The microRNA miR–34 modulates ageing and neurodegeneration in Drosophila

Nan Liu1, Michael Landreh1, Kajia Cao2,3, Masashi Abe1, Gert-Jan Hendriks1, Jason R. Kennerdell1, Yongqing Zhu1, Li-San Wang2,4,5 & Nancy M. Bonini1,6

Human neurogenerative diseases have the temporal hallmark of afflicting the elderly population. Ageing is one of the most prominent factors to influence disease onset and progression, yet little is known about the molecular pathways that connect these processes. To understand this connection it is necessary to identify the pathways that functionally integrate ageing, chronic maintenance of the brain and modulation of neurodegenerative disease. MicroRNAs (miRNA) are emerging as critical factors in gene regulation during development; however, their role in adult-onset, age-associated processes is only beginning to be revealed. Here we report that the conserved miRNA miR-34 regulates age-associated events and long-term brain integrity in Drosophila, providing a molecular link between ageing and neurodegeneration. Fly mir-34 expression exhibits adult-onset, brain-enriched and age-modulated characteristics. Whereas mir-34 loss triggers a gene profile of accelerated brain ageing, late-onset brain degeneration and a catastrophic decline in survival, mir-34 upregulation extends median lifespan and mitigates neurodegeneration induced by human pathogenic polyglutamine disease protein.

Some of the age-associated effects of miR-34 require adult-onset translational repression of Eip74EF, an essential ETS domain transcription factor involved in steroid hormone pathways. Our studies indicate that miRNA-dependent pathways may have an impact on adult-onset, age-associated events by silencing developmental genes that later have a deleterious influence on adult life cycle and disease, and highlight fly miR-34 as a key miRNA with a role in this process.

Recent evidence reveals that miRNA pathways are important in the adult nervous system, notably in the maintenance of neurons and in the regulation of genes and pathways associated with neurodegenerative disease. Given these findings, we considered that there may be a fundamental role for select miRNAs in ageing. We examined flies carrying a hypomorphic mutation in loquacious (loq), a key gene in fly miRNA processing 6 (Supplementary Fig. 1a). Flies bearing the loq mutation were viable, but detailed examination indicated a significantly shortened lifespan (Supplementary Fig. 1b). Further analysis indicated that log2 9971 flies showed late-onset brain morphological deterioration: although normal as young adults, by 25 days log2 9971 flies developed large vacuoles in the retina and lamina of the brain (Supplementary Fig. 1c). Although developmental processes may contribute to shortened lifespan, the adult-onset brain degeneration of log2 9971 mutants indicated that one or more specific miRNAs may be critically involved in age-associated events impacting on long-term brain integrity.

To explore this question, we determined whether specific miRNAs displayed age-modulated expression in the brain. RNA was isolated from dissected brains of adult flies of young (3 days), mid (30 days) and old time points (60 days). Using an array for Drosophila miRNAs, 29 were expressed in the adult brain (Fig. 1a). Whereas most miRNAs maintained a steady level or decreased with age, one miRNA, mir-34, increased (Fig. 1a). Small RNA northern blot analysis confirmed that mir-34 expression was barely detectable during development, but became high in the adult and was further upregulated with age (Supplementary Fig. 2a, b). Expression of mir-34 was affected in loq flies (Supplementary Fig. 1d). miR-34 falls into a category of Drosophila miRNAs whose processing requires the exoribonuclease nibbler (nbr). In the adult, mature miR-34 displayed three major differentially sized forms (24 nucleotides, 22 nucleotides and 21 nucleotides) with a uniform 5’ end, descending by single nucleotides at the 3’ end which result from nbr-mediated trimming; only isoform c became upregulated with age (Supplementary Fig. 2c and Fig. 1b, c; see also refs 5–7).

MiR-34 is a markedly conserved miRNA, with orthologues in fly, Caenorhabditis elegans, mouse and human showing identical seed sequence (Supplementary Fig. 2d). To define miR-34 function, flies deleted for the gene were generated (Supplementary Fig. 3a). The resulting mir-34 mutant flies retained normal wild-type expression of neighbouring genes, but selectively lacked mir-34 (Supplementary Fig. 3b).
Fig. 3b, c). To interrogate age-associated phenotypes carefully, we generated mir-34 null flies in the same uniform homogeneous genetic background (see Methods). mir-34 mutants displayed no obvious developmental defects, consistent with its adult-onset expression. However, detailed examination of adult animals indicated that mir-34 mutants, although showing normal adult appearance and early survival, displayed a catastrophic decline in viability just after 30 days (Fig. 2a and Supplementary Table 4). Analysis of age-associated functions revealed that young mutants (3 days) had normal locomotion and stress resistance, but by 20 days the mutants had dramatic climbing deficits and were markedly stress-sensitive compared to age-matched controls (Fig. 2b). Because mir-34 expression was brain-enriched, we also examined the brain. Typically, older flies show sporadic, age-correlated vacuoles in the brain—a morphological hallmark of neural deterioration8. mir-34 mutants were born with normal brain morphology, but showed dramatic vacuolization with age, indicative of loss of brain integrity (Fig. 2c). Rescue with a 9-kb genomic DNA fragment containing mir-34 and its endogenous cis-regulatory elements (Supplementary Fig. 3a, b) partially restored the age-associated expression of mir-34 to mir-34 null flies in the same homogeneous genetic background (Supplementary Fig. 3d). Although rescue was not complete, indicative of a complexity in genomic elements that regulate mir-34, rescue was sufficient to mitigate the mutant effects, indicating that mir-34 function normally underlies these age-associated aspects (Supplementary Table 1).

These data indicated that mir-34 mutants were normal as young adults, but with age developed deficits reflective of much older animals, including loss of locomotion, stress sensitivity and brain deterioration,
coupled with shortened lifespan. We therefore hypothesized that loss of mir-34 accelerated brain ageing. To address this, we transcriptionally profiled the fly brain (3 days, 30 days and 60 days) from wild-type animals. On the basis of a linear regression model, we extracted 173 probe sets from this profile the expression of which was tightly correlated with the progression of normal ageing (Fig. 2d and Supplementary Tables 2 and 3). We next made another set of brain transcriptional profiles for mir-34 mutants and controls of matched chronological age (3 days and 20 days). We measured relative changes of these probe sets between 3 days and 20 days within each genotype, and compared the extent of such changes between mir-34 mutants and controls. This indicated that the overall pattern of these probe sets was significantly different between the two genotypes ($P = 0.006$, two-sample, paired Wilcoxon test; Fig. 2d). In particular, most positively correlated probe sets displayed a faster pace of increase in mir-34 mutants compared to controls—thus showing accelerated age-associated expression changes in mir-34 mutants (Fig. 2d). This result, combined with the physiological and histological evidence of more rapid loss of age-associated functions, suggested that mir-34 mutants were undergoing accelerated brain ageing.

miRNAs function by binding to the 3' UTRs of target miRNAs and often result in downregulation of protein translation. We therefore reasoned that age-associated activities of miR-34 might be mediated through silencing of critical targets that have a negative impact on the adult animal. miRNA-target prediction algorithms indicated miR-34 binding sites within the 3' UTR of the Eip74EF gene; notably, these binding sites were conserved in the orthologous Eip74EF genes from different Drosophila species (Supplementary Fig. 4a). We confirmed the miR-34 interaction through mutations in the seed sequences of the predicted miR-34 binding sites in the 3' UTR of the Eip74EF mRNA (Supplementary Fig. 4b). The Eip74EF gene is a component of steroid hormone signalling pathways. Although such pathways have generally been studied for effects during development, data have implicated these pathways in lifespan regulation.

The Eip74EF gene encodes two major protein isoforms, E74A and E74B (referred to as the $E74A$ and $E74B$ genes, respectively); the isoforms share the same 3' UTR (Supplementary Fig. 4a). Northern blots indicated that transcription of $E74A$, but not $E74B$, persisted in adults, overlapping the time period when mir-34 is expressed (Supplementary Fig. 4c). Given this, we focused on $E74A$ as a regulated target of miR-34 in the adult. Despite robust expression of the mRNA transcript, the $E74A$ protein was expressed at low levels in adult heads throughout lifespan (Fig. 3a, b and Supplementary Fig. 4d). In flies lacking miR-34, $E74A$ protein was markedly increased (Fig. 3b); $E74A$ was also de-regulated in the log$_{10}$ mutant flies (Supplementary Fig. 1e). Genomic rescue of $miR-34$ mitigated this de-regulation of the E74A protein (Fig. 3c). Fine temporal analysis indicated that the $E74A$ protein was highly expressed in young flies, but underwent a marked decrease within a 24-h time window (Supplementary Fig. 5). This temporal pattern seemed to be mutually exclusive to that of miR-34 (see Supplementary Fig. 2a). Moreover, in flies lacking miR-34, the downregulation of E74A protein during this critical period was dampened (Supplementary Fig. 5). This evidence indicates that adult-onset expression of $miR-34$ functions, at least in part, to attenuate E74A protein expression in the young adult, and maintain that repression through adulthood (Supplementary Fig. 4d).

We next determined whether deregulated expression of $E74A$ protein contributed to the age-associated defects in $miR-34$ mutants. Because $E74A$ functionality is essential during development, with strong mutations leading to pre-adult lethality, we used the mild, but viable, $E74A^{RG01805}$ hypomorphic mutation (Supplementary Fig. 4a). When the $E74A^{RG01805}$ mutation was combined with $miR-34$ mutant flies in the same homogenous genetic background, proper regulation of $E74A$ protein was partially restored (Fig. 3d), and age-associated defects due to loss of $miR-34$, including shortened lifespan and brain vacuolation, were mitigated (Fig. 3e; f; $E74A^{RG01805}$ mutants alone have a normal lifespan (Supplementary Fig. 6a)). To assess further the adult activity of $E74A$, we upregulated $E74A$ in the adult with an $E74A$ transgene that lacks miR-34 binding sites driven by a temperature-sensitive promoter at 29°C. At 29°C, these flies demonstrated increased levels of $E74A$ expression in the adult (Supplementary Fig. 6b). Notably, these animals also showed late-onset brain degeneration (Supplementary Fig. 6c) and a significantly shortened lifespan (Supplementary Fig. 6d). These data indicate that deregulated expression of $E74A$ has a negative impact on normal ageing, and that one function of $miR-34$ is to silence $E74A$ in the adult to prevent the adult-stage deleterious activity of $E74A$ on brain integrity and viability.

Notably, during the course of these studies, we noted that $miR-34$ mutants also displayed a defect in protein misfolding—a molecular process implicated in ageing and common to many human neurodegenerative diseases. Whereas normally with age, the fly brain accumulates a low level of inclusions that immunostain for stress chaperones like Hsp70/Hsc70, $miR-34$ mutants showed a marked increase compared to control flies of matched age (30 days) (Supplementary Fig. 7). Given that $miR-34$ expression increases with age, and $miR-34$ loss slows altered chaperone accumulation, we tested whether $miR-34$ expression itself is upregulated by stresses like heat shock or oxidative toxins, but found no evidence to support this (data not shown). However, given that loss of $miR-34$ caused an increase in protein misfolding, this raised the possibility that upregulation of $miR-34$ expression might mitigate disease-associated protein misfolding. In Drosophila, expression of a pathogenic ataxin-3 polyglutamine (polyQ) disease protein (SCA3trQ78) leads to inclusion formation, a decrease in polyQ protein solubility and progressive neural loss (Supplementary Fig. 8a). Upregulation of $miR-34$ markedly mitigated polyQ degeneration, such that inclusion formation was slowed, the protein retained greater solubility, and neural degeneration was suppressed (Fig. 3g, h and Supplementary Fig. 8b–d). Lowering $E74A$ expression by heterozygous reduction in flies expressing pathogenic polyQ protein revealed a minimal effect (data not shown), indicating that $E74A$ may not be a target of miR-34 activity in this process. However, our studies with $E74A$ were of necessity limited to hypomorphic alleles that may not uncover the full extent of $E74A$ function mediated by $miR-34$. Furthermore, additional targets of $miR-34$ may be involved in different aspects of $miR-34$-directed pathways, including disease.

Given this effect to mitigate disease-associated neural toxicity with upregulation of $miR-34$, and that $miR-34$ expression naturally increases with age, we investigated whether enhanced expression of $miR-34$ in wild-type flies could modulate the ageing process. We increased $miR-34$ dosage in wild-type flies with genomic rescue transgenes, which express $miR-34$ under its endogenous regulatory elements (see Supplementary Fig. 2a). Analysis of multiple independent transgensics in the same genetic background with that of control indicated that upregulation of $miR-34$ levels with genomic constructs (≈20%, Supplementary Fig. 3d) promoted median survival rate by ≈10% compared to wild type (Fig. 3i); other traits, such as the occurrence of brain vacuolation, despite being an age-associated phenomenon, are sporadic and low in normal flies, thus were difficult to assess. Thus, upregulation of $miR-34$ expression can protect from neurodegenerative disease and extend median lifespan.

Our findings indicate that $miR-34$ in Drosophila presents a key miRNA that couples long-term maintenance of the brain with healthy ageing of the organism. $miR-34$ activity, enhanced by its age-modulated expression and processing, is critically involved in silencing of the $E74A$ transcript through adulthood and in modulation of protein homeostasis with age, as well as in polyQ disease. Select neural cell types may be especially vulnerable in ageing and disease; $miR-34$ function may have an impact on the integrity or activity of these systems. Intriguingly, $E74A$ seems to confer sharply opposing function to controls—thus showing accelerated age-associated expression changes in $miR-34$ mutants (Fig. 2d). This result, combined with the physiological and histological evidence of more rapid loss of age-associated functions, suggested that $miR-34$ mutants were undergoing accelerated brain ageing.
Supplementary Table 4). Flies raised at 29 °C. Lifespan: P < 0.0001 (log-rank test). Mean ± s.e.m., n = 150 male flies. Brain vacuoles: \( *P < 0.01 \) (one-way analysis of variance, with post test: Tukey’s multiple comparison test).

Mean ± s.e.m., n = 10 independent male animals. Genotypes as in d, g. Upregulation of mir-34 reduces accumulation of pathogenic polyQ protein inclusions. Left panels: in the retina of flies expressing SCA3trQ78 alone, pathogenic polyQ protein is initially diffuse (1 day, top), but gradually accumulates into nuclear inclusions (3 day, bottom). Right panels: upregulation of mir-34 reduces inclusion formation. DAPI staining highlights nuclei. 3 day controls show 53.75 ± 12.55 inclusions in a retinal section versus 23.67 ± 7.57 with mir-34 upregulation; mean ± s.d., n = 3 cryosections from independent experiments; P < 0.01 (t-test). Genotypes: SCA3trQ78 is w\(^{+}\); rh1-GAL4, UAS-SCA3trQ78/+. Scale bar, 0.05 mm. h. Upregulation of mir-34 prevents neuron degeneration. At 21 days, male flies expressing SCA3trQ78 show a marked loss of photoreceptor neuronal integrity (middle panel), with an average of only 2.46 ± 1.32 photoreceptors per ommatidium remaining by pseudopupil analysis. Flies with upregulated mir-34 (right panel) retain 6.90 ± 0.34 photoreceptors per ommatidium. Control (left panel) and upregulation of mir-34 alone (not shown) have normal photoreceptor numbers per ommatidium. Mean ± s.d., n = 619, 722 and 700 ommatidia, for SCA3trQ78, SCA3trQ78; mir-34 (+) and control, respectively; \( * * P < 0.0001 \) (one-way analysis of variance, with Bonferroni’s multiple comparison test).

They are not properly regulated. miRNA pathways provide a tantalizing mechanism by which to suppress potentially deleterious age-related activities of such genes; a number of miRNAs have been noted to show age-modulated expression and activity\(^{18,19}\). Roles of
select miRNAs normally expressed in the adult may be of evolutionary advantage to tune-down events that promote age-associated decline and potentially disease, in order to prolong healthy lifespan and longevity. Upregulation of lin-4, a *C. elegans* miRNA with a known developmental role, extends nematode lifespan19, raising the possibility that this upregulation, like the natural increase of *mir-34* expression in *Drosophila*, functions to silence genes that have a negative impact on ageing and potentially promote disease. Notably, *mir-34* expression is elevated with age in *C. elegans*19,20, and mammalian *mir-34* orthologues are highly expressed in the adult brain21 and have also been noted to increase with age and be misregulated in degenerative disease in humans22–26. Current data regarding *mir-34* function indicate that it is neutral or adverse in *C. elegans*19,27, and can be either protective or contributory to age-associated events in vertebrates22–26. Thus, *mir-34* seems to be a key miRNA poised to integrate age-associated physiology; the precise function will reflect the diverse spatiotemporal expression and activity of distinct orthologues, the miRNA target spectrum, as well as the complexity of the adult brain and life cycle. The conservation of *mir-34*, coupled with in-depth comparative analysis of *mir-34* expression, 3’ end processing, targets and pathways in the ageing process of nematodes, flies and mammals, make it a tempting subject for understanding features of ageing and disease susceptibility.

**METHODS SUMMARY**

Flies were grown in standard media at 25 °C unless otherwise specified. Stock lines and *GAL4* driver lines were obtained from the *Drosophila* Stock centre at Bloomington, or are described44. Deletion of the *mir-34* region was made by site-specific recombination. Fly transgenics were generated by standard procedures. Flies were generated or backcrossed a minimum of five generations into a controlled uniform homogeneous genetic background (line 5905 (FlyBase ID FBst0005905, w1118)), to assure that all phenotypes were robust and not associated with variation in genetic background. In this uniform homogeneous genetic background, the lifespan of control flies is highly uniform with repetition when 150 or more individuals are used for lifespan analysis. Negative geotaxis and thermo stress were used to examine fly locomotion and stress resistance, respectively. Adult male heads were processed for paraffin sections as described44. To determine lifespan, newly eclosed males were collected and maintained at 15 flies per vial, transferred to fresh vials every 2 days while scored for survival. A total of 150–200 flies were used per genotype per lifespan; all experiments were repeated multiple times (see Supplementary Table 4). Lifespans were analysed in Excel (Microsoft) and by Prism software (GraphPad) for survival curves and statistics. Techniques of molecular biology, western immunoblots and histology were standard. Fly brain mRNA was prepared using Trizol reagent for array and mRNA analysis, miRNA molecular biology, western immunoblots and histology were standard. Fly brain tissue was processed for paraffin sections as described14. To determine lifespan, newly eclosed individuals are used for lifespan analysis. Negative geotaxis and thermo stress were used for experiments.

**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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**Author Information** The microarray data can be found in the Gene Expression Omnibus (GEO) of NCBI through accession number GSE25009. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to N.M.B. (nbonini@sas.upenn.edu).

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of 108 bp of mir-277 sequence. In addition, a modified mir-34 genomic rescue construct was made (pCaSpeR4 vector), which contains the upstream and downstream ends of the original mir-34 genomic rescue construct, with a small deletion of mir-277 mature sequence. The genomic regulation of mir-34 seems complex, despite this standard manipulations for gene rescue, the genomic rescue expression of mir-34 and extent of phenotypic rescue of mir-34 mutants was only partial. We attempted upregulation of mir-34 with the GAL4-UAS system, including with the conditional gene switch system in adults. Uprregulation of mir-34 during development of non-germline tissues (when it normally is not expressed; Supplementary Fig. 2a) was deleterious, and we were unable to upregulate mir-34 expression more robustly than with the genomic constructs.

For western immunoblots, 10 adult male heads per sample were homogenized in 50 μl of Laemmli buffer (Bio-Rad) supplemented with 5% 2-mercaptoethanol, heated to 95 °C for 5 min and 10 μl loaded onto 4–12% Bis-Tris gels (NuPage), then transferred to nitrocellulose membrane (Biorad) and blotted by standard protocols. Primary antibodies used were anti-tubulin (1:10,000, E7, Developmental Studies Hybridoma Bank), anti-E74A (a gift of C. Thummel). Secondary antibodies for immunoblots were goat anti-mouse conjugated to HRP (1:2,000, Chemicon) and developed with chemiluminescence (ECL, Amersham). The final image was obtained by Fuji scanner (Fujifilm).

Total RNA was isolated from 50–200 male heads per genotype, by cutting off heads with a sharp razor, then putting heads into Trizol reagent. Heads were ground by pestle, then RNA was isolated following the manufacturer’s protocol (Trizol reagent, Invitrogen). 5 μg RNA was used per lane. Gel running (1% agarose) and blot transfer (nylon plus) were according to recommended procedures (NorthernMax, Ambion). The RNA blot was then used for hybridization following standard procedures at 68 °C, with pre-hybridization (−1 h), hybridization (−12 h or overnight) with P32-labelled probe, washed and exposed to Phosphoimager (Amersham). RNA probes were used that were made by in vitro transcription of cDNA templates using Maxiscript-IT in vitro transcription kit (Ambion), supplemented with P32-labeled UTP. The cDNA templates were prepared from total RNA by one-step RT–PCR (SuperScript One-Step RT–PCR with Platinum Taq, Invitrogen), with primers: E74A (5′-GTGACAGGGTGGTGGAGACAC-3′/5′-GATAATAACATCATAATAGGGGATGTCATCCTGATCTAAG-3′); E74B (5′-CATGGTGGCTCAAGTGTC-3′/5′-GATAATACGACTCACTATAGGGAGTCGTATTATC-3′). PCR products were then ligated into pGEM-T Easy vector (Promega) and transformed into E.coli DH5α. Positive transformants were selected on ampicillin plates.

Fly age-associated phenotypes. Negative geotaxis and thermo stress were used to examine fly locomotion and stress resistance, respectively. To perform negative geotaxis, groups of 15 adult male flies of indicated age were transferred into a 14-ml polystyrene round-bottom tube (Falcon), and placed in the dark for 30-min recovery. The assay was conducted in the dark, with only a red light on. Climbing ability was scored as the percentage of flies failing to climb higher than 1.5 cm from the bottom of the tube, within 15 s after gently being banded to the bottom. Three repeats were performed for each group and the result averaged. For each genotype at a given age, a minimum of 200 flies were tested. For heat sensitivity, groups of 15 adult males of indicated age were transferred into 14-ml polystyrene round-bottom tubes (Falcon) then placed in a 25 °C incubator for 30 min recovery. Heat stress was applied by immersing the vial containing the flies into a 37 °C water bath for 1 h, followed by a 30-min recovery at 25 °C, then another 1-h heat stress at 37 °C. Flies were then transferred into regular food vials and maintained at 25 °C. Dead flies were counted after 24 h. To assess brain morphology, adult male heads were processed for paraffin sections as described32, and brain vacuoles were counted through continuous sections generated from each head (n = 10 heads counted for each genotype).

Molecular biology. Fly genomic DNA was prepared from whole flies with the Puregene DNA purification kit (Qiagen). To generate mir-34 PUASt constructs, PCR amplification was conducted using genomic DNA as template, with primer pairs of PUASt mir-34-1 (286 bp, PCR primer 5′-GGGTGATACACGACT ATTCTGATTACAC-3′/5′-CCTCCTGCTATCAATTATTTCT-3′) and PUASt mir-34-2 (210 bp, PCR primer 5′-ACCTGGGGGCTTTTATTACC-3′/5′-CACTTCCTCTGTTTGTGATG-3′). PCR products were then ligated into the PUASt vector. mir-277/dfmr1 rescue construct was made in the pCaSpeR4 vector, which contained two parts. Part 1 was a genomic DNA fragment (7,530 bp) harbouring the mir-277 sequence (PCR primers: 5′-GGTGGTGATACACGACT ATTCTGATTACAC-3′/5′-GAGTTGTGGTGGTGGAGACAC-3′), and part 2 was a genomic fragment containing dfmr1 genomic sequence, derived from the pBS WTR construct (a gift from T. Jongens30), by BamH1 and Pspml. The mir-34 genomic rescue construct was also made in the pCaSpeR4 vector, with two parts. One was a genomic DNA fragment (6,855 bp) upstream of mir-34 sequence (PCR primers: 5′-GGTGGTGATACACGACT ATTCTGATTACAC-3′/5′-GAGTTGTGGTGGTGGAGACAC-3′), and the other was a genomic DNA fragment (2,111 bp) containing mir-34 sequence (PCR primers: 5′-GCAAGGAGGTAATGAGGAAATAGTGAAGGAGCTGATTATC3′/5′-CTGTTACAACATCGGAAAATCT-3′). The resultant construct, therefore, contains mir-34 sequence, including most upstream fragment, with the exclusion of...
with a ~50/50 ratio of males and females. RNA was prepared using the mirVana RNA extraction system (Ambion) yielding ~2.5 μg per 100 brains. RNA was eluted into 80 μl of RNase free water (Fisher Scientific) and stored at ~80 °C. miRNA profiling was carried out at the Penn microarray core facility using miRCURY LNA arrays (Exiqon) and protocols. Exiqon’s Hy3/H5-labelling kit was used (Exiqon). RNA samples were labelled with Hy3 and hybridized together with a Hy5-labelled common reference standard. The common reference standard consisted of equal amounts of RNA from brains of 3 days, 30 days and 60 days flies. The miRNA microarray data were analysed at the Penn Bioinformatics Core. Raw data was imported into GeneSpring 1.0 (Agilent) and normalized using a global LOESS regression algorithm (locally weighted scatterplot smoothing). Relative expression levels were calculated as the log₂ normalized signal intensity difference between the Hy3 and Hy5 intensity. Present/absent flagging was analysed by Exiqon (Exiqon). Expression levels (fold changes) for the 30 day and 60 day time point were calculated relative to the 3 day time point. The data sets were exported into Spotfire DecisionSite 9.0 (Tibco) for visualization and filtering.

**miRNA microarray analysis.** For ageing microarray analysis, fly stock Iso31 was used. For mir-34 mutant microarray analysis, mir-34 null line-1 in 5905 background was used, with fly 5905 line, as control. To generate an ageing profile, flies were aged to 3 days, 30 days and 60 days, and 30–50 brains dissected per time point, per replicate, as above (50–50 males and females). For each time point, three replicates were conducted. For mir-34 mutant microarray analysis, time points were 3 days and 20 days, and for each time point, 20 brains from male flies of the appropriate genotype were used, with five replicates in total. Microarray hybridization and reading was performed at the Penn Microarray Core Facility. For miRNA microarrays, total RNA was reverse transcribed to ss-cDNA, followed by two PCR cycles using the Ovation RNA amplification system V2 (Ovation). Quality control on both RNA and ss-cDNA was performed using an 2100 Agilent Bioanalyzer (Quantum Analytics). The CDNA was labelled using the FL-Ovation cDNA Biotin Module V2 (Ovation), hybridized to Affymetrix Drosophila 2.0 chips (Affymetrix) and scanned with an Axon Instruments 4000B Scanner using GenePix Pro 6.0 image acquisition software (Molecular Devices). Affymetrix .cel (probe intensity) files were exported from GeneChip Operating Software (Affymetrix). The .cel files were imported to ArrayAssist Lite (Agilent) in which GCRMA probe-set expression levels and Affymetrix absent/present/marginal flags were calculated. Statistical analysis for those genes passing the flag filter was performed using Partek Genomics Suite (Partek). The signal values were log₂ transformed and a 2-way ANOVA was performed. Then, a linear regression model was used to compute the significance of a correlation between age and gene expression. This approach assumes a linear relationship between age and log₂ expression level:

\[ Y_{ij} = \mu + \beta_1 A_i + \epsilon_{ij} \]

In this equation, \( Y_{ij} \) is the log₂ gene expression level of probe set \( i \) in sample \( j \), \( A_i \) is the age for individual \( j \), the coefficients \( \beta_1 \) is regression coefficients reflecting the rate of change in gene expression with respect to age. Probe sets with expression significantly correlated with age (\( P \leq 0.001 \) for \( \beta_1 \)) were determined. Then the same probe sets were used to estimate the relative expression in separate profiles of mir-34 mutants and age-matched controls. The average levels of each individual probe set were calculated for the difference between 20 day and 3 day, within the same genotype (that is, \( \Delta 20 \text{day}/\Delta 3 \text{day} \)) for each gene in controls and mir-34 mutants, respectively. These differences were then compared between genotypes (that is, mir-34 mutants — controls). The significance of the difference between genotypes was analysed using a paired Wilcoxon test. The difference between control and mutant samples in positively correlated genes (Fig. 2d) is not by chance (\( P = 0.0001 \)).

Transcriptional analysis of ageing status. We first used the wild type to extract age-associated probe sets and then compared the relative changes of these probe sets in a separate set of transcriptional profiles generated for the wild type and mir-34 mutant. For transcriptional profiles of normal aged brains, the GCRMA package RNA (J. Z. Wu, J. MacDonald and J. Gentry, GCRMA: background adjustment using sequence information, R package version 2.14) for R/Bioconductor was used to generate log₂ expression levels for probe-set IDs from the original .cel files. Then, a linear regression model was used to compute the significance of a correlation between age and gene expression. This approach assumes a linear relationship between age and log₂ expression level:

\[ Y_{ij} = \mu + \beta_1 A_i + \epsilon_{ij} \]

In this equation, \( Y_{ij} \) is the log₂ gene expression level of probe set \( i \) in sample \( j \), \( A_i \) is the age for individual \( j \), the coefficients \( \beta_1 \) is regression coefficients reflecting the rate of change in gene expression with respect to age. Probe sets with expression significantly correlated with age (\( P \leq 0.001 \) for \( \beta_1 \)) were determined. Then the same probe sets were used to estimate the relative expression in separate profiles of mir-34 mutants and age-matched controls. The average levels of each individual probe set were calculated for the difference between 20 day and 3 day, within the same genotype (that is, \( \Delta 20 \text{day}/\Delta 3 \text{day} \)) for each gene in controls and mir-34 mutants, respectively. These differences were then compared between genotypes (that is, mir-34 mutants — controls). The significance of the difference between genotypes was analysed using a paired Wilcoxon test. The difference between control and mutant samples in positively correlated genes (Fig. 2d) is not by chance (\( P = 0.0001 \)).