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The microRNA *miR-34* modulates aging and neurodegeneration in *Drosophila*

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Abstract

Human neurodegenerative diseases possess the temporal hallmark of afflicting the elderly population. Hence, aging is among the most significant factors to impinge on disease onset and progression[1], yet little is known of molecular pathways that connect these processes. Central to understanding this connection is to unmask the nature of pathways that functionally integrate aging, chronic maintenance of the brain and modulation of neurodegenerative disease. microRNAs (miRNA) are emerging as critical players in gene regulation during development, yet their role in adult-onset, age-associated processes are 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*, presenting such a molecular link between aging and neurodegeneration. Fly *miR-34* expression is adult-onset, brain-enriched and age-modulated. Whereas *miR-34* loss triggers a gene profile of accelerated brain aging, late-onset brain degeneration and a catastrophic decline in survival, *miR-34* upregulation extends median lifespan and mitigates neurodegeneration induced by human pathogenic polyglutamine (polyQ) 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. These studies indicate that miRNA-dependent pathways may impact 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

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Author contributions

N.L. and N.M.B. conceived and designed the project. N.L., M.L., M.A., G.J.H., J.K., and Y.Z. planned, executed and analyzed experiments. K. C. and L.S-W. performed aging computational modeling. N.L. and N.M.B. wrote the manuscript with input from all authors.

Recent evidence reveals that miRNA pathways are important in the adult nervous system, notably in maintenance of neurons and in regulation of genes and pathways associated with neurodegenerative disease[2,3]. Given these findings, we considered that there may be a fundamental role for select miRNAs in aging. We examined flies carrying a hypomorphic mutation in *loquacious* (*loqs*), a key gene in fly miRNA processing[4] (Supplementary Fig. 1a). Flies bearing the *loqs*^{f00791} mutation were viable, but detailed examination indicated a significantly shortened lifespan (Supplementary Fig. 1b). Further analysis indicated that *loqs*^{f00791} flies showed late-onset brain morphological deterioration: although normal as young adults, by 25d *loqs*^{f00791} 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 *loqs*^{f00791} mutants indicated that one or more specific miRNAs may be critically involved in age-associated events impacting 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 (3d), mid (30d) and old time points (60d). 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 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). *miR-34* expression was affected in *loqs*^{f00791} (Supplementary Fig. 1d). *miR-34* falls into a category of *Drosophila* miRNAs whose processing requires the exoribonuclease *nibbler* (*nbr*)[5,6] In the adult, mature *miR-34* displayed three major differentially-sized forms (21nt, 22nt and 24nt) 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; also[5–7]).

miR-34 is a markedly conserved miRNA, with identical seed sequence of orthologues among fly, *C elegans*, mouse, and human (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 neighboring genes, but selectively lacked *miR-34* (Supplementary Fig. 3b, c). To carefully interrogate age-associated phenotypes, 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 30d (Fig. 2a, Supplementary Table 4). Analysis of age-associated functions revealed that young mutants (3d) had normal locomotion and stress resistance, but by 20d the mutants had dramatic climbing deficits and were strikingly stress-sensitive compared to age-matched controls (Fig. 2b). Since *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 deterioration[8]. *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 aging. To address this, we transcriptionally profiled the fly brain (3d, 30d, and 60d) from wild type animals. Based on a linear regression model[9], we extracted 173 probesets from this profile whose expression was tightly correlated with the progression of normal aging (Fig. 2d, Supplementary Tables 2 and 3). We next made another set of brain transcriptional profiles for *miR-34* mutants and controls of matched chronological age (3d and 20d). We measured relative changes of these probesets between 3d and 20d within each genotype, and compared the extent of such changes between *miR-34* mutants and controls. This indicated that the overall pattern of these probesets was significantly different between the two genotypes (p=0.006, two-sample, paired Wilcoxon test; Fig. 2d). In particular, the majority of positively-correlated probesets 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 and Supplementary Table 2). 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 aging.

miRNAs function by binding to the 3'UTRs of target mRNAs 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 3'UTR of the *Eip74EF* gene; significantly, 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 signaling pathways. Whereas such pathways have generally been studied for effects during development, data have implicated these pathways in lifespan regulation[10].

The *Eip74EF* gene encodes two major protein isoforms, E74A and E74B (referred to as the *E74A* and *E74B* genes, respectively[11]); 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 dramatically increased (Fig. 3b); E74A was also de-regulated in the

loqs^{f00791} mutant (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 dramatic decrease within a 24h time window (Supplementary Fig. 5). This temporal pattern appeared 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 suggests 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 if deregulated expression of E74A protein contributed to the age-associated defects in *miR-34* mutants. Because E74A function is essential during development, with strong mutations leading to pre-adult lethality[11], we used the mild, but viable *E74A*^{BG01805} hypomorphic mutation (Supplementary Fig. 4a). When the *E74A*^{BG01805} mutation was combined with *miR-34* mutant flies in the same homogenous genetic background, proper regulation of E74A protein partially restored (Fig. 3d), and age-associated defects due to loss of *miR-34*, including shortened lifespan and brain vacuolization, were mitigated (Fig. 3e, f; *E74A*^{BG01805} mutants alone have a normal lifespan (Supplementary Fig. 6a)). To further assess 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[12]. At 29°C, these flies demonstrated increased levels of E74A expression in the adult (Supplementary Fig. 6b). Strikingly, 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 negatively impacts normal aging, 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.

Intriguingly, in the course of these studies, we noted that *miR-34* mutants also displayed a defect in protein misfolding—a molecular process implicated in aging and common to many human neurodegenerative diseases[13]. 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 striking increase compared to control flies of matched age (30d) (Supplementary Fig. 7). Given that *miR-34* increases with age, and loss showed altered chaperone accumulation, we tested although found no evidence that *miR-34* itself is upregulated by stresses like heat shock or oxidative toxins (data not shown). However, that loss of *miR-34* caused an increase in protein misfolding raised the possibility that upregulating *miR-34* may mitigate disease-associated protein misfolding. In *Drosophila*, expression of a pathogenic ataxin-3 polyQ disease protein (SCA3tr-Q78) leads to inclusion formation, a decrease in polyQ protein solubility, and progressive neural loss[14] (Supplementary Fig. 8a). Upregulation of *miR-34* dramatically mitigated polyQ degeneration, such that inclusion formation was slowed, the protein retained greater solubility, and neural degeneration was suppressed (Fig. 3g, h; Supplementary Fig. 8b, c, d). Lowering *E74A* expression by heterozygous reduction in flies expressing pathogenic polyQ protein revealed a minimal effect (data not shown) indicating 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*. Further, 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 aging process. We increased *miR-34* dosage in wild-type animals with genomic rescue transgenes, which express *miR-34* under its endogenous regulatory elements (see Supplementary Fig. 2a). Analysis of multiple independent transgenics 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 vacuolization, despite being an age-associated phenomenon, are sporadic and low in normal flies, thus were difficult to assess). Thus, upregulation of *miR-34* can protect from neurodegenerative disease and extend median lifespan.

In summary, our findings suggest that *miR-34* in *Drosophila* presents a key miRNA that couples long-term maintenance of the brain with healthy aging of the organism. *miR-34* activity, enhanced by its age-modulated expression and processing, is critically involved in silencing of the *E74A* gene 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 aging and disease[15]; *miR-34* function may impact the integrity or activity of these systems. Intriguingly, *E74A* appears to confer sharply opposing function on animal fitness at different life stages, being essential during pre-adult development[11], but harmful to the adult during aging (this study). This biological property—of a gene being beneficial at one life stage, but damaging at another—is referred to as antagonistic pleiotropy[16]. Genes associated with antagonistic pleiotropy are likely to be evolutionarily retained due to their earlier beneficial function[17]. Their adult-onset activities, however, antagonize the aging process if 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 lifespan[18], raising the possibility that this upregulation, like the natural increase of *miR-34* in *Drosophila*, functions to silence genes that negatively impact aging and potentially promote disease. Provocatively, *miR-34* is elevated with age in *C elegans*[19,20], and mammalian *miR-34* orthologues are highly expressed in the adult brain[21] and have also been noted to increase with age and be misregulated in degenerative disease in humans[22–26]. 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 vertebrates[22–26]. Thus, *miR-34* appears a key miRNA poised to integrate age-associated physiology; the precise function will reflect the diverse spatiotemporal expression and activity of distinct orthologues, the mRNA 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 aging process of worms, flies and mammals, makes it a tempting subject for understanding features of aging 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 center at Bloomington, or are described[14]. Deletion of *miR-34* region was made by site-specific recombination. Fly transgenics were generated by standard procedures. Flies were generated or backcrossed a minimum of 5 generations into a controlled uniform homogeneous genetic background (line 5905 (Flybase ID FBst0005905, w^{1118}), 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 described[21]. To determine lifespan, newly eclosed males were collected and maintained at 15 flies per vial, transferred to fresh vials every two days while scored for survival. A total of 150–200 flies were used per genotype per lifespan; all experiments were repeated multiple times (See Supplemental Table 4). Lifespans were analyzed 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 arrays were the miRCURY™ LNA arrays version 8.1 (Exiqon), and mRNA expression was profiled using Affymetrix *Drosophila* 2.0 chips (Affymetrix). The microarray data can be found in the Gene Expression Omnibus (GEO) of NCBI through accession number GSE25009.

Methods

Genetic background

To control for background effects, and to assess significance of all effects, flies were generated in the same uniform homogeneous genetic background (line 5905 (Flybase ID FBst0005905, w^{1118})), or backcrossed a minimum of 5 generations into this uniform genetic background. This assured that, for all phenotypes, even modest and consistent effects were associated with the gene manipulations and not a variation in background. With these carefully controlled experiments, the lifespan of control flies was highly uniform upon repetition, when 200 or more individuals were used for lifespan analysis (see Supplemental Table 4).

miR-34 deletion mutants

Deletion of *miR-34* region was made by site-specific recombination between two piggyBac insertions, using FLP-FRT mediated site-specific recombination[28]. The loss of any other genes in the region was then fully rescued back by genomic transgenes, so that a line selectively lacking only *miR-34* was generated. Two FRT-bearing insertions,

PBac[XP]d02752 and PBac[RB]*Fmr1*^{e02790}, were used (Exelixis collection, Harvard University), which encompass the *miR-34* region. Genetic crosses were made to combine these two transposon elements with heat-inducible FLP recombinase. After 48h of egg laying, parents were removed, and vials containing progeny were placed in a 37 °C water bath for a 1h-heat shock. Progeny flies were treated with daily 1h-heat shock, for an additional 4d. Young virgin female progeny flies were collected and crossed to males with 3rd chromosome balancers. In the subsequent generation, progeny males were used to generate additional progeny for PCR confirmation. Progeny flies bearing the deletion were positive for PCR verification, using primers from neighboring genomic DNA and ones from transposons (upstream insertion: 5'-GGTCGTGCATGACGAGATTA-3'/5'-TACTATTCCTTTCACTCGCACTTATTG-3'; downstream insertion: 5'-TCCAAGCGGCGACTGAGATG-3'/5'-GTGCGTTCGAAGAAATGATG-3'). Flies having the *miR-34* region deletion were viable, and were further verified for the appropriate deletion by PCR amplification, with primers for, *miR-317* (5'-CGGAAAAACGGTTTGTGTCT-3'/5'-CCCGGGAACGAGTAAACGAAATGAAAATCA-3'), *miR-277* (5'-TGATTTATGGTTTTTGTTTCAGTTG-3'/5'-TTGATATCATTTCACACTATCACAAAAATTGC-3'), *miR-34* (5'-ACCTTGAGCGCTTCAACTCT-3'/5'-CACTCTTTCTCGTTTGCATGG-3') and *dfmr1* (5'-CACACAGAGCTTCCCAGTGA-3'/5'-AGGCCCTCCTTTTTGACATT-3').

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 1.5cm from the bottom of the tube, within 15 sec after gently being banged 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 14ml 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 1h, followed by a 30 min recovery at 25°C, then another 1h heat stress at 37°C. Flies were then transferred into regular food vials and maintained at 25°C. Dead flies were counted after 24h. To assess brain morphology, adult male heads were processed for paraffin sections as described[29], 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 *miR-34* pUAST-I (286bp, PCR primer 5'-CCGTTACACACGACTATTCTCAAT-3'/5'-CCATCTGATACAGGTCCTACATTTTCTAAAA-3') and *miR-34* pUAST-II (936bp, PCR

primer 5'-ACCTTGAGCGCTTCAACTCT-3'/5'-CACTCTTTCTCGTTTGCATGG-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 (7530bp) harboring the *miR-277* sequence (PCR primers: 5'-GGTCGTGCATGACGAGATTA-3'/5'-GGATGTTTTGCGACCAACTT-3'), and part 2 was a genomic fragment containing *dfmr1* genomic sequence, derived from the pBS WTR construct (a gift from Dr. Thomas Jongens, University of Pennsylvania[30]), by BamH1 and Ppum1. The *miR-34* genomic rescue construct was also made in the pCaSpeR4 vector, with two parts. One was a genomic DNA fragment (6855bp) upstream of *miR-277* sequence (PCR primers: 5'-GGTCGTGCATGACGAGATTA-3'/5'-GGATGCATTTTATCGTTAGGC-3'), and the other was a genomic DNA fragment (2111bp) containing *miR-34* sequence (PCR primers: 5'-GCAGGAAAATGCGATAAATGA-3'/5'-TCGTTACAACATGGAAATCCTC). The resultant construct, therefore, contains *miR-34* sequence, including most upstream fragment, with the exclusion of 108bp of *miR-277* sequence. In addition, a modified *miR-34* genomic rescue construct was made (pCaSpeR4 vector), which contains same 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* appears complex, as despite these 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. Upregulation of *miR-34* during development in 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 Dr. Carl Thummel, University of Utah). Secondary antibodies for immunoblots were goat anti-mouse conjugated to HRP (1:2000, Chemicon), and developed by 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 manufacturer's protocol (Trizol reagent, Invitrogen). 5μg 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 prehybridization (~ 1 h), hybridization (~ 12 h or overnight) with P^{32} labeled probe, washed and exposed to Phosphoimager (Amersham). RNA probes were used that were made by in vitro transcription of cDNA templates using Maxiscript-T7 in vitro transcription kit (Ambion), supplemented with P^{32}-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'-GTGAACGTGGTGGTGGAAC-3'/ 5'-GATAATACGACTCACTATAGGGAGATGTCCATTCGCTTCTCAATG-3'); *E74B* (5'-CATCGCTTGTCAATGTGTCC-3'/ 5'-GATAATACGACTCACTATAGGGAGACTGCGGTAATCACTGAGCTG-3');18S rRNA loading control (5'-GATAATACGACTCACTATAGGGAGA-3'/ 5'-AGGGAGCCTGAGAAACGGCTACCACATCTAAGGAATCTCCCTATAGTGAGTCGTATTATC -3')

For small RNA northerns, total RNA was isolated from male fly heads using Trizol reagent as above. For each lane, 3μg of RNA was used, and RNA was fractionated on a 15% Tris-UREA gel (NuPage) with 1XTBE buffer. The blot transfer was performed with 0.5XTBE buffer. Prior to hybridization, the RNA blots were prehybridized with Oligohyb (Ambion), and then incubated with radioactive labeled RNA probes for ~12 h to overnight. RNA probes were used, and made by in vitro transcription of oligo templates using Maxiscript-T7 in vitro transcription kit (Ambion), supplemented with P^{32} -labeled UTP. Oligo DNA templates were prepared by annealing two single stranded DNA oligos into duplex (99°C 5min and cool down to room temperature). Oligos used for *miR-34* (5'-GATAATACGACTCACTATAGGGAGA-3'/5'-AAAAAATGGCAGTGTGGTTAGCTGGTTGTGTCTCCCTATAGTGAGTCGTATTATC -3'), *miR-277* (5'-GATAATACGACTCACTATAGGGAGA-3'/5'-TAAATGCACTATCTGGTACGACATAAATGCACTATCTGGTACGACA TCTCCCTATAGTGAGTCGTATTATC-3') and 2S rRNA (5'-GATAATACGACTCACTATAGGGAGA-3'/5'-TGCTTGGACTACATATGGTTGAGGGTTGTATCTCCCTATAGTGAGTCGTATTATC -3').

Luciferase assays were performed using standard approaches [5]. Specifically, 8×10^4 DL1 cells were plated and bathed in 30ul of serum-free medium with 60ng of dsRNA in each well of a 96 well plate. The next day, 1.6ng of pMT-Firefly, 400ng of pMT-*miR-34*, and 400ng of pMT-renilla-3'UTR (E74A wild type or mutant reporters) were transfected by Effectene (Qiagen). Two days post transfection, the expression of the reporters and *miR-34* was induced by $CuSO_4$. 24h after induction, luminescence assays were performed by Dual-Glo Luciferase Assay System (Promega). The *miR-34* seed sequences in the 3'UTR of *E74A* were mutated as noted in Supplementary Figure 3, using the Quik change mutagenesis system (Stratagene). Primers to knockdown Ago1 are described[5].

The miRNA-target prediction algorithms TargetScan (v5.1) [31] and PicTar (fly)[32] were used to determine *miR-34* target mRNA candidates.

miRNA microarray analysis

For miRNA array analysis, Iso31 flies (isogenized w^{1118}) were used. Flies were killed by brief submersion in ethanol under CO_2 anesthesia, followed by two PBS washes (Sigma). To control for circadian effects, all flies were processed between 11 am and 1 pm. Brains were removed manually and collected in an Eppendorf microcentrifuge tube stored on ice. For each miRNA microarray replicate, 200–300 brains were collected for each time point, with a ~50/50 ratio of males and females. RNA was prepared using the miRvana RNA

extraction system (Ambion) yielding ~2.5μg/100 brains. RNA was eluted into 80μl of RNAse free water (Fisher Scientific, NJ) 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-labeling kit was used (Exiqon). RNA samples were labeled with Hy3 and hybridized together with a Hy5-labeled common reference standard. The common reference standard consisted of equal amounts of RNA from brains of 3d, 30d, and 60d flies. The miRNA microarray data were analyzed at the Penn Bioinformatics Core. Raw data was imported into Gene Spring 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 analyzed by Exiqon (Exiqon). Expression levels (fold changes) for the 30d and 60d time point were calculated relative to the 3d time point. The data sets were exported into Spotfire DecisionSite 9.0 (Tibco) for visualization and filtering.

mRNA microarray analysis

For aging 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 aging profile, flies were aged to 3d, 30d and 60d, and 30–50 brains dissected per time point, per replicate, as above (50-50 males and females). For each time point, 3 replicates were conducted. For *miR-34* mutant microarray analysis, time points were 3d and 20d, and for each time point, 20 brains from male flies of the appropriate genotype were used, with 5 replicates in total. Microarray hybridization and reading was performed at the Penn Microarray Core Facility. For mRNA 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 labeled 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 probeset 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.

Transcriptional analysis of aging status

We first used the wild type to extract age-associated probesets and then compared the relative changes of these probesets 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 RMA (JZ Wu, J MacDonald, J Gentry, GCRMA: Background adjustment using sequence information, R package version 2.14) for R/Bioconductor[33] was used to generate log-2 expression levels for probeset 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[9,34]. This approach assumes a linear relationship between age and log-2 expression level:

$$Y_{ij}=\mu_i+''_{1i}A_j+!_{ij}$$

In this equation, Y_{ij} is the log-2 gene expression level of probe set i in sample j, A_j is the age for individual j. The coefficients β_{1i} is regression coefficients reflecting the rate of change in gene expression with respect to age. Probesets with expression significantly correlated with age ($p \leq 0.001$ for $\beta 1i$) were determined. Then the same probesets were used to estimate the relative expression in separate profiles of *miR34* mutants and age-matched controls. The average levels of each individual probeset were calculated for the difference between 20d and 3d, within the same genotype (i.e. $\Delta 20d/\Delta 3d$) for each gene in controls and *miR-34* mutants, respectively. These differences were then compared between genotypes (i.e. *miR-34* mutants - controls). The significance of the difference between genotypes was analyzed using a paired Wilcoxon test. The difference between control and mutant samples in positively-correlated genes (Fig. 2d) is not by chance ($p<0.0001$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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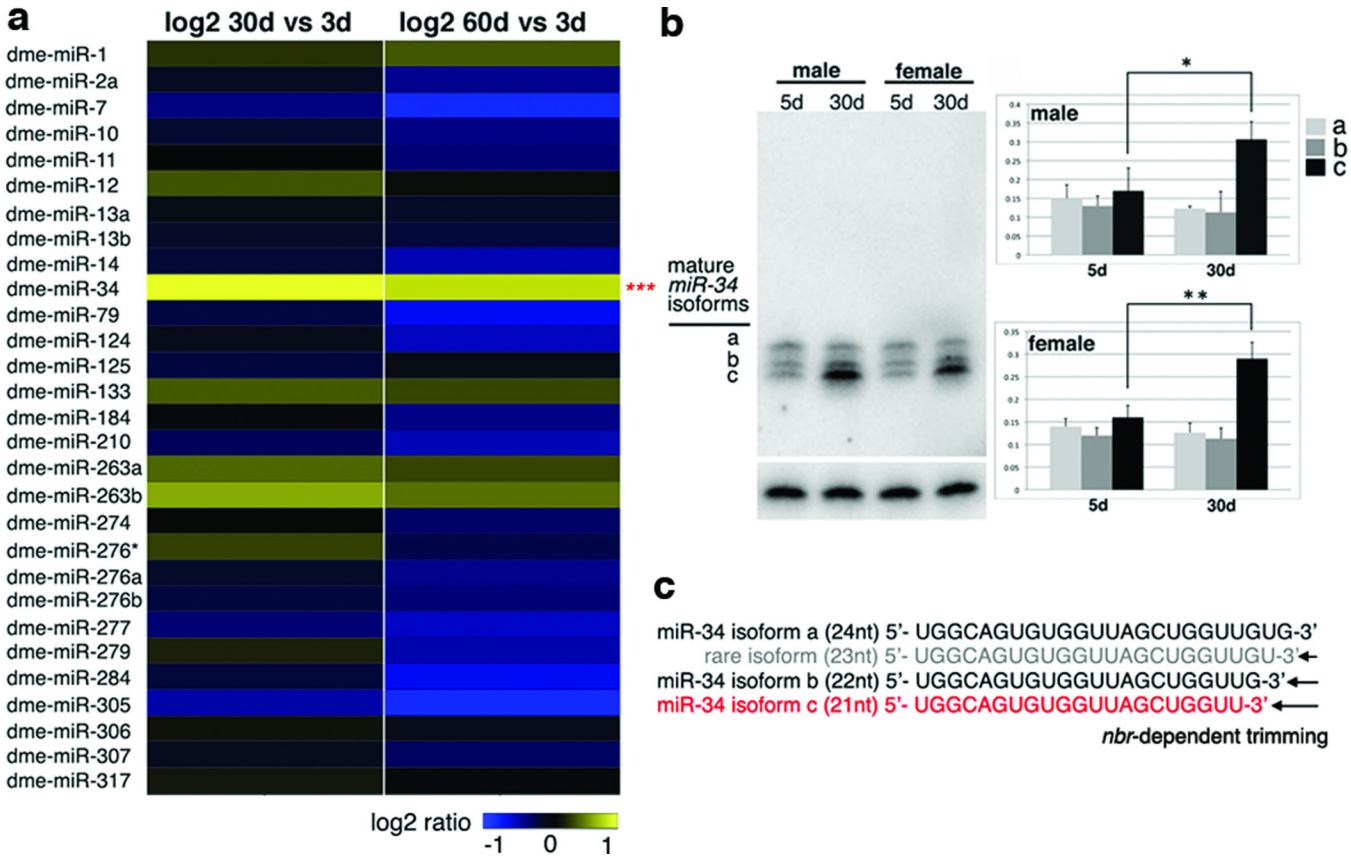

Figure 1. *Drosophila miR-34* is upregulated with age

a. Heat map of fold-change of *Drosophila* miRNAs in brains aged 3d, 30d and 60d. 29 miRNAs (shown) were flagged present of a total of 78. One-way analysis of variance defined significance for each miRNA over all time points (***=p <0.001; n=3 replicates). Genotype: iso31

b. Fly *miR-34* isoform-c shows age-modulated expression in fly heads. Left panels, *miR-34* shows three major mature forms (labeled a, b, and c), but only isoform c increases with age. Right panels, quantification of *miR-34* isoforms with age (top: male; bottom: female). n = 3 independent experiments; signal density of all isoforms normalized at the same time point to 2S rRNA loading control. (*=p < 0.01, **=p < 0.001, one-way analysis of variance, with post test: Tukey's multiple comparison test). Genotype: 5905.

c. Sequences of *miR-34* isoforms are generated through *nbr*-dependent 3'end trimming.

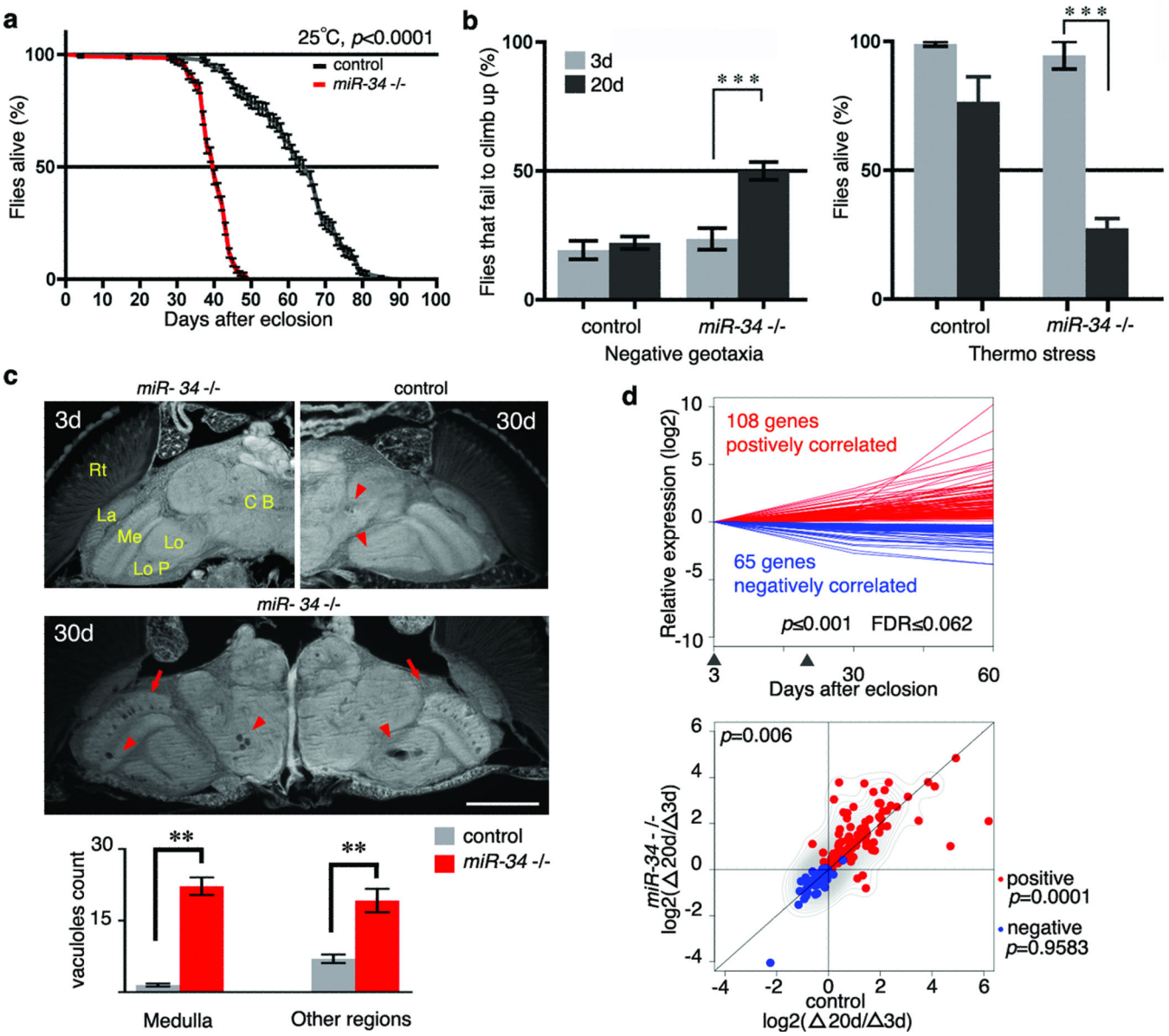

Figure 2. *miR-34* modulates age-associated processes

a. *miR-34* mutant flies have a shortened lifespan (control: 64d median, 90d maximal lifespan; *miR-34*: 40d medium, 64d maximal lifespan; $p<0.001$, log-rank test). Mean ± s.e., n≥240 male flies per curve. Genotypes: control: 5905. *miR-34* −/−: *miR-34* null-1 in 5905 homogenous genetic background.

b. *miR-34* mutant flies have late-onset behavioral deficits. Left panel, for locomotion behavior, *miR-34* mutant flies have normal climbing at 3d. At 20d, 50±3.4% *miR-34* mutant flies fail to climb; in contrast, only 22.1±2.4% of control flies have defective climbing. Mean ± s.e.m. of 3 experiments, n=120–140 male flies per experiment. Right panel, for stress resistance, *miR-34* mutant flies have normal resistance to heat stress at 3d. *miR-34* mutant flies become strikingly sensitive to heat shock with age, such that at 20d, only 27.5±3.8% survive after heat stress. In contrast, 76.7±9.6% of control flies survive after the

same treatment. Mean ± s.e.m. of 3 experiments, n=120–140 male flies. *** =p<0.0001 (two-way analysis of variance). Genotypes as in a.

c. *miR-34* mutant flies show age-associated brain degeneration. Top left panel, *miR-34* mutant flies have normal brain morphology at 3d. Major anatomical structures: CB (central brain), Lo (lobula), LoP (lobula plate), Me (medulla), La (lamina) and Rt (retina). At 3d, control flies have normal brain morphology (not shown), but develop a small number of sporadic vacuoles at 30d (top right panel, arrowheads). Middle panel, aged *miR-34* mutants (30d) show striking vacuoles in the medulla (arrows) and other regions of the brain (arrowheads). Bottom, the number of vacuoles in *miR-34* mutants is significantly higher than in controls (22.2± 1.8 vs 1.5±0.3 in medulla; 19.2±2.5 vs 7.0±0.9 in other regions of the brain; **=p < 0.001, one-way analysis of variance, with post test: Tukey's multiple comparison test). Mean ± s.e.m., n=10 independent male fly brains. Genotypes as in a. Scale bar: 0.1mm.

d. *miR-34* mutant flies have a transcriptional profile indicative of accelerated aging. Top panel, 173 age-correlated probesets were defined from a transcriptional profile of fly brains at 3d, 30d, and 60d of age. Arrowheads indicate time points (3d and 20d) at which *miR-34* mutants and controls were compared. Genotype: iso31 flies used for transcriptional profiles of normal aging brains. n=3 biological replicates for each time point. p≤0.001, FDR≤0.062, linear regression model. Bottom panel, scatter plot illustrates the relative expression of 173 probesets, which shows a significant difference between *miR-34* mutants and age-matched controls (p=0.006, two-sample, paired Wilcoxon test). Whereas the pattern for positively-correlated probesets (red), indicated by the contour lines, is significantly different (p=0.0001) between the two genotypes, and tend to show higher expression in *miR-34* mutants compared to controls, it is not for negatively-correlated probesets (blue) (p=0.9583).). Contour lines indicate that positively correlated probesets tend to show higher expression in *miR-34* mutants compared to controls. Genotypes as in a. n=5 biological replicates for each time point.

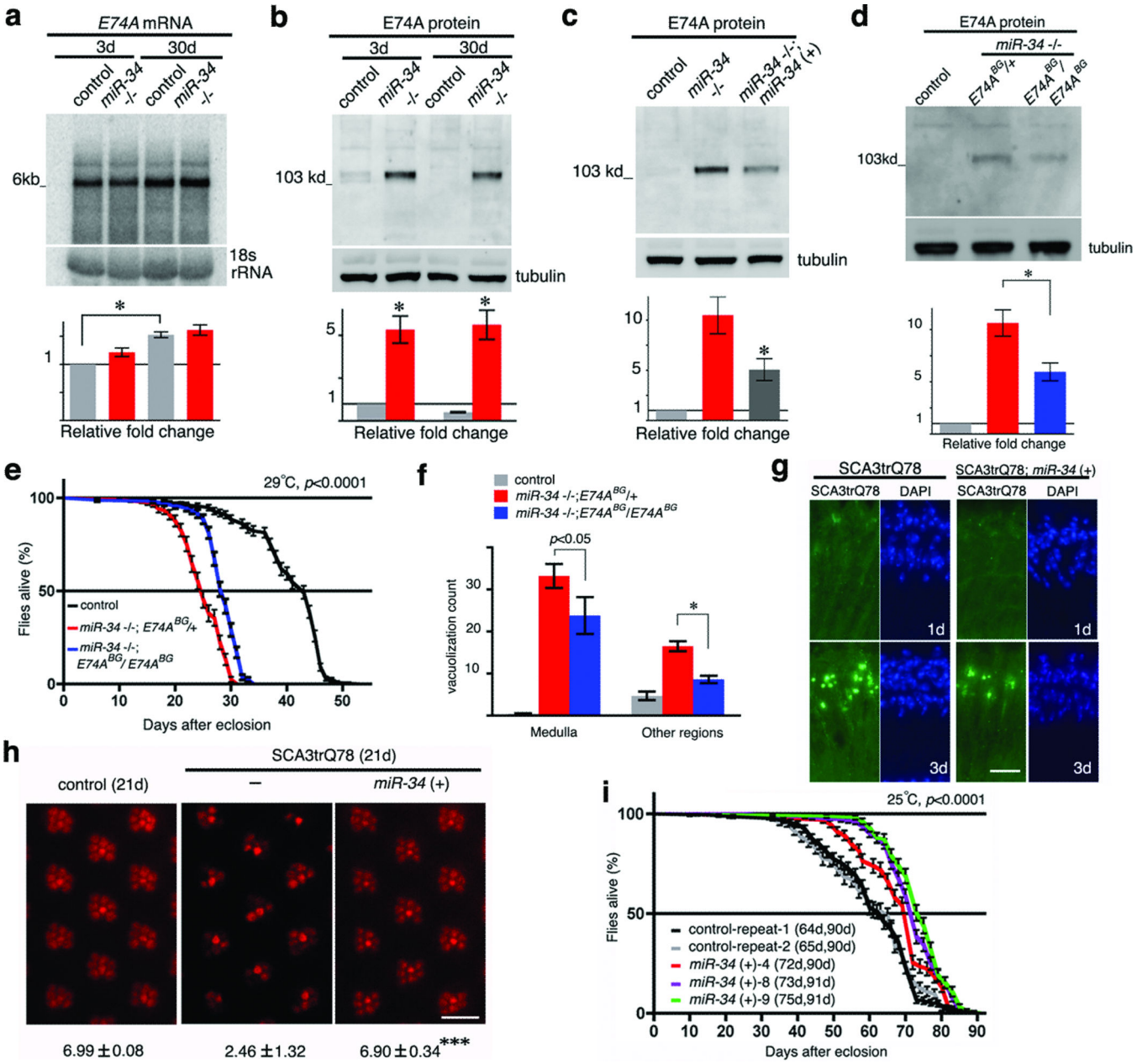

Figure 3. The *Drosophila Eip74EF* gene is a target of *miR-34* in modulation of the aging process

a. *E74A* mRNA is robustly expressed in the adult and unchanged between age-matched controls and *miR-34* mutants. In control flies, *E74A* mRNA is significantly upregulated in 30d compared to 3d animals. RNA was from male heads. Mean ± s.e.m., n = 3 independent experiments; signal density of *E74A* mRNA normalized to 18S rRNA loading control (*=$p < 0.01$, one-way analysis of variance, with post test: Tukey's multiple comparison test). Genotypes: control: 5905. *miR-34* −/−: *miR-34* null-1 in 5905 homogenous genetic background.

b. E74A protein is deregulated in *miR-34* mutants. Protein was from male heads. Mean ± s.e.m., n = 3 independent experiments; signal density of E74A protein normalized to tubulin

loading control (*=$p < 0.01$, one-way analysis of variance, with post test: Tukey's multiple comparison). Genotypes as in a.

c. De-regulation of E74A protein is diminished in *miR-34* rescue flies. Protein was from male heads. Mean ± s.e.m., n = 3 independent experiments; signal density normalized to tubulin loading control (*=$p < 0.05$, one-way analysis of variance, with post test: Tukey's multiple comparison test). Genotypes: control: 5905. *miR-34* −/−: *miR-34* null-1 in 5905 homogenous genetic background. *miR-34* −/−; *miR-34* (+): *miR-34* genomic rescue in *miR-34* null-1 in 5905 homogenous genetic background.

d. *miR-34* mutants homozygous for the $E74A^{BG01805}$ allele have lower levels of E74A protein. Protein was from male heads of 20d flies raised at 29°C. Mean ± s.e.m., n = 3 independent experiments; signal density normalized to tubulin loading control (*=$p < 0.01$, one-way analysis of variance, with post test: Tukey's multiple comparison test). Genotypes: control: 5905. *miR-34*−/−, $E74A^{BG}$/+: $E74A^{BG01805}$/+, *miR-34* null-1 in 5905 homogenous genetic background. *miR-34*−/−, $E74A^{BG}/E74A^{BG}$: $E74A^{BG01805}/E74A^{BG01805}$, *miR-34* null-1 in 5905 homogenous genetic background.

e–f. Reducing E74A protein levels in the adult mitigates age-related defects of *miR-34* mutants.*miR-34* mutants also homozygous for the $E74A^{BG01805}$ show rescued lifespan (e) and brain morphology (f), compared to *miR-34* mutants heterozygous for the $E74A^{BG01805}$ (these flies have a lifespan that is the same as *miR-34* mutants alone; see Supplemental Table 4). Flies raised at 29°C. Lifespan: $p<0.0001$ (log-rank test). Mean ± s.e., 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 (1d, top), but gradually accumulates into nuclear inclusions (3d, bottom). Right panels, upregulation of *miR-34* reduces inclusion formation. DAPI staining highlights nuclei. 3d controls show 53.75 ± 12.55 inclusions in a retinal section, vs 23.67 ± 7.57 with *miR-34* upregulation; mean ± s.d., n≥3 cryosections from independent male animals; $p<0.01$ (*t*-test). Genotypes: SCA3trQ78 is w^{+}*; rh1-gal4, UAS-SCA3trQ78/+*. SCA3trQ78*; miR34* (+) is w^{+}*; rh1-gal4, SCA3trQ78/+; UAS-miR-34/+*. Scale bar: 0.05mm.

h. Upregulation of *miR-34* prevents neural degeneration. At 21d, male flies expressing SCA3trQ78 show a dramatic 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). Genotypes as in b; control: w^{+}*; rh1-gal4/+*. Scale bar: 0.05mm.

i. Flies with upregulated *miR-34* (color) have an extended median lifespan compared to control flies (black and grey curves for repeats 1 and 2, respectively) (log-rank test). Lifespan result for each genotype is indicated in median and maximal days. Mean ± s.e., n>=150 male flies per genotype, 25°C. Three independent *miR-34* genomic transgenic lines

(4, 8, 9) were analyzed. Genotypes: control: 5905, *miR-34* (+): *miR-34* genomic rescue in 5905 homogenous genetic background.