Impact of age-associated increase in 2’-O-methylation of miRNAs on aging and neurodegeneration in Drosophila

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MicroRNAs (miRNAs) are 20- to ~24-nucleotide (nt) small RNAs that impact a variety of biological processes, from development to age-associated events. To study the role of miRNAs in aging, studies have profiled the levels of miRNAs with time. However, evidence suggests that miRNAs show heterogeneity in length and sequence in different biological contexts. Here, by examining the expression pattern of miRNAs by Northern blot analysis, we found that Drosophila miRNAs show distinct isoform pattern changes with age. Surprisingly, an increase of some miRNAs reflects increased 2’-O-methylation of select isoforms. Small RNA deep sequencing revealed a global increase of miRNAs loaded into Ago2, but not into Ago1, with age. Our data suggest increased loading of miRNAs into Ago2, but not Ago1, with age, indicating a mechanism for differential loading of miRNAs with age between Ago1 and Ago2. Mutations in Hen1 and Ago2, which lack 2’-O-methylation of miRNAs, result in accelerated neurodegeneration and shorter life span, suggesting a potential impact of the age-associated increase of 2’-O-methylation of small RNAs on age-associated processes. Our study highlights that miRNA 2’-O-methylation at the 3’ end is modulated by differential partitioning of miRNAs between Ago1 and Ago2 with age and that this process, along with other functions of Ago2, might impact age-associated events in Drosophila.

[Keywords: miRNA; 2’-O-methylation; miRNA sorting; aging; neurodegeneration]

Supplemental material is available for this article.

Received August 29, 2013; revised version accepted December 3, 2013.
associated neurodegeneration in *Drosophila* revealed an intriguing pattern of miR-34 isoforms: Although multiple-length isoforms of miR-34 are generated, only the short isoform accumulates with age (Liu et al. 2012). Furthermore, the generation of isoforms of miR-34 requires 3’ end trimming by a novel 3’-to-5’ exonuclease, Nibbler [Nbr] (Han et al. 2011; Liu et al. 2011), highlighting the potential importance of regulation of miRNA length in age-associated processes.

In addition to length and sequence heterogeneity, the 2’-OH of the 3’ terminal ribose of miRNAs can be modified by 2’-O-methylation [Zhao et al. 2012a]. In plants, this modification occurs on nearly all miRNAs and siRNAs [Yu et al. 2005; Zhao et al. 2012a,b] and protects the small RNAs from HESO1-mediated uridylation and degradation [Li et al. 2005; Yu et al. 2005; Ren et al. 2012; Zhao et al. 2012b]. In *Drosophila*, most miRNAs are loaded into Ago1 and remain unmodified (Hutvagner et al. 2001; Okamura et al. 2004; Vagin et al. 2006). However, a subset of miRNAs are found to be 2’-O-methylated [Forstemann et al. 2007; Horwich et al. 2007; Ghildiyal et al. 2008; Czech et al. 2009; Okamura et al. 2009]. Importantly, this modification is also protective in animals: Loss of 2’-O-methylation of small RNAs leads to destabilization as well as tailing and trimming of the small RNAs [Kurth and Mochizuki 2009; Ameres et al. 2010; Kammenga et al. 2010].

In *Drosophila*, 2’-O-methylation of small RNAs is associated with loading of the miRNAs into different Ago complexes: While most miRNAs are loaded into Ago1 [Ago1-RISC] and remain unmodified, those loaded into Ago2 [Ago2-RISC] are 2’-O-methylated [Czech et al. 2009; Okamura et al. 2009; Ghildiyal et al. 2010]. The miRNA/miRNA* duplex structure and 5’ nucleotide preference are two suggested mechanisms by which miRNAs are directed into the two distinct Ago complexes [Forstemann et al. 2007; Tomari et al. 2007; Okamura et al. 2009; Ghildiyal et al. 2010]. miRNAs loaded into Ago2 are also active in translational silencing of the target genes, like miRNAs loaded into Ago1, but by a potentially different mechanism and with different silencing efficiency [Czech et al. 2009; Iwasaki et al. 2009; Okamura et al. 2009]. However, despite the discovery of functional Ago2-loaded and 2’-O-methylated miRNAs in *Drosophila*, whether such loading of miRNAs to Ago2 is biologically regulated or biologically important in vivo is not yet clear.

Here, we pursued the study of several Nbr-dependent miRNAs that, like miR-34, show multiple isoforms. Examination of changes in their isoform patterns with age unexpectedly revealed a diversity of patterns. The age-associated increase of some *Drosophila* miRNAs reflected increased 2’-O-methylation of select isoforms. Associated with this increase, we found increased loading of specific miRNA isoforms into Ago2, but not Ago1, with age. Furthermore, the lack of 2’-O-methylation by Hen1 and Ago2 mutations resulted in reduced life span and brain degeneration, raising the possibility that the increased protection of small RNAs with age may impact age-associated events.

### Results

**Distinct age-associated isoform patterns of Nbr-trimmed miRNAs**

*Drosophila* miR-34-5p, which is a target of Nbr-dependent 3’ end-trimming [Han et al. 2011; Liu et al. 2011], shows an increase in the short isoform with age [Fig. 1A,B; Liu et al. 2012]. This observation led us to examine the age-associated pattern of other Nbr-trimmed miRNAs. We focused on several miRNAs that could be readily detected by Northern analysis. Northern blots of these miRNAs at 3 d and 30 d in heads revealed intriguing changes in isoform patterns. One Nbr-trimmed miRNA, miR-317-3p, increased with age like miR-34-5p [Fig. 1A,B]. The increase in both of these miRNAs reflected an increase in their short isoforms with age. In contrast, although both miR-305-5p and miR-263a-5p increased in total amount with age, their isoform patterns were opposite: These miRNAs showed an increase in the longer isoforms with age [Fig. 1C,D]. miR-11-3p showed a single isoform by Northern and increased by 30 d [Fig. 1E,F]. These observations raised the possibility that multiple mechanisms are used to generate distinct miRNA isoform patterns with age.

**Age-associated increase in 2’-O-methylation of Nbr-trimmed miRNAs**

One mechanism to explain an increase in long isoforms with age could be an increased protection of these specific isoforms with age. Almost all plant miRNAs and siRNAs [Li et al. 2005; Yu et al. 2005; Ren et al. 2012; Zhao et al. 2012b] and animal siRNAs and piRNAs are 2’-O-methylated [Horwich et al. 2007; Kirino and Mourelatos 2007a,b,c]. Recent studies also suggest that a subset of *Drosophila* miRNAs are 2’-O-methylated [Forstemann et al. 2007; Horwich et al. 2007; Czech et al. 2009; Okamura et al. 2009; Ghildiyal et al. 2010]. Importantly, 2’-O-methylation of animal small RNAs is also protective such that loss of 2’-O-methylation leads to their destabilization [Ameres et al. 2010; Kammenga et al. 2010]. Therefore, we hypothesized that the increase of the long isoforms of miR-305-5p, miR-263a-5p, and miR-11-3p may result from increased protection at the 3’ end of specific isoforms by 2’-O-methylation. To address this, we performed oxidation/β-elimination assays on total RNA from 3-d and 30-d wild-type heads and bodies. If the 3’ end ribose of small RNAs is protected by 2’-O-methylation, as for piRNAs and siRNAs, these small RNAs cannot react with sodium periodate in the oxidation/β-elimination assay and will not decrease in size. However, if the 3’ end ribose is unmodified, they can react, will be β-eliminated, and will shift down in length [Horwich et al. 2007]. Therefore, a change in the mobility of the small RNAs by Northern indicates the presence or absence of a protective modification at the 3’ end. We performed this assay and found that miR-34-5p shifted down in size and thus is not protected at the 3’ end [Fig. 2A, left panel]. In contrast, miR-305-5p, miR-263a-5p, and miR-11-3p showed an increase in protection of the long
isoforms with age [Fig. 2A, right panel, arrows]. Quantification of the ratio of protected to unprotected isoforms of miR-305-5p, miR-263a-5p, and miR-11-3p showed an increase in the ratio of protected to unprotected isoforms with age. If the increase of the protected isoforms simply reflected an increase of the overall level of these miRNAs, the ratio of protected to unprotected isoforms after oxidation/β-elimination would not change. However, the ratio increased for these three miRNAs (Fig. 2B), indicating a mechanism to increase the protection of the long isoforms of these miRNAs with age.

We next determined whether the protection of specific miRNA isoforms is due to 2′-O-methylation at the 3′ end. 2′-O-methylation of small RNAs depends on the methyltransferase Hen1. In addition, 2′-O-methylation of small RNAs requires loading into Ago2: Loss of Ago2 and Ago2-loading machinery (Dcr2 and R2D2) leads to loss of modification [Horwich et al. 2007]. Therefore, to confirm that the miRNAs were 2′-O-methylated and loaded into Ago2, we tested the effect of loss of Hen1 and Ago2 on the pattern of miR-305-5p, miR-263a-5p, and miR-11-3p with age. Hen1^00810 and Ago2^BL16608 mutant flies were aged to 3 d or 30 d, RNA was isolated, and oxidation/β-elimination was performed. This revealed that miR-305-5p, miR-263a-5p, and miR-11-3p were no longer protected but rather depended on Hen1 and Ago2 for protection at both 3 d and 30 d [Fig. 3, arrowheads]. This finding indicates that the increase of the long isoforms of these miRNAs is dependent on Hen1 and thus is due to 2′-O-methylation at the 3′ end. These data also indicate that these miRNA isoforms are loaded into Ago2 for 2′-O-methylation. We note that miR-317-3p is exceptional in that it showed protection of the long isoform from oxidation/β-elimination in both young and old flies (see Fig. 2A, arrowheads); however, to our surprise, protection persisted even after the loss of Hen1 and Ago2 [Fig. 3, arrowheads]. This suggests that miR-317-3p is protected in a manner independent of Hen1 and Ago2 function.

An age-associated increase in Ago2 loading of 2′-O-methylated isoforms of miRNAs

In Drosophila, while most miRNAs are loaded into Ago1 and thus are unmodified, a subset of miRNAs has been

Figure 1. Nbr-dependent miRNAs show distinct isoform patterns with age. Northern blots of different Nbr-dependent miRNAs with age. (A,B, left panel) miR-34-5p and miR-317-3p showed accumulation of short isoforms with age. (B, right panel) Both miR-34-5p and miR-317-3p also increased in total amount with age. (C,D, left panel) miR-305-5p and miR-263a-5p showed accumulation of long isoforms with age. (D, right panel) Both miR-305-5p and miR-263a-5p increased in total amount with age. (E,F) miR-11-3p, a single-form miRNA by Northern, accumulated with age. (B,D, left panels, F) Quantification of the different isoforms of each miRNA. [Right panels] Quantification of the total amount of each miRNA with age. Red arrowheads and arrows indicate the isoforms that increased with age. Mean ± SD. n = 3; (*) P < 0.05 (Student’s t-test).
observed to be loaded into Ago2 (Ago2-RISC) and is 2'-O-methylated (Czech et al. 2009; Okamura et al. 2009; Ghildiyal et al. 2010). Therefore, based on our findings, we proceeded to determine whether the protected isoforms of miR-305-5p, miR-263a-5p, and miR-11-3p increased their Ago2 loading with age. To do this, we immunoprecipitated Ago1 and Ago2 from whole flies and then performed Northern blots on the precipitated small RNAs, comparing 3 d with 30 d. Ago1 could be immunoprecipitated with an antibody; for Ago2, we used flies bearing a genomically tagged Flag-HA-Ago2 (Czech et al. 2008). This approach revealed a specific loading pattern for each small RNA with age in Ago1 versus Ago2. miR-34-5p, which was not protected at the 3' end (see Fig. 2A), was predominantly loaded into Ago1 and was not detectably present in Ago2 (Fig. 4A). Consistent with the age-associated pattern of miR-34-5p by Northern from total RNA (see Fig. 1A; Liu et al. 2012), the short isoforms of miR-34-5p accumulated in Ago1 with age (Fig. 4A). esi-2.1, an abundant endogenous siRNA in flies (Czech et al. 2008; Ghildiyal et al. 2008; Kawamura et al. 2008; Okamura et al. 2008), was selectively loaded into Ago2 and was not detectable in Ago1 (Fig. 4A). In contrast, miR-305-5p and miR-263a-5p showed an intriguing loading pattern: While multiple isoforms were present in Ago1 at both 3 d and 30 d, only the long isoforms (24 nt) were present in Norderns of Ago2-IP material (Fig. 4A). The single isoform of miR-11-3p was present in Ago1, however, a small portion was loaded into Ago2 (Fig. 4A). In addition to the loading of specific lengths of isoforms into Ago2, there was also a trend of increased levels of the loaded isoforms of miR-305-5p, miR-263a-5p, and miR-11-3p into Ago2 with age. To quantify this, we normalized the level of the Ago2-loaded miRNAs to Ago2-loaded esi-2.1 at the corresponding age; the total level of esi-2.1 did not change with age [Fig. 4B] and is loaded into Ago2 and not into Ago1 [Fig. 4A]. After normalizing, we confirmed a significant increase of Ago2-loaded miR-305-5p, miR-263a-5p, and miR-11-3p with age [Fig. 4C–E]. These findings suggest that the age-associated increase of 2'-O-methylation of miRNAs reflects increased loading of selective isoforms into Ago2 with age.

One possibility for increased loading into Ago2 could be an increase in the level of Hen1, Ago2, and/or the Ago2-loading machinery (Dcr2 and R2D2) with age (Liu et al. 2003; Tomari et al. 2004; Marques et al. 2010; Okamura et al. 2011; Nishida et al. 2013). To address this possibility, we performed Western immunoblots on Ago2 (Flag-HA-Ago2 flies), R2D2, and Dcr2 with age (no antibody is available for Hen1). Both Ago2 and R2D2 were unchanged with age [Fig. 4F–H], and Dcr2 was not detectable in adult flies [Supplemental Fig. S1]. To further confirm these results, we examined microarray data from aged Drosophila brains (3 d, 30 d, and 60 d) [Liu et al. 2012]. Analysis confirmed no significant increase in Hen1, Ago2, Dcr-2, or R2D2 RNA levels with age [Supplemental Table S1]. In addition, the level of Ago1 mRNA and protein was unchanged, suggesting that a decrease in Ago1 levels would not explain the relative increase in Ago2 loading of miRNAs with age [Supplemental Table S1; Supplemen-
We observed a potential increase in mRNA levels for Hsp70 (Supplemental Table S1), a component of RISC-loading machinery, in a complex with Hsp90 (Iki et al. 2010; Iwasaki et al. 2010; Miyoshi et al. 2010). However, since the Hsp90 level is unchanged, there is likely no increase in the Hsp90/Hsp70 complex with age. Together, these results suggest that the increase of Ago2 loading of miRNAs with age is independent of a change in the level of Ago2 or Ago2-loading machinery.

A global shift of Ago1 vs. Ago2-loaded miRNAs with age

To confirm the increase in miRNA loading into Ago2 with age, we immunopurified Ago1 and Ago2 from flies aged 3 d and 30 d and performed deep sequencing of the small RNA fraction. We normalized the number of observed reads to the total number of non-miRNA reads in Ago1 and to the esi-2.1 reads in Ago2. This analysis showed that the total read number of miRNAs in Ago1 was largely unchanged, while that of Ago2 increased with age [Fig. 5A]. In addition, the share of miRNAs among total small RNAs occupying Ago1 showed little change with age, while in Ago2, the miRNA reads increased dramatically with age [30.7% at 3 d to 69.6% at 30 d] [Fig. 5B]. During the analysis, we noticed that miR-263a-5p was the singular most abundant miRNA in the 3-d and 30-d Ago2 libraries. Therefore, to rule out that the increased trend of total miRNA reads and percentage in Ago2 with age simply reflected a change in miR-263a-5p, we also removed miR-263a-5p reads from 3-d and 30-d Ago2-IP libraries and recalculated the total miRNA read number (normalized to esi-2.1) and the percentage of total miRNAs in 3-d and 30-d Ago2 libraries. This confirmed that, even after removing miR-263a-5p, the trend persisted: The read number and percentage of the total miRNAs in Ago2 increased with age [Supplemental Fig. S3]. These results, together with the increased ratio of protected to unprotected isoforms of miRNAs with age [see Fig. 2], support the idea that more miRNA molecules are becoming loaded into Ago2, and not Ago1, with age.

We analyzed in detail the three miRNAs that were the focus of Northern analysis: miR-305-5p, miR-263a-5p, and miR-11-3p. Supporting the earlier observations, miR-305-5p, miR-263a-5p, and miR-11-3p showed increased association in Ago2 with age [Fig. 5C, bottom panels]. In contrast, the amount of these miRNAs in Ago1 decreased dramatically with age [30.7% at 3 d to 69.6% at 30 d] [Fig. 5B]. During the analysis, we noticed that miR-263a-5p was the singular most abundant miRNA in the 3-d and 30-d Ago2 libraries. Therefore, to rule out that the increased trend of total miRNA reads and percentage in Ago2 with age simply reflected a change in miR-263a-5p, we also removed miR-263a-5p reads from 3-d and 30-d Ago2-IP libraries and recalculated the total miRNA read number (normalized to esi-2.1) and the percentage of total miRNAs in 3-d and 30-d Ago2 libraries. This confirmed that, even after removing miR-263a-5p, the trend persisted: The read number and percentage of the total miRNAs in Ago2 increased with age [Supplemental Fig. S3]. These results, together with the increased ratio of protected to unprotected isoforms of miRNAs with age [see Fig. 2], support the idea that more miRNA molecules are becoming loaded into Ago2, and not Ago1, with age.

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with age (Fig. 5C, top panels). Together, these data support the idea that the loading pattern of miRNAs between Ago1 and Ago2 changes dramatically with age such that Ago2 loading of selective miRNA isoforms increases, while Ago1 loading of the miRNA isoforms generated from the same stem–loops decreases.

Detailed analysis of the libraries allowed identification of additional miRNAs with the same trend of an increase of select isoforms in Ago2 with age. We compared relative loading ratios [Ago2 over Ago1, $R_{21}$] (see the Materials and Methods) of the most abundant isoform for each miRNA in 30-d flies versus 3-d flies (Fig. 6A). This showed the three miRNAs that were the focus of our tests with Northerns [miR-305-5p, miR-263a-5p, and miR-11-3p] are among those preferentially loaded into Ago2 at both 3 d and 30 d (Fig. 6A).

Among the 240 Drosophila miRNA genes, 135 were detected with at least one raw read in both Ago1-IP and Ago2-IP libraries at 3 d, while 143 were detected at 30 d. Among these, 67 5p and 3p sequences had at least 1000 raw reads of at least one isoform at 3 d or 30 d, of which 15 mature and nine star sequences showed preferential loading of specific isoforms into Ago2 relative to Ago1 at 3 d ($R_{21} > 1.2$), while 16 mature and nine star sequences showed that trend at 30 d ($R_{21} > 1.2$) [Fig. 6A; Supplemental Tables S2, S3]. 5p and 3p sequences were classified as “mature” or “star” based on their relative abundance in the Ago1-IP 3-d library and a consistent behavior.

Figure 4. Increased Ago2 loading of long miRNA isoforms with age. Northern blots with quantitation of miRNAs associated with Ago1 versus Ago2 with age. (A) Northern blots for small RNAs on RNA isolated from Ago1-IP (left) and Ago2-IP (Flag-HA-Ago2) (right). (B) Quantification of esi-2.1 level with age. Mean ± SD. $n=4$; Student’s t-test confirmed no significant differences with age. There is no change in esi-2.1 levels with age. (C–E) Quantification of miRNA isoforms loaded into Ago2 with age, normalized to esi-2.1 in Ago2-IP. Mean ± SD. $n=4$; * $P < 0.05$ (Student’s t-test). For all three miRNAs, the Ago2-loaded isoforms increase with age. (F) Western immunoblot for Ago2 and R2D2 with age. (G) Quantification of Ago2 protein level with age. Mean ± SD. $n=3$, not significant by Student’s t-test. (H) Quantification of R2D2 protein level with age. Mean ± SD. $n=4$, not significant by Student’s t-test.
across all four libraries: Those with a higher read number compared with the read number from the other strand of the stem–loop were defined as “mature” [see the Materials and Methods for details].

Next, we determined whether the miRNA isoforms showing preferential loading into Ago2 compared with Ago1 at either age ($R_{21} > 1.2$ at 3 d or 30 d) (Fig. 6A) also showed an increase in loading into Ago2 with age. To assess the latter value, we calculated for each miRNA the fold change between 30 d and 3 d in normalized read numbers of the main isoform loaded into Ago2 (Fig. 6B). A change of more than one would indicate miRNAs with an increase in specific miRNA loading into Ago2 with age. We confirmed that miR-305-5p, miR-263a-5p, and miR-11-3p showed increased loading into Ago2 with age (Figs. 4A–E, 6B [red bars]). Together, these data indicate that 11 mature and eight star sequences out of 67 (5p and 3p) total sequences with >1000 raw reads of at least one isoform showed preferential loading of specific isoforms into Ago2 relative to Ago1, and such loading increased with age. We also analyzed a library from a previous study comparing Ago1-IP miRNAs to 3'9 end-protected miRNA from heads (Ghildiyal et al. 2010). This analysis also indicated a selective enrichment for particular miRNAs (Supplemental Fig. 4). From these analyses (Fig. 6B; Supplemental Fig. 4), we then performed Northern blots on several highlighted miRNAs that would be sufficiently abundant to detect in order to examine whether their protection increases with age: miR-1000, miR-100, miR-305, miR-11, miR-263a, miR-263b, and miR-8. In a combined list of mRNA targets of these miRNAs [predicted by TargetScan], the down-regulation ratio was substantially higher [104/13 = 8; $P < 0.001$; Benjamini-Hochberg FDR < 5%]; thus, these target genes are twice as likely to show stronger down-regulation with age compared with the whole genome. We further analyzed the splicing array data of Taliaferro et al. (2013) following knockdowns of Ago proteins in S2 cells. Although these arrays were designed for quantifying alternative splicing rather than gene expression, they still show trends consistent with our observations. Of 159 deregulated genes with Ago1 knockdown, eight predicted targets of the miRNAs shifted to Ago2 showed changed expression (down-regulated, except for the cal9 gene), all were also down-regulated in the aging microarray at 30 d versus 3 d. Among the 51 deregulated genes in Ago2 knockdown, four predicted targets of the miRNAs shifted to Ago2 showed a change of expression, but it was up-
regulation (except for the bbc gene), while they were down-regulated in the aging microarray at 30 d versus 3 d. Thus, for the group of genes in common, there is a striking reversal of expression following Ago2 knockdown, but not Ago1 knockdown, compared with the general down-regulation with age.

Loss of Hen1 and Ago2 is associated with shortened life span and neurodegeneration

To address the biological significance of the global shift in the population of Ago2-loaded miRNAs, we examined the impact of global loss of 2'-O-methylation of small RNAs by analyzing Hen1 and Ago2 mutant animals. Ago2 loss is known to cause defects in synaptic structure in third instar larvae [Pepper et al. 2009], indicating the importance of Ago2 on neuronal development. Here we focused on adult-specific age-associated effects. Life span analysis indicated that both Hen1f00810 and Ago2BL16608 mutants showed a shorter life span compared with genetic background-matched controls [Fig. 7D]. Detailed analysis of these animals also showed that both Hen1f00810 and Ago2 (Ago2414 and Ago2BL16608) mutants have increased brain vacuolization, indicative of brain degeneration with age [Fig. 7A–C]. While Hen1f00810 mutants showed more brain vacuolization in the optic lamina [Fig. 7B], Ago2 mutants had excessive vacuoles in the retina (Fig. 7C). Both Hen1 and Ago2 mutants are predicted to impact siRNAs, which may contribute to the effects, and the difference in phenotypes between Hen1 and Ago2 mutants might reflect a broader role of Ago2 beyond its effects on siRNAs (Taliaferro et al. 2013). However, defects in both Hen1 and Ago2 mutants suggest that 2'-O-methylation of

Figure 6. Identification of Ago2-loaded miRNA isoforms whose loading increases with age. (A) Dot plot showing the ratio of preferentially Ago2-loaded isoforms at 3 d (X-axis) to 30 d (Y-axis). We selected miRNAs with values $R_{21} > 1.2$ as candidates with greater loading into Ago2 at 3 d or 30 d (dashed lines parallel to the axes, miR-305-5p, miR-263a-5p, and miR-11-3p are seen) [full lists are in Supplemental Tables S2, S3]. miR-34-18nt, miR-276a/b-5p-17nt, and miR-981-5p-21nt showed an extremely high $R_{21}$ ratio at 3 d and/or 30 d (>16) [Supplemental Tables S2, S3]. This suggests much higher accumulation of these miRNA isoforms in Ago2-IP compared with Ago1-IP at 3 d and/or 30 d. This could happen because of biologically relevant up-regulation of these miRNA isoforms or cloning bias and degradation from the corresponding longer isoforms. These miRNA isoforms were removed from this figure because including these data obscured the distribution of the other data in the plot. miR-34-18nt was undetectable by Ago1-IP versus Ago2-IP Northerns. Therefore, most likely, miR-34-18nt is an artifact of library preparation. (B) Fold change of the main isoform of preferentially Ago2-loaded miRNA isoforms with age (30 d/3 d). Red indicates miRNAs known from our Northern analyses that show an increase in Ago2 association with age. Black arrows here and green bars in Supplemental Figure 4 indicate abundant miRNAs that were tested for accumulation in Ago2 with age in C. (C) Northern analysis [top] and quantitation [bottom] of miRNAs highlighted by the analyses in B and Supplemental Figure 4.
small RNAs, one class of which is miRNAs, is critical for age-associated processes in *Drosophila*.

**Discussion**

*Dynamic regulation of Ago1 vs. Ago2 loading of miRNAs with age*

Increasing evidence suggests that miRNAs show heterogeneity at the 5’ end and 3’ end and even in precise sequence in different cell types and biological contexts [Neilsen et al. 2012]. Despite this, it is less understood whether and how such heterogeneity is regulated biologically or its biological impact. Here, by examining the age-associated pattern of miRNA isoforms, we show that 2’-O-methylation of miRNAs changes in an age-depen-

dent manner in *Drosophila*. This change is correlated with a shift in partitioning of miRNAs into Ago2 versus Ago1 with age. These data suggest that the partitioning of miRNAs between Ago1 and Ago2 is modulated with age in *Drosophila*.

The mechanism of miRNA loading into the different Ago complexes in *Drosophila*, especially in vivo, remains unclear. Previously, miR-277 was shown to be loaded into both Ago1 and Ago2 because of the lack of an extensive central bulge in the miR-277/miR-277* duplex [Forstemann et al. 2007]. However, our analysis of the miRNAs that show higher levels of 2’-O-methylation with age failed to identify a consistent lack of central bulges on miRNA/miRNA* duplexes [Supplemental Fig. S5]. Rather, our results indicate that miRNA isoforms with the same sequence and length can be partitioned into both Ago1 and Ago2 and
that a change in this distribution is an age-associated phenomenon. This effect, at least in vivo, is likely to be driven by a bulge-independent mechanism. Bulge-independent partitioning of small RNAs into different Ago complexes is suggested from other studies as well: The loop sequence from miR-34 precursor stem–loop is abundant in some cells and loaded into Ago1 despite its single-stranded nature [Okamura et al. 2013].

**In vivo significance of differential partitioning of miRNAs between Ago1 and Ago2 with age**

Our analyses of *Hen1* and *Ago2* mutants suggest that 2′-O-methylation of small RNAs affects age-associated traits of brain degeneration and life span. In addition to effects on other small RNA classes, it is possible that the loss of 2′-O-methylation of miRNAs leads to destabilization of the miRNAs, thus affecting target silencing and contributing to accelerated age-associated defects. In this case, Ago2-loaded, but not Ago1-loaded, miRNAs would be selectively affected. Interestingly, both Ago1 and Ago2 are active in silencing miRNA targets. However, *Drosophila* Ago1 and Ago2 use different mechanisms to silence target miRNAs, as silencing by let-7 Ago1-RISC entails the removal of the polyA tail, whereas that by let-7 Ago2-RISC entails retention of the polyA tail [Iwasaki et al. 2009]. Such mechanistic differences might contribute to the differential silencing efficiency of the two Ago complexes of the reporters: let-7 Ago1-RISC is more efficient than let-7 Ago2-RISC in silencing the reporters [Iwasaki et al. 2009]. Our analysis of gene expression changes with age indicates a global down-regulation in the brain; correlating this with Ago1 versus Ago2 knockdown data in cells indicates greater down-regulation of predicted mRNA targets of miRNAs shifted to Ago2. While we cannot exclude that such changes result from other activities of Ago2 [Taliaferro et al. 2013, the observed reversal of expression is also consistent with a model of regulation resulting from increased loading of miRNAs shifted to Ago2 with age. Thus, potentially, the shift of these miRNAs into Ago2 may affect down-regulation of their targets with age, and disruption of this process in *Hen1* and *Ago2* mutants, along with effects on other small RNAs, may contribute to the life span and neurodegenerative phenotypes observed. Further experimental testing of the effects on mRNA targets by the miRNAs when loaded into Ago1 versus Ago2 may reveal specific details regarding the functional effects of the partitioning of the miRNAs between the RISCs and the biological outcomes.

An interesting possibility is that, by shifting miRNAs toward Ago2 from Ago1 with age, the organism might be adjusting the efficiency of target gene expression for ongoing or upcoming age-associated stresses. Since Ago2-mediated translational silencing causes retention of the polyA tail [Iwasaki et al. 2009], this might make it possible to respond to age-associated internal or external stresses more rapidly by reactivation of target miRNAs. Another possibility is the effect of loss of 2′-O-methylation on other classes of small RNAs, such as endo-siRNAs or piRNAs [Horwich et al. 2007, Czech et al. 2008, Ghildiyal et al. 2008, Kawamura et al. 2008, Okamura et al. 2008]. Loss of Ago2 leads to decreased production of endo-siRNAs, which is correlated with up-regulation of transposons [Czech et al. 2008, Ghildiyal et al. 2008, Kawamura et al. 2008, Okamura et al. 2008]. Recently, an age-associated increase of transposon expression and shorter life span were noted in *Ago2* mutants [Li et al. 2013]. Although *Ago2* mutants show developmental defects [Pepper et al. 2009], both *Hen1* and *Ago2* mutants are maintained in homozygous condition, and the overall brain morphology and climbing activity of these mutants when young are normal [see Fig. 7]. Although it is possible that loss of these gene activities during development might sensitize the adult state, taken together, these data suggest that the defects observed in adult *Hen1* and *Ago2* mutants more likely reflect defects in adult age-associated processes. These findings support the importance of terminal modification of small RNAs on impacting age-associated traits.

**Dynamic miRNA isoform regulation with age**

It has become evident that the pattern of miRNA isoforms varies in different biological contexts, such as different stages of development, different tissues, and disease [Fernandez-Valverde et al. 2010, Marti et al. 2010, Li et al. 2012, Neilson et al. 2012; Llorens et al. 2013]. It is also becoming evident that such isoform distributions are regulated [Neilson et al. 2012]. For example, *Nbr* defines the 3′ end of many *Drosophila* miRNAs [Han et al. 2011, Liu et al. 2011], and *Loqs-PB* (*Drosophila* homolog of TRBP) partnering with *Dcr-1* defines the cleavage position of several *Drosophila* miRNAs, which seems conserved in mammals as well [Fukunaga et al. 2012, Lee and Doudna 2012]. Our study reveals another repertoire of biological heterogeneity of miRNAs: differential 2′-O-methylation of miRNA with age. It is intriguing that the loss of such miRNA heterogeneity leads to clear biological defects [M Abe and N Bonini, unpubl.; this study]. These results raise the possibility that, in the aging adult, fine-tuning of miRNA heterogeneity might be critical for combating age-associated stresses.

**Materials and methods**

**Small RNA Northern hybridization**

Total RNA was extracted from fly tissues using Trizol reagent (#15596-018, Life Technologies) following the manufacturer's protocol. Three micrograms to 10 μg of total RNA was loaded per lane in 15% TBE-urea gel (#EC6885BOX, Life Technologies) and then transferred to nylon membrane (Hybond N+, GE Healthcare). After UV cross-linking and prehybridization (1 h at 50°C), the membranes were hybridized with P 32-labeled probes overnight at 50°C. DNA oligonucleotides used to make probes were miR-34-5p (5′-GATAATCGACTCTATATGGAGA-3′, Li et al. 2012; Neilsen et al. 2012; Llorens et al. 2013). It is also becoming evident that such isoform distributions are regulated [Neilson et al. 2012]. For example, *Nbr* defines the 3′ end of many *Drosophila* miRNAs [Han et al. 2011, Liu et al. 2011], and *Loqs-PB* (*Drosophila* homolog of TRBP) partnering with *Dcr-1* defines the cleavage position of several *Drosophila* miRNAs, which seems conserved in mammals as well [Fukunaga et al. 2012, Lee and Doudna 2012]. Our study reveals another repertoire of biological heterogeneity of miRNAs: differential 2′-O-methylation of miRNA with age. It is intriguing that the loss of such miRNA heterogeneity leads to clear biological defects [M Abe and N Bonini, unpubl.; this study]. These results raise the possibility that, in the aging adult, fine-tuning of miRNA heterogeneity might be critical for combating age-associated stresses.
CAGCAGTTCTCCCTATAGTGAGTCGTATTATC-3

rRNA (5'ACTCACTATAGGGAGA-3')

AGTCGTATTATC-3

TTGACTACATATGGTTGAGGGTTGTATCTCCCTATAGTG

GACTCACTATAGGGAGA-3'

TCCCTATAGTGAGTCGTATTATC-3

(15596-018, Life Technologies) following the manufacturer's

5'ACTATAGGGAGA-3'

purified RNA was P32-labeled as described (Kirino et al. 2011),

ATAGTGAGTCGTATTATC-3

the purified RNA was loaded onto 15% TBE-urea gel

5'ACTATAGGGAGA-3'

Sigma-Aldrich) were used for Ago1 and Ago2-IP, respectively.

TTGACTACAATATGGTTGAGGGTTGTATCTCCCTATAGTG

CAGCAGTTCTCCCTATAGTGAGTCGTATTATC-3

GACTCACTATAGGGAGA-3'

TCCCTATAGTGAGTCGTATTATC-3

immunoprecipitation was performed as described (Kirino et al.

5'ACTATAGGGAGA-3'

2011), except that 800 mM NaCl (final concentration) was used

AGTCGTATTATC-3

Preparation of Ago1 and Ago2-IP small RNA libraries

After extraction of RNA from beads as described in Kirino et al.

AGTCGTATTATC-3

2011), the purified RNA was loaded onto 15% TBE-urea gel

immunoprecipitation was performed as described (Kirino et al.

TTGACTACAATATGGTTGAGGGTTGTATCTCCCTATAGTG

CAGCAGTTCTCCCTATAGTGAGTCGTATTATC-3

GACTCACTATAGGGAGA-3'

TCCCTATAGTGAGTCGTATTATC-3

Western immunoblots

Fly tissues were resuspended in RIPA buffer followed by grinding

immunoprecipitation was performed as described (Kirino et al.

TTGACTACAATATGGTTGAGGGTTGTATCTCCCTATAGTG

CAGCAGTTCTCCCTATAGTGAGTCGTATTATC-3

GACTCACTATAGGGAGA-3'

TCCCTATAGTGAGTCGTATTATC-3

Western, Flag-HA-Ago2, and anti-rabbit IgG-HRP (1:2000; #sc-2030, Santa Cruz Biotechnology, Inc.).

immunoprecipitation was performed as described (Kirino et al.

TTGACTACAATATGGTTGAGGGTTGTATCTCCCTATAGTG

CAGCAGTTCTCCCTATAGTGAGTCGTATTATC-3

GACTCACTATAGGGAGA-3'

TCCCTATAGTGAGTCGTATTATC-3

western tempeature in the dark. After adding 1/10 vol of 100% glycerol, the samples were incubated further for 10 min at room temperature in the dark. After purifying the RNA, the RNA samples were resuspended in 1× borate/borax buffer with or without NaOAc (final concentration 25 mM) (#311448, Sigma-Aldrich) and incubated for 30 min at room temperature in the dark. After purifying the RNA, the RNA samples were resuspended in 1× borate/borax buffer and supplemented with NaOH to a final concentration of 0.1 mM. The samples were incubated for 90 min at 45°C followed by purification and analysis by Northern blots. Ten micrograms to ~20 μg of total RNA was used for each miRNA.

Fly stocks

Flies were grown in standard cornmeal molasses agar medium at 25°C. Henn100810 [FlyBase ID: FBst1016506, The Exelixis Collection at the Harvard Medical School] and Ago2-G14 [FlyBase ID: FBst0313641, Drosophila Genetic Resource Center, Kyoto Stock Center] flies were kindly gifts from the Sara Cherry laboratory. Ago2BL16608 [FlyBase ID: FBst0016608] and Flag-HA-Ago2 flies [FlyBase ID: FBst0033242] were obtained from the Bloomington Stock Center. Henn100810 and Ago2BL16608 lines were backcrossed into a homogenous wild-type background [Bloomington Stock Line 5905 [BL5905], FlyBaseID: FBst0005905, w1118] for five generations.

Life span assay

One-hundred-eighty to ~200 flies were used for each life span replicate. Male flies were collected on the day of eclosion (15 flies per vial), aged at 25°C, and transferred to new fly food vials every other day while scored for survival. The assay was repeated in triplicate and was analyzed by Excel (Microsoft) and GraphPad Prism for survival curves.

Brain paraffin sections

Adult female heads (3 d and 30 d) were used for paraffin sections as described [Li et al. 2008]. Brain vacuoles were counted for lamina or retina through 10 continuous horizontal sections, defining the center section, with esophagus being most prominent. Four heads per genotype were used for quantification.

Computational analyses

Mapping and histograms Adaptor sequences were removed from the 3′ ends of the reads in the Illumina fastq-generated files using the fastx toolkit [http://hannonlab.cshl.edu/fastx_toolkit]. The adapter sequences were as follows: 5′ adapter, 5′-GUUCA-
GAGUUCUACAGUCCGACGUA-3’; and 3’ adapter, 5’-TGGAATATTCTGCGGTTGCCAAGG-3’.

Reads were then collapsed and annotated with the number of times each was sequenced, and only unique reads were analyzed. Reads were filtered based on base-pair size such that reads <16 base pairs (bp) or >30 bp were discarded. The remaining reads were then mapped using Bowtie to the Drosophila melanogaster (dm5) genome and miRNA stem–loop sequences obtained from miRBase (release 20). Bowtie parameters were restricted to output only perfectly aligned matches to the stem–loop sequence. The reads were aligned and mapped to entire miRNA stem–loops. After aligning, each read was annotated as either a 5p or a 3p. To do this, the stem–loop sequence was split in half, with the first half designated the 5’ arm and the second half designated the 3’ arm, as the start and end positions of most miRNAs are imprecise.

Identification of preferentially Ago2-loaded miRNA isoforms at 3 d and 30 d. We identified miRNA isoforms preferentially loaded into Ago2 compared with Ago1 for flies of different ages using a measure of “Ago2/Ago1 relative load ratio” or R2. The most abundant isoform from each miRNA stem (5p or 3p) from the Ago2-IP 3-d library was selected. For all of the isoforms in each library that start with the same 5’ nucleotide position, we denoted the share of each isoform $s_{ih}$ such that $\sum_{i} s_{ih} = 1$ in each library and denoted the share of the most abundant isoform $s_{ih}$.

The age ratio for 3-d flies was $R_{3d} = \frac{S_{ih}^{Ago2}}{S_{ih}^{Ago1}}$ (Y-axis in Fig. 6A), and the age ratio for 30-d flies was $R_{30d} = \frac{S_{ih}^{Ago2}}{S_{ih}^{Ago1}}$ (Y-axis in Fig. 6A). 5p and 3p miRNA stem sequences were classified as “mature” and “star” based on their relative [high and low, respectively] deep sequencing read counts in the Ago1 sample at 3 d [strand with $S_{ih}^{Ago1}$ being the mature strand]. To compare the ratio of a specific isoform between Ago1 and Ago2, after assigning to the “mature” or “star” category, we kept only the cases with the mature and star categories consistent among all four libraries. Thus, if there was a discrepancy of the relatively more abundant stem [5p or 3p] between Ago1 and Ago2 and any of the other three libraries, those cases were removed. This was to assure that the same isoform was consistently called “mature” or “star” in all four libraries [Ago1-3d, Ago1-30d, Ago2-3d, and Ago2-30d]. We also filtered out any miRNA isoforms that were below a 1000 read count threshold. Note that miR-34-18nt, miR-276a/b-5p-17nt, and miR-981-5p-21nt showed an extremely high $R_{3d}$ ratio at 3 d and/or 30 d [16]. [Supplemental Tables S2, S3]. This suggests much higher accumulation of these miRNA isoforms in Ago2-IP compared with Ago1-IP at 3 d and/or 30 d. This could happen due to either biologically relevant up-regulation of these miRNA isoforms or cloning bias or degradation from the corresponding longer isoforms. These miRNA isoforms were removed from Figure 6 because including the dots corresponding to these miRNA isoforms obscured the distribution of the other dots in the plot [see Fig. 6A]. miR-34-18nt was undetectable by Ago1 versus Ago2-IP Northern; therefore, most likely, miR-34-18nt is an abnormal by-product of the library preparation. After identifying preferentially loaded Ago2 isoforms, we calculated a ratio of normalized reads at 30 d and 3 d for each specific isoform. The ratios [fold change] were plotted as in Figure 6B. To check for selective enrichment of particular miRNAs from the data of Ghildiyal et al. [2010], we first identified the most abundant isoform for each miRNA in the total RNA set (GSM466487). For each of the libraries [GSM466488 and GSM466489], we counted the number of reads $M_i$ for these isoforms and calculated their relative enrichment as $E = N_i / (N - M)$, where $N$ is the total number of library reads for a given miRNA. We then plotted the enrichment ratio $E_{R2} = E_{CSM466488}/E_{CSM466489}$ in Supplemental Figure 4. Note that we used both sequence analysis for discovery of new Ago2 preferably loaded miRNAs [see Fig. 6A,B, Supplemental Fig. 4] and Northern analysis [see Fig. 6C], as each technique [deep sequencing and Northern analysis] will have strengths and weaknesses. The Gene Expression Omnibus record for libraries generated here is GSE52963. We also analyzed previously published microarray data GSE25009 from Liu et al. [2012] and data from Taliaferro et al. [2013].

Acknowledgments

We thank members of the Bonini laboratory for critical reading, Xiuyin Teng for outstanding technical assistance, and Matthew Taliaferro and Dr. Donald Rio for kindly sharing data. This work was funded in part by the Ellison Medical Foundation [to N.M.B.], and the National Science Foundation [grant DBI-1126052 to A.G.].

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