Int. J. Dev. Biol. 55: 223-227 (2011)
doi: 10.1387/ijdb.103112cw

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Spatially controlled expression of the *Drosophila* pseudouridine synthase *RluA-1*

CHUNG-CHI WANG[1], JUN-CHIH LO[2], CHENG-TING CHIEN[3] and MIN-LANG HUANG*[,1]

[1]Department of Life Science, National Chung-Cheng University, [2]Graduate Institute of Natural Healing Sciences, Nanhua University and [3]Institute of Molecular Biology, Academia Sinica, Taiwan

ABSTRACT Pseudouridine (Ψ) synthases function in the formation of Ψ, the most abundant of the modified RNA residues. All Ψ synthases in *E. coli* are classified into one of five families according to their sequences. Among them, members of the RluA Ψ synthase family catalyze certain Ψ formations in ribosomal RNA. RluA family members are required for ribosomal assembly and bacterial growth. None of the RluA in multicellular organisms has been studied. In the *Drosophila* peripheral nervous system, multiple dendritic (MD) neurons are recognized by their dendritic arbors. MD neurons can also be identified by using the enhancer trap line *E7-2-36*, which expresses the *lacZ* gene in MD neurons. Here, we show that the P-element of *E7-2-36* inserts into the *Drosophila RluA-1* gene. RluA-1 is homologous to *E. coli* RluA family members and is evolutionarily conserved in multicellular organisms. *In situ* hybridization and immunocytochemistry revealed that *RluA-1* is expressed in MD neurons. We investigated the *RluA-1* enhancer responsible for MD expression and found that the membrane-tethered green fluorescent protein driven by *RluA-1-GAL4* was expressed in the dendritic arbors of MD neurons, confirming that *RluA-1* is indeed expressed in MD neurons. Thus, the expression of *RluA-1* is spatially controlled during development.

KEY WORDS: *pseudouridine synthase, multiple dendritic neuron*

Pseudouridine (Ψ) is the most common modified nucleotide in cellular RNAs (Rozenski *et al.*, 1999). Ψ is synthesized from uridine by Ψ synthases. According to the amino acid sequence, Ψ synthases have been classified into five families: TruD, TruA, TruB, RsuA, and RluA (Kaya and Ofengand, 2003, Koonin, 1996). All Ψ synthases share a conserved active site cleft containing catalytic aspartate (Hamma and Ferre-D'Amare, 2006). In *E. coli* and yeasts, it is known that each Ψ synthase functions in the synthesis of specific Ψ residues in tRNA or rRNA. For example, the RluA family member RluD in *E. coli* is responsible for the formation of Ψ residues in rRNA. Lack of RluD causes defects in ribosomal assembly and bacterial growth (Gutgsell *et al.*, 2005). In addition, Ψ synthases may have functions other than Ψ synthesis, such as being an RNA chaperone (Grosshans *et al.*, 2001). In humans, the TruB Ψ synthase dyskerin is involved in an RNP complex, which is required for rRNA processing and telomerase activity as well as Ψ synthesis (Hamma and Ferre-D'Amare, 2006). Mutation in dyskerin causes the human bone marrow failure syndrome dyskeratosis congenita (Heiss *et al.*, 1998). No RluA family Ψ synthases in multicellular organisms have been investigated.

The *Drosophila* embryonic peripheral nervous system (PNS) consists of type I and type II neurons (Jan and Jan, 1993, Jan and Jan, 1994). Type I neurons locate within external sensory (ES) and chordotonal (CH) organs. Each of the type I neurons has one dendrite, whereas each of the type II multiple dendritic (MD) neurons possesses more than one dendrite. MD neurons can be recognized in the enhancer trap line *E7-2-36*, which bears a *lacZ*-containing P-element and expresses *lacZ* in all MD neurons (Brewster and Bodmer, 1995). The *lacZ* expression in *E7-2-36* suggests the existence of a nearby gene expressed in MD neurons.

We identified that the P-element of *E7-2-36* is inserted in the *Drosophila* gene *RluA-1*. *In situ* hybridization, immunocytochemistry, and study of the enhancer revealed that *RluA-1* is expressed in MD neurons. Our data indicate that *RluA-1* expression is

Abbreviations used in this paper: PNS, peripheral nervous system; MD, multiple dendritic; Ψ, pseudouridine.

***Address correspondence to:** Min-Lang Huang. Department of Life Science, National Chung-Cheng University, Chia-Yi 621, Taiwan. Fax: +886-5-2722871. e-mail: biomlh@ccu.edu.tw - web: http://admbio.ccu.edu.tw/new/teacher_hml.html

Accepted: 22 September 2010. *Final author corrected PDF published online:* 8 June 2011.

ISSN: Online 1696-3547, Print 0214-6282

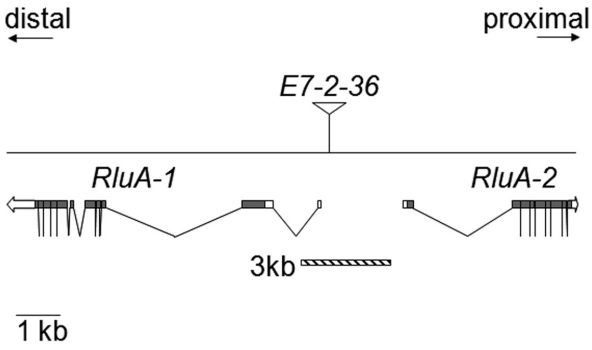

Fig. 1. Insertion of the *E7-2-36* P-element. *The P-element of* E7-2-36 *resides between* RluA-1 *and* RluA-2. *The transcripts of* RluA-1 *and* RluA-2 *are illustrated under the genomic DNA. Filled bars indicate exons. The 3 kb enhancer is shown in hatched bars.*

spatially regulated during development.

Results

Insertion of E7-2-36 *and evolutional conservation of RluA Ψ synthases*

The genomic sequence flanking the *E7-2-36* P-element insertion site was identified by plasmid rescue and sequencing. We found the P-element inserts between two genes, *RluA-1* and *RluA-2* (Fig. 1), both of which encode Ψ synthases.

Sequence analyses revealed that *Drosophila* RluA-1 and RluA-2 contain the Ψ synthase domain (InterPro IPR006225), which is found in the RluC and RluD Ψ synthases in *E. coli*. RluA-1 and RluA-2 are highly similar in the Ψ synthase domain, which is evolutionarily conserved among RluA Ψ synthases (Fig. 2A). In addition, the Ψ synthase domain contains the catalytic aspartate (Fig. 2B), which suggests that RluA-1 and RluA-2 function in the Ψ formation. On the other hand, RluA-1 is longer than RluA-2. In RluA-1, the N-terminal sequence of amino acids 1–132 exhibits no functionally conserved domain and is absent in RluA-2. Sequence analyses revealed that RluA-1 and RluA-2 have one distinct homologue, respectively, in all *Drosophila* species sequenced. However, in each of the other sequenced arthropods and mammals, only one gene homologous to RluA-1 and RluA-2 is found. The homologue is more similar to RluA-2 than to RluA-1 because of the lack of a region similar to the RluA-1 N-terminus (Fig. 2). Thus, *RluA-2* seems to be conserved across phyla, whereas *RluA-1* may be produced by gene duplication only present in *Drosophila*.

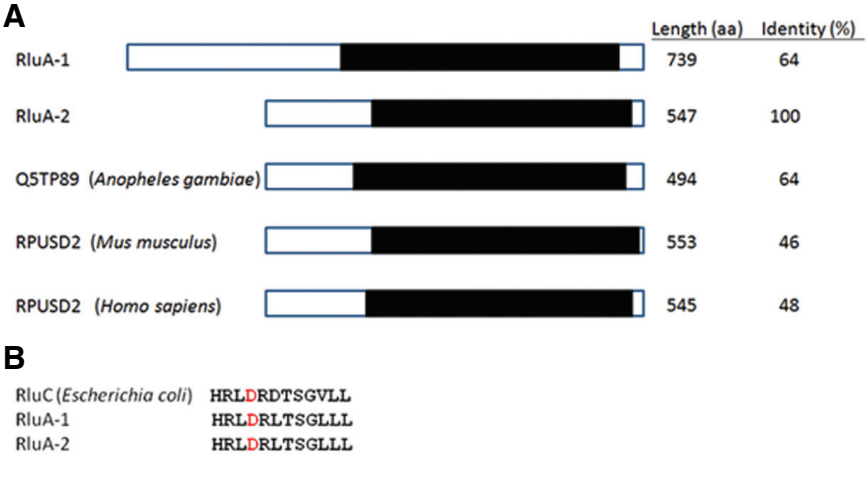

Fig. 2. Comparison of RluA and homologues. (A) *Similarity among RluA-1, RluA-2, and the homologues in mosquito, mouse, and human. Q5TP89 is the accession number on Swiss-Prot. The filled boxes indicate the Ψ synthase domain. The identities of the Ψ synthase domain compared with that of RluA-2 are indicated.* **(B)** *Alignment of the residues in the active site cleft containing the catalytic aspartate (red).*

Expression of RluA-1 *mRNA*

The insertion site of the P-element was 30 bp upstream of the *RluA-1* transcription start site, suggesting that *RluA-1* may be the candidate gene expressed in MD neurons. We performed *in situ* hybridization to analyze the expression of *RluA-1* mRNA during embryogenesis. At stage 14, *RluA-1* mRNA was expressed in the cells that appear iteratively in peripheral tissues (Fig. 3A). The signal of *RluA-1* mRNA was also detected in the dorsal pharyngeal musculature (Fig. 3B). At stage 16–17, *RluA-1* mRNA was expressed in the cells that distribute as PNS neurons (Fig. 3C and 4C). In addition, *RluA-1* mRNA was expressed in the dorsal vessel (Fig. 3D). No *RluA-1* mRNA was detected in the embryonic central nervous system (CNS). *RluA-2* mRNA was expressed ubiquitously (data not shown).

Comparison of the distribution of multiple dendritic (MD) neurons and RluA-1 expression

MD neurons are divided into three classes, da, td, and bd, according to the nature of their dendrites: da neurons exhibit large dendritic arbors, td neurons' dendrites are associated with the trachea, and bd neurons have bipolar dendrites (Brewster and Bodmer, 1995). When all PNS and MD neurons were labeled using pan-neuronal markers and *E7-2-36*, respectively, the location of MD neurons among PNS neurons were observed (Fig. 4A–C). We generated RluA-1 antibody and examined its distribution in embryos. To avoid cross-reaction with RluA-2, we chose the 20–37 amino acids, which are not homologous to RluA-2, as the antigen. Immunocytochemistry studies using anti-RluA-1 and anti-Elav, which recognizes all neuronal nuclei, revealed that RluA-1 was expressed in the subsets of PNS neurons (Fig. 4 D–G). The colocalization of their signals suggested that RluA-1 functions in the nuclei, where eukaryotic rRNA Ψ formations take place (Maden, 1990). The distribution of the RluA-1 expressing cells suggests that RluA-1 is expressed in MD neurons.

Confirmation of the RluA-1 *expression in MD neurons*

We examined whether *RluA-1* is indeed expressed in MD neurons. In wild-type embryos, the dorsal part in one abdominal hemisegment consists of 12 ES and MD neurons (Fig. 4C). The sc^{B57} allele lacks the proneural genes of the *achaete-scute complex*, which are required for ES and some MD neuron formation (Lindsley, 1992). Therefore, in sc^{B57} embryos, only two MD neurons remain in the dorsal abdominal

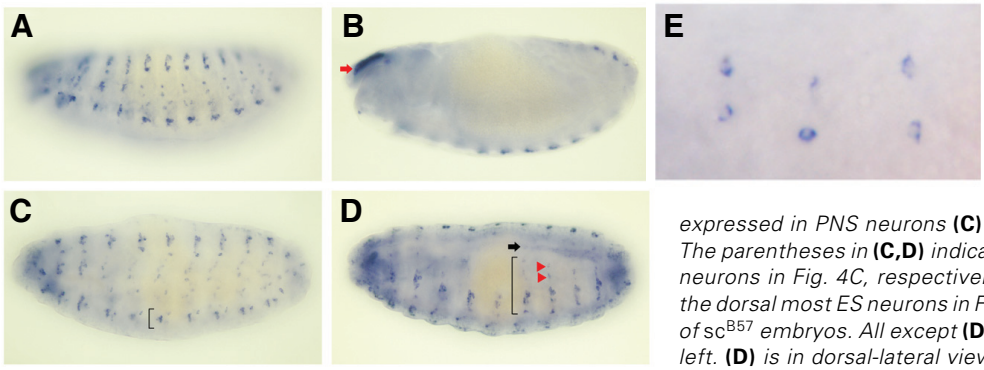

Fig. 3. *RluA-1* mRNA expression in embryos. *Whole mount* in situ *hybridization of embryos using* RluA-1 *RNA probe. At stage 14,* RluA-1 *mRNA appeared in PNS-like cells* **(A)** *and the dorsal pharyngeal musculature (red arrow)* **(B)**. *At stage 17,* RluA-1 *mRNA is likely expressed in PNS neurons* **(C)** *and in the dorsal vessel (black arrow)* **(D)**. *The parentheses in* **(C,D)** *indicate the cells similar to the ventral and dorsal neurons in Fig. 4C, respectively. Arrowheads indicate the cells similar to the dorsal most ES neurons in Fig. 4C.* **(E)** *Dorsal abdominal hemisegments of* sc^{B57} *embryos. All except* **(D)** *are in lateral view. Dorsal is up, anterior is left.* **(D)** *is in dorsal-lateral view.*

hemisegment (Huang *et al.*, 2000). We found that *RluA-1* mRNA was expressed in two cells in the dorsal abdominal hemisegment of sc^{B57} (Fig. 3E), suggesting that *RluA-1* is expressed in MD neurons. In addition, we examined RluA-1 expression in the fly whose MD neurons were labeled by the MD neuron-specific marker *GAL4 109(2)80* (Gao *et al.*, 1999). This GAL4 line starts driving transgene expression at embryonic stage 15 (Sugimura *et al.*, 2004). In stage 16 embryos bearing *GAL4 109(2)80* and *UAS-mCD8::GFP*, most MD neurons are labeled by the membrane-tethered green fluorescent protein (GFP) (Fig. 4H). Later on, at stage 17 all MD neurons are recognized by GFP. Because the RluA-1 antibody cannot label MD neurons after embryonic stage 16, we examined RluA-1 expression in the stage 16 embryos. In the embryos we found that RluA-1 was expressed in the GFP-positive cells (Fig. 4 H–I), confirming the expression of RluA-1 in MD neurons.

Observing MD neurons using an enhancer of *RluA-1*

One way to ensure that the *RluA-1*-expressing cells are MD neurons is to examine whether the cells exhibit multiple dendrites. We investigated the enhancer of *RluA-1* and used the membrane-tethered GFP, mCD8::GFP (Lee and Luo, 1999), to label cellular morphology. If *RluA-1* enhancer could drive the GFP expression in MD neurons, we would observe their dendritic arbors. Indeed, we found that the 3 kb genomic fragment including upstream sequences of *RluA-1* (Fig. 1) was such an enhancer. We used the 3 kb fragment to generate *RluA-1-GAL4*. In the fly carrying *RluA-1-GAL4* and *UAS-mCD8::GFP* (*RluA-1-GAL4>mCD8::GFP*), some MD neurons began to be labeled by GFP at embryonic stage 16. At stage 17, all MD neurons were GFP-positive (Fig. 5A). The dendritic arbors of MD neurons were clearly seen at the third instar larvae (Fig. 5 C–D). By comparing the dendritic arbor with that of the da neurons (Grueber *et al.*, 2002), we identified all da neurons. db and td neurons were also recognized by the bipolar and trachea-associated dendrites, respectively. In each abdominal hemisegment, we detected all MD except ltd and lbd neurons. ltd and lbd neurons were observed occasionally possibly because they locate deeply in the body and exhibit low levels of GFP (data not shown). In addition, GFP signals were present in the cells that were negative for anti-RluA-1 in stage 16 embryos, such as v'td2 neurons, and subsets of ES neurons and muscles (Fig. 5, 4). In these cells, *RluA-1* may be transcribed in a low level or later in embryogenesis, or only at larval stages. Consistently, *RluA-1* mRNA appeared to be expressed in the

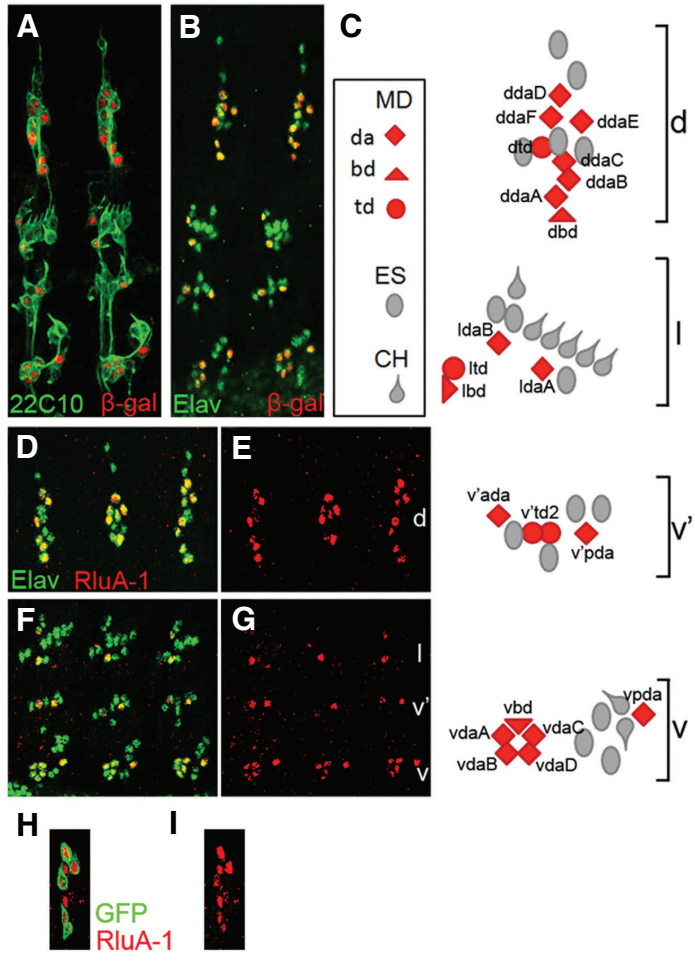

Fig. 4. RluA-1 expression in multiple dendritic neurons. (A,B) *MD neurons in stage 16* E7-2-36 *embryos were marked by β-galactosidase (red).* **(A)** *The MAb22C10 antibody (green) marks the morphology of PNS neurons.* **(B)** *Anti-Elav (green) is a pan-neuronal nuclear marker.* **(C)** *Schematic diagram of an abdominal hemisegment of the* Drosophila *embryonic PNS. MD neurons are in red, ES (oval), and CH (drop shape) neurons are in gray. da, td, and bd neurons are indicated by diamonds, circles, and triangles, respectively. MD neurons are indicated according to the nomenclature described (Brewster and Bodmer, 1995, Grueber* et al., *2002).* **(D, E, F, G)** *Stage 16 embryos stained with anti-Elav (green) and anti-RluA-1 (red). d, dorsal; l, lateral; v, ventral.* **(H,I)** *In stage 16* 109(2)80>mCD8::GFP *embryos, RluA-1 was expressed in the GFP-labeled MD neurons. Shown is the dorsal group.*

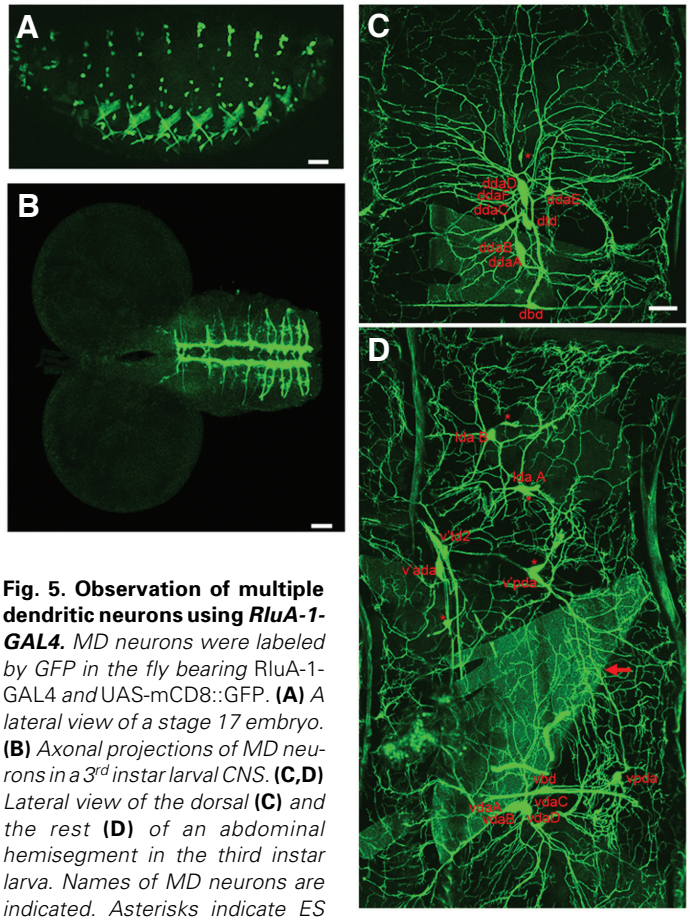

Fig. 5. Observation of multiple dendritic neurons using *RluA-1-GAL4*. *MD neurons were labeled by GFP in the fly bearing* RluA-1-GAL4 *and* UAS-mCD8::GFP. **(A)** *A lateral view of a stage 17 embryo.* **(B)** *Axonal projections of MD neurons in a 3rd instar larval CNS.* **(C,D)** *Lateral view of the dorsal* **(C)** *and the rest* **(D)** *of an abdominal hemisegment in the third instar larva. Names of MD neurons are indicated. Asterisks indicate ES neurons. Arrows indicate muscles. Anterior is left. Bar, 50 μm.*

dorsal most ES neurons at stage 17 (Fig. 3D). In the larval CNS of *RluA-1-GAL4>mCD8::GFP*, axonal projections of MD neurons were observed by GFP in the ventral nerve cord (VNC) (Fig. 5B). Although there is a low expression of β-galactosidase in the VNC of *E7-2-36* (data not shown), *RluA-1-GAL4* did not drive GFP in any cell body in the CNS and there is no expression in the CNS with the RluA-1 antibody, indicating that RluA-1 is not expressed in the CNS.

In summary, our data indicate that RluA-1 is expressed in MD neurons. Therefore, although Ψ synthases appear to function ubiquitously, the *Drosophila RluA-1* expression is spatially controlled during development.

Materials and Methods

Fly stocks and transgenic flies

Flies were raised on a standard cornmeal–yeast–agar medium at 25°C. The following flies were used: *E7-2-36* (Brewster and Bodmer, 1995); *UAS-mCD8::GFP* (Lee and Luo, 1999); sc^{B57} (Bloomington Stock Center); *Gal4 109(2)80* (Gao *et al.*, 1999). To make *RluA-1-GAL4*, the 3 kb enhancer of the fly and the *GAL4* gene of *Saccharomyces cerevisiae* were amplified from their genomic DNA by polymerase chain reaction. Primer sequences are available upon request. We removed the UAS sequence from the pUAST vector and used this modified vector as a backbone. The 3 kb enhancer and the *GAL4* gene were thus cloned into the modified pUAST vector. Transgenic flies were generated by P-element mediated germ-line transformation techniques (Spradling, 1986).

In situ *hybridization*

The procedure used for performing *in situ* RNA hybridization of *Drosophila* embryos has been described (Tautz and Pfeifle, 1989). *RluA-1* and *RluA-2* riboprobes were made by using the EST clones *RE33113* and *LD40728* (Berkeley *Drosophila* Genome Project), respectively. Embryos were visualized with a Zeiss Axioskop2 plus microscope under Normarski optics.

Immunocytochemistry

The rabbit polyclonal antibody against the RluA-1 peptide of 20–37 amino acids was generated (Quality Controlled Biochemicals). Standard embryo fixing and staining protocols (Patel, 1994) were used. The primary antibodies were: mouse MAb22C10 (1:250) and rat anti-Elav (1:100) (Developmental Studies Hybridoma Bank), rabbit anti-β-galactosidase (1:100) (Cappel), rabbit anti-RluA-1 (1:40). Images were collected on a Zeiss LSM 510 confocal microscope and merged in Adobe Photoshop.

Acknowledgements

We thank the Bloomington Stock Center for fly stocks and the Developmental Studies Hybridoma Bank for antibodies. This work was supported by grants from Academia Sinica and the National Science Council of Taiwan.

References

BREWSTER, R. and BODMER, R. (1995). Origin and specification of type II sensory neurons in *Drosophila*. *Development* 121: 2923-2936.

GAO, F.B., BRENMAN, J.E., JAN, L.Y. and JAN, Y.N. (1999). Genes regulating dendritic outgrowth, branching, and routing in *Drosophila*. *Genes Dev* 13: 2549-2561.

GROSSHANS, H., LECOINTE, F., GROSJEAN, H., HURT, E. and SIMOS, G. (2001). Pus1p-dependent tRNA pseudouridinylation becomes essential when tRNA biogenesis is compromised in yeast. *J Biol Chem* 276: 46333-46339.

GRUEBER, W.B., JAN, L.Y. and JAN, Y.N. (2002). Tiling of the *Drosophila* epidermis by multidendritic sensory neurons. *Development* 129: 2867-2878.

GUTGSELL, N.S., DEUTSCHER, M.P. and OFENGAND, J. (2005). The pseudouridine synthase RluD is required for normal ribosome assembly and function in Escherichia coli. *RNA* 11: 1141-1152.

HAMMA, T. and FERRE-D'AMARE, A.R. (2006). Pseudouridine synthases. *Chem Biol* 13: 1125-1135.

HEISS, N.S., KNIGHT, S.W., VULLIAMY, T.J., KLAUCK, S.M., WIEMANN, S., MASON, P.J., POUSTKA, A. and DOKAL, I. (1998). X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions. *Nat Genet* 19: 32-38.

HUANG, M.L., HSU, C.H. and CHIEN, C.T. (2000). The proneural gene amos promotes multiple dendritic neuron formation in the *Drosophila* peripheral nervous system. *Neuron* 25: 57-67.

JAN, Y.N. and JAN, L.Y. (1993). Functional gene cassettes in development. *Proc Natl Acad Sci USA* 90: 8305-8307.

JAN, Y.N. and JAN, L.Y. (1994). Genetic control of cell fate specification in *Drosophila* peripheral nervous system. *Annu Rev Genet* 28: 373-393.

KAYA, Y. and OFENGAND, J. (2003). A novel unanticipated type of pseudouridine synthase with homologs in bacteria, archaea, and eukarya. *RNA* 9: 711-721.

KOONIN, E.V. (1996). Pseudouridine synthases: four families of enzymes containing a putative uridine-binding motif also conserved in dUTPases and dCTP deaminases. *Nucleic Acids Res* 24: 2411-2415.

LEE, T. and LUO, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* 22: 451-461.

LINDSLEY, D.L., AND ZIMM, G.G. (1992). *The genome of Drosophila Melanogaster.* Academic press, San Diego.

MADEN, B.E. (1990). The numerous modified nucleotides in eukaryotic ribosomal RNA. *Prog Nucleic Acid Res Mol Biol* 39: 241-303.

PATEL, N.H. (1994). Imaging neuronal subsets and other cell types in whole-mount *Drosophila* embryos and larvae using antibody probes. In *Practical Uses in Cell*

and Molecular Biology., (ed. E.A. Fyrberg, L S.B.G.A.). Academic Press, San Diego, California, pp.446-485.

ROZENSKI, J., CRAIN, P.F. and MCCLOSKEY, J.A. (1999). The RNA Modification Database: 1999 update. *Nucleic Acids Res* 27: 196-197.

SPRADLING, A.C. (1986). *P element-mediated transformation in Drosophila: a practical approach*. IRL, Oxford.

SUGIMURA, K., SATOH, D., ESTES, P., CREWS, S. and UEMURA, T. (2004). Development of morphological diversity of dendrites in *Drosophila* by the BTB-zinc finger protein abrupt. *Neuron* 43: 809-822.

TAUTZ, D. and PFEIFLE, C. (1989). A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene hunchback. *Chromosoma* 98: 81-85.