Transposable element landscape changes are buffered by RNA silencing in aging Drosophila

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ABSTRACT

Genetic mechanisms that repress transposable elements (TEs) in young animals decline during aging, as reflected by increased TE expression in aged animals. Does increased TE expression during aging lead to more genomic TE copies in older animals? To answer this question, we quantified TE Landscapes (TLs) via whole genome sequencing of young and aged Drosophila strains of wild-type and mutant backgrounds. We quantified TLs in whole flies and dissected brains and validated the feasibility of our approach in detecting new TE insertions in aging Drosophila genomes when natural defenses like RNA interference (RNAi) pathways are compromised. By also incorporating droplet digital PCR to validate genomic TE loads, we confirm TL changes can occur in a single lifespan of Drosophila when TEs are not suppressed. We also describe improved sequencing methods to quantify extra-chromosomal DNA circles (eccDNAs) in Drosophila as an additional source of TE copies that accumulate during aging. Lastly, to combat the natural progression of aging-associated TE expression, we show that knocking down PAF1, a conserved transcription elongation factor that antagonizes RNAi pathways, may bolster suppression of TEs during aging and extend lifespan. Our study suggests that RNAi mechanisms generally mitigate genomic TL expansion despite the increase in TE transcripts during aging.
INTRODUCTION

All animal genomes carry the genetic burden of a sizeable reservoir of parasitic elements called transposons or transposable elements (TEs). This TE burden can range from the extreme >70% proportion of the axolotl genome [1, 2] to >50% in the human genome [3] to >10% in the Drosophila melanogaster genome [4, 5]. TEs are selfish invaders of animal genomes with some potential for stimulating more rapid gene regulatory innovations like serving as novel enhancers [6], but more frequently are detrimental to animal fitness when they insert into and disrupt expression of important genes [7]. Therefore, conserved chromatin regulation and RNA-interference (RNAi) pathways must silence TEs to ensure fertility and animal health. However, these genomic defense mechanisms also weaken during animal aging concomitant with observable decreases in genomic integrity in aging cells. This phenomenon has been articulated in the hypothesis of TEs impacting aging [8].

Initial support for this hypothesis in the model organism D. melanogaster came from studies of TE expression increasing in aging flies [9-11, 12]. For example, mutants in chromatin silencing factors and RNAi pathway genes which repress TEs have reduced lifespans [9, 10, 13-16], whereas dietary restriction and overexpressing the RNAi and chromatin factors can limit TE expression and promote longevity [13, 14]. Neurodegeneration modeled in aging flies through overexpressing aggregating proteins like TDP-43 and TAU also leads to elevated TE expression [17-20]. Additionally, there is evidence of a somatic population of Piwi proteins which can serve an additional TE defense mechanism that when mutated leads to shorter lifespan and loss of stem cell maintenance [13, 16, 21-23].
Beyond flies, mammals also must repress TEs for critical development of germ

cells, embryos and neurons. Mammals have a complex, interconnected network of

silencing pathways like the axis of SETDB1 [24, 25], KAP1 [26-28] and the HUSH

complex [29-32]; and its cooperation with histone deacetylases like SIRT6 [33, 34] and

histone methyltransferases like Suv39h1 and G9A [35-37]. In addition, there are DNA

methyltransferases that genetically interact with the piRNA pathway to target TEs for

chromatin silencing in mammalian germ cells [38-44]. In primates and mice, the most

active TE is the *LINE-L1* which is implicated in somatic genome mosaicism in

developing brains and individual neurons [45-53]. Lastly, *LINE-L1* is linked to

deleterious novel mutations in tumors and they are activated in cell culture models of

cellular senescence [54-59]. Although TE control is clearly important to mammalian

health, the large genome sizes and longer lifespans hampers comprehensive

assessments of mammalian TLs during impact aging.

Therefore, in this study we leveraged *Drosophila*’s rapid aging, its compact

genome and powerful genetic tools as significant advantages for testing how TE

landscapes may change during normal animal aging. An important goal of our study is

to address the debate of whether TLs quantitated from Whole Genome Sequencing

(WGS) of *Drosophila* genomes represent true gains in TE genomic load [60]. One

bioinformatics program called TEMP [61] has been used extensively in determining TE

insertions from *Drosophila* WGS [62, 63] but its capacity to distinguish bona fide TE

insertions from potential library sequencing artifacts has been re-examined [60]. Noting

the high degree of variability in TE insertion calls from various bioinformatics programs

applied to *Drosophila* WGS data [64], we therefore developed our own program called

the Transposon Insertion & Depletion AnaLyzer (TIDAL) to identify the tremendous
diversity of TLs across various Drosophila strains [65]. TIDAL’s increased specificity in TE determinations comes from requiring sequencing reads mapping to both sides of genomic locus flanking the TE insertion. This specificity was benchmarked against genomic PCR tests [65], and TIDAL has characterized TLs in other Drosophila studies of genetic factors regulating TE silencing [66, 67].

In this study, we demonstrate how WGS and extrachromosomal circular DNA (eccDNA) sequencing of aged and young flies can report changes in TLs during fly aging. Although TE RNA upregulation is a recurring phenotype of aging wild-type flies, we show that major changes in the genomic TLs are generally suppressed by the RNAi pathway because RNAi mutants allow TEs to expand their genomic DNA (gDNA) copy numbers. We also demonstrate that tissue-specific (i.e. fly brain tissues) gDNA sequencing can sensitize the detection of genomic TL changes; and eccDNA accumulation during aging of the ISO1 strain is an additional feature of the hypothesis of TEs impacting animal aging. Lastly, we show that genetically boosting RNAi activity in aged flies via knockdown of PAF1 can suppress TE RNAs and extend longevity. Together, these results demonstrate that the RNAi pathway buffers genomic alterations by the natural increase of TE RNAs during aging and suggest PAF1 inhibition in aging animals could be a therapeutic target in this genetic mechanism of TE repression.

RESULTS

Recurring increase of TE RNA expression during fly aging.

Although previous studies using certain control wild-type (WT) fly strains showed that TE RNAs were upregulated in aged flies [10, 14], we decided to reconfirm this
observation for three commonly used WT fly strains that would form the basis of this study. Using our lab’s standard rearing conditions, we first determined the aging curves for the ISO1 strain used for the *D. melanogaster* reference genome sequence [4], an isogenic *w1118* strain that is a common background strain in genetic studies [68], and the *Oregon-R* strain used in a series of functional genomics datasets [69]. Whereas *w1118* and *OreR* displayed lifespans typical of other WT fly strains (Figure 1A), the shorter lifespan of *ISO1* was expected because its genetic background was known to sensitize phenotypes from chemical mutagenesis screens [70].

We then followed the experimental convention of other studies [10, 14] to standardize the comparison of 30-day aged adults versus 5-day young adults, and we performed quantitative RT-PCR on a panel of TEs from total RNAs from females (Fig. 1B). We replicated many examples of TE RNAs being upregulated in the aged WT flies but noticed variability in which specific TE families were the most significantly upregulated during aging. For example, *gypsy, mdg1,* and *l-element* were up regulated at the RNA level in *ISO1* aged flies, while *copia* and *1731* RNAs were upregulated in *w1118* and *OreR* (Fig. 1B). This variability may reflect the inherently distinct TLs between these three strains [65], but the trend holds true that WT adult flies recurrently experience increased TE expression during aging.

However, some previous studies examining *Drosophila* TEs during aging mainly used a genetic reporter called the *gypsy-TRAP* to reflect increased transposition activity in aging flies [13, 14, 18, 71]. This reporter has the advantage of low cost and sensitivity of detecting small numbers of cells in a background of nonmodified cells, yet this transgenic construct is also only designed for *gypsy* to insert and activate a fluorescent protein read-out and cannot assess overall TLs. A newer and distinct TE activity
reporter called the *gypsy*-CLEVR puts the fluorescent protein expression cassette into the domain of a 5X-UAS promoter only after retrotransposition, to take advantage of cell-type or tissue specific GAL4 driver *Drosophila* strains [15]. Only one recent study we are aware of assessed TLs during fly aging by WGS of enriched αβ-Kenyon Cell neurons [60] and which argued that various pitfalls obscured the ability to observe TL increases during fly aging. For example, the study itself discussed that Multiple Displacement Amplification (MDA) required to amplify the minute amount of neuronal gDNA prior to Illumina library construction could contribute to artifactual chimeric molecules that represent false positive TE insertions [60]. Therefore, our more comprehensive effort to examine TLs through direct WGS should add valuable insight to this question.

First, some issues need to be considered in TL determinations in *Drosophila* WGS datasets, such as two different TE-insertion discovery programs, TEMP [61] and TIDAL [65] that each can yield different results from analyses of the same dataset (Figure S1). Balancing sensitivity against specificity, TIDAL has similar trends as TEMP in revealing the diversity of TLs amongst *Drosophila* samples (Fig. S1B) and both are effective at calling germline insertions, but TIDAL avoids false positive predictions that others have contended as somatic TE insertions (Fig. S1C, [60]). TIDAL handles this issue differently by computing a Coverage Ratio (CR) score for each TE insertion from pooled sequencing of a small group of flies (Fig. 1C), where TE insertion reads are divided by reference genome mapping reads and a pseudocount of 1; such as a CR of 2 that we used as an arbitrary cutoff for indicating deep penetrance of a TE insertion at a given insertion locus. When we re-analyzed the WGS datasets from [60] with TIDAL
even with the caveats of MDA, the TIDAL outputs do suggest increasing TLs within the fly aging neuron genomes (Figure S2).

Measuring TL differences by direct WGS and the TIDAL program.

To meaningfully compare TL changes during a single generation of aging flies from WGS and to avoid the genomic complications of normalizing against Y-chromosome reads that are exceptionally dense with repeats [72], we only compared samples from within the same strain in small numbers of young versus aged whole female adult flies or female brains (Fig. 1D). In our process we extracted a set amount of genomic DNA from 10 flies that allowed for reproducible WGS library construction without requiring MDA or other total DNA amplification methods. We then sequenced on the Illumina platform each fly strains’ bulk gDNA library to a minimum >~30 million 75-bp reads for >~16X fold genomic coverage of the version Dm6 genome assembly (Table S1). Each library was analyzed identically with the TIDAL program [65] and new TE insertions were counted individually and then normalized against the reads per million measurement to account for sequencing depth differences. In developing our own methodology to examine fly TLs during aging, we recall our previous study showing that each fly strain’s unique TLs depends on how inherently distinct its genetic background is from the reference genome strain ISO1 [65]. Therefore, it was expected that new TE insertions quantified and normalized against each library’s sequencing depth would yield the lowest numbers for ISO1 and the most TE insertion differences in w1118 and OreR (Figure 2A).

As expected, each of these WT flies TLs displayed completely distinct compositions of new insertions of TE families relative to the Dm6 reference genome
sequence (Fig. 2B), such as a larger proportion of hobo TEs in ISO1, major infiltration of P-elements in OreR, and several more FB, pogo and 412 TEs in w1118. Focusing on the ratio of 30-day to 5-day insertions for the specific TE families making up the bulk of these strains TLs, we could observe some increases in TE insertions in aged flies as well as decreases (Fig. 2C). This was reflected at the total TL level with modest new TE insertions in 30-day aged ISO1 flies versus 5-day young flies, with also perplexing total decreases in OreR and w1118 flies (Fig. 2A). Although the vast majority of the TE insertions were commonly detected by TIDAL in both 5-day and 30-day w1118 and OreR flies (Fig. 2D,E), there were more TE insertions only detected in these 5-day young fly genomes than of 30-day aged flies. Only a few hobo insertions were also only seen in 5-day young ISO1 flies and were no longer detected in 30-day aged flies (Fig. 2). This observation can be explained by this analysis that only focuses on TE insertion counts as quantile samplings of reads discordant from the reference genome. Thus, a new somatic transposition event in a small subset of cells could be overshadowed by a background of unmodified reference sequences and could explain a TE with a low CR score that is sampled in 5-day fly gDNA sample but then missed in the 30-day sample. This is a known limitation of the WGS approach and sacrificing sensitivity to improve specificity in the original TIDAL program [65].

Therefore, we updated TIDAL to map to Drosophila TE families consensus sequence coverage, and added arbitrarily-selected protein coding genes, analogous to the modification to TEMP to track protein-coding genes as Immobile gene elements (IGEs) [60]. We gauged a relatively low average rate (<4%, Table S1) of false positive split reads called by TIDAL in hitting IGEs, whereas these protein coding genes sequencing coverage generally also remained stable between 5-day young and 30-day
aged flies. (Figure S3A). Tracking TE consensus sequence coverage has the advantage of accounting for all accumulating TE sequences in both the mappable and unassembled and ambiguous-mapping regions of the genome. With this analysis approach, we could detect a clearer increase of total TE sequence coverage in *OreR* and *w1118* 30-day aged flies versus 5-day young flies (Fig. S3A). This overall aging-associated increase in TE consensus sequence coverage was represented by many TE families and is controlled against stable protein-coding gene coverage (Fig. S3B). However, both this coverage analysis and the quantile insertions analysis cannot discriminate between a full-length or truncated TE sequence, which we have noted in *P-elements* can have critically variable transposition activities [73].

**Resolving and validating our approaches measuring TE landscapes with RNAi mutants.**

This unresolved genomics challenge of using short read WGS data for analyzing TE sequences coverage also extends to some limitations in using droplet digital PCR (ddPCR) to precisely quantify genomic TE copies for only the isoforms covered by the short ddPCR amplicons [45]. Although the ddPCR quantifications of specific TE copies (Fig. S3C) followed the similar proportional trends of TE families called by TIDAL (Fig. 2B), the aging-associated increases in TE copies measured by ddPCR in the WT fly strains was also not detected. In questioning the accuracy of this ddPCR assay in absolute quantification of TE copies, we compared ddPCR results on *P-elements* and *I-elements* versus WGS and TIDAL determinations in two other directly matched gDNA samples (Figure S4A,B). The ddPCR copy number measurements were very similar to the WGS and TIDAL determinations, indicating both methodologies are consistent with each other in the quantifications. Furthermore, we replicated a previously reported
genetic cross [74] that in one format triggers a large burst of \textit{l-element} transposition in the embryos but in a second format maintains \textit{l-element} silencing (Fig. S4C). We reanalyzed the WGS datasets from [74] with TIDAL reporting 505 new \textit{l-element} copies versus the 3732 insertions called by TEMP in that study, with our ddPCR results leaning closer to the TIDAL count (1590 copies, Fig. S4D,E). These data reaffirm the findings from [74] that the oocyte is the critical battleground between the host and the selfish genetic element.

To explain why aging-associated TL changes seemed muted or were challenging to detect in WT fly strains, we considered two competing hypotheses: (1) non-penetrant TE insertions are masked by multiple unmodified genomic loci within the pools of sequences imposing limitations in WGS and TIDAL analysis versus (2) WT flies retain RNAi defenses like TE-targeting siRNAs [21, 22, 75-77] and piRNAs [13, 78-80] to prevent increasing TE RNAs from completing genomic transposition events. To test these hypotheses, we collected the same 5-day and 30-day aging cohorts from three sets of different mutants in the two main arms of the RNAi pathway in \textit{Drosophila} (Figure 3). We analyzed two independent mutants each in the \textit{piwi}, \textit{aubergine}, and \textit{AGO2} genes and conducted the same whole flies WGS and TIDAL analysis as the WT strains. In each of these six mutants, TLs showed dramatic increases in new TE insertions during fly aging (Fig. 3A,B,C). There was still significant variability again in the TLs between each mutant background, with no particular sets of TEs consistently exhibiting increased transposition (Fig. 3D,E,F).

The ddPCR results above affirm that genomic approaches are capable of detecting TL changes and WGS analysis of RNAi mutants demonstrate that the TL
changes can be detected during a single generation of aging flies. Therefore, we conclude that although fly aging may allow TE RNAs to become upregulated [10, 13], the RNAi pathways are still functioning in aging WT flies to mitigate TE RNAs from transposing in genomes. In essence, the RNAi mutants raise the frequency of successful TE mobilization events earlier in development so that these new insertions become highly penetrant in the pooled population of the WGS libraries. This would also suggest that successful TE insertions in WT flies are usually infrequent as to explain the modest changes in WT TLs when sequencing genomes from whole flies.

Detectable TL changes in fly brains during aging.

Perhaps new TE insertions may be better detected in specific tissues where cells that are more permanent and not turned over as frequently, such as the brain. For example, in mammalian neurons, the most active TE LINE-L1 has been implicated in transposing relatively frequently during development to give rise to genomic mosaicism in the brain [45-53]. Given the caveats of having to do prior total DNA amplification from limited gDNA from fly neurons [60], we undertook WGS from at least 50 dissected female brains to provide sufficient nucleic acid for RT-PCR confirmation of neuronal gene expression and WGS of brain DNA (Figure 4).

We successfully generated libraries directly from brains of WT fly strains and piwi and AGO2 mutants without any prior total DNA amplification, and now we could detect increases in TLs from OreR and w1118 strains (Fig. 4B). Although there may be piRNA-like small RNAs and piwi expression in fly heads [13, 21, 22], we detected increases in TLs in piwi mutants’ brains that were similar in magnitude to the WT OreR
and w1118 strains (Fig. 4C). Greater and more variable increases in TLs were apparent in the AGO2 mutants’ brains (Fig. 4D). Only the brains from the ISO1 strain were recalcitrant from showing much TE insertion increases except for a >2-fold increase in the Stalker TE (Fig. 4B). Except for ISO1, the other fly strains appear to enable increasing TL changes in the brain during fly aging.

Extra-chromosomal circular DNAs (eccDNAs) as an additional genomic cache of increasing TE sequences.

In normal and diseased animal cells, there is a cache of eccDNAs that has recently been explored by deep sequencing of DNA that is resistant to extensive exonuclease digestion [81-84]. In certain tumor samples, eccDNAs are implicated in rapid copy-number expansion of oncogenes [85], while ectopic accumulation of DNA in the cytoplasm of senescing cells might trigger aging-associated inflammation responses [86]. Several earlier studies had also found evidence of eccDNAs in Drosophila, with the copia TE as a prominent example accumulating in certain strains [87-91]. Lastly, eccDNA enriched in TE sequences and other repeats were detected in normal plants and gDNA of human tissues [81, 92], which in both of these studies required total DNA amplification prior to library construction to enrich the surviving eccDNAs after exonuclease digestion.

We investigated eccDNAs in Drosophila by optimizing our own method to purify enough eccDNAs to directly generate libraries for deep sequencing without requiring prior total DNA amplification (Figure 5A). Furthermore, we used spike-ins of cloning-vector plasmid DNAs into gDNA preparations and magnetic beads for improved recovery and quantitation of eccDNAs for comparing between different samples. To
confirm that eccDNA was recovered after two rounds of Exo5 and Exo8 exonuclease
digestion steps which only degrade linear but not circular DNA, we conducted PCR with
standard primers amplifying linear genes and TEs (F1-R1 primer pairs, Table S2), and
outward-facing primers that either generate an amplicon from a TE eccDNA or tandem
genomic copies of the same TE (P10-P11 primer pairs) (Fig. 5B). Linear gene
amplicons were significantly depleted after exonuclease digestions, while the amplicon
for the spike-in plasmid was enriched. Linear TE amplicons were also reduced while
eccDNA-targeted amplicons for the copia TE was resilient against the exonuclease
treatment. Some other TE amplicons with outward-facing primers that were reduced
after exonuclease treatment may reflect more tandem copies of these TEs.

Since the regular PCR amplicons for the copia eccDNA were readily apparent in
WT strains (Fig. 5B), we used qPCR to quantify the changes and show that copia
eccDNA copies were increased >~2-fold in 30-day aged flies compared to 5-day young
flies (Fig. 5C). This result motivated us to deeply sequence short read libraries
generated directly from those eccDNA-enriched samples which did not undergo any
total DNA amplification (Table S3). We first adapted the TIDAL scripts of mapping reads
to the TE families consensus sequences to measure sequencing coverage as well as
circular junction spanning reads against copia and observed an aging-associated
increase in copia eccDNA that was consistent with our qPCR results (Fig. 5D). We also
applied this custom eccDNA quantitation pipeline to all the other Drosophila TEs as well
as adapting the CIRCLE-Map pipeline previously used to measure mammalian
eccDNAs [81] to the Drosophila TEs. We then normalized the ratios of the eccDNA-TE
counts between 30-day aged and 5-day young flies (Fig. 5E). Although the CIRCLE-
Map pipeline was more sophisticated at providing a significance "circle score" that we
set the cutoff to be >50, our custom eccDNA quantitation pipeline’s results were notably consistent in showing overall that most eccDNAs as TEs were increasing in the libraries of 30-day aged flies (Fig. 5E, F). However, the additional normalization to the plasmid spike-ins were more informative in moderating eccDNA levels in w1118 while reaffirming the TE eccDNA increases in OreR and ISO1 (Fig. 5E, F). Thus, while ISO1 TLs did not change much at the chromosomal level during aging, ISO1 TE copy numbers may instead increase through eccDNA accumulation.

Genetically enhancing RNAi counteracts TE expression during Drosophila aging

Although TE expression still increased in WT aging flies, we hypothesized whether endogenous RNAi pathways that still limit genomic TL increases could also be genetically enhanced to mitigate the aging-associated rise of TE RNAs. To test this hypothesis, we first used a ubiquitous Tubulin-GAL4 driver to overexpress AGO2 in adults, and as expected, multiple TE RNAs had lowered expression relative to the negative control (Figure 6A). We then used the same driver to overexpress piwi, and although there was likely a silencing limit to prevalent piwi expression in the ovary, the enhancement of piwi expression and TE silencing was much more apparent in the female carcass (Fig. 6B).

These data provided a proof of principal that augmenting these RNAi pathways in adults results in improvements in TE silencing. However, inhibiting a factor that normally limits RNAi activity would be preferable from a therapeutic standpoint. Examples of endogenous negative regulation of RNAi activity include proteasome-mediated turnover of AGO2 [93], ENRI factors that negatively regulate nuclear RNAi in nematodes [94], and the RNA exosome and PAF1’s transcription elongation role
modulating RNAi silencing activity on TEs conserved in both fission yeast and flies [95-97]. Even though we were able to use siRNA knockdown of PAF1 in *Drosophila* OSS cells to demonstrate enhanced TE silencing, we recognized that genetic knockdowns of this essential modulator of RNAi would have detrimental effects on development like its requirement in ovarian development [95].

So, to circumvent developmental impacts of PAF1 knockdown in flies, we further combined the temperature-sensitive inhibitor of GAL4 expressed from a second transgene of *Tubulin-Gal80* with the *Tubulin-Gal4* driver [98]. This double-transgenic fly could then be crossed to the same UAS-PAF1-RNAi line so that flies can develop fully at the permissive temperature of 18 °C, and after eclosion be raised at 29 °C to trigger the RNAi knockdown of PAF1 (Fig. 6C). Because elevated temperature itself can affect TE silencing activity in flies [99-102], we used an *mCherry*-shRNA strain as a negative control that was also raised at 29 °C at the same time as the PAF1 knockdowns. There was appreciable enhancement of TE silencing in the whole female flies at both 5-day young and 30-day aged flies (Fig. 6C) with similar levels of TE silencing enhancement between the ovaries and the soma (Fig. 6D). We attribute the increased TE silencing during PAF1 knockdown to the reduced elongation rate of TE transcripts so that RNAi factors can better engage [95] and not from a global transcription reduction because steady state levels of control gene, TFIIIs, AGO2 and piwi were not reduced by PAF1 knockdown (Fig. 6C,E).

Since we had observed TE landscape activity in the adult fly brain (Fig. 4), we also tested a brain-specific driver, *elav-GAL4*, that was effective at triggering PAF1 knockdown and enhancing TE silencing in the 30-day aged fly brains (Fig. 6E). However, this *elav-GAL4* driver that was likely reducing PAF1 levels in all neurons
during embryonic and adult development [103] also presented some problems like reduced lifespan relative to the control, and we have not yet been able to recombine Tubulin-Gal80ts with the elav-GAL4 needed for the post-eclosion knockdown experiment. Thus, we report the measured lifespans from the PAF1 knockdown versus the mCherry-shRNA negative control with the Tubulin-Gal80ts and Tubulin-Gal4 driver cross at 29 °C (Fig. 6F). After an initial dip at 2 weeks, the PAF1 RNAi knockdown flies ended up living longer than the control and suggested that future pharmacological inhibition of PAF1 activity in maturing adult animals may be a relevant avenue of intervening with the aging-associated increase in TE expression.

DISCUSSION

In this study we conducted an analysis of WGS approaches towards assessing changing TLs during Drosophila aging, and we found that TL increases are readily detectable in the genomes of aging RNAi mutants such as piwi, aubergine and AGO2. These mutants are viable although others have shown that they have reduced longevity compared to control strains [10, 13, 16], and our data now confirms that unchecked elevation of TE transcripts can result in quantifiable genomic alterations in a single lifetime of flies. However, it was more difficult to detect new TE insertions amongst the gDNA of WT fly strains: we had to focus the TIDAL analyses on specific TE families mobilizing into uniquely-mapping sequences and also count the coverage on TE family consensus sequences (Fig. S3). After showing that an orthogonal quantitation method like ddPCR is consistent with TIDAL’s quantitation of TE copy numbers from WGS of P-elements and I-elements (Fig. S4), our parsimonious conclusion is that despite aging-
associated increases in TE expression during fly aging, the RNAi pathway still protects
the fly genomes from massively accumulating new TE insertions.

Despite the compactness and completeness of the *D. melanogaster* genome
sequence, technical challenges still remain in fully optimizing WGS approaches to
quantify TLs. For example, all current metazoan genome assemblies still suffer from
large sequencing gaps in telomeric, centromeric and other repetitive regions that remain
unanalyzable. Meanwhile, long-read sequencing like Nanopore and PacBio that could
close these gaps are still less economical and not as accurate as the Illumina
sequencing platform [104], yet library construction methods for the Illumina platform
require sufficient input material for reproducible generation of sequencing libraries.

Single-cell WGS is not yet robust enough nor has total DNA amplification approaches
been demonstrated to be unhampered by molecule bias, so our study required pools of
genomes and non-amplified input DNA samples to reduce the prior concerns. Our study
also adds a second dimension to WGS of TLs by incorporating eccDNA as an *in vivo*
cache of accumulating TE DNA sequences (Fig. 5). Intriguingly, the ISO1 strain showed
the least chromosomal TL changes yet exhibited the greatest increase in TE-eccDNAs
in the whole flies, while the OreR and w1118 strains also showed evidence of TE-
eccDNAs accumulating in the brain (*Figure S5A*).

In addition to variations in TLs between WT strains, we also observed differences
in TLs between other RNAi mutants that we cannot fully explain. For example, we
examined aging-associated TLs from two EMS-induced point mutants of *Dcr-2*
(*L811fsx*) and *Dcr-2 (R416X)* from [105]), the nuclease acting upstream of *AGO2* to
generate the siRNAs from TE dsRNAs. However, there was inconsistent and contrary
TL differences between young and aged *Drosophila* in these *Dcr-2* mutants whole flies
and brains (Fig. S5B, C) as well as in AGO3 mutants (Fig. S5D, [106]). Perhaps these sets of mutants are not as penetrant in the loss of RNAi activity as the piwi, aubergine and AGO2 mutants. Furthermore, the analysis of a partially rescuing AGO2 transgene in the AGO2 (2-5-14) null mutant did lower the initial levels of TE insertion differences noted by TIDAL, but the partial rescue still did not fully prevent aging-associated TE increases (Fig. S5E), suggesting only wild type strength RNAi can buffer aging genomes from accumulating new TE insertions.

Therefore, we propose that RNAi activity must be sustained during aging to mitigate negative effects of increased TE expression in aged flies, a phenotype that has also been frequently observed in mammals [34, 55, 107, 108]. To combat TEs’ impact on aging, some therapeutic approaches have used reverse transcriptase inhibitors and drugs that inhibit LINE-L1 activity [33], while other studies showed that dietary restriction and prolonged exercise in animals can reduce aging-associated increases in TE expression [14, 54, 109]. Our study proposes an additional therapeutic target of augmenting the RNAi pathway’s response to TEs by inhibiting PAF1, which has a conserved impact on limiting RNAi from silencing TE transcripts [95, 96]. Perhaps therapeutic siRNAs against PAF1 transcripts can be hypothesized as a feed-forwarding therapeutic agent to augment RNAi activity in aging animal cells.

A final question to resolve in the future is what cascade of epigenetic and chromatin landscape changes during animal aging consistently leads to increases in TE expression? Given the pleiotropic nature of the animal aging process, we anticipate that there will also be multiple genomic mechanisms that will vary in impact between different genetic backgrounds. For example, we describe variation amongst three WT *Drosophila* strains in the level of accumulating eccDNAs containing TE sequences (Fig.
5), while others have shown increased in polyploidy in adult *Drosophila* brains [110] as well as somatic genome instability in regions of the *Drosophila* genome [111] that might contribute to changes at the level of TE consensus sequence coverages (Fig. S3B).

Lastly, during fly aging there are also gross-level changes in histone marks typically associated with chromatin silencing [12, 14], which may precede the increase TE expression, so the future extension of this work will be to add epigenetic and chromatin accessibility landscapes to TLs during *Drosophila* aging.
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MATERIALS AND METHODS

Drosophila strains, genetic crosses and aging curves

All flies were raised at 25 °C on standard cornmeal food. For fly aging analyses, newly eclosed female flies were harvested from bottles and mated with males for two days. These females were then divided into ~20 individuals per vial and flipped to new vials every 2-3 days to mitigate crowding stress according to this protocol [112]. Surviving flies were counted at each flip, and the percentage of cumulative survival rate at each time point was plotted against its corresponding age (date of counting subtracted by date of eclosion).

The isogenized ISO1 fly strain for the Dm6 reference genome sequence was obtained from Susan Celniker [4]; the w1118 is an isogenized strain and was a gift from R. Scott Hawley [68]; and the OreR from the ModEncode project was a gift from Terry Orr-Weaver [69]. The RNAi pathway null mutant strains piwi-(g1), aubergine-(g1), aubergine-(g2), AGO3-(g1) and AGO3-(g2) were a gift from Julius Brennecke [106]. An additional mutant strain of Piwi-[HDR-4xP3-mCherry] [113] was a gift from Eric Lai. The null AGO2 mutants deletion strains of AGO2-[2-5-14] and AGO2-[2-16-4] and Ago2-WT-rescue stocks were generated by CRISPR Cas9 approaches as described in [114, 115]. The strains with active and inactive I-elements and spermless males were a gift from Zhao Zhang [74]. The UAS-Ago2-HA strain was a gift from Arno Muller lab [116] and the UASp-3xHA-Piwi was a gift from the Ruth Lehman lab [117]. The driver strains of Tubulin-Gal4 and elav-Gal4 were a gift from Leslie Griffith [118]. In addition to a Tubulin-Gal80gs strain we received from the Griffith lab, we also obtained a second Tubulin-Gal80gs stock from the Bloomington Drosophila Stock Center (BDSC#7018) and combined with Tubulin-Gal4 for further experiments. The PAF1 knockdown RNAi line was obtained from the Vienna Drosophila Resource Center (VDRC#108826) and an mCherry shRNA control line was obtained from the Harvard TRiP resource (BDSC#35785).

To quantify I-element copies by ddPCR, fly cross schemes from [74] were replicated (Fig. S4C). Parental crosses between the w1118 strain with active I-elements and wk strain with inactive I-elements were performed reciprocally to generate many virgin F1 females where one strain enables I-element transposition (“invaded” from wk as the maternal parent) versus a control that maintains I-element silencing (w1118 as the maternal parent). These F1 females were then crossed to sperm-less males that were obtained as F1 male progenies from the parental cross of w1118 virgin females with XY attached male. F2 oocytes were collected overnight and DNA was extracted for ddPCR against the I-element and Rp49.

Fly brain isolation, genomic DNA extraction, WGS library construction and deep sequencing.

Fly brains were dissected from at least 50 females per age group, following a procedure laid out in [119]. Eye disks and other tissues were removed from heads with forceps, and brain lobes were dissected into tubes with ice-cold PBS before freezing once at -20 °C. Whole female flies and fly brains were homogenized in a standard DNA digestion buffer (1% SDS, 50 mM Tris-HCl pH 8.0, 100 mM EDTA, 100 mM NaCl, 0.5mg/ml Proteinase K) overnight at 50 °C, and then extracted using standard phenol chloroform extraction, ethanol precipitation, and resuspending gDNA pellets in pure water.
WGS of whole flies began with the circa 2014 Nextera Tn5 tagmentation kit (Illumina) using an input of 50 ng gDNA and outputs were purified with AMPure XP beads (Beckman Coulter). WGS libraries were quality controlled with the high-sensitivity DNA kit on the Bioanalyzer (Agilent), selecting for size distributions of 300bp to 1kb and concentrations over 1 nM. Multiplexed libraries were sequenced on Illumina Nextseq500 high-output flow cells using 75 bp paired-end and single end kits. All WGS libraries were sequenced to a minimum depth of 35 million reads (Table S1, S2). After determining that some whole fly libraries made using NEBNext Ultra-II DNA library prep kit for Illumina (NEB) were as complete and had better yields than the then discontinued Nextera kit, we completed the fly brain gDNA libraries with the NEBNext kit and sequenced them to similar depths as above.

RNA extraction, quantitative RT-PCR, digital droplet PCR (dd-PCR) and TE copy number estimation

Total RNA was extracted from 5-10 female flies harvested at corresponding age with TRI-reagent (MRC, Inc.). Reverse transcription (RT) was performed using random primers, ProtoScript II (NEB), and 1 μg input of total RNA. Quantitative PCR (qPCR) with the Luna Sybr-Green mastermix (NEB) used primer sequences in Table S3 and 2 μL of a 1:10 dilution of the cDNA. Relative changes in gene expression were calculated using the 2^ΔΔCt method with Rp49 as a housekeeping gene for normalization.

Droplet digital PCR (ddPCR) was conducted on a QX200 instrument with the Evagreen assay reaction (Biorad). Copy number measurements from specific TE primers (Table S3) were normalized to Rp49 as a diploid gene, starting first at 2 ng of gDNA as input per 20 μL ddPCR for droplet generation for most TEs. For TEs with very high copy numbers that saturate the droplets, input gDNA was diluted further to 2 ng into the ddPCR mix prior to droplet generation. At least 10,000 droplets were required to achieve good statistical estimation of the concentration calculated by Poisson distribution using Quantasoft Analysis Pro (Biorad). TE copy numbers per genome was determined by dividing against half of the measured Rp49 copies.

Extracellular circular DNA isolation and sequencing

To quantify eccDNAs during fly aging, 30 female flies were harvested from 5-days and 30-days post eclosion, and a fixed amount off pre-extraction plasmids was added prior to cell lysis: ~80 pg of ~7kb-pGL3-DmPiwipro1 and ~50 pg of ~11kb-pCas9 prior to cell lysis. About 30 ug of total gDNA was recovered from using MasterPure™ Complete DNA and RNA Purification kit (Lucigen), and 0.5ug-1ug gDNA was checked on a 1% agarose gel for integrity and quality. Good gDNA primarily migrated at >10kb and to 20 μg gDNA we added 40ul of a second plasmid cocktail: (1ng/ul of the 2.7kb pUC19, 0.1ng/ul of the 3.5kb pMaxGFP, 0.01ng/ul of the 5.2kb pGSH0 and 0.001ng/ul of the 6.3kb pCENPm3) and split equally to two reactions: Exo5/8 non-treated control versus Exo5/8 treated samples. We conducted a first round of Exo5/Exo8 (NEB) treatment at 37 °C overnight, then an additional 2-hour treatment with freshly replenished buffer, ATP and enzymes. The reaction was stopped and purified using AMPure XP beads (Beckman Coulter) and eluted in 50 ul of water.

To check the efficiency of Exo5/8 treatment, 10 ul of the eluate from untreated versus treated samples were loaded on 1% agarose gel to visualize complete digestion of gDNA. We quality controlled Exo5/8 treatments by performing qPCR against rp49, ND5 and various
plasmid primers including pUC19, pGL3piwipro and pCas9 and Ct values were compared between untreated versus treated samples. Mitochondria was not a reliable circular molecule because of the high variability of ND5 Ct values across multiple sample preps. Comparing between treated and untreated sample, the plasmids Ct values were generally stable (<2 Ct difference), and much higher for rp49 (>5 Ct difference) indicating the Exo5/8 treatments were effective at removing linear chromosomal DNA and not affecting the circular plasmids. Half of the Exo5/8 treated sample (25 ul out of 50 ul purified elute) was used as template for library construction using NEBNext UltraII library prep kit as stated above. Libraries were single end (75bp) or pair-end sequenced at 36 bp by 36 bp on a Nextseq550 flow cell (Illumina).

For eccDNA sequencing from brains, 200 female brains were dissected and added with half the volume of pre-extraction plasmids as whole flies, and gDNA concentration was measured by the Qubit 4 Fluorometer (Thermofisher). To 100 ng of brain gDNA, we mixed 20 ul of the plasmid spike-in cocktail and a tenth of the Exo5/8 enzyme as whole flies gDNA. At least 10 million eccDNA reads were required for analysis.

**TIDAL updates with total TE consensus and gene mapping strategies**

TE insertion analysis was carried out with an updated version of our previously developed TIDAL program (original code available on the Github repository at: [https://github.com/laulabbrandeis/TIDAL](https://github.com/laulabbrandeis/TIDAL) [65]. In this study, the updated version of TIDALv1.2 is also posted to Github at [https://github.com/laulabbumc/TIDAL1.2](https://github.com/laulabbumc/TIDAL1.2). These scripts carry out the analysis run the same way as the original TIDAL, but we incorporated two additional features. First, for the euchromatic TE insertions we selected 22 arbitrarily selected protein coding gene (Immobile gene elements IGEs) that are computed along with consensus TE sequence to benchmark noise in detection of genetic elements. The algorithm used to identify transposon insertion sites based on consensus transposon sequence is then applied on these 100 IGE sequence to determine their insertion sites. Second, for the total reads mapped to consensus TE sequences, here we added 100 IGEs are computed by mapping reads with bowtie2 using parameters "--sensitive --end-to-end" and custom shell, Perl, C-code, and R-code scripts all accessible from [https://github.com/laulabbumc/TIDAL1.2](https://github.com/laulabbumc/TIDAL1.2).

**TEMP v1.05 code was acquired from the GitHub repository at:** [https://github.com/JialiUMassWengLab/TEMP](https://github.com/JialiUMassWengLab/TEMP), and was run with default parameters except "-x 30, -m 3 -f 500". These parameters were chosen to ensure that TEMP results are consistent with analysis shown in [60, 62].

**Bioinformatics counting of eccDNA from TEs and spike-in plasmids using a custom pipeline and CIRCLE-Map program.**

In our first look at the eccDNA reads, we inputted them into an existing bioinformatics pipeline already developed for mapping *Drosophila* small RNA counts to TEs [120]. Reads were first checked by the Cutadapt program to see if adaptor sequences at the 3' end needed to be removed, and then we indexed the reads to the *Drosophila* genome assembly file by running BWA version 1 [121] and formatdb from NCBI. Using Bowtie1 with 2 mismatches [122], reads were mapped to genome to get the genic and intergenic counts using the genome GTF file. The total number of reads mapped to the *Drosophila* genome was derived by subtracting the total
number of reads not mapped to the *Drosophila* genome from the total number of reads. The total number of mapped reads was used as the basis for normalization of TE counts and spike-in plasmid counts.

Plasmid sequences were treated as linear entries in the FASTA file database similar to the TE family consensus sequences. The raw read counts from TE mapping were further normalized by the total number of reads mapping to the *Drosophila* Dm6 genome assembly. For spike-in plasmid counting, because several plasmids share the same backbone with different inserts, read frequencies were normalized by the total plasmid mapping sites as well as by the total number of *Drosophila* genome-mapping reads.

To execute the CIRCLE-Map program for repeats [81], we indexed the *Drosophila* genome FASTA file by BWA. We then used the MEM algorithm under BWA to align reads against the *Drosophila* genome FASTA file. Next, we sorted the reads by alignment position within the resulting BAM file and indexed the resulting BAM file. Finally, we detected the circles by calling CIRCLE-Map program. The CIRCLE-Map program for repeats yields an output for reads with two high scoring alignments as these ones are indicative of circles formed from regions with homology.
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FIGURE LEGENDS

Figure 1. Overview of study to examine whether TE-DNA copy numbers change during fly aging.
(A) Survival curves of the three wild-type fly strains carried out in this study, indicating the selection of 30-day adults as a representative timepoint of aging onset. (B) Validation of TE transcript expression increases during fly aging through qRT-PCR of TE RNAs normalized to rp49 transcripts. Error bars are propagated standard deviations of delta-CT values from three replicates. (C) Overview of TE detection strategy from Whole Genome Sequencing (WGS) data using updated TIDAL-Fly and extra-chromosomal circular DNA (eccDNAs) detection scripts. (D) Study designed for comparing TE load between 5-day young and 30-day aged flies within each wild-type and mutant strain.

Figure 2. WGS analysis of TE insertion numbers between 5-day young versus 30-day aged wild-type fly strains.
(A) Quantification of new TE insertions as compared to the reference genome using the TIDAL-fly program. Categories of total TE insertions broken by the Coverage Ratios (CR) of CR>2 and CR<=2. (B) Within each strain, TE families’ percentages are ordered by the color legend. (C) Ratios of the 30-day versus 5-day of normalized TE insertions from panels D-F. Note the w1118 strain has the greatest number of distinct TE insertions detected by TIDAL. (D-F) Number of unique TE insertions (filled bars) present in 5-day and 30-day relative to common insertions present in both samples (open bar) of w1118, OreR and ISO1 fly strains. These panels display only the TE families that
were detected by TIDAL be at least 1% of total number of TE families (i.e. all the TEs not lumped into the “Others” category of Fig. 2B).

**Figure 3. WGS analysis of TE insertion numbers between Young (5-day) versus Aged (30-day) RNAi mutants fly strains.**

(A-C) Quantification of new TE insertions as compared to the reference genome using the TIDAL-fly program. Categories of total TE insertions broken by the coverage ratios (CR) of CR>2 and CR<=2. Dashed arrows highlight the accumulation of TE insertions in a single generation of aging in two distinct strains of each RNAi null mutants in piwi-/-, aub-/-, and AGO2-/- genes. (D-F) Ratios of the 30-day versus 5-day of normalized TE insertions from panels A-C. These panels display only the TE families that were detected by TIDAL be at least 1% of total number of TE families (i.e. all the TEs not lumped into the “Others” category of Fig. 2B).

**Figure 4. Aging-associated TE landscape changes in fly brains of WT and RNAi mutant strains.**

(A) Validation of fly brain dissections by microscopy and RT-PCR of brain-specific gene expression. TIDAL analysis of WGS for new TE insertions in the brains of (B) Wild-type (WT) strains, (C) piwi mutants, and (D) Ago2 mutants. The bar graphs on the left represent categories of total TE insertions broken by the Coverage Ratios (CR) of CR>2 and CR<=2. Dashed arrows highlight the accumulation of TE insertions in a single generation of aging flies. The dot graphs to the right show the ratios of the 30-day versus 5-day of normalized TE insertions from left panels B-D. These panels display
only the TE families that were detected by TIDAL be at least 1% of total number of TE families, hence only three blue dots for ISO1 are visible in (B).

Figure 5. Aging Drosophila display increases in TEs existing as extrachromosomal circular DNA (eccDNA).

(A) Diagram of methodology to enrich and purify eccDNAs for direct library construction and sequencing without requiring prior amplification. (B) Genomic PCR from WT flies demonstrating the depletion of linear gDNA and enrichment of eccDNA with TE sequences during exonucleases treatments. Inset diagram explains configuration of PCR primers. Left diagram explains configuration of PCR primers, L=DNA ladder. (C) qPCR validation of spike-in plasmids and copia eccDNA after exonucleases treatments of ISO1 gDNA from young versus aged adult flies. (D) Ratio of the read coverage just across the copia consensus sequence comparing young versus aged flies. (E) Box plots of 30-day/5-day ratios of read coverage for eccDNA TE sequences rated by the CIRCLE-Map pipeline with significant “circle score” >50 (Moller et al, 2018); and for our own custom quantitation pipeline that uses a TE-mapping scripts previously used for small RNA analysis. (F) Dot graphs highlighting specific TE eccDNAs whose 30-day/5-day sequencing ratios are normalized to the RPM library size or further normalized to the plasmid spike-ins from (E). These panels display only the TE families that had “circle score” >50 (left) or displayed a measurable 30-day/5-day ratio from the custom analysis pipeline (right).

Figure 6. Genetic interventions of TE expression in adult Drosophila.
(A) Overexpressing AGO2 [Tub>Gal4;UAS-HA-AGO2]/[Tub>Gal4] and (B) overexpressing PIWI [Tub>Gal4;UAS-3X-HA piwi]/[Tub>Gal4] results in a reduction of TE RNA expression in 5-day young adult Drosophila. Left graphs confirm gene overexpression and right graphs detail TE RNA expression measured by RT-qPCR of the target gene compared to the rp49 housekeeping gene and with error bars representing propagated standard error of triplicate measurements. (C) Adult-specific knockdown of PAF1 in 5-day young females qualitatively assessed in the gel (left) and RT-qPCR (middle), which reduces TE RNA expression (right). The genes TFIIs and piwi are controls suggesting that TE RNA reduction is distinct from a concern that PAF1 RNAi would simply be causing global reduction in transcription. Examining the effect of TE RNA reduction in the PAF1 knockdown in the ovary (D) and brain (E) of adult Drosophila with TEs and PAF1 in left graph and control genes in the right graph. (F) Life span comparison between control versus PAF1 RNAi knockdown of adult female flies upon raising them at 29 °C to release the GAL80\textsuperscript{ts} inhibitor to induce RNAi from the Tub>GAL4. PAF1 RNAi n=112, Control RNAi n=170.

SUPPORTING ONLINE MATERIALS LIST

Supplementary Text, Supplementary Figures and Tables legends.

Figures S1-S5.

Tables S1-S3.
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(A) Survival curves of the three wild-type (WT) fly strains carried out in this study, indicating the selection of 30-day adults as a representative timepoint of aging onset. (B) Validation of TE transcript expression increases during fly aging through qRT-PCR of TE RNAs normalized to rp49 transcripts. Error bars are propagated standard deviations of delta-CT values from three replicates. (C) Overview of TE detection strategy from Whole Genome Sequencing (WGS) data using updated TIDAL and extra-chromosomal circular DNA (eccDNAs) detection scripts. (D) Study designed for comparing TE load between 5-day young and 30-day aged flies within each WT and mutant strain.
Figure 2. WGS analysis of TE insertion numbers between 5-day young versus 30-day aged wild-type fly strains.

(A) Quantification of new TE insertions as compared to the reference genome using the TIDAL program. Categories of total TE insertions broken by the Coverage Ratios (CR) of CR>2 and CR<=2. (B) Within each strain, TE families' percentages are ordered by the color legend. (C) Ratios of the 30-day versus 5-day of normalized TE insertions from panels D-F. Note the w1118 strain has the greatest number of distinct TE insertions detected by TIDAL. (D-F) Number of unique TE insertions (filled bars) present in 5-day and 30-day relative to common insertions present in both samples (open bars) of w1118, OreR and ISO1 fly strains. These panels display only the TE families that were detected by TIDAL be at least 1% of total number of TE families (i.e. all the TEs not lumped into the “Others” category of Fig. 2B).

Figure 2
Figure 6. Genetic interventions of TE expression in adult Drosophila.
(A) Overexpressing AGO2 [Tub>Gal4, UAS-HA-AGO2] or [Tub>Gal4] and (B) overexpressing Piwi [Tub>Gal4, UAS-3X-HA-piwi] results in a reduction of TE RNA expression in 5-day young adult Drosophila. Left graphs confirm gene overexpression and right graphs detail TE RNA expression measured by RT-qPCR of the target gene compared to the rp49 housekeeping gene and with error bars representing propagated standard error of triplicate measurements. (C) Adult-specific knockdown of PAF1 in 5-day young females qualitatively assessed in the germ (left) and RT-qPCR (middle), which reduces TE RNA expression (right). The genes Tflits and pmwi are controls suggesting that TE RNA reduction is distinct from a concern that PAF1 RNAi would simply be causing global reduction in transcription. Examining the effect of TE RNA reduction in the PAF knockdown in the ovary (D) and brain (E) of adult Drosophila with TEs and PAF1 in left graph and control genes in the right graph. (F) Life span comparison between control versus PAF1 RNAi knockdown of adult female flies upon raising them at 20 °C to release the GAL80 inhibitor to induce RNAi from the Tub>Gal4. PAF1 RNAi n=112, Control RNAi n=170.
Figure 3. WGS analysis of TE insertion numbers between 5-day young versus 30-day aged RNAi mutant fly strains. (A-C) Quantification of new TE insertions as compared to the reference genome using the TIDAL program. Categories of total TE insertions broken by the coverage ratios (CR) of CR>2 and CR<2. Dashed arrows highlight the accumulation of TE insertions in a single generation of aging in two distinct strains of each RNAi null mutants in piwi-/-, aub-/-, and AGO2-/- genes. (D-F) Ratios of the 30-day versus 5-day of normalized TE insertions from panels A-C. These panels display only the TE families that were detected by TIDAL be at least 1% of total number of TE families (i.e. all the TE families not lumped into the “Others” category of Fig. 2E).
Figure 4. Aging-associated TE landscape changes in fly brains of WT and RNAi mutant strains.

(A) Validation of fly brain dissections by RT-PCR of brain-specific gene expression. TIDAL analysis of WGS for new TE insertions in the brains of (B) Wild-type (WT) strains, (C) piwi mutants, and (D) Ago2 mutants. The bar graphs on the left represent categories of total TE insertions broken by the coverage ratios (CR) of CR>2 and CR<2. Dashed arrows highlight the accumulation of TE insertions in a single generation of aging flies. The dot plots to the right show the ratios of the 30-day versus 5-day of normalized TE insertions from left panels B-D. These panels display only the TE families that were detected by TIDAL be at least 1% of total number of TE families, hence only three blue dots for ISO1 are visible in (B).

Figure 4
Figure 5. Aging *Drosophila* display increases in TEs existing as extra-chromosomal circular DNA (eccDNA).

(A) Diagram of methodology to enrich and purify eccDNAs for direct library construction and sequencing without requiring prior amplification. 

(B) Genomic PCR from WT flies demonstrating the depletion of linear gDNA and enrichment of eccDNA with TE sequences during exonuclease treatments. Left diagram explains configuration of PCR primers. L=DNA ladder (C) qPCR validation of spike-in plasmids and copia eccDNA after exonuclease treatments of *ISO1* gDNA from young versus aged adult flies. (D) Ratio of the read coverage just across the copia consensus sequence comparing young versus aged flies. (E) Box plots of 30-day/5-day ratios of read coverage for eccDNA TE sequences rated by the CIRCLE-Map pipeline with significant “circle score” >50 (Moller et al. 2018); and for our own custom quantitation pipeline that uses a TE-mapping scripts previously used for small RNA analysis. (F) Dot graphs highlighting specific TE eccDNAs whose 30-day/5-day sequencing ratios are normalized to the RPM library size or further normalized to the plasmid spike-ins from (E). These panels display only the TE families that had “circle score” >50 (left) or displayed a measurable 30-day/5-day ratio from the custom analysis pipeline (right).