MiR-34 inhibits polycomb repressive complex 2 to modulate chaperone expression and promote healthy brain aging

Jason R. Kennerdell1, Nan Liu1,2 & Nancy M. Bonini1

Aging is a prominent risk factor for neurodegenerative disease. Defining gene expression mechanisms affecting healthy brain aging should lead to insight into genes that modulate susceptibility to disease. To define such mechanisms, we have pursued analysis of miR-34 mutants in Drosophila. The miR-34 mutant brain displays a gene expression profile of accelerated aging, and miR-34 upregulation is a potent suppressor of polyglutamine-induced neurodegeneration. We demonstrate that Pcl and Su(z)12, two components of polycomb repressive complex 2 (PRC2), are targets of miR-34, with implications for age-associated processes. Because PRC2 confers the repressive H3K27me3 mark, we hypothesize that miR-34 modulates PRC2 activity to relieve silencing of genes promoting healthful aging. Gene expression profiling of the brains of hypomorphic mutants in Enhancer of zeste (E(z)), the enzymatic methyltransferase component of PRC2, revealed a younger brain transcriptome profile and identified the small heat shock proteins as key genes reduced in expression with age.
AGING IS AN IMPORTANT MAJOR RISK FACTOR FOR DEVELOPING NEURODEGENERATIVE DISEASE. however, molecular pathways that confer susceptibility to aged individuals are still being explored. several genetic manipulations can produce long-lived animals; for example, daf-2 mutations extend lifespan in C. elegans, as does upregulation of a sirtuin in both C. elegans and S. cerevisiae. Non-genetic interventions also extend lifespan, including caloric restriction and ablation of the reproductive tissue in C. elegans. Better understanding of mechanisms that confer a healthy brain could lead to insight for treatments for age-associated brain diseases.

The microRNA miR-34 is a post-transcriptional regulatory molecule that impacts age-associated outcomes in Drosophila. MiR-34 becomes upregulated in the fly brain in an age-associated manner and is functionally required for healthy brain aging. The mutants undergo brain degeneration and die prematurely; transcriptional profiling demonstrates an accelerated age-associated profile of transcriptional changes. As such, miR-34 mutant animals display normal age-associated processes, but at an accelerated pace. Consistent with a role in healthy brain aging, upregulation of miR-34 in the fly extends median lifespan and potently mitigates degeneration in a model of polyglutamine disease. Elucidating the targets of miR-34 that help confer these functions would lead to insight into mechanisms of healthy brain aging.

MicroRNAs affect protein abundance by binding select target mRNAs containing sequence complementarity within their 3’ UTR. This results in decreased translation or degradation of the mRNA, leading to a downregulation of the protein. Several activities of miR-34 can be attributed to regulation of Eip74EF, a component of steroid hormone signaling pathways. However, Eip74EF does not affect polyglutamine toxicity, suggesting that other targets of miR-34 are involved in regulating age-associated susceptibility to brain disease. Identification of additional targets of miR-34 should provide additional insight into aging of the brain, and define mechanisms that can delay or prevent age-associated diseases. A number of observations have implicated miR-34 in the response to stress: miR-34 mutants are sensitive to stress in flies and in C. elegans. In flies, miR-34 brain tissue also accumulates inclusions of misfolded protein, and upregulation of miR-34 activates innate immunity pathways and promotes survival from infection. These data suggest that miR-34 function should impact stress-associated genes important for maintaining health of the brain.

The importance of epigenetic modulation of the genome has been well established for developmental events; however, the role and impact on aging and age-associated diseases is only recently gaining attention. Histone modification pathways affect lifespan in yeast and mutations in components of the H3K4 trimethylation complex extend lifespan in C. elegans. Mutation in components of polycomb repressive complex 2 (PRC2), which confers the H3K27me3 mark associated with decreased gene expression, extends lifespan and promotes resilience to stresses. PRC2 is elevated in a mouse model of ataxia-telangiectasia, and inhibiting PRC2 activity suppresses neurodegeneration in this model. Despite these findings, little is known mechanistically regarding epigenetic modulation in the brain with age, yet such modulations could have a vast impact on healthy aging, stress resistance, and disease susceptibility.

In pursuing additional targets of miR-34 that may contribute to healthy brain aging, we reveal that miR-34 regulates two components of the PRC2 complex—Su(z)12 and Pcl—to convey resistance to polyglutamine toxicity. By study of potential epigenetically modulated genes in the brain with age normally and upon PRC2 reduction, we reveal that small heat shock proteins (sHSPs) may be critical players in healthy brain aging. Protein chaperones, including the sHSPs, have been shown to be particularly important in protection from neurodegeneration, stress, and age-associated processes in both flies and C. elegans.

Detailed transcriptomic analysis further reveals that reduction of PRC2 function in the fly brain confers a chronologically “younger” transcriptomic profile, opposite to that of the miR-34 mutant, highlighting that approaches to modulate the epigenome may impact healthy aging of the brain.

RESULTS

MiR-34 regulates the Pcl and Su(z)12 3’ UTRs. To define targets of miR-34 that might be important to brain aging, we scanned the lists of potential target genes using multiple microRNA prediction algorithms (Supplementary Table 1). We noted that two components of the same complex, PRC2, are targets: Polycomblike (Pcl), a peripheral component of PRC2, is a predicted target in Drosophila and vertebrates; and a gene encoding a protein within the core PRC2 complex, Su(z)12. Polycomb group proteins function together as members of several repressive complexes called PRC1, PRC2, and PRC2-Pcl, of which the protein subunits can vary. Methylation of lysine 27 of histone H3 by the enzymatic methyltransferase component of PRC2, Enhancer of zeste (E(z)), recruits PRC1 to select loci typically resulting in transcriptional silencing, although it may also result in increased transcription depending on the distribution of the epigenetic mark. The miR-34 binding site appears conserved in all Drosophila examined, and is also conserved in one of the three human orthologues of Pcl, PHE19 (Fig. 1a). To determine whether miR-34 can regulate Pcl through this site, we used a luciferase cell culture reporter assay. A Renilla luciferase reporter utilizing the 3’ UTR of Pcl was used to assess the sensitivity of the 3’ UTR to miR-34 in SL2 cells. Upregulation of miR-34 was achieved with an expression construct driven by an inducible metallothionein promoter. Upregulation of miR-34 resulted in a 56% decrease in reporter expression with the 3’ UTR of Pcl when compared to a control inducing expression of GFP (Fig. 1c, left; also Supplementary Figure 1). To determine whether the decrease in reporter activity was dependent upon the miR-34 seed region, we generated a seed mutant that disrupted strand complementarity (Fig. 1a, bottom). The seed mutation reduced the ability of miR-34 to regulate the Pcl 3’ UTR (Fig. 1c, right). These data indicate that the Pcl 3’ UTR can be regulated by miR-34, at least in part through the seed region defined.

In parallel, we examined miR-34 regulation of Su(z)12, the rate limiting component of PRC2. Previous work investigating the reliability of a microRNA prediction algorithm demonstrated that upregulation of miR-34 decreases translation of a reporter bearing the 3’ UTR of Su(z)12. MiR-34 regulation is not predicted in mammalian SUZ12 by common algorithms; however, RNAhybrid showed a potential site in the 3’ UTR of vertebrate SUZ12 (Fig. 1b). Targeting of SUZ12 through this cryptic site would require wobble base pairing which is tolerated in some miRNA/target pairings. We confirmed that miR-34 regulates the 3’ UTR of fly Su(z)12 and determined that this regulation was dependent on the miR-34 seed sequence. Upregulation of miR-34 resulted in a 33% decrease in the reporter utilizing the 3’ UTR of Su(z)12 (Fig. 1d, left; also Supplementary Figure 1), consistent with the previous findings. A Su(z)12 reporter with a mutated 3’ UTR that disrupted strand complementarity between miR-34 and the 3’ UTR of Su(z)12 (Fig. 1b, bottom) yielded an attenuated response. These data suggest that miR-34 regulation requires, at least in part, the seed sequence present in the 3’ UTR of Su(z)12.

If this regulation of the Pcl and Su(z)12 3’ UTR reporter constructs occurs upon upregulation of the mature miR-34 miRNA, then we would expect interfering with the miRNA
Fig. 1 miR-34 targets 3′ UTRs of Su(z)12 and Pcl. a, b Pcl and Su(z)12 are predicted targets of miR-34. The seed sequence of miR-34 has complementarity with the (a) Pcl 3′ UTR and the (b) Su(z)12 3′ UTR. The seed sequence is conserved in various homologs. Bottom, an engineered mutation in the seed sequence was also generated, red letters indicate base-pairing retained in the seed mutation, those with disrupted base-pairing are in black. c, d Luciferase assays confirm that miR-34 targets Pcl and Su(z)12. A reporter construct containing the 3′ UTR of (c) Pcl or (d) Su(z)12 fused to Renilla luciferase was expressed in Drosophila S2 cells and tested for a response to miR-34 upregulation. Luciferase activity decreases in reporters containing the (c, left) Pcl 3′ UTR and the (d, left) Su(z)12 3′ UTR. Regulation of the Pcl 3′ UTR by miR-34 was attenuated by a seed sequence mutation (c, right). Regulation of the Su(z)12 3′ UTR by miR-34 was also attenuated by the seed sequence mutation (d, right). Two-way ANOVA indicated a significant interaction term for the Pcl 3′ UTR (F1,8 = 8.5, p < 0.02), and Su(z)12 3′ UTR (F1,8 = 17.5, p < 0.01). *p < 0.05, two-way ANOVA with Tukey post-test. Mean ± SEM, n = 3 wells.

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miR-34 regulates Pcl and Su(z)12 levels in the aging brain. Given this regulation in cells, we extended the findings to the brain in vivo. MiR-34 is expressed in the Drosophila brain in an age-dependent manner, with low expression in young adult animals and increased expression with age. We confirmed specificity of an anti-Pcl antibody by verifying that an immunoband present in brain tissue was depleted upon hypomorphic mutation or knockdown of Pcl (Supplementary Figure 2). Using this antibody, we examined the levels of Pcl protein in young (3d) and older (20d) fly brain tissue. As wild-type animals age, the level of Pcl protein in the brain decreased dramatically, dropping by 43% from 3d to 20d in the wild-type body, we examined the levels of Pcl protein tagged by a C-terminal HA epitope. To assess Su(z)12 protein levels within its normal genomic context we included promoter, introns, and UTRs in the transgene. As with the Pcl protein, Su(z)12 protein levels normally decreased with age, becoming reduced by 37% from 3d to 20d in the wild-type brain (Fig. 3a, b; Supplementary Figure 3). In miR-34 mutant tissue, the levels of HA-tagged Su(z)12 protein became elevated compared to age-matched controls, by 39% at 3d and 47% at 20d, indicating that Su(z)12 is also an in vivo target of miR-34 activity.
Fig. 3 Su(z)12 is a target of miR-34 in the brain. a, b Su(z)12 protein levels are deregulated in miR-34 mutants. Su(z)12 protein was detected using a transgene containing the Su(z)12 genomic region tagged with the HA epitope on the C-terminus of the predicted protein (Su(z)12-C-term HA). Su(z)12 levels decreased with age in wild-type, but remained elevated in miR-34 mutants. Protein samples from dissected brains. Su(z)12-C-term HA protein levels were normalized to tubulin loading control. Mean ± SEM, n = 3 biological replicates. Significant main effects were observed (genotype: F_{1,8} = 68, p < 0.001 age: F_{1,8} = 101, p < 0.001). *(p < 0.05, **p < 0.01, two-way ANOVA with Tukey post-test). c, d The regulation of Su(z)12 protein levels by miR-34 is dependent upon the miR-34 seed sequence in the 3′UTR of the Su(z)12 transcript. The Su(z)12-C-term HA-mutant 3′UTR transgene contains a mutation in the miR-34 seed sequence that relieves the transcript from regulation by miR-34 (see Fig. 1d). Su(z)12-C-term HA-mutant 3′UTR protein levels still decrease with age, but are no longer increased in miR-34 mutants. The difference between wild-type at 20d and miR-34 mutant at 20d is not significant. Protein samples from dissected brains. Quantification of Su(z)12-C-term HA-mutant 3′UTR protein levels using Western immunoblotting, normalized to tubulin loading control. Mean ± SEM, n = 3 biological replicates. Significant main effects were observed (genotype: F_{1,8} = 17, p < 0.01 age: F_{1,8} = 28.5, p < 0.001). *(p < 0.05, two-way ANOVA with Tukey post-test).

Having shown that Su(z)12 protein levels are higher in miR-34 mutants, we determined whether this regulation was dependent on the miR-34 seed sequence within the 3′UTR of the Su(z)12 transcript. A mutation was introduced into the transgene to disrupt the base-pairing of miR-34 in the 3′UTR (Fig. 1b, bottom) and transgenic animals generated. The mutated transgene was then crossed into wild-type and miR-34 mutant male flies at 20d and miR-34 mutant at 20d is not significant. Protein samples from dissected brains. Quantification of Su(z)12-C-term HA-mutant 3′UTR protein levels using Western immunoblotting, normalized to tubulin loading control. Mean ± SEM, n = 3 biological replicates. Significant main effects were observed (genotype: F_{1,8} = 68, p < 0.001 age: F_{1,8} = 101, p < 0.001). *(p < 0.05, **p < 0.01, two-way ANOVA with Tukey post-test).

Brain histone H3K27me3 levels are elevated in miR-34 mutants. A key function of the PRC2 complex is to silence gene expression by methylating proximal histones with H3K27me3.27 Thus, the increases in Su(z)12 and Pcl protein that we observed in miR-34 mutants could result in downstream changes in H3K27me3. To examine this, we purified histones from young (3d) and aged (20d, 40d) Drosophila heads of wild-type and miR-34 mutant animals, and used Western immunoblotting to assay total histone H3 and H3K27me3 modification levels. In wild-type animals, the levels of H3K27me3 rose steadily with age, increasing by 105% at 40d (Fig. 4a, b). In miR-34 mutant animals, H3K27me3 also increased with age, but the increase was accelerated (67% from 3d to 20d for miR-34, compared to the wild-type trend of 35% from 3d to 20d). MiR-34 mutant animals at 20d had H3K27me3 levels comparable to those of wild-type animals at 40d, and these levels were more than twice the levels of 3d wild-type animals (Fig. 4a, b).

We refined these observations by limiting the analysis to brain tissue. The brain is enriched for miR-34 compared to heads.6 We carefully dissected brains from the head capsule to assess levels in central brain plus outer optic regions, lacking the eyes and lamina. Interestingly, when examined in this manner, the levels of H3K27me3 in wild-type animals did not increase with age, but rather decreased (Fig. 4c, d). However, the increase of H3K27me3 in mutant compared to wild-type brains was still observed, with mutants displaying a 76% increase over age-matched wild-type brains. These data indicate that the increase in H3K27me3 observed in heads with age may be largely due to changes outside the central brain region dissected. We also addressed the specificity of histone modulation with age by assessing an additional histone silencing mark, H3K9me3, which, interestingly, was modestly affected by mutation of miR-34 (Fig. 4e, f).

Taken together, these data suggest that Pcl and Su(z)12 are in vivo targets of miR-34, and that the H3K27me3 activity of the PRC2 complex with which these proteins are associated is modulated with age in the brain. H3K27me3 levels are also higher in miR-34 mutants with age than in wild-type tissue. These findings raise the possibility that modulation of PRC2 and H3K27me3 may be a molecular mechanism that impacts susceptibility of the brain to degenerative disease with age, given that miR-34 mutants undergo brain degeneration and display an accelerated aging profile, and that upregulation of miR-34 robustly prevents neurodegeneration.3 Therefore, miR-34 levels may increase with age to help minimize age-related elevations in H3K27me3 and negative impacts of this histone modification on pathways associated with neurodegeneration.

Pcl and Su(z)12 mutations mitigate polyglutamine toxicity. Expression of a pathogenic form of human ATXN3 with an expanded polyQ repeat (SCA3trQ78) in photoreceptor cells of the fly eye induces age-dependent neural loss that can be readily visualized by the pseudopupil imaging technique (Fig. 5a).13 Whereas flies normally retain 7 ± 0 (mean ± SEM) photoreceptors (PR) per ommatidial unit with age, expression of pathogenic SCA3trQ78 results in age-dependent photoreceptor loss to 1.72 ± 0.06 PR/ommatidial unit in flies aged 21d (Fig. 5a). Given that our data indicate that Pcl and Su(z)12 are targets of miR-34, we hypothesized that decreasing the activity of these genes may produce effects similar to upregulation of miR-34 to suppress polyglutamine-mediated neurodegeneration.6 To assess the impact of these PRC2 components, we reduced their levels by introducing one copy of the null mutations Su(z)122 or Pcl– into flies expressing SCA3trQ78. When Pcl activity was decreased, photoreceptor cell loss was strongly mitigated: while animals expressing SCA3trQ78 on its own had an average of fewer than two photoreceptors per ommatidium (1.72 ± 0.06) at 21d, animals expressing SCA3trQ78 and heterozygous for the null allele Pcl– retained 4.02 ± 1.10 PR/ommatidial unit (Fig. 5a, b). Similarly, animals expressing SCA3trQ78 and heterozygous for the null allele Su(z)122 retained 4.93 ± 0.11 PR/ommatidial unit (Fig. 5a–c). Several other alleles of Pcl and Su(z)12 were also tested, and all protected from polyglutamine-mediated toxicity (Supplementary Figure 4). We also confirmed that Pcl and Su(z)12 mutations did not affect the steady-state level of the SCA3trQ78 mRNA (Supplementary Figure 4). These data indicate that reduction of Pcl or Su(z)12 function mitigates polyglutamine degeneration.

Pcl and Su(z)12 mutations mitigate polyglutamine aggregation. To investigate biological mechanisms through which genetic reduction of these PRC2 components impact neural degeneration, we conducted a detailed analysis of the aggregation of SCA3trQ78 protein in photoreceptor neurons. To assess inclusions, we examined tissue by immunohistochemistry. Normally, the polyglutamine protein accumulates rapidly, generating abundant, large nuclear inclusions. At 3d, many polyglutamine inclusions were visible in sections of the retinal neurons (38.0 ± 2.6) (Fig. 6a–d). When Pcl activity was reduced, the number of the inclusions decreased by 65%, to just 13.2 ± 3.4 (Fig. 6b–d). Additionally, there was a 39% decrease in the size of the
remaining inclusions (Fig. 6e). Reduction of Su(z)12 acted similarly, with the number of inclusions decreased by 60% to 15.1 ± 1.5 and the size of the remaining inclusions being smaller by 28% (Fig. 6c–e). These data suggest that the targets of the PRC2 complex that are critical to the effects of miR-34 on polyglutamine toxicity may include genes that regulate protein misfolding and turnover, given these dramatic effects on protein aggregation and toxicity.

**Transcriptome profiling of the brains of E(z) mutants.** As PRC2 functions as a histone modification complex, we anticipated that a consequence of higher PRC2 activity in aged miR-34 mutants should be alterations in gene expression; the identification of these genes should include targets of the complex that are critical to healthy brain aging. For transcriptome analysis, we focused on mutants of the E(z) gene which encodes the catalytically active methyltransferase of PRC2: homozygous reduction-of-function mutants compared to age-matched wild-type controls were differentially expressed at both the permissive and restrictive temperatures. This finding indicates that the E(z)61/E(z)731 transheterozygote combination has significant consequences for gene expression even at normal cultivation temperatures, consistent with our observations that mild removal of PRC2 activity mitigates polyglutamine toxicity (Fig. 5). We selected lists of genes differentially expressed using a Benjamini false discovery rate of p < 0.05, and a log2 fold-change of more than 0.5 (for upregulated genes) or less than −0.5 (for downregulated genes). This defined 343 upregulated and 290 downregulated genes upon reduced E(z) function (Supplementary Data 1). We examined GO Terms, Kegg Pathways, and InterPro motifs that were enriched in these lists of genes using DAVID. Because we were interested in genes that would normally be targets of the H3K27me3 silencing mark and PRC2 is normally downregulated by miR-34, we first investigated genes that became upregulated with E(z) loss at 20d. GO terms associated with these genes included several relevant to age-associated processes, such as oxidoreductase activity (Supplementary Table 2). Additionally, the genes upregulated in E(z) mutants were enriched in the motifs Cytochrome P450 and Alpha-crystallin/HSP family. We noted that the Hsp70ba gene was also upregulated, which is a chaperone of the Hsp70 family, genes that are known to convey resistance to neurodegenerative disease.

To define gene expression changes with age, we compared the transcriptomes of young and older wild-type brains, using a false discovery rate of p < 0.05 and an absolute log fold-change of more than 0.5. These criteria defined 672 downregulated and 959 upregulated genes (Supplementary Data 1). Again, due to our interest in genes that may normally be silenced by PRC2 with age, we focused on those genes that became downregulated with age in the brain. GO Terms associated with these genes included ATP
synthesis coupled proton transport, among others (Supplementary Table 2). Notably, genes downregulated with age also included the Alpha-crystallin/sHSP category that was upregulated in the brain upon reduced function of $E(z)$. We expected that target(s) of PRC2 relevant to healthy brain aging would be genes that normally become downregulated with age in the wild-type brain. We therefore compared the overlap of aging-induced genes with those that are downregulated with age to those that are upregulated in the $E(z)$ mutant. This identified four categories: GO:0005887—integral component of the plasma membrane, dme01100:Metabolic pathways, IPR002068:Alpha crystallin/Hsp20 domain, and IPR001436:Alpha crystallin/Heat shock protein. Intriguingly, sHSPs were common to both sets of genes (Fig. 7a, Supplementary Figure 5, Supplementary Table 2). Five alpha-crystallin/sHSP genes were upregulated in $E(z)$ mutants: Hsp23, Hsp26, Hsp27, CG13133, and CG7409. An MA plot of the mutant brain tissue may be a measuring stick for the levels of these probes to determine whether the mutant brain was assessed for the levels of these probes to determine whether the mutant brain displayed the same, a more advanced or a less advanced age. The mutant brain was assessed for the levels of these probes to determine whether the mutant brain displayed the same, a more advanced or a less advanced age. The mutant brain also displayed the same, a more advanced or a less advanced age.

The $E(z)$ mutant brain shows a youthful transcriptome. MiR-34 mutant brains have a transcriptional profile consistent with advanced age beyond their chronological age. To determine this, we had defined 173 probe sets as highly correlated with age in the brain using linear regression statistical methods and then the transcriptional profile of the miR-34 mutant brain was assessed for the levels of these probe sets to determine whether the mutant displayed the same, a more advanced or a less advanced age. The genes corresponding to these probe sets define a “measuring stick” of brain age, to which we could compare our current transcriptomic data. We thus used the genes corresponding to these probe sets to determine whether the $E(z)$ mutant brain also impacted the transcriptional profile in a way that indicated an altered age. Because miR-34 mutants display a premature-aged gene expression profile, we considered that $E(z)$ brain tissue may...
correspond to a younger age profile, if targets of PRC2 relevant to aging of the brain are impacted.

Hierarchical clustering of the positively age-correlated measuring stick genes across the samples identified two clusters of genes that were downregulated in E(z) mutant brains (Fig. 8a). pression in the adult brain with age, acts to help reduce PRC2, \( E(z) \), and reduction in PRC2 activity have inverse effect of a brain of less advanced age. Collectively, these findings indicate that \( E(z) \) reduction-of-function mutants have several gene expression characteristics that are conducive to healthy brain aging: an overall transcriptome signature of age consistent with a more youthful brain, as well as elevated sHSP chaperone expression, and elevated Hsp70 expression.

**Discussion**

We present an age-associated gene expression pattern of the brain that can be both accelerated (in the miR-34 mutant, resulting a gene expression profile consistent with advanced age), as well as decelerated, resulting in an expression profile consistent with a more youthful age (in the \( E(z) \) mutant, Fig. 8b, c). These gene expression changes are a result of manipulations of a naturally occurring regulatory circuit in which miR-34, which increases in expression in the adult brain with age, acts to help reduce translation of the PRC2 components \( Su(z)12 \) and Pcl. In the absence of miR-34, \( Su(z)12 \) and Pcl protein levels are more elevated in the brain (Figs. 2, 3), and the PRC2 associated chromatin mark H3K27me3 is higher (Fig. 4). Decreasing the activity of components of PRC2 results in resistance to neurodegeneration and stress, and lifespan extension (Fig. 5). Importantly, expression profiling of mutants in the catalytic component of PRC2, \( E(z) \), reveals decreases in expression of genes positively correlated with age (Fig. 8b, c). These experiments reveal that miR-34 mutants and reduction in PRC2 activity have inverse
The small HSP chaperone family is regulated with age and by E(z). RNA-seq was performed on 20d brain tissue from animals transheterozygous for the E(z)F1 and E(z)C27 alleles, and on wild-type. Because the E(z)F1 allele is temperature-sensitive, two temperatures were used, 25 °C (permissive) and 29 °C (restrictive). Wild-type brains, 3d (young) and 20d (aged) 25 °C, were also dissected and RNA-seq performed. Genes were selected as differentially expressed using a Benjamini-Hochberg p < 0.05 and at least a 0.5 absolute log2 fold-change. a Gene overlap. Twenty genes had an expression pattern consistent with being a target of E(z) with age in the brain: upregulated in E(z) and downregulated with age. b, c Differential gene expression analysis of sHSP family genes. Gray data points are genes not differentially expressed. Black data points are genes with significant changes in expression (p<0.05, DESeq2 negative binomial generalized linear model, Benjamini-Hochberg correction). Red circles mark the sHSP family members Hsp23, Hsp26, Hsp27, Hsp67Ba, Hsp67Bc (C27), CG4461, CG7409, CG13133, and CG14207. Hsp22 was not included because no reads mapped uniquely to this gene. b Small HSP genes are upregulated in 20d E(z) mutant brains. Among these, Hsp23, Hsp26, Hsp27, and CG7409 were significantly elevated in E(z) mutants (p < 0.05, DESeq2 negative binomial generalized linear model, Bonferroni correction), compared to 20d wild-type. c Small HSPs are downregulated in the brain with age. Hsp23, Hsp26, Hsp27, Hsp67Ba, and Hsp67Bc were significantly decreased (p < 0.05, DESeq2 negative binomial generalized linear model, Bonferroni correction) with age comparing 20d to 3d wild-type. d, e Sequence coverage at specific loci indicates silencing of sHSPs with age, and release from silencing in E(z) mutants. Read coverage at the individual sHSP loci (d) Hsp26 and (e) Hsp27 demonstrates a decrease in expression of sHSPs with age. Young wild-type animals express high levels (blue) while aged wild-type animals have less sHSP expression (yellow). E(z) mutation (purple) results in increased sHSP levels compared to age and temperature matched controls (orange). Y-axis indicates sequence read coverage from one biological replicate from each cohort. Each library shown had similar depth of sequencing (24–26 × 10⁶ reads). X-axis indicates position along the gene. Expression levels in E(z) are high, similar to young brain tissue.
transcriptome profile. Small HSP proteins have been shown to suppress polyglutamine aggregation and extend lifespan, which are two phenotypes associated with reduction of PRC2 components (Figs. 5, 6). Several sHSP proteins are also upregulated in the long-lived 

**miR-34 modulation**, versus elimination of PRC2 function in the brain. Mice with severe mutation for PRC2 activity in the striatum suffer from neurodegeneration. In those experiments, PRC2 was conditionally inactivated using null mutations in both mouse homologs of E(z) (EZH1 and EZH2). Although it was proposed from that finding that PRC2 activity could normally be protective against neurodegeneration, null mutations in PRC2 component genes including mammalian EZH2 and SUZ12 are embryonically lethal. Thus, critical cellular and/or developmental functions are provided by this complex. We suggest that null mutations will result in stronger and perhaps more gene expression changes that eliminate critical functions, compared to the situation of reduction—but not elimination—of PRC2 activity achieved in our studies and by miR-34. Consistent
The possibility that mitigating PRC2 activity, through gene or small molecule approaches, may lead to healthier brain aging and protection from associated deleterious consequences, such as cognitive decline and neurodegenerative disease.

Methods

Fly strains. Drosophila were maintained at 25°C, unless otherwise indicated, and raised on standard molasses fly meal. For GeneSwitch experiments, 30 μl ethanol (vehicle control) or 50 μl 4 mg/ml RU486 dissolved in ethanol was added to food vials and allowed to dry with gentle rocking for 24 h. E(z) alleles were backcrossed at least five times to the homozygous w1118 stock (Bloomington line 5905) wild-type control, selecting for heterozygous animals using restriction fragment length polymorphisms (RFLP), (Supplementary Table 4). Male flies were used for all experiments. Complete genotypes and cross details of flies used are provided (Supplementary Table 7).

Brain dissections. In many experiments, brains were carefully dissected from the head capsule for use due to enrichment of miR-34. Flies were mildly anesthetized using CO2 and decapitated using a razor blade. The head was placed posterior side down and the proboscis was then removed using Dumont #5 forceps (Fine Science Tools, Foster City, CA). The head was then simultaneously squeezed from both lateral sides and the posterior resulting in slight protrusion of the brain through the proboscis cavity. Forceps were then used to drag it out of the head capsule and place it in ice-cold PBS in a glass dissection well. Further dissection of remaining eye and trachea tissue was under cold PBS. Brains were then transferred to a microtube using a Pasteur pipet, PBS was aspirated, and brains were grounded in Laemmli Buffer (1–2 μl per brain, at least 20 brains for each sample) for Western immunoblotting, or Trizol for RNA-seq analysis (see below).

Luciferase assays. All primers for constructs used in these experiments are listed in Supplementary Table 5. Su(z)12 luciferase reporters were constructed using PCR primers to amplify 953 nucleotides of the 3' UTR from Su(z)12. The miR-34–3'UTR was cloned into the BamHI/Sall sites in pMT-Remilla. The pC3 3'UTR was prepared using overlap extension of synthesized oligos utilizing Klenow fragment to produce a 211 bp 3'UTR which was cloned into the BamHI/Sall sites in pMT-Remilla. Mutations were induced to disrupt the seed sequence using the Quikchange mutagenesis system (Stratagene, La Jolla, CA).

For the luciferase reporter assay, cells were plated at 1 × 105 cells/mL, in a total of 2 ml for each transfection in a six-well plate. Cell lines from the DGRC. Transfection of pMT-Remilla luciferase reporter containing the 3'UTR of interest, a transfection efficiency marker (pMT-Firefly), and a plasmid upregulating miR-34 (pMT-miR-34) or a control (pMT-GeP), was performed the next day using Effectene (Qiagen, Germantown, MD). For SL2 cells, amounts of plasmids transfected per well were 100 ng, 40 ng, and 2 ng, respectively. For SL2 cells, amounts used were 1067 ng pMT-Remilla reporter, 4 ng pMT-Firefly, and 1007 ng pMT-miR-34 or pMT-miR-277 control. 1 day after transfection of SL2 cells, the cells were treated with media (wheat and/or 100 μl of complete media was added to each well. 3 days later, CuSO4 was added to a final concentration of 1 mM. 1 day later, Firefly and Remilla luciferase levels were measured using Dual-Glo Luciferase Assay System (Promega, Madison, WI) with an Analyst HT luminometer.

For the dsRNA experiments in SL2 cells, 1 day after transfection cells were instead treated with dsRNA to the AGO1 gene, or an E. coli beta-galactosidase control (previously described). For dsRNA treatment, cells were harvested, pelleted, and resuspended in 5.5 ml serum-free media. DsRNA (0.75 ng) was placed in each well of a 96-well plate, and 50 μl of cells were added. After 45 min, 100 μl of complete media was added to each well. 3 days later, CuSO4 was added to a final concentration of 1 mM, and the next day luciferase levels were measured as above.

Western immunoblotting. Conditions were optimized for each antigen/antibody pair. Antibodies and conditions are listed in Supplementary Table 6. HRP signal was developed using ECL (Amersham, Piscataway, NJ) and imaged on an Ameri- sham Image 600. Histones derived from heads were purified using Histone Purification Mini Kit (Active Motif, Carlsbad, CA). Quantification of bands was performed with ImageJ. Uncropped Western images with size markers are presented in Supplementary Fig. 6.

Su(z)12-HA-tag construction. One copy of the HA tag sequence, YPDVDPVDY, was fused in frame to the coding region of Su(z)12 just prior to the endogenous stop codon, followed by a BamHI cloning site. Fragments of the Su(z)12 3'UTR derived from the luciferase reporters were cloned into pCSpE4, one with a wildtype 3'UTR, and one with the mutated seed 3'UTR. The promoter and coding sequence were amplified using primers listed in Supplementary Table 5, the fragment was then cloned into the pCSpE4 vectors already containing the wild-type 3'UTR from Su(z)12, and the mutated 3'UTR from Su(z)12 to produce Su(z)12-Ctermin-HA (pRK228) and Su(z)12-Cterm-HA-NsiI (pRK235).
Pseudopupil assay. Flies were mildly anesthetized using CO₂ and decapitated using a razor blade. Heads were mounted onto a microscope slide to allow illumination from the posterior along the axis of the ommatidia, resulting in visualization of photoreceptors. Heads were positioned using a thin line petroleum jelly, positioning the head in the jelly such that the light path travels through a minimum of head tissue, and does not travel through petroleum jelly. The head was then covered in immersion oil suitable for an oil immersion objective (100×). The photoreceptors are then visible with bright field imaging, with the condenser adjusted for high intensity, highly focused light centered beneath the ommatidia in the field of view. Experiments were done with blinding to genotype.

Immunocytochemistry. Flies were mildly anesthetized using CO₂ and decapitated using a razor blade. The head was placed posterior side down and the proboscis and air sac were removed using forceps. The head was then placed on Tissue Freezing Medium (TFM) (Electron Microscopy Sciences, Hatfield, PA), and the proboscis cavity was filled with TFM. The head was then submerged and any bubbles were removed. The head was oriented inside an Embedding Mold (Poly-science, Warrington, PA) to allow for coronal sections, and frozen in a dry ice/ethanol bath. Fly heads were then cut to 12µ sections on a CM3005S cryostat (Leica, Buffalo Grove, IL). Sections were placed on Colorfrost Plus Microscope Slides (Fisher Scientific, Pittsburgh, PA), and kept at −80 °C until all replicates were finished.

Frozen sections were then warmed to 37 °C for 30 min. Sections were outlined with a PAP pen (Daido Sangyo Co., Tokyo, Japan). Sections were then fixed in 0.5% paraformaldehyde in phosphate buffered saline (PBS) for 30 min at room temperature (RT), then washed 3 times for 5 min each in PBS. Sections were blocked in PBSG (1% goat serum, 0.2% Bovine Serum Albumin, and 0.01% saponin in PBS) for 1 h at RT, followed by incubation with primary antibody (rat anti-HA monoclonal antibody, 3F10, Roche 11867423001, 1:2 dilution in PBSG) overnight in a humidified chamber at 4 °C. Primary antibody stained sections were then washed 3 times for 5 min each in PBSG, followed by secondary antibody (AffiniPure goat anti-Rat conjugated FITC, 1:20 dilution in PBSG, Jackson ImmunoResearch) incubation for 1 h at RT. Sections were then rinsed 3 times for 5 min each in PBS, followed by staining with Hoechst 33342 stain (Molecular Probes) for 10 min, followed by 3 more PBS washes for 5 min each. Sections were then covered in vechstahl mounting medium (Vector Laboratories, Burlingame, CA), and a coverslip was applied.

Statistics. Statistical tests were done in R or with Prism (GraphPad Software, La Jolla, CA). One-way or two-way ANOVA was performed followed by Tukey’s post hoc test for multiple comparisons. For each ANOVA, the Shapiro-Wilk test was chosen for normality testing, as determined by the DESeq2 package. Age-correlated probes were translated to Ensemb genes using the biomart R package. Only probesets that overlapped genes expressed in brain by the RNA-seq data were used in the analysis. This, along with redundancy in some of the probesets, resulted in condensing the 108 positively age-correlated probesets to 102 ENSEMB genes (Supplementary Data 2). RNA-seq data and count matrices are accessible under Gene Expression Omnibus (GSE110135).

Code availability. The R code used for all analyses is available upon request.

Data availability. The datasets generated during the current study are available under accession GSE110135 in NCBI GEO (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110135) and upon request.

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RNA sequencing. Once at least 20 brains were collected as described above, brains were transferred to an Eppendorf tube and homogenized in 50 µL Trizol (Thermo Fisher Scientific, Waltham, MA) using a pestle. An additional 200 µL of Trizol was added to the tissue Trizol mixture, and then frozen at −80 °C. RNA was extracted using the manufacturer recommended procedure for Trizol reagent. Any residual DNA was removed using a DNase digestion (Ambion, Grand Island, NY). Total RNA was then cleaned up using RNeasy columns (Qiagen, Germantown, MD). The quality of the resulting mRNA was validated using a Bioanalyzer and quantified with Qubit. 95–200 ng of total RNA was used for TruSeq stranded mRNA library preparation according to the manufacturer’s protocol (Illumina, San Diego, CA). Libraries were quantified using both Qubit and Kapa Quanti-fluor to allow for coronal sections, and frozen in a dry ice/ethanol bath. Fly heads were then cut to 12µ sections on a CM3005S cryostat (Leica, Buffalo Grove, IL). Sections were placed on Colorfrost Plus Microscope Slides (Fisher Scientific, Pittsburgh, PA), and a coverslip was applied.

RNA-seq data analysis. Reads were mapped to the Drosophila Genome Release 6.15 with STAR using the default parameters. Reads were assigned to exons in the FlyBase version15.1 GTF file using the hiseq-count Python package. Reads per gene were then analyzed using the DESeq2 R package from Bioconductor. Gene expression changes observed in E(z) mutants were not restricted to animals cultivated at the restrictive temperature of 29 °C: only three genes showed a temperature-dependent gene expression change consistent with the reported temperature-sensitive nature of the allele, as indicated by testing for a significant interaction of temperature and genotype. As such, genes upregulated and downregulated in 20d E(z) mutant brains relative to 20d wild-type were selected using a two-way statistical model including both temperature and genotype as independent categorical variables. When determining fold change and p values of wild-type vs. E(z) mutant, the comparison was made using both 25 °C and 29 °C samples; sampling on temperature was used to determine gene expression changes that were a consequence of E(z) mutation.
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Author contributions

J.R.K., N.L. and N.M.B. conception and design. J.R.K performed the experiments and analyzed the data. J.R.K and N.M.B interpreted the data. J.R.K and N.M.B wrote the paper.

Additional information

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