Activation of transposable elements during aging and neuronal decline in *Drosophila*

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We found that several transposable elements were highly active in *Drosophila* brain during normal aging. In addition, we found that mutations in *Drosophila Argonaute 2* (Ago2) resulted in exacerbated transposon expression in the brain, progressive and age-dependent memory impairment, and shortened lifespan. These findings suggest that transposon activation may contribute to age-dependent loss of neuronal function.

Transposable elements are mobile genetic elements that constitute a large fraction of most eukaryotic genomes1,2. Retrotransposons, which replicate through an RNA intermediate, represent approximately 40% and 30% of the human and *Drosophila* genomes, respectively. Mounting evidence suggests that transposable elements can be active not only in the germline, but also in the brain. LINE-1 elements, for instance, are actively mobilized in normal mammalian brains during neurogenesis3–5, leading to genetic instability as well as RNA-level toxicity. Transposable element activation has been correlated with several neurodegenerative disorders6–13.

We examined transposon expression in *Drosophila*, where it is feasible to manipulate their control mechanisms and to measure physiological effects on the nervous system. We first used quantitative real-time PCR (QPCR) for several transposon transcripts in head tissues during normal aging, comparing transcript levels from 2–4-d-old adult wild-type flies with those of ~14-, ~21- and ~28-d-old counterparts. Transcripts from R2, a LINE-like element, and gypsy, an LTR element, were markedly elevated in aged, relative to young, flies (Fig. 1a). R1, another LINE-like element, also showed elevated expression with age (see below). Although we did not exhaustively examine expression of the transposable element families in the *Drosophila* genome, the age-dependent expression may affect certain transposons specifically, as we did not see effects on gypsy4 or Zam (data not shown). In addition to the effects on transcripts from gypsy, R1 and R2, we detected an age-dependent increase in expression of the gypsy membrane glycoprotein ENV using immunohistochemical staining in whole-mount brains (Fig. 1b). The ENV signal was most intense in the cortical regions that contain most of the cell bodies, but was also detected in neuropil, areas containing axons and dendrites (Fig. 1b). This age-dependent de-repression was not a result of loss of expression of either Dicer-2 or AGO2, which are required for transposable element silencing in somatic tissues14 (Supplementary Fig. 1).

To determine whether expression of gypsy in older flies is associated with physical transposition, we designed a gypsy-TRAP reporter system to detect *de novo* gypsy integration events, adapting a system that was previously established for detecting gypsy integration in the germline15. In the gypsy-TRAP reporter, insertions of either Dicer-2 or AGO2, which are required for transposable element silencing in somatic tissues14 (Supplementary Fig. 1).

We used this system to screen for *de novo* gypsy integration events in the brain by focusing on neurons of the mushroom body, for which highly specific and strongly expressing transformant lines was sufficient to silence GFP (Fig. 2a). GAL80 expression from our MB247 GAL4 line16, which is known to label about 800–2,500 mushroom body Kenyon cell neurons per brain hemisphere (Fig. 2a). GAL80 expression from our gypsy-TRAP (Tubp-OvoSite-GAL80) transformant lines was sufficient to silence GFP (Fig. 2 and Supplementary Fig. 2). In fact, we did not observe any labeled neurons in 2–4-d-old flies containing this construct (0 of 26 brains; Fig. 2b, c, Supplementary Fig. 2 and Supplementary Table 1). However, we did observe sparse GFP-labeled mushroom body Kenyon cells at later ages in each of two transformant lines containing gypsy-TRAP, often in multiple neurons (14 of 39 brains labeled from 28–35-d-old flies; Fig. 2e, Supplementary Fig. 2 and Supplementary Table 1). This effect of age was significant.
Figure 2  gypsy-TRAP reporter detects de novo integration in neurons in aged flies. In the presence of gypsy integration into the gypsy-TRAP, GAL80 expression was blocked, and UAS::mCD8::GFP was turned on (Supplementary Fig. 2). (a) Approximately 800 mushroom body Kenyon cell neurons per brain hemisphere were labeled by MB247-GAL4-driven UAS::mCD8::GFP. (b) An example brain from a 2–4-d-old mutated gypsy-TRAP; UAS::mCD8::GFP/+; MB247/+ fly. No GFP-labeled neurons were observed. (c) An example brain from a ~28-d-old mutated gypsy-TRAP; UAS::mCD8::GFP/+; MB247/+ fly. No GFP-labeled neurons were observed. (d) An example brain from a 2–4-d-old gypsy-TRAP; UAS::mCD8::GFP/+; MB247/+ fly. No GFP-labeled neurons were observed. (e) Example brains from ~28-d-old gypsy-TRAP; UAS::mCD8::GFP/+; MB247/+ flies. Several GFP-labeled mushroom body neurons were observed in each brain. See Supplementary Table 1 and Supplementary Figure 2 for statistical summary and more images.

(χ² test, P < 0.01). The labeling appeared to be stochastic, as we observed both intra- and interhemisphere variation. The accumulation of GFP-positive neurons also required the five Ovo binding sites, as is true for gypsy insertions in the germline, as we found no GFP-labeled cells in control transformant lines containing a gypsy-TRAP with an ovo fragment in which the binding sites were mutated (UBiP-MutatedOvoSite-GAL80, χ² test, P < 0.001; Fig. 2b, Supplementary Fig. 2 and Supplementary Table 1). These results strongly support the conclusion that gypsy is not only expressed in the neurons of aging flies, but is actively mobile in an age-dependent manner (Supplementary Fig. 3).

We next used genetic manipulations of Ago2 to create a situation in which transposons are unleashed prematurely in young flies. In both animals and plants, transposable element control is mediated by Argonaute proteins guided by small regulatory RNAs14. Germline tissues are protected against transposable elements by the concerted action of Argonaute proteins of the PIWI clade and their small RNA partners, the piRNAs14. Although control of transposable elements in somatic tissues in Drosophila is dependent on AGO2, guided by endogenous small interfering RNAs, a different Argonaute protein in flies, AGO1, preferentially loads the miRNAs that target cellular mRNAs, but has no known effect on transposable elements. Thus, it is possible to use Ago2 mutants to create a condition in which the somatic transposable element control mechanism is selectively disrupted.

Although Ago2 mutants have been shown to exhibit elevated transposable element expression in somatic tissue, the phenotypic consequences of such mutations on aging are not known. We found that R2 and gypsy transcripts were substantially elevated in head tissue from young Ago2414 and Ago2518 mutant flies, as well as in transheterozygous Ago2414/518 flies (Fig. 3a and Supplementary Fig. 4a). In addition, the age-dependent elevation of both R2 and gypsy expression was accelerated in the Ago2 mutants, such that transcript levels of both R2 and gypsy in 2–4-d-old mutant flies were comparable to those seen in ~28-d-old wild-type flies. At the protein level, we observe an accelerated age-dependent increase in ENV in Ago2 mutants (the Ago2414 and Ago2518 hypomorphic alleles and the Ago2254 null allele) in both whole-mount brains (Fig. 3b and Supplementary Fig. 5b) and western blots from adult heads (Fig. 3c and Supplementary Fig. 5a). Furthermore, elevated expression of gypsy in Ago2 mutants was also associated with de novo insertions into the ovo locus, as detected by genomic PCR and sequencing (Supplementary Fig. 3).

To investigate the correlation between age-dependent neuronal decline and transposable element activation, we used a Pavlovian learning and memory assay that is well established in Drosophila17. We compared 24-h long-term memory (LTM) performance in flies that were trained when they were young (2–4 d) or when they were at an intermediate age (~20 d). Ago2 mutants exhibited a partial reduction in memory by 2–4 d (Fig. 3d, Supplementary Table 2 and Supplementary Fig. 4c,d), an effect that was rescued by neuronal expression of an Ago2 transgene (Supplementary Fig. 4e). The mild defect seen in young flies became markedly worse in 20-d-old adults (Fig. 3d). In contrast, wild-type flies trained at ~20 d showed LTM equivalent to that seen in 2–4-d-old wild-type flies (Fig. 3d), and only developed impairment at ~28 d of age (Supplementary Fig. 6b).

We also examined the effects of Ago2 mutations on longevity and found that Ago2414, Ago2518 and Ago2254 mutants exhibited shorter lifespans than their wild-type counterparts (Fig. 3e and Supplementary Fig. 5c). This finding is consistent with reports that mutations in Dicer-2 (ref. 18) and loquacious19, other components of the somatic small RNA–dependent transposable element silencing pathway, also result in short lifespan. Although Ago2 mutants are susceptible to exogenous viruses, viral infections did not contribute to the age-dependent decline in these mutants and did not cause the observed induction of transposons (Supplementary Fig. 7).

Transposable element activation in the germline is sufficient to cause sterility, at least in part by triggering Checkpoint kinase 2 (Chk2)-mediated DNA damage–induced apoptosis. In fact, disruption of Chk2 in the germline prevents cells from undergoing programmed cell death, which is sufficient to suppress transposable element–dependent sterility20. To test whether DNA damage leading to Chk2 signaling also contributes to age-dependent mortality in wild-type flies, we used an RNAi transgene to target loki, the Drosophila ortholog of chk2. Notably, we found that disrupting loki function exclusively in neurons by expressing loki RNAi under the control of the pan-neuronal elav-GAL4 delayed mortality (Fig. 3f and Supplementary Fig. 7a). This disruption of loki function also yields a modest delay in age-dependent memory impairment (Supplementary Fig. 6b,c). Although a causal
role for transposable element activation and transposable element–
induced DNA damage in age-related neuronal decline remains to be
demonstrated, the effects of disruption of Chk2 signaling are at least
consistent with this interpretation.

There is accumulating evidence that transposable elements actively
mobilize in neurons3,4. This phenomenon has led to the suggestion
that regulated transposable element jumping provides a source of
somatic mosaicism that may contribute to normal brain physiology,
although such functional effects remain to be established. On the other
hand, LINE, SINE and LTR elements are de-repressed in a variety
of neurodegenerative diseases6–13, suggesting that misregulation of
transposable elements is detrimental. Our results, together with pre-
vious studies, suggest that transposable element activation with age
or disease may contribute to neuronal decline.

METHODS

Methods and any associated references are available in the online
version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

W.L., L.P. and J.D. conceived and designed the project and analyzed the
experiments. W.L. performed the behavior experiments and western blots. The
QPCR and lifespan analyses were performed by L.P. with assistance from W.L.,
L.K. and D.T. L.P., N.C. and S.G. did the imaging. W.L., L.P. and J.D. wrote the
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The authors declare competing financial interests: details are available in the online
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ONLINE METHODS

Fly stocks. The wild-type flies that we used were w^1118 (isoCJ1), a Canton-S derivative^{21}. The Ago2 mutants and UAS::Ago2 transgenic strains were back-crossed to the w^1118 (isoCJ1) flies for at least five generations. Flies were cultured in standard fly food at 22.5 °C. The gypsy-TRAP transgenic flies were made by cloning a ~500-bp Ovo binding site^{15} into the NotI site between α-tubulin promoter and GAL80 gene in the Tubg-GAL80 in pcSpE84 plasmid. The resulting construct was injected into w^1118 (isoCJ1) recipient embryos and transformant lines were isolated by standard procedures (BestGene). The mutated gypsy-TRAP transgenic flies were made by injecting a similar construct bearing mutations in Ovo binding sites^{15}. The MB247, Repo and Elav-GAL4 lines are as reported previously^{22}.

Behavioral assays. Aversive Pavlovian olfactory task was performed by training flies in a T-maze apparatus using a Pavlovian conditioning procedure. Approximately 50–100 flies were loaded into an electrifiable training grid. For a single training session, flies were exposed sequentially to one odor (the conditioned stimulus), which was paired with a 60-V electric shock and then a second odor (the unconditioned stimulus) without shock. The flies were tested 2 min after this training session and allowed to choose between the two odors. A half performance index was calculated by dividing the number of flies that chose correctly, minus the flies that chose incorrectly, by the total number of flies in the experiment. The same protocol was then performed with another group of 50–100 flies and reciprocal odor presentation. The final performance index was calculated by averaging both reciprocal half performance indices. The LTM experiment was an adaptation of this training protocol. Flies were subjected to ten such training sessions in robotic trainers spaced out with a 15-min rest interval between each. Flies were then transferred into food vials and incubated at 18 °C until testing 24 h after the training. All genotypes were trained and tested in parallel, and rotated between all the robotic trainers to ensure a balanced experiment. Odor pairs and concentrations used for these behavior procedures were 3-octanol (1.5 × 10^{-3}, vol/vol) and benzaldehyde (0.5 × 10^{-3}, vol/vol). Pure odors were made with Bonferroni corrections.

Lifespan. Lifespan was measured with ~50–150 flies per genotype. Equal numbers of male and female flies were used for each genotype. Survival analyses were performed with the Kaplan-Meier method. Log-rank test and Gehan-Breslow-Wilcoxon test were used to compare survival curves. Pair-wise comparisons were made with the Kaplan-Meier method. Log-rank test and Gehan-Breslow-Wilcoxon test were used to compare survival curves. Pair-wise comparisons were made with Bonferroni corrections.

QPCR. QPCR was performed according to the assay manual. In brief, massive numbers of fly heads were collected for each genotype and total RNA was purified with Trizol (Invitrogen) and treated by DNase I (Promega). Total RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer. For the reverse transcription reaction, each 20-µl reaction was performed with 2 µg of total RNA using the High capacity RNA-to-cDNA kit (Applied Biosystems). The QPCR reactions for each assay were carried out in duplicate, and each 20-µl reaction mixture included 1 µl of previous reverse transcription products. The QPCR reaction was carried out and analyzed in an Applied Biosystems 7900HT Fast Real-Time PCR System in 96-well plates at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Linearity tests were performed on all custom-designed primers and probes to ensure linearity.

Custom TaqMan probes. All TaqMan Gene Expression Assays (Applied Biosystems) used the FAM Reporter and MGB Quencher. TaqMan probes for each transcript were designed following the vendor’s custom assay design service manual. The customized gene-specific TaqMan probes and inventoried TaqMan probes had the following sequences and assay IDs: R1-ORF2 (assay ID AID1DT0, F8Gn0003968; probe: 5’-ACATAAGCCGAGTAGCTCG-3’), R25 (assay ID A0V12, F8Gn0003949; probe: 5’-GAATGCGCTTCCCAAATGAGGACC-3’), R23 (assay ID A1Y9XVU, F8Gn0003939; probe: 5’-TGAAATAATTTGTCGGAACAGTTAGTGTAGGCCTCGCG-3’), DBV (assay ID AIV13Y; probe: 5’-CCATATTAGTGATCGGCTCGCG-3’), DTRV (assay ID AIS0573; probe: 5’-CTTGGATCCCGAGTAGCTCG-3’), DAV (assay ID A1X00AZ; probe: 5’-AAGTGTAGGTTCAATTGTCTGG-3’), Sigma V (assay ID AIW14R; probe: 5’-CCGAGTCTCCGATGTTCC-3’), Nora V (assay ID AIQ19N; probe: 5’-CTGAGGCTCTTGGTGTATAT-3’), DCV (assay ID IA699F; probe: 5’-TCTAGATGATCTGAAATTT-3’), ANV (assay ID AIT95SB; probe: 5’-CAGACAATTTTCCTCGAATCAT-3’), Act5 (assay ID DM01811114_g1), Loki (assay ID DM01805432_g1 and DM01805433_g1) and Dcr-2 (assay ID DM01821537_g1 and DM01821540_g1).

Western blots. We homogenized ~15 adult fly heads per sample in 20 µl of Nupage sample loading buffer, heated them to 95 °C for 5 min and loaded 10 µl onto Nupage 4–12% Bis-Tris gels, followed by transferring to PVDF membrane (Invitrogen) and blotting using standard protocols. We used antibodies to tubulin (1:10,000, catalog #E7-a, Developmental Studies Hybridoma Bank), the ENV monoclonal antibody (clone 7B3)^{24} was used at 1:5,000. The WesternBreeze Chemiluminescent Kit–Anti-Mouse system was used to visualize the blotted bands on films.

Bleach treatment of embryos. To remove virus infection in fly stocks, we collected 2-h embryos from wild-type controls and Ago2 mutants and treated them with 50% bleach twice, 20 min each time. Treated embryos were then grown in a virus-free clean room equipped with ultraviolet lamps to sterilize surfaces. Expanded fly stocks after bleach treatment are proven to be virus free. All strains were grown on a rotating set of six antibiotics.

Immunohistochemistry and GFP imaging. Dissection, fixation, immunolabeling and confocal imaging acquisition were performed as previously described^{23}. Ascites containing monoclonal antibody to gypsy ENV was prepared from the gypsy ENV 7B3 hybridoma cell line^{24}. A 1:100 dilution of ENV primary monoclonal antibody and a 1:200 dilution of secondary antibody of Cy3-conjugated goat antibody (Molecular Probes, A10521) to mouse IgG were used. We used 2 µM DiIC18(5)-DS lipophilic dye solution (Molecular Probes) to label cell membranes throughout the brain as counterstaining. For ENV immunolabeling, we imaged multiple brains of each genotype and age. Total numbers imaged for wild type were 6 (0–4 d), 14 (14 d), 16 (21–28 d) and 4 (70 d). For Ago2^{21}, the total numbers imaged were 8 (14 d) and 7 (21–28 d). For Ago2^{21}, the total numbers imaged were 5 (0–4 d), 16 (14 d), 6 (21–28 d) and 3 (70 d). For Ago2^{21}, the total numbers imaged were 9 (0–4 d), 13 (14 d) and 6 (21–28 d).

Nested PCR. Standard DNA was extracted from ~300 fly heads of the indicated ages. Standard PCR was performed in nested fashion with the first round of PCR using primers 1 and 3 followed by a second round of PCR with primers 2 and 4 (primer 1: CAACCTCGACCAACGACTA; primer 2: CACACACCCATGGAATAGGAA; primer 3: CAGCAGAAGGTGACATCTC; primer 4: GGTCATGCGGTTAACCAT). Nested PCR was then run on 0.9% agarose gel (Sigma) and size was estimated according to 1 kb plus DNA ladder (Invitrogen). The PCR product was then gel purified using illustra GFX PCR DNA and Gel Band Purification Kit from GE Healthcare. The fragment was cloned using the TOPO TA Cloning Kit for sequencing from Invitrogen and sequenced by ELIM BIOPHARM using the Sanger sequencing method. MacVector was used to display sequencing results.

Statistical testing. Behavioral data from the Pavlovian memory task were normally distributed^{21}. For these data, one-way ANOVA and post hoc analyses were performed. For the lifespan curves, survival analyses were performed with the Kaplan-Meier method. Log-rank test and Gehan-Breslow-Wilcoxon test were used to compare survival curves. Pair-wise comparisons were made with Bonferroni corrections.

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