide scale, resulting in
673 a large number of TE insertions significantly varying in frequency between control and
674 selected populations. We found only a minor fraction of TE insertions in Fabian2018
675 and Hoedjes2019, but not in the other two studies, with significantly different
676 frequencies between the regimes that are in or close to <100 genes (**Fig. 3** and **Table**
677 **S5**). The small fraction of TE insertions with a higher frequency in selected populations
678 found in only two studies together with little differences in frequency of TE families
679 suggests that standing genetic variation presented by TEs plays a role in the evolution
680 of aging, but it is unlikely to be a major driver of TE abundance differentiation.
681 Yet, we found a signal indicating potentially improved telomere maintenance – a key
682 hallmark of aging and lifespan (López-Otín et al. 2013) – as an exciting mechanism
683 possibly adaptive during the evolution of aging. Telomere shortening coincides with
684 aging and has been associated to mortality, diseases and the rate of senescence in
685 several organisms (Canela et al. 2007; López-Otín et al. 2013; Dantzer and Fletcher
686 2015; Foley et al. 2018; Whittemore et al. 2019). In *D. melanogaster*, three TE families
687 exclusively found in telomeres comprise most of their DNA-content, and are further
688 essential for their enzymatic maintenance (Casacuberta 2017). In all four studies, we
689 found that long-lived populations had on average an increased genomic abundance
690 of *HeT-A*, which is thought to be the most common telomeric TE, although the
691 difference was less clear in two studies after correcting for TE content **(Fig. 3A** and
692 **Fig. S1)**. Moreover, the very few TEs transcriptionally upregulated in males and
693 females of long-lived populations in Carnes2015 were almost exclusively telomeric
694 elements (**Fig. 4B**). Our study therefore provides the first indication of a genomic and
695 transcriptional response of telomeric TEs to selection for postponed reproduction and
696 longevity. Previous studies, however, failed to establish a connection between
697 telomere length and lifespan. For instance, telomere length was not associated with

698 survival in *D. melanogaster* and *C. elegans*, but might affect other traits such as
699 fecundity (Raices et al. 2005; Walter et al. 2007). Moreover, in several species the
700 rate of telomere shortening rather than the initial length itself was a better predictor for
701 lifespan (Whittemore et al. 2019). Another complication yet to be addressed in
702 *Drosophila* concerns the phenomenon of 'intergenerational plasticity' of telomere
703 length observed in several mammals including humans, where increased paternal age
704 at reproduction causes longer telomeres in offspring for at least two generations
705 (Eisenberg et al. 2012; Eisenberg and Kuzawa 2018). The exact impact of telomere
706 length on evolutionary fitness and aging therefore remains to be poorly understood.
707
708 **Is TE expression detrimental for longevity?**
709 At the transcriptional level, age-dependent TE misregulation thought to be resulting
710 from a gradual decline in heterochromatin maintenance has been proposed to be
711 harmful for lifespan in *Drosophila* (Chen et al. 2016; Wood et al. 2016; Brown and
712 Bachtrog 2017), mice (De Cecco et al. 2019), and humans (Bogu et al. 2019). Our
713 RNA-seq analysis in whole flies did not identify a TE-wide decrease in expression in
714 selected populations of Carnes2015 (**Fig. 4** and **Table S7**). In fact, TE expression
715 appeared to be more strongly influenced by sex and age compared to selection
716 regime. Nevertheless, TEs with a significant regime effect tended to be downregulated
717 in selected populations, and this effect was even more apparent after we corrected for
718 genomic copies. Our results are in line with the notion that transcription of several TEs
719 is detrimental and reducing their expression could be beneficial for longevity (Li et al.
720 2013; Wood et al. 2016; Guo et al. 2018). Together with the weak association between
721 genomic abundance and expression (**Fig. S10**), this further proposes that lowering
722 expression of TEs might be more important than purging TEs from the genome in the
723 evolution of longevity.
724 Interestingly, the tendency towards lower TE expression in late-breeding populations
725 as well as the age-related increase in expression with age was more pronounced in
726 male flies. Almost all TEs had a higher expression in males relative to females (**Fig. 4**
727 and **Fig. S7**). These findings are consistent with recent work showing that males suffer
728 more from TE derepression during aging due to their entirely repetitive,
729 heterochromatin-rich *Y* chromosome (Brown and Bachtrog 2017). If a systemically
730 increased TE expression in males contributes to sexual dimorphism in lifespan is yet
731 to be confirmed. DNA-sequencing of male flies in the four experimental evolution

732 studies would be necessary to determine if selection for postponed senescence had
733 similarly strong effects on TE copy number of the *Y* chromosome.
734
735 **Did selection lead to differentiation in genes related to TE regulation?**
736 We also hypothesized that potential detrimental effects of TEs on longevity should be
737 reflected by selection on genes related to TE regulation and transposition (**Table 2**
738 and **Table S11**). Across the four studies, 3 to 10 out of 96 TE regulation genes have
739 been reported as genetically differentiated candidates, of which only *E2f1* and *Hsp83*
740 were shared between two studies. There was further little transcriptional differentiation
741 between control and selected populations of Carnes2015 and Remolina2012 and no
742 overlaps of these genes have been observed. The absence of a strong, shared signal
743 of selection on these genes and missing GO enrichment associated to regulation of
744 TEs allows us to hypothesize that improvement of chromatin
745 structure/heterochromatin maintenance, piRNA-mediated silencing and modulators of
746 transposition are generally not prime targets of selection during the evolution of
747 longevity. This, however, does not preclude that other means of TE protection have
748 evolved. It is becoming increasingly evident that TE expression acts as a causative
749 agent of inflammation and immune activation in mammals (Kassiotis and Stoye 2016;
750 De Cecco et al. 2019). Interestingly, Carnes2015, Fabian2018, and Remolina2012 all
751 found significant divergence in innate immunity genes, whereas Fabian et al. (2018)
752 demonstrated an improved survival upon infection and alleviated immunosenescence
753 in the long-lived populations. Rather than reducing TE copy number and expression,
754 selection might preferentially act on immunity genes to reduce TE-mediated
755 inflammation and increase tolerance to TEs with improved lifespan as a consequence.
756 It remains yet to be explored to what degree innate immune pathways other than the
757 RNAi machinery contribute to TE regulation in *D. melanogaster*.
758
759 **Is reproduction at old age associated with an increased TE content?**
760 Our findings suggest that neither genetic drift nor pervasive selection on TEs or genes
761 related to TE regulation are predominant drivers of the differences in TE abundance.
762 The most parsimonious explanation for our results therefore is that postponed
763 reproduction increases the chance of many TEs to be inserted into the germline and
764 passed on to the next generation. TEs of low transpositional activity in particular might
765 need a prolonged chronological time offered by late-life reproduction to achieve a

766 successful genomic insertion (**Figure 2**). Over many generations, flies breeding at old
767 age would have accumulated more TEs in the genome than populations reproducing
768 early in life. Supporting this hypothesis, it has been demonstrated that most TE
769 families had a higher rate of insertions in the ovaries of older relative to young *P-*
770 *element* induced dysgenic hybrids, even though at the same time fertility was restored
771 and improved with age (Khurana et al. 2011). However, if this applies to non-dysgenic
772 fruit flies and whether it can result in a larger number of TEs over multiple generations
773 has to our knowledge not yet been observed. Thus, TE accumulation in late-breeding
774 populations could be similar to the regularly observed positive correlation between
775 parental age and number of *de novo* mutations in offspring (Goldmann et al. 2019;
776 Sasani et al. 2019). In line with this, genome-wide measures of nucleotide diversity
777 were also repeatably larger in late-breeding populations across four experiments
778 (**Table S4**), although we have not ruled out that this was driven by genetic drift or
779 balancing selection as proposed by one study (Michalak et al. 2017).
780 Opposing our hypothesis, two recent studies in termites (Elsner et al. 2018) and *D.*
781 *melanogaster* (Erwin and Blumenstiel 2019) suggest that the germline is protected
782 from TE invasions through increased transcription of the piRNA machinery. Indeed,
783 our expression analysis confirms that many genes associated with transcriptional and
784 post-transcriptional TE silencing tend to be upregulated with age. Despite this, TE
785 families had a generally higher copy number in populations reproducing late in life. It
786 therefore remains to be determined whether this age-dependent upregulation of TE
787 regulation genes really equates to reduced insertional activity, since potential and
788 realized TE repression might not necessarily match. The observation that these genes
789 also tended to be more expressed in controls relative to selected flies in Carnes2015
790 further poses the question whether there is a trade-off between TE silencing in the
791 germline and lifespan, which could be another mechanism explaining the rising TE
792 abundance in the genomes of long-lived flies.
793
794 Altogether, our work presents a novel viewpoint on the poorly understood role of TEs
795 in aging and longevity that is largely, but not exclusively, neutral. However, the caveat
796 remains that we are unable to rule out that survival of selected populations would be
797 further extended if they had a reduced TE content and expression. In-depth studies
798 tracking piRNA production in the germline together with direct measures of TE
799 transposition rates throughout life or measuring longevity upon knockdown and

overexpression of TEs would be crucial experiments to obtain a more complete picture.

MATERIALS & METHODS

Datasets

We utilized genomic data from four independent studies performing laboratory selection for postponed reproduction on wild-derived replicate populations by only allowing flies of relatively old age to contribute to subsequent generations, whereas controls reproduced early in life (Remolina et al. 2012; Carnes et al. 2015; Fabian et al. 2018; Hoedjes et al. 2019) (**Table S1**). The experimental designs of the studies were overall comparable, but notable differences include the mode of selection, maintenance of controls, variable source populations, number of replicate populations and generations at sequencing. Moreover, Hoedjes2019 performed the selection for postponed senescence on three varying larval diets ranging from low to high sugar/protein content. The genomic analysis was based on available raw fastq files from whole-genome pool-sequencing of 100 to 250 females. RNA-seq data from Carnes et al. (2015) consisted of raw fastq files from pools of 50 flies. The study included transcriptomes of all selected and control populations, for which both sexes at two ages 3-5 days (young) and 26-35 days of age (old) have been sequenced in replicates. Microarray expression data from Remolina et al. (2012) are derived from heads and abdomens from females at the age of 1, 5, 15, 30, and 50 days of age from the three control and selected populations. See methods in the publications of each study for details on experimental design and **Table S1** for a summary. For simplicity, we refer to Carnes et al. (2015) as Carnes2015, Fabian et al. (2018) as Fabian2018, Hoedjes et al. (2019) as Hoedjes2019, and Remolina et al. (2012) as Remolina2012 throughout this report.

Genome-wide TE abundance

To quantify the number of genomic insertions for each TE family in selected and control populations we used the assembly-free tool deviaTE (Weilguny and Kofler 2019). In brief, deviaTE maps raw reads to a library of known TE sequences and normalizes the sequencing depth of each position within a TE to the average depth of multiple single-copy genes. The obtained distribution of normalized values reflects

coverage fluctuations within a TE, where averaging over all positions of a TE gives the mean abundance per haploid genome. We used raw fastq files as input and ran deviaTE with options: --read_type phred+33 (phred+64 for Fabian2018), --min_read_len 50, --quality_threshold 18, --hq_threshold 20, --min_alignment_len 30, --threads 20, --families ALL, --single_copy_genes Dmel_rpl32,Dmel_RpII140,Dmel_Act5C,Dmel_piwi,Dmel_p53. TE abundance was estimated considering reads with a mapping quality of >=20 by adding the "hq_cov" and "phys_cov" column from the deviaTE output. Due to sequence similarity within and between TEs, we further repeated most analyses using a less conservative mapping approach without filtering for mapping quality by using values from the "cov" column instead of "hq_cov". We restricted our downstream analysis to TEs that had a study-average of >=0.5 insertions for at least 80% of the positions within a TE sequence, thereby excluding all TEs with very low abundance and potentially wrongly mapped reads.

We then investigated if the number of genomic abundance per TE varies between control and selected populations using three different approaches (**Fig. S1**). First, we tested each study (and diet regime for Hoedjes2019) independently and fit a model for every detected TE as: *NormalizedCoverage ~ Regime + Population[Regime]*, where *NormalizedCoverage* are the normalized coverage values reflecting insertion estimates of each position within a TE, allowing us to take the variance of insertion abundance within a TE into account. *Regime* is the effect of selection regime (levels: selected and control), and *Population[Regime]* is the effect of the different replicate populations nested within Regime. To obtain the overall effect of breeding regime in Hoedjes2019, we fit: *NormalizedCoverage ~ Diet + Regime + Diet x Regime*, where *Diet* corresponds to the three larval diets the flies were raised on (low, medium, high). To correct for multiple testing and to define significant differences in TE abundance, we applied a Bonferroni cut-off at $\alpha = 0.01$.

To visualize differences in genomic abundance of TEs shown in **Fig. 1**, we first calculated the average number of insertions per TE for each breeding regime. We then took the absolute difference per TE by subtracting control from selected insertion values (δInsertions, upper panel in **Fig. 1**). We used Pearson's correlation to show that δInsertions from each TE were expectedly correlated to its copy number in the genomes. To correct for this, we divided the selected by control number of insertions

per TE and took the $\log_2$ of these values to obtain the $\log_2$ fold change ($\log_2$ (S / C), lower panel in **Fig. 1**). We further used *SuperExactTest* (Wang et al. 2015) to analyze if the overlap of TEs with a significantly higher genomic abundance in selected ("S>C") or control populations ("C>S") between postponed senescence studies is expected by chance.

In our second approach, we combined all four studies and only considered a single average insertion value per TE instead of taking all positions into account. We then summed up all TE insertion values within a population to obtain the total genomic TE content. Because the total insertion TE content was significantly different between studies (see **Fig. S3** and methods below), we normalized the insertion counts of each TE by the total insertions in the corresponding population, and fit a combined model as: asin(sqrt(*NormalizedInsertions*) ~ *Study* + *Regime* + *Regime*Study*, where asin(sqrt(*NormalizedInsertions*) is the arcsine square root transformed proportion of insertions per TE relative to the total genome-wide TE content in each population. The *Study* term had four levels (Carnes2015, Fabian2018, Hoedjes2019, Remolina2012), whereas *Regime* is as described above. As not all positions within a TE are considered in this model, statistical power is lower than in the previous approach above. We therefore performed a less stringent multiple testing correction by employing an FDR cut-off of 0.05 using the "p.adjust" function with method "fdr" in *R*.

Third, we identified all TEs that showed a consistent increase or decrease in the genomic abundance within all selected populations relative to all control populations of a given study and within diet regimes of Hoedjes2019.

To analyze if the total genomic TE content varies between control and selected populations, we calculated the total TE load across the whole genome, and TE subclasses of Long Terminal Repeat (LTR), Non-Long Terminal Repeat (non-LTR), and Terminal Inverted Repeat (TIR) by summing up all insertion values of each TE family per population. We then used these sums as dependent variables in a statistical model with *Study*, *Regime* and the *Study x Regime* interaction as factors.

All statistics were done in *R* using in-built functions unless otherwise stated. We did not consider the effect of diet in the models combining all studies.

Genomic TE locations and approximate mobility

To identify the exact genomic positions and population frequency of TE insertions, we used the program PoPoolationTE2 (Kofler et al. 2016). We first quality trimmed paired-

end raw reads using cutadapt (v.2.0) (Martin 2011) with options: --minimum-length 50 -q 18 and also --quality-base=64 for Fabian2018. Next, we masked the *D. melanogaster* reference (v.6.27) for TEs present in the deviaTE library using RepeatMasker (v.4.0.9) (Smit et al. 1996) with the RMBlast (v.2.9.0) search engine and set options as recommended in the PopoolationTE2 manual (-gccalc -s -cutoff 200 -no_is -nolow -norna -gff -u -pa 18). Trimmed reads were then mapped against the masked reference genome using bwa bwasw (v.0.7.17) (Li and Durbin 2009). We restored paired-end information as outlined in the PopoolationTE2 pipeline (function *se2pe*) and generated a ppileup file (function *ppileup*) applying a mapping quality cut-off of 15. TE insertions were then identified using the functions *identifySignatures* (--signature-window fix500, --min-valley fix150; and due to differences in the number of replicate populations we chose --min-count 3 for Remolina2012 and Fabian2018, --min-count 5 for Carnes2015, or --min-count 12 for Hoedjes2019). Finally, we applied the *frequency* and *pairupSignatures* (--min-distance -200 --max-distance 300) functions and filtered identified TEs using *filterSignatures* (--max-otherte-count 2 --max-structvar-count 2) to create the final set of TE insertions. We only included sites covered in all populations within a study and restricted our analysis to chromosomes *X*, *2, 3,* and *4*. For each TE family, we calculated the average population frequency across all of its identified genomic locations within a population as a proxy for active or recent transposition events (Kofler et al. 2015). We used Spearman's correlation analysis to compare average frequency values of each study to average frequencies from a natural South African (SA) population sequenced to a high genomic coverage (Kofler et al. 2015), and to correlated TE abundance with average frequency. We employed t-tests to analyze if average population frequency from the SA population varies between TEs more abundant in selected or controls, and also performed this analysis using only the top10 TEs with the largest $\log_2$ FC values of abundance change.

Genome-wide nucleotide diversity

We mapped trimmed paired-end reads against a fasta file including the repeat-masked v6.27 reference genome (see above), TE library from deviaTE (Weilguny and Kofler 2019), *Wolbachia pipientis* (NC_002978.6), and two common gut bacteria *Acetobacter pasteurianus* (AP011121.1), and *Lactobacillus brevis* (CP000416.1) using bwa *mem* (v.0.7.17) (Li and Durbin 2009), and then created sorted bam files with the samtools

(v.1.9) (Li et al. 2009) functions *view* (-Sb) followed by *sort*. Duplicates were removed using PicardTools (v.2.18.27) function *MarkDuplicates* with REMOVE_DUPLICATES=true. For Remolina2012, we merged files from the same population sequenced on multiple lanes using samtools *merge*. We then filtered and created pileup files using samtools *mpileup* with option -q 20 --rf 0x2 --ff 0x4 --ff 0x8 -f (and -6 for Fabian2018). Average coverage per study was determined only using major chromosomal arms. We detected reads mapping to the genome of the intracellular bacterium *Wolbachia* for all populations. To calculate nucleotide diversity π and Watterson's θ across non-overlapping 100kb windows we used *Variance-sliding.pl* implemented in PoPoolation (Kofler et al. 2011) with parameters --min-count 2 --min-coverage 5 --window-size 100000 --step-size 100000 --pool-size 100 --measure pi (or theta) --fastq-type sanger --min-covered-fraction 0.6. We set the maximum coverage threshold (--max-coverage) to two times the average genome coverage of each population. The range of cut-offs used in each study was 20-74 in Carnes2015, 286-355 in Fabian2018, 67-107 in Remolina2012 and 166-228 in Hoedjes2019. We then analyzed variation in π and θ using ANOVA models including the factors *Chromosome* (*X, 2L, 2R, 3L, 3R, 4*), *Diet* (low, medium, high), *Regime* (control, selected) and the *Diet x Regime* interaction for Hoedjes2019, and *Population[Regime]*, *Chromosome*, and *Regime* for all other studies.

Genetic drift simulations

As the analyzed studies did not include data from the ancestral populations, we used average TE frequencies from the South African population (Kofler et al. 2015) as a starting point in our simulations. To simulate frequency change of each TE, we set population sizes (N), generation times and number of replicate populations as mentioned in the original publications (**Table S1**). As some studies reported a range of population sizes, we performed simulations using the lower and upper limits. We used the *rbinom* function in *R* and drew from a size of 2N considering the diploid genome and given the average frequency of a TE as probability to be drawn. The number of successful draws was divided by 2N to obtain the new TE frequency and used as input for the next draw. We repeated this process until the generation times at sequencing of control and selected populations were reached. Using the simulated TE frequencies in the last generation, we calculated average TE frequency across all

967 simulated replicates within breeding regime. Next, we obtained the proportion of TEs
968 with a higher (S>C) and lower frequency (C>S) in selected populations and calculated
969 the $\log_2$ relative proportion (i.e. $\log_2$ of S>C proportion divided by C>S proportion). As
970 an additional approach, we also acquired the $\log_2$ relative proportion by using the
971 number of TEs with consistent differences in frequency between all the populations of
972 the regimes instead of averaging. We performed 5,000 simulations to get a distribution
973 of relative proportions expected under genetic drift. P-values were calculated by
974 dividing the number of simulations that resulted in a larger or equal proportion as
975 observed in the actual analysis by the total number of simulations.
976
977 **TE frequency differences**
978 We further asked if certain genomic TE insertions are putatively involved in lifespan
979 and aging as might be indicated by a consistent TE frequency differences at any
980 genomic site between selected and control populations. We used the identified TE
981 insertions and frequency estimates from PopoolationTE2 (see above). Significant
982 difference in frequency between selected and control populations were determined by
983 t-tests analyzing differences in arcsine square root transformed frequencies between
984 regimes for Carnes2015, Fabian2018, and Remolina2012. For Hoedjes2019, we used
985 a linear model with *Diet*, *Regime*, and *Diet x Regime* as factors. Bonferroni correction
986 at $\alpha = 0.05$ was used to correct for multiple testing. Functional annotations were
987 supplemented using SnpEff (v.4.0e, Cingolani et al. 2012) and the *D. melanogaster*
988 reference v6.27 considering TE insertions within 1000 bp of the 5' and 3' UTR as
989 upstream or downstream of a gene.
990 We analyzed if each TE family varies in frequency between regimes by fitting the
991 factors of *Diet*, *Regime, and Diet x Regime* for Hoedjes2019, or *Regime* and
992 *Population[Regime]* for all other studies on arcsine square root transformed insertion
993 site frequencies. FDR values were obtained by using "p.adjust" in *R* and TE families
994 considered significant at FDR<0.05.
995
996 **RNA-seq analysis**
997 RNA-seq data from Carnes et al. (2015) consisted of two replicates of young and old
998 males and females from all control and selected populations (**Table S1**). Raw reads
999 were filtered to remove remaining adapter sequences and to exclude low quality

positions using cutadapt (v.2.0) (Martin 2011) with options: -a AGATCGGAAGAGC --minimum-length 75 -q 20. Filtered reads were then mapped to the repeat-masked reference genome (see above), the TE library from deviaTE, *Wolbachia pipientis*, *Acetobacter pasteurianus*, and *Lactobacillus brevis* (see above) using STAR (Dobin et al. 2013) with options --alignIntronMin 23 --alignIntronMax 268107 –– outFilterMultimapNmax 10 --outReadsUnmapped FastX --outSAMstrandField intronMotif --outSAMtype BAM SortedByCoordinate. Read counts were obtained using the command-line version of featureCounts (Liao et al. 2013) with options -t exon -g gene_id --extraAttributes gene_symbol. We next pre-filtered the read count data by excluding all genes and TEs that did not have a sum of 400 counts across all 80 samples (i.e. on average 5 counts per sample). Five TEs that are not known to occur in *D. melanogaster* passed this filter and were excluded. For simplicity, the analysis was performed on average read counts from two replicates, as all replicates were highly significantly correlated (Pearson's r ranging from 0.95 to 1, significant after Bonferroni correction). To analyze differential expression, we fit two models using read counts of genes and TEs with DESEq2 in *R* (Love et al. 2014). First, a full model with *Regime* (selected vs control), *Sex* (male vs female), *Age* (young vs old) and all interactions were fit. As the sex term was significant for most TEs, we decided to analyze males and females separately and by fitting models with *Regime*, *Age* and the *Regime x Age* interaction. We obtained $\log_2$ fold change values for each factor and the normalized read counts from DEseq2 for further analysis. To correct for the effect of copy number on expression, we divided read counts of TEs by the number of insertions observed in each population, assuming that genes and 13 TEs that did not pass out filters in the genomic analysis have a single copy in the genome.

Evolution of TE regulation genes

The list of genes involved in TE regulation consisted of piRNA pathway genes also analyzed in Erwin and Blumenstiel 2019 and Elsner et al. 2018, and genes involved in heterochromatic and chromatin structure from Lee and Karpen 2017. We further added 7 additional genes involved in these functions, and genes annotated to "regulation of transposition" (GO:0010528) and "transposition" (GO:0032196) according to FlyBase so that we ended up with a total of 96 genes (**Table S11**). We then screened the published genomic candidate gene lists from Carnes2015, Fabian2018, Hoedjes2019 and Remolina2012 for these genes. We also compared TE

regulation genes to differentially expressed genes from the RNA-seq analysis of Carnes2015 (see above). We further obtained normalized microarray expression data from Remolina2012 of female flies at 1, 5, 15, 30, and 50 days of age (**Table S1**). Notably, the expression data were created from flies at 40 generations of selection compared to 50 generations in the genomic analysis. We fit a mixed effects model similar to the one used in the original publication with *Age*, *Regime*, and *Age x Regime* as fixed and *replication within population-age combination* as random effect. The two available tissues (heads and abdomens) were analyzed separately. A gene was considered to be differentially expressed if it had an FDR < 0.05 unless otherwise stated.

DATA ACCESSIBILITY

All data used in this study have been previously published. Accession numbers to the raw genomic and transcriptomic data can be found in **Table S1** and in the original studies (Remolina et al. 2012; Carnes et al. 2015; Fabian et al. 2018; Hoedjes et al. 2019). RNA-seq data were obtained directly from the authors. Additional raw data and results files on TE abundance, TE positions and frequency differentiation, nucleotide diversity, RNA-seq results for genes and TEs, and microarray results are available at: *to be made available upon publication*.

ACKNOWLEDGEMENTS

We thank Robert Kofler, Andrea Betancourt, Frank Jiggins, Lukas Weilguny and Suse Franssen for helpful comments and discussions. This work was supported by the Wellcome Trust (WT098565/Z/12/Z to J.M.T. and L.P.), EMBL (H.M.D. and J.M.T.), and Comisión Nacional de Investigación Científica y Tecnológica – Government of Chile (CONICYT scholarship to M.F.). We are further grateful for financial support from the Society for Molecular Biology & Evolution enabling us to present this work at the annual meeting (SMBE 2019, Carer travel award & registration award to D.F.).

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FIGURE LEGENDS

Figure 1. Difference in TE abundance between regimes. Upper row: δinsertions (Y-axis) were obtained by subtracting the average genomic insertions of the controls from the selected populations. Bottom row: $\log_2$ fold change between selected and control populations. The dashed line indicates no difference between regimes. >0 denote TEs with a larger abundance in selected populations, while <0 TEs with more insertions in controls. TE subclasses are given in different colors. Selected flies had more genomic insertions than controls for most TE families (also see Table 1).

Figure 2. Differences in average TE frequency. Average TE frequency from the South African population separated into C>S (blue) and S>C TEs (red) are shown on the Y-axis. Differences are shown considering all C>S and S>C TEs ("All") or only the top 10 TEs with the biggest differences in $\log_2$ FC of insertions ("Top 10"). t-tests were used to assess statistical significance. ns, not significant; * P < 0.05; *** P < 0.001.

Figure 3. Selection on TE abundance and insertions. (A) Sharing of TEs with significant differences in abundance between regimes of each study. S>C and C>S denote TEs with a higher abundance in selected or control populations, respectively. Red bars indicate a significant overlap at P < 0.05. (B) Boxplots of the number of genomic insertions relative to the total genomic content of the 2 significantly shared C>S TEs. (C) Genome-wide differentiation in TE insertion frequency between selected and control populations in Fabian2018 and (D) Hoedjes2019. Every point indicates the $-\log_{10}$ P-value of a TE insertion across chromosomal arms (alternating black and grey color). The solid orange line corresponds to the Bonferroni cut-off at $\alpha = 0.05$. Red points denote TEs with a significantly higher frequency in selected relative to control populations. More details including exact positions, frequency and annotation of candidate TE insertions can be found in **Table S5**.

Figure 4. Multiple factors influence TE expression. (A) Proportions of differentially expressed TEs at adjusted P < 0.05 and directionality relative to 123 TEs with detectable expression for factors from statistical models on pre-filtered read counts in DESeq2 (also see **Table S7**). "Sex" refers to the results of the full model including Sex (M, males; F, females), Age (young; old), Regime (C, control; S, selected), and all

interactions. “Regime”, “Age” and “RxA” (i.e. *Regime x Age* interaction) refer to results from model fits with males and females separately analyzed. The absolute number of TEs for factor levels are given above or below bars. (B) Log_2 fold change of regime (selected vs control) and (C) age (young vs old) for males and females. Colors designate TEs significant only in males (blue), or females (red), or shared between both sexes (orange). Not significant TEs are in grey. (D and E) Log_2 fold changes across regime against age differences in males and females. Colors designate TEs significant only for regime (blue), or age (red), or for both factors (orange). Not significant TEs are in grey. (F) Normalized expression counts were significantly correlated to the number of insertions in the genome. Expression values for each TE were averaged across all conditions (sex, age, regime). Separating samples into different conditions resulted in similar significant correlation coefficients (**Table S8**). Colors correspond to TEs with a significantly higher genomic abundance in selected (red) or control populations (blue). (B to E): r, Pearson’s correlation coefficient; (F): r, Spearman’s correlation coefficient; * $P < 0.05$, *** $P < 0.0001$.

TABLES

Table 1. Number of detected and significant TE families with percentage of TEs more abundant in selected (S>C) or controls (C>S) or not different between regimes (n.s.) from models analyzing studies independently or combined.

Study	N	N (sign.)[a]	S>C	C>S	n.s.
Carnes2015	112	107	77%	19%	4%
Fabian2018	110	85	46%	31%	33%
Hoedjes2019	115	94	58%	24%	18%
Remolina2012	110	76	57%	12%	31%
Combined Model	103	Regime: 41 Study: 101 Study x Regime: 65	33%	7%	60%

[a]Significant after Bonferroni correction at α=0.01 in single studies or FDR<0.05 in combined model.

Table 2. TE regulation genes identified by each study as genetically differentiated candidate genes for lifespan combined with differential expression in Carnes2015 and Remolina2012 (see **Table S11** for a full summary of all 96 genes).

		Genetic Differentiation				Differential expression		
Gene	**Type**	**Carnes 2015**	**Fabian 2018**	**Hoedjes 2019**	**Remolina 2012**	**Direction of Expression[a]**	**Sex**	**Study**
E2f1	epigenetic	✓	✓	-	-	Old>Young	M	Carnes
Hsp83	transposition	✓	-	-	✓	Old>Young/RxA	M	Carnes
csul	piRNA	✓	-	-	-	Old>Young	M	Carnes
fs(1)Yb	piRNA	✓	-	-	-	-	-	-
G9a	epigenetic	✓	-	-	-	Old>Young	M	Carnes
nub	epigenetic	✓	-	-	-	Old>Young	M	Carnes
piwi	epigenetic & piRNA	✓	-	-	-	S>C	F	Carnes
Su(var)2-HP2	epigenetic	✓	-	-	-	Old>Young	M	Carnes
tej	piRNA	✓	-	-	-	Old>Young/RxA	F	Carnes
wapl	epigenetic	✓	-	-	-	Old>Young	F	Remolina
tj	piRNA	-	✓	-	-	Young>Old/RxA	F	Carnes
vas	piRNA	-	✓	-	-	Old>Young	F	Remolina
del	piRNA	-	-	✓	-	Old>Young	F	Remolina
Hen1	piRNA	-	-	✓	-	C>S/Old>Young/RxA	F	Carnes
kis	epigenetic	-	-	✓	-	Old>Young Young>Old/RxA	M F	Carnes
mino	piRNA	-	-	✓	-	C>S Old>Young/RxA	M&F M	Carnes
Nbr	piRNA	-	-	✓[b]	-	Old>Young	M&F	Carnes
qin	piRNA	-	-	✓	-	C>S Old>Young	M F	Carnes Remolina
Su(var)3-7	epigenetic	-	-	✓	-	Old>Young	F	Remolina
AGO3	piRNA	-	-	-	✓	Old>Young RxA	M&F F	Carnes
armi	piRNA	-	-	-	✓	-	-	-
CG8108	epigenetic	-	-	-	✓	Old>Young	F	Remolina
moon	piRNA	-	-	-	✓	-	-	-
nos	piRNA	-	-	-	✓	C>S Old>Young	M F	Carnes Remolina

[a]Padj.<0.05 for *Age*, *Regime* and interaction of both (RxA), where ">" indicates larger. [b]*P-element* frequency divergence found in the present study.

FIGURE 1

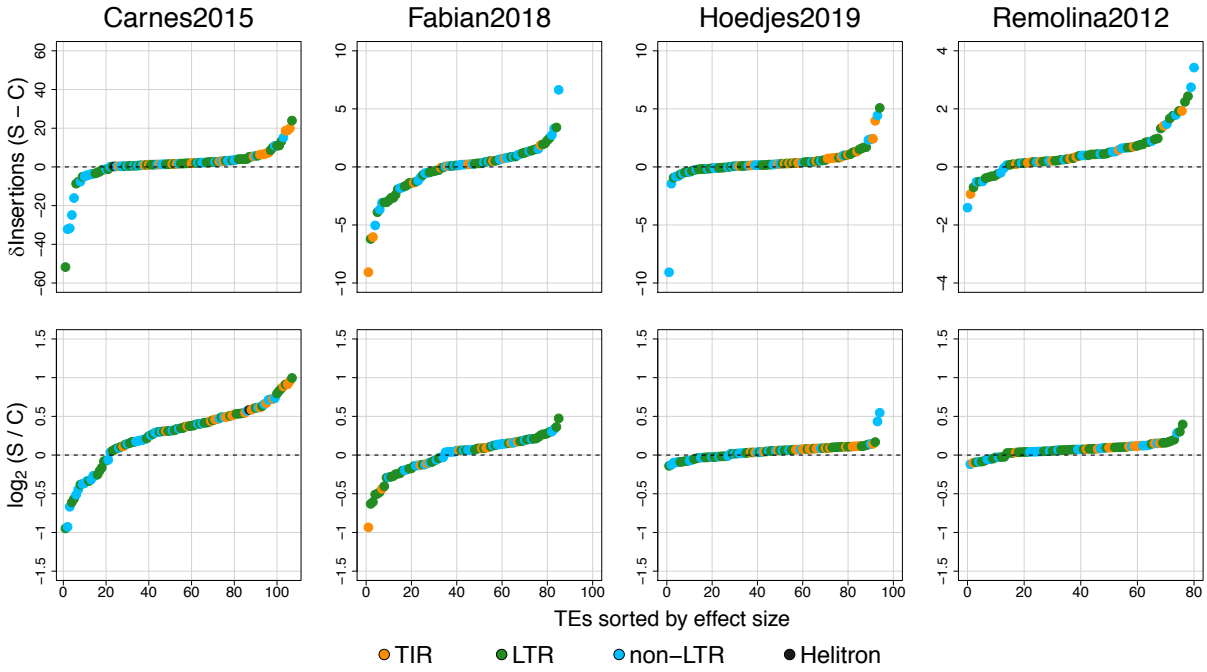


FIGURE 2

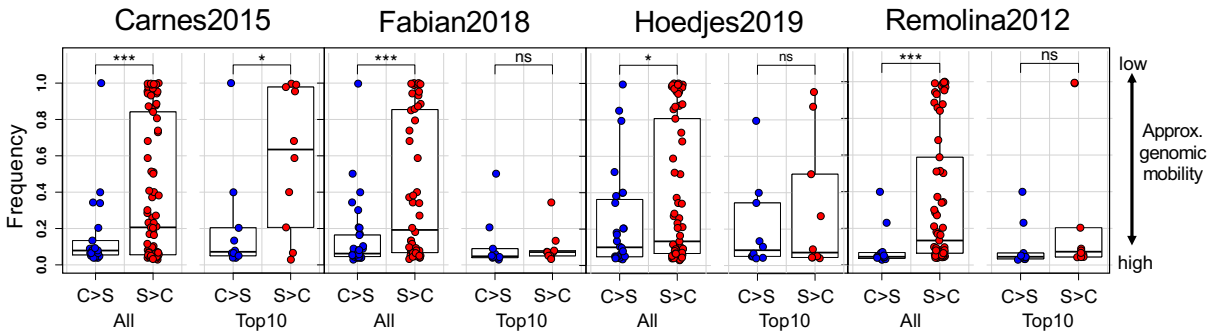
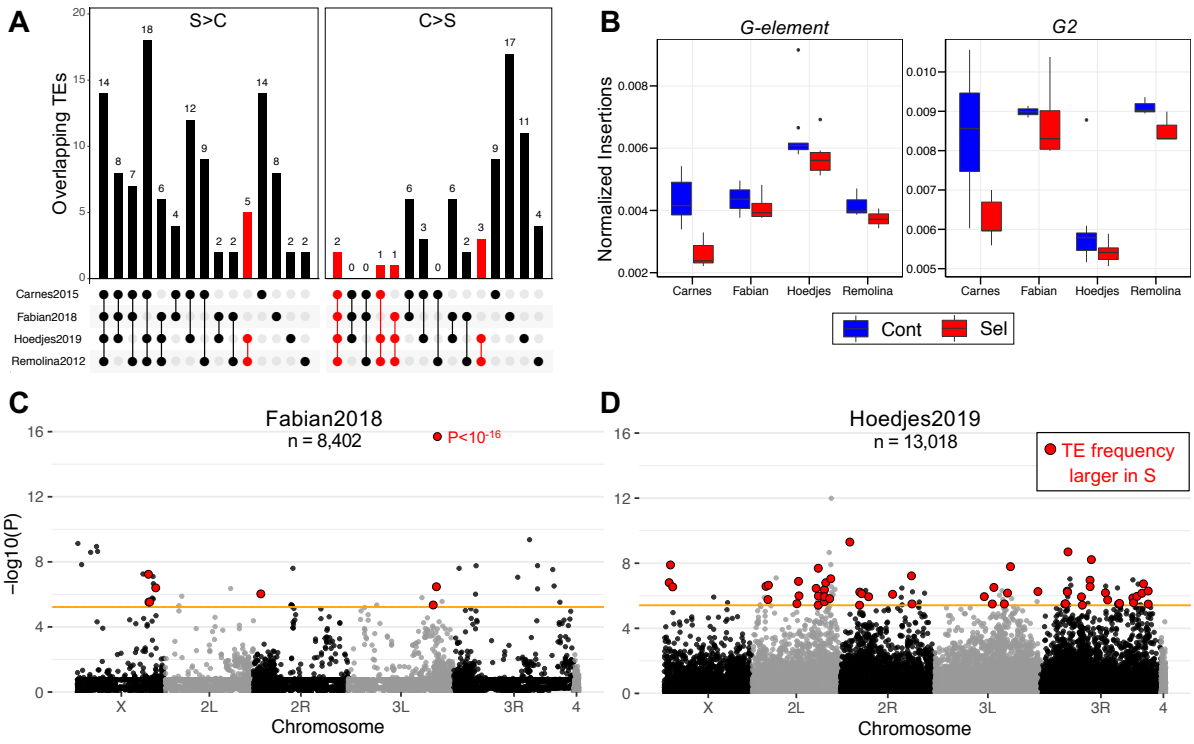
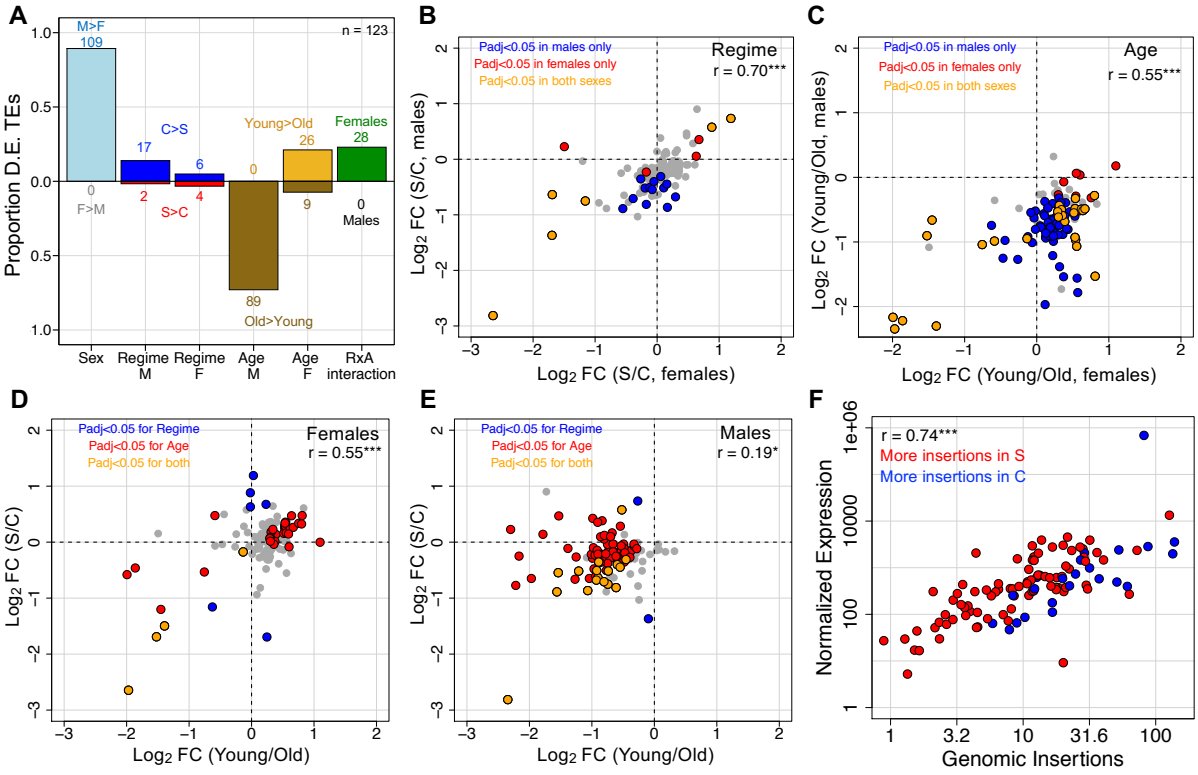


FIGURE 3



1514 **FIGURE 4**

1515



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