A let-7-to-miR-125 MicroRNA Switch Regulates Neuronal Integrity and Lifespan in Drosophila

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Abstract

Messenger RNAs (mRNAs) often contain binding sites for multiple, different microRNAs (miRNAs). However, the biological significance of this feature is unclear, since such co-targeting miRNAs could function coordinately, independently, or redundantly with one another. Here, we show that two co-transcribed Drosophila miRNAs, let-7 and miR-125, non-redundantly regulate a common target, the transcription factor Chronologically Inappropriate Morphogenesis (Chinmo). We first characterize novel adult phenotypes associated with loss of both let-7 and miR-125, which are derived from a common, polycistronic transcript that also encodes a third miRNA, miR-100. Consistent with the coordinate upregulation of all three miRNAs in aging flies, these phenotypes include brain degeneration and shortened lifespan. However, transgenic rescue analysis reveal separable roles for these miRNAs: adult miR-125 but not let-7 mutant phenotypes are associated with ectopic Chinmo expression in adult brains and are suppressed by chinmo reduction. In contrast, let-7 is predominantly responsible for regulating chinmo during nervous system formation. These results indicate that let-7 and miR-125 function during two distinct stages, development and adulthood, rather than acting at the same time. These different activities are facilitated by an increased rate of processing of let-7 during development and a lower rate of decay of the accumulated miR-125 in the adult nervous system. Thus, this work not only establishes a key role for the highly conserved miR-125 in aging. It also demonstrates that two co-transcribed miRNAs function independently during distinct stages to regulate a common target, raising the possibility that such biphasic control may be a general feature of clustered miRNAs.

Author Summary

Deregulation of mRNAs that are targeted by multiple miRNAs is a common feature of a number of diseased states including neurodegenerative disorders. The currently accepted
model is that the combined action of all binding miRNAs ensures target repression. Here, we show that two co-expressed miRNAs exert distinct outcomes on a common target. While miR-125 extends lifespan by repressing its target, chinmo, in adult brains, let-7 downregulates Chinmo in developing animals. Our results indicate that differential processing and turnover rates of let-7 and miR-125 contribute to this switch in miRNA activity. This study has identified the physiological relevance of the targeting of a single mRNA by multiple miRNAs in a scenario where each miRNA exerts a distinct and non-overlapping outcome.

Introduction

RNA-mediated post-transcriptional mechanisms regulate the accumulation and homeostasis of proteins not only during animal development but also during adulthood [1–3]. These mechanisms include regulation by microRNAs (miRNAs), a class of small non-coding RNAs that usually silence messenger RNAs (mRNAs) by binding to partially complementary sequences frequently found in the target 3’ untranslated (3’UTR) sequence [4]. Some miRNAs are known to affect lifespan by post-transcriptionally silencing mRNAs that play critical, beneficial roles at early stages of the life cycle but are deleterious when expressed inappropriately at later stages [1, 2, 5–7]. For example, loss of C. elegans lin-4, the first miRNA to be functionally characterized for its role in lifespan, leads to shortened lifespan due to the persistence of its target, lin-14 [2, 8]. Similarly, the adult onset of Drosophila miR-34 promotes longevity and maintains neuronal homeostasis by repressing Eip74EF, a transcription factor required for progression through earlier life stages [1, 3]. Although loss of other miRNAs like Drosophila miR-1000 lead to shortened lifespan [3], the complete repertoire of miRNAs that regulate aging processes remains uncharacterized [9].

Understanding the role of miRNAs in the adult nervous system is particularly relevant to aging, since the nervous system is a key coordinator of age-related changes in overall organismal physiology [1, 10, 11]. For example, the ablation of specific neurons in both worms and flies extends lifespan [12, 13]. In addition, conserved mechanisms that regulate organismal aging, including insulin signaling and mitochondrial function, modulate the pathology of neurodegenerative disease models [14–18]. Since premature loss of miRNAs has been linked to defective neuronal function and survival as well as the accumulation of disease related proteins, miRNA regulatory networks likely constitute an important component of the normal aging process in the brain [3, 19–21]. Thus, exploring the functional roles of miRNAs and their mRNA targets in the adult brain is necessary to understand the mechanisms involved in the onset and progression of late onset neurodegenerative diseases.

Multiple miRNAs are frequently predicted to regulate the same mRNA indicating that miRNA activity within tissues such as the nervous system is coordinated. Bioinformatic analyses estimate that greater than 70% of targeted human mRNAs and between 30 to 50% of targeted Drosophila mRNAs have sites for two or more miRNAs [22–24]. The Drosophila predictions are likely underestimates of the frequency of co-targeting in the nervous system, since they were generated prior to the discovery of dozens of Drosophila miRNAs as well as the 3’UTR extensions of numerous neural mRNAs [25–27]. Recent analyses have found that co-targeting is particularly prevalent for clustered miRNAs, which are likely to be co-transcribed and therefore co-expressed [28]. Based on reporter assays showing a positive correlation between the number of miRNA sites in a 3’UTR and the degree of its repression [29, 30], the current model suggests that miRNA activity is additive and predicts that spatially overlapping
combinations of miRNAs—presumably including those that are co-transcribed—lead to greater target repression [31, 32]. However, there are very few published investigations that have tested this model directly by delineating the individual activities of multiple co-targeting miRNAs. Here, we re-evaluate this model by distinguishing the effects of two co-transcribed neural miRNAs, let-7 and miR-125, on a common target mRNA during development and adulthood.

**Results**

**let-7-Complex miRNAs modulate age-associated processes in the brain**

The let-7-Complex (let-7-C) locus in Drosophila encodes an evolutionarily conserved cluster of three co-transcribed miRNAs: miR-100, let-7 and miR-125, the orthologue of C. elegans lin-4 [33, 34]. Although the levels of processed let-7 are known to increase with age in testes and ovaries [35, 36], the relative expression levels of all three miRNAs have not been characterized in aging flies. To address this, we performed Northern blot and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses of whole animals (Fig 1A and 1B, left). These analyses revealed an age-dependent increase in all three let-7-C miRNAs in both adult males and females, suggesting a role for this miRNA cluster in aging-related processes.

To characterize the role of this age-dependent increase in let-7-C miRNAs, we analyzed a let-7-C hypomorphic (let-7-Chyp) strain in which let-7-C miRNAs were expressed during development [37] but not maintained during adulthood (Fig 1B, right). This hypomorphic strain was trans-heterozygous for two let-7-C null alleles but also harbored a single copy of a minimal let-7-C rescuing transgene that contained regulatory elements required for onset of pri-let-7-C (let-7-Cp^3kb::cDNA) during development but lacked elements needed for its post-developmental maintenance. Consistent with our previous analysis [37], young let-7-Chyp mutant males expressed reduced levels of miR-100 (9.2±4.6% of control), let-7 (22±10.2% of control) and miR-125 (33±7.2% of control) that decreased further as the adults aged (Fig 1B). We therefore performed survival analysis of these let-7-Chyp mutant males and found that they died prematurely relative to control males (Fig 1C, compare black and red curves; w1118: median survival 74d, maximum lifespan 98d; let-7-Chyp: median survival 36d, maximum lifespan 56d). Prompted by this reduced viability, we assayed the let-7-Chyp strain for additional functional and morphological age-dependent phenotypes. Young let-7-Chyp mutants climbed normally, indicating that the levels of let-7-C miRNAs they express during metamorphosis and early adulthood is sufficient for general adult function. However, aged let-7-Chyp mutants displayed a steep reduction in this ability (Fig 1D). These results indicated that persistent expression of one or more of the three let-7-C miRNAs specifically during adulthood was required for normal adult healthspan.

Given the neural expression of let-7-C miRNAs [33, 37, 38], we next looked for age-associated deterioration in brain morphology. Brain degeneration has been anatomically characterized by an age-dependent increase in the number of scattered vacuoles that mark cells undergoing necrotic cell death [39]. Sections of 40-day old control and let-7-C^hyp brains revealed a sharp increase in vacuole number in mutant brains (Fig 1E–1G). As with the climbing defect described above, this phenotype had an adult onset since the brains of young mutant flies contained hardly any vacuoles (0 vacuoles in w1118, 0.6 ± 0.9 vacuoles in let-7-C^hyp, n = 5). Importantly, a let-7-C transgene that substantially restored miR-100 levels (59.4±11.1% of control), let-7 levels (50.3±24% of control) and miR-125 levels (108±28%) in 3-day old adults (let-7-C^hyp rescue in Fig 1B) rescued the lifespan and age-dependent climbing defects as well as the brain deterioration of let-7-C^hyp mutants (Fig 1C, 1D and 1G). Since our qRT-PCR analysis indicated that rescued let-7-C^hyp mutants express a constant level of let-7-C miRNAs during adulthood (Fig 1B), we inferred that the age-dependent increase in let-7-C miRNAs detected in
wildtype adults was not absolutely required for their pro-survival and neuroprotective roles. Taken together, these results confirmed a role for let-7-C miRNAs in the aging processes that occur in the brain.

miR-125 and let-7 mutants display reduced lifespan and neurodegeneration

In order to distinguish the roles of the three let-7-C miRNAs, we generated a set of rescuing transgenes with either miR-100, let-7 or miR-125 deleted. These transgenes were inserted into identical chromosomal locations using phiC31-mediated integration [40] and crossed into a trans-heterozygous let-7-C null background, yielding strains we referred to as ΔmiR-100, Δlet-7 and ΔmiR-125 single mutants, respectively (see S1 Fig for our crossing scheme that ensured

Fig 1. let-7-C mutants are short-lived and display adult onset defects in motility and brain morphology. (A) Northern blots of total RNA from w1118 (wt) males and females that were aged for 3 or 40 days and probed for let-7, miR-125, and miR-100. Blots were also probed with U6 as a loading control. (B) Quantitation of miR-100, let-7, and miR-125 in 3 or 40 day w1118 (wt), let-7-Chyp rescue, and let-7-Chyp adult males (n = 3). Processed miRNA levels were normalized to 28S rRNA. (C) let-7-C mutant males displayed a reduced lifespan (w1118: median survival 74d, maximum lifespan 98d, n = 210 male flies; let-7-C null: median survival 12d, maximum lifespan 26d, n = 210 male flies; let-7-Chyp: median survival 36d, maximum lifespan 56d, n = 210 male flies; let-7-Chyp rescue: median survival 66d, maximum lifespan 110d, n = 210 male flies). P value of the lifespan curves were calculated by log-rank test. (D) Climbing activity of 3 or 30 day w1118, let-7-Chyp rescued and let-7-Chyp adult males. At 3 days, the climbing ability of let-7-Chyp (80 ± 5.8%) was comparable to the control w1118 (81.33 ± 2.3%). However, at 30 days, only 10.7 ± 6.18% of let-7-Chyp males were able to climb as opposed to 68.72 ± 4.67% of w1118 males. The age related climbing defects of let-7-Chyp were almost completely rescued by a single copy of the let-7-C rescue transgene (64.66 ± 3.69%). Mean ± S.D of three experiments, n = 15 male flies in each experiment, *** = p value <0.001. (E, F) Brain sections of 40d let-7-Chyp rescue (E) and let-7-Chyp mutant males (F) (arrows point at vacuoles). (G) Quantification of vacuole number. See S1 Table for detailed genotypes. Data represent mean ± S.D (B, D, G). doi:10.1371/journal.pgen.1006247.g001

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that single mutant strains were otherwise as close to identical as possible). Unlike previously
generated strains with P-element rescue transgenes [33], differences between these single
mutants could be attributed to loss of an individual miRNA rather than to position effects.

Quantitative RT-PCR analysis of miR-100, let-7 and miR-125 confirmed the absence of
miRNA expression in each of the deletion lines (Fig 2A). However, this analysis also revealed
cross-regulatory relationships between the three miRNAs: loss of let-7 resulted in reduced lev-
els of both miR-100 (0.29 fold relative to control) and miR-125 (0.35 fold relative to control),
while loss of miR-100 and miR-125 resulted in increased levels of let-7 (2.5 fold relative to con-
trol) and miR-100 (2.5 fold relative to control), respectively (Fig 2A). To assess the cause of
these changes, we turned to a cell culture assay in which we could quantify the activity of each
miRNA in cells transfected with altered let-7-C versions. MiRNA activity was quantified as the
fold repression in luciferase levels produced by previously validated “sensors” for each let-7-C
miRNA [38]. Individual sensors were co-transfected along with UAS-let-7-C cDNA constructs
into Kc-167 cells that do not ordinarily express let-7-C miRNAs [37]. First, confirming the
effect of let-7 deletion on miR-100 and miR-125 levels, we found that miR-100 and miR-125
activity reporters were less repressed in cells transfected with a let-7-C cDNA lacking the let-7
hairpin (miR-100: 5.6 ± 0.56 fold repression in Δlet-7 compared to 10.48 ± 1.7 in wild type; 
Δlet-7-C: 8.3 ± 0.66 fold in Δlet-7 compared to 13.8 ± 1.97 fold in wild type). Then, to test
whether this effect was due to the absence of mature let-7 or some other cause (e.g. altered
RNA conformation of the Δlet-7 primary transcript that reduced miR-100 and miR-125 pro-
cessing), we generated a chimeric UAS let-7-C cDNA construct in which the Drosophila let-7
hairpin was replaced with the human let-7-a2 hairpin that encoded the same mature let-7 but
has a different hairpin structure. While the human let-7-a2 hairpin restored the let-7 mediated
repression of its sensor, it did not restore miR-100 and miR-125 mediated repression (Fig 2B,
construct 5). These data indicated that processed let-7 miRNA did not directly regulate the pro-
cessing of miR-100 or miR-125. Instead, we favor a model where the rate of let-7 processing has
an effect on the rate of miR-100 and miR-125 processing, a model consistent with processing of
other polycistronic microRNAs [41]. We note that Truscott et al. also recently found evidence
for cross-regulatory interaction between let-7-C miRNAs [41], although their results were
slightly different—deletion of let-7 and miR-100 but not let-7 alone reduced miR-125 levels—
probably due to technical differences in the constructs used. We also evaluated ΔmiR-100 or
ΔmiR-125 let-7-C cDNA constructs in this cell culture assay, but detected no enhancement in
miRNA activity (Fig 2B), suggesting that the changes in miRNA levels detected in tissue (Fig
2A) may not be functionally significant. Taken together, these results indicated that the set of
ΔmiR-100, Δlet-7 and ΔmiR-125 strains described above would allow the dissection of the indi-
vidual contributions of the three miRNAs since neighboring miRNAs continued to be
expressed when individual miRNAs were deleted, albeit at altered levels in some cases.

We then used the ΔmiR-100, Δlet-7 and ΔmiR-125 single mutant lines to analyze the conse-
quences of deleting each miRNA on age-associated brain degeneration and behavioral defects.
Δlet-7 and ΔmiR-125 single mutant flies displayed significantly reduced longevity compared to
control or ΔmiR-100 flies (Fig 2C). In addition, while young Δlet-7 and ΔmiR-125 mutants had
normal climbing behavior and brain morphology, a significant decrease in climbing ability as
well as a marked increase in vacuole number was observed in both mutants with age (Fig 2E
and 2F). The vacuoles in both Δlet-7 and ΔmiR-125 mutants appeared to be scattered through-
out the central brain region and some enrichment was also seen in the retina (S2 Fig). These
data indicated that loss of either let-7 or miR-125 but not miR-100 caused behavioral and mor-
phological changes that were normally seen in much older flies and were indicative of rapid
aging of the brain.
Fig 2. ΔmiR-125 mutant aging phenotypes are suppressed by loss of chinmo. (A) Quantitative RT-PCR analysis of miR-100, let-7 and miR-125 in 3d old ΔmiR-100, Δlet-7 and ΔmiR-125 and rescue adult males. MiRNA levels were normalized to 2S rRNA. P values: ** < 0.01; **** < 0.0001. (B) Fold repression of miR-100, let-7 and miR-125 luciferase sensors in Kc-167 cells transfected with UAS-let-7-C constructs (schematic on left). Assays were performed in triplicate and the results were represented as Mean ± S.D. The data was statistically analyzed by an unpaired t-test. (C) Δlet-7 and ΔmiR-125 mutant males have a shortened life span (let-7-Cnull rescue: median survival 72d, maximum lifespan 114d, n = 272; ΔmiR-100: median 76d, maximum lifespan 118d, n = 317; Δlet-7: median 36d, maximum lifespan 62d, n = 202; ΔmiR-125: median 38d, maximum lifespan 68d, n = 337 p values were calculated by Log-rank (Mantel-Cox) test. (D) Reducing one copy of chinmo or knock down of chinmo by RNAi rescue the lifespan defects of ΔmiR-125 mutant but not Δlet-7 mutant males (chin1; wt: median
miR-125 and let-7 enhance neurodegeneration at distinct stages during the life cycle

Given that loss of let-7 and miR-125 triggered physiological processes involved in aging, we tested whether inhibition of individual let-7-C miRNAs enhanced the neurodegeneration of a disease model of fragile X-associated tremor/ataxia syndrome (FXTAS) [42]. FXTAS is a late onset human neurodegenerative disease that is characterized by the presence of ubiquitin positive nuclear inclusions containing RNAs with expanded CGG repeats (rCGG) in neurons and astrocytes [43]. Ectopic expression of transcripts with artificial expansion of these repeats in the fly retina causes a pathology similar to human FXTAS, including photoreceptor degeneration and disorganization of the ommatidia [42]. Using miRNA “sponge” constructs designed to individually inhibit miR-100, let-7, or miR-125 (miR-100SP, let-7SP, or miR-125SP), we tested whether loss of any of these miRNAs’ activities enhanced the retinal degeneration in the FXTAS model. We found that driving let-7SP or miR-125SP but not miR-100SP specifically in the eye throughout development and adulthood resulted in significant enhancement of the rCGG phenotype (S3 Fig). This result indicated that, in addition to their role in modulating lifespan, let-7 and miR-125 promoted disease pathogenesis while miR-100 did not.

To pinpoint the specific stage during which let-7 and miR-125 activity were involved in FXTAS disease pathogenesis, we utilized a temperature sensitive allele of Gal80 (tubP-Gal80ts). This approach allowed temporal control of both the UAS-rCGG90 transgene as well as the UAS-miRNA sponges in the eye, since animals at 29°C express UAS transgenes but animals at 18°C do not [44]. We reared strains to control expression in three ways: no expression (18°C), constant expression (29°C), or expression during development but not adulthood (29°C→18°C) (Fig 3A–3L). As expected, constant expression of either let-7SP or miR-125SP enhanced the rCGG90 phenotype whereas no expression did not (Fig 3A–3F). However, let-7SP and miR-125SP behaved differently from one another in the 29°C→18°C regimen: let-7SP enhanced rCGG90 retinal degeneration while miR-125SP did not (compare Fig 3G–3I). This result, along with the observation that let-7SP animals reared at 29°C→18°C looked no worse than those reared at 29°C→29°C, suggested that let-7’s main contribution to disease progression occurred during development. Conversely, miR-125SP animals reared at 29°C→18°C looked no worse than those reared at 18°C, suggesting that the phenotypes displayed by those reared at 29°C→29°C was a specific consequence of adult miR-125SP expression. The reciprocal experiment involving adult-only transgene expression was not informative because none of the 18°C→29°C animals displayed a phenotype, even when aged up to 20 days, perhaps because the underlying rCGG90 phenotype was at least partially of developmental origin. These data indicated that let-7 and mir-125 functioned during distinct temporal periods to effect disease progression, and
| +rCGG90 transgene | -rCGG90 transgene |
|-------------------|-------------------|
| **18°—18° (no expression)** | **29°—29° (constant expression)** |
| 29°—18° (developmental expression) | **29°—29° (constant expression)** |

| no sponge | let-7 sponge | miR-125 sponge |
|-----------|-------------|---------------|
| ![Image A](image1.png) | ![Image B](image2.png) | ![Image C](image3.png) |
| ![Image D](image4.png) | ![Image E](image5.png) | ![Image F](image6.png) |
| ![Image G](image7.png) | ![Image H](image8.png) | ![Image I](image9.png) |

Biphasic Regulation of *chinmo* by *let-7* and *miR-125*
Fig 3. Loss of miR-125 and let-7 enhance rCGG90 mediated retinal degeneration during different stages of the life cycle. Scanning electron microscope eye sections from 10 day old flies harboring a GMR-Gal4 transgene (all panels), a tubP-Gal80\textsuperscript{ts} transgene (all panels), a UAS-rCGG90 transgene (A-I), a UAS-let-7SP sponge transgene (B, E, H, K), and/or a UAS-miR-125SP sponge transgene (C, F, I, K) and reared under one of three conditions: 18°C during development and adulthood (18→18, panels A-C), 29°C during development and adulthood (29→29, panels D-F and J-L), or 29°C during development and 18°C during adulthood (29→18, panels G-I). Scale bars, 100\mu m.

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that the retinal degeneration caused by a decline in let-7 activity was of a developmental origin while that caused by inhibition in miR-125 activity was due to degeneration of adult brains.

miR-125 regulates aging via repression of chinmo

Since miRNAs function by repressing target mRNAs, we investigated whether the age-associated Δlet-7 and ΔmiR-125 phenotypes were due to the elevated expression of chronologically inappropriate morphogenesis (chinmo), the only verified target of both let-7 and miR-125 in flies [38]. Chinmo is a transcription factor that controls neuronal fate in a dosage-sensitive manner. In the mushroom body lineages in the central brain, for example, Chinmo is expressed at high levels early in development to promote early born cell fates and its post-transcriptional downregulation leads to the production of later born fates [45]. The chinmo 3'UTR contains multiple let-7 and miR-125 binding sites and is regulated by let-7-C miRNAs during development [38]. To address whether the adult phenotypes of Δlet-7 and ΔmiR-125 mutants were due to elevated Chinmo, we reduced the dosage of chinmo in these mutants by either removing one copy of chinmo using a null chinmo\textsuperscript{Δ} mutation [45] or by knocking down chinmo using a RNAi transgene that we verified in vivo (S4A–S4L Fig). Lowering chinmo levels dramatically suppressed the premature death (Fig 2D), climbing defects (Fig 2E), and brain necrosis (Fig 2F and S4M Fig) of ΔmiR-125 mutants but not Δlet-7 mutants. This result indicated that elevated Chinmo was responsible for ΔmiR-125 phenotypes but that other factors were responsible for Δlet-7 phenotypes. This distinction also indicated that the Δlet-7 phenotypes described above (reduced longevity, climbing and neurodegeneration) were not solely due to the reduction in miR-125 levels observed in these mutants (Fig 2A) and implied the de-repression of other, currently unidentified, mRNAs.

To test whether ectopic Chinmo was sufficient to cause the neurodegenerative phenotypes associated with loss of miR-125, we ectopically expressed a chinmo transgene in adult brains using an inducible neural GAL4 driver (Fig 4A). This forced expression resulted in a drastic reduction in both lifespan (Fig 4B) and climbing ability (Fig 4C) along with a dramatic increase in brain vacuole numbers in 20d aged flies (Fig 4D and 4E). Interestingly over-expression of Chinmo in neurons showed an increased localization of vacuoles in the lamina and central brain regions, indicating that neurons in the lamina region were more sensitive to deregulation of Chinmo (Fig 4D and 4E and S2E Fig). These experiments confirmed that deregulated expression of Chinmo in adult neurons results in premature neurodegeneration in adult flies and supported the genetic suppression evidence above that silencing of this protein by miR-125 was critical for maintaining neuronal integrity and viability in adult flies.

Biphasic regulation of chinmo by let-7 and miR-125

Messenger RNAs frequently contain binding sites for multiple miRNAs, which may repress common targets in an additive manner [31]. However, our analysis raised the possibility that the chinmo mRNA might be regulated by miR-125 but not let-7 in adult brains despite containing verified functional binding sites for let-7 [38]. Intriguingly, these experiments were supported by immunostaining of Chinmo in Δlet-7 and ΔmiR-125 adult brains. The degree of de-
repression of Chinmo was more widespread and starkly higher in ΔmiR-125 brains when compared to Δlet-7 (S5D Fig). In contrast, Δlet-7 mutant brains displayed a much weaker immunostaining signal of Chinmo (S5C Fig). To distinguish the contributions of let-7 and miR-125 on chinmo repression, we compared the levels of Chinmo protein in immunostained brains of control, Δlet-7, ΔmiR-125, and let-7-C null adults. Shortly after let-7-C activation at 24 hours after puparium formation (APF), elimination of both miRNAs resulted in much higher levels of Chinmo than loss of either miRNA alone (Fig 5A–5I), indicating that both let-7 and miR-125 contributed to chinmo repression at this time-point. Three days later, however, let-7 played the predominant role in silencing chinmo, since Δlet-7 mutant brains expressed 86.7 ± 4.8 arbitrary units (AU) of Chinmo while ΔmiR-125 mutant brains expressed only 43.3 ± 5.2 AUs of Chinmo (Fig 5A–5D and Fig 5I). A complete reversal in the relative contributions of let-7 and miR-125 occurred during the pupal-to-adult transition; Δlet-7 mutant adult brains expressed...
38.5 ± 10.3 AUs while ΔmiR-125 mutant brains expressed 72.9 ± 9.2 AUs, indicating that miR-125 was primarily responsible for silencing chinmo in adults (Fig 5E–5I and S5A–S5D Fig). To support this data, we also quantified the levels of chinmo mRNA in late pupal and adult Δlet-7 and ΔmiR-125 mutant heads, since miRNA regulation is known to cause mRNA destabilization. Consistent with our quantification of Chinmo protein levels, loss of let-7 affected chinmo mRNA levels in late pupae but not adults, while loss of miR-125 affected chinmo mRNA levels in adults but not pupae (Fig 5J). Together, these data indicated that let-7 and miR-125 predominantly regulated chinmo during development and adulthood, respectively.

Since the derepression of Chinmo during development was higher in Δlet-7 than ΔmiR-125 mutants, we tested whether chinmo-dependent developmental defects were more severe in Δlet-7 than ΔmiR-125 single mutants. To examine this possibility, we investigated the relative roles of let-7 and miR-125 in fate transitions in MB neuronal temporal identity. The MB is composed of four subtypes of neurons that are generated in a sequential manner (γ → α1′/β) →
pioneer α/β → α/β) by neuroblasts [45–47]. High levels of Chinmo specify early born cell fates (γ, α/β), while low levels of Chinmo specify later born cell fates (pioneer α/β, α/β). Altered dosages of chinmo lead to changes in the total numbers of these various neuronal classes so that, for example, elevated chinmo is associated with a smaller population of pioneer α/β neurons [38, 45]. Therefore, to evaluate the relative contributions of let-7 and miR-125 chinmo regulation during neural development, we counted the number of pioneer α/β neurons in adult brains. As expected, Δlet-7 mutants displayed greater reduction in pioneer α/β neuron number than ΔmiR-125 mutants and, furthermore, Δlet-7, miR-125 double mutants showed a reduction similar to Δlet-7 single mutants (Fig 5K). Thus, regulation of chinmo was more dependent on let-7 than miR-125 during the larval-to-adult transition but was more dependent on miR-125 in aging adults.

To assess whether let-7 and miR-125 had different strengths of repression that might contribute to their differential activities, we examined the expression of luciferase reporters containing a previously characterized 1.4kb 3' UTR fragment of chinmo that harbors six let-7 and four miR-125 binding sites that are conserved between Drosophila species [38]. Overexpression of the entire let-7-C primary transcript in Kc-167 cells repressed the wild type reporter 17-fold (Fig 5L). In contrast the fold repression of mutants lacking let-7 binding sites or miR-125 sites or both was reduced 6.9-, 14.0- and 4.8-fold, respectively. While these data indicated that let-7 was a stronger repressor of chinmo than miR-125, it was not clear whether the greater decrease in fold repression was due to a greater number of let-7 sites. To circumvent this issue, we designed and quantified the degree of repression of a luciferase reporter that contained a single verified miRNA binding site for let-7 and a single miR-125 site that had comparable base pairing characteristics (S6 Fig). Ectopic expression of let-7-C miRNAs resulted in a 12-fold repression of the wild type reporter while deletion of the let-7 seed sequence, the miR-125 seed sequence or both sequences resulted in a 2.9-, 4.6- and 0.7-fold repression of luciferase activity respectively (Fig 5M). Together these data confirmed that both let-7 and miR-125 were capable of silencing the luciferase sensor individually but maximum repression was achieved when both sites were functional and that let-7 was a stronger post-transcriptional repressor than miR-125.

Differential processing and turnover rates of let-7 and miR-125 direct a switch in miRNA targeting activity during the larval-to-adult transition

To investigate the basis for the sequential repression of Chinmo by let-7 and miR-125, we first compared the rate of let-7 and miR-125 production in the developing and adult nervous system. To do so, we quantified the ratio of processed miRNA to precursor miRNA for let-7 and miR-125 in staged nervous system samples (Fig 6A). The let-7/pre-let-7 ratio was significantly higher than the miR-125/pre-miR-125 prior to 72h APF. In contrast, the miR-125/pre-miR-125 ratios were higher than the let-7/pre-let-7 ratios after 72h APF and into adulthood (Fig 6A). The temporal dynamics of let-7 and miR-125 production in the nervous system correlated with their relative roles in chinmo regulation, suggesting that the basis for their sequential activity may involve differential processing and/or turnover.

To determine the basis for this switch in the relative expression of let-7 and miR-125, we first investigated the possibility that let-7 and miR-125 might be differentially processed. To do so, we again took advantage of the Kc-167 embryonic cell line. As mentioned above, the let-7-C locus is not ordinarily transcribed in this cell line, but it is activated in response to the Drosophila steroid hormone 20-hydroxycedysone (20E) [37]. Kc-167 cells were treated with 20E for 24h to induce primary let-7-C transcript and the levels of let-7 and miR-125 were monitored at different time intervals after washing off the steroid hormone. To measure the relative rates of
processed let-7 and miR-125 production, we performed qRT-PCR on 20E-treated Kc-167 samples using a standard curve to extrapolate absolute miRNA levels. Forty-eight hours after the...
20E treatment, we detected 1024±440 copies of let-7 per nanogram (ng) of total RNA but only 3±1 copies of miR-125 per ng of total RNA, indicating that let-7 was processed more efficiently than miR-125 (Fig 6B). Incubation of cells for 120 hours after the 20E pulse resulted in a 3–4 fold increase in the copy number of let-7 (3872±1365 copies/ng of total RNA) and a ~30 fold increase in the copy number of miR-125 (101±24 copies/ng of total RNA). The greater increase in the miR-125 copy number at later time points suggested that processed miR-125 persisted longer than let-7.

In order to identify the key steps in miRNA biogenesis that contributed to the inefficient processing of miR-125, we performed in vitro Drosha and Dicer processing assays (Fig 6C and 6D). We first examined the rate of generation of precursor miRNAs (pre-miRNA) from longer primary miRNA (pri-miRNA) transcripts by the Drosha-Pasha complex. In initial experiments, we found that pri-miR-125 processing was extremely inefficient (S7A Fig). Therefore, we compared the processing of pri-let-7 to the processing of chimeric constructs in which either the pri-let-7 terminal loop or its stem-base were replaced with the pri-miR-125 loop (pri-let-7\emph{miR-125L}) or pri-miR-125 stem base (pri-let-7\emph{miR-125B}), respectively (Fig 6B and S7B Fig).

The rates of processing of these three transcripts were examined by incubating with Drosha-Pasha complexes immunoprecipitated from Kc-167 cells (S7B Fig). Substituting either the terminal loop or stem base of pri-miR-125 in pri-let-7 resulted in a dramatic reduction in Drosha processing. While 15% of the unmodified let-7 primary transcript was processed within 30 minutes, only 5% of pri-let-7\emph{miR-125L} was cleaved by Drosha. Incubation with Drosha-Pasha complex for 120 minutes increased the percentage of precursor to 30% and 7% for pri-let-7 and pri-let-7\emph{miR-125L}, respectively. However, substituting the stem-base of pri-miR-125 in pri-let-7 completely abolished its Drosha processing (Fig 6C). Thus, both the terminal loop and stem base sequence determinants of pri-miR-125 contributed to its inefficient processing by Drosha in vitro, raising the possibility that other post-transcriptional mechanisms may facilitate miR-125’s processing in vivo.

To evaluate the Dicer-1 processing of pre-let-7 and pre-miR-125, we also performed in vitro processing assays with Flag-tagged Dicer-1 that was, like the Drosha-Pasha complexes described above, purified from Kc-167 cell extracts (S7B Fig). In these assays, pre-miR-125 displayed a significantly lower kinetics of processing than pre-let-7 (Fig 6C). Within 10 minutes of incubation with Dicer-1, 23±3.6% of pre-let-7 and 15±0.6% of pre-miR-125 were processed to their mature forms. After 60 minutes of incubation, the percentage diced was 71±7.2% and 47±0.4% for pre-let-7 and pre-miR-125, respectively (Fig 6D and 6E). Thus, this higher kinetics of processing of let-7 by both Drosha and Dicer likely contributed to its rapid accumulation during metamorphosis.

While differential processing was consistent with the more rapid accumulation of let-7, we hypothesized that the temporal dynamics of miR-125 accumulation (Fig 6A) might also reflect an increased stability. In order to monitor the persistence of let-7 and miR-125 in adult nervous system tissue, we measured the expression of these miRNAs after blocking Dicer-1 activity. Total RNA was extracted from heads of 10d-old adult flies that expressed one of two Dicer-1 shRNA constructs, and the levels of let-7 and miR-125 were quantified by Taqman miRNA assays (Fig 6D and S7C Fig). Knockdown of Dicer-1 resulted in a greater reduction of let-7 relative to miR-125 (Fig 6D): expression of Dicer-1\emph{shRNA1} or Dicer-1\emph{shRNA2} resulted in 0.23±0.1 or 0.31±0.37 fold expression of let-7 relative to control but 0.62±0.18 or 0.58±0.18 fold expression of miR-125 relative to control, respectively. These data indicated that the decay rate of let-7 was significantly higher than that of miR-125 in the adult nervous system.

Finally, to assess whether miR-125 had a longer half-life than let-7, we measured the decay rates and half-lives of let-7 and miR-125 by analyzing Kc-167 cells transfected with synthetic miRNA duplexes. Cells were washed with fresh medium 5 hours after transfection, and samples were collected at the indicated times for total RNA preparation followed by quantitation of
let-7 and miR-125 by Taqman assays. Half-lives were inferred from fitted exponential curves (S7D Fig). As expected, the half-life of miR-125 ($T_{1/2}$, 2.7h) was significantly higher than that of let-7 ($T_{1/2}$, 2h). Taken together these experiments suggested a mechanistic basis for the switch in the relative abundance of the two miRNAs during adulthood. This differential temporal expression contributes to the distinct functions of the two miRNAs during the life of a neuron: let-7 fine-tunes the gradient of the dosage-sensitive transcription factor chinmo to control temporal cell fate determination during neural stem cell (NSC) division (for example, adjusting $\gamma, \alpha'/\beta'$, and $\alpha/\beta$ identities in the mushroom body lineage) while miR-125 ensures the complete silencing of chinmo during adulthood to promote neuron maintenance. Such phasic control may be a general feature of clustered miRNAs.

**Discussion**

**Summary and model**

let-7 and miR-125 have distinct and non-overlapping functions, despite being co-transcribed and sharing the same target. Loss of either miRNA alone leads to shortened lifespan and premature deterioration of health, as indicated by age-dependent climbing defects and brain degeneration. The aging defects caused specifically by loss of miR-125 are associated with high levels of Chinmo in adult brains, and can be rescued by reducing chinmo levels in the $\Delta$miR-125 mutant. In contrast, Chinmo is substantially lower in $\Delta$let-7 mutant adult brains and it appears not to contribute to adult $\Delta$let-7 mutant phenotypes: neither adult $\Delta$let-7 mutant climbing defects, brain vacuolization, nor reduced longevity are suppressed by chinmo reduction. Instead, let-7 predominates during development: pupal Chinmo expression is higher and associated defects in neuronal identity are worse in $\Delta$let-7 mutants than $\Delta$miR-125 mutants. Although deletion of let-7 reduces miR-125 levels, the differences in the $\Delta$let-7 and $\Delta$miR-125

Fig 7. Biphasic action of two co-transcribed microRNAs. Pre-let-7 and pre-miR-125 are co-transcribed, but mature let-7 accumulates more rapidly than mature miR-125 during development due to its higher rate of processing by Drosha and Dicer. The enhanced stability of miR-125 leads to a switch in the relative abundance of the two miRNAs during adulthood. This differential temporal expression contributes to the distinct functions of the two miRNAs during the life of a neuron: let-7 fine-tunes the gradient of the dosage-sensitive transcription factor chinmo to control temporal cell fate determination during neural stem cell (NSC) division (for example, adjusting $\gamma, \alpha'/\beta'$, and $\alpha/\beta$ identities in the mushroom body lineage) while miR-125 ensures the complete silencing of chinmo during adulthood to promote neuron maintenance. Such phasic control may be a general feature of clustered miRNAs.

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phenotypes indicate that the Δlet-7 phenotypes are not simply due to loss of miR-125. In support of this, distinct temporal periods of let-7 and miR-125 activity were also identified using sponges, an independent method for disrupting miRNA activity in which let-7 interference does not affect miR-125 activity. Based on these results, we conclude that a let-7-to-miR-125 switch during the pupal-to-adult transition ensures chinmo repression in adults, maintaining neuronal integrity and promoting life span.

Our results illuminate a function of miRNA co-targeting that we term “phasic control,” which indicates that co-targeting can reflect non-redundant regulation during distinct phases of a cell’s life, from its birth to its death. Rather than simply reinforcing silencing, such repression at different times may have distinct functions, based not only on the changing status of the cell but also on differences in miRNA:mRNA interactions (e.g. base-pairing characteristics, trans-acting factors, etc). Highlighting such phasic control, we propose a model in which let-7-C miRNAs collectively function as both a rheostat and as a switch but at distinct times (Fig 7). According to this model, let-7 predominates during nervous system formation, where it shapes the temporal gradient of Chinmo. let-7-dependent attenuation of this dosage-sensitive transcription factor is responsible for the establishment of proper cell fate as neural progenitors divide. Subtle alterations in the rate of let-7 accumulation may adjust the neuronal classes that comprise structures like the mushroom body, whose composition is known to be sensitive to environmental cues [48]. While let-7 adjusts chinmo, miR-125 in contrast switches chinmo off in post-mitotic neurons throughout the adult nervous system. This silencing of a juvenile neuronal marker maintains adult neuronal integrity, since forced Chinmo expression in adults leads to brain deterioration. While our model proposes that miR-125 ensures complete silencing of chinmo, we cannot rule out the possibility that miR-125 repression is alleviated under certain conditions in the adult (e.g. injury-induced repair) so that Chinmo can reprogram neurons to a juvenile state that is needed for certain adult functions. Thus, by independently regulating the same target during two different periods, let-7 and miR-125 miRNAs control cell fate establishment and maintenance, respectively.

Our model is based in part on results that chinmo repression is achieved predominantly by miR-125 in adult brains, even though let-7 is present. What accounts for the muted let-7 activity that, while present, is not responsible for repression of a verified target? Perhaps let-7 has many more targets than miR-125 in the adult brain, since miRNAs with a larger repertoire of target genes have a weaker effect on each individual target [49, 50]. In addition, let-7 targets may be highly expressed in the adult brain, thereby titrating away functional let-7 and leading to its reduced effect on all its targets, including those that are co-targeted by both let-7 and miR-125. Such a scenario is supported by increasing evidence that the effectiveness of a particular miRNA is influenced by the cellular concentration of available miRNA binding sites [51, 52]. Alternatively, perhaps let-7 silencing requires cofactors that are only expressed during development. Future studies focused on the identification and characterization of the miR-125-independent targets of let-7 should provide insight into the networks of let-7 targets in adults.

Significance and scenarios of miRNA co-targeting

While an overarching feature of miRNA regulation is that mRNAs are responsive to multiple miRNAs, our understanding of the biological significance of co-targeting is rudimentary. Supporting the apparently abundant co-targeting identified by miRNA binding site predictions [22–24, 28, 53], there are plenty of examples of multiple miRNAs that can when expressed one-by-one repress the same 3’UTR reporter. Such examples include repression of mtpn 3’UTR by any one of a trio of miRNAs (miR-375, miR-125, and let-7b) and repression of cdkn1A/p21 3’UTR by any one of a staggering 28 different miRNAs [53, 54]. Since the effects
of combinations of miRNAs are rarely tested, such studies suggest that multiple miRNAs limit the spatial and/or temporal expression of targets and, in cells where they are co-expressed, may function redundantly with one another.

In addition to this simple scenario, there are hints of more complex combinatorial scenarios involving either cooperation or competition between co-targeting miRNAs. In the cooperative scenario, miRNAs with overlapping expression patterns lead to enhanced repression of co-targeted mRNAs in cells where they are expressed together. Examples of this include miR-25 and miR-221/222 co-repression of p57 and miR-148a and the miR-206 co-repression of dmpk [55, 56], although it is worth noting that the additive effects of these pairs of miRNAs is small though significant. Supporting such cooperative action, additional studies report that multiple binding sites, especially when they are within 15–35 bp of one another, lead to enhanced reporter repression [29, 57, 58]. Co-expressed miRNAs can also act competitively, as shown for miR-184 and miR-205 regulation of ship2 [59]. In this scenario, miR-184 does not have repressive activity itself but alleviates the repressive ability that miR-205 exerts via an overlapping binding site, as shown by comparative analysis of miR-205 alone versus in combination with miR-184 as well as analysis of mutated 3' UTR reporters containing intact miR-205 but mutant miR-184 sites. In light of this miR-184 function, systematic assays that have found that many miRNAs when expressed individually have no effect on a 3' UTR reporter do not rule out the possibility that these miRNAs function competitively with others [60, 61].

Phasic control expands the repertoire of known co-targeting functions, and emphasizes that co-targeting miRNAs may function at different times from one another and for different purposes. Phasic control may be particularly relevant to clustered miRNAs, since clustered miRNAs are enriched for co-targeting relationships [22, 28]. For example, the vertebrate miR-17~92 cluster, like other miRNA clusters, targets multiple components of related networks and pathways of genes, including the TGF-β pathway [62, 63]. As with the let-7-C cluster in flies, members of polycistronic clusters, including the miR-17~92 and miR-1/miR-133 clusters, are differentially processed [64, 65]. The resulting differential accumulation of these co-targeting miRNAs, along with differential base pairing and turnover of co-transcribed miRNAs, may lead to the distinct temporal accumulations of processed miRNAs that are indicative of phasic control. Thus, the staggered accumulation of different miRNAs processed from the same polycistronic transcript over time may be an important feature controlling the progression of temporal features of cell and organismal biology.

Conservation of co-targeting by let-7-C miRNAs

The biphasic regulation by let-7-C miRNAs may also be relevant to mRNAs co-targeted by let-7-C orthologues in other animals. These include lin-28 and lin-41, which were originally identified in C. elegans as potential targets of both let-7 and lin-4, the C. elegans miR-125 orthologue [66, 67]. These co-targeting relationships are conserved to vertebrates, since clustered miRNAs are enriched for co-targeting relationships [22, 28]. For example, the vertebrate miR-17~92 cluster, like other miRNA clusters, targets multiple components of related networks and pathways of genes, including the TGF-β pathway [62, 63]. As with the let-7-C cluster in flies, members of polycistronic clusters, including the miR-17~92 and miR-1/miR-133 clusters, are differentially processed [64, 65]. The resulting differential accumulation of these co-targeting miRNAs, along with differential base pairing and turnover of co-transcribed miRNAs, may lead to the distinct temporal accumulations of processed miRNAs that are indicative of phasic control. Thus, the staggered accumulation of different miRNAs processed from the same polycistronic transcript over time may be an important feature controlling the progression of temporal features of cell and organismal biology.
equally good amino acid similarity between Chinmo and a number of mammalian BTB-ZFs including Hic2, the conservation of let-7 and miR-125 sites in the hic2 3’UTR but not other BTB-ZF 3’UTRs suggests that Hic2 is the mammalian orthologue of Chinmo. Thus, our results predict that mammalian let-7 and miR-125 regulate hic2 in a biphasic manner.

**let-7-C miRNAs in aging and neurodegeneration**

The persistence and gradual increase of let-7-C miRNAs during adult life may balance the various cellular demands needed for proper tissue and organismal homeostasis over time. Thus, the increasing levels of let-7 that dampen stem cell function in aging tissue, found both in the mouse nervous system and the fly testis [35, 75], may be part of a general program that includes the neuronal maintenance function that this study explores. A conserved role for let-7-C miRNAs in such cell maintenance during adult life is supported by the requirement of lin-4 for proper lifespan in *C. elegans* [2], since nematodes, like fly brains, exhibit limited cell proliferation during adulthood. In addition, *Drosophila* let-7 and its target the *dp* transcription factor promote the maintenance of dopaminergic neurons in the adult brain by regulating the expression of pathogenic Leucine-Rich Repeat Kinase 2 [76]. Interestingly, changes in miR-125b have been linked to Alzheimer’s disease and vertebrate cerebellar neurodegeneration, although the molecular mechanisms underlying these changes have not been addressed [77, 78]. Taken together, this mounting evidence indicates that let-7 and miR-125 play critical neuroprotective roles in the aging brain so understanding their post-developmental functions in greater detail may be relevant to therapies for human neurodegenerative diseases, including Parkinson’s and Alzheimer’s diseases. In summary, our work has identified a novel in vivo mechanism by which multiple miRNAs repress a common target during distinct stages. Such differential regulation by subsets of co-expressed miRNAs should be considered for designing therapeutic strategies to treat diseases that are frequently caused by de-regulation of highly targeted mRNAs.

**Materials and Methods**

**Drosophila husbandry**

All flies were cultured on standard cornmeal medium at 25°C under 12 h light, 12 h dark cycles, except for flies analyzed in the temperature sensitive experiment presented in Fig 3. These flies were cultured in one of three regimens: at 18°C, at 29°C, and at 18°C until eclosion and then at 29°C thereafter. For steroid mediated UAS-transgene control using the Gene-Switch driver, flies were fed food containing 200 μM RU-486 (Mifepristone, Cayman Chemicals, Ann Arbor MI). Staging of pupae and MARCM clone induction was performed as previously described [37, 38]. Unless otherwise noted, adult male flies of indicated ages were used for experiments.

**Drosophila genetics and let-7-C mutant strain construction**

Detailed genotypes of all strains as well as the sources of the genetic mutations and transgenes used in the study are listed in S1 and S2 Tables, respectively. Transgenesis was performed by Rainbow Transgenic Services (Camarillo, CA) and BestGene, Inc. (Chino Hills, CA). The let-7-C mutant strains analyzed in this study (including let-7-Cnull, let-7-Ckop, let-7-Ckop rescue, ΔmiR-100, Δlet-7 and ΔmiR-125 strains) were generated by crossing w1118; let-7-CGKI / CyO strains to w1118; let-7-CKO2, P{neoFRT}40A / CyO strains in which one or both of these strains contained a rescuing transgene inserted on the third chromosome. Since this approach generated trans-heterozygous let-7-CGKI / let-7-CKO2, P{neoFRT}40A animals, it eliminated the effect of any confounding recessive background mutations that might have accumulated on those chromosomes. In addition, because the differing rescuing transgenes were inserted at identical
positions on the third chromosome, this approach ensured the pairwise comparison of strains that were otherwise as genetically similar to one another as possible. The detailed genetic scheme for generation of the transgenic samples is described in S1 Fig.

Climbing and lifespan analyses
Climbing assays were performed as described previously [33]. Lifespan analysis was performed as previously reported [1, 3, 79] using let-7-C mutant flies that were generated as described above. Fifteen male flies (0–1 day old) were transferred to each vial. Flies were transferred to fresh food every 3 days at which time dead flies were counted and removed. The survival curves were plotted using Microsoft Excel. Statistical analysis was performed with the Online Application for the Survival Analysis of lifespan assays (OASIS) [80] and the p values were calculated using the log-rank (Mantel-cox) test. The number of flies used for each experiment are noted in the figure legends, and also included along with the median and maximum lifespans of the tested strains in S3 Table. S4 Table indicates the p values for curves shown in one or more panels. The numbers of flies used for each experiment have been noted in the figure legends. Experiments usually included two independent controls: w1118 as well as a let-7-C mutant strain containing a fully rescuing transgene. The w1118 survival curve was generated with flies that had been back crossed five times.

Immunofluorescence
Immunofluorescence was performed as described previously [37, 38]. Primary antibodies included rat anti-Chinmo [38] (1:500), chicken anti-GFP (Rockland Immunochemicals, 1:4000), rabbit anti-Woc [81] (gift from Maurizio Gatti 1:1000), rat anti-Elav (DSHB, 1:250) and mouse anti-Dachshund (DSHB, 1:100). For quantitating Chinmo levels, pixel intensity of 30 individual cells in single confocal sections of 5 independent dissected brains stained with anti-Chinmo and anti-Woc antibodies were quantified using ImageJ software. The expression of Chinmo was normalized to the pixel intensity of Woc and the average pixel intensity of one Δlet-7-C confocal section showing the highest pixel intensity was designated as 100 Arbitrary Units (AU). Samples whose staining was directly compared were prepared and imaged in parallel and under identical conditions.

For c708a neuron counts, mushroom bodies were optically sectioned in 0.5 μm increments, and the total number of neurons was determined by manually counting the number of GFP-positive cells section by section, ensuring that cells present on consecutive sections were counted only once. Statistical analysis was performed and histograms generated using GraphPad Prism software. P values were calculated using a two-tailed paired t test. Values are presented as mean ± SEM. All images were collected on a Leica SP5 confocal microscope (Light Microscopy Imaging Center, Indiana University, Bloomington IN). Confocal stacks were merged using Leica LSM software.

Northern blot analysis and quantitative real time PCR
Total RNA was extracted with Trizol and treated with DNase I. The reverse transcription was performed as described previously [82]. For analysis of miRNA copy number in Fig 4A, Kc-167 cells were incubated with 20E (5×10⁻⁶ M) at 25⁰ C for 24h before being washed with fresh medium. Reverse transcription (RT) was carried out on 25ng of total RNA using the Reverse Transcription miRNA Taqman assays (Applied Biosystem, Foster City, CA) specific for the miRNA (dme-let-7 and dme-miR-125). Each cDNA sample was diluted 1:25 and real-time quantitative PCR (qPCR) was performed in duplicate using miRNA-specific primers/probe on a StepOnePlus Real Time PCR System (Applied Biosystem, Foster City, CA). For
determination of copy number, we generated a standard curve for let-7 and miR-125 using a synthetic let-7 and miR-125 HPLC-purified RNA oligonucleotides synthesized by Integrated DNA Technologies (Coralville, IA) corresponding to the 22 nucleotide miR-125-5p (5'-rUrCrCrUrGrArGrArCrCrUrGrArGrArCrUrUrGrUrArArCrUrUrGrUrArArCrUrU-3') and 21 nucleotide let-7-5p (5'-rUrGrArGrGrUrArGrUrArGrGrUrUrGrUrArUrArGrU-3'). For fold change analysis, individual values were normalized to 2S rRNA for Taqman miRNA assays and kinesin levels for Sybr green assays. For qRT-PCR analysis, oligos 2515, 2516, 2599, 2530, 2728, 2729 listed in S5 Table were used. Northern blot analysis was performed as described previously [37].

Plasmid and transgenes

**Chinmo transgenes.** pP[w+, UAS-chin::SV40] contained the Chinmo open reading frame (ORF) flanked by hsp70 5'UTR and SV40 3'UTR and under the control of UAS sites. It was generated by PCR amplifying the Chinmo ORF with oligos 136 and 137 (see S5 Table for sequences) from reverse transcribed RNA generated from CNS tissue, then sequence verifying and subcloning the resulting fragment into the BglII and NotI restriction enzyme sites of plasmid pUAST. pP[w+, UAS-chin::RNAi 148] encoded a short hairpin RNA (TGTGGGCTTTGAATACTACGC) targeting chinmo, designed based on rules described previously [83] and under the control of UAS sites. It was generated by subcloning the annealed oligos 1004 and 1005 (see S4 Table for sequences) into the EcoRI and NheI sites of plasmid pWalium20 (TRiP at Harvard Medical School).

**Sponge transgenes.** Sponge constructs targeting miR-100, let-7 and miR-125 were designed based on Loya et al. [84] and Bejarano et al. [85]. A silencing cassette for each miRNA was synthesized by BioBasic, Inc that contained twenty miRNA complementary sequences separated by variable four-nucleotide linker sequences (see S6 Table for complete sequences). The entire cassette was subcloned into the NotI and XbaI sites of a modified pValium10 plasmid (TRiP at Harvard Medical School, Boston, MA) and inserted into both attP40 and attP2 sites using phiC31 site-specific genomic integration. Resulting transformants were identified using vermillion as a transformation marker.

**let-7-C locus transgenes.** New transgenes were generated using previously reported P-element based transgenes containing either the full-length let-7-C locus or variants in which miR-100, let-7, and/or miR-125 were specifically deleted [33]. These ~18kb fragments were excised using unique restriction sites AvrII and XbaI and subcloned into the XbaI site of a modified pValium10 plasmid (TRiP at Harvard Medical School, Boston, MA). Resulting plasmids were inserted into attP2 sites using phiC31 site-specific genomic integration and transformants were identified using vermillion as a transformation marker.

**UAS-let-7-C constructs.** These constructs were generated using the previously generated full length pri-let-7-C cDNA construct [82]. The hairpins corresponding to pri-miR-100, pri-let-7 and pri-miR-125 were deleted using splicing by overlap extension PCR. The oligo pairs 937/938, 939/940 and 941/942 were used to generate ΔmiR-100, Δlet-7 and ΔmiR-125 constructs, respectively. The pri-let-7-C cDNA was subcloned as an XhoI-KpnI fragment into pUAST-attB using the oligo pair 935/936. The oligo pair 1070/1071 was annealed to generate pri-let-7 human let-7a2 and cloned into the XbaI site created after deleting Drosophila melanogaster pri-let-7. All PCRs were done with Pfu Polymerase.

**Luciferase reporters.** The nonmutated chinmo 3'UTR sensor has been described previously [38]. The constructs containing seed deletions of the four miR-125 or six let-7 sites were generated using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) and oligonucleotides 978–981 and 972–977 respectively (see S5 Table for oligonucleotide sequences). The synthetic luciferase sensors were generated by
annealing oligonucleotides with NotI and XhoI compatible ends (3046/3047, 3048/3049, 3050/3051 and 3052/3053). The annealed oligonucleotides (sequences in **S5 Table**) were cloned into a modified version of pSiCheck2 (Promega Life Science, Madison WI). Fold repression was calculated relative to control samples transfected with empty vector instead of miRNA-encoding plasmids. The luciferase reporters used in **Fig 2B** were psiCHECK plasmids bearing six perfect sites for either miR-100, let-7 or miR-125 downstream of a Renilla luciferase gene were used and have been described previously [38].

**Tagged protein plasmids.** Plasmids encoding N-terminal Flag tagged version of Dicer was generated by recombining **pENTR-Dicer 1** (kind gift from Mikiko C. Siomi) with the pAFW gateway plasmid (T. Murphy; obtained from the Drosophila Genome Resource Center) using the LR Clonase enzyme (Life Technologies, Carlsbad CA), respectively. Flag tagged Drosha and Pasha constructs have been described previously [82].

### Histochemistry and scanning electron microscopy

For histochemistry, heads were fixed for 3h in AAF buffer (10% Formaldehyde, 5% Acetic Acid, and 85% Ethanol). The fixed tissue was serially passaged through 70% ethanol, 95% ethanol, 100% ethanol, and twice in Xylene for 45 minutes each. Following these incubations, the tissues were embedded in paraffin followed by sectioning. The 7 μm tissue sections were mounted on superfrost-plus slides (VWR International, Radnor PA) and processed for hematoxylin-eosin staining. For scanning electron microscopy, adult flies were serially passaged through 25% ethanol (10h), 50% ethanol (2h), 75% ethanol (2h), 100% ethanol (2h), 50% ethanol: 50% hexamethyldisilazane (HMDS)(3minutes) and 100% HMDS (3 minutes), coated with gold-palladium and viewed with a JEOL 5800LV SEM microscope.

### Luciferase reporter assays

*Drosophila Kc-167* cells were cultured in CCM3 at 23°C. Cells were transfected in 48-well plates with 25 ng of **tub-Gal4** plasmid DNA, 25 ng **UAS-miRNA** plasmid DNA, and 25 ng of 3’UTR-containing sensor plasmid DNA using Effectene (Qiagen). Luciferase assays were performed using the Dual-Luciferase reporter system (Promega Life Science, Madison WI). Transfections were performed in triplicates and resulting luciferase levels were averaged. Fold repression was calculated by dividing the ratio of Renilla luciferase and firefly luciferase in cells transfected with an empty pUAST attB plasmid with the ratio of Renilla luciferase and firefly luciferase in cells transfected with pUAST attB plasmid containing let-7-C cDNAs.

### MiRNA duplex transfection and determination of half-life of miRNAs

Si-miRNA duplexes were synthesized as single-stranded RNAs by Integrated DNA Technologies (Coralville, IA) with HPLC purification, and resuspended in duplex buffer (100mM potassium acetate, 30mM HEPES, pH 7.5) to a concentration of 100μM. Annealing was performed by incubating 50 μM complementary single-stranded RNAs at 92°C for 2 min and leaving them for 30 min at room temperature [86].

- si miR-125 sense; 5’UCCCUUGAGACCCUAACUUUGUGAUU
- si miR-125 antisense; 5’UCACAAAGUAGGGUCUCAGGACU
- si let-7 sense; 5’UGAGGUAGGUGUUAAGCU
- si let-7 antisense; 5’UCAUAUACACCUArCrUrArCrUrArCrUrArCrUr

The miRNA duplexes were transfected using Dharmafect duo according to manufacturer’s instructions (GE Life Sciences, Lafayette CO). Briefly, 2.25 μl of mi-siRNA molecules (diluted to 4 μM in duplex buffer) was added such that the final concentration of each siRNA was 5 nM per well (the volumes indicated are for biological triplicate) in a 24 well plate. The cells were
incubated at 25°C for 5 h before being washed with fresh medium. Quantitative real time PCR was performed to measure the relative levels of the miRNAs in total RNA extracted from transfected cells at different time points. The half lives of let-7 and miR-125 were determined by exponential regression curve fitting using GraphPad Prism version 6 software.

**In vitro Drosha and Dicer processing assays**

Pri-let-7, and pri-miR-125 were generated by annealing oligos cloned into pLitmus 28i. DNA templates for transcription were generated by PCR with the T7 and 2162 oligo and were transcribed and labelled with 32UTP (Perkin Elmer, Waltham MA) using the T7 Megashortscript Kit (ThermoFisher, Cambridge MA). The transcript was purified by running the DNAsel treated reaction on a 4% denaturing PAGE gel and the gel piece corresponding to the labeled transcript was excised from the gel and eluted in a Eppendorf Thermomixer set at 400rpm and 37°C in a buffer containing 0.3M Sodium acetate, 0.2% Sodium dodecyl sulphate, and 1mM EDTA. The supernatant was precipitated in Ethanol. The precipitated RNA was refolded by heating at 95°C for 2 minutes followed by 37°C for 1 hour. A typical 25μL reaction contained 15μL of the Flag-Drosha-Pasha beads immunoprecipitate, 6.4mM MgCl₂, 1 U/μL of Ribonuclease Inhibitor (ThermoFisher, Cambridge MA), and the refolded labeled transcripts (0.5 × 10⁶ cpm). The reaction mixture was incubated at 26°C for 30 to 90 min, and RNA was extracted by phenol followed by ethanol precipitation and analyzed on a 10% denaturing polyacrylamide gel.

In vitro dicing assays were typically carried out in 25μl lysis buffer, containing 5% (v/v) glycerol, 1 mM DTT, 0.1unit μL⁻¹ RNasin Plus RNase Inhibitor (ThermoFisher, Cambridge MA), 1 nM 5' -radiolabeled substrate RNAs (GE Life Sciences, Lafayette CO; sequences listed below) and 25 nM Flag-tagged Dicer proteins. The reaction products were resolved by electrophoresis on 10% denaturing Page gel, detected by Typhoon phosphorimager and quantified by ImageQuant software (GE Life Sciences, Lafayette CO).

**Supporting Information**

**S1 Fig. Scheme for Generation of Experimental Samples.** This study compared flies that were generated using a scheme that ensured that they had similar genetic backgrounds. Flies that were analyzed (F14) were trans-heterozygous for two different let-7-C null alleles (indicated by red and yellow bars), ensuring that phenotypes were not due to recessive mutations on either let-7-C mutant chromosome. In addition, third chromosomes that contained differing rescuing transgenes (indicated by green bar) were derived in parallel from the same population of flies. Finally, all flies had a common X-chromosome (blue bar), derived from an isogenized stock. (S1-1) All rescuing transgenes, including the wildtype rescuing transgene as well as let-7 and miR-125 deleted versions, were injected into embryos from the same population of stock BL#25710 from the Bloomington Drosophila Stock Center. Resulting progeny were backcrossed twice to BL#32261 in order to select and balance vermilion+ transformants (F1 and F2). Single transformants were subsequently backcrossed to an isogenized version of BL#3703 three times (F3-F5) in order to make balanced stocks with isogenized X chromosomes (F6). (S1-2) Stacks with differing rescuing transgenes were crossed to the same population of a stock that contained the let-7-C KO2 chromosome, an isogenized X chromosome, and two 3rd chromosome balancers. The let-7-C KO2 stock used in F7 was generated in a similar fashion as the rescuing transgenes stocks, by backcrossing three times to an isogenized version of BL#3703. Resulting stocks (F8) had common X (blue), 2nd (yellow) and 3rd (green) chromosomes, and were used in F13 to generate the experimental strains. (S1-3) A second let-7-C allele, let-7-C GKI, was prepared by outcrossing twice to an isogenized stock, and then crossed to
an isogenized stock containing a T(2;3) CyO-TM6b compound chromosome. The let-7-C allele was selected based on mini-white, and the T(2;3) CyO-TM6b balancer was selected based on the dominant Humoral marker. The resulting stock with a fixed second and third chromosome was amplified and used as the source for all virgins in the crosses that yielded the flies for analysis. (S1-4) Flies for analysis were generated by crossing virgins of the stock generated in F12 with males of stocks generated in F8 that harbored differing rescuing transgenes.

(TIF)

S2 Fig. The vacuoles in aged Δlet-7 and ΔmiR-125 loss-of-function and Chinmo gain-of-function strains are predominantly localized to the central brain. (A) Depiction of major anatomical structures in a 3d aged w^{118} brain. CB (central brain), Lo (lobula), LoP (lobula plate), Me (medulla), La (lamina) and Rt (retina). Scale bar: 20μm. (B-E) Quantitation of vacuoles in 40d aged brains of (B) w^{118} (wt), let-7-ΔC90p, let-7-ΔC90p rescue strains, (C) let-7-ΔCnull rescue, ΔmiR-100, Δlet-7, and ΔmiR-125 mutant strains, (D) chinmo1; let-ΔCnull rescue, chinmo1; ΔmiR-100, chinmo1; Δlet-7, chinmo1; ΔmiR-125, chinmoRNAi; ΔmiR-125 mutant strains, and (E) elavGS; UAS-Chinmo (-RU-486) and elavGS; UAS-Chinmo (+RU-486) strains.

(TIF)

S3 Fig. Loss of miR-125 and let-7 enhance rCGG90 mediated retinal degeneration. (A-E) Scanning electron microscope (SEM) eye sections from 7d GMR-Gal4 flies harboring a (A) miR-125 sponge (miR-125SP), (B) a rCGG90 transgene (rCGG90), (C) a rCGG90 transgene along with a miR-100 sponge (miR-100 SP + rCGG90), (D) a let-7 sponge (let-7SP + rCGG90), or (E) a miR-125 sponge (miR-125 SP + rCGG90).

(TIF)

S4 Fig. ΔmiR-125 mutants display late onset brain degeneration that is rescued by reducing chinmo levels. (A-L) Verification of chinmo knock down in chinmoRNAi transgenic line. (A-F) Confocal images of 3d old adult brains immunostained for Chinmo (green) and Woc (red). The intensity of Chinmo immunostaining is reduced in brains harboring the chinmoRNAi transgene (D-F) relative to the control (A-C). The genotype of the control in A-C is let-7-ΔGKI / let-7-ΔC90P; P[neoFRT]40A; [v+, let-7-ΔCΔmiR-125]attP2 / +, and the genotype displayed in D-F is let-7-ΔGKI / let-7-ΔC90P, P[neoFRT]40A; [v+, let-7-ΔCΔmiR-125]attP2 / P[w+, UAS-chinmoRNAi 148] VK00033. (G-L) Elav-Gal4, UAS-mCD8::GFP labeled wild type (G) and UAS-chinmoRNAi (L) third instar larval clones generated in newly hatched larvae using the mosaic analysis with represible cell marker (MARCM) technique and stained with Chinmo antibody. Absence of Chinmo staining in clones confirmed knockdown of chinmo. The genotype in G-I is P[w+]elav [C155], P[UAS-mCD8::GFP.L];LA, P[tubP-GAL80];LL10 P[neoFRT]40A / P[neoFRT]40A; + and the genotype in J-L is P[w+]elav[C155], P[UAS-mCD8::GFP.L];LA, P[tubP-GAL80];LL10 P[neoFRT]40A / P[neoFRT]40A; P[w+, UAS-chinmoRNAi 148] VK00033 / +. (M) Reducing dosage of chinmo decreases brain vacuolization in ΔmiR-125 mutants. Histochemistry was performed on brain sections of 40d old ΔmiR-125 mutants and chin1, ΔmiR-125 mutants and the number of vacuoles were scored to assess brain morphology. Representative examples of brains sections are shown (vacuoles indicated by blue arrows) and the total vacuole number quantified from such sections of five independent brains is presented in Fig 2D.

(TIF)

S5 Fig. miR-125 is the predominant miRNA that silences chinmo in adult flies. (A-D) Confocal images of 3d old adult brains immunostained for Chinmo (green). No Chinmo expression was detected in brains of flies harboring either the wild type or the ΔmiR-100 transgene (panels A and B). The level of Chinmo expression in ΔmiR-125 mutants is much higher than in Δlet-7 mutant adult flies (compare panels C and D). Genotypes used are the same as those listed for
S6 Fig. Schematic of let-7 and miR-125 binding sites in the luciferase reporter. (A, B) Sequences and predicted base-pairing of let-7 (green) and miR-125 (red) binding sites in the luciferase sensors. Numbering is relative to the first nucleotide in the 3’UTR. Yellow boxes indicate the sequences that were deleted in the mutant constructs. The sites were designed so that the binding pattern was comparable between the miRNAs and the target sites. The nucleotides 1–9, 11–15, 19–21(let-7) and 19–22(miR-125) formed base-pairing interactions with the 3’UTR. The minimum free energy (mfe) calculated by RNAhybrid is indicated on the right of each binding site.

S7 Fig. The decreased rate of processing of miR-125 is compensated by its lower rate of decay. (A) Primary transcripts expressing wild type pri-miR-125 does not undergo Drosha processing in vitro. In vitro processing of pri-let-7 and pri-miR-125 with purified Flag tagged Drosha-Pasha complex. The primary transcript (pri), 5’ flank (5’F), 3’ flank (3’F) and precursor (pre) are indicated on the right. Quantitation of the fraction processed is calculated as precursor/primary +5’F+3’F+precursor. (B) Western blot analysis of purified Flag tagged Drosha-Pasha(top panel) and Flag tagged Dicer 1(middle panel) used in Drosha and Dicer processing assays, respectively. (C) Expression analysis of Dicer 1 in UAS Dicer RNAi lines as determined by quantitative real time PCR of total RNA extracted from 10d old adult fly heads. Rp49 was used as a control for normalization. P-values determined by two-tailed paired t-test are denoted on top of the histogram. Assays were performed in triplicate for each experiment. Error bars, S.D. (D) MiRNA decay was calculated by quantitating the relative miRNA levels in Kc-167 cells transfected with miRNA duplexes and fitted exponential regression curve for let-7 and miR-125 indicates that the half life for let-7 is lower than the half life of miR-125. The decay constant (λ) was extrapolated from the exponential decay curves of each biological replicate (n = 3 per time point), and the mean ± S.D is shown. The half-life in hours (hr) was calculated by the formula ln2/λ.

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