Targeted Downregulation of kdm4a Ameliorates Tau-engendered Defects in Drosophila melanogaster

Sung Yeon Park 1,2, Jieun Seo 2,3 and Yang-Sook Chun 1,2,3

1Ischemic/Hypoxic Disease Institute, Seoul National University College of Medicine, Seoul, Korea
2Department of Physiology, Seoul National University College of Medicine, Seoul, Korea
3Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul, Korea

ABSTRACT

Background: Tauopathies, a class of neurodegenerative diseases that includes Alzheimer’s disease (AD), are characterized by the deposition of neurofibrillary tangles composed of hyperphosphorylated tau protein in the human brain. As abnormal alterations in histone acetylation and methylation show a cause and effect relationship with AD, we investigated the role of several Jumonji domain-containing histone demethylase (JHDM) genes, which have yet to be studied in AD pathology.

Methods: To examine alterations of several JHDM genes in AD pathology, we performed bioinformatics analyses of JHDM gene expression profiles in brain tissue samples from deceased AD patients. Furthermore, to investigate the possible relationship between alterations in JHDM gene expression profiles and AD pathology in vivo, we examined whether tissue-specific downregulation of JHDM Drosophila homologs (kdm) can affect tau R406W-induced neurotoxicity using transgenic flies containing the UAS-Gal4 binary system.

Results: The expression levels of JHDM1A, JHDM2A/2B, and JHDM3A/3B were significantly higher in postmortem brain tissue from patients with AD than from non-demented controls, whereas JHDM1B mRNA levels were downregulated in the brains of patients with AD. Using transgenic flies, we revealed that knockdown of kdm2 (homolog to human JHDM1), kdm3 (homolog to human JHDM2), kdm4a (homolog to human JHDM3A), or kdm4b (homolog to human JHDM3B) genes in the eye ameliorated the tau R406W-engendered defects, resulting in less severe phenotypes. However, kdm4a knockdown in the central nervous system uniquely ameliorated tau R406W-induced locomotion defects by restoring heterochromatin.

Conclusion: Our results suggest that downregulation of kdm4a expression may be a potential therapeutic target in AD.

Keywords: Drosophila melanogaster; Tauopathy; Alzheimer’s Disease; Heterochromatin; JHDM; kdm

INTRODUCTION

Tauopathies, a class of neurodegenerative diseases that includes Alzheimer’s disease (AD), are characterized by the deposition of neurofibrillary tangles composed of hyperphosphorylated tau protein in the human brain. 1 However, the underlying mechanism of tauopathy remains unclear, and deciphering the process is crucial for developing effective
biomarkers and identifying potential therapeutic targets of AD. Abnormal alterations of histone acetylation and methylation have been documented in patients with AD and in a transgenic animal model of AD. In early development, several epigenetic alterations may contribute to AD, whereas certain epigenetic shifts may occur downstream of AD pathology. In particular, histone acetylation is indicative of late onset cognitive loss, and has been used to develop biomarkers of AD.

Tau phosphorylation is an important pathogenic event in AD. Targeted expression of human tau in the central nervous system (CNS) of Drosophila recapitulates several characteristics in human AD, such as neuronal loss in the form of prominent vacuoles, compromised life span, and locomotor and cognitive impairments. Therefore, to provide a useful AD model, transgenic Drosophila were generated using an FTDP-17 mutant form of human tau (tauR406W) that was subcloned downstream of the yeast upstream activator sequence (UAS), tissue-specific expression of the transgene was achieved by regulating the availability of Gal4 transcription factor with different Gal4 drivers.

Analyses of tissues from AD patients and a transgenic mouse model of AD consistently show decreased histone acetylation. Accordingly, histone deacetylase (HDAC) inhibitors have been proposed as potential procognitive agents for the treatment of AD. Indeed, Tip60 histone acetyltransferase (HAT) in human tau overexpressing Drosophila has been proposed to play a neuroprotective role in impaired cognition during early development of AD. Reciprocally, Drosophila genetic screens have shown that HDAC6 null mutation and pharmacological inhibition can rescue tau-induced microtubule defects in muscles and neurons. Furthermore, histone methylation has a marked influence on synaptic plasticity and cognition.

Recently, Mastroeni and colleagues reported increased histone H3K4 trimethylation in the cytoplasm and significant colocalization with hyperphosphorylated tau tangles, but decreased in the nuclei of AD brains compared to non-demented controls. Moreover, the total levels of histone H3K9 dimethylation and HP1α were decreased and heterochromatin relaxed, resulting in aberrant neuronal gene expression in Drosophila, mouse, and human tauopathies. However, no studies have investigated the involvement of histone demethylases in tauopathies.

In this study, bioinformatic analyses revealed significantly higher expression of several Jumonji domain-containing histone demethylase (JHDM) genes in postmortem brain tissue from patients with AD than from non-demented controls. In addition, our results suggest that the downregulation of kdm4a (JHDM3A) expression may be a potential therapeutic target in AD, providing new insight into the role of histone demethylases in tauopathies.

**METHODS**

**Bioinformatics analyses**

Expression levels of histone demethylases in postmortem brain tissue from patients with AD were evaluated using the publically available Gene Expression Omnibus (GEO, GSE33000) (Table 1). The dataset included patients with AD (n = 310) and non-demented controls (n = 157). JHDM1A (10025907153_at probe), JHD1B (10025912776_at probe), JHDM2A (10023810558_at probe), JHDM2B (10025911102_at probe), JHDM3A...
(10025904057_at probe) and JHDM3B (10025924555_at probe) mRNA expression levels were compared between groups using unpaired, two-sided Student’s $t$-tests.

**Drosophila strains and genetics**

Fruit flies were maintained on standard media, and genetic crosses were performed at 25°C unless specified otherwise. All genetic crosses were performed at least in triplicate, and percentages represent the means of the replicates. The following *Drosophila* strains were used: UAS- $kdm2$ RNAi (P[KK101783]VIE-260B) (Vienna *Drosophila* RNAi Center [VDRC], Vienna, Austria), UAS- $kdm3$ RNAi (y[1] v[1]; P[y[+]t7.7] v[+t1.8] =TRiP.HMJ22328) [attrP40] (Bloomington *Drosophila* Stock Center, Indiana University, Bloomington, IN, USA), UAS- $kdm4a$ RNAi lines (w$^{1118}$; P[GD9133]v32652 hereafter referred to as UAS- $kdm4a$ RNAi and w$^{1118}$, P[GD9133]v32650/CyO hereafter referred to as UAS- $kdm4a$ RNAi 2), UAS- $kdm4b$ RNAi (P[KK102089]VIE-260B) (VDRC), elav- Gal4 lines (P[w[+mW.hs]=GawB]elav[C155] and P[w[+mC]=Gal4-elav.L]2/CyO, #B8765) (Bloomington *Drosophila* Stock Center), UAS- $kdm4a$-HA,FLAG$_2$ (w;P[w[+mC]=[UAS- $kdm4a$-HA,FLAG$_2$]) hereafter referred to as UAS- $kdm4a$) (Jerry L. Workman, Stowers Institute for Medical Research, Kansas, MO, USA),$^{22}$ BL2 chromatin reporter (y$^{+}$-w$^{+}$/Dp(3;Y)BL2, P[HS-lacZ.scs]65E) (Bloomington *Drosophila* Stock Center), UAS-tau$^{R406W}$ (Mel B. Feany, Harvard Medical School, Boston, MA, USA),$^{5}$ GMR-Gal4 (Deborah A. Hursh, CBER/FDA, Silver Springs, MD, USA).$^{23}$ The wild-type strain was *w$^{1118}$* (Seungbok Lee, Seoul National University, Seoul, Korea).$^{24}$

**Locomotor behavioral assay**

Flies were collected in vials on the day of eclosion and then transferred without anesthesia to fresh vials every other day. The locomotor assay was performed in the afternoon on day 10. Ten flies were placed in each plastic vial marked with 15 cm height and gently tapped to the bottom. The percentage of flies that climbed to the height of 15 cm in 10 seconds at each consecutive 5-day interval was calculated for each genotype unless specified otherwise. The assay was performed for 20 days after eclosion.

**Histochemical detection**

For histochemical detection of β-galactosidase activity, larvae were heat-shocked for 45 minutes at 37°C, followed by recovery for 1 hour at room temperature. Larval brain lobes were dissected in phosphate-buffered saline (PBS), fixed in 3.7% formaldehyde in PBS for 5 minutes, washed in PBS, transferred to X-gal solution,$^{25}$ and incubated overnight at 37°C. Larval brain lobes were mounted in 80% glycerol and examined using an Olympus DP71 microscope.

**Statistical analyses**

All data were analyzed using Microsoft Excel 2007 and expressed as the means and standard deviations. Continuous variables were analyzed using Student’s $t$-tests when data were normally distributed. All statistical tests were two-sided. $P$ values < 0.05 were considered to indicate statistical significance.
RESULTS

Increased expression of several JHDM genes in postmortem brain tissue from patients with AD

To investigate the potential relationship between alterations in the expression of JHDM genes and AD pathology in vivo, we used bioinformatics analyses to examine differences in the expression levels of JHDM1A/1B, JHDM2A/2B, and JHDM3A/3B, which have Drosophila homologs (Tables 1 and 2). The expression levels of JHDM1A, JHDM2A/2B, and JHDM3A/3B were significantly higher in postmortem brain tissue from AD patients than from non-demented controls (average log2 value, 0.0221 ± 0.00337 vs. −0.0333 ± 0.00451; 0.0064 ± 0.00516 vs. −0.0794 ± 0.00543; 0.00837 ± 0.00276 vs. −0.0179 ± 0.00342; 0.0471 ± 0.00251 vs. 0.0256 ± 0.00326; 0.1403 ± 0.0155 vs. −0.0220 ± 0.0171, respectively; P < 0.001), whereas the JHDM1B mRNA levels were significantly downregulated in brain tissues from AD patients (average log2 value, −0.1300 ± 0.00603 vs. −0.0161 ± 0.00826; P < 0.001) (Fig. 1).

Table 2. List of genes for the study

| Fly symbol | Fly gene ID | Human gene symbol | Human gene ID | DIOPT score | Weighted score | Rank |
|------------|-------------|-------------------|--------------|-------------|----------------|------|
| kdm2       | cg11033     | JHDM1A/KDM2A      | 22992        | 14 of 15    | 13.85          | High |
|            |             | JHDM1B/KDM2B      | 84678        | 11 of 15    | 10.97          | Moderate |
| kdm3       | cg8165      | JHDM2A/KDM3A      | 55818        | 9 of 15     | 8.75           | Moderate |
|            |             | JHDM2B/KDM3B      | 57780        | 10 of 15    | 9.76           | High |
| kdm4a      | cg15835     | JHDM3A/KDM4A      | 9682         | 10 of 15    | 9.74           | High |
|            |             | JHDM3B/KDM4B      | 23030        | 10 of 15    | 9.74           | High |
| kdm4b      | cg33182     | JHDM3A/KDM4A      | 9682         | 10 of 15    | 8.79           | Moderate |
|            |             | JHDM3B/KDM4B      | 23030        | 9 of 15     |                |      |

*Drosophila RNAi Screening Center Integrative Orthologue Prediction Tool (ver 7.1).

Fig. 1. Increased expression of JHDM genes in postmortem brain tissue from patients with AD. Scatter diagrams representing mRNA expression levels of JHDM genes in postmortem brain tissue from patients with AD. Dot plots showing JHDM1A/1B, JHDM2A/2B, and JHDM3A/3B mRNA levels in non-demented healthy controls and AD patients. JHDM = Jumonji domain-containing histone demethylase, AD = Alzheimer’s disease. *P < 0.001 between the indicated groups.
Knockdown of JHDM genes ameliorates the morphological deficit induced by overexpressing tau^{R406W} in Drosophila eyes

To examine the effect of JHDM gene knockdown in an AD background, tau^{R406W}-expressing flies were crossed with UAS-RNAi strains expressing RNAi specific for each of the JHDM fly homologs (kdm2, JHDM1; kdm3, JHDM2; kdm4a, JHDM3A; and kdm4b, JHDM3B) (Table 2) in Drosophila eye using the GMR-Gal4 driver. Neurodegeneration can be easily monitored when tau^{R406W} is overexpressed in the fly eyes, resulting in eye morphological defects such as reduced size, loss of bristles, disordered ommatidia and roughened eye surfaces.\(^{26,27}\) The observed eye defects can be categorized into four classes according to their severity: class 1, normal; class 2, roughness in the eyes with size reduction and some ommatidial disruption; class 3, collapsed eye tissue with size reduction; and class 4, black spots of apoptotic tissue in the collapsed eye (Fig. 2A). Approximately 74% of flies overexpressing tau^{R406W} have class 3 + 4 eyes. In contrast, knockdown of kdm2, kdm3, kdm4a or kdm4b genes in tau^{R406W}-overexpressing flies ameliorated the tau^{R406W}-engendered eye defects, resulting in only 8%, 37%, 40%, or 21% class 3 + 4 eyes, respectively (Fig. 2B). No eye defects were detected in

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**Fig. 2.** Knockdown of any of the JHDM genes ameliorates the morphological defects induced by overexpressing tau^{R406W} in Drosophila eyes. (A) Bright field images of 10-day-old adult Drosophila eyes of control (w1118) and tau^{R406W}-transgenic flies heterozygous for knockdown of any of the kdm genes using the GMR-Gal4. Images were taken using an Olympus SZ61 stereo zoom binocular microscope equipped with an eXcope XCAM1080 digital camera. (B) kdm knockdown flies that overexpressed tau^{R406W} showed a shift toward less severity in the distribution of phenotype categories in a population of flies (n = 30–73). JHDM = Jumonji domain-containing histone demethylase.
flies overexpressing each of the UAS-RNAi strains of kdm alone (data not shown). Therefore, knockdown of the kdm genes ameliorated the tau<sup>R406W</sup>-induced eye phenotype, resulting in less severe phenotypes.

**Targeted downregulation of kdm4a ameliorates tau<sup>R406W</sup>-induced locomotion defect**

To evaluate the neuronal effect of kdm gene knockdown in flies overexpressing tau<sup>R406W</sup> in their CNS via pan-neuronal elav-Gal4, we performed the climbing assay, a behavioral readout used for monitoring neurotoxic deficits in *Drosophila*. Whereas w1118 control flies retained 90% of their climbing ability on day 10, only 45%–50% of tau<sup>R406W</sup>-expressing flies climbed up. Inconsistent with the eye analysis results, knockdown of either kdm2 or kdm3 in flies overexpressing tau<sup>R406W</sup> alone had no significant effect on the climbing ability of flies overexpressing tau<sup>R406W</sup> alone (Fig. 3A and B). Interestingly, flies that overexpressed kdm2 RNAi alone exhibited a locomotion defect, showing 60% climbing ability compared to 90% climbing ability in control flies. This finding indicates that kdm2 itself may play a role in neuronal function. However, knockdown of kdm4a in tau<sup>R406W</sup>-overexpressing flies caused a mild but significant amelioration of the locomotion defect, with flies showing 80% climbing ability compared to the 50%–60% climbing ability of flies overexpressing tau<sup>R406W</sup> alone (Fig. 3C). Importantly, overexpression of kdm4a RNAi alone had no significant effect on locomotion, indicating that the neuroprotective effect may be specific to the context of tauopathy. In contrast, knockdown of kdm4b in flies that overexpressed tau<sup>R406W</sup> exacerbated the locomotion defect, and they showed 30% climbing ability compared to 50% in flies overexpressing tau<sup>R406W</sup> alone. Moreover, flies expressing kdm4b RNAi alone exhibited the locomotion defect, showing 60% climbing ability compared to 90% in control flies, indicating that kdm4b itself may be involved in neuronal function (Fig. 3D). Therefore, tau<sup>R406W</sup> overexpression may aggravate the neuronal defect in kdm4b-deficient flies during development.

To ascertain the neuronal protective effect of kdm4a knockdown in tau<sup>R406W</sup>-overexpressing flies, we performed the climbing assay with different kdm4a RNAi strain or wild-type kdm4a transgenic flies crossed to tau<sup>R406W</sup>-overexpressing flies. Crossing the kdm4a RNA<sup>2</sup> line with flies overexpressing tau<sup>R406W</sup> in their CNS ameliorated the locomotion defect, and they showed 70% climbing ability compared to 50% in flies that overexpressed tau<sup>R406W</sup> alone (Fig. 3E), whereas exogenously overexpressing wild-type kdm4a in tau<sup>R406W</sup>-overexpressing flies exacerbated the locomotion defect, as they showed 10% climbing ability compared to 40% in flies overexpressing tau<sup>R406W</sup> alone (Fig. 3F). Accordingly, in the eye, kdm4a knockdown with the RNA<sup>2</sup> strain in tau<sup>R406W</sup>-overexpressing flies resulted in 58.5% class 3 + 4 eyes compared to 87% class 3 + 4 eyes in flies overexpressing tau<sup>R406W</sup> alone, whereas overexpressing kdm4a in tau<sup>R406W</sup>-overexpressing flies showed no significant difference in eye morphology compared to those overexpressing tau<sup>R406W</sup> alone (Fig. 3G). Taken together, kdm4a knockdown ameliorated tau<sup>R406W</sup>-engendered deficits in both neurons and eyes.

**Targeted downregulation of kdm4a reduces heterochromatic loss induced by tau<sup>R406W</sup> overexpression**

Given that tau promotes neuronal death through heterochromatin loss, global chromatin relaxation, and aberrant transcriptional activation in *Drosophila*, mouse, and human tauopathies, we investigated whether knockdown of kdm genes alters heterochromatin loss in flies overexpressing tau<sup>R406W</sup> in their CNS via pan-neuronal elav-Gal4. We measured reporter expression in larval brain lobes using a transgenic fly strain (BL2 reporter) that has a LacZ reporter gene embedded in and silenced by heterochromatin. After 45 min heat
Fig. 3. Targeted downregulation of kdm4a ameliorates tau<sup>R406W</sup>-induced locomotion defect. (A–E) Locomotor activity in control and tau<sup>R406W</sup> transgenic flies heterozygous for knockdown of any of the kdm genes using pan-neuronal elav-Gal4 (#8765) was measured on day 10 post-eclosion (10 flies and 10 repeats per group, respectively; 15 cm/10 sec). (F) Locomotor activity in control and tau<sup>R406W</sup> transgenic flies heterozygous for overexpression of wild-type kdm4a transgene using pan-neuronal elav-Gal4 (#c155) was measured on day 7 post-eclosion (10 flies and 10 repeats per group, respectively; 8 cm/20 sec). (G) A shift in the distribution of eye phenotype categories in populations of flies with kdm4a knockdown or overexpression in tau<sup>R406W</sup>-overexpressing flies using GMR-Gal4 (n = 78–89). *P < 0.05 and n.s. denotes P > 0.05 between the indicated groups.
shock pulse at 37°C, this LacZ reporter was silenced in controls (Fig. 4A), but abnormally expressed in tauR406W-overexpressing fly brain due to heterochromatin loosening (Fig. 4B). kdm2 knockdown enhanced the expression of the heterochromatin-embedded reporter compared to overexpressed tauR406W alone (Fig. 4C and F). Expectedly, RNAi-mediated reduction of kdm4a decreased the number and intensity of the expression foci of the heterochromatin-embbedded reporter in tauR406W-overexpressing flies (Fig. 4D and F), whereas kdm4b knockdown had no significant alteration on the expression of the heterochromatin-embedded reporter in flies overexpressing tauR406W alone (Fig. 4E). Therefore, neuronal-specific downregulation of kdm4a may suppress tauR406W-engendered locomotion impairment by restoring heterochromatin.

**DISCUSSION**

We used bioinformatics analyses to detect alterations in JHDM gene expression in AD pathology. The expression levels of JHDM1A, JHDM2A/2B, and JHDM3A/3B were significantly higher in postmortem brain tissue from AD patients than from non-demented controls. In *Drosophila*, CNS-specific knockdown of kdm4a (JHDM3A) ameliorated tauR406W-induced locomotion defects, in agreement with eye analysis results.
Recently, Frost et al.\(^5\) reported that oxidative stress and subsequent DNA damage can substantially alter chromatin structure, inducing heterochromatin loss and aberrant transcriptional activity, causing neurodegeneration in tauopathies. The authors reported that tau\(^{R406W}\) overexpressing adult fly brain had decreased total levels of H3K9 dimethylation and heterochromatin protein 1α (HP1α), which are normally enriched in heterochromatin. In addition, loss of function mutations in HP1α or Su(var)3-9, which encodes a histone methyltransferase responsible for H3K9 dimethylation, exacerbated tau\(^{R406W}\)-engendered locomotion deficits. Given that KDM4A also directly interacts with HP1α and is essential for heterochromatin organization and function via both enzymatic and structural mechanisms in Drosophila,\(^22,34,35\) it is plausible that KDM4A may be associated with heterochromatin alteration in tauopathies. Intriguingly, kdm4a knockdown with either of two RNAi strains ameliorated tau\(^{R406W}\)-induced deficits in the eye and in neurons. Conversely, wild-type kdm4a overexpression in flies overexpressing tau\(^{R406W}\) in the CNS exacerbated tau\(^{R406W}\)-induced locomotion defects. Importantly, overexpression of kdm4a RNAi alone had no significant effect on locomotion in flies, indicating that the neuroprotective effect was specific for the AD pathological context. Consistent with these results, Lorbeck et al.\(^36\) reported that flies containing the P-element suppressor mutant of kdm4a (kdm4a\(^{P-supp}\)) showed no significant loss in climbing ability. According to the BL2 transgenic reporter assay, RNAi-mediated reduction of kdm4a in the CNS reduced heterochromatin relaxation, thus ameliorating the tau\(^{R406W}\)-engendered locomotion defect. Further studies are needed to elucidate the underlying mechanism of KDM4A involvement in the heterochromatic organization and transcriptional regulation of tauopathies.

kdm4b knockdown in the CNS exacerbated the tau\(^{R406W}\)-induced locomotion defect. Interestingly, flies that overexpressed kdm4b RNAi alone exhibited the locomotion defect, showing 60% climbing ability compared to 90% in control flies. This finding indicates that KDM4B may play a role in neuronal function in Drosophila. Consistent with this result, neuronal-specific kdm4b-deficient mice exhibit hyperactive behavior, sustained hyperactivity in a novel environment, deficits in working memory, and spontaneous epileptic-like seizures, indicating impaired neurodevelopment.\(^37\) This finding suggests that KDM4B plays a crucial role in the formation of functional neuronal networks. Therefore, neuronal defects in kdm4b-deficient flies during development may exacerbate tau\(^{R406W}\)-engendered locomotion impairment. However, the underlying mechanism does not appear to involve altered heterochromatin structure, as kdm4b knockdown had no significant effect on heterochromatin relaxation induced by tau\(^{R406W}\) overexpression.

Inconsistent with the eye analysis results, knockdown of either kdm2 or kdm3 in tau\(^{R406W}\)-overexpressing flies had no significant effect on climbing ability in flies that overexpressed tau\(^{R406W}\) alone. However kdm2 knockdown tended to slightly exacerbate the tau\(^{R406W}\)-induced locomotion defect, particularly in young flies (2–3 days after eclosion), but not significantly in older flies (data not shown). Furthermore, flies expressing kdm2 RNAi alone exhibited the locomotion defect, showing 60% climbing ability compared to 90% in control flies, indicating that KDM2 may play a role in neuronal function. In agreement with this result, kdm2 null mutant adult flies displayed defects in circadian locomotor behavior.\(^38\) Thus, a neuronal defect in kdm2-deficient flies during development may exacerbate the tau\(^{R406W}\)-engendered locomotion impairment in young flies. Furthermore, kdm2 knockdown in the larval brain lobe can enhance the number and intensity of expression foci of the heterochromatin-embedded reporter in tau\(^{R406W}\)-overexpressing flies. These results indicate that neuronal-specific downregulation of kdm2 may exacerbate tau\(^{R406W}\)-induced locomotion.
impairment by enhancing heterochromatin loss during early development; however, the underlying mechanism remains to be elucidated.

Using bioinformatics analyses, we revealed significantly increased expression of JHDM1A, JHDM2A/2B, and JHDM3A/3B in postmortem brain tissue from patients with AD compared to non-demented controls, whereas JHDM1B mRNA levels were downregulated in the brains of AD patients. Using tau<sup>R406W</sup>-induced transgenic flies as an AD model, we showed that kdm4a knockdown in the CNS ameliorated tau<sup>R406W</sup>-induced locomotion defects by restoring heterochromatin. These results suggest that the downregulation of kdm4a expression could be a potential therapeutic target in AD.

**ACKNOWLEDGMENTS**

We thank Vincenzo Pirrotta, Deborah Hursh, Brian Stultz, Jerry L. Workman, Mel B Feany, Seungbok Lee, the Bloomington Stock Center, and the Vienna RNAi stock for supplying stocks. Vincenzo Pirrotta made valuable comments that greatly improved the manuscript. Jieun Seo received a scholarship from the BK21-plus education program of the National Research Foundation of Korea.

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