A concerted increase in readthrough and intron retention drives transposon expression during aging and senescence

Authors: Kamil Pabis 123, Diogo Barardo 23, Kumar Selvarajoo 5678, Jan Gruber 14, Brian K. Kennedy 123

1 Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore
2 Healthy Longevity Translational Research Programme, Yong Loo Lin School of Medicine, National University of Singapore
3 Centre for Healthy Longevity, National University Health System
4 Science Divisions, Yale-NUS College, Singapore, 138527, Singapore
5 Bioinformatics Institute, Agency for Science, Technology and Research (A*STAR), Singapore, Singapore.
6 Singapore Institute of Food and Biotechnology Innovation, Agency for Science, Technology and Research (A*STAR), Singapore, Singapore.
7 NUS Synthetic Biology for Clinical and Technological Innovation (SynCTI), National University of Singapore, Singapore, Singapore.
8 School of Biological Sciences, Nanyang Technological University, Singapore, Singapore.

Aging and senescence are characterized by pervasive transcriptional dysfunction, including increased expression of transposons and introns. Our aim was to elucidate mechanisms behind this increased expression. Most transposons are found within genes and introns, with a large minority being close to genes. This raises the possibility that transcriptional readthrough and intron retention are responsible for age-related changes in transposon expression rather than expression of autonomous transposons.

To test this, we compiled public RNA-seq datasets from aged human fibroblasts, replicative and drug-induced senescence in human cells and RNA-seq from aging mice and senescent mouse cells.

Indeed, our reanalysis revealed a correlation between transposons expression, intron retention and transcriptional readthrough across samples and within samples. Both intron retention and readthrough increased with aging or cellular senescence and these transcriptional defects were more pronounced in human samples as compared to those of mice. In support of a causal connection between readthrough and transposon expression, analysis of models showing induced transcriptional readthrough confirmed that they also show elevated transposon expression.

Taken together, our data shows that elevated transposon reads during aging seen in various RNA-seq dataset are concomitant with multiple transcriptional defects. Intron retention and transcriptional readthrough are the most likely explanation for the expression of transposable elements that lack a functional promoter.

38 **INTRO**

39 Repetitive DNA makes up at least 50% of the human genome (**de Koning et al. 2011**)
40 and has been linked to genomic instability, cancer and – somewhat less consistently –
41 species longevity (**Pabis et al. 2021, Khristich and Mirkin 2020**). Transposons are one
42 particularly abundant class of repetitive sequences found in the nuclear genome of
43 eukaryotes and are strongly associated with cancer-causing structural variants like DNA
44 deletions, insertions or inversions (**Rodriguez-Martin et al. 2020**). Although transposons
45 are often considered parasitic, harmful or, at best, neutral recent evidence suggests
46 they may contribute to genomic diversification on evolutionary timescales and to the
47 cellular stress-response by providing transcription factor binding sites (**Villanueva-Cañas**
48 **et al. 2019**).

49 The major transposon families in the human genome are long interspersed nuclear
50 element (LINEs), short interspersed nuclear element (SINEs), long terminal repeat
51 transposons (LTRs) and DNA transposons comprising 21%, 11%, 8% and 3% of the
52 human genome, respectively (**Kazazian and Moran 2017**).

53 In recent years, transposon expression, particularly of elements belonging to the LINE-1
54 family, has been hypothesized to be a near universal marker of aging. Several lines of
55 evidence support a link between transposon reactivation and aging (**Gorbunova et al.**
56 **2021**).

57 Using RNA-seq, for example, to quantify reads mapping to transposable elements it was
58 shown that expression of these was higher in 10 vs 1 day-old nematodes (**LaRocca,**
59 **Cavalier, Wahl 2020**), 40 vs 10 day-old flies (**Wood et al. 2016**) and in muscle and liver
60 of aged mice (**De Cecco et al. 2013**). Transposon expression also increases during in vitro
61 senescence (**Colombo et al. 2018**) and in fibroblasts isolated from people between the
62 ages of 1 and 96 years (**LaRocca, Cavalier, Wahl 2020**), whereas lifespan extending
63 mutations and interventions in mice reduce expression of transposons (**Wahl et al. 2021**)

64 However, not all studies report such consistent age-related increases across all
65 transposon classes (**Ghanam et al. 2019**). Moreover, it would be a mistake to conflate
66 changes in RNA-seq-, RNA-, DNA- and protein-based measurements of transposon
67 expression as evidence for one and the same phenomenon.

68 Quantification of transposon expression using RNA-seq or PCR techniques remains
69 challenging given their repetitive nature and the fact that 98 to 99% of all transposons
70 are co-expressed with neighboring transcriptional units (**Deininger et al. 2017, Stow et**
71 **al. 2021**). In fact, only a small fraction of LINE-1 and SINE elements, the latter relying on
72 co-transposition by LINE elements, are expressed from a functional promoter. It is

believed that such “hot” LINE-1 loci drive most transposition events and genomic instability (**Deininger et al. 2017, Rodriguez-Martin et al. 2020**).

The large number of co-expressed transposons would likely mask any signal from autonomous elements in standard RNA-seq experiments. Therefore, alternative explanations for increased transposon expression in such datasets are needed and these could include age-related changes in transposon adjacent genes e.g. intron retention and transcriptional readthrough.

Most eukaryotic genes contain introns which need to be removed during a complex co-transcriptional process called splicing. Defects in splicing are the cause of many, often neurologic, hereditary conditions (**Scotti and Swanson 2016**). One such type of splicing defect is intron retention. Although basal levels of intron retention may be benign or even physiologic, high levels are considered harmful (**Zheng et al. 2020**). Aging is accompanied by many changes to splicing patterns (**Wang et al. 2018**), including intron retention, which is increased during cellular senescence (**Yao et al. 2020**) and in aging drosophila, mouse hippocampus and human prefrontal cortex. In addition, levels in human AD brain tissues are elevated even further than in the aged brain (**Adusumalli et al. 2019**).

Transcription is terminated by the cleavage and polyadenylation machinery when the polymerase transcribes through the polyadenylation signal leading to pre-mRNA cleavage, polymerase pausing, conformational change and eventually polymerase detachment (**Rosa-Mercado et al. 2021**). However, this termination may fail leading to so called readthrough transcription which increases during stress conditions (**Rosa-Mercado and Steitz 2022**) and during cellular senescence, at least for a subset of genes (**Muniz et al. 2017**).

Emerging evidence suggests that intron retention, readthrough and transposon expression are linked (**Rosa-Mercado et al. 2021, Hadar et al. 2022**), but this has not been studied in the context of aging. In this manuscript we show that intron retention and readthrough taken together can explain most of the apparent increase in transposon expression seen in RNA-seq datasets of aging.

RESULTS

1. Genomic location of transposons implicates them as a marker of multiple transcriptional defects

During aging and senescence expression of all four major transposon families (LTR, DNA, LINE, SINE) increases (**Table S1**). However, such a global increase in transposon expression cannot be explained by transposon biology alone since most transposons are ancient, and, having accumulated many mutations, lack a functional promoter (**Özgen et al. 2019).** Instead, we reasoned that transposon expression is associated with the expression of nearby genes.

Consistently, out of 4.5 million annotated transposons 60% were located within genes (most of them intronic), 10% within 5 kb of a gene and the remaining 30% were between genes, albeit usually still within 100 kb (**Table 1**). For transposons whose expression significantly changed with age or senescence, the number of intronic and downstream-of-gene transposons is even higher.

Based on these findings we studied intronic transposons as a potential marker for age-related intron retention and downstream-of-gene transposons as a marker of age-related readthrough transcription.

Dataset	Genic	Intronic	ds	us	ds	us	n
all transposons	0.604	0.493	0.045	0.049	0.187	0.174	4520928
Fleischer et al. (aging, significant)	0.857	0.564	0.082	0.025	0.105	0.037	7673
Colombo et al. (senescence, significant)	0.698	0.540	0.107	0.053	0.177	0.118	8730
	within genes		within 5kb		within 100kb		

Table 1.
Fraction of genic or intronic transposons and of transposons downstream (ds) or upstream (us) of genes in two different datasets.

2. Elevated transposon expression and intron retention with age

First, we confirmed that age-related transposon expression is indeed elevated as shown by **LaRocca et al. (2020).** This analysis is based on a dataset originally published by **Fleischer et al. (2018)**, who performed RNA-seq of 143 fibroblasts isolated from donors between the ages of 0 to 96. This dataset will be hereafter referred to as the “aging dataset”. We find that transposon expression increases slowly and non-linearly during aging, with a sudden and sharp increase around 80 years of age (**Fig. 1A, Fig. S1**). Next, we showed that intron retention is also elevated with aging as shown by **Yao et al. (2020).** We find that intron retention increases with age (**Fig. 1B**) and that samples with high transposon expression also showed elevated intron retention (**Fig. 1C**).

134 In our analysis we corrected for gene co-expression by normalizing the expression levels
135 of introns and transposons to the expression levels of the closest gene. However,
136 another complementary approach would be to plot and compare the raw, unadjusted
137 log-fold changes for genes, introns, transposons and other elements. In this analysis, we
138 found that, on average, age-related upregulation of transposons and introns is more
139 pronounced than upregulation of genes (**Fig. S2A**), consistent with **Fig. 1A** and **1B**.

140 Next, we re-analyzed a dataset originally published by **Colombo et al. (2018)** which will
141 be referred to as the "senescence dataset". Here, we tested whether MDAH041 cells
142 induced into senescence with H2O2, 5-azacytidine, adriamycin or serial passaging show
143 elevated transposon expression and intron retention. As expected, all these treatments
144 increased the expression of transposons (**Fig. 1D**, **Fig. S2B**), as documented before in
145 this dataset (**Colombo et al. 2018**). The retention of introns was also increased (**Fig. 1E**)
146 and expression levels of introns and transposons correlated with each other (**Fig. 1F**).

147 Having established that transposons reads and retained introns are elevated with aging
148 and senescence, we tested whether this is conserved between mouse and human.
149 Transposon expression ($p<0.0001$) was elevated, and intron retention showed a trend
150 towards elevation ($p=0.14$), in the liver of 26 month-old mice, suggesting these age-
151 related transcriptional defects might be conserved between species (**Fig. S3A, B**). In
152 contrast, we found that increased expression of transposons and intron retention in
153 models of cellular senescence is not conserved between species. To the contrary, early
154 passage mouse fibroblasts showed elevated transposon expression and intron retention
155 compared to senescent fibroblasts (**Fig. S3C, D**), which contrasts with the mouse liver
156 and human senescence data.

157

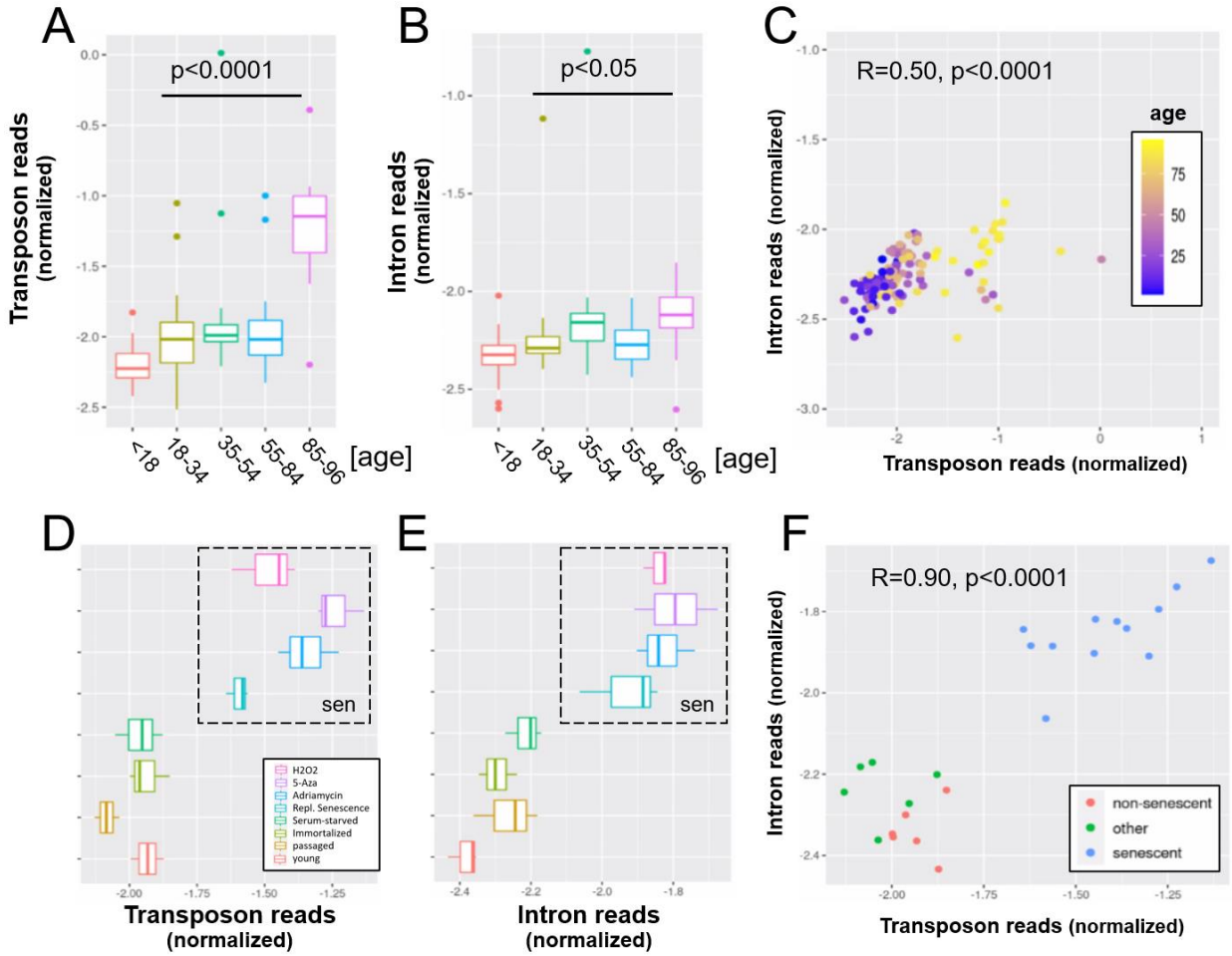


Fig. 1. A concerted increase in transposon and intron expression with aging and senescence.

Transposon (A) and intron (B) expression is increased in skin fibroblasts isolated from aged individuals. What is more, expression of both transposons and introns shows a significant correlation (C). Similarly, transposon (D) and intron (E) expression is increased in MDAH041 cells induced into senescence (sen) by treatment with drugs or via passaging. Normalized reads from the top 1000 differentially expressed genes, transposons and introns were used in this analysis.

A) Transposon reads normalized by the expression of adjacent genes plotted across five age groups (adolescent n=32, young n=31, middle-aged n=22, old n=37 and very old n=21).

B) Intron reads normalized by the expression of adjacent genes plotted across five age groups (adolescent n=32, young n=31, middle-aged n=22, old n=37 and very old n=21).

C) Scatterplot between transposon and intron expression (normalized as in A and B) for all 143 samples. Each sample is colored by age.

D) Transposon reads normalized by the expression of adjacent genes plotted across four

175 senescent conditions (H2O2, 5-azacytidine, adriamycin, replicative senescence) and four
176 other conditions (serum-starved, immortalized, intermediate passage and early passage).
177 N=3 per group.
178 E) Intron reads normalized by the expression of adjacent genes plotted across four
179 senescent conditions (H2O2, 5-azacytidine, adriamycin, replicative senescence) and four
180 other conditions (serum-starved, immortalized, intermediate passage and early passage).
181 N=3 per group.
182 F) Scatterplot between transposon and intron expression (normalized as in E and F) for
183 all 24 samples. Each sample is colored by senescence status.

184 **3. Transposon expression as a marker of intron retention**

185 So far these age-related events have been considered independently, but given the
186 localization of transposons being mostly intragenic we reasoned that intron retention
187 could bias the transposon findings. Indeed, we found that samples with high intron
188 retention also show high transposon expression. This is true both in the aging dataset
189 (**Fig. 1C**) and in the cellular senescence dataset (**Fig. 1F**), where intronic reads accounted
190 for most of the observed variability in transposon expression (or vice versa). Looking at
191 individual genomic loci, we also found a strong correlation between the log-fold change
192 of a transposon and the log-fold change of the intron it is located in (**Fig. S4**).

193 As a more direct test of this hypothesis, we determined the correlation between the
194 counts of retained introns and the counts of intronic transposons for each sample
195 individually. Non-intronic transposons and randomized transposons served as a negative
196 control. Consistent with the log-fold change data, we found a specific correlation
197 between intronic transposon and intron counts in both datasets, with stronger
198 correlations in the senescence dataset (**Fig. S5**).

199 Altogether this data suggests that intron retention contributes to the age-related
200 increase in the expression of transposons (**Fig. 2**).

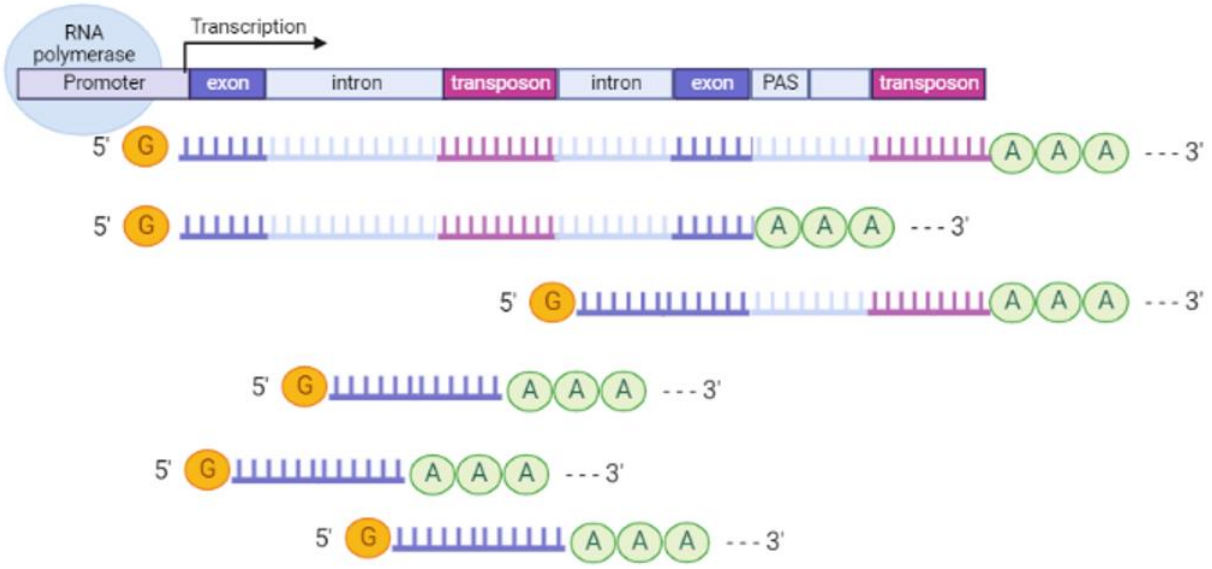


Fig. 2. A schematic model of transposon expression.
In our model, transcription can give rise to mRNAs and pre-mRNAs that contain retained introns when co-transcriptional splicing is impaired, as seen during aging and senescence, and these often contain transposon sequences. In addition, transcription can give rise to mRNAs and pre-mRNAs that contain transposon sequences towards the 3'-end of the mRNA when co-transcriptional termination at the polyadenylation signal (PAS) is impaired as seen with aging and senescence. Some of these RNAs may be successfully polyadenylated whereas others will be subject to nonsense mediated decay. Image created by Biorender.

4. Expression of extragenic transposons as a marker of readthrough transcription

Most transposons showing increased expression with aging or cellular senescence mapped within genes or introns and their expression changes may be attributed to intron retention. How can we explain expression of the remaining 14 to 30% of transposons? (**Table 1**)

To answer this question, we analyzed the distribution of transposons mapping to extragenic regions. Out of all the transposons, showing significantly changed expression during aging or cellular senescence, most within 5 kb of genes and they were found predominantly downstream of genes (**Fig. 3A**). The distribution of transposons in the aging and senescence dataset was very similar except for a wider distribution of senescence-associated transposons within a 100 kb window around genes (**Fig. 3B**). This

contrasts with the almost completely even distribution of randomly permuted transposons.

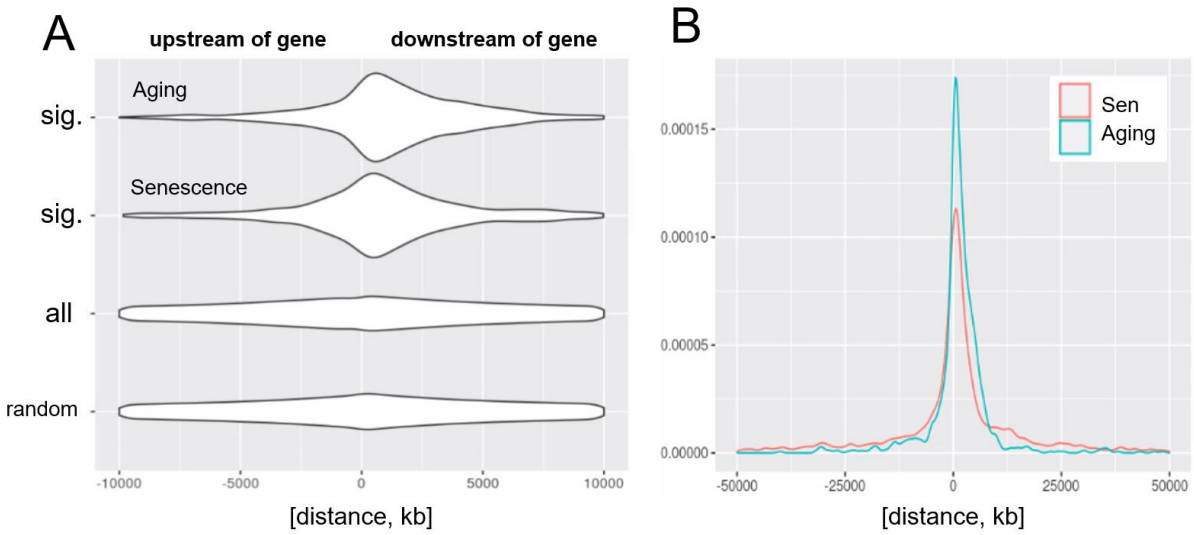


Fig. 3. Biased distribution of extragenic transposons relative to genes.
(A) Extragenic transposons with significantly (sig.) changed expression during aging or cellular senescence show a biased distribution, when mapped back onto the genome, with a preference towards a 5 kb region at the 3'-end of genes when compared to all annotated transposons (all) or randomly permuted transposons (random). (B) Extragenic transposons whose expression changes during cellular senescence are spread out further from genes compared to aging-associated transposons.

Given the proximity of significantly changed transposons and genes, this suggested that an age-related increase in transcriptional readthrough could explain elevated transposon expression. To test this, we next quantified readthrough in a 10 to 20 kb region downstream of genes. We chose a region not immediately adjacent to genes to avoid biases through basal levels of readthrough, although the data was comparable for other distances (**Fig. S7**).

Indeed, readthrough transcription was increased with aging (**Fig. 4A, B**), and in the senescence dataset, levels of readthrough transcription increased with replicative or chemically induced senescence (**Fig. 4D, E**). Transposon expression correlated with readthrough levels on a per sample basis in both the aging and the senescence dataset (**Fig. 4C, F**).

As shown before for intron retention, these findings may not translate to other species. We found that readthrough was unchanged in the liver of 26 month-old mice (p=0.22,

Fig. S6A). In addition, readthrough decreased with replicative senescence of mouse fibroblasts, contrary to our expectations (**Fig. S6C, D**).

Next, we quantified readthrough across a 100 kb region downstream of genes. We found that both aging and senescence lead to readthrough across the whole region, although senescence was associated with more extensive readthrough further downstream of genes (**Fig. S7, Fig. 3B**). As another line of evidence we quantified transcription upstream of genes, which is called read-in. This kind of transcription is often attributed to widespread readthrough extending from the end of a gene to the start of another (**Roth et al. 2020**). Consistent with the extensive readthrough levels observed we found elevated read-in transcription during cellular senescence (**Fig. S8A**) but not aging (**Fig. S8B**).

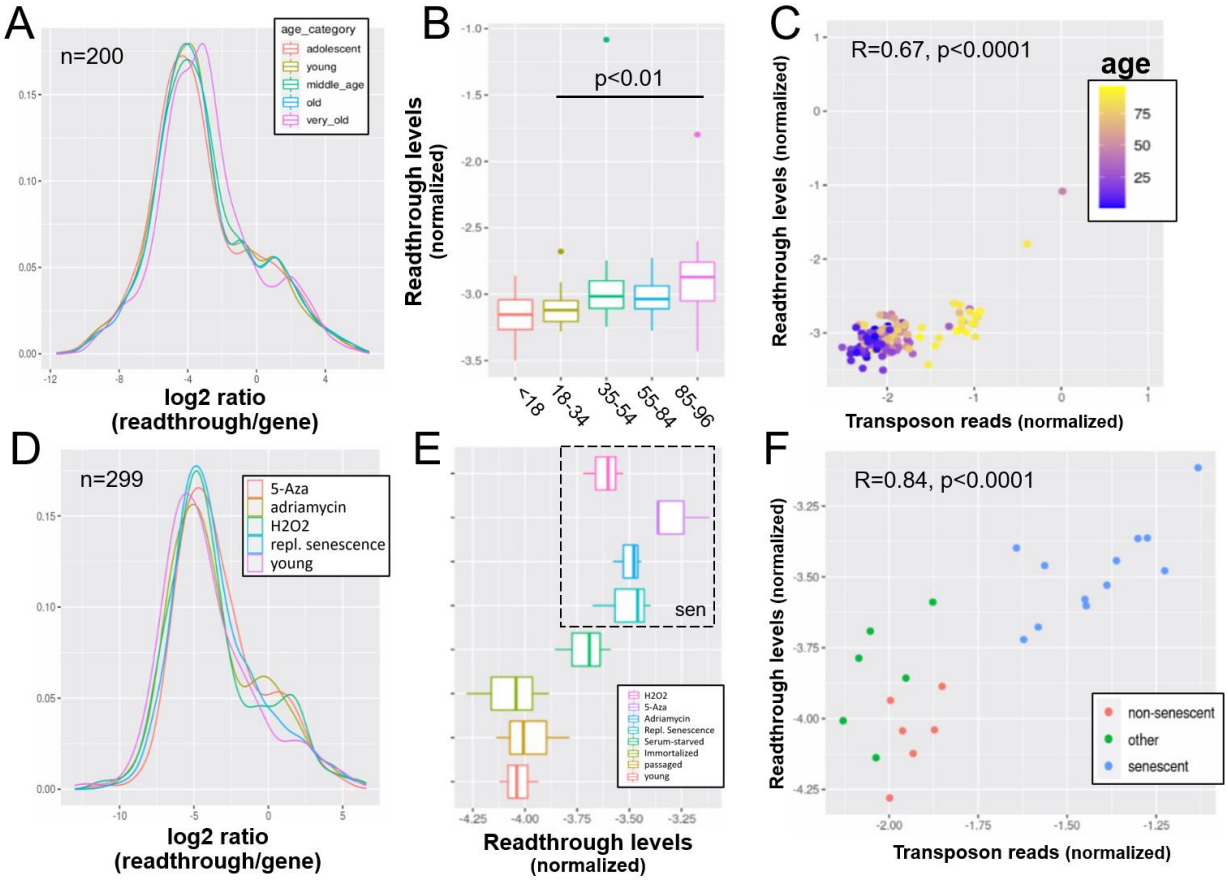


Fig. 4. Increased readthrough levels with aging and senescence.
Readthrough transcription is increased in fibroblasts isolated from the very old (A, B) and after induction of senescence in vitro (D, E). Readthrough transcription is correlated

with transposon expression (C, F).
A) Readthrough was determined in a region 10 to 20 kb downstream of genes for a subset of genes that were at least 20 kb away from the nearest neighboring gene (n=206 regions). The log2 ratio of readthrough to gene expression is plotted across five age groups (adolescent n=32, young n=31, middle-aged n=22, old n=37 and very old n=21).
B) As in (A) but data is plotted on a per sample basis.
C) Scatterplot between transposon expression and readthrough levels (normalized as in A and Fig. 1) for all 143 samples. Each sample is colored by age.
D) Readthrough was determined in a region 10 to 20 kb downstream of genes for a subset of genes that were at least 20 kb away from the nearest neighboring gene (n=299 regions). The log2 ratio of readthrough to gene expression is plotted across four senescent conditions (H2O2, 5-Aza, Adriamycin, replicative senescence) and for early passage cells. N=3 per group.
E) As in (D) but data is plotted on a per sample basis and for additional control datasets (serum-starved, immortalized, intermediate passage and early passage). N=3 per group.
F) Scatterplot between transposon expression and readthrough levels (normalized as in A and Fig. 1) for all 24 samples. Each sample is colored by senescence status.

Having shown that samples with higher levels of readthrough also have higher transposon expression (per sample correlation), we asked whether transposons are more often than expected located in readthrough regions (per locus correlation). Indeed as expected, in both datasets, transposons significantly changed with age or senescence were more often found in significant readthrough regions compared to all transposons (**Table S2**). Moreover, there was a significant correlation between the log-fold changes of transposons downstream of genes with the log-fold changes of readthrough downstream of the same genes (**Fig. S9**). Transposons located elsewhere showed a weaker correlation with readthrough (**Table S3**).

To further test whether these correlations between log-fold changes are also reflected on the count level, we determined the correlation between readthrough counts and the counts of transposons downstream of genes for each sample individually. Transposons located upstream of genes and randomized transposons served as a negative control. Consistent with the above data, we found a specific correlation between transposon and readthrough counts in both datasets (**Fig. S10**).

Finally, we asked whether readthrough levels are predictive of transposon expression on a per sample basis independently of intron retention. Using the aging dataset with 143 samples we compared a linear model just including intron retention with one also including readthrough and found the second model to perform significantly better

298 (p<0.0001, **Table S4**). In addition, we found that the correlation between readthrough
299 levels and transposon expression remained significant even when correcting for intron
300 retention using partial correlation (R=0.53, p<0.0001). We did not test this in the
301 senescence dataset due to its limited size.
302

303 **5. Readthrough is a causal inducer of transposon expression**

304 Based on this correlative evidence (**Fig. 4, Fig. S9**), we hypothesized that specific
305 inducers of readthrough would also increase transposon expression. To test this, we re-
306 analyzed literature data from studies involving multiple treatments that promote
307 readthrough. These included viral infection, hyperosmotic stress (KCl treatment), heat
308 shock and oxidative stress.

309 We found that KCl treatment of HEK293 cells for 1 hour (**Rosa-Mercado et al. 2021**)
310 indeed strongly induced both readthrough (**Fig. 5A**) and transposon expression (**Fig. 5B**)
311 as determined by RNA-seq and TTLseq. Consistent with this, in our reanalysis of a study
312 using mNET-seq profiling of nascent mRNA (**Bauer et al. 2018**), KCl treatment of HEK293
313 cells also induced readthrough (**Fig. 5D**) and transposon expression (**Fig. 5E**), whereas
314 influenza infection caused an intermediate phenotype. Finally, we analyzed RNA-seq
315 data from NIH-3T3 mouse fibroblasts subjected to three stressors over two hours
316 (**Vilborg et al. 2017**). Heatshock caused extensive readthrough (**Fig. S11A**) and
317 transposon expression (**Fig. S11B**), whereas hydrogen peroxide treatment and KCl
318 caused moderate expression of these.

319 We also reasoned that readthrough should specifically increase the expression of
320 transposons that are close to genes. Consistent with this notion, induced readthrough
321 led to preferential expression of gene proximal transposons (i.e. those within 25 kb of
322 genes), when compared with senescence or aging (**Fig. S12A**). Moreover, the fraction of
323 significantly upregulated transposons found outside of genes was higher with induced
324 readthrough than with senescence or aging (**Fig. S12B**).

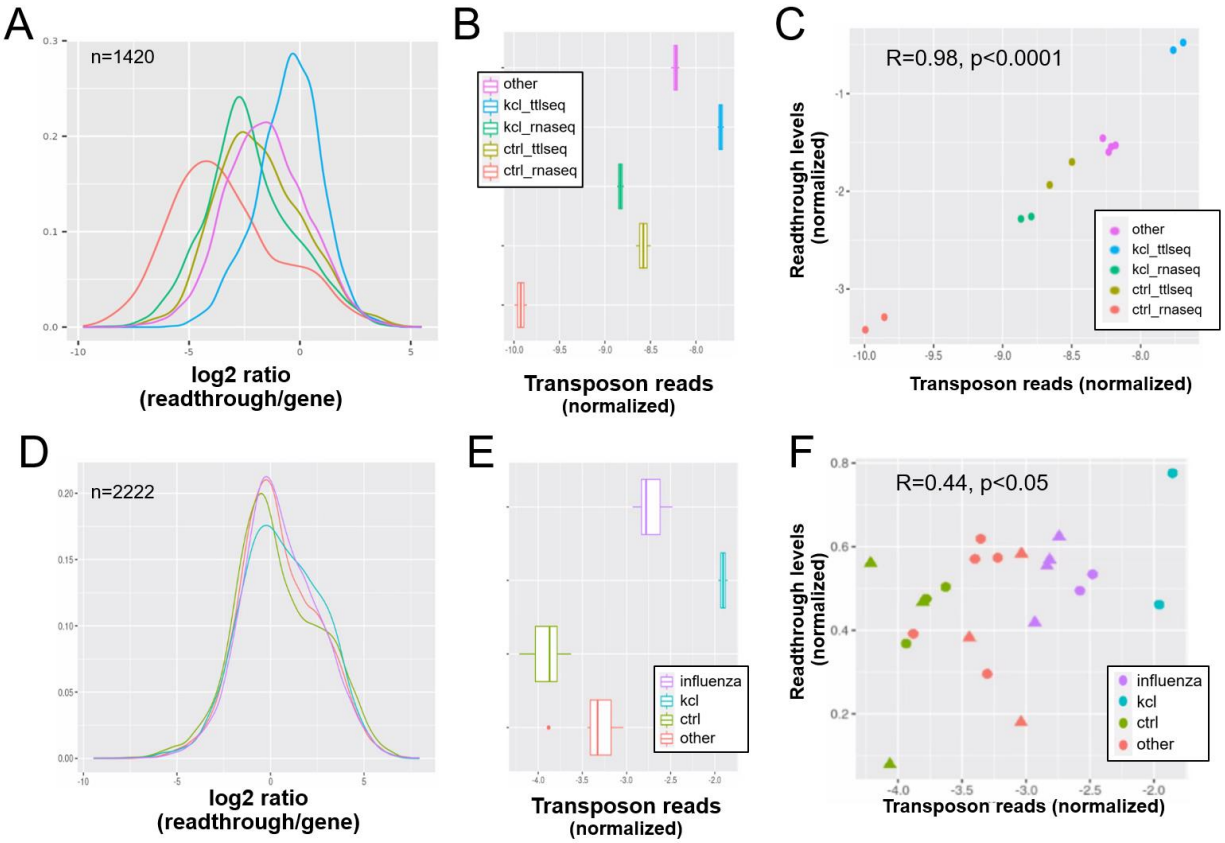


Fig. 5. Elevated transposon expression after induced readthrough.
Readthrough transcription is increased after KCl treatment of HEK293 cells (A, D) and to a lesser extent during influenza infection (D). These readthrough inducing treatments also promote increased transposon expression (B, E). Giving rise to a correlation between readthrough and transposon expression on a per sample basis (C, F). Data in (A-C) is from **Rosa-Mercado et al. (2021)**. In this study HEK293 cells were subjected to 1 hour of hyperosmotic stress (KCl) after which RNA-seq and TTLseq were performed. Data in (D-F) is from **Bauer et al. (2018)**. In this study HEK293 cells were subjected to 1 hour of hyperosmotic stress (KCl) after which mNETseq was performed.
A) Readthrough was determined in a region 10 to 20 kb downstream of genes for a subset of genes that was at least 20 kb away from the nearest neighbouring gene (n=1420 regions).
B) To normalize transposon expression counts for each transposon were corrected for the expression of the nearest gene. Normalized transposon counts for each sample are shown as box-whisker plot.
C) Normalized transposon counts (as in B) and readthrough levels are plotted for each sample (n=12).
D) Readthrough was determined in a region 10 to 20 kb downstream of genes for a subset of genes that was at least 20 kb away from the nearest neighbouring gene

(n=2222 regions).
E) To normalize transposon expression counts for each transposon were corrected for the expression of the nearest gene. Normalized transposon levels for each sample are shown as box-whisker plot.
F) Normalized transposon counts (as in B) and readthrough counts are plotted for each sample (n=22). The shapes indicate the cell type (circles=HEK293, triangles=A549).

6. Increased expression of autonomous LINE-1 elements with senescence but not aging

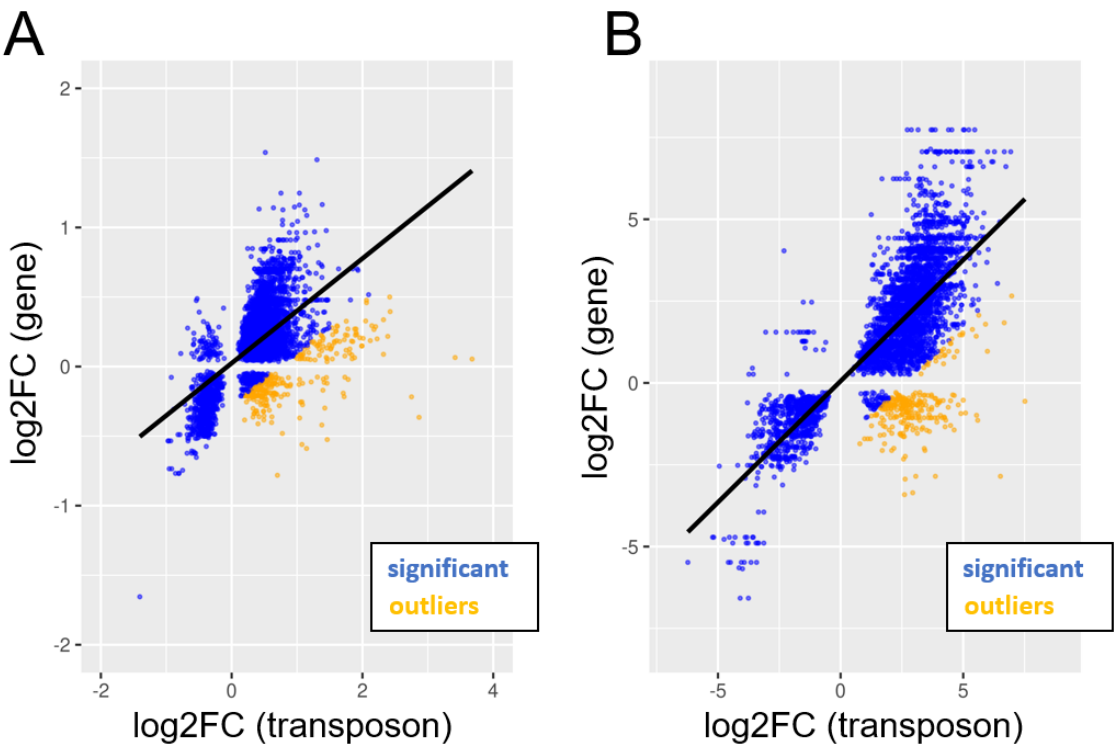
Many expressed LINE-1 elements are found within genes (**Table S5**), which poses a challenge for the quantification of active LINE-1 elements. Given this proximity it is unsurprising that a substantial correlation between the expression of transposons and neighboring genes (**Fig. 6**) is seen in RNA-seq.

To mitigate this problem, we selected 250 transposons that showed higher than expected expression levels compared to the nearest gene. We reasoned that LINE-1 elements with a functional promoter ("active" LINE-1s) would be more likely autonomous and should be enriched among transposons that show a divergent expression profile from their neighboring genes.

We found no enrichment for active LINE-1 elements in the aging dataset (**Fig. 6A**). In contrast, transposons significantly upregulated with senescence and, among those, especially the ones with higher-than-expected expression, showed significant enrichment for active LINE-1 elements (**Fig. 6B**). Moreover, we found that almost 100% of active LINE-1 elements were increased with senescence which was higher than for all LINE-1 elements and other transposons (**Table S5**).

Finally, we redid the same analysis with a different definition of active LINE-1 elements as those potentially encoding functional ORF1p and ORF2p. In this analysis, we included all elements encoding an open reading frame > 900 nt with high sequence similarity to ORF1p and >3400 nt with high sequence similarity to ORF2p ($p<0.05$ for pairwise alignment). Out of 122626 annotated LINE-1 elements 585 encoded a putative ORF1p and 133 encoded an ORF2p.

ORF1p-encoding LINE-1 elements were significantly enriched among transposons with higher-than-expected expression and among transposons significantly changed with senescence or aging (**Table S7**). These findings were more robust for senescence than for aging. ORF2p-encoding LINE-1 elements, in contrast, were too rare to be studied reliably as to their enrichment.



	active LINE1	LINE1	active%	p value
outliers	2	79	2.53	NS
significant	55	2820	1.95	NS
all expressed	549	12214	4.49	

	active LINE1	LINE1	active%	p value
outliers	21	91	25.61	<0.0001
significant	340	2506	13.57	<0.0001
all expressed	1538	27167	5.66	

Fig. 6. Increased expression of active LINE-1 elements with senescence but not aging. A scatterplot between the log2-fold change of every transposon ($p<0.05$) differentially expressed with age or senescence and the log2-fold change of the nearest gene. The percentage of active LINE-1 elements among transposons with higher-than-expected expression levels is unchanged in aging (A) and increased during senescence (B). For the tables, we compared the percentage of active LINE-1 elements among all LINE-1 elements for 250 transposons with higher-than-expected expression levels (in orange; labeled "outliers"), all significantly changed transposons (blue plus orange; labeled "significant") and all transposons passing read filtering (not plotted; labeled "all expressed"). The p-value is based on the comparison with "all expressed".

DISCUSSION

394 We show that transposons would serve as a useful biomarker of aging, even if most of
395 the transposon signal in RNA-seq was due to co-expression with nearby transcriptional
396 units, because they capture different kinds of transcriptional dysfunction.

397 **Introns**

398 Intron retention is one prominent example of dysfunctional splicing during aging, but
399 the association with age-related transposon expression has not been evaluated. A
400 recent study by **Gualandi et al. (2022)** using RNA-seq data from the 1000 Genomes
401 project found a strong per sample correlation between intron and transposon
402 expression in the general population. In line with these results, we showed that intron
403 retention may explain a large fraction of transposon reads in aging and senescence
404 datasets (**Fig. 1**).

405 **Readthrough, read-in and transposons**

406 In a study of oncogene-Induced senescence, **Muniz et al. (2017)** found increased
407 readthrough transcription at 91 so called START loci, which was associated with
408 suppression of neighboring genes through the production of antisense RNAs and several
409 of these antisense RNAs were necessary for the maintenance of the senescent state.
410 Our data is consistent with their model, although we find even more extensive
411 readthrough transcription. Analyzing hundreds of loci we show that senescence
412 promotes transcriptional readthrough leading to the overexpression of intergenic
413 transposons (>25 kb from the nearest gene) and read-in at neighboring genes (**Fig. 3B,**
414 **Fig. S8B**)**.** In contrast, aging-related readthrough was qualitatively different, producing
415 shorter readthrough and no read-in (**Fig. 3B, Fig. S8A**).

416 Our correlative data suggested that readthrough transcription could account for the
417 expression of many transposons that are outside of genes and thus cannot be explained
418 by intron retention (**Fig. 4C, Fig. 4F**). To test this hypothesis, we reanalyzed data from
419 models of induced readthrough showing that readthrough is indeed a causal inducer for
420 the expression of gene proximal transposons after hyperosmotic stress and viral
421 infection (**Fig. 5**). Given the technical challenges of measuring readthrough and the
422 unreliability of intron retention detection tools (**David et al. 2022**) our results are likely
423 underestimating the true magnitude of correlation between readthrough and
424 transposon expression.

425 In a previous study it was observed that senescence leads to increased expression of
426 transposons and of genes with low abundances (**Zhang et al. 2021**). This “leakage” was
427 attributed to loss of H3K9me3 histone marks and increased heterochromatin
428 accessibility, but is also consistent with readthrough transcription, as we observed.

429 Indeed, it has been suggested that readthrough is the most likely cause for apparent
430 intergenic transcription (**Agostini et al. 2021**).

431 **Is there genuine expression of autonomous transposons during aging?**

432 The distribution of transposons showing apparent age-related expression within the
433 genome is an important question since intragenic transposons are more likely to be co-
434 expressed with genes. Using Sanger sequencing of long-range amplicons to study
435 replicative senescence, **De Cecco et al. (2019)** found a large fraction of transposons
436 mapping to intergenic (69%) regions and few to intragenic/gene-proximal regions (31%).
437 In contrast using RNA-seq data, we find that most transposons are located within genes
438 (≥70%; **Table 1**), although, there are more intergenic transposons significantly changed
439 with senescence than with aging (**Fig. 3**). One explanation for this discrepancy is the
440 mapping approach used. Since their analysis did not use an expectation-maximization
441 algorithm to map back transposons onto the genome, it may underestimate the number
442 of intragenic and gene-adjacent transposons in contrast to our analysis using TELocal.

443 Given that inactive LINE-1 loci greatly outnumber active loci (**Deininger et al. 2017, Stow**
444 **et al. 2021**), and we saw only limited evidence for active LINE-1 transcription in
445 fibroblasts isolated from donors of varying ages (**Fig. 6A**), our data appears to question
446 the role of transposons in organismal aging. An intriguing explanation is provided by **De**
447 **Cecco et al. (2019)**, who found that LINE-1 ORF1 protein-positive cells colocalize with a
448 small subset of senescent cells. Most likely, such changes would not be obvious from
449 bulk sequencing, perhaps explaining why we did not see elevated LINE-1 expression in
450 the aging dataset. In contrast, the signal would be readily detectable in cultures induced
451 into senescence where most cells do enter senescence, which is consistent with our
452 findings (**Fig. 6B**).

453 As for another explanation, we speculate that LINE-1 elements expressed through intron
454 retention and readthrough are compatible with translation via leaky scanning or re-
455 initiation (**Mouilleron et al. 2015**), accounting for increased LINE-1 ORF1 protein levels
456 detected in senescent cells, even if leaky scanning is orders of magnitude less efficient
457 than normal initiation.

458 **Summary**

459 Taken together, our work and existing literature suggest that RNA-seq, and by extension
460 qPCR, of transposable elements overestimates their true expression and their age-
461 related changes. Such data alone is insufficient as support for a “transposon hypothesis
462 of aging” that postulates an increase in autonomous expression of such elements.
463 Instead, a concerted increase in transposon expression, intron retention and

transcriptional readthrough points to a global increase in transcriptional dysfunction during aging and senescence, at least in human cells.

Nevertheless, there are several other techniques such as immunoblotting for ORF1 protein, rapid amplification of cDNA ends (RACE), or genomic PCR that may (and do) provide evidence in favor of age-related reactivation of potentially autonomous transposons, specifically LINE-1 elements.

Although we have shown increased transcriptional dysfunction with aging and cellular senescence, so far we lack a model to explain these findings. In this context it is noteworthy that the elongation rate of Pol II is correlated with readthrough transcription (**Fong et al. 2015**), intron processing, and may increase with aging (**Debès et al. 2019**), potentially providing a unifying explanation.

METHODS

Datasets, read preprocessing and alignment

We searched the SRA for suitable datasets that used total RNA extraction because this improves the detection of non-canonical transcripts that may be degraded before successful polyadenylation. Publicly available human RNA-seq datasets of aging and senescence, respectively, were obtained from Colombo et al. 2018 (GSE60340) and Fleischer et al. 2018 (GSE113957). Human RNA-seq and TTLseq data was obtained from Rosa-Mercado et al. 2021 (GSE152059), human mNET-seq data from Bauer et al. 2018 (PRJNA432639) and mouse RNA-seq data from Vilborg et al. 2017 (GSE98906).

Mouse liver RNA-seq data from ad libitum and dietary restricted mice was obtained from Hahn et al. 2017 (GSE92486) and mouse replicative senescence data from Wang et al. 2022 (GSE179880).

Reads were filtered, adaptor-trimmed and repaired using fastp (**Chen et al. 2018**) with a min Phred score of 25. Afterwards reads passing these filters were aligned to the human genome (GRCh38) or mouse genome (GRCm39) using STAR 2.7.8a (**Dobin et al. 2013**) in single-pass mode. To optimize the quantification of transposons the STAR parameters --outFilterMultimapNmax and --winAnchorMultimapNmax were set to 100. The full pipeline with all the configuration files is available on github (pabisk/aging_transposons).

Detection of readthrough and intron retention

We employed a modified Automatic Readthrough Transcription Detection (ARTDeco) pipeline, using their provided annotations (**Roth et al. 2020**) but with a different quantification approach. Readthrough candidate regions in the ARTDeco pipeline were

497 defined based on the longest transcript to exclude the effects of differential 3'-UTR
498 usage. Genic and readthrough reads were counted using featureCounts from the
499 Rsubread package in 10 kb bins downstream of genes, in a window between 0 to 100 kb.
500 Readthrough regions with low coverage, in close proximity to, or overlapping, nearby
501 protein-coding genes were excluded from the analysis. Intron retention was detected
502 using the intron REtention Analysis and Detector (iREAD), which is a tool that detects
503 retention events based on both splice junction reads and intron expression levels (**Li et**
504 **al. 2020**). All samples were treated as unstranded.

505 **Locus specific detection of transposon reads and transcripts**

506 We used TELocal v1.1.1 (mhammell-laboratory/TElocal) to quantify transposon reads,
507 which is functionally similar to TETranscripts (**Jin et al. 2015**) except it provides locus-
508 specific expression levels. Both tools use an expectation maximization algorithm to
509 assign ambiguous reads. Annotation files were downloaded from the mhammell lab and
510 from genecode (v39 for human and M27 for mouse). TELocal was also used to quantify
511 transcriptomic counts. Again, all samples were treated as unstranded.

512 **Expression and normalization of genes, introns, transposons and readthrough**
513 **transcripts**

514 Transcripts and elements with low expression counts were filtered. After filtering, intron,
515 readthrough, read-in or transposon elements were combined with transcript counts for
516 the analysis in DESeq2. For the aging fibroblast dataset age was normalized and
517 centered and the design model considered age+gender+ethnicity. For the induced
518 senescence dataset all four inducers were included together and the remaining
519 treatment groups were considered non-senescent.

520 In order to study expression of these elements unbiased by gene coexpression, we
521 selected the top 1000 overexpressed elements and divided their read counts by the
522 read counts of the nearest gene. When not specified in the annotation the nearest
523 genes or elements were identified with the bedtools closest or intersect function, as
524 appropriate. Finally, to provide a single expression value for each sample we used the
525 mean log of the normalized expression values.

526 **Expression of potentially active and protein-coding LINE-1 elements**

527 We defined potentially active LINE-1 elements as those that have a functional promoter
528 (**McKerrow and Fenyö 2020**). Whereas to define potentially protein-coding LINE-1
529 elements we predicted open reading frames for each annotated element using the
530 predORF function from the systemPipeR package. Global pairwise alignment to ORF1p

531 (uniprot: Q9UN81) and ORF2p (uniprot: O00370) was performed and only elements with
532 a significant alignment were included for further analyses ($p<0.05$).

533

534 **ACKNOWLEDGEMENTS**

535 We would like to thank Juliane Liepe, Marco Malavolta and Chin-Tong Ong for
536 support and/or their help in improving this manuscript and VitaDAO for financial
537 support.

538

539 **REFERENCES**

540 Adusumalli, S., Ngian, Z.-K., Lin, W.-Q., Benoukraf, T., & Ong, C.-T. (2019). Increased intron retention is a
541 post-transcriptional signature associated with progressive aging and Alzheimer's disease. Aging Cell,
542 18(3), e12928. https://doi.org/10.1111/acel.12928

543 Agostini, F., Zagalak, J., Attig, J., Ule, J., & Luscombe, N. M. (2021). Intergenic RNA mainly derives from
544 nascent transcripts of known genes. Genome Biology, 22(1), 136. https://doi.org/10.1186/s13059-021-
545 02350-x

546 Bauer, D. L. V., Tellier, M., Martínez-Alonso, M., Nojima, T., Proudfoot, N. J., Murphy, S., & Fodor, E.
547 (2018). Influenza Virus Mounts a Two-Pronged Attack on Host RNA Polymerase II Transcription. Cell
548 Reports, 23(7), 2119-2129.e3. https://doi.org/10.1016/j.celrep.2018.04.047

549 Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). fastp: An ultra-fast all-in-one FASTQ preprocessor.
550 Bioinformatics, 34(17), i884–i890. https://doi.org/10.1093/bioinformatics/bty560

551 Colombo, A. R., Elias, H. K., & Ramsingh, G. (2018). Senescence induction universally activates
552 transposable element expression. Cell Cycle, 17(14), 1846–1857.
553 https://doi.org/10.1080/15384101.2018.1502576

554 David, J. K., Maden, S. K., Wood, M. A., Thompson, R. F., & Nellore, A. (2022). Retained introns in long
555 RNA-seq reads are not reliably detected in sample-matched short reads (p. 2022.03.11.484016). bioRxiv.
556 https://doi.org/10.1101/2022.03.11.484016

557 De Cecco, M., Ito, T., Petrashen, A. P., Elias, A. E., Skvir, N. J., Criscione, S. W., Caligiana, A., Brocculi, G.,
558 Adney, E. M., Boeke, J. D., Le, O., Beauséjour, C., Ambati, J., Ambati, K., Simon, M., Seluanov, A.,
559 Gorbunova, V., Slagboom, P. E., Helfand, S. L., … Sedivy, J. M. (2019). L1 drives IFN in senescent cells and
560 promotes age-associated inflammation. Nature, 566(7742), Article 7742.
561 https://doi.org/10.1038/s41586-018-0784-9

562 Debès, C., Papadakis, A., Grönke, S., Karalay, Ö., Tain, L., Mizi, A., Nakamura, S., Hahn, O., Weigelt, C.,
563 Josipovic, N., Zirkel, A., Brusius, I., Sofiadis, K., Lamprousi, M., Lu, Y.-X., Huang, W., Esmaillie, R., Kubacki,
564 T., Späth, M. R., … Beyer, A. (2022). Aging-associated changes in transcriptional elongation influence
565 metazoan longevity (p. 719864). bioRxiv. https://doi.org/10.1101/719864

566 Deininger, P., Morales, M. E., White, T. B., Baddoo, M., Hedges, D. J., Servant, G., Srivastav, S., Smither,
567 M. E., Concha, M., DeHaro, D. L., Flemington, E. K., & Belancio, V. P. (2017). A comprehensive approach
568 to expression of L1 loci. Nucleic Acids Research, 45(5), e31. https://doi.org/10.1093/nar/gkw1067

569 Deniz, Ö., Frost, J. M., & Branco, M. R. (2019). Regulation of transposable elements by DNA
570 modifications. Nature Reviews Genetics, 20(7), Article 7. https://doi.org/10.1038/s41576-019-0106-6

571 Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras,
572 T. R. (2013). STAR: Ultrafast universal RNA-seq aligner. Bioinformatics, 29(1), 15–21.
573 https://doi.org/10.1093/bioinformatics/bts635

574 Fleischer, J. G., Schulte, R., Tsai, H. H., Tyagi, S., Ibarra, A., Shokhirev, M. N., Huang, L., Hetzer, M. W., &
575 Navlakha, S. (2018). Predicting age from the transcriptome of human dermal fibroblasts. Genome
576 Biology, 19(1), 221. https://doi.org/10.1186/s13059-018-1599-6

577 Fong, N., Brannan, K., Erickson, B., Kim, H., Cortazar, M. A., Sheridan, R. M., Nguyen, T., Karp, S., &
578 Bentley, D. L. (2015). Effects of Transcription Elongation Rate and Xrn2 Exonuclease Activity on RNA
579 Polymerase II Termination Suggest Widespread Kinetic Competition. Molecular Cell, 60(2), 256–267.
580 https://doi.org/10.1016/j.molcel.2015.09.026

581 Ghanam, A. R., Cao, J., Ouyang, X., & Song, X. (2019). New Insights into Chronological Mobility of
582 Retrotransposons In Vivo. Oxidative Medicine and Cellular Longevity, 2019, e2818415.
583 https://doi.org/10.1155/2019/2818415

584 Gorbunova, V., Seluanov, A., Mita, P., McKerrow, W., Fenyö, D., Boeke, J. D., Linker, S. B., Gage, F. H.,
585 Kreiling, J. A., Petrashen, A. P., Woodham, T. A., Taylor, J. R., Helfand, S. L., & Sedivy, J. M. (2021). The
586 role of retrotransposable elements in ageing and age-associated diseases. Nature, 596(7870), Article
587 7870. https://doi.org/10.1038/s41586-021-03542-y

588 Gualandi, N., Iperi, C., Esposito, M., Ansaloni, F., Gustincich, S., & Sanges, R. (2022). Meta-Analysis
589 Suggests That Intron Retention Can Affect Quantification of Transposable Elements from RNA-Seq Data.
590 Biology, 11(6), Article 6. https://doi.org/10.3390/biology11060826

591 Hadar, S., Meller, A., Saida, N., & Shalgi, R. (2022). Stress-induced transcriptional readthrough into
592 neighboring genes is linked to intron retention. IScience, 25(12), 105543.
593 https://doi.org/10.1016/j.isci.2022.105543

594 Hahn, O., Grönke, S., Stubbs, T. M., Ficz, G., Hendrich, O., Krueger, F., Andrews, S., Zhang, Q., Wakelam,
595 M. J., Beyer, A., Reik, W., & Partridge, L. (2017). Dietary restriction protects from age-associated DNA
596 methylation and induces epigenetic reprogramming of lipid metabolism. Genome Biology, 18(1), 56.
597 https://doi.org/10.1186/s13059-017-1187-1

598 Jin, Y., Tam, O. H., Paniagua, E., & Hammell, M. (2015). TEtranscripts: A package for including
599 transposable elements in differential expression analysis of RNA-seq datasets. Bioinformatics, 31(22),
600 3593–3599. https://doi.org/10.1093/bioinformatics/btv422

601 Kazazian, H. H., & Moran, J. V. (2017). Mobile DNA in Health and Disease. New England Journal of
602 Medicine, 377(4), 361–370. https://doi.org/10.1056/NEJMra1510092

603 Khristich, A. N., & Mirkin, S. M. (2020). On the wrong DNA track: Molecular mechanisms of repeat-
604 mediated genome instability. Journal of Biological Chemistry, 295(13), 4134–4170.
605 https://doi.org/10.1074/jbc.REV119.007678

606 Koning, A. P. J. de, Gu, W., Castoe, T. A., Batzer, M. A., & Pollock, D. D. (2011). Repetitive Elements May
607 Comprise Over Two-Thirds of the Human Genome. PLOS Genetics, 7(12), e1002384.
608 https://doi.org/10.1371/journal.pgen.1002384

609 LaRocca, T. J., Cavalier, A. N., & Wahl, D. (2020). Repetitive elements as a transcriptomic marker of aging:
610 Evidence in multiple datasets and models. Aging Cell, 19(7), e13167. https://doi.org/10.1111/acel.13167

611 Li, H.-D., Funk, C. C., & Price, N. D. (2020). iREAD: A tool for intron retention detection from RNA-seq
612 data. BMC Genomics, 21(1), 128. https://doi.org/10.1186/s12864-020-6541-0

613 McKerrow, W., & Fenyö, D. (2020). L1EM: A tool for accurate locus specific LINE-1 RNA quantification.
614 Bioinformatics, 36(4), 1167–1173. https://doi.org/10.1093/bioinformatics/btz724

615 Mouilleron, H., Delcourt, V., & Roucou, X. (2016). Death of a dogma: Eukaryotic mRNAs can code for
616 more than one protein. Nucleic Acids Research, 44(1), 14–23. https://doi.org/10.1093/nar/gkv1218

617 Muniz, L., Deb, M. K., Aguirrebengoa, M., Lazorthes, S., Trouche, D., & Nicolas, E. (2017). Control of Gene
618 Expression in Senescence through Transcriptional Read-Through of Convergent Protein-Coding Genes.
619 Cell Reports, 21(9), 2433–2446. https://doi.org/10.1016/j.celrep.2017.11.006

620 Pabis, K. (2021). Triplex and other DNA motifs show motif-specific associations with mitochondrial DNA
621 deletions and species lifespan. Mechanisms of Ageing and Development, 194, 111429.
622 https://doi.org/10.1016/j.mad.2021.111429

623 Retained introns in long RNA-seq reads are not reliably detected in sample-matched short reads |
624 bioRxiv. (n.d.). Retrieved December 27, 2022, from
625 https://www.biorxiv.org/content/10.1101/2022.03.11.484016v1.abstract

626 Rodriguez-Martin, B., Alvarez, E. G., Baez-Ortega, A., Zamora, J., Supek, F., Demeulemeester, J.,
627 Santamarina, M., Ju, Y. S., Temes, J., Garcia-Souto, D., Detering, H., Li, Y., Rodriguez-Castro, J., Dueso-
628 Barroso, A., Bruzos, A. L., Dentro, S. C., Blanco, M. G., Contino, G., Ardeljan, D., … Tubio, J. M. C. (2020).
629 Pan-cancer analysis of whole genomes identifies driver rearrangements promoted by LINE-1
630 retrotransposition. Nature Genetics, 52(3), Article 3. https://doi.org/10.1038/s41588-019-0562-0

631 Rosa-Mercado, N. A., & Steitz, J. A. (2022). Who let the DoGs out? – Biogenesis of stress-induced
632 readthrough transcripts. Trends in Biochemical Sciences, 47(3), 206–217.
633 https://doi.org/10.1016/j.tibs.2021.08.003

634 Rosa-Mercado, N. A., Zimmer, J. T., Apostolidi, M., Rinehart, J., Simon, M. D., & Steitz, J. A. (2021).
635 Hyperosmotic stress alters the RNA polymerase II interactome and induces readthrough transcription
636 despite widespread transcriptional repression. Molecular Cell, 81(3), 502-513.e4.
637 https://doi.org/10.1016/j.molcel.2020.12.002

638 Roth, S. J., Heinz, S., & Benner, C. (2020). ARTDeco: Automatic readthrough transcription detection. BMC
639 Bioinformatics, 21(1), 214. https://doi.org/10.1186/s12859-020-03551-0

Scotti, M. M., & Swanson, M. S. (2016). RNA mis-splicing in disease. Nature Reviews Genetics, 17(1), Article 1. https://doi.org/10.1038/nrg.2015.3

Stow, E. C., Kaul, T., deHaro, D. L., Dem, M. R., Beletsky, A. G., Morales, M. E., Du, Q., LaRosa, A. J., Yang, H., Smither, E., Baddoo, M., Ungerleider, N., Deininger, P., & Belancio, V. P. (2021). Organ-, sex- and age-dependent patterns of endogenous L1 mRNA expression at a single locus resolution. Nucleic Acids Research, 49(10), 5813–5831. https://doi.org/10.1093/nar/gkab369

Vilborg, A., Sabath, N., Wiesel, Y., Nathans, J., Levy-Adam, F., Yario, T. A., Steitz, J. A., & Shalgi, R. (2017). Comparative analysis reveals genomic features of stress-induced transcriptional readthrough. Proceedings of the National Academy of Sciences, 114(40), E8362–E8371. https://doi.org/10.1073/pnas.1711120114

Villanueva-Cañas, J. L., Horvath, V., Aguilera, L., & González, J. (2019). Diverse families of transposable elements affect the transcriptional regulation of stress-response genes in Drosophila melanogaster. Nucleic Acids Research, 47(13), 6842–6857. https://doi.org/10.1093/nar/gkz490

Wahl, D., Cavalier, A. N., Smith, M., Seals, D. R., & LaRocca, T. J. (2021). Healthy Aging Interventions Reduce Repetitive Element Transcripts. The Journals of Gerontology: Series A, 76(5), 805–810. https://doi.org/10.1093/gerona/glaa302

Wang, K., Wu, D., Zhang, H., Das, A., Basu, M., Malin, J., Cao, K., & Hannenhalli, S. (2018). Comprehensive map of age-associated splicing changes across human tissues and their contributions to age-associated diseases. Scientific Reports, 8(1), Article 1. https://doi.org/10.1038/s41598-018-29086-2

Wang, Y., Liu, L., Song, Y., Yu, X., & Deng, H. (2022). Unveiling E2F4, TEAD1 and AP-1 as regulatory transcription factors of the replicative senescence program by multi-omics analysis. Protein & Cell, 13(10), 742–759. https://doi.org/10.1007/s13238-021-00894-z

Wood, J. G., Jones, B. C., Jiang, N., Chang, C., Hosier, S., Wickremesinghe, P., Garcia, M., Hartnett, D. A., Burhenn, L., Neretti, N., & Helfand, S. L. (2016). Chromatin-modifying genetic interventions suppress age-associated transposable element activation and extend life span in Drosophila. Proceedings of the National Academy of Sciences, 113(40), 11277–11282. https://doi.org/10.1073/pnas.1604621113

Yao, J., Ding, D., Li, X., Shen, T., Fu, H., Zhong, H., Wei, G., & Ni, T. (2020). Prevalent intron retention fine-tunes gene expression and contributes to cellular senescence. Aging Cell, 19(12), e13276. https://doi.org/10.1111/acel.13276

Zhang, X., Liu, X., Du, Z., Wei, L., Fang, H., Dong, Q., Niu, J., Li, Y., Gao, J., Zhang, M. Q., Xie, W., & Wang, X. (2021). The loss of heterochromatin is associated with multiscale three-dimensional genome reorganization and aberrant transcription during cellular senescence. Genome Research, 31(7), 1121–1135. https://doi.org/10.1101/gr.275235.121

Zheng, J.-T., Lin, C.-X., Fang, Z.-Y., & Li, H.-D. (2020). Intron Retention as a Mode for RNA-Seq Data Analysis. Frontiers in Genetics, 11. https://www.frontiersin.org/articles/10.3389/fgene.2020.00586